Application Note

EVOSEP

Cost-efficient and scalable end-to-end workflow on the Opentrons OT-2 for neat plasma utilizing **Evotip Pure**

Highlights

- Fully automated workflow from raw plasma to ready-to-analyze Evotips
- Miniaturized sample preparation for sustainable and scalable workflows

1. Introduction

Plasma proteomics stands at the forefront of biomedical research as it offers invaluable insights into disease progression and holds immense potential for minimally invasive diagnostics. With recent advancements toward faster LC-MS analysis, the demand for robust, high-throughput, and cost-efficient sample preparation has increased. To address this, we present a fully automated plasma sample preparation workflow on the Opentrons OT-2 (OT-2) liquid handler directly integrated with Evotip Pure, enabling complete hands-off sample preparation of raw plasma to ready-to-analyze Evotips. The workflow employs Protein Aggregation Capture (PAC)[1] on hyper-porous magnetic beads for fast and efficient removal of detergent and plasma contaminants followed by on-bead digestion. Leveraging the properties of Evotip Pure, this workflow includes immediate and online desalting and storage of digested peptides, ensuring consistent and seamless integration between sample preparation and LC-MS analysis. The workflow is easily accessible through a HTML-form user interface for custom experiment design, standardized sample preparation and simplicity for operators to implement protocols within and across laboratories. A resulting custom Python script is easily executed through the Opentrons app. Tailored for high-throughput analysis, the workflow allows for the processing of 192 samples in just eight hours, significantly reducing manual handling while increasing experimental robustness. The neat plasma workflow includes a functional module and can be considered a complement to the existing Evotip-based workflows on the OT-2.

Figure 1: Consistency, cost- and time-per-sample are essentials for scalable plasma proteomics.

2. Method details

A detailed step-by-step guide “IN-004A 24/05” is available online at *www.evosep.com/support/automation-opentrons-ot2*. Briefly, 1 µl plasma was added to a 96-well plate (0030129512, Eppendorf) and transferred to the OT-2. Simultaneous reduction and alkylation were carried out in a one-pot solution (1% SDS, 5 mM TCEP, 10 mM CAA in 50 mM TEAB) with 1 hour incubation at ambient temperature. The samples were diluted and the workflow continued with the incubation of approximately 2 µg of plasma protein with 5 µl MagReSyn Hydroxyl magnetic beads (ReSyn Biosciences). On-bead protein capture was initiated by adding acetonitrile to a final concentration of 80%. After a single wash using 100% acetonitrile, digestion was carried out for 4 hours at ambient temperature with a combination of 10 ng LysC (129-02541, Wako Fujifilm) and 40 ng trypsin (T6567, Sigma Aldrich) in 50 mM TEAB. Following digestion, 40% of the resulting digest was loaded directly on Evotips on the OT-2. Samples were analyzed with either the 100 SPD using EV1109 Performance column, (Evosep), operated at 40°C with the 200 SPD method using the EV1107 Endurance column, (Evosep), operated at ambient temperature coupled to a timsTOF HT mass spectrometer (Bruker). Spray voltage was set to 1400 V. The mass spectrometer was operated in dia-PASEF mode using the default “short gradient method”. Data was analyzed using DIA-NN (version 1.8.1) in library-free mode against the reviewed human proteome (Uniprot, Oct 2020, 20,600 entries) with trypsin/P as digestion enzyme allowing 2 missed cleavages. All conditions were searched separately with match between runs exclusively enabled across replicates within identical conditions.

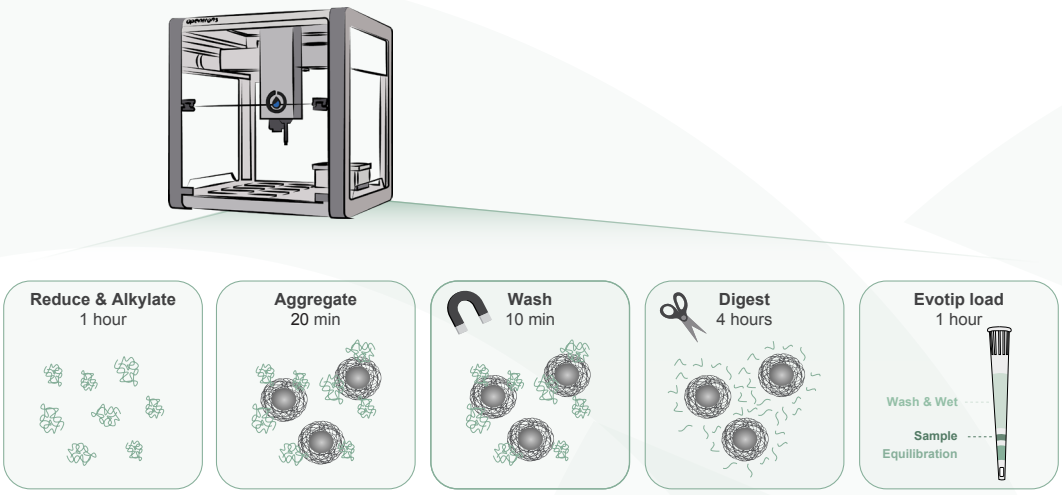

Figure 2: Schematic representation of the standardized HTML-form generated neat plasma workflow.

3. Technical validation

To accommodate high-throughput applications, the workflow contains on-deck reduction and alkylation, as well as sample dilution. This minimizes manual preparation and enzyme consumption, ensuring time- and cost-efficient processing of large sample cohorts. We evaluated the performance of the workflow by loading 40% of the resulting peptides on Evotip and analyzed the samples using the 100 SPD method and the timsTOF HT mass spectrometer. With an approximate 2 µg plasma proteins after dilution, we carried out digestion using just 10 ng lys-C and 40 ng trypsin with four hours of incubation at ambient temperature. We observed a reproducible digestion efficiency across the 16 replicate digestions with approximately 65% fully cleaved peptides leading to the identification of 4000 precursors corresponding to 400 protein groups (Figure 3). The workflow exhibits excellent precision with a median coefficient of variation (CV) of 13.4%.

The method identifies proteins within four orders of magnitude with wide representation of soluble, highly abundant plasma proteins. Several of these are among FDA-approved biomarkers, where the majority are quantified with a CV below 20%, enabling efficient monitoring through this fully automated sample preparation workflow.

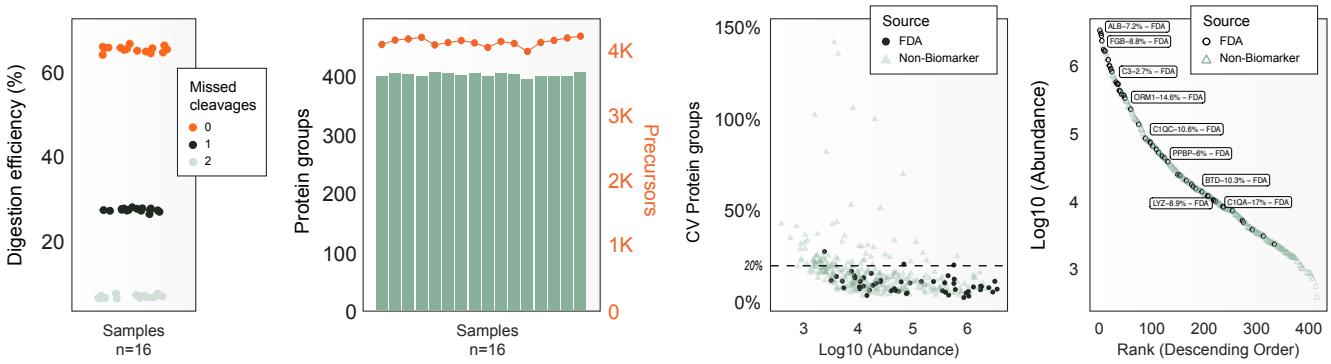

Figure 3: Digestion efficiency, proteome coverage with associated CVs on protein level and dynamic range plot. ~40% of each peptide digest was loaded on Evotips and analyzed with 100 SPD.

4. A standardized workflow

A 96-well plate-based workflow is efficient for scalability as it enables simultaneous processing of samples. However, it is critical to avoid any positional bias in the plate, which is often associated with these workflows. To monitor this, a full plate was prepared with the workflow, starting from raw plasma. Following 40% sample load on Evotip, the samples were analyzed using the 200 SPD method on the timsTOF HT. The analysis revealed a coverage of approximately 300 proteins per sample and around 250 proteins identified in more than 95% of samples. A principal component analysis (PCA) was carried out to investigate positional bias across the plate. The samples were grouped based on rows and columns, respectively. Reproducibility is ensured through careful sequential timing throughout the protocol and immediate Evotip loading after digestion for efficient storage until LC-MS analysis. The absence of clustering shows no correlation between initial position and sample characteristics.

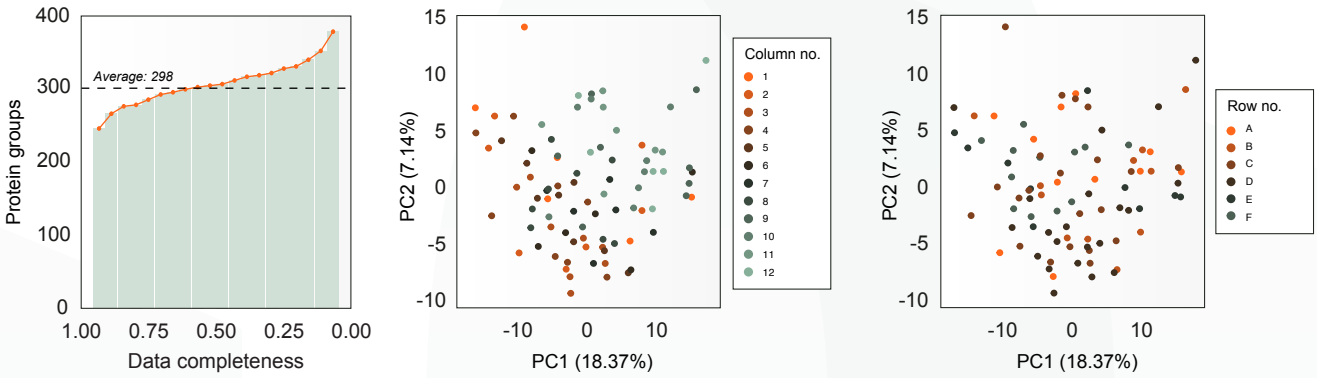

Figure 4: Data completeness assessment on protein level across one plate analyzed with 200 SPD and PCA analysis showing potential bias of column and row number in the plate.

5. Conclusion

The OT-2 protocol is accessible via a user-friendly interface, enabling customization of the experimental setup for any combination of the Evosep method and mass spectrometer. This fully automated workflow, integrated with Evotip Pure and Opentrons OT-2, provides a robust solution for high-throughput plasma proteomics. While requiring just 1 µl of plasma and performing a short 4 hours digestion at ambient temperature, a significant number of FDA-approved biomarkers are identified with excellent quantification using the 100 SPD method. Notably, the analysis did not reveal any positional biases during sample preparation, ensuring reproducible analysis across a sample plate. The workflow has a capacity to process 192 samples in parallel driving scalability using all available deck space on the robot. Importantly, it is cost-efficient by significantly reducing the use of enzymes, which is critical when analyzing large sample cohorts. In conclusion, this standardized workflow streamlines sample preparation while also establishing a foundation for future adaptations of functional modules for the Opentrons OT-2 for continued innovation in biomedical research and therapy. This concept can be easily transferred to other robotic platforms which may allow for higher throughput and improved digestion using temperature control.

References

1. Batth TS., Tollenaere M., Rüther P., Gonzalez-Franquesa A., Prabhakar BS., Bekker-Jensen S., Deshmukh AS., Olsen JV (2019) Protein Aggregation Capture on Microparticles Enables Multipurpose Proteomics Sample Preparation. Mol Cell Proteomics., mcp.TIR118.001270