Mitochondria Isolation Kit

Cat. No. MII-324K **Lot. No.** (See product label)

SPECIFICATION

Product Overview	The Mitochondria Isolation Kit provides a fast and easy isolation of an enriched mitochondrial fraction from animal tissues as well as the testing of the electrochemical proton gradient of the inner mitochondrial membrane
Usage	1 Kit is sufficient for extraction of up to 10-20 g of animal tissue and 50 JC-1 assays of 2 ml.
Storage	Store the kit at -20 centigrade. When stored unopened, the components in this kit are stable for 24 months.
Kit Components	Extraction Buffer A, 5× 50 ml Extraction Buffer B, 5× 50 ml Storage Buffer, 5× 25 ml Albumin Solution 10 ml JC-1 Stain 25 mg JC-1 Assay Buffer, 5× 25 ml Trypsin 50 mg
Materials Required but Not Supplied	· 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 (for small scale) or PTFE pestle and 45 ml glass tube (for large scale) · Overhead electric motor · Spectrofluorometer with a suitable cuvette

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	· Scalpel and glass plate · Ice bath · 2 ml Eppendorf tubes · Ultrapure water · Dimethyl sulfoxide (DMSO)
Preparation	Use ultrapure water for the preparation of reagents. 1× Extraction Buffer A (isotonic solution, 10 mM HEPES, pH 7.5, containing 200 mM mannitol, 70 mM sucrose, and 1 mM EGTA) - Defrost Extraction Buffer A at 37 centigrade. 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 centigrade before use. The concentrated buffer may be refrozen. 1× Extraction Buffer B (ionic solution, 20 mM MOPS, pH 7.5, containing 110 mM KCl and 1 mM EGTA) - Dilute an aliquot of Extraction Buffer B 5-fold with water. Keep the diluted buffer at 4 centigrade before use. The concentrated buffer may be refrozen. 1× Storage Buffer (10 mM HEPES, pH 7.4, containing 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K2HPO4, and 1 mM DTT) - Dilute an aliquot of the 5× Storage Buffer 5-fold with water. Keep the diluted buffer at 4 centigrade 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 ml of dry DMSO (D 8418). This will give a 1 mg/ml solution (1.53 mM; MW 652.2). The solution may be stored at -20 centigrade at this concentration. For the assay, dilute an aliquot of the reconsituted solution 5-fold with DMSO. 1× JC-1 Assay Buffer (20 mM MOPS, pH 7.5, containing 110 mM KCl, 10 mM ATP, 10 mM MgCl2, 10 mM sodium succinate, and 1 mM EGTA) - Dilute an aliquot of the buffer 5-fold with water. Keep the diluted buffer at 4 centigrade 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 centigrade until needed.

Separation Protocol	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) and 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), 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 5–10 times. 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 and centrifuge the sample at 600 × g for 5 minutes. 7. Carefully transfer the supernatant liquid into a new tube. Centrifuge it 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 ml 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. Preparation of mitochondria from hard tissues (skeletal or heart muscle) Note: For heart muscle, use 1× Extraction Buffer A and 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) and 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 and then spin down the tissue for a few seconds in the centrifuge.
7. Remove the supernatant by aspiration and 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 to a final concentration of 10 mg/ml to quench the proteolytic reaction, mix, and then spin down the tissue for a few seconds in the centrifuge.
10. Remove the supernatant by aspiration, wash the pellet with 8 volumes of the appropriate extraction buffer, and spin down the tissue for a few seconds in the centrifuge.

11. Remove the supernatant by aspiration and 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 ml 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 u

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ptake (see separate section). For assaying the outer membrane integrity use the Cyto chrome c Oxidase Assay Kit.

JC-1 uptake in mitochondria

The integrity of the inner mitochondrial membrane may be measured by observing the potential gradient (Dy) over this membrane. This can be achieved by measuring the u ptake of the cationic carbocyanine dye JC-1 (5,5¢,6,6¢-tetrachloro-1,1¢,3,3¢-tetraethy lbenzimidazolcarbocyanine iodide) into the matrix.2 The mitochondrial membrane pot ential, across the inner membrane, determines the redistribution of this dye. The distri bution depends on the transmembrane electric field (negative inside) and the concent ration gradient of the dye.

The fluorophore JC-1 has the property that when excited at 490 nm, the emission spe ctrum will be dependent on the concentration of the molecule. In dilute solutions of 30 0 nM it will give a green fluorescence at 527 nm, but when the concentration is greate r than 1 mM, a very strong red-orange fluorescence will occur at 590 nm. This is due t o the formation of aggregates of the dye, named J-aggregates. In a mitochondrial mat rix, bounded by an inner membrane with a large Dy, the dilute external concentration of the dye is concentrated in the matrix to a level that enables the formation of J-aggr egates. Thus, observation of the fluorescence at 590 nm is a simple and convenient i ndicator of the Dy. JC-1 is the probe of choice for this work, since it is extremely sensi tive to Dy as shown by depletion of the potential over the inner membrane in the pres ence of K + /valinomycin.

This procedure is a fixed-point assay measuring the uptake of JC-1 with formation of t he J-aggregates. It is also possible to follow the uptake with time using a kinetic progr am.2 The observed fluorescence of the solution will rise to a plateau after 5–10 minut es. Compounds that cause a blocking of the electron transport chain, followed by dee nergization of the mitochondria and significant lowering of the Dy, such as Antimycin A at 2 mM or Pyrrolnitrin at 3–12 mM, will show a drastic decrease in the JC-1 fluores cence due to equalization of the JC-1 concentrations inside and outside the mitochon drial matrix.

1. Prepare the JC-1 Stain and 1× JC-1 Assay Buffer as described under Preparation I nstructions.
2. Prepare a suitable mitochondrial suspension. Dilute this suspension with 1× Storag e Buffer to 1 mg of protein (mgP) per ml and then take samples of 5–40 mg protein fo r the JC-1 assay.
3. Prepare the samples as follows: Add 1.9 ml of 1× JC-1 Assay Buffer to a tube. The n add an appropriate sample and bring the volume to 2 ml with 1× Storage Buffer.
4. Start the reaction by addition of 2 ml of JC-1 Stain and mix by inversion.
5. Leave the tubes at room temperature in the dark (cover with aluminium foil to avoid photo-bleaching) for 7 minutes to allow complete uptake of the dye into the mitochond ria.
6. Read the fluorescence of the sample in a spectrofluorometer with settings as follow s: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 (Prep arations, step 11 or 16) per mg mitochondrial protein (FLU/mgP).

Calculation

FLU/mgP = ((DFL) × dil)/(V × C)

FLU= fluorescence units

mgP= milligram protein

DFL = FLsample - FLblank

dil = dilution factor to prepare 1 mg/ml suspension (JC-1 uptake, step 2)

C = mgP/ml

V = volume of mitochondrial sample in ml

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