Application Note

EVOSEP

A complete, automated Opentrons OT-2 loading protocol for **simplified sample loading of Evotip Pure**

1. Introduction

The growing interest for high-throughput proteomics requires standardization and simplified workflows. This is enhanced by automation to remove human errors and decrease data variation. The Opentrons OT-2 (OT-2) liquid handling robot is an excellent example of a low-cost and open-source platform that easily enables the development and integration of end-to-end workflows. Other liquid handling robots are often expensive, and their proprietary design limits the integration of specific applications. As a step towards end-to-end workflows, we have developed a simple and automation friendly protocol for sample loading on Evotips, which makes use of a layered sandwich approach with defined airgaps between the layers (Figure 1). This is then pushed through the Evotip with the OT-2 pipette for 100 seconds leaving the Evotip ready for injection on the Evosep One. The automated sample loading protocol relies on a defined sample volume of 20 µl and solvent A volumes of 15 µl and 150 µl respectively to keep the sandwich intact through loading. The protocol has been converted into an easy-to-use HTML form, that generates a complete python script for use in the Opentrons app. It allows the user to load from 8 to 288 Evotips in a single run.

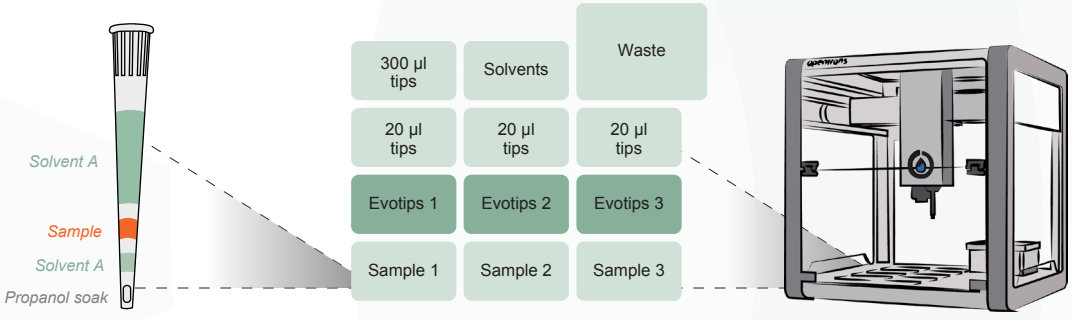

Figure 1: Layout of OT-2 deck for automation of Evotip Pure sample loading.

2. Method details

HeLa cells were cultured in DMEM media and harvested in boiling 5% sodium dodecyl sulfate (SDS) buffer. Sample preparation was performed using protein aggregation capture (PAC) on magnetic microparticles, followed by on-bead trypsin digestion. Peptide concentration was estimated using nanodrop A280 nm. 30 µl of sample were transferred to all wells in a 96 well plate (Eppendorf, 0030129512). Up to three plates were placed in deck positions 4,5 and 6 on the OT-2 depending on the number of Evotips to load.

A solvent plate was placed in a 12-well reservoir 22 ml (USA Scientific, 1061-8150) in deck position 11. 18 ml of solvent A was transferred to column 1 in the solvent plate to load one box of Evotips. When the sample loading is extended to two boxes, another 18 ml of solvent A is transferred to column 2 and column 3 in case of three boxes. This is also the case for 2-propanol, where 6 ml is added to columns 12, 11 and 10 for loading of 1, 2 and 3 boxes of Evotips respectively. Corresponding Evotips were placed in deck positions 1, 2 and 3 using the Evotip loading kit (OT-2) adapters and trays (Evosep, EV1164). Opentrons 300 µl tips Opentrons, 999-00009) were placed in deck position 10 and Opentrons 20 µl tips (Opentrons, 999-00007) were placed in deck positions 7-9. The P20 8-channel electronic pipette GEN2 (Opentrons, 999-00005) was used to place the samples and the P300 8-channel electronic pipette GEN2 (Opentrons, 999-00006) was used to perform the remaining liquid transfers and push the sandwich liquid through the Evotips.

A dilution curve was measured with the Whisper 40 SPD method using the Aurora Elite column (IonOpticks, AUR3-15075C18-CSI) operated at 50 degrees. A comparison between two Opentrons robots were measured with the 100 SPD method using a EV1109 column (Evosep) operated at 40 °C and a robustness study were measured with the 500 SPD method using a EV1107 column (Evosep) at ambient temperature. All samples were analyzed on a timsTOF Pro 2 mass spectrometer (Bruker) with dia-PASEF and analyzed with DIA-NN (version 1.8.1) in library-free mode against the reviewed human proteome (UniProt, Nov 2021, 20,360 entries without isoforms) with trypsin/P as digestion enzymes and MBR enabled.

3. Sensitivity

We initially assessed the sensitivity of our new generic sample loading strategy against the standard sample loading protocol using a centrifuge. We loaded quadruplicates of 1, 5, 10, 20 and 50 ng HeLa peptides respectively with both protocols and used the Whisper 40 SPD method for validation. The two loading protocols provided a similar proteome coverage at all peptide loads with an average of 3,100 proteins quantified from 1 ng peptide input and 7,000 proteins quantified from 50 ng peptide input. This revealed similar sensitivity of the automated sample loading protocol compared to the manual loading protocol (Figure 2).

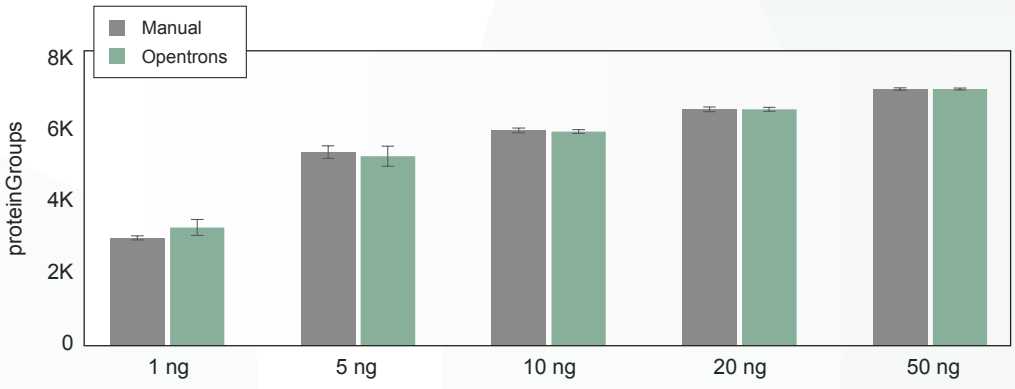

Figure 2: Sensitivity of automated sample loading on the OT-2, analyzed with the Whisper 40 SPD method.

4. Robustness

Next, we evaluated the robustness and reproducibility of the protocol when loading to the full capacity of 288 Evotips in a single run on the OT-2. Evotips were loaded with 50 ng of HeLa using the automated loading protocol and analysed using our fastest methods with a throughput of 500 samples per day. The data revealed a robust level of proteome coverage with an average of 2,500 protein groups identified from each of the 288 Evotips (Figure 3). Furthermore, no column or row specific biases were observed in the data.

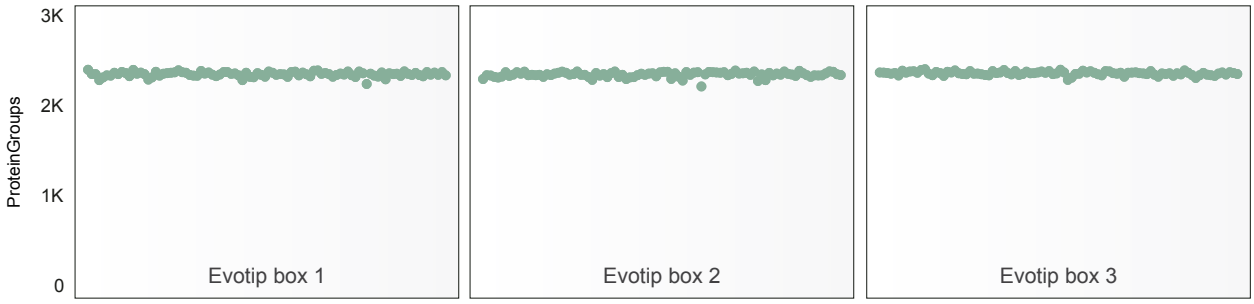

Figure 3: Robustness of three boxes loaded with 50 ng HeLa on the OT-2, and analyzed with the 500 SPD method on a tims TOF Pro 2.

5. Robot-to-robot reproducibility

As great robustness and reproducibility was observed using one OT-2, we investigated if this would be the case for a different OT-2. Thus, an additional comparison was made using two different OT-2. Six replicates with 50 ng HeLa using the automated loading protocol were prepared on two different Opentrons-2 and analyzed using the 100 SPD method. An average of 33,000 identified precursors are identified across replicates and OT-2 platforms (Figure 4). This comparison revealed that the difference between the two OT-2 robots is below 10% indicating a good robot-to-robot reproducibility suggesting that the automated loading protocol is a robust option for reproducible loading of Evotips across different OT-2.

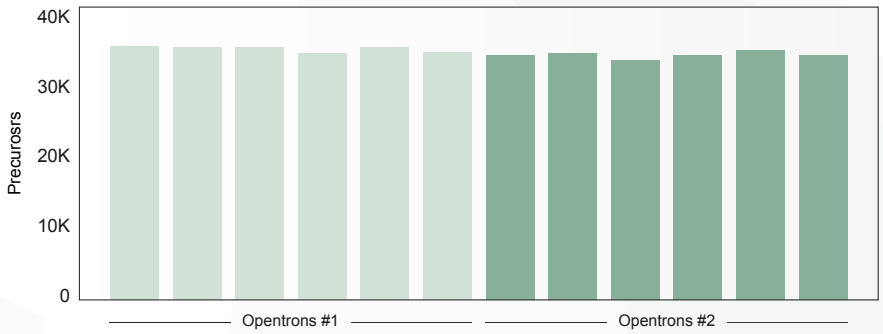

Figure 4: Comparison of two OT-2 robots using 50 ng HeLa and the 100 SPD method.

6. Conclusion

The combination of an automated loading protocol, using the OT-2, and the Evosep One enables an even higher degree of robustness and reproducibility in high-throughput analysis. The presented data highlights that the automated loading protocol is a viable solution for robust and reproducible loading of Evotip Pure. We show that the automated loading protocol has excellent sensitivity, yielding similar results to the manual loading protocol with an average of 3,100 quantified proteins from 1 ng peptide input, and 7,000 proteins quantified from 50 ng peptide input with the Whisper 40 SPD method on the Bruker timsTOF Pro2. The automated loading protocol features a current capacity of up to 288 Evotip (3 Evotip boxes) in one run with a high degree of reproducibility. Lastly, when comparing the robot-to-robot reproducibility, we find the difference to be below 10% in identified precursors between two OT-2 platforms. This automated loading protocol not only aids standardization of sample loading but also opens the door for combined end-to-end workflows.

Availability of automated Opentrons-2 loading protocol

The Evotip loading kit adapters and trays (Evosep, EV1164) are required to run the protocol. One can load up to three boxes of Evotips in one run. A protocol generator allows you to choose the amount of Evotips, which should be loaded, sample tip location and whether those should be reused and lastly solvent tip location. The generated protocol can be imported into and run via the Opentrons app. The generator and information can be found online at *www.evosep.com/support/automation*.