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Using timsTOF Ultra 2 with optimized dda-PASEF method and TIMSrescore for high-sensitivity class I immunopeptidomics analysis in discovery workflows

Marx K[1], Pandey K[2], Purcell AW[2], Vitko D[3], Hornburg D[4], Mueller T[1], Krieger J[5]

[1]Bruker Daltonics GmbH & Co. KG, Bremen, Germany; [2]Department of Biochemistry and Molecular Biology, Infection and Immunity Program, Biomedicine Discovery Institute, Monash University, Clayton 3800 Victoria, Australia.[3]Bruker Scientific LLC, Billerica, USA; [4]Bruker Scientific LLC, San Jose, USA; [5]Bruker Ltd., Milton, Ontario, Canada

Introduction:

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 and development of the corresponding immunotherapies. However, the identification of low-abundance peptides and their diverse sequences is still a challenge. In recent years, the tremendous increase of MS instruments sensitivity, coming along with more effective, multi-dimensional ("4D") acquisition modes allowing to efficiently target singly and multiply- charged precursors, has boosted the number of immunopeptides that can be detected in an immunopeptidomics experiment.

Here we present 4D acquisition methods on a timsTOF Ultra 2 with optimized ion transfer parameters to further overcome these challenges which provides an extremely sensitive setup for the analysis of a wide range of different sample loads.

Methods:

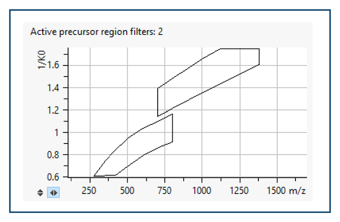
Human elastase digest: 1 ng/µl for parameter optimization. A dilution series (15.6 pg – 1 ng) was used to test the optimized parameters on different sample amounts.

Pierce peptide RT calibration mix (PRTC): For parameter optimization the sample has been diluted to 5 fmol/µl or 1 fmol/µl.

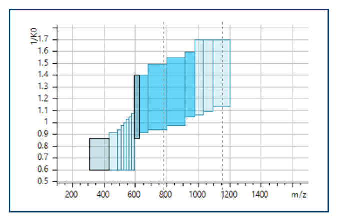
MHC-I: Combination of three cell lines (THP-1, MV411.1 and HL60). A dilution series ranging from 62.5 pg – 10 ng has been measured with the optimized method.

LC: nanoElute 2 with an Aurora Ultimate column coupled to a CaptiveSpray Ultra ionization source on a timsTOF Ultra 2; gradient time: 22 min (triplicates).

dda-PASEF: Using optimized MS/MS parameters (Bruker application note LCMS-226)



dia-PASEF: Use of same parameters as in dda experiment with optimized dia isolation windows (py_diAID).



Processing: Human elastase digest and MHC-I: dda-PASEF raw data were processed with Bruker ProteoScape using the TIMSrescore algorithm. dia-PASEF raw data were processed in Spectronaut 19.5 using the library approach with default settings (library containing of 58.444 precursors and 33,080 precursors, respectively). PRTC peptides were analyzed with Skyline.

Results:

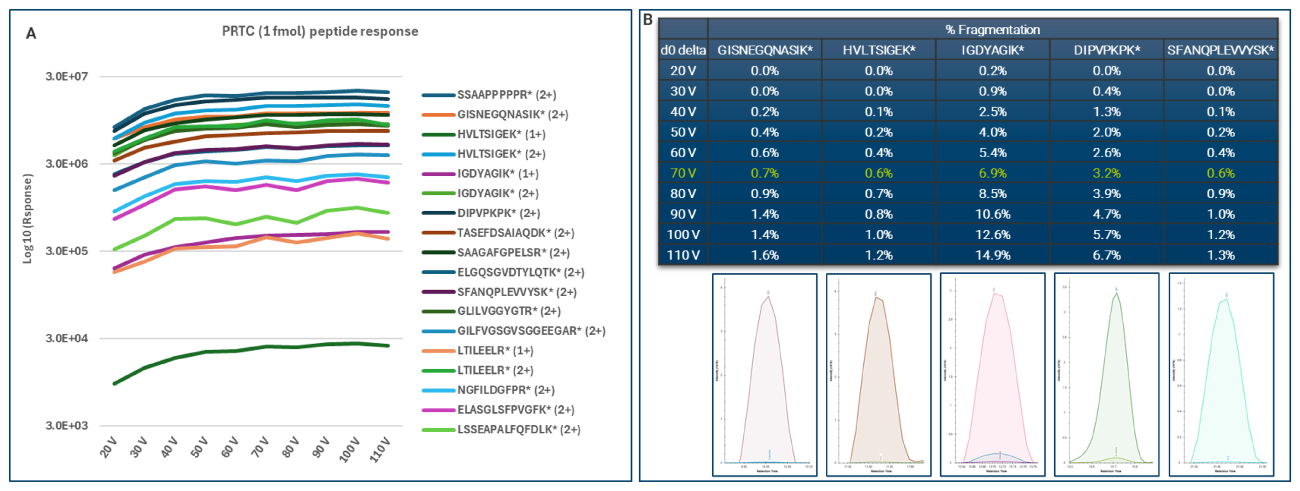
Optimization of source setting: Capillary voltage and dry temperature

The ionization of peptides is directly influenced by the source settings. Capillary voltages of 1600 V, 1500 V, 1400 V, and 1300 V in combination with dry temperatures of 160 °C, 180 °C, and 200 °C were tested. For human elastase digest and PRTC peptides only minor changes were observed across different source settings, which are likely within the normal variation of measurement accuracy and integration. For the following experiments a capillary voltage of 1500 V and a dry temperature of 180 °C was used as this has slightly higher identification rates.

Optimization of ion transfer settings: deflection 0 delta

The deflection 0 delta affects the orthogonal deflection of the ions into the funnel 0. This parameter should be balanced for a soft ion transfer without loss of ions. Voltages from 110 V to 20 V were tested. In contrast to the source settings, the deflection 0 delta has a major impact. Figure 1A provides an overview of the PRTC peptide responses which remain relatively constant between 110 V and 70 V but with a loss < 70V.

Simultaneously, the percentage of fragmentation for five particularly labile peptides decreases (Figure 1B).



	% Fragmentation				
d0 delta	GISNEGQNASIK*	HVLTSIGEK*	IGDYAGIK*	DIPVPKPK*	SFANQPLEVVYSK*
20 V	0.0%	0.0%	0.2%	0.0%	0.0%
30 V	0.0%	0.0%	0.9%	0.4%	0.0%
40 V	0.2%	0.1%	2.5%	1.3%	0.1%
50 V	0.4%	0.2%	4.0%	2.0%	0.2%
60 V	0.6%	0.4%	5.4%	2.6%	0.4%
70 V	0.7%	0.6%	6.9%	3.2%	0.6%
80 V	0.9%	0.7%	8.5%	3.9%	0.9%
90 V	1.4%	0.8%	10.6%	4.7%	1.0%
100 V	1.4%	1.0%	12.6%	5.7%	1.2%
110 V	1.6%	1.2%	14.9%	6.7%	1.3%

Fig. 1: The effect of reduction of deflection 0 delta voltage on the fragmentation and abundance of PRTC peptides.

Human elastase digest behaves analogue (Figure 2A). Figure 2B illustrates the charge state distribution. Within the voltage range of 110 V to 60 V, only minor changes were observed: the frequency of singly charged precursors decreased slightly (from 25.6% to 24.7%), while the frequency of doubly (from 49.7% to 49.9%) and triply charged precursors (from 9.3% to 9.8%) increased slightly. Figure 2C shows the typical peptide length distribution, with most peptides consisting of 9 amino acids and a Gaussian distribution up to approximately 20 amino acids. This distribution remained constant across all measurements, regardless of the applied deflection 0 delta voltage.

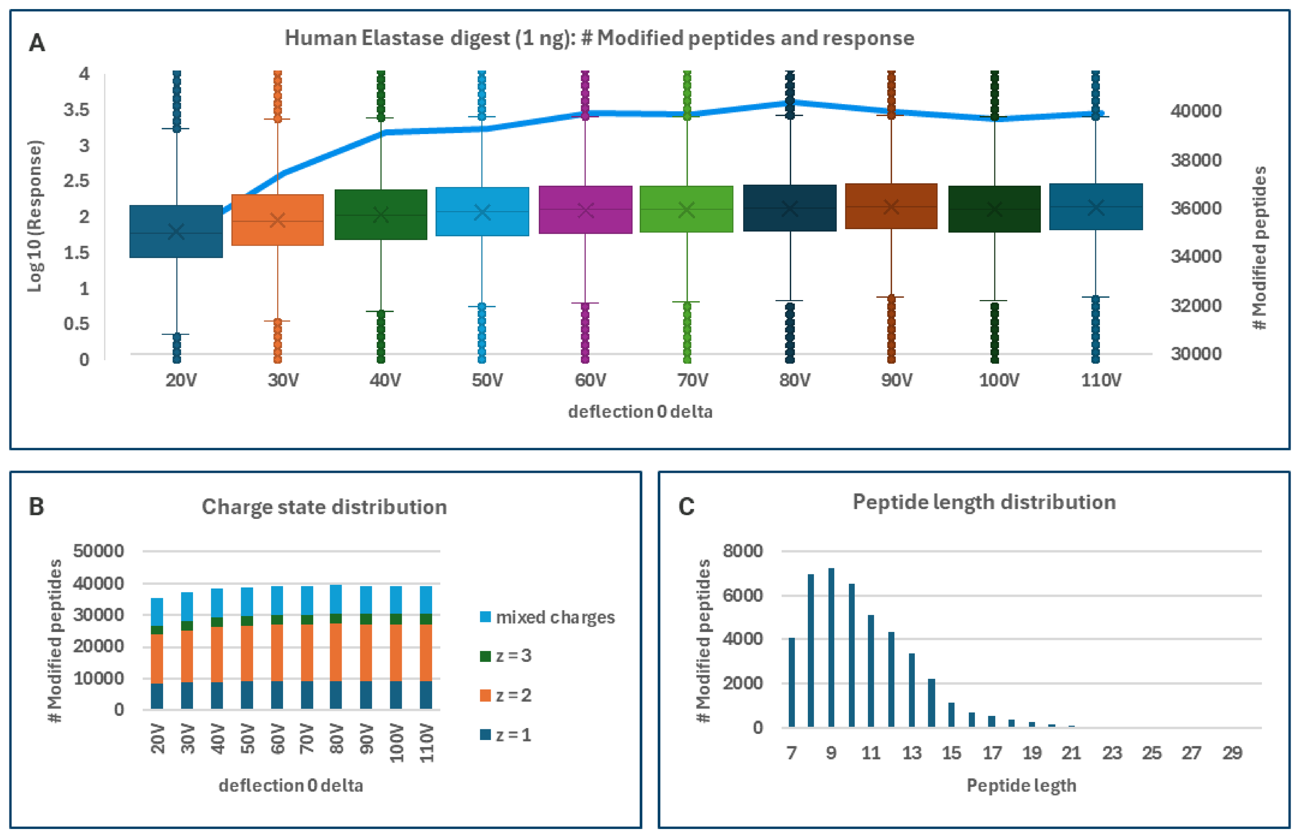


Fig. 2: The effect of reduction of deflection 0 delta voltage on the number of peptide identifications and abundance of unspecific peptides from human elastase digest.

In summary, a deflection 0 delta of 70 V appears to be an optimal setting, ensuring soft ion transfer without compromising overall signal intensities. For the next optimization steps, a deflection 0 delta voltage of 70 V has been used.

Optimization of ion transfer settings: delta t6 and delta t4

When working with especially fragile components, it may be preferable to reduce the values for delta t4 and delta t6 so that these molecules are exposed to the lowest possible potential differences. Voltages from 55 V to 20 V (delta t6) and 120 V to 20 V (delta t4) were tested.

Peptides from human elastase digest show decreased response when lowering delta t6 voltages. This trend is observed across all peptide identifications, including 7 to 15 mers and 9 mers .The number of peptide identifications remains constant between 50 V and 60 V, with a significant drop below 40 V (data not shown).

Further analysis reveals an effect on the charge state when changing the voltage. Charge state 2 is only slightly affected, whereas charge state 3 shows a much better response at higher voltages. Conversely, the transfer of singly charged peptides is more pronounced at lower voltages. A complete overview of the charge state distribution is provided in figure 3.

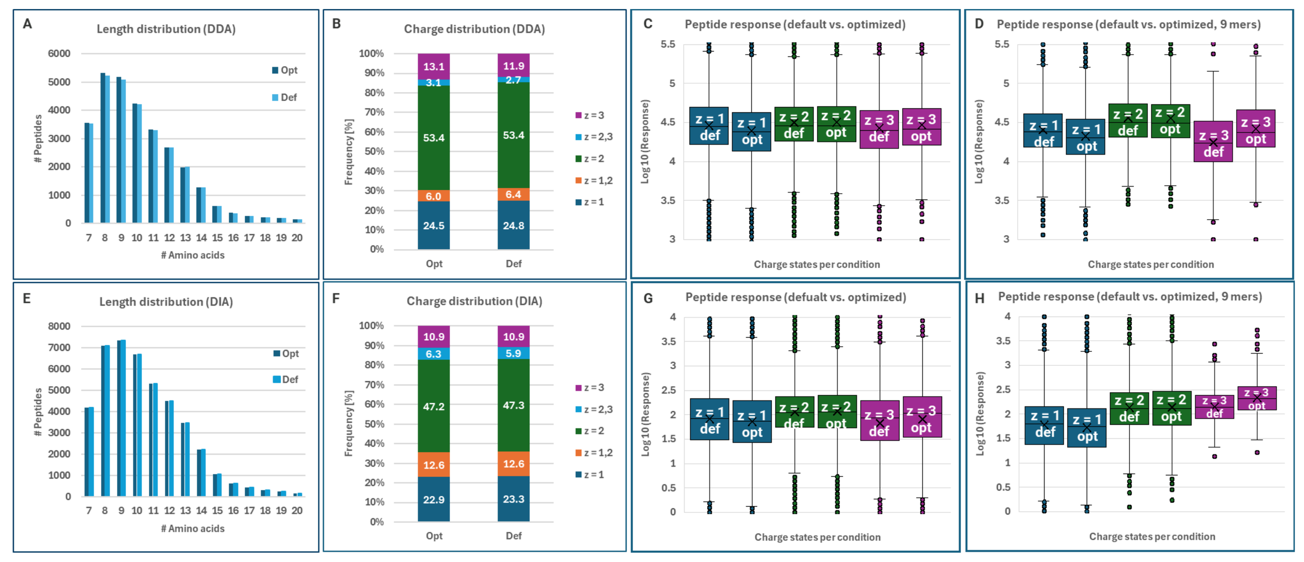


Fig. 3: Comparison of default parameters with optimized parameters in terms of peptide length and charge distribution and peptide response (1 ng human elastase digest).

Compared to the default setting of 55 V, 60 V gives slightly higher peptide identifications which is used for the next optimization step.

The number of identified peptides, their response, and the peptide length and charge distribution are not affected by different delta t4 voltages. Comparing two different voltages results in a peptide overlap of more than 90% (data not shown)

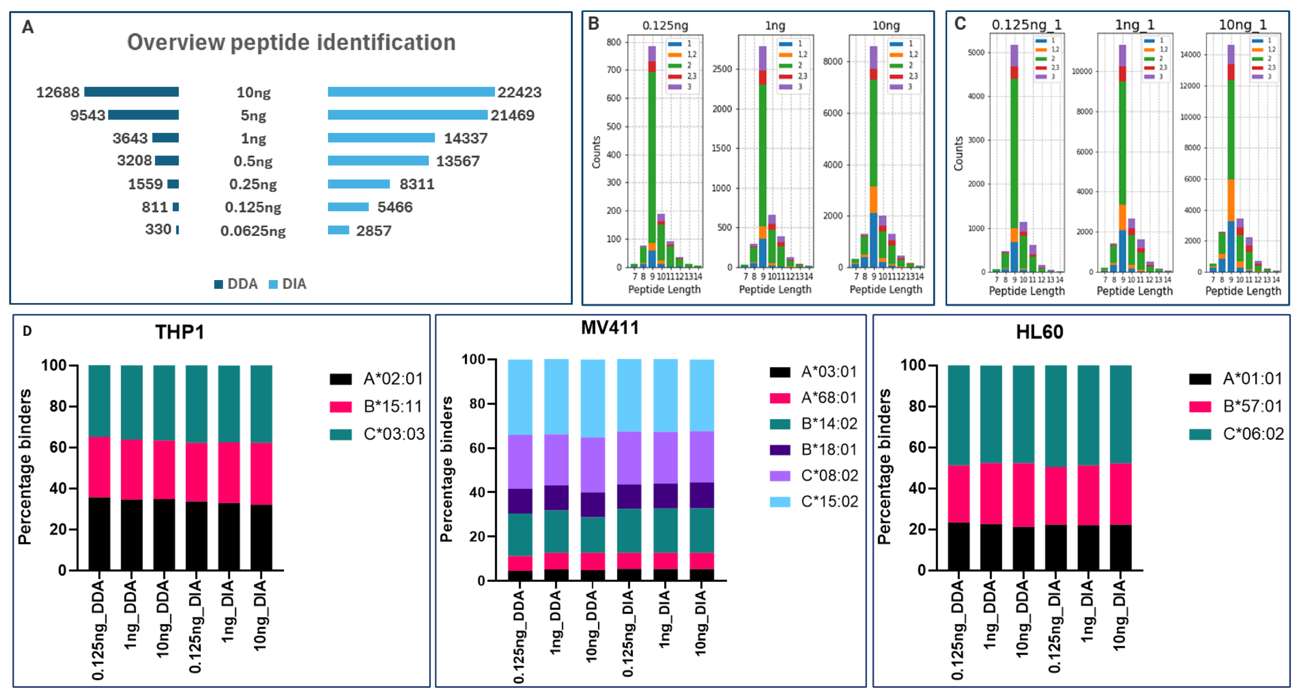


Fig. 4: Dilution series of MHC-I standard A) The bar plot shows the average number of peptides identified. Peptide length distribution of three conditions is shown for dda-PASEF (B) and dia-PASEF (C). D) Binder comparison in percent.

Summary of optimization steps and final comparison

To translate these results into a laboratory setting, a serial dilution (62.5 pg - 10 ng) of an MHC-I ligand standard prepared from THP-1, MV4-11, and HL60 cells was analysed. An overview over the identification rates is given in Figure 4A. As expected, the majority of identified immunopeptides had a peptide length of 8-12 amino acids (Figures 4B-C). Based on binding affinity there was not much difference in percentage binders across different injection concentrations regardless of whether the sample was acquired by DDA or DIA (Figure 4D).

Conclusion:

- The dda- and dia-PASEF methods have been further optimized in terms of ionization and ion transfer to provide an extremely sensitive setup for the analysis of a wide range of different sample loads.
- dia-PASEF offers comprehensive data collection with less bias, resulting in increased identifications of low abundance ions and better reproducibility.

Immunopeptidomics

Conflict-of-interest

Kristina Marx, Dijana Vitko, Daniel Hornburg, Torsten Mueller and Jonathan Krieger are employees of Bruker. Kirti Pandey and Anthony W Purcell are employees of Monash University.