SureQuant Targeted Mass Spectrometry Standards and Assay Panel for Quantitative Analysis of Phosphorylated Proteins from Multiple Signaling Pathways

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ABSTRACT

Purpose: The study of phosphorylation changes in signaling pathways is a critical process to understand for both normal cellular growth and when disruptions cause unregulated growth leading to cancer.

Methods: New Thermo Scientific™ SureQuant™ phosphopeptide standards Multipathway Phosphopeptide Standard (PN# A51745) and SureQuant Phosphopeptide Suitability Standard (PN# A51746) were utilized to assess instrument suitability and quantitate phosphopeptide changes.

Results: The Phosphopeptide Suitability standard is an excellent tool to assess LC-MS system performance. Spiked -in heavy labeled Multipathway Phosphopetide standard allows for the detection of 131 different phosphorylation events.

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 Thermo Scientific™ EasyPep™ technology, phosphopeptide enrichment, validated multipathway AQUA™ heavy-labeled phosphopeptide standards, and SureQuant™ targeted MS to quantitate changes in phosphorylated protein abundance across multiple stimulated cell lines. Specific phosphopeptide standards were chosen representing phosphosites from several different pathways including EGFR/HER, RAS-MAPK, PI3K/AKT/mTOR, AMPK, death and apoptosis, and stress (p38/SAPK/JNK) signaling. This novel workflow enables targeted quantitation of biologically relevant phosphorylation sites with high accuracy, precision, and specificity.

MATERIALS AND METHODS

Cell Culture, MS Sample Preparation, and Phosphopeptide Enrichment

Multiple cell lines (MCF7, HCT116, A431, LNCAP, HepG2) cells were grown with different stimulation conditions (hIGF-1/hEFG). Cells were harvested and lysed with EasyPep lysis buffer containing Thermo Scientific™ Halt™ phosphatase inhibitor. Thermo Scientific™ EasyPep™ Maxi MS Sample Prep kit reagents (A45734) were used to prepare digests. One milligram of each digest spiked-in with 1pmol of multipathway phosphopeptides standard was subjected to phosphopeptide enrichment using the new Thermo Scientific™ Hi-Select™ Fe-NTA magnetic agarose (PN# A52284). Additionally, we compared this new method with the Sequential Metal Oxide Affinity Chromatography (SMOAC) method and competitor Fe-NTA method for phosphopeptide enrichment. Replicate samples for all phosphopeptide enrichment methods were combined into separate pooled samples. After SMOAC, phosphopeptides were cleaned off-line using Thermo Scientific™ Pierce™ Peptide Desalting Spin Columns (PN# 89852). Samples were processed using EasyPep Maxi (PN A45734) and Multipathway Phosphopeptide Standard (PN# A51745) peptide mixture was spiked in either before or after phosphoenrichemnt.

Liquid Chromatography and Mass Spectrometry

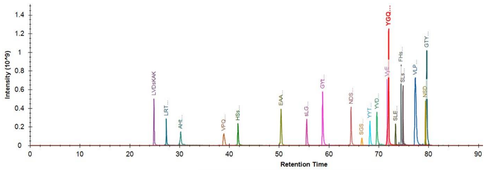
Phosphopeptide Suitability Standard (PN# A51746) was used to assess system performance of the nanoLC-MS system prior to running unknown samples. Each sample was then desalted on-line using the Thermo Scientific™ Acclaim™ PepMap 100 C18 Trap Column (PN#164564) followed by seperation using a Thermo Scientific™ EASY-Spray™ C18 column (PN#E903). For discovery MS and targeted PRM-MS analysis, the samples were analyzed using the Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System and Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer. Verified instrument acquisition methods were used, as well as inclusion lists. For SureQuant LC-MS analysis, Spectra were acquired on an Thermo Scientific™ EASY-nLC™ 1200 system coupled to an Orbitrap Exploris 480 mass spectrometer. 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 their detection, dynamically performs a high-resolution high-sensitivity MS2 analysis of the corresponding endogenous target (Figure 3).

MS 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 modifications included carbamidomethylation (C). Dynamic modifications included heavy R, K, methionine oxidation and phosphorylation (S,T,Y). For targeted PRM or SureQuant data analysis, Skyline software (University of Washington) was used to process Survey Run files and measure light/heavy ratios from samples.

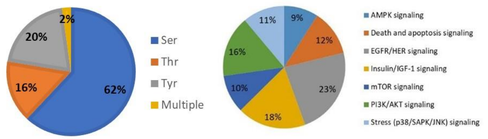
RESULTS

Figure 1. SureQuant Phosphopeptide Suitability Standard



Skyline analysis of 200 fmol on column SureQuant Phosphopeptide Standard separated using an Acclaim PepMap RSLC C18 2µm x 75µm x 50cm column (Product No. ES903) with a 2-35% gradient (A:0.1% FA in water, B: 0.1% FA in 100% acetonitrile) at 300nL/min for 120 minutes and detected on a Q Exactive HF mass spectrometer.

Figure 2. SureQuant Multipathway Phosphopeptide Standard



Classification of localized phosphosites (Left) and represented signaling pathways (Right)

Figure 3. SureQuant IS-Triggered, Data-Aware Acquisition

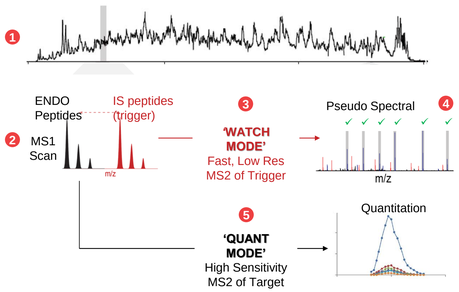
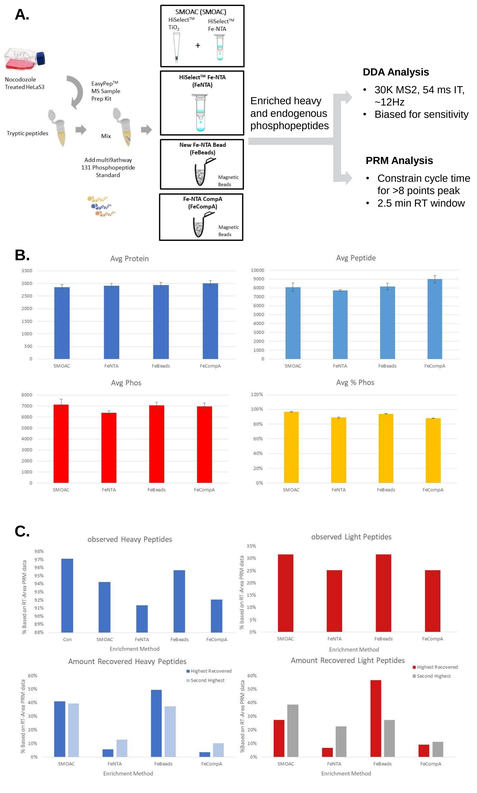
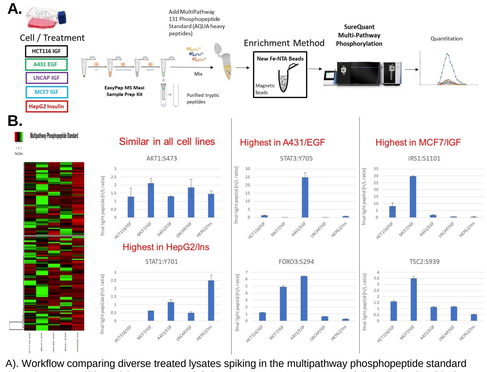


Figure 4. Evaluation of phosphopeptide enrichment method using multipathway phosphopeptide Standard



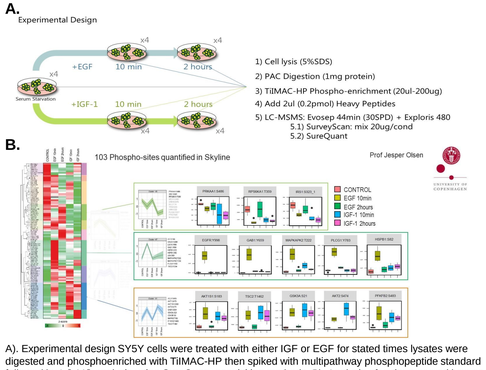
A). Workflow comparing different phosphopeptide enrichment methods followed by LC-MS analysis using DDA and PRM acquisition methods. B). Proteome discoverer data analysis showed comparative protein ID, peptide ID, phosphopeptide ID and % phosphopeptide across all methods. C). Analysis of different phosphoenrichment methods using multipathway phosphopeptide standard and targeted PRM analysis in Skyline showed higher heavy and light phosphopeptides for SMOAC and new Hi-Select Fe-NTA magnetic beads.

Figure 5. Multipathway Phosphopeptide Standard Method Optimization – Spike in Prior to Magnetic Fe-NTA bead enrichment



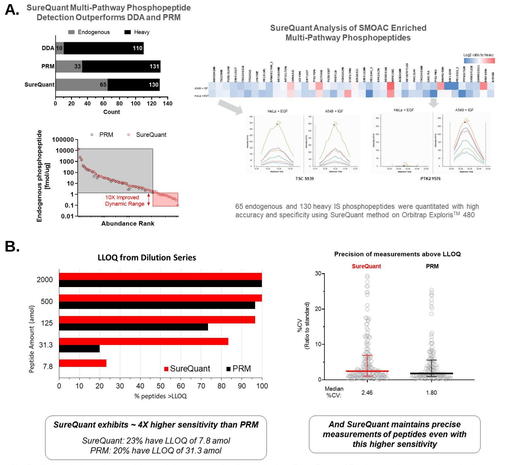
A). Workflow comparing diverse treated lysates spiking in the multipathway phosphopeptide standard then phosphopeptide enrichment using Hi-Select Fe-NTA magnetic agarose followed by LC-MS/MS analysis using PRM acquisition methods. B). Analysis of various treated lysates using the multipathway phosphopeptide standard, phosphoenrichment and targeted PRM analysis in Skyline revealed alterations in phosphorylation as depicted in the heat map on the left and specific PRM target results on the right.

Figure 6. Multipathway Phosphopeptide Standard Method Optimization – Spike in After Magnetic Ti-IMAC bead enrichment



A). Experimental design SY5Y cells were treated with either IGF or EGF for stated times lysates were digested and phosphoenriched with TiIMAC-HP then spiked with multipathway phosphopeptide standard followed by LC-MS analysis using SureQuant acquisition methods. B). Analysis of various treated lysates using the multipathway phosphopeptide standard, phosphoenrichment and targeted SureQuant analysis in Skyline revealed alterations in phosphorylation as depicted in the heat map on the left and specific SureQuant target results on the right.

Figure 7. Assessment of multipathway phosphopeptide standard with various acquisition methods



A). Optimized phosphopeptide enrichment method coupled to SureQuant IS-triggered targeted analysis enabled higher detection and quantitation of low-abundant phosphorylated signaling pathway proteins than PRM or DDA due to significantly improved measurement sensitivity. B). SureQuant method enables the 4x higher sensitivity than PRM assay while enabling equally high precision.

CONCLUSIONS

- SureQuant Phosphopeptide system suitability standard enables complete assessment of LC-MS/MS system for both discovery and targeted phosphoproteomics workflows.
- The multipathway phosphopeptide panel coupled with standardized sample preparation, phosphoenrichment using multiple methods and IS-triggered acquisition provides a turnkey approach for studying signaling pathway analysis.
- SureQuant IS-triggered targeted analysis enabled higher detection and quantitation of low-abundant phosphorylated signaling pathway proteins than PRM or DDA due to significantly improved measurement sensitivity. Since the reference standard was detected by the SureQuant method, the undetected endogenous targets are not likely phosphorylated under these conditions or are below the limit of detection.

ACKNOWLEDGEMENTS

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TRADEMARKS/LICENSING

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