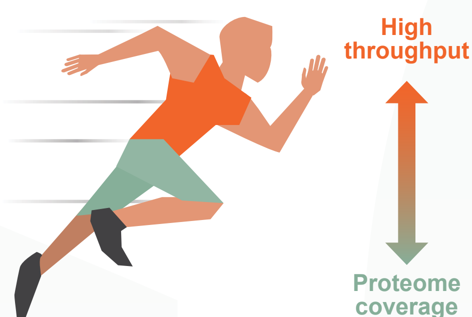
Application note

EVOSEP

Evosep One solves the proteomics dilemma - covering high throughput analysis and proteome depth

1. Introduction

Mass spectrometry (MS)-based proteomics is fast growing and provides a powerful set of technologies, with the potential to revolutionize health care and enable precision medicine. This is powered by a leap of performance over the last years including improved mass accuracy and significantly increased scan speed as well as sensitivity of modern mass spectrometers. To further integrate the technology into clinical settings, the Evosep One features a novel and extremely robust way to pre-form and elute one of six different gradients. The corresponding six standard methods ensure throughput, robustness, reproducibility and ease of use. They cover a range of use cases from ultra high throughput analysis with 500 samples per day to more comprehensive proteome analysis with only 30 samples analyzed per day (Figure 1).



	Gradient Length (min)	Cycle Time (min)	Flow Rate (µl/min)
500	2.2	2.9	4.0
300	3.2	4.8	4.0
200	5.6	7.2	2.0
100	11.5	14	1.5
60	21.0	24.0	1.0
30	44.0	48.0	0.5

Figure 1: The six standard methods with a throughput of up to 500 samples analyzed per day.

2. Minimal overhead time

The Evosep One is designed for throughput and robustness, while maintaining sensitivity for proteomics applications. Low-pressure pumps (ABCD) elute the sample from a disposable trap column, simultaneously forming a chromatographic gradient that is stored in a long storage loop. An auxiliary gradient creates an offset, ensuring efficient re-focusing of the peptides before the separation on the analytical column by a single high-pressure pump (HP) , that pushes the preformed gradient containing the analytes through the column. This simplified design enables robust operation over tens of thousands of sample injections. Furthermore, the steps between injections are performed in parallel, reducing overhead time to only 0.7 minutes on the shortest methods and 4 minutes on the longer gradients allowing for very high MS utilization time (Figure 2).

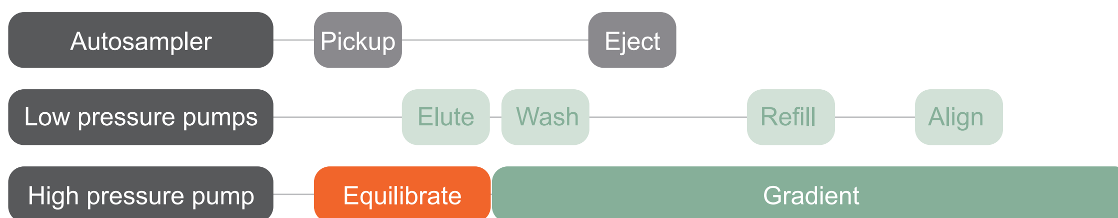
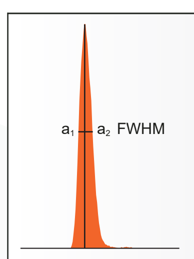


Figure 2: The steps between injections are performed in parallel to reduce overhead time.

3. High separation power

Peak capacity is the most common measure of chromatographic performance and is defined as the number of peaks, that can be resolved over a gradient. Here, we defined the peak capacity as the total number of peaks, that can be separated at full-width-half-maximum (FWHM) within an elution window, which is defined by the retention time window from the first to the last identified precursor. Longer gradients and longer columns are generally used to increase peak capacity (Figure 3), but there is not a linear correlation, and the longer gradient times reduce the sample throughput, which is a crucial factor in clinical research. For high throughput analysis of complex samples, highest peak capacity in the shortest time is a relevant measure. This is achieved by ultra fast methods employing short columns and high flow rates providing the best separation power per gradient minute (Figure 3) made affordable by the low overhead time. Importantly, the narrower peaks greatly reduce redundant sampling thus suggesting that the sensitivity of the analysis is boosted by employing the faster standard methods.



$$\text{Peak capacity} = 1 + \frac{\textit{retention time window}}{\textit{median FWHM}}$$

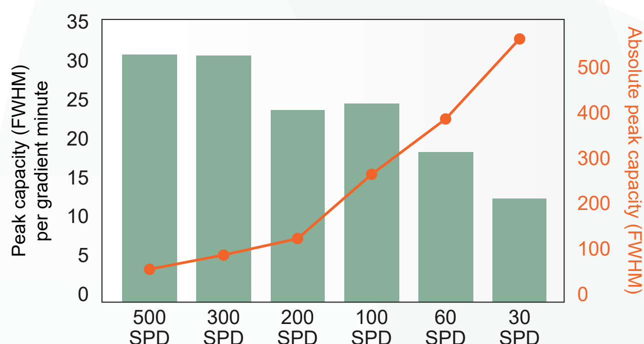


Figure 3: Peak capacity per gradient minute for each method (green) and absolute peak capacity for each method (orange).

4. Method details

HeLa digests were generated using Protein Aggregation Capture (PAC) based on-bead protein binding, clean-up and digestion with peptide amount estimated using a NanoDrop device. 200 ng were loaded on Evotips in quadruplicates and analyzed with each of the standard methods. The EV1107 Endurance colum was operated at ambient temperature (23 °C in this case) and used for the 500, 300 and 200 SPD methods. The EV1109 Performance column was used for the 100 and 60 SPD methods, while the EV1137 Performance column was used for the 30 SPD method. Both performance columns were operated at 40 °C. Samples were measured on a timsTOF Pro 2 (Bruker) mass spectrometer using the "dia-PASEF - short gradient method". Data from each peptide load was processed independently using DIA-NN (version 1.8.1) in library-free mode against the reviewed human proteome (UniProt, Nov 2021, 20,360 entries without isoforms) with MBR (match-between-runs) enabled across technical replicates. Identifications represent protein groups and precursors as stated in the pr_matrix.tsv and pg_matrix.tsv files.

5. Excellent proteome depth

Good chromatographic quality is essential, and especially to fully employ the high MS/MS scan rates on modern instruments. While the mass spectrometer accounts for mass accuracy and cycle times, the applied chromatographic performance determines the number of peptides available for ionization. Here the number of co-eluting peptides additionally impact the efficiency of the electrospray ionization (ESI) due to competition for space and charge leading to suppressive effects. All these factors directly affect the amount of detectable peptides and, hence, the number of identifiable proteins. Longer gradients increase the number of precursors with close to 70,000 identified from 200 ng of HeLa peptides analyzed with the 30 SPD method on a timsTOF Pro 2 (Bruker) with dia-PASEF (Figure 4). Whereas in the past, maximum proteome coverage was usually the goal, faster instrumentation has shifted the priorities toward analysis throughput with more biological replicates. This underlines the perspectives of proteome depth versus sample throughput. More than eight thousand precursors

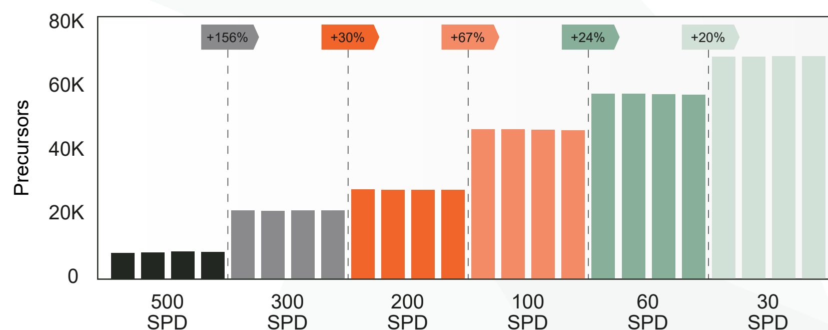


Figure 4: Precursor identifications from quadruplicate samples loaded with 200 ng peptide. Percentage bars represent increase compare to the faster method before.

are identified in only 2,2 min gradient time (500 SPD). A significant increase in precursor depth of 156% is observed when the slightly longer, 300 SPD method is employed, and only an additional 30% precursors are identified with the 200 SPD method. A linear trend is observed throughout the standard methods (Figure 4). This translates into a corresponding trend for protein groups identifications, where just above 2,000 protein groups are identified with the 500 SPD method and then gradually increasing to more than 7,000 protein groups as the throughput decreases with the 30 SPD method (Figure 5). The 100 and 200 SPD methods both identify more than 6,000 protein groups. This data shows that the overall proteome coverage is stable throughout the range of SPD methods, making the fast methods attractive to use for large screening

assays, where a certain proteome coverage is needed, while sample throughput is still critical. These numbers represent data achieved by library-free searches and can potentially be boosted further by using project specific spectral libraries. Notably, the coefficient of variations (CVs) are excellent with a median CV below 10% for all the methods, whereas the 30 SPD method achieves a remarkable CV below 5% for all identified protein groups (Figure 5).

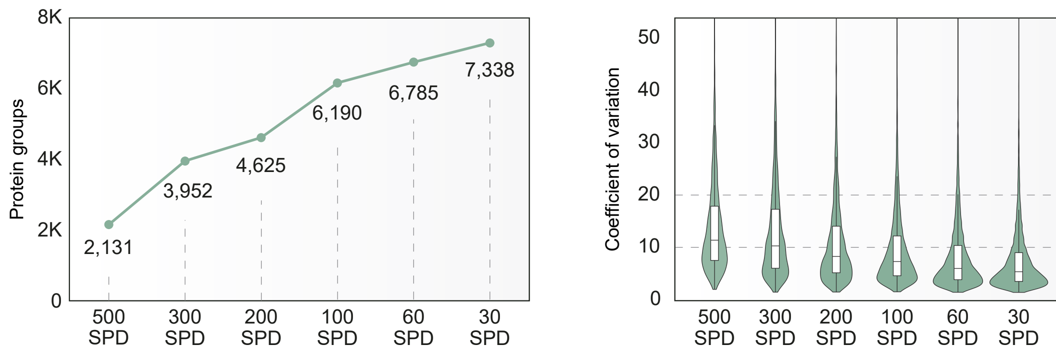


Figure 5: Protein group identifications and CVs with 200 ng peptide.

To achieve the highest protein coverage with short LC-MS time, multidimensional fractionation strategies can be employed with the fastest standard methods. In general, running many fractions on short gradients is the best compromise between instrument time used and sequencing depth obtained. For high-throughput applications, the 300 samples per day method is an excellent candidate to take advantage of the high sequencing speed, which is reflected by the number of precursors and proteins identified per minute (Figure 6).

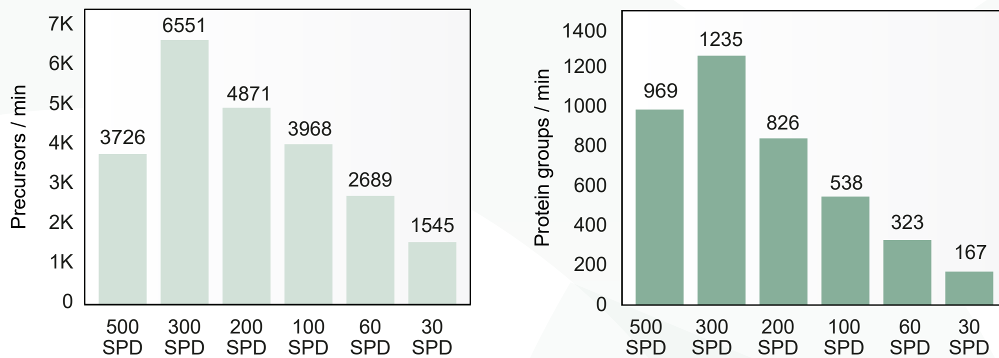


Figure 6: Precursor and protein identifications per minute from samples loaded with 200 ng peptide.

7. Conclusion

With the six standard methods, the Evosep One covers a range of use cases from comprehensive proteome analysis with fractionation strategies to ultra high-throughput single-shot analysis. With each method presented in relation to each other in terms of identifications of precursors and proteins, it is important for the individual researcher to carefully review the optimal level of proteome coverage in relation to sample throughput before starting a series of experiments. As mass spectrometers are likely to become faster and even more sensitive in the future, we expect high-throughput applications to benefit from the higher efficient peak capacity per gradient minute of the shorter methods.

EVOSEP

AN-011B 23/05