In-gel Digestion protocol for Mass Spec Analysis

Wash two sets of 500 µl snap cap micro centrifuge tubes prior to use with 2X 500 µl Methanol and 500 µl Milli-Q water rinse. Cut the protein band as small as possible using razor and take the band into the micro centrifuge tube.

A. RINSING

1) If gel slices are still wet with destain /etc. Rinse with 25 mM ammonium bicarbonate (ABC).

B. WASHING AND DEHYDRATION

1) Dehydrate gel with 50 µl of solution [A] ([A] = 2:1 mixture of Acetonitrile (ACN): 50 mM ABC) for 5 min. ABC = Ammonium Bicarbonate
2) Remove supernatant and add 50 µL of 25 mM ABC for 2 min.
3) Dehydrate gel slice with 50 µL of solution [A] for 5 min.
4) Remove supernatant and add 50 µl of 25 mM ABC for 2 min.
5) Dehydrate gel slice with 50 µL of solution [A] for 5min.

C. REDUCTION/ALKYLATION

1) After step B (Two or three cycles of dehydration/rehydration) rehydrate gel slice in 20-50 µL of 10-20 mM Dithiothreitol (DTT)
2) Place gel slice at 60 ^{0}C for 1 hour
3) Remove DTT, and rinse gel slice with 50 µl 25 mM ABC
4) Add 20-50 µL of 50-100 mM Iodoacetamide (IAA).
5) Incubate gel slice at RT in the dark for 20 min.
6) Remove IAA, and rinse gel slice with 50 µL ABC.

D. DEHYDRATION (Continued)

1) Dehydrate gel slice with 50 µL solution A for 5 min.
2) Remove supernatant and add 50 µl of 25 mM ABC for 2 min.
3) Optional (repeat dehydration and rehydration 1 more time)
4) Remove final supernatant and dry spots in a speed vac (ca. 5-15min).

E. DIGESTION

1) Prepare trypsin stock by adding 1 mL of ice-cold 1 mM HCl to a standard 20 µg trypsin vial (Sigma/Promega MS grade trypsin). Store on ice. Make aliquots of 5 µL. For each gel band, add 95 µL of 25 mM ABC to the stock (concentration is 100 ng)
2) Rehydrate the gel slice with trypsin, (100 µL) at 0 ^{0}C on ice.
3) Incubate gel slice on ice 20-30 min until trypsin is absorbed.
4) After gel slice is completely rehydrated, add just enough 25 mm ABC to cover gel slice in tube
5) Incubate at 37 °C for at least 16 hours, typically overnight. (24 hrs recommended)
6) Periodically check gel slice to make sure it is still just covered with 25 mM ABC, add more if needed.

F. EXTRACTION

1) Remove supernatant, and vortex gel slice with 2x extractions of 100 µl extraction solution (50:50 ACN:H_2O, 0.1% Trifluoroacetic Acid), add these extractions to supernatant. Collect supernatant of step 1, 2 and 3 and reduce the volume in speed vac. (Total volume approximately 500µl)
2) Speed vac the mixture to complete dryness and resuspend in 10 µL of 0.1% TFA in water. This solution is used for mass spec after ZIP Tip.

REAGENTS PREPARATION:

1) Preparation of 25 mM ABC
   Add 0.1977g NH_4HCO_3 to 100 ml Milli-Q water and mix until dissolved.
2) Preparation of 50 mM ABC
   Add 0.395g NH_4HCO_3 to 100 ml Milli-Q water and mix until dissolved.
3) Preparation of 10 mM DTT
   Add 1.5 mg DTT to a 1.5 ml Eppendorf tube.
   Add 1 ml of 50 mM ammonium bicarbonate and mix until dissolved.
4) Preparation of 50 mM Iodoacetamide
   Add 10 mg IAA to a 1.5 ml Eppendorf tube.
   Add 1 ml of 50 mM ammonium bicarbonate.
5) Preparation of 0.1% TFA
   Add 100 µl of TFA to 99.9 ml Milli-Q water. Mix it.

CHEMICALS AND CONSUMABLES:

1) Ammonium Bicarbonate: Sigma Cat # A6141
2) Trifluoroacetic acid: Sigma Cat # 299537-100G
3) Acetonitrile: Sigma Cat # 34967
4) Trypsin: Sigma Cat # T6567
5) Zip tips: Sigma Cat # Z720070-96EA
6) IAA: Sigma Cat # I6125-25G
7) DTT: Sigma Cat # D9779-5G
8) Promega Trypsin: Promega Cat # V5111

Zip Tip C18 Protocol for MS Analysis:

I. Materials

• **Wetting solution** (100% HPLC-grade Acetonitrile)
• **Washing solution** (0.1% TFA in Milli Q water)
• **Sample preparation**: Adjust sample to 0.1% TFA
• **Elution solution** (50% Acetonitrile in 0.1% TFA):
300 µL HPLC-grade Acetonitrile, 240 µL Milli Q water, 60 µL 1% TFA in Milli Q water

II. Procedure

Note: Resin bed provides back pressure, so set pipette to 10 µL, depress plunger to dead stop and slowly release or dispense plunger throughout operation.

1. Equilibrate:

Aspirate 10 µL wetting solution into tip and dispense to waste. Repeat. Aspirate washing solution into tip and dispense to waste. Repeat.

2. Bind & wash:

Bind peptides to Zip Tip pipette tip by aspirating and dispensing 8-10 cycles (simple mixtures), up to 15 cycles (complex). Aspirate **washing solution** and dispense to waste. Repeat wash 5 times.
Note: A 5% methanol in 0.1% TFA/water wash can improve desalting efficiency.

3. Elute:

Dispense 10µL of **elution solution** into clean 0.5 mL Eppendorf micro centrifuge tube using a standard pipette tip. Aspirate and dispense eluent through Zip Tip at least 8-10 times without introducing air.

Reference:

Shevchenko, A.; Tomas, H.; Havlis, J.; Oslen, J. V.; Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. ***Nature Protocols*** (2006) VoL-6, 2856-2860.