EVOSEP

Step-by-step guide

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

This end-to-end workflow facilitates the processing of cell lysate or plasma to fully digested samples loaded on Evotips. The method is carried out on the Opentrons OT-2 (OT-2) liquid handling system and combines protein aggregation capture digestion with seamless Evotip loading based on a unique layered sandwich approach. 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 process from 8 to 192 samples in a single run within 5-8 hours and is optimized for digestion ranging from 1 to 20 µg protein starting amount.

SAMPLE PREPARATION

Protein extraction is performed 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). The lysate was boiled for an additional 10 min. Plasma is reduced and alkylated using the same buffer and incubating 30 min at room temperature.

1. Transfer 5 µl MagReSyn Hydroxyl magnetic beads (20 mg/ml) to a 96 well sample plate (Eppendorf, 00300129512).
2. Transfer 5 µl - 15 µl of cell lysate or plasma to a 96-well plate with a total protein amount of 1 µg, 5 µg, 10 µg, 15 µg, or 20 µg.
3. Add acetonitrile to a final concentration of 80%. The total volume in the well (referred to as the aggregation volume from here on) should be in the range of 40 µl to 100 µl.

Table 1: Contains examples of aggregation volumes

Magnetic beads (µl)	Lysate (µl)	100% Acetonitrile (µl)	Aggregation volume (µl)
5	5	40	50
5	10	60	70
5	15	80	100

DECK LAYOUT

4. Place 96-well sample plate with pre-loaded beads, sample and acetonitrile from step 3 on top of a magnetic module GEN2 (Opentrons, 999-00098) in position 7.
5. Place a box with Opentrons 300 µl tips (Opentrons, 999-00009) in position 8.
6. Place Evotips within Evotip loading kit (OT-2) adapter and tray (Evosep, EV1144) in position 9.
Note: If more than 96 samples are processed, place an Eppendorf sample plate with cell lysates and beads, Opentrons 300 µl tips and Evotips in positions 4, 5 and, 6, respectively.
Note: If more than 1 µg sample is digested, additional 96-well plates are needed for dilution. This is placed in position 1, when up to 96 samples are digested, and one additional is placed in position 2, when more than 96 samples are digested.
7. Place a 12-well reservoir 22 ml solvent plate (USA Scientific, 1061-8150) in position 10 as well as another box of Opentrons 300 µL tips in position 11.

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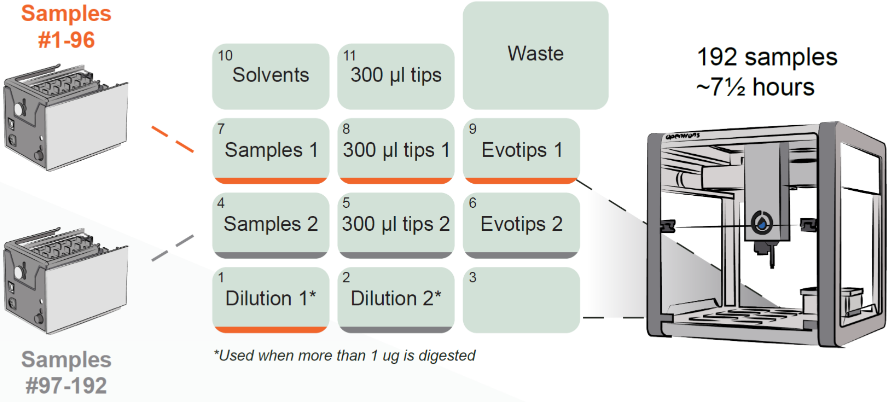

*Used when more than 1 ug is digested

SOLVENT PLATE LAYOUT

Note: The volumes listed below are sufficient to process up to 192 samples with solvents in positions 2, 6 and 9 utilized for a sample plate in deck position 7, whilst solvents in positions 3, 7, and 10 are used to process samples in deck position 4.

8. Transfer 18 ml of acetonitrile to each of columns 2 and 3.
9. Fill columns 4 with 10 ml of digestion buffer: 50 mM Triethyl ammonium bicarbonate (TEAB) digestion buffer with freshly added trypsin (1.33 µg/ml)(Sigma T6567) and Lys-C (0.33 µg/ml) (WAKO 121-05063).
10. Fill columns 6, 7, 9, and 10 each with 18 ml Solvent A (MS-grade water with 0.1% Formic Acid, JT Baker P/N: JT-9834-2 or equivalent).
11. Fill column 12 with 5 ml MS-grade propanol.
12. When more than 1 µg of protein is digested, fill column 11 with 18 ml Solvent A

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Waste	Acetonitrile 1	Acetonitrile 2		Lys-C/Trypsin in 50 mM TEAB	Solvent A 1	Solvent A 2		Solvent A 1	Solvent A 2	Solvent A 1+2	Propanol

LIQUID HANDLING

The P300 8-channel electronic pipette GEN2 (Opentrons, 999-00006) is used for all liquid handling steps and pushing samples through Evotips during sample loading. The Opentrons 300 µl tips in deck positions 5 and 8 are each used for mixing, transferring, and waste disposal of a single sample. The Opentrons tips in deck position 10 are used for the transfer of solvents from the solvent plate to the sample plate. Pipette tips in columns 1-3 are used for acetonitrile, digestion buffer, and solvent A, respectively.

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HTML FORM CONFIGURATION

13. Go to https://ot2-evotip-loading.evosep.com/pac.php

14. Complete the HTML method generation form by navigating through the following options:

  a. Sample positions: Indicate the number of samples to be processed, the magnetic deck position on which the sample plate is placed i.e. position 4 or position 7, and finally indicate the sample positions (columns 1-12).
  b. Select the sample input: Amount of protein to be digested.
  c. Select the aggregation volume: Total volume including beads, protein and acetonitrile.
  d. Select digestion time: The duration of digestion.
  e. Select amount of peptides to be loaded on Evotips post digestion completion. This is the theoretical peptide load, based on 100% recovery.
  f. Select if adapter magnets are used, as this influences the engage height in the magnetic module.

Note: The optimal peptide load on Evotips will be a function of recovery, Evosep method and the mass spectrometer used and should be tested accordingly.

  g. Indicate the placement of P300 tips to be used for solvents.
  h. Indicate in which position the P300 pipette is mounted.

15. Click “Download” to download the OT-2 compatible python script.

16. For the Opentrons app. versions 6.3.0 and higher, additional files as indicated are required to be downloaded and imported into the Opentrons app.

17. Open the Opentrons App and go to the ‘Protocols’ tab. Import and run the generated protocol.

18. Choose the Opentrons to use for the protocol. Calibrate pipettes if needed (Step 1).

19. Navigate to Labware setup (Step 2). Click ‘Apply stored data’ and run ‘Labware Position Check’.

20. Perform labware position check.

21. Click ‘Start run’ to execute script on OT-2 which will run 6-8 hours digestion for 2x 96 samples.

22. Once completed, the Evotips can be transferred to the Evotip box, submerged in solvent A and stored at 4 °C until analysis.