Single Tube Solid Phase Sample Preparation for Proteomic analysis (SP^3).

Protocol is based on the protocol form Krijgsveld's lab with some modifications.
(Hughes CS, Foehr S, Garfield DA, Furlong EE, Steinmetz LM, Krijgsveld J. Ultrasensitive proteome analysis using paramagnetic bead technology. *Molecular Systems Biology*. 2014; 10(10):1-10. doi:10.15252/msb.20145625).

Note: All reagents, solutions and vessels should be of high purity and keratin free to obtain optimal results.

Used buffers are based on 100 mM TEAB.

Never let beads without liquid, otherwise they can get dry (except in steps where that is required). It is recommended removing liquid from a tube and immediately adding fresh liquid.

Starting material:

10 µg of protein in 100 µl buffer. SP3 is done in 1.5 ml Eppis, using 1000 µL of liquid for binding to beads.

Reagents & Solutions

- Sera-Mag Speed Beads A and Sera-Mag Speed Beads B
- 100 mM TEAB pH 8.5
- 100% acetonitrile (ACN)
- 20x TCEP (57,4 mg/ml) in 50 mM TEAB = 200 mM TCEP
- 20x CAA (74,8 mg/ml) in 50 mM TEAB = 800 mM CAA
- 10% SDS
- TFA stock solution
  40% Trifluoroacetic acid in H_2O
- Lysis-buffer: 100 mM TEAB / 1%SDS / 10 mM TCEP/ 40 mM CAA
- Binding-buffer: 40% Lysis-buffer containing proteins / 60% ACN
- Wash buffer: Same as the binding buffer w/o SDS, TCEP and CAA, means in this case 60% ACN and 40% 100 mM TEAB.
- 80% Ethanol in dH_2O.
- Lys-C stock solution
  200 ng/µl solution (in 0.01% TFA), store at -20°C
- Trypsin stock solution
  200 ng/µl solution (in 0.01% TFA), store at -20°C

Procedure

Bead Preparation.

Use 2µL of bead mix for sub microgramme-20µg of protein amount.

1. To prepare beads, remove from fridge and keep at room temperature for 10 minutes, suspend well using a vortex to ensure you have a homogenous slurry. Combine 20 µL of Sera-Mag A and 20 µL of Sera-Mag B. Critical: Store the beads in the fridge. Never freeze the beads.
2. Add 160 µL of dH_2O.
3. Place the tube with beads on a magnetic rack and let beads settle for about2 minutes, subsequently remove and discard supernatant.
4. Rinse beads with 200µL of water by pipette mixing (take off magnetic stand, put back on before removing supernatant). Repeat this twice.
5. Store beads in 20µL of dH_2O (100 µg/µl total bead concentration) in the fridge for up to 2 weeks.
6. Use 2µL of bead-mix per sample; make sure the beads are well resuspended in the solution (pipette mix) before adding to a sample.

Reduction & Alkylation

We use 10mM TCEP and 40mM CAA (final concentration) for reduction/alkylation.
Set up samples as follows:

100 µl starting material
\+ 20 µl 20x TCEP
\+ 20 µl 20x CAA
\+ 40 µl 10% SDS
<u>+ 220 µl 100 mM TEAB</u>

400 µl total volume

Incubate at 95°C for 5 minutes, then 25 minutes at 70°C in the shaker for reduction/alkylation. Cool down to room temperature.

SP3 Protein Clean-up and digestion

1. Add 600µl ACN to each sample and then 2µl SP3-beads. Mix
2. Incubate for 20 minutes at room temperature in the shaker.
3. Centrifuge briefly to bring all beads and liquid from the lid of the vial, then place on the magnetic rack and incubate for 2 minutes. Remove and collect supernatant, store as SP3 FT1 in -20°C.
4. Add 200µl wash buffer, mix by pipetting.
5. Sonicate for 7 minutes in the ultrasound bath.
6. Incubate for 20 minutes at room temperature in the shaker.

7. Spin samples to bring all beads and liquid from the lid of a vial. Place on the magnetic rack, incubate for another 2 minutes at room temperature. Remove supernatant. It is stored as SP3 FT2 at -20°C.
8. Add 200µl of 80% ethanol in Biosolve-H_2O, mix by pipetting and incubate for 2 minutes on the magnetic rack. Remove and discard supernatant.
9. Repeat step 8.
10. Add 180µL of 100% ACN, mix by pipetting and incubate for 2 minutes on the magnetic rack. Remove and discard supernatant.
11. Dry beads in 37 °C incubator for some minutes. Ensure beads change from a wet dark rust color to a dry light rust color, to ensure all acetonitrile has really evaporated.
12. Add 100µL of 100mM TEAB to beads.
13. Sonicate for 7 min and quickly spin down.
14. Add 1 µl proteinase Lys-C (200ng/µl). Mix by pipetting, thereby removing residues of the beads from the wall and incubate for 4h at 37°C in the shaker.
15. Add 1µl trypsin (200ng/µl). Mix by pipetting and incubate over night at 37°C in the shaker.

Next day

16. Allow the digest to cool down to RT. Spin samples in the centrifuge to bring all beads and liquid from the lid of a vial.
17. Stop the digestion by adding 3µl 40%TFA. Verify that pH is < 2. Place on the magnetic rack and incubate for another 2 minutes at room temperature.
18. Transfer supernatant to fresh Eppis.

Proceed with Stage tip desalting of supernatant and LC-MS analysis