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TMT proteomic analysis of purified proteasomes or other purified protein complexes

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We use this protocol and it's working

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Abstract

This protocol described a general protocol for TMT labeling of diverse samples, with the proteasome as a featured complex. The approach is useful as well for immune complexes, isolated organelles, or whole cell lysates. Inclusion of appropriate controls is important to consider.

Materials

Reagents:

- Phosphate Buffered Saline: powder for 5 L of 10X **Santa Cruz Biotechnology Catalog #**sc-24947
- TCEP-HCl **Gold Biotechnology Catalog #**TCEP2
- Acetonitrile **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34851
- Sodium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S9888
- Lysyl EndopeptidaseR (Lys-C) **Wako Catalog #**129-02541
- EPPS **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E9502
- 2-Chloroacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**C0267
- Pierce™ High pH Reversed-Phase Peptide Fractionation Kit **Thermo Fisher Catalog #**84868
- TMT10plex™ Isobaric Label Reagent Set **Thermo Fisher Scientific Catalog #**90406
- Bio-Rad Protein Assay Dye Reagent Concentrate **Bio-Rad Laboratories Catalog #**5000006
- Sep-Pak C18 1 cc Vac Cartridge 50 mg Sorbent per Cartridge 55-105 µm 100/pk **Waters Catalog #**WAT054955
- 3M™ Empore™ C18 47 mm Extraction Disc Model 2215 20 pack 3 packs per case **3M corporation Catalog #**2215

	A	B	C
	REAGENT or RESOURCE	SOURCE	IDENTIFIER
	Chemicals, Peptides, and Recombinant Proteins		
	KCL	Sigma-Aldrich	P9541
	PBS (10x)	Santa Cruz	sc-24947
	TCEP	Gold Biotechnology	TCEP2
	Formic Acid (FA)	Sigma-Aldrich	94318
	Acetonitrile (ACN)	Sigma-Aldrich	34851
	Sodium Chloride	Sigma-Aldrich	S9888
	Trypsin	Promega	Custom order
	Lys-C	Wako Chemicals	129-02541
	EPPS	Sigma-Aldrich	E9502
	2-Chloroacetamide	Sigma-Aldrich	C0267

	A	B	C
	Critical Commercial Assays		
	Pierce‰ High pH Reversed-Phase Peptide Fractionation Kit	Thermo Fisher Scientific	84868
	Tandem Mass Tags	Thermo Fisher Scientific	90406
	Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	5000006
	Other		
	Sep-Pak C18 1cc Vac Cartridge, 50 mg		
	Empore‰ SPE Disks C18	3M Bioanalytical Technologies	2215
	TMTPro 18Plex tandem mass tags	Thermo Fisher Scientific	A52045
	TMTPro 16Plex tandem mass tags	Thermo Fisher Scientific	A44520
	TMTPro 10Plex tandem mass tags	Thermo Fisher Scientific	A58332

Proteasome or Other Protein Samples

1 It is often useful to perform Tanden Mass Tagging based proteomics on various types of samples, including purified proteins, purified organelles, or whole cell lysates. This protocol for tagging of peptides with TMT reagents has proven useful across diverse sample types, including proteasomes and organelles.

Proteasomes are purified as described previously using a GST-UBL resin and elution from the resin with a peptide containing the UIM motif, which competes the proteasome off of the resin. The method is reported in: C. L. Kuo, G. A. Collins, A. L. Goldberg, Methods to Rapidly Prepare Mammalian 26S Proteasomes for Biochemical Analysis. *Methods Mol Biol* **1844**, 277-288 (2018). [10.1007/978-1-4939-8706-1_18]. Eluted proteasomes are used for trypsinization and subsequent analysis by proteomics. If desired, samples of purified proteasomes can be examined by immunoblotting with specific antibodies (e.g. PSMA3) prior to proteomic analysis. Typically, one would have 2-4 replicate purified samples, which would be processed for trypsinization and TMT labeling in parallel.

This protocol is also suitable for other protein samples such as protein complexes purified by immunoprecipitation, for example, specific immune complexes from expressed genes (e.g. HA-tagged proteins) or entire organelles purified by tagged proteins under non-denaturing conditions (e.g. Endo-IP or Lyso-IP).

The amount of protein to be used will depend of the experiment. Five to 25 micrograms per sample is typically suitable for most applications. Note that for most comparisons by TMT, proper negative controls are needed.

Protein digestion (trypsinization)

2 Reduce lysates for 00:30:00 at 25 °C (Room temperature) with [M] 5 millimolar (mM) TCEP.

3 Alkylate cysteine residues with [M] 20 millimolar (mM) Chloroacetamide for 30m 00:30:00 at Room temperature .

Note

Note: Alternatively, after this step proteins can be digested using S-trap sample preparation following micro-spin column digestion protocol (version 4.7) as provided by the manufacturer (Protifi, C02-micro-80).

4 Add TCA to eluates to a final concentration of 20% and place On ice at 4 °C for at least 01:00:00 .

5 Pellet the proteins for 00:30:00 at maximum speed at 4 °C .

6 Aspirate supernatant carefully and leave ~ 30 µL - 40 µL of solution so as to not disturb the pellet.

Note

Note: It is common not to observe a visible pellet.

7 Resuspend the pellets in 4 volumes of ice cold 10% TCA and pellet by centrifugation at 4 °C for 00:10:00 at maximum speed. Aspirate as before.

8 Resuspend the pellets in 4 volumes of ice cold methanol and pellet by centrifugation at 4 °C for 00:10:00 at maximum speed. Aspirate as before.

9 Repeat the methanol wash.

10 Aspirate methanol as before and air dry the remaining 30 µL - 40 µL of solution (speed-vac can also be used to dry sample).

11 Resuspend the dried pellets in 50 µL , [M] 200 millimolar (mM) EPPS, pH 8.0 .

12 Carry the peptide digestion out using LysC (0.25 µg) for 02:00:00 at 37 °C followed by trypsin (0.5 µg) overnight at 37 °C .

TMT Labelling

13 Add 3 µL - 4 µL of the TMT reagent and 15 µL of 100% ACN to each 50 µL sample.

14 Incubate for 01:00:00 at Room temperature .

15 Stop the reaction with 4 µL of hydroxylamine 5% for 00:15:00 at Room temperature .

16 Combine samples and dry in a speed-vac.

Basic-pH RP peptide fractionation kit (follow manufacturer's instructions)

17 Follow manufacturer’s instructions (Thermo Cat# 84868).

18 Use elution: 17.5% ACN, 20% ACN, 22.5% ACN, 25% ACN, 27.5% ACN and 70% ACN.

19 Speed vac individual samples to dryness.

Stage Tip

20 Resuspend samples in 100 µL of 5% FA, 5% ACN. Check to ensure that the pH of the samples is ~pH3 (or lower) using pH strips.

21 Perform C-18 cleanup:

21.1 a. Wash C-18 with 100 µL of 100% methanol.

21.2 b. Equilibrate C-18 with 50 µL of 50% ACN 5% FA.

21.3 c. Equilibrate C-18 with 100 µL of 5% ACN 5% FA.

21.4 d. Load sample on to C-18 to bind peptides.

21.5 e. Collect flow through and freeze.

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

21.7 g. Elute peptides off C-18 with 50 µL of 75% ACN/5 % FA.

22 3. Dry down eluted peptides in speed-vac.

23 4. Re-constitute peptides in 10 µL of 5% ACN 5% FA.

Mass spectrometry

24

Note

The analysis of 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.

Instrument settings

25 Collect mass spectrometry data 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).

26 Separate the peptides on a 100 µm inner diameter microcapillary column packed in house with ~ 30 cm of Accucore C18 resin (2.6 µm , 150 Å, ThermoFisher Scientific, San Jose, CA) with a gradient consisting of 5%–21% (ACN, 0.1% FA) over a total 01:30:00 run at ~ 500 nL/min . 1h 30m

Note

Details of typical instrument parameters are provided below. For Multi-Notch MS3-based TMT analysis, the scan sequence began with an MS1 spectrum (Orbitrap analysis; resolution 60,000; mass range 375–1500 m/z; standard automatic gain control (AGC); auto maximum injection time).

27 Select the precursors for MS2 analysis using a Top10 method.

Note

MS2 analysis consisted of collision-induced dissociation (quadrupole ion trap analysis; Turbo scan rate; AGC $2x10^4$; isolation window 0.7 Th; normalized collision energy (NCE) 35; maximum injection time 35 ms).

28 Use the monoisotopic peak assignment and exclude the previously interrogated precursors using a dynamic window and perform the dependent scans on a single charge state per precursor.

29 Following acquisition of each MS2 spectrum, collect a synchronous-precursor-selection (SPS) MS3 scan on the top 10 most intense ions in the MS2 spectrum.

30 Fragment the MS3 precursors by high energy collision-induced dissociation (HCD) and analyze using the Orbitrap (NCE 55; AGC 1.5×10^5; maximum injection time 150 ms, resolution was 50,000).

Data Analysis

31

Note

Data analysis will be platform and purpose specific.

32 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) set as variable modifications

33 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.

Note

This process will depend on the type of analysis software employed with the specific MS platform being used.

Protocol references

C. L. Kuo, G. A. Collins, A. L. Goldberg, Methods to Rapidly Prepare Mammalian 26S Proteasomes for Biochemical Analysis. *Methods Mol Biol* **1844**, 277-288 (2018). [10.1007/978-1-4939-8706-1_18].

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