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

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Abstract

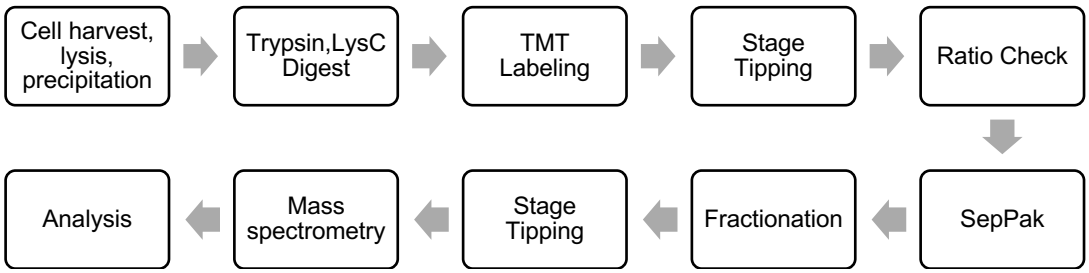
The analysis of relative protein abundance has emerged as an important tool in cell biology. Typically, it is possible to quantify >8000 proteins under standard conditions. Tandem Mass Tags (TMT) are isobaric reagents that contain a set of isotopically distinct reporter ions, which can be used to quantify 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 ug) makes application of this approach simple for many different types of cells

Materials

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Protease Inhibitor Cocktail	Roche	11873580001
PBS (10x)	Santa Cruz	sc-24947
tris(2-carboxyethyl)phosphine (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
Sequencing grade Trypsin	Promega	V5111
Lys-C	Wako Chemicals	129-02541
EPPS	Sigma-Aldrich	E9502
2-Chloroacetamide	Sigma-Aldrich	C0267
Protein A Plus Ultralink resin	Thermo-Fisher Scientific	53142
Sodium metaborate	Sigma-Aldrich	S0251
Aeris peptide XB-c18 column	Phenomenex	00G-4507-E0
Dimethyl pimelimidate dihydrochloride (DMP)	Sigma-Aldrich	D8388
Critical Commercial Assays		
Tandem Mass Tags	Thermo Fisher Scientific	90406
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	5000006
Software		
SEQUEST	Eng et al., 1994	N/A
COMET	Eng et al., 2013	http://comet-ms.sourceforge.net/
Other		
Sep-Pak C18 1cc Vac Cartridge, 50 mg	Waters	WAT054955

Empore™ SPE Disks C18	3M Bioanalytical Technologies	2215
Orbitrap Fusion Lumos Mass Spectrometer	ThermoFisher Scientific	Cat#IQLAAEGAAPF ADBMBHQ
high-pH reverse-phase HPLC - LC1260	Agilent	N/A
Protein Lo-Bind Tubes		

WORKFLOW:



BUFFERS:

1. Urea lysis buffer:

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. Phosphate buffered saline (pH 7.4)

Harvest, precipitation and digestion

1. For whole proteome analysis, 50 µg of protein lysate is required for each replicate. Cells are lysed in lysis buffer and passed through a 21G needle 10 times. Alternatively, cells are lysed by sonication as per manufactures instructions
2. Suspensions are centrifuged at 13k rpm (high speed) for 10 min at 4°C and the supernatant is collected.
3. Quantify protein lysate concentration and 50 µg of lysate for each sample is transferred to a clean 1.5 mL protein Lo-Bind Eppendorf tubes. Lysates are reduced for 20 min at room temperature with 5 mM TCEP, and cysteine residues are then alkylated with 20mM Chloroacetamide (room temperature, 30 min).
4. Extract protein content by methanol-chloroform precipitation and subsequent MeOH washes.

    a. Add 4x volumes of MeOH and vortex
    b. Add 1x volume of chloroform and vortex
    c. Add 3x volume of water and vortex

d. Spin down at RT for 5 min at high speed.
e. 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).
f. Add 4x volumes of MeOH and vortex
g. Spin down at RT for 5 min at high speed
h. Aspirate and discard supernatant. Do not disturb the protein pellet at the bottom of the tube.
i. Repeat MeOH wash
j. Air dry (or speed-vac) protein pellet down to remove all traces of MeOH.

5. Protein pellets are resuspended 100 µl of 200 mM EPPS buffer (pH 8.5).
6. Samples are then digested at 37°C for 2 hr with endoproteinase Lys-C (Wako, Japan) at a 1/200 enzyme/protein ratio.
7. Digest with Trypsin (1:100) for 6 hr at 37ºC.

TMT-Labeling of samples

8. Add 5 µl of the TMT reagent to each sample. TMT reagents are solubilized in ACN as per manufacturer's instructions and 5 µl of TMT label is used for every 50 µg of protein lysate. TMT labeling is performed in a final concentration of 20-25% ACN. Add 20 µL of ACN to bring the reaction volume ot 125 µL. The number of samples, and hence the number of individual TMT reagents, will depend upon the design of the experiment.
9. Incubate for 1 hour at room temperature to label the samples.
10. Combine 1% of each labeled sample together in a tube, quench the reaction with 4 µL of 5% hydroxylamine for 15 min 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.

Stage tip for Ratio Check

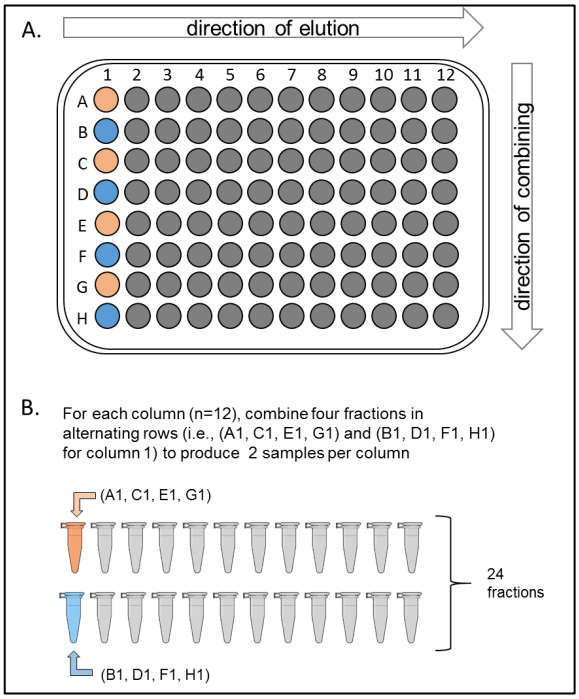
11. Resuspend the dried sample in 100 uL of 5% FA/5% ACN. Check to ensure that the pH of the sample is ~pH 3 (or lower) using pH strip
12. Make stage tip by placing 6-8 "cookies" of C-18 embedded membranes in 200µl Rainin tip.
13. Perform C-18 cleanup:
    a. Equilibrate C-18 with 100 uL of 100% methanol.
    b. Wash C-18 with 50 µL of 50% ACN/5% FA.
    c. Wash C-18 with 100 µL of 5% ACN/5% FA.
    d. Load sample on to C-18 to bind peptides
    e. Wash bound peptides on C-18 with 50 µL of 5% ACN/5% FA
    f. Elute peptides off C-18 with 50 µl 75% ACN/ 5% FA in to a mass spec vial
14. Dry down eluted peptides in speed-vac.
15. Reconstitute peptides in 10 µl 5% ACN/5% FA.
16. Perform ratio check by analyzing the total amount of reporter ions present, as measured by mass spectrometry, for each TMT reporter ion channel.
17. Quench the entire volume of each sample using 8 µL of 5% hydroxylamine for 15 min at room temperature. Combine samples in 2 ml Eppendorf tube to equal amounts, based on normalization values you obtain from the ratio check.
18. Dry down labeled, combined sample in speed-vac
19. Re-constitute the sample in 750 µL of 5% ACN/5% FA

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

20. Place SepPak column into vacuum slot on a vacuum manifold.
21. Fill SepPak with 1ml 100% MeOH.
22. 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.
23. Fill SepPak with 1ml 5% FA/50% ACN, Flow Liquid through.
24. Fill SepPak with 1ml 5% FA/5% ACN, Flow Liquid through, repeat 2 more times.
25. Replace tube with 2 ml collection tube.
26. Add Peptides, Flow Liquid through (~750µl).
27. Wash with 1ml 5% FA/5% ACN, Flow Liquid through, 2 times.
28. Replace 15 ml collection tube with 2ml collection tube.
29. Elute with 750µl 75% ACN/5% FA. Since there is residual liquid left in the SepPak, ensure that all the liquid flows through SepPak.
30. Dry down in SpeedVac @ RT over night or 30°C.

Fractionation

31. Resuspend sample in 100µl of 10 mM NH_4HCO_3 pH8
32. Fractionate using pH reverse-phase HPLC
    a. Samples are fractionated by high-pH reverse-phase HPLC (Agilent LC1260) into 96 fractions over a 90 min run.
    b. Fractions are run through an Aeris peptide XB-c18 column (Phenomenex; 250 mm x 3.6 mm), with mobile phase A containing 5% ACN and 10 mM NH_4HCO_3 pH8 and phase B containing 90% ACN and 10 mM NH_4HCO_3 pH8 (all in LC-MS grade H_20).
33. Combine fractionated samples (either 12 or 24 fractions) in a non-continuous manner into individual 1.5ml Eppendorf tube (see outline below form Paulo et al., 2016).



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

Stage tip for proteomics sample

37. Stage tip each fraction.
    c. Make stage tips and equilibrate. Spin down at 3000 rpm for 3min.
    d. Perform C-18 cleanup:
    e. Wash with 50µl 50% ACN /5% FA.
    f. wash with 100µl 5% ACN /5% FA.
    g. Load sample
    h. Collect flow through and freeze.
    i. Wash with 50µl of 5% FA/5% ACN.
    j. h. Elute with 50µl 75% ACN/1% FA in mass spec vial.
38. Dry down in SpeedVac.
39. Reconstitute pellet in 10µl 5% FA / 5% ACN.
40. Freeze sample at -20°C until ready to run proteomics.

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^1 spectrum (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^2 analysis were selected using a Top 4 sec method. MS^2 analysis 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^2 spectrum, a synchronous-precursor-selection (SPS) MS^3 scan was collected on the top 10 most intense ions in the MS^2 spectrum (McAlister et al., 2014). MS^3 precursors 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).

REFERENCES

- 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).

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