

# Protocol for sample preparation for mass spectrometry analysis by nanoflow LC-MS/MS

Jeroen Demmers | Karel Bezstarosti | Dick Dekkers

Proteomics Center | [www.proteomicscenter.nl](http://www.proteomicscenter.nl)  
Erasmus University Medical Center, Office Ee-679A | Lab Ee-626  
Dr Molewaterplein 50  
3015 GE Rotterdam  
The Netherlands  
P: +31-(0)10-7038124  
E: [j.demmers@erasmusmc.nl](mailto:j.demmers@erasmusmc.nl)

## **Standard procedures:**

If you want to analyze proteins from a gel, go to [Protocol A](#)

If you want to analyze proteins bound to beads, go to [Protocol B](#)

## **Protocol A:**

**We strongly recommend that you use commercial pre-cast protein gels** (Pierce, Invitrogen, etc.). These gels tend to contain less keratin contaminants than in-house poured gels. Be sure that all equipment that you use for running gels (trays, boxes, dishes, tips, etc.) is clean and try to keep equipment that you use for gels for mass spec apart from other electrophoresis equipment in your lab (e.g. sample loading buffer). The more keratin contaminants, the less protein identifications in the end! Also, run controls on a separate gel, so that no interference with your sample takes place.

## **Materials and chemicals**

Ammonium bicarbonate ('ABC'   $\text{NH}_4\text{HCO}_3$ )	50 mM = 4 mg/ml
Dithiothreitol (DTT) in ABC	100 mM = 15.4 mg/ml ABC
2-Chloroacetamide (CAA) in ABC	200 mM = ... mg/ml

*Use highest quality of chemicals and  $\text{qH}_2\text{O}$ . CAA can be obtained from the Proteomics Center upon request.*

## **Pretreatment of samples for SDS-PAGE (alkylation/carbamidomethylation)**

1. Add DTT from the 100 mM stock solution into the protein sample to a final concentration of 5 mM. The protein sample should be a clear solution with  $<100\mu\text{g}$  of protein, in a buffer with a pH  $\sim 8$ .
2. Mix and heat the sample for 30 min at 60 °C.
3. Add CAA from the 200 mM stock solution to a final concentration of 10 mM. Mix and leave in the dark at RT for 30 min.

**Important notice 1:** Tick the checkbox on the submission form at [www.proteomicscenter.nl](http://www.proteomicscenter.nl) to indicate that cysteines in your proteins have been alkylated already.

**Important notice 2:** If **ubiquitination** of proteins will be studied, contact the Proteomics Center as the protocol needs to be adapted.

The sample is now ready for analysis by SDS-PAGE. Continue running the gel using your own standard procedure or see below. Once you have run the gel, stain with Coomassie, destain and leave the gel in  $\text{qH}_2\text{O}$  in a clean cell culture dish. Make a medium resolution

picture of the gel using a digital camera. E-mail the picture to the Proteomics Center ([pictures@proteomicscenter.nl](mailto:pictures@proteomicscenter.nl)) and bring the gel to the Center (Room Ee-679A).

### Running the gel

4. Mix 9  $\mu\text{L}$   $\text{qH}_2\text{O}$  and 10  $\mu\text{L}$  2X TCEP sample buffer (625  $\mu\text{L}$  Tris-HCl 2M, 500  $\mu\text{L}$  0.1% BPB, 400  $\mu\text{L}$  1M Tris base, 500  $\mu\text{L}$  0.2 M (60 mg/ml) TCEP-HCl (tris(carboxyethyl)phosphine) in  $\text{qH}_2\text{O}$ , 2000  $\mu\text{L}$  glycerol, 4000  $\mu\text{L}$  10% SDS, store at  $-20^\circ\text{C}$  for approx. 2 months), heat to  $95^\circ\text{C}$  for 5 min, spin down for 20 sec at max speed, cool to RT while making fresh CAA.
5. Resolve sample with a pre-cast gel (depending on your MW region of interest use 4-12% or 4-20% gradient gels).
6. Fix gel in a clean tip box or petridish.
7. Fill box or dish  $\frac{1}{2}$  way with  $\text{qH}_2\text{O}$  and microwave 80 sec, pour off, re-fill with  $\text{H}_2\text{O}$ , microwave 80 sec, pour off, re-fill with  $\text{qH}_2\text{O}$  and rock for 5 min, pour off.
8. Stain the gel with colloidal CBB G-250 or Pierce GelCode Blue Stain reagent. Destain and store in  $\text{qH}_2\text{O}$ .

### Make gel picture

9. Make a medium resolution picture of the gel using a digital camera (go to Reproduction Department in room Ee 1638) or, alternatively, scan the gel in a plastic sheet. E-mail the picture to the Proteomics Center ([pictures@proteomicscenter.nl](mailto:pictures@proteomicscenter.nl))

### Excision of bands from the gel

10. Wash the gel twice with  $\text{qH}_2\text{O}$ .
11. Cut out the lane of interest using clean razor blade and tweezers and put the complete lane onto two wetted filter papers (1.5 X 10 cm). If necessary, also cut out the negative control lane.
12. Clean the razor blade of the Mickle gel slicer with MeOH and  $\text{qH}_2\text{O}$ .
13. Put the filter paper with the gel lane on top onto the sled of the Mickle gel slicer and start cutting the lane into slices of 1 mm each.
14. Depending on the complexity of the protein mixture in the gel lane, transfer 2 or 3 adjacent slices to 1.5 ml eppendorf tubes that contain 600  $\mu\text{l}$  of a 1:1 (v:v) solution of 100 mM  $\text{NH}_4\text{HCO}_3$  / acetonitrile, so that you divide the complete lane over 20-30 sample tubes.

### Destaining and washing of gel pieces

15. Destain the gel slices by shaking at  $4^\circ\text{C}$  o/n (a few hrs is sufficient depending on time of day)
16. Aspirate off wash solution with gel loading tip, replace with fresh 0.5 ml of wash and shake for 1hr at  $4^\circ\text{C}$
17. Wash with 200  $\mu\text{l}$   $\text{qH}_2\text{O}$  once and with 200  $\mu\text{l}$  100 mM  $\text{NH}_4\text{HCO}_3$  / acetonitrile (1:1, v/v) twice for 15 min.
18. Shrink the gel pieces in 200  $\mu\text{l}$  100 % acetonitrile and flick until the gel pieces turn white. Incubate for 5 min.
19. Aspirate off acetonitrile, let air dry for 5 min.

### Reduction and alkylation of proteins (skip steps 18-23 if you have alkylated the proteins before running the gel!)

20. Swell the gel pieces in 200  $\mu\text{l}$  freshly prepared 10 mM DTT in 50 mM  $\text{NH}_4\text{HCO}_3$ .
21. Incubate for 1 hour at  $37^\circ\text{C}$ .
22. Remove the DTT solution and add 200  $\mu\text{l}$  freshly prepared 55 mM CAA in 50 mM  $\text{NH}_4\text{HCO}_3$ .

23. Incubate for 1 hour at RT in the dark.
24. Wash two times with 200  $\mu$ l 100 mM  $\text{NH}_4\text{HCO}_3$  / acetonitrile (1:1, v/v) for 15 min.
25. Shrink the gel pieces in 200  $\mu$ l 100 % acetonitrile.

### **In-gel digestion**

26. To one vial (100  $\mu$ g) of trypsin sequencing grade (Promega), add 1 ml of a 1 mM HCl solution. Aliquot 10  $\mu$ l and store at  $-80^\circ\text{C}$ . Just before use, dilute the trypsin stock solution 10 times in 50 mM  $\text{NH}_4\text{HCO}_3$ .
27. Add a sufficient amount of the diluted trypsin solution to fully immerse the gel pieces (use about 30  $\mu$ l for 3 x 1 mm bands).
28. Leave for 30 min at RT and then check whether the gel pieces are still fully covered by the trypsin solution. If not, add some more of the diluted trypsin solution to fully cover the gel pieces.
29. Incubate overnight at  $37^\circ\text{C}$ ; shaking not necessary.

### **Extraction of peptides from the gel and solution**

30. Spin down tubes for 10 s.
31. Add 50  $\mu$ l of a 0.5 % formic acid solution in 30% acetonitrile, sonicate in a ultrasonic bath for 2 min and then incubate for 30 min at RT.
32. Transfer the supernatant to a clean eppendorf tube or into a 96 well plate.
33. Repeat steps 29-30 twice.
34. Dry the combined supernatants in a speedvac (Room 681).
35. Label the sample tubes or well plate with the appropriate indexing number and store the dry peptides at  $-20^\circ\text{C}$  until LC-MS/MS analysis.

## **Protocol B:**

### **On-bead digestion protocol**

1. Follow the standard procedure for your co-IP
2. Wash the beads with the buffer you normally use solution
3. Wash 2 x with 50mM  $\text{NH}_4\text{CO}_3$  solution
4. Add 200-300  $\mu$ l of 50mM  $\text{NH}_4\text{CO}_3$  sol'n and 24  $\mu$ l of the stock trypsin solution (which is about 100ng/ $\mu$ l – see Promega or Roche instruction manual for details on how to prepare the stock solution)
5. Digest o/n @  $37^\circ\text{C}$  while shaking gently
6. Pellet the beads, bring the supernatant or whole digestion mixture to the Proteomics Center