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On beads digestion (immunoprecipitate)

Protocol starts after an IP have been performed in precleared lysate (lysed in immunoprecipitation buffer, ex NEB buffer: 50mM NaCl, 10mM Tris-HCl pH 7.4, 1mM EDTA, 1mM EGTA pH 8.0, 0.2 mM sodium ortho-vanadate (phosphatase inhibitor, 0.2M Phenylmetanesulfonyl fluoride (serine protease inhibitor), 1% Triton, 0.5% NP-40), adding Protein A/G Magnetic Beads, and beads washed 3 times with immunoprecipitation buffer.

Equipment needed: Magnetic rack, Eppendorf shaker with temperature control.

Bead cleaning

All traces of immunoprecipitation buffer (detergents) need to be removed

1. Place the Eppendorf tube on the magnetic rack for 1 minute until beads have settled on the wall of the Eppendorf tube and remove the buffer.
2. Add 200 µl PBS to the magnetic beads *(see right panel)*, pipette wash and place the tube on the magnetic rack.
3. Remove the supernatant to waste, repeat wash with PBS twice (total of three times)

Reduction/alkylation/digestion

4. Add 40 µl of 50mM Tris-HCl buffer *(see right panel)*.
5. Add 4 µl of 0.1M DTT *(see right panel)*, and heat at 95˚C for 5 minutes to denature and break disulfide bonds.
6. Let the solution cool until RT is reached.
7. Add 5 µl of 0.2M IAA *(see right panel)* to alkylate cysteines and incubate in RT for 1h in the dark.
8. Add 0.8 µl 0.1M DTT and incubate for 10 minutes.
9. Add trypsin *(see right panel)* at a concentration of 50 (25) times lower than the amount of protein in the sample. If the sample contains approx. 100 µg protein, add 2 (4) µg of protease. Measure pH using an indicator paper (litmus paper or similar) and incubate samples at 37 ˚C overnight on a shaker.
10. Add 5 µl of 10% TFA (trifluoroacetic acid) and check pH for acidic reaction, and proceed with desalting (OASIS) before LC-MSMS analysis.

1L PBS (Phosphate Buffered Saline) pH 7.4

Dissolve 8g NaCl, 200mg KCl, 1.44g Na2HPO4, and 245 mg KH2PO4 in 800 ml distilled water. Adjust pH to 7.4 and top up to 1L with water.

50mM Tris-HCl buffer/1mM $CaCl_2$ pH 7.8-8:

*Add **0.61g Tris** (art. no. 252859, Sigma-Aldrich) and **15mg $CaCl_2$ x $2H_2O$** (art. no. 21097, Sigma-Aldrich) to about 90ml dH_2O. Correct the pH to 7.8-8 with HCl and adjust the volume to 100ml. Store the solution at 4 ℃.*

100 mM DTT in MillQ water:

*Add **15.4 mg DTT** (DiThioThreitol, art. no. D-9163, Sigma-Aldrich) to 1ml dH_2O*

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).*

2µg Trypsin Porcine (4µl) (Promega, art. no. V 5111):

*Dissolve each ampoule (**20 µg trypsin porcine**) in **40 µl 50 mM acetic acid** (resuspension buffer supplied from Promega with the trypsin powder). The trypsin concentration in this stock solution is then 0.5 µg/µl*