Digging deeper into the plasma proteome using Mag-Net enrichment and Zeno SWATH data-independent acquisition (DIA)

High-throughput plasma proteomics using the Evosep One and ZenoTOF 7600 systems

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This technical note describes the high-throughput analysis of Mag-Net enriched plasma protein digests using the Evosep One system combined with Zeno SWATH DIA on the ZenoTOF 7600 system. Approximately 1,600 protein groups and 12,500 precursors were identified with excellent quantitative reproducibility from enriched plasma digests at a throughput of 100 samples per day (SPD), increasing to 3,500 protein groups and 30,000 precursors identified at 30 SPD. Mag-Net enrichment increases the sensitivity and dynamic range of protein detections in plasma. Combined with high-throughput separation on the Evosep One system and the high sensitivity and quantitative capabilities of Zeno SWATH DIA, the methods described here enable robust plasma analysis that can be automated for the study of large-scale cohorts.

Key features of enriched plasma proteomics using Zeno SWATH DIA

- **Capitalizing on the qualitative and quantitative power of Zeno SWATH DIA:** The ZenoTOF 7600 system provides the speed, selectivity and sensitivity to identify >3,500 protein groups from 4 µL of Mag-Net-enriched plasma with high quantitative reproducibility
- **Robust, high-throughput separation using the Evosep One system:** Plug-and-play methods enable LC separations from 30 to 500 samples per day to cover throughput requirements
- **Increasing the depth of plasma proteome coverage using Mag-Net enrichment:** Identify and quantify lower abundance proteins in plasma using MagReSyn SAX bead capture and depletion

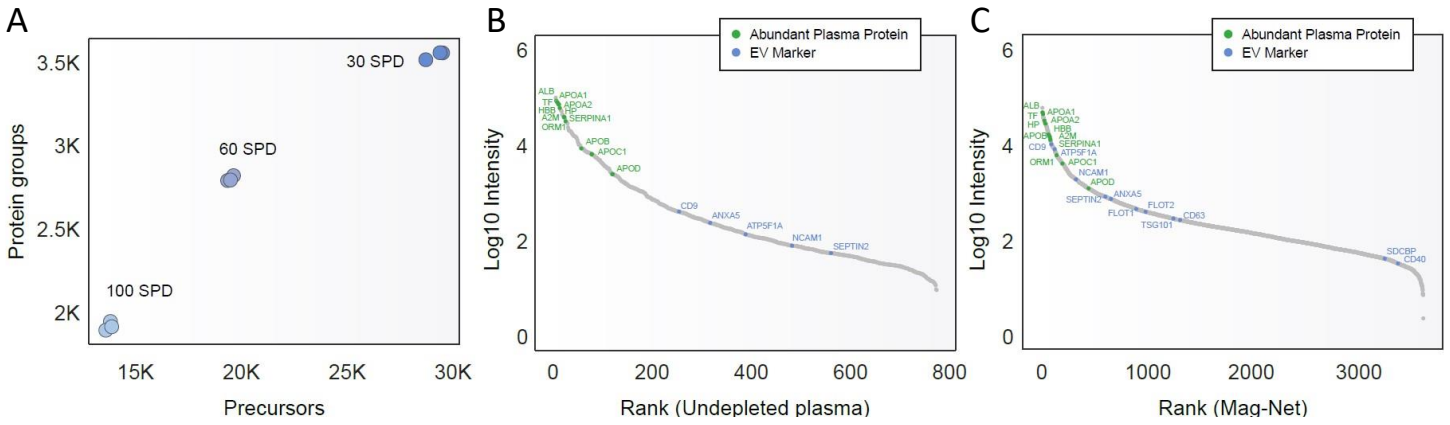

Figure 1. Detection of >3,500 proteins in enriched plasma using Zeno SWATH DIA. Mag-Net enriched plasma protein digests were analyzed using Zeno SWATH DIA following Evosep One separation at 100, 60 and 30 SPD and the numbers of protein groups and precursors identified are shown for the different throughput levels (A). Protein identifications and dynamic range are shown separately for undepleted (B) and Mag-Net enriched plasma (C) at 30 SPD, highlighting the improvement in plasma protein identification depth from Mag-Net enrichment followed by Zeno SWATH DIA analysis.

Introduction

Plasma is one of the most common biological matrices for disease research. Plasma proteomics seeks to characterize the relative abundances of plasma proteins, and how these change as a function of disease, to identify potential clinical biomarkers. The large dynamic range of protein concentrations in plasma (>10 orders) poses a significant challenge to the identification and quantitation of mid- to low-abundance plasma proteins. In recent years, numerous workflows have been created to address the limitations in developing reliable, automated methodologies that can be utilized for analyzing large sample cohorts. These workflows help in the deeper characterization of plasma biomarkers which ultimately supports the development of therapeutic treatments. The Mag-Net workflow has been demonstrated to be an efficient method for the enrichment of membrane-bound particles and simultaneously depletion of high-abundance proteins [1]. This method has been adapted for a complete end-to-end workflow including sample loading on Evotips in a fully automated manner on the Opentrons OT-2 liquid handler. Following this sample preparation method, LC-MS detection of potential plasma biomarkers requires high analysis speed, selectivity and sensitivity. High-throughput proteomics has been demonstrated previously by loading digested peptides onto Evotips for efficient concentration and cleanup followed by chromatographic separation on the Evosep One system for sample analysis at desired throughput levels ranging from 30 SPD to 500 SPD [2]. Previous studies have highlighted the substantial qualitative and quantitative protein characterization depth and robustness achieved by using Zeno SWATH DIA [3-5]. In this technical note, the combination of Mag-Net plasma enrichment, followed by high-throughput LC-MS with the Evosep One and ZenoTOF 7600 systems is described.

Methods

Sample preparation: Three different plasma centrifugation preparation methods were tested with samples of human plasma: the Early Detection Research Network SOP (1x1200g for 15 min), platelet-poor plasma (2x1500g for 10 min) and platelet-rich plasma (2x400g for 10 min). The preceding three plasma preparation techniques were analyzed further by LC-MS without any additional enrichment or depletion. In parallel, simultaneous enrichment of membrane-bound particles and depletion of abundant plasma proteins was carried out using MagReSyn strong anion exchange (SAX) beads as previously described [1]. Automated Mag-Net enrichment, extraction, digestion and Evotip loading were performed on 4 µL of prepared plasma samples using an Opentrons OT-2 robotic liquid handling system. Technical replicates were prepared for all samples in a 96-well plate format on the Opentrons system.

Chromatography: LC separation was performed on an Evosep One system using the methods for 30, 60, 100 and 200 SPD, with the LC columns matching the corresponding methods as recommended by Evosep [2]. Mobile phases consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, with all components being LC-MS grade.

Mass spectrometry: Zeno SWATH DIA data was acquired on a ZenoTOF 7600 system using an OptiFlow Turbo V ion source with the 1-10 µL/min microflow electrode in the vertical probe position. The parameters and methods employed for the various SPD modes were as previously described [2].

Data processing: Zeno SWATH DIA data were processed with DIA-NN software version 1.8.1, using a direct-DIA approach against the predicted UniProt human protein database [6]. Replicates for a given condition were searched separately, with match between runs selected. Principle component analysis (PCA) and hierarchical clustering analysis of the identified protein groups from the various plasma preparation methods were done using Enrichr software.

Sample preparation is critical to unlocking the depth of the plasma proteome

To compare the effectiveness of different plasma preparation techniques for LC-MS analysis, three different methods were evaluated: Early Detection Research Network SOP (1x1200g), platelet-poor plasma (2x1500g) and platelet-rich plasma (2x400g). Plasma protein preparations using the above methods were subsequently tested with or without Mag-Net enrichment. Digested

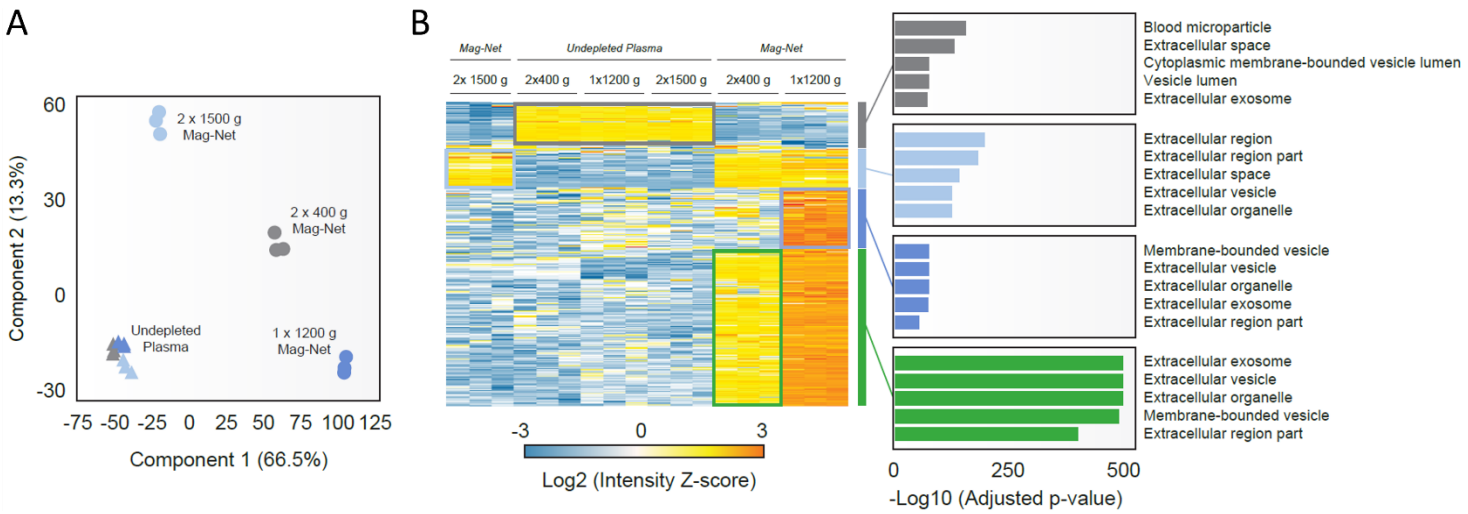

Figure 2. Effects of plasma preparation on the depletion and identification of plasma proteins. Three different plasma centrifugation preparations were tested, either with or without Mag-Net enrichment: Early Detection Research Network SOP (1x1200g for 15 min), platelet-poor plasma (2x1500g for 10 min) and platelet-rich plasma (2x400g for 10 min). Following preparation, digestion and Evotip loading, samples were analyzed using Zeno SWATH DIA at 100 SPD. Data was processed with DIA-NN software, followed by PCA analysis (A) and hierarchical clustering (B) using Enrichr software. The 1x1200g preparation method, followed by Mag-Net enrichment and LC-MS analysis with Zeno SWATH DIA, enabled the greatest depth of enrichment of the plasma protein components, as shown in the heatmap.

protein samples were analyzed using Zeno SWATH DIA and data analysis with DIA-NN software. PCA and hierarchical clustering analysis of the identified protein groups were done using Enrichr software to compare the levels of plasma proteome enrichment. PCA analysis demonstrated that the undepleted plasma preparations all cluster together, regardless of the centrifugation method, while the results for different plasma preparation methods cluster separately following Mag-Net enrichment (Figure 2). As indicated in the PCA plot in Figure 2A, these results suggest that different plasma preparation methods result in different subsets of proteins being isolated. This is also visualized in the hierarchical clustering highlighting different selectivity depending on the plasma preparation, as shown in Figure 2B. Importantly, undepleted plasma yielded only the expected high-abundance protein elements, regardless of the plasma centrifugation preparation technique. The 1x1200g preparation, followed by Mag-Net enrichment, resulted in the most efficient detection of lower-abundance plasma components. The subsequent results described in this technical note were generated from plasma protein samples prepared using the 1x1200g method.

Zeno SWATH DIA enables in-depth, high-throughput identification and quantitation of enriched plasma proteins

To further validate the reproducibility of the automated plasma workflows, a set of sample preparations using both the undepleted and Mag-Net enrichment methods were performed, followed by Zeno SWATH DIA analysis using the 200 SPD method. Precursor and protein group identifications are summarized in Figure 3. The identified precursors increased from approximately 2,000 with undepleted plasma to >8,000 with Mag-Net enriched plasma. The quantitative reproducibility also improved with Mag-Net enrichment: Figure 3B shows that the median CV for identified precursors decreased following Mag-Net enrichment, with a tightening of the overall distribution. The number of protein groups identified increased from approximately 250 with undepleted plasma to >1,200 with Mag-Net enrichment at 200 SPD with Zeno SWATH DIA.

To determine the overall improvement in identification, as well as the dynamic range of plasma proteins identified, the list of protein groups identified from Mag-Net enrichment followed by Zeno SWATH DIA at 200 SPD were compared against known extracellular vesicle (EV) protein components as catalogued in Vesiclepedia

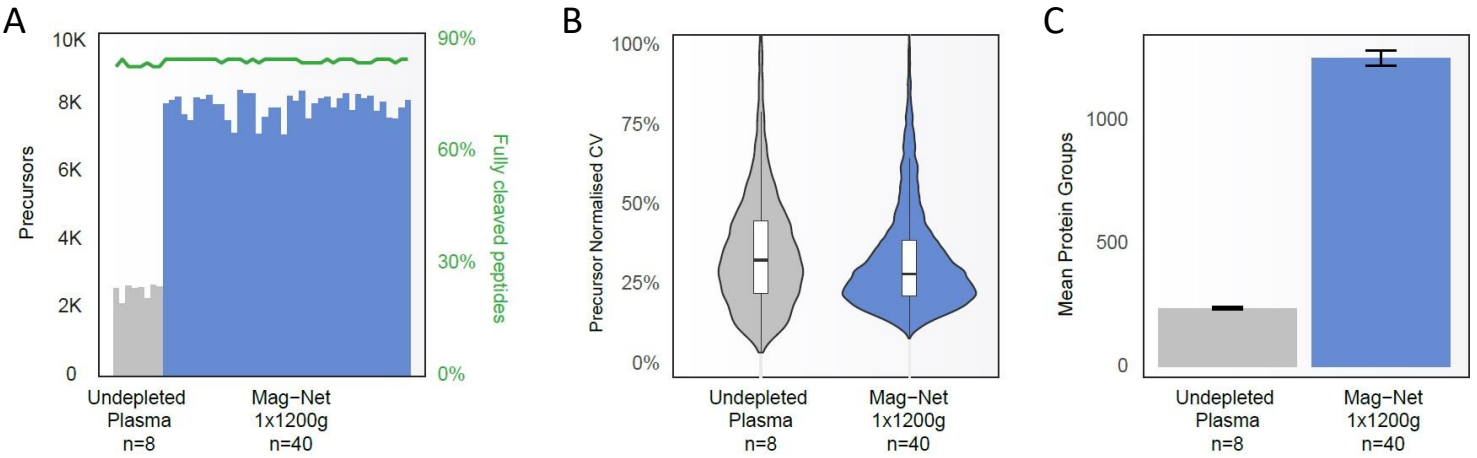

Figure 3. Identification and quantitation of plasma proteins at 200 SPD using Zeno SWATH DIA. Plasma proteins were prepared using the 1x1200g centrifugation protocol, either with or without subsequent Mag-Net enrichment. Digested protein samples were analyzed using LC separation on the Evosep One system at 200 SPD followed by Zeno SWATH DIA. Mag-Net enrichment resulted in an increase in precursor detections from approximately 2,000 to >8,000 relative to undepleted plasma (A). The median normalized coefficient of variation (CV) distribution for the identified precursors was also reduced because of the improved quantitative reproducibility (B). The number of protein group detections improved from 250 with undepleted plasma to >1,200 with Mag-Net enriched plasma (C).

(http://www.microvesicles.org/). Figure 4A shows that 80% of known EV protein markers and 100% of abundant plasma markers were identified with Mag-Net enrichment. A comparison of undepleted and Mag-Net enriched plasma (Figures 4B and 4C, respectively) indicates that the proportion of plasma protein signal intensities increased following Mag-Net enrichment, with a greater number of EV markers (including SEPTIN2, FLOT2, PDCD6IP, FLOT1, TSG101 and CD63) subsequently identified.

To increase the overall number of identifications, lower-throughput methods (100, 60 and 30 SPD) were also tested, comparing undepleted and Mag-Net enriched plasma using Zeno SWATH DIA (Figure 1). Figure 1A shows that at 30 SPD, >3,500 protein groups and approximately 30,000 precursors were identified from Mag-Net enriched plasma using Zeno SWATH DIA. The dynamic range compression for the protein group identifications using Mag-Net enrichment, compared to undepleted plasma, is shown in Figures 1B and 1C. The

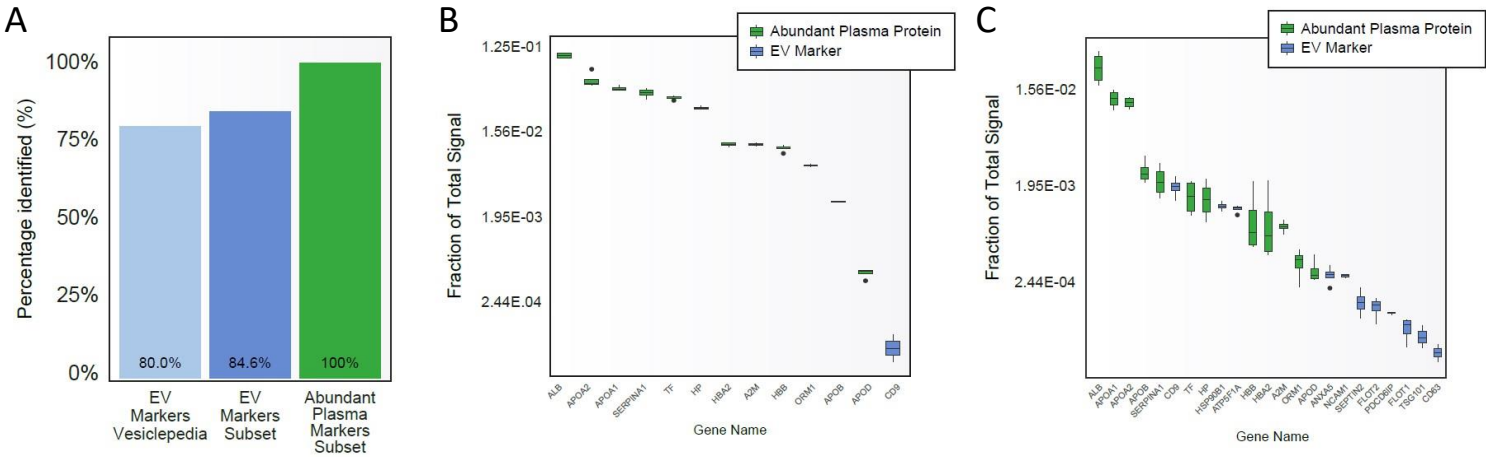

Figure 4. Improvement in depth of plasma proteome detection using Mag-Net enrichment followed by Zeno SWATH DIA. Plasma proteins were prepared using the 1x1200g centrifugation protocol, either with or without subsequent Mag-Net enrichment. Digested protein samples were analyzed using LC separation on the Evosep One system at 200 SPD followed by Zeno SWATH DIA. Plasma protein identifications were compared against the known proteins present in the extracellular vesicles (EV) components as documented in the Vesiclepedia (http://www.microvesicles.org/). Mag-Net enrichment resulted in the identification of 80% of catalogued EV markers and 100% of known high-abundance plasma markers (A). The fraction of total signal intensities of selected abundant proteins in plasma were compared between the undepleted plasma (B) and the Mag-Net enriched plasma (C). The dynamic range of detections was significantly improved using Mag-Net enrichment.

plots show Log10 intensities of protein groups as a function of protein group identification rank, indicating a 4-fold increase in the number of identified protein groups for Mag-Net enriched plasma relative to undepleted plasma.

These results demonstrate significant gains in plasma protein identification using Mag-Net enrichment relative to undepleted plasma and highlight the robust qualitative and quantitative power of high-throughput proteomics using the combination of the Evosep One and ZenoTOF 7600 systems. The entire workflow can be fully automated, enabling plasma proteomics for large-scale biomarker research.

Conclusions

- >3,500 protein groups and 30,000 precursors were identified in Mag-Net enriched plasma using Zeno SWATH DIA on the ZenoTOF 7600 system at a throughput of 30 SPD
- Mag-Net enrichment enables dynamic range compression and subsequently enhanced detection of lower abundance plasma proteins
- The Evosep One system allows for robust analysis over a wide range of desired throughput levels
- Fully automated Mag-Net plasma protein enrichment, digestion with direct sample loading on Evotips, combined with Evosep One separation and Zeno SWATH DIA analysis on the ZenoTOF 7600 system, can be applied toward the analysis of large-scale clinical cohorts for biomarker research

References

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Acknowledgments

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