Evaluation of Parallel Reaction Monitoring assays at discovery scale on a new hybrid nominal mass instrument for phosphoproteomics studies

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Abstract

Purpose: Development of discovery-scale quantitative PRM-based approaches for phosphoproteomics studies.

Methods: Spectral libraries were previously generated on a high-resolution accurate mass (HRAM) mass spectrometer using synthetic SIL peptides. The precursor list from these libraries was imported to create targeted parallel reaction monitoring (PRM) assays on a newly developed hybrid high-speed nominal mass instrument.The SIL peptides mixture on neat solution was then measured to determine retention times in a 30-minute gradient method. Scheduled targeted MS^2 and MS^3 (tMS2 and tMS3) assays were created using PRM conductor, a new Skyline-based plugin tool. This new tool also added the corresponding endogenous peptides which led to a final assay of 670 phosphopeptides in both tMS2 and tMS3 methods. The performance of the methods was evaluated by measuring the phosphopeptides in a mixture of five cancer cell lines.

Results: Assessment of the limit of quantitation and linearity of both tMS2 and tMS3 methods was performed. For this, a serial dilution of cell lysate with SIL peptides into cell lysate was carried out. Preliminary results demonstrated excellent sensitivity in the atto-mole range of peptide amount on column. Additionally, results also indicated that enhanced selectivity can be achieved with tMS3 acquisition. Sensitivity was also evaluated in a mixture of five cancer cell lines.

Introduction

The measurement of phosphosignatures is crucial to better understand cellular mechanisms, as protein phosphorylation is well known to be a strong mediator of cellular signaling. MS-based proteomics approaches have led to the discovery of thousands of known human phosphosites, however discovery approaches fail in detecting uniformly any given phosphosite across an entire sample cohort. Hence, there is a need for more accurate, comprehensive methods for phosphopeptide analysis to enhance our understanding of cellular processes and disease mechanisms, potentially aiding the development of more effective therapeutic strategies.

Recently, a 300-plex phosphopeptide targeted MS assay (SigPath assay) suitable for both discovery and preclinical studies was developed using triple quadrupole technology[1]. Herein we evaluate Parallel Reaction Monitoring-based approaches using a new hybrid high-speed nominal mass instrument. Both tMS2 and tMS3 approaches were assessed for the absolute quantitation of more than 300 phosphopeptides in cancer cell lines using synthetic stable isotope labeled (SIL) peptides.

Materials and methods

Sample Preparation

LOQ and linearity assessment: Synthetic SIL phosphopeptides were spiked into 200 ng/µL HeLa digest ranging from 0.018 to 40 fmol/µL.

Titration curve experiment: Five digested cancer cell lines were equally combined to create an endogenous peptide mix, spiked with 30 fmol SIL peptides and enriched by IMAC prior LC-MS/MS analysis as described by Keshishian et al.[1] Sample input amounts ranged from 25 to 500 µg.

Data Acquisition

1µL of sample was loaded on an Thermo Scientific™ Easy-Spray™ PepMap™ Neo 2 µm C18 75 µm X 150 mm (ES75150PN), held at 45 C with a flow rate of 350 nL/min, delivered by Thermo Scientific™ Vanquish™ Neo UHPLC system using 30 min gradient. Data was acquired on Thermo Scientific™ Stellar™ mass spectrometer, a new hybrid nominal mass instrument, utilizing both tMS2 and tMS3 scan functions and HCD and CID fragmentation.

Figure 1. LC-MS setup for the targeted phosphoproteomics study.

Data Analysis

The acquired data was processed using Skyline-Daily software.

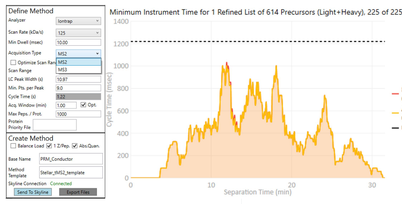
Results

PRM Conductor

Setting up a tMS2 method (PRM)

Precursor lists of the spectral libraries previously generated on HRAM instrument,[1,2] were exported to create 4 unscheduled PRM methods using a 30-min gradient to establish the retention times of 335 SIL phosphopeptides. PRM conductor, a new Skyline-based plugin tool, was then used to create a scheduled PRM-based instrument method. By defining the expected peak width, minimum number of points across the peak and retention time window, graphically displays the amount of instrument time required for any given scan cycle. This can be used to fine tune method parameters (Figure 2). By selecting Abs. Quan box, PRM conductor will include endogenous (light) phosphopeptides to the final precursor list. Moreover, if an instrument method template containing LC and MS settings is available, the PRM conductor will export a PRM instrument method ready to be browsed into the run sequence.

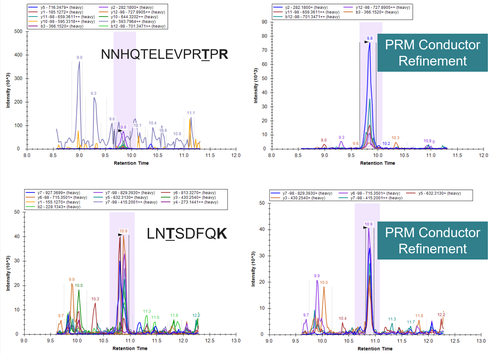
Figure 2. PRM Conductor - Define Method. Automatically creates acquisition methods. On right hand side, it is indicated the required cycle time for all scans. Dashed horizontal line, indicates maximum cycle time that will not impact instrument performance.



Refinement of Transitions

PRM Conductor automatically refines the transition list based on user defined minimum peak area values, S/N ratio, ion ratio, peak shape correlation and peak width (Figure 3).

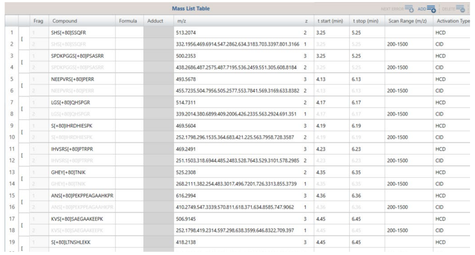
Figure 3. Comparison of effect of PRM Conductor on transition selection, for two SIL phosphopeptides spiked at 1.48 fmol into 200 ng/µL HeLa digest. On the left the initial PRM transitions, on the right after PRM Conductor refinement. PRM Conductor removes interferences and noisy transitions.



Setting up a targeted-MS3 method

PRM conductor also enabled the creation of tMS3 instrument methods with Synchronous Precursor Selection (SPS) option to improve sensitivity. Additionally, the versatility of the instrument control software of Stellar MS allows the user to define a combination of different activation types for tMS3 experiments for optimized results (Figure 4).

Figure 4. Fraction of a mass list table used for tMS3 experiments. The list of precursors includes more than 600 phosphopeptides (30-min gradient).



PRM-based approaches: Quantitative performance

Calibration curves were generated by diluting SIL phosphopeptides in a 200 ng/µL HeLa background and using both tMS2 and tMS3 acquisition approaches. The concentration of SIL phosphopeptides ranged from 18 amol to 40 fmol on column. Good linearity with good accuracy and reproducibility were achieved for most of SIL phosphopeptides. Additionally, an improvement in LOQ using a tMS3 approach was observed for some of SIL phosphopeptides (Figure 5).

Figure 5. Calibration curves of a SIL phosphopeptide using tMS2 and tMS3 approaches.

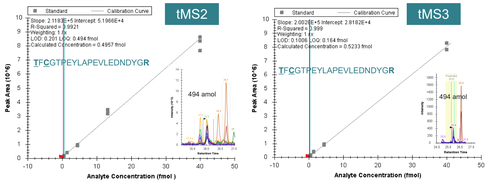


Figure 6. Reproducibility at 494 amol on column of SIL phosphopeptides using a tMS2 approach.

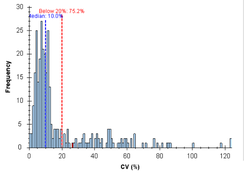
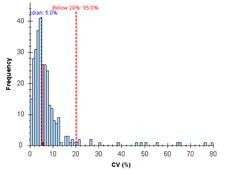


Figure 7. Reproducibility at 13.3 fmol on column of SIL phosphopeptides using a tMS3 approach.



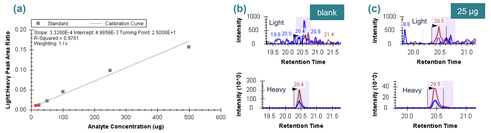
Titration curve experiment

Evaluation of sensitivity of the PRM assay was performed in a mixture of 5 cancer cell lines to maximize detection of endogenous phosphopeptides. IMAC enrichment was performed with 5 different input amounts ranging from 25 to 500 µg. One hundred thirteen endogenous light peptides were detected in this experiment.

Table 1. Number of detected endogenous light peptides at different input sample levels.

Input sample	Number of detected endogenous light phosphopeptides
25 µg	113
50 µg	121
100 µg	148
250 µg	171
500 µg	193

Figure 8. IADPEHDHTGFLT(+80)EY(+80)VATR: (a) Plot of response ratio for light and heavy phosphopeptide over an input amount range from 25 to 500 µg. (b) EIC of a blank sample spiked with SIL peptides only. (c) EIC of lowest input sample 25 µg.



Conclusions

Both PRM and tMS3 assays were developed to measure a large panel of phosphopeptides in a 30-min gradient:

- Good linearity with good accuracy and reproducibility was achieved for most of SIL phosphopeptides.
- PRM conductor enabled the creation of scheduled PRM and tMS3 assays.
- Improvement of specificity and sensitivity using a tMS3 approach was observed for some of SIL phosphopeptides.

The sensitivity of PRM assay was assessed in a mixture of 5 cancer cell lines:

- Over 190 endogenous light peptides were detected in this mixture after enrichment using IMAC.
- PRM method could detect over 100 light peptides in the lowest input sample of 25 µg.

These findings suggest an improvement of at least 4-fold in sensitivity and approximately 5-fold in sample analysis throughput compared to the previous triple quadrupole-based method.

Future experiments with cancer cell lines samples:

- Assessment of sensitivity in an input amount of 10 µg
- Assessment of reproducibility
- Assessment of sensitivity of tMS3 assay

References

1. Keshishian *et al.*, Mol Syst. Biol., 17(9), 2021.
2. Abelin *et al.*, Mol. Cell Proteomics, 15(5), 2016.

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