



High sensitivity class I immunopeptidomics on the timsTOF Ultra mass spectrometer

The identification of peptide antigens presented by the major histocompatibility complex (MHC) provides important insights for the understanding of cancer, infectious or autoimmune diseases and for the design, development and application of the corresponding immunotherapies.

Abstract

The analysis of low-abundance peptides and their diverse sequences is still a challenge. In recent years, the tremendous increase in the sensitivity of MS instruments, along with more effective, multi-dimensional ("4D") acquisition modes that efficiently target both singly and multiply charged precursors, has boosted the number of immunopeptides that can be detected in an immunopeptidomics experiment [1,2,3,4].

Keywords:
Immunopeptidomics,
timsTOF Ultra,
nanoElute 2

Here we present how the combination of sensitivity, selectivity, and speed of the timsTOF Ultra makes it possible to overcome these challenges. The combined TIMS and TOF separation enables better discrimination of all peptides, even with the use of short gradients. Co-eluting isobaric antigens can be separated and uniquely sequenced. In addition, singly charged MHC class I antigens can be analysed in parallel with doubly charged antigens, excluding singly charged background noise. All peptides are coded with their CCS (Collisional Cross Section) value, which increases confidence in identification and sequencing, especially for low intensity signals.

Introduction

Immunopeptidomics, a field dedicated to elucidating the landscape of endogenous peptide presentation by major histocompatibility complex (MHC) proteins, stands as a cornerstone in understanding adaptive immunity. By investigating the repertoire of peptides showcased by MHC proteins, this discipline not only deepens our comprehension of disease pathogenesis but also paves the way for targeted therapeutic interventions.

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The timsTOF mass spectrometers, powered by trapped ion mobility spectrometry (TIMS) technology and their unprecedented robustness have set a new standard for highly sensitive proteomics applications.

TIMS separates ions based on their mobility, enhancing sensitivity and peak capacity. It enables precise targeting of low-abundant MHC class I-presented and accurate identification of highly similar peptides, both while operating in DDA (PASEF®) or DIA (dia-PASEF®) acquisition modes.

While DIA approaches are becoming standard acquisition modes for discovery proteomics in general, the difficulty to generate comprehensive antigen sequence libraries – either by prediction, sequencing, or extensive DDA acquisition – leads to use DDA as a standard approach for discovery immunopeptidomics. Moreover, the superior selectivity obtained with DDA approaches (especially on timsTOF instruments, where narrow quadrupole isolation is combined with TIMS separation) enables DeNovo sequencing on detected peptides, allowing for direct sequence information of peptides that may not have been detected or were inaccurately predicted before. This is of specific interest for allergies or infection related studies, for which the variety of potentially presented antigens makes it unrealistic to work with a library-based approach.

For these reasons, we have focused the present study on the optimization of PASEF acquisition modes (DDA), which can be run at up to 300 Hz on the latest timsTOF instruments. Note that other studies [5,6] are describing a successful use of dia-PASEF for immunopeptidomics approaches.

In our prior application note (LCMS-203), we introduced the application of the timsTOF SCP instrument, highlighting a new era of sensitivity and precision in immunopeptidomic research. Leveraging TIMS technology, the timsTOF SCP demonstrated remarkable capability in identifying low-abundance MHC class I-presented peptides amidst the complex environment of cellular proteomes.

Building upon this foundation, we now introduce the evolution of our approach with the successor, namely the timsTOF Ultra. This next-generation instrument integrates optimized components, including an enhanced conductive glass capillary for superior ion transfer, augmented ion collection optics, and dual orthogonal deflection mechanisms for the highest robustness. This is complemented by the advancements in ionization efficiency and signal-to-noise ratios offered by CaptiveSpray Ultra as well as the latest TIMS XR cell generation and the HDR 14-bit digitizer, offering high quantitative dynamic range and highest quantitative precision. Altogether, the timsTOF Ultra redefines the limits of sensitivity in proteomics and pushes the boundaries for in-depth immunopeptidomics analysis.

In this updated application note, we present optimized MS parameters tailored for the detection and identification of MHC class I-presented, while concurrently streamlining gradient times to enhance analysis by a factor of 3. By harnessing the power of the timsTOF Ultra and refined methodologies, we enable a deeper analysis of the immunopeptidome, enabling novel insights into immune responses, disease mechanisms, and therapeutic avenues.

Material and Methods

Peptide standards for immunopeptidomics

1. We used a HeLa elastase protein lysate as immunopeptidome-like peptide standard which already showed good method optimization capabilities on the timsTOF SCP. More than 85% of the resulting peptides have an amino acid length of 8-14 indicating that the standard provides a reasonable surrogate to the class I immunopeptidome. HeLa protein lysate was reduced, alkylated, and digested with elastase (cleaves at C-terminal of Ala, Val, Ser, Gly, Leu and Ile) at 37°C for 3 h. Peptides were purified by SEP-PAK reversed phase SPE [7].
2. Varying concentrations (1e5 – 5e7 cells) of acute myeloid leukemia cell line EoL (HLA-A24:02; HLA-A29:02; HLA-B35:03; HLA-B44:03; HLA-C04:01; HLA-C16:01): HLA class I ligands were isolated via immunoaffinity chromatography using the pan HLA class I-specific W6/32 monoclonal antibody as previously described [8].

- Varying concentrations (1e6 – 1e7 cells) of A375 human melanoma cell line (HLA-A01:01; HLA-A02:01; HLA-B44:03; HLA-B57:01; HLA-C06:02; HLA-C16:02) were cultured to provide final cell pellets of 5e7, 2e7, 1e7 and 4e6 cells. Cells were lysed and HLA proteins immunoprecipitated using W632-sepharose beads. Peptides were purified by 5 KDa MWCO filtration and C18 stage tips prior to analysis.

LC-MS and Data analysis

Chromatographic separation was performed using a nanoElute 2 equipped with an Aurora column (pre-fitted nanoZero™ connection and integrated emitter tip, 25 cm x 75 μm, 1.7 μm, C18; IonOpticks, Australia) with gradient times of 22 min (5-35% acetonitrile in 0.1% formic acid; 250 nL/min) and 45 min (5-37% acetonitrile in 0.1% formic acid; 250 nL/min) coupled to a CaptiveSpray Ultra ionisation source on a timsTOF Ultra. Samples of acute myeloid leukemia cell line EoL were loaded on a PepMap™ Neo Trap-Cartridge (Thermo Fisher Scientific), whereas the other samples were directly loaded on the separation column. Method optimization was performed in PASEF mode. Static settings (not affected by hardware changes) already optimized for immunopeptidomics class I analysis on the timsTOF SCP: Ion mobility range (1/k₀=0.7-1.7), optimized collision energies (1/k₀ 0.7 à 20 eV, 1/k₀ 1.06 à 30 eV, 1/k₀ 1.1 à 40 eV, 1/k₀ 1.34 à 40 eV, 1/k₀ 1.68 à 70 eV), intensity threshold of 500 and target intensity of 20,000, charge states 1-3 were fragmented. Precursor selection includes multiply charged precursors from 300-800 m/z and singly charged precursors from 700-1375 m/z (see Figure 1).

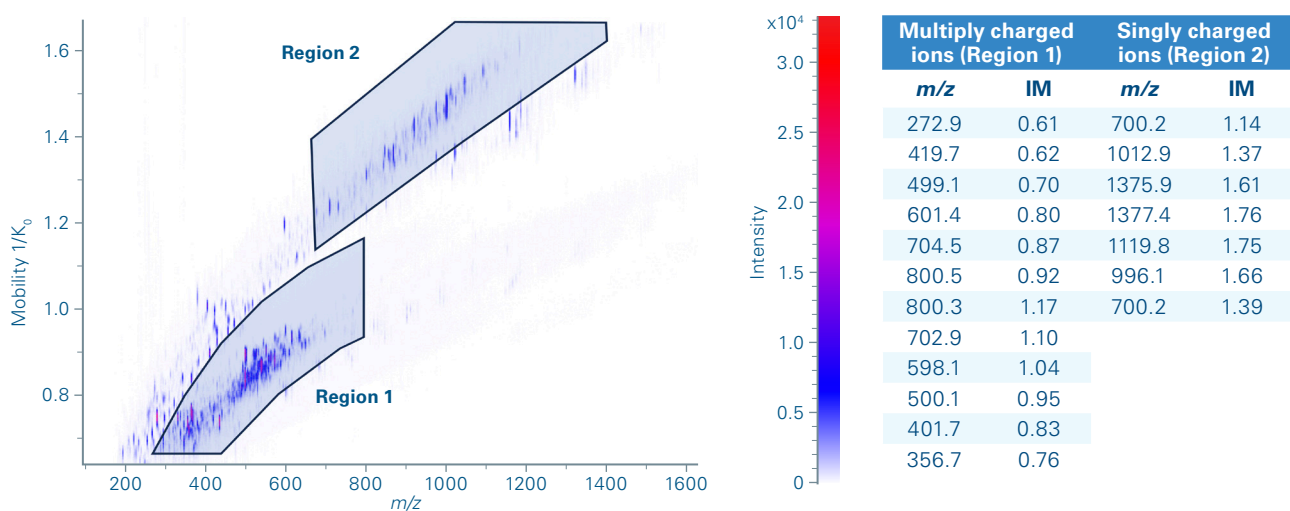


Figure 1

Precursor selection includes multiply charged precursors from 300-800 m/z (region 1) and singly charged precursors from 700-1375 m/z (region 2)

As the timsTOF Ultra was optimized to gain higher sensitivity and speed the optimal accumulation times in combination with the number of PASEF ramps were evaluated:

- 100 ms – 5/10 PASEF ramps
- 150 ms – 5/10 PASEF ramps
- 200 ms – 4/8 PASEF ramps

The raw data were processed with MSFragger (4.0) using an HLA workflow in FragPipe (21.1). Rescoring with MSBooster was applied.

General search parameters: Human uniprot database with 20,606 reviewed entries, 15 ppm precursor m/z tolerance; 30 ppm fragment m/z tolerance; enzyme: nonspecific; peptide length 7-30 amino acids; fixed modifications: carbamidomethylation(C) (only for human elastase digest); variable modifications: oxidation (M). Peptides were reported at a peptide and PSM FDR of <1% and filtered for an amino acid length of 8-14.

Results and discussion

Cycle time

In 2023, several publications on the optimisation of parameters that influence the sensitivity of immunopeptide measurements were published [2-4].

High accumulation times (166 min to 300 ms) are used to achieve highest sensitivity, which at the same time entails higher cycle times, so that gradient lengths of about 60 min are required depending on the complexity of the sample. Due to hardware changes, the timsTOF Ultra with the CaptiveSpray Ultra offers higher sensitivity than the other timsTOF mass spectrometers. Therefore, 1 ng and 5 ng of human elastase digest were initially tested with different accumulation times (100 ms, 150 ms, 200 ms) in combination with two combinations of the number of PASEF ramps (see "LC-MS and data analysis") on a 22 min and 45 min gradient.

For all accumulation times, the lower number of PASEF ramps directly translated into shorter cycle times resulted in equivalent or higher sequence identification (up to 5%) for both sample loads and gradients (data not shown).

Sensitivity

After optimising the cycle time, the overall sensitivity of the timsTOF Ultra was evaluated by serial dilution of the human elastase digest (62.5 pg to 5 ng) using a 22 min gradient and a 45 min gradient, respectively.

Figure 2 shows the comparison of 100 ms, 150 ms and 200 ms accumulation time. Regardless of the gradient length and sample amount, the 200 ms accumulation time resulted in fewer sequence identifications. This is most likely related to the longer cycle time (1.04 seconds) in combination with fewer PASEF ramps, which reduces the number of possible precursors that can be selected.

100 ms and 150 ms accumulation time resulted in almost similar sequence identifications. With increasing sample amount and complexity (5 ng), the short cycle time (0.64 s, 100 ms accumulation time) yields more identifications.

In terms of gradient length, improved sensitivity is observed with shorter gradients for < 500 pg sample amount. The reason for this lies in the sharper peak shapes. Peptides eluted from short gradients have a higher peak intensity and a narrower peak width, while peptides eluted from long gradients have a lower peak intensity and a wider peak width.

We were able to detect 964 sequences (8-14 amino acids) from as little as 62.5 pg of peptides loaded on the column, and up to 17,707 sequences (8-14 amino acids) with 5 ng of sample loading.

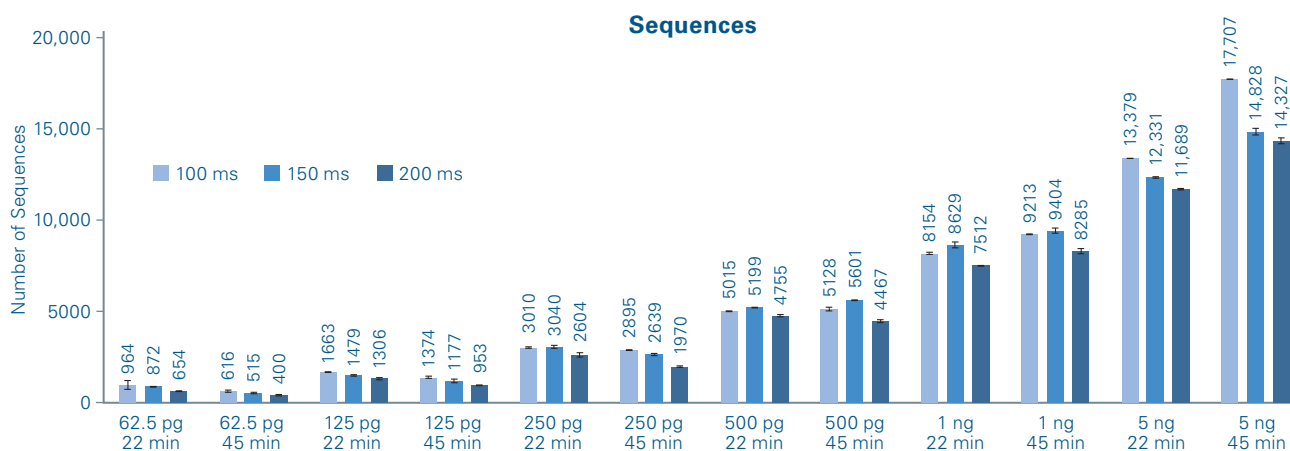


Figure 2

A dilution series starting from 62.5 pg to 5 ng of a human elastase digest was measured in duplicates with a 22 min gradient and a 45 min gradient using varying accumulation times. The bar plot shows the total number of sequences (8-14 amino acids) identified.

Validation and assessment of acute myeloid leukemia cell line EoL

Although the amino acid length distribution of the human elastase digest is comparable to that of MHC class I-presented, with most peptides having 8-14 amino acids, it differs from true immunopeptidomics samples in the proportion of specific amino acid lengths and their complexity. Therefore, different concentrations ($1e5$ - $5e7$ cells) of the acute myeloid leukaemia cell line EoL were tested with 100 ms and 150 ms accumulation time and a 22 min and 45 min gradient.

Figure 3 gives an overview of the identified sequences. Short gradients with higher peak intensities and sharper peak shapes are particularly advantageous for small sample amounts as described above, here $< 5e6$ cells. Higher sample amounts benefit from longer gradients, which reduce the complexity and saturation of the column. Another trend can be seen in the accumulation time. Sample amounts $< 5e6$ cells benefit from a longer accumulation time (higher sensitivity), while higher sample amounts benefit from the shorter cycle time when using an accumulation time of 100 ms (0.64 s vs. 0.94 s). We were able to detect 607 sequences from as little as $1e5$ cells and up to 21,614 sequences with $5e7$ cells loaded (8-14 amino acids in both cases).

Figure 4A shows the number of identified peptides of a certain amino acid length and a certain charge state of $5e6$ cells (150 ms accumulation time and 45 minutes gradient) from the acute myeloid leukaemia cell line EoL. Approximately 31% of all peptides were present as singly charged precursors.

A cell line specific allele distribution of the same sample is shown in Figure 4B, with a relative distribution of HLA-A24:02=19%; HLA-A29:02=9%; HLA-B35:03=30%; HLA-B44:03=31%; HLA-C04:01=4%; HLA-C16:01. A detailed motif analysis of $5e6$ cells is demonstrated in Figure 4C.

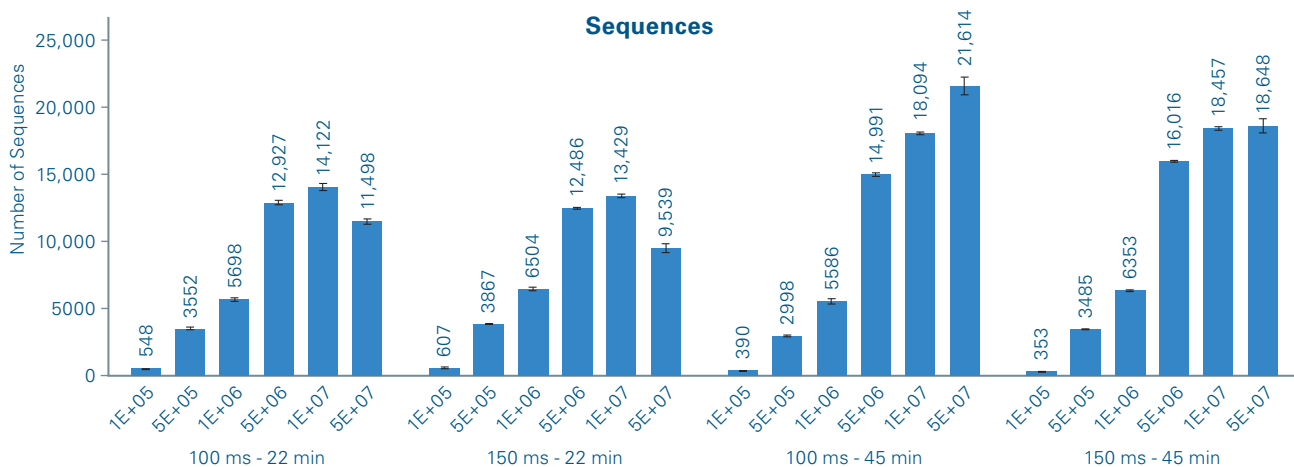


Figure 3
Dilution series of acute myeloid leukemia cell line EoL ($1e5$ – $5e7$ cells) measured with a 22 min and 45 min gradient (triplicates) using varying accumulation times.

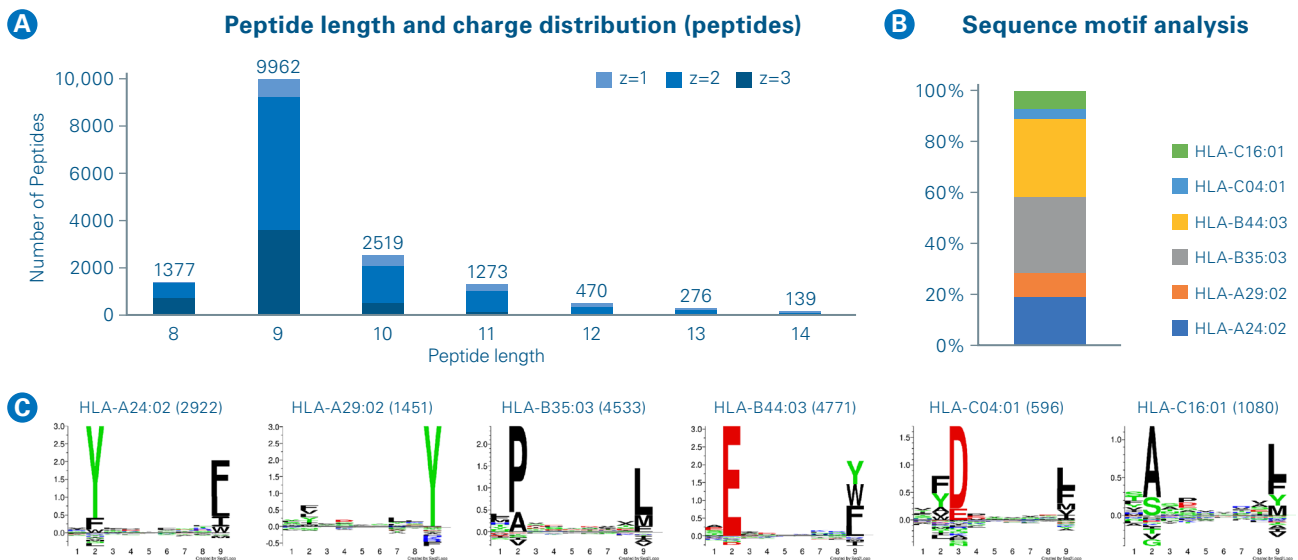


Figure 4
A Number of peptide sequences identified of a given amino acid length and charge state of 5e6 cells (150 ms, 45 min gradient) from acute myeloid leukemia cell line EoL. Approximately 31% of all peptides were present as singly charged precursor. **B** Cell line specific allele distribution of the same sample. **C** Detailed motif analysis of 5e6 cells.

Validation of the method using the example of A375 human melanoma cells

In a second approach, A375 human melanoma cells (1e6 - 1e7 cells) were tested with 100 ms accumulation time and a 22-minute gradient.

The total number of sequences identified is shown in Figure 5A, with a maximum of 12,419 sequences for 1e7 cells. Figure 5B gives an overview of the number of identified peptides with a specific amino acid length and charge state from 5e6 cells. Approximately 33% of all peptides were present as singly charged precursors. The cell line specific allele distribution is shown for all 3 samples (Figure 5C). A total of 7157 sequences (1e6), 11,272 sequences (5e6) and 9542 sequences (1e7) could be assigned to specific alleles. The relative distribution is not influenced by different sample quantities: HLA-A01:01=19%, HLA-A02:0=24%, HLA-B44:03=32%, HLA-B57:01=11%, HLA-C06:02=4%, HLA-C16:02=8%.

A detailed motif analysis of 5e6 cells is shown in Figure 5D.

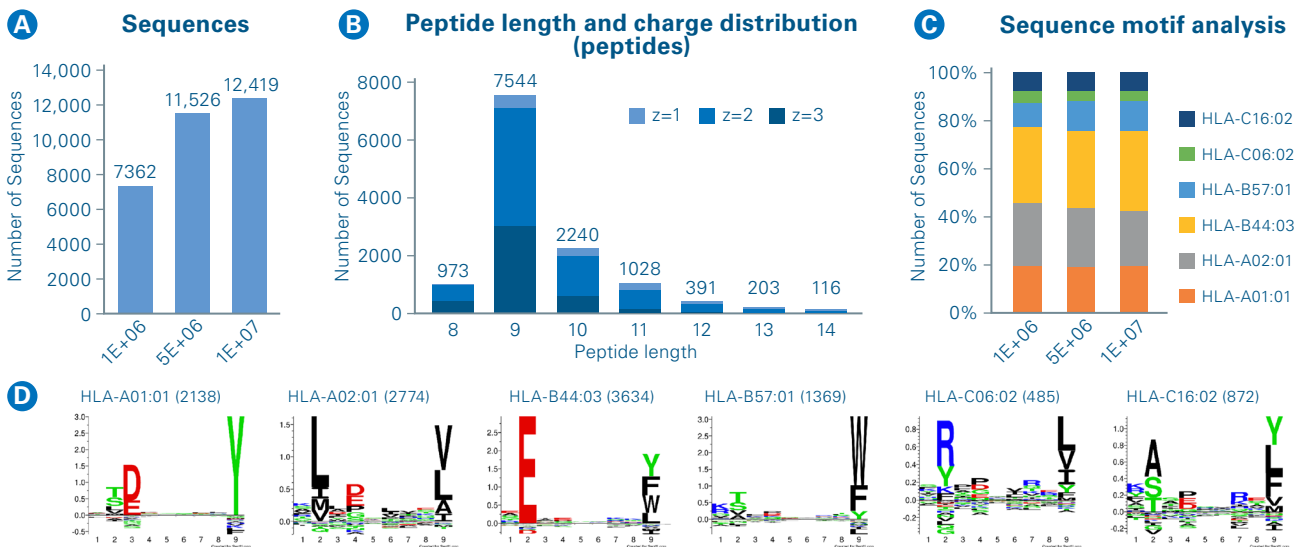


Figure 5
A Number of sequences identified from 1e6, 5e6 and 1e7 A375 human melanoma cells (100 ms accumulation time, 22 min gradient) **B** Number of peptides identified of a given amino acid length and charge state of 5e6 cells. Approximately 33% of all peptides were present as singly charged precursor. **C** Cell line specific allele distribution. **D** Detailed motif analysis of 5e6 cells.

Conclusions

- Inclusion of singly charged precursors and optimized collision energies were adopted from previous method developments.
- Optimization focused on reduction of gradient length (22 min and 45 min) with optimized cycle times (< 1 sec) while maintaining sensitivity.
- The timsTOF Ultra provides a robust and sensitive platform for immunopeptidomics.
- The recent launch of the timsTOF Ultra 2 with improved sensitivity and a dynamic control of the tims cell filling (ICC2) will allow to further improve the performance observed with the timsTOF Ultra.

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Further reading



Immunopeptidomics ebook

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Precision targeting of single charged species	TIMS polygon filtering to allow homing in on relevant precursor ions, maximizing your capture of MHC class I peptide ions, even those that don't play by the usual rules
Ultimate performance	CSI ion sources: CaptiveSpray Ultra moves boundaries and delivers robust performance and simplicity for outstanding sensitivity.
Higher throughput, deeper profiles	TIMS spreads sample complexity across the mobility dimension for fast turnaround, without compromise.
Enhanced confidence with TIMS	Mobility offset, mass aligned (MOMA) for confident identification and quantification of isobaric or nearly isobaric peptides.
Powerful insights	Bruker ProteoScape™ (BPS) : Real-time data processing for large experiments.
Maximum speed	BPS Novor enabling de novo sequencing on the fly at 1000 spectra per second from a 300 Hz dda-PASEF® acquisition. Specifically trained on immunopeptide data.
Limitless processing	Spectronaut® simplifies complex DIA workflows with unmatched sensitivity and accelerated data analysis.

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