

Yeast Mitochondria Isolation Kit

Cat. No. YMI-329K **Lot. No.** (See product label)

SPECIFICATION

Product Overview	The kit is used for yeast cell wall lysis by lyticase, followed by cell membrane lysis/breakage and mitochondria isolation. The kit was tested on <i>Saccharomyces cerevisiae</i> , <i>Pichia pastoris</i> , and <i>Schizosaccharomyces pombe</i> .
Usage	The kit is sufficient for 40 procedures using 20 OD culture preparations (see Procedure, sample preparation).
Storage	Store the kit at -20 centigrade. When properly stored, the components of this kit are stable for at least 2 years.
Kit Components	Buffer A, 10× 10 ml Buffer B, 2× 30 ml 1 M Dithiothreitol Solution 1 ml Storage Buffer, 5× 30 ml Lyticase from <i>Arthrobacter luteus</i> 10,000 units Protease Inhibitor Cocktail for use with fungal and yeast extracts 1 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 or equivalent · Dulbecco's Phosphate Buffered Saline (PBS) · Ultrapure water · 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
- Percoll for additional purification on density gradient, optional
- Tributylphosphine (TBP) Solution for two dimensional (2D) gel analysis
- Iodoacetamide, alkylating reagent for 2D gel analysis
- CellLytic M, for functional tests, optional
- Trichloroacetic acid solution, ~100% (w/v) , optional

Preparation

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× and dilute an aliquot of the buffer 10-fold with ultrapure water. Just prior to use, add 10 ml of the 1 M Dithiothreitol (DTT) Solution 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× and 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× 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 and mix until homogenous. Dilute the required volume of the Cell Lysis Solution 200-fold with 1× Storage Buffer with Protease Inhibitor Cocktail, e.g., add 5 ml of the Cell Lysis Solution to 1 ml of 1× Storage Buffer with Protease Inhibitor Cocktail. Mix well by vortexing and keep on ice until use. Prepare ~1 ml of Lysis Buffer for each sample. Note: For each yeast strain,

it is recommended to optimize the dilution of the Cell Lysis Solution (in the range of 100 to 800-fold dilution) for obtaining 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 and needs to be warmed to 20-25 centigrade to entirely dissolve the solids. A 30 centigrade water bath will aid in dissolving the material. Do not allow the material to heat above 30 centigrade, since this product may begin to form cyanates, which are detrimental to the proteins. Freeze any unused solution in working aliquots at -20 centigrade for further use.

Lyticase Solution - The vial of enzyme can be reconstituted with 100 ml of 50% glycerol solution. The Lyticase Solution is stable for at least 6 months at -20 centigrade.

Separation Protocol

The described procedures are for a small cell sample (yeast culture of 20 OD600). For larger scale preparation (~200 OD600 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 ($600 \times g$) and high speed ($6,500 \times g$) 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 and high speed centrifugation steps are changed to $1,000 \times g$ and $3,500 \times g$, respectively, so the mitochondrial enriched fraction is obtained as the $3,500 \times g$ pellet. The drawback of this method is a lower yield of mitochondria.

Sample Preparation

Grow yeast cells into log phase and determine the OD of the culture at 600 nm. Calculate the total OD600 of the sample as follows: multiply the OD600 of 1 ml by the total volume (ml) of the culture. For example, a 100 ml culture with OD600 of 0.2 is a 20 OD culture sample. Use a 20 OD600 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. Moreover, in order to isolate respiring mitochondria from yeast cells grown in aerobic conditions in which ethanol serves as the carbon source, one should take into consideration that cells grow very slowly and develop a thickened wall.

Yeast Spheroplast Formation

1. Centrifuge the yeast cells at $3,000 \times g$ for 5 minutes and discard the supernatant.
2. Resuspend the cell pellet in 5–6 volumes of water. Centrifuge at $3,000 \times g$ for 5 minutes and discard the supernatant.
3. Resuspend the cell pellet in 2 ml of $1\times$ Buffer A.
4. Incubate for 15 minutes at 30 centigrade with gentle shaking.
5. Centrifuge the cells at $1,500 \times g$ for 5 minutes and discard the supernatant.
6. Resuspend the cell pellet in 1 ml of $1\times$ Buffer B.
7. Add 10 ml of the resuspended cell sample to 990 ml of ultrapure water and read the OD at 600 nm. Calculate the total OD of the cell suspension, at this stage, to be used 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.
9. Place the cells at 30 centigrade with gentle shaking. During the incubation, measure the OD every 5 minutes; transfer 10 ml of the sample to 990 ml of ultrapure water and read the OD at 600 nm. When the OD decreases to 30–40% of the initial value (pre-lysed sample, step 7), stop the reaction by centrifuging at $1,200 \times g$ for 5 minutes at 2–8 centigrade. 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 using one of two separate methods for spheroplast membrane disruption:

- homogenization or
- detergent lysis

A separate procedure is presented for each method of spheroplast membrane disruption.

All isolation procedures should be performed at 2–8 centigrade with ice-cold solutions, homogenizer (when used), and tubes.

A. 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 2 ml microcentrifuge tube and add 1 ml of 1× Storage Buffer.

4. Centrifuge the homogenate at $600 \times g$ for 10 minutes at 2–8 centigrade. Carefully transfer the supernatant to a fresh tube.

5. Centrifuge at $6,500 \times g$ for 10 minutes at 2–8 centigrade. 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:

- For applications requiring intact mitochondria (e.g., measurement of JC-1 uptake, citrate synthase activity, or cytochrome c oxidase activity) add 150-250 ml of 1×

Storage Buffer.

· 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 (i.e., 5–20 mg per 200 ml 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).

· For further fractionation add 150–250 ml of 1× Storage buffer.

· For mitochondrial protein characterization or functional assays, add 150–250 ml of CellLytic M with Protease Inhibitor Cocktail added (1:100 [v/v]).

B. 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 cause disruption of 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 (i.e., perform a 1:100 dilution) and if the yield is very high, but the mitochondria intactness is low, decrease the concentration of the Cell Lysis Solution (i.e., 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 and centrifuge the mixture at $600 \times g$ for 10 minutes at 2–8 centigrade.
4. Carefully transfer the supernatant liquid to a fresh tube and centrifuge at $6,500 \times g$ for 10 minutes at 2–8 centigrade.
5. Carefully remove and discard the supernatant, and 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 ml of 0.2 M TBP Solution to 200 ml of protein sample and incubate for 30 minutes.

2. Alkylate the solution with 15 mM iodoacetamide. Add 6 ml of prepared 0.5 M iodoacetamide solution to 200 ml 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 A.

Further Purification of the Mitochondrial Fraction on a Percoll Density Gradient

The mitochondrial pellet (Procedure A, step 5 or Procedure B, step 5) can be further fractionated by layering onto a Percoll density gradient. Further purification using a Percoll density gradient decreases the overall yield of mitochondria.⁵ 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 (Procedure A, step 5 or Procedure B, step 5) in ~0.85 ml of 1× Storage Buffer.
2. Add 150 ml of Percoll, resulting in 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, and a top layer of 1 ml of the mitochondrial suspension in 1× Storage Buffer containing 15% (v/v) Percoll.
4. Centrifuge the gradient in a swinging bucket rotor at $\sim 31,000 \times g$ for 10 minutes at 2–8 centigrade.
5. Harvest the mitochondria that band at the lowest interface and dilute them with 4 volumes of ice-cold 1× Storage Buffer.
6. Centrifuge the mitochondria in a fixed angle rotor at $\sim 17,000 \times g$ for 10 minutes at 2–8 centigrade.
7. Remove and discard the supernatant and suspend the mitochondrial pellet in 1×

Storage Buffer at a protein concentration of 1–5 mg/ml.

B. TCA Lowry Determination of Protein Concentration

1. Precipitate the protein from the sample by adding ice cold TCA solution to give a final concentration of 8–10% (w/v) and then centrifuging at $11\text{--}14,000 \times g$ for 10 minutes at 2–8 centigrade.
2. Wash the pellet with ice cold 10% TCA solution and dissolve the pellet in 0.1 ml of 0.1 N NaOH.
3. Determine the protein concentration using the Lowry method⁶ in a total reaction volume of 1 ml and using a standard curve with BSA in the range of 5–20 mg.

C. 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. Important factors are: partial oxygen pressure, glucose concentration, presence of unfermentable substrates, and availability of sterols, fatty acids, and divalent metal ions (e.g., Mg^{2+}).

Species	Carbon source	Lyticase units/OD	Approximate time for spheroplast formation (minutes)
<i>S. Cerevisiae</i>	Ethanol	10–15	40
<i>S. Cerevisiae</i>	Glucose	1–2	10
<i>P. Pastoris</i>	Glycerol	0.5–1	5
<i>S. Pombe</i>	Glucose	5–8	30

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