Product Information

Yeast Mitochondria Isolation Kit

Sufficient for 40 applications (using 20 OD culture preparations), for isolation of an enriched mitochondrial fraction from yeast cells

MITOISO3

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] Isolated mitochondria are useful tools for *in vitro* studies like respiration and energy production studies, apoptosis, mtDNA and mtRNA, and for mitochondrial protein profiling.

This kit enables fast and easy yeast cell wall lysis, and isolation of an enriched mitochondrial fraction from yeast 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).[1] The integrity of the inner membrane can be assessed by measuring citrate synthase activity (Cat. No. CS0720, Citrate Synthase Assay Kit).[1,2] The inner membrane integrity can also be assessed by testing the electrochemical proton gradient (ΔΨ) of the inner mitochondrial membrane by measuring uptake of the fluorescent dye JC-1 into the mitochondria (Cat. No. CS0760, Isolated Mitochondria Staining Kit).[3]

The kit contains all the reagents required for yeast cell wall lysis by lyticase (spheroplast formation), followed by cell membrane lysis/breakage and mitochondria isolation. MITOISO3 also includes an extraction buffer for mitochondrial protein profiling to be used in proteome studies and a storage buffer for use with intact mitochondria. The kit was tested on *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Schizosaccharomyces pombe*. Several publications[4-7] and dissertations[8-12] have cited use of MITOISO3.

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.

Components

The reagents are sufficient for 40 procedures using 20 OD culture preparations (see Procedure, "Sample Preparation" section).

- Buffer A, 10× (Component B3311): 10 mL
- Buffer B, 2× (Component B3186): 30 mL
- 1 M Dithiothreitol Solution (Component D7059): 1 mL
- Storage Buffer, 5× (Component S9689): 30 mL
- Lyticase from *Arthrobacter luteus* (Cat. No. L2524): 10,000 units
- Protease Inhibitor Cocktail for use with fungal and yeast extracts (Cat. No. P8215): 1 mL
- Cell Lysis Solution (Component C1242): 0.6 mL
- Protein Extraction Reagent Type 4 (Component C0356): 1 bottle (1 BTL)

Reagents and Equipment Required

(Not provided)

- Table-top centrifuge
- Cooled microcentrifuge
- Dulbecco's Phosphate Buffered Saline (DPBS, Cat. No. D8537)
- Ultrapure water
- 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)
- Percoll® (Cat. No. P1644) for additional purification on density gradient (optional)

- Tributylphosphine (TBP) Solution (Cat. No. T7567) for two-dimensional (2D) gel analysis
- Iodoacetamide, alkylating reagent (Cat. No. A3221) for 2D gel analysis
- CelLytic™ M (Cat. No. C2978), for functional tests, optional
- Trichloroacetic acid solution, ~100% (w/v) (Cat. No. T0699; optional)

Storage/Stability

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

Preparation Instructions

Use ultrapure water for the preparation of reagents. These instructions are for the preparation of reagents suitable for a 20 OD sample:

1× Buffer A

- Thaw Buffer A, 10× (Component B3311).
- Dilute an aliquot of the buffer 10-fold with ultrapure water.
- Just prior to use, add 10 µL of the 1 M Dithiothreitol (DTT) Solution (Component D7059) to each mL of diluted buffer to give a final DTT concentration of 10 mM.
- Prepare 2 mL of 1× Buffer A for each sample.

1× Buffer B

- Thaw Buffer B, 2× (Component B3186).
- Dilute an aliquot of the buffer 2-fold with ultrapure water under sterile conditions.
- Prepare 1 mL of 1× Buffer B for each sample.

1× Storage Buffer

- Dilute an aliquot of the Storage Buffer, 5× (Component S9689) 5-fold with ultrapure water.
- Prepare 2-3 mL of 1× Storage buffer for each sample, according to the protocol used.

1× Storage Buffer with Protease Inhibitor Cocktail

- Dilute an aliquot of the Protease Inhibitor Cocktail (PIC) 100-fold with the 1× Storage Buffer. The presence of the PIC in the appropriate buffer is important for the first step of both methods of the Cell Membrane Disruption procedure.
- The 1× Storage Buffer with Protease Inhibitor Cocktail is used in Step 1 of the Homogenization method and/or in Step 1 of the Detergent Lysis method.
- Since the volume of the Protease Inhibitor Cocktail is limited, it is recommended to prepare 1-2 mL of 1× Storage Buffer with Protease Inhibitor Cocktail.

Lysis Buffer

- Before use, thaw the Cell Lysis Solution (Component C1242).
- Mix until homogenous.
- Dilute the required volume of the Cell Lysis Solution 200-fold with 1× Storage Buffer with Protease Inhibitor Cocktail.
  - For example, add 5 µL of the Cell Lysis Solution to 1 mL of 1× Storage Buffer with Protease Inhibitor Cocktail.
- Mix well by vortexing. Keep on ice until use.
- Prepare ~1 mL of Lysis Buffer for each sample.
- **Note**: For each yeast strain, it is suggested to optimize the dilution of the Cell Lysis Solution (in the range of 100-fold to 800-fold dilution) to obtain the best yield of intact mitochondria.

Protein Extraction Reagent Type 4

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

Lyticase Solution

- The vial of lyticase can be reconstituted with 100 µL of 50% glycerol solution.
- The Lyticase Solution is stable for at least 6 months at –20 °C.

Procedure

The described procedures are for a small cell sample (yeast culture of 20 OD_{600}).

For larger-scale preparation (~200 OD_{600} per sample), calculate the volumes of reagents required for the procedure accordingly.

After yeast cell wall lysis using lyticase, the mitochondria can be easily isolated from the spheroplasts by a simple method of homogenization or lysis with the use of a detergent, followed by low (600 × g) and high speed (6,500 × g) centrifugation. The final pellet represents a crude mitochondrial fraction that may be used for further experiments.

Another option is isolation of 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.

Sample Preparation

- Grow the yeast cells into log phase.
- Determine the OD of the culture at 600 nm.
- Calculate the total OD_{600} of the sample as follows:
  - Multiply the OD_{600} of 1 mL by the total volume (mL) of the culture.
  - For example, a 100 mL culture with OD_{600} of 0.2 is a 20 OD culture sample. Use a 20 OD_{600} aliquot for each preparation.

Note: Cell stress leads to alteration of polysaccharide levels in the yeast cell wall. The cell wall of stressed cells, such as stationary phase cells, is thicker. These cells are resistant to digestion by enzymatic activities like lyticase. Therefore, it is important to start the growth with a freshly seeded plate (from a frozen stock) and also not to grow the yeast cell culture to the late log phase or close to the stationary phase.[13] Moreover, in order to isolate respirating mitochondria from yeast cells grown in aerobic conditions in which ethanol serves as the carbon source, one should take into consideration that the cells grow very slowly and develop a thickened wall.

Yeast Spheroplast Formation

1. Centrifuge the yeast cells at 3,000 × g for 5 minutes. Discard the supernatant.
2. Resuspend the cell pellet in 5-6 volumes of water.
   2.1. Centrifuge at 3,000 × g for 5 minutes.
   2.2. Discard the supernatant.
3. Resuspend the cell pellet in 2 mL of 1× Buffer A.
4. Incubate for 15 minutes at 30 °C with gentle shaking.
5. Centrifuge the cells at 1,500 × g for 5 minutes and discard the supernatant.
6. Resuspend the cell pellet in 1 ml of 1× Buffer B.
7. Add 10 µL of the resuspended cell sample to 990 µL of ultrapure water and read the OD at 600 nm.
   7.1. Calculate the total OD of the cell suspension at this stage, for use as a reference value.
8. Add the Lyticase Solution to the sample suspension. For each yeast strain, it is recommended to optimize the lyticase concentration. Recommended concentrations for different yeast species are given in Table 1.

Table 1. Suggested units of lyticase and lysis time required for spheroplast preparation from different yeast species.

Species	Carbon source	Lyticase units/OD	Approximate time for spheroplast formation (minutes)
S. cerevisiae	Ethanol	10-15	40
S. cerevisiae	Glucose	1-2	10
P. pastoris	Glycerol	0.5-1	5
S. pombe	Glucose	5-8	30

Sample calculation: According to Table 1, for a 20 OD *S. cerevisiae* culture grown on ethanol, 200-300 units (20 × 10-15 units) of lyticase are required.

9. Place the cells at 30 °C with gentle shaking.
   9.1. During the incubation, measure the OD every 5 mL.
   9.2. Transfer 10 µL of the sample to 990 µL of ultrapure water.
   9.3. Read the OD at 600 nm.
   9.4. When the OD decreases to 30-40% of the initial value (pre-lysed sample, Step 7), stop the reaction by centrifuging at 1,200 × g for 5 minutes at 2-8 °C.
   9.5. After centrifugation, discard the supernatant. Keep the tubes on ice.

Notes:

- It is advisable to stop spheroplast formation at an OD of 30-40% of the original value, since preparations with lower OD values may contain severely disrupted mitochondria.
- Formation of spheroplasts may also be assessed by microscopy:
  - When yeast cells are observed under a light microscope, they appear as bright cells.
  - When spheroplasts are formed, they can be distinguished from yeast cells by their darker appearance.

Cell Membrane Disruption

At this stage, the mitochondria can be released from the spheroplast by one of two methods for spheroplast membrane disruption:

- Homogenization
- Detergent lysis

A separate procedure is presented for each method of spheroplast membrane disruption. All isolation procedures should be done at 2-8 °C with ice-cold solutions, homogenizer (when used), and tubes.

Homogenization

Excess homogenization increases the total protein level in the sample but may cause disruption of mitochondrial membranes.

1. Resuspend the spheroplasts in 1 ml of 1× Storage Buffer with Protease Inhibitor Cocktail.
2. Homogenize the spheroplasts on ice with ~10 strokes using a 2 mL glass-glass homogenizer (Dounce homogenizer, tight pestle). Some yeast strains may require the number of strokes be optimized for obtaining the best yield of intact mitochondria.
3. Transfer the homogenate to a 2 mL microcentrifuge tube. Add 1 mL of 1× Storage Buffer.
4. Centrifuge the homogenate at 600 × g for 10 minutes at 2-8 °C. Carefully transfer the supernatant to a fresh tube.
5. Centrifuge at 6,500 × g for 10 minutes at 2-8 °C. Carefully remove and discard the supernatant.
6. Suspend the pellet in a buffer suitable for your application. The following are suggestions for the volume and type of buffer required for different applications. If the volume is not appropriate for your system, adjust accordingly:
   6.1. For applications that require intact mitochondria (such as measurement of JC-1 uptake, citrate synthase activity, or cytochrome c oxidase activity), add 150-250 µL of 1× Storage Buffer.
   6.2. For protein profiling (2D gel) analysis, it is recommended to suspend the mitochondrial pellet to a concentration of 0.025-0.1 mg/mL in Protein Extraction Reagent Type 4 (such as at 5-20 µg per 200 µL of Protein Extraction Reagent Type 4).

   Note: it is highly recommended to use the TCA Lowry method for the determination of the amount of protein suspended in this buffer (see Appendix).
   6.3. For further fractionation, add 150-250 µL of 1× Storage Buffer.
   6.4. For mitochondrial protein characterization or functional assays, add 150-250 µL of CelLytic™ M (Cat. No. C2978) with Protease Inhibitor Cocktail added (1:100 [v/v]).

Detergent Lysis

The procedure of cell lysis using a detergent requires optimization of the amount of detergent used. Excess detergent will increase the total amount of mitochondria but may disrupt the mitochondrial membranes. Therefore, for each yeast strain, it is recommended to optimize the concentration of the Cell Lysis Solution in the Lysis Buffer to obtain the best yield of intact mitochondria. Begin with a dilution of 1:200 (see Preparation Instructions).

- If the mitochondria yield is too low, increase the concentration of the Cell Lysis Solution (for example, perform a 1:100 dilution).
- If the yield is very high, but the mitochondria intactness is low, decrease the concentration of the Cell Lysis Solution (for example, perform a 1:300 – 1:800 dilution).

1. Resuspend the spheroplasts prepared from a 20 OD culture to a uniform suspension in 0.65-1 mL of Lysis Buffer.
2. Incubate on ice for 5 minutes. During the 5-minute incubation, mix the spheroplasts every minute by a single inversion of the tube.
3. At the end of the incubation, add 2 volumes of 1× Storage Buffer. Centrifuge the mixture at 600 × g for 10 minutes at 2-8 °C.
4. Carefully transfer the supernatant liquid to a fresh tube. Centrifuge at 6,500 × g for 10 minutes at 2-8 °C.
5. Carefully remove and discard the supernatant. Suspend the pellet in a suitable buffer (see Section A, Step 6 for buffer suggestions).

Preparation of Sample for 2D Gel Electrophoresis

1. Reduce the protein extract prepared for profiling in Protein Extraction Reagent Type 4 with 5 mM Tributylphosphine (TBP). Add 5 µL of 0.2 M TBP Solution to 200 µL of protein sample and incubate for 30 minutes.
2. Alkylate the solution with 15 mM iodoacetamide. Add 6 µL of prepared 0.5 M iodoacetamide solution to 200 µL of protein sample and incubate 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 (from "Homogenization", Step 5 or "Detergent Lysis", Step 5) can be further fractionated by layering onto a Percoll® (Cat. No. P1644) density gradient. Further purification using a Percoll® density gradient decreases the overall yield of mitochondria.[14] Therefore, it is recommended to use a larger initial cell sample in order to obtain a significant quantity of mitochondria.

The following procedure is for an initial 200 OD culture sample:

1. Suspend the mitochondrial pellet ("Homogenization", Step 5 or "Detergent Lysis", Step 5) in ~0.85 mL of 1× Storage Buffer.
2. Add 150 µL of Percoll®, to give a final Percoll® concentration of 15% (v/v) in the sample.
3. Use the mitochondrial suspension to form a Percoll® density gradient. The 5 mL gradient consists of:
   - A bottom layer of 2 mL of 40% (v/v) Percoll® in 1× Storage Buffer
   - A middle layer of 2 mL of 23% (v/v) Percoll® in 1× Storage Buffer
   - A top layer of 1 mL of the mitochondrial suspension in 1× Storage Buffer that contains 15% (v/v) Percoll®
4. Centrifuge the gradient in a swinging bucket rotor at ~31,000 × g for 10 minutes at 2-8 °C.
5. Harvest the mitochondria which band at the lowest interface. Dilute these mitochondria with 4 volumes of ice-cold 1× Storage Buffer.
6. Centrifuge the mitochondria in a fixed angle rotor at ~17,000 × g for 10 minutes at 2-8 °C.
7. Remove and discard the supernatant. Suspend the mitochondrial pellet in 1× Storage Buffer at a protein concentration of 1-5 mg/mL.

TCA Lowry Determination of Protein Concentration

1. Precipitate the protein from the sample as follows:
   1.1. Add ice-cold TCA solution (Cat. No. T0699) to give a final concentration of 8-10% (w/v).
   1.2. Then centrifuge at 11,000 – 14,000 × g for 10 minutes at 2-8 °C.
2. Wash the pellet with ice cold 10% TCA solution. Dissolve the pellet in 0.1 ml of 0.1 N NaOH.
3. Determine the protein concentration using the Lowry method[15] in a total reaction volume of 1 mL and using a standard curve with BSA in the range of 5-20 µg.

The Effect of Nutrients on Yeast Mitochondria

Yeast mitochondria are dynamic structures whose size, shape, and number can vary greatly according to strain, cell cycle phase, and growth conditions (such as shown in Table 2). Important factors include:

- Partial oxygen pressure
- Glucose concentration
- Presence of unfermentable substrates
- Availability of sterols, fatty acids, and divalent metal ions (such as Mg^{2+}).

Table 2. Effect of nutrients on the number and morphology of yeast mitochondria

Nutrient	Concentration	Oxygen	Respiration	Morphology
Glucose	Excess	+	Repressed	Few large
Glucose	Excess	–	Repressed	Few large
Glucose	Limited	+	Activated	Many small
Glucose	Limited	–	Repressed	Few large
Ethanol	Excess	+	Activated	Many small

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