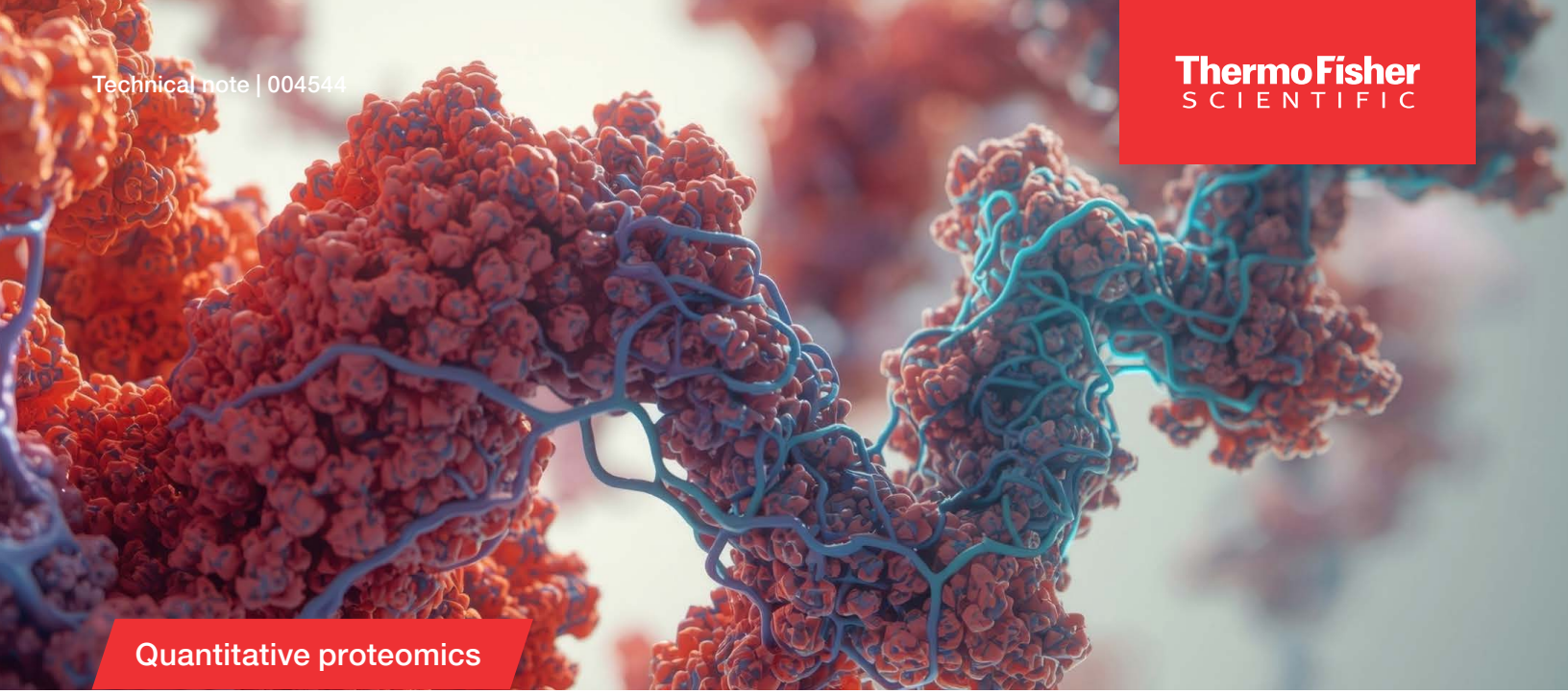


Technical note | 004544

Quantitative proteomics

High-throughput quantitative proteomics with Evosep Eno and the Orbitrap Astral Zoom Mass Spectrometer

Authors

William J Comstock[1], Iulia Macavei[2], Brett Larsen[3], Tabiwang N. Arrey[4], Till Reinhardt[4], Eugen Damoc[4], Nicolai Bache[2], Dorte Bekker-Jensen[2], Tonya Pekar-Hart[1], Amirmansoor Hakimi[1]

[1]Thermo Fisher Scientific, San Jose, CA, USA

[2]Evosep Biosystems, Odense, Denmark

[3]Thermo Fisher Scientific, Mississauga, ON, Canada

[4]Thermo Fisher Scientific, Bremen, Germany

Keywords

Orbitrap Astral Zoom, Evosep Eno, high-throughput proteomics, narrow-window data-independent acquisition, nDIA, 500 SPD

Goal

To assess proteome coverage and quantitative performance of the Thermo Scientific™ Orbitrap™ Astral™ Zoom Mass Spectrometer coupled with the Evosep™ Eno liquid chromatography (LC) system for high-throughput proteomics using narrow-window data-independent acquisition (nDIA).

Introduction

High-throughput proteomics is a critical enabling technology for large clinical studies, high-content drug screening, and systems biology research, allowing for comprehensive and quantitative analysis of complex proteomes across large sample cohorts within practical timeframes. As biological and translational studies expand in scale and complexity, the ability to generate deep and reproducible proteome measurements across thousands of samples has become increasingly essential for robust biomarker discovery, network-level systems modeling, and downstream analyses. In these contexts, accurate and precise protein quantitation must be maintained without sacrificing throughput, placing stringent demands on the performance and robustness of both LC instruments and mass spectrometers.

To meet these increasing analytical demands, the Evosep Eno was developed as a high-throughput LC system that combines robustness, ease of use, and highly reproducible chromatography. It offers six standardized methods: 30, 60, 100, 200, 300, and 500 samples per day (SPD), allowing users to select the throughput that aligns best with their study goals.[1] These predefined methods ensure consistent gradient delivery, highly stable retention times, and reproducible system performance across instruments and laboratories.[2] By reducing LC-related variability and simplifying everyday operation, the Evosep Eno offers a strong and consistent foundation for large-scale proteomics workflows, particularly those relying on short gradients and high reproducibility required for downstream quantitative analysis.

Label-free analyses using short chromatographic gradients enabled by LC systems like the Evosep Eno are often paired with data-independent acquisition (DIA) due to high reproducibility and data completeness along with deep proteome coverage. Short gradients generally feature many peptides coeluting within a short timeframe, requiring narrow precursor isolation DIA windows to preserve precursor selectivity. nDIA uses isolation windows (IW) more similar to that of data-dependent acquisition (DDA) methodology, leading to reduced incidence of chimeric spectra and enabling more confident peptide identification. However, sampling narrow windows across a broad mass range within short chromatographic timescales can result in prohibitive cycle times and compromised quantitation. The Orbitrap Astral Zoom Mass Spectrometer addresses these requirements by combining exceptional acquisition speed, sensitivity, and dynamic range, enabling routine nDIA analyses at scale.

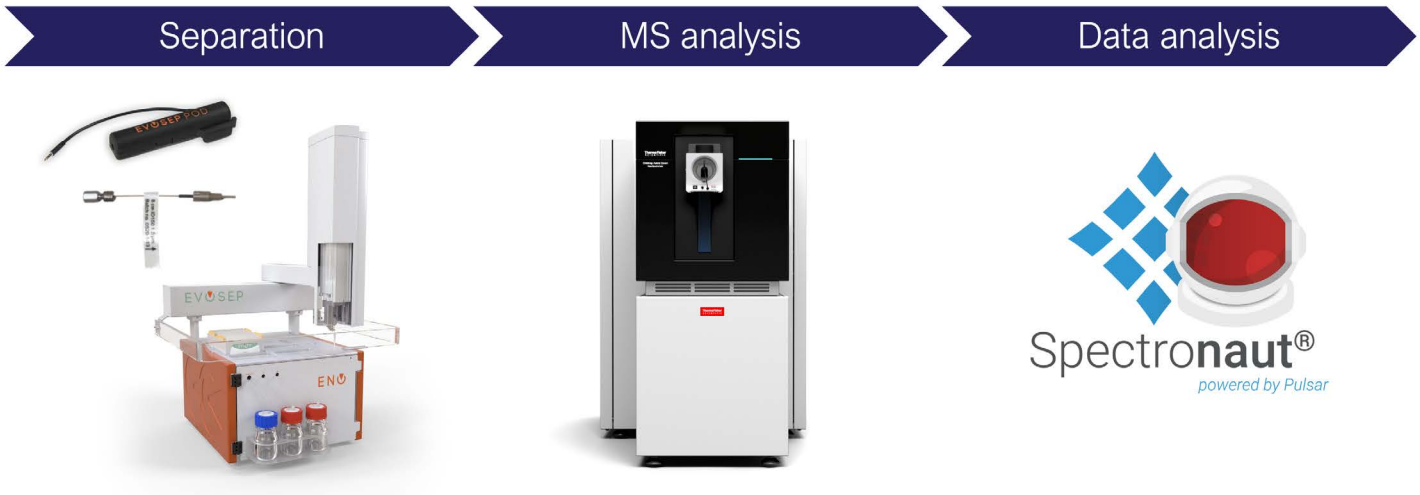


Figure 1. High-throughput nDIA workflow using Evosep Eno coupled to the Orbitrap Astral Zoom Mass Spectrometer.

When coupled, the Evosep Eno system and Orbitrap Astral Zoom Mass Spectrometer present a powerful, robust, high-throughput solution for complex proteome analysis at scale. Here, we demonstrate how this setup enables deep and reproducible proteome profiling across 30, 60, 100, 200, 300, and 500 SPD. With the ultra-high throughput of 500 SPD, more than 6,800 protein groups and 74,000 modified peptides were identified from five replicates of 200 ng Thermo Scientific™ Pierce™ HeLa Protein Digest Standard, with effective injection-to-injection run times of 2.9 minutes. High precision and consistent chromatographic performance were maintained across runs, with retention time variation below 1 second, highlighting the platform's ability to deliver reliable, high-quality quantitative proteomics at ultra-high throughput.

Experiment

Recommended consumables

- Fisher Chemical™ Optima™ LC-MS Grade Water with 0.1% Formic Acid (FA), (Part No. LS118-500)
- Fisher Chemical™ Optima™ LC-MS Grade 80% Acetonitrile (ACN with 0.1% FA (Part No. LS122500)
- Fisher Chemical™ Optima™ LC-MS Acetonitrile (Part No. A955-1)
- Fisher Chemical™ Optima™ LC-MS Isopropanol (Part No. A461-212)
- Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard (Part No. A47996)
- Pierce HeLa Protein Digest Standard (Part No. 88328)
- Thermo Scientific™ Pierce™ Peptide Retention Time Calibration Mixture (Part No. 88321)
- Evosep™ Evotip™ Pure (Part No. EV2011)

LC analytical columns and emitters

- Evosep™ Performance column, 200/300/500 SPD (Part No. EV1182)
- Evosep Performance column, 60/100 SPD (Part No. EV1109)
- Evosep Performance column, 30 SPD (Part No. EV1137)
- Evosep™ Stainless steel emitters, ID 30 µm (Part No. EV1086)

Instrumentation

- Evosep Eno
- Orbitrap Astral Zoom Mass Spectrometer
- Thermo Scientific™ EASY-Spray™ Ion Source
- Evosep™ Pod for EASY-Spray Ion Source (Part No. EV1187)

Software

- Biognosys Spectronaut® 19.7 software
- MacCoss Lab Software Skyline-Daily V25.1

Sample preparation

For nDIA optimization and final benchmark data acquisition, lyophilized Pierce HeLa Digest/PRTC Standard (10 µg/vial) was reconstituted in H_2O + 0.1% FA by sonication at room temperature for 3 minutes to reach a final peptide concentration of 10 ng/µL. For quantitative assessment of 500 SPD nDIA methods, 0.5 pmol/µL Pierce Peptide Retention Time Calibration Mixture was spiked into HeLa digest standard such that 20 µL of the final solution contained 200 ng of HeLa digest and peptide retention time calibration (PRTC) loads ranging from 1 fmol to 100 fmol. For all samples analyzed, 20 µL of peptide solution was loaded onto Evotips after conditioning with acetonitrile + 0.1% FA, wetting with isopropanol, and equilibration with 0.1% FA, after which tips were washed with 0.1% FA and stored wet at 4 °C until mass spectrometry (MS) analysis.[3]

LC-MS analysis

Samples were measured on the Orbitrap Astral Zoom Mass Spectrometer in DIA mode. Samples were separated using an Evosep Eno running standard high-performance LC methods for each SPD. The recommended Performance column was used for each SPD as shown in Table 1. Columns were connected online via an EASY-Spray Ion Source to the mass spectrometer, where the source was outfitted with an Evosep Pod to contain and heat the columns to 40 °C.[4] MS method parameters are listed in Table 2. The DIA isolation window schemes and maximum injection times (IT) selected for each SPD are listed in Table 3.

Data processing and analysis

Pierce HeLa Digest/PRTC Standard data were analyzed in triplicate for each injection during parameter optimization and in five replicates for final analyses using optimal MS parameters. Replicates of each SPD were analyzed together using the Biognosys directDIA® workflow with default settings in Spectronaut 19.7 software. The data were searched against the *Homo sapiens* SwissProt database, including verified isoforms (TaxID = 9606: 42,252 entries) and contaminants. Metrics regarding identified precursors, protein groups, and modified peptides were acquired from Average Profiles in search reports. PRTC peak metrics and quantitation were performed by importing DIA runs into MacCoss Lab Skyline software.

Table 1. Evosep analytical columns used for each standard method.

SPD	Column length (cm)	Evosep Part No.
500, 300, 200	4	EV1182
100, 60	8	EV1109
30	15	EV1137

Table 2. SPD-independent MS method parameters.

Global parameters (source and MS)	
Positive ion voltage (V)	1,900
Ion transfer tube temperature (°C)	290
Expected peak width (s)	6
Default charge state	2
Orbitrap full scan properties	
Orbitrap resolution	240,000
RF lens (%)	40
Scan range (*m/z*)	See Table 3
Normalized AGC target (%)	500
Maximum IT (ms)	See Table 3
Microscans	1
Data-independent acquisition properties	
Precursor mass range (*m/z*)	See Table 3
Isolation width	See Table 3
Window placement optimization	On
Detector type	Astral
HCD collision energy (%)	25
Scan range (m/z)	150–2,000
RF lens (%)	40
Pre-accumulation	On
Maximum IT (ms)	See Table 3
AGC target (%)	500
Loop control	Time
Time (s)	0.6

Table 3. SPD-dependent MS parameters.

SPD	IW width (Th)	Orbitrap max. IT (ms)	Astral max. IT (ms)	Precursor mass range (*m/z*)	DIA scan range (*m/z*)
500	4	3	3	400–800	400–800
300	4	3	3	400–800	400–800
200	3	3	3	400–800	400–800
100	3	3	3	400–800	400–800
60	3	3	3	400–800	400–800
30	2	5	3.5	380–980	380–980

Results

To investigate the performance of the Orbitrap Astral Zoom Mass Spectrometer using standard Evosep Eno LC methods ranging from 30 to 500 SPD, we analyzed HeLa sample loads of 200 ng containing 100 fmol of heavy-labeled PRTC peptides. Given the differences in elution profile across the six standard gradient lengths tested, different isolation widths and maximum IT were found to be optimal for each standard method. Optimal parameters were chosen based on the number of protein groups identified while maintaining exceptional quantitative precision.

Optimizing MS parameters for MS²-based quantitation at high throughput

Using the fastest chromatographic gradient, many combinations of IW and IT were tested. Figure 1 shows the proteome coverage and precision of the 500 SPD method as a function of IW and Astral IT. While 2 Th windows allowed for exceptionally high protein group IDs, fewer data points per peak (DPPP) resulted, leading to higher quantitative variation. By widening the IW to 3–6 Th, the cycle time was reduced leading to better peak sampling and improved protein and peptide quantitative precision. With respect to fill time, 5 ms IT helps low-abundance precursors reach the automatic gain control (AGC) target, while 3 ms IT reduces cycle times and increases peak sampling to keep pace with rapid peptide elution during the 2.3-minute acquisition window. At 500 SPD, 4 Th IW with a 3 ms maximum IT provided the best balance between proteome depth and quantitative precision, yielding 6,579 protein groups and 71,279 modified peptides (200 ng HeLa; n = 3) with median coefficient of variation (CVs) of 4.4% and 9.6%, respectively.

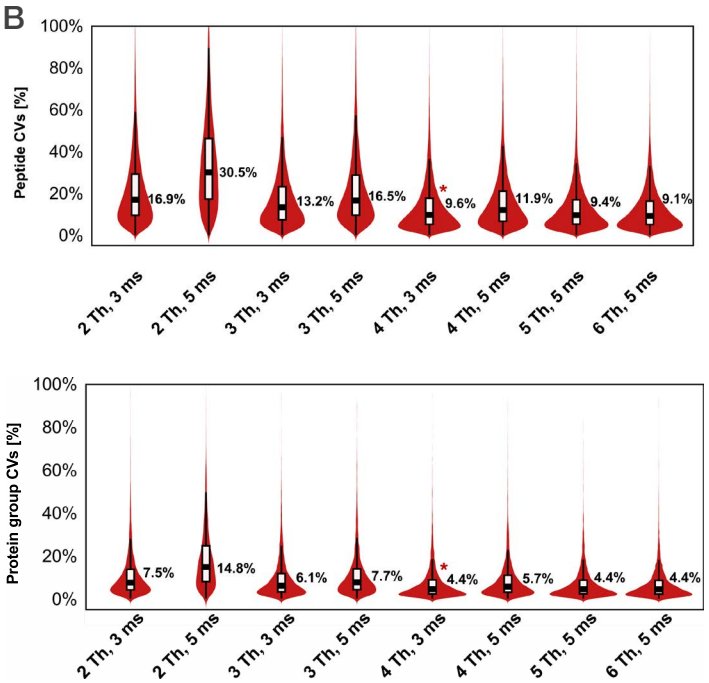
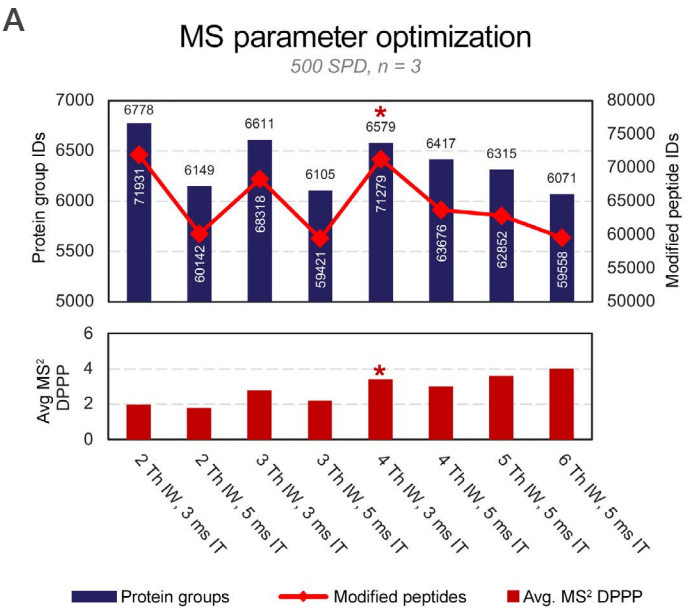


Figure 2. Optimization of MS parameters for standard high-throughput 500 SPD Evosep Eno methods. (A) Number of average identified protein groups and modified peptides using different nDIA isolation widths and maximum IT. Each method tested featured three replicates of 200 ng HeLa/PRTC standard searched together. Median MS/MS DPPP are shown below. (B) Median peptide and protein group percent CVs shown above and below, respectively. In both panels, the chosen optimal MS method is denoted with an asterisk.



Evaluating MS²-based quantitative performance from 30 to 500 SPD

In addition to the ultra-high throughput 500 SPD method, five other standard Evosep Eno methods were tested using the Orbitrap Astral Zoom Mass Spectrometer ranging from 300 to 30 SPD (Figure 3A). As the gradient length increased, increases in precursor, peptide, and protein group identifications (Figure 3B) were observed. With five replicates of 200 ng HeLa/PRTC standard injections per method, up to 190,732 modified peptides and 10,595 protein groups were identified using the 30 SPD method (Figure 3B). MS parameters also maintained enough DPPP to maintain low CVs across all methods, with no median protein group CVs exceeding 5.2% and peptide CVs exceeding 13%, respectively (Figure 3C). All methods preserved dynamic range with no loss of signal fidelity across all throughputs (Figure 4).

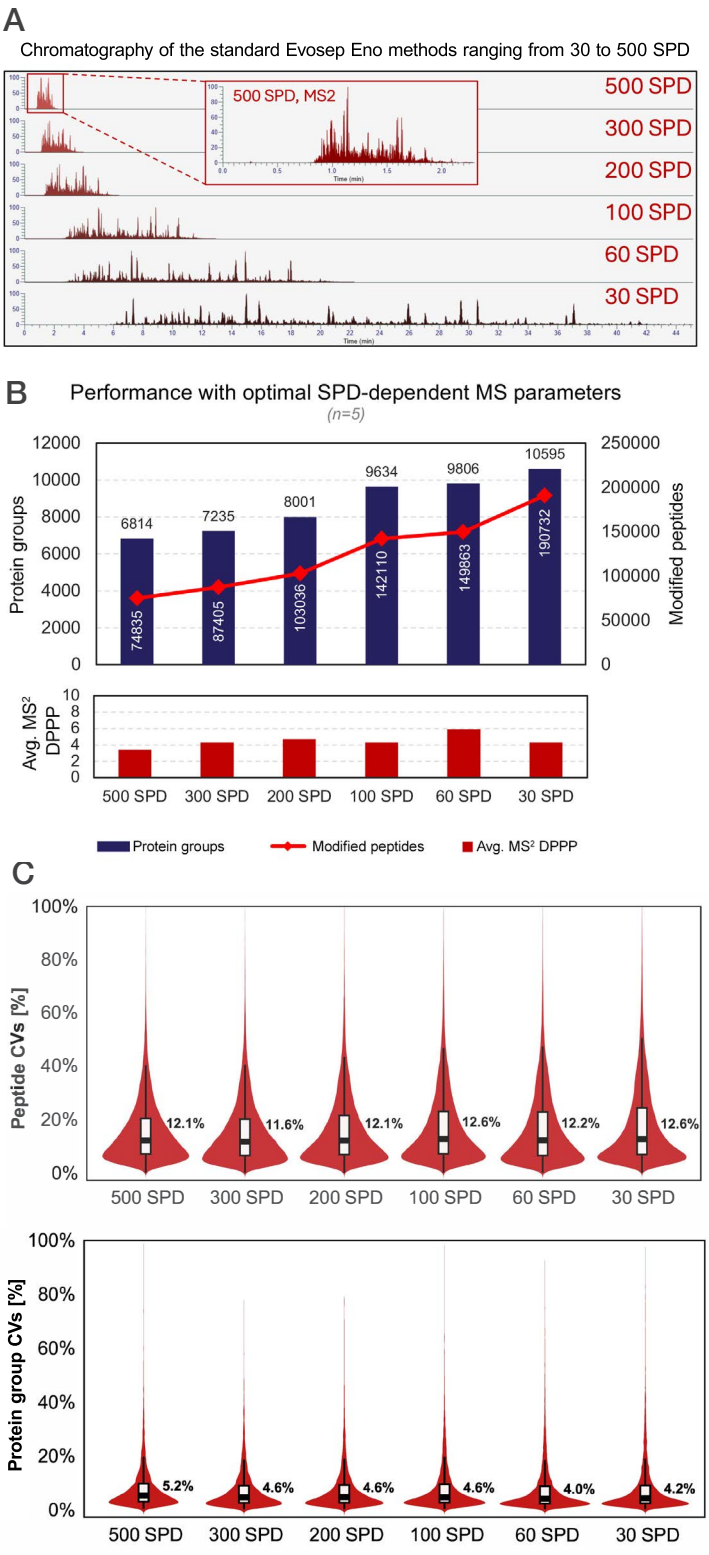


Figure 3. Quantitative performance and precision across sample throughputs from 30 to 500 SPD with 200 ng HeLa/PRTC standard digest. (A) Base peak chromatograms of the six standard Evosep Eno gradients. (B) Average protein group and modified peptide identifications (n = 5) across the six standard Evosep Eno methods using optimized MS parameters detailed in Tables 2 and 3. Median data points per DPPP are shown below. (C) Median peptide and protein group CVs for each throughput method above and below, respectively.

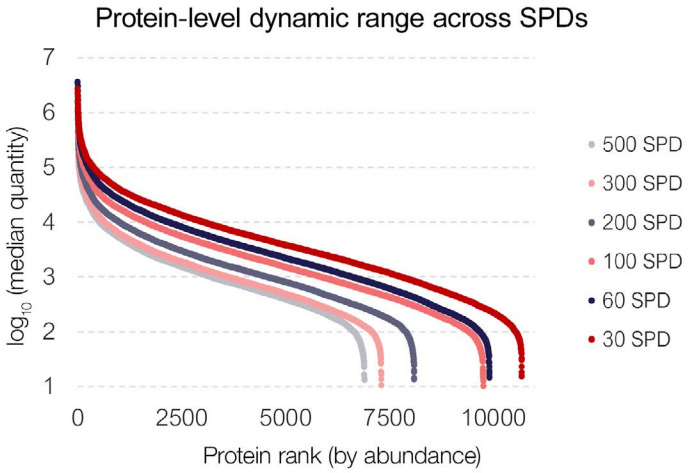


Figure 4. Protein-level rank intensity plot across 30 to 500 SPD methods showcasing preservation of dynamic range on the Orbitrap Astral Zoom Mass Spectrometer with predictable depth-throughput tradeoffs and no loss of high-abundance signal fidelity.

Evaluation of chromatographic reproducibility and quantitative accuracy at 500 SPD

Given the high performance of the Evosep Eno and Orbitrap Astral Zoom Mass Spectrometer, maintaining robust and consistent performance across large sample sets is essential, particularly at the highest throughput. Continuous sample acquisition has the potential to exhibit chromatographic stochasticity depending on the robustness of instrument fluidics, configuration, and columns. To assess reproducibility of the Evosep Eno and Orbitrap Astral Zoom Mass Spectrometer, the elution profiles of the Pierce PRTC peptides within 50 ng of HeLa/PRTC standard were monitored using Skyline software across 94 LC-MS runs performed consecutively using DIA isolation widths of 5 *m/z* and maximum ITs of 6 ms.

The 11 isotopically labeled PRTC peptides observed within the chosen scan range (400–800 *m/z*) eluted between 0.7 and 1.9 minutes within the 2.3-minute 500 SPD active gradient (Figure 5A). The retention times for each respective PRTC peptide remained consistent across all 94 runs, with all peaks featuring a standard deviation of less than 0.4 seconds (Figures 5B and 5C). These peaks exhibited a median full width at half maximum (FWHM) of 1.2 seconds, high peak symmetry, and an average of six data points were captured per peak (Figure 5D).

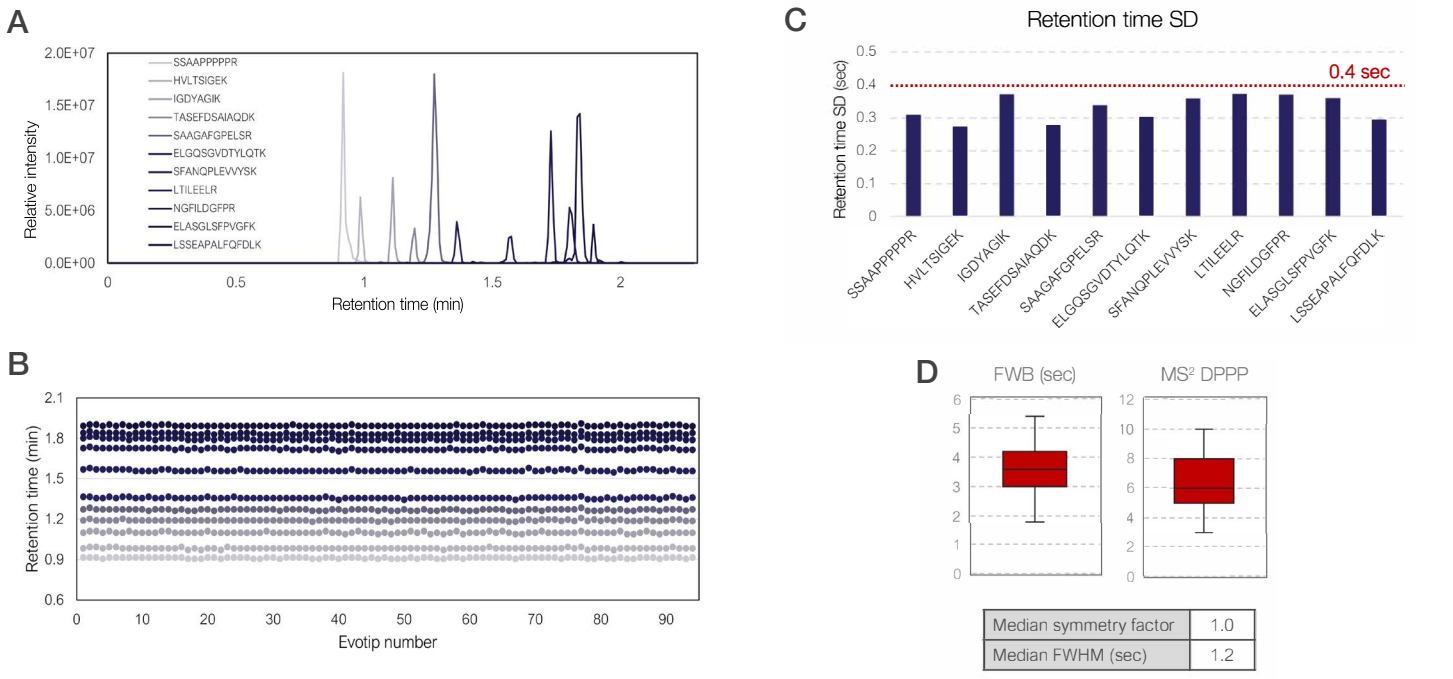


Figure 5. Chromatographic reproducibility across 94 replicates at 500 SPD. (A) XIC of 11 PRTC peptides spiked into 50 ng HeLa digest standard. (B) PRTC peptide retention times as a function of LC-MS run time. (C) Retention time standard deviations in seconds shown per PRTC peptide across the 94 LC-MS runs. (D) Peak metrics showing full width at base (FWB), MS² DPPP, median peak symmetry factor, and median FWHM across the 94 LC-MS runs.

To further evaluate the quantitative performance of the Orbitrap Astral Zoom Mass Spectrometer nDIA methods at high throughputs enabled by Evosep Eno, PRTC mixture was spiked into 200 ng of HeLa digest at five different concentrations ranging from 1 to 100 fmol before loading onto Evotips, equating to a range of 66.7 amol to 6.7 fmol per individual peptide. Five replicates of each sample were prepared, totaling 25 runs. Across all runs and PRTC concentrations, each PRTC peptide featured retention time standard deviations of under 0.5 seconds. Untargeted nDIA analysis showed high sensitivity and a linear response observed for peptides across the chromatographic gradient, demonstrating robust peptide quantitation at 500 SPD.

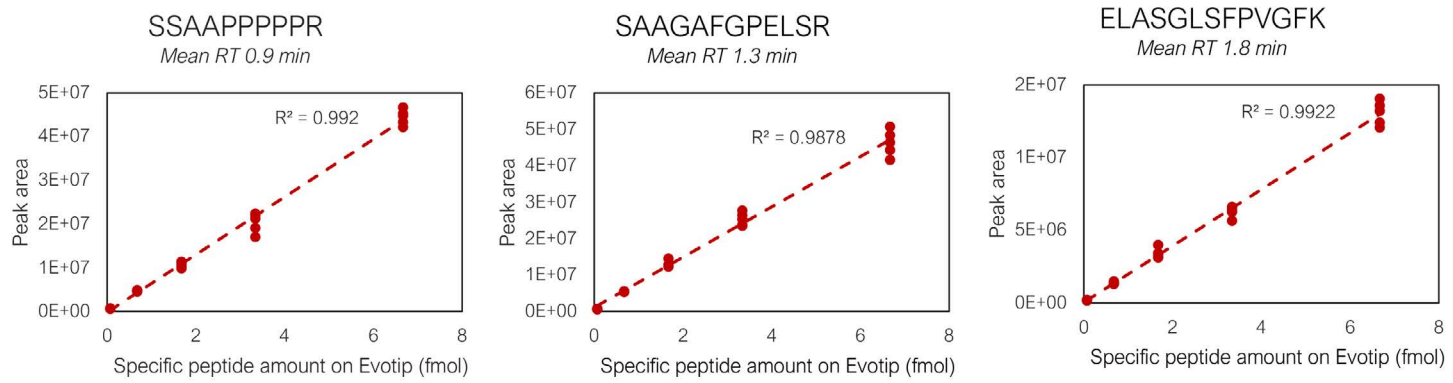


Figure 6. Standard curves of three PRTC peptides selected across the 500 SPD chromatographic gradient.

Summary

Seamless and easy integration for high-throughput proteomics

Evosep Eno integrates seamlessly with the Orbitrap Astral Zoom Mass Spectrometer, supported by EASY-Spray source-compatible columns, emitters, and the Evosep Pod column oven for a simple, streamlined, high-throughput workflow.

Deep proteome coverage at 500 SPD

Optimized MS parameters for the standard 500 SPD Evosep Eno gradient enabled identification of more than 6,500 protein groups and 71,000 modified peptides from three replicates of 200 ng Pierce HeLa Digest/PRTC Standard.

Reproducible quantitation across standard gradients

Across all standard Evosep Eno gradients, the workflow delivered highly reproducible label-free quantification, with median protein group %CVs at or below 5.2%, while identifying more than 10,500 protein groups and 190,000 modified peptides from five replicates of 200 ng Pierce HeLa Digest/PRTC Standard at 30 SPD.

Robust chromatography at ultra-high throughput

Chromatographic performance remained stable and robust at 500 SPD, with nearly 100 consecutive runs showing PRTC retention time standard deviations below 0.4 seconds and a median of 6 DPPP.

Strong linearity for confident quantitation

Quantitative performance remained strong at 500 SPD, with PRTC peptides showing excellent linearity across the active gradient (R^2 >0.98) when spiked from attomolar to femtomolar concentrations.

References

1. The six standard methods: Built for standardization. https://www.evosep.com/evosep-eno-methods/standard-methods/
2. Reproducible, inter-laboratory, scalable, and routine LC-MS-based proteomics with the Evosep Eno. https://www.evosep.com/wp-content/uploads/2025/06/AN-043B_Eno_multi-lab-study_web.pdf
3. Loading protocol for Evotip Pure. https://www.evosep.com/wp-content/uploads/2024/03/PR-001D-Sample-loading-protocol_WEB.pdf
4. Evosep Pod user manual. https://www.evosep.com/wp-content/uploads/2026/01/UM-002C_Evosep-Pod_User-Manual_Web.pdf

Learn more at **thermofisher.com/OrbitrapAstralZoom**

TN-004544-NA-EN 0526