Please cite this article as: Ignacio *et. al.*, (2016). Isolating Liver Mitochondria by Differential Centrifugation, *Bio-protocol* 6 (10): e1809. DOI: 10.21769/BioProtoc.1809.

bio-protocol http://www.bio-protocol.org/e1809 Vol 6, Iss 10, May 20, 2016

Isolating Liver Mitochondria by Differential Centrifugation

Ignacio Amigo[1, *], Javier Traba[2,*] and Carlos B. Rueda[3, 4, *]

[1]Departamento de Bioquímica, Instituto de Química, Universidade de, Sao Paulo, Brazil; [2]Cardiovascular and Pulmonary Branch, NHLBI, NIH, Bethesda, USA ; [3]Department of Neurology, Columbia University, New York, USA; [4]Center for Motor Neuron Biology and Disease, Columbia University, New York, USA

[*]For correspondence: iamigo@iq.usp.br; javier.trabadominguez@nih.gov; cbr2126@cumc.columbia.edu

[Abstract] In addition to methods aimed at the study of mitochondrial function in-situ, a full understanding of mitochondrial function requires their purification from cells or tissues under specific physiological or pathological conditions. This protocol illustrates a sequential procedure to obtain functional mitochondria with high yield from mice liver tissue. Mitochondria obtained with this method can be used to assess different mitochondrial parameters, including oxygen consumption, membrane potential and calcium retention capacity.

Materials and Reagents

1. Centrifuge tubes
2. Mice
3. Potassium salts
4. Sodium salts
5. Sucrose (Sigma-Aldrich, catalog number: 84100)
6. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A6003)
7. Disodium ethylenediaminetetraacetate dihydrate (EDTA) (Sigma-Aldrich, catalog number: ED2SS)
8. Ethylene glycol-bis(2-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA) (Sigma-Aldrich, catalog number: E4378)
9. Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: 646563)
10. HEPES (Thermo Fisher Scientific, Gibco™, catalog number: 15630-080)
11. Protease inhibitors (100x) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 78429)
12. D-Mannitol (Sigma-Aldrich, catalog number: M4125)
13. Magnesium chloride hexahydrate ($MgCl_2$) (Sigma-Aldrich, catalog number: M9272)
14. Potassium hydroxide (KOH) (Sigma-Aldrich, catalog number: 221473)
15. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: S5881)
16. Extraction buffer (see Recipes)

Please cite this article as: Ignacio *et. al.*, (2016). Isolating Liver Mitochondria by Differential Centrifugation, *Bio-protocol* 6 (10): e1809. DOI: 10.21769/BioProtoc.1809.

Note: Approximately 100 ml extraction buffer per liver.

Equipment

1. Dounce homogenizer and pestles (A and B)
2. Small scissors
3. Tweezers

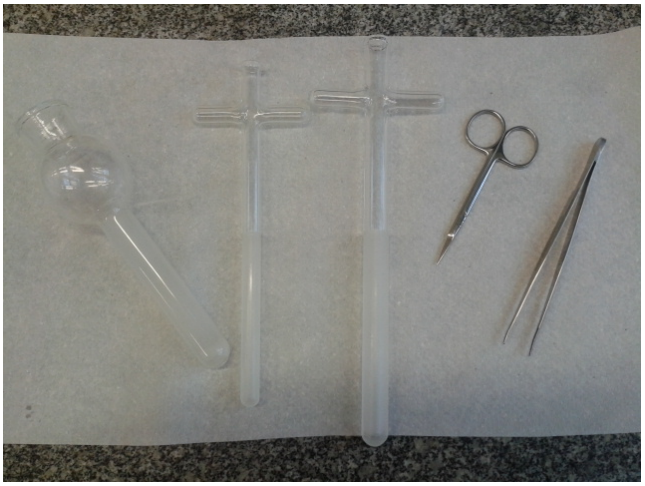

Figure 1. Tools for mincing and homogenate the tissue. Dounce homogenizer and pestles (A and B), Small scissors and tweezers.

Procedure

Notes:

a. *To obtain a high yield of functional mitochondria the procedure must be done as fast as possible (preferably in less than 1.5 h) and the samples maintained on ice at all times.*
b. *The procedure described here refers to mice but can be used in rats by adapting the volumes. Functional assays should be carried out within 3-4 h of isolation.*
c. *Buffers can be made using potassium or sodium salts depending on the use of the mitochondria.*
d. *If potassium can affect the subsequent mitochondrial experiments, KCl can be replaced by mannitol.*
e. *Animal protocols must meet local ethics standards.*

A. Preparation

1. Starve animals overnight (8-16 h during the dark cycle)
   Note: Starvation is optional but leads to more reproducible results, especially in the liver.

Please cite this article as: Ignacio *et. al.*, (2016). Isolating Liver Mitochondria by Differential Centrifugation, *Bio-protocol* 6 (10): e1809. DOI: 10.21769/BioProtoc.1809.

2. Prepare a container with ice and place:
   a. Dounce homogenizer and pestles
   b. Three centrifuge tubes per sample (20-50 ml)
   c. One beaker containing extraction buffer per sample
3. Cool centrifuge and rotor to 4 °C. Cool on ice Dounce and pestles (Figure 1).
4. Animal dissection material (Figure 1)
   a. Small scissors
   b. Tweezers

B. Procedure

1. Sacrifice the mouse by cervical dislocation, immediately remove the liver and place it in the ice-cold beaker with extraction buffer.
2. Rinse the liver by adding and removing cold fresh extraction buffer until most of the blood is removed (5-6 washes).
3. Mince the liver in the beaker in ice extensively using small scissors until homogeneus (Figure 3)
4. Transfer the minced liver into a Dounce homogenizer and add approximately 3 ml of cold extraction buffer.
5. With the homogenizer placed in the ice container, gently grind the tissue ten times with the A pestle (looser) and another ten with the B pestle (tighter). Avoiding the formation of bubbles is critical to obtain high quality mitochondria (Figure 4).
6. Transfer the homogenate into a centrifuge tube (Figure 5) and complete to 30-40 ml with fresh cold extraction buffer. Follow the differential centrifugation steps (Figure 2)
7. Centrifuge 10 min at 700 *x g* and 4 °C. Pour supernatant to a new ice-cold tube and discard the pellet containing nuclei and intact cells (Figure 6).
8. Repeat the operation centrifuging again at 700 *x g* for 10 min at 4 °C and subsequently pouring the supernatant to a new ice-cold tube.
9. Centrifuge at 10,000 *x g* for 15 min at 4 °C. Discard the supernatant and re-suspend the pellet (Figure 7) in ice-cold extraction buffer.
10. Centrifuge at 10,000 *x g* for 15 min at 4 °C, discard the supernatant and re-suspend the final pellet in the minimal possible volume (around 0.3 ml) of extraction buffer or the specific experimental buffer (Figure 8).

    Notes:

    a. After isolation, protein concentration is determined by standard methods. Typically, around 30-40 mg of mitochondrial protein are obtained from one liver.

    b. The quality of the isolated mitochondria can be determined their respiratory control ratio (RCR) using an oxygen electrode and measuring their oxygen consumption rate in the presence and in the absence of ADP. RCR should range 4-6 with pyruvate plus malate and 1.5-3 with succinate plus rotenone.

Please cite this article as: Ignacio *et. al.*, (2016). Isolating Liver Mitochondria by Differential Centrifugation, *Bio-protocol* 6 (10): e1809. DOI: 10.21769/BioProtoc.1809.

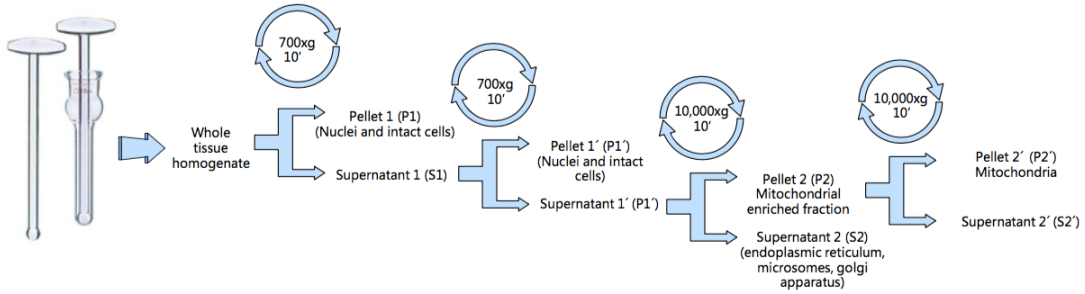

Figure 2. Mitochondrial isolation by differential centrifugation. The whole protocol must be carried at 4 °C. Avoid excessive pipetting, transfer supernatants by inversion.

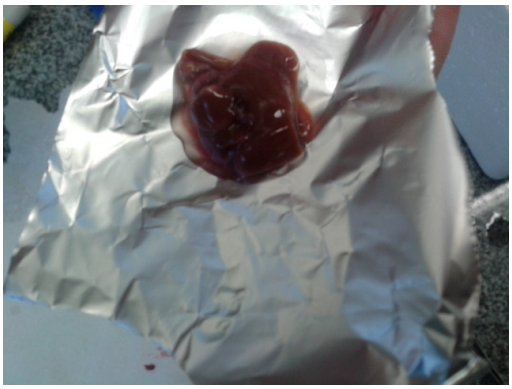

Figure 3. Extracted liver

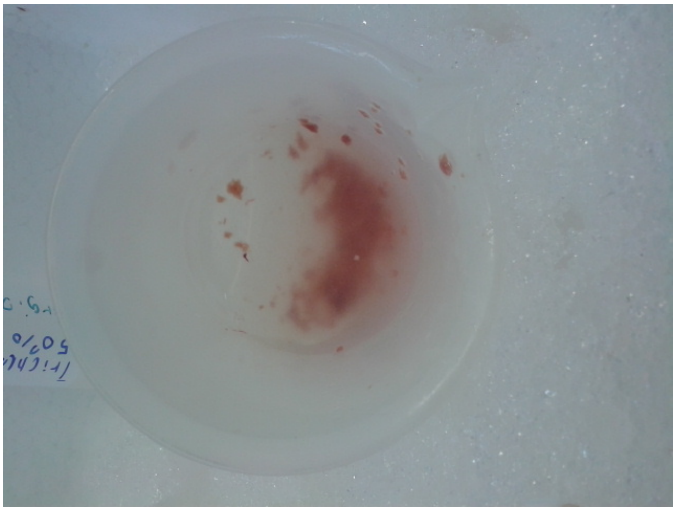

Figure 4. Minced liver

Please cite this article as: Ignacio *et. al.*, (2016). Isolating Liver Mitochondria by Differential Centrifugation, *Bio-protocol* 6 (10): e1809. DOI: 10.21769/BioProtoc.1809.

Figure 5. Homogenized liver

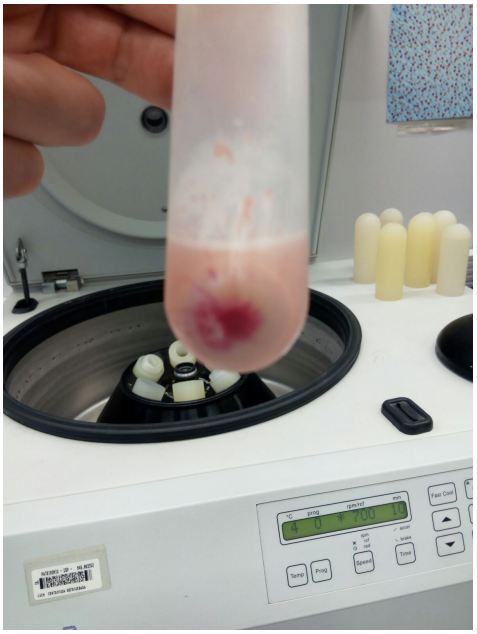

Figure 6. Pellet 1 (P1, nuclei and intact cells)

Please cite this article as: Ignacio *et. al.*, (2016). Isolating Liver Mitochondria by Differential Centrifugation, *Bio-protocol* 6 (10): e1809. DOI: 10.21769/BioProtoc.1809.

Figure 7. Pellet 2 (P2, mitochondrial enriched fraction)

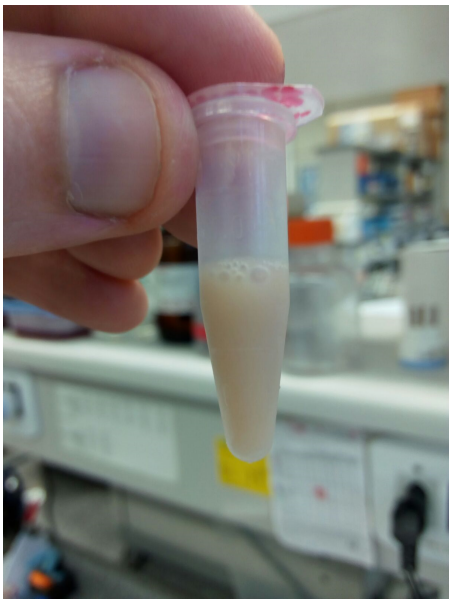

Figure 8. Resuspended mitochondria

Recipes

1. Extraction buffer (freshly prepared)
   250 mM sucrose
   250 mM mannitol
   25 mM HEPES

Please cite this article as: Ignacio *et. al.*, (2016). Isolating Liver Mitochondria by Differential Centrifugation, *Bio-protocol* 6 (10): e1809. DOI: 10.21769/BioProtoc.1809.

10 mM KCl
0.25 mM EDTA
10 mM EGTA
1.5 mM $MgCl_2$
1 mM DTT
0.1% BSA
1x protease inhibitors
pH 7.4 with KOH or NaOH
Note: The type of salt used can interfere with some functional assays. KOH is recommended for calcium handling experiments, as it prevents the efflux of calcium from the mitochondria through the Na^+/Ca^{2+} exchanger. For membrane potential experiments using safranin O, NaOH is recommended to allow calibration with KOH and valinomycin.

Acknowledgments

We thank Dr. Jorgina Satrustegui, in whose laboratory our previous work was carried out, and Dr. Araceli del Arco, for constant help, guidance, support and critical comments.

References

1. Amigo, I., Traba, J., Gonzalez-Barroso, M. M., Rueda, C. B., Fernandez, M., Rial, E., Sanchez, A., Satrustegui, J. and Del Arco, A. (2013). Glucagon regulation of oxidative phosphorylation requires an increase in matrix adenine nucleotide content through Ca2+ activation of the mitochondrial ATP-Mg/Pi carrier SCaMC-3. *J Biol Chem* 288(11): 7791-7802.
2. Frezza, C., Cipolat, S. and Scorrano, L. (2007). Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. *Nat Protoc* 2(2): 287-295.

