In-solution Chymotrypsin Enzymatic Digestion

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)

6. This protocol is optimized for 15µg of protein.

Guidelines for sample submission

1. Provide 10ul of samples in total 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 MALDI-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_3CN, HPLC or LC-MS grade)

50% Acetonitrile

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

100 mM ammonium bicarbonate (NH_4HCO_3, MW 79.06)

-0.79 g NH_4HCO_3 in 100 ml MilliQ water

-Store at -20°C in aliquots of 10ml

50mM acetic acid

-Prepare 50 mM acetic acid solution by adding 287.36 µL of glacial acetic acid to 100 mL of ultrapure water.

50mM ammonium bicarbonate

-Dilute 100 mM NH_4HCO_3 stock solution 1:1 using MilliQ water

1M DTT (Dithiothreitol, $HSCH_2(CHOH)_2CH_2SH$, MW 154.24)

- 0.77 g DTT in 5 ml water MilliQ

- Store at -20°C in aliquots of 500 µl

85mM DTT (To reduce the proteins: *in-gel* reduction is recommended even if the proteins were reduced prior to an electrophoresis run)

-Dilute 1M IAA stock solution using 50mM ammonium bicarbonate

110 mM IAA (Iodoacetamide, C_2H_4INO, MW184.96)

-Dissolve 68 mg of IAA in 3327 µl of water MilliQ

- Store at -20°C in aliquots of 250 µl

55 mM IAA (To prevent the re-formation of disulphide bridges)

-Dilute 110mM IAA stock 1:1 using 50mM ammonium bicarbonate

25 µg/µl of Chymotrypsin Endoproteinase MS Grade, ThermoScientific

(Other enzymes with the same pH tolerance as trypsin can be substituted without modifying conditions. These enzymes include Trypsin, Asp-N, Glu-C and Lys-C)

- Reconstitute lyophilized chymotrypsin using 50mM acetic acid to 1mg/mL (i.e., add 25µL of 50mM acetic acid to 25µg of lyophilized chymotrypsin).

Dilute 1mg/mL chymotrypsin stock solution to 0.01mg/mL using 50mM ammonium bicarbonate.

Always work with the chymotrypsin in an ice bucket to prevent auto-proteolysis

Procedure

Reduction and alkylation

1. Typically, samples should have a protein concentration of [Protein]=1mg/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 56ºC**
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 **162µl of ice-cold acetone, vortex and incubate overnight at -20ºC**
    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**
    d. Resolubilize the pellet in **27µl of 50mM Ambic**

Note: Point 6, just need to be performed if the proteins are from cell or tissue extraction.

Chymotrypsin digestion

1. Perform **in-solution chymotrypsin digestion**

Add chymotrypsin in a **1:20 to 1:50** (W/W) ratio regarding the total protein content of your sample. Mix carefully and carry out the digestion **overnight at 37 °C**.

2. Stop the chymotrypsin digestion by **adding up to 5% FA**

3. **Dry the digested sample to completion** using vacuum centrifuge

4. **Resolubilize** the sample peptides:
   4.1 For **MALDI-TOF/TOF** analysis re-dissolve in 10-20 µl of 0.1% **Trifluoroacetic Acid (TFA)** and use Zip Tip to clean up the sample (please see separate protocol)
   4.2 For **LC-MS/MS** analysis re-dissolve in 10-20 µl of 0.1% of **Formic Acid (FA)** and use Zip Tip to clean up the sample (please see separate protocol)