Application Note

End-to-end, fully automated digestion protocol and Evotip Pure workflow on the Opentrons OT-2

1. Introduction

The field of proteomics is rapidly being redefined by technological advancements towards faster and increasingly robust 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. The Evosep One provides a standardized and user-friendly platform that enables routine analysis of hundreds of proteomes per instrument per day, which has shifted the bottleneck for realizing high-throughput proteomics towards sample preparation.

To address this, we have developed a fully automated Evotip loading protocol, based on a unique layered sandwich approach, which is the central component of a modular automation strategy that allows for addition of functional modules such as digestion and enrichment steps.

Here, we introduce a fast and efficient, automated sample preparation protocol on the Opentrons OT-2 (OT-2) utilizing protein aggregation capture (PAC) on magnetic microparticles in an end-to-end standardized hands-off-workflow, starting from protein lysate to peptide loaded on Evotips. This is made possible due to the essential properties of the Evotip, where the entire sample is efficiently loaded in a high volume just after preparation, minimizing losses by removing unnecessary sample handling steps. Furthermore, the Evotip serves as a highly efficient, temporary storage device, allowing for complete sample handling on the robot and transfer of the prepared Evotips to the LC/MS instrumentation, once available or whenever convenient.

The presented protocol can digest and load up to 192 samples on Evotips in less than eight hours, enabling a throughput of up to 384 samples prepared in a work day. The protocol was developed to digest an unusual small amount of protein starting material, i.e. from 1 to 20 µg. This results in a very cost-efficient sample preparation strategy for deep proteome profiling and with more than 7,000 proteins identified from just 100 ng of input material it is remarkably sensitive as well. The protocol is available in an easy-to-use HTML form that generates complete scripts that can be downloaded from the Evosep website and directly imported into the Opentrons app.

2. Method details

For information about how to setup the protocol on the OT-2, including deck layout, solvent plate layout, and liquid handling, please see the step-by-step guide for the protocol, "IN-003A 23/05", 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. 1 µg HeLa lysate, 5 µl MagReSyn Hydroxyl (Resyn Biosciences), and 80% acetonitrile was transferred to each well of the sample plate(s). 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 for analyses are calculated based on the initial amount of protein assuming 100% recovery.

The digestion time and reproducibility experiments were carried out with the standard 100 SPD method using the EV1109 column (Evosep) operated at 40 °C. The maximum depth experiment was carried out with the 30 SPD method using the EV1137 column (Evosep) operated at 40 °C. 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 (Uniprot, Oct 2020, 20,570 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. Digestion time

We initially assessed the digestion efficiency and proteome coverage of this protocol with 1, 2, 3, and 4 hours of digestion time against a standard overnight digestion at ambient temperature. For each condition, we digested eight replicates of 1 µg HeLa lysate and loaded 20% (corresponding to a theoretical 200 ng peptide amount) of the resulting peptides, based on volume, of each sample on Evotips. All samples were analyzed in a randomized order with the 100 SPD method. The different digestion times were evaluated based on the number of identified precursors, protein groups and the digestion efficiency, as estimated by the number of missed cleavages at precursor level. With increasing digestion times, the digestion efficiency and recovery also increases compared to shorter digestion times. This trend continues until 4 hours of digestion, at which point the number of identified precursors and digestion efficiency reaches a level similar to that of an overnight digestion (Figure 1). Therefore, the automated protocol was fixed at 4 hours of incubation time to accommodate a complete workflow to process 192 samples in 7.5 hours with a possible sample preparation throughput of 384 in one day, by running two consecutive workflows.

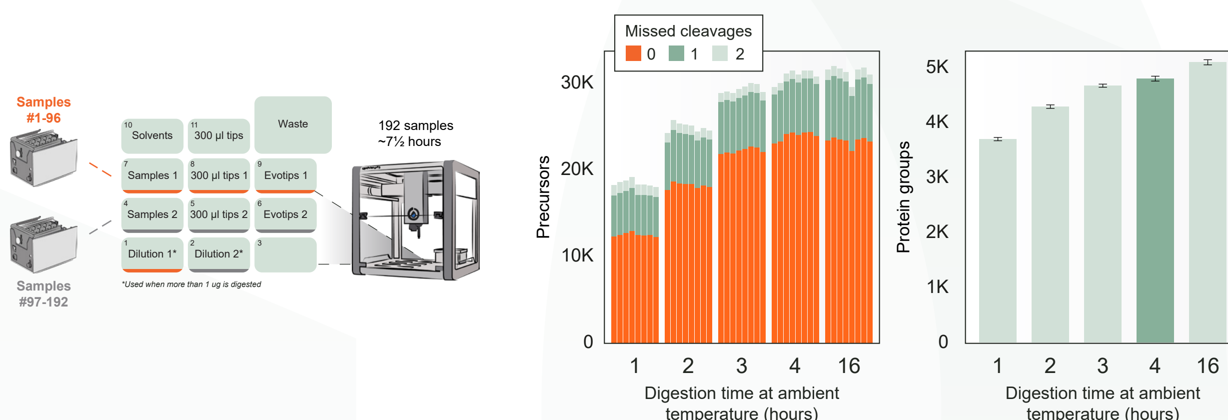

Figure 1: Layout of the OT-2. Identifications and digestion efficiency, measured at different time points based on 1 µg lysate starting amount. ~20% of resulting peptides loaded on Evotips and analyzed with 100 SPD.

4. Reproducibility

The automated PAC protocol is designed to use just a single pipette tip per sample for all sample handling steps and four columns of common tips for transferring solvents and buffers. This strategy ensures an economical and environmentally friendly approach to sample preparation while minimizing the risk of cross-contamination between samples. The reproducibility and sample-to-sample carry-over of the protocol was evaluated by digesting a full plate of samples with eight randomized blanks. Analysis reproducibly identified over 30,000 precursors across all samples and a digestion efficiency of 73% fully cleaved peptides (Figure 2). Each blank resulted in the identification of fewer than 500 peptides, validating the approach. The precision in the sample data had median CVs of 22% and 13% at the precursor and protein group levels, respectively. Finally, samples from the same digestion were stored for ten days at 4 °C post Evotip loading and analyzed to demonstrate the stability of peptides once loaded on the Evotip Pure (Figure 2). This property of the Evotip Pure is essential to the concept of high throughput proteomics, as it ensures that samples remain stable in the time between sample preparation and LC/MS analysis.

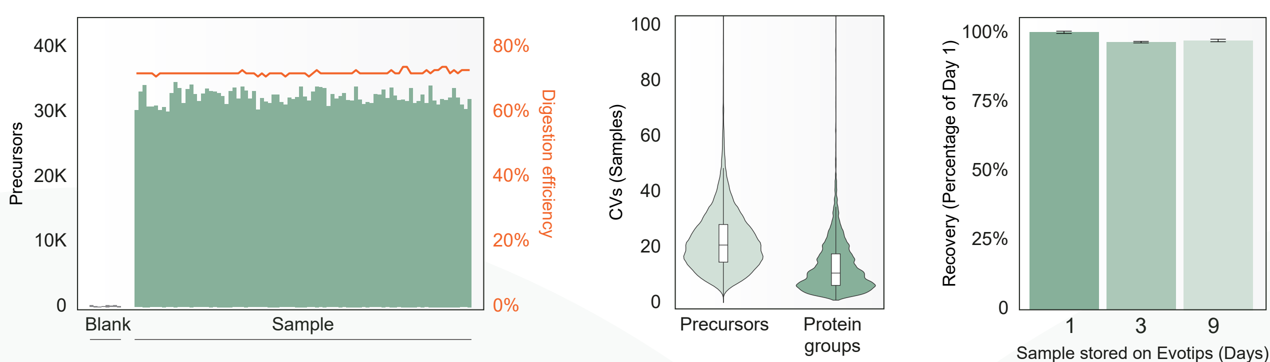

Figure 2: Identifications, CVs from replicate digestions of 1 µg HeLa and effect of storage of digested samples on the Evotip. ~20% of each peptide digest was loaded on Evotips and analyzed with 100 SPD. Recovery was calculated based on total number of precursors in relation to Day 1.

5. Maximum Depth

The protocol is optimized for excellent proteome depth, while keeping the sample input low in the range of 1 µg - 20 µg protein. To assess the achievable proteome depth at low sample input material, 1 µg, 5 µg, and 15 µg of HeLa lysate were digested in triplicates. From 1 µg of starting material, ~20% (low load) and ~50% (high load) of the resulting peptides were loaded onto Evotips. The same relative amounts based on volume, were loaded from the 5 and 15 µg digestions. All samples were analyzedwith the 30 SPD method. When low peptide amount is loaded, it is attractive to digest more material, whereas this effect is balanced once a higher peptide amount is analyzed (Figure 3). This difference is caused by lower intensities of the identified precursors, likely due to the increased impact of peptide loss to non-specific binding at

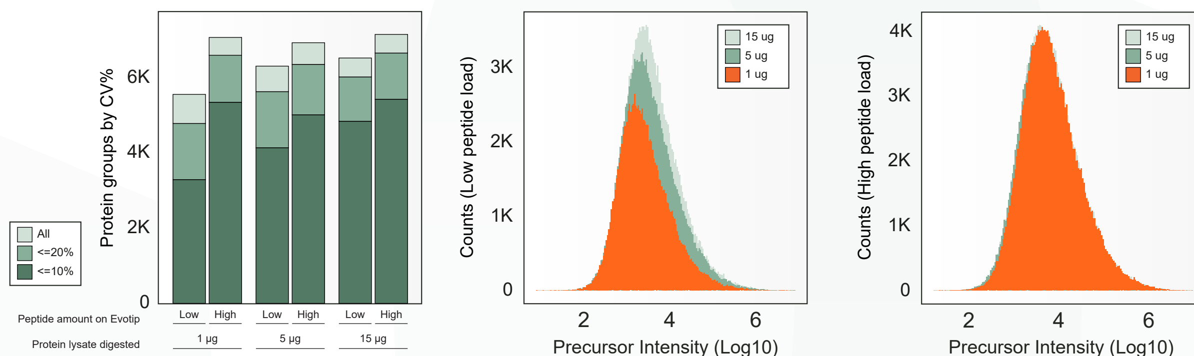

Figure 3: Proteome coverage and precursor abundance distribution from 1, 5 and 15 µg protein starting amount with 30 SPD.

sample input. Overall, this results in the same deep proteome coverage despite the protein starting amount digested. Notably, the cost of digesting 1 µg is 10 times less per sample than digesting 15 µg, making the method very cost-efficient when analyzing large sample cohorts.

6. Sensitivity

Finally, the sensitivity of the protocol was benchmarked by preparing serial dilutions of 125 ng of HeLa lysate, down to 0.98 ng HeLa lysate upon which all were digested in quadruplicate. ~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 chemical 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.

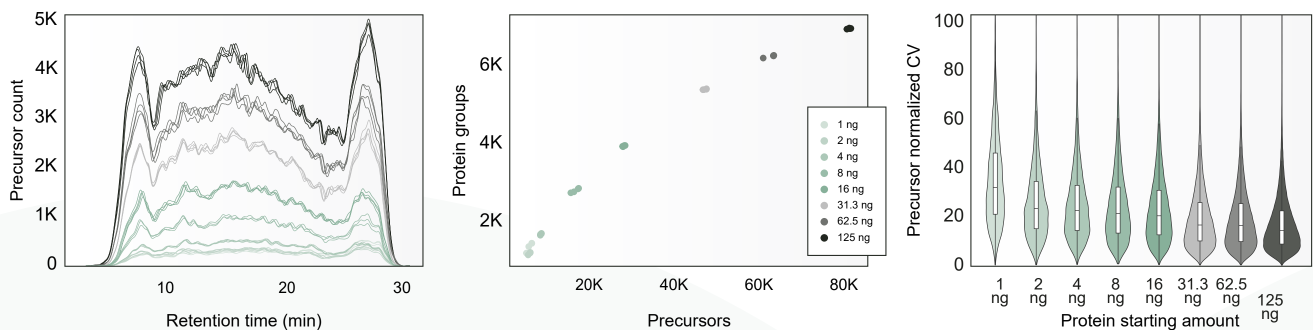

Figure 4: Identifications and CVs for protein lysate dilution curve. ~70% of the resulting peptide is analyzed with Whisper 40 SPD.

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. Low protein starting amounts provide remarkable proteome depth with the digestion of 1 µg yielding 7,000 proteins. Furthermore, excellent sensitivity is proven with a digestion of a dilution series down to 1 ng protein, which was analyzed with the Whisper 40 SPD method Importantly, the protocol demonstrates excellent reproducibility and no sample-to-sample carryover, ensuring reliable identification and quantification of proteins. Overall, the success of this workflow is driven by the key features of the Evotip, where it is possible to concentrate large sample volumes on the Evotip directly on the robot and store the samples efficiently on the Evotip until analysis.

Notably, by lowering the protein starting material and carefully considering the use of plastic-ware, the cost for consumables is significantly decreased and the workflow therefore greatly enables the analysis of sample cohorts of 10,000s of samples.

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*.

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