Application Note

EVOSEP

Using a cost-efficient and scalable plasma proteomics pipeline to analyze more than **1,100 patient samples**.

Highlights

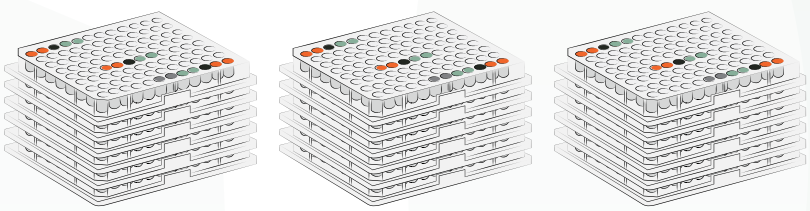
- Evotip Pure ensures ultra-low carryover (<0.2%) across 1,100+ plasma samples.
- Unmatched stability and reliability for consistent cohort analysis.

1. Introduction

Plasma offers valuable insights into disease mechanisms and therapeutic targets. However, scaling plasma proteomics studies to thousands of patient samples presents significant challenges, both in terms of costs associated with sample handling, and the need for robust, reproducible sample preparation pipelines to ensure reliable outcomes. To overcome these challenges, automated and miniaturized workflows are required to reduce costs, ensure consistency and enhance scalability, all while maintaining high-quality results, that can be delivered through direct workflow integration with Evotip Pure.

Here, we evaluated the robustness and scalability of our automated, high-throughput plasma proteomics workflow leveraging Evotip Pure for miniaturized and streamlined sample handling, and Evosep One for robust liquid chromatography (LC) performance.

With the capability to process 192 samples on the Opentrons OT-2 and parallel LC-MS analysis using the 200 SPD method, we showcase the scalability and robustness of this workflow by analyzing 1,122 patient plasma samples, and a total of 1,392 samples including controls in under seven days using a single- instrument platform.



	Samples
Evotip Blank (EB)	30
System Suitability Control (SSC)	88
Plate Blank (PB)	64
Common plasma reference (CR)	88
Patient plasma sample	1,122
Total # samples	*1,392*

Figure 1: Study overview with indication of controls across the 15 sample plates.

2. Method details

For each patient sample, 1 µl plasma was added to a 96-well plate (0030129512, Eppendorf) and transferred to the OT-2 robot (Opentrons). Reduction and alkylation were performed simultaneously in a one-pot solution (1% SDS, 5 mM TCEP, 10 mM CAA in 50 mM TEAB) with 1 hour incubation at ambient temperature. Following dilution, 1.8 µg of plasma protein was incubated with 5 µl MagReSyn Hydroxyl magnetic beads (ReSyn Biosciences). On-bead protein capture was initiated by adding acetonitrile to a final concentration of 80%, followed by a single 100% acetonitrile wash. Proteins were then digested for 4 hours at ambient temperature (22 degrees C) using 10 ng LysC (129-02541, Wako Fujifilm) and 40 ng trypsin (T6567, Sigma Aldrich) in 50 nM TEAB.

Following digestion, 40% of the resulting peptide digest was loaded directly onto Evotip Pure on the OT-2. Samples were analyzed using the Evosep One 200 SPD method with an EV1107 Endurance column (Evosep) at ambient temperature, coupled to a timsTOF HT mass spectrometer (Bruker). The instrument was operated in dia-PASEF mode with the default "short gradient method" and a spray voltage of 1400 V. Data was processed using DIA-NN (version 1.9.2) in library-free mode against the reviewed human proteome (Uniprot, Oct 2020, 20,600 entries) with trypsin/P as the digestion enzyme, allowing two missed cleavages. Conditions were analyzed separately, with match-between-runs enabled only across replicates within identical conditions.

3. Optimized for large cohorts

When analyzing large plasma sample cohorts, the durability and reliability of the LC instrumentation is critical to ensure continuous operation and keep high data integrity. However, maintaining consistent performance across expanding cohorts remains challenging as LC instability can lead to variability in retention times (RT), quantitative precision, and separation efficiency. To evaluate the performance of our plasma workflow across the patient cohort, we implemented a systematic approach using a range of QC samples for real time monitoring of key metrics. These included column monitoring and LC carry-over assessment using blank Evotips (EB); evaluation of LC-MS stability using Evotips preloaded with 50 ng HeLa digest; robotic precision assessment by monitoring cross-well contamination through plate blank (PB) samples; and measuring overall workflow and inter-plate variability using common plasma reference samples (CR). Each 96-well plate contained 17 QC samples, enabling the analysis of 79 patient samples per plate with a total of 15 plates covering all patient samples.

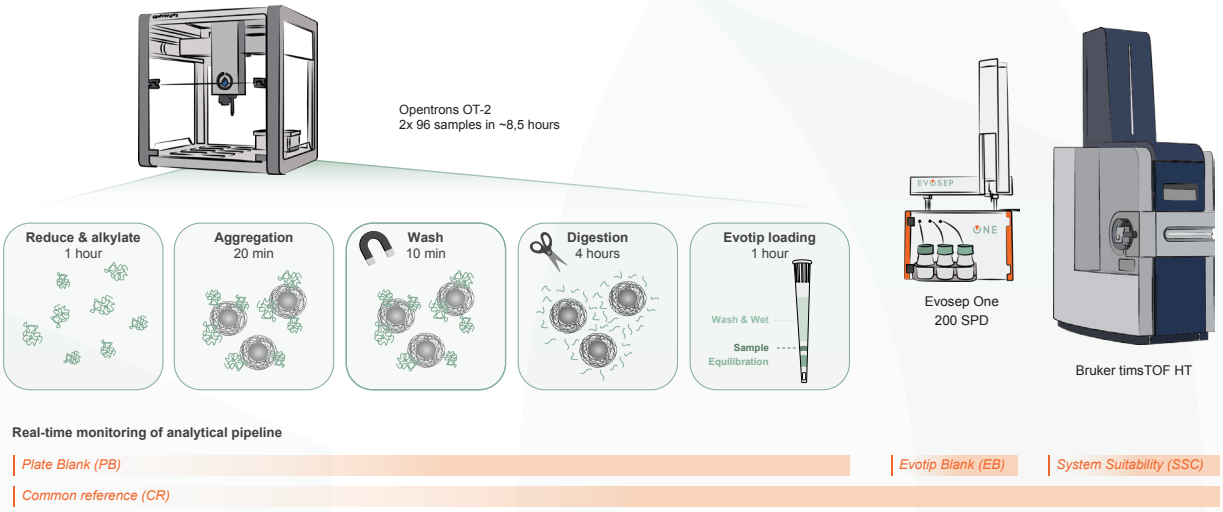


Figure 2: Schematic representation of the fully automated digestion workflow for 1 to 192 samples, and overall analytical workflow including controls for each step.

4. Reliable and robust solution

Throughout the analysis of patient and common reference (CR) plasma samples (1,210 in total), the Evotip blanks (EB) demonstrated less than 0.2% column carryover, confirming that the use of Evotip Pure effectively protects the LC system and analytical column. Consistent plasma intensities across patient and CR samples, coupled with negligible plate blank (PB) signals further confirmed that the entire workflow was free from cross-well contamination and positional bias. Notably, the minimal detectable PB signal is attributed to the enzymes and reagents introduced into PB samples during the robotic workflow.

To ensure consistent LC-MS performance, 50 ng HeLa samples (SSC) were deposited across well plates to assess reproducibility in proteome coverage and quantitative precision. Additionally, pooled patient plasma (CR) samples were included to validate the accuracy of the entire workflow. The high reproducibility observed with the SSC samples confirmed the robustness of the LC-MS performance across the cohort. Similarly, CR samples demonstrated consistent performance with minor variations compared to SSC performance reflecting robotic variation. Overall, excellent quantitation is observed across both HeLa and plasma proteins. The median protein coefficient of variation (CV) for all SSC samples and for plasma proteins identified across all CR samples were 15.1% and 15.6%, respectively. Both represent excellent consistency across the 15 sample plates.

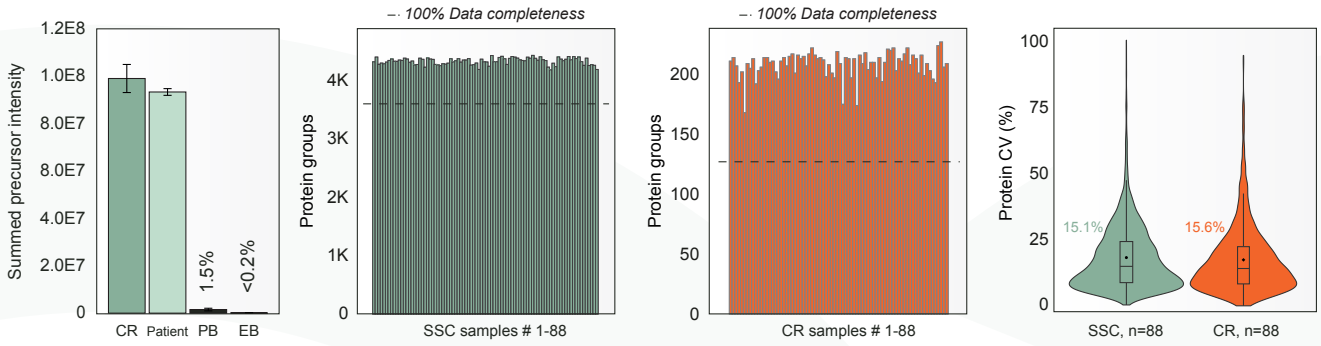


Figure 3: Summed precursor intensity across sample types (CR n=88, Patient n=1,122, PB n=64, EB n=30), and proteome coverage from SSC and CR samples across the cohort with associated protein CVs.

5. Standardized performance

The Evosep One system demonstrated exceptional stability and reliability for large-scale plasma proteomics. It processed a total 1,392 sample injections (1,122 patient + 270 QC samples) with a consistent pressure profile (<1% CV) and no systematic pressure buildup, confirming its suitability for continuous high-throughput operation. The stable pressure profile is a further testament to the advantages of the Evotip Pure which functions as a disposable trap column that together with partial elution safeguards the LC system and chromatographic column.

To monitor LC-MS performance, we included system suitability controls (SSC) consisting of 50 ng HeLa peptides and evaluated the chromatographic performance, by extracting 10 diagnostic HeLa peptides selected across the gradient. Across all 88 SSC samples, spanning the entire cohort of 1,392 samples, these peptides exhibited minimal retention time variation (<2% CV) and high peak area accuracy (<13% CV). This showcases the reproducible chromatographic separation across all SSC runs and the stable performance of the Evosep column. All samples were analyzed consecutively using a single EV1107 Endurance column without downtime, underscoring the system's durability and efficiency across thousands of injections.

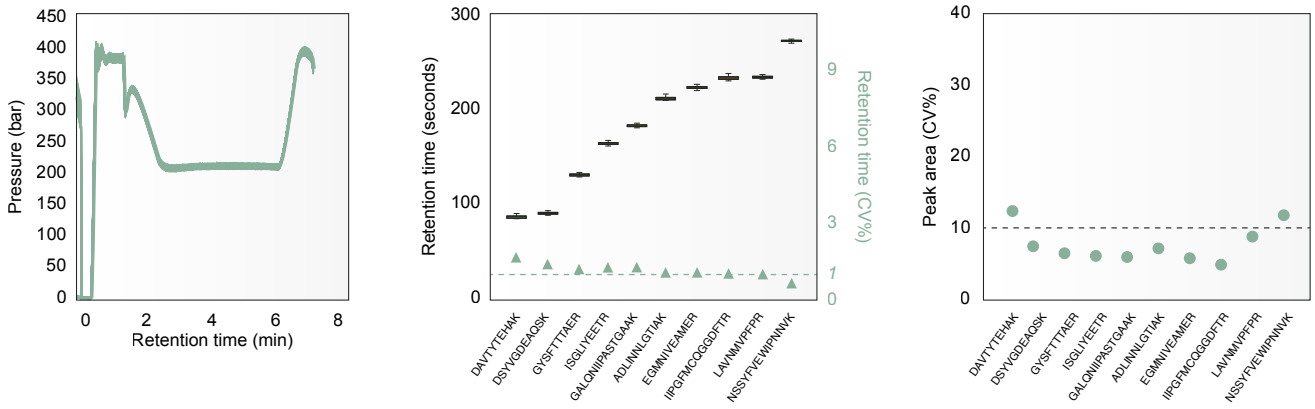


Figure 4: Pressure profiles for all samples analyzed with 200 SPD (n=1,392). Boxplot of retention times for diagnostic peptides and associated CVs for retention times and peak areas across the SSC samples (n=88)

6. Conclusion

Sample preparation is a critical component of large-scale proteomics, and automated sample handling workflows are becoming increasingly valuable as cohort sizes grow. Hence, maintaining workflow stability and ensuring consistent LC performance across large-scale experiments become even more essential.

Here we detail the performance of Evosep based workflows, and showcase the exceptional robustness and stability of the Evosep One system for large-cohort analyses. A key advantage is the automated Evotip loading, which seamlessly integrates sample preparation with LC-MS analysis and enables simple, cost-efficient, miniaturized sample handling. This improves both reliability and reproducibility as evidenced by high inter- and intra-plate reproducibility with no cross-contamination across a large patient plasma cohort. Additionally, the Evotip Pure functions as a disposable trap column, leveraging partial elution to safeguard the LC system and analytical column while minimizing column carryover. The design ensures sustained chromatographic performance and LC stability, as demonstrated in the analysis of more than 1,100 patient plasma samples.

Collectively, the workflow demonstrated robust performance over 1,392 injections confirming its high reliability, reproducibility, and positions the Evosep One as a highly robust and scalable solution for large plasma proteomics studies.

Evosep One is for General Laboratory Use.