In-Gel Digestion for proteomics

*Guidelines for sample preparation*

*(How to protect your samples from contamination with keratin)*

1. Try to avoid any contact of samples and solutions with dust, skin or hair

2. Clean your bench

3. Wear gloves at all times

4. All reagents should be prepared fresh or aliquots could be used if stored at -20°C (the stock solution validity is 6 months if the validity of the reagent itself is not lower)

5. Use ultra-pure water for all solutions (MilliQ water)

*Guidelines for sample submission*

1. Provide 10ul of samples in no recovery vials* or vials with insert for small volumes for LC-MS/MS analysis

*Autosampler vials appropriate for analysis*

Waters Total Recovery (part number: 186000385C.)

![Figure 1: Waters Total Recovery Vial](image)

2. Provide samples in 1.5 ml eppendorf tube for MADI-TOF/TOF analysis.

3. Label your tube with the sample ID.

3. Fill in online sample submission form to provide us with more information about your sample
Solutions of reagents

100% Acetonitrile (CH\textsubscript{3}CN, HPLC or LC-MS grade)

50% Acetonitrile

-Dilute a volume of 100% ACN 1:1 in MilliQ water

100 mM ammonium bicarbonate (NH\textsubscript{4}HCO\textsubscript{3}, MW 79.06)

-0.79 g NH\textsubscript{4}HCO\textsubscript{3} in 100 ml MilliQ water
-Store at -20°C in aliquots of 10ml

50mM ammonium bicarbonate

-Dilute 100 mM NH\textsubscript{4}HCO\textsubscript{3} stock 1:1 in MilliQ water

1M DTT (Dithiothreitol, HSCH\textsubscript{2}(CHOH)\textsubscript{2}CH\textsubscript{2}SH, MW 154.24)

- 0.77 g DTT in 5 ml water MilliQ
- Store at -20°C in aliquots of 500 µl

85mM DTT in 50mM ammonium acetate (To reduce the proteins: \textit{in-gel} reduction is recommended even if the proteins were reduced prior to an electrophoresis run)

110 mM IAA (Iodoacetamide, C\textsubscript{2}H\textsubscript{4}INO, MW184.96)

-Dissolve 56 mg of IAA in 3327 µl of water MilliQ
- Store at -20°C in aliquots of 250 µl

55 mM IAA in 50mM ammonium acetate (To prevent the re-formation of disulphide bridges)

-Dilute 110mM IAA stock 1:1 in 50mM ammonium acetate

20 ng/µl of Trypsin (Other enzymes with the same pH tolerance as trypsin can be substituted without modifying conditions. These enzymes includes Chymotrypsin, Asp-N, Glu-C and Lys-C)

- Add 1 ml of ice-cold 50mM ammonium bicarbonate to 20 µg trypsin vial

IMP: always work with the trypsin in an ice bucket to prevent auto-proteolysis
Procedure

Excision of protein bands from polyacrylamide gels

1. Wash the gel slab with water (2 changes, 10 min each)
2. To excise the bands/spots from the gel use clean nitrile gloves and scalpel
3. Cut as close to the protein band as possible to reduce the amount of background gel
4. Excise a gel piece of roughly the same size from a non-protein containing region of the gel for use as a control
5. Cut the gel pieces into roughly 1mm$^3$ cubes
6. Put the gel pieces in clean 0.5 or 1.5 ml eppendorfs

De-staining gel pieces from *Coomassie stained bands/spot

1. Wash the gel pieces with water (15 min shaking)
2. Add 100 µl of 100mM 100 mM ammonium bicarbonate (10 min shaking)
3. Remove buffer with pipette and add 50 µl of 1:1 50 mM ammonium bicarbonate / ACN to gel pieces (10 min shaking)
4. Repeat step 2 and 3
5. If gel pieces are still blue, repeat steps 2 and 3
6. Remove all liquid and add 100% ACN, enough to cover the gel pieces (30 min at 37°C shaking)
7. After the gel pieces have shrunk (they become white and stick together) remove the acetonitrile

IMPORTANT: Solvent volumes used in the washing steps should roughly equal 5 times the gel volume

De-staining gel pieces from *Silver stained bands/spot

1. Add 200µl of 1:1 potassium ferricyanide/sodium thiosulfate and agitate 20 min in the dark at RT
2. Remove all the liquid

3. Add 200µl of MilliQ water and agitate 20 min at RT

4. Remove all the liquid

5. Repeat this procedure until the bands/spots are transparent

6. Add 30µl of MilliQ water and agitate for 20 min at RT

7. Remove all the liquid

8. Add 30µl of 100% of ACN and agitate for 30 min at 37°C

9. Remove all liquid

**IMPORTANT: Solvent volumes used in the washing steps should roughly equal 5 times the gel volume**

**Reduction and alkylation**

1. Swell gel pieces in 30 µl of 10mM DTT in 100mM NH₄HCO₃

2. Reduce for 45 min at 56°C with agitation

3. Remove excess liquid

4. Add 30 µl of 55mM of iodoacetamide in 100mM NH₄HCO₃

5. Alkylate in the dark for 30 min at room temperature with agitation

6. Remove all the liquid

7. Wash with 100 µl of 100mM NH₄HCO₃ for 5 min at room temperature and agitation

8. Remove all the liquid

9. Wash with 100 µl of 100%ACN for 10 min at room temperature and agitation

10. Remove all the liquid

11. Dry completely in a vacuum centrifuge

**Trypsin digestion**
1. Rehydrate the gel pieces with 30 µl (add enough liquid to cover the gel pieces) of digestion buffer containing 50mM NH₄HCO₃ and 20ng/µl of trypsin, for 30 min and at 4°C

2. After 15 min, add more digestion buffer if the initial volume has been absorbed by the gel pieces

3. Remove excess gel enzyme solution

4. Add sufficient 50mM NH₄HCO₃ buffer to cover the gel pieces.

5. Incubate overnight at 37°C

6. After overnight incubation, recover the digest to a new tube and add formic acid to each sample that final formic acid concentration is 5.0% to stop enzymatic reaction

**Extraction of peptides from the gel** (normally used to extract big peptides that don’t leak from the gel easily)

1. Add 100 µl of 1% formic acid and incubate for 15 min at RT with shaking, then transfer to the vial containing supernatant

2. Add the same volume of 100%ACN and incubate for 15 min at RT with shaking, pipette off and save the supernatant

3. Add 100ul of 1:1 ACN/Water, leave for 5 min, then transfer to the vial containing supernatant.

4. Add 100ul of 1% formic acid in ACN, leave for 5 min, then transfer to the vial containing supernatant

5. Dry extracted peptides

6. **Resolubilize** the sample peptides:

   6.1 For **MALDI-TOF/TOF** analysis re-dissolve in 10-20 ul of 0.1% of formic acid and use Zip Tip to clean up the sample

   6.2 For **LC-MS/MS** analysis re-dissolve in 10-20 ul of 0.1% of formic acid and use Zip Tip to clean up the sample
ZipTip Protocol

_Solutions Required_

**Buffer A**
- 98% Milli-Q water
- 2% ACN
- 0.1% FA*

**Buffer B**
- 80% ACN*
- 20% Milli-Q water
- 0.1% FA
*Use HPLC-grade Acetonitrile and FA, and MilliQ-H20

Matrix CHCA (concentration: 10 mg/mL)
- 10mg CHCA in 1ml of 0.1 % of FA in ACN

_Procedure_

1. Acidify sample (Vol 20-100 μl ) by adding TFA or FA (0.1 % final concentration)

2. ZipTip equilibration
   - Aspirate **Buffer B** (10 μl) into the tip. Dispense into waste. Repeat.
   - Aspirate **Buffer A** (10 μl) into the tip. Dispense into waste. Repeat.

3. Bind and Wash the peptides/proteins
   - Take 10 μl of sample. Aspirate and dispense the sample (repeat 10 x). Dispense.
   - Wash with **Buffer A** (10 μl). Dispense into waste. Repeat 4

4. Elution:
   **LC-MS/MS analysis**
   - Elute with 10 μl with **Buffer B** in new tube.
   - dry in vaccum centrifuge
   - resuspend in 10 μl **Buffer A**.

   **MALDI MS/MS analysis**
   - Pipette 1-2 μl of matrix (CHCA)
   - Spot the sample on MALDI plate. Leave to dry