Sample preparation for MS analysis

(max. 4 samples at the same times = 40 tubes)

ELECTROPHORESIS

Solutions / reagents :

100mM DTT = dithiotheritol, Sigma D9779 (aliquots stored at -20°C)

550 mM IAA = iodo-acetamide, Sigma I1149 (aliquots stored at -20°C, max. 3x thawing up!)

Running Buffer = 1x Running Buffer prepared with ultrapure, distilled water
- 20x MOPS, Invitrogen NP0001
- or 20x MES, Invitrogen NP0002

NuPAGE antioxidant, Invitrogen NP0005

Precast gels :
- NuPAGE 4-12% Bis-Tris Gel, 1.5mm x 10 well, 10 gels, Invitrogen NP0335
- or NuPAGE 4-12% Bis-Tris Gel, 1.5mm x 15 well, 10 gels, Invitrogen NP0336

Protein ladder = PageRuler Plus Prestained Protein Ladder, Pierce 26620

6x SDS loading buffer = 0.35M Tris-HCl pH6.8, 30% glycerol, 10% SDS, bromophenolblue

1x SDS loading buffer = 0.06M Tris-HCl pH6.8, 5% glycerol, 1.7% SDS, bromophenolblue

Fixing Solution = 50%MeOH, 10% acetic acid
- for 1 gel = 10 ml ddH_2O + 12.5 ml MeOH + 2.5 ml acetic acid
- for 2 gels = 20 ml ddH_2O + 25 ml MeOH + 5 ml acetic acid

Staining solution = Colloidal Blue Staining Kit, Invitrogen LC6025

Prepare sample for gel electrophoresis :

Add 10µl of 6 x SDS loading buffer (50µl sample + 10µl 6x SB).

REDUCTION OF THE SAMPLE * (to break S-S bridges):
Add 1µl of 100mM DTT (or add 1/100 vol. of DTT if sample >100µl).
Heat at 75°C while shaking at ~ 800 – 1000 rpm for 10 minutes.

ALKYLATION OF THE SAMPLE *:
Let the sample cool down.
Add 1µl of 550 mM IAA (or add 1/100 vol. of IAA if sample >100µl).
Alkylate in the dark for 10 min at RT while shaking at ~ 800 – 1000 rpm.

*sometimes, when you receive samples from collaborations (pieces of gels), reduction and alkylation have not been done… -> go directly to "gel pieces washing/dehydratation"

It is sometimes necessary to concentrate the samples with a Vivaspin to reduce its volume
=> after reduction/alkylation, load the sample on the Vivaspin
=> spin 14'000g up to a volume between 35 and 80µl (not less than 30µl) and directly load the sample.

Electrophoresis :

Prepare running buffer for electrophoresis (for Life technologies migration tank : 400ml/gel).

Remove white isolation strip from the precast gel.

Clip the gel in the chamber.

Fill the middle part of the chamber with 1x Buffer and add 500µl of antioxidant.

Fill the middle part up to the top, and add the remaining 1x Buffer in the other part of the chamber.

Load 5-6µl of protein ladder.

Load samples : If possible leave one lane empty in between.

Fill the empty lanes with 1x SDS loading buffer (or 2-4x SDS loading buffer if the samples have been concentrated by Vivaspin).

Perform electrophoresis at 140 V for 5 min
=> optional for big volumes : stop and reload the second part of the sample
then at 200 V for ~ 45 min (or until the running front has reached the end).

Gel staining :

Prepare Fixing Solution
- *for 1 gel = 10 ml ddH_2O + 12.5 ml MeOH + 2.5 ml acetic acid*
- *for 2 gels = 20 ml ddH_2O + 25 ml MeOH + 5 ml acetic acid*
=> Incubate the gel in this solution for 15 min at RT on a rocking platform

Prepare staining solution
- *for 1 gel = 13.75 ml ddH_2O + 5ml Stainer A + 5 ml MeOH*
- *for 2 gels = 27.5 ml ddH_2O + 10 ml Stainer A + 10ml MeOH*
=> Incubate the gel in this solution for 15 min at RT on a rocking platform

Add Stainer B (shake well before pipetting => 1.25ml for 1 gel, 2.5ml for 2 gels)
=> Stain until protein bands become visible (minimum 5 min. -> max. 2-3 hours !).

Discard fixing-staining solutions to the organic waste container.

Destain with ultrapure, distilled water, using non-fuzzing paper towels to soak Coomassie off the solution. Change water and towels several times.
=> It takes generally about 1h. If necessary destain over night at 4°C on a rocking platform. It can also stay up to 4 days at 4°C before cutting bands.

Cut the gel :

Put the gel on a plastic foil or a glass plate.

Before cutting the gel, take a picture.

Work on clean bench or on a very clean area, wear gloves and coat, tie back long hair, clean scalpels regularly with ethanol.

For each sample, prepare 10 x 1.5ml Low Binding Protein ED-tubes (not autoclaved).

With a clean scalpel cut off the running front and the ladder.

Cut lane into 10 pieces according to the protein amount. Avoid to split one major band in two different fractions.

Cut each of the 10 pieces into smaller pieces (1mmx1mm) and put those into an ED-tubes. Use one reaction tube per gel slice.

Clean the scalpel and glass surface with 70% ethanol in-between cutting different samples.

Gel pieces can be stored at 4°C for few days or for longer periods after dehydration with EtOH at -20°C.

IN GEL DIGESTION => Day 1 :

Solutions / reagents :

ABC-Buffer = 100mM Ammonium bicarbonate, pH7.5

Ethanol (ultra pure grade)

Gel pieces washing/dehydration:

Add 100 – 150 µl ABC-Buffer and incubate for 10 min at RT with 1'000 rpm shaking.
=> Discard SN.

Add 100 – 150 µl EtOH and incubate for 10 min at RT with 1'000 rpm shaking.
=> Discard SN.

Repeat 2x more both cleaning (total : ABC=>EtOH=>ABC=>EtOH=>ABC=>EtOH)

If your pieces are still blue, you can repeat 1 more wash: add ABC, heat at 30°C (max. 37°C), then EtOH.

*when not reduced and not alkylated pieces of gels : second wash: ABC + DTT (1:10): 10' shaking at 56°C -> EtOH-> ABC + IAA (1:10): 10' shaking in the dark at room temperature -> EtOH

After the last EtOH step, remove EtOH => Gel pieces should be white. If not, repeat EtOH step once more.

Pieces can be stored at -20°C.

Trypsin digestion :

Prepare Trypsin (80µl/slice => 800µl/sample => 1 Trypsin tube for 2 samples)
=> 1 aliquot of 20µg is diluted in 1,6 ml ABC-buffer and kept on ice.
=> if you use several aliquots of trypsin, it's better to pool before adding on gel pieces

Add 80 µl trypsin/tube (pieces must be covered) and let liquid be soaked into the gel pieces for 10-15 min at RT.

Add the minimum volume of ABC buffer to cover gel pieces (generally 40-60µl), vortex and spin quickly.
=> Digest over night at 37°C in incubator.

IN GEL DIGESTION => Day 2 :

Solutions / reagents :

FROM THE „TRYPSIN DIGESTION" STEP ONWARDS, YOU NEED HPLC OR MS GRADE CHEMICALS/SOLVANTS !!!

H_2O (HPLC or MS Grade)

Ethanol (HPLC or MS Grade)

Acetonitrile (HPLC or MS Grade)

Acetic acid 99% (VW Ref. 84874.260)

Trifluoracetic acid, TFA (Sigma Ref. 302031)

Trypsin = Sequencing Grade Modified Trypsin, Promega Ref. V5113

2% TFA in H_2O = trifluoroacetic acid (!!! No plastic!!!)
=> 49ml H_2O + 1ml TFA

buffer A = 0.5 % acetic acid in H_2O
=> 50ml H_2O + 250µl acetic acid

buffer A = 3% Acetonitrile, 0,3% Trifluoracid (TFA) (!!! No plastic!!!)*
=> 41ml H_2O + 1.5ml acetonitrile + 7.5ml TFA 2%

buffer B = 80% Acetonitrile, 0.5% acetic acid, 20% H_2O
=> 10ml H2O MS Grade + 40 ml Acetonitrile + 250µl Acetic. Acid

buffer A/ buffer A = 30% A*, 70% A, <1% Acetonitrile*
=> 15ml buffer A + 35ml buffer A*

Peptides extraction :

Add 50 µl 2% TFA to the gel pieces incubate for 10 min at RT with 1000rpm shaking.
=> Transfer SN to a fresh 1.5ml "low retention" reaction tube.

Add 100-150 µl EtOH to the gel pieces incubate for 10 min at RT with 1000rpm shaking.
=> Transfer SN to the first SN.

Add 100-150 µl EtOH to the gel pieces incubate for 10 min at RT with 1000rpm shaking.
=> Transfer SN to the first SN.
(Keep the gel pieces at 4°C until samples has been run on MS.)

Evaporate volatile components of the solution and decrease volume to 50µl using the speed vac :
- new speed-vac : automatic run, around 1h (can take up to 4-5 hours), at 35°C, max.45°C
- old speed-vac : medium heat, around 1h (can take up to 4-5 hours)
It's important to evaporate all the organic solvents, otherwise you will loose your peptides at the "C18 column step". But don't evaporate completely your samples, otherwise your peptides may stick to the tube and won't be solubilize with Buffer A. => if your sample is completely dry, add 5µl of Buffer B, vortex, spin and continue (+ 200µl Buffer A, etc.)

Add 200 µl Buffer A

STAGE TIPS:

Prepare stage tips with C18 material *(X20 membrane empore C18 47mm, Supelco ref. 2215)*.
Label the tips!

*Next spinning steps can be performed in “customized” centrifuge or in a “normal” Eppendorf centrifuge, using ED collector tubes, at 3’000rcf (max 4’000rcf).

Equilibrate C18 stage tips with 50 µl Buffer B => spin* 2-3min, RT.

Wash twice with 50 µl buffer A => spin 2-3min, RT.

Load sample (around 250µl) to the STAGE tip=> spin* 2-3min, RT.

Wash with 100 µl buffer A=> spin* 2-3min, RT.

If you don’t have the time to finish, you can load the sample into the stage tip, centrifuge (not too long to leave some liquid under the tip and avoid membrane to dry) => your peptide will stick to the C18 => very stable => it can be stored for 1-2 weeks at 4°C.

Elute peptides with 50 µl buffer B :
- put a small 0.2 ml labeled tube into a 1.5ml ED tube with a hole (screwdriver) in the cap
- or use a 1.5ml ED-tube with a hole (screwdriver) in a removable cap, without small tube inside
=> put the column into the corresponding tube
=> centrifuge 2-3’, 1’000rcf (not faster), Eppendorf centrifuge

Evaporate organic solvents and decrease volume to 5 µl (between 1-10µl) using the speed-vac :
- new speed-vac : automatic run, 5-10min (sometimes longer), at 35°C
- old speed-vac : medium heat, 5-10min (sometimes longer), at 35°C
Here also it's important to evaporate all the organic part to avoid loosing your peptides, but avoid to dry completely.
If nothing left into the tube, add 3µl of Buffer A* => vortex well to resuspend the peptides.

Fill up with buffer A*/buffer A to a final volume of 20 µl and store samples at -80°C.