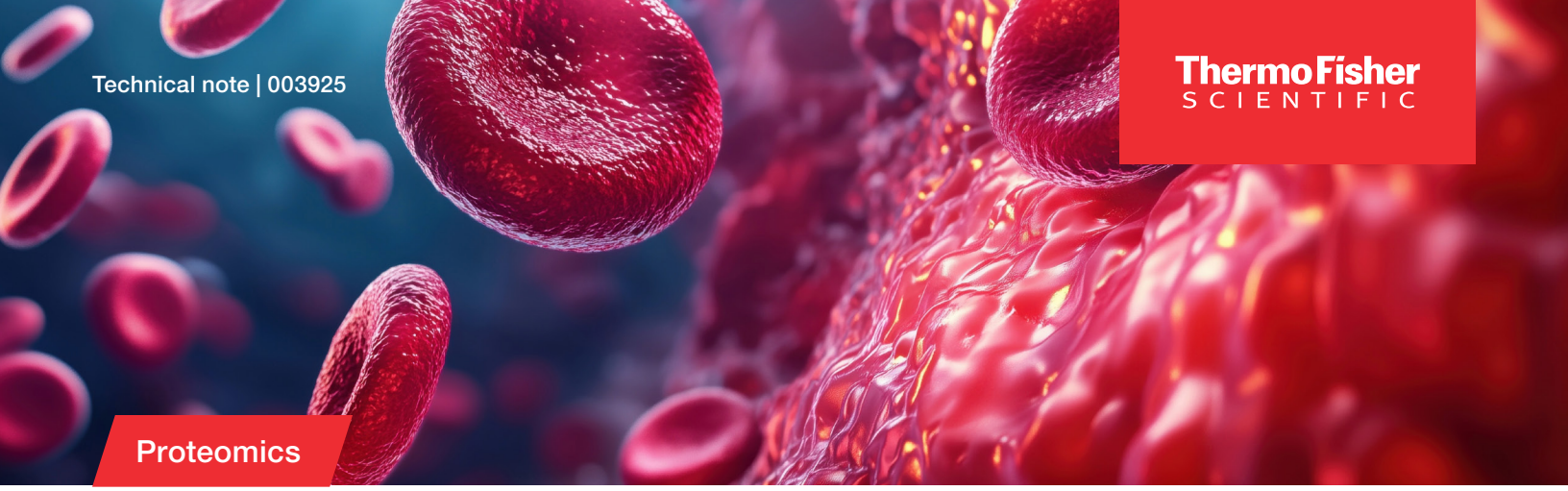

Technical note | 003925

Proteomics

Advancing plasma proteomics: Quantitative and high-throughput EV analysis with automated Mag-Net workflow, Evosep One, and Orbitrap Astral mass spectrometer

Authors

Jared Deyarmin,
Khatereh Motamedchaboki,
Stephanie Samra

Thermo Fisher Scientific,
San Jose, CA USA

Keywords

Plasma, extracellular vesicle, blood, translational proteomics, Mag-Net, Evosep One, Orbitrap Astral mass spectrometer

Goal

Demonstrate an automated solution to address the dynamic range challenges in plasma proteomics with extracellular vesicle (EV) enrichment. By integrating the Mag-Net workflow with the Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer, we aim to significantly enhance throughput, sensitivity, and reproducibility of this blood-based plasma sample analysis while also achieving deeper coverage of the plasma proteome.

Introduction

Plasma proteomics, a pivotal field in translational research, focuses on analyzing the protein composition of blood plasma to identify biomarkers and elucidate disease mechanisms. Despite its transformative potential, this field faces substantial challenges. Achieving high throughput, exceptional sensitivity, and unwavering reproducibility are critical yet difficult objectives. The inherent complexity of plasma, characterized by a vast array of proteins and other biomolecules, presents significant hurdles. Additionally, the presence of numerous interfering substances and the wide dynamic range of protein concentrations, spanning several orders of magnitude,[1] further complicate the analysis. Overcoming these challenges is essential for advancing our understanding of disease processes and for the development of precise diagnostic and therapeutic strategies.

To overcome detection challenges faced by conventional plasma proteomics and capture deeper biological insights, we focus on a specific dimension of the plasma proteome through the enrichment of extracellular vesicles (EVs), which carry proteins, nucleic acids, and lipids reflective of the physiological or pathological state of their cells of origin.[2] Plasma is readily accessible through routine blood collection, making EV proteomics a minimally invasive approach suitable for early disease detection, longitudinal monitoring, and large-scale cohort studies. Proteomic analysis of EVs

provides insights into molecular mechanisms relevant to both diagnosis and therapy, supporting the discovery of biomarkers and the development of EV-based treatments, including personalized strategies informed by disease-specific protein signatures.[2] Overall, plasma EV proteomics offers a powerful platform for advancing our understanding of disease biology, improving diagnostic precision, and guiding therapeutic innovation.

To address the challenges in plasma proteomics, we introduce a comprehensive end-to-end solution that combines the Mag-Net plasma EV enrichment protocol[3] adapted for miniaturization and complete automation through Evotip™ peptide loading with robust Evosep One™ separation in combination with enhanced sensitivity of the Orbitrap Astral mass spectrometer. This integration is designed to significantly enhance the throughput and depth of proteome coverage in plasma proteomics studies through extracellular vesicle enrichment. The automated Mag-Net workflow combined with the automated Evotip loading streamlines sample preparation, while the Orbitrap Astral MS provides unparalleled sensitivity and resolution (Figure 1).

Experimental

Plasma preparation

To assess the quantitative precision and accuracy of the Mag-Net workflow, calibration curves were generated by mixing pooled human plasma (IPLAWBK2E50ML, Innovative Research) and chicken plasma (IGCHPLAK2E50ML, Innovative Research) at eight dilution ratios (100:0,70:30, 50:50, 30:70, 10:90, 5:95, 1:99, 0:100) with five replicates per ratio.[4] The mixed plasma samples were then split and digested by the neat digestion or Mag-Net protocols.

For the neat workflow, 1 µL of plasma was diluted 180 times in a buffer containing 1% SDS, 5 mM TCEP, and 10 mM CAA in 50 mM TEAB. Subsequently, 6 µL of the diluted plasma, corresponding to ~1.8 µg plasma protein was added to 5 µL of MagReSyn™ Hydroxyl magnetic beads (ReSyn Biosciences).

Acetonitrile was added to each well of the sample plate to achieve a final concentration of 80%, facilitating on-bead protein capture. After a 10-minute binding step, the beads were washed once with 100% acetonitrile. Proteins were then digested for 4 hours using a combination of 10 ng LysC and 40 ng Trypsin. Post-digestion, 40% of the digest was directly loaded onto Evotips using the OT-2 (Opentrons) liquid handler.

For the Mag-Net workflow, 4 µL of plasma was mixed with 4 µL of bind buffer (100 mM Bis-Tris Propane, 150 mM NaCl, pH 6.5) and 1 µL of MagReSyn™ SAX beads (ReSyn Biosciences), and then diluted with 32 µL of wash buffer (50 mM Bis-Tris Propane, 150 mM NaCl, pH 6.5). Three sequential 12-minute binding steps were performed, followed by three washes with 100 µL of wash buffer. Proteins were solubilized, reduced, and alkylated using a one-pot buffer (1% SDS, 10 mM TCEP, 5 mM CAA in 50 mM Tris-HCl, pH 8.5) during a one-hour on-deck incubation. Proteins were re-captured using acetonitrile-induced on-bead aggregation, followed by a single wash. Digestion was carried out for 4 hours using a combination of 75 ng LysC and 300 ng Trypsin. Following digestion, 40% of the resulting digest was loaded onto Evotips.

Analysis by Orbitrap Astral MS

Samples were analyzed on the Evosep One by the Whisper™ Zoom 40 SPD (Aurora Elite™ XT column, IonOpticks) and 60 SPD methods for quantitative precision and accuracy assessment of the workflow. The analytical column temperature was set at 40 °C. The Orbitrap Astral MS was used with the following settings: spray voltage set to 1,900 V, heated capillary temperature at 275 °C, full MS resolution of 240,000, full scan range of 380–980 *m/z*, and full MS AGC set to 500%. MS/MS scans were recorded with a 3 Th isolation window, 7 ms maximum ion injection time, and a scan range of 380–980 *m/z*. Fragmentation was performed using higher-energy collisional dissociation (HCD) with 25% normalized collision energy (NCE).

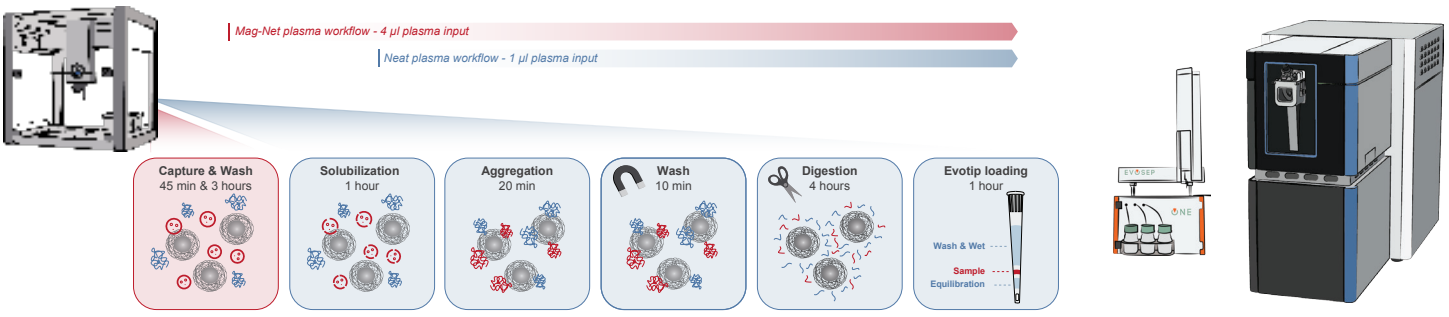

Figure 1. Schematic representation of the OT-2 workflows for neat and Mag-Net plasma sample preparation and analysis

Data processing

Raw LC-MS (liquid chromatography coupled to mass spectrometry) data was analyzed using DIA-NN (version 1.9.2) software in library-free mode against the reviewed human proteome (Uniprot, October 2020, 20,600 entries) with Trypsin/P as the digestion enzyme, allowing for up to two missed cleavages. Each condition was analyzed separately, with the "match between runs" feature enabled only across replicates within the same condition.

Results and discussion

Quantitative assessment

The neat workflow identified over 950 unique protein groups and more than 7,000 precursors across all replicate samples for 100% human plasma with 60 SPD method and as much as 750 protein groups for 1% human plasma (Figure 2). The automated Mag-Net workflow excelled even further and identified over 4,000 protein groups and 27,000 precursors across all replicate samples for 100% human plasma for 60 SPD and 5,000 protein groups and 35,000 precursors for the Whisper Zoom 40 SPD method (Figures 2 and 3).

Quantitative accuracy experiments evaluated the empirical vs. theoretical $\log_2$ ratios of human plasma percentages at 60 SPD and the Whisper Zoom 40 SPD method across different injection replicates of varying ratios of human and chicken plasma samples. Both neat and Mag-Net workflows showcased excellent precision for precursors (Figure 4), with a median CV of less than 16% for 60 SPD and less than 15% for Whisper Zoom 40 SPD methods at the protein group level.

The enhanced proteome coverage and quantitative performance from the Mag-Net workflow are due to EV enrichment by Mag-Net beads and the automation, which minimizes manual handling and reduces variability, ensuring consistent and reproducible data crucial for large-scale proteomics studies. The on-bead digestion and subsequent direct loading onto Evotips streamline sample preparation, enhancing throughput without compromising sensitivity and quantitative accuracy.

Combined with the Orbitrap Astral mass spectrometer's high resolution and fast acquisition rates, this workflow delivers deep and accurate mass spectrometry data utilizing EV enrichment in biofluids like plasma, enabling the detection of low-abundance EV

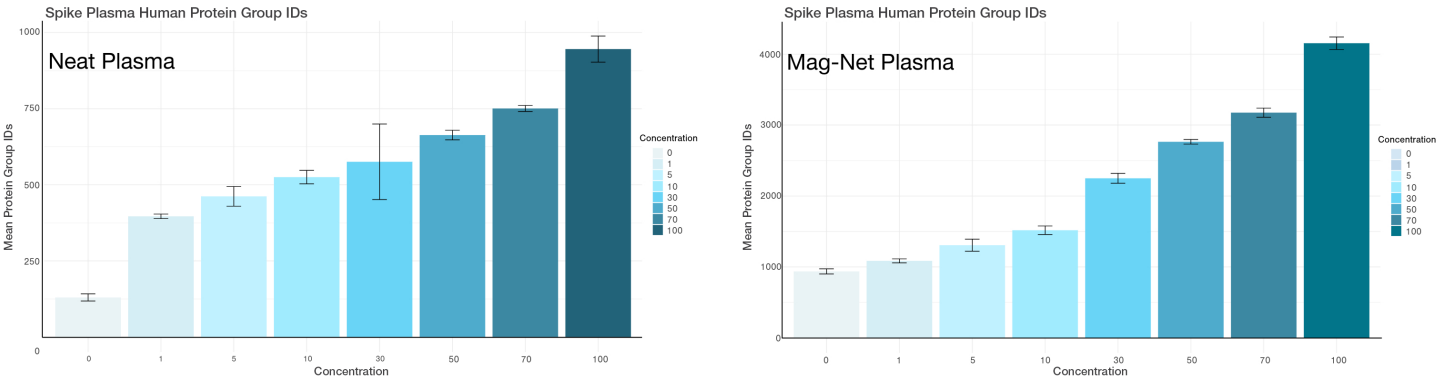

Figure 2. Protein Group IDs for 60 SPD from neat and Mag-Net enriched plasma. Samples are reported at the protein level for five replicates per condition and % human ratio.

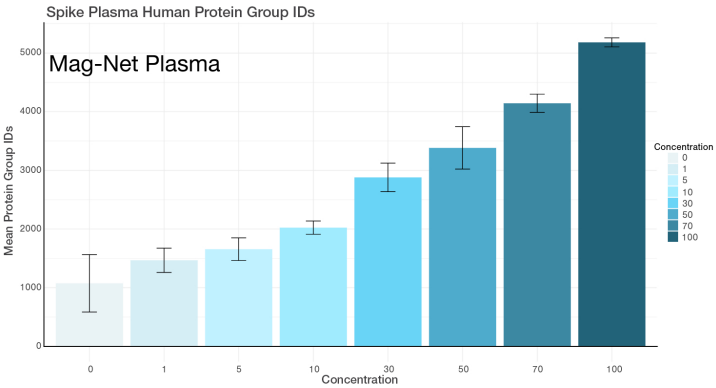

Figure 3. Protein Group IDs for 40 SPD Whisper Zoom from Mag-Net enriched plasma. Samples are reported at the protein level for five replicates per condition and % human ratio.

proteins essential for deep plasma proteomics. The flexibility of both the Evosep One and the Orbitrap Astral MS allows the user to prioritize either high-throughput or maximum identifications for their workflows. However, due to the industry-leading performance of the Orbitrap Astral MS, the identifications observed at higher throughput ranges, such as 100 SPD, are still deep, with the tradeoffs being minimal as observed between the 60 SPD and Whisper Zoom 40 SPD methods.

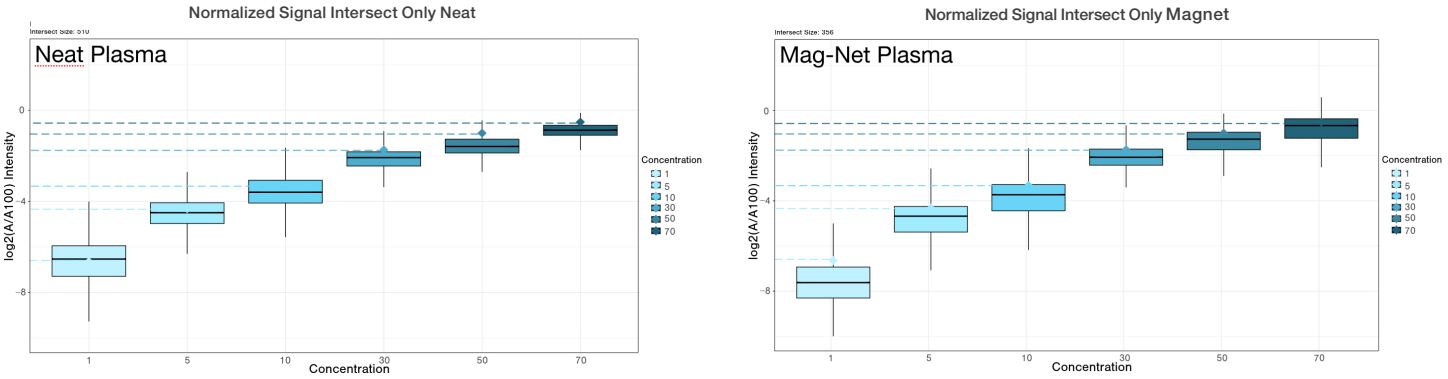

Figure 4. Quantification accuracy and precision across a large dynamic range for 60 SPD throughput using neat and Mag-Net enriched plasma. Samples were analyzed at a peptide level with the mean signal intensity of each precursor compared with the mean signal intensity of the same precursor in the 100% human condition. Only precursors identified in every condition except 0% human were included. The expected value was calculated as $\log_2$ (Plasma ratio).

Conclusion

Overall, the integration of the Mag-Net workflow with the Evosep One and Orbitrap Astral MS offers a powerful and efficient platform for proteomic analysis of extracellular vesicle enrichment in plasma. This combination addresses many inherent challenges of routine plasma analysis, providing a high-throughput, sensitive, and reproducible solution well-suited for large-scale studies and clinical research. By enabling deeper proteome coverage and reliable EV biomarker discovery, the Mag-Net workflow significantly advances the field of plasma proteomics, offering researchers an end-to-end solution that can be seamlessly adopted into their laboratories.

The Orbitrap Astral MS consistently delivered high-quality data with enhanced precision, sensitivity, and dynamic range across both analytical throughputs tested (60 SPD, Whisper Zoom 40 SPD methods). It has proven to be an invaluable tool for quantitative proteomics, as demonstrated by experiments where accuracy and precision were thoroughly tested by a matrix-matched experiment with varying the ratios of human and chicken plasma. The presented workflows offer a solution for producing robust and reproducible measurements with unprecedented speed, empowering researchers to accelerate discoveries and advance biological insights.

The advancements in mass spectrometry technology embodied by the Orbitrap Astral MS significantly enhance the accuracy and comprehensiveness of proteome-wide studies, ensuring precise and thorough analysis crucial for groundbreaking discoveries. This optimized workflow not only elevates the capabilities of plasma proteomics focusing on the EV proteome but also paves the way for transformative discoveries in clinical research and biomarker identification, driving scientific progress towards understanding the complex nature of human health and disease while providing novel insights.

Acknowledgements

We appreciate Evosep™ for their support and assistance with data analysis using DIA-NN software and data acquisition, which were essential to this project.

References

1. Anderson, N.L.; Anderson, N.G. The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell Proteomics*. 2002 Nov, 1(11), 845–67.
2. Xu R.; Greening, D.W.; Zhu, H.J.; Takahashi, N.; Simpson, R.J. Extracellular vesicle isolation and characterization: toward clinical application. *J. Clin. Invest.* 2016 Apr 1, 126(4), 1152-62.
3. Wu, C.C.; Tsantilas, K.A.; Park, J.; Plubell, D.; Naicker, P.; Govender, I.; Buthelezi, S.; Stoychev, S.; Jordaan, J.; Merrihew, G.; Huang, E.; Parker, E.D.; Riffle, M.; Hoofnagle, A.N.; MacCoss, M.J. Mag-Net: Rapid enrichment of membrane-bound particles enables high coverage quantitative analysis of the plasma proteome. *BioRxiv* 2024 [Preprint]. 2024 Apr 2:2023.06.10.544439.
4. Pino, L.K.; Searle, B.C.; Yang, H.Y.; Hoofnagle, A.N.; Noble, W.S.; MacCoss, M.J. Matrix-matched calibration curves for assessing analytical figures of merit in quantitative proteomics. *J. Proteome Res.* 2020, 19, 3, 1147–1153.

Learn more at **thermofisher.com/translationalresearch**

General Laboratory Equipment – Not For Diagnostic Procedures. © 2025 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. Evosep, Evosep One, Evotip, and Whisper are trademarks of Evosep. MagReSyn is a trademark of ReSyn Biosciences (Pty) Ltd. Aurora Elite is a trademark of IonOpticks. This information is presented as an example of the capabilities of Thermo Fisher Scientific products. It is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details. **TN003925-0625S**