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# TMTpro HUNTER N-terminomics

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1 *Works for me* This protocol is published without a DOI.

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ABSTRACT

Protocol for N-terminomic analysis of protease substrates. This method is an adaption of the Weng et al. 2019 Mol. Cell. Proteomics (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6823850/) method for N-terminomics, modified to allow sample multiplexing and quantification using TMTpro reagents rather than the original implementation from the Lange lab which used dimethyl or DIA-based quantification. This method was used in Meyer et al. 2020 - https://www.biorxiv.org/content/10.1101/2020.09.16.297945v1.

If you use this protocol, please cite both the Meyer et al. and Weng et al. publications.

This adaption has advantages for the analysis of protease substrates, permitting sample multiplexing early in the protocol and allowing all samples to be processed subsequently as a single sample. This protocol has disadvantages compared to its parental protocol for analysis of native N-termini where blocking of the native N-terminus by acetylation or pyroglutamine prevents TMT labelling, and thus quantification of non-lysine-containing peptides. As neo-N-termini generated by viral proteases are not expected to possess natively-blocked N-termini (aside from a possible minority of pyroglutamine N-termini), these are not a consideration here.

In brief:
1. Cells are lysed in SDS and heated
2. Samples are reduced and alkylated.
3. Protein is precipitated on SP3 beads
4. Protein is resolubilised and TMTpro labelling is performed at the **protein level** to block N-termini and lysine residues
5. Unreacted TMTpro reagent is quenched and washed away.
6. Samples are pooled.
7. Overnight Tryptic digestion.
8. Unblocked N-termini generated by tryptic digestion are hydrophlabelled with undecannal.
9. Undecannal-labelled N-termini are depleted by passing the peptides over C18 resin in 50% ethanol, resulting in flow-through containing natively-blocked and TMTpro-labelled N-termini.

This protocol can be accomplished in aproximately two days.

EXTERNAL LINK

http://emmottlab.org

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

https://www.biorxiv.org/content/10.1101/2020.09.16.297945v1

PROTOCOL CITATION

Edward Emmott 2020. TMTpro HUNTER N-terminomics. **protocols.io**
https://protocols.io/view/tmtpro-hunter-n-terminomics-bi44kgyw

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

https://www.biorxiv.org/content/10.1101/2020.09.16.297945v1

EXTERNAL LINK

http://emmottlab.org

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Proteomics, Mass Spectrometry, N-terminomics, Protease



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DISCLAIMER:

> It works for me (TM).

## Day 1

1

One T25 dish of ACE2-A549 cells/sample was collected by centrifugation, washed 3x with PBS, and the pellet frozen in a 5mL low-bind Eppendorf for use the next day.

## Day 2 - Sample cleanup

2

Cell pellets resuspended in 200uL lysis buffer consisting of:
- 1% SDS
- 2x Thermo HALT protease inhibitor
- 100mM HEPES, pH8
- 1% NP40

3 5m

Sample heated to 95C for 5 minutes

4 5m

Sample chilled on ice for 5 minutes

5

Sample briefly centrifuged to collect condensation

6 30m

Benzonase added at 1 in 200 dilution and incubated at 37C for 30 minutes

7

A protein assay should be performed e.g. BCA. Normalise sample volumes to 110uL, containing 25ug of material

8 30m

2uL of 1M DTT added and incubated at 37C for 30 minutes
Note: DTT should be made up fresh from stock

9 30m

**11.2**uL of **0.5M** 2-chloroacetamide added, incubated at RT in the dark for 30 minutes.
Note: 2-CAA should be made up fresh from stock

10 20m

6uL of 1M DTT added to quench CAA, incubated at RT in the dark for 20 minutes.

11

2.5uL of previously prepared SP3 beads was added to each low-bind tube.
Note 1:10 protein:bead ratio with beads at **20**ug/uL

12

Check volume: If you have been following the instructions above, each sample should be 141uL volume. If not, adjust volume with HPLC-grade water

13 18m

Add 564uL 100% ethanol to initiate binding, incubate for 18 minutes at RT.

14 5m

Incubate on magnetic stand for 5 minutes.

15

Beads should have collected at the side of the tube, and the supernatent should be clear. Remove supernatent

16

Wash beads twice with 400uL 90% ethanol.
Note: do not disturb beads

17

Briefly centrifuge beads and pipette off any remaining liquid

Day 2 - Sample Labelling

18

Resuspend beads in:

22.5uL 6M GuCL
30uL 0.5M HEPES pH8
4.5uL TCEP to 10mM final (diluted 1 in 3.5 from 500mM stock)

19

Incubate beads at room temperature for 30 minutes
During this incubation, prepare the TMT in the following two steps

20 10m

Remove the TMTpro from the -80C, and allow it to equilibrate to room temperature before opening

21 40m

Dissolve TMTpro labels in 62uL of anhydrous DMSO, mix well. The TMTpro can take a while to dissolve
Note - seriously, use the GOOD anyhydrous DMSO. TMT aint cheap!

22 5m

Add the 57uL TMTpro/DMSO to each 57uL sample. Mix well by pipetting.
Note - it is good practice to randomise the allocation of TMT/TMTpro labels to samples.

23 1h 30m

Incubate at RT in the dark for 1.5h.

24 45m

Add **13**uL of 1M ethanolamide, mix well and incubate for 45 min to quench unreacted TMT labels.

25

Combine samples into a single tube. For a full 16plex TMTpro experiment this will give a volume of 16 * 133uL = 2128uL, containing ~400ug total protein

Day 2 - Post-label cleanup

26

To ease sample handling. Divide sample into 6 * 354uL aliquots in 2mL tubes

27

Add 13uL of SP3 beads (20ug/ul) per aliquot

28

1468uL 100% ethanol added

29 15m

Incubate beads at RT off the magnetic stand for 15 minutes.

30 5m

Incubate beads on stand for 5 minutes (or until clear)

31

Remove supernatant.

32

Wash beads twice 600uL 90% ethanol.
Note: do not disturb beads

33

Briefly centrifuge beads and pipette off any remaining liquid

## Day 2 - Overnight digest 13h

34

Recombine the Six aliquots by resuspending in **400uL** of 200mM HEPES, pH 8

35

Add 20ug Trypsin Gold in 10uL of 200mM HEPES pH8.

36 13h

Incubate sample **overnight** at 37C.
Note - minimum 13h

## Day 3 - Sample cleanup 1h 6m 45s

37 5m

Place sample onto magnetic rack for 5 minutes.

38

Retain 10% of the sample (40uL) to assess protein labelling and protein abundance (unenriched sample)

39 Remove sample from magnetic rack.

40

Add 240uL 100% ethanol resuspending the beads
Note: should take to 40% ethanol

41 30s

Tap the tube and sonicate for 30s to mix.

42

Add 18uL undecanal
Note: this is ~97% undecanal, and assumes the protocol has 400ug total protein (-10%) in the sample

43 30s

Tap the tube and sonicate for 30s to mix.

44

Add 18.5uL of 1M sodium cyanoborohydride
Note: Gives 30mM final
Note: make 1M sodium cyanoborohydride fresh

45 30s

Tap the tube and sonicate for 30s to mix.

46

Confirm pH7-8 by spotting 1uL of sample onto pH paper

47 1h

Incubate at 37C for 1h.

48 15s

Sonicate in waterbath for 15s to mix

49

Bind tube to magnetic stand foor 1 minute.

50

Transfer the supernatent to a fresh tube

Day 3 - enrichment 6m

51

Acidify supernatant with 5% TFA in 40% ethanol to pH3-4.

52

Confirm pH3-4 by spotting 1uL onto filter paper

53 

Adjust final volume to 1600uL with 1% TFA in 40% ethanol. Set tube to one side.

54 

2m

Add 400uL methanol to each of 4 macrospin columns to condition them. Centrifuge 2m, 300g

55 

2m

Add 400uL 0.1% TFA in 40% ethanol to each of the 4 macrospin columns. Centrifuge 2m, 300g

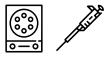
56 

2m

Repeat previous step

57 

Place each macrospin column in a fresh 2mL collection tube

58 

Load 400uL of the acidified sample to each of the 4 macrospin columns. Centrifuge 2m, 300g.

59 

Discard the macrospin column, retain the flow-through liquid in the 2mL collection tube. This contains your N-terminally enriched peptides.

Day 3 - post-enrichment cleanup

60 Dry sample on speed-vac, resuspend in 0.1% TFA.

61 Sample should be desalted by standard methods (e.g. macrospin column). N-terminal identifications can be improved by further sample fractionation after desalting, for example basic reverse phase.