

Real-time, High Density Monitoring of pTyr Signaling Targets in Human Tumors Using Heavy Peptide Triggered Targeted Quantitation

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ABSTRACT

Purpose: We sought to develop a universal internal standard (IS)-guided LC-MS acquisition workflow for reproducible, sensitive and high-density absolute quantification of phosphotyrosine (pTyr) signaling.

Methods: We leveraged two-stage selective phosphotyrosine peptide enrichment, isotopically-labeled trigger peptides and the Thermo Scientific™ SureQuant™ IS targeted protein quantitation method to detect and quantify 356 pTyr targets per analysis using Thermo Scientific™ Orbitrap Exploris™ 480 and Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ Mass Spectrometers.

Results: Compared to traditional discovery-based proteomics approaches for pTyr analysis, the SureQuant pTyr acquisition workflow delivered more reproducible quantitation profiles across samples along with the ability to determine absolute concentrations of measured signaling components with high sensitivity.

INTRODUCTION

Efforts to understand PTM signaling networks that govern cellular deregulation and disease have harnessed LC-MS based proteomics to catalog several thousand phosphorylation sites across many functional protein categories. Protein tyrosine phosphorylation (pTyr) is of great interest, being highly correlated to the known repertoire of human kinase oncogenes despite only accounting for <1% of total phosphorylation events. Methods to monitor tyrosine phosphorylation networks are balanced between broad-coverage discovery proteomics or traditional targeted approaches which focus on limited subsets of the entire pTyr network. We describe a novel, high-density targeted approach that combines selective pTyr peptide enrichment, and leverages isotopically-labeled trigger peptides, to efficiently guide LC-MS acquisition in real-time, allowing reliable quantitation of several hundred pTyr targets per analysis for research studies.

To circumvent the drawbacks of typical quantitative data-dependent discovery proteomics (i.e., stochasticity, bias towards abundant targets, poor reproducibility), we employed an internal-standard guided targeted quantitation approach for systematic monitoring and measurement of pTyr peptides in human colorectal tumor specimens. In contrast to traditional targeted acquisition schemes which are often a compromise between: (i) number of peptides that can be reliably measured, and (ii) sensitivity and selectivity of those measurements, the method described herein allows unprecedented breadth of coverage to be achieved while retaining exquisite quantitative performance. This workflow was adapted from the internal standard triggered parallel reaction monitoring method (IS-PRM); however, the adapted method works with the native instrument control software of the commercially available Orbitrap instruments. In the current operation, the instrument alternates between a “watch mode”, in which IS representing the targets of interest were continuously measured at fast scanning rates, and a “quantitative mode”, which is triggered by real-time standard peptide identification that enables highly selective and sensitive measurement of endogenous peptides serially over their elution profile.

MATERIALS AND METHODS

Sample Processing

Proteins were extracted from previously characterized human colorectal tumor specimens and subjected to reduction, alkylation, trypsin digestion and cleanup¹. A 1 pmol mixture of 356 stable isotopically-labeled peptides, corresponding to several hundred pTyr targets, was spiked into the tumor samples. The endogenous and IS forms of the peptide targets were enriched by multiplexed pTyr immunoprecipitation (PT66, PY100, and 4G10 antibodies) followed Hi-Select Fe-NTA (Thermo Fisher Scientific) enrichment.

Test Methods

LC-MS analysis was performed with a Thermo Scientific™ EASY-nLC™ 1200 system 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. 1 pmol of the pTyr IS mixture enriched from an EGF-stimulated A549 cell line digest was used for the survey run analysis to determine intensity thresholds for subsequent SureQuant tumor analysis.

Data Analysis

Survey run and SureQuant data analysis was performed using Skyline software.

RESULTS

SureQuant IS triggered global pTyr profiling methodology

To demonstrate the application of the SureQuant IS targeted quantitation method on a biologically relevant sample cohort, we developed a method to quantify several hundred pTyr sites across a panel of colorectal tumors (Figure 1). The method consists of spiking-in pTyr IS into experimental samples followed by antibody enrichment and metal affinity chromatography to selectively enrich for low abundant IS and endogenously expressed pTyr peptides. IS peptide-triggered and guided acquisition maximizes the efficiency of targeted quantification by enhancing the detectability of targets, significantly improving measurement reproducibility across analysis (Figure 2). The improved acquisition efficiency allows greater number of target multiplexing to be achieved without compromising duty cycle time and quantitative performance such as sensitivity and precision. A curated list of functionally relevant pTyr peptide targets was selected from previously collected reference datasets of quantitative phospho-tyrosine LC-MS analysis (Figure 3). 385 selected pTyr peptide targets were synthesized and characterized to evaluate optimal charge states, fragment ions, and signal intensity thresholds for pTyr triggering and quantitation. Of this initial panel, 356 peptides were reproducibly detected in pilot experiments and utilized for subsequent tumor profiling experiments.

Figure 1. Tumor specimen cohort characteristics

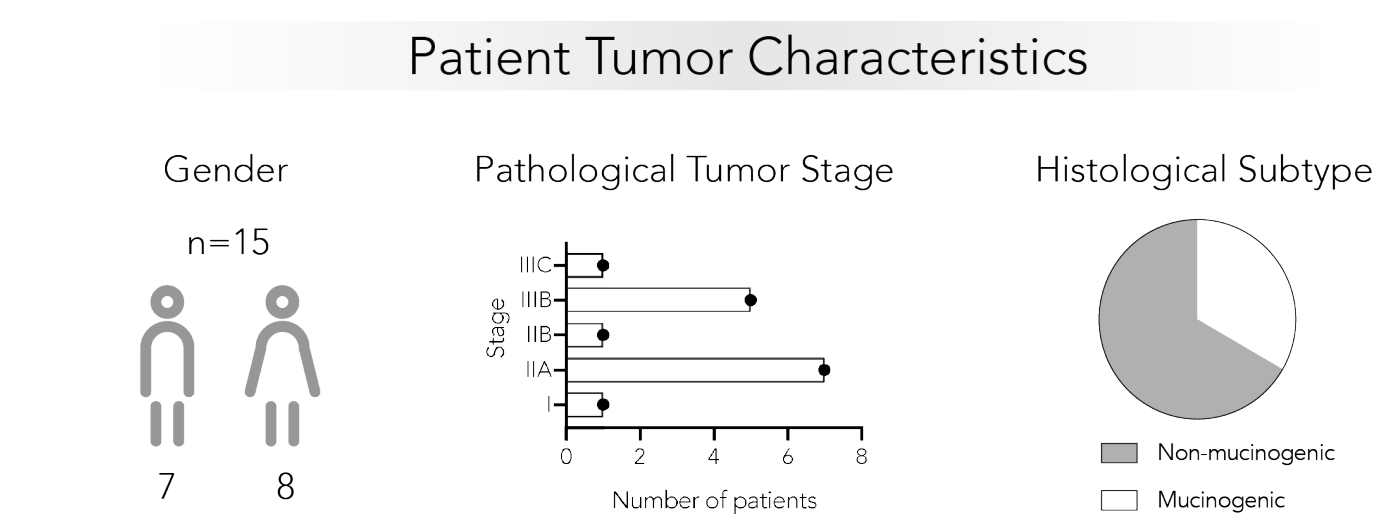


Figure 2. Workflow steps for the SureQuant IS targeted pTyr acquisition

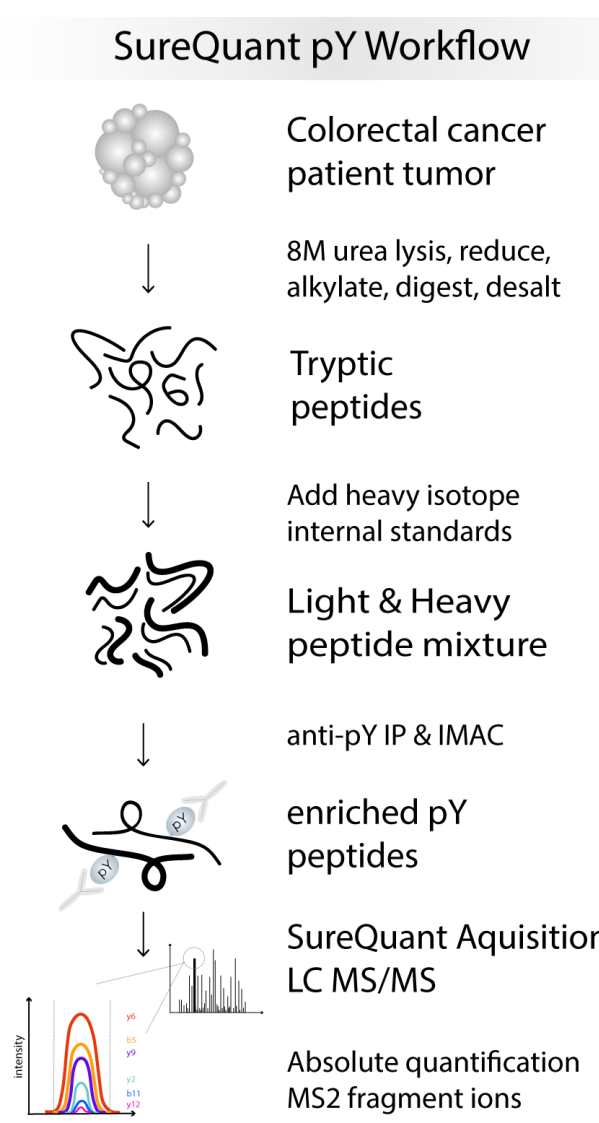
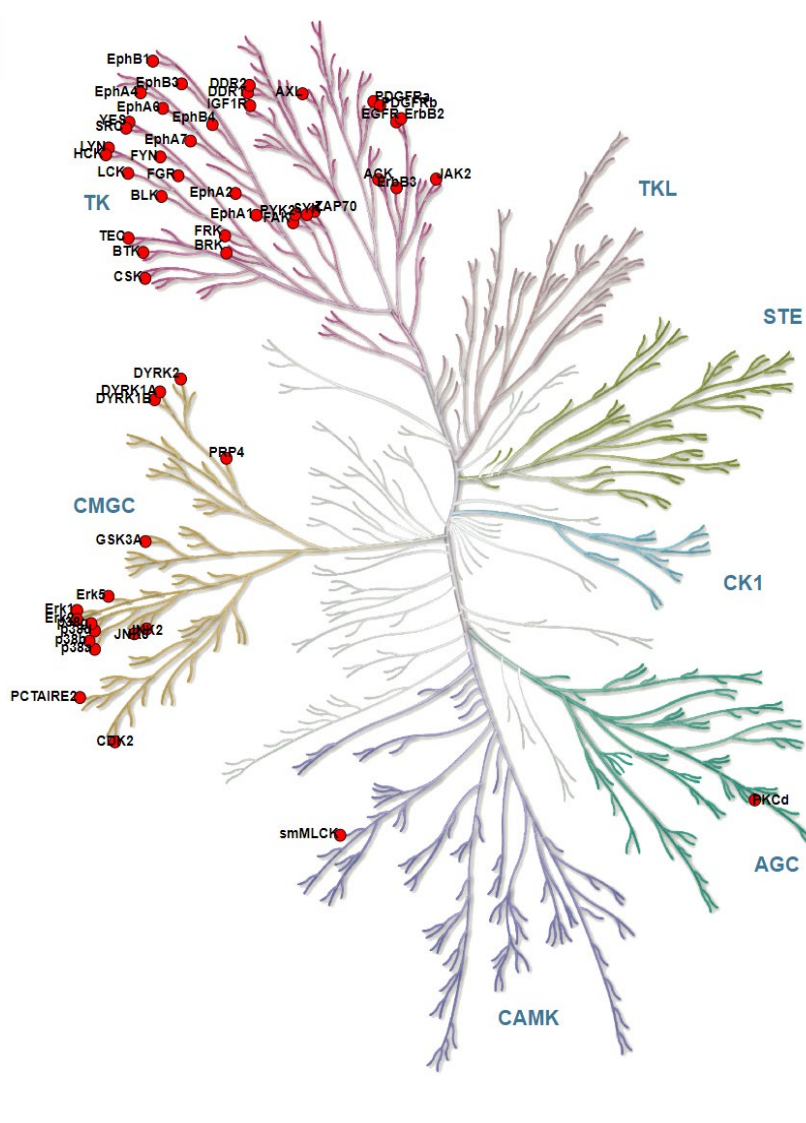


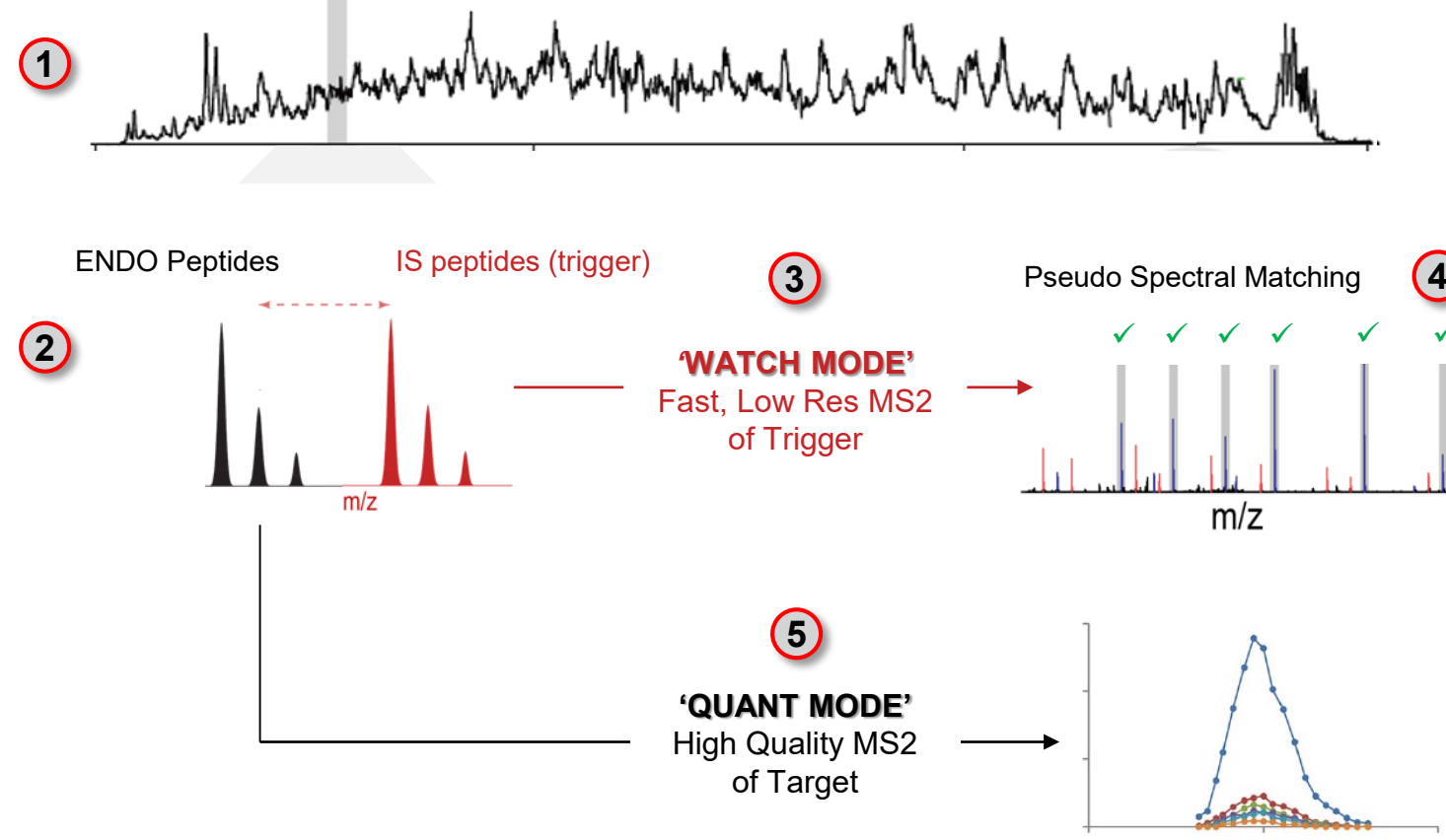
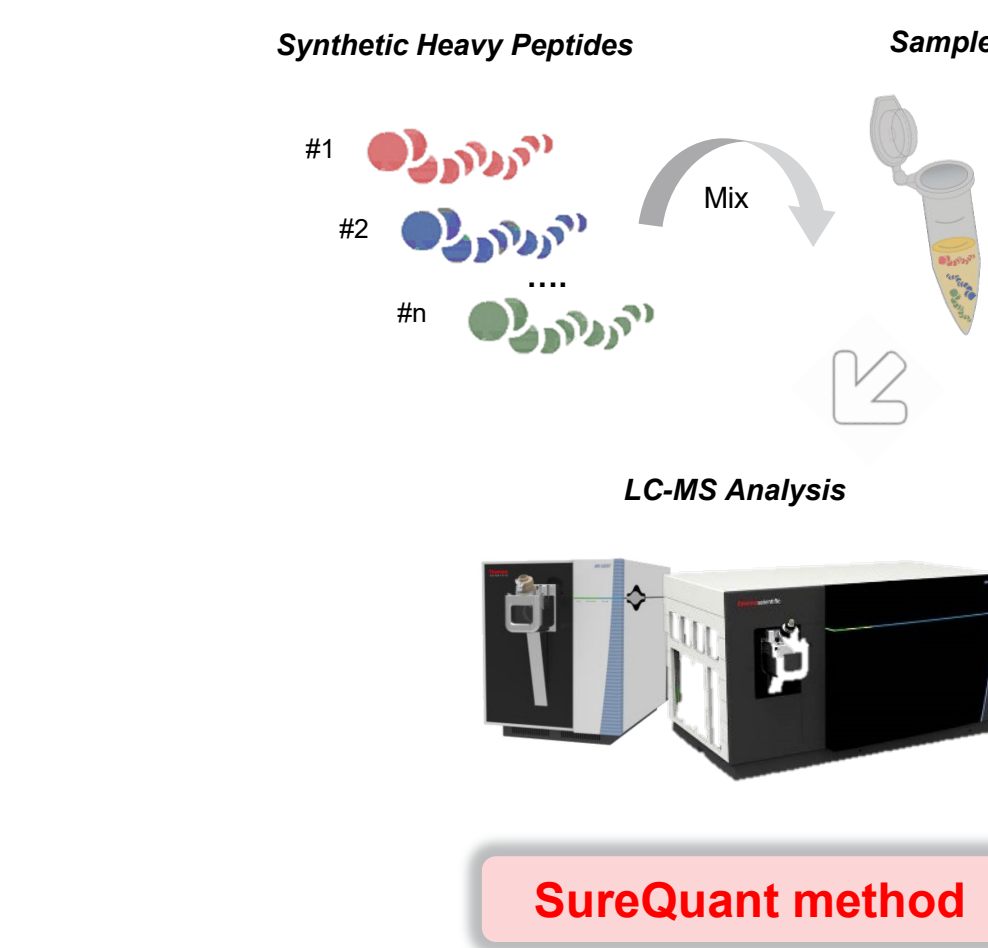
Figure 3. Representation of 55 kinases included in the 385 pTyr internal standard panel.



SureQuant maximizes quantitative performance for targeted proteomics analysis

SureQuant is novel quantitation paradigm leveraging IS to automatically guide targeted proteome analysis in real-time (Figure 4). In the SureQuant method, the mass spectrometer is programmed to monitor reference IS in the sample and dynamically adjust scan parameters such as fill time and resolution, upon their detection, to enhance the data quality for endogenous peptides. The mass spectrometer is programmed to alternate between a “watch mode”, in which IS representing the targets of interest were continuously measured at fast scanning rates, and a “quantitative mode”, which is triggered by real-time standard peptide identification that enables highly selective and sensitive measurement of endogenous peptides serially over their elution profile. Balancing the maximum number of protein targets with highest sensitivity and quality presents a formidable challenge in traditional protein quantitation methods. Detection of the IS proxies ensures optimal targeted scan settings for the corresponding targets of interest are enabled at precisely the right time and without the need for scheduling. The real-time management of acquisition time maximizes the time devoted to analyte quantitation allowing greater number of targets to be reliably detected and unrivaled productivity for targeted proteomics experiments with a “load-and-play” setting in a single analytical run. The built-in positive IS control provides a definitive LOD measure for the presence or absence of proteins in the sample addressing the common need to assess protein copy number expression in many molecular biology experiments.

Figure 4. SureQuant acquisition delivers intelligent detection of targets and maximizes productivity and quantitative certainty



SureQuant analytical attributes enable high target detectability, suitable peak sampling rates, and high measurement sensitivity

A preliminary set of survey runs were conducted to determine the optimal amount of pTyr IS peptide mixture needed to ensure sufficient recovery following the pTyr enrichment steps. Ultimately, 1 pmol of the IS mixture allowed facile detectability during LC-MS analysis (Figure 5). Additionally, the dynamic management of acquisition parameters allows high quality MS2 quantitation for hundreds of targets without a penalizing sampling rates (Figure 6).

Figure 5. Elution distribution of pTyr IS and easily detectable apex signal by LC-MS

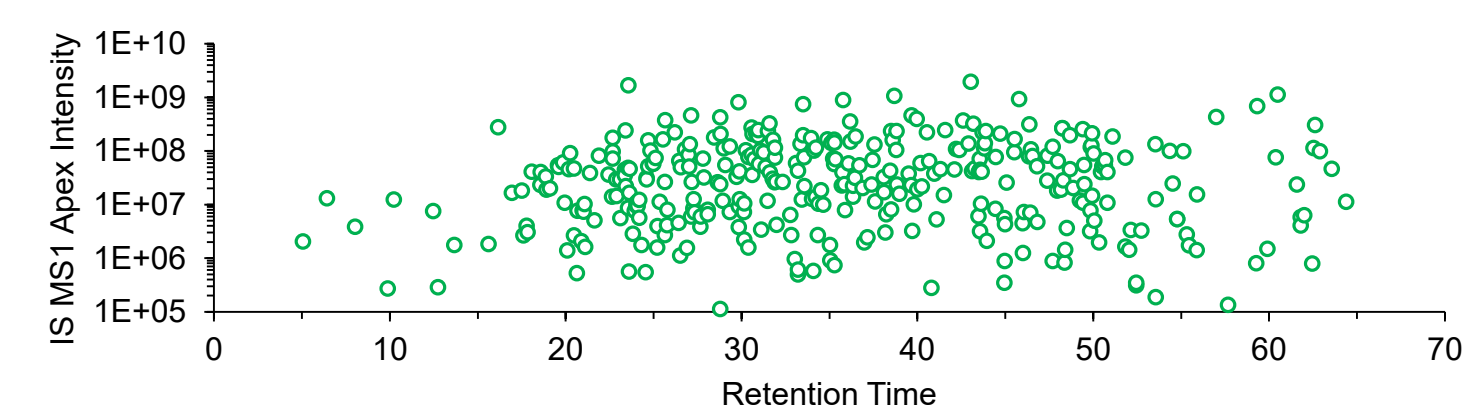
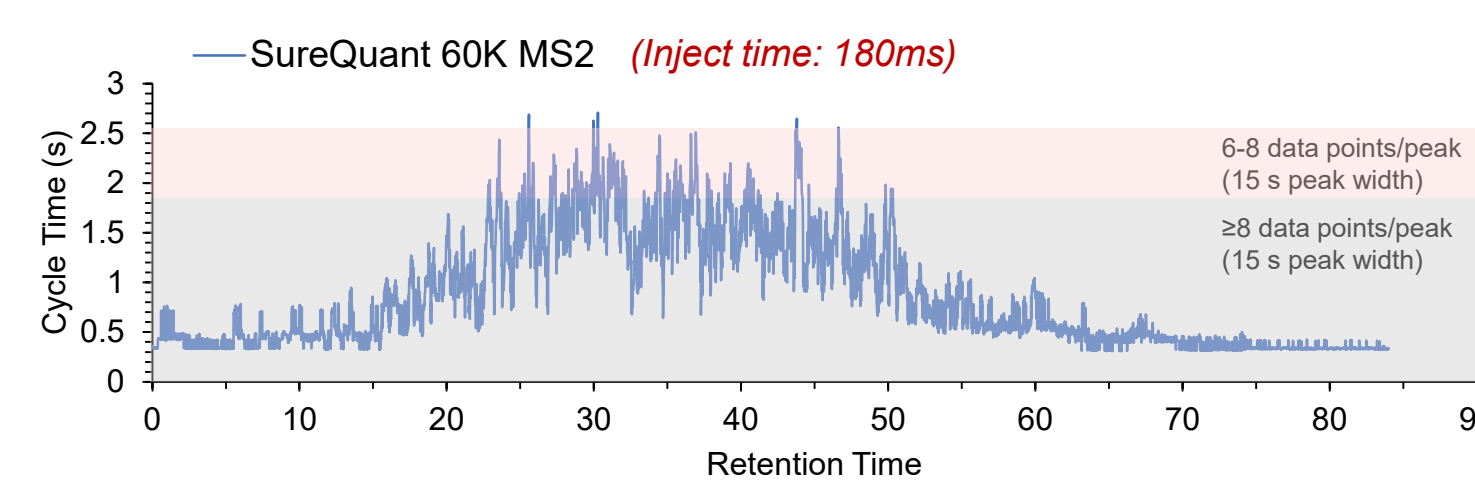


Figure 6. Representative SureQuant cycle time profile (tumor sample 19) highlights appropriate sampling rates despite MS2 parameters favoring high sensitivity



Targeted pTyr profiling reveals tumor specific phosphorylation differences

The SureQuant approach permitted the reproducible monitoring of hundreds of signaling nodes within the cellular pTyr network with the ability to determine absolute expression levels through one-point calibration to the IS of known concentration (Figure 7). In this way, absolute quantification of tumor specific phosphorylation signatures were revealed (Figure 8).

Figure 7. Example of quantification of phosphorylation of EGFR Y1173 (GSTAENAEyLR) from tumor 19

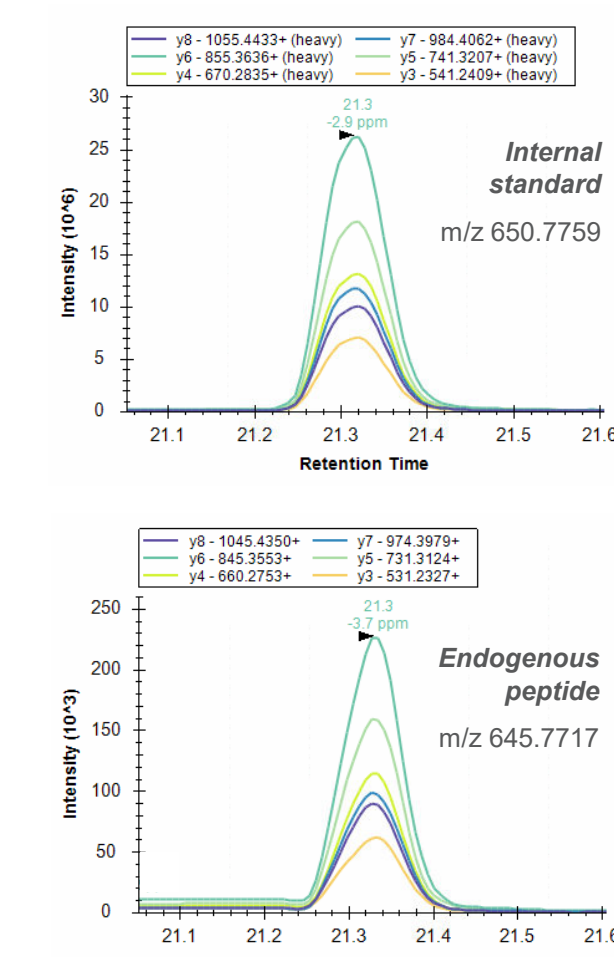
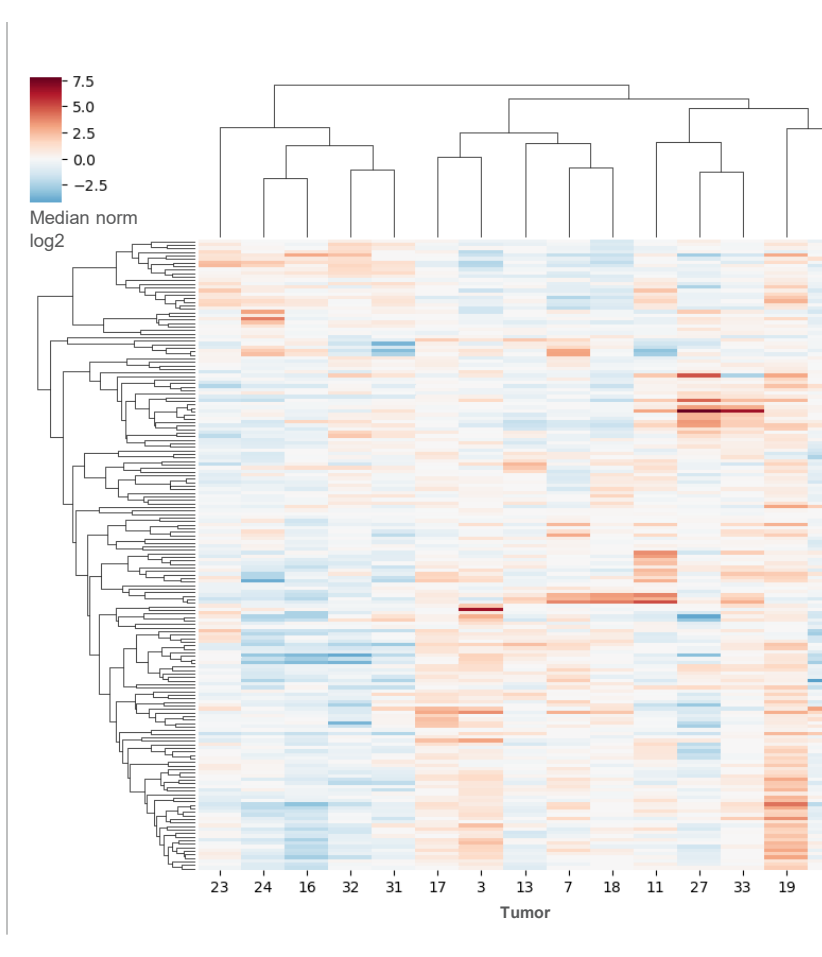


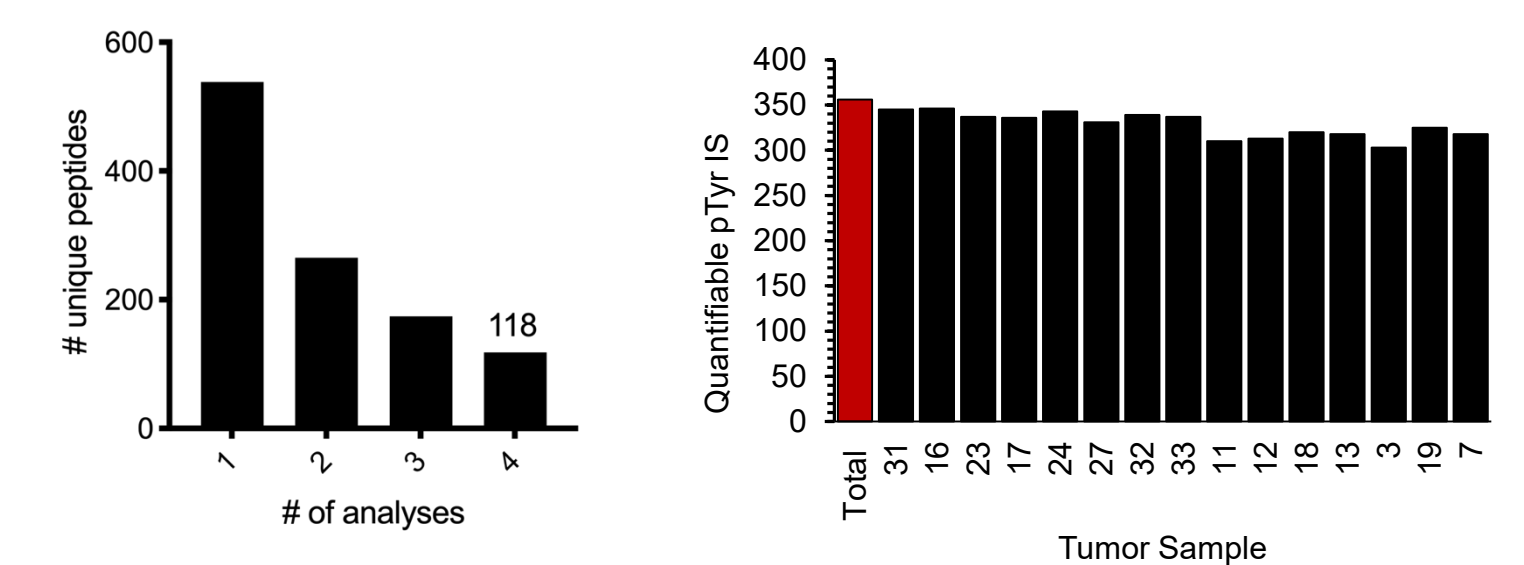
Figure 8. Tumor specific tyrosine phosphorylation signatures can be detected within the CRC cohort



SureQuant facilitates highly reproducible measurements across many samples

Compared to traditional DDA discovery LC-MS analysis where peptides are stochastically selected for MS/MS identification and quantitation, SureQuant measurements are efficiently directed by the IS spiked into the experimental sample towards the relevant precursor targets. For comparison, the same cohort of tumor samples analyzed by SureQuant underwent TMT based pTyr LC-MS analysis where multiple 10-plex analysis were performed (Figure 9). When the number of reproducibly measured peptides was evaluated between the independent TMT analysis, <25% of measurements were found across all samples. SureQuant targeted pTyr measurements by comparison were highly reproducible, with >80% of all targets observed across all of the tumor samples.

Figure 9. Differences in measurement reproducibility for DDA (left) and SureQuant (right) based pTyr quantitation approaches.



CONCLUSIONS

- SureQuant IS triggered global pTyr profiling methodology utilizes the presence of synthetic isotopically labeled heavy peptide surrogates to enable efficient, sensitive, and reproducible target multiplexing measurements for hundreds of pTyr targets in a single analysis with absolute quantitation of phosphosite abundance.
- The combination of pTyr enrichment strategies and IS guided acquisition presents a new paradigm for signaling network proteomics involving PTM analysis.
- In principle, SureQuant global profiling methodology can be applied for the analysis of a variety of phosphorylated (ie. pSer or pThr) or PTM containing peptides, as well as disease or pathway specific protein panels.

REFERENCES

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