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

Bhavin Patel[1]; Penny Jensen[1]; Aaron S. Gajadhar[2]; Sebastien Gallien[3]; Jae Choi[1]; Romain Huguet[2]; Graeme McAlister[2]; Derek Bailey[2]; Shannon Eliuk[2]; Markus Kellmann[4]; Tabiwang N. Arrey[4]; Alexander Harder[4]; Andreas Huhmer[2]; Kay Opperman[1]; John C Rogers[1]

[1]Thermo Fisher Scientific, Rockford, IL; [2]Thermo Fisher Scientific, San Jose, CA; [3]Thermo Fisher Scientific, Precision Medicine Science Center, Cambridge, MA; [4]Thermo Fisher Scientific, Bremen, Germany

ABSTRACT

Introduction

There is broad interest in quantifying dynamic protein phosphorylation states in cellular signaling pathways under different conditions. We have combined SMOAC (Sequential enrichment of Metal Oxide Affinity Chromatography), 146 AQUA™ heavy-labeled phosphopeptide standards, and internal standard triggered 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.

Methods

We developed an assay containing a pool of 146 AQUA heavy-labeled phosphopeptides from 89 signaling proteins. HeLa/A549 cells were grown with different stimulation conditions (hIGF-1/hEFG) before in-solution digestion. One milligram of each cell digest spiked with phosphopeptides standard was subjected to Thermo Scientific™ Hi-Select TiO_2 phosphopeptide enrichment kit (PN#A32993). TiO2 flow-through/wash fractions were enriched with the Hi-Select Fe-NTA phophopeptide enrichment kit (PN#A32992). Both eluents were combined before LC-MS analysis using Thermo Scientific™ Dionex™ nanoLC™ system coupled to modified Orbitrap mass spectrometers. To ensure optimal measurement of each target, a novel targeting Thermo Scientific™ 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.

Preliminary Data

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 (96 serine, 26 threonine and 36 tyrosine modified peptides). More than 80% of peptides were quantified with SureQuant method. The phosphopeptide standards spiked into stimulated HeLa and A549 cell digest, followed by enrichment using the SMOAC method, allowed quantitation of endogenous phosphopeptides by a directed discovery (DDA with inclusion list and DIA) method. With an adapted internal standard triggered PRM method (SureQuant), using the modified Orbitrap MS instruments, we quantified multiple phosphopeptides in the SMOAC enriched HeLa and A549 stimulated digest. This SIL-triggered targeted analysis allowed much better quantitation of signaling pathway phosphorylated proteins by enhancing the detectability of targets and significantly improving measurement reproducibility across the different stimulation conditions. This targeted phosphopeptide assay coupled with SMOAC method and novel MS acquisition approach provided excellent quantitation, specificity and selectivity for signaling pathway analysis.

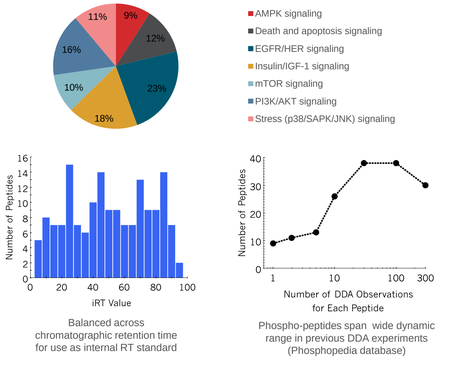
Novel Aspect

This phosphopeptide standard with novel targeted MS analysis allowed quantitation of phosphorylation changes from 89 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 triggered PRM (SureQuant) analysis.

Figure 1. Generating a 146 Synthetic Phosphopeptide Standard for Multipathway Analysis



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 hour 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

Thermo Scientific™ EasyPep™ Mini MS Sample Prep kit reagents (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 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 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 cleaned off-line using the Pierce C18 Spin Tips (PN#84850) followed by peptide quantitation using the Thermo Scientific Pierce Colorimetric Peptide Assay (PN#23275).

LC-MS Analysis

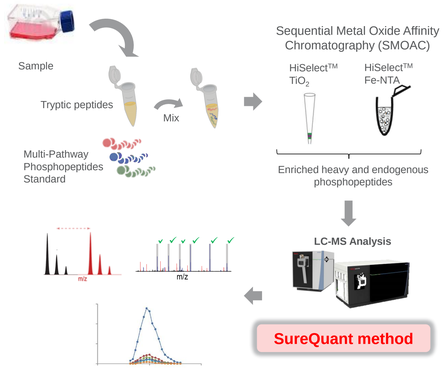
For the LC-MS analysis using DDA or DIA method, Thermo Scientific™ EASY-Spray™ C18 LC column (2 µm particle size) to separate peptides with a 5-30% acetonitrile gradient over 120 minutes at a flow rate of 300 nL/min. Spectra were acquired on an Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System 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 Thermo Scientific™ Orbitrap Exploris™ 480 and Thermo Scientific™ 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.

Figure 2. SMOAC Enriched Multi-Pathway Phosphopeptide SureQuant Profiling



RESULTS

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

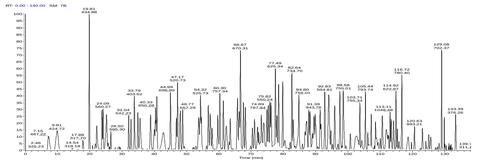
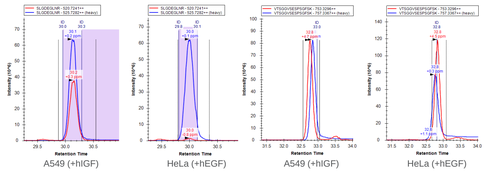
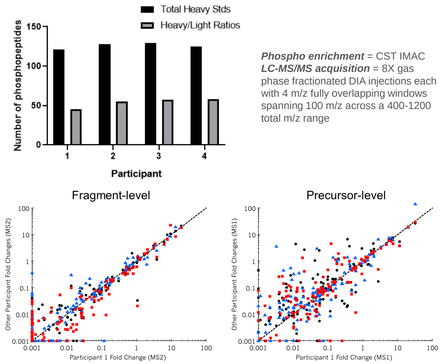


Figure 4. Data Dependent Analysis (DDA) of SMOAC Enriched Multi-Pathway Phosphopeptides



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.

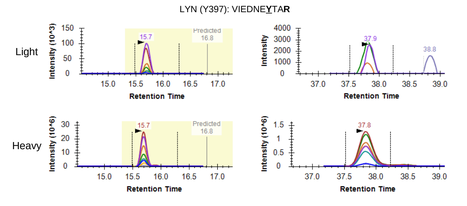
Figure 5. Data Independent Analysis (DIA) of IMAC Enriched Multi-Pathway Phosphopeptides



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 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

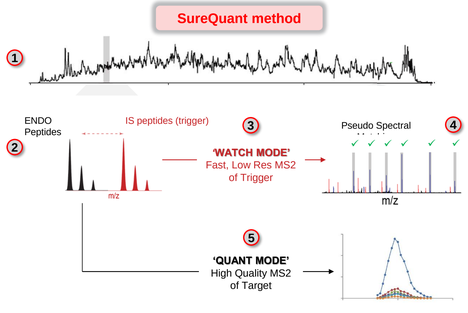
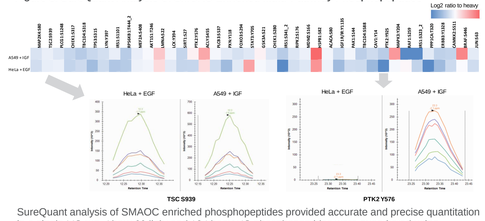
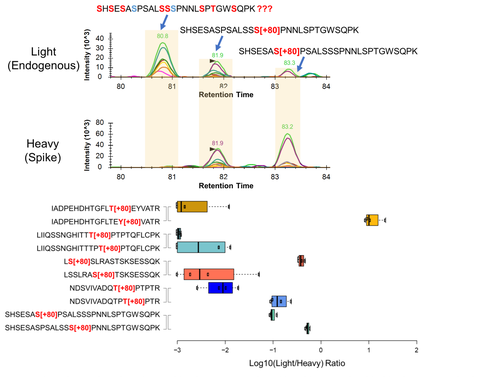


Figure 8. SureQuant Analysis of SMOAC Enriched Multi-Pathway Phosphopeptides



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 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 134 phosphopeptides 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 of complex phosphopeptide signatures and positional isomers.

REFERENCE

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.

ACKNOWLEGEMENT

We would like to acknowledge ABRF sPRG group members and 2018-2019 study participants/sponsors for providing DIA data.

For Research Use Only. Not for use in diagnostic procedures.

TRADEMARKS AND LEGAL INFORMATION

PO65550-EN0519S

ThermoFisher SCIENTIFIC