Targeted Proteomics

Ultra-sensitive and rapid method development for targeted immunopeptidomics using the Stellar mass spectrometer

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Abstract

Purpose: Development of a high-throughput, ultra-sensitive mass spectrometry method for quantifying immunopeptides, to enable advancement of cancer and autoimmune therapies.

Methods: A dilution series of 48 isotopically labeled AQUA peptides was spiked into the equivalent of 1e6 HCT116 cell immunopeptide material, with concentrations ranging from 0.001 to 100 fmol. Labeled and endogenous immunopeptides were quantified using parallel reaction monitoring (PRM) on the Thermo Scientific™ Stellar™ mass spectrometer, incorporating tMS3 for enhanced data acquisition.

Results: The method, utilizing a 13-minute gradient, achieved a limit of quantitation as low as 1 amol (~100 copies per cell) with high linearity and low coefficients of variation. Timed-MS³ (tMS3) improved peptide identification by 14%, enabling precise quantification across a broad dynamic range. Method development was completed in under one week, with fewer than nine injections, offering at least an 11x time-saving compared to traditional workflows.

Introduction

Immunopeptidomics focuses on identifying peptides presented by major histocompatibility complex (MHC) molecules, critical for immune system activation. While discovery mass spectrometry methods have identified immunogenic and tumor-specific peptides, translating these into high-throughput, targeted mass spectrometry (MS) methods is essential for precise quantification to support therapeutic development. Traditional triple quadrupole MS faces challenges such as noise and lengthy method development. The Stellar MS, a quadrupole ion trap, overcomes these issues by offering ultra-sensitive, high-throughput quantification with parallel reaction monitoring (PRM) and tMS³. This study presents a targeted proteomics approach that enables sensitive and rapid immunopeptide quantitation, streamlining the transition from discovery to clinical applications.

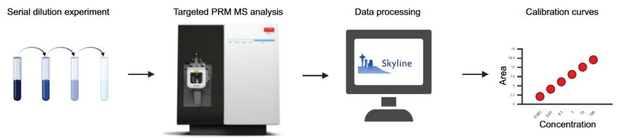


Figure 1. Experiment outline to assess sensitivity and method development ease using the Stellar MS

Materials and methods

Sample Preparation

Class I MHC peptides were obtained by immunocapture with W6/32-conjugated resin on 100 million HCT-116 cells. 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 1amol to 100 fmol (Figure 1).

LC-MS/MS conditions

Samples were analyzing using a Thermo Scientific™ EASY-Spray™ HPLC column (P/N ES906) connected to a Thermo Scientific™ 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. Data was acquired with a tMSn scan. For tMSn, peptide elution time was scheduled, based on optimization.

Data Analysis

PRM data was processed in Skyline software daily (V24.1.1.284). The PRM Conductor tool 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

Absolute quantitation of immunopeptides using the Stellar MS

Coefficients of variation (CVs) were below 10% on average for both light and heavy peptides at 100 fmol (Figure 7), 45 AQUA peptides were detected and 27 were quantified across the entire dilution series from 0.001 to 100 fmol (Figure 8), and an example dilution curve is shown for heavy and light peptides across a heavy peptide dilution series (Figure 9).

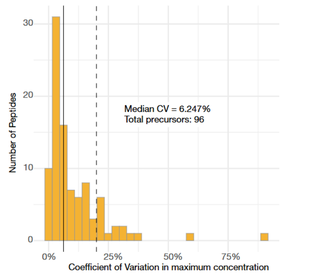


Figure 2. Coefficient of variation in maximum concentration for all monitored peptides. 48 heavy peptides and 48 light peptides were monitored. The dotted line represents 20% CV. The solid line represents median CV.

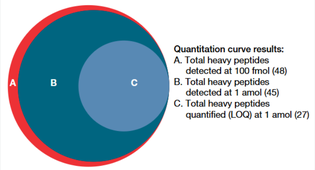


Figure 3. Number of peptides detected and quantified at different concentrations. (A) Total heavy peptides detected at 100 fmol. (B) Total heavy peptides detected (LOD) at 1 amol. (C) Total heavy peptides quantified (LOQ) at 1 amol. LOD and LOQ as defined by Skyline output.

Key Takeaway
13 minute gradient, 1 amol sensitivity

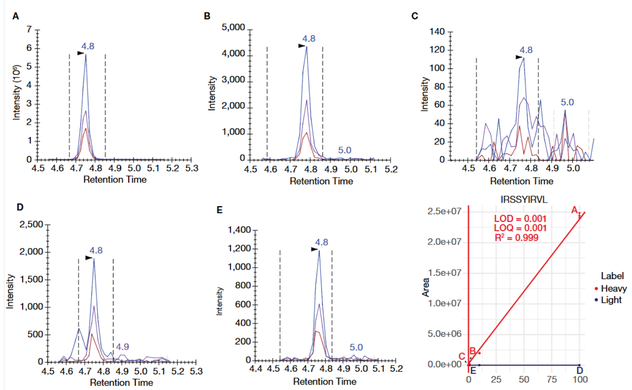


Figure 4. Example dilution curve. Heavy (A, B, C at the respective spike in concentration: 100 fmol, 0.1 fmol, and 1 amol) and light peptides (D and E) are shown across heavy peptide dilution series.

Utilization of MS³ to reduce noise and increase sensitivity

Key Takeaway
Utilize MS³ to increase peptide detection

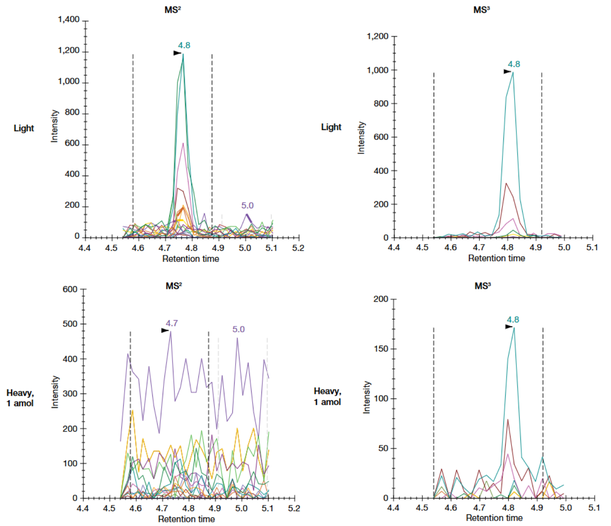
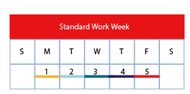


Figure 5. Example peptide for MS² and MS³

Comparative product ion XIC analysis showed that tMS3 reduced matrix interference compared to tMS2, improving peptide limit of quantitation (LOQ) (Figure 5).

Efficiency of targeted proteomics method development



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

Figure 6. Targeted proteomics workflow steps achieved within the standard work week

Method developed in one week (Figure 6), eliminating transition selection speeds development by 11x (Figure 7).

Key Takeaway
>11x time savings when compared to QQQ

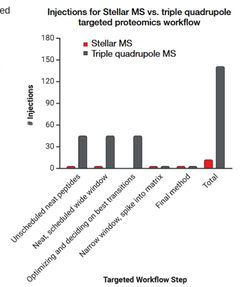
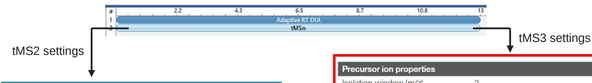


Figure 7. Number of injections comparison for targeted method setup between Stellar MS and triple quadrupole mass spectrometer

Method settings



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

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

Conclusions

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

- **1 attamole 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.
- **tMS3 enhancements in quantitation and peptide confirmation:** Utilizing tMS3 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.

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