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Proteomics workflow for whole cell lysate, endosome, and lysosome fractions V.2

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We present a protocol for sample preparation for LC-MS analysis of whole cell lysates and for lysosomal and endosomal fractions purified by Lyso-IP and Endo-IP. Protocols for purification of lysosomes and endosomes is provided in protocol dx.doi.org/10.17504/protocols.io.byi9puh6 using cells that express endogenously tagged TMEM192-HA and stably expressing FLAG-EEA1 as descrbed in dx.doi.org/10.17504/protocols.io.byi7puhn.

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
TMTProTM 16Plex Label Reagent set	Thermo Fisher Scientific	A44520
Pierce™ High pH Reversed-Phase Peptide Fractionation Kit	Thermo Fisher Scientific	84868
Tris(2-carboxyethyl)phosphine hydrochloride solution	Sigma-Aldrich	646547
Iodoacetamide	Sigma-Aldrich	I1149
Trichloroacetic acid solution 6.1 N	Sigma-Aldrich	T0699
Trifluoroacetic acid	fisher scientific	A11650
Hydroxylamine solution 50 wt. %	Sigma-Aldrich	438227
Formic Acid	Sigma-Aldrich	5330020050
Pierce Trypsin Protease, MS grade	Thermo Fisher Scientific	90305
Lysyl endopeptidaseR (Lys-C)	Wako	129-02541
Urea	Sigma-Aldrich	U5378
EPPS 0.2M buffer solution, pH 8.5	Alfa Aesar	J61476.AE
Empore C18 47 mm Extraction Disc, Model 2215	3M	98060402173
Sep-Pak C18 1 cc Vac Cartridge	Waters	WAT054955
Instrument		
Orbitrap Eclipse Tribrid Mass Spectrometer	Thermo Fisher Scientific	FSN04-10000
Agilent 1260 Infinity HPLC	Agilent	G1311B, G1316A, G1329B, G1315D
Aeris 2.6 mm PEPTIDE XB-C18 100 Å, LC column	Phenomenex	00G-4505-E0

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Whole cell global proteomics

1 Seed each cell line in one 15cm dish (293 or 293 cells stably expressing TMEM192-HA and FLAG-EEA1). At ~80% confluency, collect cells by scraping in DPBS

2 At ~80% confluency, harvest cells on ice by scraping in 2-3 mL DPBS and pellet at 1,000xg for 2 min at 4 ºC. Discard supernatant.

3 Wash once with DPBS, resuspend cell pellets with 8 M urea buffer supplemented with protease and phosphatase inhibitors and lyse by sonication.

4 Centrifuge lysates at 17,000xg for 8 min at 4 ºC, and collect the supernatant.

5 Determine total protein concentration using a BCA assay, and aliquot 50 µg of the proteins.

6 Add TCEP to 5 mM final and incubate at 25 ºC for 30 min.

7 Alkylate cysteines by addition of iodoacetamide to 15 mM final and incubate at 25 ºC for 30 min protected from light.

8 Add EPPS buffer to dilute urea to1 M final concentration.

9 Precipitate protein by addition of 6.1 N TCA solution to 20% final and incubate at 4 ºC for 1h.

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10 Centrifuge samples at 20,000xg for 7 min at 4 ºC, and remove supernatants.

11 Wash pellets twice with ice-cold acetone by centrifuging at 20,000xg for 10 min at 4 ºC.

12 Briefly dry pellets in a SpeedVac.

13 Resuspend pellets in 50 µL of 8 M urea buffer, sonicate in a water bath sonicator, and dilute urea by adding of 50 µL of 200 mM EPPS, pH 8.5.

14 Digest peptides with 1 µg of LysC and incubate at 30 ºC for 4h with shaking. Further dilute urea to 1.6 M final by addition of 200 mM EPPS.

15 Further digest peptides with 1 µg trypsin and incubate at 37 ºC overnight with shaking.

16 The next day, add acetonitrile (ACN) to 30% final, and label the peptides with 10 µL of the TMT reagents (10 µg/µL in anhydrous ACN) or TMTpro reagents (12.5 µg/µL in anhydrous ACN) for 1h at 25 ºC. Quench labeling with hydroxylamine at 0.5% final followed by incubation at room temperature for 15 min.

17 Pool TMT-labeled samples with 1:1 ratio and dry.

18 Desalt the sample with C18 solid-phase extraction using Sep-Pak, and dry the eluate.

19 Resuspend samples in 110 µL of 10 mM ammonium bicarbonate, 5% ACN solution and filter through a 0.2 µm PTFE centrifugal filter.

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20 Pre-fractionat samples by high-pH reverse-phase HPLC (Agilent 1260 Infinity) with an Aeris C18 column (250 mm 4.6 mm) with a gradient of mobile phase A (10 mM ammonium bicarbonate, 5% ACN) and mobile phase B (10 mM ammonium bicarbonate, 90% ACN). Collect 96 fractions between 10 min (10% mobile phase B) and 72 min (100% mobile phase B) at a flow rate of 0.6 mL/min, and concatenate into 24 fractions. Dry fractions by SpeedVac and desalt by C18 StageTip. Resuspend alternating 12 fractions out of the 24 fractions in 3% ACN, 1% FA for LC-MS analysis.

21 Perform LC-MS analysis with an Orbitrap Eclipse coupled with a Proxeon EASY-nLC1200 liquid chromatography pump. Separate peptides on a microcapillary column (100 m inner diameter) packed with ~ 35 cm of Accucore150 resin (2.6 m, 150 Å, Thermo Fisher Scientific) with 8-23% (3-73 min), 23-30% (73-80 min), 30-100% (83-86 min) gradient of ACN (0.1% of FA) at 550 nL/min flow rate. For analysis, use multi-notch MS3-based TMT method coupled with Real Time Search algorithm (REF). The scan sequence starts with MS1 spectra analyzed by Orbitrap (resolution 120,000 at 200 Th, 400-1500 m/z, automatic gain control (AGC) target 2105, maximum injection time 50 ms). Monoisotopic peaks should be assigned, use precursor fit filter (70% for a fit window of 0.5 Th), and apply dynamic exclusion window (90 s, ± 10 ppm). Analyze MS2 spectra by quadrupole-ion trap with collision-induced dissociation (Rapid scan rate, AGC 1.0104, isolation window 0.5 Th, normalized collision energy (NBE) 34, maximum injection time 80 ms). Synchronous precursor selection (SPS) API-MS3 scan should collect top 10 most intense b- or y-ions matched with the real-time search algorithm (REF). Fragment MS3 precursors by high energy collision-induced dissociation (HCD) and analyze with the Orbitrap (NCE 45, AGC 2.5105, maximum injection time 200 ms, resolution 50,000 at 200 Th). Set closeout at two peptides per protein for each fraction.

22 Convert RAW files to mzXML files. Construct the searching database from Swiss-Prot human database, which is appended with common contaminants and reversed for target-decoy false discovery rate (FDR) estimation. Perform a database searching with a 20-ppm precursor ion tolerance and 1.0005 Da product ion tolerance. Include static modifications with carbamidomethylation at cysteine (+57.021 Da), TMT labeling at lysine (+229.162 Da for TMT or +304.207 Da for TMTpro) while including variable modifications with oxidation at methionine (+15.995 Da) and TMT labeling at peptide N-termini (+229.162 Da for TMT or +304.207 Da for TMTpro). Filter peptide-spectrum matches (PSM) using a linear discriminant analysis algorithm while considering XCorr, ΔCn, missed cleavages, peptide length, charge state, and precursor mass accuracy. Control the identified peptides at 1% false discovery rate (FDR). Do a protein assembly by parsimony principle, and apply 1% FDR to the protein level. For reporter ion quantification, extract signal-to-noise (S/N) ratio for each TMT channel with an integration tolerance of 0.003 Da. Quantify proteins by summing the reporter ion counts across all matching PSMs. Adjust S/N of each channel using the isotopic impurity table of TMT reagents provided by the vendor.

23 Export protein quantitation values to csv file, and analyze by R 3.6.3. Normalize protein abundances according to the total reporter values in each channel assuming equal amount of loading. Calculate Pearson's correlation coefficient by the basic R function, cor(). For the classification by subcellular locations, use proteins annotated as "very high" or "high" from Itzhak et al (2016).

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Lysosomal and endosomal proteomics

24 Purify lysosomal and endosomal fractions as described (dx.doi.org/10.17504/protocols.io.byi9puh6).

25 Reduce samples with TCEP at 5 mM final and incubate at 25 ºC for 30 min.

26 Alkylate cysteines with iodoacetamide at 15 mM final and incubate at 25 ºC for 30 min and protect from light.

27 Dilute samples with EPPS buffer for 1 M urea final concentration.

28 Precipitate proteins by addition of 6.1 N TCA solution to 20% final and incubate at 4 ºC for 1h.

29 Centrifuge samples at 20,000xg for 15 min at 4 ºC, and remove supernatants.

30 Wash samples twice with ice-cold acetone by centrifuging at 20,000xg for 10 min at 4 ºC.

31 After final wash, briefly dry pellets in a SpeedVac.

32 Resuspend pellets in 5 µL of 8 M urea buffer, sonicate in a water bath sonicator, and dilute urea with 5 µL of 200 mM EPPS.

33 Digest peptides with 0.2 µg of LysC and incubate at 30 ºC for 4h with shaking. Further dilute urea to 1.6 M final by addition of 200 mM EPPS.

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34 Further digest peptides with 0.2 µg trypsin and incubate at 37 ºC overnight with shaking.

35 The next day, add ACN to 30% final, and label the peptides with 10 µL of the TMTpro reagents (12.5 µg/µL in anhydrous ACN) for 1h at 25 ºC.

36 Quench labeling with hydroxylamine to 0.5% final and incubate at room temperature for 15 min.

37 Pool sample, dry by SpeedVac, and pre-fractionate using Pierce High pH Reversed-Phase Peptide Fractionation Kit following the manufacturer's instructions.

38 Concatenate gradient eluates to four final fractions, then desalt with C18 StageTip.

39 Perform LC-MS with an Orbitrap Eclipse tribrid mass spectrometer coupled with a Proxeon EASY-nLC1200 liquid chromatography pump. Separate peptides on a microcapillary column (100 m inner diameter) packed with ~ 35 cm of Accucore150 resin (2.6 µm, 150 Å, Thermo Fisher Scientific) with 5-30% (3-108 min), 30-99% (108-113 min) gradient of ACN (0.1% of FA) at 550 nL/min flow rate. For analysis, use FAIMS Pro Interface, and multi-notch MS3-based TMT method coupled with Real Time Search algorithm. The scan sequence starts with MS1 spectra analyzed by Orbitrap (resolution 120,000 at 200 Th, 400-1500 m/z, automatic gain control (AGC) target $4*10^5$, maximum injection time 50 ms). Assign monoisotopic peaks, use precursor fit filter (70% for a fit window of 0.5 Th), and apply dynamic exclusion (120 s, ± 7 ppm). Select precursor ions using a cycle type of 1.25 sec/CV with FAIMS CV of -40/60/80. Analyze MS2 spectra by quadrupole-ion trap with collision-induced dissociation (Rapid scan rate, AGC $1.0*10^4$, isolation window 0.5 Th, normalized collision energy (NBE) 34, maximum injection time 86 ms). Use synchronous precursor selection (SPS) API-MS3 scan to collect top 10 most intense b- or y-ions matched with the real time search algorithm. Fragment MS3 precursors by high energy collision-induced dissociation (HCD) and analyze with the Orbitrap (NCE 45, AGC 2.5105, maximum injection time 200 ms, resolution 50,000 at 200 Th). Set closeout at two peptides per protein for each fraction.

40 Convert RAW files to mzXML files. Construct the searching database from Swiss-Prot human database, which is appended with common contaminants and reversed for target-decoy false discovery rate (FDR) estimation. Perform a database searching with a 20-ppm precursor ion tolerance and 1.0005 Da product ion tolerance. Include static modifications with carbamidomethylation at cysteine (+57.021 Da), TMT labeling at lysine (+229.162 Da for TMT

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or +304.207 Da for TMTpro) while including variable modifications with oxidation at methionine (+15.995 Da) and TMT labeling at peptide N-termini (+229.162 Da for TMT or +304.207 Da for TMTpro). Filter peptide-spectrum matches (PSM) using a linear discriminant analysis algorithm while considering XCorr, ΔCn, missed cleavages, peptide length, charge state, and precursor mass accuracy. Control the identified peptides at 1% false discovery rate (FDR). Do a protein assembly by parsimony principle, and apply 1% FDR to the protein level. For reporter ion quantification, extract signal-to-noise (S/N) ratio for each TMT channel with an integration tolerance of 0.003 Da. Quantify proteins by summing the reporter ion counts across all matching PSMs. Adjust S/N of each channel using the isotopic impurity table of TMT reagents provided by the vendor.

41 Export protein quantitation values to csv file, and analyze by R 3.6.3. Perform two-sided Student's *t*-test by *t_test()* function in the *rstatix* package version 0.7.0. Adjust individual *p*-values for multiple testing correction by *mt.rawp2adjp()* function with two-stage Benjamini & Hochberg (2006) step-up FDR-controlling procedure in the *multtest* package version 2.42.0.

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