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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

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.

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Methods

Leveraged Sequential Metal Oxide Affinity Chromatography (SMOAC) phosphopeptide enrichment, isotopically-labeled phosphopeptides and Thermo Scientific™ SureQuant™ IS-triggered acquisition to detect and quantify 138 pSTY targets simultaneously using Thermo Scientific™ Orbitrap Exploris™ 480 Mass Spectrometer.

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.

Orbitrap Exploris 480 MS

Introduction

Challenge

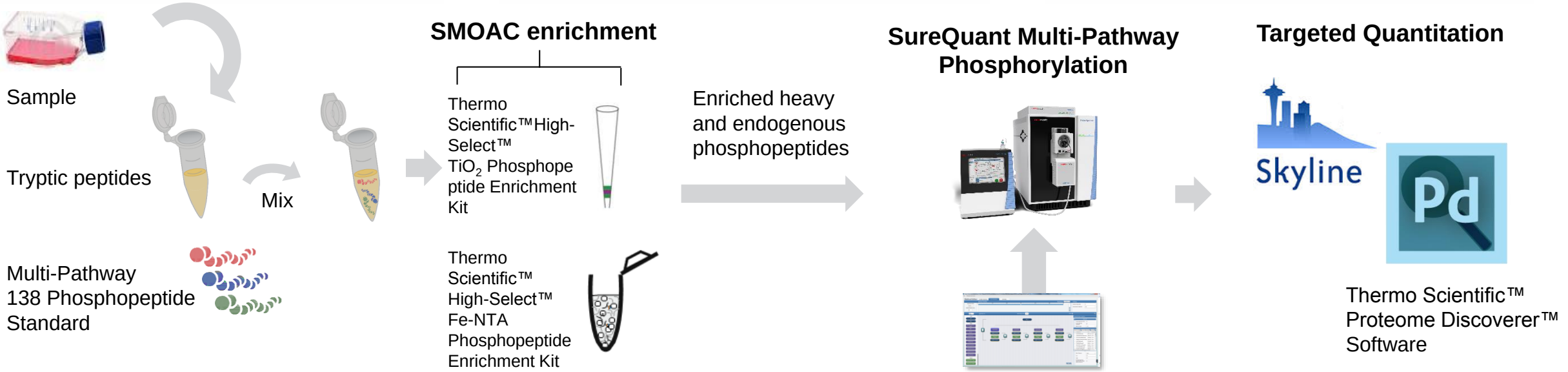
- Phosphopeptide analysis of multiple signaling pathways is analytically challenging.
- Phosphopeptides account for <1% of total peptides in a sample and require enrichment!

Methods

- Currently, low-yield, low specificity of phosphopeptide enrichment, and irreproducible detection of functionally important phosphopeptides prohibits routine pathway proteomics.

New Approach

- SureQuant IS-triggered targeted detection strategy
- 138 phosphopeptide internal standard panel
- SMOAC



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Materials and Methods

Sample Preparation

- 2 mg HeLa + Nocadozole-treated cell line
- Thermo Scientific™ EasyPep™ Maxi MS Sample Prep kit
- 1 pmol of 138 heavy-labeled Multi-Pathway Phosphopeptide Standard
- High-Select TiO2 Kit and High-Select Fe-NTA Kit
- Thermo Scientific™ Pierce™ Peptide Desalting Spin Columns
- 20% of total phospho-enrichment elution was used per injection

LC/MS Analysis

- Column: Thermo Scientific™ EASY-Spray™ C18 HPLC columns (ES804)
- HPLC: Thermo Scientific™ EASY-nLC™ 1200 system
- Gradient: 60 minutes
- MS: Orbitrap Exploris 480 MS
- Method: SureQuant IS-triggered targeted acquisition
- Method: Data-dependent acquisition for discovery run
- Method: PRM targeted analysis for benchmarking

Data Analysis

- Proteome Discoverer software for DDA analysis
- Skyline software for PRM and SureQuant analysis

Results – Multi-Pathway Phosphopeptide Profiling

Compare DDA, PRM and SureQuant quantitation of 138 functionally-relevant phosphopeptide targets

- Replicate injections of same phosphopeptide elution to compare methods
- DDA: 30K MS2, 54 ms IT, ~12Hz, favoring sensitivity
- PRM: Constrain cycle time for 8 points peak, 2.5 min RT window
- SureQuant: Constrain cycle time for 8 points peak, No RT scheduling needed

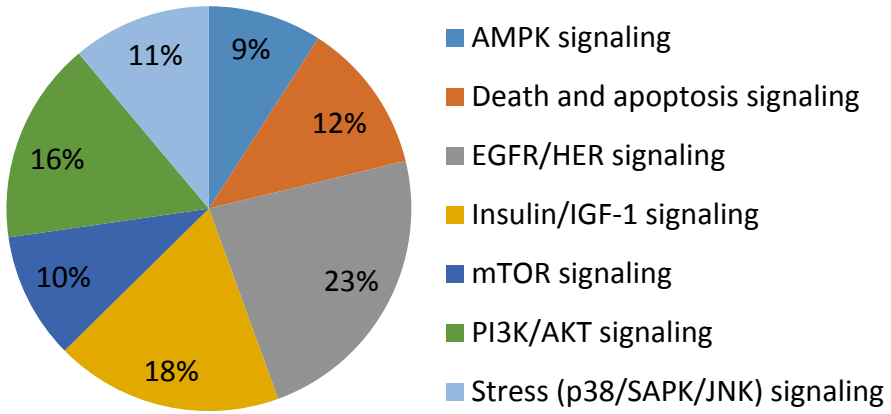
Multi-Pathway 138 Phosphopeptide Standard

AQUA Ultimate Grade, Heavy labeled

Sites	**Occupancy**
88 Serine	132 single
25 Threonine	5 double
29 Tyrosine	1 triple

85 Unique Proteins
142 Phosphorylation Sites

Cellular Phosphorylation Networks



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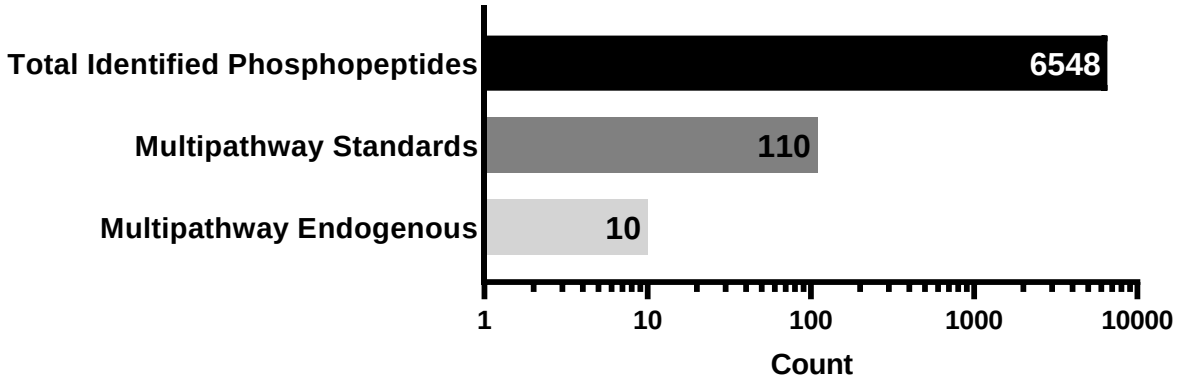
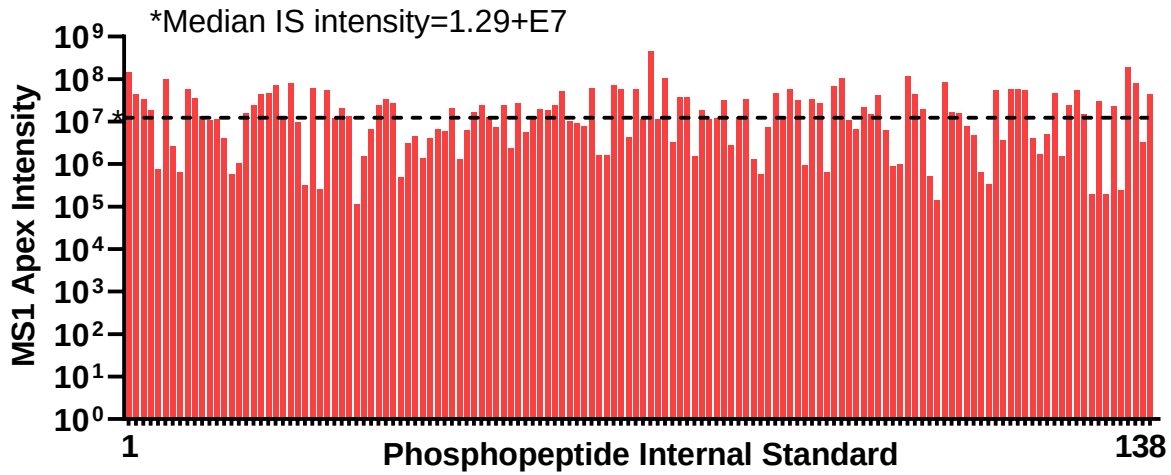
Results – Discovery Acquisition Schemes Do Not Adequately Monitor Pathway Targets

DDA Misses Most Multi-pathway Standards and Endogenous Targets Despite Detectable Amounts

- SMOAC enrichment captures broad pool of phosphopeptides.
- Poor detection of functionally relevant endogenous phosphopeptides from the multi-pathway panel.
- DDA stochastic sampling likely resulted in missed detection of desired multi-pathway targets.





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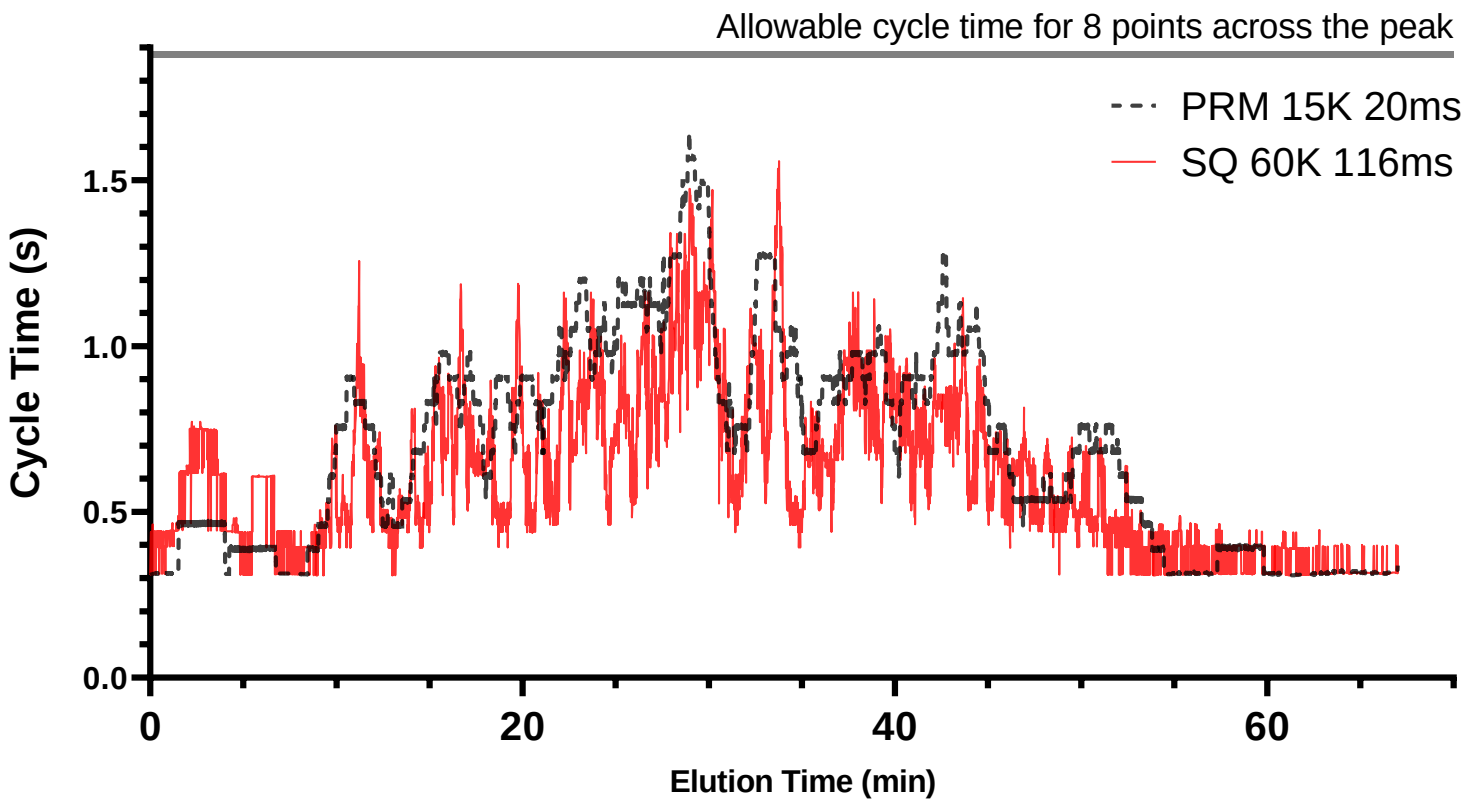
Results – Typical Targeted Methods Don’t Provide Sensitivity For High-Density Panels

SureQuant Dynamic MS2 Control Allows ~6X More Fill Time and Maintains Appropriate Sampling Rates

- Real-time recognition of target elution time.
- Dynamic control of high-sensitivity, high-fill time MS2 scans at the precise time of target elution.
- Dynamic fill-time management allows duty cycles to be maintained within appropriate ranges.



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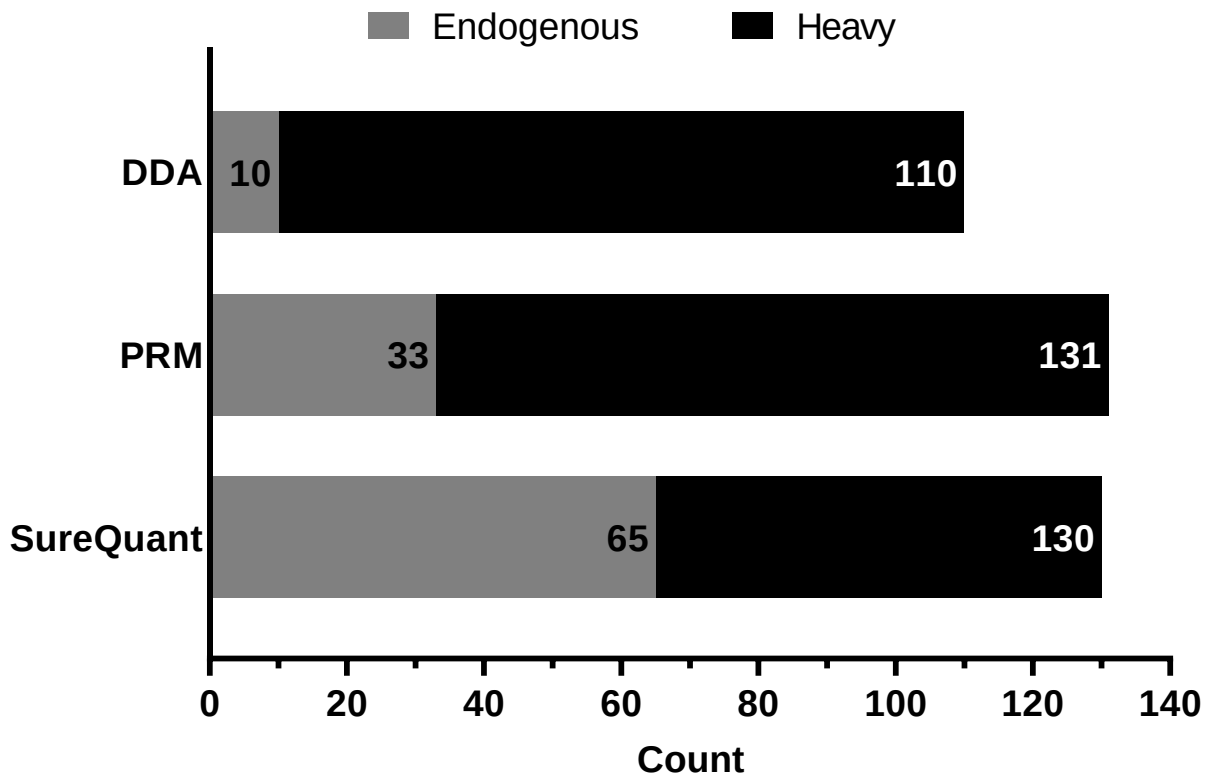
Results – Increased Fill Time = Higher Measurement Sensitivity

SureQuant Multi-Pathway Phosphopeptide Detection Outperforms DDA And PRM

- SMOAC enrichment provides reproducible enrichment of multi-pathway targets.
- SureQuant targeted analysis enabled most comprehensive detection.
- Reference standard detection provides clarity about ‘missing values’ for endogenous targets.



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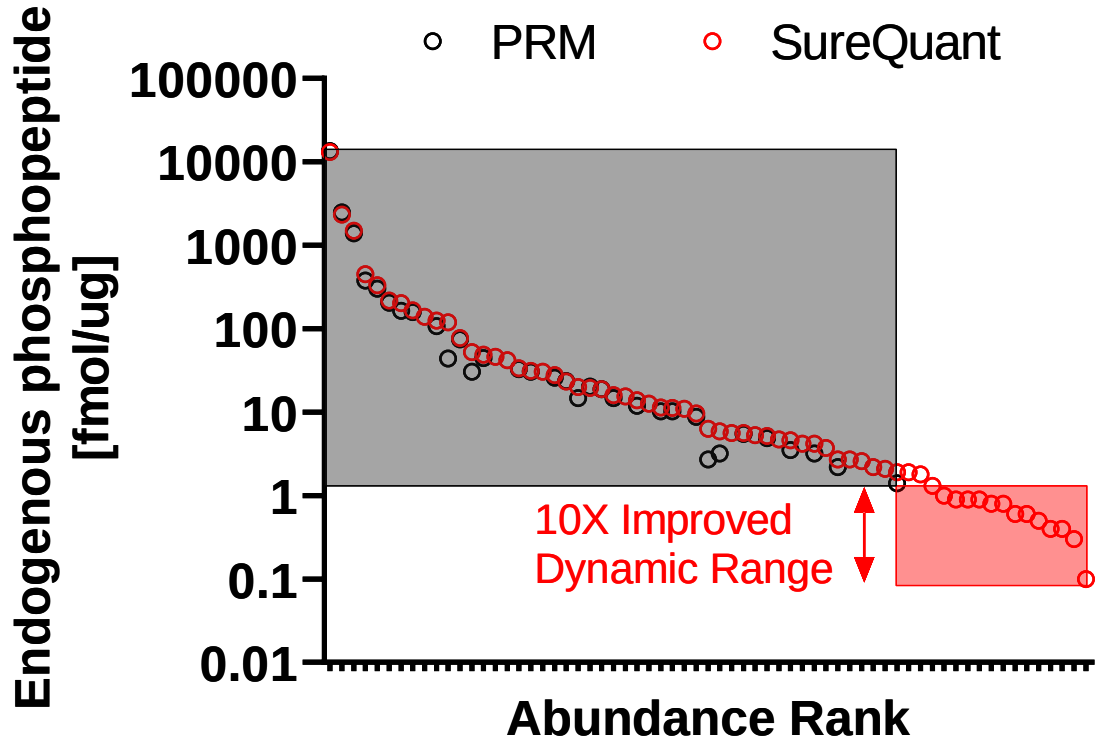
Results – SureQuant Measurement Sensitivity More Sample Insight

Endogenous phosphopeptides can be quantified at 10X lower levels than PRM

- SureQuant targeted acquisition enabled quantitation of more low-abundant phosphorylated signaling pathway proteins than PRM.
- Due to significantly improved measurement sensitivity.
- Amol/ug levels of detection.



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Conclusions

- SureQuant IS-triggered targeted acquisition on Orbitrap Exploris 480 MS facilitates reliable and robust quantitative measurements.
- The multi-pathway phosphopeptide panel coupled with standardized sample preparation, SMOAC enrichment, and SureQuant acquisition provides a turnkey approach for comprehensive signaling pathway analysis.
- Modular combination of SMOAC enrichment, IS phosphopeptide panels, and SureQuant targeting creates the opportunity of fit-for-purpose signaling pathway assays.