In-Gel Digestion

- In-Gel Digestion for Coomassie Stained Gels
- Silver Stain In-Gel Digestion

Notes:

- Always wear gloves and change them often
- Use MilliQ water or HPLC Grade water to making all solutions
- 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 possible.
- **Trypsin**: Promega sequencing grade modified trypsin (V511A), 20 ug lyophilized powder, can be stored in solution for several weeks at -20°C.
- Always have a negative control.

Chemical Reagents:

- **Trypsin**: Promega sequencing grade modified trypsin (V511A), 20 ug lyophilized powder, can be stored in solution for several weeks at -20°C.
- **Endoproteinase ASP-N**: SIGMA (P 3303).
- **Glutamic Acid-C:** Princeton Separations Sequencing grade modified Glutamic Acid-C (EN-140).
- **ddH2O**: MilliQ water or HPLC water.

Solutions:

1 M Dithiothreitol (DTT)

154.3 mg of DTT +1000 uL ddH2O

1 M ammonium bicarbonate (NH4HCO3)

79.06 mg of NH4HCO3 + 1000 uL ddH2O

50% Acetonitrile (ACN) in ddH2O:

250 uL of ACN + 250 uL of ddH2O

100 mM NH4HCO3:

100 uL of 1 M NH4HCO3 in 900 uL of ddH2O

10 mM DTT in 100 mM NH4HCO3:

10 uL of 1 M DTT + 890 uL ddH2O+ 100 uL 1 M NH4HCO3

55 mM iodoacetamide in 100 mM NH4HCO3:

10 mg of iodoacetamide + 900 uL ddH2O + 100 uL 1 M NH4HCO3

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

Trypsin digestion solution

25 mM NH4HCO3 + 2.5 mM $CaCl_2$ with 12.5 ng/mL of Trypsin

2.5 uL of 1 M $CaCl_2$ + 25 uL of 1 M NH4HCO3 + 972.5 uL of ddH2O

Take100 uL of above solution add 1.25 uL of 1 mg/mL Trypsin solution (Trypsin usually prepared in 100 mM NH4HCO3)

25 mM NH4HCO3

25 uL of 1 M NH4HCO3+ 975 uL of ddH2O

ASP-N digestion solution

100 mM NH4HCO3 (or 100mM Tris-Cl pH 8.5) with 12.5 ng/mL of ASP-N.

Glutamic Acid-C digestion solution

50 mM NH4HCO3 (or 50mM Tris-HCl pH 8.0) with 12.5 ng/mL of Glutamic Acid-C.

10 mM of DTT in ddH2O

10 uL of 1 M DTT + 990 uL of ddH2O

5% formic acid

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

0.1% Trifluoroacetic (TFA)

1 uL of TFA + 999 uL of ddH2O

In-Gel Digestion for Coomassie Stained Gels

1. Excision protein bands or spot from gels

a. Rinse the gel with 2 aliquots of water, shaking for 10 min each.
b. Transfer the gel into a suitable size tissue culture dish.
c. 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.
d. Cut the same size gel piece from a blank region (i.e. no protein) of the gel for a negative control.
e. Chop the excised bands into cubes (about 1x1x1 mm). Transfer gel particles into a siliconized tube (0.5 mL Eppendorf).

2. Washing (With occasional vortexing, then centrifuge for seconds before removing the liquid)

a. To each of the chopped up bands, add 100 uL of ddH2O and incubate for 15 min.

b. Discard ddH2O, then add 40 uL 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).
c. Discard solution, then add 40 uL of acetonitrile. Incubate until gel pieces are white and sticky (approx. 5 min.)
d. Discard solution, then add 40 uL of 100 mM NH4HCO3. Incubate for 5 minutes to rehydrate the gel pieces.
e. Add 40 uL of ACN to make 1:1 solution of ACN:100 mM NH4HCO3. Incubate 15 minutes.
f. Discard solution, then dry samples in a speedvac until completely dry.
g. Gel pieces can be stored at 4°C or -20°C for overnight.

3. Reduction and Alkylation

a. Remove samples from the speedvac and let it cool.

b. To each tube add 40 uL of 10 mM DTT/100 mM NH4HCO3 **(For cysteine-rich proteins, we suggest to use 50 mM DTT instead)** then incubate in a water bath at 56°C for 45 minutes.

c. Remove samples from bath and let cool.

d. Discard solution and immediately add 40 uL of 55 mM iodoacetamide/100 mM NH4HCO3 **(For cysteine-rich proteins, we suggest to use 200 mM iodoacetamide instead),** then incubate at room temperature for 30 minutes in the dark.

e. At this point, the stain is totally removed and the gel should be clear.

f. Discard solution, then wash with 100 uL of 100 mM NH4HCO3 and incubate for 5 minutes.

g. Discard solution, then add 100 uL 50% ACN in water and incubate for 15 minutes.

h. Discard solution, then add 40 uL of acetonitrile. Incubate until gel pieces are white and sticky. (2 to 3 minutes)

i. Discard solution, then add 40 uL of 100 mM NH4HCO3. Incubate 5 minutes to rehydrate the gel pieces.

j. Add 40 uL of ACN to make 1:1 solution, then incubate for 15 minutes.

k. Discard solution, then dry samples in a speed vacuum until completely dry.

l. The gel can store at 4°C overnight or -20°C until digestion occurs.

4. Digestion

a. Add 10-20 uL (enough to cover pieces) of the appropriate enzyme solution (see table 1, and table 2), then incubate for 45 min at 4°C (ice bath). Add more solution if the pieces absorb all of the liquid.

Table 1.

Protein quantity	>1ug protein	100-1000ng protein	< 100ng protein
Enzyme (ng/ uL)	25	12.5	6.25
Digestion solution without enzyme (uL)	10	10	10

b. Remove excess solution and discard. Add 20-30uL of same enzyme digestion buffer without enzyme (enough to cover gel pieces), then incubate overnight at suitable temperature (see table 2).

Table 2.

Enzyme	Company	Digestion Buffer	Digestion temperature
Sequencing grade modified trypsin	Promega	25 mM NH_4HCO_3 and 2.5mM $CaCl_2$, pH 7.8	37°C
Endoproteinase ASP-N	SIGMA	100 mM NH_4HCO_3 (or 100mM Tris-HCl) pH 8.5	37°C
Sequencing grade modified Glutamic Acid-C	Princeton Separations, Inc.	50 mM NH_4HCO_3 (or 50mM Tris-HCl) pH 8.0	30°C

5. Extraction of peptides from gel

a. The next morning (after about 16-17 hours of digestion), sonicate the tubes for 5 min in cool water. Remove supernatant and save.
b. To the gel in tubes add 20-30 mL of 25 mM NH4HCO3 and incubate 15 minutes. (Occasionally sonicate for 2 to 3 minutes. Add some ice to the sonication water to prevent over heating.)
c. Add 20-30 uL ACN to make a 1:1 solution of NH4HCO3/ACN and incubate for 15 minutes. Remove supernatant and combine wtih the solution saved in step 5a.
d. To the gel add 20-30mL of 5% formic acid, then incubate for 15 minutes.
e. Add the same amount of ACN, then incubate for 15 minutes.
f. Remove supernatant, then combine with solution from step 5a.
g. Repeat steps d) to f) once. Total extraction is about 140-210 uL.
h. To the pooled solution from 5a add 100 mM DTT to give a final concentration of 1 mM DTT. About 1.4-2.1 uL
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 uL of 0.1% FA, vortex and then incubate at least 10 minutes proceed to the mass spectrometric analysis.

Silver Stain In-Gel Digestion

1. **Excision band follow the procedure outlined in coomassie staining**

2. **Decolorization / Destaining (only for silver stained gels)**

    1. Prepare the following two stock solutions just prior to use:
        A) 2 mL of 100 mM sodium thiosulfate: 49.6 mg in 2mL HPLC water.
        B) 2 mL of 30 mM potassium Ferricyanide: 19.75 mg in 2ml HPLC water.
    2. Combine solution A and B in a 1:1 ratio to make 4ml working solution labeled as "reducing solution".
    3. Add 200 uL reducing solution to gel tube, destain until brownish color has disappeared, approx 2 to 3 minutes.
    4. Quickly discard the reducing solution from the tube, add 500 uL of HPLC water to wash. Repeat wash 2 times, with 5 min each.
    5. Dry samples in speed vacuum for approximately 15 minutes (until completely dry).

3. **Reduction and alkylation**
   Follow procedure as outlined for Coomassie stained gel.
4. **Digestion same as Coomassie stain.**
   Follow procedure as outlined for Coomassie stained gel.