Quantitative analysis of signaling pathways using TMT 11plex reagents and comprehensive phosphopeptide enrichment strategies

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ABSTRACT

Purpose: There is broad interest in quantifying protein phosphorylation alterations in cellular signaling pathways under different conditions. The transient nature and low abundance of many phosphorylation sites makes this challenging. We have combined SMOAC (Sequential enrichment of Metal Oxide Affinity Chromatography) method with Tandem Mass Tag (TMT) isobaric labeling reagents to evaluate changes in phosphorylated proteins expressions under different stimulation conditions.

Methods: HeLa cells were grown with 10 different conditions of starvation/stimulations (Nocodazole/TPA/hIGF-1/hEFG/hPDGF-bb/FBS) before being subjected to in-solution digestion. Thermo Scientific TMT10plex tags plus a novel TMT11-131C reagent were used to label 0.45mg of HeLa digest for each condition. 5mg of combined TMT11plex labeled peptides was subjected to Thermo Scientific Pierce Hi-Select™ TiO2 phosphopeptide enrichment kit (PN#A32993). TiO_2 flow-through/wash fractions were enriched with the Thermo Scientific Pierce Hi-Select™ Fe-NTA phophopeptide enrichment kit (PN#A32992). Both eluents were combined and fractionated using the Thermo Scientific Pierce™ High pH Reversed-Phase Peptide Fractionation Kit (PN#84868) before LC-MS analysis using Thermo Scientific Orbitrap ™ Fusion™ instrument. Thermo Scientific Proteome Discoverer 2.2 software was used to localize the phosphorylation sites.

Results: TMT11plex with the SMOAC method allowed comprehensive identification and quantitation of phosphopeptides across different conditions. A peptide assay before TMT labeling and after phosphopeptide fractionation enabled normalization of peptide amounts from different condition. High pH reversed-phase fractionation after phosphopeptide enrichment resulted in better throughput and proteome depth for profiling changes in phosphopeptide expressions. Excellent selectivity and specificity for phosphopeptides were achieved with this improved SMOAC workflow.

INTRODUCTION

Post translational modifications (PTMs) are crucial in controlling key aspects of protein function, including interactions in signaling pathways. Identification and quantitation of the phosphorylation state of proteins involved in cell progression, metabolism, growth, and disease is critical for the continued elucidation of cellular function[1]. Despite improvements in new MS instrumentation, phosphoproteomic analyses still face challenges including low yield/specificity of phosphopeptide enrichment, poor assignment of phosphorylation sites and low phosphorylation site stoichiometry. Advances in multiplexed quantitation utilizing isobaric tags (e.g., TMT) have the potential to create a new paradigm in quantitative phosphoproteomics[2]. Amine-reactive isobaric TMT tags enable concurrent identification and multiplexed quantitation of proteins in different samples using tandem mass spectrometry[3-4]. The SMOAC (Sequential enrichment of Metal Oxide Affinity Chromatography) method was optimized in which the phosphopeptides were enriched by TiO_2 first and the TiO_2 flow-through (FT) and wash fraction were pooled and subjected to Fe-NTA. In this comprehensive phosphoproteomics study, we have combined TMT11plex quantitation, SMOAC method, and high pH reversed phase fractionation to evaluate network dynamics in HeLa cells under different stimulation conditions.

Figure 1. Structures of the TMT10plex reagent set plus TMT11-131C reagent with isotope positions noted (*).

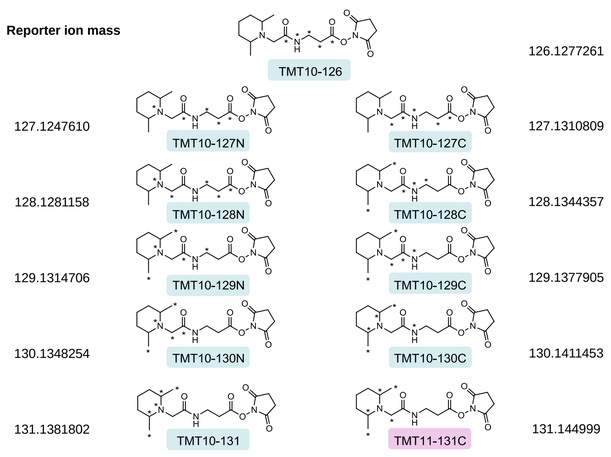
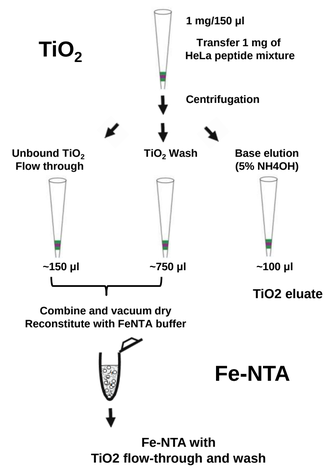


Figure 2. Sequential enrichment of Metal Oxide Affinity Chromatography (SMOAC) for phosphopeptide enrichment.



MATERIALS AND METHODS

Cell Culture

HeLa cells from the ATCC (PN#CCL-2) were cultured in Life Technologies™ Gibco™ DMEM with high glucose/10% Fetal Bovine Serum/1XPenicillin-Streptamycin complete media. All stimulants and drugs were acquired from Cell Signaling Technology. HeLa cells at approximately 70% confluency were treated with the following conditions for 15 minutes after 24 hr serum starvation with DMEM plus high glucose/0.1% charcoal-stripped FBS: no treatment, 100ng/mL hIGF-1 (PN#8917LF), 50ng/mL hEGF(PN#8916SF), 50ng/mL hPDGF-bb(PN#8912SF), 10% FBS, or 400nM TPA(PN#4174S). HeLa cells at approximately 70% confluency were treated with 100ng/mL hIGF-1 for 15 minutes or without treatment after serum starvation for 1 hour with DMEM plus high glucose/0.0% charcoal-stripped FBS. HeLa cells at approximately 70% confluency were treated with the following conditions without serum starvation in complete media: no treatment or 100ng/mL Nocodazole (PN#2190S) for 24 hours. This resulted in 10 different conditions with HeLa cultured cells. Cells were lysed by mechanical scraping with 100mM TEAB/1.0% SDS/1X Thermo Scientific™ Halt™ phosphatase inhibitor (PN#1861281) as lysis buffer, followed by sonication, centrifugation to remove the pellets, and Thermo Scientific™ Pierce™ BCA Protein Assay (PN#23225) was performed for protein quantitation.

MS Sample Preparation and TMT11plex

Equivalent amounts of lysate from each condition were processed using Pierce™ Mass Spec Sample Prep Kit for Cultured Cells (PN#84840). The digested samples were quantitated with the Thermo Scientific™ Pierce™ Fluorometric Peptide Assay (PN#23290) and were labeled with TMT10plex (PN#90406) reagents and TMT11-131C according to manufacturer's instructions. Aliquots of each sample were mixed in equimolar ratios before sample desalting. TMT11-131C was used to label an equimolar pool of all samples which was combined with TMT10plex samples. Samples were desalted with Waters™ tC18™ 500mg SepPak™ columns (PN#WAT043425). Samples were quantitated with the Thermo Scientific™ Pierce™ Colorimetric Peptide Assay (PN#23275) & aliquoted into 1mg portions.

Phosphopeptide Enrichment and High pH Reverse Phase Fractionation

The optimized SMOAC method was used for phosphopeptide enrichment. One milligram per replicate of 11-plex HeLa digest was subjected to Thermo Scientific™ Pierce™ HiSelect™ TiO_2 phosphopeptide enrichment kit (PN#A32993) and the TiO_2 eluent was saved for MS analysis. The TiO_2 flow-through and wash fractions were pooled, and the phosphopeptides were enriched by Thermo Scientific™ Pierce™ HiSelect™ Fe-NTA phosphopeptide enrichment kit (PN#A32992). Replicate samples for all TiO_2 enrichment steps and Fe-NTA enrichment steps were combined into separate pooled samples. After SMOAC, phosphopeptides were quantitated using the Thermo Scientific™ Pierce™ Colorimetric Peptide Assay (PN#23275) and fractionated with the Thermo Scientific™ Pierce™ High pH Reverse-Phase Peptide Fractionation Kit (PN#84868).

LC-MS Analysis

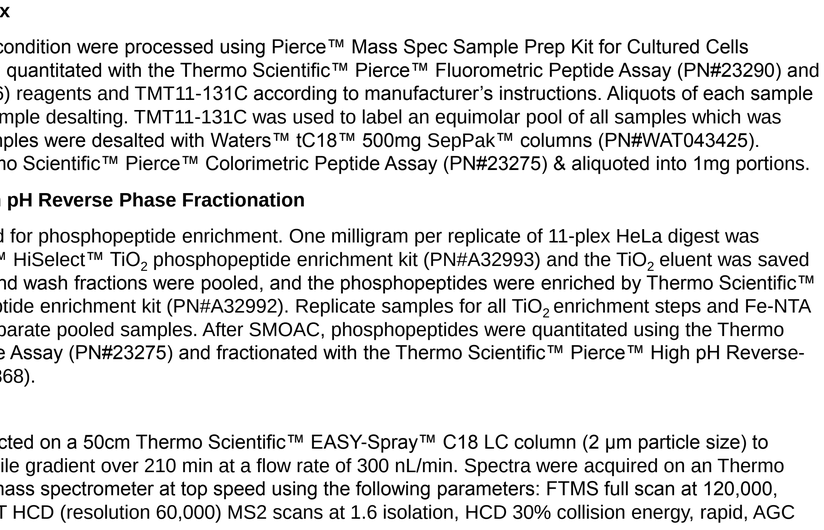
For the LC-MS analysis, 1~2 ug was injected on a 50cm Thermo Scientific™ EASY-Spray™ C18 LC column (2 µm particle size) to separate peptides with a 5-30% acetonitrile gradient over 210 min at a flow rate of 300 nL/min. Spectra were acquired on an Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer at top speed using the following parameters: FTMS full scan at 120,000, AGC 4e5, IT 50ms followed by $ddMS^2$ OT HCD (resolution 60,000) MS2 scans at 1.6 isolation, HCD 30% collision energy, rapid, AGC target 5.0e4, IT 50ms.

Data Analysis

Thermo Scientific™ Proteome Discoverer™ 2.2 software was used to search MS/MS spectra with the Byonic™ or Sequest™ HT search engine with a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da. Static modifications included carbamidomethylation (C) and TMTsixplex (N-terminal, K). Dynamic modifications included methionine oxidation and phosphorylation (S,T,Y). PhosphoRS node was used for site localization. Data was searched against a Swiss-Prot® human database with a 1.0% FDR criteria using Percolator. Resulting peptide hits were filtered for maximum 1% FDR using Percolator[5]. A custom TMT11plex quantification method was used to calculate the reporter ratios with mass tolerance ±10 ppm and to apply isotopic correction factors.

Figure 3. Schematic of TMT reagent sample preparation, phosphopeptides enrichment and fractionation followed by LC-MS analysis.

Sample	Serum-starved	Treatment
1	-	-
2	+ (1 hr)	-
3	+ (24 hr)	-
4	+ (24 hr)	+ FBS
5	-	+ NOC
6	+ (24 hr)	+ TPA
7	+ (24 hr)	+ EGF
8	+ (1 hr)	+ IGF-1
9	+ (24 hr)	+ IGF-1
10	+ (24 hr)	+ PDGF
11	Pool	Pool

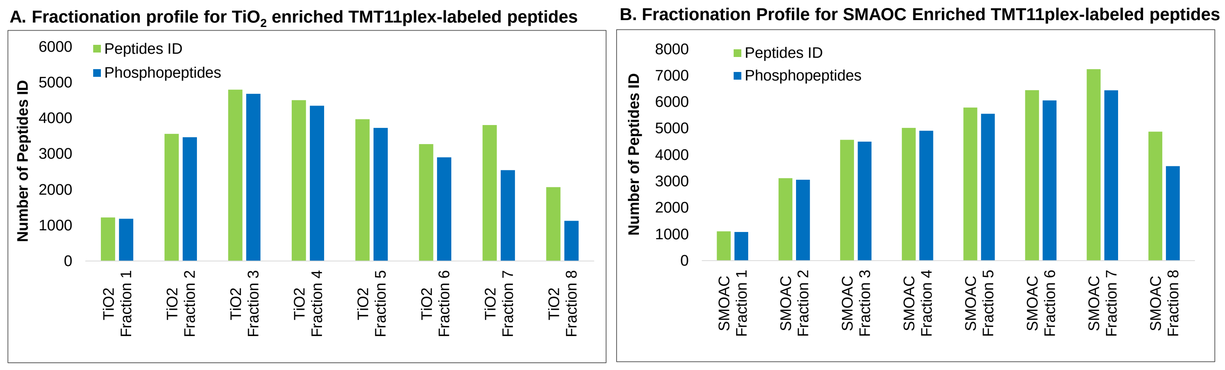


Proteins from different conditions were extracted, reduced, alkylated, and digested before being labeled with TMT10plex reagents. TMT11-131C was used to labeled a pooled sample of all 10 conditions. Labeled peptides from each treatment condition were combined, cleaned with C18 and further enriched by optimized SMOAC method followed by high pH reverse phase chromatography fractionation before subjected to LC-MS/MS analysis.

Results

Previously, we expanded isobaric tandem mass tag multiplexing from 6plex to 10plex using high resolution mass spectrometry (>50K at m/z 200) to separate 15N and 13C stable isotope variants. Using the same principle, we synthesized the full 13C isotope variant of the TMT-131 reporter, called TMT11-131C (Figure 1). This tag increases isobaric tag multiplex quantitation to 11 samples in a single LC-MS analysis without any changes in reagent structure or LC-MS/MS analysis (Figure 3). HeLa cell lysates prepared with 10 different conditions were labeled with each reagent before the SMOAC phosphopeptide enrichment followed by high pH RP fractionation. This optimized workflow resulted in identification of 33,000 phosphopeptides including ~ 24,000 with quantitation and site localization (confidence score >90) (Table 1). Furthermore, this comprehensive phosphoproteomics analysis allowed quantitation of phosphorylation changes for multiple signaling pathways proteins under different conditions (Figure 5).

Figure 4. Analysis of TMT11plex-labeled phosphopeptides enriched using TiO_2 (A) and SMOAC (B) using high pH reversed phase fractionation.



Each fraction was assessed by total number of peptides identified in order to determine an appropriate custom gradient using high pH RP fractionation. Number of phosphopeptides per fraction was compared to total number of peptides identified in order to assess specificity of each phosphoenrichment method in each fraction.

Table 1. Summary of phosphopeptide enrichment data analysis using the Byoinc search node.

	TiO_2 Only	SMOAC (Fe-NTA)	SMOAC Total
Protein groups	3,831	5,288	5,763
Unique peptides	21,596	32,936	42,431
Unique Phosphopeptides	14,572	28,211	33,135
Quantified phosphopeptides	11,100	19,137	23,983

Data was analyzed with Thermo Scientific™ Proteome Discoverer™ 2.2 using the Byonic search node and PhosphoRS. Total number of phosphopeptides and quantified phosphopeptides were recorded to assess specificity of phosphopeptide enrichment methods.

Figure 5. KEGG pathway analysis of quantified proteins

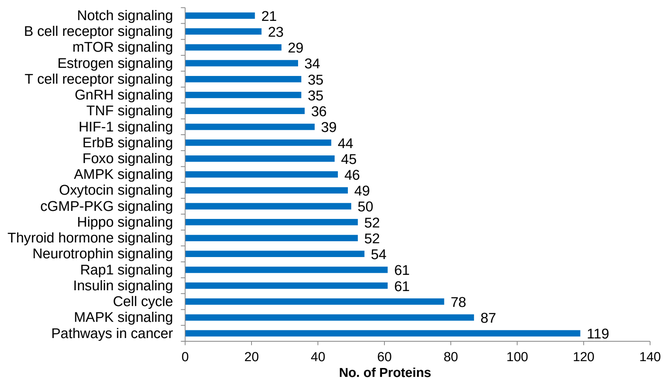
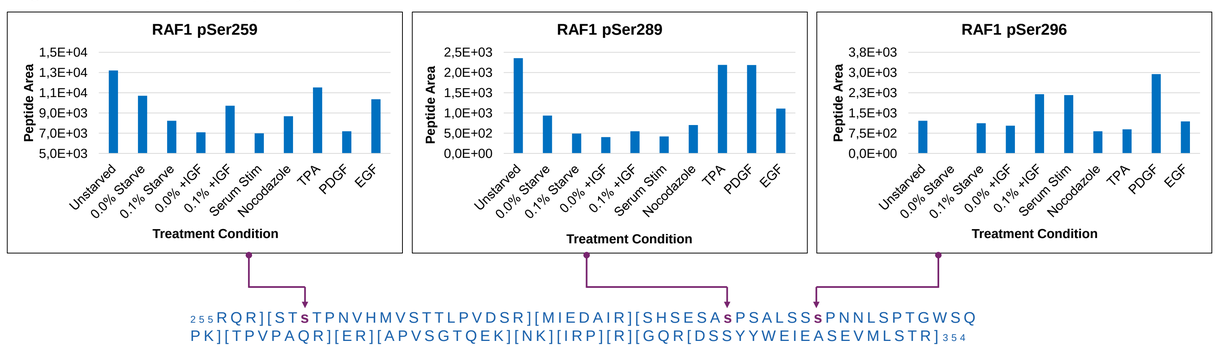
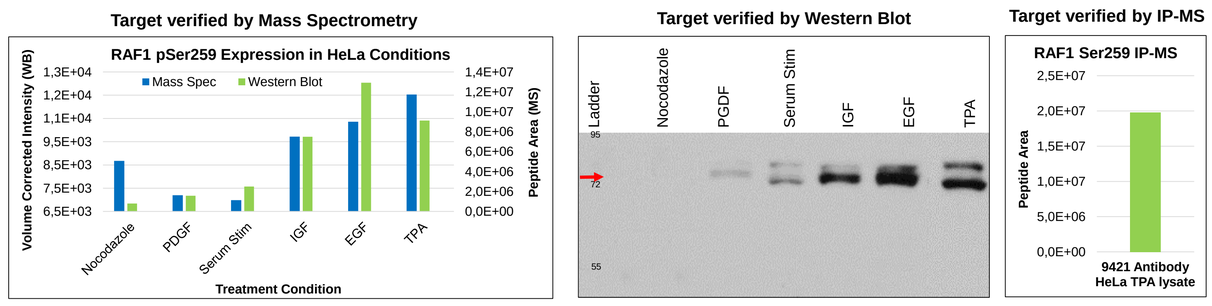


Figure 6. Differential phosphorylation of RAF1 with various cell treatments.



Differential expression of three phosphoserines in RAF1 protein indicates that specific treatment conditions are optimal in a site-dependent manner.

Figure 7. Verification of RAF1 pSer259 differential expression by MS and Western Blot.



Six conditions of HeLa lysates were tested for relative expression of phospho-RAF1 (Ser259). TMT 11plex data was extracted for these six conditions to compare phosphopeptide (Ser259) expression by mass spectrometry and Western blot. Western blot was analyzed using MyImage™ Analysis software, and the volume-corrected density of each band was determined for comparison to peptide intensity data by mass spectrometry analysis. Positive control antibody against phospho-RAF1 (Ser259) was verified to pull down target protein in HeLa TPA lysate (serum starved at 0.1% FBS for 24 hours followed by 400nM TPA stimulation for 15 minutes).

CONCLUSIONS

- This new SMOAC method coupled with high pH fractionation greatly enhances the TMT-based proteomics workflow, gaining more biological insight from complex samples.
- Global protein profiling using TMT reagents enables protein profiling from multiple samples in a single LC-MS run. TMT workflow enabled quantification of nearly 24,000 phosphopeptides in 11 different samples in less than 48hrs of LC-MS instrument time.
- Phosphopeptide enrichment with SMOAC method followed by high-pH reversed-phase fractionation resulted in 2X phosphopeptide identifications and quantitation compared to using only TiO_2 enrichment.
- Overall, good correlation observed with differential expression of the phosphorylated residues in RAF1 protein between MS and Western blot analysis.

REFERENCES

1. Lombardi B, Rendell N, Edwards M, Katan M, Zimmermann JG. EuPA Open Proteom. 2015 Mar 1;6:10-15.
2. Erickson BK1, Jedrychowski MP, McAlister GC, Everley RA, Kunz R, Gygi SP. Anal Chem. 2015 Jan 20;87(2):1241-9.
3. Viner, R.; Scigelova, M.; Zeller, M.; Oppermann, M.; Moehring, T.; Zabrouskov, V. Thermo Fisher Scientific Application Note #566, 2012
4. McAlister, G., Huttlin, E.L.; Haas, W.; Ting, L.; Jedrychowski, M.P.; Rogers, J.C.; Kuhn, K.; Pike, I.; Grothe, R.A,; Blethrow, J.D.; Gygi, S.P. Anal Chem. 2012. 84: 7469-7478.
5. Kall, L. Canterbury, J, Weston, J., Noble, W.S., MacCoss, M. Nature Meth. 2007, 4: 923-925.

TRADEMARKS/LICENSING

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