

SDS-PAGE of Captured Proteins and LC-MS Analysis

NOTE: Before proceeding this protocol, please read carefully the instruction in the handbook “Guideline for CCMS”! This is only a brief description of the procedure!

SDS-PAGE

Add 10 µl 2x Laemmli sample buffer to the Streptavidin-magnetic-beads-protein-complexes collected after the last wash step, homogenize, incubate 10 min at 95 °C and load the gel with the whole suspension. Alternatively, the beads may be separated by using the caproMag and only the supernatant may be loaded on the gel.



Note: When the whole suspension was loaded wash the slots after the run with water before proceeding fixation the gels.

Recommended downstream analysis:

SDS-Page: Pre-cast gels from OLS (1 mm mini gels, 4-20 %, 12 lanes). Run the gel at 120 V and 4 °C.

Silver staining: ProteoSilver™ Plus Silver Stain Kit from Sigma.

In-gel Tryptic Digestion

Prepare all reagents freshly prior to use.

Excision of protein bands

- Wash the gel for 10 min with LC-MS-grade water, discard water and repeat washing step
- Cut as close as possible to the protein band to reduce the amount of "background" gel
- **Optional:** fragment the excised pieces into nearly 1 mm³ cubes and transfer them to a clean siliconised 0.5 ml PCR tube.

Washing of gel pieces

- Wash the gel pieces each 15 min and discard supernatant after each step:
 - 100 µl water
 - 100 µl 50 % ethanol (v/v)
 - 100 µl water

- 100 µl 50 % ethanol (v/v)
- Add 50 µl 100 % ethanol and incubate for ~5 min to shrink the gel pieces (visual inspection), discard supernatant

Optional: Reduction and alkylation

- Re-swell the gel pieces in 50 µl 10 mM dithiothreitol (DTT)/100 mM NH_4HCO_3 and incubate for 45 min at 56 °C
- Cool down tubes to room temperature
- Remove DTT solution
- Incubate gel pieces in 50 µl 55 mM iodoacetamide (IAA)/100 mM NH_4HCO_3 for 30 min at room temperature in the dark
- Remove IAA solution

Washing of gel pieces

- Wash the gel pieces each 15 min and discard supernatant after each wash step:
 - 100 µl water
 - 100 µl 50 % ethanol (v/v)
 - 100 µl water
 - 100 µl 50 % ethanol (v/v)
- Add abs. ethanol (50 µl) and incubate 5 min to shrink the gel pieces (visual inspection), discard supernatant

Digestion

- Rehydrate gel pieces in a solution of 12.5 ng/µl of trypsin/50 mM NH_4HCO_3 on ice, start with 10 µl, incubate for 20 min on ice
- Add more buffer if the initially added volume has been absorbed by the gel pieces and further incubate up to 45 min incubation on ice
- Replace supernatant by 5-20 µl (depending on size of the gel pieces) of 50 mM NH_4HCO_3 without trypsin
- Close the lids of the tubes well, wrap the whole rack with the tubes in parafilm and incubate over night at 37 °C

Extraction of peptides

- Transfer supernatant with peptides to a clean 0.5 ml siliconised tube
- Add enough of 5 % formic acid to cover the gel pieces (~ 20 µl), do **NOT** discard supernatant
- Add the same volume of acetonitrile and incubate for 15 min
- Remove supernatant into the clean 0.5 ml siliconised tube above
- Add enough of 5 % formic acid to cover the gel pieces (~ 20 µl), do **NOT** discard supernatant
- Add the same volume of acetonitrile and incubate for 15 min
- Remove supernatant into the clean 0.5 ml siliconised tube above
- Dry the sample in a vacuum centrifuge

Optional: Desalting

- Re-dissolve the peptides in 5-10 µl of 5 % formic acid, sonicate briefly
- For desalting we recommend Stage tips® available from Proxeon Biosystems (www.proxeon.com)
- Follow the manufacturer's instruction

Mass spectrometry

- Re-dissolve the peptides in 5-10 µl of 0.1 % formic acid, sonicate briefly
- **Optional:** Centrifuge for 10 min at 12.000 x g
- Inject peptide extract into MS

"OnBead" Digestion

Washing of proteins

- Wash the Streptavidin magnetic beads six times with 200 µl 80 % acetonitrile
Note: During the first washing steps the bead suspension could agglutinate, resuspend well by using a pipette
- Wash the Streptavidin magnetic beads one time with 200 µl LC-MS-grade water, discard supernatant

Optional: Reduction and alkylation

- Add 20 μ l of 10 mM DTT/100 mM NH_4HCO_3 , mix the sample by gently vortexing and incubate for 1 h at 56 °C
- Remove DTT solution
- Add 20 μ l of 55 mM IAA/100 mM NH_4HCO_3 , mix the sample and incubate for 30 min at room temperature in the dark
- Remove IAA solution

Digestion

- Add 10 μ l of 50 mM NH_4HCO_3 /0.5 mM CaCl_2 , 200 ng/ μ l trypsin,
- Close the lids of the tubes well, wrap the whole rack with the tubes in parafilm and incubate over night at 37 °C on a temperature controlled shaker

Optional: Desalting

- Re-dissolve the peptides in 5-10 μ l of 5 % formic acid, sonicate briefly
- For desalting we recommend Stage tips® available from Proxeon Biosystems (www.proxeon.com)
- Follow the manufacturer's instruction

Mass spectrometry

- Re-dissolve the peptides in 5-10 μ l of 0.1 % formic acid, sonicate briefly
- **Optional:** Centrifuge for 10 min at 12.000 x g
- Inject peptide extract into MS

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