COVID-19-MSC Serum Proteomics Protocol

(v0.2: 2020/05/12)

This protocol takes samples from serum to evotip loading in 96-well format, ready for label-free quantitation using an Evosep 1/Q Exative-HF platform and subsequent LC-MS/MS analysis and data searching. Sample preparation can be completed in one day, and yields sufficient samples for four days of MS run time.

Overall protocol flow:

1. Serum depletion – 96-well format (spin plates)
2. SP3 sample processing – 96-well, magnetic format
3. Evosep tip preparation (includes desalting, sample finishes ready to load for MS analysis, 96-well format)
4. LC-MS/MS analysis on Q Exactive-HF
5. Initial data searching using MaxQuant

Step 1: Serum depletion

Note: this is Thermo's recommended 96-well format protocol for use with the High Select Top14 resin. Volumes reduced by ⅓, in-line with those used in the mini column format.

Step 1 timing: ~1-1.5h

Materials and equipment for step 1:

- High select Top14 depletion resin (Thermo, A36372 – 50mL) : note ~60mL required per 96 well plate
- 96 well spin filtration plates (e.g. Thermo Pierce)
- 96 well collection plates for spin plates
- Self-adhesive foil lids for PCR plates (e.g. Fisher, 10130853) or equivalent sealing system.
- Centrifuge for 96 well plates (preferable refrigerated) OR 96 well vacuum plate filtration unit.
- Multichannel/electronic pipettes for dispensing resin and lysis buffer
- 5 x Lysis buffer: 5% SDS, 500mM Ammonium bicarbonate pH8.5

Note:

Steps 4 to 10 must be conducted in a BSL2 hood under COVID19 sample handling conditions.

The virus is inactivated in the lower plate at step 9 and this can be removed from Cat 2(+) containment for further processing. The upper filter plate may still contain live virus and should be disposed of accordingly.

Method for step 1:

1. Add 50 µL of 5 x Lysis buffer (5% SDS, 500mM Ammonium bicarbonate pH8.5) to each well of the 96 well collection plate.

2. Invert the bottle of Top14 end-over-end until slurry is homogeneous.

3. Add 200 µL of 50% Top14 resin slurry to each of the wells in the 96-well plate making sure to dispense the resin to the bottom of the well.

In a BSL2 hood:

4. Add 10 µL of serum sample to each of the wells. Note: This represents 600-800 µg of total protein

5. Seal the plate with a foil lid.

6. Place the filter plate on top of a 96-well collection plate, and place the plates on a plate shaker with gentle shaking (optional) or agitate manually occasionally for 10-20 minutes.

7. Place the plates in a centrifuge and centrifuge at 300- g for 10 minutes (at 4 °C if possible). The use of a sealed rotor is recommended.

8. Leave plate to stand in centrifuge for 10 minutes after the centrifuge has stopped to allow for inactivation.

9. Remove the plates from the centrifuge. Remove the top plate and bag and transfer for autoclaving to deactivate any remaining virus using local protocols

10. The depleted sample (flow-through), will be in the collection plate in a volume of approximately 200µL and is virus deactivated.

    Note: *The top14 kit removes the proteins which make up 95% of the protein within serum. Following depletion, approximately 40ug should be present. (Thermo product sheet)*

    This should yield ~3x the amount of material needed for downstream processing (redundancy).

The sample plate can be remove from BSL2 containment at this stage.

11. Seal the sample plate with a foil lid and freeze at -80 °C.

12. Transfer to analysis lab on dry ice. Store at -80 °C.

Step 2: SP3 sample processing
Note: includes reduction and alkylation. Method taken from:
https://www.embopress.org/doi/10.15252/msb.20199111

Step 2 timing: 4h (<1h hands-on)

Final 10mM TCEP, 40mM CAA.

Assumes starting sample volume of 40uL in lysis buffer. (Scaled for typical serum depletion yield).

Materials and equipment for step 2:

- 96 well magnetic plate stand (e.g. Thermo, AM10027)
- 96 well plates (e.g. Fisher, E0030129504)
- Foil lids for PCR plates (e.g. Fisher, 10130853)
- Multichannel pipettes
- Reagent reservoirs
- SP3 beads (50:50 mix of):
  Sera-Mag SpeedBeads (GE Healthcare, cat. no. 45152105050250)
  Sera-Mag SpeedBeads (GE Healthcare, cat. no. 65152105050250)
  Reconstitute beads in HPLC-grade water at 50 mg/mL.
- Heat block or PCR machine with 96-well adaptor.
- Timer for incubation steps
- 3 x CAA (2-chloroacetamide)/TCEP mix (30 mM TCEP, 120 mM CAA)
- 2 x Lysis buffer (2% SDS, 200 mM ammonium bicarbonate pH 8.5)
- 80% Ethanol
- 100% Acetonitrile

Method for step 2:

1. Thaw the sample plate on ice
2. To each well add 30 µL of 3x TCEP/CAA mix (30 mM TCEP, 120 mM CAA: *for 10 mM TCEP, 40 mM CAA final concentration*)
3. Mix by pipetting 3x
4. Cover plate with foil lid, making sure that wells are sealed.
5. Move plate to heat block, incubate at 95C for 5 minutes.
6. Add 30uL of SP3 bead slurry per well with a multichannel, taking care that the bead slurry remains well-mixed during the dispensing process
7. Add 90uL of 100% acetonitrile to each well and mix by pipetting 3x.
8. Leave on bench for 2 minutes.
9. Add to magnetic rack and leave for 5 minutes to allow beads to settle.
10. Discard supernatant using multichannel pipette

Volumes from here remain the same irrespective of input adjustments.

11. Leave plate on magnetic rack, add 200 µL of 80% ethanol to each well. Mix by pipetting
12. Allow beads to settle for 2 minutes on the magnetic rack.
13. Discard supernatant using multichannel pipette
14. Leave plate on magnetic rack, add 200 µL of 80% ethanol to each well. Mix by pipetting
15. Allow beads to settle for 2 minutes on the magnetic rack.
16. Discard supernatant using multichannel pipette
17. Leave plate on magnetic rack, add 170 µL of 100% acetonitrile to each well. Mix by pipetting
18. Allow beads to settle for 2 minutes on the magnetic rack.
19. Discard supernatant using multichannel pipette
20. Add 40 µL of Trypsin mix (40 µL of 100 mM Ammonium bicarbonate containing 250 ng Trypsin – Promega Gold, 1:40 Trypsin to Protein ratio).
21. Cover plate with foil lid, making sure that wells are sealed.
22. Transfer plate to heat block with heated lid and incubate at 37 °C for 3 hours.
23. Allow plate to cool, transfer to magnetic rack and allow beads to settle for 5 minutes.
24. Transfer to fresh 96 well PCR plate containing 10 µL of 5% Formic acid/well.
    a. Note – check pH of top left and bottom right wells to confirm acidification. Samples should be 10 µg in 50 µL volume.

Step 3: Evotip loading (and sample desalting)

Note: this protocol is the manufacturers recommended protocol for Evotip loading, and is unmodified from: https://www.evosep.com/wp-content/uploads/2020/03/Sample-loading-protocol.pdf

Step 3 timing: <1h (<1h hands-on)

Materials and equipment for step 3:

- Box of Evotips
- Mass spec grade 1-propanol, alternatively 2-propanol
- Solvent A: Mass Spec Grade Water with 0.1% Formic Acid, JT Baker P/N: JT-9834-2 or equivalent
- Solvent B: Mass Spec Grade Acetonitrile with 0.1% Formic Acid, JT Baker P/N: JT-9832-2 or equivalent

- Multichannel pipette
- Solvent containers for multichannel pipette, e.g. Dual Solution Reservoir HEA20281A from Heathrow Scientific *www.heathrowscientific.com/dual-solution-reservoir-i-hea20821a*
- 96 well Microtiter plate (MTP), e.g. 249946, 96 well Conical Btm PP Plt Natural from Thermo Scientific, *www.thermofisher.com/order/catalog/product/249946*
- Lids for 96 well MTP, e.g. 263339 NuncTM Microplate Lids, *www.thermofisher.com/order/catalog/ product/263339*
- Evotip adapter rack (EV1068)
- Gloves
- Table centrifuge
- Fume hood

Centrifugation steps should be conducted at room temperature.

Method for step 3:

1. Add 100uL 1-propanol to each well of 96 well MTP.
2. Set MTP to one side.
3. Add 20uL solvent B to all evotips using a multichannel pipette.
4. Place the evotips in a centrifuge (in their container) and centrifuge at 700 x g for 90 seconds at room temperature.
5. Discard the flow through
6. Place the evotip adaptor on top of the MTP.
7. Soak evotips in the 1-propanol-containing MTP for 60 seconds.
8. Visually inspect tips to confirm all tips are pale white following soaking.
9. With the tips still in the adaptor rack, add 20uL solvent A to all evotips using a multichannel pipette.
10. Place the evotips in a centrifuge and centrifuge at 700 x g for 60 seconds at room temperature.
11. Place evotips back in the original container
12. Add 20uL of sample to each tip.
    a. Note: this should represent 1ug of peptide material
13. Place the evotips in a centrifuge and centrifuge at 700 x g for 60 seconds at room temperature.
14. Transfer 20ul of solvent A to all evotips using a multichannel pipette
15. Place the evotips in a centrifuge and centrifuge at 700 x g for 60 seconds at room temperature.
16. Transfer 100uL of solvent A to all evotips using a multichannel pipette

17. Place the evotips in a centrifuge and centrifuge at 700 x g for 10 seconds at room temperature to keep tips wet.
18. Add enough solvent A to the bottom of the tray to that the bottom of the evotips are submerged, and store cold (4C) with a lid on until analysis.

Step 4: LC-MS/MS method on Q Exactive-HF

TBC

Step 5: Initial data analysis in Maxquant

TBC