Application Note

EVOSEP

Fully automated, end-to-end digestion workflow with Evotip Pure loading on the Opentrons OT-2

Highlights

- Completely hands-off workflow for preparation of 384 samples in a workday
- A sustainable and cost-efficient approach leveraged by the Evotip Pure

1. Modular sample prep workflows

The field of proteomics is being rapidly redefined by technological advancements towards increasingly robust and faster instrumentation. This development is driving a new era where the combination of high-throughput and excellent performance paves the way for novel proteomics applications across various fields. To address this, we have established the framework for a versatile, modular, and fully automated sample preparation strategy based on magnetic beads integrated with Evotip Pure sample loading. Here we present the workflow on the Opentrons OT-2 liquid handler (OT-2) for efficient and robust preparation of ready-to-analyze peptides from extracted proteins with just 4 hours of digestion at room temperature. For ease-of-use, we have designed a HTML-based user interface that allows for easy design of experiments, and produces a Python script that can be directly imported into the Opentrons app. The unique properties of the Evotip Pure allow for immediate and online desalting and storage of digested peptides for a lossless integration between sample preparation and LC-MS analysis. This framework presents a solid foundation on which functional modules for versatile proteomics applications can be added in the future (Figure 1).

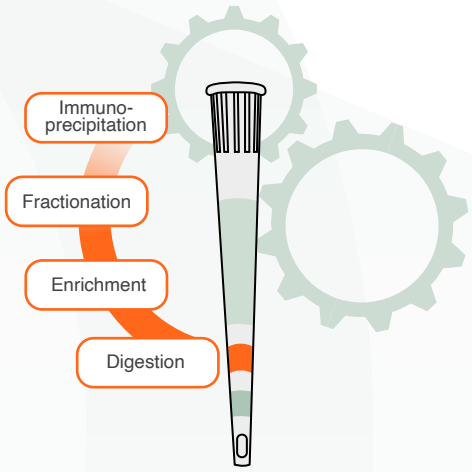

Figure 1: Modular sample preparation strategy.

2. Method details

For information about how to set up the protocol, please see the step-by-step guide for the protocol, “IN-003C 24/02”, which can be found online at; www.evosep.com/support/automation. HeLa cells were cultured in DMEM media and harvested in boiling 5% sodium dodecyl sulfate (SDS) buffer. Protein aggregation capture (PAC) assisted digestion was prepared from 1 µg HeLa lysate and 5 µl MagReSyn Hydroxyl (Resyn Biosciences), both transferred to each well of the sample plate(s) along with acetonitrile to a final concentration of 80%. Two mixing steps were carried out to facilitate on-bead aggregation for 10 minutes, followed by a single wash in acetonitrile. Digestion was carried out at ambient temperature with a ratio of 1:100 Lys-C:protein and 1:25 trypsin:protein for 4 hours. Following digestion, samples were diluted and loaded onto Evotips. Peptide loads are stated as a combination of the starting protein amount and the percentage of the resulting peptides. The digestion time and reproducibility experi- ments were carried out with the standard 100 SPD method using the EV1109 column (Evosep) operated at 40 °C. The different loads experiment additionally uses the 200 SPD method with the EV1107 column (Evosep) operated at ambient temperature. The sensitivity experiment was carried out with the Whisper 40 SPD method using the Aurora Elite column (IonOpticks, AUR3-15075C18-CSI) operated at 50 °C. All samples were analyzed on a timsTOF Pro 2 mass spectrometer (Bruker) with dia-PASEF and 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 match between runs enabled across replicates within the same condition.

3. Sustainable by design

The automated PAC protocol uses just a single pipette tip per sample for all sample handling steps and 24 common tips for dispensing solvents and buffers. This strategy ensures a sustainable and cost-efficient approach to sample preparation while minimizing the risk of cross-contamination between samples.
To optimize the throughput of the protocol, while considering the limited deck space on the OT-2, we assessed the proteome coverage and digestion efficiency of short digestion times at ambient temperature and thereby avoiding the need for a heating block on the deck. For each condition, we digested 8 replicates of 1 µg HeLa lysate and loaded 20% of the resulting peptides from each sample on Evotips. All samples were analyzed using the 100 SPD method in a randomized order. After only 4 hours of digestion, we achieved an analysis depth and digestion efficiency similar to those of a standard overnight digestion. The resulting throughput is 192 samples in 6.5 hours and 384 samples in a single workday per OT-2, enabling high-throughput scalable proteomics workflows.

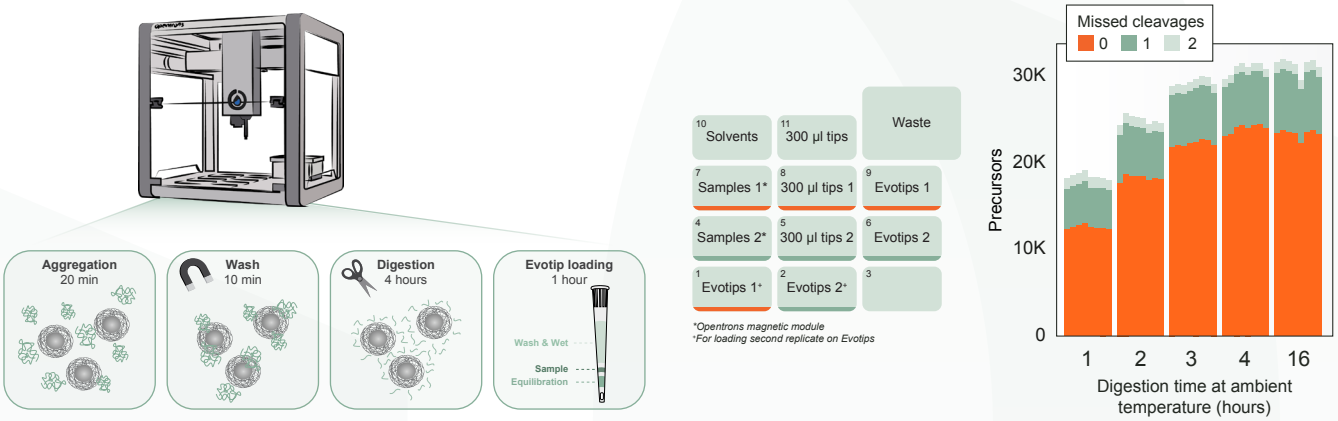

Figure 1: Layout of the OT-2 workflow. Precursor identifications and digestion efficiencies at different time points based on 1 µg lysate starting amount. ~20% of the resulting peptides were loaded on Evotips and analyzed with 100 SPD.

4. Reproducible and robust

The workflow utilizes efficient binding of peptides on Evotip Pure to reduce sample losses, manual intervention and carryover. A sample plate with 8 dispersed blanks was digested to evaluate reproducibility and cross-contamination. The analysis reproducibly identified over 30,000 precursors across all samples with a digestion efficiency of 73% (Figure 2). Each blank resulted in the identification of fewer than 500 precursors. Samples showed good quantitative precision with median CVs of 22% and 13% for identified precursor and protein groups, respectively. Finally, samples were stored for up to 9 days at 4 °C post Evotip loading, and analysed to show the stability of peptides once loaded on Evotip Pure (Figure 2). This property is essential for high-throughput proteomics, as it ensures that samples are preserved from sample preparation to LC/MS analysis.

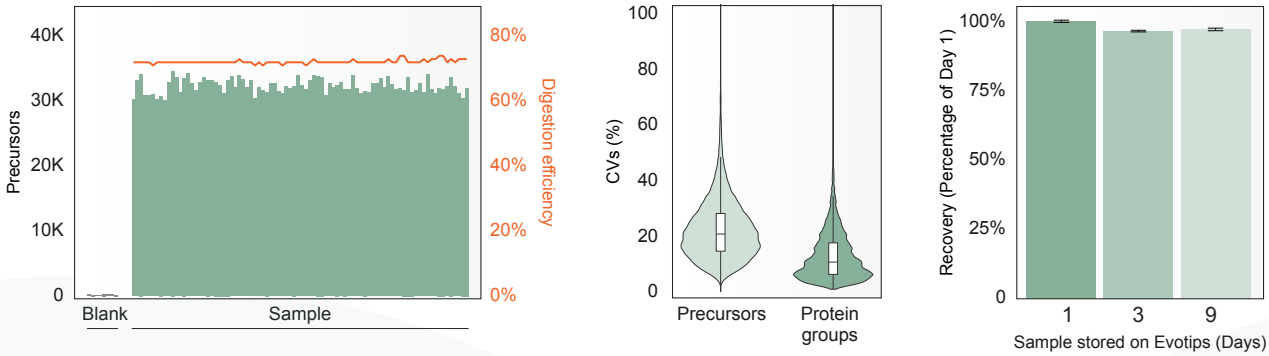

Figure 2: Workflow reproducibility and peptide digest sample storage on Evotips. Staring amount was 1 µg lysate and ~20% of the resulting peptides were loaded on Evotips and analyzed with 100 SPD.

5. Standardized, yet flexible

The protocol is accessed through a user-friendly interface allowing customizing the experimental setup. With the option to digest between 1 and 5 µg of protein and load 20% to 80% of the resulting peptides, one can decide for the optimal load for any combination of Evosep method and mass spectrometer. We digested 1 µg HeLa and analyzed different loads using the 100 SPD and 200 SPD methods on a timsTOF Pro 2. Increasing loads result in more protein identifications with both methods, but the faster 200 SPD method suffers a loss in quantitative precision at the highest load (Figure 3). Additionally, the protocol design facilitates loading of two technical replicates from the same digest, when less than 50% is selected as sample load to enable the

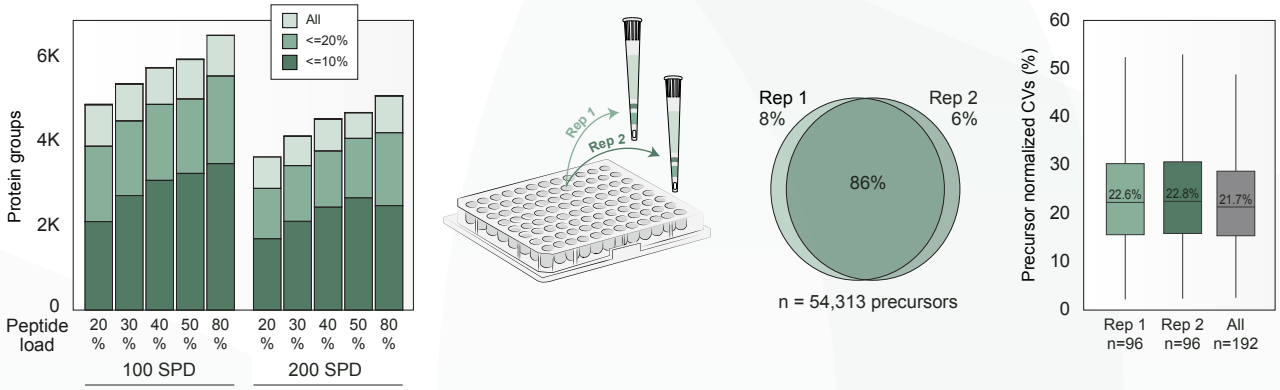

Figure 3: Identified protein groups by CVs based on digestion of 1 µg HeLa with varying peptide loads analyzed with 100 SPD and 200 SPD. Technical replicates generated from 96 samples from 1 µg HeLa digestions with 30% peptide load, visualized with overlap in identified precursors and CVs.

possibility to use the entire sample for analysis. This feature was tested by digesting 96 replicates of 1 µg of HeLa lysate and loading two different Evotips with 30% load. The two precursor populations were very similar with 86% overlap and equal quantification (Figure 3).

6. High sensitivity

Finally, the sensitivity of the protocol was benchmarked by digesting serial dilutions of HeLa lysate from 125 ng to 0.98 ng in quadruplicates. ~70% of the resulting peptides were loaded on Evotips and analyzed with the Whisper 40 SPD method. With more than 1,000 and 7,000 proteins identified at 1 ng and 125 ng input material respectively, the protocol showed outstanding performance for sensitive applications (Figure 4). The reduction in identified precursors in diluted samples occurred evenly across the gradient indicating that the performance is not biased towards peptides with specific physiochemical properties at reduced loads. These results showcase the potential of the protocol for high-sensitivity applications with robust quantification of precursors as evidenced by median CVs below 25% for all samples where more than 1 ng was digested.

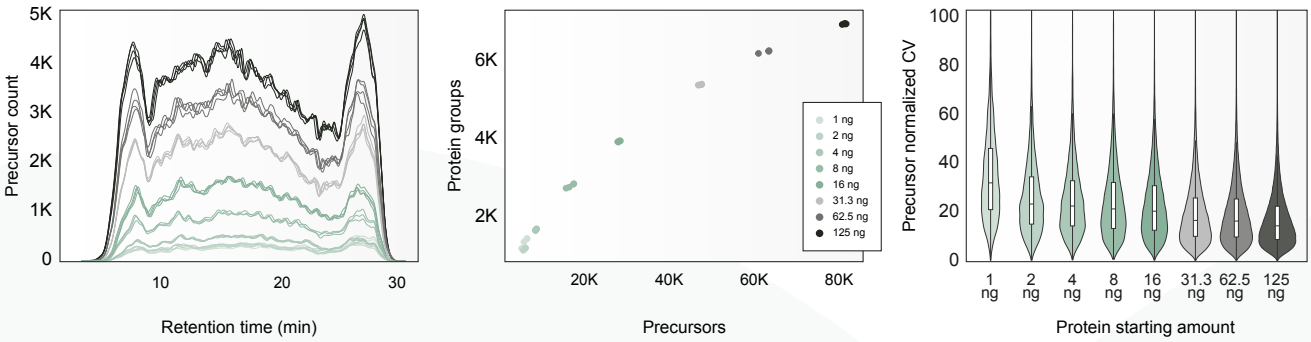

Figure 4: Identifications and CVs for protein lysate dilution curve. ~70% of the resulting peptide is analyzed with Whisper 40 SPD.

7. Conclusion

We have successfully developed a rapid and robust end-to-end proteomics workflow for PAC digestion and subsequent sample loading on Evotips, where even the inexpensive OT-2 liquid handling platform can prepare 384 samples per day. The protocol utilizes short digestion times at ambient temperature and yet provides comparable performance to overnight digestion. With a simple user interface, the versatile features of the protocol are easily customized to address a wide variety of needs, including robust sample preparation, diverse protocol generation, and sensitive applications. Careful design balances capacity for high throughout with low costs per sample to facilitate cost-effective analysis of large sample cohorts. Overall, the success of this workflow demonstrates how the Evotip Pure seamlessly connects sample preparation and LC-MS analysis. The combination of PAC digestion and automated sample loading lays a strong foundation on which more functional modules for sample preparation will be added.

The Evosep One instrument is for Research Use Only.

Availability of the automated PAC digestion protocol for the Opentrons-2
A protocol generator and information can be found online at *www.evosep.com/support/automation*.