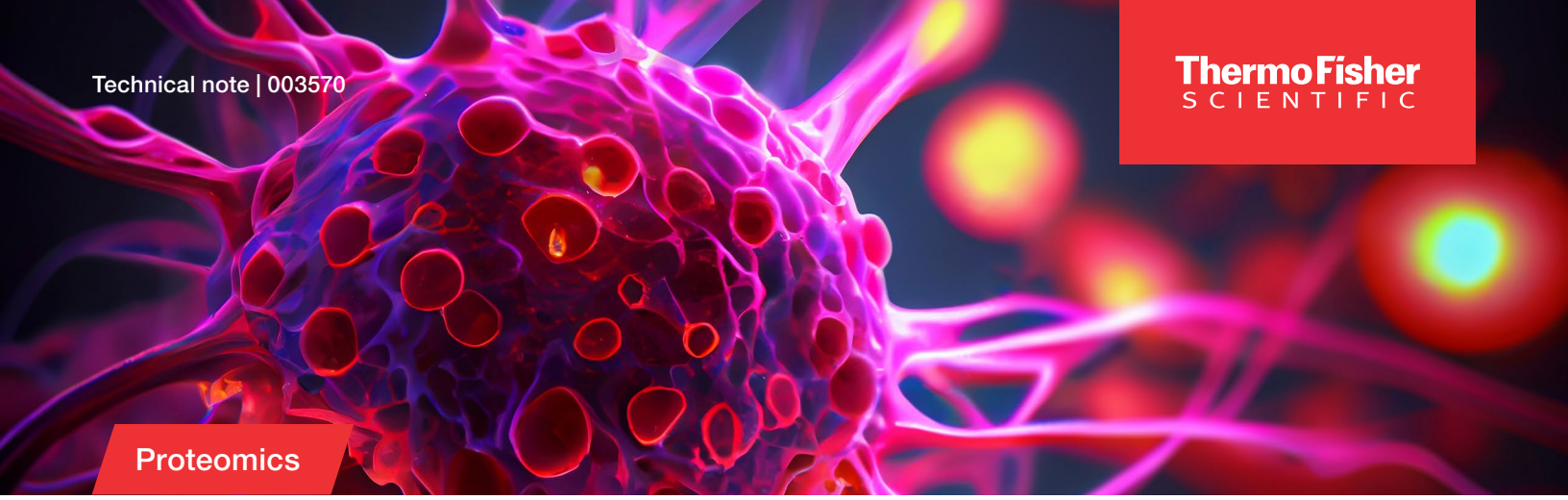

Technical note | 003570

Proteomics

Unprecedented depth and data quality in immunopeptidome profiling with the Orbitrap Astral mass spectrometer

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Keywords

Immunopeptidomics, Orbitrap Astral MS, FAIMS, Vanquish Neo UHPLC system, MHC Class I peptides

Goal

To assess depth and quality of immunopeptidome coverage using data-dependent acquisition (DDA) on the Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer.

Introduction

Immunopeptidomics is a rapidly evolving field that encompasses the identification and characterization of peptides presented by major histocompatibility complex (MHC) molecules on the surface of cells. These MHC-peptide complexes play a crucial role in the immune system by presenting peptide antigens to T cells, thereby initiating immune responses against foreign pathogens or abnormal cells. This field has drawn interest for many reasons, notably because the ability to identify tumor-specific antigens (TSAs) can revolutionize personalized medicine. Mass spectrometry (MS)-based techniques are the sole technology capable of directly identifying and quantifying peptides presented on the cell's surface. By coupling MS with liquid chromatography (LC), researchers can detect and sequence a wide range of MHC-peptides. This technology enables the identification of both abundant and rare peptides, offering a comprehensive view of the immunopeptidome. However, identifying TSAs from biological samples is analytically challenging because these peptides are often of low abundance. Immunopeptides also lack standard enzymatic cleavage sites, which complicates database searching, and often must be enriched from limited and precious tumor biopsy samples. Recent advances enabled by the Orbitrap Astral MS have allowed unprecedented depth of coverage and accuracy required to take the field to the next level. Here, we describe the setup and method for analysis of MHC Class I peptides using the Thermo Scientific™ Vanquish™ Neo UHPLC system and the Orbitrap Astral MS.

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Experimental

Recommended consumables

- Fisher Scientific™ LC-MS grade water with 0.1% formic acid (FA) (P/N LS118-500)
- Fisher Scientific™ LC-MS grade 80% acetonitrile (ACN) with 0.1% formic acid (P/N LS122500)
- Fisher Scientific™ LC-MS grade formic acid (P/N A117-50)
- Fisher Scientific™ Optima™ LC-MS grade water (P/N 10505904)
- Fisher Scientific™ Optima™ LC-MS acetonitrile (P/N A955-1)
- Fisher Scientific™ Optima™ LC-MS isopropanol (P/N A461-212)

LC columns

- IonOpticks™ Aurora Ultimate™ XT 25 cm × 75 µm C18 column (AUR3-25075C18-XT)
- Thermo Scientific™ PepMap™ Neo Trap Cartridge (P/N 174500)
- Thermo Scientific™ PepMap™ Neo Trap Cartridge Holder (P/N 174502)

UHPLC system

- Vanquish Neo Pump/Autosampler (P/N VN-S10-A-01)
- Vanquish Column Compartment (P/N VN-C10-A-01)

Mass spectrometer

- Orbitrap Astral MS
- Thermo Scientific™ FAIMS Pro Duo interface
- Thermo Scientific™ EASY-Spray™ ion source

Data analysis

PEAKS™ Studio 12 software

HCT-116 samples

Class I MHC peptides were obtained by immunocapture with a pan-specific MHC class I antibody, W6/32-conjugated resin on 100 million HCT-116 cells. After cleanup on stage tips, the starting material was diluted 100x with 0.1% formic acid. Samples were diluted to represent the equivalent of E+5 (100,000), E+6 (1,000,000), and E+7 (10,000,000) cells of extracted immunopeptides.

HeLa samples

Cells were lysed in a buffer consisting of 0.1 M Tris–HCl, pH 8.0, 0.1 M DTT, and 2% SDS at 99°C for 10 min. Lysate were loaded on 10 kDa MWCO centrifugal filters following FASP protocol[1] for protein digestion with elastase.

LC conditions

The Vanquish Neo UHPLC system was configured to run a trap-and-elute workflow.

The UHPLC conditions are described in Table 1, with the gradient details in Table 2.

Table 1. UHPLC conditions

UHPLC method parameters	
Mobile phase A	0.1% formic acid (FA) in water
Mobile phase B	0.1% FA in 80% acetonitrile (ACN)
Flow rate	0.2 µL/min
Column	Aurora Ultimate XT 25 cm
Colum temperature	50 °C
Autosampler temperature	7 °C
Injection wash solvents	Strong wash: 0.1% FA in 80% ACN Weak wash: 0.1% FA in water
Needle wash	Enabled after-draw
Trap-elute operation	Backward flush mode

Table 2. LC method gradient

Time (min)	Duration (min)	%B	Flow rate (µL/min)
Run			
0.0	0.0	2.0	0.5
1.0	1.0	5.0	0.5
1.1	0.1	5.0	0.2
61.1	60.0	35.0	0.2
Column wash			
63.1	2.0	70.0	0.2
67.1	4.0	99.0	0.2
67.2	0.1	99.0	0.5
72.0	4.8	99.0	0.5
Stop run			
Column equilibration			

MS parameters

The Orbitrap Astral MS was operated in DDA mode using one or multiple field asymmetric ion mobility spectrometry (FAIMS) compensation voltages (CVs). Each MS experiment utilizes one FAIMS CV (Figure 1). Spectrum filters shown in Figure 2 were used for each MS experiment. Settings for full scan, spectrum filters, and data-dependent MS^2 are described in Table 3.

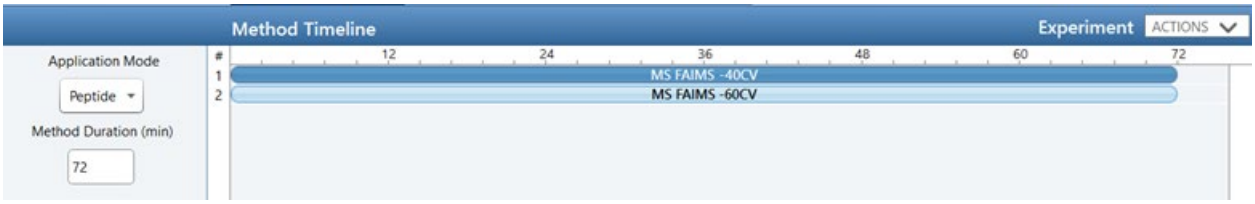

Figure 1. Method editor: one MS experiment for each FAIMS CV

Table 3. MS parameters

Source parameters	
Spray voltage (kV)	2.0
Capillary temperature (°C)	280
FAIMS CV	Single CVs: -80 to -10 (for optimization) or double CVs: -60 and -50 (optimized for HCT-116)
FAIMS mode	Standard resolution
Total carrier gas flow (L/min)	3.5
Orbitrap MS full scan parameters	
Resolution	120,000
Scan range (*m/z*)	350–800
RF lens (%)	45
AGC target (%)	300
Maximum injection time (ms)	100
MIPS properties	
Monoisotopic peak determination	Peptide
Charge state	
Include charge states	2–4 or 1–4 (for optimization Figure 3)
Dynamic exclusion	
Exclude after n times	1
Exclusion duration (s)	15
Mass tolerance (ppm)	Low: 5 High: 5
Exclude isotopes	Yes
Perform dependent scan on single charge state per precursor only	Yes
Astral MS² scan parameters	
Isolation window (*m/z*)	1 *m/z* – for >E+6 load 1.2 *m/z* – for E+5 load
HCD collision energy (%)	29
Scan range (*m/z*)	110–1,500
Normalized AGC target (%)	30
Maximum injection time (ms)	100
Microscans	1
Data dependent properties	
Data dependent mode	Cycle time
Time between master scans (s)	0.6

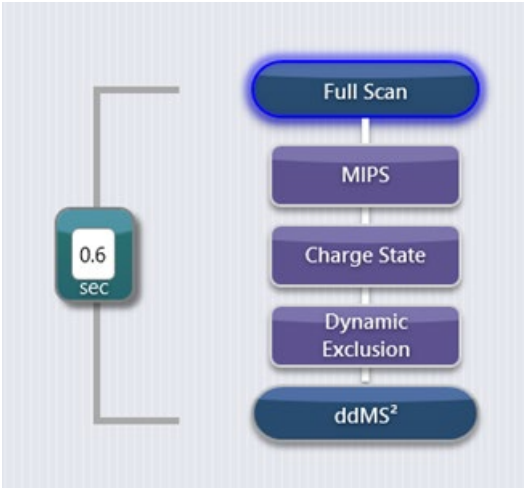

Figure 2. Method structure of each MS experiment

Data processing parameters

The data analysis was performed using PEAKS 12 Studio software with the DeepNovo Peptidome workflow for database search and de novo peptides identification. Spectra were searched against the UniProt human database (20,607 sequences) with the no-enzyme option. Variable modifications: oxidation (M) and carbamidomethylation (C). Precursor mass error tolerance was set to 10 ppm and fragment error tolerance to 0.02 Da. FDR control at peptide level was set to 1% and DeepNovo score was ≥70%.

Results and discussion

FAIMS optimization

Gas phase separation of ions using FAIMS can significantly enhance sensitivity by increasing the signal-to-noise ratio (S/N), which is particularly important for low input samples such as immunopeptidome samples.[2] Also, FAIMS enhances selectivity and specificity by separating ions in the gas phase, improving analysis depth. An additional benefit of FAIMS is the reduction of contaminants entering the instrument, which increases time between cleanings even when running complex tissue samples that may contain polymers, intact proteins, and a variety of small molecule contaminants.

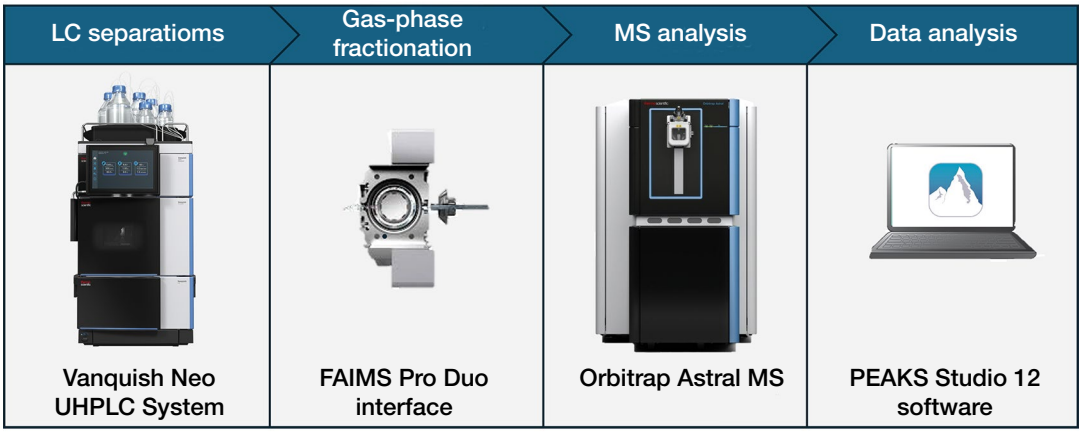

Figure 3. Immunopeptidomics workflow on the Orbitrap Astral MS

In this study, we demonstrate how FAIMS CV can be optimized to improve the identification of immunopeptides. Various methods were developed using single FAIMS CVs ranging from -80 to -10 to illustrate how the choice of FAIMS CV can impact the results. Figure 4 presents peptide identification for each FAIMS CV method using MHC-class I derived peptides and HeLa non-tryptic peptides obtained from elastase digestion. For the MHC sample, FAIMS CVs of -60 and -50 resulted in the highest numbers of peptides identified, together accounting for 67% of the total peptides combined from different injections (Figure 4A), with only 22% of peptides detected in both CVs (Figure 4B). In contrast, CVs of -50 and -40 identified the highest number of unique HeLa peptides (Figure 4D). This indicates that FAIMS can be optimized based on the sample composition/type. The distribution of different ion populations separated and analyzed varies with the FAIMS CV, influencing the charge state distribution (Figure 4C). Higher FAIMS CVs (-30 to -10) result in higher proportions of +1 ions, while lower CVs (< -40) yield higher proportions of multiply charged ions, especially +2. This data underscores the importance of optimizing FAIMS CVs to enhance peptide identification based on the specific characteristics of the sample.

Another round of injections was conducted combining different FAIMS CVs to demonstrate the effect on the peptidome coverage. Figure 5 illustrates the number of peptides identified using different FAIMS CV combinations and no FAIMS. The combination of -60 and -50 FAIMS CVs resulted in the highest number of unique peptides detected, with 9,340 peptides. This finding highlights the potential of utilizing optimized FAIMS CV combinations to enhance the depth of peptidome analysis and improve the overall identification of unique peptides. If time and sample permits, we recommend testing a range of CVs on a variety of representative samples to select the best combination of CVs for that particular sample type. However, if this is not feasible, we have found that -40 and -60 CVs give high coverage for a wide range of immunopeptidomics samples.

Isolation window optimization

The isolation window on the quadrupole can significantly affect peptide-spectrum match (PSM) rates depending on the sample loading. Narrower isolation windows reduce spectral complexity and chemical noise, which results in higher PSM rates. However, very narrow (<1.5 Th) isolation windows can reduce ion transmission, which may hurt performance when the sample is very limited. Figure 6 demonstrates how PSM rates are influenced by small changes in the isolation window. Generally, for optimized results, the higher the sample load, the narrower the isolation window should be.

While the number of peptides identified is only slightly impacted by changes in the isolation window, the PSM:MS^2 ratio can experience a notable reduction (Figure 6). For high sample loads (E+7), the PSM:MS^2 ratio dropped from 48% to 31% (Figure 6). This highlights the importance of carefully selecting the isolation window based on the sample load to maximize the efficiency and accuracy of peptide identification.

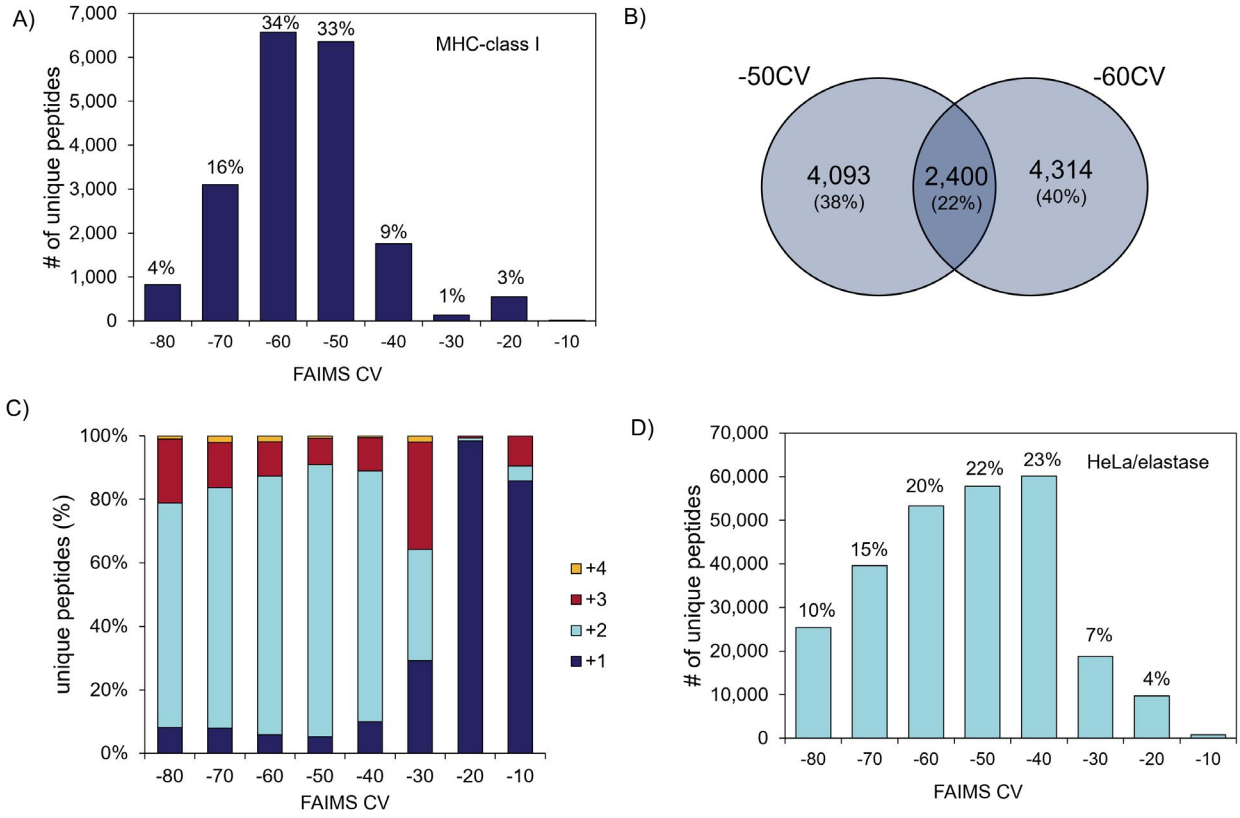

Figure 4. Optimization of FAIMS CVs for immunopeptidome analysis. (A) MHC-class I unique peptides derived from HCT-116 cells identified using E+6 cell equivalent injections; (B) Venn diagram of peptides identified from E+6 injections of MHC-class I sample using -50 and -60 FAIMS CVs; (C) charge state distribution of each injection using a different FAIMS CV; and (D) 50 ng peptide injections from HeLa proteins digested with elastase. Percentages (%) represent the portion of peptides identified on a specific FAIMS CV from the total number of peptides considering all FAIMS CVs.

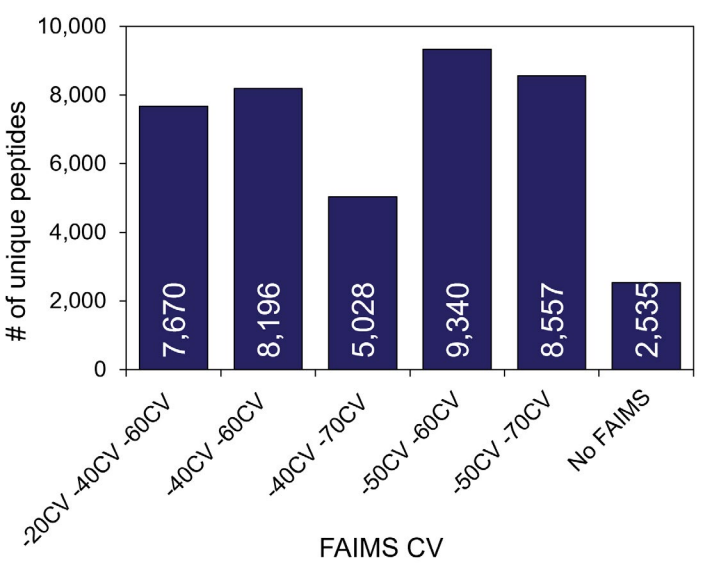

Figure 5. Combination of multiple FAIMS CVs to maximize the immunopeptidome coverage. This chart represents the number of peptides identified (y-axis) using triple FAIMS CVs (-20, -40 and -60) or double FAIMS CVs combinations (x-axis) using E+6 cell equivalent injections. The combination of two CVs (-40/-60 or -50/-60) is usually enough for deep coverage of a wide variety of immunopeptidomic samples. Here, for MHC-I peptidome from HCT116 cells, the highest coverage is obtained with the combination of –50 and –60 CVs.

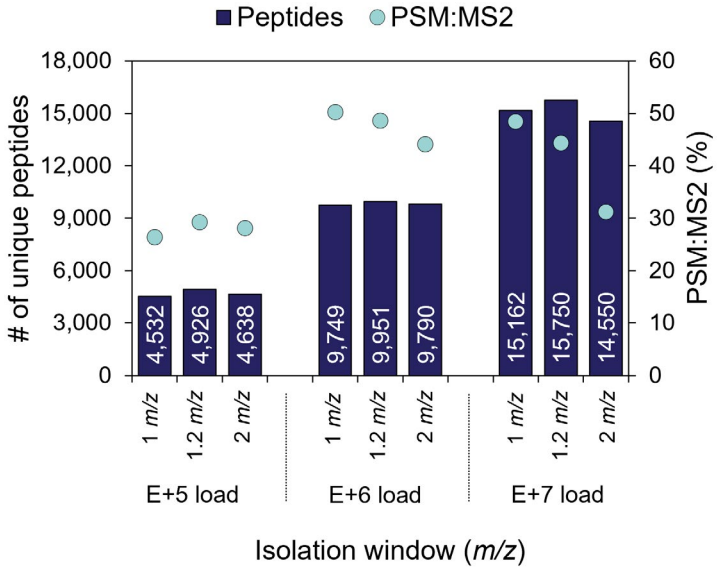

Figure 6. Optimization of quadrupole isolation window (1 *m/z*, 1.2 *m/z*, and 2 *m/z*) in DDA mode for different sample loading (E+5, E+6, and E+7 cell equivalent) to enhance PSM success rate (PSM:MS², %). Dark blue bars represent the number of unique peptides identified and light blue dots represent the PSM:MS².

Using optimized methods for each loading, the samples were analyzed in triplicates, with the results shown in Figure 7. All samples were analyzed with two FAIMS CVs (-60 and -50), and the isolation window was adjusted according to the sample loading (1.2 *m/z* for E+5 loads and 1 *m/z* for E+6 and E+7 loads).

As anticipated, the peptide identification increased with higher sample loading (Figure 7). On average (n=3), 5,299, 9,867, and 15,501 peptides were identified from E+5, E+6, and E+7 cell equivalent injections, respectively (Figure 7A). This progressive increase in the number of unique peptides identified indicates that the LC-MS methods were effective for the sample loading range used in Figure 7A.

An important characteristic of the immunopeptidome is the specificity of peptide length. Most Class I peptides range from 8 to 14 amino acids in length.[3] Figure 7B shows that most peptides detected in all loadings fall within the 8–9 amino acid range, which is consistent with expectations. This observation aligns with the known characteristics of Class I peptides, further validating the effectiveness of the optimized methods in accurately identifying immunopeptides.

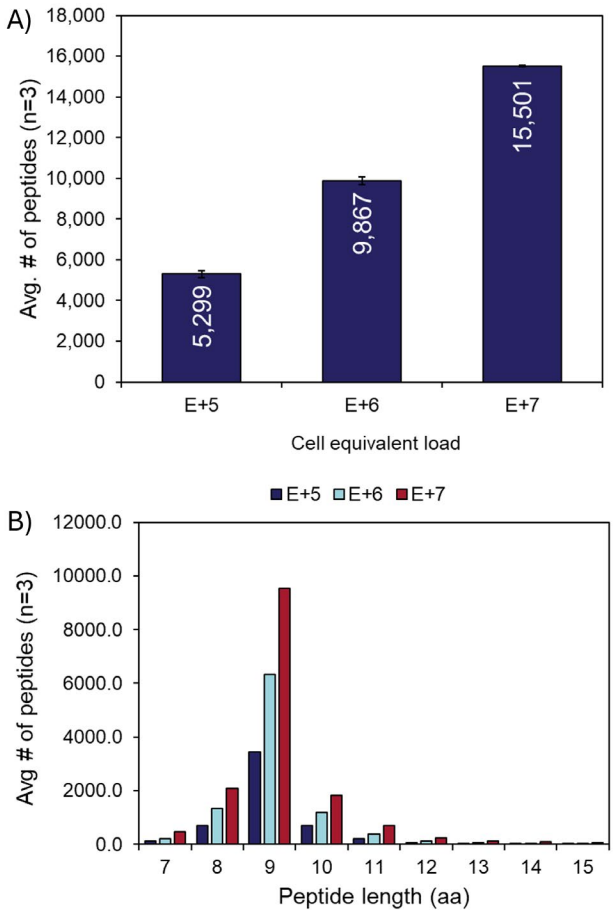

Figure 7. Optimized method for immunopeptidome analysis of MHC-class I peptides derived from HCT 116 cells at different loads. (A) Average number of identified peptides (n=3), (B) peptide length distribution.

The abundance of immunopeptides can vary significantly, ranging from a single copy to over 10,000 copies per cell.[4] This underscores the necessity for MS instrumentation with high sensitivity and a wide dynamic range. By employing the workflow described herein, the observed dynamic range extended up to seven orders of magnitude (Figure 8).

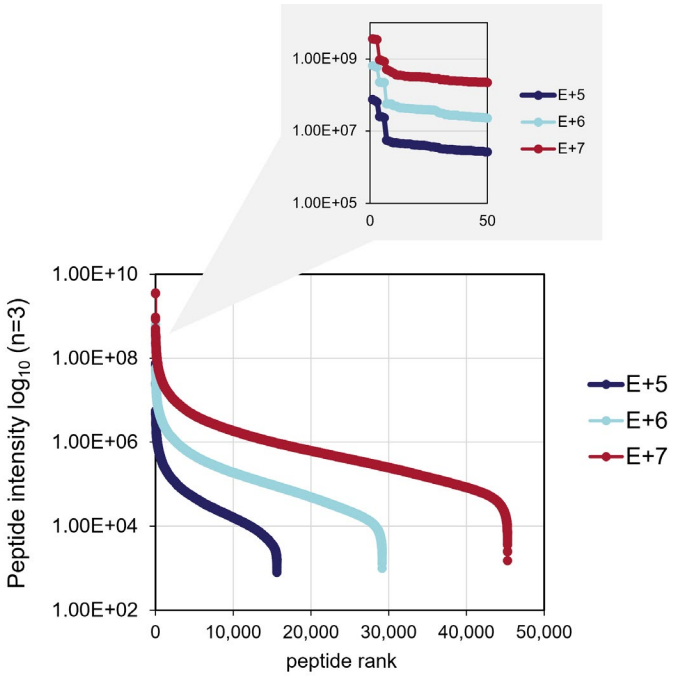

Figure 8. Dynamic range spans up to seven orders of magnitude depending on the amount of sample injected.

While the number of peptides that can be identified is an important high-level metric, a large number of peptides identified means very little if the spectral quality is too low to confidently assign the complete sequence. Therefore, manual viewing of individual spectra is an important step in method evaluation to ensure that the data quality is up to standard. As an example, we randomly selected a moderately abundant peptide that was identified across all three loading levels to highlight the spectral quality of the peptide IDs. To this end, annotated spectrum charts of the same peptide at different sample loadings are shown in Figure 9. Regardless of the loading, spectral quality remained consistent across all loads, demonstrating quantitative linearity at the spectral level. This consistency and quality are enabled by automatic gain control (AGC), which automatically adjusts the amount of time the instrument fills to ensure the same number of ions is measured per spectrum regardless of loading level. In this way, AGC ensures that high quality spectra are acquired across a wide inter- and intra-sample dynamic range without any manual optimization.

Additionally, the peak areas of the SADPGNLKY peptide spanned a linear range from 1.66E+4 to 1.57E+6 (Figure 10), illustrating the method's robustness and its ability to maintain high spectral quality and accurate quantification across varying sample amounts. As shown in Figure 9, the Orbitrap Astral MS can provide extended spectra information below 200 *m/z* allowing detection of small fragments and/or immonium ions that might be critical for identity confirmation of immunopeptides.

Another easy way to assess spectral quality is to examine the quality of matching scores provided by the search engines. Figure 11 shows the distribution of -10LgP and CAA (confident amino acid) scores provided by PEAKS. The distribution shows high quality matching of peptides detected.

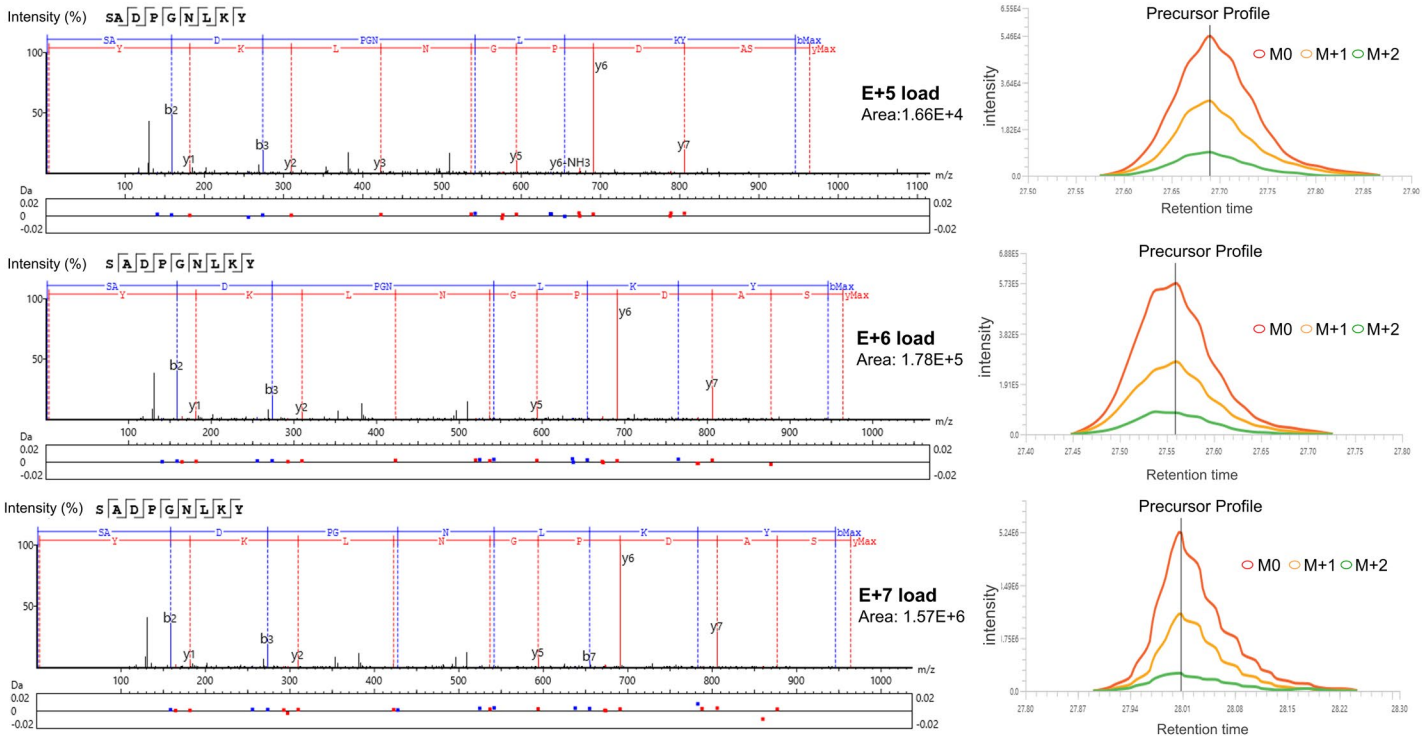

Figure 9. Spectral quality is maintained from ultra-low to higher loads. On the left, annotated spectrum charts of the same peptide (SADPGNLKY) in different loadings. From top to bottom: E+5, E+6, and E+7 cell equivalent injections. On the right, the precursor profile of the same peptide is represented for all three loads.

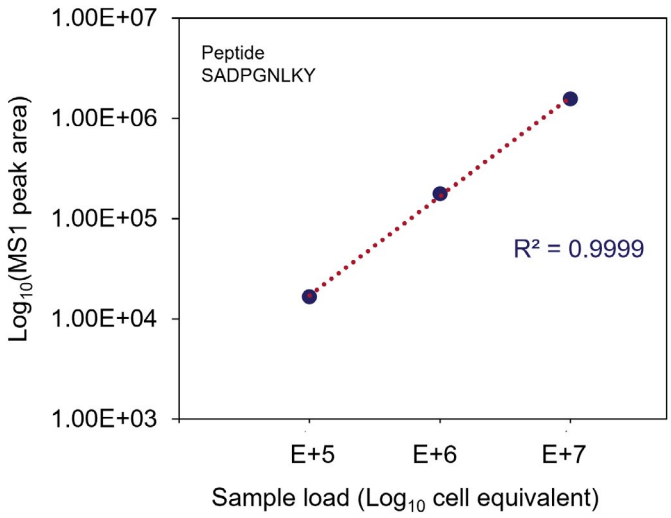

Figure 10. Linearity of MS¹ peak area of peptide across all three orders of magnitude measured. The peak area of SADPGNLKY peptide across E+5, E+6, and E+7 cell equivalent loadings.

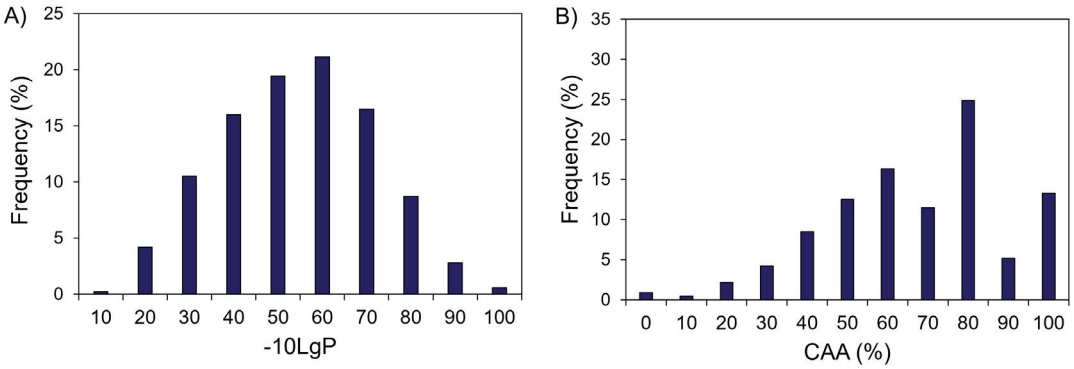

Figure 11. Spectral quality measured by search engine. Distribution of quality matching scores from PEAKS Studio 12 (DeepNovo peptidome workflow). 10LogP score indicates the scoring significance of a peptide-spectrum-match (A). CAA (%) represents the matching quality between a de novo sequence tag and a database peptide (B).

Summary

- An Orbitrap Astral MS equipped with a FAIMS Pro Duo interface and coupled to a Vanquish Neo UHPLC system provides high sensitivity and the dynamic range necessary for deeper coverage of immunopeptidomics samples.
- The high sensitivity of this workflow allows compatibility with low levels of material equivalent to samples extracted from tissue biopsy samples.
- FAIMS enables expanded dynamic range of analysis for sample characterization with the flexibility to customize acquisition settings to enrich target ion populations.
- The versatility of the Orbitrap Astral MS enables extensive optimization of many method parameters. Isolation window width is an example of a parameter that can be optimized to maximize coverage. Notably, all isolation widths tested yielded good coverage for all loads, suggesting that a single method generally can be used to accommodate a wide variety of samples and loading levels.
- AGC modulates injection times for each peptide, producing high quality spectra across a wide range of loading levels. This spectral quality is crucial for downstream analysis and confident identification of novel TSAs.

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