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Mito-IP: Immunopurification of Mitochondria from MitoTag Cultured Cells

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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. 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 cultured cells. The same steps apply to the immunopurification of other organelles when the HA-epitope tag is present on the organelle of interest.

Guidelines

INTRODUCTION:

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 **(Figure 1)**. 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 (LysoIP), Golgi (GolgiIP), and peroxisomes (PeroxoIP). The following optimised protocol details the immunoprecipitation of mitochondria from Mito-Tag cultured cells. The same steps apply to the immunopurification of other organelles when the HA-epitope tag is present on the organelle of interest.

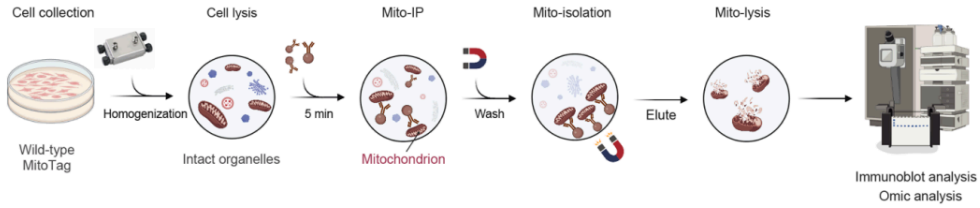

Fig. 1. Schematic of the Mito-IP method. Cells are collected and homogenized using a cell homogenizer in KPBS buffer. After the release of intact organelles from the cells, the homogenate is incubated with anti-HA magnetic beads for 5 minutes, allowing the beads to bind to the mitochondria. Using a magnetic 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

MATERIALS & REAGENTS

- **Cell lines:**

1. HEK Invitrogen™ 293FT Cell Line **Invitrogen - Thermo Fisher Catalog #**R70007
2. The cell line of interest for introducing the Mito-Tag.

- **Plasmids:**

1. Control-Tag construct: pLJC5-KOZAK-3HA-Empty (DU70022 available at MRCPPU depository at MRCPPUreagents@dundee.ac.uk)
2. Mito-Tag construct: pMXs-3XHA-EGFP-OMP25 **addgene Catalog #**83356 or available at MRCPPU depository at MRCPPUreagents@dundee.ac.uk)
3. pVSVG - lentivirus envelope plasmid: Lenti-X HTX packaging system (Clonetech, #631247)
4. pGag/Pol - Lentivirus Gag/Pol plasmid: Lenti-X HTX Packaging system (Clonetech, #631247)
5. pVSVG - retrovirus envelope plasmid: Addgene plasmid #8454)
6. pGag/Pol - retrovirus plasmid: Addgene plasmid #8449)

- **Growth Media:**

1. DMEM (Gibco™ #11960-085) **Gibco - Thermo Fisher Scientific Catalog #**11960085
2. 10% Fetal Bovine Serum **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F7524
3. 1% Penicillin-Streptomycin **Gibco - Thermo Fisher Scientific Catalog #**15140122
4. 1% L-Glutamine **Gibco - Thermo Fisher Scientific Catalog #**25030024

- Selection Media: Growth Media with 2µg/ml Puromycin dihydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9620
- Transfection media: Opti-MEM **Gibco - Thermo Fisher Scientific Catalog #**31985062 .
- PEI MAX® - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40000) **Polysciences, Inc. Catalog #**24765-1
- Polybrene Infection/Transfection reagent **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**TR-1003-G
- DPBS no calcium no magnesium **Gibco - Thermo Fisher Scientific Catalog #**14190169
- KPBS Buffer: 136mM KCl, 10mM KH_2PO_4. 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 **Thermo Scientific** **Catalog #**23227 , lot# VA294738)
- NuPAGE 4xLDS sample buffer (Invitrogen, #1941674)
- 2-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M6250

EQUIPMENT

- Cell homogeniser (Isobiotec).
- Orbiter (The Belly Dancer Shaker; IBI Scientific, #BDRAA115S)

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

Magnet	NAME
DynaMag™-2 Magnet	TYPE
Thermo Fisher	BRAND
12321D	SKU
https://www.thermofisher.com/order/catalog/product/12321D	LINK

- Microcentrifuge (VWR Micro Star 17R. S/N 42209232, # 521-1647).

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).
- Incubator with FPI-sensor system and display controller MB1 (BINDER GmbH. Model: CB150. Power Output: 1.40kW, 230V, 6.1 Amp).

CONSUMABLES

- Pierce™ Anti-HA Magnetic Beads **Thermo Fisher Catalog #**88837
- 10cm and 15cm tissue culture Petri Dishes (ThermoFisher, #172931 and 16838).
- Syringe filter (0.45µm. Sartorius, #ST16537-Q)
- Syringes (10ml) (Medicina, #19111004).
- Fisherbrand™ Cell Lifters **Thermo Fisher Scientific Catalog #**08-100-240
- Eppendorf Tubes® 3810X **Eppendorf Catalog #**0030125150
- 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).

Generation of MitoTag or ControlTag Retrovirus (Timing: 3d)

2d 0h 35m

1

Note

! Caution: Work under sterile conditions in a category 2 biological safety hood.

Plate 2 10cm Petri dishes of HEK293FT cells to 60% confluency in Growth media (one dish to generate the MitoTag retrovirus and the other one the ControlTag lentivirus).

Note

- Different batches of HEK293T cells vary in their ability to produce high-tier viruses and can be sensitive to overcrowding, media changing and trypsin digestion.
- An alternative ControlTag for the organelleIP can be the: 3xMyc-EGFP-OMP25.
- The presence of GFP in the MitoTag can help with immunofluorescence (IF) and cell sorting experiments. If not needed, the MitoTag^{Lite} construct (3xHA-OMP25) can be used instead.

1.1 MitoTag^{Lite} construct: pBabeD-3XHA-OMP25 (DU71376, available at MRCPPU depository at MRCPPUreagents@dundee.ac.uk).

2 Prepare a transfection mix for each plate in a 1.5ml Eppendorf tube.

	A	B
	Component	**per 10cm dish**
	pGag/Pol plasmid	3.8 µg
	pVSVG plasmid	2.2 µg
	Mito-Tag or ControlTag plasmid	6 µg
	Opti-MEM	300 µl

Note

Plasmids were purified using a NucleoBond Xtra Midi kit (ref 740410.50) following the manufacturer's protocols and instructions.

3 Prepare PEI mixture in a 1.5ml Eppendorf tube.

	A	B
	Component	**per 10cm dish**
	PEI (1mg/ml, dissolved in dH2O)	40 µl
	Opti-MEM	600 µl

4 Incubate each mixture from steps 2 and 3 separately for 00:05:00 at Room temperature . 5m

5 Add 300 µL PEI Mixture (step 3) to the MitoTag and ControlTag tube (step 2).

6 Mix and incubate each mixture at Room temperature for 00:30:00 . During this time, carefully replace the medium of each plate of HEK293T cells with 10 mL of fresh Growth Media. 30m

7 Add each mixture dropwise into a HEK293FT plate and gently swirl to mix.

8 Incubate cells at 37 °C for 24:00:00 . 1d

9 Replace the medium of each dish with 10 mL fresh Growth Media and incubate cells at 37 °C for a further 24:00:00 . 1d

10 Harvest the virus by collecting the medium and passing it through a 0.45µm syringe filter. This infection medium can be used immediately or stored at -80 °C .

Note

Critical step: The virus can be stored at -80 °C for years. Because viruses are sensitive to freeze-thaw cycles, multiple aliquots of 3 mL can be made.

Note

Note: If more than 10 mL of infection media will be needed, this procedure can be scaled up by plating more than one dish per condition. In the end, infection media from different plates of the same condition can be combined and aliquoted before freezing down. Alternatively, after collecting the infection media, add 10 mL fresh Growth Media to the plates and recollect after 24h (second harvest).

Generation of Epitope-Tagged Mitochondria (Transduction and Selection) (Timing: 2d)

2d

11

Note

! Caution: Work under sterile conditions in a category 2 biological safety hood.

Mix 3 mL of infection media with 7 mL fresh Growth Media.

12 Add Polybrene to the Mix using a stock of 10 µL (dissolved in MilliQ water and sterile filtered) to a final concentration of 10 µL .

13 Gently add 10 mL of the mix to a 10cm plate of cells already at 60% confluency.

Note

Mouse and human cell types can be infected with this media (e.g., mouse embryonic fibroblasts; MEF, A549, etc). The amount of virus needed per infection is dependent on the titer of the virus and the infectability of the cell line being used. The expression levels of the MitoTag can affect the yield and purity of mitochondrial capture. It is recommended to use different dilutions of virus:media (e.g. 2:8, 4:6, 6:4) and proceed with the ones that show optimal Mito-IP results by biochemical analysis. Lower expression of the construct is generally better, achieving amounts of mitochondrial capture similar to those of higher expressing systems while substantially reducing contamination with organelles such as the peroxisome. It is important to note that mitochondria can directly interact with certain organelles, such as the endoplasmic reticulum and peroxisomes, in living cells; the strength of these interactions may vary among cell types and lead to different amounts of contamination in immunopurified mitochondria, but there should generally be an enrichment of mitochondrial markers in the IP lysates as compared with whole-cell lysates. If the used construct contains EGFP, FACS can be used to sort and obtain cells with an appropriate amount of EGFP signal (preferably the ones expressing low to mid-levels of EGFP).

14 Incubate at 37 °C for 24:00:00 . 1d

15 Change media to Growth Media and incubate at 37 °C for another 24:00:00 . 1d

16 To select cells stably expressing the MitoTag or ControlTag, replace media with 10 mL freshly prepared selection media. After 24-48h, there will be observable dead cells in the cultures.

Note

Different cell lines have varying sensitivities to antibiotics, and it is important to empirically determine the working concentration needed to completely kill uninfected cells (kill curve). Puromycin selection requires around 48h while blasticidin selection can take up to 7 days.

17 Change Selection Media every 48h for 4-7 days (depending on the efficiency of the transduction process). At this stage cells stably expressing the MitoTag or ControlTag should be at 90% confluency.

Note

If the cell colonies do not grow or do not grow fast enough, move the cells from a 10cm dish to a 6cm dish or optimise the infection/ virus production steps. Sometimes the transfection efficiency is high and there are no dead cells.

18 Split the cells into more dishes while maintaining them in selection Media. Cells can be frozen down and stored long-term in liquid nitrogen.

Note

Cells should be grown only in selection media, especially before freezing them down. The Selection Media can be replaced by Growth Media only when thawed to help them recover the first 24-48h or before an experiment.

Cell Preparation for MitoTag and ControlTag Immunoprecipitation (2-3 days prior to immunoprecipitation)

19 Split cells stably expressing MitoTag or ControlTag in a 15cm Petri dish. Allow to grow near confluency (approximately 48h depending on the seeding density).

Note

Using a 15cm instead of a 10cm dish significantly increases the Mito-IP yield.

DAY OF THE IMMUNOPRECIPITATION Pre-clearing of Anti-HA Beads (Timing: 5 min)

2m 30s

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

21 Pipette 200 µL anti-HA bead slurry into a tube (100 µL per sample).

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

30s

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

24 Repeat the wash step (22+23) 2 more times. 1m

24.1
- Place the tube onto a magnet for 00:00:30 and carefully remove the overlying solution.
- Remove the tube from the magnet, add 1 mL of cold KPBS and gently resuspend 3 times to disperse any clumps. (1/2)

30s

24.2
- Place the tube onto a magnet for 00:00:30 and carefully remove the overlying solution.
- Remove the tube from the magnet, add 1 mL of cold KPBS and gently resuspend 3 times to disperse any clumps. (2/2)

30s

25 After the last wash, resuspend the beads in 200 µL of KPBS, split it into 2 tubes of 100 µL (one for the Mito-IP and one for the Control-IP) and keep them On ice.

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

Cell Collection and Homogenisation (Timing: 10min) 4m

26 Place cells On ice and remove the media by pouring it into a container.

27 Wash the plates twice with 10 mL cold PBS (pour it in and out if the cells are well attached to increase the speed of the workflow. Alternatively use pipettes).

28 Aspirate all of the PBS and add 1 mL of KPBS.

29 Scrape the cells while On ice with a cell lifter.

30 Carefully transfer scrapped cells using a P1000 pipette into a 1.5ml (or 2ml) tube.

31 Spin down at 1000 x g, 4°C, 00:02:00 . 2m

32 Discard the supernatant and resuspend the pellet in 950 µL of KPBS.

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

34 Move the cells in a 1ml syringe with a 21G needle and place it on the homogeniser.

Note

Wash, assemble and prechill the homogeniser beforehand. Insert in the stainless-steel block a ball that leaves a 10µm gap and screw the lids on tightly. Place it On ice and with the help of two 1ml syringes pass through the device 1ml of KBPS a couple of times (to fill the device with KPBS instead of water and remove any bubbles).

35 With the help of a second 1ml syringe pass the cells 20 times through the bore.

Note

Ensure you hold the syringes firmly while passing the homogenate through the device, as the build-up pressure can force the syringes out of the openings, resulting in the loss of the homogenate. If excessive pressure prevents the homogenate from passing through the bore, adjust the size of the ball used to leave a bigger gap and the number of passes.

36 Transfer the homogenate into a new 1.5ml tube.

Note

To collect the remaining sample in the device, inject some air into one of the openings using a syringe and collect the sample from the other opening with a second syringe.

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

Note

This step ensures the removal of the non-lysed cells, nuclei and debris such as cell membranes and leave the intact organelles in the supernatant.

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

39 Carefully move the supernatant (700 µL - 800 µL) into the tube containing the prewashed beads. Ensure that the bead clumps are dispersed by gently pipetting up and down 3 times.

Note

Critical step: Be very careful not to collect any of the pellet as it will affect the purity of the IP. For some cell homogenates, the separation between the pellet and the supernatant is not very clear. In this case, collect less supernatant.

Note

Continue in the cold room until the detergent lysis step

Immunoprecipitation (Timing: 9min) 5m 30s

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

Note

- Incubation time can be adjusted.
- In the meantime, you can wash and prepare the cell homogeniser for the next sample. Open it from one side, remove the ball, wash it by passing some ultrapure water through it and close it. After the processing of the last sample, wash and clean each part with 70% (v/v) ethanol and leave them to dry before assembling the device back to avoid developing rust.

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

Note

Residual material left on the cap can be brought down with a pulse spin (< 1s) before placing the tubes on the magnet.

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

43 Wash beads with 1000µl 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.

44 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:

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

Elusion and Lysis (Timing: 25-45min) 40m 30s

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

46 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

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

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

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

50 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 2 µL of undiluted IP samples and 2 µL of diluted (1:10) input samples. Quantification should be done in triplicate or duplicate.

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

52 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, MFN).

53 **For sample preparation 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