

Enhanced Sensitivity and Reproducibility for HLA-Class I & II Immunopeptidomics Utilizing dia-PASEF Workflow

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Introduction

Mass spectrometry (MS) is key to the study of immunopeptides. While data dependent acquisition (DDA) is the most common acquisition method for immunopeptidomics, data independent acquisition (DIA) has gained significant interest due to the sensitivity, reproducibility, and speed. It has proven to be an attractive method for label free quantification in global proteomics but its potential for immunopeptides has not been fully explored. In this work, we aimed to evaluate the performance of DIA in combination with ion mobility (dia-PASEF) for high-sensitivity identification of HLA peptides.

Methods

Immunopeptides (Class I and Class II) were enriched from an IM9 cells, a B lymphocyte cell line, and analyzed by coupling nanoElute 2 to a trapped ion mobility spectrometry – quadrupole time of flight mass spectrometer Bruker timsTOF Ultra 2 (Figure 1). Data were acquired in dda-PASEF with gradients of 60 minutes and dia-PASEF with a 45 min gradient. dia-PASEF schemes were optimized for HLA Class I and II, covering distinct m/z and mobility ranges as demonstrated in figure 2. dia-PASEF data were processed with Bruker ProteomeXis for dda-PASEF runs, Spectronaut 19 (Biognosys) using spectral a library created from the dda runs and Spectronaut 20 using directDIA+.

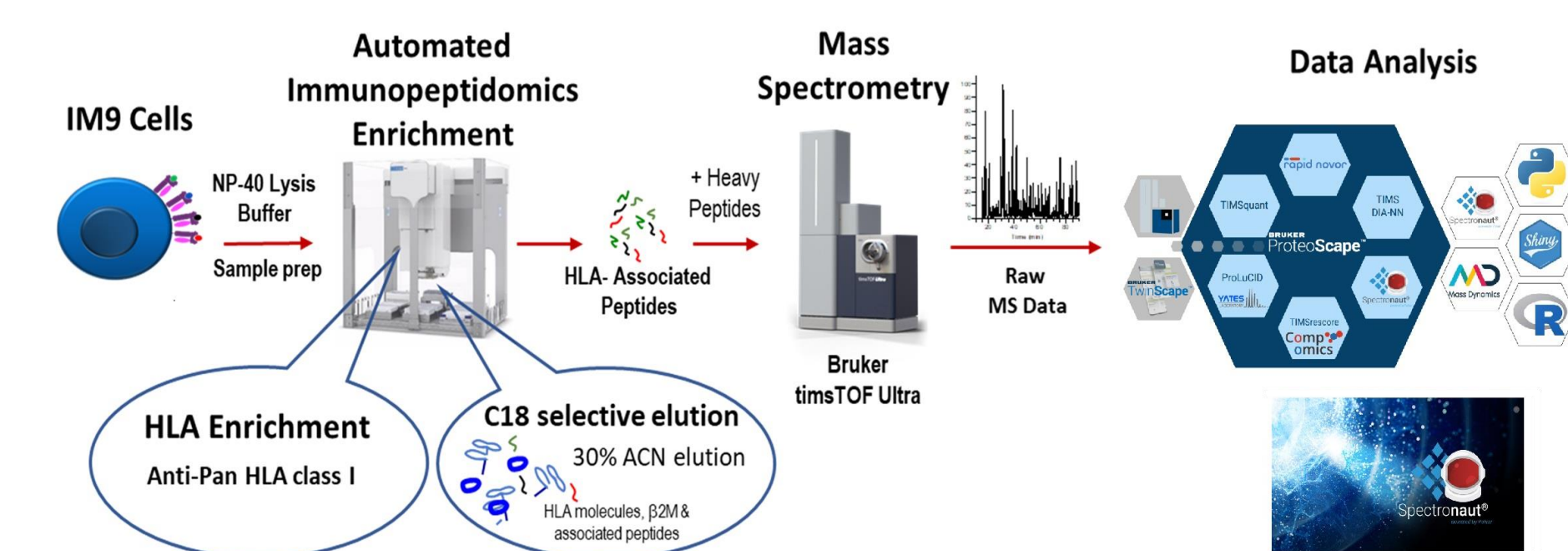


Figure 1: An overview of platform used in this study showing immunopeptides extraction, LCMS system and the data analysis options which include real time data processing. Here, TIMSrescore was used for dda-PASEF runs while Spectronaut was used for the library construction and dia-PASEF runs. All data and plots were created using a shiny python app (<https://github.com/zack-kirsch/timsplot>).

Results

Experiments were designed to compare differing cell inputs using either library free or spectral library approaches. Equivalent injections from 1e6 to 5e6 cells were acquired resulting in a range of 10-15k identified ninemers from dda-PASEF runs, respectively. After combining dda-PASEF runs, a spectral library was created on Spectronaut 19 (Pulsar Search) resulting in about 15k ninemers. Based on the spectral library and aiming 9mers, a dia-PASEF window scheme was created focusing on singly charged ions with a m/z greater than 600 and multiply charged ions with m/z below 700. Thus, using this small library, the number of ninemers was around 13.5 k for all injections. However, when applying directDIA+ on Spectronaut 20, the number of 9mers increased to around 17k ninemers from 5e6 cells (figure 3A) surpassing the number of IDs from dda runs in all dilutions.

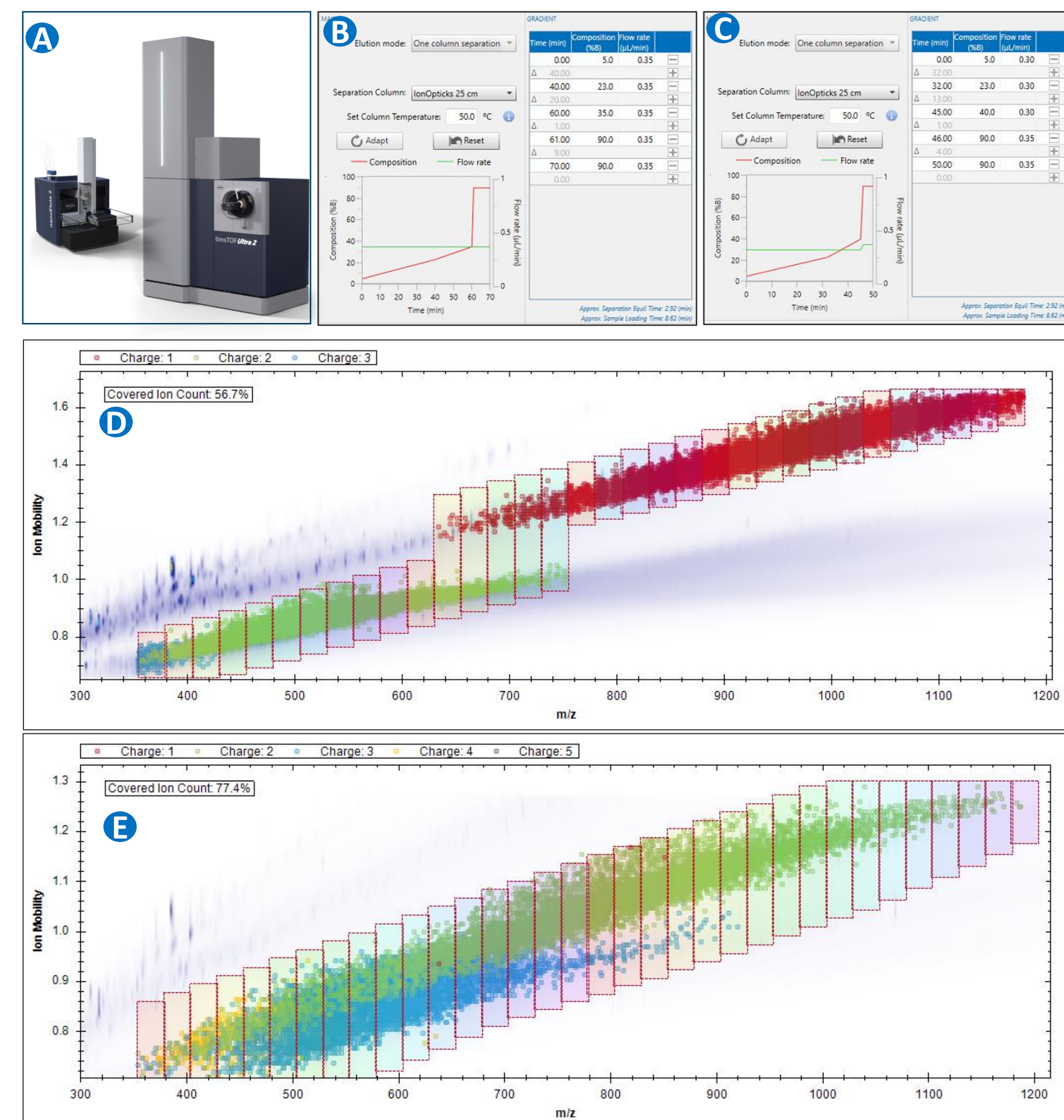


Figure 2: A) nanoElute and timsTOF Ultra 2 used on the study. B) 60 minutes LC gradient with DDA acquisition. C) 45 minutes LC gradient with dia-PASEF acquisition. D) and E) - Heat map (m/z versus mobility 1/K0) from Spectronaut 20 showing peptide distribution for an entire run where colored diamonds indicate identified precursors and charge states. D) represents dia-PASEF scheme for HLA Class I (33 windows in 1.0 sec total cycle time, 80 ms as ramp and accumulation time, mobility range from 0.68 to 1.7 1/K0) and E) represents the scheme for HLA Class II peptides (50ms accumulation time, 34 windows in 12 mobility scans, cycle time of 1.1 seconds, mobility range from 0.70 to 1.3 1/K0). m/z range from 100 to 1700 for both methods.

Additionally, a second library was created with runs collected from several dda-PASEF acquisitions from the same line at different times containing about 27k ninemers. The library-based search showed about 18.5k precursors. Remarkably, the directDIA+ search identified around 17k precursors. This represents a great advance in identifications and time saving compared to constructing a spectra library. Applying the same approach to HLA-II enriched peptides from the same IM9 cell line, which represents a more challenging class of peptides, the number of identified peptides from dia-PASEF library-based was higher. directDIA+ wasn't performed on HLA Class II yet (Figure 3B).

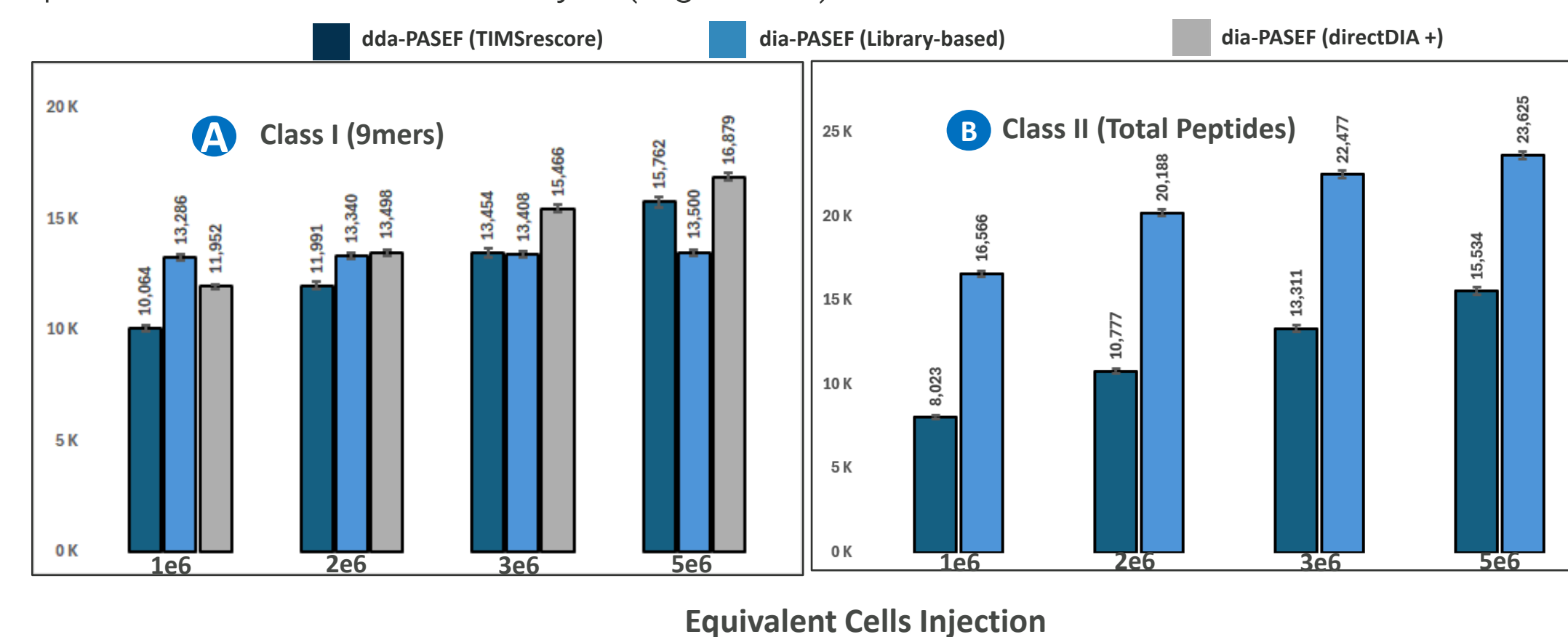


Figure 3: Number of identified Class I ninemers peptides (A) and Class II total peptides (B) from IM(cell line in dda-PASEF mode (TIMSrescore), dia-PASEF library based and dia-PASEF using directDIA+.

Besides of increasing the number of IDs, dia-PASEF results also show an excellent overlap between different workflows and search engines (figure 4). Furthermore, sequence logo motifs are very similar (figure 5).

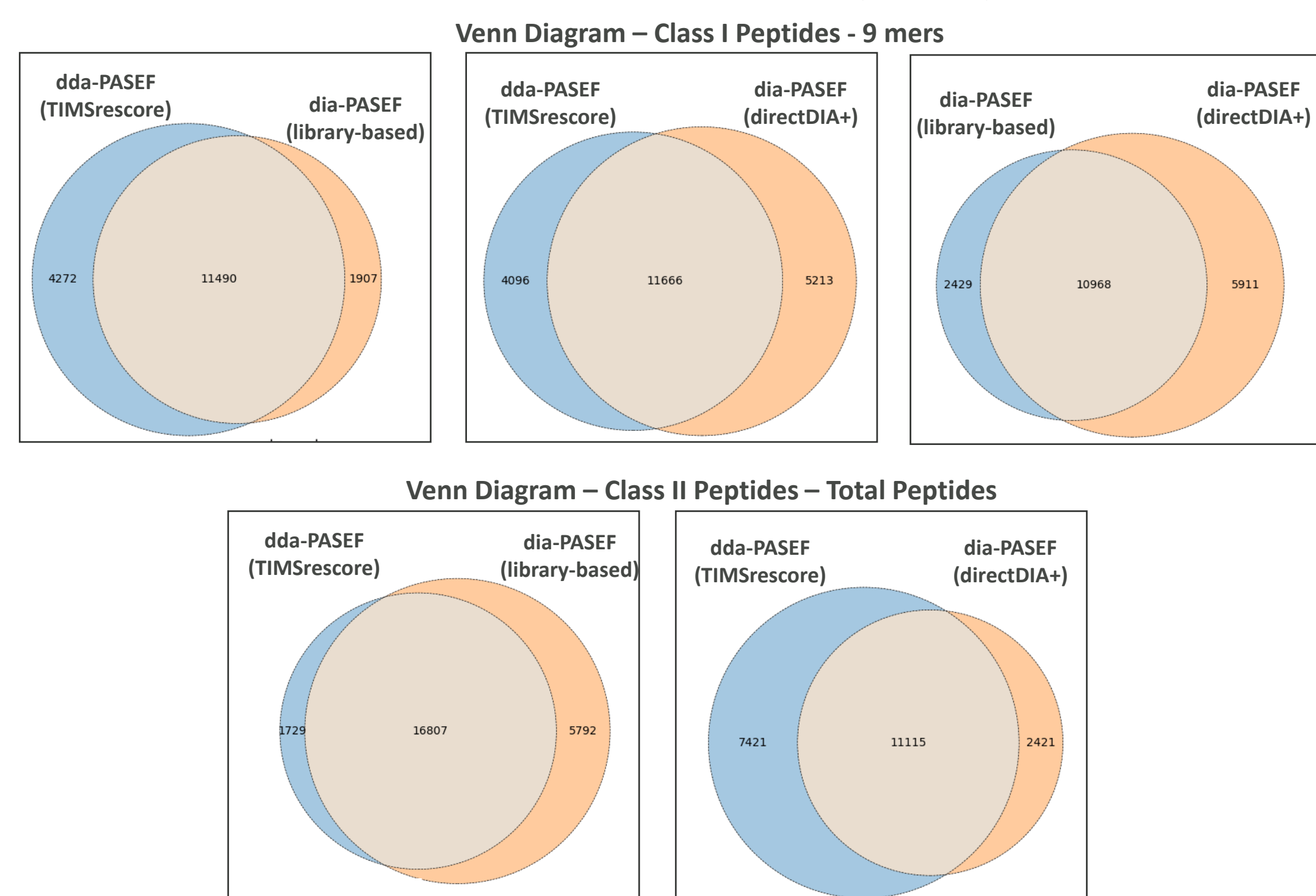


Figure 4: Venn Diagrams comparing Class I and Class II immunopeptides. Injections of 5e6 cells were plotted.

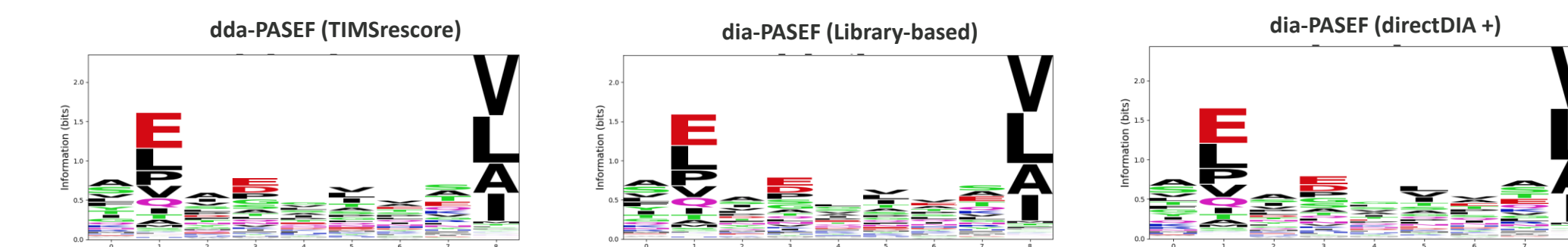


Figure 5: Sequence logo motif detected from different PASEF workflows.

Similar to dda-PASEF, taken as the standard acquisition mode for immunopeptides, dia-PASEF either in library mode or directDIA+ shows a very consistent charge and peptide distributions (Figure 6).

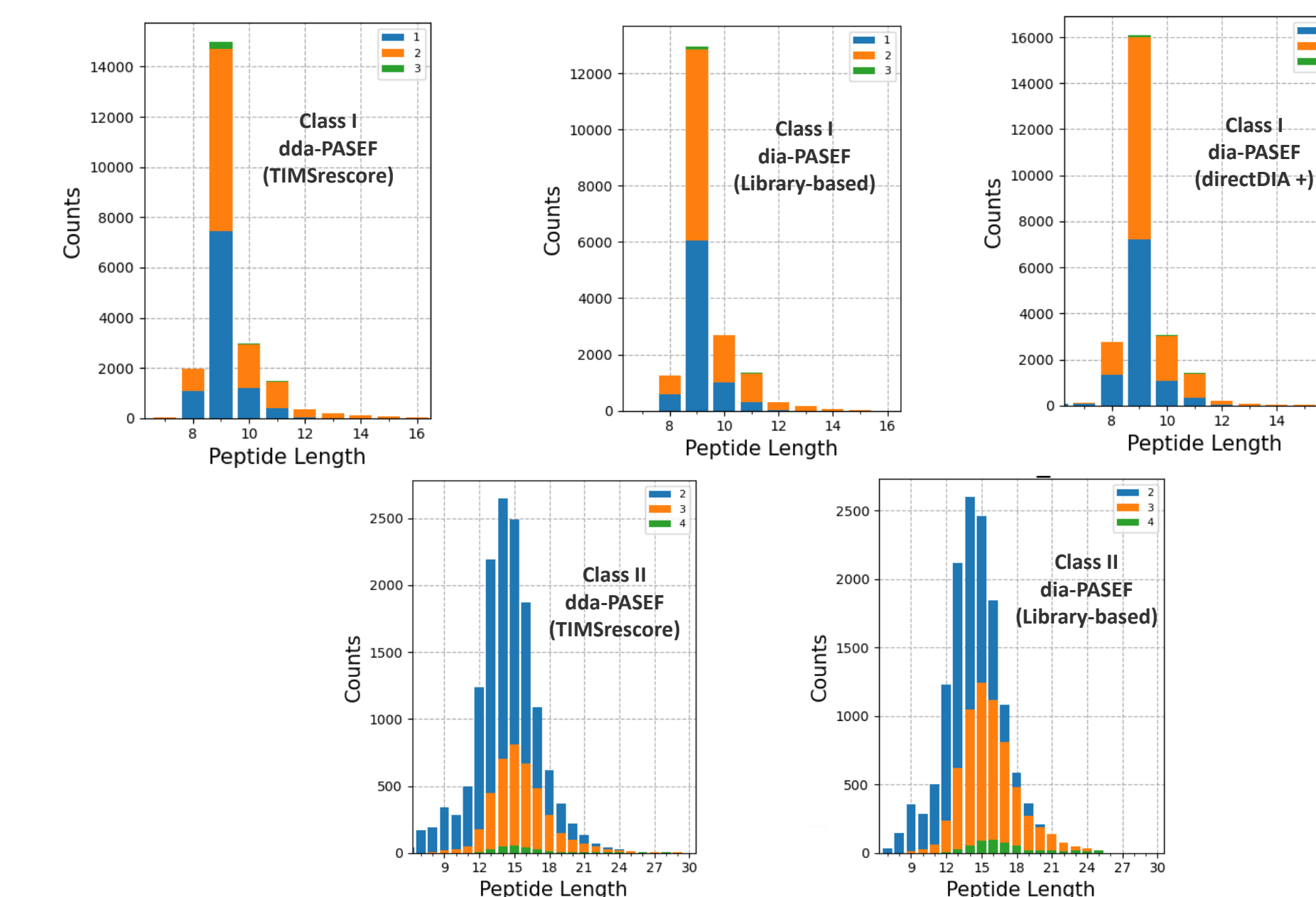


Figure 6: Charge State and Peptide Length Distribution. Comparisons done at 5e6 cells.

The impressive number of high confidence identifications is a result of a combination high quality MS/MS spectra and improvements in the data processing, especially dia-PASEF and direct-DIA+ (Figure 7).

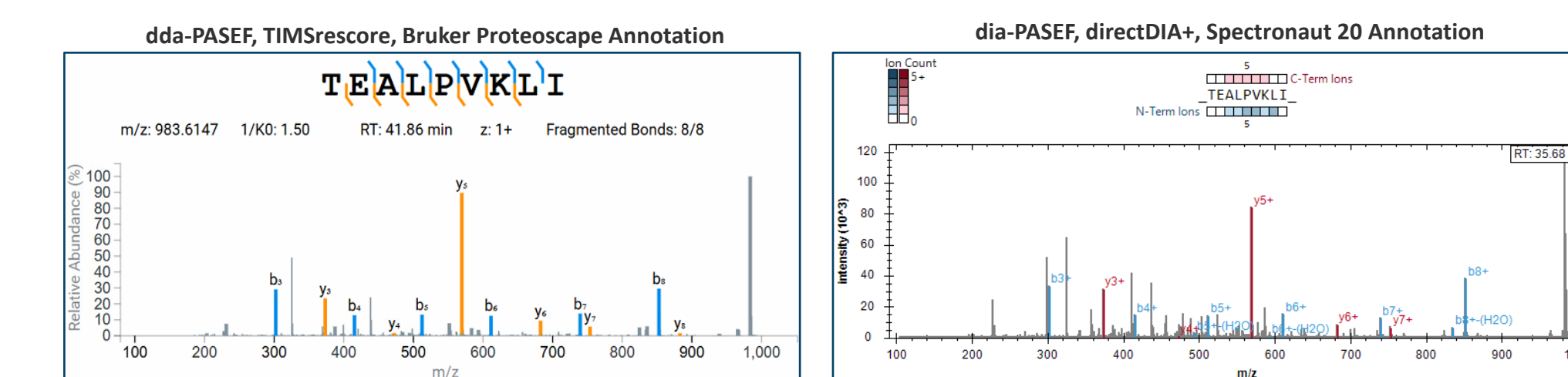


Figure 7: Comparison of annotated MS/MS spectrum of HLA Class I peptide, demonstrating same ions and intensities between dda and dia-PASEF. Despite of collected diagnostic immonium ions are not demonstrated in these annotations.

Conclusion

- Analysis in dia-PASEF mode for HLA peptides on timsTOF Ultra2 increases peptidome coverage for lower sample input using shorter gradients keeping a high identification confidence.

timsTOF Ultra 2