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Lysosome immunopurification (LysoIP) protocol for subcellular metabolite profiling

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ABSTRACT

Lysosomes function as metabolic hub in the cell by degrading and recycling biomolecules. Despite its critical cellular function, there has been challenges to quantitatively assess lysosomal metabolite profiles. To overcome this hurdle, we developed a rapid harvesting and purification method using immunoprecipitation (LysoIP). This protocol provides details for preparing LysoIP samples for metabolite profiling.

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lysosomes, immunoprecipitation, metabolomics, lipidomics

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MATERIALS TEXT

Consumables

- Marker pen
- Pipette set (1000 µl)
- PPE kit (Lab coat, gloves, safety glasses)
- Ice and ice bucket
- 1.5 ml Eppendorf tubes rack

Reagents

- Anti-HA magnetic beads (Thermo Fisher Scientific, cat. no. 88837)
- Optima LC/MS water (Fisher, cat. no. W6-4)
- Optima LC/MS methanol (Fisher, cat. no. A456-4)
- KPBS (136 mM KCl, 10 mM KH2PO4, pH 7.25 using KOH in Optima LC/MS water)
- Isotopically labeled amino acids (Cambridge Isotope Laboratories, cat. no. MSK-A2-S)
- LysoTracker Red DND-99 (ThermoFisher Scientific, cat. no. L7528)

Equipment

- Glass Vessel: (VWR, cat no. 89026-386)
- Tissue Grinder: (VWR, cat no. 89026-398)
- Benchtop centrifuge (VWR)
- Milli-Q water system
- ID-X Orbitrap Tribrid Mass Spectrometer

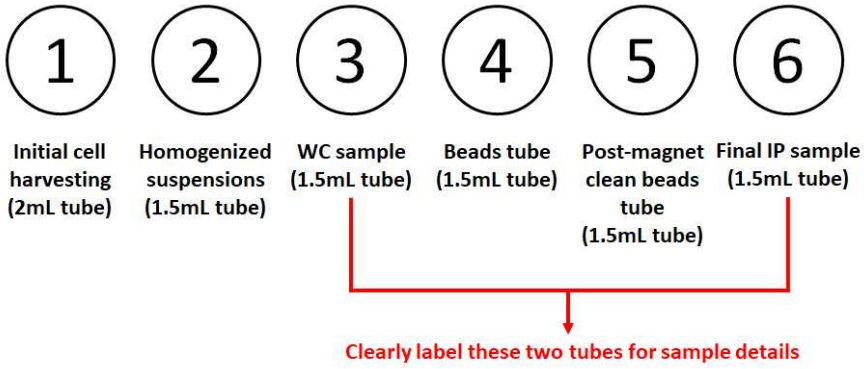
SAFETY WARNINGS

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

Preparation of homogenizers and sample tubes

1 Wash the glass vessel homogenizer with MilliQ Water, 10 times each. wash the tissue grinder homogenizer thoroughly with DI Water and MilliQ Water, especially the gap between the white parts, don't touch the part that goes into the glass vessel. Then dry upside-down using paper towels. Carefully place the glass vessels against something to prevent falling down. Minimize any contact between the grinder and anything else.

2 Prepare microcentrifuge tubes as follows on a metal rack on ice (for each sample, from left to right): ① 2 mL tube for cell suspension from harvesting; ② 1.5 mL tube for post-homogenization cell suspension (organelles in supernatant, membranes in pellet); ③ 1.5 mL tube for whole cell sample; ④ 1.5 mL tube for beads; ⑤5 mL tube for post-magnetic samples; ⑥ 1.5 mL tube for final lysoIP samples. Carefully label tubes ③ and ⑥ with detailed samples and experiments names.



Preparation of Anti-HA beads

3 Pool all required volumes together (**100 µl** / plate, e.g. 800 uL total for 8 plates, extra is not needed).

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4 Shake bottle very well before removing as beads tend to sink to the bottom.

5 

Wash 3 x with the same volume cold clean MS grade KPBS, after settling on magnet. Remove the holder from the magnet itself before dispending washing KPBS to avoid wetting the magnet.

6 Resuspend with KPBS with same amount of volume originally removed from bottle.

7 Aliquot **100 µl** into each 1.5 mL labeled tubes ④.

Cell preparation before harvesting

8 

Wash the first set of 15cm plates (each set has two plates) with **10 mL** of DMEM/plate (for HEKS, use no serum + no antibiotics).

9 Replace with **10 mL** of DMEM/plate (+ **40 µl** of 1:1000 diluted LysoTracker) for an hour.

10 **10 mL** The second set of plates will be washed **00:20:00** later after the first set and so on. 20m

Note: it is very important to maintain the schedule of the plate washing --> Lyso IP. If there are any deviations to the schedule for any reason, note them as it may affect results of experiment.

11 One hour after DMEM wash, take the first set of plates from incubator to bench and place on ice.

12 

Decant the media. Then Wash the cells twice by pouring ~ **5 mL** cold clean MS grade PBS on the edge of the plate, decant the first time and then aspirate the second time.

Cell harvesting

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13 Add **950 µl** of cold KPBS to each 15-cm dish.

14 Scrape the cells down to the bottom of your plates with a cell lifter and transfer the cell suspension into the 2ml tube ①.
Note: this step should be carefully accounted for and done the same between plates. Visually check (with an angle) that all cells have been harvested. We are using a 2mL tube since 950 uL KPBS + cells gives around 2mL volume.

15  2m

Spin at **1000 x g** for **00:02:00** at **4 °C** .

Note: centrifuge must already be cooled to **4 °C** at this point.

16 Aspirate the supernatant and resuspend the pellets with **950 µl** cold KPBS.

17 From this resuspended sample, take **25 µl** for whole cell in the 1.5 mL tube ③.
Note: if pellet mixer is used instead of douncer, resuspend the pellets with **100 µl** cold KPBS in step 16, homogenize cells and then replenish to 950 uL and follow step 17.

Homogenization and LysoIP

18 Transfer the remainder (**925 µl**) of cells into a clean and pre-chill douncer. Dounce the cells 25 times (for 293 T cells, other cells need to be optimized) gently on ice and avoid making bubbles.

Note: count and repeat for each of the samples (both in number and speed).

19 Use serological pipet to transfer sample from douncer into the 1.5 mL tubes ②.

20  2m

Spin 1,000g for **00:02:00** at **4 °C** .

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21

a. Wash douncers during this spin for subsequent harvesting

22 Put the remaining supernatant (**it contains the organelles**) on the 1.5 ml tube ④ with beads and resuspend by pipetting up and down ONE TIME (take **800 µl** when using pellet mixer) .

Note: leave the pellet and make sure not to accidently suck up any of the pellet as that can negatively affect experiment.

23 Rock in cold room for **00:03:00** (everything from now on is in the cold room). 3m

Note: Max speed. Set timer for 3 mins.

24 Put the ④ tube on magnet. Count at least **00:00:25** to allow for beads to be pulled by magnets. 25s

Note: it is important to keep this count the same between each wash and each sample for consistency I.e. 25 seconds each time .

25

Wash the bound fraction 3 times with **1 mL** cold KPBS. Then aspirate all cold KPBS.

Note: during the first wash, make sure to aspirate any liquid trapped on the inner side of the cap. Pipet up and down 2 or 3 times and keep consistent each wash, each sample. After the second wash, resuspend and then switch to the clean 1.5 mL tube ⑤ for the third wash (this step helps give cleaner results) .

Processing of polar metabolite samples 20m

26 Resuspend the IP samples in **50 µl** of freezing cold [M] **80 % (v/v)** MeOH with isotopically labeled amino acids (500 nM) as internal standards.

Note: it is difficult to resuspend. Can begin flushing on the side of beads stuck on the tube, then gradually move inward.

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27 Place samples in ice and start Lyso IP for the next one (remember you are on a strict timed schedule).

28 At this point you should have WC samples (**25 µl** from step 17) in the 1.5 mL tube ③ and IP samples (**50 µl** from step 26) in the 1.5 mL tube ⑤ with beads still in it.

29 After **00:10:00** finishing the last IP, place IP samples in the tube ⑤ on the magnet, collect supernatant, and transfer to the 1.5 mL tube ⑥. 10m

30 *For WC samples,* add **225 µl** freezing cold **80 % (v/v)** MeOH with isotopically labeled amino acids to tube ③. Then vortex briefly.

31 *For WC and LysoIP samples,* centrifuge at top speed (**15000 rpm** , **00:10:00** , **4 °C**) and transfer the supernatant to a set of new tubes. Store WC an IP samples in these new tubes (from step 30) at **-80 °C** . On the day of LC/MS measurement, vortex samples for **00:10:00** at **4 °C** and centrifuge at top speed (**15000 rpm** , **00:10:00** , **4 °C**). Then transfer supernatant to autosampler vials. 30m

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