Published in final edited form as:

Anal Chem. 2015 October 20; 87(20): 10222–10229. doi:10.1021/acs.analchem.5b02983.

Multiplexed, Scheduled, High-Resolution Parallel Reaction Monitoring on a Full Scan QqTOF Instrument with Integrated Data-Dependent and Targeted Mass Spectrometric Workflows

Birgit Schilling[†], Brendan MacLean[‡], Jason M. Held[§], Alexandria K. Sahu[†], Matthew J. Rardin[†], Dylan J. Sorensen[†], Theodore Peters[†], Alan J. Wolfell^{||}, Christie L. Hunter[⊥], Michael J. MacCoss[‡], and Bradford W. Gibson^{*,†,#}

[†]Buck Institute for Research on Aging, 8001 Redwood Boulevard, Novato, California 94945, United States

[‡]Department of Genome Sciences, University of Washington School of Medicine, Foegen Building S113, 3720 15th Avenue NE, Seattle, Washington 98195, United States

[§]Departments of Medicine and Anesthesiology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, Missouri 63110, United States

^{||}Department of Microbiology and Immunology, Stritch School of Medicine, Health Sciences Division, Loyola University Chicago, 2160 South First Avenue, Maywood, Illinois 60153, United States

[⊥]SCIEX, 1201 Radio Road, Redwood City, California 94065, United States

[#]Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143, United States

Abstract

Recent advances in commercial mass spectrometers with higher resolving power and faster scanning capabilities have expanded their functionality beyond traditional data-dependent acquisition (DDA) to targeted proteomics with higher precision and multiplexing. Using an orthogonal quadrupole time-of flight (QqTOF) LC-MS system, we investigated the feasibility of implementing large-scale targeted quantitative assays using scheduled, high resolution multiple reaction monitoring (sMRM-HR), also referred to as parallel reaction monitoring (sPRM). We assessed the selectivity and reproducibility of PRM, also referred to as parallel reaction monitoring, by measuring standard peptide concentration curves and system suitability assays. By

*Corresponding Author: bgibson@buckinstitute.org. Phone: 415-209-2032. Fax: 415-209-2231.

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.5b02983.v

Supplementary Methods and Figures S-1 to S-7 (PDF)

Table S-1 (XLSX)

Table S-2 (XLSX)

Table S-3 (XLSX)

Table S-4 (XLSX)

evaluating up to 500 peptides in a single assay, the robustness and accuracy of PRM assays were compared to traditional SRM workflows on triple quadrupole instruments. The high resolution and high mass accuracy of the full scan MS/MS spectra resulted in sufficient selectivity to monitor 6–10 MS/MS fragment ions per target precursor, providing flexibility in postacquisition assay refinement and optimization. The general applicability of the sPRM workflow was assessed in complex biological samples by first targeting 532 peptide precursor ions in a yeast lysate, and then 466 peptide precursors from a previously generated candidate list of differentially expressed proteins in whole cell lysates from *E. coli*. Lastly, we found that sPRM assays could be rapidly and efficiently developed in Skyline from DDA libraries when acquired on the same QqTOF platform, greatly facilitating their successful implementation. These results establish a robust sPRM workflow on a QqTOF platform to rapidly transition from discovery analysis to highly multiplexed, targeted peptide quantitation.

Graphical Abstract

Quantitative proteomics has advanced rapidly in recent years with developments of newer generation mass spectrometers that provide improved precision, mass accuracy and resolution, dynamic range, and scan speed. Data-dependent acquisition (DDA) quantitative workflows often include metabolic labeling, such as stable isotope labeling by amino acids in cell culture (SILAC),^{1–3} or postmetabolic, isobaric chemical labeling, such as an isobaric tag for relative and absolute quantitation (iTRAQ) or a tandem mass tag (TMT).^{4,5} However, label-free workflows offer effective alternatives and do not require additional sample preparation steps.^{6–8} More recently, global data-independent acquisition (DIA) workflows, such as SWATH-MS, have attracted attention for large-scale targeted data analysis, attempting to monitor larger parts of the proteome.^{9–11}

Traditionally, targeted mass spectrometric methods for candidate verification and large-scale clinical studies have employed selected reaction monitoring (SRM) assays on triple quadrupole instruments. The SRM methodology has been considered the “gold standard” for mass spectrometric quantitation,¹² and it was declared “Method of the Year 2012” by *Nature Methods*.^{13,14} Modern triple quadrupole instrumentation has undergone significant technical improvements, such as in scan speed and sensitivity, that allow for highly multiplexed SRM studies.^{15,16} For example, Burgess and colleagues were able to monitor 800 peptides (400 pairs of light and heavy peptides) with a total of 2400 transitions in a systematic study of plasma samples using long gradients (~4 h).¹⁵ The number of targeted SRM analytes that

can be multiplexed has increased substantially by taking advantage of retention time scheduling.^{17,18} Nevertheless, SRM assays usually still require significant assay development time, and the number of multiplexed transitions/peptides can be limiting in large-scale studies.

Technological improvements in high-resolution, full scanning instrumentation have led to several new DIA and targeted acquisition methods that have challenged the dominance of triple quadrupoles for targeted mass spectrometry. One approach, originally described as high resolution MRM or MRM-HR, although now generally referred to as parallel reaction monitoring (PRM), has emerged in which a peptide precursor ion is isolated in Q1 and fragmented in Q2, and subsequently, all generated MS/MS fragment ions are monitored in parallel on a high resolution, accurate mass, full scan mass spectrometer.^{19–21} Recently, PRM has been demonstrated in several biological applications,^{22–24} also implementing retention time (RT) scheduling of analytes to increase the number of analytes analyzed during a single LC-MS run.^{23,25,26} Moreover, Creech et al. employed a high-throughput PRM approach to monitor modifications of core histones of chromatin in human cell lines and mouse embryonic cell lines,²⁷ while others targeted ~600 biomarker candidates by PRM in human plasma and urine samples.²⁸ Additional methodological advances have been reported, such as using Internal Standard triggered Parallel Reaction Monitoring (IS-PRM) to optimize fill times and data quality while maintaining high-throughput.²⁹

Most of the PRM studies have used Orbitraps or hybrid Q-Exactive instruments (Thermo) for data acquisition, although, to our knowledge, this is the first large-scale PRM study carried out on an orthogonal quadrupole time-of-flight instrument (QqTOF), or TripleTOF 5600 (SCIEX). Initially, our PRM experiments were directed toward assessing dynamic range and linearity by acquiring response curves in simple and complex matrices. To further assess the performance of the PRM workflow, we performed a system suitability study originally developed for triple quadrupole instruments.³⁰ We also employed retention time scheduling of PRM (sPRM) for greater multiplexing, achieving ~500 peptide analytes in a single acquisition. Integrated software solutions are also presented using the Skyline software environment for fast and large-scale sPRM assay development, as well as rapid postacquisition data processing.

EXPERIMENTAL SECTION

Sample Preparations

Response Curve for a Set of Spiked Acetylated Peptides in a Simple Matrix—

Six lysine-acetylated synthetic peptides containing $^{13}\text{C}_6^{15}\text{N}_2$ -Lys and $^{13}\text{C}_6^{15}\text{N}_4$ -Arg were used to generate standard concentration curves in a simple matrix, a predigested six-protein mix (matrix at $0.3\ \mu\text{g}/\mu\text{L}$ and $0.3\ \mu\text{g}$ on column), with 6 spiked concentration points at 0.063, 0.125, 0.625, 1.25, 2.5, and $25\ \text{fmol}/\mu\text{L}$ (loading $1\ \mu\text{L}$ of sample on-column). Three replicate concentration curves were acquired on the TripleTOF 5600 in PRM mode.

Response Curve for Spiked Digested Six Protein Mix in Complex Matrix (C. Elegans Whole Cell Lysate)—A mixture of six predigested proteins was spiked into the digested *C. elegans* cell lysate (complex matrix at $1\ \mu\text{g}/\mu\text{L}$ and $1\ \mu\text{g}$ on column) at 8

concentrations: 0, 0.015, 0.061, 0.244, 0.975, 3.9, 15.6, and 62.5 fmol/ μ L (loading 1 μ L of sample on-column). The spike level “0 fmol/ μ L” was used as “blank” measurement. Two replicate concentration curves were acquired in parallel on the TripleTOF 5600 in PRM mode, and on the QTRAP 5500 in SRM mode.

Digested Whole Cell Lysate from Yeast—Reproducibility Assessment and Highly Multiplexed Scheduled sPRM Experiments—BY4743 yeast strain samples were grown at 30 °C in synthetic complete media³¹ (for sample workup see Supplementary Methods, Supporting Information).

Whole Cell Lysates from *E. coli* Mutant (*ackA*) and WT Strains—Scheduled PRM, Differential Protein Expression—*E. coli* WT and several isogenic mutant strains cells were grown at 37 °C in TB7 (1% tryptone buffered at pH 7.0 with 100 mM potassium phosphate) supplemented with 0.4% glucose. Cell pellets were processed as previously described.³² Tryptic digestion was performed using urea denaturation.³³

Mass Spectrometry

TripleTOF 5600—DDA Acquisition and Subsequent PRM or sPRM Assays—Samples were analyzed by reverse-phase HPLC-ESI-MS/MS using an Eksigent Ultra Plus nano-LC 2D HPLC system connected to a quadrupole time-of-flight TripleTOF 5600 (QqTOF) mass spectrometer. Typically, the mass resolution for MS1 scans and corresponding precursor ions was ~35,000 while the resolution for MS/MS scans and the resulting fragment ions (PRM transitions) was ~15,000.⁸ Initially, data acquisition was performed in DDA mode to obtain MS/MS spectra for the 30 most abundant precursor ions (50 ms per MS/MS) following each survey MS1 scan (250 ms) with a cycle time of 1.8 s. The PRM and sPRM acquisitions consisted of 1 MS1 scan (250 ms) followed by the targeted MS/MS scans with cycle times between 1.3 and 3.3 s depending on project and target numbers (see Supplementary Methods, Supporting Information). As an example, the sPRM study examining 503 peptides (532 precursor ions) from a yeast protein hydrolysate was designed with a total cycle time of 1.6 s, yielding ~15 data points measured across the chromatographic peaks.

QTRAP 5500—Response Curve SRM-Assays—The SRM analysis was performed using the QTRAP 5500, a hybrid triple quadrupole/linear ion trap (SCIEX). Chromatography was performed on a NanoLC-Ultra 2D LC system. The optimized assay transitions (45 precursor-to-product ion, Q1/Q3 transitions for 15 peptides) for the spiked predigested six protein mix in *C. elegans* lysate are listed in Table S-2E (Supporting Information).

Raw Data Accession and Panorama Public Spectral Libraries

The mass spectrometric raw data associated with this manuscript may be downloaded from MassIVE at massive.ucsd.edu (MassIVE ID: MSV000079077). Spectral libraries and quantitative sPRM data processed in Skyline were uploaded to Panorama Public (https://panoramaweb.org/labkey/TripleTOF5600_MRMHR.url).³⁴

RESULTS AND DISCUSSION

Multiple experiments were performed to demonstrate linearity, robustness, and validity of high resolution PRM assays. Specific emphasis was directed to easy and fast assay development, as well as to high sPRM multiplexing generating retention time scheduled assays.

Concentration Curves—Linearity and Reproducibility of PRM Assays Using a TripleTOF 5600 Mass Spectrometer

PRM standard concentration curves were acquired for a set of several synthetic, stable isotope-labeled and acetyllysine (Kac)-containing peptides, spanning a concentration range from 63 attomol/ μL to 25 femtomol/ μL (loading 1 μL of sample on-column) spiked into a simple matrix consisting of six digested proteins. Figure 1A shows data from three replicate curves for stable isotope-labeled acetyl peptide LVSSVSDLPKacR* in a simple matrix (25 fmol/ μL digested six protein mix) acquired on a TripleTOF 5600 in PRM mode. Peak areas were extracted for 10–13 fragment ions per peptide precursor ion; however, only the top 5 MS/MS fragment ions (transitions) were selected for further data processing by summing the peak areas of the top 5 fragment ions per peptide within each replicate acquisition. The acetylated peptide LVSSVSDLPKacR* showed excellent peak area linearity across the concentration range with a linear regression slope of 0.9855 and an R^2 value of 0.9977. Several other peptides were similarly monitored and had R^2 values near 1 with excellent linearity (Table S-1, Supporting Information). In addition, the percent coefficient of variation (CV) of the peak area for each of the six analytes was calculated at the different concentrations acquired in triplicates (Figure 1B), and it was typically $\sim 20\%$, demonstrating very good reproducibility. Underlying data, such as CV values, peak area means, and standard deviations can be viewed in Table S-1B (Supporting Information).

Next, response curves were acquired for a predigested “six protein mix” spiked into a complex matrix, a *C. elegans* lysate (1 $\mu\text{g}/\mu\text{L}$), following 15 peptides by PRM. Eight concentrations, spanning a range from 15 attomol/ μL to 62.5 femtomol/ μL on column (loading 1 μL of sample on-column), were measured in duplicate on the TripleTOF 5600 in PRM mode (full scan MS/MS monitoring all fragment ions), and in parallel on the QTRAP 5500 in SRM mode (with 3 transitions per peptide). Representative peptides displayed strong linearity, selectivity, and reproducibility across a large dynamic range for both instruments (Figure 2A and Table S-2A-B, Supporting Information). Both slopes and R^2 values of these response curves demonstrated a high degree of linearity with slopes very close to 1 for the PRM assays. In addition, we determined the limits of detection (LOD) and limits of quantitation (LOQ) for each of the 15 peptides monitored in this study spiked into the complex *C. elegans* lysate (1 $\mu\text{g}/\mu\text{L}$) using a method based on measurements of the blank and low concentration samples as previously described.¹⁵ LODs and LOQs are reported as fmol/ μL as listed in Table S-2C (Supporting Information) with 1 μL of sample loaded on column per injection. Peptide LOD values were determined, with a mean LOD of 153 amol/ μL averaging across peptides for the TripleTOF 5600, and a mean LOD of 172 amol/ μL for the QTRAP 5500. In this targeted response curve experiment performed in a *C. elegans* matrix, LOD and LOQ performance was very similar between the full scan QqTOF and the

triple quadrupole instrument. However, the PRM method employed on the TripleTOF 5600 provided additional, valuable postacquisition assay refinement options, when compared with SRM. For example, using the peak area sum of the “best”, i.e. top 3 fragment ions per peptide, compared to taking the sum of the peak area of “all” 10–24 measured fragment ions, improved both the slopes and R^2 values slightly in the curves acquired on the TripleTOF 5600: for example, linear regression slopes improved from 0.9 to 1.0 (VLDALDSIK⁺), and from 0.81 to 0.94 (YSTDVSDEVK²⁺); see Table S-2-A (Supporting Information). This indicates a unique advantage of monitoring a full scan MS/MS during PRM acquisitions, as assays can be easily refined postacquisition and apparent interferences or low intensity fragment ions can be eliminated. This postacquisition data processing option can be applied in a nonbiased, systematic approach, and will be discussed in detail below.

In a related experiment, the same predigested 6 protein mix was spiked into a *C. elegans* matrix (1 $\mu\text{g}/\mu\text{L}$) at 50 fmol/ μL (loading 1 μL on column), and LC-MS data was acquired in triplicate using the PRM assay on the TripleTOF 5600. Peak area CV plots were generated for all monitored fragment ions (Figure 2B), and the majority of these transitions (216 out of 240) showed CVs < 20%. All underlying CV values, peak area means, and standard deviations are listed in Table S-2F (Supporting Information). The few transitions with higher CVs all displayed very low peak area abundance measurements, and they would be significantly deemphasized when forming weighted peak area sums per peptide. For additional systematic assessment of this data set, including Skyline Transition Selection filter settings for automatic elimination of these few less robust transitions, see Figure S-1 of the Supporting Information.

For the TripleTOF 5600, we typically did not need to further optimize collision energies for full-scan PRM acquisition methods, and thus, PRM assay development time can be held to a minimum. PRM assay collision energies (CE) were determined using the CE equations based on m/z and z values (Supplementary Methods, Supporting Information).

Spectral Library-Informed Transition Selection for PRM Assays Using Skyline

For PRM assays the full-scan MS2 tandem mass spectral data can be acquired without first assigning the set of fragment ions that will be extracted for quantitation, allowing fast implementation of the assay. However, peptide fragment ion/transition selection is required for PRM data processing, and we and others²⁸ have found it advantageous to use existing spectral library data from data-dependent acquisitions for these selections as opposed to extracting all theoretically possible fragment ions for a given precursor ion. The criteria for transition selections are similar to rules applied to SRM assay development. For PRM, typically 6–10 fragment ion transitions were chosen from the spectral library based on ion abundance ranking and adjustable Skyline Transition Filter Settings, and low m/z product ions (<300 m/z) were automatically excluded. These automated selection criteria generally eliminate very low abundance transitions from being chosen, thus reducing background noise. For example, Figure S-1 (Supporting Information) shows a systematic comparison between spectral library-informed PRM assay results and results from assays constructed using all theoretical MS/MS fragment ions (no transition prefiltering). Interestingly, the

spectral library-informed assays revealed overall better peak area CVs for the preselected transitions.

The presence of 6–10 extracted fragment ions per precursor ion as part of the acquired PRM data processing allows for appropriate target identification and confirmation, as well as automated Skyline/mProphet false discovery rate (FDR) determination.^{35,36} Typically, we subsequently use a subset of the most robust fragment ion transitions with the lowest CVs for final quantitation, i.e., summing extracted peak areas of the top 3 (or top 5) ranked fragment ions per each precursor ion, thus providing some weighting of the contribution from the different transitions for quantitation.

Metrics Assessment, Statistical Comparisons to Other Triple Quadrupole Instruments

Recent work in the LC-SRM-MS and biomarker field has established the importance of system suitability checks and controls through the use of quantitative metrics gauging instrument performance.^{30,37,38} One of these studies³⁰ that we participated in described a system suitability SOP where multiple MS and HPLC metrics parameters were assessed across 13 triple quadrupole mass spectrometers from 4 vendors. To examine these metrics on the TripleTOF 5600 in PRM mode, we followed the same system suitability SOP. Data were acquired in 10 replicates of a digested 6-protein mix, and processed as described.³⁰ The metrics measured for the TripleTOF 5600 (site “52B”, Figure S-2, Supporting Information) were well within the previously suggested guidelines for triple quadrupole instruments.³⁰ We show that peak area, peak width, and retention time stability plots, from the PRM assays acquired on the TripleTOF 5600, were practically indistinguishable from the published 13 CPTAC triple quadrupole SRM assays, emphasizing the analytical robustness of full scan PRM assays (Figure S-2D-F, Supporting Information). Overall, response curves and system suitability measurements demonstrated that the PRM assays performed on the TripleTOF 5600 were highly reproducible, robust, and linear over a dynamic range of 3–3.5 orders of magnitude. These results clearly indicate PRM methods as a reasonable alternative to SRM assays, particularly if acquired on the same instrument used for DDA-based discovery workflows, thus eliminating the time-consuming process of SRM development on a triple quadrupole instrument, or when higher selectivity is required due to interferences that may be encountered at lower resolutions.

High-Throughput, Multiplexed sPRM Assays Assessing >500 Peptides in Whole Yeast Lysates

The major advantage of performing PRM assays on high resolution, full scan instruments results from having fragment ion information at high mass resolution, as well as being able to monitor the entire set of MS/MS fragment ions simultaneously without having to preselect them in an assay development stage. The latter reduces assay development time and accelerates transitioning from DDA results to a targeted assay on the same instrument platform.

PRM assays on a TripleTOF 5600 can be highly multiplexed by scheduling based on retention time information on the peptide precursor ions identified during initial discovery-

type workflows. As the chromatographic setup is identical for the discovery analysis and sPRM, additional acquisitions are not required for optimizing elution times.

Using the Skyline software, a retention time scheduled PRM (sPRM) assay can be easily implemented (Figure 3A) by importing spectral libraries for target peptides and obtaining peptide RT information from previous DDA acquisitions.⁸ The RT scheduling window width was estimated in Skyline and adjusted to obtain less than 50 concurrent precursor ions at any given time point, allowing us to maintain MS/MS accumulation times with sufficient ion statistics and ~9–10 measurement points across an eluting targeted peptide. Accumulation times between 50 and 60 ms typically provide good data quality and signal-to-noise. The sPRM assay generated in Skyline can be directly exported to the TripleTOF 5600 Analyst acquisition software (Figure S-3, Supporting Information). In cases of retention time drift, Skyline offers a means to rapidly “reschedule” the analyte retention times during the course of long studies. Altogether, over 500 individual peptide precursor ions were monitored per assay. For the yeast data set, sPRM triplicate acquisitions targeting 532 precursor ions (503 peptides) were processed in Skyline extracting MS2 fragment ion chromatograms. Figure 3B displays a typical sPRM extracted ion chromatogram (XIC) for 8 fragment ions (peptide DFIGITTLYMGR) that were preselected based on the spectral library MS/MS. The peak area replicate view shows the good agreement between the expected fragment ion distribution from the spectral library and the XIC peak areas measured in sPRM acquisitions (Figure 3B).

Next, we examined the reproducibility of peak areas in sPRM assays. The 503 quantifiable peptide precursor ions in the yeast lysates were processed in Skyline where 3830 fragment ions were extracted with 8–10 transitions per precursor ion, for all three replicates. The CVs across the triplicate acquisitions were plotted for each individual fragment ion (Figure 3C; Table S-3, Supporting Information). Although the great majority of data points showed CVs < 20%, we observed a number of fragment ions with higher CVs. However, when processing the sPRM data set, the extracted fragment ions (up to 10) that populate the Skyline “target tree” were used to confirm that the peptide analyte was observed confidently in the PRM assay. We subsequently took this data set and generated the sum of the peak areas of only the top 5 fragment ions for each of the precursor ions, and the corresponding peak area CVs dropped below 20% for 98% of all peptides monitored by sPRM (Figure 3D), with 60% of all peptides now showing CVs less than 10% (also see Figure S-4 and Table S-3, Supporting Information). It is worth noting that, while PRM provides the ability to extract chromatograms for much larger sets of fragment ions per precursor than are typically used in SRM experiments, it is still advisable to base quantification on a limited subset of the most intense ions. In general, PRM provides additional postacquisition flexibility in deleting weak and interference-prone fragment ions and focusing on stronger interference-free ions for final quantitative data processing. In this yeast data set, however, no significant interferences were detected. Overall, these data clearly demonstrated that sPRM had a comparable level of selectivity and reproducibility to scheduling in SRM assays on a triple quadrupole.

Skyline mProphet for Target Confirmation, FDR Determination and Interference Analysis

Interference and MS2 transition outlier analysis for PRM data was typically performed using mProphet,³⁶ an algorithm that recently has been implemented into Skyline.³⁵ Originally designed as a chromatogram peak identification probability model for SRM analysis to determine false discovery rates for peptides, mProphet has been adapted to process MS2-full scan data-independent acquisition data, and it is available as part of OpenSWATH,³⁹ SWATHProphet,⁴⁰ and Skyline.³⁵ All high-throughput sPRM data sets described in this study were processed with the mProphet tool in Skyline to generate false discovery rates (Q -values < 0.01 required).

A critical concern in any quantitative study is to detect and eliminate potential interferences. Interference removal algorithms for SRM data sets have been described, including AuDIT⁴¹ and other tools.⁴² Similarly, algorithms have been recently developed for interference removal in full scan MS2 quantitative data sets in PRM²⁸ and SWATH⁴⁰ data sets. For our yeast and *E. coli* PRM data, after removal of a few peptides that were not identified (Q -values > 0.01) and/or could not be quantified, no significant transition interferences were detected for the analytes. This was likely a consequence of using defined Skyline transition filter settings and transition selection criteria based on pre-existing spectral libraries, which had already eliminated most or all low abundance and interference prone transitions.

To better demonstrate our unbiased interference detection and removal strategy, we processed an MS2 full scan mitochondrial lysate data set with Skyline mProphet. Both peptide FDR and Q -values were determined with a statistical score cutoff of $Q < 0.01$. One key component of mProphet scoring is the ion dot-product,⁴³ which indicates the degree of the match between spectral library MS/MS and the extracted ion chromatograms of the corresponding transitions, a value that is particularly helpful in indicating the presence of interfering signals. If the Q -value was greater than 0.01, in most cases either an incorrect peak was selected or an interfering ion was present. If ion interference was suspected, we systematically eliminated all transition(s), alone or in combination, and recalculated the mProphet Q -values (Figure S-5, Supporting Information). Q -values that now fell below the threshold of 0.01 indicated successful removal of compromised transitions. However, peptides with Q -values that remained high after this process would be disqualified. This iterative, and mostly automated, process in Skyline provides an efficient, objective means to refine the postacquisition PRM assays applying similar rules that are currently used in classical preacquisition SRM assay development.

Searching Targeted sPRM Data with Database Search Engines

sPRM signal detection in Skyline is performed using well established algorithms also used for triple quadrupole SRM assays,⁴⁴ where Skyline matches the integrated XICs from observed fragment ions against existing peptide spectral libraries. However, for sPRM acquisitions, the measured MS/MS spectra are also directly searchable with traditional database search engines. For example, in a multiplexed sPRM acquisition, out of 492 monitored unique peptides, ProteinPilot searches confidently identified 471 target peptides (96%).

Multiplexed, Retention Time Scheduled sPRM Assay Comparing WT vs *ackA* Mutant Whole Lysates in *E. coli*

Next, a set of biologically relevant samples was chosen to apply the sPRM methodology to confirm a candidate list of several proteins that had been previously shown to be differentially expressed in whole lysates between several *E. coli* strains in DDA acquisitions quantified using MS1 Filtering. In this earlier study, we reported changes in lysine acetylation between an *ackA* mutant and its WT parent³² and examined changes in protein expression levels by comparing DDA results from total protein lysates by MS1 Filtering. As a follow-up here, we used a candidate list of proteins that showed significant changes in the *ackA* mutant vs WT strain to develop a highly multiplexed sPRM assay. We first generated an unscheduled PRM method from Skyline to monitor only 2 of these candidate proteins, AckA and OsmY, with a total of 19 peptides. The results of these PRM assays confirmed the statistically significant up-regulation of OsmY and the “absence” of AckA itself in the *ackA* mutant lysate compared to the WT strain. Ultimately, a highly multiplexed sPRM assay was performed in triplicate, monitoring 410 peptides derived from a total of 48 proteins by sPRM (466 precursor ions, 2694 Skyline selected transitions) using a 2 min RT scheduling window. The high multiplexing did not negatively impact the sPRM assay results, as both the unscheduled and scheduled results for the mutant (*ackA*)/WT ratios were highly similar for proteins that were acquired with both methods. For example, protein OsmY was significantly upregulated, 17.9-fold ($q = 0.0012$) in the unscheduled PRM assay, and it was measured as 17.5-fold upregulated ($q = 4.5 \times 10^{-5}$) in the highly multiplexed sPRM assay. All data details can be viewed in Table S-4 (Supporting Information). The RT scheduling sPRM method allowed considerably more proteins and peptides to be monitored compared to unscheduled methods. Indeed, subsequent processing of the sPRM assays in Skyline confirmed that 9 proteins and their underlying peptides significantly changed between the *ackA* mutant and its WT parent. Figure S-6 (Supporting Information) shows a representative peptide SGSGTLTVSNTTLTQK from Antigen 43 (AG43) which showed a 22.2-fold increase in abundance ($p\text{-value} = 2.18 \times 10^{-6}$) in the *ackA* mutant in all sPRM replicates. In fact, 24 additional AG43-derived peptides could be quantitated, confirming this change.

Lastly, this sample set allowed us to directly compare our PRM assays to a comprehensive SWATH (or DIA) method, as we collected data sets using both of these acquisition methods. Although one cannot generalize from a single comparison, the narrower precursor ion selection in the PRM assay (1 m/z) compared to the much larger window employed for the SWATH acquisition (here 25 m/z) produced several cases where PRM had reduced MS2 ion interferences for the same peptide analyte (Figure S-7, Supporting Information). However, smaller m/z windows can also be implemented in a SWATH assay if desired to eliminate such interferences.

CONCLUSION

With recent advances in mass spectrometry-based proteomics aimed at developing new targeted acquisition workflows, PRM has gained interest as an alternative to traditional SRM.^{20,21,23–26} However, most reports have utilized Orbitrap or hybrid Quadrupole Orbitrap (Q-Exactive) mass spectrometers. In this study, we have expanded this type of acquisition to

systematically evaluate the performance of a QqTOF platform (TripleTOF 5600), with an emphasis on comparisons with traditional SRM, on scheduled sPRM performance in conducting highly multiplexed assays, and postacquisition analysis features available in Skyline. Indeed, we showed that when PRM is carried out with retention time scheduling, up to 500 peptide analytes could be easily monitored in a single assay. Over the past few years, retention time scheduling of targeted acquisitions has become much more robust. Modern chromatographic systems often provide “heating” for chromatographic columns to ensure temperature stability to reduce retention time drift, and HPLC Chip-based systems have been introduced with high column batch to batch reproducibility. Nevertheless, in cases where one experiences a chromatographic shift, such as in assays conducted over several weeks or when introducing a new column, there are software algorithms available, including ones in Skyline, that offer retention time monitoring and rescheduling. Alternatively, when changing columns or gradients, one can employ the Skyline tool “iRT”, which uses normalized retention times and RT regressions to accurately predict analyte RTs.⁴⁵

The response curve assessment shown in Figure 2 revealed that our PRM experiments had an overall dynamic range of 3 to 3.5 orders of magnitude in complex samples. Both LOD and LOQ values for these PRM assays were calculated from response curves for analytes spiked into the *C. elegans* lysate matrix, and mean LODs were determined for the investigated and monitored peptides at 153 amol/ μ L with corresponding mean LOQs of 460 amol/ μ L. Comprehensive comparisons of the QqTOF PRM assays with assays on triple quadrupole instruments showed very similar performance in the dynamic range, LOD/LOQ, and other assay metrics, as described recently by the CPTAC consortium.³⁰ In contrast to SRM, PRM experiments allow for higher assay selectivity with higher resolution and mass accuracy and greater number of MS/MS fragment ion transitions monitored. Postacquisition refinements of the PRM assays using Skyline were also examined, such as removing weak and/or noisy signals, as well as the elimination of transitions with interferences. Indeed, PRM data can be graphically assessed in Skyline, and data processing and postacquisition refinement is relatively straightforward. In fact, if a full-scan MS2 interference analysis is performed in an objective, automated, and reproducible way, such as by employing algorithms that can systematically assess signal quality and remove interfering fragment ions,^{28,35,36,39,40} the postacquisition refinement options of PRM assays can provide greater assay flexibility in case interferences do occur. This feature is particularly useful when analyzing complex samples, such as one often encounters with clinical specimens.

In conclusion, we have shown that a comprehensive set of tools exist in Skyline that are capable of providing a means for the rapid implementation of these highly multiplexed targeted PRM assays. In addition, sPRM assays on a QqTOF platform provide an excellent opportunity and alternative for rapid verification of sets of interesting peptide or protein targets in large-scale targeted assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the support of instrumentation from the NCRR shared instrumentation grants S10 RR027953 and 1S10 OD016281 (both to B.W.G.), as well as the DOE grant (Systems Biology of Protein Acetylation in Fuel-Producing Microorganisms, to B.W.G. and A.J.W.) for the *E. coli* data. We thank David Cox for interfacing Skyline with Analyst acquisition software.

References

1. Mann M. *Methods Mol Biol.* 2014; 1188:1–7. [PubMed: 25059600]
2. Merrill AE, Hebert AS, MacGilvray ME, Rose CM, Bailey DJ, Bradley JC, Wood WW, El Masri M, Westphall MS, Gasch AP, Coon JJ. *Mol Cell Proteomics.* 2014; 13:2503–2512. [PubMed: 24938287]
3. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. *Mol Cell Proteomics.* 2002; 1:376–386. [PubMed: 12118079]
4. Dayon L, Turck N, Kienle S, Schulz-Knappe P, Hochstrasser DF, Scherl A, Sanchez JC. *Anal Chem.* 2010; 82:848–858. [PubMed: 20058875]
5. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhász P, Martin S, Bartlett-Jones M, He F, Jacobson A, Pappin DJ. *Mol Cell Proteomics.* 2004; 3:1154–1169. [PubMed: 15385600]
6. Cox J, Hein MY, Lubner CA, Paron I, Nagaraj N, Mann M. *Mol Cell Proteomics.* 2014; 13:2513–2526. [PubMed: 24942700]
7. Gonzalez-Galarza FF, Lawless C, Hubbard SJ, Fan J, Bessant C, Hermjakob H, Jones AR. *OMICS.* 2012; 16:431–442. [PubMed: 22804616]
8. Schilling B, Rardin MJ, MacLean BX, Zawadzka AM, Frewen BE, Cusack MP, Sorensen DJ, Bereman MS, Jing E, Wu CC, Verdin E, Kahn CR, Maccoss MJ, Gibson BW. *Mol Cell Proteomics.* 2012; 11:202–214. [PubMed: 22454539]
9. Gillet LC, Navarro P, Tate S, Rost H, Selevsek N, Reiter L, Bonner R, Aebersold R. *Mol Cell Proteomics.* 2012; 11(O111):016717. [PubMed: 22261725]
10. Held JM, Schilling B, D'Souza AK, Srinivasan T, Behring JB, Sorensen DJ, Benz CC, Gibson BW. *Int J Proteomics.* 2013; 2013:791985. [PubMed: 23710360]
11. Rosenberger G, Koh CC, Guo T, Röst HL, Kouvonen P, Collins BC, Heusel M, Liu Y, Caron E, Vichalkovski A, Faini M, Schubert OT, Faridi P, Ebhardt HA, Matondo M, Lam H, Bader SL, Campbell DS, Deutsch EW, Moritz RL, Tate S, Aebersold R. *Sci Data.* 2014; 1:140031. [PubMed: 25977788]
12. Lange V, Picotti P, Domon B, Aebersold R. *Mol Syst Biol.* 2008; 4:222. [PubMed: 18854821]
13. Editorial. *Nat Methods.* 2013; 10:1–1. [PubMed: 23547284]
14. Gillette MA, Carr SA. *Nat Methods.* 2013; 10:28–34. [PubMed: 23269374]
15. Burgess MW, Keshishian H, Mani DR, Gillette MA, Carr SA. *Mol Cell Proteomics.* 2014; 13:1137–1149. [PubMed: 24522978]
16. Kennedy JJ, Abbatiello SE, Kim K, Yan P, Whiteaker JR, Lin C, Kim JS, Zhang Y, Wang X, Ivey RG, Zhao L, Min H, Lee Y, Yu MH, Yang EG, Lee C, Wang P, Rodriguez H, Kim Y, Carr SA, Paulovich AG. *Nat Methods.* 2014; 11:149–155. [PubMed: 24317253]
17. Huttenhain R, Soste M, Selevsek N, Rost H, Sethi A, Carapito C, Farrah T, Deutsch EW, Kusebauch U, Moritz RL, Nimeus-Malmstrom E, Rinner O, Aebersold R. *Sci Transl Med.* 2012; 4:142–194.
18. Stahl-Zeng J, Lange V, Ossola R, Eckhardt K, Krek W, Aebersold R, Domon B. *Mol Cell Proteomics.* 2007; 6:1809–1817. [PubMed: 17644760]
19. MacLean, B., Bereman, MS., Ham, AJL., Schilling, B., Thompson, JW., Dubois, LG., Held, JM., Cusack, MP., Bock, T., Wollscheid, B., Moseley, A., Gibson, BW., Liebler, DC., MacCoss, MJ. 59th Annual ASMS Conference on Mass Spectrometry; Denver, CO. June 5 – 9, 2011;
20. Peterson AC, Russell JD, Bailey DJ, Westphall MS, Coon JJ. *Mol Cell Proteomics.* 2012; 11:1475–1488. [PubMed: 22865924]

21. Sherrod SD, Myers MV, Li M, Myers JS, Carpenter KL, Maclean B, Maccoss MJ, Liebler DC, Ham AJ. *J Proteome Res.* 2012; 11:3467–3479. [PubMed: 22559222]
22. Gallien S, Duriez E, Crone C, Kellmann M, Moehring T, Domon B. *Mol Cell Proteomics.* 2012; 11:1709–1723. [PubMed: 22962056]
23. Tang H, Fang H, Yin E, Brasier AR, Sowers LC, Zhang K. *Anal Chem.* 2014; 86:5526–5534. [PubMed: 24823915]
24. Tsuchiya H, Tanaka K, Saeki Y. *Biochem Biophys Res Commun.* 2013; 436:223–229. [PubMed: 23726910]
25. Gallien S, Bourmaud A, Kim SY, Domon B. *J Proteomics.* 2014; 100:147–159. [PubMed: 24200835]
26. Peach SE, Rudomin EL, Udeshi ND, Carr SA, Jaffe JD. *Mol Cell Proteomics.* 2012; 11:128–137. [PubMed: 22442256]
27. Creech AL, Taylor JE, Maier VK, Wu X, Feeney CM, Udeshi ND, Peach SE, Boehm JS, Lee JT, Carr SA, Jaffe JD. *Methods.* 2015; 72:57–64. [PubMed: 25448295]
28. Gallien S, Domon B. *Methods.* 2015; 81:15–23. [PubMed: 25843604]
29. Gallien S, Kim SY, Domon B. *Mol Cell Proteomics.* 2015; 14:1630–1644. [PubMed: 25755295]
30. Abbatiello SE, Mani DR, Schilling B, Maclean B, Zimmerman LJ, Feng X, Cusack MP, Sedransk N, Hall SC, Addona T, Allen S, Dodder NG, Ghosh M, Held JM, Hedrick V, Inerowicz HD, Jackson A, Keshishian H, Kim JW, Lyssand JS, Riley CP, Rudnick P, Sadowski P, Shaddox K, Smith D, Tomazela D, Wahlander A, Waldemarson S, Whitwell CA, You J, Zhang S, Kinsinger CR, Mesri M, Rodriguez H, Borchers CH, Buck C, Fisher SJ, Gibson BW, Liebler D, Maccoss M, Neubert TA, Paulovich A, Regnier F, Skates SJ, Tempst P, Wang M, Carr SA. *Mol Cell Proteomics.* 2013; 12:2623–2639. [PubMed: 23689285]
31. Sherman F. *Methods Enzymol.* 1991; 194:3–21. [PubMed: 2005794]
32. Kuhn ML, Zemaitaitis B, Hu LI, Sahu A, Sorensen D, Minasov G, Lima BP, Scholle M, Mrksich M, Anderson WF, Gibson BW, Schilling B, Wolfe AJ. *PLoS One.* 2014; 9:e94816. [PubMed: 24756028]
33. Keshishian H, Addona T, Burgess M, Kuhn E, Carr SA. *Mol Cell Proteomics.* 2007; 6:2212–2229. [PubMed: 17939991]
34. Sharma V, Eckels J, Taylor GK, Shulman NJ, Stergachis AB, Joyner SA, Yan P, Whiteaker JR, Halusa GN, Schilling B, Gibson BW, Colangelo CM, Paulovich AG, Carr SA, Jaffe JD, MacCoss MJ, MacLean B. *J Proteome Res.* 2014; 13:4205–4210. [PubMed: 25102069]
35. MacLean B., Marsh D., Röst H., Espona Pernas L., Rosenberger G., Aebersold R., Maccoss M. 61th Annual ASMS Conference on Mass Spectrometry; Minneapolis, MN. June 8–13, 2013;
36. Reiter L, Rinner O, Picotti P, Huttenhain R, Beck M, Brusniak MY, Hengartner MO, Aebersold R. *Nat Methods.* 2011; 8:430–435. [PubMed: 21423193]
37. Bereman MS, Johnson R, Bollinger J, Boss Y, Shulman N, MacLean B, Hoofnagle AN, MacCoss MJ. *J Am Soc Mass Spectrom.* 2014; 25:581–587. [PubMed: 24496601]
38. Briscoe CJ, Stiles MR, Hage DS. *J Pharm Biomed Anal.* 2007; 44:484–491. [PubMed: 17433601]
39. Rost HL, Rosenberger G, Navarro P, Gillet L, Miladinovic SM, Schubert OT, Wolski W, Collins BC, Malmstrom J, Malmstrom L, Aebersold R. *Nat Biotechnol.* 2014; 32:219–223. [PubMed: 24727770]
40. Keller A, Bader SL, Shteynberg D, Hood L, Moritz RL. *Mol Cell Proteomics.* 2015; 14:1411–1418. [PubMed: 25713123]
41. Abbatiello SE, Mani DR, Keshishian H, Carr SA. *Clin Chem.* 2010; 56:291–305. [PubMed: 20022980]
42. Bao Y, Waldemarson S, Zhang G, Wahlander A, Ueberheide B, Myung S, Reed B, Molloy K, Padovan JC, Eriksson J, Neubert TA, Chait BT, Fenyo D. *Methods.* 2013; 61:299–303. [PubMed: 23707623]
43. Toprak UH, Gillet LC, Maiolica A, Navarro P, Leitner A, Aebersold R. *Mol Cell Proteomics.* 2014; 13:2056–2071. [PubMed: 24623587]
44. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. *Bioinformatics.* 2010; 26:966–968. [PubMed: 20147306]

45. Escher C, Reiter L, MacLean B, Ossola R, Herzog F, Chilton J, MacCoss MJ, Rinner O. *Proteomics*. 2012; 12:1111–1121. [PubMed: 22577012]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

Standard concentration curves for stable isotope-labeled and acetyllysine-containing peptides. (A) Stable isotope-labeled acetylated peptide LVSSVSDLPKacR* (HMGCS2) was monitored spanning a concentration range from 63 attomol/ μ L to 25 femtomol/ μ L (m/z 626.86 $^{2+}$), acquiring three replicates (1 μ L sample on column). Peptides were spiked into a simple matrix containing a six digested protein mix at 25 fmol/ μ L each, and acquired on a TripleTOF 5600 mass spectrometer (QqTOF) in PRM mode in triplicate. Extracted peak areas for the top 5 MS/MS fragment ions were summed per peptide. (B) Peak area CVs across three replicates for each concentration point (0.063–25 fmol/ μ L) are shown for six targeted acetyllysine peptides with 20% CV cutoff indicated (Table S-1B, Supporting Information); K* = $^{13}\text{C}_6^{15}\text{N}_2$ -Lys and R* = $^{13}\text{C}_6^{15}\text{N}_4$ -Arg.

Figure 2.

Response curve for a digested 6 protein mix spiked into a complex matrix (*C. elegans* whole cell lysate). (A) The mixture of six predigested proteins was spiked into digested *C. elegans* whole cell lysate (1 μg/μL and 1 μg on column) at 8 concentrations from 15 attomol/μL to 62.5 femtomol/μL (0 = blank, 0.015–62.5 fmol/μL, loading 1 μL sample on column). Two replicate concentration curves were acquired on the TripleTOF 5600 (QqTOF in PRM mode, top) and on the QTRAP 5500 (QQQ in SRM mode, bottom), respectively. Peak areas of extracted fragment ion transitions are summed per peptide. Curves are displayed for peptides HLVDEPQNLIK²⁺ (*m/z* 653.36) and YSTDVSVDEVK²⁺ (*m/z* 621.30), respectively. (B) Peak area CV display for triplicate acquisitions of a digested 6 protein mix (50 fmol/μL) spiked into a complex *C. elegans* matrix (1 μg/μL) monitoring 15 peptides in PRM mode with 10–24 transitions per peptide (50 fmol analyte injected). Each extracted fragment ion transition is displayed as a vertical colored line. 216 of 240 transitions showed CVs < 20% (Table S-2F, Supporting Information).

Figure 3.

Workflow for highly multiplexed, RT scheduled sPRM assay development. (A) Spectral libraries were built from database searches from data-dependent acquisitions of a yeast extract. DDA files were imported into Skyline, and RTs are determined using MS1 Filtering for peptides to be targeted in the PRM assay. Skyline generates an instrument method with 2 min scheduling windows per peptide. sPRM acquisitions were imported into Skyline extracting fragment ion peak areas. (B) Extracted ion chromatogram of PRM fragment ions for DPGITTLYMGR, spectral library MS/MS, and the corresponding peak area replicate view displaying the theoretical fragment ion distribution as simulated from the spectral library, and fragment ion XICs from one targeted sPRM acquisition. (C, D) Acquisition of three sPRM replicates monitoring >500 peptides from yeast lysate: (C) Peak area CVs from 3830 individual fragment ions when extracting peak areas from up to 10 fragment ions from each of the 503 precursor ions monitored. (D) Peak area CVs for the sum of the top 5 fragment ions from each of 503 precursor ions (Table S-3A-B, Supporting Information).