In-Gel Tryptic Digestion Protocol

General Reagents

- **100 mM Ammonium Bicarbonate**: Dissolve .79g in 100mL MilliQ water. Place in a tightly capped bottle. **Replace after 1 month**.
- **Acetonitrile**
- **45 mM Dithiothreitol** (DTT 154.2g/mol): Make solution in a 1.5mL tube by weighing less than 10 mg DTT and adding appropriate amount of MilliQ water. **Make fresh, keep refrigerated**
  - **Example:** 7.6mg of DTT with 1080µL water.
- **100 mM Iodoacetamide** (IA 185g/mol): Make solution in a 1.5mL tube by weighing the appropriate amount of IA and adding appropriate amount of MilliQ water. **Make fresh, keep out of light**
  - **Example:** 11.2mg IA with 680µL water.
- **Triflouroacetic Acid:** In a fume hood measure 1mL TFA. Mix 50:50 with MilliQ water for a 50% stock solution; then dilute 1:5 in milliQ water to make a 10% working stock solution. **All solutions can be stored at -20°C**.
- **Modified Trypsin:** (Promega cat #V528A; Trypsin Gold Mass Spectrometry Grade; 100µg/vial) Carefully dissolve one pellet in the vial with 1000µL of 50 mM ammonium bicarbonate to make a working 0.1mg/mL stock solution. **Aliquot into 0.5mL tubes, store in -20°C. Limit freeze-thaw cycles to 5.**
- **50 mM Ammonium Bicarbonate, 50% Acetonitrile:** Mix equal volumes 100Mm ammonium bicarbonate and 100% Acetonitrile. **Best if made fresh.**
- **25 mM Ammonium Bicarbonate:** Mix 250µL 100mM ammonium bicarbonate with 750µL MilliQ water. **Best if made fresh.**
- **0.1% Triflouroacetic Acid (TFA):** Dilute 10µL 10% TFA into 990 µL MilliQ water. **Best if made fresh.**
- **60% Acetonitrile, 0.1% TFA:** Mix 600µL acetonitrile, 10µL 10%TFA, and 390µL MilliQ water. **Best if made fresh.**

Silver-Stained Specific Reagents

- **Potassium ferricyanide/ Sodium thiosulfate:** Dissolve 50mg potassium ferricyanide and 80mg sodium thiosulfate in 5mL MilliQ water, in a clean 15mL tube. **Make fresh, use within 30 minutes**

Peptide Clean-Up and Concentration Materials

- 10µL pipettor (or one that will hold 10µL tips)
- ZipTipC18 pipet tips (Millipore cat # ZTC18S096)

Procedure

Pipet tips do not need to be changed between samples for steps 1-6. There is minimal risk of cross contamination of samples until trypsin protease has entered the gel slices.

Shaking/ sonication not necessary

1. **Band Excision**:
    a. For wet gels, insert the corner edge of a straight razor blade, or the tip of a dissecting blade, at the top corner of the band and chop down along the length of the band (do not slice razor through gel). It is best to minimize excess gel and better to waste a bit of protein if necessary-helps reduce background and increase overall protein concentration. Chop sides of band.
    b. Once band is removed cut band into **1mm** cubes and place into a 0.5mL tube.
    c. For gels archived in acetate sheets, slice band using the corner edge of a straight razor and place into a 0.5mL tube.
2. **Equilibration**
    a. Use 100µL of 100mM ammonium bicarbonate for 15 minutes
    b. For gels archived in acetate sheets, removes acetate pieces using forceps, remove re-swelled gel slice, cut into 1mm cubes and place back into tube.
    c. Discard wash
3. **Silver Removal** This step is for silver stained gels only. A separate step to de-stain coomassie blue-stained gels is not needed. Sufficient de-staining is accomplished in steps 4-5.
    a. Add 100µL of the potassium ferricyanide/ sodium thiosulfate solution to each tube containing silver-stained gel slices.
    b. Let sit for a minimum of 5 minutes,
    c. Discard solution and replace with 250µL of 100mM ammonium bicarbonate.
    d. Let sit for 10 minutes.
    e. Remove and repeat ammonium bicarbonate wash until the yellow coloration is gone from the gel slices.
    f. Discard last wash.
4. **Reduction/ Alkylation** This step is not needed for gels that come from a 2D protocol; which already includes reduction and alkylation.
    a. Add 150µL 100mM ammonium bicarbonate to each sample
    b. Add 10µL 45mM DTT
    c. Incubate at 50°C for 15 minutes
    d. Add 10µL 100mM IA to the same tube
    e. Place in the dark at room temperature for 15 minutes

    This results in a carbamidomethylation of the cystein residues with a net addition of 57 Daltons to each cystein residue.

5. **Equilibration/Dehydration**
    a. Remove liquid and replace with 100µL 50% acetonitrile, 50mM ammonium bicarbonate
    b. Let sit for 15 minutes
    c. Repeat to remove any residual coomassie stain, if needed
    d. Remove liquid and replace with 50-100µL 100% acetonitrile
    e. Let sit for 10 minutes or until all gel slices are white
    f. Remove liquid and desiccate in a vacuum centrifuge for 5 minutes
    g. Dry gels can be stored at -20°C for months
6. **Digest**
    a. Dilute 0.1mg/mL stock trypsin 1:10 into 25mM ammonium bicarbonate
    b. Add 10-15µL of diluted trypsin into each tube-Use only enough to cover the gel slices. It is better to have all the trypsin enter the gel than to have excess remaining.
    c. Incubate for 2-24 hours at 37°C
7. **Overlay**—*Starting with this step, use a fresh pipet tip for each sample*
    After trypsin has entered the gel add 25mM ammonium bicarbonate, to each tube, in 5µL intervals. **Do not add excess**
8. **Peptide Extraction**
    a. Remove the supernatant to a new labeled 0.5mL tube. Supernatant may contain peptides that have diffused out of the gel slices.
    b. Extract peptides by adding 15-25µL of 60% acetonitrile, 0.1%TFA to each tube containing gel slices
    c. Let sit for 15 minutes
    d. Remove extract and combine with the supernatant in the new tube.
    e. Repeat extraction, let sit for 15 minutes
    f. Remove extract and thoroughly mix with previous extract by pipetting up and down a few times
    g. Dry extract/supernatant, in a vacuum centrifuge, to dryness but not too long after dryness. *Times can vary from 30-60 minutes depending on the centrifuge used. Check samples as necessary.*
9. **Reconstitution and Mass Analysis**
    a. For samples analyzed by ***LC/MS*** reconstitute peptides in 10µL 0.1% FA.
    b. For samples analyzed by ***MALDI-TOF*** reconstitute peptides in 20µL 0.1% TFA, 50% acetonitrile
10. **Peptide Clean-Up** *For less abundant samples*
    Use a ZipTipC18 pipet tip for this process
    a. Equilibrate tip in 10µL 60% acetonitrile, 0.1% TFA, twice
    b. Wash in 10µL 0.1% TFA, twice
    c. Load up to 20µL of sample with repetitive pipetting (about 5 times)
    d. Wash in 10µL 0.1%TFA, twice
    e. Elute into 5µL 60% acetonitrile, 0.1% TFA with repetitive pipetting (5µL placed into fresh tube prior to elution)

Tech Notes:

- Silver stained gels do not produce the best results.