PP Proteomics SS2017

SDS PAGE and in-gel-digestion

Notes:

- Always wear gloves and change them often
- Preferable to work in a clean hood. If unable to work in clean, protected area, wear a solid hair net and mask to avoid contamination of the samples.
- Incubations are at room temperature unless otherwise noted.
- Always centrifuge sample prior to removing supernatant.
- Larger bands will require more solution volume.
- It is very important to use siliconized tubes to prevent sample loss.
- It is preferable to have a positive control (i.e. known protein) if at all

Reagents (all reagents are freshly prepared and ready to use)

SDS polyacrylamide electrophoresis:

4 X Running gel buffer (LOWER GEL BUFFER): 0.4% SDS, 1.5 M Tris-HCl pH 8.8

36.4 g Tris
0.8 g SDS
add 150 ml of dH_2O
add 6 ml of concentrated HCl
adjust volume to 200 ml
filter and store at 4°C

4 X Stacking gel buffer (UPPER GEL BUFFER): 0.4% SDS, 0.5 M Tris-HCl pH 6.8

6.06 g Tris
0.4 g SDS
add 60 ml of dH_2O
add 2.9 ml of concentrated HCl
adjust volume to 100 ml
filter and store at 4°C

10 X electrode buffer (10x TGS): 2% SDS, 0.25 M Tris-glycine, pH 8.6

30.0 g Tris
144 g glycine
10 g SDS
dissolve and adjust volume to 1 liter, do not adjust pH

1 X sample buffer: 62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% SDS, 5% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue

0.4 g Tris (MW 121.1)
12.5 g glycerol (10 ml)
1 g SDS (MW 288)
2 ml 2-mercaptoethanol
1 mg bromophenol blue
dissolve and bring volume to near 40 ml
adjust pH to 6.8 with HCl
adjust volume to 40 ml
store in 1 ml aliquots at -20°C

10% (w/v) Ammonium persulfate (APS):

0.2 g ammonium persulfate
add dH_2O to bring weight to 2.0 g

Sensitive Coomassie Blue Staining of polyacrylamide gels:

Stock solution:

0.1% (w/v) CBB G-250 in:
2% (w/v) phosphoric acid
10% (w/v) ammonium sulfate.

For 1 liter of stock solution dissolve 100 g ammonium sulfate in 2% (w/v) phosphoric acid (20 g of 85% phosphoric acid in 980 ml dH_2O), followed by the addition of dye from a solution (1 g dye in 20 ml dH_2O). This stock solution should not be filtered.

Staining solution:

40% (v/v) methanol
10% (v/v) acetic acid
0.1% (w/v) coomassie staining R-250
in ddH_2O

Destaining solution:

40% (v/v) methanol
2% (v/v) acetic acid
in ddH_2O

In gel Digestion:

1 M Dithiothreitol (DTT)

154.3 mg of DTT +1000 µl ddH2O

1 M ammonium bicarbonate (AmBic; NH4HCO3)

79.06 mg of NH_4HCO_3 + 1000 µl ddH2O

50% Acetonitrile (ACN) in ddH2O:

250 µl of ACN + 250 µl of ddH2O

100 mM NH4HCO3:

100 µl of 1 M NH_4HCO_3 in 900 µl of ddH2O

25 mM NH4HCO3

25 µl of 1 M NH_4HCO_3 + 975 µl of ddH2O

10 mM DTT in 100 mM NH4HCO3:

10 µl of 1 M DTT + 890 µl ddH2O+ 100 µl 1 M NH_4HCO_3

55 mM iodoacetamide in 100 mM NH4HCO3:

10 mg of iodoacetamide + 900 µl ddH2O + 100 µl 1 M NH_4HCO_3

1 M Tris-Cl, pH 7.5

121.1 g Tris base in 900 mL of ddH2O. Adjust the pH to the desired value by adding concentrated HCl. Adjust volume to 1000 mL

5% formic acid 57 µl of stock formic acid (88%) + 943 µl of ddH2O

Method:

1) Polyacryalmide gel electrophoresis:

SDS PAGE is the most commonly used low-level protein separation and purification technique in biological research. The reason of using this technique in LC MS is to reduce sample complexity and to purify samples contaminants as detergents, salts, DNA and other non-protein componds.

A number of staining procedures is available for the visualisation of protein bands or spots on polyacrylamide gels. Out of these, Coomassie Brilliant Blue is probably the most commonly used when protein amounts in excess of 100 ng (0.5 to 5 pmole) are available.

Procedure SDS-PAGE

1. Clean glass plates with isopropanol/di H_2O and assemble in the casting stand as described in the manufacturers instructions.
2. The following volumes are sufficient for two 8 X 6 cm minigels (for example Bio-Rad Mini Protean II). These volumes are for gels of 0.75 mm thickness, adjust as necessary for different width gels.

Minigel: 8 X 6 cm

	15%	13%	12,5%	10%	7%
dH_2O (mL)	4.5	3.2	3.95	4.2	5.2
4 X Lower gel buffer (mL)	3	2.5	2.9	2.5	2.5
30% acrylamide (mL)	4.5	4.3	4.73	3.3	2.3
10% APS (µL)	60	30	54.6	30	30
TEMED (µl)	12	9	7.29	9	9
Total (ml)	12	10	~12	10	10

3. Add APS, swirl to mix (without introducing bubbles) then add TEMED.
4. Pour to the desired height. Cover with 3 mm of water saturated butanol. The gel will polymerize within 30 minutes, allow 60 minutes for complete polymerization.

5 Wash the top surface of the gel to remove all of the butanol. Remove all excess dH_2O by blotting with a Kimwipe.

Stacking gel: large or minigel

dH_2O (ml)	3.5
Stacking buffer (ml)	1.25
30% acrylamide (ml)	0.65
10% APS (µl)	50
TEMED (µl)	5
Total (ml)	~5.5

7. Mix the stacking buffer, acrylamide, and dH_2O
9. Add the APS, swirl, then add the TEMED. With a pasteur pipette add the stacking gel to the polymerized running gel.
8. Insert the comb into between the glass plates at an angle to avoid trapping air. The stacking gel will polymerize within 5 minutes, allow 20 minutes for complete polymerization.

10. Remove the comb, rinse the wells with electrode buffer (1x TGS). Wipe excess polyacrylamide from the glass plates.
11. Boil samples (~10 µg) for 1 minute (95°C) in loading buffer at 25 µL (note: urea sample must be dilutet 1:5 with 5xSDS loading buffer), centrifugate 6000g 10min. at room temperature. Load samples and mass reference ladder in the gel pockets. Samples may be stored in sample buffer at -20°C and should be boiled again prior to electrophoresis.
12. Run the gel. For the 10 X 10 cm gel using a Hoefer Minive run the gel at constant voltage (80 V for the stacking gel, 120V for the running gel) for 1-2 gels at each chamber. Approximate running time: 2 hours.

2) *Sensitive Coomassie Blue Staining of polyacrylamide gels:*

Sensitivity with this one-step staining method is increased up to 10 times over conventional Coomassie staining .

1. Gently shake the gel in a sealed container with staining solution for 30 min.
2. Destaining for 80 min. Replace the destaining solution every 20 min.
3. Wash the gel in ddH_2O for 30 min

3) *In-gel Digestion:*

Excision protein bands from gels

a. Transfer the gel into a suitable size tissue culture dish.

b. Excise bands of interest with clean scalpel, cutting as close to the edge of the band as possible. It is important to reduce the volume of background gel.

c. Chop the excised bands into cubes (about 1x1x1 mm). Transfer gel particles into a siliconized tube (0.5 mL Eppendorf).

d. add 40 µl of 50/50 acetonitrile (ACN)/ddH2O and incubate 15 minutes (Repeat this step 2 to 3 times; some strongly stained bands will still be light blue).

e. Discard solution, then add ~100 µl (enough to cover pieces) of 100% acetonitrile. Incubate until gel pieces are white and sticky (approx. 5 min.)

f. Discard solution, then add 40 µl of 100 mM NH_4HCO_3. Incubate for 5 minutes to rehydrate the gel pieces.

g. Add 40 µl of ACN to make 1:1 solution of ACN:100 mM NH_4HCO_3. Incubate 15 minutes.

h. Discard solution, then dry samples in a speedvac until completely dry.

i. Gel pieces can be stored at 4°C or -20°C for overnight.

Reduction and Alkylation

j. Add 100µl of 100 mM AmBic, 10 mM DTT, incubate 45 min at 56°C; cool to RT

k. Replace solution with IAA solution.

l. Incubate at RT in dark 30 min. with occasional vortexing.

m. Remove liquid, wash gel pieces with 200µL 100mM AmBic, 10 min while shaking at RT.

n. Add directly 200µL ACN, 10min., shaker

o. Wash 2x5min. with 200 µL of 50% ACN in AmBic

q. Remove all liquid, dry speedvac for exactly 15 min.

Digestion

a. Add 10-20 µl of the appropriate enzyme solution (see table 1), then incubate for 45 min at 4°C (ice bath). Add more solution if the pieces absorb all of the liquid.

b. Remove excess solution and discard. Add 20-30µl of same enzyme digestion buffer without enzyme (enough to cover gel pieces), then incubate overnight at suitable temperature (see table 1).

Table 1. Enzyme	Digestion Buffer	Digestion temperature
Sequencing grade modified trypsin (12,5ng/µl)	25 mM NH_4HCO_3 10% ACN, 5mM $CaCl_2$, pH 7.8	37°C

Extraction of peptides from gel:

a. add 100 µL 5% FA, vortex 5 min, remove liquid into fresh low binding tube

b. add 100 µL 1% FA, 5% ACN, vortex 45 min, remove liquid into same tube

c. add 100 µL 1% FA, 50% ACN, vortex 45 min, remove liquid into same tube

d. add 100 µL 1% FA, 90% ACN, vortex 45 min, remove liquid into same tube

e. dry down in speedvac

f. Resuspend the sample in 5-10 µl of 0.1% FA, vortex and then proceed to peptide desalting.

3) Peptide desalting with STAGE-Tips:

a. Attach a clean STAGE-Tip to a 100µl Pipette.

b. Wash STAGE-Tips with 100% MetOH (+0,1% FA).

c. Equilibrate STAGE-Tips with 100µl 0,1% FA. Repeat procedure. .

d. Pull softly and repeated times the resuspended sample with the pipette.

e. Wash the Tip again with 100µl 0,1% FA from a fresh Eppi. Repeat procedure.

f. Eluate the desalted peptides from the STAGE-Tips with 100µl 100% MetOH.

g. Pipette the eluted MetOH in a fresh low-binding 500µl Eppi.

h. Dry the solution in a speed vacuum.

i. Completely dry the solution in a speed vacuum. It should take 4-5 hours at medium temperature.

j. Resuspend the sample in 5-10 µl of 0.1% FA, vortex, incubate at least 10 minutes and spin 10' 6000g, and proceed to the mass sp