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Turnkey, Multi-pathway Signaling Analysis Using a Synthetic Phosphopeptide Panel, Standardized Sample Preparation Kits and SureQuant Internal Standard Targeted Quantitation

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ABSTRACT

Purpose: We sought to develop a universal phosphopeptide enrichment and internal standard (IS)-guided LC-MS acquisition workflow for reproducible, sensitive and high-density absolute quantification of biologically relevant phosphorylation sites in multiple biological pathways.

Methods: We leveraged Sequential Metal Oxide Affinity Chromatography (SMOAC) for selective phosphopeptide enrichment, isotopically-labeled trigger peptides and the Thermo Scientific™ SureQuant™ IS targeted protein quantitation method to detect and quantify 138 pSTY targets per analysis using Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometers.

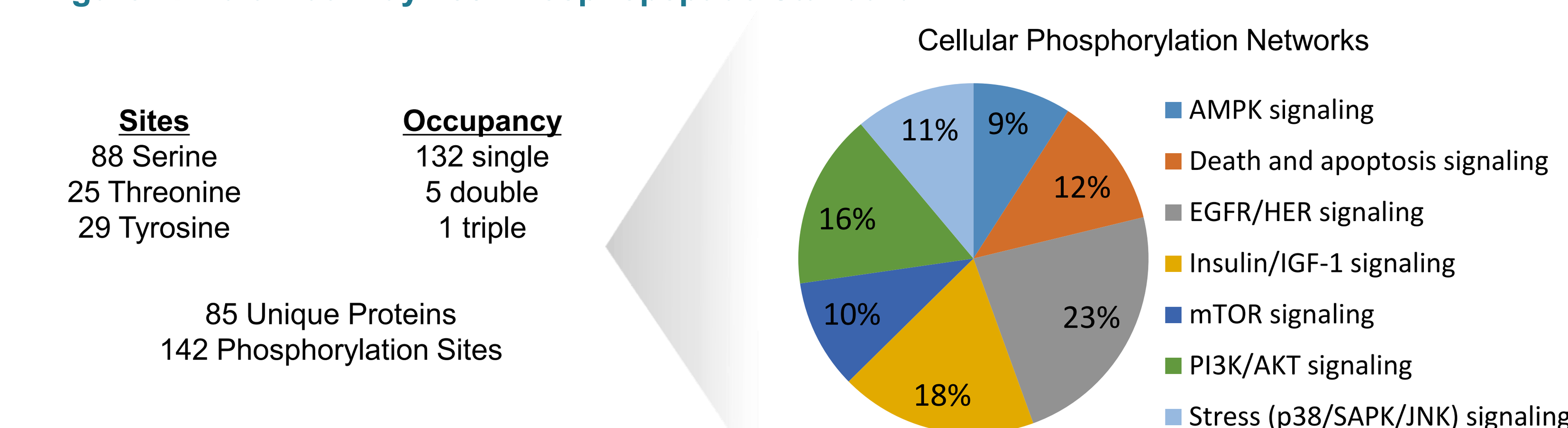
Results: Compared to traditional discovery-based proteomics approaches for pSTY analysis, the SureQuant multi-pathway phosphopeptide acquisition workflow outperformed DDA and PRM for detection of the multi-pathway phosphopeptides panel.

INTRODUCTION

Quantitative measurements of signal transduction pathway proteins and their post-translational modifications such as phosphorylation, are necessary for classifying disease states and uncovering novel signaling mechanisms. Despite improvements in new MS instrumentation, phosphoproteomic analyses still face challenges including low-yield/specificity of phosphopeptide enrichment, and irreproducible detection of functionally important phosphopeptides. We have developed a SureQuant internal standard (IS)-triggered targeted strategy using a pool of phosphopeptide reference internal standards and SMOAC (Sequential enrichment of Metal Oxide Affinity Chromatography) to purify and quantify phosphorylation abundance. 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. The proposed turnkey workflow enables reliable targeted quantitation for routine phosphoproteomics of biologically relevant phosphorylation sites.

In this study, we developed a targeted assay based upon 138 AQUA heavy-isotope phosphopeptide standards (Figure 1). Importantly, this workflow allows reliable enrichment, detection and quantification of multiple signaling pathways component simultaneously. For proof of concept, the entire workflow was demonstrated using a HeLa cell line treated with a microtubule polymerization inhibitor. A performance comparison of DDA, PRM and SureQuant acquisition was conducted for the detection of heavy IS and endogenous phosphopeptides in the multi-pathway panel.

Figure 1. Multi-Pathway 138 Phosphopeptide Standard



MATERIALS AND METHODS

Cell Culture, MS Sample Preparation, and Phosphopeptide Enrichment

HeLa S3 cells were cultured in S-MEM/glutamate/10% FBS media and treated with nocodazole (0.1 µl/mL) for 18 hours to achieve homogeneous mitotic arrest. 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 from 2mg of HeLa + nocodazole treated cell lysate. The optimized SMOAC method was used for phosphopeptide enrichment. Briefly, 1pmol of the 138 phosphopeptide standard was spiked-in to one milligram per replicate of treated HeLa digest. Spiked-in digest was subjected to Thermo Scientific™ High-Select™ TiO_2 phosphopeptide enrichment kit (A32993) and the TiO_2 eluent was saved for MS analysis. The TiO2 flow-through and wash fractions were pooled, and the phosphopeptides were enriched by High-Select Fe-NTA phosphopeptide enrichment kit (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 Thermo Scientific™ Pierce™ Peptide Desalting Spin Columns (89852).

LC-MS Analysis

For the DDA, PRM and SureQuant LC-MS analysis Thermo Scientific™ EASY-Spray™ C18 LC columns (ES804) were used to separate peptides with a 2.4-34% acetonitrile gradient over 60 minutes at a flow rate of 300 nL/min. 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 2). For DDA, PRM, and SureQuant analysis, 20% of total SMOAC elution was used per injection to compare the methods. MS parameters are shown in Figure 3.

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.

Figure 2. SureQuant IS-Triggered, Data-Aware Acquisition

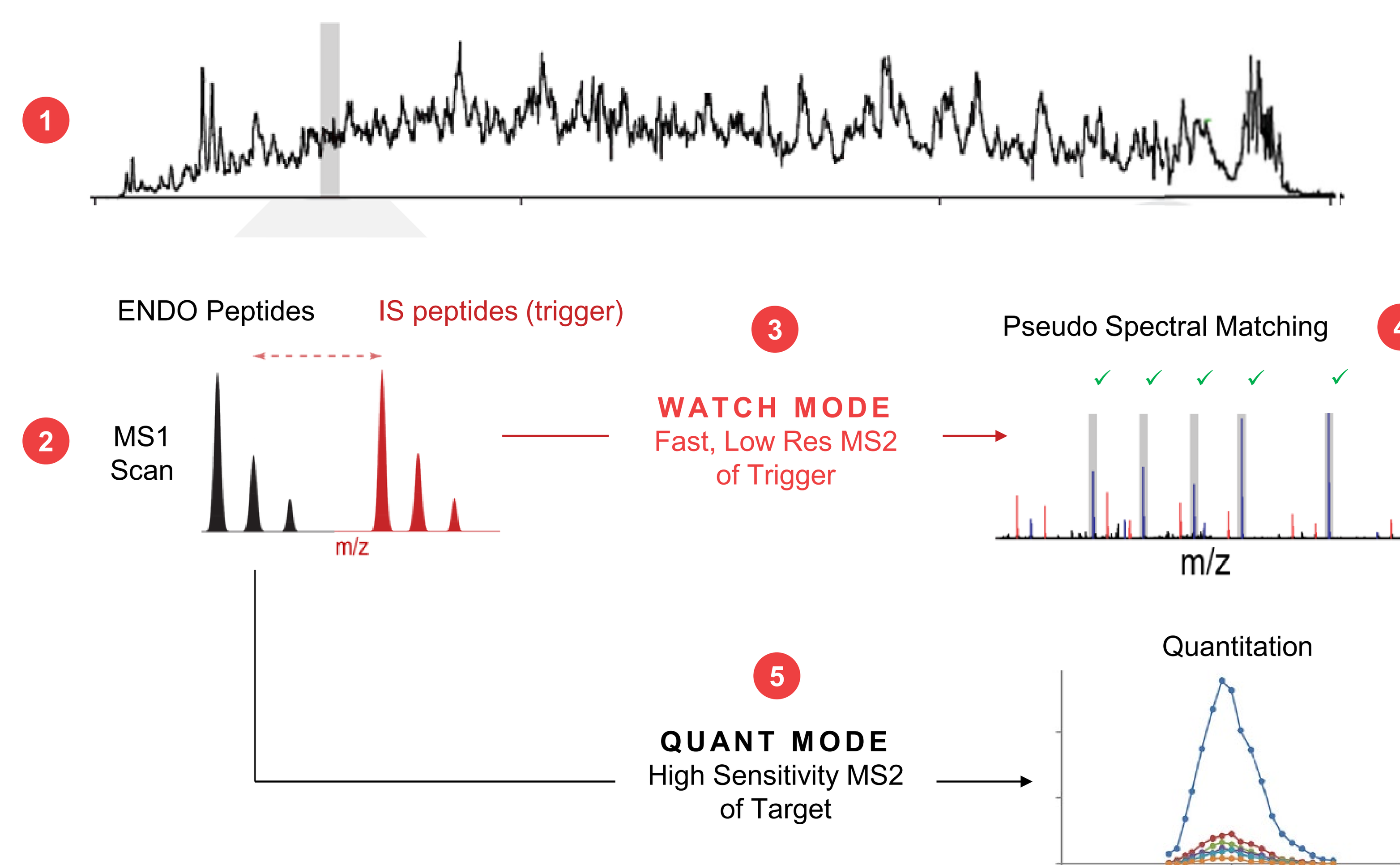
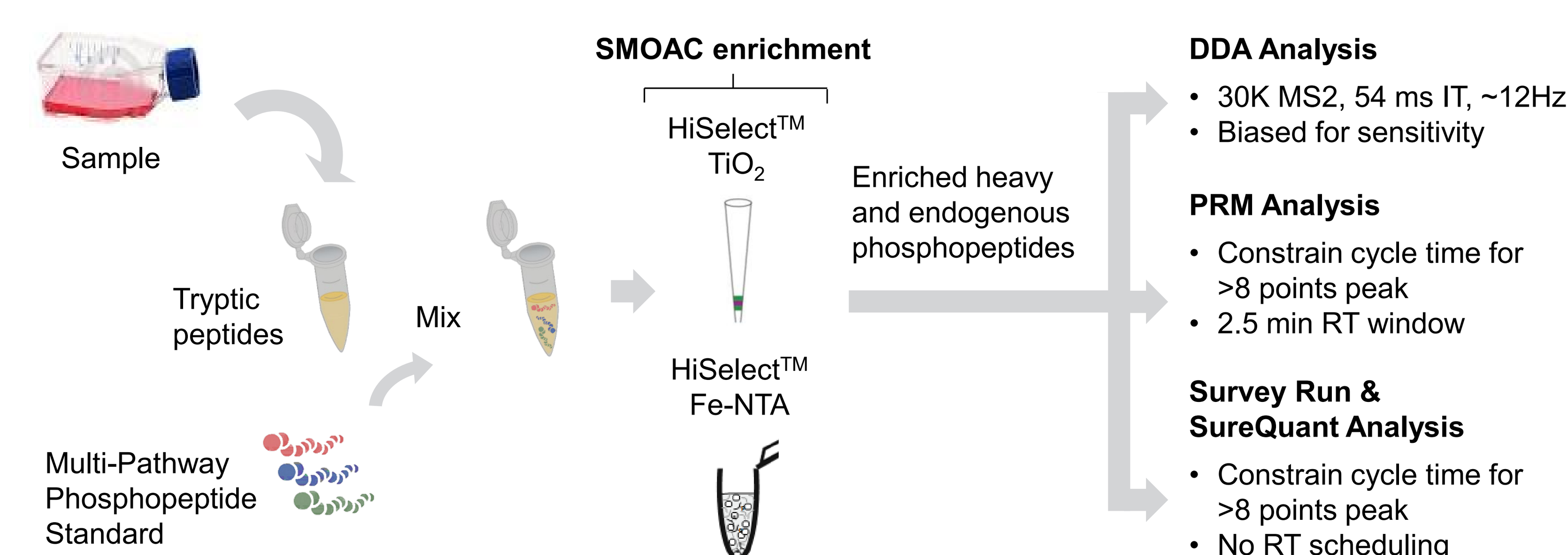
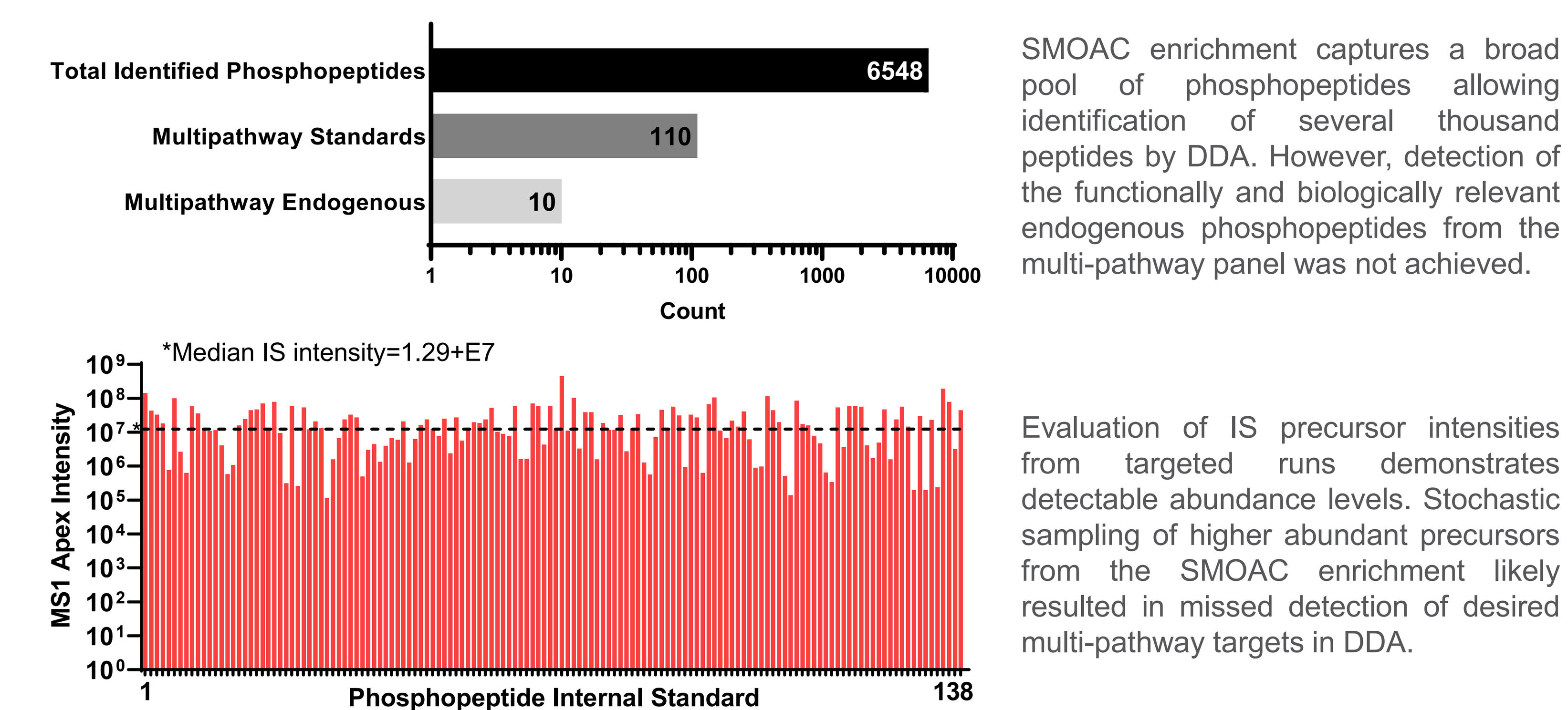


Figure 3. Multi-Pathway Phosphopeptide Enrichment and Analysis Workflow



RESULTS

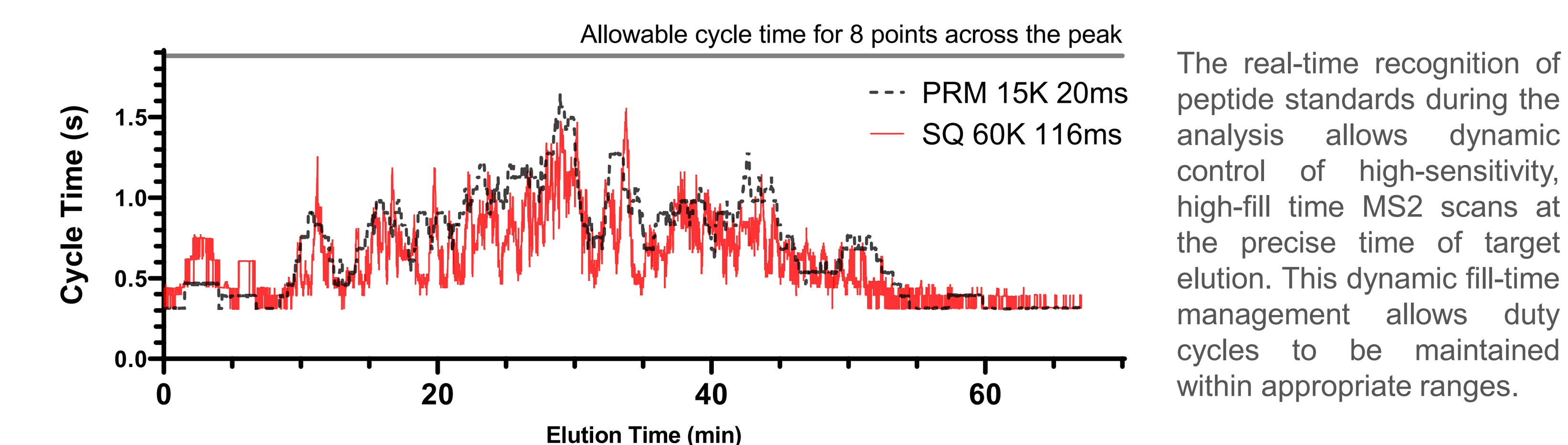
Figure 4. DDA Misses Most Multi-pathway Standards and Targets Despite Detectable Amounts



SMOAC enrichment captures a broad pool of phosphopeptides allowing identification of several thousand peptides by DDA. However, detection of the functionally and biologically relevant endogenous phosphopeptides from the multi-pathway panel was not achieved.

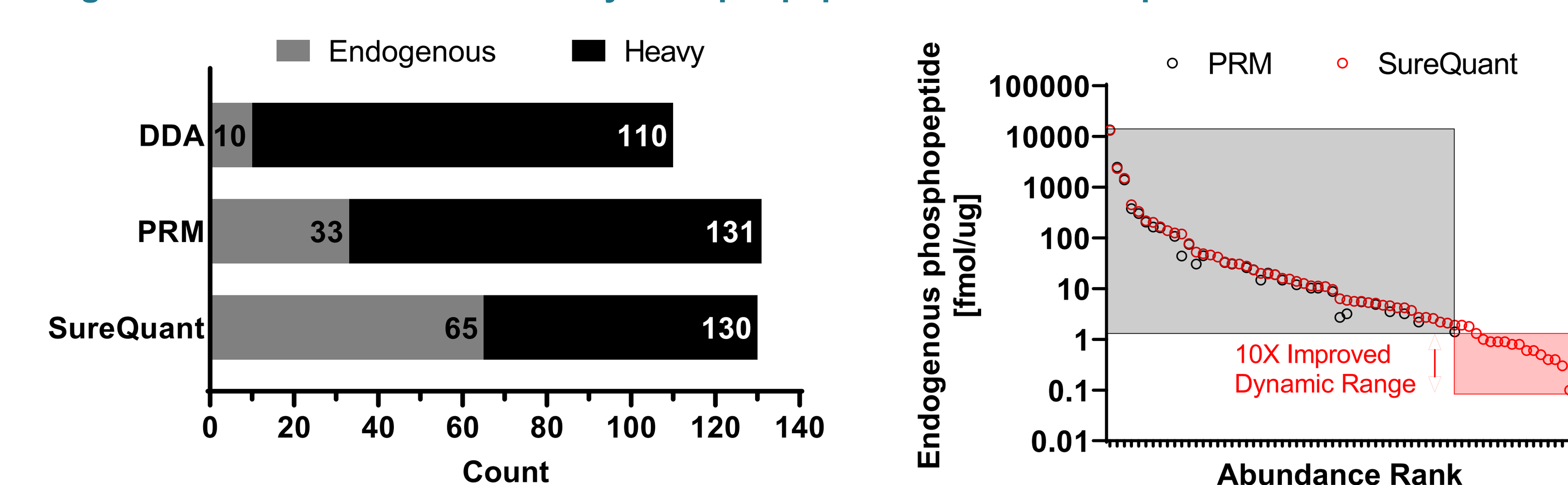
Evaluation of IS precursor intensities from targeted runs demonstrates detectable abundance levels. Stochastic sampling of higher abundant precursors from the SMOAC enrichment likely resulted in missed detection of desired multi-pathway targets in DDA.

Figure 5. SureQuant Dynamic MS2 Control Allows ~6X More Fill Time and Desired Duty Cycle



The real-time recognition of peptide standards during the analysis allows dynamic control of high-sensitivity, high-fill time MS2 scans at the precise time of target elution. This dynamic fill-time management allows duty cycles to be maintained within appropriate ranges.

Figure 6. SureQuant Multi-Pathway Phosphopeptide Detection Outperforms DDA and PRM



CONCLUSIONS

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 SureQuant, the undetected endogenous targets are not likely phosphorylated under these conditions or are below the limit of detection.

The multi-pathway phosphopeptide panel coupled with standardized sample preparation, SMOAC enrichment, and IS-triggered acquisition provides a turnkey approach for signaling pathway analysis.

TRADEMARKS/LICENSING

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