In solution digestion of proteins

IMPORTANT:

- Discuss your project and experimental design with the Proteomics Core Facility **before** starting your experiments!
- fill out the submission form online at www.proteomics-cologne.com

Reagents & Solutions

- 50x Protease Inhibitor cocktail (Roche) *
- Triethylammoniumbicarbonate (TEAB), 50 mM *
- Urea buffer: 8M Urea in 50 mM TEAB *
- Benzonase HC nuclease *
- Dithiothreitol (DTT), 100 mM stocks available*
- Chloroacetamide, 550 mM stocks available*
- Trypsin protease, 1 µg/µL or 0.1 µg/µL *
- Lysyl Endopeptidase (Lys-C), 0.5 µg/µL *
- Formic acid, 10% in water *

* available in the core facility

Equipment

- Benchtop Eppendorf centrifuge *
- Eppendorf thermo mixer *
- Diagenode Bioruptor
- Vacuum concentrator (Speedvac) *
- Vortex *

* available in the core facility

Protocol - 1st Day

Please work under the clean bench, wear gloves & work as clean as possible!

IMPORTANT: If your sample possibly contains detergents (e.g. Triton, NP40, Tween, Glycerol, PEG, SDS etc.) protein precipitation has to be performed before continuing with the in-solution protocol

1. Prepare Urea lysis buffer by adding 50x Protease inhibitor to the 8M Urea/50 mM TEAB buffer. For example, add 20 µL 50x Protease inhibitors to 1 mL of 8M Urea[1]

2. Sample lysis

    a. CELL CULTURE. Lyse cell pellet in Urea buffer. As a starting point it is recommended to start with approx. 1.000.000 cells.[2] If proteins are already in solution add an appropriate amount of Urea buffer or crystalline Urea to reach ≥ 6 M Urea[3]
    b. TISSUES. Grind a frozen piece of tissue under liquid nitrogen in a clean mortar. Resuspend the tissue powder directly with Urea lysis buffer

3. Degrade chromatin using a Bioruptor (10 min, cycle 30/30 sec) or equivalent sonifier.
   ALTERNATIVE: Completely degrade all nucleic acids by Benzonase HC. Use **25 Units per 500.000** cells.[4] Mix briefly and incubate for 30 min at 37°C. Check the viscosity of the sample to ensure that the chromatin has been completely degraded

4. Centrifuge for 15 min at 20.000 × g, remove cell debris if visible. Measure protein concentration by your assay of choice[5]

5. Transfer 50 µg per sample to a new 1.5 mL tube

6. Add DTT to a final concentration of 5 mM, vortex and incubate at 25°C for 1 h

7. Add CAA to a final concentration of 40 mM, vortex and incubate in the dark for 30 min

8. Add **Lys-C** protease at an enzyme:substrate ratio of **1:75** and incubate at 25°C for 4 h[6]

9. Dilute the sample with 50 mM TEAB to achieve a final concentration of Urea ≤ 2M[7]

10. Add **Trypsin** at an enzyme:substrate ratio of **1:75** and incubate at 25°C overnight[6]

Protocol – 2nd Day

11. Acidify the sample to stop enzymatic digestion. Add formic acid to a final concentration of 1%. Continue with SDB RP StageTip purification

Notes

[1] Always use freshly prepared Urea buffer. Aliquots are available in the core facility. Buffers containing urea should not be stored at room temperature for more than 24h. Never incubate Urea buffer containing samples at temperatures > 37°C.

[2] Rule of thumb: Add one volume of lysis buffer to one volume of sample.

[3] Try to keep the volumes small. If the volume gets too large (i.e. ≥ 150 µl) perform Acetone precipitation first, then resuspend the pellet in Urea buffer.

[4] Use 50 units per 100 µg of tissue.

[5] Samples can be frozen and stored at -20°C at this step.

[6] it has been shown that digestion at room temperature is superior: https://pubs.acs.org/doi/10.1021/acs.jproteome.8b00228

[7] This step is crucial to ensure optimal trypsin acitivity.