Application Note

EVOSEP

Evosep Eno - high sensitivity and quantitative accuracy for plasma proteome analysis at scale

Highlights

- Low amol detection of standard peptides with >5 orders of dynamic range.
- >90% accuracy from serially diluted plasma sample preparation workflow.

1. Targeted plasma proteomics

Recent advancements in sample preparation and LC-MS technology are enabling deeper coverage of the plasma proteome, with the potential to detect over 7,000 proteins in single-shot analyses.

While shotgun proteome profiling remains a powerful approach for biomarker discovery, targeted workflows are essential for translating these discoveries into clinically relevant applications. By focusing on specific peptides, targeted assays offer increased sensitivity. Combined with robust and sensitive low-flow LC-MS, targeted MS applications provide complementary and additional insights to immunoassay based technologies.

Scalable sample preparation is essential and should be seamlessly integrated with LC-MS to enable high-throughput quantitative measurement of captured plasma biomarkers. This is made possible by the Evosep Eno, a robust, standardized and high-throughput separations platform. Scalable and targeted workflows are enabled due to the unique features of the platform including the Evotip Pure, allowing robust and reproducible processing of thousands of samples. High retention time stability supports tight method scheduling, while defined and symmetric peak shapes ensure high sensitivity and minimal matrix interference effects.

Here, we describe a fully automated plasma workflow, coupled to a scheduled Multiple Reaction Monitoring (MRM) targeted assay via the Evosep Eno. The workflow seamlessly processes samples from raw plasma to ready-to-analyze digests in an automated manner.

Figure 1: Targeted LC-MS with Evosep Eno

2. Method details

Assay robustness was assessed by processing 200 Evotips loaded with 50 ng plasma digests. Promega LC-MS/MS Peptide Reference Mix of 6 stable-isotope labelled standard (SIS) peptides, each with 5 isotopologues, were used to gauge the quantitative performance of the Evosep Eno targeted assay. These were diluted, using neat plasma as background matrix, to span a range from 500 fmol to 2 amol.

Matrix Matched Calibration Curves (MMCC) were generated from a mixture of human plasma serially diluted with chicken plasma from 100% to 5%. 1 µl from each mixture was further diluted ~200X, as well as reduced, and alkylated. Approximately 2 µg total plasma protein was used for fully automated PAC based digestion workflow using a Biomek i7 MC liquid handler (Beckman Coulter Life Sciences) as previously described[1]. 40% of the resulting peptides were loaded onto Evotips on the liquid handler.

LC-MS/MS analyses were performed at 200 SPD, using the Evosep Eno coupled to a Waters Xevo TQ-Absolute mass spectrometer via a 4 cm Performance column operating at 40 °C (EV1182, Evosep) using a custom column oven and ESI adaptor equipped with 30 µm stainless steel emitter (EV1086, Evosep). The NanoFlow ESI source was operated at 150 °C, with capillary and cone voltages set to 3.1kV and 20V, respectively. Gas flow rates were adjusted to 350L/h for the purge and 150L/h for the cone.

The analyzer settings were as follows: LM Res1 at 2.65, HM Res1 at 15.33, LM Res2 at 2.66, and HM Res2 at 15.28. Data acquisition was performed through MassLynx software using a scheduled MRM assay monitoring 14 plasma peptides, representative of 11 plasma proteins. Enabling the automatic dwell time assignment option allowed for the optimal selection of dwell, interchannel, and interscan delay times. Skyline software was used to optimize collision energies and cone voltage settings for each peptide. These parameters were then used to develop MRM methods for initial retention time screening. The method was subsequently refined with a tightly scheduled retention time window, using a 24-second overlap.

3. Robust performance

Repeatability of the assay was monitored over 200 sequential injections of 50 ng plasma and showed excellent retention time stability with average SD of <0.4 seconds using the 200 SPD method. Excellent peak definition and symmetry was observed with mean peak symmetry of 1.1, mean

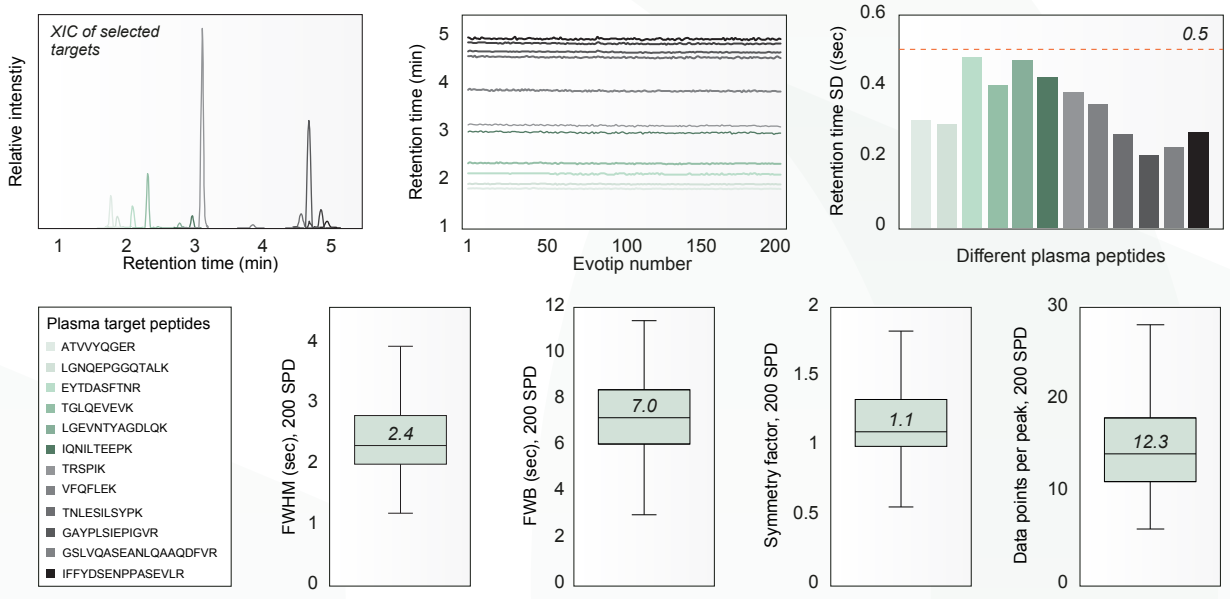

Figure 2: Chromatographic performance at 200 SPD - XIC of plasma targets (n=1), and retention time stability (n=200) with 200 SPD. Associated FWHM, FWB, symmetry factor and data points per peak.

Full Width at Half Maximum (FWHM) of 2.4 seconds and mean Full Width at Base (FWB) of 7 seconds across all target peptides. Ultimately, the robust chromatographic performance of the Evosep Eno, with a clinically relevant sample source such as plasma, allows for improved assay development with tight retention time scheduling windows leading to measurement of more targets without sacrificing cycle time and thus data points per peak (DPP). In this experiment, the mean DPP across the target peptides was 12.3, made possible due to scheduling windows of just 24 seconds.

4. Sensitivity at scale

The quantitative performance of the Evosep Eno for targeted analysis was evaluated using serially diluted, heavy labelled, peptide standards spiked in neat plasma matrix. The multiplexed MRM assay showed high sensitivity down to 2 amol and a linear response of 4 to 5 orders of magnitude, spanning the amol to fmol range. This was observed for early, middle as well as late eluting peptides thus allowing for accurate quantification across the full chromatographic gradient.

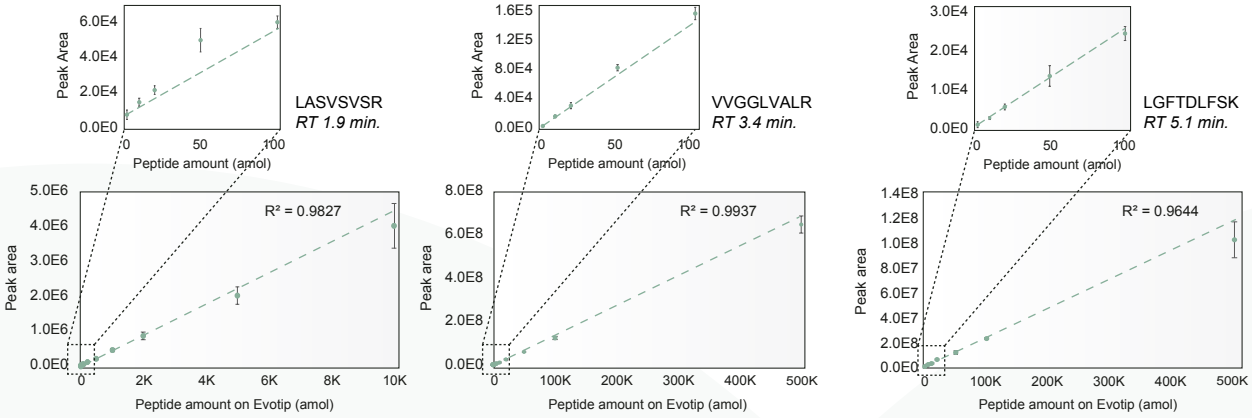

Figure 3: Standard curves of plasma spiked SIS peptides spanning the 200 SPD method with insets showing the signal response in the low amol region.

5. Quantitative accuracy

Successful quantification of clinically relevant plasma proteins requires a robust and sensitive LC-MS setup, but also seamless integration with front-end sample preparation that retains quantitative performance. MMCC were generated from serially diluted human plasma in the background of chicken plasma. This approach enables assessment of linearity in a real-world scenario where the background signal remains constant across dilutions. This allows assessing

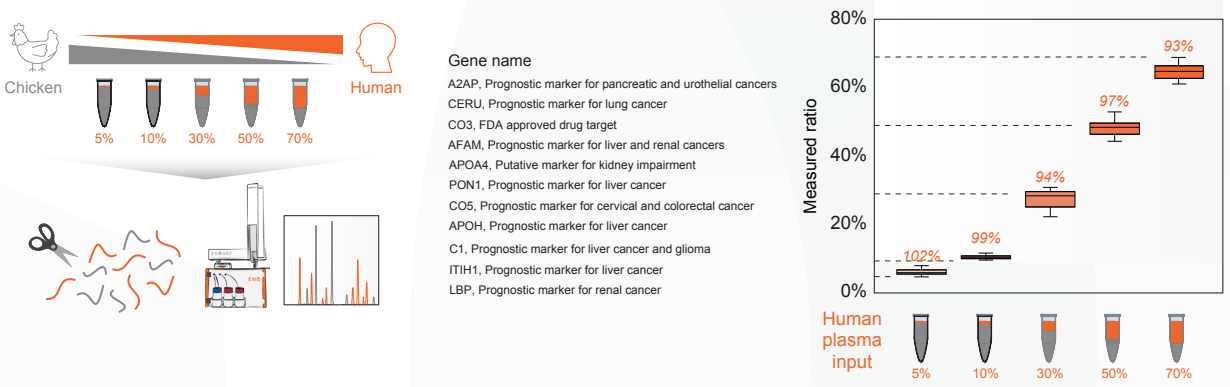

Figure 4: Experimental design for 200 SPD MRM assay monitoring 11 target proteins. Dilution series results of all measured ratios (box plot) against expected (dotted lines). Orange numbers indicate accuracy.

accuracy of the full workflow, rather than the LC-MS component alone. Raw plasma samples were digested using the Biomek i7 liquid handler running a fully automated version of the PAC workflow[1]. Summed transition areas of the target peptides, normalized to the undiluted human plasma fraction, showed a linear response across the dilution series of 93% to 102% accuracy from 70% to 5% dilution (see above). Standard curves of the target plasma peptides were constructed by plotting the expected (target) ratio against the experimentally measured ratio. Three representative peptides, that span the early, mid and late parts of the 200 SPD method (Figure 5) showing linear response (R^2 > 0.99) with high accuracy (93% - 110%) across the dilution series, confirming the quantitative performance of the complete workflow, from raw plasma to LC-MS.

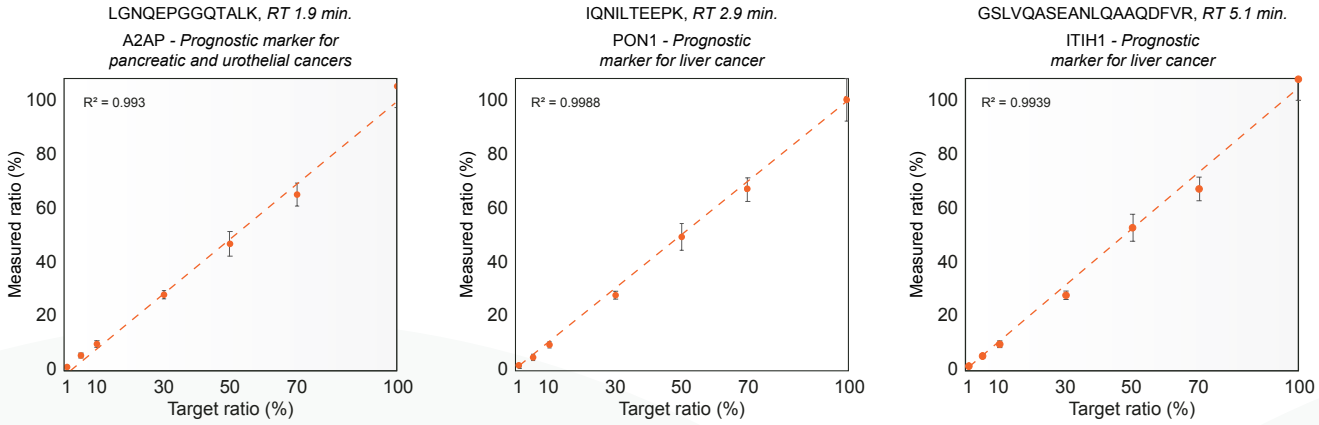

Figure 5: Experimental (target) ratio versus measured ratio across the dilution series for three plasma peptides.

6. Conclusion

Here we showcase a complete solution for accurate, precise and robust quantification of clinically relevant plasma proteins.
A high-throughput and fully automated plasma sample preparation workflow was coupled to a scheduled MRM targeted assay using the 200 SPD method with the Evosep Eno. The system showed high chromatographic stability across the 200 plasma samples with minimal RT variability (SD < 0.5), highly symmetrical peaks (mean symmetry of 1.1 and FWHM of 2.4 seconds), allowing for tight scheduling of target peptides. The highly sensitive setup is capable of measuring targets down to low amol levels across a wide dynamic range of >5 orders of magnitude. Complete workflow integration with efficient and fully automated sample preparation allows for accurate and precise quantification of clinically relevant target proteins in a cost-effective manner at scale.

Evosep Eno is for General Laboratory Use.

References

1. Evosep application note, AN-034 (2024) Scalability for high-throughput proteomics - Evotip Pure integration with the Biomek i5 liquid handler