Protocol 1 - In-solution digestion for cells/tissues

Sample requirements and precautions

- **Preferred amount of sample is 10 µg of total protein**
- Samples are not radioactive
- Samples are detergent-free (e.g. Triton X-100, PEG-44)
- Salt concentrations should be less than 500 µM
- Glycerol concentration should be less than 2.5 %
- 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)
- **Recommended**: Sample Grinding kit (80-6483-37, GE Healthcare)
- Pierce detergent removal spin columns, 1 column/sample: article# 87777
- Agilent Bond Elut C18 Omix tips, 1 tip/sample, article# A57003100
- 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 grade	-	-
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 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. **8M Urea in 10 mM Tris pH 8.0**
   Dissolve 24.024 gram 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. **0.5µg/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 50ml with Milli-Q.

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

Procedure

Digestion

1. Lyse cells using the grinding kit, sonication or snap freezing.
   (In our experience the grinding kit gives the best results using 8M urea/10mM Tris as lysis buffer)
2. Determine protein concentration
   (In our experience the 2D-Quant kit gives the best results)
3. **We recommend to use 10 µg of total protein for digestion.**
4. Dilute samples 1:1 with 8 M urea/10 mM Tris pH 8.0 (solution 5). For optimal digestion of the sample, the final urea concentration should be 4 M and the pH should be near pH 8.
5. 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)
6. Add 1 µl alkylation buffer (solution 3) for every 50 µg sample protein and incubate 20 min at room temperature **in the dark.**
7. Add 1 µg LysC/50 µg total protein and incubate for at least 3 hours at room temperature.
8. Dilute sample 4x with 50 mM ABC (solution 1).
9. Add 1 µg trypsin/50 µg total protein and incubate overnight at 37 °C.
10. After incubation, spin down the water droplets condensed inside the lid of the test tube.
11. Proceed to Sample cleanup.

Sample cleanup

For more detailed information see the Pierce Detergent Removal Column manual

1. Remove bottom closure from Pierce Detergent Removal Column and loosen cap (do not remove cap).
2. Place column into a collection tube.
3. Centrifuge at 1500 x *g* for 1 minute to remove storage solution, **do not exceed the indicated speed!**

Note: When using fixed-angle rotors, place a mark on the side of the column where the compacted resin is slanted upward. Place column in centrifuge with the mark facing outward in all subsequent centrifugation steps. Improper orientation will result in reduced detergent removal efficiency.

4. Add 400 µl 50 mM ABC (solution 1) and centrifuge for 1 minute at 1500 x *g*. Discard the buffer and repeat this step two additional times.
5. Place column in a new 1.5 ml collection tube. Slowly apply sample (max 100 µl) to the top of the compact resin bed and incubate for 2 minutes at room temperature.
   (*If sample volume exceeds 100 µl use a speedvac to concentrate or apply sample to more columns and pool afterwards)*
6. Centrifuge at 1500 x *g* for 2 minutes to collect the detergent-free sample. Discard the used column.
7. 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 sample 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 the 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.

Related 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