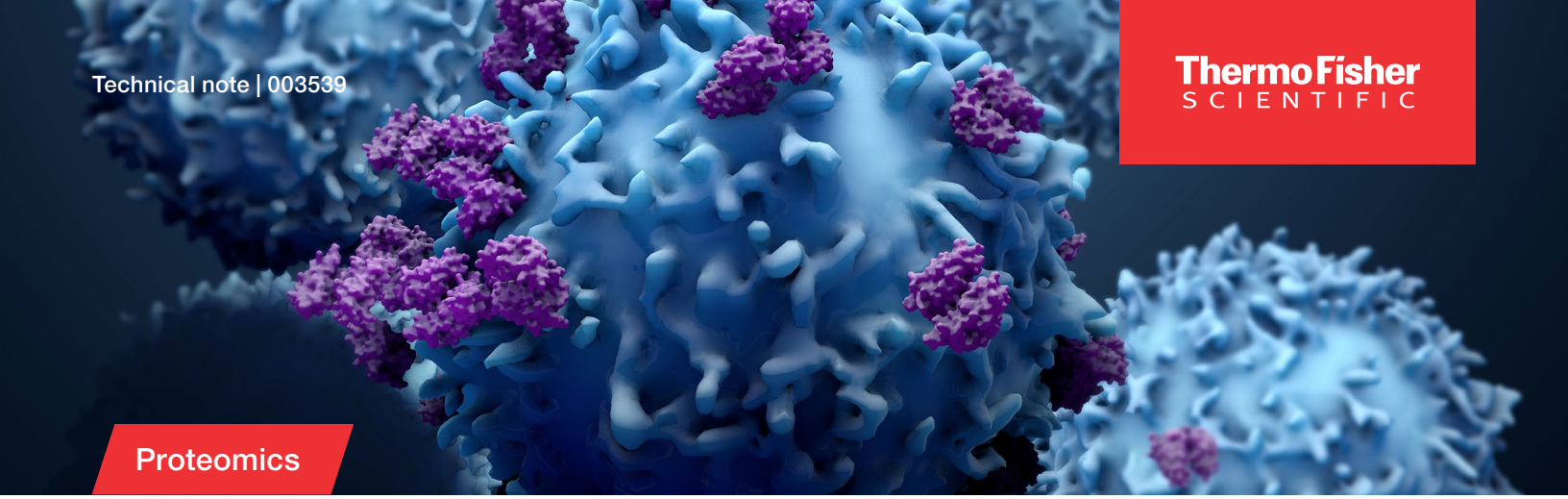

Technical note | 003539

Proteomics

Ultra-sensitive absolute quantitation and high-throughput method development for immunopeptidomics using the Stellar mass spectrometer

Authors

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Keywords

Immunopeptidomics, Stellar mass spectrometer, major histocompatibility complex, MHC, PRM, Heavy Peptide AQUA, Vanquish Neo UHPLC, Skyline, adaptive retention time, data-independent acquisition, DIA, wide-window acquisition

Goal

The primary objective of this study is to evaluate the sensitivity, throughput, and ease of method development using the Thermo Scientific™ Stellar™ mass spectrometer for targeted immunopeptidomics mass spectrometry (MS). Specifically, we aim to demonstrate the precise quantitation of immunopeptides. By developing and validating an ultra-sensitive, high-throughput method, we seek to provide a framework for researchers to efficiently quantify low-abundance peptides, with method development and data acquisition completed within one week. This work aims to support the advancement of personalized cancer and autoimmune therapies by offering a robust and versatile tool for immunopeptidomics research.

Introduction

Immunopeptidomics is the study of peptides displayed by major histocompatibility complex (MHC) molecules on the surface of cells, which play a critical role in activating the immune system. Recent studies have shown that identifying immunogenic peptides is crucial for designing targeted cancer and autoimmune therapies. MS proteomics is the only technology capable of experimentally identifying and quantifying thousands of these peptides simultaneously through immunopeptidomics workflows (Figure 1).

Over the past decade, MS-based methods have achieved significant success in discovering immunopeptides, including the identification and validation of immunogenic peptides from tumor samples. However, high-throughput quantitation of these peptides remains essential for vaccine and cell therapy development, providing insights into treatment response, preferred drug delivery methods, and the presence of immunopeptides across large patient cohorts.

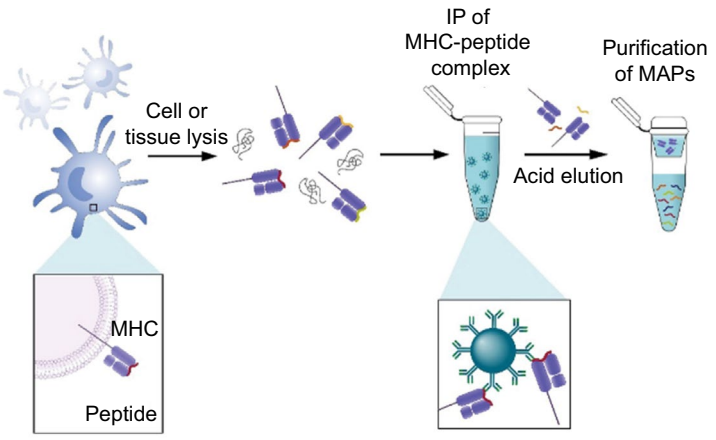


Figure 1. General sample preparation workflow for immunopeptidomics

Targeted MS proteomics can meet the high-throughput, absolute quantitation needs of immunopeptidomics. However, specific challenges prevent the straightforward adoption of traditional triple quadrupole mass spectrometer methods using selected reaction monitoring (SRM). One major challenge is the presence of low abundance immunopeptides, typically ranging from 10 to 1,000 copies per cell, which makes accurate quantitation difficult when dealing with high background noise. Another challenge is the extensive method development required for SRM. For instance, monitoring more than 50 precursors on a triple quadrupole mass spectrometer would demand more replicate injections and longer method development times, which can be impractical, especially for precious patient biopsy samples.

The Stellar MS addresses the needs of targeted immunopeptidomics by enabling absolute quantitation, leveraging advanced capabilities currently unavailable with triple quadrupole platforms. Stellar MS technology facilitates parallel reaction monitoring (PRM), a technique that targets and monitors all transitions per peptide in one scan event (Figure 2). This approach eliminates the need for extensive transition selection, optimization, and testing as required for SRM, thereby streamlining the development of targeted MS methods. Monitoring all transitions in a targeted proteomics experiment can provide the flexibility to adapt to different sample matrices and tumor microenvironments without the need for extensive method re-optimization followed by sample re-analysis, if there is sample remaining. PRM is particularly valuable to enable built-in confirmation of sequence identity for immunopeptides during targeted quantification, to reduce ambiguity that the desired target is quantified in the method. Additionally, the Stellar MS offers versatile options, including different activation types (HCD and resonance CID) and MS^n capabilities. These features enable highly customizable methods with simple drop-down method selection steps to achieve the utmost sensitivity and precision in targeted assays. This study demonstrates the use of the Stellar MS to develop an ultra-sensitive, high-throughput method capable of identifying and quantifying peptides down to 1 amol, with method development and data acquisition completed within one week (Figure 3).

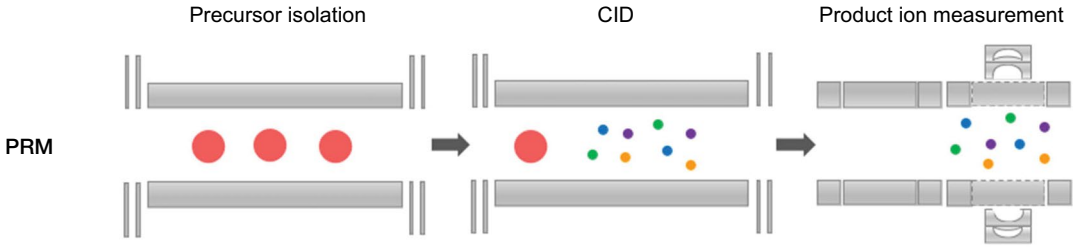


Figure 2. In PRM, precursor ions are selected, fragmented, and all resulting product ions are simultaneously measured in one scan event, thus resulting in a full scan MS².[1]

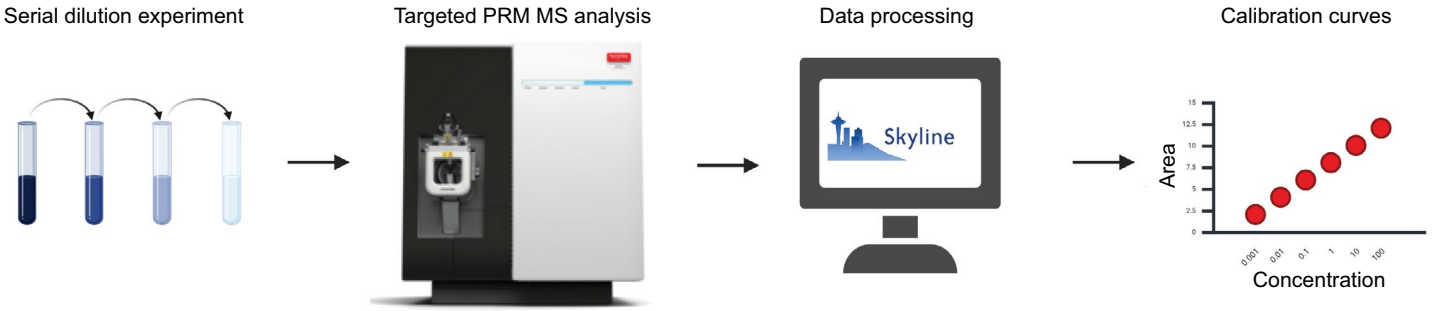


Figure 3. Experiment outline to assess sensitivity and method development ease using the Stellar MS

Experimental

Recommended consumables

- Thermo Scientific™ Pierce™ C18 stage tips
- Thermo Scientific™ Heavy Peptide™ AQUA Custom Synthesis Services isotopically labeled AQUA-grade peptides
- Other chemicals and reagents (e.g., LC-MS grade water, acetonitrile, formic acid)
- Mobile phase A: water + 0.1% formic acid
- Mobile phase B: 80% acetonitrile + 0.1% formic acid
- Thermo Scientific™ SureSTART™ 0.2 mL amber TPX screw top vials, 9 mm short thread conical glass insert, 110/pack (P/N 60180-1655)
- Thermo Scientific™ SureSTART™ 9 mm screw caps, Level 3 high performance applications (P/N 6PSC9STB1)

Samples

- Cayman Chemical™ HCT 116 HLA Enriched Sample, contracted from Cayman Chemical
- Isotopically labeled AQUA-grade peptides

LC columns

- Thermo Scientific™ EASY-Spray™ HPLC column (P/N ES906)
- Thermo Scientific™ PepMap™ Neo trap cartridge (P/N 174500 and 174502)

UHPLC system

- Thermo Scientific ™ Vanquish ™ Neo UHPLC system (P/N VN-S10-A-01)
- Thermo Scientific Vanquish™ user interface (P/N 6036.1180)

Mass spectrometer

- Stellar MS
- Thermo Scientific™ Easy-Spray™ source (P/N ES081)

Data analysis software

- MacCoss Lab Software Skyline™ V23.1

MHC standard preparation

Class I MHC peptides were obtained by immunocapture with W6/32-conjugated resin on 100 million HCT-116 cells (Figure 1). After cleanup using stage tips, the starting material was diluted 100x with 0.1% formic acid. A dilution series was prepared by spiking 48 synthetic heavy labeled AQUA peptide standards into the MHC peptide sample at concentrations ranging from 1 amol to 100 fmol.

LC and MS conditions

Samples were analyzing using an EASY-Spray HPLC column (150 µm I.D. × 150 mm) connected to a Vanquish Neo UHPLC system and Stellar MS. A gradient length of 13 minutes was used. The LC was operated in the trap-and elute workflow for desalting and to protect the separation column.

Table 1. HPLC conditions

Parameter	Value
Mobile phase A	100% H_2O with 0.1% FA
Mobile phase B	80% ACN / 20% H_2O (v/v) with 0.1% FA
Flow rate	1.8 µL/min
Trap column	PepMap Neo trap cartridge (300 µm I.D. × 5 mm)
Trap loading volume	Automatic (5 µL)
Trap column fast wash and equilibration	Check
Trap column wash factor	Automatic
Trap column equilibration factor	Automatic
Trap column mode	CombinedControl
Trap column flow	200
Trap column pressure	800
Trap column flush direction	Backward
Analytical column	EASY-Spray HPLC column (P/N ES906)
Separation column fast equilibration	Check
Separation column mode	Pressure Control
Separation column pressure	1,000
Separation column equilibration factor	2
Analytical column temperature	40 °C
Autosampler temperature	7 °C
Inner needle wash/ metering device (MD)	Weak: Water with 0.1% FA Strong: ACN 80% with 0.1%FA
Outer needle wash/ wash port (W)	Weak: Water with 0.1% FA Strong: ACN 80% with 0.1%FA

Table 2. HPLC gradient

Run start time (min)	Duration (min)	%B	Flow rate (µL/min)
0.0	0.0	1.0	1.8
0.7	0.7	4.0	1.8
1.0	0.3	8.0	1.8
7.7	6.7	22.5	1.8
11.4	3.7	35.0	1.8
11.8	0.4	55	2.5
Column wash			
12.3	0.5	99	2.5
13	0.7	99	2.5

Table 3. Global MS parameters

Source parameters	
Spray voltage (positive)	1,200
Capillary temperature	275
Sweep gas	2
MS global settings	
Expected LC peak width(s)	8 (MS^2 method) or 9.09 (MS^3 method)
Default charge state	2
Collision cell gas pressure (mTorr)	8

Targeted MS^2 experiment setup

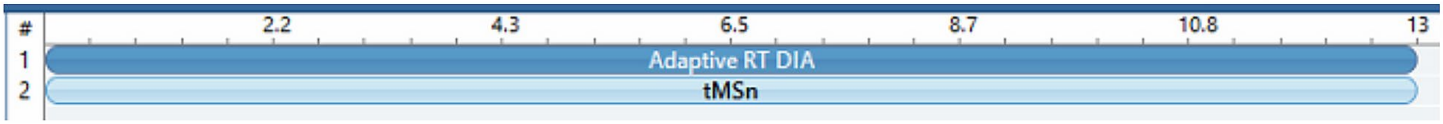


Figure 4. Targeted MS^2 experiment setup

The adaptive retention time data-independent acquisition (RT DIA) experiment is optional. This experiment will create a .RTbin file that can be used to shift expected retention time windows based on matrix background. The .RTbin file will only be created if the "Acquire reference" box is checked. Tables 4 and 5 describe the Targeted MS^2 method setup for Adaptive RT DIA and tMS^n experiments.

The target peptide list consisted of 48 endogenous peptides with their corresponding isotopically labeled AQUA-grade counterparts. The peptide elution time was scheduled, based on prior run optimization (Figure 12, Results and discussion section).

Table 4. Adaptive RT DIA

Data-independent analysis properties	
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
Polarity	Positive

Table 5. tMS^n: MS^2 Targeted mass spectrometry experiment

MS scan properties	
Isolation window (*m/z*)	1
Activation type	HCD
HCD collision energy type	Normalized
HCD collision energy/energies (%)	30
Scan rate (kDa/s)	125
Scan range mode	Define *m/z* Range
Scan range (*m/z*)	200–1,500
Use multi-stage fragmentation	No check
RF lens (%)	30
AGC target	Custom
Normalized AGC target (%)	200
Maximum injection time mode	Dynamic
Cycle time (s)	1.2
Points per peak	7
Data type	Centroid
Polarity	Positive
Source fragmentation (V)	0
Loop control	All
Time mode	Start/End Time
Dynamic time scheduling	Adaptive RT
Reference file	Load appropriate .RTbin file

Targeted MS^3 experiment setup

Adaptive RT DIA settings are shown in Table 4.

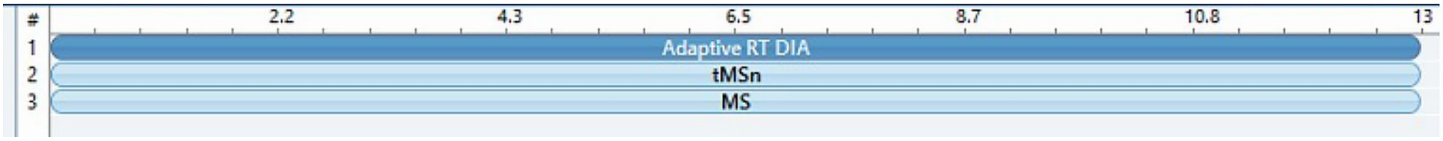


Figure 5. tMS^n: MS^3 targeted mass spectrometry experiment

Tables 6 and 7 describe the Targeted MS^3 method setup for tMS^n and MS experiments.

Table 6. tMS^3: Targeted MS^n scan properties

Precursor ion properties	
Isolation window (*m/z*)	2
Activation type	HCD
HCD collision energy type	Normalized
HCD collision energy/energies (%)	30
Product ion properties	
Activation type	HCD
HCD collision energy type	Normalized
HCD collision energy/energies (%)	30
Use multistage fragmentation	Check
MS^2 scan rate (kDA/s)	125
MS^2 scan range mode	Define *m/z* Range (defined in table)
MS^3 scan rate (kDA/s)	125
MS^3 scan range mode	Define *m/z* Range (defined in table)
RF lens (%)	30
AGC target	Custom
Normalized AGC target (%)	200
Maximum injection time mode	Dynamic
Cycle time (s)	1.52
Points per peak	6
Data type	Centroid
Source fragmentation (V)	0
Loop control	All
Time mode	Start/End Time
Dynamic time scheduling	Off

Table 7. MS: MS scan settings (optional). These settings can be included or not, depending on if there is extra room in the cycle time. They can be helpful if testing a method in a new matrix and a peak may elute outside of targeted retention time.

MS scan properties	
Scan range (*m/z*)	350–1,250
Scan rate (kDa/s)	67
RF lens (%)	30
AGC target	Standard
Maximum injection time mode	Auto
Data type	Centroid
Polarity	Positive
Source fragmentation (V)	0

The target peptide list consisted of 48 endogenous peptides with their corresponding isotopically labeled AQUA-grade counterparts. The peptide elution time was scheduled, based on prior run optimization (Figure 12, Results and discussion section). The target peptide list from Table 5 was used as a base for creating the tMS^3-specific target peptide list, with the selected MS^2 fragment ions undergoing further fragmentation added by the user in the method editor.

Data processing parameters

PRM data was processed in Skyline software daily (V24.1.1.284). PRM Conductor selected the best transitions for quantification, and the area under the curve (AUC) was calculated from the raw fragment area of these transitions using Skyline software. For figures of merit calculation, the regression was fit to bilinear turning point for limit of detection (LOD) and max CV <20% for limit of quantitation (LOQ).

Results and discussion

Absolute quantitation of immunopeptides using the Stellar MS

To evaluate the quantitative performance of the dual-pressure linear ion trap in the Stellar MS, we conducted a dilution series using 48 isotopically labeled AQUA-grade peptides spiked into a background of 1% immunopeptide sample derived from 1e8 HCT116 cells, equivalent to the immunopeptide material from 1e6 cells. AQUA-grade peptides (heavy) were spiked at concentration levels ranging from 0.001 to 100 fmol with three replicates. A total of 96 light and heavy peptides and all subsequent transitions were monitored (Figure 6).

At high concentrations, the coefficients of variation (CVs) were below 10% on average for both light and heavy peptides (Figure 7).

Across the entire dilution series, 45 AQUA peptides were detected (limit of detection) from 0.001 to 100 fmol (Figure 8). Additionally, 27 heavy peptides were quantified across the entire spiked dynamic range (Figure 8).

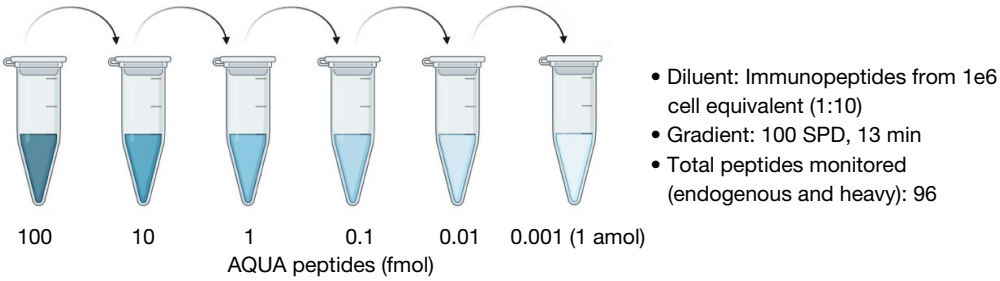


Figure 6. Dilution scheme setup for targeted immunopeptidomics calibration curve experiment

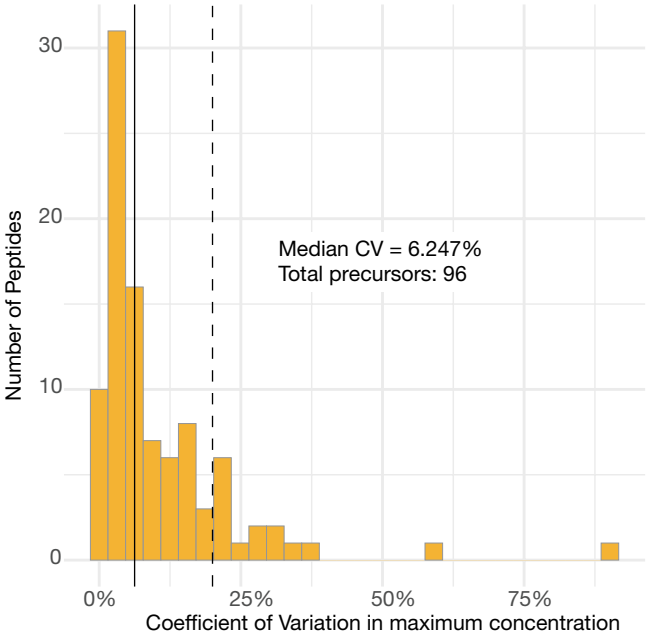


Figure 7. Coefficient of variation in maximum concentration for all monitored peptides. 48 heavy peptides and 48 light peptides were monitored. The dotted line represents 20% CV. The solid line represents median CV.

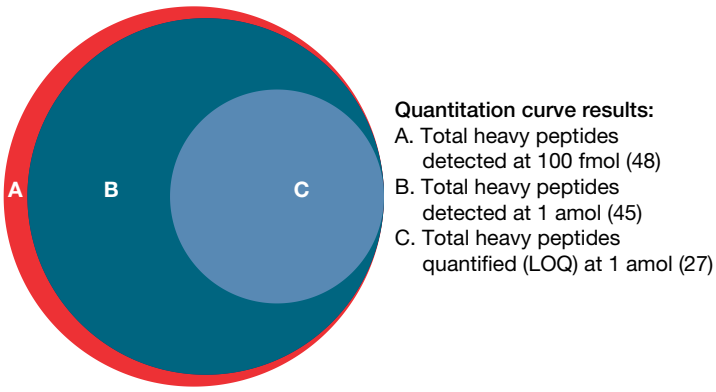


Figure 8. Number of peptides detected and quantified at different concentrations. (A) Total heavy peptides detected at 100 fmol. (B) Total heavy peptides detected (LOD) at 1 amol. (C) Total heavy peptides quantified (LOQ) at 1 amol. LOD and LOQ as defined by Skyline output.

The data demonstrated high quality, with LOD and LOQ values determined based on high linearity (R^2 > 0.9) and CVs (n=3) below 20%, while light peptides remained constant in the background matrix (Figure 9).

Utilization of MS^3 to reduce noise and increase sensitivity

To assess the utility of tMS^3 capabilities in the linear ion trap on the Stellar MS, we repeated the dilution curve and collected tMS^3 data for each peptide. Targeted-MS^3 acquisition improved the number of detected peptides across the full dilution range by 14% and increased the LOQ determination to 0.001 fmol for five peptides (Figure 10).

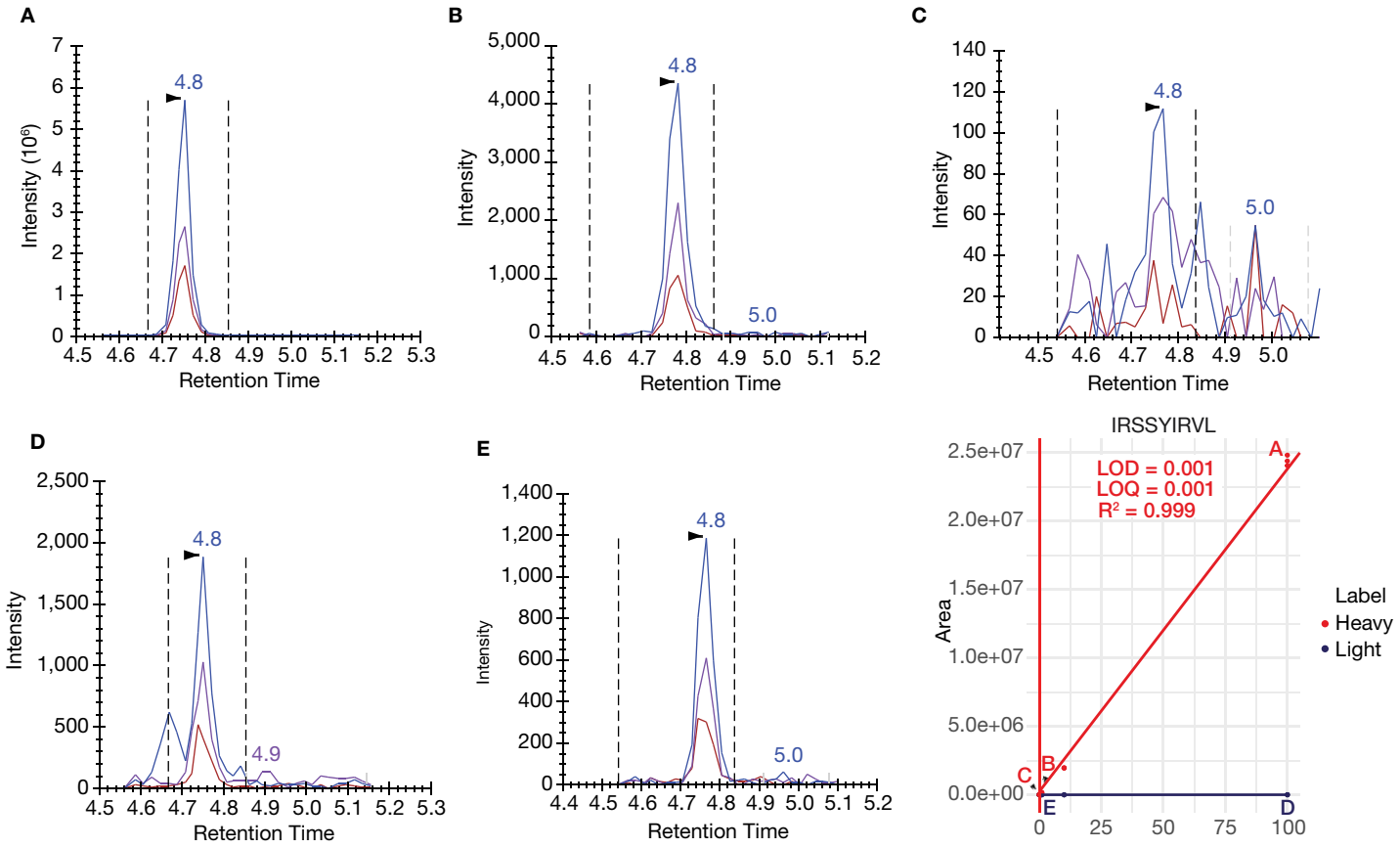


Figure 9. Example dilution curve. Heavy (A, B, and C at the respective spike in concentration: 100 fmol, 0.1 fmol, and 1 amol) and light peptides (D and E) are shown across heavy peptide dilution series

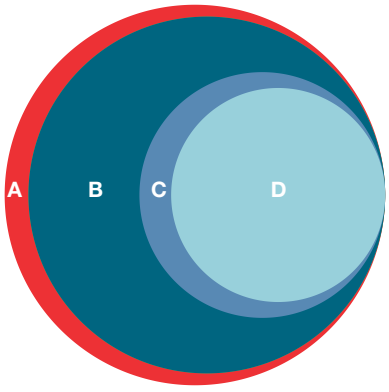


Figure 10. Number of peptides detected and quantified at different concentrations leveraging MS^2 and MS^3. (A) Total heavy peptides detected at 100 fmol. (B) Total heavy peptides detected at (LOD) at 1 amol. (C) Total heavy peptides quantified (LOQ) at 1 amol with MS^2 and MS^3 combined. (D) Total heavy peptides quantified (LOQ) at 1 amol with MS^2 only. LOD and LOQ as defined by Skyline output.

Quantitation curve results:

A. Total heavy peptides detected (48)
B. Total heavy peptides detected at 1 amol (45)
C. Total heavy peptides quantified (LOQ) at 1 amol with MS^2 +MS^3 (31)
D. Total heavy peptides quantified (LOQ) at 1 amol with MS^2 only (27)

Comparative product ion XIC analysis showed tMS^3 reducing matrix interference using tMS^3 as compared to tMS^2 chromatograms for both light and heavy peptides (Figure 11).

Efficiency of targeted proteomics method development

Absolute quantitation of heavy and light peptides was achieved within a week of combined method development and instrument time. The method development steps for the targeted proteomics experiment were performed on the Stellar MS (Figure 12).

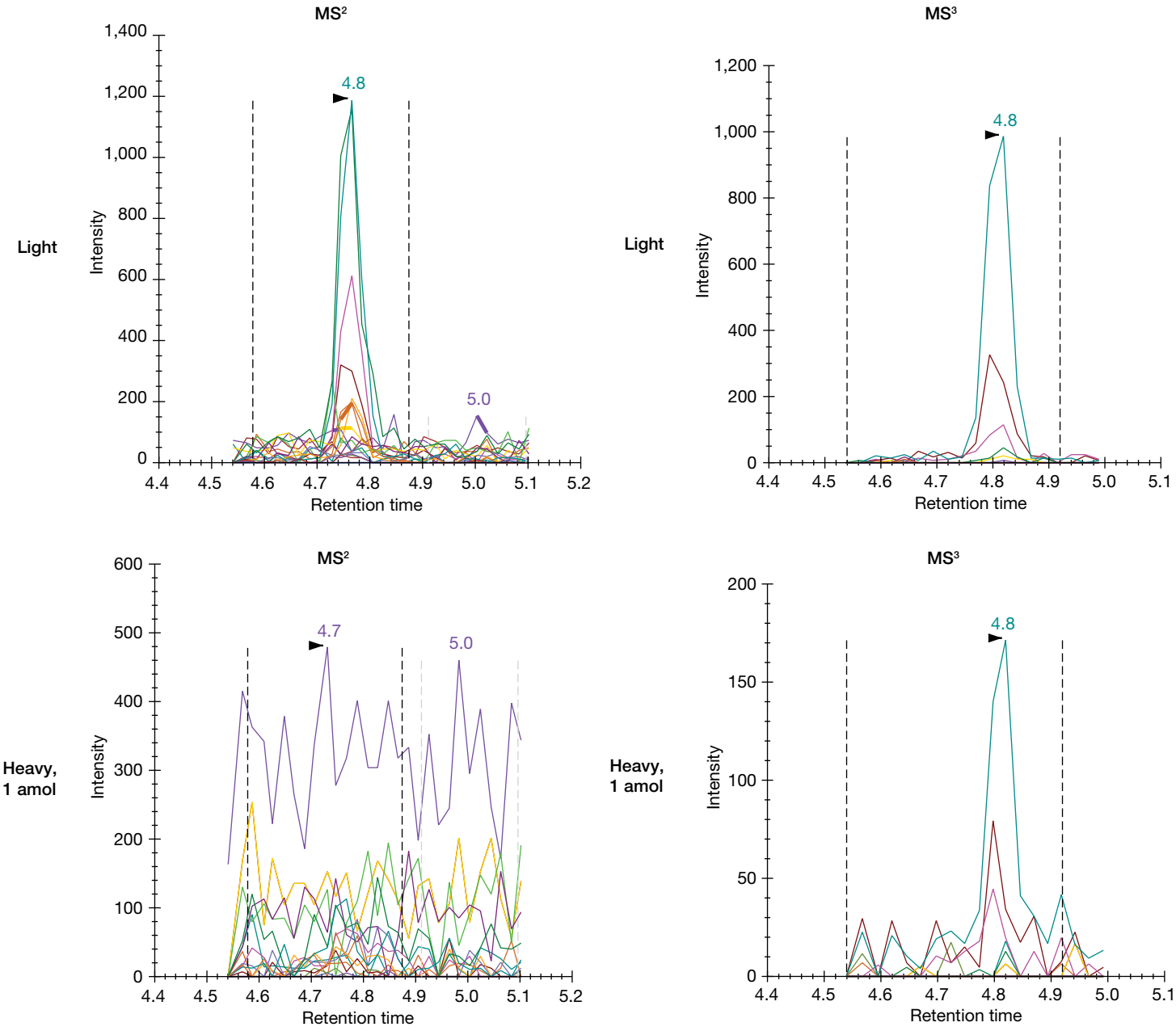
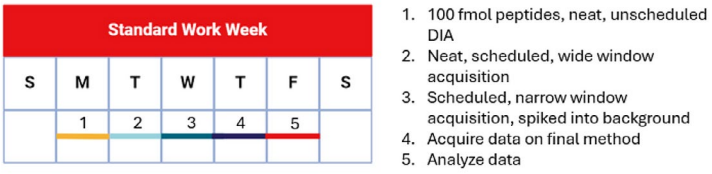


Figure 11. Example peptide for MS^2 and MS^3 chromatograms

Figure 12. Targeted proteomics workflow steps achieved within the standard work week



1. 100 fmol peptides, neat, unscheduled DIA
2. Neat, scheduled, wide window acquisition
3. Scheduled, narrow window acquisition, spiked into background
4. Acquire data on final method
5. Analyze data

The method development steps were as follows:

(1) Initial setup: A 100 fmol equal molar mixture of isotopically labeled AQUA-grade peptides was analyzed using unscheduled DIA windows to identify the LC and MS settings for the final quantitative methods. Three replicate injections were performed to determine retention time, precursor charge state, *m/z* values, and product ion XIC for all detected ions per peptide. In comparison, developing the quantitative method using triple quadrupole mass spectrometers would require many additional replicate injections. Each precursor would need to be characterized with six to eight SRM transitions, resulting in 276 to 368 total transitions per peptide charge state. Due to the limited number of unscheduled transitions that can be monitored simultaneously on a triple quadrupole mass spectrometer, this would require dividing the peptides into groups and conducting additional replicate injections to cover all the transitions. This results in at least a 15x time savings when using the Stellar MS for initial method development, which can effectively monitor a larger number of unscheduled precursors with multiple charge states and no pre-selection of transitions.

(**2) Wide-window acquisition:** A neat, scheduled, wide-window acquisition was performed on the heavy peptides, again achieving a 15x savings in instrument time.

(3) Narrow-window acquisition: A scheduled, narrow-window acquisition was conducted with peptides spiked into the background, requiring only three triplicate injections to ensure peptides were monitored in the correct windows.

(4) Final method: After the previous three steps, taking a total of nine injections, the method was now developed and ready to acquire final, absolute targeted proteomics data. Unlike triple quadrupole mass spectrometer based workflows, there was no need to optimize and select the best transitions, as all transitions could be considered even in the final method. This comprehensive approach enabled researchers to have full peptide information at their fingertips (Figure 13).

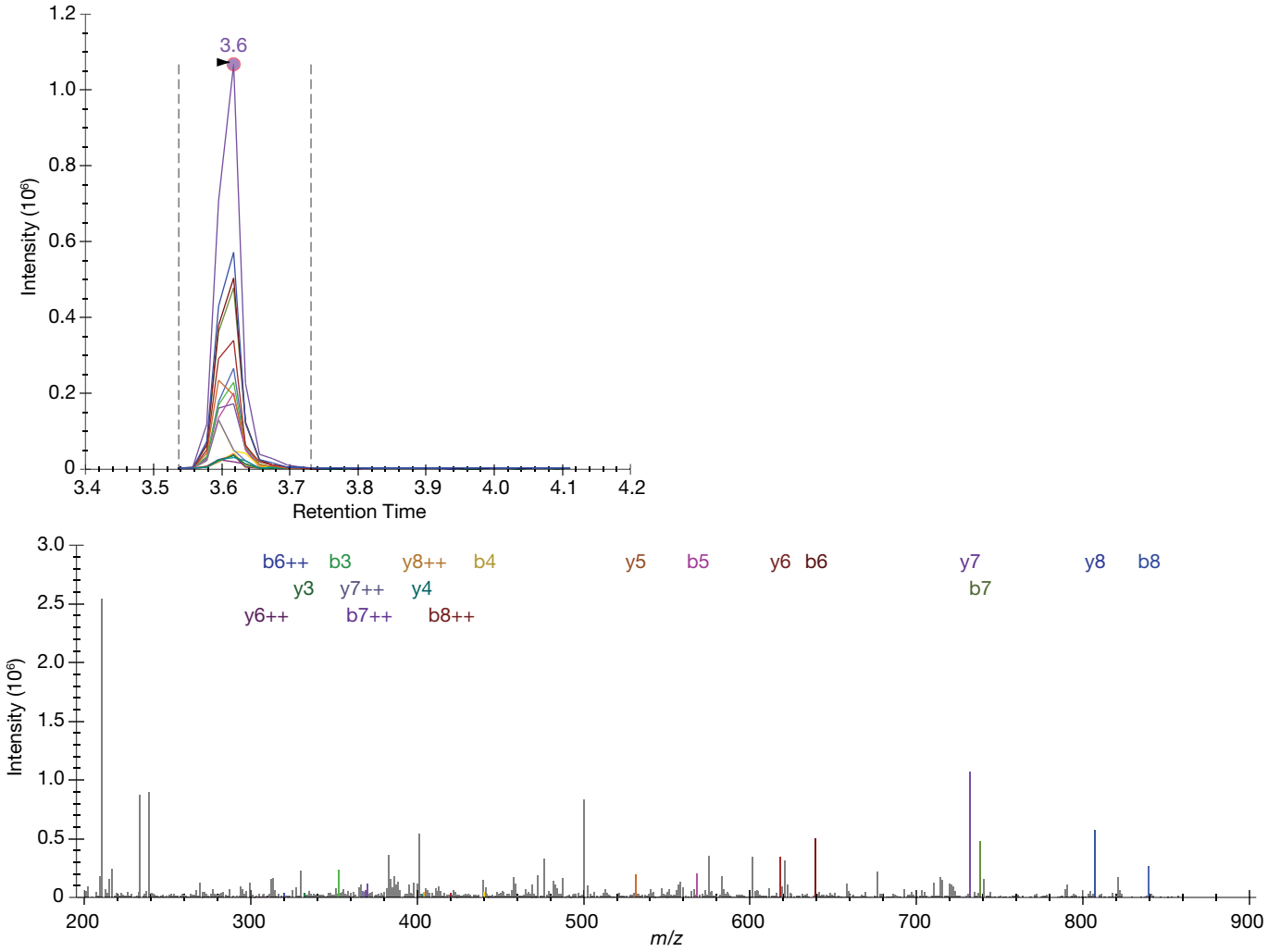


Figure 13. Example PRM chromatogram (top) and spectrum (bottom) at the apex of the heavy peptide peak, illustrating built-in verification of immunopeptide sequence identity within the targeted quantification method. B and Y ions are labeled directly above their location on the spectrum and demonstrate confident identification of the peptide sequence based on backbone fragmentation.

Overall, an 11-fold reduction in injections and mass spectrometer time was achieved in method development using the Stellar MS (Figure 14).

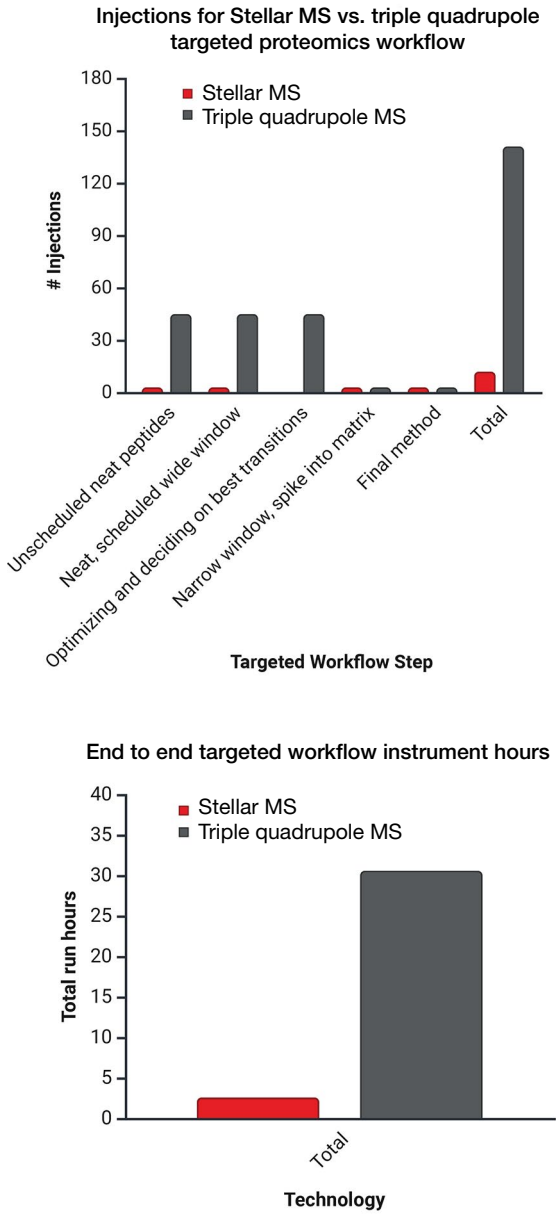


Figure 14. Injection and time comparison for targeted method setup between Stellar MS and triple quadrupole mass spectrometer

Summary

This study demonstrates the significant capabilities of the Stellar MS in the field of immunopeptidomics. Key findings include:

- **1 attomole sensitivity:** The Stellar MS achieved ultra-sensitive detection of immunopeptidomics samples with a sensitivity of 1 amol on a microflow, 13-minute gradient. This level of sensitivity corresponds to approximately 100 copies per cell in a 1e6 background, allowing for effective quantitation of low abundance immunopeptides.
- **tMS^3 enhancements in quantitation and peptide confirmation:** Utilizing tMS^3 capabilities increases the number of identified peptides across the full concentration range by 14% while improving the LOQ for four peptides to 1 amol.
- **Efficient method development:** Method development was completed in less than one week with fewer than nine injections. This represents an 11-fold time savings compared to traditional triple quadrupole SRM workflows, facilitating a rapid transition from discovery to targeted data acquisition and clinical decision-making.
- **Comprehensive peptide profiling:** The final method allowed for monitoring all transitions without extensive optimization. This flexibility accommodates precursor and transition variability in patient samples, tumor microenvironments, and sample matrices, ensuring consistent and reliable quantitation across diverse patient samples.

These advancements highlight the Stellar MS as a robust and versatile tool for immunopeptidomics research, with significant implications for the development of personalized cancer and autoimmune therapies.

Reference

1. Plubell, D.L. et al. Constructing targeted proteomics assays using the Thermo Scientific™ Stellar™ mass spectrometer, Thermo Fisher Scientific, On-demand webinar series: Breaking barriers for biomarker translation toward validation, Sept. 18, 2024.

Learn more at **thermofisher.com/immunopeptidomics**

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