IN-GEL DIGESTION AND EXTRACTION PROTOCOL

Solutions Required:

- 1% Acetic Acid
- 100% Acetonitrile (ACN), HPLC Grade
- 10mM Dithiothreitol (DTT)
- 55mM iodoacetamide (IAA), prepared immediately prior to use, protect from light
- 25mM NH_4HCO_3
- Gel Wash Solution
    - 25mM NH_4HCO_3
    - 50% ACN
- Enzyme of interest

Protocol:

Part A: Excision & De-stain of gel bands

A1. Excise protein bands of interest from de-stained gel with sharp scalpel. Ensure to cut as close to the band of interest on all 4 sides to reduce the amount of gel. Optimal results are achieved when minimal gel is present. Place one single band in a microfuge tube for processing. *Do not place more than one band in a tube*.

A2. De-stain gel band further in microfuge with vigorous shaking using appropriate de-stain solution for 30 minutes.

A3. Remove de-stain solution by gentle aspiration.

A4. If gel band is still blue, replace solution with fresh de-stain solution and repeat steps A2 & A3 until stain is no longer observed.

A5. Cover gel band with 1% acetic acid. At this point, bands in acetic acid may be stored indefinitely at 4°C or they may be processed immediately. Do not freeze gel bands, as this will reduce yield.

Part B: Reducing Disulfide Bonds & Alkylation of free cysteine residues

B1. Dehydrate gel pieces with 500µL of 100% ACN for 10 minutes

B2. Remove acetonitrile and dry gel pieces in vacuum centrifuge for 10 minutes.
B3. Add 100μL of 10mM DTT to the gel piece, and incubate for 45 minutes at 55°C.
Dithiothreitol (DTT) is a reducing agent that converts the cysteine's disulfide bond into free sulfhydryl groups.
B4. Remove DTT solution by gentle aspiration and add 100μL of freshly prepared 55mM iodoacetamide (IAA), and incubate for 20 minutes at room temperature in the dark.
Iodoacetamide (IAA) is an alkylating reagent that reacts with the free sulfhydryl groups of cysteine residues. This reaction forms S-carboxyamidomethyl-cysteine, which cannot be re-oxidized to form disulfide bonds. This step is important to allow the digesting enzyme maximum access to cleavage sites within the protein. If this step is not done, peptides containing cysteine residues will not be identified in the same capacity as if this step is done.
B5. Remove IAA solution by gentle aspiration
B6. Add 500μL of Gel Wash Solution, and incubate at room temperature with gentle shaking for 15 minutes.
B7. Repeat step B6 twice.
B8. Dehydrate gel pieces with 500μL of 100% ACN for 15 minutes.
B9. Remove acetonitrile and dry gel pieces in vacuum centrifuge for 10 minutes.

Part C: Enzymatic Digestion

C1. Dilute enzyme stock solution with 25mM ammonium bicarbonate to obtain a 10-20 ng/μL working solution.
C2. Add sufficient amount of the gel enzyme to cover gel pieces (typically 20-30μL), and incubate on ice for 1 hour.
C3. Add sufficient amount of 25mM ammonium bicarbonate to cover the gel pieces.
The enzyme diffuses into the gel that contains the reduced and alkylated protein and acts to cleave the protein into peptides in the gel. These peptides then get extracted from the gel in the following steps.
C4. Incubate at 37°C overnight.

Part D: Peptide Extraction

From this point forward, all supernatant collected should be transferred to a new, clean microfuge tube. Supernatant from each step can be combined with the previous step's supernatant for each individual sample.
D1. Remove any supernatant (which will contain peptides) and transfer to a new clean tube.
D2. Add 50μL of 5% (v/v) formic acid, and allow gel band to incubate for 15 minutes at room temperature
D3. Remove formic acid and combine with previous supernatant.
D4. Add 50μL of 100% Acetonitrile, and allow gel band to incubate for 15 minutes at room temperature
D5. Remove formic acid and combine with previous supernatant.
D6. Repeat Steps D2-D5.
D7. Dry combined supernatants using a vacuum centrifuge.