

Optimizing diagonal-PASEF with Retention Time Summation for High-Throughput Proteomics

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Introduction

Proteomics complements genomics by directly measuring proteins, capturing regulation, signaling, and drug responses that nucleic acids cannot. Recent advances in LC-MS and AI-driven analysis have transformed the field, enabling fast, high-coverage, high-throughput workflows.

Data-independent acquisition (DIA) has become the standard for robust, unbiased identification and quantitation, and the introduction of TIMS and PASEF further improved ion efficiency and added CCS information. Combining DIA with TIMS led to dia-PASEF and its variants, offering faster cycles and deeper coverage.

Diagonal-PASEF methods such as synchro-PASEF and midia-PASEF synchronize quadrupole windows with ion mobility, improving peak sampling and quantitative performance. Although software support is growing (e.g., Spectronaut, DIA-NN, alphaDIA), optimization remains ongoing. In this study, we develop improved Spectronaut algorithms and demonstrate enhanced diagonal-PASEF performance on the timsTOF HT.

Methods

Tryptic digests of human cell lysate, *C. elegans*, *E. coli*, and *S. cerevisiae* (all in-house) were used to benchmark diagonal-PASEF. Peptides were separated on an IonOpticks Aurora CSI 75µm×250mm column using an EASY-nLC 1200 coupled to a timsTOF HT with a 17-min nonlinear gradient. The reference dia-PASEF method (12 ramps, 50 variable boxes, 0.5 Th overlap) was generated in-house and operated at a 0.99 s cycle time. Diagonal-PASEF methods were created using the timsControl Window Editor with a 200m/z width, resolved into 1–12 slices; cycle time scaled with slice number. All data were processed in Spectronaut v20.2 using directDIA, with each condition searched separately and replicates grouped per search.

Results

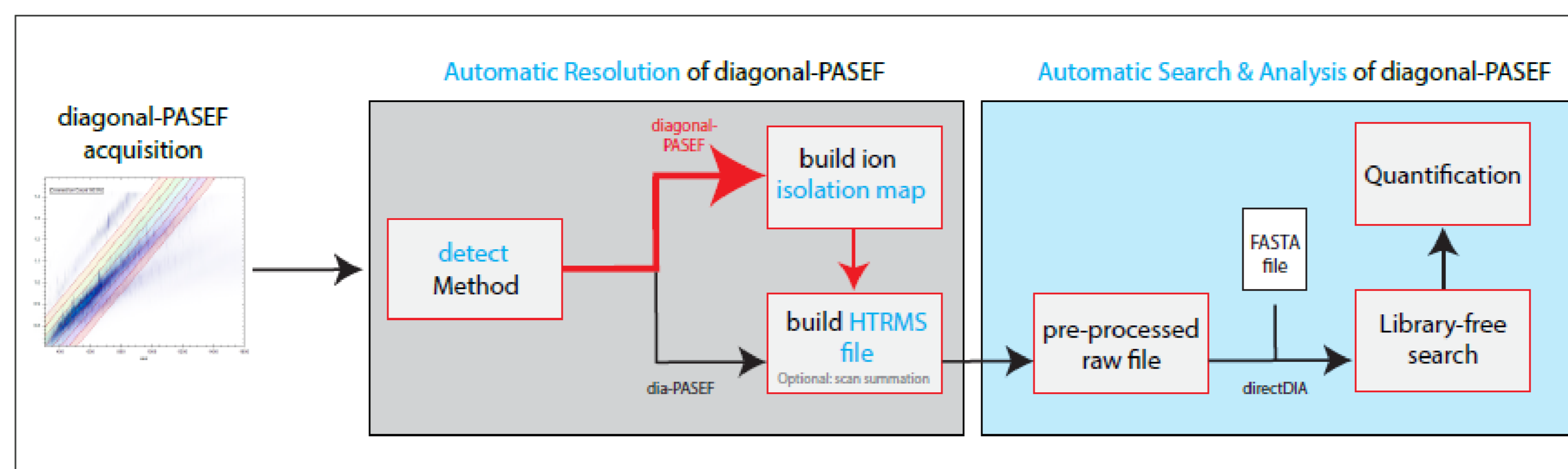


Figure 1: Spectronaut (version 19 or later) allows for the seamless integration of diagonal-PASEF workflows into the existing analysis pipeline. Shown is a simplified schematic visualization of a directDIA search and analysis workflow.

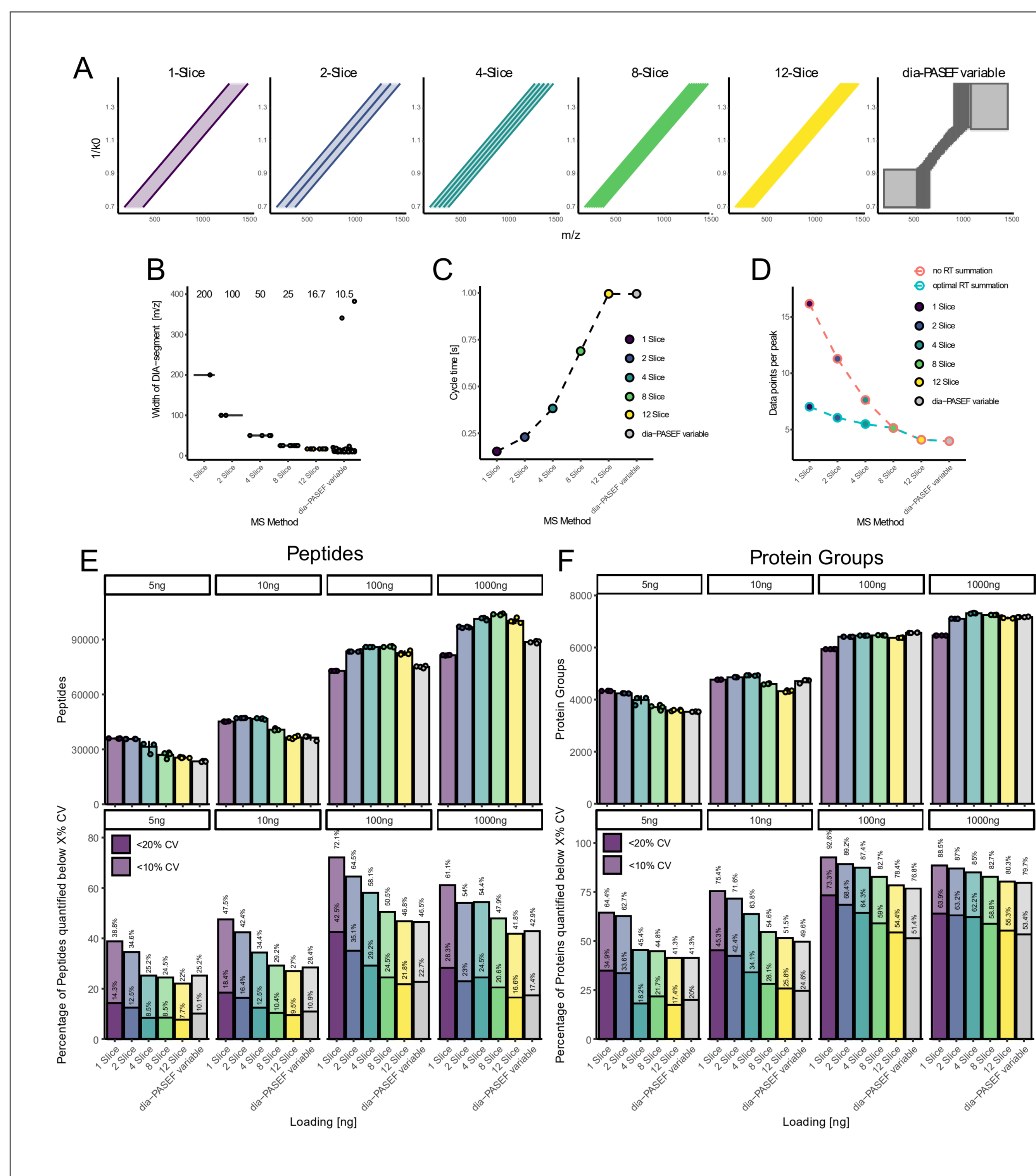


Figure 2: Diagonal-PASEF achieves higher overall identification and precision compared to dia-PASEF. A: schematic of diagonal-PASEF and dia-PASEF methods used. B: width of the DIA-segments of each applied method for tested methods from A. Each datapoint indicates an individual slice or DIA-segment. Boxplots have been added behind the data points to visualize the distribution of the DIA segment width. C: cycle time of tested methods for loading ramp experiment depicted in A. D: data points per peak of tested methods for loading ramp experiment pre (red) or post (blue) retention time summation. For the RT summation, the ideal value has been applied as follows: 1-slice: 4xRTs, two slice: 3xRTs, 4-slice: 2xRTs, 8-slice: 1xRTs, 12-slice: 1xRTs. E–F, results of loading ramp. Top: Average peptide (E, left) or protein groups (F, right) identifications obtained for all tested methods across the indicated loadings. Error bars indicate standard deviation of mean. Each datapoint indicates an individual acquisition (n = 4). Bottom: Percentage of peptides (E, left) or protein groups (F, right) quantified below 10% or 20% coefficient of variation (CV) against overall identifications. For panels D and E, the optimal retention time summation was applied to all diagonal-PASEF acquisitions.

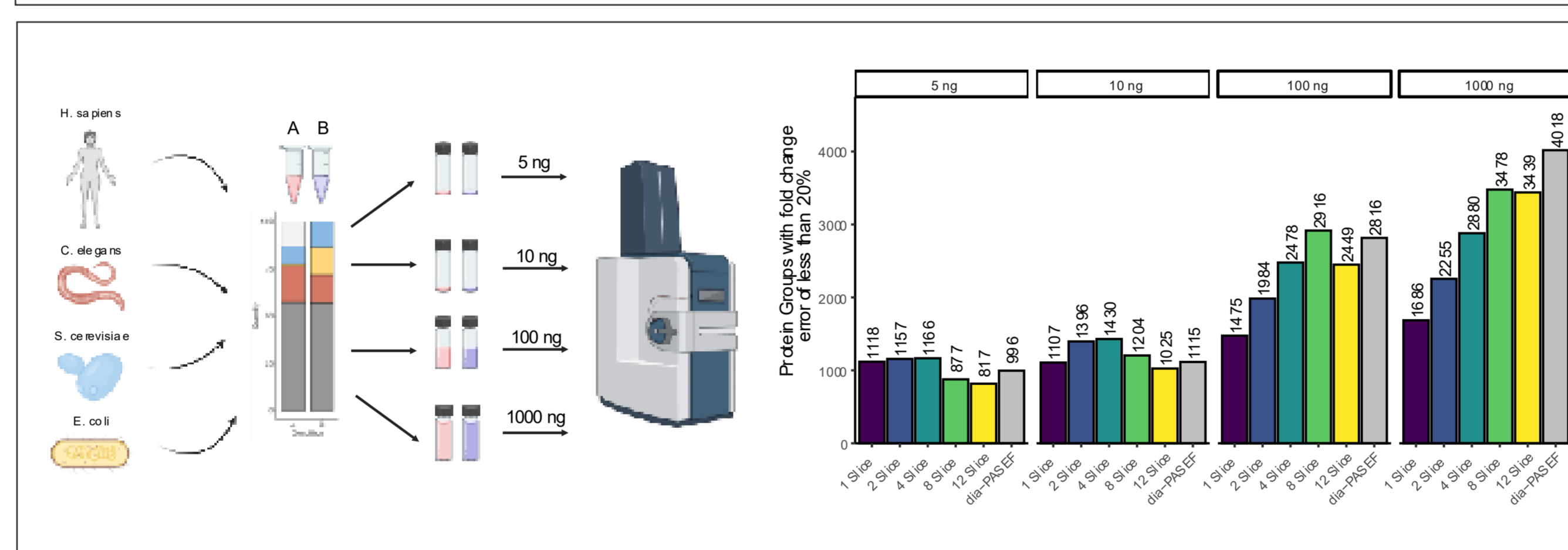


Figure 3: Experimental approach conducted for the controlled quantitative experiment (CQE) and composition of samples “A” and “B” based on protein amount. Interpretation of the data. Number of protein groups quantified with a foldchange error of less than 20% are shown.

Summary

Diagonal-PASEF enables highly efficient ion utilization by covering the TIMS elution space with very few slices, resulting in exceptionally short cycle times (~0.05–0.5 s). These fast methods allow oversampling of chromatographic peaks, and we demonstrate that summing consecutive MS1 and MS2 scans along the retention time (RT) dimension substantially improves signal-to-noise ratios. This effect is most pronounced for low sample loads (5–10 ng), where few-slice methods combined with RT summation yield notably higher peptide identifications and precision compared to methods with many slices. Across HeLa datasets, diagonal-PASEF with RT summation consistently outperformed dia-PASEF

at the peptide level and achieved similar or slightly higher protein identifications. The improved signal-to-noise and precision translated into broader sequence coverage and thus can help for enhanced detection of peptide-level features such as PTMs, isoforms, and proteoforms. In controlled quantitative experiments, diagonal-PASEF also recovered more statistically significant differentially abundant peptides and proteins below a 1% error rate, highlighting its superior statistical power. While complex mixtures at very high loadings still favor dia-PASEF, diagonal-PASEF shows clear advantages for the majority of proteomics workflows that focus on a single organism and require high-quality peptide-level data.

These results establish diagonal-PASEF as a powerful acquisition strategy for proteomics.

Conclusion

- Spectronaut fully supports all diagonal-PASEF methods.
- Diagonal-PASEF in combination with retention time summation results in increased identification rates and enhanced quantitative precision across varying input amounts.

Diagonal-PASEF

