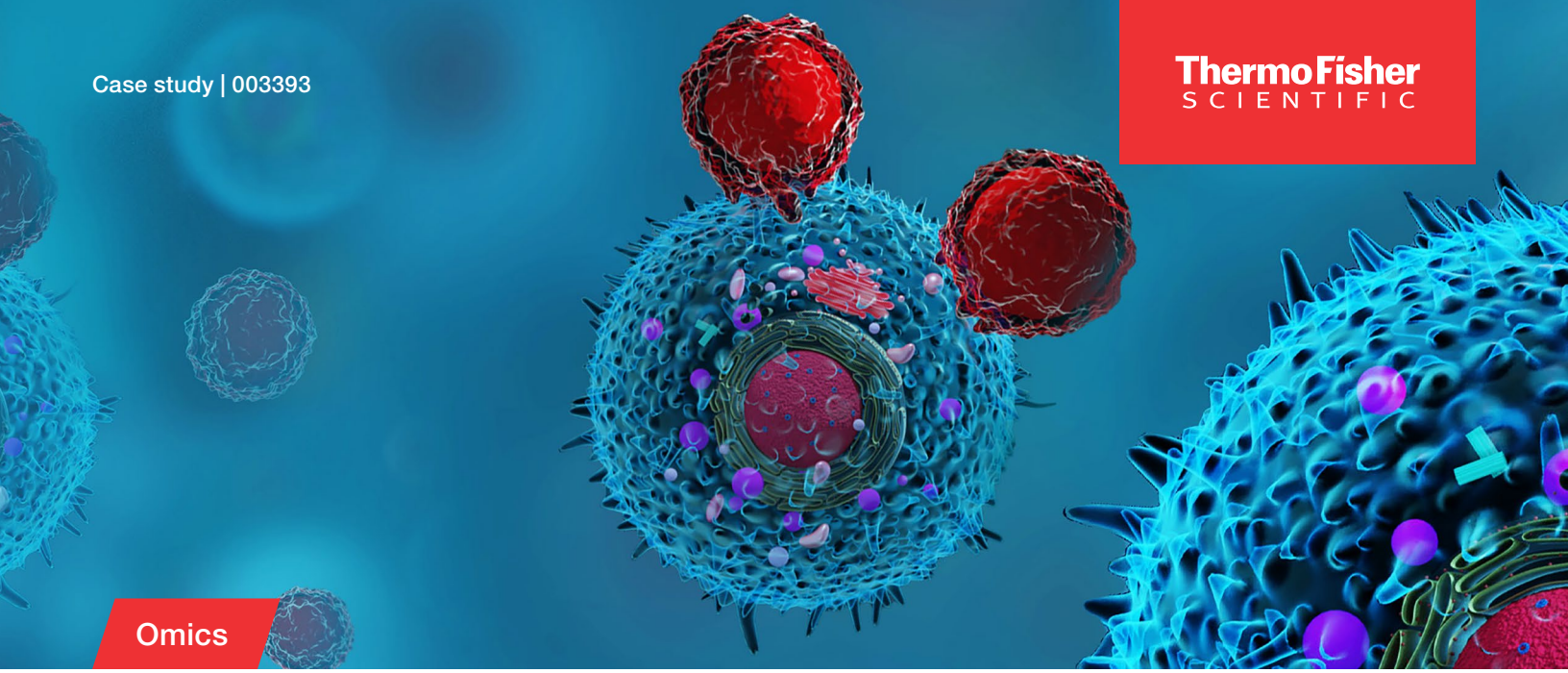

Case study | 003393

Omics

Immunopeptidomics: Harnessing cutting-edge technology for unprecedented depth and accessibility in analysis to leverage the immune response for precision therapy

Introduction

Immunopeptidomics is the study of the peptides presented by major histocompatibility complex (MHC) molecules on the surface of cells. These MHC peptides have major implications for many areas of research, including immunotherapy and personalized medicine. For example, many studies in this field aim to identify low-level tumor specific antigens (TSAs) with the goal of developing personalized immunotherapies to target cancerous cells with a high degree of specificity. Mass spectrometry (MS) allows for direct immunopeptidomics analysis, enabling simultaneous identification and quantification of thousands of MHC peptides in a single run. The recently developed Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer has enabled new levels of sensitivity and selectivity to provide deeper insights into the immunopeptidome. In this study, we utilized the Orbitrap Astral MS to characterize the immunopeptidome extracted from IM-9 human Multiple Myeloma B-lymphocyte cells to support the detection and annotation of potential neoantigens.

Rethink what is possible

The Orbitrap Astral MS sets a new standard for high-sensitivity, direct measurements of MHC Class I and Class II immunopeptides extracted from cell culture or tissues, achieving deeper coverage from less material at higher throughput. The Orbitrap Astral MS redefines performance and discovery by integrating three mass analyzers: a quadrupole mass analyzer for high selectivity and efficient ion transmission, an Orbitrap mass analyzer for a wide dynamic range and high-resolution measurements, and a new Thermo Scientific™ Astral™ analyzer designed for rapid and sensitive measurements with seamless synchronization of ion transfer and processing across the instrument. This synchronization, coupled with the parallel handling of five separate ion packets simultaneously, allows each mass analyzer to be utilized concurrently for optimal performance. The Orbitrap Astral MS significantly expands experimental capabilities, offering up to four times faster throughput, up to twice the depth of proteome coverage, and enhanced sensitivity with accurate and precise quantitation.

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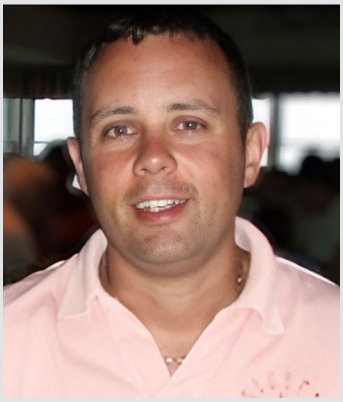
An interview with Dr. Robert Salzler, Regeneron Pharmaceuticals



Robert Salzler, Senior Principal Scientist and Immunopeptidomics Lead in the Department of Therapeutic Proteins at Regeneron Pharmaceuticals Inc., Tarrytown, New York.

REGENERON
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Q: Can you describe your role at Regeneron?

A: My role is lead of the immunopeptidomics team in the department of Therapeutic Proteins. We work closely with departments at Regeneron working on oncology, infectious disease, autoimmunity, metabolism, allergies, aging, and ophthalmology.

Q: What is your research focus and what type of information are you looking for when studying the immunopeptidome?

A: Our research focus has been mainly oncology, but we are moving more in the direction of infectious diseases and autoimmunity. We are interested in HLA-Class I, HLA-Class II, as well as HLA-E associated targets. We are exploring TCR mimic, TCR and CAR-T-cell, and vaccine approaches as therapeutic approaches associated with these diseases.

Q: What are the main challenges with your work?

A: The biggest challenge is sample size, since we need to sequence peptides in the groove of HLA-molecules. Mass spectrometry is really the only tool to map the immunopeptidome of a tissue, and often tissue size is limiting. The ultimate goal would be to perform immunopeptidomics on biopsy size tissues on an out-patient basis without the need for surgery to determine the best form of targeted therapy.

Q: Can you share how newly developed analytical tools like the Orbitrap Astral mass spectrometer would help address these challenges?

A: The increased sensitivity of the Orbitrap Astral MS allows us to get closer to the goal of immunopepdome mapping from biopsy-size tissues. Currently, we often need a chunk of frozen tissue around 500 mg in order to have a robust immunopeptidome on our current Thermo Scientific™ Orbitrap™ instruments with FAIMS. With the Orbitrap Astral MS, we will be able to get the same results with just 25 mg of tissue—getting us into that biopsy range!

Q: Could you share any opinions you have on future directions or discoveries in your work that will have an impact?

A: One of our goals would be to have off-the-shelf immunotherapies, which could treat the patient as soon as we knew which antigens in the context of HLA were being presented on the surface. With our current workflow, we would be able to determine the correct antigens to target within 3 days. To combat a tumor indication, we may have to have multiple hits on goal since tumors are heterogenous. It could be a combination of TCR mimics, Car-T or TCR therapeutics, as well as a vaccine approach to help limit reoccurrence. Also, with a biopsy-like method, as soon as we see the current therapy may be failing, we could re-biopsy and see how the immunopeptidome has changed with treatment and design a new course of therapy to get ahead of the tumor.

Exemplary results

Sample preparation

HLA-associated peptides, also known as immunopeptides (IMP), were enriched from 5e8 cells of IM-9 human B-lymphocyte. The AssayMAP™ Bravo automated system was used after lysis with NP-40 lysis buffer. HLA-peptide complexes were captured using Pan-HLA Class I and Class II antibodies (W6/32 for Class I, IVA12 for Class II). Glycine-eluted complexes were further fractioned on a Sep-Pak™ C18 cartridge using 30% acetonitrile to harvest peptides, leaving proteins captured to the C18. Samples were diluted to represent the equivalent of 5e5 to 1e7 cells of extracted IMP.

Test method(s)

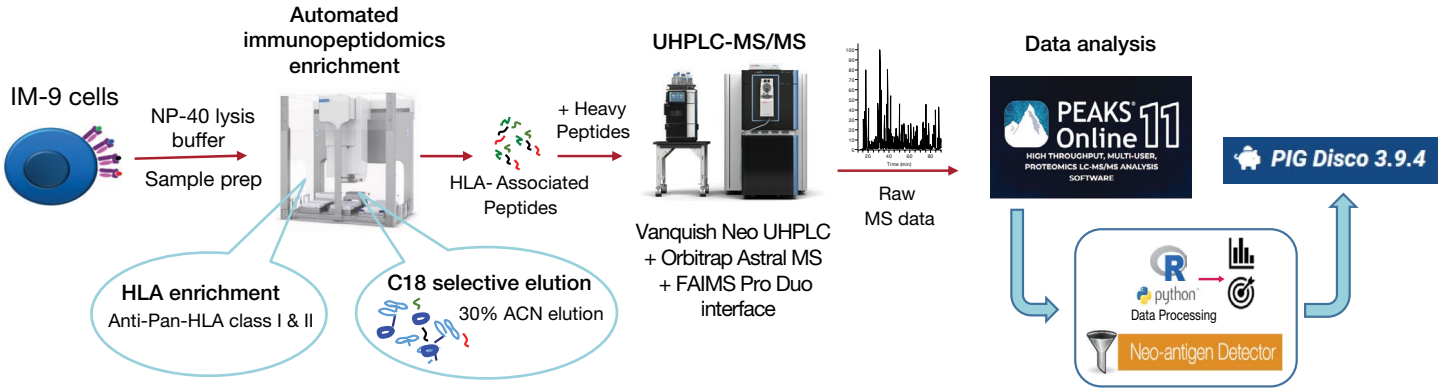
IMP extracts were analyzed using data-dependent acquisition (DDA) LC-MS/MS analysis on an Orbitrap Astral MS interfaced with a Thermo Scientific™ Vanquish™ Neo UHPLC System and Thermo Scientific™ FAIMS Pro Duo interface. IMP were separated on a 25 cm x 75 µm C18 column packed with 1.7 µm particles via reversed phase chromatography with variable gradient separations at 200 nL/min (Figure 1). DDA acquisition methods were used with ion cloud separations enabled by field asymmetric ion mobility (FAIMS). Three FAIMS compensation voltages (CV) (-25, -50, and –70 V) were employed for HLA Class I peptides, and two FAIMS CV (-40 and -60 V) for HLA Class II peptides analysis. Full MS scans were acquired with Orbitrap detection. MS/MS scans were acquired with Astral detection.

Data analysis

The data analysis was performed using PEAKS™ Studio software (ver. 11.5) for database search and de novo peptides identification. Spectra were searched against the UniProt™ human database (20,607 sequences) with the no-enzyme option. Data were searched in parallel for both DB search and De Novo search. The sequence motif and binding properties of 9-mer peptides were analyzed using MHCMotifDecon 1.0 and NetMHCpan 4.0.

Results

MHC class I peptides isolated from IM-9 cells were analyzed at different cell line equivalent dilutions to produce identification of up to 40,551 peptides with 8–12 amino acid (AA) and 26,745 9 AA length from 1e7 cells equivalent input (Figure 2).



IM-9	HLA-A	HLA-B	HLA-C
Allele 1	*02:01	*49:01	*01:02
Allele 2	*02:05	*56:01	*07:01

Figure 1. Overview of analytical workflow for this study, depicting immunopeptide extraction and nano-UHPLC-FAIMS-MS/MS analysis

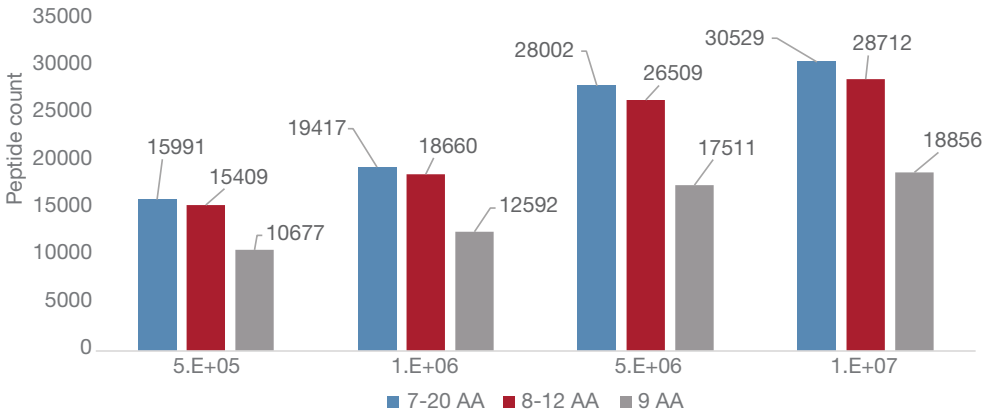


Figure 2. IM-9 HLA Class I peptides annotated from 5e5 to 1e7 cell line equivalent loads separated over 60-minute gradient. Outputs generated with DeepNovo Workflow from PEAKS software.

The number of 9-mer peptides constituted ≈65% of total class I peptides identified (Figures 3 and 4). The Orbitrap Astral mass spectrometer with FAIMS Pro Duo interface enabled detection of HLA peptides of different charge states (Figure 4).

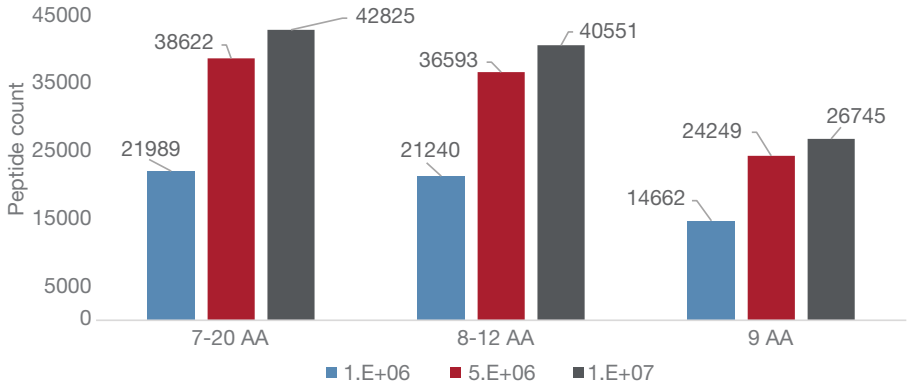


Figure 3. IM-9 HLA Class I peptides annotated from 1e6 to 1e7 cell line equivalent loads separated over 120-minute gradient. Outputs generated with DeepNovo Workflow from PEAKS software.

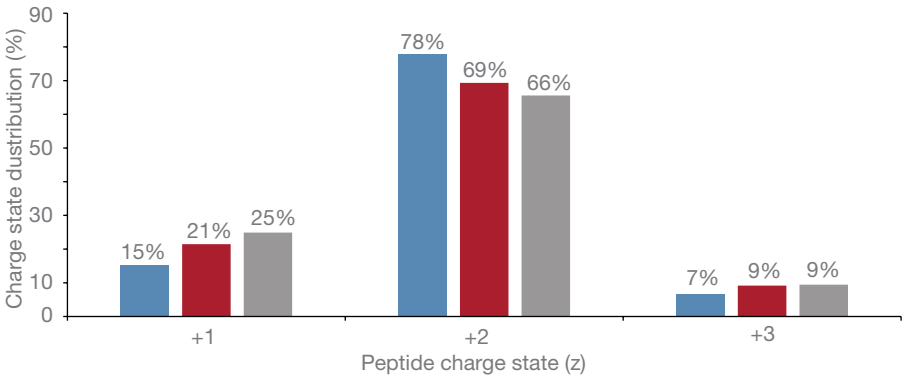


Figure 4. Relative charge distribution of annotated IM-9 Class I HLA peptides from 5e6 and 1e7 cell line equivalent loads separated over 60-minute gradient. Outputs generated with DeepNovo Workflow from PEAKS software.

The resulting data were analyzed for sequence motifs, and the results were consistent with known HLA alleles (Figure 5).

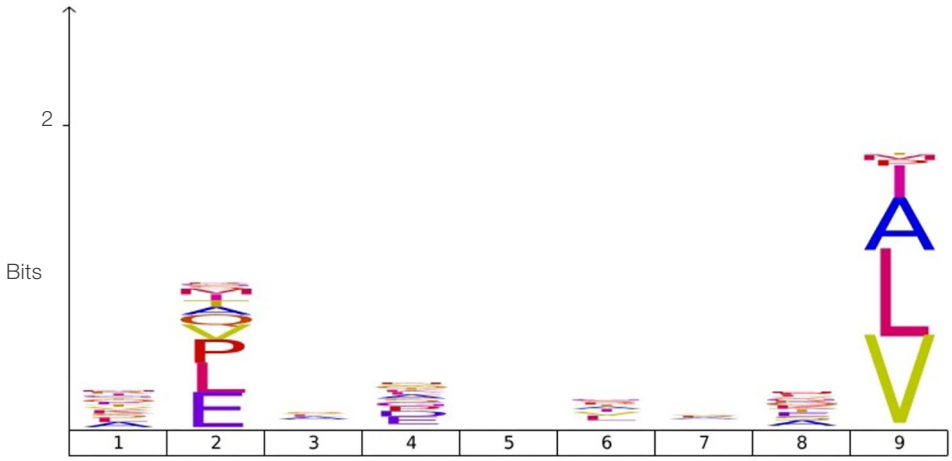


Figure 5. Sequence logo motif detected for 9-mer (9 amino acid length) peptides

The depth and accuracy of these results were enabled by features of the Orbitrap Astral MS that are ideally suited for immunopeptidome analysis. Specifically, the high dynamic range MS1 survey scan enhances acquisition sensitivity (Figure 6).

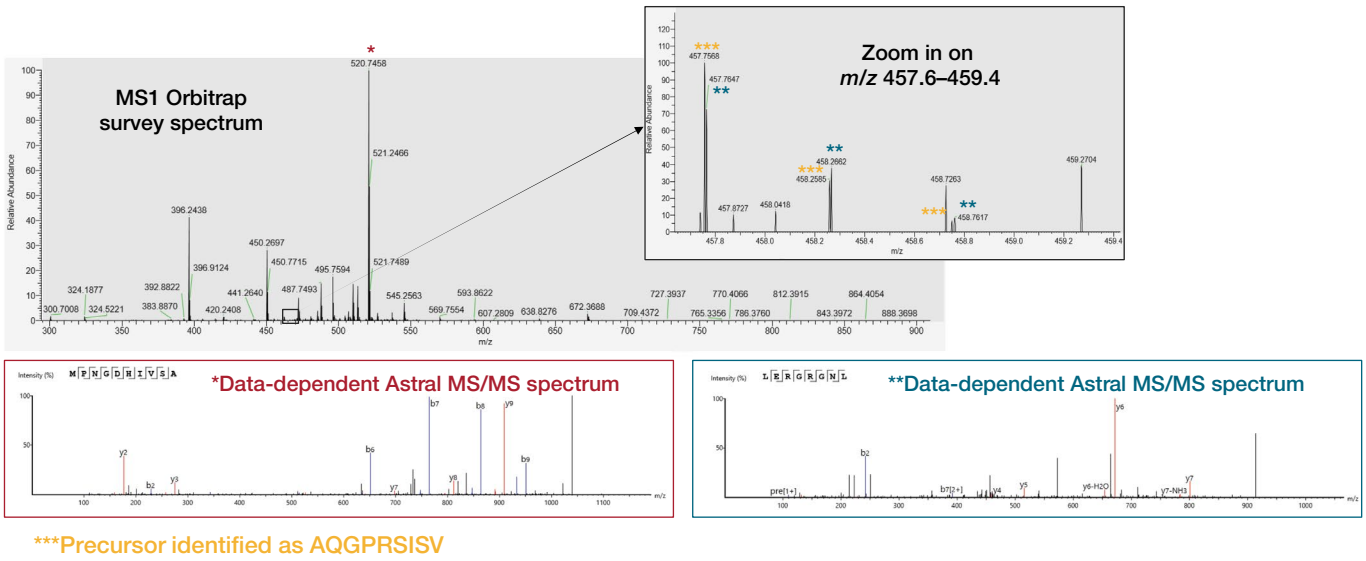


Figure 6. Example of spectral quality of the Orbitrap Astral mass spectrometer MS/MS scans to demonstrate low abundance precursor isolation from high dynamic range Orbitrap Full scans for HLA Class I peptide annotations

Confident sequence assignment was achieved because the Orbitrap Astral MS acquires high quality MS/MS spectra across the complete ion series (Figure 7) with very high mass accuracy (Figure 8). Approximately 70% of 9-mers from the 5e6 cell sample input data were predicted binders to at least one of the representative HLA supertypes, with the largest proportion predicted to bind HLA-A02.

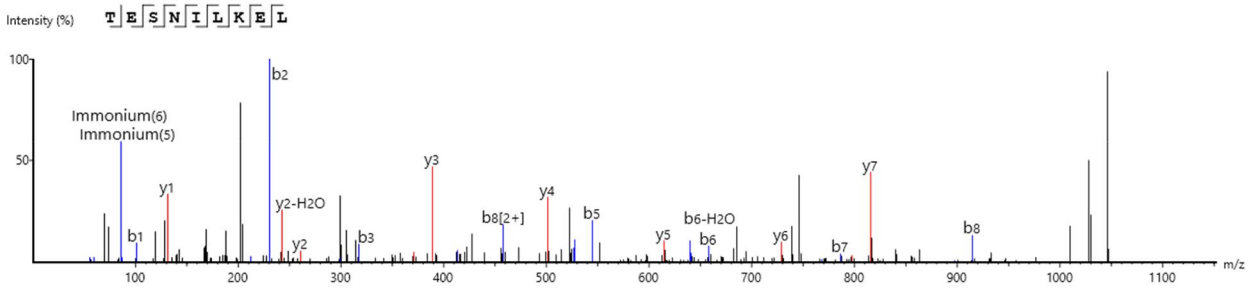


Figure 7. Annotated MS/MS spectrum of HLA Class I peptide, demonstrating full mass range of detection to capture diagnostic immonium ions, b1 and y1 ions, valuable for de novo sequencing annotation

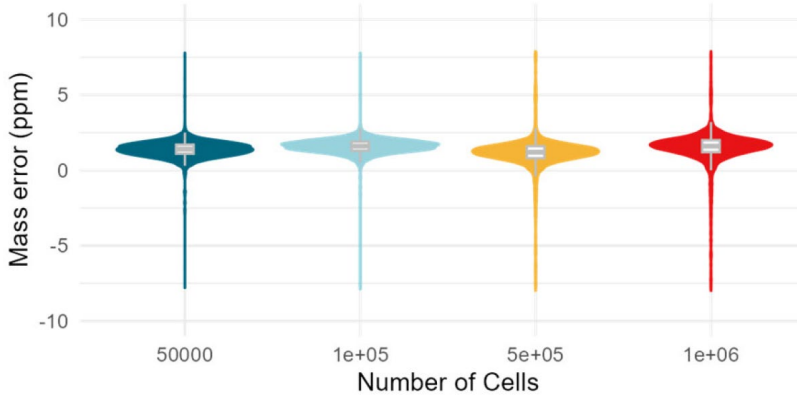


Figure 8. Plot of accurate mass measurement for precursors of all identified HLA Class I peptides from each cell line equivalent dilution

Similar to MHC class I, the analysis of different cell equivalent dilutions of MHC class II peptides resulted in the identification of 8,927 to 23,435 peptides from as low as 2e5 to 5e6 cells equivalent units (Figure 9) with the great majority of peptides ranging from 13 to 17 AA in length (Figure 10).

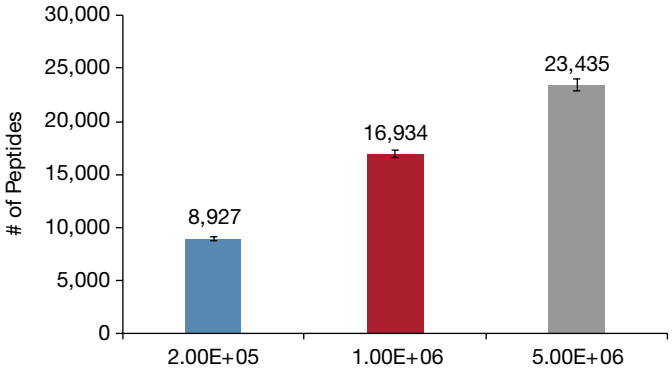


Figure 9. IM-9 HLA Class II peptides annotated from 2e5 to 5e6 cell line equivalent loads separated over 60-minute gradient. Outputs generated with PEAKS DB Workflow from PEAKS software.

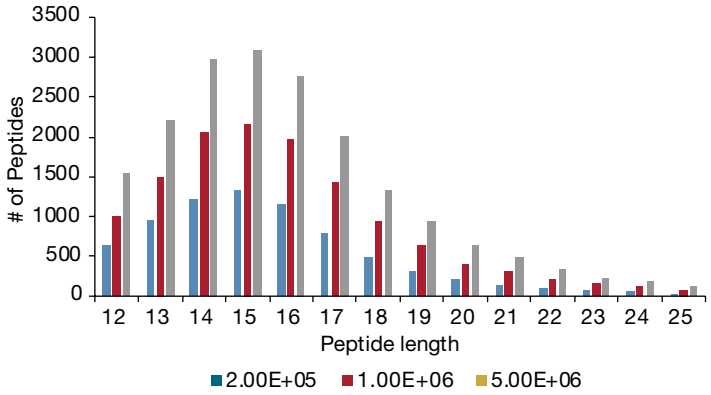


Figure 10. IM-9 HLA Class II peptide length distribution annotated from 2e5 to 5e6 cell line equivalent loads separated over 60-minute gradient. Outputs generated with PEAKS DB Workflow from PEAKS software.

Summary

- The integration of the Vanquish Neo UHPLC system with an Orbitrap Astral mass spectrometer, featuring the FAIMS Pro Duo interface, enhances sensitivity and dynamic detection range in immunopeptide analysis. This configuration enables greater depth of coverage and increased analysis throughput.
- Increased sensitivity allows for compatibility with low levels of material equivalent to samples extracted from tissue biopsy samples.

Acknowledgements

Nicholas Cheung[1], Fernanda Salvato[2], Lilian Heil[2], Tonya Pekar Hart[2], Santosh Renuse[2], Mick Greer[2], Jie Qian[2] and Robert R. Salzler[1]

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