thermoscientific

Quantitative, comprehensive multi-pathway signaling analysis using an optimized phosphopeptide enrichment method combined with an internal standard triggered targeted MS assay

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ABSTRACT

Introduction:

There is broad interest in quantifying dynamic protein phosphorylation states in cellular signaling pathways under different conditions. Enrichment is necessary for better detection of the low abundant phosphorylated proteins, and multiplexed quantitation reagents parallelize processing across a multitude of experimental conditions. We have combined SMOAC (Sequential enrichment of Metal Oxide Affinity Chromatography), 146 AQUATM heavy-labeled phosphopeptide standards, and SureQuantTM targeted MS to evaluate changes in phosphorylated protein abundance under different stimulation conditions. The specific phosphopeptides have been chosen to cover biologically interesting phosphosites from several different signaling pathways.

Method:

HeLa/A549 cells were grown with different stimulation conditions (hIGF-1/hEFG) before in-solution digestion. One milligram of each digest spiked with phosphopeptides standard was subjected to SMOAC analysis using the Thermo Scientific Pierce Hi-SelectTM TiO2 and Fe_NTA phosphopeptide enrichment kits. Both eluents were combined before LC-MS analysis using Thermo Scientific Dionex nanoLC™ system coupled to Thermo Scientific™ Orbitrap Exploris™ 480 or Orbitrap Eclipse™ Tribrid™ Mass Spectrometers. To ensure optimal measurement of each target, a novel SureQuant method was performed where real-time heavy peptide detection triggered high-sensitivity measurement of endogenous targets. Data analysis was performed with Proteome Discoverer and Skyline software.

Results:

We have previously described our optimized SMOAC phosphopeptide enrichment method and we have shown with that method significant improvement in the number of phosphopeptides identified. In this study, we developed a targeted assay based upon 146 AQUA heavy-isotope phosphopeptide standards. More than 90% of heavy peptides were quantified with high sensitivity and reproducibility across different MS acquisition methods. The phosphopeptide standards spiked into stimulated HeLa/A549 cell digest, followed by enrichment using the SMOAC method, allowed quantitation of about 60 endogenous phosphopeptides and 134 heavy phosphopeptides by PRM or SureQuant method.

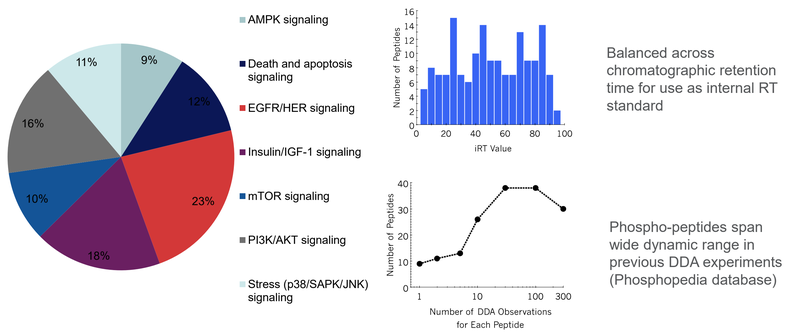
Conclusion:

This phosphopeptide standard with novel targeted MS analysis allowed quantitation of phosphorylation changes from >80 signaling pathway proteins.

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. We have constructed a pool of about 146 heavy-labeled phosphopeptides from seven different signaling pathways that will enable the quantitation of 146 phosphopeptides in a single analysis using the optimized SMOAC phosphopeptide enrichment method couples to internal standard trigger PRM (SureQuant) analysis.

Figure 1. Generating a 146 Synthetic Phosphopeptide Standard for Multi-Pathway Analysis



Balanced across chromatographic retention time for use as internal RT standard

Phospho-peptides span wide dynamic range in previous DDA experiments (Phosphopedia database)

MATERIALS AND METHODS

Cell Culture

HeLas3 (PN#CCL-2.2) and A549 (PN#CCL-185) cells were purchased from ATCC and cultured in Life Technologies™ Gibco™ SMEM or FK-12 with 10% Fetal Bovine Serum complete media. hIGF-1 was acquired from Thermo Fisher Scientific and hEGF was acquired from Cell Signaling Technology. HeLaS3 and A549 cells at approximately 80% confluency were treated for 15 minutes with hEGF and hIGF-1 respectively, after 24 hours serum starvation using appropriate media plus 0.1% charcoal-stripped FBS. Cells were lysed with TEAB/SDS plus universal nuclease as lysis buffer. Thermo Scientific™ Pierce™ BCA Protein Assay (PN#23225) was performed for protein quantitation.

MS Sample Preparation and Phosphopeptide Enrichment

EasyPepTM Mini MS Sample Prep kit reagents (Thermo Fisher Scientific, PN#A40006) with modified scale up protocol was used to prepare digest from HeLa (+hEGF) and A549 (+hIGF-1) cell lysate. The optimized SMOAC method was used for phosphopeptide enrichment. Briefly, 100fmol of 146 phosphopeptides standard was spiked-in to one milligram per replicate of stimulated A549 or HeLa digest. Spiked-in digest was subjected to Thermo Scientific™ Pierce™ HiSelect™ TiO2 phosphopeptide enrichment kit (PN#A32993) and the TiO2 eluent was saved for MS analysis. The TiO2 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 TiO2 enrichment steps and Fe-NTA enrichment steps were combined into separate pooled samples. After SMOAC, phosphopeptides were cleaned off-line using the PierceTM C18 Spin Tips (PN#84850) followed by peptide quantitation using the Thermo Scientific™ Pierce™ Colorimetric Peptide Assay (PN#23275).

LC-MS Analysis

For the LC-MS analysis using DDA or DIA method, Thermo Scientific™ EASY-Spray™ C18 LC column (2 µm particle size) to separate peptides with a 5-30% acetonitrile gradient over 120 minutes at a flow rate of 300 nL/min. Spectra were acquired on an Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System or EASY-nLC™ 1200 system coupled to Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer.

LC-MS analysis of PRM or SureQuant method was performed with an EASY-nLC™ 1200 coupled to Orbitrap Exploris™ 480 and Orbitrap Eclipse™ Tribrid™ Mass Spectrometers. The overall SureQuant workflow consists of two steps: (i) A 'Survey run' experiment to determine optimal precursor charge states, establish corresponding fragment ions, and determine the apex intensity of the IS, (ii) SureQuant experiments where the instrument monitors for the optimal m/z and triggering intensity (1% of apex) of the IS trigger peptides and upon its detection, dynamically performs a high-resolution high-sensitivity MS2 analysis of the corresponding endogenous target. For both Survey and SureQuant analysis, 60 min gradients, at 400nL/min were performed. 600fmol of the 146 phosphopeptide IS mixture enriched from stimulated A549 or HeLa digest was used for the survey run analysis to determine intensity thresholds for subsequent SureQuant analysis.

Data Analysis

For DDA data analysis, Thermo Scientific™ Proteome Discoverer™ 2.2 software was used to search MS/MS spectra with the Sequest™ HT search engine with a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da. Static modification included carbamidomethylation (C). Dynamic modifications included methionine oxidation and phosphorylation (S,T,Y). PhosphoRS node was used for site localization. For targeted PRM or SureQuant data analysis, Skyline software (University of Washington) was used to develop targeted assay and measure light/heavy ratio and calculate concentrations from unknown samples.

RESULTS

Figure 2. SMOAC Enriched Multi-Pathway Phosphopeptide SureQuant Profiling

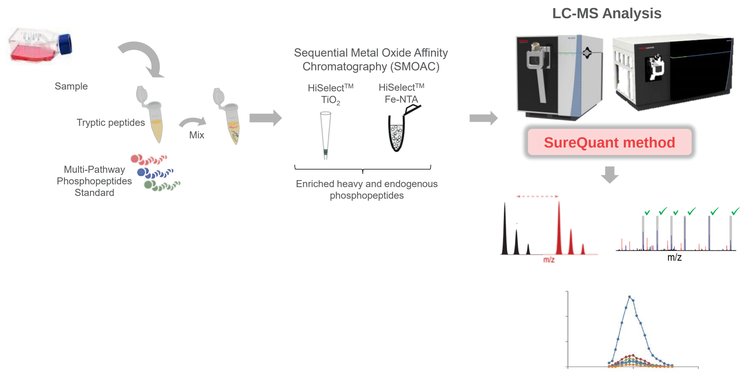


Figure 3. LC-MS Analysis of 146 Synthetic Phosphopeptide Standard

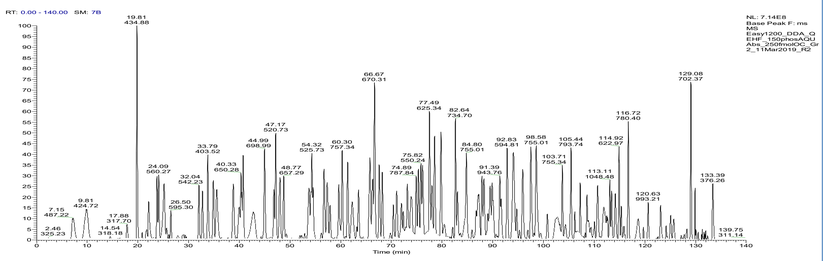
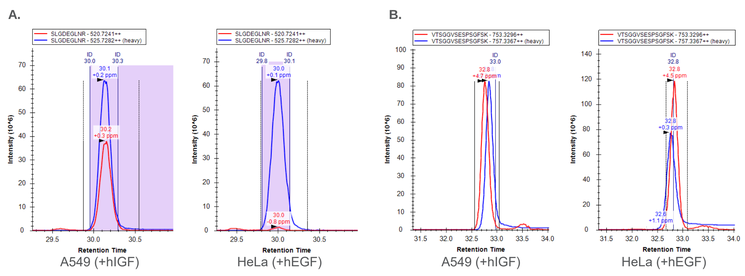
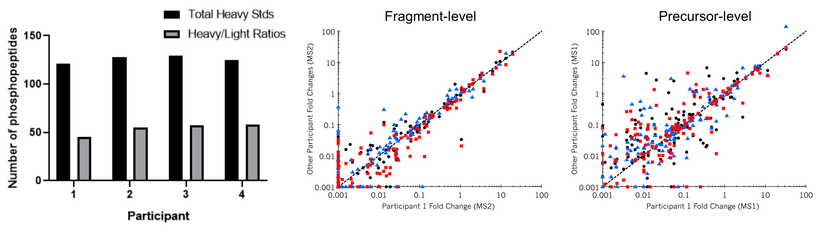


Figure 4. Data Dependent Analysis (DDA) of SMOAC Enriched Multi-Pathway Phosphopeptides



SMOAC enriched phosphopeptides from A549 (+hIGF) and HeLa (+hEGF) and nanoLC-MS analysis using the DDA with inclusion list method resulted in identification of 114 heavy IS phosphopeptides. Examples of phosphopeptides without interference (A) and with interference (B) from enriched samples.

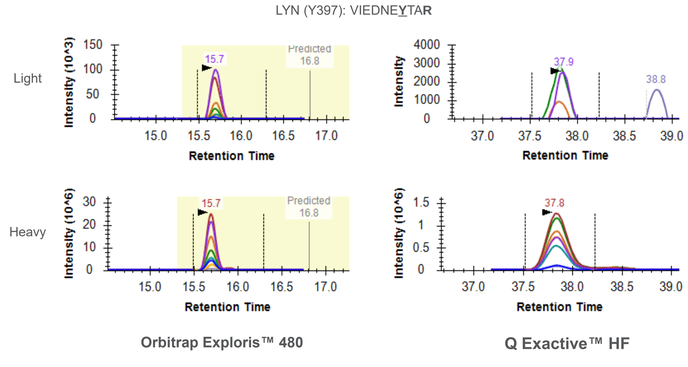
Figure 5. Data Independent Analysis (DIA) of IMAC Enriched Multi-Pathway Phosphopeptides



Phospho enrichment = CST IMAC
LC-MS/MS acquisition = 8X gas phase fractionated DIA injections each with 4 m/z fully overlapping windows spanning 100 m/z across a 400-1200 total m/z range

DIA analysis of IMAC enriched phosphopeptides from HeLa (+hEGF) showed better quantitation compared to precursor-level (DDA) analysis. About 50 light and 124 heavy IS phosphopeptides were quantitated across 4 different core labs using standardized DIA workflow (sPRG 2018-2019 study).

Figure 6. PRM Analysis of SMOAC Enriched Multi-Pathway Phosphopeptides



PRM analysis of SMOAC enriched phosphopeptides from A549 (+hIGF) and HeLa (+hEGF) resulted in accurate and precise quantitation of about 60 light and 134 heavy IS phosphopeptides. Improved signal to noise and sensitivity was observed with new Orbitrap Exploris™ 480 (Figure 6). Differential expression of many phosphopeptides observed between two cancer cell lines (data not shown).

Figure 7. SureQuant Acquisition Method Delivers Intelligent Detection of Targets

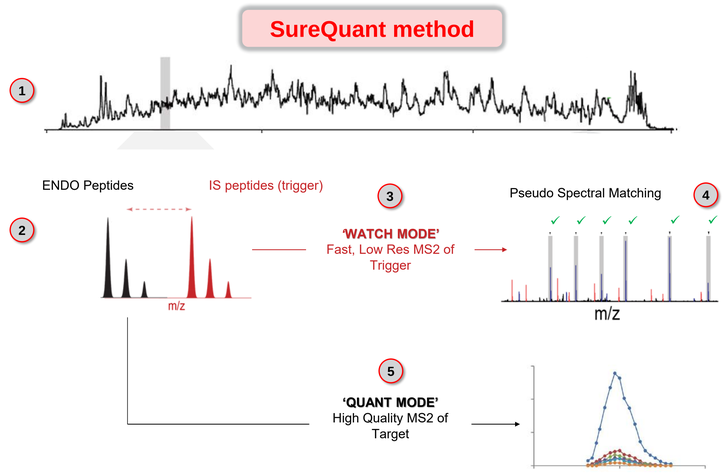
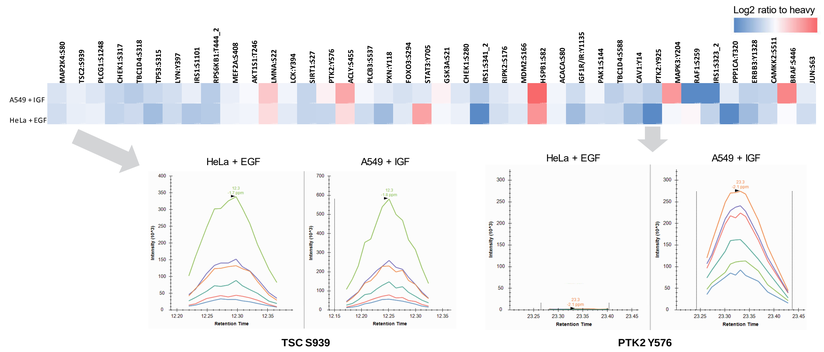
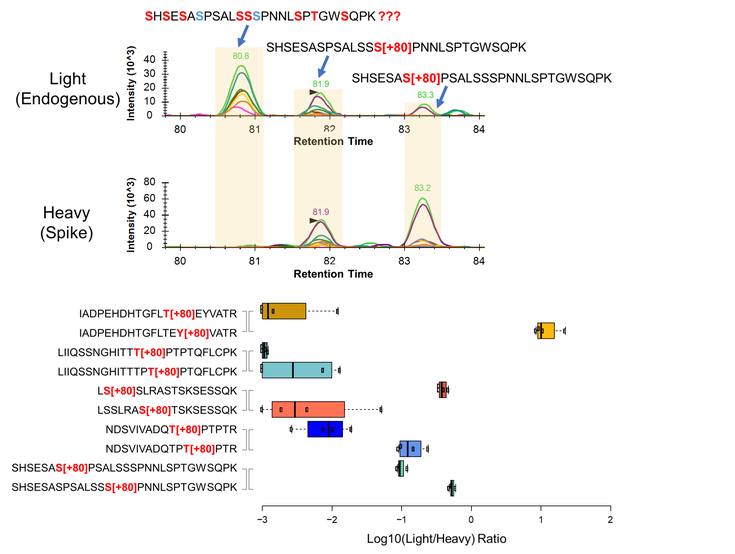


Figure 8. SureQuant Analysis of SMOAC Enriched Multi-Pathway Phosphopeptides



SureQuant analysis of SMAOC enriched phosphopeptides provided accurate and precise quantitation in a single MS run. About 60 light and 134 heavy IS phosphopeptides were quantitated with high accuracy and specificity using SureQuant method on Orbitrap Exploris™ 480.

Figure 9. Benefits of Heavy Multi-Pathway Phosphopeptides Standard



Interpretation of complex phosphopeptide signatures and positional isomers is aided by heavy standards and DIA/targeted MS acquisition methods.

CONCLUSIONS

- Multi-pathway phosphopeptide profiling with SureQuant IS triggered method utilizes the presence of synthetic isotopically labeled heavy peptides to enable sensitive and reproducible target multiplexing measurement of about 146 phosphopetides in a single analysis with accurate and precise quantitation.
- The combination of EasyPep MS sample prep kit and SMAOC phosphopeptide enrichment method followed by IS guided SureQuant method presents a new paradigm for signaling pathway analysis involving PTMs.
- Multi-pathway phosphopeptide quantitation using the spiked-in internal standard and targeted MS method provides easy interpretation and quantitation of complex phosphopeptide signatures and positional isomers.

REFERENCES

1. Logue JS, Morrison DK. Complexity in the signaling network: insights from the use of targeted inhibitors in cancer therapy. Genes Dev. 2012 Apr 1; 26(7):641-50.

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TRADEMARKS/LICENSING

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