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Workflow for proteomic analysis of purified lysosomes with or without damage V.2

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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. The Lyso-IP is adapted from a previous reported method (Wyant et al., 2018). Here we also 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.

ATTACHMENTS

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Wyant, G.A., Abu-Remaileh, M., Frenkel, E.M., Laqtom, N.N., Dharamdasani, V., Lewis, C.A., Chan, S.H., Heinze, I., Ori, A., and Sabatini, D.M. (2018). Nufip1 is a ribosome receptor for starvation-induced ribophagy. Science 360, 751–758.

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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
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MATERIALS TEXT

Reagents:

- L-Leucyl-L-Leucine methyl ester (hydrochloride) **Cayman Chemical Company Catalog #16008**
- Phosphate Buffered Saline: powder for 5 L of 10X **Santa Cruz Biotechnology Catalog #sc-24947**
- Gly-Phe-β-naphthylamide **Cayman Chemical Company Catalog #14634**
- TCEP-HCl **Gold Biotechnology Catalog #TCEP2**
- Urea **Sigma Aldrich Catalog #U5378**
- Acetonitrile **Sigma Aldrich Catalog #34851**
- Sodium Chloride **Sigma Catalog #S9888**
- 3-(N-Morpholino)propanesulfonic acid 4-Morpholinepropanesulfonic acid (MOPS) **Millipore Sigma Catalog #M1254**

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Lysyl EndopeptidaseR (Lys-C) **Wako Catalog #129-02541**

EPPS **Sigma Aldrich Catalog #E9502**

2-Chloroacetamide **Sigma – Aldrich Catalog #C0267**

Sodium metaborate tetrahydrate **Sigma Aldrich Catalog #S0251**

Dimethyl pimelimidate dihydrochloride **Sigma Aldrich Catalog #D8388**

Pierce™ High pH Reversed-Phase Peptide Fractionation Kit **Thermo Fisher Catalog #84868**

TMT10plex™ Isobaric Label Reagent Set **Thermo Fisher Scientific Catalog #90406**

Bio-Rad Protein Assay Dye Reagent Concentrate **Bio-rad Laboratories Catalog #5000006**

Sep-Pak C18 1 cc Vac Cartridge 50 mg Sorbent per Cartridge 55-105 µm 100/pk **Waters Catalog #WAT054955**

3M™ Empore™ C18 47 mm Extraction Disc Model 2215 20 pack 3 packs per case **3M corporation Catalog #2215**

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A	B	C
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

A	B
BUFFERS:	
1. Urea lysis buffer:	
Compound	**[Compound]final**
Urea	8 M
NaCl	75 mM
EPPS pH 8.5	50 mM
Protease Inhibitors	1 x

DISCLAIMER:

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Cell culture 1h 15m

1 Grow the appropriate cells (e.g. HEK293T) expressing TMEM192-3xHA in DMEM containing 5% FBS

One 15 cm plate of cells (80% confluence) is used per replicate.

2  1h 15m

To damage lysosomes, add GPN **0.2 mM**) or LLoMe (**0.5 Milimolar (mM)** - **1.0 Milimolar (mM)**) to cells for **00:15:00** to **01:00:00** .

The length of time employed depends on the desired level of lysosomal damage desired.

Lyso-IP 30m

3 All buffers were supplemented with protease inhibitors.

4 After treatment of cells at 80% confluency with or without lysosomal damage, cells were harvested on ice by scraping and washed once with Phosphate buffered saline (PBS) containing protease inhibitors (Roche).

5 The cells were pelleted at **300 x g** for **00:05:00** at **4 °C** . 5m

6 Cells were washed once with KPBS buffer (**136 mM** KCL, **10 mM** KH2PO4, **50 mM** Sucrose, pH **7.2**).

7 The cell pellet was resuspended in **1 mL** KPBS and lysed using 30 strokes in a **2 mL** Potter-Elvehjem homogenizer.

8 The lysed cells were spun down at **1000 x g** for **00:05:00** at **4 °C** . 5m

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9 The pellet was discarded and the protein concentration of the lysate was determined by Bradford assay.

10 After normalizing the protein concentration to be equal across all replicates, 5% of the input sample was saved and **50-100 µl** of anti-HA magnetic beads was added the remainder of the sample.

11 The lysate/magnetic bead mixture was placed on gentle rotation for **00:20:00** , at **4 °C** and beads were separated from the lysate using a magnetic stand. 20m

12 The beads were washed twice with KPBS containing **300 mM** NaCl and once with KPBS buffer.

13 30m

Elute each sample with **100 µl** KPBS containing **0.5 % (v/v)** NP-40 in thermo mixer at **4 °C** for **00:30:00** .

Elutes were snap frozen in liquid nitrogen and stored in **-80 °C** until further processing.

Trypsinization 4h 50m

14 Reduce lysates for **00:30:00** at **25 °C** (**Room temperature**) with **5 Milimolar (mM)** TCEP. 30m

15 Alkylate cysteine residues with **20 Milimolar (mM)** Chloroacetamide for **00:30:00** at **Room temperature** . 30m

16  1h

Add TCA to eluates to a final concentration of 20% and place **On ice** at **4 °C** for at least **01:00:00** .

17 Pellet the proteins for **00:30:00** at maximum speed at **4 °C** . 30m

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18 Aspirate supernatant carefully and leave ~ **30 µl** - **40 µl** of solution so as to not disturb the pellet.

> **Note**: It is common not to observe a visible pellet.

19 

10m

Resuspend the pellets in 4 volumes of ice cold 10% TCA and pellet by centrifugation at **4 °C** for **00:10:00** at maximum speed. Aspirate as before.

20 

10m

Resuspend the pellets in 4 volumes of ice cold methanol and pellet by centrifugation at **4 °C** for **00:10:00** at maximum speed. Aspirate as before.

21 

Repeat the methanol wash.

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

23 Resuspend the dried pellets in **50 µl** , **[M]200 Milimolar (mM)** EPPS, **pH8.0** .

24 

2h

Carry the peptide digestion out using LysC (**0.25 µg**) for **02:00:00** at **37 °C** followed by trypsin (**0.5 µg**) overnight at **37 °C** .

Labeling 1h 15m

25 

Add **3 µl** - **4 µl** of the TMT reagent and **15 µl** of 100% ACN to each **50 µl** sample.

26 

1h

Incubate for **01:00:00** at **Room temperature** .

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15m

27 Stop the reaction with **4 µl** of hydroxylamine 5% for **00:15:00** at **Room temperature** .

28 Combine samples and dry in a speed-vac.

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

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

30 Use elution: 17.5% ACN, 20% ACN, 22.5% ACN, 25% ACN, 27.5% ACN and 70% ACN.

31 Speed vac individual samples to dryness.

32 Proceed to stage-tip.

Stage TiP

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

34 Perform C-18 cleanup:

34.1 

a. Wash C-18 with **100 µl** of 100% methanol.

34.2 b. Equilibrate C-18 with **50 µl** of 50% ACN 5% FA.

34.3 c. Equilibrate C-18 with **100 µl** of 5% ACN 5% FA.

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

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34.5 e. Collect flow through and freeze.

34.6 

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

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

35 3. Dry down eluted peptides in speed-vac.

36 4. Re-constitute peptides in **10 µl** of 5% ACN 5% FA.

Mass spectrometry

37 

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.

38 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

39 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 2h 30m

40 Collect mass spectrometry data 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).

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41 Separate the peptides 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 **02:30:00** run at ~ **500 nL/min** .

2h 30m

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.

42 Select the precursors for MS2 analysis 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).

43 Use the monoisotopic peak assignment and exclude the previously interrogated precursors using a dynamic window (150 s ± 7898 ppm) and perform the dependent scans on a single charge state per precursor.

44 Following acquisition of each MS2 spectrum, collect a synchronous-precursor-selection (SPS) MS3 scan on the top 10 most intense ions in the MS2 spectrum.

45 Fragment the MS3 precursors by high energy collision-induced dissociation (HCD) and analyze using the Orbitrap (NCE 65; AGC 3Å~105; maximum injection time 150 ms, resolution was 50,000 at 200 Th).

Data Analysis

46

Data analysis will be platform and purpose specific.

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