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SP3, Single-pot, solid-phase-enhanced sample Preparation

Sera-Mag SpeedBeads, 50mg/ml (GE Healthcare, cat. no. 45152105050250)
Sera-Mag SpeedBeads, 50mg/ml (GE Healthcare, cat. no. 65152105050250)

Reagents and concentrations compatible with the SP3 approach

Reagent type	Concentration range	Reagent name	SP3 considerations
Detergents	0–20%	SDS Triton X-100 NP-40 Tween 20 Deoxycholate CHAPS RapiGest	We recommend keeping the total detergent concentration in the range of 0–10% (wt/vol or vol/vol depending on the detergents used).
Chaotropes	0–8 M	Urea (up to 8 M) Guanidinium hydrochloride and Isothiocyanate (up to 4 M)	High concentrations of chaotropes in the presence of the solvent used for binding can disrupt the interactions between SP3 beads and the proteins. Therefore, we recommend testing SP3 with your desired lysis solution formulation before further application.
Salts	0–1 M	A wide range of salts have been tested.	It is recommended to keep the final salt concentration in the lysate <1 M when using solvents for binding. High salt concentrations in the presence of binding solvent can disrupt the ability of proteins to efficiently localize on the SP3 bead surface.
Solvents	0–50%	Acetonitrile, acetone, isopropanol, ethanol, trifluoroethanol, and xylene.	It is recommended to keep the final solvent concentration in the lysate before binding <25% (vol/vol). If high solvent concentrations are present, the amount of ethanol added during SP3 can be scaled to achieve the desired final solvent concentration (e.g., 70% (vol/vol) final).

1 ml RIPA lysis buffer

Reagent name	Final concentration	Volume
H2O		130 µl
NP-40	1 %	10µl
10% SDS	0.1 %	10 µl
Sodium deoxycholate (5% SDC)	0.5 %	100 µl
Tris (0.5M, pH 7.6)	50mM	100 µl
NaCl (1 M)	150 mM	150 µl
2x proteaseinhibitor (cOmplete)	1x	500 µl

Alternative lysis buffers for SP3:

- *50 mM HEPES pH 8, 1% SDS, 1% Triton X100, 1% NP-40, 1% Tween 20, 1% deoxycholate, 5 mM EDTA, 50 mM NaCl, 1% glycerol, 1x protease inhibitor, 5 mM dithiothreitol*

4-5% SDS / 5 mM TCEP / 10 mM CAA / 0.1 M Tris-HCl pH 8.5

Sample homogenization and denaturation

BCA/Bradford assay

After sample denaturation, protein concentration needs to be measured.

For SP3, use 20 µg protein in 20-30 µl lysis buffer

Reduction and alkylation

1. Add **2-3µl 100 mM DTT** *(see right panel)*, and incubate for 20 min. at 60 ˚*C*. (Urea lysis buffer, 1 hour at room temperature)

2. Add **3-4µl 200 mM IAA** *(see right panel)* for cystein alkylation, and incubate for 1 h at RT (dark).

100 mM DTT in MillQ water:

*Add **15.4 mg DTT** (DiThioThreitol, art. no. D-9163, Sigma-Aldrich) to 1ml dH_2O (may be aliquoted as a 1M solution, and kept in freezer).*

200 mM IAA in MilliQ water:

*Add **18.5mg IAA** (Iodoacetamide, art. no. I-6125, Sigma Aldrich) to 0.5ml dH_2O (must be freshly made and kept in the dark).*

Homogenization

Tissue homogenization in lysis buffer can be performed with a precellys homogenizer using glass beads followed by DNA shearing using ultrasound rod (minimum volume = 150µl). Cells are lysed without the homogenization step, but DNA shearing is still necessary. Protein pellets and body fluids will dissolve and denaturate using only lysis buffer.

Tissues; 10µl lysis buffer per mg tissue, minimum 15 mg

Cells; For 500 000 cells, use 150µl lysis buffer (below 100 to 200 µg protein depending on cell type). Ideally use 100µl per 1 mill, cells.

Body fluids: Freezedry before adding lysisbuffer.

***Note:** Alkylation and reduction are not needed if the sample is boiled in SDS buffer containing TCEP and CAA*

SP3

Bead preparation, 20 µg protein

Sera-Mag beads contain sodium azide that needs to be removed. SP3 is generally performed at a (total)bead/protein ratio of 10:1 (wt/wt), with a minimum volumetric concentration of 0.5 µg/µL. With **20 µg** of protein we therefore need **200 µg** of beads, 2 µL from each of the 50 mg/mL vendor stocks. Prepare working solution by adding 20 µl from each stock solution, and add 160 µl water, pipette-mix.
Place the tube on a magnetic rack until the beads have settled to the tube wall, and remove the supernatant, repeat (**total of 2 times**)
Off the magnetic rack, reconstitute the beads in 200 µL of water and pipette-mix.
Reconstitute the beads at a suitable working concentration, e.g. 20µl water (100 mg/mL).
Prepared bead stocks can be stored at 4 °C for 1 month.

Protein cleanup and Digestion

1. Add 200 µg of prepared SP3 beads and pipette-mix to homogenize the solution. This will be **2 µL** of a prepared 100 µg/µL SP3 bead working solution. Adjust the volume to 40µl with lysis buffer, and mix by gentle pipetting 4-5 times.
2. To induce binding of the proteins to the beads, add **100%** ethanol to the Protein mixture containing the SP3 beads to a final concentration of 70% *(see right panel)*. Pipette-mix or carefully shake the tube to homogenize.
3. Incubate the binding mixture in a ThermoMixer at **24 °C (RT) for 7 min at 1,000 rpm**.
4. After the binding is complete, place the tube in a magnetic rack and incubate it until the beads have migrated to the tube wall.
5. Remove and discard the unbound supernatant in an appropriate waste container.
6. Remove the tube from the magnetic rack, and add **180 µL of 80%** ethanol SP3 rinse solution *(see right panel)* and pipette-mix to reconstitute and rinse the beads.
7. Place the tube on the magnetic rack and incubate until the beads have migrated to the tube wall.
8. Remove the supernatant, taking care to not disrupt the beads.
9. Repeat Steps **6-8 two further** times to completely rinse the proteins bound to the SP3 beads.

Absolute ethanol (Sigma, cat. no. 34852)

70% final conc calculation:
X=volume absolute ethanol to add.
S.vol= Sample Volume (e.g. 40µl)

$$(X * 100)/(S.vol + X)=70$$

80% ethanol wash solution:
Mix 80ml Absolute ethanol and 20ml water. Prepare fresh weekly and store at room temperature.

10. **PROBE users are advised to perform two extra washes in a fresh tube to fully remove traces of SDS**
11. Remove the tube from the magnetic rack and add **50 µL** of digestion solution *(see right panel)* containing **0.8 µg** of trypsin (**1:25**)
12. Using a micropipette with a 200-µL tip, gently push the beads that are not covered by liquid along the tube wall into the digestion solution. Do not attempt to pipette the mixture.
13. Sonicate for **30 s** in a water bath to fully disaggregate the beads and incubate for **12-18 h at 37 °C** in a ThermoMixer at **1,000 rpm** mixing overnight.

Peptide extraction

14. After the digestion is complete, centrifuge the tube at **13 000 rpm at 24 °C for 3 min.**
15. Place the tube on a magnetic rack until the beads have settled onto the tube wall and remove the supernatant to a fresh tube.
16. Remove the tube from the magnetic rack, and add 50µl 0.5M NaCl *(see right panel)*, pipette-mix.
17. Sonicate for **30 s** in a water bath, and centrifuge the tube at **13 000 rpm at 24 °C for 3 min.**
18. Place the tube on a magnetic rack until the beads have settled onto the tube wall and add the supernatant to new tube (2).
19. Measure peptide concentration on Nanodrop at 280 nm (optional if your sample is free of nucleic acids).
20. Desalt samples using 2 mg OASIS C18 96-well plates

Digestion solution; 1 ml 100mM AmBic/1mM $CaCl_2$: Add 7.9 mg AmBic (art. no. 09830, Merck) to 10µl 100mM $CaCl_2$ x $2H_2O$ (art. no. 21097, Merck, stabilize trypsin) and about 990µl dH_2O. Prepared fresh daily.

0.8µg Trypsin Porcine (4µl) (Promega, art. no. V 5111): Dissolve each ampoule (20 µg trypsin porcine) in 100 µl 50 mM acetic acid (resuspension buffer supplied from Promega with the trypsin powder). The trypsin concentration in this stock solution is then 0.2 µg/µl

*0.5M NaCl in MilliQ water: Add **29.2mg NaCl** in 1ml MilliQ Water (NaCl, art. no. S7653, Merck)*