Protocol 5 - In-solution digestion protocol for human body fluids (plasma, serum, CSF)

This protocol is only intended for samples of human origin. For samples of other species follow the in-gel digestion protocol (protocol 2) or use a depletion column designed for the organism.

Sample requirements and precautions

- **Preferred amount of total protein in a sample before depletion is 100µg**
- Samples are not radioactive
- Samples are detergent-free (e.g. Triton X-100, PEG-44)
- Salt concentrations should be less than 500 µM
- No organic solvents present
- Special care must be taken to avoid contamination with keratins from skin or hair (**wear gloves, lab coat at all times and clean equipment vigorously**)

Equipment

- **Recommended:** 2D-Quant kit for protein concentration determination (#80-6483-56 GE Healthcare)
- MARS14 depletion spin column (Multiple Affinity Removal Spin cartridge , Hu-14, #5188-6560, Agilent)
- Agilent Bond Elut C18 Omix tips, 1 tip/sample, article# A57003100
- 3kDa MWCO cut off filter (Amicon Ultra, UFC500396, Merck Millipore)
- Micro centrifuge suitable for 1.5 ml reaction vials
- Incubator for digestion at 37 °C
- Adjustable pipettes
- Speedvac

Recommended Reagents

Name	Supplier	Article number
2-chloroaetamide (CAA)	Sigma	22790-250G-F
Urea	GE Healthcare	17-1319-01
Dithiotreitol (DTT)	Sigma	D9163-5G
Trishydroxymethylaminomethane (Tris)	GE Healthcare	17-1321-01
Lysyl endopeptidase C (LysC)	WAKO Chemicals	125-02543
Sequencing grade modified trypsin C=0.5µg/µl	Promega	V-5113-5
Water, Milli-Q	-	-
Ammonium bicarbonate (ABC)	Sigma	A-6141
Trifluoroacetic acid (TFA)	Pierce	9470
Acetonitrile HPLC-S grade (ACN)	Biosolve	01200702
Formic Acid (FA)	Merck	1.00264.10000

Solutions

Adjust volumes if necessary

1. **50 mM Ammonium bicarbonate (ABC)**
   Dissolve 200 mg in 50 ml Milli-Q. Prepare fresh, discard remaining solution.

2. **10 mM dithiothreitol (reduction buffer)**
   Dissolve 7.7 mg in 5 ml Milli-Q. Prepare fresh, discard remaining solution.

3. **50 mM 2-chloroacetamide in 50 mM ABC (alkylation buffer)**
   Dissolve 23.35 mg in 5 ml solution 1. CAA is light sensitive so store the solution in the dark.
   Prepare fresh, discard remaining solution.

4. **10 mM TRIS-HCl pH 8.0**
   Dissolve 60.57 mg Tris in 45 ml Milli-Q. Adjust to pH 8.0 with HCl. Adjust volume to 50 ml with Milli-Q.
   Store 10 ml aliquots in -20°C up to 1 year.

5. **8 M Urea in 10 mM TRIS pH 8.0**
   Dissolve 24.024 g urea in 50 ml solution 4. Prepare fresh, discard remaining solution.

6. **2% Trifluoroacetic acid**
   Dilute 1 ml TFA with 49 ml Milli-Q. Store in glass, shelf life 12 months.

7. **0.5 µg/µl Lysyl endopeptidase C (LysC)**
   If no aliquots are available, make aliquots:
   - Dissolve the stock-vial of Lys-C to a final concentration of 0.5µg/µl in buffer A.
   - The amount of Lys-C in the vial can be found in the certificate of analysis. If not available, this can be ordered via mail from Wako (see Wako website for contact details).
   - After diluting, prepare aliquots of 50 or 100 µl and store these in -80°C.

   Using an aliquot: after first use, store in -20°C for further usage (maximally: 1 year).

8. **Buffer A: 0.1% FA in H_2O (Milli-Q)**
   50 µl FA filled up till 50 ml with water.

9. **Buffer B: 0.1% FA in ACN**
   50 µl FA filled up till 50 ml with ACN.

Procedure

Depletion and digestion

1. Determine protein concentration.
   (In our experience, the 2D-Quant kit gives the best results)
2. **We recommend using 100 µg of total protein/sample as starting material. Be sure to use the same protein amount for all samples**.
3. Apply sample to MARS14 spin column, following the manufacturer's instructions.
4. Concentrate sample on 3kDa MWCO cutoff filter and wash with 500 µl MQ for extra desalting.
5. **Optional**: Check depletion by running samples before and after depletion on SDS-PAGE and stain with Coomassie staining.
6. Dilute samples 1:1 with 8M urea/10 mM Tris pH 8.0 (solution 5) after depletion. For optimal digestion of the sample, the final urea concentration should be 4 M and the pH should be near pH 8.
7. Add 1 µl reduction buffer (solution 2) for every 50 µg sample protein and incubate 30 min at room temperature.
   (In this procedure all steps prior to digestion are done at room temperature to reduce unwanted derivatization of amino acid side-chains by the denaturants)
8. Add 1 µl alkylation buffer (solution 3) for every 50 µg sample protein and incubate 20 min at room temperature **in the dark**.
9. Add 1 µg LysC/50 µg total protein and incubate for at least 3 hours, room temperature.
10. Dilute sample 4x with 50 mM ABC (solution 1).
11. Add 1 µg trypsin/50 µg total protein and incubate overnight at 37 °C.

12. After incubation, spin down the water droplets condensed inside the lid of the test tube.
13. Proceed to Sample desalting and concentration.

Sample desalting and concentration

For more detailed information see the OMIX tips manual. ***Maximum loading capacity is 10 µg!*** *Divide sample over more tips if necessary.*

1. Dilute samples 1:1 with 2% TFA (solution 6)
2. Prepare Omix tip (1 tip/sample):
    - Aspirate 100 µl buffer B and discard solvent, repeat 1x
    - Aspirate 100 µl buffer A and discard solvent, repeat 1x
3. Aspirate up to 100 µl sample. Dispense and aspirate 5 times then discard liquid. If sample volume is more than 100 µl: repeat until all sample has been passed through the tip.
4. Aspirate 100 µl buffer A and discard solvent, repeat 1x.
5. Aspirate 100 µl buffer B and dispense in a new collection tube. Discard the OMIX tip.
6. Speedvac sample to a volume of 2 µl.
7. Add buffer A to obtain a total volume of 20 µl.
8. Store sample in -20 °C until shipment.

Literature

Kinter, M., and Sherman, N. E. 2000, Protein sequencing and identification using tandem mass spectrometry. JohnWiley & Sons, Inc. pp.161-163

Nielsen ML, Vermeulen M, Bonaldi T, Cox J, Moroder L & Mann M, Iodoacetamide-induced artifact mimics ubiquitination in mass spectrometry. Nature Methods 2008, 5

Pierce Detergent Removal Column manual, available from the Pierce website or in the package containing the columns

Agilent Bond Elut Omix C18 manual, available from the Agilent website or in the package containing the tips