PROTEOMICS
CORE FACILITY
COLOGNE

In gel 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
- **DON'T** use overhead transparencies or parafilm to wrap gels
- **DON'T** incubate gels in the microwave to expedite coomassie staining
- **DON'T** use commercial kits containing glutaraldehyde or other aldehydes/crosslinkers for staining
- We recommend the use of commercially available not pre-stained gels
- Only use deionized water or MilliQ
- All homemade buffers should be made of solvents of at least HPLC grade

Reagents & Solutions

- Dithiothreitol (DTT), 100 mM stocks available*
- Chloroacetamide, 550 mM stocks available*
- Commassie Brilliant Blue staining solution
- Fixing solution (10% Acetic acid / 20% Methanol in water)
- Acetonitrile (ACN) *
- Ammoniumbicarbonate (ABC), 50 mM *
- 50 mM ABC/50% ACN *
- Trypsin protease, 1 µg/µL or 0.1 µg/µL *
- Peptide extraction buffer: 30% Acetonitrile / 3% Trifluoroacetic acid in water *
- Formic acid, 10% in water *

* available in the core facility

Equipment

- Glass plate (thoroughly clean with detergent and 70% Ethanol prior use) *
- New razor blade or scalpel *
- Eppendorf Thermo Mixer *
- 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!

1. Reduce samples by addition of DTT to a final concentration of 5 mM, incubate at 56°C for 30 min[1]

2. Alkylate cysteines by addition of CAA to a final concentration of 40 mM, vortex and incubate for 30 min at room temperature in the dark[2]

3. Load and run the sample on a SDS-PAGE gel[3]. Precast commercial gels (not pre-stained) are preferred. Stain the gel with Coomassie staining solution. If no staining is desired fix the gel for 1h in fixing solution

4. Excise stained gel bands and chop each band into smaller pieces (~ 1mm per side) and place them in clean 1.5 mL Eppendorf tubes[4]

5. Add 100 µL 50 mM ABC/50% ACN[5]. Vortex and incubate for 20 min at RT. Discard solution afterwards

6. Repeat step 5 once

IMPORTANT: If you have not reduced & alkylated (step 1 and 2) your samples already before running the gel perform the following steps. Otherwise directly continue with step 7.

   a. Cover the gel pieces with 10 mM DTT in water, vortex, incubate 30 min at 55°C while shaking gently
   b. Discard DTT solution
   c. Cover the gel pieces with Acetonitrile[6], vortex for 1 min, incubate 15 min at RT and discard solution afterwards
   d. Cover the gel pieces with 55 mM CAA in water, vortex, incubate for 30 min at room temperature in the dark
   e. Discard CAA solution
   f. Cover the gel pieces with Acetonitrile, vortex for 1 min, incubate 15 min at RT and discard solution afterwards

7. Cover the the gel pieces with 100 µL Acetonitrile. Vortex, incubate for 10 min. Discard solution afterwards & dry the gel pieces in the Speedvac for approx. 5 min (or bench for approx. 15 min)

IMPORTANT: Perform the following steps to completely destain the gel pieces. If you have not stained the gel directly proceed with step 10.

8. Destaining, washing & dehydration:
    a. Add 100 µL 50 mM ABC. Vortex and incubate for 15 min at RT. Discard solution afterwards
    b. Cover the gel pieces with Acetonitrile, vortex for 1 min, incubate 15 min at RT and discard solution afterwards
    c. Repeat these steps until gel pieces are destained

9. dry the gel pieces in the Speed Vac for 5 min

10. Prepare digestion solution: 10 ng/µL of Trypsin in 50 mM ABC

11. Add the digestion solution to cover the gel pieces. Let the gel pieces swell for 30 min at 4°C.

12. Remove excess Trypsin solution

13. Add 50 mM ABC to cover the gel pieces completely. Incubate overnight at 37°C while shaking at 750 rpm

Protocol - 2nd Day

14. IMPORTANT: Transfer the supernatant of the gel pieces into new 1.5 mL Eppendorf tubes

15. Extraction of peptide digestion products:
    - Cover the gel pieces with 100 µL 30% ACN/3% TFA. Vortex and incubate for 20 min at RT
    - Take off the extract and combine it with the supernatant from step 14
    - Cover the gel pieces with 100 µL 100% ACN. Vortex and incubate for 20 min at RT
    - Take off the extract and combine it with the supernatant from step 14

16. Dry down the collected supernatants in the Speedvac to remove the organic solvent. The remaining volume will have a volume of approximately <50 µL[7]

17. Acidify the samples by addition of formic acid to a final concentration of 1% and continue with SDB RP StageTip purification protocol.

Notes

[1] Disulfide bridges can also be reduced by alternatives such as ß-mercaptoethanol or TCEP, for example as component of Laemmli buffer.

[2] In case a reducing agent other than DTT was used please make sure at this step that CAA is added in excess compared to the reducing agent. If concentration of the reducing agent is too high or unknown skip this step. Cycsteines alkylation can also be performed at a later stage (after step 6).

[3] If no protein separation/fractionation is necessary just let the sample run for 1-2 cm into the gel.

[4] Avoid scratching the plastic tubes with the razor or scalpel. It may contaminate the sample with polymers or plasticizers.

[5] It is important to **completely** cover the gel pieces with the solvents in this and all other steps. A volume of 100 µL is a good estimate for single bands. Larger pieces may require larger volumes.

[6] Addition of Acetonitrile will dehydrate the gel pieces, they will shrink and turn white. This process will take significantly longer if the amount of acetonitrile is not sufficient.

[7] Do not use temperatures > 30 °C in the Speedvac