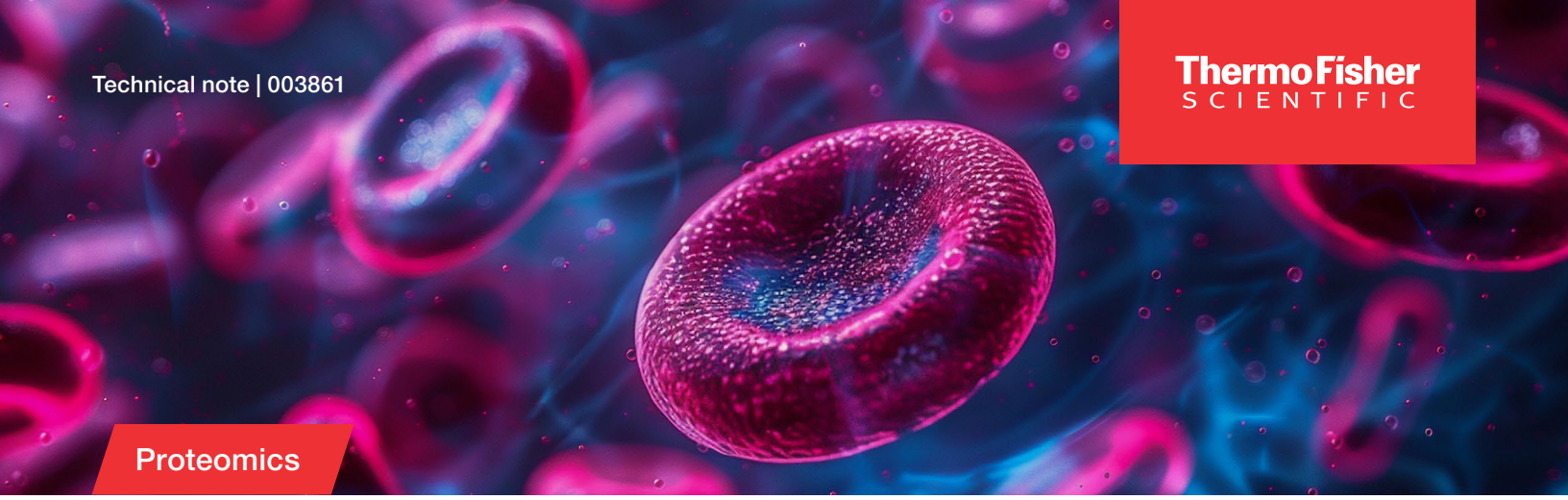

Technical note | 003861

Proteomics

New paradigm for plasma proteomics biomarker research

Large-scale targeted PRM proteomics assays enabled by the Stellar mass spectrometer

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Keywords

Stellar mass spectrometer, Skyline software, Vanquish Neo HPLC, plasma, targeted proteomics, PRM method, peptide quantitation, large scale proteomics, disease biomarkers, biomarker verification

Goal

Demonstrate an efficient and scalable biomarker verification workflow using the Thermo Scientific™ Stellar™ mass spectrometer. Showcase the streamlined end-to-end process, from generating unscheduled methods to creating a scheduled parallel reaction monitoring (PRM) method, generating and processing data, and performing data analysis for over 570 proteins in plasma using Biognosys™ PQ500™ reference peptides. Illustrate the capabilities of starting a new PRM assay, profiling hundreds of biomarker candidates, and achieving processed and analyzed targeted quantification results within three days.

Introduction

Targeted large-scale plasma proteomics is critically important in translational and clinical research. A significant challenge is the complexity and dynamic range of the plasma proteome, which requires sensitive technologies to detect low-abundance proteins among more abundant ones. By focusing on specific proteins of interest within the complex plasma proteome, this approach allows for the accurate quantification and monitoring of biomarkers associated with various diseases.

The innovative advancements introduced with the Stellar MS are geared towards large-scale quantitative targeted-MS^n (tMS^n) experiments. The hardware enhancements enable faster data acquisition rates compared to previous generations of Thermo Scientific™ linear ion traps (LITs) (Figure 1), providing flexible capabilities and higher target capacity to build biomarker verification workflows in translational and clinical research using biological matrices such as plasma.

Biomarker verification workflows on the Stellar MS provide increased sensitivity, greater specificity, and extended quantitative accuracy on a larger scale with unmatched productivity. Maximum injection times are dynamically adjusted based on assay concurrency, ensuring the longest possible injection times while maintaining the optimal

points-per-peak sampling rate. Chromatographic retention time (RT) shifts are managed with Adaptive RT real-time chromatogram alignment, which provides real-time adjustment for each tMS^2 acquisition cycle. Additionally, software has been developed to expedite the creation of targeted assays by automating the gas-phase fractionation (GPF) data-independent acquisition (DIA) or transition data to PRM strategy (Figure 2), implemented in a Skyline software external tool called PRM Conductor.

Materials and methods

Consumables and chemicals

- Fisher Scientific™ LC-MS grade water with 0.1% formic acid (P/N LS118-500)
- Fisher Scientific™ Optima™ LC-MS grade 80% acetonitrile with 0.1% formic acid (P/N LS122500)
- Thermo Scientific™ SureSTART™ 9 mm screw caps (P/N 6PSC9STB1)
- Thermo Scientific™ SureSTART™ 0.2 mL TPX screw top microvial with glass insert (P/N 60180-1655)
- Thermo Scientific™ EASY-Spray™ HPLC Column, 2 µm C18, 150 µm × 15 cm (P/N ES906A, equivalent to ES906)
- Thermo Scientific™ PepMap™ Neo trap cartridge, 5 µm C18 300 µm × 5 mm (P/N 174500)
- Biognosys™ PQ500™ reference peptide kit (Biognosys AG)
- Thermo Scientific™ Pierce™ neat digested plasma, 100 µg per vial

Instrumentation

- Thermo Scientific™ Vanquish™ Neo™ UHPLC system
- Thermo Scientific™ Stellar™ mass spectrometer
- Thermo Scientific™ Easy-Spray™ source

Data analysis

Skyline (ver.23.1.1.503) software, University of Washington, MacCoss Lab

Sample preparation

For PRM method development, PQ500 reference peptides were obtained from Biognosys and diluted following the manufacturer's instructions. A Pierce neat human plasma digest sample was diluted to 300 ng/µL with 0.1%FA in H_2O.

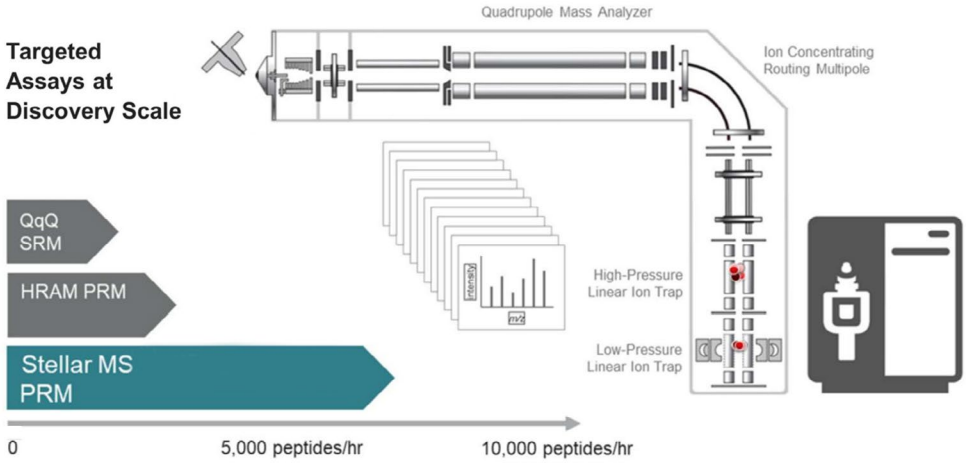


Figure 1. Stellar hybrid quadrupole linear ion trap mass spectrometer enables highly multiplex targeted proteomics

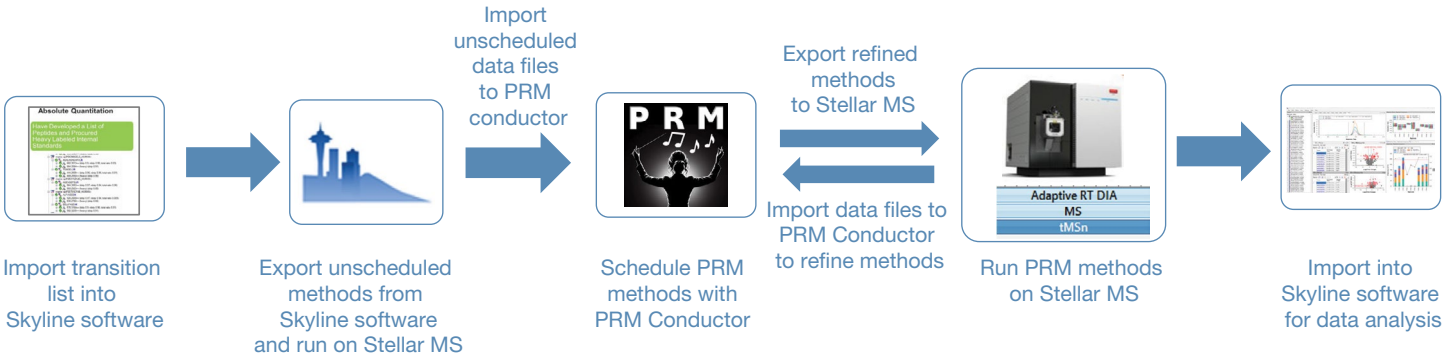


Figure 2. Targeted assay development workflow using the Stellar MS and Skyline software streamline large-scale tMS^n method creation and data acquisition management

To verify linearity, limit of detection (LOD), and limit of quantification (LOQ), peptides were diluted into 300 ng/µL digested human plasma using a 3x serial dilution. The dilution curve covered a wide dynamic range for different heavy peptides, from 1x manufacturer's concentration = 100% to 0.005% with a 100% plasma blank for the final level. 1 µL of peptides were injected onto the column.

LC-MS analysis

The Vanquish Neo UHPLC system was used with the EASY-Spray HPLC column with a trap-and-elute configuration on 60 samples-per-day (SPD) and 100 SPD methods. HPLC gradients and parameters were optimized and are shown in Tables 1A and 1B. The PRM targeted method included three sequential experiments (Figure 2):

1. Adaptive RT DIA experiment, which was used to acquire untargeted product ion spectra for real-time alignment.
2. Full scan MS experiment, while not strictly needed for quantitative success, enables the TIC normalization feature in Skyline software to be implemented.
3. The tMS^n experiment, which included the PRM mass list table.

Table 1A. HPLC conditions for 60 SPD method

Gradient		
Time (min)	*% Mobile phase B*	*Flow (µL/min)*
0	4	3.0
0.5	4	1.3
0.6	8	0.8
0.9	8	0.8
13.9	22.5	0.8
20.8	35.0	0.8
21.2	55.0	2.0
21.7	99.0	3.0
24	99.0	3.0
LC parameters		
Column temperature	45 °C	
Fast loading/equilibration	Pressure Control	
Pressure for loading/equilibration/wash	Max Pressure	
Equilibration factor	2	
Sampler temperature	7 °C	
Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in 80% acetonitrile	
Analytical column	EASY-Spray ES906A column, 2 µm C18, 150 µm × 15 cm	

Table 1B. HPLC conditions for 100 SPD method

Gradient		
Time (min)	*% Mobile phase B*	*Flow (µL/min)*
0	1	1.8
0.7	4	1.8
1.0	8	1.8
7.7	22.5	1.8
11.4	35.0	1.8
11.8	55.0	2.5
12.3	99.0	2.5
13.0	99.0	2.5
LC parameters		
Column temperature	45 °C	
Fast loading/equilibration	Pressure Control	
Pressure for loading/equilibration/wash	Max Pressure	
Equilibration factor	2	
Sampler temperature	7 °C	
Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in 80% acetonitrile	
Analytical column	EASY-Spray ES906A column, 2 µm C18, 150 µm × 15 cm	

The Adaptive RT DIA, MS, and tMS^n mass spectrometer experimental parameters are shown in Tables 1C–1E. The scan range was set to *m/z* 200–1,500 for the 60 SPD methods and customized in PRM Conductor by selecting Optimize Scan Range for the 100 SPD method. The eluted peptides were analyzed on a Stellar MS using both methods.

Table 1C. Mass spectrometer parameters in Adaptive RT DIA experiment

Adaptive RT DIA parameters	
Precursor mass range (*m/z*)	400–1,000
Isolation window (*m/z*)	50
Scan rate (kDa/s)	200
Scan range (*m/z*)	200–1,000
HCD collision energy (%)	30
RF lens (%)	30
AGC target	Standard
Maximum injection time mode	Auto
Window placement optimization	Off
Acquire reference	Checked in scheduled PRM methods to generated reference file

Table 1D. Mass spectrometer parameters in MS¹ experiment

MS experiment parameters	
Scan rate (kDa/s)	125
Scan range (*m/z*)	350–2,000
RF lens (%)	30
AGC target	Standard
Maximum injection time mode	Auto

Table 1E. Mass spectrometer parameters in tMS² experiment

tMS² experiment parameters	
Isolation window (*m/z*)	1
Activation type	HCD
HCD collision energy (%)	30
AGC target	Standard
Maximum injection time mode	Dynamic
Points per peak	7
Loop control	All
Time mode	Start/End Time
Dynamic time scheduling	Adaptive RT (Off at step 2; check at final step)
Reference file	Generated when export method from PRM conductor or when Acquire Reference is checked in "Adaptive RT DIA parameters" Note: Can be left blank when building a template method
Scan rate (kDa/s)	125
RF lens (%)	30
Scan range	200–1,500

Workflow to build the PRM method on the Stellar MS¹

Step 1 (Day 1): Create unscheduled PRM methods using the transition list of neat heavy PQ500 peptides

In the first step, the PQ500 peptide transition list (provided by Biognosys) was imported into Skyline software to generate a Skyline file and spectral library containing 804 PQ500 peptides and 14 PRTC peptides. An Indexed Retention Time (iRT) calculator was created using PRTC as the reference peptide list. The unscheduled isolation lists or methods were then exported from Skyline software to create a set of unscheduled PRM methods. These unscheduled PRM methods were divided into 10 fractions for both 60 and 100 SPD gradients to analyze the 804 PQ500 peptides (Figure 3).

Step 2 (Day 2): Schedule a wide RT window PRM using neat plasma

After data acquisition, the unscheduled PRM results were imported into Skyline software as multi-injection replicates to evaluate retention time and peptide intensities. The iRT calculator and spectral library were used to assist peak picking.

In this step, the PRM Conductor (Figure 4A) was installed and applied to filter precursor ions and transitions based on parameters such as signal-to-noise ratio (S/N), peak area, retention time, and charge state (Figure 4B). The method was refined using parameters including points across the peak, peak width, scan rate, acquisition window width, and scan range. After filtering precursors and transitions, 7,601 and 7,651 heavy peptide transitions were retained for the 60 SPD and 100 SPD methods, respectively. Acquisition RT windows were set at 1.8 minutes for both 60 SPD and 100 SPD assays.

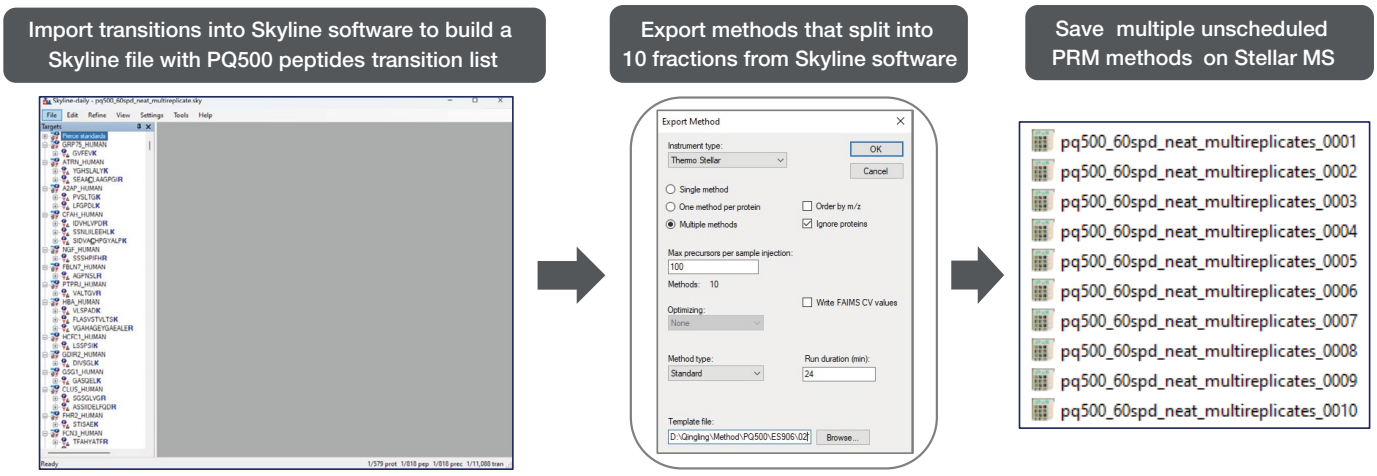


Figure 3. Importing the PQ500 peptide transition list into Skyline software to build unscheduled PRM methods

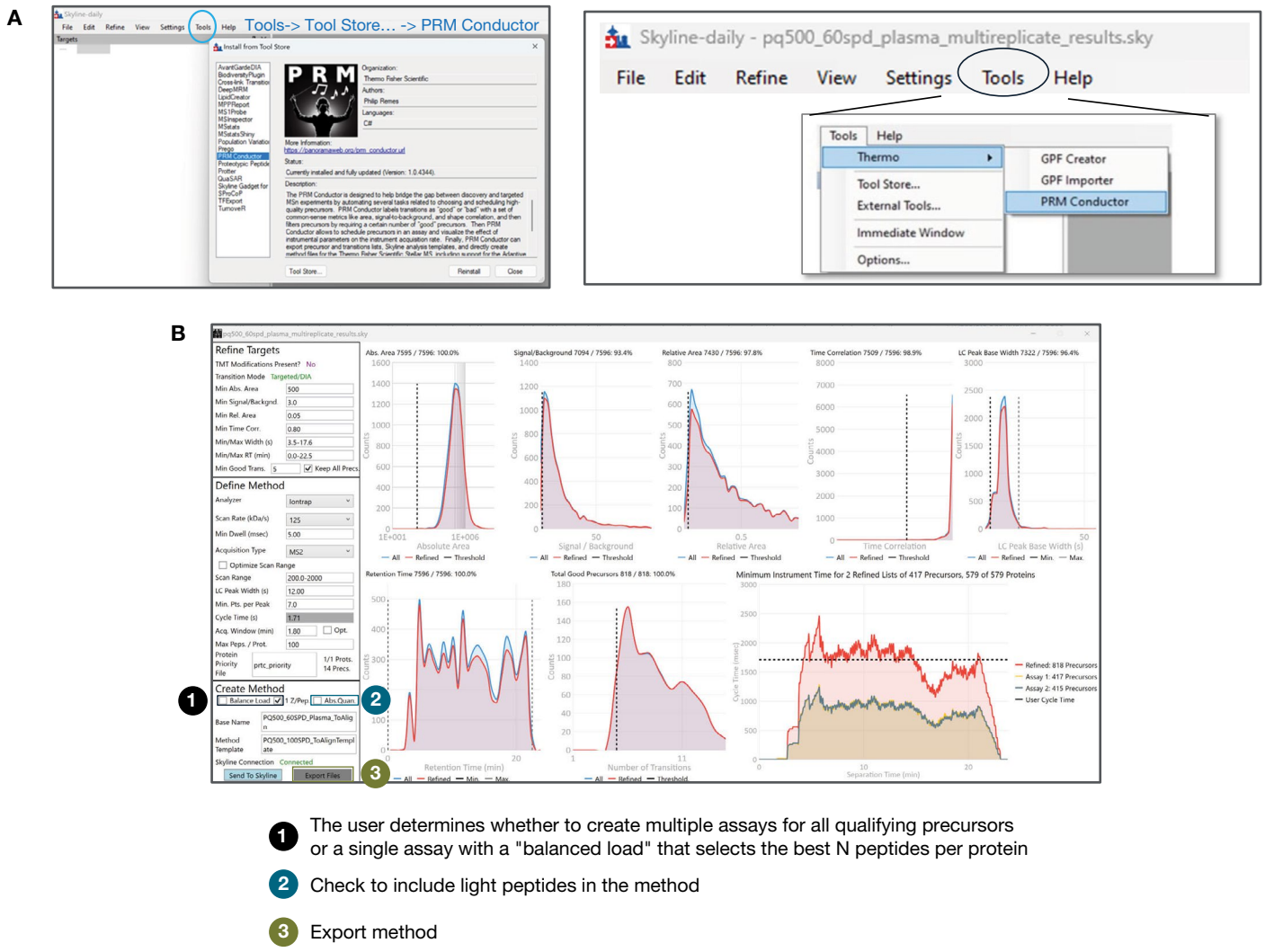


Figure 4. (A) PRM Conductor installation in Skyline software; (B) PRM Conductor peptides and transitions filtering

Without selecting the Balance Load box, precursors were split into two assays for the 60 SPD method and three assays for the 100 SPD method. The wide-window PRM methods were then exported to verify peptide retention times in plasma. Dynamic Time Scheduling was turned off at this step.

After the methods were ready, PQ500 peptides spiked into 300 ng of digested plasma matrix were analyzed using wide acquisition window PRM methods. After data acquisition, the result files were imported into Skyline software to schedule PRM methods with a narrow acquisition RT window.

The final step (Day 3): Schedule a PRM method with narrow RT window to analyze both heavy and light peptides in plasma

To finalize the method, the wide window results were imported into Skyline software for an additional round of filtering. The acquisition windows in PRM Conductor were narrowed to 0.6 minutes for 60 SPD and 0.35 minutes for 100 SPD gradients. The Opt. box, Balance Load, 1 Z/Pep., and Abs. Quan. boxes were selected. The Abs. Quan. option instructed the Export Files command to include light targets for each heavy target. Coefficient of variation (CV) values can also be used as a threshold to filter transitions with poor precision (Refine\ Advanced…\Consistency).

The LC Peak Width was adjusted to 20 seconds to ensure all targets were exported in one assay in PRM Conductor (Figure 5A), and then reverted to 11 seconds after the instrument method was created. Ultimately, there were 1,622 precursors and 13,876 transitions for the 60 SPD method and 1,622 precursors and 13,699 transitions for the 100 SPD method (Figure 5B). The final refined method was exported using a user-defined template method based on the parameters in Tables 1C to 1E. Dynamic Time Scheduling was set to Adaptive RT in the template method. Adaptive RT real-time chromatogram alignment was included in the exported method file (Figure 5C).

Data analysis and post-processing

The acquired LC-MS data was processed by Skyline software (ver. 23.1.1.503). LOQ and LOD were determined offline by selecting the set of transitions (≥3) that provided the lowest LOQ.[2] This technique has been implemented in Skyline software but has not yet been released. For the PQ500 assay, the concentration of individual peptides was calculated based on the amount of each peptide supplied by the manufacturer and the volume of diluent used for resuspension.

Results and discussion

The retention times of 804 peptides were identified using unscheduled assays. High-quality transitions were selected using PRM Conductor in Skyline software. The developed 60 SPD and 100 SPD methods included 1,622 precursors with 13,876 transitions and 1,622 precursors with 13,699 transitions using 24-minute and 14-minute gradients, respectively. The chromatograms for all peptides are shown in Figures 6A and 6B.

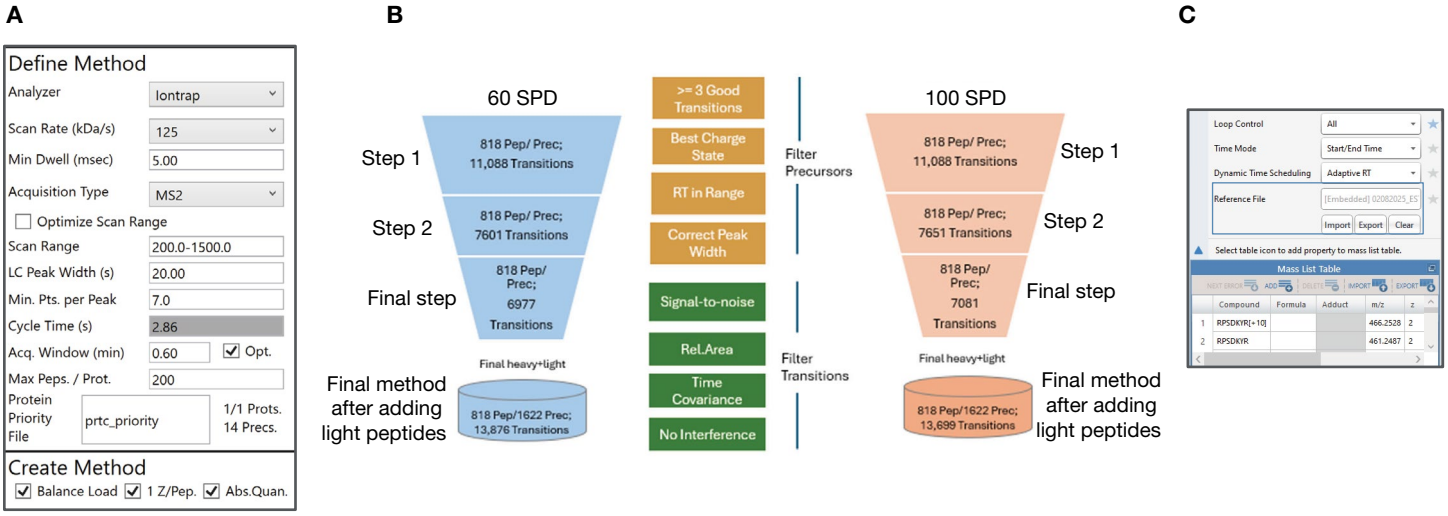


Figure 5. (A) The defined method parameters for final targeted PRM method; (B) summarized precursors and transitions filtering workflow; (C) importing the reference file in the final method to enable Adaptive RT real-time alignment

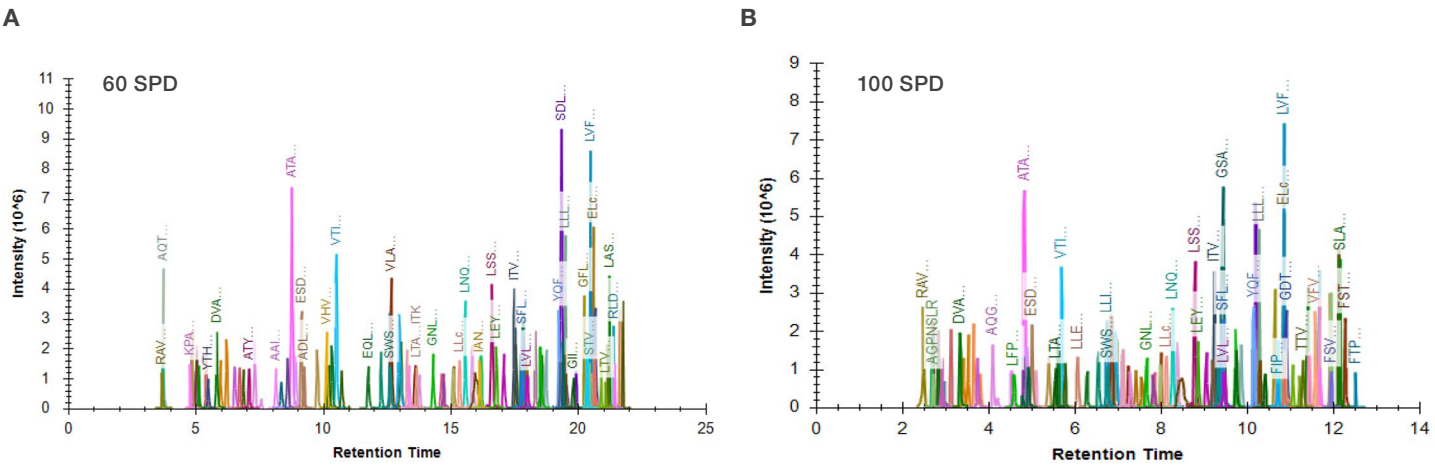


Figure 6. (A) Chromatogram of 804 heavy peptides from 60 SPD method; (B) chromatogram of 804 heavy peptides from 100 SPD method

After identifying the retention times of the heavy peptides, both light and heavy peptides were included in the final method. Experiments were performed to determine the assay's sensitivity and feasibility for peptide quantitation.[2] The analysis included characterizing the number of acquired points per LC peak, LOD, LOQ, linearity, and the CVs of the measured peptide abundances. The 60 SPD and 100 SPD assays had median points per peak of 7.1 and 6.8, respectively (Figure 7A), exceeding the generally accepted minimum of 6 points, which meets the Nyquist criteria for a Gaussian peak. More than 717 peptides had at least 7 points per peak (Figure 7B). The CV% values for 10 replicate injections were 3.8% and 4.8% for the 60 SPD and 100 SPD assays, respectively (Figure 8A). For the 60 SPD method, 93.7% of the peptides had CV values below the 20% cutoff, while 94% of peptides met this criterion for the 100 SPD method (Figure 8B).

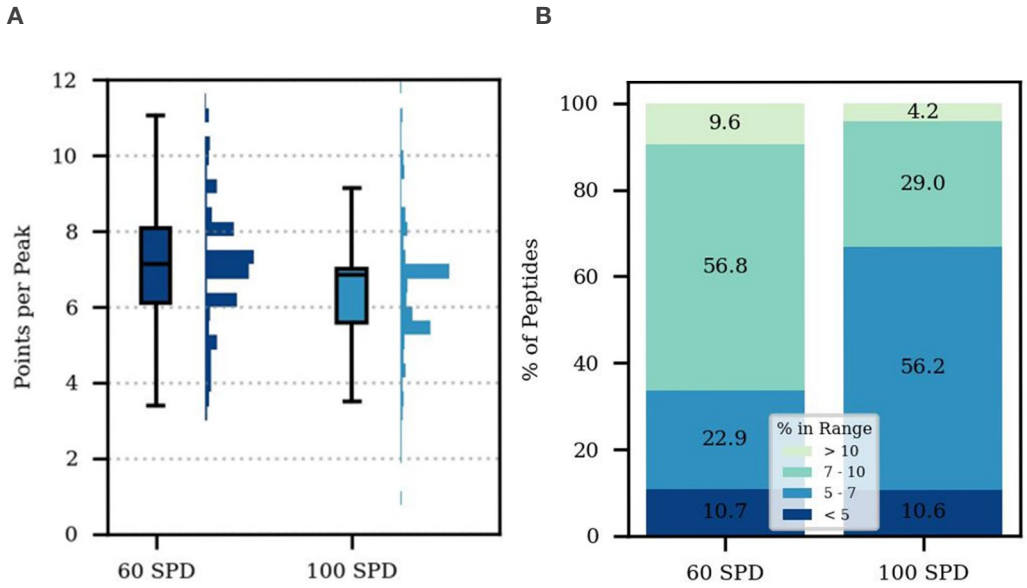


Figure 7. (A) Data points per peak of all peptides for 60 and 100 SPD methods; (B) summary of peptide percentage with different data points per peak for 60 and 100 SPD methods

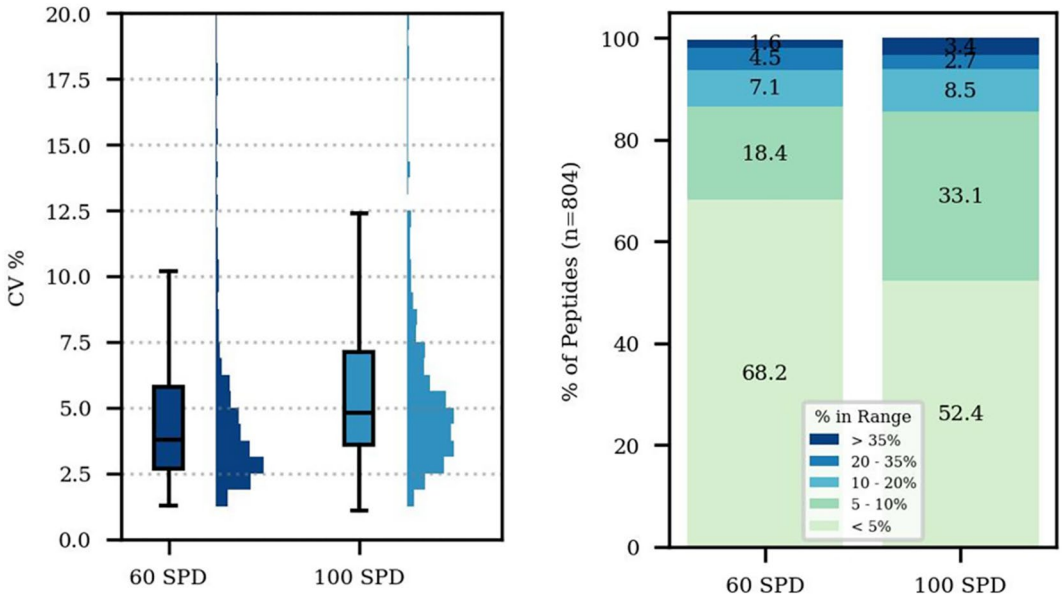
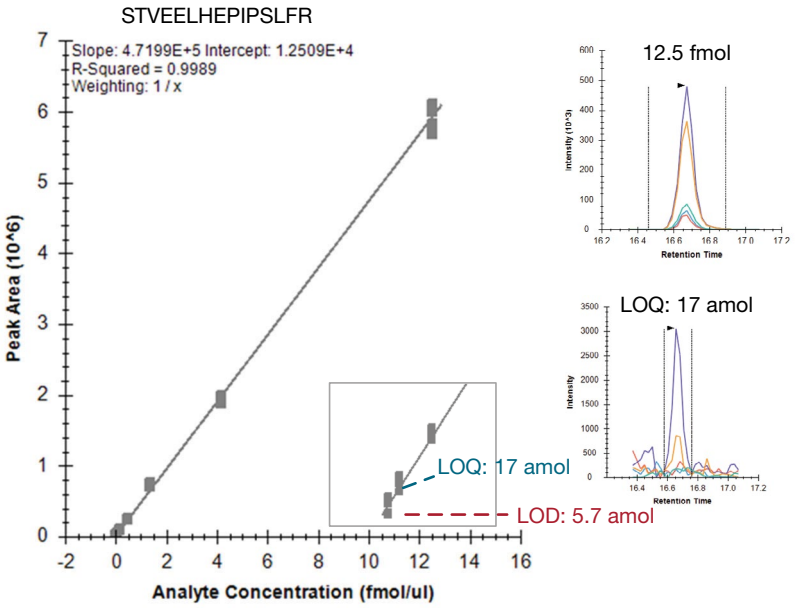


Figure 8. (A) CV% of all peptides in box-whisker plot (n=10); (B) the percentage of peptides within different CV% ranges (n=10)

Dilution curves of the PQ500 in 300 ng of human plasma were used to assess LOD and LOQ. Calibration curves were plotted with peptide concentrations on the x-axis and peak areas on the y-axis. The LODs were calculated as the bilinear turning points of the calibration curves. Peptides showed good linearities on the Stellar MS across a wide dynamic range. A sample calibration curve for peptide STVEELHEPIPSLFR is shown in Figure 9, with an estimated LOD of 5.7 attomoles and LOQ of 17 attomoles on column. Among the 804 peptides, more than 722 peptides in the 60 SPD method and 684 peptides in the 100 SPD method had an LOD less than 50 attomoles (Figure 10A). For both assays, most peptides had an LOQ less than 50 attomoles, with approximately 85–87% of peptides having an LOQ < 500 attomoles (Figure 10B). LODs and LOQs were about 1.6x lower in the 60 SPD compared to the 100 SPD method (Figures 11A and 11B).



Concentration level (fmol on column)	Accuracy (%)	CV (%)
0.017	117.8	20.6
0.052	104.1	6.5
0.15	110.2	4.4
0.46	108.3	2.6
1.39	108.9	2.5
4.17	97.8	2.2
12.52	99.3	2.9

Figure 9. Calibration curve of the STVEELHEPIPSLFR peptide

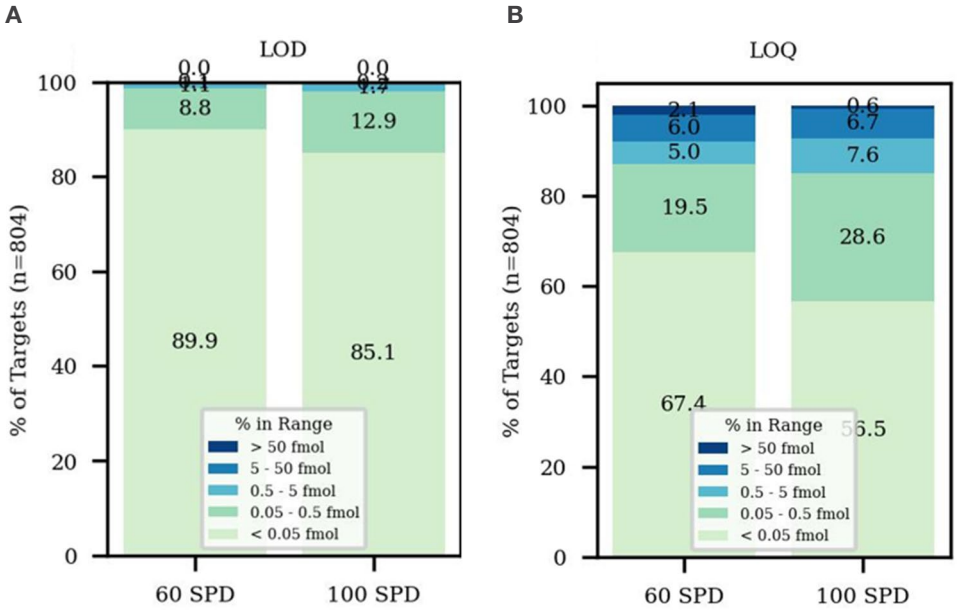


Figure 10. (A) Summary of limits of detection for all the peptides; (B) summary of limits of quantitation

Using this established workflow and heavy-labeled peptides, we identified endogenous proteins, such as alpha-2-macroglobulin (A2MG)—a potential biomarker for Alzheimer's disease[3] and cardiac disease[4], as well as other disease-relevant proteins from neat plasma samples (Figure 12). This highlights the ability to detect low-abundance proteins in complex mixtures with the streamlined targeted workflows on the Stellar MS, providing valuable insights into disease mechanisms and potential diagnostic markers.

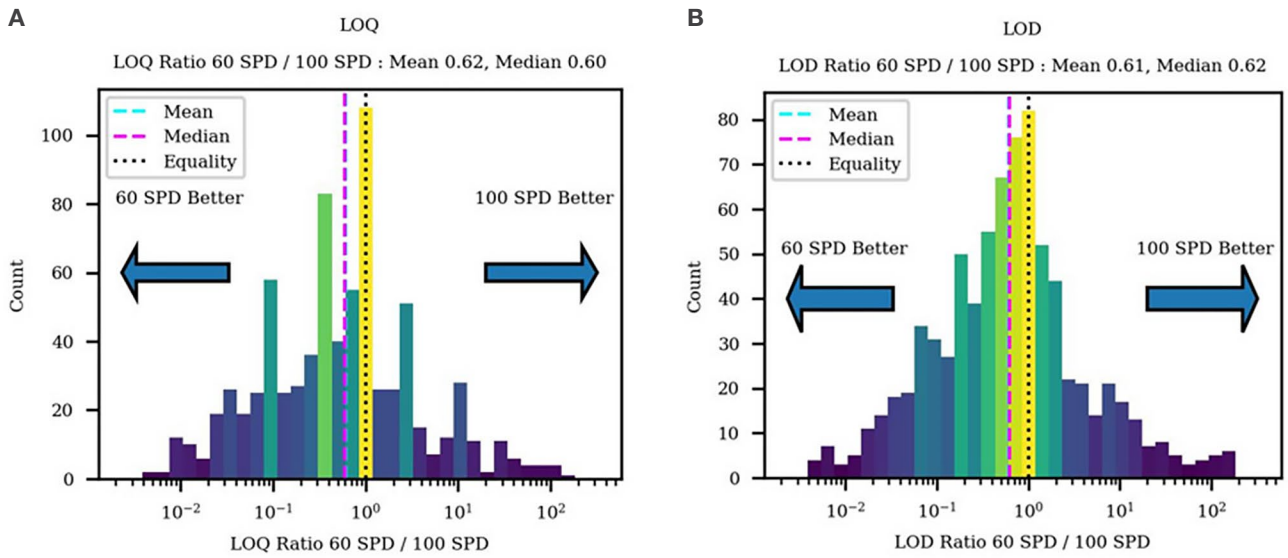


Figure 11. (A) LOQ ratio between 60 SPD and 100 SPD methods; (B) LOD ratio between 60 SPD and 100 SPD methods

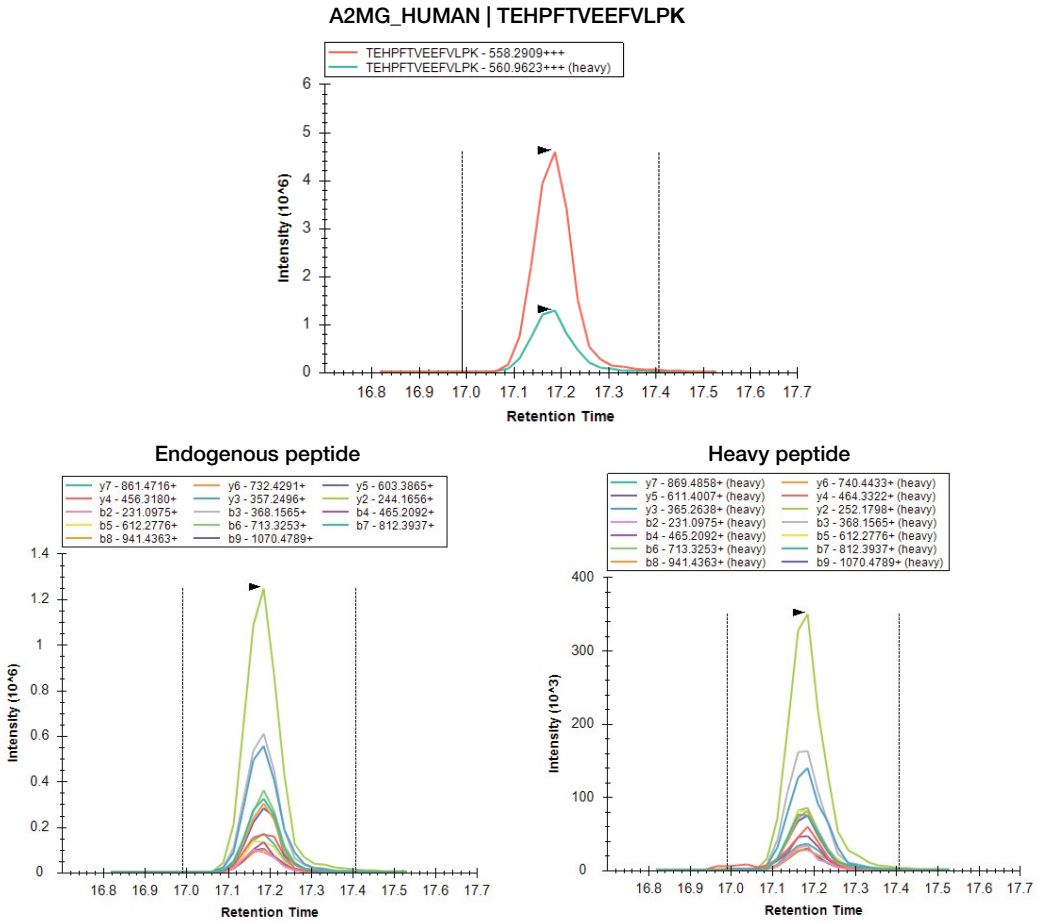


Figure 12. Detected heavy and endogenous A2MG in neat plasma

Conclusion

A streamlined targeted mass spectrometry workflow using the Stellar MS was developed within three days for the quantitation of more than 800 peptides using 100 and 60 SPD methods (14- and 24-minute gradients, respectively). Adaptive RT alignment was applied to eliminate missing peaks due to potential retention time shifts. The combination of Skyline software and the PRM Conductor tool enabled the creation of the assay without the use of spreadsheets or other manual efforts. These advancements demonstrate the Stellar MS's significant advantages in sensitivity, time efficiency, and capacity for multiplexed large-scale targeted peptide quantitation, enabling a new era of enhanced, scalable, and accessible biomarker verification assays that can revolutionize translational and clinical research.

Highlights of this study using the Stellar MS

1. **Discovery to validation at unprecedented scale:** The Stellar MS can scale to thousands of targets within 14- to 24-minute methods by utilizing scheduled narrow retention time windows and Adaptive RT alignment.
2. **Improved sensitivity:** The Stellar MS detected peptides at levels as low as a few attomoles. Over 85% of peptides had detection limits below 50 attomoles.
3. **Quantitative accuracy with speed, precision, and accuracy:** The developed 60 SPD and 100 SPD methods incorporated 1,622 precursors with 13,876 and 13,699 transitions, respectively, utilizing 24-minute and 14-minute gradients. Additionally, 93% of peptides achieved less than 20% CV with accuracy greater than 90% for all quantitative levels.
4. **Adaptive RT manages shifted RT window:** Adaptive RT bypasses the cost and time associated with method refinement using spiked iRT standards. With hyper-fast acquisition speeds, Stellar MS manages shifted retention times with Adaptive RT, maintaining the sensitivity and speed necessary to handle numerous concurrent targets. This eliminates the need for manual retention time adjustment within and across studies and decreases missingness from peaks that would shift out of retention time boundaries using conventional targeted methods.
5. **Improved workflow efficiency:** The combination of Skyline software and PRM Conductor enabled the creation of the PRM assay without the need for spreadsheets or other manual efforts. This streamlined workflow allowed a new method to be developed within just three days and offers customization and scalability for any target list of interest.

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