Dia-PASEF for in-depth immunopeptidomics analysis: Challenges and new opportunities

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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. In recent years, the tremendous increase of MS instrument 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 detected in an immunopeptidomics experiment. Here we demonstrate the use of dia-PASEF methods to further deepen the analysis of immunopeptides.

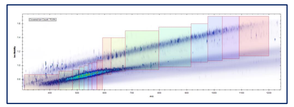
Methods

Samples: 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.

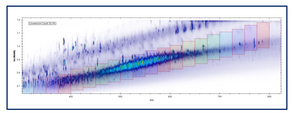
LC: Chromatographic separation was performed using a nanoElute 2. Samples were loaded on a PepMap™ Neo Trap-Cartridge and separated with an Aurora Ultimate column with gradient times of 22 and 45 min coupled to a CaptiveSpray Ultra ionization source on a timsTOF Ultra.

DDA: Ion mobility range:1/k0=0.64-1.7; accumulation time: 100 ms; optimized collision energies: 1/k0 0.7 → 20 eV, 1/k0 1.06 → 30 eV, 1/k0 1.1 → 40 eV, 1/k0 1.34 → 40 eV, 1/k0 1.68 → 70 eV; intensity threshold: 500; target intensity: 20,000, charge states: 1-3 precursor selection includes multiply charged precursors from 300-800 m/z and singly charged precursors from 600-1,300 m/z.

DIA+/++: Use of same parameters as in DDA experiment with optimized dia isolation windows (py_diAID).



DIA++: Use of same parameters as in DDA experiment with restricted ion mobility range (1/k0=0.64-1.2) and optimized dia isolation windows only for multiply charged precursors.



Processing: Spectronaut 19 with a directDIA approach and library extension runs (generated from DDA measurements) has been used. Default search settings were adjusted to: Unspecific search (7-15 amino acids), variable modification: oxidation (max 1), database: human Uniprot database. Identification Qvalues were left as in the default settings.

Method evaluation: Charge distribution

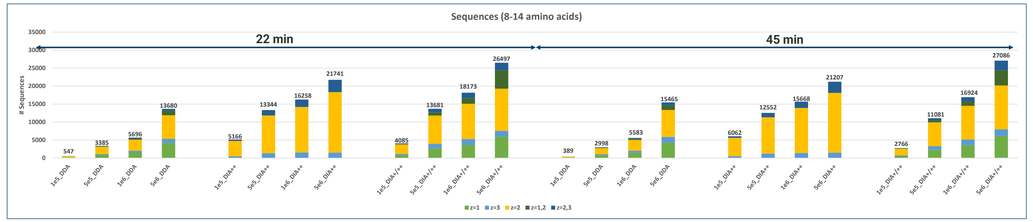
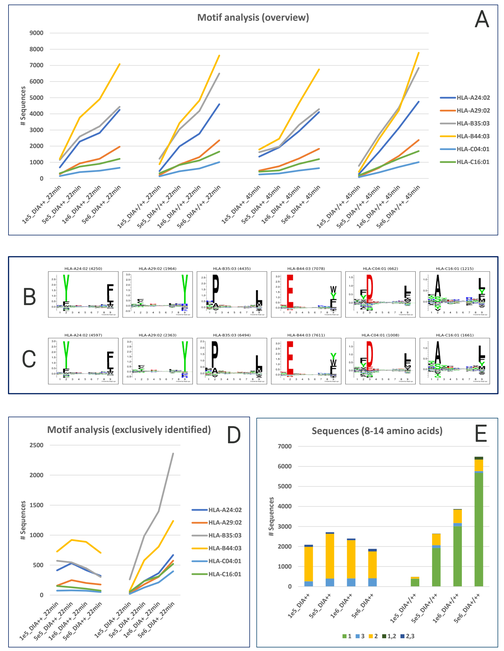


Figure 1 shows the number of peptide sequences identified with the corresponding charge state measured with DDA, DIA++, and DIA+/++ (22 min and 45 min gradient) from acute myeloid leukemia cell line EoL.

Short gradients are especially advantageous for low sample amount injections as peptides eluted by short gradients have a higher peak intensity and narrow peak width which is translated into sensitivity.

Approx. 30% of all peptides measured with DDA were only present as singly charged precursor, and approx. 20% measured with DIA+/++, respectively.

Detailed motif analysis



In Figure 2A the cell line specific allele distribution is shown for four different sample loads (1e5 – 5e6 cells), two gradients (22 min and 45 min) and the two different DIA methods (DIA++ and DIA+/++). Only peptides identified in all triplicate injections were used.

A detailed motif analysis of 5e6 cells is shown in figure 2B (DIA++) and Figure 2C (DIA+/++).

As known from previous studies, HLA-C04:01 and HLA-C16:01 are only expressed at low levels.

Inclusion of singly charged precursors is especially beneficial for HLA-B35:03 detection. This is further demonstrated in Figure 2D. Here, motif analysis is based on peptides exclusively identified with a specific method. Intriguingly, nearly all additional HLA-B35:03 peptides are only present as singly charged precursors (Figure 2E). This observation is also true for the additional identifications of HLA-B44:03 peptides.

Exclusive peptide identifications using method DIA++ show no difference with rising sample load. A detailed inspection showed very low quantities for these peptides and only small overlaps of peptide identifications between different sample loads.

Summary

- Samples from acute myeloid leukemia cell line EoL were measured with DDA and two different DIA methods.
- As stated in literature, inclusion of singly charged precursors is beneficial for class I immunopeptidomics. Depending on the analyzed cell line approx. 15 – 30% of HLA peptides are only present as singly charged ions.
- Here, we demonstrated first DIA approaches for improved sensitivity of immunopeptidomics samples.
- A combination of directDIA and a spectral library generated from DDA runs was used in a hybrid approach.
- Triplicate injections of DIA++ vs. DDA showed an improvement of sequence identifications of a factor of 1.6 → 9.4 (5e6 → 1e5 cells), DIA+/++ vs. DDA a factor of 1.9 → 7.5 (5e6 → 1e5 cells), respectively.
- HLA-C04:01 and HLA-C16:01 are only low expressed.
- Exclusive identifications measured with DIA++ are of very low abundance and do not reflect a specific motif.
- Inclusion of singly charged precursors in DIA methods yielded 20% more HLA sequence identifications, mainly coming from HLA-B35:03 and HLA-B44:03 alleles.

Conclusion

- Optimization focused on reduction of gradient length while maintaining sensitivity.
- Inclusion of singly charged precursors is advantageous for identification of HLA-B35:03 and HLA-B44:03 peptides.
- The timsTOF Ultra provides a robust and sensitive platform for immunopeptidomics.

Immunopeptidomics