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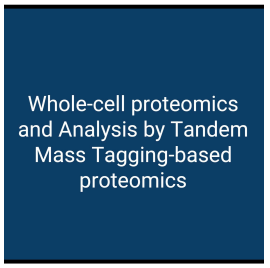
# Multiplexed TMTpro proteomic sample preparation of whole cell HeLa lysates from various growth conditions ± FAC




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

In 1 collection

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- 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).



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## Disclaimer



## 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

## Attachments

PDF

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 $MS^3$-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 $1\times10^6$; 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 $1\times10^5$; 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 $2\times10^5$;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 **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 **Catalog #**2215
- Orbitrap Fusion Lumos Mass Spectrometer, ThermoFisher Scientific, Cat#IQLAAEGAAPFADBMBHQCat#IQLAAEGAAPFADBMBHQ
- high-pH reverse-phase HPLC - LC1260, Agilent
- Protein Lo-Bind Tubes

**BUFFERS:**

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<br>1 x |

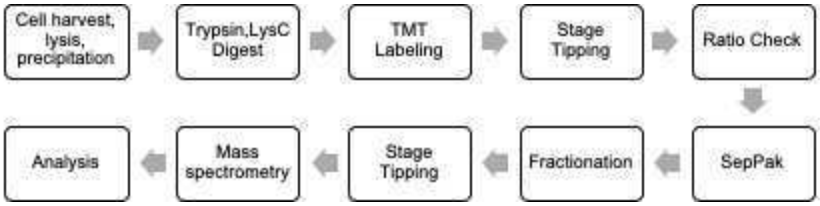
2. EPPS buffer (50 mM EPPS, pH 8.5)
3. Phosphate buffered saline (pH 7.4)

## Safety warnings

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

## Before start

**WORKFLOW:**



## Cell Culture & Treatment

1 HeLa cells are grown in tissue-vessel of choice. For galactose conditions, aspirate Glucose media, wash cells with PBS and culture for indicated times in Galactose medium. If necessary 10µM FAC was added to the media.

## Harvest, precipitation and digestion

9h 10m

2 For whole proteome analysis, 50 µg of protein lysate is required for each replicate. Lyse cells in lysis buffer and pass them through a 21G needle 10 times. Alternatively, lyse cells by sonication as per manufactures instructions.

3 Centrifugate suspensions at 13000 rpm, 4°C , (high speed) for 00:10:00 and collect the supernatant.

10m

4 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

5 Extract protein content by methanol-chloroform precipitation and subsequent MeOH washes.

5.1 Add 4x volumes of MeOH and vortex.

5.2 Add 1x volume of chloroform and vortex.

5.3 Add 3x volume of water and vortex.

5.4 Spin down at Room temperature for 00:05:00 at high speed.

5m

5.5 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).

5.6 Add 4x volumes of MeOH and vortex.

5.7 Spin down at Room temperature for 00:05:00 at high speed. 5m

5.8 Aspirate and discard supernatant. Do not disturb the protein pellet at the bottom of the tube.

5.9 Repeat MeOH wash.

5.10 Air dry (or speed-vac) protein pellet down to remove all traces of MeOH.

6 Resuspend protein pellets 100 µL of [M] 200 millimolar (mM) EPPS buffer ( pH 8.5 ).

7 Digest samples at 37 °C for 02:00:00 with endoproteinase Lys-C (Wako, Japan) at a 1/200 enzyme/protein ratio. 2h

8 Digest with Trypsin (1:100) for 06:00:00 at 37 °C . 6h

## TMT-Labeling of samples 1h 15m

9 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.

10 Incubate for 01:00:00 at Room temperature to label the samples. 1h

11 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 15m

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

15m

12 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.

13 Make stage tip by placing 6-8 “cookies” of C-18 embedded membranes in 200 µL Rainin tip .

14 Perform C-18 cleanup:

14.1 Equilibrate C-18 with 100 µL of 100% methanol .

14.2 Wash C-18 with 50 µL of 50% ACN/5% FA .

14.3 Wash C-18 with 100 µL of 5% ACN/5% FA .

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

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

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

15 Dry down eluted peptides in speed-vac.

16 Reconstitute peptides in 10 µL 5% ACN/5% FA .

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

18 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

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

20 Re-constitute the sample in 750 µL of 5% ACN/5% FA .

## SepPak clean-up (C18 solid-phase extraction (SPE)) 15m

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

22 Fill SepPak with 1 mL 100% MeOH .

23 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.

24 Fill SepPak with 1 mL 5% FA/50% ACN , Flow Liquid through.

25 Fill SepPak with 1 mL 5% FA/5% ACN , Flow Liquid through, repeat 2 more times.

26 Replace tube with 2 ml collection tube.

27 Add Peptides, Flow Liquid through (~ 750 µl).

28 Wash with 1 mL 5% FA/5% ACN , Flow Liquid through, 2 times.

29 Replace 15 ml collection tube with 2 ml collection tube.

30 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.

31 Dry down in SpeedVac Room temperature Overnight or 30 °C. 15m

## Fractionation 1h 30m

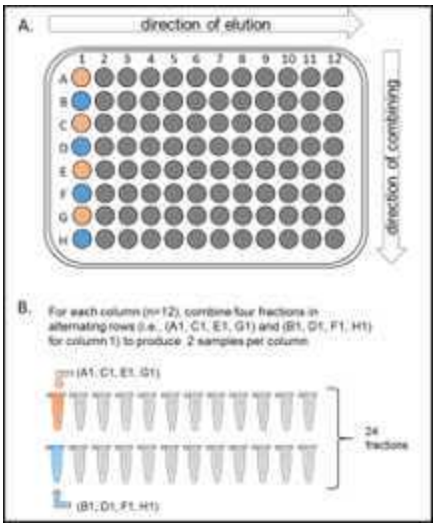
32 Resuspend sample in 100 µL of 10 mM NH4HCO3 pH 8.

33 Fractionate using pH reverse-phase HPLC:

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

33.2 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 $H_20$).

34 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**).




35 Dry down in SpeedVac.

36 Resuspend peptides in 100 µL 5%FA /5% ACN .

37 Check pH (~3.5) with pH indicator strips.

## Stage tip for proteomics sample

3m

38 Stage tip each fraction.

38.1 Make stage tips and equilibrate. Spin down at 3000 rpm, 00:03:00 . 3m

38.2 Perform C-18 cleanup:

38.3 Wash with 50 µL 50% ACN /5% FA .

38.4 Wash with 100 µL 5% ACN /5% FA .

38.5 Load sample

38.6 Collect flow through and freeze.

38.7 Wash with 50 µL 50% ACN /5% FA .

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

39 Dry down in SpeedVac.

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

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