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Whole-cell proteomics and Analysis with or without nutrient stress by Tandem Mass Tagging-based proteomics V2

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We use this protocol and it's working

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Abstract

The analysis of relative protein abundance has emerged as an important tool in cell biology. Typically, it is possible to quantify >8000 proteins under standard conditions. Tandem Mass Tags (TMT) are isobaric reagents that contain a set of isotopically distinct reporter ions, which can be used to quantify individual peptides in distinct samples through multiplexing (**McAlister et al., 2014**). Because the TMT analysis is performed in multiplexed format (up to 18 plex), it is possible to examine the effect of different perturbations (treatments, time courses, etc) on the total abundance of the proteome and include replicate samples as desired. This protocol is applicable to many different cell types, although the number of proteins quantified may differ, depending on the complexity of the proteomes in individual cell types. The small amount of protein needed (50–100 µg) makes application of this approach simple for many different types of cells. This protocol explicitly is used to examine the effects of nutrient stress on protein abundance in cell lines with or without autophagy.

Attachments



Whole-cell proteomic...

191KB

Guidelines

Mass spectrometry:

The analysis of TMT-labelled 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) and di-glycine (K) 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.

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 3%–26% (0–100 min), 26–32% (100–110min) (ACN, 0.1% FA) over a total 120 min run at ~400 nL/min. For analysis, we loaded 1/3 of each fraction onto the column. Each analysis used the Multi-Notch MS³-based TMT method ([McAlister et al., 2014](#)). The scan sequence began with an MS¹spectrum (Orbitrap analysis; resolution 120,000 at 200 Th; mass range 400–1250 m/z; automatic gain control (AGC) target 1×10⁶; maximum injection time 100 ms). Precursors for MS²analysis were selected using a Top 4 sec method. MS²analysis consisted of collision-induced dissociation (quadrupole Orbitrap analysis; AGC 1×10⁵; isolation window 0.7 Th; normalized collision energy (NCE) 35; maximum injection time 300 ms resolution was 7,500 at 200 Th). Monoisotopic peak assignment was used, and previously interrogated precursors were excluded using a dynamic window (120 s ± 7 ppm). Following acquisition of each MS²spectrum, a synchronous-precursor-selection (SPS) MS³scan was collected on the top 10 most intense ions in the MS²spectrum ([McAlister et al., 2014](#)). MS³precursors were fragmented by high energy collision-induced dissociation (HCD) and analyzed using the Orbitrap (NCE 65; AGC 2×10⁵; maximum injection time 500 ms, resolution was 50,000 at 200 Th).



Materials

Chemicals, Peptides, and Recombinant Proteins

- ☒ Protease Inhibitor Cocktail **Roche Catalog #11873580001**
- ☒ PBS (10x) **Santa Cruz Catalog #sc-24947**
- ☒ tris(2-carboxyethyl)phosphine (TCEP) **Gold Biotechnology Catalog #TCEP2**
- ☒ Formic Acid (FA) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #94318**
- ☒ Urea **Merck MilliporeSigma (Sigma-Aldrich) Catalog #U5378**
- ☒ Acetonitrile (ACN) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #34851**
- ☒ Sodium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S9888**
- ☒ MOPS **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1254**
- ☒ Sequencing grade Trypsin **Promega Catalog #V5111**
- ☒ Lys-C **Wako Chemicals Catalog #129-02541**
- ☒ EPPS **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E9502**
- ☒ 2-Chloroacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C0267**
- ☒ Protein A Plus Ultralink resin **Thermo Fisher Scientific Catalog #53142**
- ☒ Sodium metaborate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S0251**
- ☒ Aeris peptide XB-c18 column **Catalog #00G-4507-E0**
- ☒ Dimethyl pimelimidate dihydrochloride (DMP) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D8388**

Critical Commercial Assays

- ☒ Tandem Mass Tags **Thermo Fisher Scientific Catalog #90406**
- ☒ Bio-Rad Protein Assay Dye Reagent Concentrate **Bio-Rad Laboratories Catalog #5000006**

Software

- SEQUEST Eng et al., 1994
- COMET Eng et al., 2013 <http://comet-ms.sourceforge.net/>

Other

- ☒ Sep-Pak C18 1cc Vac Cartridge 50 mg **Waters**
- ☒ Empore™ SPE Disks C18 **Catalog #2215**

- Orbitrap Fusion Lumos Mass Spectrometer, ThermoFisher Scientific, Cat#IQLAAEGAAPFADMBMBHQCat#IQLAAEGAAPFADMBMBHQ
- high-pH reverse-phase HPLC - LC1260, Agilent
- Protein Lo-Bind Tubes

BUFFERS and MEDIA:

1. Urea lysis buffer:

	A	B
	Compound	[Compound]final
	Urea	8 M
	NaCl	75 mM
	Tris pH 7.4	150 mM
	Protease Inhibitors Phosphatase Inhibitors	1 x 1 x

2. EPPS buffer (50 mM EPPS, pH 8.5)

3. PBS; Phosphate buffered saline: ThermoFisher (#14040133)

4. EBSS (Sigma- Aldrich Cat#E3024).

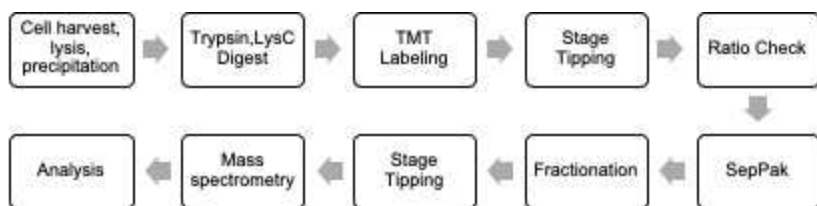
5. Dulbecco's MEM (DMEM), high glucose, pyruvate (Gibco / Invitrogen, 11995)

Safety warnings

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

Before start

WORKFLOW:





Cell culture and nutrient stress treatments

- 1 Wild-type HEK293 (human embryonic kidney, fetus, ATCC CRL-1573, RRID: CVCL_0045) cells or the analogous cells lacking either ATG7 or FIP200 (see DOI: XXX) were grown in Dulbecco's modified Eagle's medium (DMEM, high glucose and pyruvate) supplemented with 10% fetal calf serum and maintained in a 5% CO₂ incubator at 37°C. Cells were maintained at <80% confluency throughout the course of experiments.
- 2 DMEM was removed and cells were washed 3 times with PBS followed by resuspending cells in EBSS or DMEM lacking amino acids prepared according to H. Chino, et al Mol Cell **74**, 909-921 e906 (2019). The investigator can select the length of time for starvation but this is typically 10-18 hours.

Harvest, precipitation and digestion

9h 10m

- 3 For whole proteome analysis,  50 µg of protein lysate is required for each replicate. Cells from step 2 are washed with PBS three times. Cells were lysed by in UREA denaturing buffer (8M Urea, 150mM NaCl, 50mM EPPS pH8.0, containing mammalian protease inhibitor cocktail (Sigma), and Phos-STOP) Cell lysates were collected by cell scrapers and sonicated on ice for 10 seconds.
- 4 Centrifuge suspensions at  13000 rpm, 4°C , (high speed) for  00:10:00 and collect the supernatant. 
- 5 Transfer quantified protein lysate concentration and  50 µg of lysate for each sample to a clean 1.5 mL protein Lo-Bind Eppendorf tubes. Reduce lysates for  00:20:00 at  Room temperature with  5 millimolar (mM) TCEP , and alkylate cysteine residues with  20 millimolar (mM) Chloroacetamide ( Room temperature ,  00:30:00). 
- 6 Extract protein content by methanol-chloroform precipitation and subsequent MeOH washes. 
- 6.1 Add 4x volumes of MeOH and vortex.



- 6.2 Add 1x volume of chloroform and vortex.
- 6.3 Add 3x volume of water and vortex.
- 6.4 Spin down at Room temperature for 00:05:00 at high speed. 5m
- 6.5 Aspirate and discard the upper aqueous phase. Do not disturb the protein disc at the interface of the aqueous phase (top) and organic phase (bottom).
- 6.6 Add 4x volumes of MeOH and vortex.
- 6.7 Spin down at Room temperature for 00:05:00 at high speed. 5m
- 6.8 Aspirate and discard supernatant. Do not disturb the protein pellet at the bottom of the tube.
- 6.9 Repeat MeOH wash.
- 6.10 Air dry (or speed-vac) protein pellet down to remove all traces of MeOH.
- 7 Resuspend protein pellets 100 μ L of [M] 200 millimolar (mM) EPPS buffer (8.5).
- 8 Digest samples at 37 °C for 02:00:00 with endoproteinase Lys-C (Wako, Japan) at a 1/200 enzyme/protein ratio. 2h
- 9 Digest with Trypsin (1:100) for 06:00:00 at 37 °C . 6h



TMT-Labeling of samples

1h 15m

- 10 Add  5 μL of the TMT reagent to each sample. Solubilize TMT reagents are in ACN as per manufacturer's instructions and  5 μL of TMT label is used for every  50 μg of protein lysate. Perform TMT labeling in a final concentration of 20–25% ACN. Add  20 μL of ACN to bring the reaction volume at 125 μL . The number of samples, and hence the number of individual TMT reagents, will depend upon the design of the experiment.
- 11 Incubate for  01:00:00 at  Room temperature to label the samples.
- 12 Combine 1% of each labeled sample together in a tube, quench the reaction with  4 μL of 5% hydroxylamine for  00:15:00 at  Room temperature, and dry down using the speed-vac. This combined sample is used to perform the ratio check to test labeling efficiency. The remaining amount of each sample can be stored in the freezer.

1h



15m

Stage tip for Ratio Check

15m

- 13 Resuspend the dried sample in  100 μL of 5% FA/5% ACN. Check to ensure that the pH of the sample is ~  3 (or lower) using pH strip.
- 14 Make stage tip by placing 6–8 “cookies” of C-18 embedded membranes in  200 μL Rainin tip.
- 15 Perform C-18 cleanup:
- 15.1 Equilibrate C-18 with  100 μL of 100% methanol.
- 15.2 Wash C-18 with  50 μL of 50% ACN/5% FA.
- 15.3 Wash C-18 with  100 μL of 5% ACN/5% FA.





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

15.5 Wash bound peptides on C-18 with  50 μ L of 5% ACN/5% FA .



15.6 Elute peptides off C-18 with  50 μ L 75% ACN/ 5% FA into a mass spec vial.

16 Dry down eluted peptides in speed-vac.

17 Reconstitute peptides in  10 μ L 5% ACN/5% FA .

18 Perform ratio check by analyzing the total amount of reporter ions present, as measured by mass spectrometry, for each TMT reporter ion channel.

19 Quench the entire volume of each sample using  8 μ L of 5% hydroxylamine for  00:15:00 at  Room temperature . Combine samples in 2 ml Eppendorf tube to equal amounts, based on normalization values you obtain from the ratio check.

15m

20 Dry down labeled, combined sample in speed-vac.

21 Re-constitute the sample in  750 μ L of 5% ACN/5% FA .

SepPak clean-up (C18 solid-phase extraction (SPE))

15m

22 Place SepPak column into vacuum slot on a vacuum manifold.

23 Fill SepPak with  1 mL 100% MeOH .

24 Start the vacuum, gently, try to ensure that the pressure gauge is below 10. This can be achieved by opening one of the valves gently. The fluid should pass through slowly, drop



wise.

- 25 Fill SepPak with  1 mL 5% FA/50% ACN , Flow Liquid through.
- 26 Fill SepPak with  1 mL 5% FA/5% ACN , Flow Liquid through, repeat 2 more times.
- 27 Replace tube with 2 ml collection tube.
- 28 Add Peptides, Flow Liquid through (~ 750 µl).
- 29 Wash with  1 mL 5% FA/5% ACN , Flow Liquid through, 2 times. 
- 30 Replace 15 ml collection tube with 2 ml collection tube.
- 31 Elute with  750 µL 75% ACN/5% FA . Since there is residual liquid left in the SepPak, ensure that all the liquid flows through SepPak.
- 32 Dry down in SpeedVac  Room temperature  Overnight or  30 °C . 15m

Fractionation

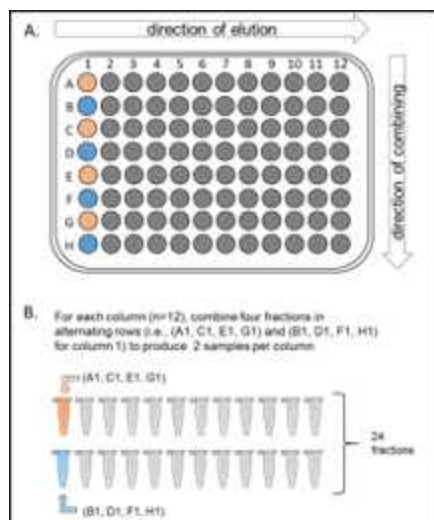
1h 30m

- 33 Resuspend sample in  100 µL of 10 mM NH₄HCO₃  8 .
- 34 Fractionate using pH reverse-phase HPLC:
 - 34.1 Fractionate samples by high-pH reverse-phase HPLC (Agilent LC1260) into 96 fractions over a  01:30:00 run. 1h 30m
 - 34.2 Fractions are run through an Aeris peptide XB-c18 column (Phenomenex; 250 mm x 3.6 mm), with mobile phase A containing  5 Mass Percent ACN and

[M] 10 millimolar (mM) NH_4HCO_3  and phase B containing

[M] 90 Mass Percent ACN and [M] 10 millimolar (mM) NH_4HCO_3  (all in LC-MS grade H_2O).

- 35 Combine fractionated samples (either 12 or 24 fractions) in a non-continuous manner into individual 1.5 ml Eppendorf tube (see outline below from [Paulo et al., 2016](#)).



- 36 Dry down in SpeedVac.
- 37 Resuspend peptides in  100 μL 5%FA /5% ACN .
- 38 Check pH (~3.5) with pH indicator strips.

Stage tip for proteomics sample

3m

- 39 Stage tip each fraction.

- 39.1 Make stage tips and equilibrate. Spin down at  3000 rpm, 00:03:00 .

3m





39.2 Perform C-18 cleanup:

39.3 Wash with  50 μ L 50% ACN /5% FA .



39.4 Wash with  100 μ L 5% ACN /5% FA .



39.5 Load sample

39.6 Collect flow through and freeze.

39.7 Wash with  50 μ L 50% ACN /5% FA .



39.8 Elute with  50 μ L 75% ACN/1% FA in mass spec vial.

40 Dry down in SpeedVac.

41 Reconstitute pellet in  10 μ L 5% FA / 5% ACN .

42 Freeze sample at -  -20 $^{\circ}$ C until ready to run proteomics.