Application Note

EVOSEP

Unlocking the plasma proteome with Evotip Pure based, standardized and scalable workflows

Highlights

- Fully automated, end-to-end workflow for neat and deep plasma profiling
- Standardized and cost-efficient strategy to monitor disease at scale

1. Introduction

Plasma stands out as one of the most readily accessible biofluids, with an estimated presence of over 10,000 distinct proteins. A significant portion of these are released from organs and tissues. The ability to measure such proteins in a robust manner holds tremendous potential for advancing next-generation multiplexed biomarker discovery. However, methods must be able to deal with dynamic range of 10-12 orders of magnitude, and at the same time provide sufficient throughput in a robust and cost-effective manner. While a standardized approach is needed for wide utilization in the field, complementary strategies are also relevant to apply, depending on the use case. Here, we describe a fully automated workflow on the Opentrons OT-2 liquid handling robot (OT-2) for neat plasma preparation based on Protein Aggregation Capture (PAC)[1], and an approach for deeper profiling of a sub-proteome based on the MagNet method[2]. The workflows are completely hands-off, incorporating all steps from raw plasma to peptides loaded on Evotips. This is driven by efficient protocols based on hyper-porous magnetic beads directly followed by concentration of the digested peptides on Evotips to minimize losses otherwise associated with additional desalting steps. The standardized workflows can robustly process up to 192 samples in less than 10 hours with high precision and sensitivity. Close to 1,500 protein groups were quantified from neat plasma, increasing to more than 5,000 protein groups from the MagNet workflow with a throughput of 100 samples analyzed per day using the Orbitrap Astral MS.

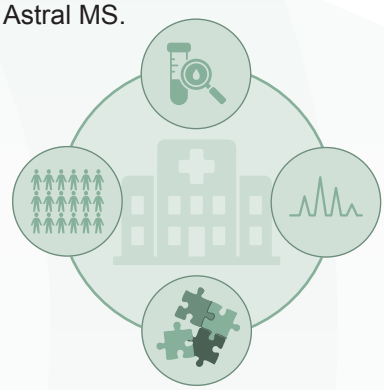

Figure 1: Plasma proteomics has many applications and shows great promise.

2. Method details

Plasma was extracted as per the Early Detection Research Network (EDRN) SOP[3]. For the neat workflow, 1 µl plasma was diluted 180X in one-pot lysis, reduction, and alkylation buffer (1% SDS, 5 mM TCEP, 10 mM CAA in 50 mM TEAB) and subsequently 6 µl was added to 5 µl MagReSyn Hydroxyl magnetic beads (ReSyn Biosciences). Acetonitrile was transferred to each well of the sample plate to a final concentration of 80% driving on-bead protein capture. Post 10 min. bind step, and a single wash using 100% acetonitrile, digestion was carried out for 4 hours using a combination of 10 ng LysC and 40 ng Trypsin. Following digestion, 40% of the digest was directly loaded on Evotips on the OT-2. In the case of the Mag-Net workflow, 4 µl plasma was mixed with 4 µl bind buffer (100 mM Bis-Tris Propane, 150 mM NaCl pH 6.5) and 1 µl MagReSyn SAX beads (ReSyn Biosciences), and diluted with 32 µl wash buffer (50 mM Bis-Tris Propane, 150 mM NaCl pH 6.5). Three sequential 12 min. bind steps was followed by three washes using 100 µl wash buffer. Next one-pot buffer (1% SDS, 10 mM TCEP, 5 mM CAA in 50mM Tris-HCl pH 8.5) was used to solubilize, reduce, and alkylate proteins during an hour on-deck incubation.

The remainder of the steps followed the neat workflow. Proteins were re-captured using acetonitrile induced on-bead aggregation followed by a single wash and digestion for 4 hours using a combination of 75 ng LysC and 300 ng Trypsin. 40% of the resulting digest was loaded on Evotip. Samples were analyzed with the 100 SPD method (EV1109 column, Evosep, operated at 40 °C) coupled to an Orbitrap Astral mass spectrometer (Thermo Scientific). Spray voltage was set to 1900 V, and heated capillary temperature at 275 °C. The mass spectrometer was operated at a full MS resolution of 240,000 with a full scan range of 380 − 980 m/z. The full MS AGC was set to 500%. MS/MS scans were recorded with 3 Th isolation window, 7 ms maximum ion injection time. MS/MS scanning range was from 380-980 m/z were used. The isolated ions were fragmented using HCD with 25% NCE. Data was extracted via 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 only enabled across replicates within the same condition.

3. Maximizing efficiency

The end-to-end MagNet workflow was adapted from the original publication[2] to facilitate integrated Evotip loading and downscaling of the plasma input to reduce costs for expensive reagents such as enzymes by only digesting what is needed for a single LC-MS injection. This was implemented on the OT-2, where digestion was carried out for 4 hours at ambient temperature to ensure throughput despite the limited deck space on the robot. Specifically, 1 µl of plasma was used as input for the neat plasma workflow, from which ~1 µg was digested using a protein aggregation capture assisted digestion, whilst the MagNet workflow used 4 µl plasma as input (Figure 2). The protocol utilizes direct loading of Evotips post digestion, which leads to increased sensitivity compared to a conventional elutionion, dry-down, and resuspension steps. In addition to the reagents, the protocol is sustainable by design, as just a single pipette tip is used for all sample transfer steps throughout.

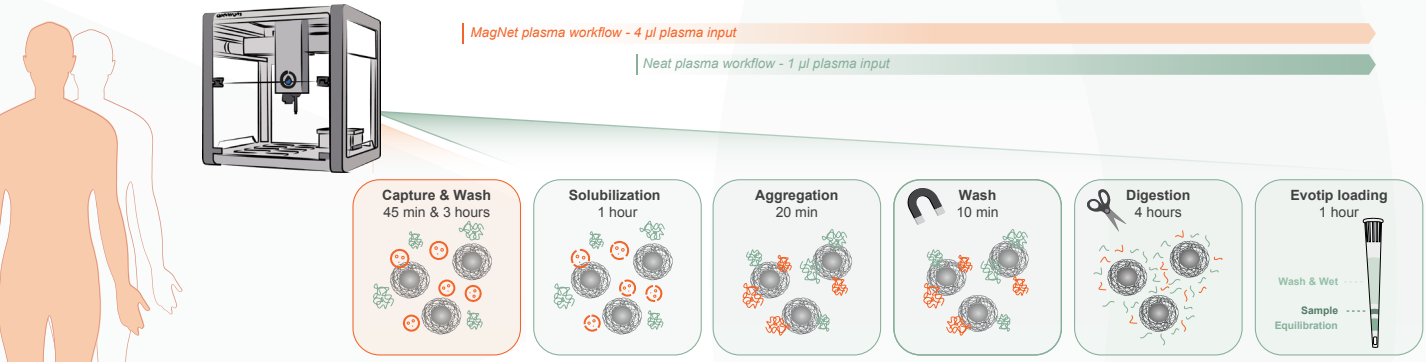

Figure 2: Schematic representation of the OT-2 workflows for neat and MagNet plasma profiling.

4. Reproducible workflows

We assessed the robustness of both workflows across two plates on the same OT-2, with a total of 24 samples of both neat and MagNet, respectively (Figure 3). Using the 100 SPD method, the neat workflow consistently identified over 1,400 unique protein groups and >15,000 precursors. Importantly, 1,033 protein groups and 6,519 precursors were quantified in all samples. Using the automated MagNet workflow, more than 5,000 protein groups and 45,000 precursors were quantified. The complete dataset revealed 25,264 precursors leading to 4,060 protein groups. The precision in the data was excellent with a median coefficient of variation (CV) on protein level of less than 10% for both neat and MagNet workflows. In context to this, the technical variability in the LC-MS setup contributed with a median CV of less than 4%, providing a solid platform for robust high-throughput analysis (Figure 3).

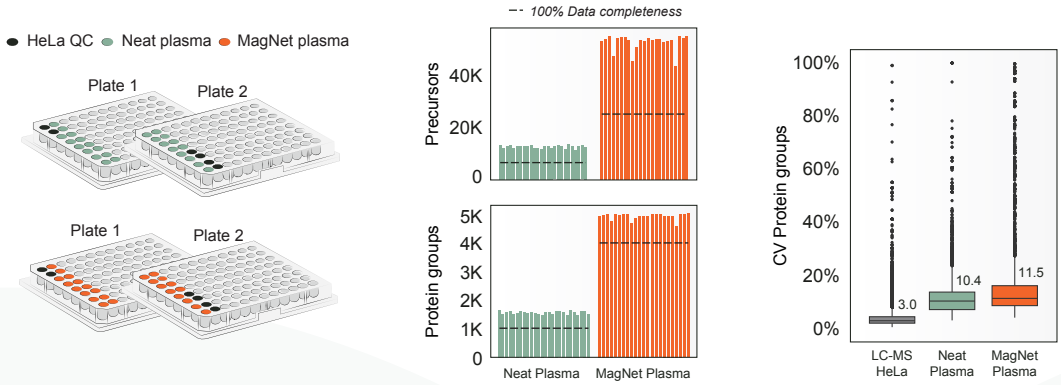

Figure 3: Experimental setup and precursor and protein ID levels. CVs representing LC-MS and workflow reproducibility. ~40% of each peptide digest was loaded on Evotips and analyzed with 100 SPD.

An asset of the presented strategy, is the option to process both the neat and MagNet based plasma in parallel from just 5 µl of plasma. Besides the obvious depth in the datasets, the two workflows are complementary from a biological perspective. The neat workflow predominantly identifies soluble, high-abundant plasma proteins. While the dynamic range of these are more than 10-fold, the sensitivity in the method enables the identification of proteins in the ng/ml range, based on estimated protein concentration levels reported in Human Disease Blood Atlas (HBDA)[4] (Figure 4). This enables the monitoring of numerous relevant proteins, including 52% (59 of 113) of FDA approved plasma biomarkers, where 72% were quantified with a CV<20%.

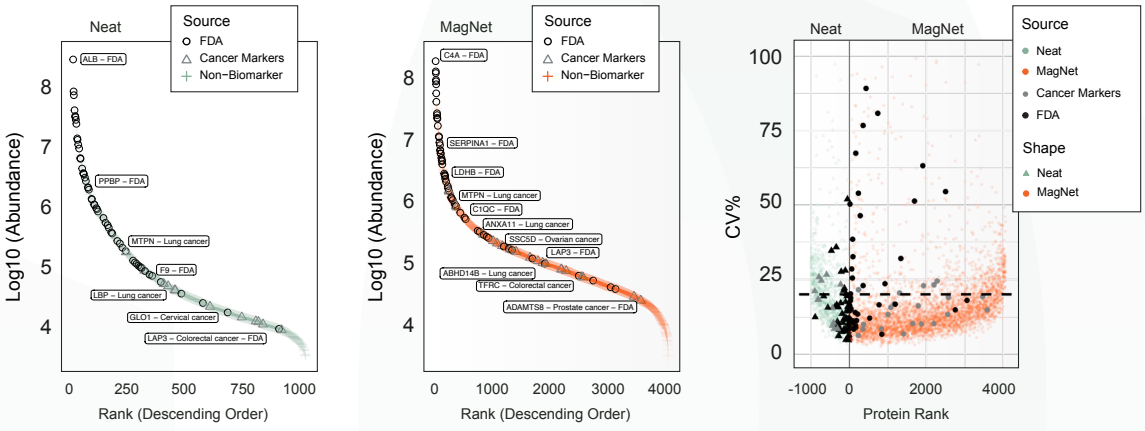

Figure 4: Neat and MagNet plasma dynamic range plot (descending) of 100% coverage proteins, annotated with CV% and isoform specific FDA and HDBA putative biomarkers.

The MagNet workflow, on the other hand reduces the dynamic range in the enriched sub-proteome by depleting soluble high-abundant plasma proteins whilst enriching for membrane bound vesicles. 96 of the Top100 extracellular vesicle marker proteins listed in Vesiclepedia were identified in the MagNet dataset, where 82% of these were quantified with a CV<20%. Further numerous proteins listed as putative markers in the Human Disease Blood Atlas with estimated blood concentrations in the low pg/ml range were readily measured by this workflow with CVs<20%. In combination, these proteins could be utilized as reporters of nine different cancer types in relevant cancer cohorts and studies (Figure 4).

5. Conclusion

Here, we describe the development of a complete, fully automated plasma profiling pipeline, seamlessly integrated with high-throughput analysis using the Evosep One and cost-efficient by design. This enables the processing of 192 samples in short time - from raw plasma to ready-to-analyze Evotips. Both workflows, for neat and membrane-bound vesicle enrichment, respectively provide good quantitative precision with median protein CVs of less than 10%. These can be processed independently of each other or within one OT-2 with just 5 µl of plasma required to for both methods. Coupling this sensitive, end-to-end analytical pipeline to the rapid 100 SPD method delivers deep plasma proteome profiles with more than 5,300 protein groups identified.This depth allowed the monitoring of low abundant biomarkers.

The ability to tackle large clinical cohorts in a robust, cost-effective, high-throughput and sensitive manner opens the door to next-generation protein biomarker discovery as well as expediting clinical implementation of existing marker panels by providing an efficient orthogonal assay to be used for biomarker validation.

The Evosep One instrument is for Research Use Only.

References

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