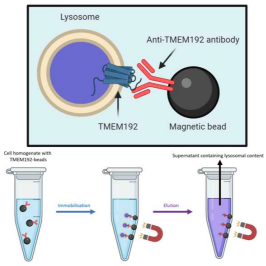
May 09, 2024 Version 1

Isolation of lysosomes using the Tagless LysoIP method in PBMCs V.1



DOI
dx.doi.org/10.17504/protocols.io.x54v9yp51g3e/v1

Daniel Saarela[1,2], Esther Sammler[1,2], Dario R Alessi[1,2], Francesca Tonelli[1,2]

[1]Aligning Science Across Parkinson's;
[2]Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK

Dario R Alessi
Dundee

DOI: dx.doi.org/10.17504/protocols.io.x54v9yp51g3e/v1

Protocol Citation: Daniel Saarela, Esther Sammler, Dario R Alessi, Francesca Tonelli 2024. Isolation of lysosomes using the Tagless LysoIP method in PBMCs. **protocols.io https://dx.doi.org/10.17504/protocols.io.x54v9yp51g3e/v1**

Protocol status: Working
We use this protocol and it's working

Created: March 07, 2022

Last Modified: May 31, 2024

Protocol Integer ID: 59146

Keywords: Purification of lysosomes, Isolation and homogenisation of PBMCs, Tag-less lysosomes, TMEM192 immunoprecipitation, ASAPCRN

Funders Acknowledgement:
Aligning Science Across Parkinson's
Grant ID: ASAP-000463

Abstract

Molecular homeostasis in cells is regulated in part by protein degradation, which is facilitated by the proteasome and lysosomal proteolysis. Lysosomes are membrane bound organelles involved in the turnover of proteins, metabolites and lipids. Recent literature implicates lysosomal dysfunction to be a feature of many a disease, including neurodegenerative diseases. Focused investigation of lysosomal content (proteome/lipidome/metabolome) in disease states could lead to the discovery of novel therapeutics and disease mechanisms. Here we describe our method to isolate peripheral blood mononuclear cells (PBMCs) and perform rapid isolation of intact, tag-less lysosomes from PBMC homogenates using ball-bearing cell breakers and anti-TMEM192 -antibody coupled to magnetic beads. First, cells are broken with physical sheering force as the cell suspension passes through a narrow gap within the cell-breaker, leading to plasma membrane rupture but due to their small size, lysosomes remain intact (Figure 1). Then, the cell homogenate is incubated with the antibody-coupled magnetic beads to allow for rapid immunopurification of lysosomes by binding to the transmembrane protein of lysosomes TMEM192 (Figure 3). The purified lysosomes can be processed and analysed with a variety of techniques including immunoblotting analysis, proteomic/lipidomic/metabolomic tools, fluorescence-activated cell sorting and GCase activity assay. The immunopurification protocol is very fast, less than 15 minutes from start of the incubation with the beads to washed, pure lysosomes. The same protocol can also be used to immunopurify lysosomes from commonly cultured cells such as mouse embryonic fibroblast, HEK293 and A549 cells.

Image Attribution

Figure 1 and Figure 3 were created using BioRender.com

Guidelines

Supporting figures

Figure 1

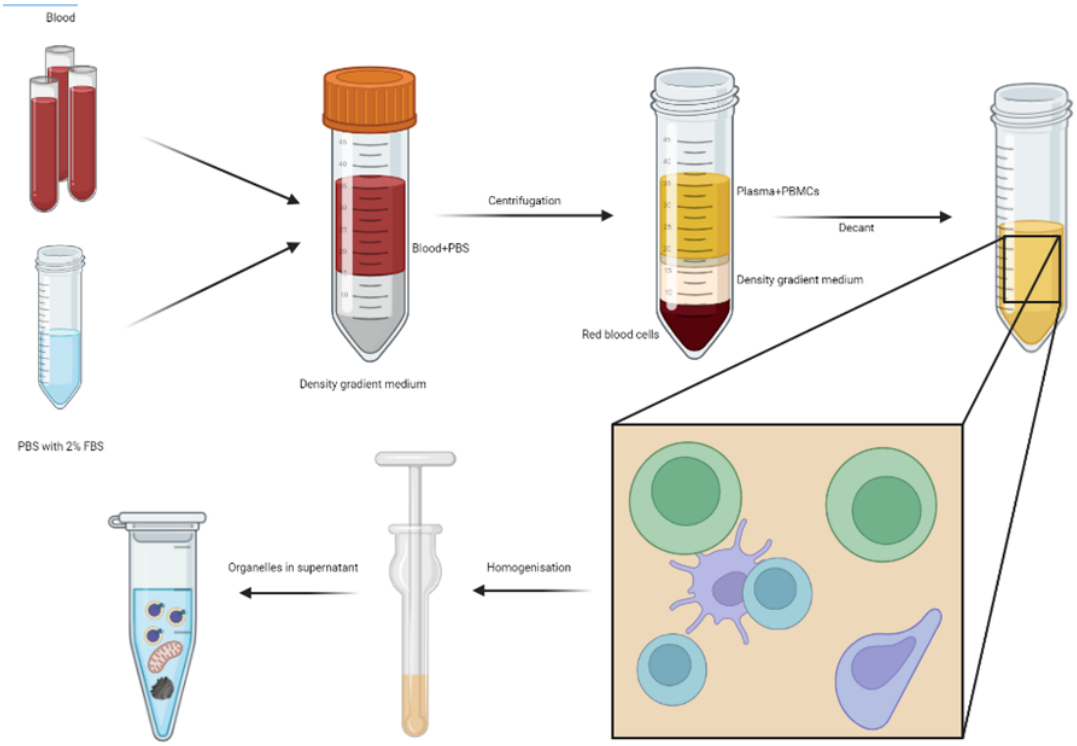


Figure 1. Isolation and homogenisation of PBMCs.

Graphical overview of the workflow of PBMC isolation and homogenisation. Note that images are not to scale, and the homogenising is performed with a different instrument, as outlined in Figure 2.

Figure 2.

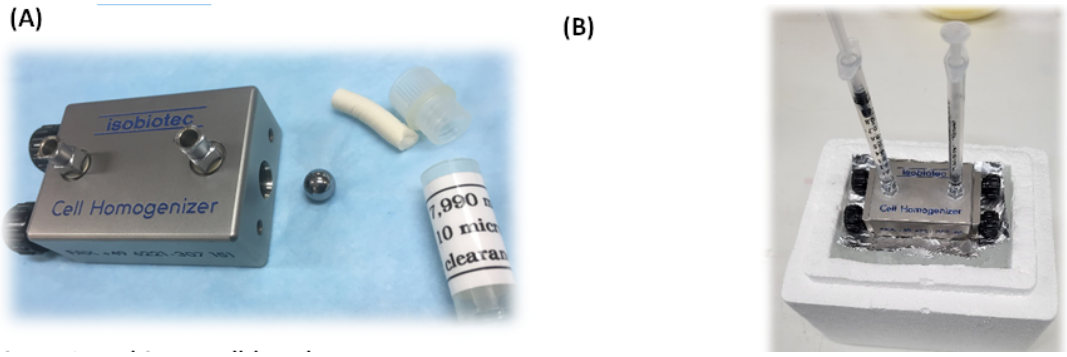


Figure 2. Isobiotec cell-breaker.

(A) Isobiotec cell-breaker with one side panel open and a 10 µm-clearance metal ball with its container.
(B) Example of assembled cell-breaker being used to homogenise cells On ice .

Figure 3.

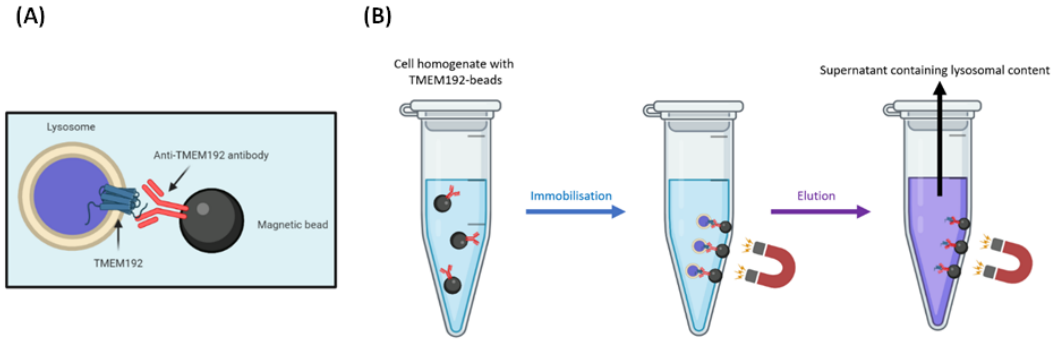


Figure 3. TMEM192 immunoprecipitation from PBMC homogenates.

(A) Graphic describing the design of anti-TMEM192 antibody-bound beads attached to a lysosome.
(B) Beads are incubated with cell homogenate containing lysosomes for 00:05:00 . After immunoprecipitation, the beads are immobilised with magnets and washed with KPBS. Lysosomes attached to the beads are lysed and the supernatant containing lysosomal contents is collected for analysis.

Figure 4.

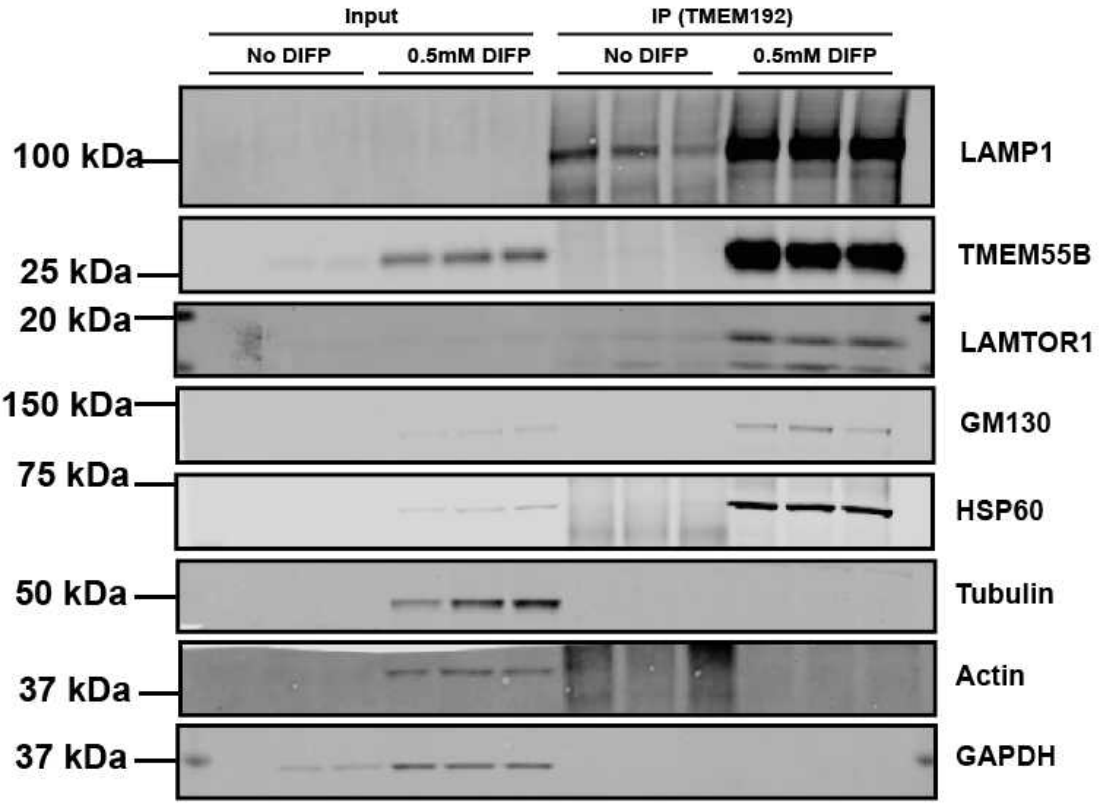


Figure 4. Immunoblot analysis of Tagless LysoIP in PBMCs

Immunoblot analysis confirming enrichment of lysosomes with Tagless Lyso-IP in PBMCs. PBMCs were treated with 0.5 mM diisopropylfluorophosphate (DIFP) to prevent rapid degradation of proteins. Whole-cell lysates (2 µg) as well as the resuspended immunoprecipitates (IPs) (2 µg) were subjected to immunoblotting with the lysosomal (LAMP1, TMEM55B, LAMTOR1), Golgi (GM130), cytosolic (α-tubulin, GAPDH) and mitochondrial (HSP60) markers. The data shown is from 3 healthy male donors.

Materials

Reagents

1. Gibco™ DPBS no calcium no magnesium **Thermo Fisher Scientific Catalog #**14190169
2. Foetal Bovine Serum
3. Dynabeads™ MyOne™ Epoxy, for OEM and industrial use only **Thermo Fisher Catalog #**34001D coupled to anti-TMEM192 antibody.
4. Ficoll-Paque PREMIUM density gradient media **Cytiva Catalog #**17544652
5. Diisopropyl fluorophosphate (DIFP) Diisopropylfluorophosphate **Millipore Sigma Catalog #**D0879
6. KPBS Buffer (homogenisation buffer):

	A	B
	KCL	136 mM
	KH2PO4	10 mM

Adjust to pH 7.25 with KOH.

Note

Note: On the day of use, add Roche cOmplete protease inhibitor cocktail tablet (REF# 11873580001) and Roche PhosSTOP tablet (REF# 04906837001).

Roche cOmplete™ EDTA-free Protease Inhibitor Cocktail **Millipore Sigma Catalog #**11873580001

Roche PhosSTOP™ **Millipore Sigma Catalog #**4906837001

Equipment

Equipment

BELLY DANCER ORBITAL SHAKER	NAME
Shaker	TYPE
ORBITAL	BRAND
BDRAA115S	SKU
https://www.ibisci.com/products/belly-dancer-shaker	LINK

Equipment

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

1. Isobiotec Cell-Breaker, isobiotec Vertriebs UG
2. Stripetter/stripette gun and stripettes
3. Set of Gilson pipettes P10, P200, P1000
4. Microcentrifuge with thermostat

Equipment

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

Consumables

1. SafeSeal reaction tube 1.5 ml PP PCR Performance Tested Low protein-binding **Sarstedt Catalog #**72.706.600

.

2. Conical tube, 15 ml **greiner bio-one Catalog #**188271 .
3. 50 ml centrifuge tubes **greiner bio-one Catalog #**227261 .
4. Standard 1ml and 200µl Pipette tips (Greiner bio-one. Catalog# 686271 and PIPETTE TIP 10 - 100 µL SUITABLE FOR EPPENDORF 96 PIECES / ST RACK **greiner bio-one Catalog #**685261 , respectively).
5. Syringe Filter Minisart High Flow PES 28 mm 0.45µm non-sterile 500 pc/PAK **Sartorius Catalog #**ST16537-Q)
6. 1ml Terumo Syringe without needle (Terumo® Syringe 3-part Syringe **Terumo Catalog #**MDSS01SE)
7. Becton Dickinson Disposable needles 21G x 1 1/2 inch **Becton-Dickinson Catalog #**304432
8. Syringe without needle (10ml) (Medicina. REF# IVS10. LOT# 19111004)
9. Syringe PP/PE without needle **Sigma Aldrich Catalog #**Z116866
10. SepMate™-50 (IVD) 100 Tubes **Stemcell Technologies Catalog #**85450
11. BD Vacutainer 10ml Glass Sodium Heparin Tubes (100) **Becton-Dickinson Catalog #**368480

Methods

26m 30s

1

Note

According to local UK regulation, we undertake all manipulations and pipetting of human blood in category 2 biological safety cabinet.

Prepare a [M] 0.5 Molarity (M) stock solution of DIFP by diluting in isopropanol under a fume hood. Stock solution can be stored in -80 °C until needed.

Note

Note: DIFP is extremely toxic and should be handled with extra care, under a fume hood and wearing double layers of gloves. All waste from this step must be disposed of into 2% (w/v) NaOH solution in distilled water, which inactivates DIFP.

2 Collect blood into a BD Vacutainer sodium heparin 10-mL tubes.

Note

Note: 17 mL of blood is the maximum amount that fits a single SepMate tube.

3 Add density gradient medium to the SepMate tube using a 20-mL syringe with a large bore needle (at least 21G) by carefully dispensing 15 mL through the central hole of the SepMate insert.

Note

Note: The density gradient should settle slightly above the insert.

4 Transfer the blood from the BD Sodium heparin tubes into a 50-mL Falcon tubes.

5 Dilute the blood by adding an equivalent volume of PBS containing 2% (v/v) FBS to achieve a 1:1 ratio. Mix gently by inversion.

6 Gently pipette the blood into the SepMate tube containing the density gradient medium.

7 Centrifuge for 1200 x g, 4°C, 00:10:00 .

8 Decant the top layer (containing PBMCs) to a new 50-ml Falcon tube, leaving the red bottom layer in the tube.

Note

Note: Decant quickly to avoid contamination of your sample with red blood cells.

9 Top up the Falcon tube from Step 8 with PBS up to 50 mL and centrifuge the cells at 450 x g, 4°C, 00:05:00 . Discard the supernatant.

10 Repeat Step 9: Top up the Falcon tube from Step 8 with PBS up to 50 mL and centrifuge the cells at 450 x g, 4°C, 00:05:00 . Discard the supernatant.

Note

Note: This step is performed to wash out the remaining plasma.

11 After last wash with PBS, resuspend the pellet in 1 mL KPBS and transfer to a low-binding Eppendorf tube.

12

Note

Steps from here on are performed on standard bench. This work should be done On ice .

Aliquot 100 µL of pre-prepared TMEM192-beads per IP into a fresh Eppendorf tube.

Note

Note: A protocol for preparing anti-TMEM192 antibody-conjugated beads is available on protocols.io.

13 Immobilize the beads by placing the tubes from Step 12 into a Dyna-Mag tube holder for 00:00:30 .

14 Remove the supernatant using a pipette and gently resuspend the beads in 1 mL of KPBS.

15 Repeat Step 13: Immobilize the beads by placing the tubes from Step 12 into a Dyna-Mag tube holder for 00:00:30 .

16 Remove the supernatant using a pipette.

17 Gently resuspend the beads in n x 100 µL of KPBS, where n = number of samples to be subjected to immunoprecipitation.

18 Aliquot the beads from Step 17 into fresh low-binding Eppendorf tubes (100 µL of slurry each). Leave the tubes On ice until use (Step 27).

19 Centrifuge the cells from Step 11 at 1500 x g, 4°C, 00:02:00 to compare total cell pellets between samples. Note down the size and colouring of the pellets.

Note

Note: The colour of cell pellets is an indication of red blood cell contaminants; the size of cell pellets is an indication of the expected total protein yield.

20 Resuspend the cells in 800 µL KPBS and add DIFP to a final concentration of [M] 0.5 millimolar (mM) (1:1000 dilution of stock solution). Leave the tubes On ice .

Note

Note: DIFP is extremely toxic. This step must be performed under a fume hood and with double layers of gloves. All waste from this step must be disposed of into 2% (w/v) NaOH solution in distilled water, which inactivates DIFP.

21 Transfer 50 µL of whole cell sample (WC) from Step 20 to a fresh Eppendorf tube and place On ice .

22 To prepare the Isobiotec cell-breaker, assemble it by inserting the ball with the size of your choosing inside the machine and screw the lids on tightly. For PBMCs 12 µm gap is effective. Place on aluminium foil On ice and push 3 mL of KPBS through the machine to wash it. Carefully tap dry.

Note

Note: There will be residual KPBS left in the cell-breaker (approximately 200 µL): this is optimal.

23 Transfer the PBMCs from Step 20 into a KPBS rinsed, ice-cold Isobiotec cell-breaker with gap-size of 12 µm using a 1-ml syringe and 21G needle. Homogenise the cells with 15-20 passes through the cell breaker using two 1-ml syringes.

Note

Note: The homogenisation requires more force with more passes. Pay extra care to make sure the syringes are securely in their seals and that the sample doesn't leak out. If you encounter too much pressure for passing the homogenate through the cell-breaker, consider using a ball that leaves a larger gap.

24 Collect the homogenate from the cell breaker into a fresh Eppendorf tube using a 1-ml syringe.

Note

Note: To extract as much sample as possible from the cell-breaker post-homogenisation, push air into the cell-breaker using a syringe and collect from the other seal using another syringe.

25 Centrifuge at 1500 x g, 4°C, 00:02:00 .

26 Transfer the supernatant to a fresh Eppendorf tube On ice .

Note

Note: The pellet contains fragments of plasma membrane and the nucleus and non-homogenised cells.

27 Add the supernatant from Step 26 to 100 µL of the prewashed beads (from Step 18). Mix by pipetting gently three times, then place on a belly-dancer orbiter for 00:05:00 at 4 °C .

Note

Note: Make sure the homogenate/bead slurry is in constant motion.

28

Note

The following steps would optimally be performed in a 4 °C cold room. If not available, then keep working On ice .

Place the IPs from Step 27 on a Dyna-Mag tube holder for 00:00:30 to immobilise the beads. Discard the supernatant or collect as flowthrough sample.

Note

Note: The flowthrough now contains cell debris and other organelles, and any lysosomes that remained unbound to the beads.

29 Resuspend the beads from Step 28 in 1 mL of KBPS and immobilise the beads by placing the tubes in a Dyna-Mag tube holder for 00:00:30. Discard the supernatant.

30 Repeat Step 29: Resuspend the beads in 1 mL of KBPS and immobilise the beads by placing the tubes in a Dyna-Mag tube holder for 00:00:30. Discard the supernatant.

31 Resuspend the beads in 1 mL of KPBS and transfer to a new Eppendorf tube On ice.

32 Place the tubes in a Dyna-Mag tube holder for 00:00:30. Discard the supernatant.

33 The lysosomes attached to the dry beads from Step 32 can now be either eluted off the beads with SDS or Triton-X100 based lysis buffer or stored as they are at -80 °C for later use.
Alternatively, they can be immediately processed for:
1. Immunoblotting analysis
2. Lipidomic analysis
3. Metabolomic analysis
4. Proteomic analysis

34

Note

The following steps instruct how to wash the Isobiotec cell-breaker between samples and at the end of the experiment.

Open the cell-breaker from one side.

35 Take the metal ball out and rinse with MilllQ-water.

36 Flush the cell breaker thoroughly with MilliQ-water.

Note

Note: Use 5-ml syringes filled with MilliQ-water to flush through both of the syringe inlets whilst covering the opening in the side of the cell breaker from Step 34.

37 Reassemble the cell-breaker by re-inserting the metal ball into the instrument and close the side panel tightly using the screws.

38 Flush the cell breaker through the syringe inlets with 5 mL of KPBS for both inlets using 5-ml syringes.

Note

Note: There will be residual KPBS left in the cell-breaker (approximately 200 µL), this is optimal.

39 Proceed to homogenise the next sample.

40 Once done using the homogeniser for the experiment, repeat Step 36.

41 Take all pieces apart (both side panels, panel screws and the metal ball).

42 Clean each part with generous amount of 70% (v/v) ethanol in MilliQ-water.

43 Wipe all parts dry and leave pieces apart to air-dry Overnight.

Note

Note: Packing up the cell-breaker before it is dry will lead to development of rust and colouring of the metal parts.