Whole-cell proteomics and Analysis by Tandem Mass Tagging-based proteomics

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MANUSCRIPT CITATION:
- 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). - Paulo, J.A., et al. Quantitative mass spectrometry-based multiplexing compares the abundance of 5000 S. cerevisiae proteins across 10 carbon sources. J Proteomics 148, 85-93 (2016a).

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

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

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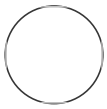
ASAP Collaborative Research Network



Kelsey Hickey

DISCLAIMER

Protocol status: Working
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 proteomics and Analysis by Tandem Mass Tagging-based proteomics.pdf

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 MS^3-based TMT method(McAlister et al., 2014). The scan sequence began with an MS^1spectrum (Orbitrap analysis; resolution 120,000 at 200 Th; mass range 400–1250 m/z; automatic gain control (AGC) target 1×10^6; maximum injection time 100 ms). Precursors for MS^2analysis were selected using a Top 4 sec method. MS^2analysis consisted of collision-induced dissociation (quadrupole Orbitrap analysis; AGC 1×10^5; 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 MS^2spectrum, a synchronous-precursor-selection (SPS) MS^3scan was collected on the top 10 most intense ions in the MS^2spectrum(McAlister et al., 2014). MS^3precursors were fragmented by high energy collision-induced dissociation (HCD) and analyzed using the Orbitrap (NCE 65; AGC 2×10^5;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) **Sigma – Aldrich Catalog #94318**
- Urea **Sigma – Aldrich Catalog #U5378**
- Acetonitrile (ACN) **Sigma – Aldrich Catalog #34851**
- Sodium Chloride **Sigma – Aldrich Catalog #S9888**
- MOPS **Sigma – Aldrich Catalog #M1254**
- Sequencing grade Trypsin **Promega Catalog #V5111**
- Lys-C **Wako Chemicals Catalog #129-02541**
- EPPS **Sigma – Aldrich Catalog #E9502**
- 2-Chloroacetamide **Sigma – Aldrich Catalog #C0267**
- Protein A Plus Ultralink resin **Thermo Fisher Scientific Catalog #53142**
- Sodium metaborate **Sigma – Aldrich Catalog #S0251**
- Aeris peptide XB-c18 column **Contributed by users Catalog #00G-4507-E0**
- Dimethyl pimelimidate dihydrochloride (DMP) **Sigma – Aldrich Catalog #D8388**

Critical Commercial Assays

- Tandem Mass Tags **Thermo Fisher Scientific Catalog #90406**
- Bio-Rad Protein Assay Dye Reagent Concentrate **BIO-RAD 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 **Contributed by users Catalog #2215**

- Orbitrap Fusion Lumos Mass Spectrometer, ThermoFisher Scientific, Cat#IQLAAEGAAPFADBMBHQCat#IQLAAEGAAPFADBMBHQ
- 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

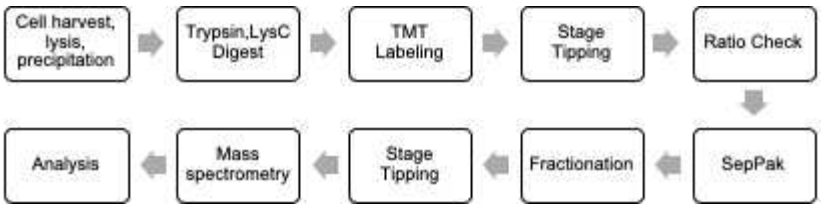
2. EPPS buffer (50 mM EPPS, pH 8.5)
3. PBS; Phosphate buffered saline: ThermoFisher (#14040133)
4. EBSS (Sigma- Aldrich Cat#E3024).
5. Dulbecco's MEM (DMEM), high glucose, pyruvate (Gibco / Invitrogen, 11995)

SAFETY WARNINGS

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

BEFORE START INSTRUCTIONS

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% CO_2 incubator at 37^{o}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 Centrifugate suspensions at 13000 rpm, 4°C , (high speed) for 00:10:00 and collect the supernatant. 10m

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 [M] 5 millimolar (mM) TCEP, and alkylate cysteine residues with [M] 20 millimolar (mM) Chloroacetamide (Room temperature, 00:30:00). 50m

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 µL of [M] 200 millimolar (mM) EPPS buffer (pH 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. Performe 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. 1h

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. 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 ~ pH 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 µL of 5% ACN/5% FA .

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

16 Dry down eluted peptides in speed-vac.

17 Reconstitute peptides in 10 µL 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 µL 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 µL 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

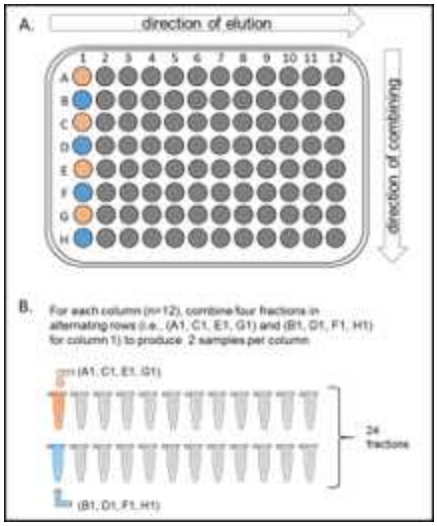
33 Resuspend sample in 100 µL of 10 mM NH4HCO3 pH 8.

34 Fractionate using pH reverse-phase HPLC:

34.1 Fractionate samples by high-pH reverse-phase HPLC (Agilent LC1260) into 96 fractions over 1h 30m 01:30:00 run.

34.2 Fractions are run through an Aeris peptide XB-c18 column (Phenomenex; 250 mm x 3.6 mm), with mobile phase A containing [M] 5 Mass Percent ACN and [M] 10 millimolar (mM) NH4HCO3 pH 8 and phase B containing [M] 90 Mass Percent ACN and [M] 10 millimolar (mM) NH4HCO3 pH 8 (all inLC-MS grade H_20).

35 Combine fractionated samples (either 12 or 24 fractions) in a non-continuous manner into individual 1.5 ml Eppendorf tube (see outline below form 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 µL 50% ACN /5% FA .

39.4 Wash with 100 µL 5% ACN /5% FA .

39.5 Load sample

39.6 Collect flow through and freeze.

39.7 Wash with 50 µL 50% ACN /5% FA .

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

40 Dry down in SpeedVac.

41 Reconstitute pellet in 10 µL 5% FA / 5% ACN .

42 Freeze sample at -20 °C until ready to run proteomics.