

Preparation of mitochondria from mouse tissue with enzymatic treatment

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1. Description

1.1 Background information

Mitochondria dysfunction has been shown to be involved in neurodegenerative diseases such as Alzheimer or Parkinson, but also play a pivotal role in cancer, heart, kidney or liver diseases. To study mitochondria function from mitochondria isolated from mouse tissue a standardized protocol has been established using the gentleMACS™ Dissociator and the Mitochondria Isolation Kit, mouse tissue (# 130-096-946). The protocol takes advantage of the time-saving and reliable features of the gentleMACS Dissociator for the disruption of mouse tissue, e.g., muscle, heart, liver, brain, or kidney, in combination with the efficiency of the mouse Mitochondria Isolation Kit for the isolation of pure and high yield mitochondria. Taken together this will allow for efficient functional mitochondria analysis in downstream applications.

This protocol is designed for the use of gentleMACS Program m_mito_tissue_01.

1.2 Reagent and instrument requirements

Preparation and homogenization of tissue

- Mouse tissue
- Dulbecco's phosphate-buffered saline (DPBS) without Ca²⁺ and Mg²⁺ (10×) (Invitrogen™, # 14200-067)
- Disodium ethylenediaminetetraacetic acid (EDTA; Fluka, # 03609)
- 2.5% Trypsin (10×) (Invitrogen, # 15090-046)
- Protease inhibitors (complete, Mini, EDTA-free,) (Roche™, # 04 693 159 001)

- Bovine serum albumin (BSA), fatty acid free (Bovogen, Bovostar, # BSAS)
- Cell culture dishes (ø 10 cm)
- Scissors and forceps
- gentleMACS Dissociator (# 130-093-235) or gentleMACS Octo Dissociator (# 130-095-937)
- gentleMACS C Tubes (# 130-093-237)
- Pre-Separation Filters, 70 µm (# 130-095-823)

Mitochondria isolation

- Mitochondria Isolation Kit, mouse tissue (# 130-096-946), containing: Anti-TOM22 MicroBeads, mouse, Lysis Buffer, 10× Separation Buffer, Storage Buffer, LS Columns, and Pre-Separation Filters, 30 µm
- Cooled table-top centrifuge
- MACSmix™ Tube Rotator (# 130-090-753)
- MACS® MultiStand (# 130-042-303)
- MidiMACS™ Separation Unit (# 130-042-302) or QuadroMACS™ Separation Unit (# 130-090-976)
- 15 mL propylene round bottom tubes
- 1.5 mL microfuge tubes

2. Protocol for the preparation of mitochondria from mouse tissue with enzymatic treatment

2.1 Preparation of reagents

2.1.1 Pre-treatment of buffers included in the Mitochondria Isolation Kit

▲ All buffers should be pre-cooled to 4 °C before use.

Lysis Buffer/protease inhibitors

To avoid activity of damaging proteases it is advisable to add protease inhibitors to PBS/10mM EDTA during the lysis step. Dissolve 1 tablet of Protease Inhibitor Cocktail in 10 mL of Lysis Buffer. Prepare 1 mL aliquots and store at -20 °C for up to 1 month.

Separation Buffer

Bring 10× Separation Buffer to room temperature and aliquot directly after arrival. Prepare 2.3 mL aliquots (required volume per reaction is 2.25 mL) and store at -20 °C.

Dilute 10× Separation Buffer with double-distilled water to achieve 1× Separation Buffer. Prepare 1× Separation Buffer on the same day of experiment and store at 4 °C.

2.1.2 Preparation of additional buffers

0.5 M EDTA, pH 7.5

Place 700 mL of double-distilled water in a volumetric flask. Add 56 g of sodium hydroxide pellets under agitation. After sodium hydroxide pellets have dissolved, add 146.1 g of EDTA stepwise to the solution. Adjust pH to 7.5 using sodium hydroxide pellets. Adjust volume to 1 liter with double-distilled water. Sterilize by autoclaving. Store at room temperature.

▲ **Note:** Note that pH depends on temperature. Measure the pH of the solution at 25 °C.

PBS/10 mM EDTA

Mix 50 mL of 10× PBS and 10 mL of 0.5 M EDTA pH 7.5. Adjust volume to 0.5 liter with distilled water. Store at 4 °C.

PBS/10 mM EDTA/0.05% Trypsin

Mix 1mL of 10× PBS with 0.2mL of 0.5 M EDTA pH7.5 and 0.2mL of 2.5% Trypsin. Adjust volume to 10 mL with distilled water. Prepare buffer at the same day of the experiment and store at 4 °C.

PBS/10 mM EDTA/protease inhibitors

▲ To avoid activity of damaging proteases it is advisable to add protease inhibitors to PBS/10 mM EDTA during the homogenization step.

Dissolve 1 tablet of Protease Inhibitor Cocktail in 10 mL of PBS/10 mM EDTA pH 7.5. Prepare the solution immediately before starting the experiment. Store solution at 4 °C.

PBS/2 mM EDTA/0.05% BSA (PEB)

Dissolve 0.5 g fatty acid free BSA in 10 mL of 10× PBS. Add 0.4 mL of 0.5 M EDTA pH 7.5 to the solution. Adjust volume to 100 mL with distilled water. Prepare buffer at the same day of the experiment and store at 4 °C.

▲ **Note:** Use fatty acid free BSA. Fatty acids can cause uncoupling of mitochondria.

2.2 Protocol overview

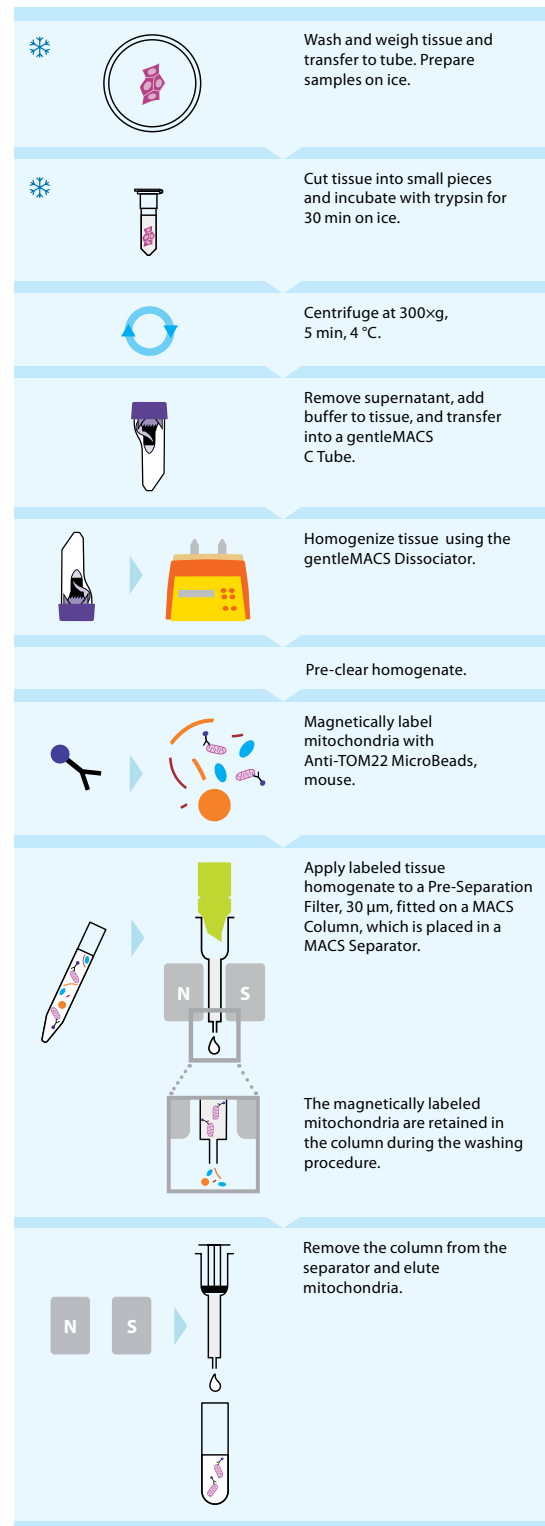


Figure 1: Isolation of mitochondria from mouse tissue.

2.3 Tissue processing

2.3.1 Preparation of tissue

1. Wash tissue with ice-cold PBS/10 mM EDTA twice.
▲ Note: It is known that Mg²⁺ can influence mitochondrial functions. Because Mg²⁺ is extremely abundant in muscle tissue, it is important to bind these ions with the use of the chelator EDTA.
2. Fill ice-cold PBS/10 mM EDTA into a cell culture dish and place the dish on ice. Transfer tissue into the cell culture dish. The tissue should be covered with buffer.
3. Resect fat, ligament, and connective tissue using a forceps and pair of scissors. Cut the tissue into pieces of approximately 5 mm³.
4. Weighing of tissue: Fill a cell culture dish with ice-cold PBS/10 mM EDTA and use this dish as tare weight. Take a piece of tissue and quickly remove excessive buffer by a paper towel. Put the tissue in the prepared culture dish. Measure the weight of the tissue.

2.3.2 Homogenization of tissue

▲ For details on the use of the gentleMACS Dissociator, refer to the gentleMACS Dissociator user manual.

▲ Use a total volume of 500 µL PBS/10 mM EDTA/0.05% Trypsin buffer/50 mg tissue. For higher tissue amounts, scale up buffer volume accordingly.

▲ Use a total volume of 2 mL of PBS/10 mM EDTA/protease inhibitor buffer for 50–100 mg of muscle and heart tissue or for 50–200 mg of other tissue. For higher tissue amounts, scale-up buffer to 10 mL/g tissue.

▲ A maximum of 1 g of mouse tissue can be dissociated per gentleMACS C Tube.

5. Transfer the pre-weight tissue to a 1.5 mL microcentrifuge tube filled with ice-cold PBS/10 mM EDTA/0.05% Trypsin.
6. Quickly mince tissue into small pieces of approximately 1–2mm³ using a pair of scissors. Incubate for 30 minutes on ice.
7. Centrifuge at 300×g for 5 minutes at 4 °C and remove the supernatant.
8. Thoroughly add 1 mL ice-cold PBS/10 mM EDTA/protease inhibitor to the loose pellet and pour suspension into a pre-cooled gentleMACS C Tube. Rinse the microcentrifuge tube with an additional 0.5 mL of the same buffer and pour the suspension into the same gentleMACS C tube.
▲ Note: Instead of pouring the suspension you can also use a glass Pasteur pipette to transfer the small tissue pieces. Use of a plastic pipette tip is not recommended as the tissue easily sticks to the walls of the tip.
9. Tightly close C Tube and attach it upside down onto the sleeve of the gentleMACS Dissociator.
▲ Note: It has to be ensured that the sample material is located in the area of the rotator/stator.
10. Run the gentleMACS Program **m_mito_tissue_01**.
11. Once the program is finished, perform a short centrifugation step at 200×g for 30 seconds at 4 °C to collect the sample material at the tube bottom.

2.3.3 Pre-clearing of tissue homogenate

14. Put a 15 mL conical tube on ice and place a Pre-Separation Filter, 70 µm on it. Pipette the homogenate into the reservoir of the filter and allow the homogenate to run through.
15. Wash filter with 2× 1 mL ice-cold PEB buffer. Centrifuge the filtrated homogenate at 500–1,000×g for 5 minutes at 4 °C. Thoroughly collect the supernatant and store it on ice.
▲ Note: Do not centrifuge with higher g force as this will decrease mitochondria yield.

2.4 Mitochondria isolation

16. Transfer pre-cleared homogenate corresponding to 50–100 mg of tissue to a 15 mL conical tube.
17. Add ice-cold 1× Separation Buffer to a total volume of 10 mL. Mix well.
18. Add 50 µL Anti-TOM22 MicroBeads, mouse to magnetically label the mitochondria.
19. Mix well and incubate for 1 hour in the refrigerator (2–8 °C) with gentle shaking using the MACSmix Tube Rotator.
20. Place one LS Column per separation in the magnetic field of a suitable MACS Separator.
21. Place a Pre-Separation Filter, 30 µm on the LS Column.
22. Prepare column and filter by rinsing with 3 mL of 1× Separation Buffer.
23. After labeling incubation has finished apply the homogenate onto the column stepwise (3×3.3 mL) and let the homogenate run through.
24. Wash column with 3×3 mL of 1× Separation Buffer. Perform washing steps by adding buffer aliquots only when the column reservoir is empty.
25. Remove column from the separator and place it on a 15 mL round bottom tube.
26. Pipette 1.5 mL of 1× Separation Buffer onto the column. Immediately flush out the magnetically labeled mitochondria by firmly pushing the plunger into the column.
27. Proceed with downstream analysis.

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