Application Note

EVOSEP

Efficient proteomics with an automated sample preparation strategy leveraged by **Evotip Pure** and the **AssayMAP Bravo**

Highlights

- A sustainable and cost-efficient strategy leveraged by the Evotip Pure
- Robust and easy-to-use digestion workflow for up to 96 samples at the time

1. Introduction

Proteomics is undergoing a significant shift and rapidly evolving towards more efficient and user-friendly technologies that can handle higher quantities of samples. The Evosep One, combined with improvements in high-end mass spectrometry, has played a vital role in transforming this field, making it possible to analyze hundreds of proteomes daily. Consequently, the bottleneck in high-throughput proteomics workflows has shifted towards sample preparation with demand for a robust pipeline with fully automated end-to-end workflows. We present a complete digestion workflow, including in-line Evotip loading of up to 96 samples using the Agilent AssayMAP Bravo liquid handling robot. With the possibility to select the digestion time, the workflow can be performed in less than one hour or extended to overnight for improved digestion efficiency.

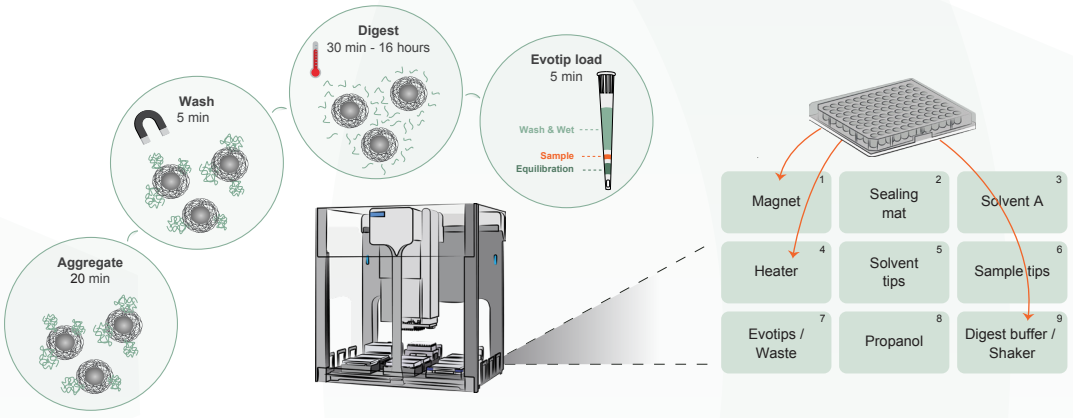

Figure 1: Schematic representation and deck layout of the fully automated digestion workflow for up to 96 samples.

2. Method details

Experiments were conducted using HeLa cells, harvested in boiling lysis buffer (5% sodium dodecyl sulfate (SDS), 5 mM tris(2-carboxyethyl)phosphine (TCEP), 10 mM chloroacetamide (CAA), 100 mM Tris, pH 8.5). The complete and automated sample preparation utilizes protein aggregation capture (PAC) on magnetic hydroxyl microparticles (Resyn Biosciences), using a single wash step with isopropanol. Digestion was carried out with a protease mix of of 1:100 Lys-C to protein and 1:25 trypsin to protein ratio for 30 min to 16 hours, shaking at 37°C. A lid was placed on top of the sample plate during all incubation steps to reduce evaporation. Resulting peptides were loaded directly on Evotip Pure using the AssayMAP Bravo (VWorks 13) and a layered sandwich approach with defined air gaps between the layers. The layers were subsequently pushed through using the AssayMAP Bravo 96-Head leaving the Evotips ready for injection on the Evosep One. The digestion time experiment was analyzed with the 100 SPD method using the EV1109 Performance column (Evosep) operated 40°C. The robustness and reproducibility experiments were conducted using the 500 SPD method with the EV1107 Endurance column (Evosep) operated at ambient temperature on a timsTOF HT (Bruker) using the “standard DIA-PASEF - short gradient” MS method. The sensitivity experiment was performed on a timsTOF HT using the Whisper™ Zoom 40 SPD method with the Aurora Elite column (IonOpticks) and Whisper Zoom 80 SPD with the Aurora Rapid75 column (IonOpticks), both operated at 50°C with the “standard DIA-PASEF - long gradient MS method”. A targeted HeLa dynamic MRM method was used for the digestion time experiment using a QQQ 6495C (Agilent).
Data was analyzed with DIA-NN (version 1.8.1) in library-free mode against the reviewed human proteome database (Uniprot, Oct 2020, 20,600 entries without isoforms) with trypsin/P as digestion enzyme allowing 2 missed cleavages. All conditions were searched separately with MBR enabled across replicates within the same condition. Targeted data was analyzed with Skyline (21.2.0.425).

3. Standardized flexibility

To allow for an easy-to-use and flexible workflow, a customer-friendly user interface was developed allowing for simple selection of digestion time to balance throughput and depth. Four replicates of 1 µg HeLa lysate was digested at each time point, and 40% of the resulting peptides were loaded on two replicate Evotips. The first set of samples were analyzed using the 100 SPD method with the timsTOF HT to monitor digestion efficiency on a global level. This revealed that overnight digestion for 16 hours yielded an efficiency with ~90% fully cleaved peptides and excellent quantitative precision with ~5% median CVs on protein level. By pushing the throughput with shorter digestion time of 2 hours, the digestion efficiency was reduced to 73%, while still maintaining excellent CVs well below 10% illustrating high reproducibility in the sample preparation protocol. The second set of samples were analyzed with the 100 SPD method using the QQQ 6495C and a targeted method monitoring 31 HeLa peptides spread

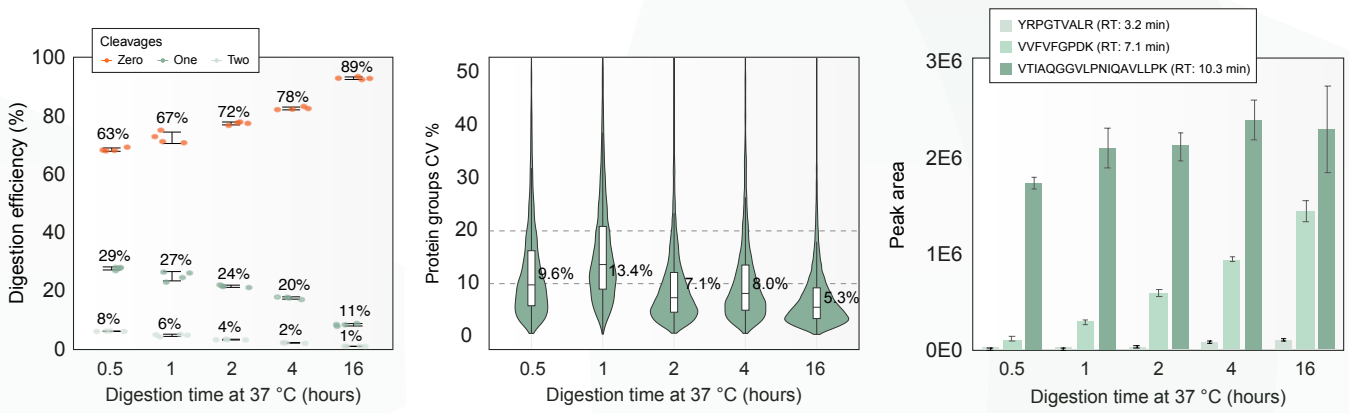

Figure 2: Digestion efficiency for 30 min to 16 hours digestion and corresponding protein group CVs per condition. Peak area of selected targets at each digestion time. ~40% of each peptide digest was loaded on Evotips and analyzed with the100 SPD method.

across the gradient including targets with missed cleaved versions. This analysis revealed peptide specific digestion patterns as some peptides were fully digested after just 30 minutes, and other peptides required overnight digestion. This suggests the need to include target dependent optimization of digestion time to obtain reproducibility in the complete workflow.

4. Maximized utilization

The throughput on the AssayMAP Bravo has been maximized by multi-purposing deck positions, including the heater and shaker modules. This enables the sequential processing of up to 480 samples in less than a single workday by using 30 minutes digestion time. To highlight the robustness and reproducibility, of this approach, 480 samples were prepared in one day using 30 minutes digestion at 37°C and analyzed using the 500 SPD method and the timsTOF HT. The analysis showed great reproducibility across all samples with a mean digestion efficiency of 67%. This was also similar across the plates with less than 7% CV. Globally, the intra plate variability was low, showed by tight clusters in the PCA analysis.The inter plate variability also revealed high reproducibility with the highest explained variance of 16.5%. The reproducibility and robustness highlighted here are essential components to pursue high-throughput proteomics.

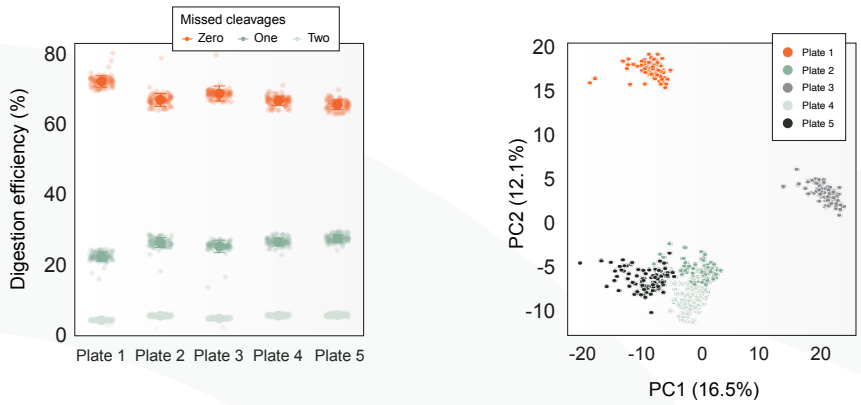

Figure 4: Digestion efficiency (30 min, 37°C) across five plates and a PCA analysis to measure variation across the plates. Samples were analyzed with the 500 SPD method.

5. Excellent sensitivity

The workflow is designed for scalability, considering the protein starting amount and cost-per-sample for the entire workflow. This is enabled by high sensitivity in all parts of the workflow and especially the direct loading on the Evotip post digestion, ensures seamless integration and optimal storage conditions in preparation for LC-MS analysis. To showcase the sensitivity, a serial dilution of HeLa lysate from 1000 ng to 5 ng starting material was used to perform eight replicate digestions overnight at 37°C. 80% of the resulting peptides were loaded on Evotips and analyzed using the Whisper Zoom (40 & 80 SPD) methods coupled with the timsTOF HT. We identified around 8,000 precursors and nearly 2,000 proteins from just 5 ng input and more than 90,000 precursors and 8,000 proteins from 1000 ng input material with the Whisper Zoom 40 SPD method. By increasing the throughput to 80 SPD, 5,000 precursors and around 1,500 proteins from 5 ng input were identified, and nearly 70,000 precursors and more than 7,200 proteins from 1000 ng input material. Importantly, the protein CVs were outstanding with less than 20% for 10 ng input and ~6-7% for 1000 ng input for both Whisper Zoom methods. These results reinforce the potential of the workflow to be used for highly sensitive applications.

Figure 4: Identification of precursors, proteins, and CVs of a protein lysate dilution curve. ~80% of each peptide digest was loaded on Evotips and analyzed with Whisper Zoom 40 & 80 SPD.

5. Conclusion

We have successfully developed a rapid and robust end-to-end proteomics workflow for PAC digestion in-line with sample loading on Evotips, on the AssayMAP Bravo platform that allows for the preparation of up to 500 samples per day dependent on the selected digestion time. The protocol utilized the heater and shaker modules on the AssayMAP Bravo allowing short digestion times but also flexibility to increase depth with overnight digestion. This allowed close to complete digestion efficiency with 89% fully cleaved peptides. Using short digestion time of 30 minutes, this was decreased to 65%, but importantly very reproducible with a 7% CV based on digestion efficiency across 480 technical workflow replicates. The workflow demonstrated high sensitivity with the identification of more than 8,000 proteins from just 1 ug of input material using the Whisper Zoom methods. This is another great example of how the Evotip Pure can easily be integrated into various workflows regardless of the robotic platform. Moreover, it shows how Evotip Pure seamlessly connects sample preparation with LC-MS analysis. By combining PAC digestion and automated sample loading, this workflow lays a solid foundation for adding other functional sample preparation modules.

The Evosep One instrument is for Research Use Only.

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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