

EVOSEP

Step-by-step guide

End-to-end, fully automated digestion protocol on the Opentrons OT-2

This workflow creates ready-to-analyze loaded Evtips from extracted, reduced, and alkylated proteins (i.e. cell lysate, plasma, or homogenized tissue). The method is carried out on the Opentrons OT-2 (OT-2) liquid handling system and combines protein aggregation capture (PAC) digestion with a unique one-push sample loading method on Evtips, that simultaneously loads and cleans up the sample. 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 for the digestion of 1-5 µg of input protein and loading of up to 80% of the resulting peptides to ensure minimal sample waste, enzyme usage, and optimal sample load on the Evtip. The workflow processes 192 samples in a single 6.5 h run and has a built-in option for loading an additional replicate of each sample. An overview of run times for a select number of samples is visualized in table 1.

Table 1: Running time table

Number of samples	Approximate run time	With two technical replicates
24	4 h, 40 min	4 h, 50 min
48	5 h	5 h, 20 min
96	5 h, 30 min	6 h, 20 min
144	6 h	7 h, 20 min
192	6 h, 30 min	8 h, 20 min

The protocol requires a P300 8-channel electronic pipette GEN2, which is used for all liquid handling steps. The protocol utilizes a single sample-specific Opentrons 300 µl tip per sample in deck positions 5 or 8, and shared liquid handling pipette tips for fresh solvents in deck position 10.

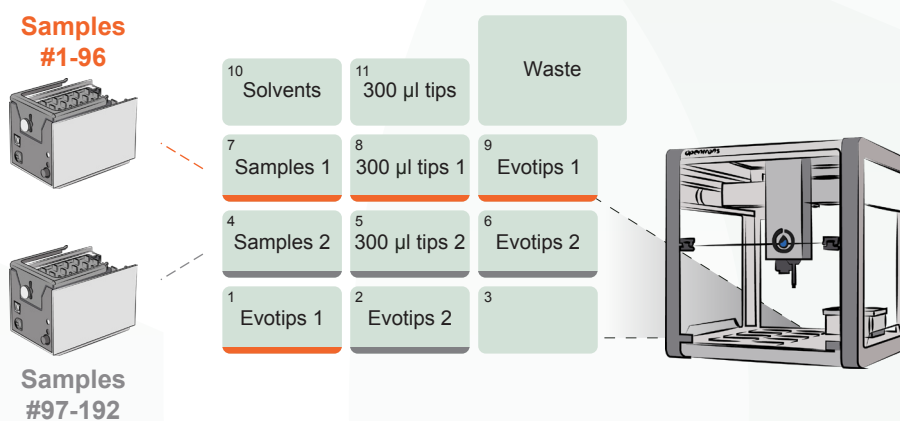


Figure 1: Layout of OT-2 deck.

HTML FORM CONFIGURATION

- 1 Go to <https://ot2-evotip-loading.evosep.com/pac.php> and select the parameters for the protocol by navigating through the options:

SAMPLE POSITIONS - Select the number of samples to be processed and the chosen deck positions for the magnetic rack with the sample plate i.e. positions 4 and/or 7, and the starting position for the first sample column.

SAMPLE INPUT - Select the amount of protein to be digested.

SAMPLE LOAD - Select the percentage of resulting peptides to be loaded on Evotips. The optimal peptide load is a function of recovery, Evosep method and the mass spectrometer used.

LIQUID HANDLING TIPS - Select the placement of P300 tips to be used for solvents.

P300 PIPETTE MOUNTING - Select the position of the P300 pipette on the Opentrons.

USE ADAPTER MAGNETS - Select if adapter magnets are used as this influences the engage height in the magnetic module.

MAKE REPLICATES - Load an additional Evotip from each well in the sample plate.

- 2 Click 'Download'. For the Opentrons app, version 6.3.0 and higher, additional files as indicated are required to be downloaded and imported into the app.
- 3 Once the python script has been downloaded, click 'Get protocol'. This will load a new tab with a customized protocol with exact volumes etc. for the downloaded script.
- 4 Open the Opentrons App and go to the 'Protocols' tab. Import the generated protocol.
- 5 Connect the magnetic module(s) needed to run the protocol to power and to the Opentrons.
- 6 Open the protocol in the Opentrons app, click 'start setup', select your Opentrons and click 'Proceed to setup'. Calibrate pipettes if needed. The protocol will now be analyzed on the robot, which can take up to 10 minutes.

DECK LAYOUT

- 7 Place a case of Opentrons 300 µl tips (999-00009, Opentrons) in deck position 11).
- 8 Place a magnetic module (999-00098, Opentrons), Opentrons 300 µl tips, and an Evotip loading kit (EV1144, Evosep) with Evotips (EV2011, Evosep) in deck positions 7, 8 and 9, respectively.
- 9 If more than 96 samples are processed, place another magnetic module, Opentrons 300 µl tips, and Evotip loading kit with Evotips in deck positions 4, 5 and 6, respectively.
- 10 If two replicates are loaded on Evotips, place another Evotip loading kit with Evotips in deck position 1 for samples in deck position 7, and in deck position 2 for samples in deck position 4.

SOLVENTS AND SAMPLES

- 11 Add solvents to a 12-well reservoir 22 ml solvent plate (1061-8150, USA Scientific) as indicated on the figure in the customized protocol, downloaded previously. The script ensures the reservoir wells contain extra solvents to ensure there is sufficient volume for executing the protocol.
- 12 Mix the digestion buffer and add it to the solvent tray before placing it in deck position 10.
- 13 Transfer 5 μl of reduced and alkylated sample to a 96-well plate with a total protein amount of either 1, 2, 3, 4 or 5 μg . The protocol is optimized for lysates prepared in boiling lysis buffer (1-5% sodium dodecyl sulfate (SDS), 5 mM tris(2-carboxyethyl)phosphine (TCEP), 10 mM chloroacetamide (CAA), 100 mM Tris, pH 8.5).
- 14 Place sample plate(s) on top of the magnetic module(s) on the Opentrons.

PROTOCOL EXECUTION

- 15 Navigate to Labware setup (Step 2). Click 'Apply stored data' and run 'Labware Position Check'.
- 16 Perform labware position check.
- 17 Click 'Start run' to execute the script on OT-2 which will run for 6-8 hours when digesting 1-2x 96 samples.
- 18 Once the protocol is completed, the Evtips can be transferred to the Evtip box, submerged in solvent A, and stored at 4 °C until analysis.

PROTOCOL DETAILS

SAMPLE AGGREGATION - Two steps of mixing samples with magnets disengaged to facilitate protein aggregation on the hydroxyl beads.

SAMPLE WASH - The aggregation volume is removed while aggregated proteins are retained on the magnetic beads with the magnet engaged. The samples are subsequently washed once with acetonitrile.

DIGESTION - After removal of the wash solution, 30 μl * of digestion solution is added to the sample. The digestion solution contains an enzyme:protein w/w ratio of 1:25 for trypsin and 1:100 for LysC in 25 mM TEAB. In between adding digestion buffer to each column, the Opentrons will wait the amount of time it takes to load Evtips to ensure identical digestion time across the entire plate. Digestion is carried out at ambient temperature for 4 hours in total.

DILUTION - Samples are diluted with an appropriate amount of solvent A to obtain the desired % of the peptides loaded on the Evtip.

EVOTIP LOADING - 20 μl of sample is loaded on Evtips by building a layered sandwich with sample and solvent A. Evtips are soaked in isopropanol and loaded using positive air pressure.

**when 80% of the peptides are loaded digestion occurs in 25 μl .*