Protocol_In-solution digest

Step	Purpose
Denaturation	Denaturation buffer solubilizes and starts to unfold proteins for efficient digestion
Reduction with DTT	Dithiothreitol (DTT) is a reducing agent that converts the cysteine's disulfide bond into free sulfhydryl groups
Alkylation with IAA	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
Precipitate	Removes contaminants (e.g. SDS)
Enzymatic Digestion	The enzyme acts to cleave the protein into peptides
C18 cleanup	Removes salts (e.g. urea) and concentrates your sample

Reagents:

- Use only HPLC grade reagents, including water to prepare reagents. Wear gloves and work on a clean surface to avoid contamination with non-target proteins (e.g. keratin)
- Denaturation buffer: 6M urea/2M thiourea
    - To make 10mL: dissolve in ~4mL HPLC grade water, 3.6g urea, 1.52g thiourea, 23.83mg HEPES. Mix vigorously (may need prolonged mixing my vortex). pH with NaOH. Add HPLC grade water to a final volume of 10mL. Centrifuge 5000g x 10min to remove insoluble particle and store in small aliquots at -80. Do not refreeze once thawed
- 50mM ammonium bicarbonate
- Reducing solution: 100mM dithiothritol (DTT) in 50mM ammonium bicarbonate
    - prepare fresh before digestion
- Alkylating solution: 200mM iodoacetamide (IAA) in in 50mM ammonium bicarbonate
    - prepare fresh before digestion
    - protect from light
- 100% Acetone, cooled to -20C
- Enzymes: 20ug of trypsin is reconstituted in 40ul of ammonium bicarbonate. Concentration of stock= 0.5ug/ul

Denaturation	1. Resolubilize proteins to standard concentration in denaturation buffer (e.g. 1mg/mL). For general proteomics, 50 micrograms of protein (total) is sufficient
Reduction	1. Add 1ul of 100mM DTT for every 10ul of sample. Final concentration is 10mM 2. Incubate 30min at room temperature
Alkylation	1. Add 1ul of 200mM IAA for every 10ul of sample. Final concentration is 20mM 2. Incubate 30min at room temperature in the dark
Precipitate	1. Cool required volume of acetone to -20°C 2. Transfer protein sample to acetone-compatible tube and add minimum 6X volume cold acetone 3. Vortex and incubate at -80°C for 60 min 4. Centrifuge @12,000*g* for 10 min, 4C 5. Decent and properly dispose of supernatant, keeping in mind to not disturb the protein pellet. Allow pellet to air dry for 10-30 min in an uncapped tube. Do not over-dry as it may not dissolve properly, but do ensure that there is not acetone remaining
Enzymatic Digestion	1. Resuspend the sample in 50mM ammonium bicarbonate, e.g. 25-50ul 2. Add enzyme at a 1:50-1:100 ratio to your starting protein concentration. E.g. if you started with 50ug of protein, add 1-2ul of the stock 3. Incubate overnight (16-20hr) at 37C 4. At end of digestion, dry samples by vacuum centrifugation
C18 cleanup	1. Use ZipTips, STAGE tips, or C18 columns to clean and concentration your sample. 2. Samples should be submitted to the MSF as dried peptides with the concentration noted on the sample and submission form