Product Information

Mitochondria Isolation Kit

Sufficient for 10-20 g (animal tissue), sufficient for 50 assays (2 mL), isolation of enriched mitochondrial fraction from animal tissues

MITOISO1

Product Description

This kit enables the fast and easy isolation of an enriched mitochondrial fraction from animal tissues. Most of the isolated mitochondria will contain intact inner and outer membranes. In addition, the kit enables the assessment of the mitochondrial inner membrane integrity by testing of the electrochemical proton gradient ($\Delta\Psi$) of the inner mitochondrial membrane.[1] This may be achieved by measuring the uptake of the fluorescent carbocyanine dye JC-1 (supplied in this kit) into the mitochondria.[2,3]

The outer membrane integrity may be measured by observing cytochrome c oxidase activity, such as with the Cytochrome c Oxidase Assay Kit, Cat. No. CYTOCOX1. The CYTOCOX1 kit measures the activity in the presence and absence of the detergent n-dodecyl β-D-maltoside. The ratio of the two activities provides a measure of the integrity of the outer membrane.

Mitochondria-mediated apoptosis studies are among the potential uses of this kit.[4-6] This kit may be also used to isolate mitochondrial proteins for proteome studies.[7,8] Several theses[9] and dissertations[10-17] have cited use of product MITOISO1 in their protocols.

Components

The reagents are sufficient for extraction of up to 10-20 g of animal tissue and for 50 JC-1 assays of 2 mL.

- Extraction Buffer A, 5×, 50 mL (Component E2778): 50 mM HEPES (pH 7.5), 1 M mannitol, 350 mM sucrose, 5 mM EGTA
- Extraction Buffer B, 5×, 50 mL (Component E6028): 100 mM MOPS (pH 7.5), 550 mM KCl, 5 mM EGTA
- Storage Buffer, 5×, 25 mL (Component S9689): 50 mM HEPES (pH 7.5), 1.25 M sucrose, 5 mM ATP, 0.4 mM ADP, 25 mM sodium succinate, 10 mM K_2HPO_4, 5 mM DTT
- Albumin Solution, 10 mL (Component A0474): 50 mg/mL delipidated bovine serum albumin (Cat. No. A7511) in water
- JC-1 Stain, 25 µg (Component J4519)
- JC-1 Assay Buffer, 5×, 25 mL (Component J4394): 100 mM MOPS (pH 7.5), 550 mM KCl, 50 mM ATP, 50 mM $MgCl_2$, 50 mM sodium succinate, 5 mM EGTA
- Trypsin, 50 mg (Component T9201)

Reagents and Equipment Required

(Not provided)

- Cooled Eppendorf centrifuge (for small scale), **or** Sorvall RC-5C centrifuge with SS-34 head (for large scale)
- PTFE pestle and 3 mL glass tube (Cat. No. P7734) (for small scale), **or** PTFE pestle and 45 mL glass tube (Cat. No. P7984) (for large scale)
- Overhead electric motor
- Spectrofluorometer with a suitable cuvette
- Scalpel and glass plate
- Ice bath
- 2 mL Eppendorf tubes
- Ultrapure water
- Dimethyl sulfoxide (DMSO, such as Cat. No. D8418)

Storage/Stability

Store the kit at −20 °C. When stored unopened, the components in this kit are stable for 24 months.

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: 10 mM HEPES (pH 7.5), 200 mM mannitol, 70 mM sucrose, 1 mM EGTA]:

- Defrost Extraction Buffer A at 37 °C. A short heating time (15 seconds in a microwave oven) may be needed to achieve a clear solution.
- Dilute an aliquot of the buffer 5-fold with water.
- Keep the diluted buffer at 4 °C before use.
- The concentrated buffer may be refrozen.

1× Extraction Buffer B

[Ionic solution: 20 mM MOPS (pH 7.5), 110 mM KCl, 1 mM EGTA]:

- Dilute an aliquot of Extraction Buffer B 5-fold with water.
- Keep the diluted buffer at 4 °C before use.
- The concentrated buffer may be refrozen.

1× Storage Buffer

[10 mM HEPES (pH 7.4), 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K_2HPO_4, and 1 mM DTT]:

- Dilute an aliquot of the 5× Storage Buffer 5-fold with water.
- Keep the diluted buffer at 4 °C before use.
- The concentrated buffer may be refrozen.

Albumin Solution (50 mg/mL)

- Dilute as needed in the appropriate buffer (see Procedure).

JC-1 Stain

- Dissolve the vial in 25 μL of dry DMSO. This will give a 1 mg/mL solution (1.53 mM; Molecular Weight 652.2).
- The solution may be stored at −20 °C at this concentration.
- For the assay, dilute an aliquot of the reconstituted solution 5-fold with DMSO.

1× JC-1 Assay Buffer

[20 mM MOPS (pH 7.5), 110 mM KCl, 10 mM ATP, 10 mM $MgCl_2$, 10 mM sodium succinate, 1 mM EGTA]:

- Dilute an aliquot of the buffer 5-fold with water.
- Keep the diluted buffer at 4 °C before use.
- The concentrated buffer may be refrozen.

Trypsin

- Dissolve an aliquot of trypsin in the appropriate 1× Extraction Buffer at 0.25 mg/mL.
- Keep at 4 °C until needed.

Procedure

General Notes

- Mitochondria can be prepared easily from animal tissues by a simple method of homogenization followed by low-speed (600 × g) and high-speed (11,000 × g) centrifugation.[18] The final pellet represents a crude mitochondrial fraction that may be used as the basis for further experiments.
- For a more purified "heavy" mitochondrial fraction that will be enriched in mitochondria (as opposed to lysosomes and peroxisomes that normally contaminate this fraction), the low-speed and high-speed centrifugation steps can be changed to 1,000 × g and 3,500 × g, respectively.[19]
- This kit supplies the necessary solutions for the isolation of mitochondria from "soft" tissues such as liver or brain, or from "hard" tissues such as skeletal muscle or heart muscle.
  - The "soft" tissues are extracted in the presence of delipidated BSA, which removes free fatty acids present in the tissue that cause uncoupling of respiration in the mitochondria.[19] EGTA is also present in the buffer to chelate Ca^{2+} ions that cause mitochondrial swelling.
  - "Hard" tissues cannot be homogenized easily without pretreatment with a protease, such as trypsin, to promote breakdown of the cellular structure. The myofibrils in skeletal muscle tend to give a gelatinous consistency to the homogenate in non-ionic media (isotonic sucrose), and thus must be isolated in an ionic medium, such as 1× Extraction Buffer B.[20]
- The mitochondrial pellet may be suspended in either:
  - 1× Storage Buffer (for experiments that need respiring mitochondria),
  - or in 1× Extraction Buffer A
- Perform all the isolation procedures at 4 °C with ice-cold solutions.

Note: The procedures described here are for small amounts of tissue samples (50-200 mg). The centrifugation is performed using a cooled micro-centrifuge. The homogenizer used is a PTFE pestle and 3 mL glass tube (Cat. No. P7734).

For larger-scale work (3-10 g of tissue), use a centrifuge with a SS-34 head. The homogenizer used is a PTFE pestle and 45 mL glass tube (Cat. No. P7984). When using larger amounts of tissue, the centrifugation time should be increased to 10 minutes at 600 × g, and to 15 minutes at 11,000 × g.

Preparation of mitochondria from soft tissues (liver or brain)

1. Use a fresh tissue sample (obtained within one hour of sacrifice) kept on ice. Do not freeze.
2. Wash the sample twice with 2 volumes of 1× Extraction Buffer A.
3. Cut small portions of the tissue (50-100 mg). Weigh in an Eppendorf tube.
4. Further cut the tissue with the aid of a scalpel and cooled glass plate to even smaller slices.
5. Homogenize the sample with 10 volumes of 1× Extraction Buffer A containing 2 mg/mL albumin (use Albumin Solution, Component A0474), using a 3 mL volume homogenizer powered by an overhead electric motor (~200 rpm).
   5.1. Ensure total homogenization of the sample by moving the pestle up and down 5-10 times.
   5.2. Keep the homogenate on ice.

   Note: The BSA is added to the 1× Extraction Buffer A to remove lipids, which may be present in the tissue.
6. Transfer the homogenate to a 2 mL Eppendorf tube. Centrifuge the sample at 600 × g for 5 minutes.
7. Carefully transfer the supernatant liquid into a new tube. Centrifuge the supernatant at 11,000 × g for 10 minutes.
8. Remove the supernatant and resuspend the pellet in 10 volumes of 1× Extraction Buffer A.
9. Repeat Steps 6 and 7.
10. Suspend the pellet in 1× Storage Buffer (~40 μL per 100 mg tissue).
11. This sample may now be assayed for protein concentration. The expected concentration should be approximately 10-25 mg/mL.
12. Assay for mitochondrial potential (inner membrane integrity) by measuring JC-1 uptake (see separate section). For assaying the outer membrane integrity, use the Cytochrome c Oxidase Assay Kit (Cat. No. CYTOCOX1).

Preparation of mitochondria from hard tissues (skeletal or heart muscle)

Notes:

- For heart muscle, use 1× Extraction Buffer A.
- For skeletal muscle, use 1× Extraction Buffer B.

1. Use a fresh tissue sample (obtained within one hour of sacrifice) kept on ice. **Do not freeze**.
2. Wash the sample with 2 volumes of the relevant extraction buffer.
3. Cut small portions of the tissue (50-100 mg). Weigh in an Eppendorf tube.
4. Further cut the tissue with the aid of a scalpel and glass plate to even smaller slices.
5. Suspend the sample with 10 volumes of the appropriate extraction buffer containing 0.25 mg/mL trypsin in a 2 mL Eppendorf tube.
6. Incubate on ice for 3 minutes. Then spin down the tissue for a few seconds in the centrifuge.
7. Remove the supernatant by aspiration. Add 8 volumes of the appropriate extraction buffer containing 0.25 mg/mL trypsin.
8. Incubate on ice for 20 minutes.
9. Add the Albumin Solution (Component A0474) to a final concentration of 10 mg/mL, to quench the proteolytic reaction.
   9.1. Mix.
   9.2. Then spin down the tissue for a few seconds in the centrifuge.
10. Remove the supernatant by aspiration.
    10.1. Wash the pellet with 8 volumes of the appropriate extraction buffer.
    10.2. Spin down the tissue for a few seconds in the centrifuge.
11. Remove the supernatant by aspiration. Add 8 volumes of the appropriate extraction buffer.
12. Homogenize using a 3 mL volume homogenizer powered by an overhead electric motor (~200 rpm). Ensure total homogenization of the sample by moving the pestle up and down 20-30 times.
13. Centrifuge the sample at 600 × g for 5 minutes.
14. Transfer the supernatant liquid to a new tube. Centrifuge it at 11,000 × g for 10 minutes.
15. Suspend the pellet in 1× Storage Buffer (~40 μL per 100 mg tissue).
16. This sample may now be assayed for protein concentration. The expected concentration should be approximately 10-20 mg/mL.

17. Assay for mitochondrial potential (inner membrane integrity) by measuring JC-1 uptake (see separate section). For assaying the outer membrane integrity, use the Cytochrome c Oxidase Assay Kit (Cat. No. CYTOCOX1).

JC-1 uptake in mitochondria

The integrity of the inner mitochondrial membrane may be measured by observing the potential gradient (ΔΨ) over this membrane. This can be achieved by measuring the uptake of the cationic carbocyanine dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) into the matrix.[2] The mitochondrial membrane potential, across the inner membrane, determines the redistribution of this dye. The distribution depends on the transmembrane electric field (negative inside) and the concentration gradient of the dye.

The fluorophore JC-1 has the property that when excited at 490 nm, the emission spectrum will be dependent on the concentration of the molecule:

- In dilute solutions of ≤ 300 nM, JC-1 will give a green fluorescence at 527 nm.
- However, when the concentration is > 1 mM, a very strong red-orange fluorescence will occur at 590 nm.

This is due to the formation of aggregates of the dye, named J-aggregates. In a mitochondrial matrix, bounded by an inner membrane with a large ΔΨ, the dilute external concentration of the dye is concentrated in the matrix to a level that enables the formation of J-aggregates. Thus, observation of the fluorescence at 590 nm is a simple and convenient indicator of the ΔΨ. JC-1 is the probe of choice for this work, since it is extremely sensitive to ΔΨ, as shown by depletion of the potential over the inner membrane in the presence of K^+/valinomycin.[3]

This procedure is a fixed-point assay that measures JC-1 uptake with formation of the J-aggregates. It is also possible to follow JC-1 uptake with time using a kinetic program.[2] The observed fluorescence of the solution will plateau after 5-10 minutes. Compounds that cause a blocking of the electron transport chain, followed by de-energization of the mitochondria and significant lowering of the ΔΨ, such as Antimycin A (Cat. No. A8674) at 2 µM or Pyrrolnitrin (Cat. No. P8861) at 3-12 µM, will show a drastic decrease in the JC-1 fluorescence because of equalization of the JC-1 concentrations inside and outside the mitochondrial matrix.

1. Prepare the JC-1 Stain and 1× JC-1 Assay Buffer as described under Preparation Instructions.
2. Prepare a suitable mitochondrial suspension.
    2.1. Dilute this suspension with 1× Storage Buffer to 1 mg of protein (mgP) per mL.
    2.2. Then take samples of 5-40 µg protein for the JC-1 assay.

Table 1. Golgi Density Gradient Preparation

Sample	Assay Buffer	Storage Buffer	Sample	JC-1 Stain
Blank	1.9 mL	100 µL	–	2 µL
Mitochondrial Fraction	1.9 mL	100-x µL	x µL	2 µL

Note: The total volume of each reaction is 2 mL.

3. Prepare the samples as follows:
    3.1. Add 1.9 mL of 1× JC-1 Assay Buffer to a tube.
    3.2. Then add an appropriate sample.
    3.3. Bring the volume to 2 mL with 1× Storage Buffer.
4. Start the reaction by addition of 2 µL of JC-1 Stain. Mix by inversion.
5. Leave the tubes at room temperature in the dark (cover with aluminum foil to avoid photobleaching) for 7 minutes, to allow complete uptake of the dye into the mitochondria.
6. Read the fluorescence of the sample in a spectrofluorometer, with settings as follows:[2]
    - Excitation wavelength = 490 nm; slit = 5 nm
    - Emission wavelength = 590 nm; slit = 7.2 nm
7. Calculate the fluorescence produced in the original mitochondria suspension (Preparations, Step 11 or 16) per mg mitochondrial protein (FLU/mgP).

Calculation:

FLU / mg Protein = [(ΔFLU) × dil] / (V × C)

- FLU = fluorescence units
- mgP = milligram protein
- $\Delta FLU = FLU_{sample} - FLU_{blank}$
- dil = dilution factor to prepare 1 mg/mL suspension (JC-1 uptake, Step 2)
- C = mgP/mL
- V = volume of mitochondrial sample in mL

References

1. Gross, A. *et al*., *J. Biol. Chem*., **274(2)**, 1156-1163 (1999).
2. Reers, M. *et al*., *Biochemistry*, **30(18)**, 4480-4486 (1991).
3. Salvioli, S. *et al*., *FEBS Lett*., **411(1)**, 77-82 (1997).

4. Rampino, N. *et al*., *Science*, **275(5302)**, 967-969 (1997).
5. Wallace, D.C., "A Mitochondrial Paradigm for Degenerative Diseases and Ageing", in *Ageing Vulnerability: Causes and Interventions* (Bock, G., and Goode, J.A., eds.). *Novartis Foundation Symposia*, **235**, 247-263 (2001).
6. Adrain, C., and Martin, S.J., *Trends in Biochem. Sci*., **26(6)**, 390-397 (2001).
7. Lopez, M.F., *et al*., *Electrophoresis*, **21(16)**, 3427-3440 (2000).
8. Rabilloud, T. *et al*., *Electrophoresis*, **19(6)**, 1006-1014 (1998).
9. Storrie, B., and Madden, E.A., *Methods Enzymol*., **182**, 203-225 (1990).
10. Graham, J.M., *Methods Mol. Biol*., **19**, 29-57 (1993).
11. Lee, C.P., *Biochem. Biophys. Acta*, **1271(1)**, 21-28 (1995).
12. Esterhuizen, Nanike, "Effects of thermal history on temperature-dependent flight performance in insects: *Ceratitis capitata* (Diptera: Tephritidae) as a model organism". Stellenbosch University, M.Sc. thesis, p. 20 (2013).
13. Mohan, Anand, "Myoglobin redox form stabilization: role of metabolic intermediates and NIR detection". Kansas State University, Ph.D. dissertation, p. 221 (2009).
14. Fett, Marieke, "Funktionelle Analyse von Parkin im Zebrafisch" ("Functional analysis of Parkin in zebrafish"). Ludwig-Maximilians-Universität München, Ph.D. dissertation, pp. 122, 143 (2010).
15. Ali, Mohammad Abdel-Motaal Mohammad, "Novel intracellular role of matrix metalloproteinase-2 in cardiac cell injury". University of Alberta, Ph.D. dissertation, p. 235 (2012).
16. Schauwecker, Katrin Martina, "Mitochondriale Wirkung von Sauerstoff und Kohlenstoffdioxid in der Postkonditionierung von humanen Kardiomyozyten nach unterschiedlicher Hypoxiedauer" ("Mitochondrial effects of oxygen and carbon dioxide in the postconditioning of human cardiomyocytes after different durations of hypoxia"). Albert-Ludwigs-Universität Freiburg im Breisgau, Dr. med. dissertation, p. 40 (2014).
17. Rodríguez, Sirena Soriano, "Study of frataxin deficit in *Drosophila*". Universitat de València, Ph.D. dissertation, p. 42 (2016).
18. Waters, Caroline T., "A Mitochondrial Profile in a Mouse Model of Dominant Optic Atrophy". Cardiff University, Ph.D. dissertation, p. 52 (2015).
19. Mejia, Edgard Marcello, "Understanding the Role of MLCL AT-1 and Tafazzin in Mitochondrial Function". University of Manitoba, Ph.D. dissertation, p. 42 (2016).
20. Zhang, Li, "Analyzing the function of TRAP1 in models of Parkinson's disease". RWTH Aachen University, Dr. rer. nat. dissertation, p. 17 (2016).

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MITOISO1pis Rev 09/22 ESS,NDH,IEG,ALC,GCY,MAM

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