Application Note

EVOSEP

BECKMAN COULTER Life Sciences

Scalability for high-throughput proteomics - **Evotip Pure** integration with the **Biomek i5** liquid handler

Highlights

- Fully automated sample preparation of up to 576 samples in parallel
- An end-to-end solution solving the sample preparation bottleneck

1. Scalable sample preparation

With the development of faster and more sensitive mass spectrometers, proteomics has become an essential tool across diverse applications in both academia and industry.

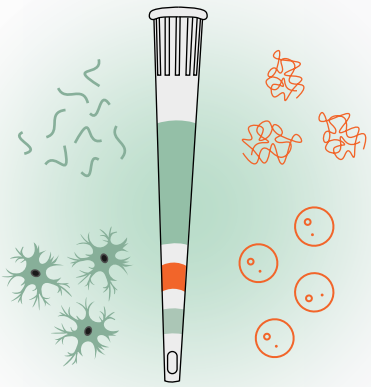
As proteomics studies become larger and analyses become more routine, the demand for standardized and scalable solutions is increasing. To address this need, we present four fully automated workflows developed for the Biomek i5 Automated Liquid Handler, seamlessly integrated with Evosep technology to ensure efficient and reproducible end-to-end sample preparation. The liquid handler can process up to six plates in parallel, handling 576 samples in a single run and leaving them ready for analysis on Evotips. The process can be optimized for speed, requiring 3 hours to digest six plates or 18 hours if digestion is performed overnight.

Evotip Sample Loading

This workflow uses an Evotip sealing mat for Biomek enabling automated desalting and loading on the Evotip for efficient sample storage prior to downstream LC-MS analysis.

Digestion workflow

The PAC[1] digestion workflow leverages protein aggregation on magnetic beads, followed by direct on-bead digestion of reduced and alkylated protein lysate.



Neat Plasma workflow

Combining steps for reduction and alkylation, this workflow utilizes PAC digestion as a streamlined approach for processing large plasma sample cohorts.

Mag-Net Plasma workflow

For deep plasma proteomics, the Mag-Net[2] workflow combines enrichment of membrane-bound vesicles with PAC digestion.

2. Method details

HeLa cells were cultured in DMEM media with 10% FBS and harvested in boiling 5% SDS buffer. The Biomek i5 MC (Beckman Coulter Life Sciences) liquid handler was used with a 1200 µl multichannel head, the BioShake (D30-T elm, Q-Instruments) and the Magnum FLX magnet plate (Alpaqua) modules.

For PAC digestion, 1 µg HeLa lysate was mixed with 5 µl MagReSyn hydroxyl beads (Resyn Biosciences). Isopropanol was added to 80% and mixed on the Bioshake module. After protein aggregation and a single isopropanol wash, overnight (16 hours) digestion was performed at 37˚C or ambient temperature (inter-plate variation experiment) with 40 ng trypsin (T6567, Sigma Aldrich) and 10 ng Lys-C (129-02541, Wako Fujifilm). Samples were diluted to the appropriate concentration before loaded on Evotips.

Plasma was extracted per the Early Detection Research Network (EDRN) SOP[3]. For neat plasma analysis, 1 µl was lysed, reduced, and alkylated before being diluted and approximately 2 ug protein was used for digestion. PAC digestion was initiated by adding 5 µl hydroxyl beads and 60 µl isopropanol. After digestion, 40% of the resulting peptides were loaded onto Evotips. For Mag-Net enrichment, 4 µl plasma was mixed 1:1 with binding buffer and 1 µl MagReSyn SAX beads (ReSyn Biosciences). Following three wash steps and one-pot lysis, reduction and alkylation, PAC initiation was carried out as described above. After overnight digestion at 37˚C, 80% of the resulting peptides were loaded on Evotips. This requires an Evotip loading kit for Biomek i5/i7 1200 µL 96-Channel head (EV1183, Evosep).

Inter-plate variation samples were analyzed with the 500 SPD method and all other samples were analyzed with the 200 SPD method. The EV1107 Endurance column (Evosep), operated at ambient temperature, was used for all experiments on a timsTOF HT mass spectrometer (Bruker) with the default “short gradient dia-PASEF MS method”. Data was analyzed with DIA-NN (version 1.8.1) in library-free mode against the human proteome database (Uniprot, Oct 2020, 20,600 entries without isoforms) with trypsin/P as protease allowing 2 missed cleavages. All conditions were searched separately with match-between-runs enabled across replicates within the same condition.

3. Built for large scale analysis

The workflows are designed to facilitate efficient and cost-effective analysis of large sample cohorts. With Evotip loading as the final step of each workflow, samples are safely stored on Evotip Pure until LC-MS analysis. This approach utilizes and analyses close to 100% of the digested sample by only digesting what is analyzed, minimizing the cost-per-sample by reducing both enzyme and sample consumption. Furthermore, using a single sample-specific tip and shared pipette tips for solvents, the workflow ensures sustainable sample preparation. Incubation steps are utilized to process multiple 96-well plates in a staggered manner, significantly increasing overall throughput. As a result, each workflow takes less than two minutes of total processing time per sample when six plates with 96 samples are managed simultaneously, for a total of 18 hours of processing.

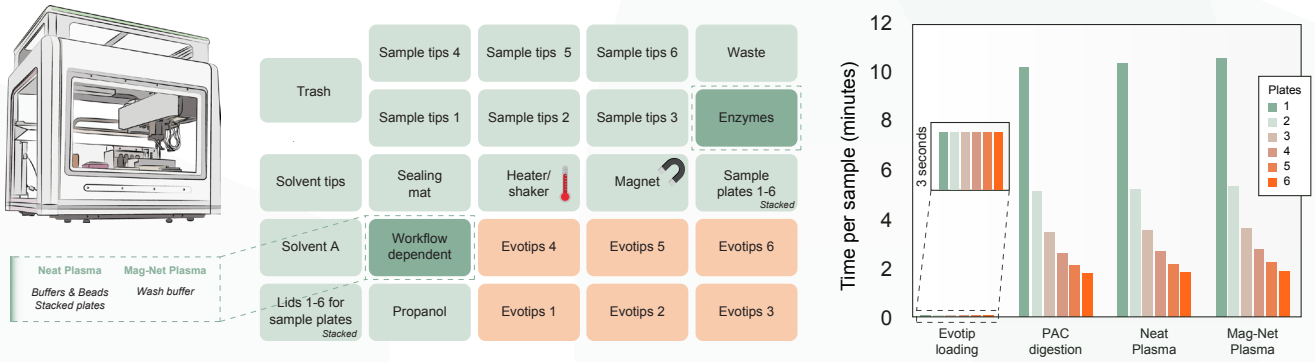


Figure 1: Schematic representation of deck layout on the Biomek i5 and throughput based on how much time is spent per sample when 1-6 plates are processed with each workflow.

4. Automation without compromise

The data presented utilizes overnight digestion at 37˚C on the BioShake module to ensure optimal digestion and tightly fitting lids to prevent sample evaporation. Shorter digestion times can be accommodated as well. We processed one sample plate with two different HeLa input amounts and two types of blanks and found no cross contamination as evidenced by negligible intensities in the blanks. Using the 200 SPD method on a timsTOF HT, we identified 5,500 proteins per sample from 1 µg protein input and 4,900 per sample from 500 ng protein input.

When processing 6 plates, using overnight digestion at ambient temperature with 1 µg HeLa input, there was no inter-plate variation, as evidenced by high Pearson correlations at the protein level. Additionally, we observed excellent and reproducible digestion efficiency, and a median coefficient of variation (CV) on protein level below 20% for all workflows. The combination of Evosep technology and the Biomek i5 liquid handler seamlessly integrates scalable sample preparation and LC-MS analysis, ensuring high quality and reproducible results.

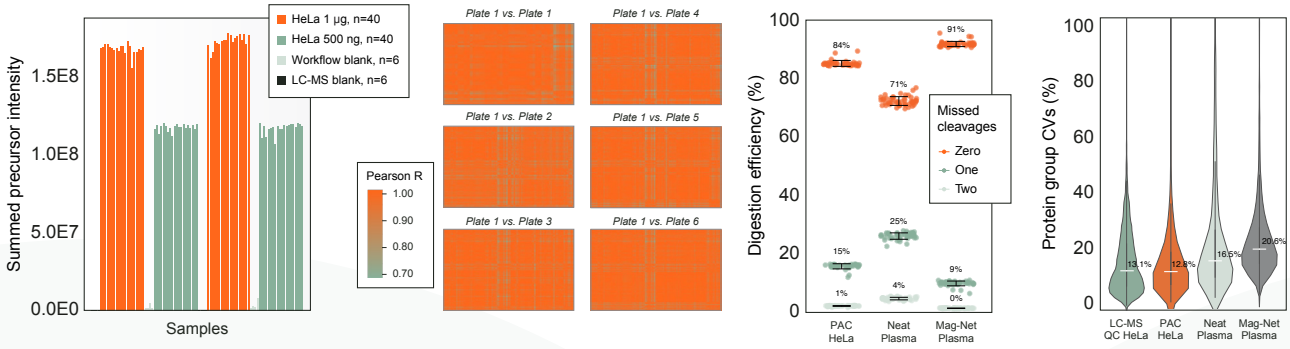


Figure 2: Summed intensities from PAC HeLa plate (80% load and 200 SPD) and heatmap of protein Pearson correlations for 6 plates (40% load and 500 SPD). Digestion efficiency across the workflow and protein group CVs for all workflows analyzed at 200 SPD.

5. Outstanding plasma proteomics

Non-targeted plasma proteomics holds immense potential in clinical research monitoring the onset and progression of diseases. With disposable trap columns, the Evosep is an ideal platform for handling challenging sample types like plasma. As such, the described workflows for neat plasma analysis and Mag-Net enrichment provide powerful tools for large-scale biomedical applications. To demonstrate the efficacy of the workflows, we prepared 40 samples of neat plasma using 1 µl of plasma and 40 samples of Mag-Net enrichment using 4 µl of plasma input. The neat plasma analysis exhibited excellent depth, identifying 500 proteins using the standard 200 SPD method.

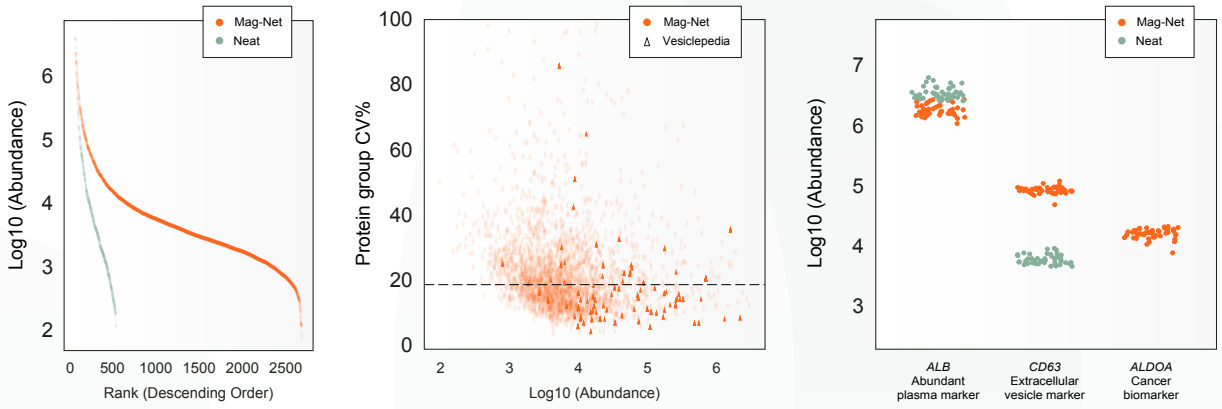


Figure 3: Dynamic range for neat plasma (1 µl, 40% load) and Mag-Net plasma (4 µl, 80% load). Scatter plot of protein groups CVs against abundance and abundance against rank for selected proteins. Samples were analyzed with 200 SPD.

The Mag-Net enrichment successfully compressed the dynamic range, resulting in more-than a 5-fold increase with 2,700 proteins identified using the 200 SPD method. Among the identified proteins were 40 listed in Vesiclepedia, the majority of which were identified with CVs below 20%, validating the workflow. Finally, we selected one abundant plasma protein (ALB), one EV marker (CD63) and a known cancer biomarker (ALDOA) and plotted their abundancies in all 40 replicates from both conditions. The high similarity across replicates demonstrates the robustness of the workflow across replicates.

7. Conclusion

The integration of the Biomek i5 Automated Liquid Handler with Evosep technology offers a robust and scalable solution for high-throughput proteomics. Our automated workflows, including automated sample loading, PAC digestion, neat plasma analysis, and Mag-Net enrichment, demonstrate exceptional throughput, reproducibility, and depth of analysis. These workflows enable the processing of large sample cohorts with minimal manual intervention and reduced costs, making them ideal for both routine analyses and large-scale studies. By combining cutting-edge technologies in automation with liquid chromatography, these workflows address the growing demand for standardized and scalable proteomics solutions. These protocols pave the way for high-quality proteomics applications in clinical research and industrial applications.

Evosep One is for General Laboratory Use.

Evosep assumes no responsibility and shall have no liability for any damage or loss of samples, material and hardware that may arise from use of or in connection with Evosep recommended protocols and SOP's.

Biomek Automated Workstations are not intended or validated for use in the diagnosis of disease or other conditions.

References

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