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

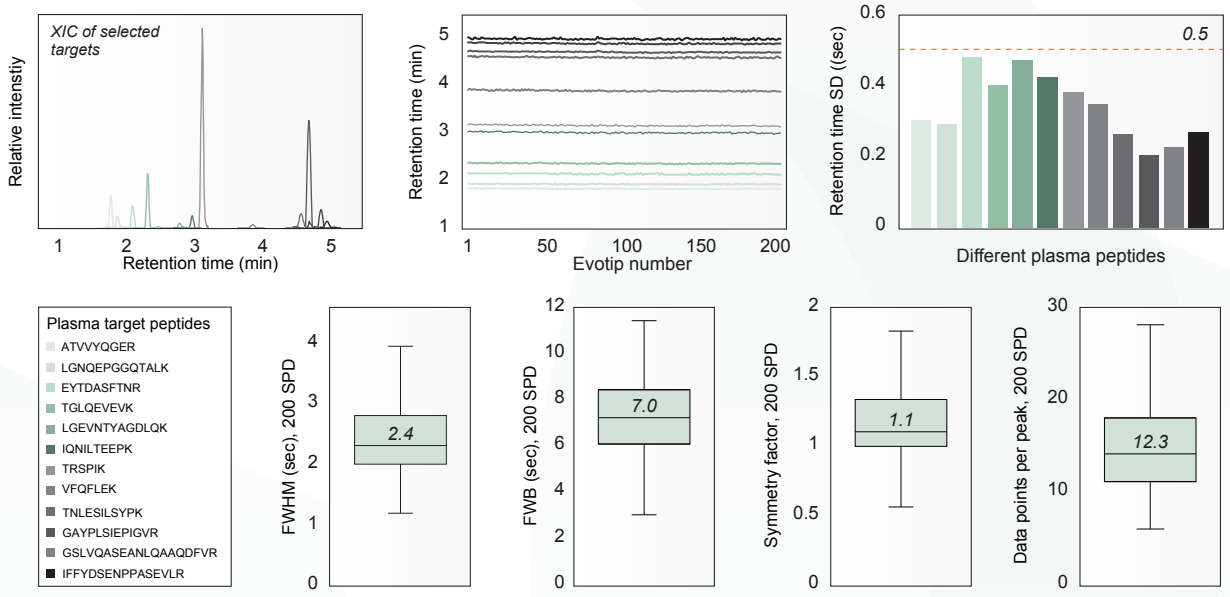


*Figure 2:* Chromatographic performance at 200 SPD - XIC of plasma targets (n=1), and retention time stability (n=200) with 200 SPD. Associated FWHM, FWB, symmetry factor and data points per peak.

## 4. Workflow reproducibility

In a controlled study, we assessed the effect of automated sample preparation using the PAC workflow and two different OT-2s. To evaluate LC-MS performance during the assessment, two sample plates were prepared simultaneously on a single OT-2 and analyzed at two independent laboratories (Figure 3). Additional variables such as input cell line, protein extraction conditions, operators, as well as source of proteolytic enzymes were kept constant at each site. Evotip blanks (EB), demonstrated less than 0.05% carryover calculated as relative precursor signal from parent sample, confirming that the Evotip Pure technology effectively protects the LC system and the analytical column. Plate blanks (PB) confirmed that the entire workflow was free from cross-well contamination (<0.20%) with minimal detectable signal in PBs attributed to trace amounts of enzymes and reagents introduced during the robotic processing steps.
The experiment also captured the variability introduced during the PAC workflow by different liquid handling robots, with coefficients of variation (CV) of 15.7% and 13.7%, respectively. Comparable CVs were obtained when identical samples were analyzed on two different LC-MS systems (13.1% and 14.1% respectively).

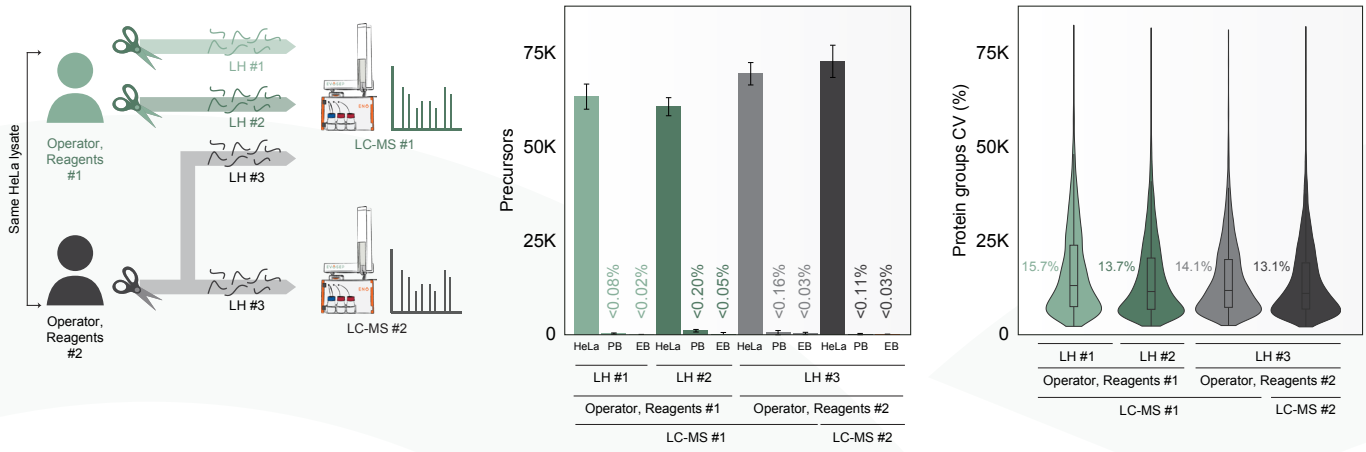


*Figure 3:* Complete workflow comparison with identified precursors, workflow QCs, and variance across identified proteins.

## 5. Inter-laboratory workflows

A comprehensive reproducibility study of the end-to-end workflows were conducted across the three different laboratories. To this end each lab prepared 84 replicate samples using automated digestion with 1 µg HeLa lysate for each sample following the OT-2 PAC protocol. Sites 1 and 2 used the same commercially available cell lysate, while site 3 utilized an internally cultured cell lysate. 50% of the resulting digest was automatically loaded onto Evotips at each site and analyzed using the 300 SPD method of Evosep Eno coupled to an Astral mass spectrometer. Comparison of digestion efficiency showed only minor differences despite the variation in lysate source and the multi laboratory setup. Across all three laboratories, 5795 protein groups were consistently identified, with approximately 75% of these quantified with a CV <20%, demonstrating strong inter-site reproducibility, even when using independently prepared HeLa digests.
The PAC workflow exhibited a dynamic range spanning 6 orders of magnitude for commonly identified proteins. High sensitivity and robustness were further demonstrated by consistent quantification (CV <20%) across a wide range of protein abundances.

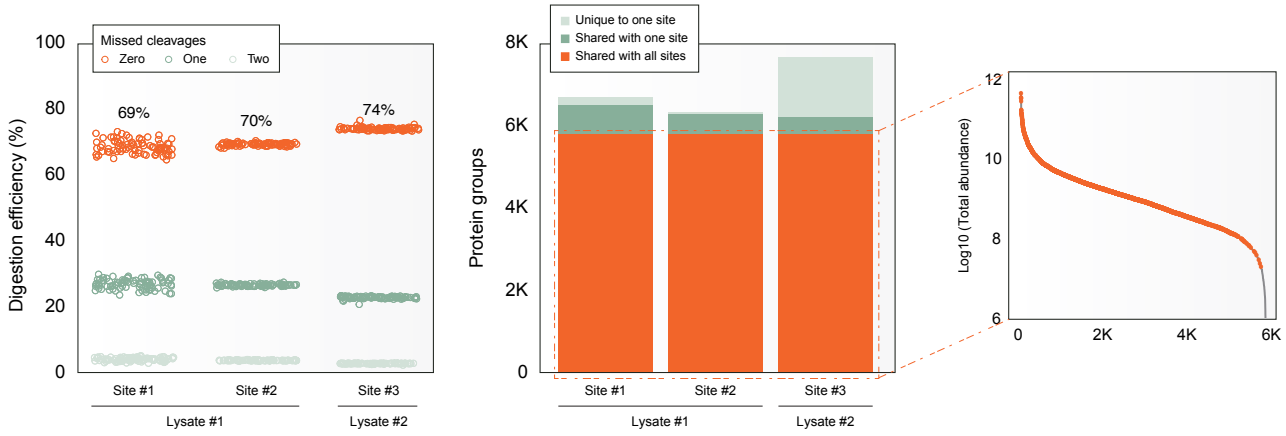


*Figure 4:* PAC workflow digestion efficiency, identification of unique and common proteins for each site and dynamic range plot of shared proteins annotated as proteins with CV below 20% in orange.

## 6. Conclusion

The complete, end-to-end workflow, multi-laboratory comparison highlights that high-throughput proteome profiling can be achieved with excellent reproducibility, enabled by the robustness of Evosep Eno and ease-of-use of the automated Evotip Pure loading.

Consistent retention times and FWHM for five diagnostic peptides across all three laboratories underscore the highly robust chromatographic performance of the Evosep Eno. The PAC protocol featuring short digestion time (4h) at ambient temperature combined with the high-throughput 300 SPD method allows for ultra-fast turnaround from sample to LC-MS readout. Despite differences in sample preparation and LC-MS systems across sites, the workflow delivered consistent protein identification rates with low CVs.

Overall, the success of the end-to-end workflow across three independent sites, despite independent sample preparations, emphasizes its reproducibility, robustness, and suitability for routine use.

The study confirms that standardized, high throughput applied proteomics - without compromising depth and sensitivity - is not only achievable, but also scalable and transferable across laboratories. This will only be improved as standardization is subjected to the complete workflow solution.

*Evosep Eno and Evosep Pod are for General Laboratory Use.*

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