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Tissue Mito-IP: Immunopurification of Mitochondria from MitoTag Mice

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We use this protocol and it's working

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Abstract

Cell organelles represent a minor fraction of the total cellular content, making whole-cell profiling inadequate for monitoring changes in the mitochondrial proteome, metabolome, and lipidome. Traditional techniques for purifying mitochondria have inherent limitations, often compromising organelle purity, isolation time, or viability. Additionally, the components of conventional organellar isolation buffers, such as sucrose, can interfere with mass spectrometry (MS) profiling. To overcome these challenges, a novel method called 'Mito-IP,' was developed which facilitates the rapid immunopurification of pure and intact mitochondria. This method enables mitochondrial isolation within 10 minutes and supports various downstream applications, including immunoblotting, proteomic, metabolomic, and other -omic analyses. The method employs a chimeric protein comprising of three HA epitope tags fused to the outer mitochondrial membrane protein OMP25 (rat, aa109-145). An LC/MS-compatible buffer (termed 'KPBS') was also developed containing only KCl and KH_2PO_4, significantly improving performance and mitochondrial viability. Following the success of the Mito-IP method, the same epitope-tagged concept is being extended to the isolation of other organelles, including lysosomes (Lyso-IP), Golgi (Golgi-IP), and peroxisomes (Peroxo-IP). The following optimised protocol details the immunoprecipitation of mitochondria from tissues of MitoTag mice. The same steps apply to the immunopurification of other organelles when the HA-epitope tag is present on the organelle of interest.

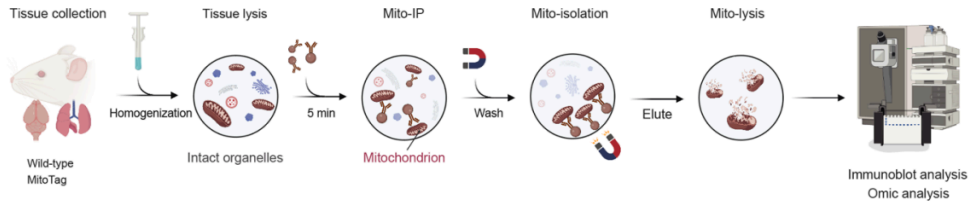

Fig. 1. Schematic of the Mito-IP method.

Tissues are collected and homogenized using a dounce homogenizer in KPBS buffer. After the release of intact organelles from the cells, the homogenate is incubated with anti-HA magnetic beads for 5 minutes, allowing the beads to bind to the mitochondria. Using a magnetic separator, the mitochondria-bound beads are drawn to the side of the tube. Following three washes, an appropriate lysis buffer is added for downstream analysis.

Materials

- DPBS no calcium no magnesium **Gibco - Thermo Fisher Scientific Catalog #**14190169
- KPBS Buffer:

	A	B
	KCl	136mM
	KH2PO4	10mM

Adjust to pH 7.25 with KOH and filter (0.22µm conring). On the day of use, add cOmplete™ EDTA-free Protease Inhibitor Cocktail **Roche Catalog #**11873580001 and Roche PhosSTOP tablet (#04906837001).

- Lysis Buffer
- MS grade water (Fisher, 11947199)
- Pierce™ BCA Protein Assay Kit (#23227, lot# VA294738).

Pierce BCA protein assay **Thermo Scientific Catalog #**23227

- NuPAGE 4xLDS sample buffer (Invitrogen, #1941674).
- 1% β-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M6250

Equipment:

Equipment

VWR GLASS TISSUE GRINDER 2ML	NAME
Homogenizer	TYPE
neobits	BRAND
89026-386-EACH	SKU
https://www.neobits.com/vwr_89026_386_each_vwr_glass_tissue_grinder_2ml_p3461292.html?srsltid=AfmBOopMUFg2KjilCr83nFVhOC03xxabSjciG3yYHqPT5OE6wEAVZpBQ	LINK

Equipment

VWR PL TISSUE GRINDER 2ML (Each)	NAME
plain plunger	TYPE
neobits	BRAND
89026-398-EACH	SKU
https://www.neobits.com/vwr_89026_398_each_vwr_pl_tissue_grinder_2ml_each__p3461298.html	LINK

•

Equipment

The Belly Dancer Shaker (Orbiter)	NAME
The Belly Dancer®	BRAND
BDRAA115S	SKU
https://www.ibisci.com/products/belly-dancer-shaker	LINK

• DynaMag™ Magnet. (Thermofisher scientific, #12320D or #12321D).

Equipment

DynaMag™- Spin Magnet
NAME

Invitrogen™
BRAND

12320D
SKU

https://www.thermofisher.com/order/catalog/product/12320D
LINK

Equipment

DynaMag-2
NAME

Magnet
TYPE

Invitrogen
BRAND

12321D
SKU

https://www.thermofisher.com/order/catalog/product/12321D#/12321D
LINK

•

Equipment

Microcentrifuges, Micro Star 17R (VWR #521-1647)	NAME
Microcentrifuges	TYPE
Micro Star 17R	BRAND
521-1647	SKU
https://in.vwr.com/store/product/8306728/microcentrifuges-ventilated-refrigerated-micro-star-17-17r	LINK

- Bioruptor NGS Sonication System (UCD-600).
- Dissecting tools.

Consumables:

- Pierce™ Anti-HA Magnetic Beads **Thermo Fisher Catalog #**88837
- SafeSeal reaction tube 1.5 ml PP PCR Performance Tested Low protein-binding **Sarstedt Catalog #**72.706.600
- 15 ml centrifuge tubes **greiner bio-one Catalog #**188271
- 50 ml centrifuge tubes **greiner bio-one Catalog #**227261
- TipOne bevelled 1000µl, 200µl and 20µl pipette tips (Starlab).

(A) Pre-clearing of anti-HA magnetic beads

5m

1 Resuspend beads by shaking the bottle until there is a homogeneous suspension.

2 Pipette 200 µL of anti-HA bead slurry into a 1.5ml tube (100 µl/sample).

> Note
>
> The volume of beads used per sample can be adjusted.

3 Place the tube onto a magnet for 00:00:30 and remove the overlying solution.

30s

4 Remove the tube from the magnet, add 1 mL of cold KPBS and gently resuspend 3 times to disperse any clumps.

5 Repeat the wash step (3+4) 2 more times.

6 After the last wash, resuspend the beads in 200 µL KPBS, split them into two tubes of 100µl (one for the Mito-IP and one for the Control-IP) and keep them On ice.

(B) Tissue collection and homogenisation

10m

7 Euthanize the mouse by cervical dislocation.

8 Dissect and collect the tissue of interest in a tube containing cold PBS.

> Note
>
> Make sure to remove any fat tissue connected to it if applicable.

9

Note

❄❄❄ **Continue in the cold room until the detergent lysis step** ❄❄❄

Place the tissue in the prechilled homogeniser and add 1 mL of KPBS.

10 Homogenise the tissue with 25 strokes without rotation.

Note

Wash the douncer 10 times with milliQ water before using it again and always start with the Control-IP to avoid any contamination.

11 Move 25 µL into a new tube (**whole-cell input**) and keep it On ice.

12 Spin down at 2000 x g, 4°C, 00:02:00. This step is to remove any non-lysed cells, nuclei and debris such as cell membranes and leave the intact organelles in the supernatant. 2m

Note

Critical step: Optimise the homogenisation process by following the steps of section D [**Optimization of tissue homogenisation**].

13 Move 25 µL to a new tube (**IP input**) and keep it On ice.

(C) Immunoprecipitation 9m

14 Move the homogenate into the tube containing the HA-beads. Ensure that the bead clumps are dispersed by gently pipetting up and down 2-3 times.

Note

Critical step:

Be very careful not to take any of the pellet as it will affect the purity of the IP. For some tissue homogenates, the separation between the pellet and the supernatant is not very clear. In this case, is preferred to spin down the homogenate at 2000xg instead and take less supernatant. See section D [**Optimization of tissue homogenisation**].

15 Incubate the homogenate with the beads for 00:05:00 on an orbiter. 5m

Note

Incubation time can be adjusted.

16 Separate the beads by putting the tube on the magnet for 00:00:30 .

Note

Residual material left on the cap can be brought down with a pulse spin (00:00:01) before placing the tubes on the magnet.

17 Collect 25 µL of flow-through into a new tube (**FT input**) and keep it On ice .

18 Wash beads with 1000 µL of cold KPBS 3 times.

Note

Move the beads to a new tube after every wash to minimise contamination from cell extracts sticking to the tube.

19 The isolated mitochondria on the beads can be either stored at -80 °C for later use or eluted off the beads using an appropriate lysis buffer for:

- Immunoblot analysis.
- Proteomic analysis.
- Lipidomic analysis.
- Metabolomic analysis.
- Other.

(D) Elusion and lysis

40m 30s

20 Incubate the beads with 50 µL lysis buffer for 00:10:00 . 10m

21 Place the tube on the magnet for 00:00:30 and collect the supernatant into a new tube. Repeat this step one more time to ensure total removal of the beads. 30s

22 For the input samples, add 100 µL of lysis buffer, resuspend and incubate for 00:20:00 . 20m

23 Leave samples On ice and continue with the next round of IP.

24 Spin down input samples at 13000 x g, 4°C, 00:10:00 and move supernatant into a new tube. 10m

25 For samples intended for immunoblot or proteomic analysis: Sonicate the IP and input samples for maximal protein extraction (15 cycles – 30sec on, 30sec off). Determine the protein concentration of the IP and inputs using a micro BCA Protein Assay Kit. Use 2µl of undiluted IP samples and 2µl of diluted (1:10) input samples. Quantification should be done in triplicate or duplicate.

25.1 Samples can be stored at -80 °C for future application.

25.2 For immunoblot analysis: samples are diluted into 4xLDS loading buffer supplemented with fresh 5% (by vol) 2-mercaptoethanol prior to analysis on SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Loading 1 µg - 2 µg of sample is enough to detect the mitochondrial markers (such as HSP60, CS, VDAC, CISD1, OPA1, MFN1/2).

26 **For samples intended for proteomic analysis, use the protocol**

'Sample preparation for proteomic analysis of isolated mitochondria and whole-cell extracts'
dx.doi.org/10.17504/protocols.io.3byl4wrj8vo5/v1

(E) Optimization of tissue homogenisation

8m

27 Determining the minimum amount of homogenization necessary is critical. Poor homogenization leads to inadequate amounts of mitochondria being available for immunocapture. However, excessive homogenization can damage the organelles released from cells. This section provides troubleshooting guidance for various mouse tissues to ensure the successful isolation of pure mitochondria.

28 **Brain**

28.1 Place the tissue at the bottom of the homogeniser and add 1 mL KPBS.

28.2 Homogenise the tissue using 40 strokes while avoiding introducing any bubbles.

28.3 Pour the homogenate into a 1.5ml tube slowly, as it will be viscous.

28.4 Spin down at 2000 x g, 4°C, 00:02:00 .

2m

28.5 Move 750 µL of supernatant into the tube with the beads. There will be a big pellet.

28.6 Continue with the steps of section C [**Immunoprecipitation**].

> Note
>
> Homogenising just one hemisphere or both separately, improves the homogenisation efficiency and Mito-IP purity.

29 **Lung**

29.1 Place the tissue at the bottom of the homogeniser and add 200 µL KPBS.

29.2 Homogenise the tissue with an initial 5-7 strokes until there are no big tissue pieces.

29.3 Add the remaining 800 µL KPBS and perform another 25 strokes. With each stroke, move the probe all the way out of the buffer and then all the way back to the bottom of the homogeniser.

29.4 Pour the homogenate into a 1.5ml tube.

29.5 Spin down at 2000 x g, 4°C, 00:02:00 . 2m

29.6 Move 900 µL of supernatant into the tube with the beads.

29.7 Continue with the steps of section C [**Immunoprecipitation**].

30 **Heart**

30.1 Place the tissue at the bottom of the homogeniser and add 1 mL KPBS.

30.2 Homogenise the tissue using 25 strokes. Make sure you push the probe all the way to the bottom of the homogeniser with the first stroke forcing the tissue to the sides of the probe.

30.3 Pour the homogenate into a 1.5ml tube slowly.

30.4 Spin down at 2000 x g, 4°C, 00:02:00 .

30.5 Move 900 µL of supernatant into the tube with the beads.

30.6 Continue with the steps of section C [**Immunoprecipitation**].

31 **Kidney**

31.1 Place the tissue at the bottom of the homogeniser and add 1 mL KPBS.

31.2 Homogenise the tissue using 40 strokes while avoiding introducing any bubbles.

31.3 Pour the homogenate into a 1.5ml tube slowly.

31.4 Spin down at 2000 x g, 4°C, 00:02:00 .

31.5 Move 900 µL of supernatant into the tube with the beads.

31.6 Continue with the steps of section C [**Immunoprecipitation**].

> Note
>
> Homogenising only one kidney or both separately, improves the homogenisation efficiency and Mito-IP purity.

32 **Spleen**

32.1 Place the tissue at the bottom of the homogeniser and add 1 mL KPBS.

32.2 Homogenise the tissue using 25 strokes. Make sure you push the probe all the way to the bottom of the homogeniser with the first stroke forcing the tissue to the sides of the probe.

32.3 Pour the homogenate into a 1.5ml tube slowly.

32.4 Spin down at 2000 x g, 4°C, 00:02:00 .

32.5 Move 900 µL of supernatant into the tube with the beads.

32.6 Continue with the steps of section C [**Immunoprecipitation**].

33 **Liver**

33.1 Place the tissue at the bottom of the homogeniser and add 1 mL KPBS.

33.2 Homogenise the tissue using 25 strokes. Make sure you push the probe all the way to the bottom of the homogeniser with the first stroke forcing the tissue to the sides of the probe.

33.3 Pour the homogenate into a 1.5ml tube slowly.

33.4 Spin down at 2000 x g, 4°C, 00:02:00 . 2m

33.5 Move 900 µL of supernatant into the tube with the beads.

33.6 Continue with the steps of section C [**Immunoprecipitation**].

Note

Homogenising only one liver lobe or more separately, improves the Mito-IP purity.

34 **Skeletal muscle (gastrocnemius and soleus)**

34.1 Place the tissue at the bottom of the homogeniser and add 200 µL KPBS.

34.2 Homogenise the tissue with an initial 5-7 strokes until there are no big tissue pieces.

34.3 Add the remaining 800 µL KPBS and perform another 40 strokes. With each stroke, move the probe all the way out of the buffer and then all the way back to the bottom of the homogeniser.

34.4 Pour the homogenate into a 1.5ml tube.

34.5 Spin down at 2000 x g, 4°C, 00:02:00 . 2m

34.6 Move 900 µL of supernatant into the tube with the beads.

34.7 Continue with the steps of section C [**Immunoprecipitation**].

> Note
>
> Homogenising the skeletal muscles of both the hindlimbs together can improve the Mito-IP efficiency. Skeletal muscles are more difficult to homogenise and will require to apply more force, especially during the initial strokes.

35 **Intestine (small or large)**

35.1 Dissect a 5-6 cm segment and remove its contents using an angled probe. Intestines are surrounded by membranes. If not removed, they will not be able to be pelleted down and will affect the Mito-IP purity.

35.2 Place the tissue at the bottom of the homogeniser and add 1 mL KPBS.

35.3 Homogenise the tissue using 40 strokes. Make sure you push the probe all the way to the bottom of the homogeniser with the first stroke forcing the tissue to the sides of the probe.

35.4 Pour the homogenate into a 1.5ml tube slowly.

35.5 Spin down at 5000 x g, 4°C, 00:02:00 .

35.6 Move 800 µL of supernatant into the tube with the beads.

35.7 Continue with the steps of section C [**Immunoprecipitation**].