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Workflow for proteomic analysis of purified lysosomes in cells lacking GRN V.3

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

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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 HeLa cells lacking the GRN gene. Such cells can be produced using the following protocol: DOI: dx.doi.org/10.17504/protocols.io.4r3l2oxqqv1y/v1.

Attachments

d3×7bhjdf.docx

26KB

Guidelines

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).

Materials

Reagents:

- Phosphate Buffered Saline: powder for 5 L of 10X **Santa Cruz Biotechnology Catalog #**sc-24947
- TCEP-HCl **Gold Biotechnology Catalog #**TCEP2
- Acetonitrile **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34851
- Sodium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S9888
- Lysyl EndopeptidaseR (Lys-C) **Wako Catalog #**129-02541
- EPPS **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E9502
- 2-Chloroacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**C0267
- 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

	A	B	C
	REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
	Chemicals, Peptides, and Recombinant Proteins		
	KCL	Sigma-Aldrich	P9541
	PBS (10x)	Santa Cruz	sc-24947
	TCEP	Gold Biotechnology	TCEP2
	Formic Acid (FA)	Sigma-Aldrich	94318
	Acetonitrile (ACN)	Sigma-Aldrich	34851
	Sodium Chloride	Sigma-Aldrich	S9888
	Trypsin	Promega	Custom order
	Lys-C	Wako Chemicals	129-02541
	EPPS	Sigma-Aldrich	E9502
	2-Chloroacetamide	Sigma-Aldrich	C0267

	A	B	C
	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		
	Empore™ SPE Disks C18	3M Bioanalytical Technologies	2215

	A	B
	BUFFERS:	
	1. KPBS buffer:	
	Compound	**[Compound]final**
	KH2PO4	10 mM
	KCl	136 mM
	Phosphatase Inhibitors	1 x
	Protease Inhibitors	1 x

Troubleshooting

Cell culture

1 Grow the appropriate cells (e.g. HeLa with or without the GRN gene created by gene editing, see DOI: dx.doi.org/10.17504/protocols.io.4r3l2oxqqv1y/v1) expressing TMEM192-3xHA in DMEM containing 10% FBS

Note

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

Lyso-IP

30m

2 All buffers were supplemented with protease and phosphatase inhibitors (Roche).

3 Cells at 80% confluency were harvested on ice by scraping and washed once with Phosphate buffered saline (PBS) containing protease inhibitors (Roche).

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

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

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

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

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

9 After normalizing the protein concentration to be equal across all replicates, 5% of the input sample was saved and 30-50 µL of anti-HA magnetic beads was added the remainder of the sample.

10 The lysate/magnetic bead mixture was placed on gentle rotation for 01:00:00, at 4 °C and beads were separated from the lysate using a magnetic stand. 1h

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

12 30m

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

Note

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

Trypsinization 4h 50m

13 Reduce lysates for 00:30:00 at 25 °C (Room temperature) with [M] 5 millimolar (mM) TCEP. 30m

14 Alkylate cysteine residues with [M] 20 millimolar (mM) Chloroacetamide for 00:30:00 at Room temperature. 30m

15 Add TCA to eluates to a final concentration of 20% and place On ice at 4 °C for at least 01:00:00. 1h

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

17 Aspirate supernatant carefully and leave ~ 30 µL - 40 µL of solution so as to not disturb the pellet.

Note

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

18 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. 10m

19 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. 10m

20 Repeat the methanol wash.

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

22 Resuspend the dried pellets in 50 µL , [M] 200 millimolar (mM) EPPS, pH 8.0 .

23 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 . 2h

Labeling 1h 15m

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

25 Incubate for 01:00:00 at Room temperature . 1h

26 Stop the reaction with 4 µL of hydroxylamine 5% for 00:15:00 at Room temperature . 15m

27 Combine samples and dry in a speed-vac.

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

28 Follow manufacturer's instructions (Thermo Cat# 84868).

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

30 Speed vac individual samples to dryness.

31 Proceed to stage-tip.

Stage TiP

32 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.

33 Perform C-18 cleanup:

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

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

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

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

33.5 e. Collect flow through and freeze.

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

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

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

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

Mass spectrometry

36

Note

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.

37 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

38 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.

Note

This process will depend on the type of analysis software employed with the specific MS platform being used.

Instrument settings

2h 30m

39 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).

40 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

Note

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.

41 Select the precursors for MS2 analysis using a Top10 method.

Note

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).

42 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.

43 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.

44 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

45

Note

Data analysis will be platform and purpose specific.