In-Solution 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

2. Provide samples in 1.5 ml eppendorf tube for MADI-TOF/TOF analysis.
3. Label your tube with the sample ID.
4. Fill in online sample submission form to provide us with more information about your sample
Solutions of reagents

**100% Acetonitrile** (CH₃CN, HPLC or LC-MS grade)

**50% Acetonitrile**

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

**100 mM ammonium bicarbonate** (NH₄HCO₃, MW 79.06)

-0.79 g NH₄HCO₃ in 100 ml MilliQ water

-Store at -20°C in aliquots of 10ml

**50mM ammonium bicarbonate**

-Dilute 100 mM NH₄HCO₃ stock 1:1 in MilliQ water

**1M DTT** (Dithiothreitol, HSCH₂(CHOH)₂CH₂SH, 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: *in-gel* reduction is recommended even if the proteins were reduced prior to an electrophoresis run)

**110 mM IAA** (Iodoacetamide, C₂H₄INO, MW 184.96)

-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**

**Reduction and alkylation**

1. Typically samples should have a protein concentration of \([\text{Protein}]=1\text{mg/ml}\)
2. Take an aliquot of **15µl** (15µg of protein)
3. Reduce with **2µl of 85mM DTT** in 50mM ammonium bicarbonate (Ambic)10mM (final concentration) DTT for **40min at 56°C**
4. Alkylate with **7µl of 55mM IAA** in 50mM Ambic 20mM (final concentration) IAA for **30min in the dark at RT**
5. Reduce the sample again with **3µl of 85mM DTT** in 50mM Ambic for **10min in the dark at RT**, in order to eliminate excess IAA
6. Precipitate the sample with 6 volumes of ice-cold acetone or using the 2-D clean-up Kit (Code number: 80-6484-51, from GE Healthcare)
   a. Add **126µl of ice-cold acetone**, vortex and incubate overnight at **-20°C**
   b. **Centrifuge at 15,000 x g for 10 min**, in a previous cooled rotor
   c. Remove the supernatant and allow the pellet to dry for no more than **5min**
7. Resolubilize the pellet in **27µl of 50mM Ambic**

**Trypsin digestion**

1. Perform **in-solution trypsin digestion**
   a. Quant Prot=**15µg**
      
      \[
      \frac{1}{20} \times 15 = 0.75 \text{ µg} \leftarrow 750\text{ng}
      \]
      
      Quant needed Trypsin=750ng
      
      Hence, add **3µl of 1µg/µl Trypsin in 50mM ambic** and incubate overnight at **37°C**

2. Perform a **second in-solution trypsin digestion in 80% ACN**
   b. Quant Prot=**15µg**
      
      \[
      \frac{1}{100} \times 15 = 0.15 \text{ µg} \leftarrow 150\text{ng}
      \]
      
      Quant needed Trypsin=150ng
Hence, from the [Trypsin] take 1.65µl and add 1.35µl of 50mM ambic in order to achieve the 550ng/µl of trypsin. In order to achieve the 80% ACN environment (it represents 4/5 of the final sample volume), add 132µl of 100% ACN (LC-MS) and incubate at 37°C for 3 hours.

8. Stop the trypsin digestion by adding up to 5% FA
9. Dry the digested sample to completion using the SpeedVac
10. Resolubilize the sample peptides:

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

   10.2 For LC-MS/MS analysis re-dissolve in 10ul 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% CAN*  
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