

# Discovery and Interrogation of Functional Protein Modifications by Hotspot Thermal Profiling

Jun X. Huang

University of Chicago <https://orcid.org/0000-0002-1318-8562>

Gihoon Lee

University of Chicago

Raymond E. Moellering (✉ [rmoellering@uchicago.edu](mailto:rmoellering@uchicago.edu))

University of Chicago

---

## Method Article

**Keywords:** Proteomics, Post-Translational Modifications, Biophysics, Mass Spectrometry, Signaling, Thermal Profiling

**Posted Date:** August 8th, 2019

**DOI:** <https://doi.org/10.21203/rs.2.10602/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

Hundreds of protein posttranslational modification types have been reported across diverse organisms, however we still lack methods to systematically predict, or even prioritize, which modification sites may perturb protein function under specific cellular contexts. This protocol describes a method to detect the effects of site-specific protein phosphorylation on the thermal stability of thousands of native proteins in live cells. This mass spectrometry-based protocol measures shifts in overall protein stability in response to site-specific phosphorylation sites. The resulting dataset can enable discovery of intrinsic changes to protein structure as well as extrinsic changes to protein-protein, and protein-metabolite interactions, and can help prioritize site-specific study in a high-throughput and unbiased fashion. This approach takes several days complete, can be performed with multiple samples in parallel and is applicable to diverse organisms, cell types and posttranslational modifications.

## Introduction

Recent advances in global profiling methods have greatly accelerated the discovery and study of endogenous protein posttranslational modification (PTM)<sup>1</sup>, which represents a central signaling mechanism in all aspects of cellular function. Chief among these technologies are liquid chromatography-mass spectrometry (LC-MS) proteomics platforms, which are typically integrated with front-end, PTM-specific enrichment protocols to detect modified peptides and proteins from biological samples. Coupled with data-dependent and de-novo PTM discovery algorithms, mass spectrometry-based studies have discovered tens of thousands unique PTM sites on proteins from diverse organisms<sup>2-4</sup>. More recently, quantitative proteomic methods have enabled the dynamics of these sites to be studied in diverse cell states and responses to stimuli<sup>5-7</sup>. A critical question that remains, however, is how minute changes to chemical structure alters the biophysical properties of a much larger protein, and how these changes lead to signaling and phenotypic consequences. The traditional approach to address this problem involves identifying specific modification sites associated with a phenotype of interest, and then laboriously studying the properties of mutant proteins that attempt to mimic the modified protein one-by-one. Because we lack general methods with which to predict, or even prioritize, which modification sites are likely to be functional in the proteome, the downstream study of PTMs is intrinsically inefficient and low-throughput.

Here we sought to address these limitations by developing a quantitative proteomic method that enables direct, high-throughput interrogation of altered protein stability in response to endogenous, site-specific posttranslational modifications for thousands of proteins in parallel. This method, Hotspot Thermal Profiling (HTP), couples PTM-specific enrichments and isotopic labeling with thermal proteomic profiling to globally detect and quantitatively measure the thermal stability of endogenous proteins and their site-specific, modified proteoforms, which we refer to herein as “modiforms,” from *in vitro* and *insitu* samples. We establish and apply this approach to a ubiquitous yet diminutive PTM – phosphorylation – and

demonstrate that global thermal profiling of the modified proteome enables specific detection of both inter- and intramolecular interactions on wide range of proteins.

Our approach is applicable to study posttranslational modifications in diverse organisms and cell types. Before embarking on this protocol, one should first optimize enrichment strategies for the PTM of interest using the appropriate cell lines. Often a large amount of bulk cell lysates is needed for sufficient enrichment. Since our protocol has ten temperature fractions and each fraction is enriched independently before pooling, variable input proteome quantities should be tested for optimal results. Other step-by-step experimental considerations are described in the protocol.

## Reagents

HEK293T (ATCC)

Iodoacetamide (Sigma Aldrich, I1149)

Lactic Acid (Sigma Aldrich, 69785)

Hydroxylamine (Thermo Fisher, 467804)

Pyrrolidine (Sigma Aldrich, 83240)

Acetonitrile (Fisher Chemical, 75-05-8 )

Formic Acid (Fluka Analytical, 94318)

Trifluoroacetic Acid (Alfa Aesar, 44630)

EDTA-free protease inhibitor tablet (Roche, 4693132001)

Phosphatase inhibitor cocktail 3 (Sigma Aldrich, P0044)

Pierce™ Trypsin Protease, MS Grade (Thermo Pierce, 90057)

Sep-Pak tC18 cartridges, 50mg (Waters, WAT200685)

TiO<sub>2</sub> spin tips (Thermo Pierce, 88303)

ZipTip C18 tips, 100 µL bed (Millipore, 87784)

TMT10plex™ Isobaric Label Reagent Set (Thermo Fisher, 90406)

Integrated Proteomics Pipeline ([Intergratedproteomics.com](http://Intergratedproteomics.com))

R Studio (Rstudio.com)

TPP Package for R ([bioconductor.org/packages/release/bioc/html/TPP.html](https://bioconductor.org/packages/release/bioc/html/TPP.html))

## Equipment

Q Exactive HF (Thermo Fisher)

Easy-nLC 1200 (Thermo Fisher)

Table top centrifuge

Thermal cycler

Lyophilizer

## Procedure

### Cell Preparation

1. Propagate HEK293T cell in RPMI 1640 media supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a 5% CO<sub>2</sub> humidified incubator until five 15-cm cell culture treated plates are grown to 90% confluence.

[Suggestion] Five 15-cm plates of HEK293T provide ~10 mg of protein lysates. Different cell lines may require different number of plates to provide the desired amount of proteome.

2. Remove media and wash cells once with DPBS.
3. Add 3 mL of DPBS to each plate and gently collect cells from plates with sterile scraper.
4. Wash cells two more times with DPBS by centrifugation at 200*g* for 2 minutes.
5. Resuspend cells in 4 mL of DPBS with EDTA-free protease inhibitor tablet and 1:100 phosphatase inhibitor cocktail 3.
6. Divide live cells into ten aliquots of 400 mL each (or 40 aliquots of 100 mL each if thermal cycler tubes are used).

## *In Situ* Thermal Pulse

7. Expose each 400 mL of cell lysates to a steady temperature between 37°C to 67°C for 3 mins in parallel (eg. aliquot #1 at 37°C for 3 mins, aliquot #2 at 40°C for 3 mins, etc.)

[Caution] Cells must be exposed to temperature treatment immediately after collection from plates and all aliquots must be treated in parallel to avoid sample variation due to time delay.

8. After heat treatment, cool and incubate cells at 25°C for another 3 mins.

9. Immediately lyse cells by rapid freeze-thawing three times with liquid nitrogen.

10. Remove insoluble proteins and cell debris by centrifugation at 17,000*g* for 10 mins.

11. Set aside a small amount of supernatant from the 37°C aliquot for protein quantification by Bradford assay.

[Caution] For high efficiency phosphopeptide enrichment, at least 700 mg of proteins (preferably 1 mg) should be present in the 37°C aliquot.

12. Collect 360 mL of lysate from each aliquot. Be careful not to disturb the pellet.

## Trypsin Digestion

13. Denature lysates in 8 M urea, followed by disulfide reduction with DTT (10 mM, 30 mins, 65°C), alkylation (iodoacetamide, 15 mM, 30 min, room temperature, protected from light) and quenching (DTT, 5 mM, 10 mins, room temperature).

14. Dilute lysates 4-fold in ammonium bicarbonate solution (50 mM, pH 8.0).

15. Add CaCl<sub>2</sub> (final concentration 1 mM) and sequencing grade trypsin (~1:100 enzyme/protein ratio) to each aliquot.

16. Rotate at 37°C while rotating overnight.
17. Quench peptide digestion reaction by acidification to pH 2-3 with 1% formic acid.
18. Desalt peptides with Sep-Pak tC18 cartridges according to manufacturer's protocol.
19. Dry eluted peptides under vacuum and resuspend in 1 mL of LC-MS grade water.
20. Reserve 50 mL or 5% from each aliquot for bulk unmodified proteome thermal profiling.
21. Freeze and lyophilize the two sets of peptides (95% and 5%).

### Phosphopeptide Enrichment

22. Resolubilize the major fraction of lyophilized peptides (95%) in 28.5% lactic acid, 57% acetonitrile, 0.28% TFA, and applied to TiO<sub>2</sub> spin tips (88303, Thermo Pierce) for phosphopeptide enrichment according to manufacturer's protocol.
23. Acidify eluted phosphopeptides with 1% formic acid until pH 2-3.
24. Desalt acidified phosphopeptides with ZipTip C18 tips according to manufacturer's protocol.
25. Freeze and lyophilize desalted phosphopeptides.

[Suggestion] Desalted peptides could be stored -20°C for months but modified peptides are more sensitive to storage and freeze-thaw cycles. For best results, modified peptides should be analyzed right away.

### TMT Labeling

26. Reconstitute TMT reagents to 8 mg/mL with anhydrous acetonitrile.
27. Re-dissolve lyophilized peptides in 100 mL of 200 mM HEPES buffer, pH 8.0. Sonicate with water bath to help with reconstitution.

28. Add 10 mL of reconstituted TMT reagents to each aliquot of peptides. Each aliquot should be labeled by a different isobaric TMT tag.
29. Incubate for 1 hour in room temperature with gentle shaking.
30. Quench reaction by adding 5 mL of 5% hydroxylamine solution to each tube.
31. Incubate for 15 mins in room temperature with gentle shaking.
32. Combine all 10 TMT-labeled phosphopeptides into one tube, and combine all 10 TMT-labeled non-enriched peptides into another tube.
33. Acidify the peptide solution by adding formic acid until pH 2-3.
34. Desalt using ZipTip C18 tips according to manufacturer's protocol.
35. Freeze and lyophilize.

#### Proteomic Analysis by LC-MS/MS

36. Dissolve processed peptides in LC-MS/MS Buffer A (LC-MS/MS grade H<sub>2</sub>O with 0.1% formic acid). Sonicate for 5 mins to help peptides dissolve if needed.
37. Perform LC-MS/MS with an Easy-nLC 1000 ultra-high pressure LC system (ThermoFisher) using a PepMap RSLC C18 column (75 µm x 50 cm; 3 µm, 100 Å, Thermo Scientific) heated to 40°C coupled to a Q Exactive HF orbitrap and Easy-Spray nanosource (ThermoFisher).
38. Separate samples using the following gradient of buffer B (0.1% Formic acid acetonitrile) at 300 nL/min: 0-10% buffer B in 5 minutes, 10-40% buffer B in 240 mins, 40-90% buffer B over 6 mins, and hold at 90% for 20 mins.
39. Collect MS/MS spectra from 0 to 250 mins using a data-dependent, top-10 ion setting with the following settings: acquire full MS scans at a resolution of 120,000, scan range of 375-1500 m/z, maximum IT of 60 ms, AGC target of 1e6, and data collection in profile mode. Perform MS2 by HCD fragmentation with a resolution of 60,000, AGC target of 1e5, maximum IT of 60 ms, NCE of 30, and data type in centroid mode. Set isolation window for precursor ions to 1.0 m/z with an underfill ratio of 0.5%. Exclude peptides with charge state 1 and undefined and set dynamic exclusion to 20 seconds. Set S-lens RF level to 60 with a spray voltage value of 2.60kV and ionization chamber temperature of 300°C.

[Suggestion] Chromatography and MS should be optimized for the PTM of interest. Further off-line fractionation might be needed to provide deep proteome coverage if PTM of interest is low in abundance.

## Proteomics Data Analysis

40. Generate a concatenated target/decoy database from UniProt in Fasta format.

41. Generate MS2 files and search data using the ProLuCID algorithm in the Integrated Proteomics Pipeline (IP2) software platform. Basic searches were performed with the following search parameters: HCD fragmentation method; monoisotopic precursor ions; high resolution mode (3 isotopic peaks); precursor mass range 600-6,000 and initial fragment tolerance at 600 p.p.m.; enzyme cleavage specificity at C-terminal lysine and arginine residues with 3 missed cleavage sites permitted; static modification of +57.02146 on cysteine (carboxyamidomethylation), +229.1629 on N-terminal and lysine for TMT-10-plex tag; 4 total differential modification sites per peptide, including oxidized methionine (+15.9949), and phosphorylation (+79.9663) on serine, threonine, and tyrosine (only for phospho-enriched samples); primary scoring type by XCorr and secondary by Zscore; minimum peptide length of six residues with a candidate peptide threshold of 500; minimum of one peptide per protein and half-tryptic peptide specificity;  $\Delta$ mass cutoff = 10 p.p.m. with modstat, and trypstat settings; false-discovery rates of peptide (sfp) at 1%. Perform TMT quantification using the isobaric labeling 10-plex labeling algorithm, with a mass tolerance of 5.0 p.p.m. or less in cases where co-eluting peptide interfere. Use reporter ions 126.127726, 127.124761, 127.131081, 128.128116, 128.134436, 129.131417, 129.13779, 130.134825, 130.141145, and 131.13838 for relative quantification.

## Melting Curve Analyses

42. Export peptide quantitative results from IP2 in Excel format.

43. Organize results in excel into 15 columns: one column displaying sequence peptide, one column displaying the custom identifier (Gene\_pSite) that localizes the phosphosite in the protein, one column displaying number of spectra detected for that peptide sequence, 10 columns displaying TMT reporter ion intensities of the 10 different temperature fractions, one column displaying protein accession, and one column displaying protein description.

44. Use the consolidate function in Excel, integrate TMT reporter ion intensities of the same temperature fraction for peptides that map to the same phosphosite(s) using Gene\_pSite column as filter.

45. Calculate fold change values by dividing all 10 reporter ion intensities by the reporter ion intensity of the 37°C fraction.

46. Generate unmodified protein and phosphomodiform melt curves, fit relative fold changes as a function of temperature to the equation derived from the chemical denaturation theory using the R package developed by Savitski et al (Science, 2014).  $T_m$  values are calculated at which the sigmoidal curve crosses the 0.5-fold change level.

47. Remove  $T_m$  values from curves with sigmoidal curve  $R^2 < 0.8$ .

48. Calculate  $\Delta T_m$  by subtracting  $T_m$  of the bulk, unmodified protein from that of the phosphomodiform.  $\Delta T_m$ s could only be calculated for phosphomodiforms belonging to proteins that are also detected in the unmodified proteome.

## Troubleshooting

### Problem

Very few modified peptides detected.

### Reason

Not enough bulk peptides are used for enrichment.

### Solution

Use more cells so that more raw proteome can be collected. Use at least 700  $\mu$ g of peptides in the lowest temperature fraction prior to enrichment.

### Reason

Low enrichment efficiency.

### Solution

Try a different brand/manufacturer of modification enrichment reagent. Experiment with different enrichment buffer conditions and protocol timing.

### Reason

MS and/or chromatography not optimized for modified peptides.

#### Solution

Optimize methods for TMT-or specific PTM-modified peptide detection to account for unique physicochemical properties. Alter chromatography settings or chemistry. Alter ionization settings.

#### Reason

Peptides degraded in storage.

#### Solution

Perform LC-MS/MS experiments as soon as samples are finished processing.

## Time Taken

Cell preparation: 1 hour

*In situ* thermal pulse workflow: 2 hours

Trypsin digestion: 2 hours for pre-digestion sample preparation and 16 hours overnight incubation

Peptide desalting: 2-3 hours using gravity elution, overnight lyophilization

Phosphopeptide enrichment: 4 hours sample preparation, overnight lyophilization

TMT labeling: 4 hours sample preparation, overnight lyophilization

LC-MS/MS analysis: 6-7 hours total running time per injection including 4 hours of analytical gradient and 2-3 hours of instrument equilibration

ProLuCID search: 0.5 to 1 day per MS run

Data analysis: 1 day

Time for the entire procedure: 7 days; samples can be processed in parallel.

## Anticipated Results

Depending on LC-MS/MS instrumentation and settings this method should yield >4,000 proteins from combined replicates of the bulk, unmodified proteome and >10,000 TMT-labeled phosphopeptides from

the phospho-enriched proteome.

## References

1. Aebersold, R. & Mann, M. Mass-spectrometric exploration of proteome structure and function. *Nature***537**, 347-355 (2016).
2. Huttlin, E.L., *et al.* A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell***143**, 1174-1189 (2010).
3. Olsen, J.V. & Mann, M. Status of large-scale analysis of post-translational modifications by mass spectrometry. *Molecular & cellular proteomics : MCP***12**, 3444-3452 (2013).
4. Walther, T.C. & Mann, M. Mass spectrometry-based proteomics in cell biology. *The Journal of cell biology***190**, 491-500 (2010).
5. Humphrey, S.J., Azimifar, S.B. & Mann, M. High-throughput phosphoproteomics reveals in vivo insulin signaling dynamics. *Nature biotechnology***33**, 990-995 (2015).
6. Olsen, J.V., *et al.* Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci Signal***3**, ra3 (2010).
7. Tsai, C.F., *et al.* Large-scale determination of absolute phosphorylation stoichiometries in human cells by motif-targeting quantitative proteomics. *Nat Commun***6**, 6622 (2015).

## Acknowledgements