Protocol 2 - In-gel digestion for cell/tissues (+/- fractionation)

Sample requirements and precautions

- **Load 20 µg (protein) sample on gel for fractionation**
- Samples are not radioactive
- 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**)
- **Do NOT use silverstaining**! Only coomassie!
- Only cut gels on a clean glass plate
- Use a new scalpel blade

Equipment

- **Recommended:** 2D-Quant kit for protein concentration determination (#80-6483-56 GE Healthcare)
- **Recommended:** Sample Grinding kit (80-6483-37, GE Healthcare)
- **Recommended:** BioRad Laemmli Sample Buffer 2x or 4x (161-0737, 161-0747, BioRad)
- **Recommended:** Mini-Protean 12% TGX pre-cast gel (4561044, Bio-Rad)
- **Recommended:** Colloidal Coomassie Blue stain (30-38-10, Severn Biotech)
- 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 pipets
- Speedvac

Reagents

Name	Supplier	Article number
2-chloroaetamide (CAA)	Sigma	22790-250G-F
Dithiotreitol (DTT)	Sigma	D9163-5G
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. **2% Trifluoroacetic acid**

Dilute 1 ml TFA with 49 ml Milli-Q. Store in glass, shelf life 12 months.

5. **12,5 ng/µl trypsin in 50 mM ABC (Digestion buffer)**
   Dilute 1 µl trypsin with 39 µl 50 mM ABC.
   Alternatively, for concentrated samples in a small total gel volume, 100 ng trypsin/sample in 50 mM ABC can be used. Use immediately.

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

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

Procedure

Digestion

All volumes are for reference purposes only; please adjust the volumes so that all gel particles are covered with liquid.

1. Lyse cells by use of 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 load 20µg sample for fractionation, or 10µg if no fractionation is needed**. Add Laemmli sample buffer to the samples and heat samples 5 min at 95 °C.
4. Run samples on 12% SDS gel:
   (We recommend to use pre-cast gels like the 12% TGX gel from Bio-Rad)
   For fractionation: run the gel until front reaches the end of the gel.
   If no fractionation is needed: (SDS-PAGE used as sample cleanup only): run sample approx. 1-2 cm into the gel.
5. Stain and destain gel **(Only Coomassie stainings!)**.
   (We recommend the ready to use Colloidal Coomassie Blue from Severn Biotech)
6. **Fractionate** the gel in the desired amount of fractions (Fraction sizes may vary, try to keep abundant bands (e.g. albumin), if present, in 1 separate fraction).
   In case **no fractionation** is performed, cut out the lane until the sample front.
7. Chop the excised fractions in pieces of approximately 1 x 1 mm with use of a clean scalpel. Transfer the gel pieces into clean microcentrifuge tubes. Any excess gel will lead to background noise, so cut precisely.
8. Add 100 µl 50 mM ABC. Incubate for 5 minutes at room temperature. Discard all liquid.
9. Add 100 µl 50% ACN/50% H2O Incubate 5 minutes at room temperature. Discard all liquid.
10. Add 100 µl acetonitrile. Incubate for 5 minutes at room temperature. Discard all liquid.
11. **Repeat steps 8 to 10 twice.**
12. Add 100 µl reduction buffer (solution 2) and incubate for 20 minutes at 56 °C. Discard all liquid.
13. Add 100 µl acetonitrile. Incubate for 5 minutes at room temperature. Discard all liquid.
14. Add 100 µl alkylation buffer (solution 3) and incubate 20 min at room temperature **in the dark.**
15. Add 100 µl acetonitrile. Incubate for 5 minutes at room temperature. Discard all liquid.
16. Add 100 µl 50 mM ABC. Incubate for 5 minutes at room temperature. Discard all liquid.
17. Add 100 µl acetonitrile. Incubate for 5 minutes at room temperature. Discard all liquid.
18. **Repeat steps 16 and 17.**
19. Add digestion buffer (solution 5) until all gel pieces are covered. Incubate for 15-20 minutes at room temperature. Add more digestion buffer if the digestion buffer is completely absorbed. Incubate for 30 minutes at room temperature. Add 10-20 µl 50 mM ABC to cover all gel pieces and keep them wet during digestion.

20. Incubate overnight at 37 °C.
21. After incubation, spin down the water droplets condensed inside the lid of the test tube.
22. Add 2% TFA (solution 4) 1:1 and incubate 20 minutes, shaking at room temperature.
23. Transfer supernatant to a new tube.
24. Repeat steps 22 and 23 with buffer B (solution 7) and pool supernatants after incubation.
25. Speedvac samples to ensure all ACN has been evaporated .
    (tip: set a mark on the new tube after transferring the first supernatant. When during speedvac the level of sample reaches the mark, as the second supernatant consists of ACN, almost all ACN has been evaporated)
26. Dilute sample 1:1 with 2% TFA (solution 4).
27. 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. 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
2. Aspirate up to 100µl 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.
3. Aspirate 100 µl buffer A and discard solvent, repeat 1x.
4. Aspirate 100 µl buffer B and dispense in a new collection tube. Discard the OMIX tip.
5. Speedvac sample to a volume of 2 µl.
6. Add buffer A to obtain a total volume of 20 µl.
7. Store sample in -20 °C until shipment.

Literature

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

Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S. et al., Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature 1996, 379, 466–469

Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes Nature Protocols (2006), 1(6), 2856-2860

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