**Workflow for proteomic analysis of purified lysosomes with or without damage**

**Sharan Swarup1 and J. Wade Harper1**

**1Department of Cell Biology**

**Harvard Medical School**

**Boston MA 02115**

**Abstract**

Lysosomes are a major degradative organelle within eukaryotic cells. Previous work has developed a method wherein the TMEM192 protein is tagged on its C-terminus with an epitope tag in order to immunopurify (IP) lysosomes from cell extracts.1 This process is referred to as Lyso-IP. Such lysosomes can be used for proteomic analysis or for metabolomic analysis. A detailed protocol has been described by Dong et al (2021)2 for the isolation of lysosomes (<https://protocols.io/view/sample-preparation-protocol-for-lipidomics-harvest-br9ym97w>), with an emphasis on downstream analysis by metabolomics. Here we describe processing steps using proteomics after lysosome purification in the context of lysosomal damaging agents. Agents such as L-Leucyl-L-Leucine methyl ester (hydrochloride) (LLoMe) and Gly-Phe-β-naphthylamide (GPN) induce lysosomal damage, leading to the degradation of damaged lysosomes by lysophagy. This adaptation of Lyso-IP provides a route to identify proteins that are recruited to damaged lysosomes using quantitative proteomics.

|  |  |  |
| --- | --- | --- |
| **REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER** |
| **Chemicals, Peptides, and Recombinant Proteins** |
| L-Leucyl-L-Leucine methyl ester (hydrochloride) (LLoMe) | Cayman Chemicals | 16008 |
| PBS (10x) | Santa Cruz | sc-24947 |
| Gly-Phe-β-naphthylamide (GPN) | Cayman Chemicals | 14634 |
| TCEP | Gold Biotechnology | TCEP2 |
| Formic Acid (FA) | Sigma-Aldrich | 94318 |
| Urea | Sigma-Aldrich | U5378 |
| Acetonitrile (ACN) | Sigma-Aldrich | 34851 |
| Sodium Chloride | Sigma-Aldrich | S9888 |
| MOPS | Sigma-Aldrich | M1254 |
| Trypsin | Promega | Custom order |
| Lys-C | Wako Chemicals | 129-02541 |
| EPPS | Sigma-Aldrich | E9502 |
| 2-Chloroacetamide | Sigma-Aldrich | C0267 |
| Sodium metaborate | Sigma-Aldrich | S0251 |
| Dimethyl pimelimidate dihydrochloride (DMP) | Sigma-Aldrich, | D8388 |
| Critical Commercial Assays |
| Pierce™ High pH Reversed-Phase Peptide Fractionation Kit | Thermo Fisher Scientific | 84868 |
| Tandem Mass Tags | Thermo Fisher Scientific | 90406 |
| Bio-Rad Protein Assay Dye Reagent Concentrate | Bio-Rad | 5000006 |
| Other |
| Sep-Pak C18 1cc Vac Cartridge, 50 mg | Waters | WAT054955 |
| Empore™ SPE Disks C18 | 3M Bioanalytical Technologies | 2215 |

**BUFFERS:**

1. Urea lysis buffer:

|  |  |
| --- | --- |
| **Compound**  | **[Compound]final**  |
| Urea  | 8 M  |
| NaCl  | 75 mM  |
| EPPS pH 8.5  | 50 mM  |
| Protease Inhibitors  | 1 x  |

**Cell culture**

1. The appropriate cells (e.g. HEK293T) expressing TMEM192-3xHA, as described1, are grown as described in <https://protocols.io/view/sample-preparation-protocol-for-lipidomics-harvest-br9ym97w>. One 15 cm plate of cells (80% confluence) is used per replicate.

2. To damage lysosomes, GPN (0.2 mM) or LLoMe (0.5-1.0 mM) is added to cells for 15 minutes to 1 hour. The length of time employed depends on the desired level of lysosomal damage desired.

**Lyso-IP**

1. Detailed steps for cell lysis and Lyso-IP are provided in <https://protocols.io/view/sample-preparation-protocol-for-lipidomics-harvest-br9ym97w>. The protocol is followed through step 25, to yield purified lysosomes.

2. Each sample was then eluted with 100μl KPBS containing 0.5% NP-40 in thermo mixer at 4oC for 30 minutes. Elutes were snap frozen in liquid nitrogen and stored in -80oC until further processing

**Trypsinization**

1. Reduce lysates for 30 minutes at 25oC (room temperature) with 5mM TCEP.

2. Alkylate cysteine residues with 20mM Chloroacetamide for 30 minutes at room temperature.

3. TCA was added to eluates to a final concentration of 20% and placed on ice at 4oC for at least 1 hour. Proteins were pelleted for 30 minutes at maximum speed at 4oC. Aspirate supernatant carefully and leave ~30-40μL of solution so as to not disturb the pellet.

Note: It is common not to observe a visible pellet

4. Pellets are resuspended in 4 volumes of ice cold 10% TCA and pelleted by centrifugation at 4oC for 10 minutes at maximum speed. Aspirate as before.

5. Pellets are resuspended in 4 volumes of ice cold methanol and pelleted by centrifugation at 4oC for 10 minutes at maximum speed. Aspirate as before.

6. Repeat the methanol wash.

7. Aspirate methanol as before and air dry remaining 30-40uL of solution (speed-vac can also be used to dry sample)

8. Dried pellets were then resuspended in 50μL, 200 mM EPPS, pH8.0.

8. Peptide digestion was carried out using LysC (0.25μg) for 2 hours at 37oC followed by trypsin (0.5μg) overnight at 37C.

# Labeling

1. Add 3-4μl of the TMT reagent and 15μL of 100% ACN to each 50μL sample.
2. Incubate for 1 hour at room temperature.
3. Stop the reaction with 4μl of hydroxylamine 5% for 15 minutes at room temperature
4. Combine samples and dry in a speed-vac.

# Basic-pH RP peptide fractionation kit (follow manufacturer's instructions)

1. Follow manufacturer’s instructions (Thermo Cat# 84868)

2. Elution used: 17.5% ACN, 20% ACN, 22.5% ACN, 25% ACN, 27.5% ACN and 70% ACN

3. Speed vac individual samples to dryness.

4. Proceed to stage-tip

# Stage TiP

1. Resuspend samples in 100μL of 5% FA, 5% ACN. Check to ensure that the pH of the samples is ~pH3 (or lower) using pH strips

2. Perform C-18 cleanup:

a. Wash C-18 with 100μL of 100% methanol.

b. Equilibrate C-18 with 50μL of 50% ACN 5% FA.

c. Equilibrate C-18 with 100μL of 5% ACN 5% FA.

d. Load sample on to C-18 to bind peptides.

e. Collect flow through and freeze.

f. Wash bound peptides on C-18 with 50μL of 5% ACN 5% FA.

g. Elute peptides off C-18 with 50μL of 75% ACN/5 % FA.

3. Dry down eluted peptides in speed-vac.

4. Re-constitute peptides in 10μL of 5% ACN 5% FA

**Mass spectrometry:**

The analysis of peptides by mass spectrometry will depend on the type of instrument/platform used. Typical instrument settings for analysis on a Thermo Fusion Lumos instrument are provided in the following section.

Inject 3 μl for each LC–MS/MS analysis using available mass spectrometer with a 120-minute online LC separation.

Search raw data against UniProt human protein database using any proteomic analysis software with the following parameters:

* Up to 3 missed cleavages allowed for trypsin/LysC digestion
* Carbamidomethyl (C), TMT (N-term peptide and K) set as a fixed modification
* Oxidation (M) set as variable modifications

Extract signal to noise intensity values of each TMT reporter and identified proteins, and further calculate the ratio of each condition to the control sample’s intensity. This process will depend on the type of analysis software employed with the specific MS platform being used.

**Instrument settings:**

Mass spectrometry data were collected using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to a Proxeon EASY-nLC1200 liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were separated on a 100 μm inner diameter microcapillary column packed in house with ~35 cm of Accucore150 resin (2.6 μm, 150 Å, ThermoFisher Scientific, San Jose, CA) with a gradient consisting of 5%–21% (ACN, 0.1% FA) over a total 150 min run at ~500 nL/min.3 Details of typical instrument parameters are provided below. For Multi-Notch MS3-based TMT analysis3, the scan sequence began with an MS1 spectrum (Orbitrap analysis; resolution 60,000 at 200 Th; mass range 375−1500 m/z; automatic gain control (AGC) target 5Å~105; maximum injection time 50 ms) unless otherwise stated in the instrument parameters in each supplemental table. Precursors for MS2 analysis were selected using a Top10 method. MS2 analysis consisted of collision-induced dissociation (quadrupole ion trap analysis; Turbo scan rate; AGC 2.0Å~104; isolation window 0.7 Th; normalized collision energy (NCE) 35; maximum injection time 90 ms). Monoisotopic peak assignment was used and previously interrogated precursors were excluded using a dynamic window (150 s ± 7898 ppm) and dependent scans were performed on a single charge state per precursor. Following acquisition of each MS2 spectrum, a synchronous-precursor-selection (SPS) MS3 scan was collected on the top 10 most intense ions in the MS2 spectrum. MS3 precursors were fragmented by high energy collision-induced dissociation (HCD) and analyzed using the Orbitrap (NCE 65; AGC 3Å~105; maximum injection time 150 ms, resolution was 50,000 at 200 Th).

**Data Analysis**

Data analysis will be platform and purpose specific.

REFERENCES

1. Abu-Remaileh M, Wyant GA, Kim C, Laqtom NN, Abbasi M, Chan SH, Freinkman E, Sabatini DM. Lysosomal metabolomics reveals V-ATPase- and mTOR-dependent regulation of amino acid efflux from lysosomes.Science. 2017 Nov 10;358(6364):807-813. doi: 10.1126/science.aan6298. Epub 2017 Oct 26. PMID:29074583; PMCID: PMC5704967.

2. Wentao Dong, Nouf Laqtom, Monther Abu-Remaileh. Sample preparation protocol for lipidomics harvesting using lysosome immunoprecipitation (Lipidomics LysoIP, updated 02/09/21). protocols.io https://protocols.io/view/sample-preparation-protocol-for-lipidomics-harvest-br9ym97w

3. McAlister, G. C. *et al.* MultiNotch MS3 Enables Accurate, Sensitive, and Multiplexed Detection of Differential Expression across Cancer Cell Line Proteomes. *Analytical chemistry* **86**, 7150-7158 (2014).