Protocol_In-Gel Digest

Overview:

Step	Purpose
Gel electrophoresis	Sample buffer denatures (SDS) and reduces (beta-merc) the proteins, which are then separated based on molecular weight via gel electrophoresis
Reduction with DTT	Dithiothreitol (DTT) is a reducing agent that converts the cysteine's disulfide bond into free sulfhydryl groups. At this point in the protocol, proteins may be fully or partially reduced from 2-mercaptoethnaol in PAGE sample buffer, so a lower concentration of DTT can be used (vs. in-solution)
Alkylation iwth 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
wash and dehydrate	Prepares gel for digestion reaction
Enzymatic Digestion	The enzyme diffuses into the gel that contains the reduced and alkylated protein and acts to cleave the protein into peptides in the gel
Peptide Extraction	Peptides are extracted into the supernatant and collected for LC-MS

Pros:

- reduces sample complexity
- removes some types of contaminants/interfering high-abundance proteins

Cons:

- time
- extraction/recovery of peptides is lower than in-solution digestion
- Other resources: https://proteomicsresource.washington.edu/protocols03/ingeldigestion.php

Reagents:

- ddH20
- 100% ACN

- 25mM NH_4HCO_3 in 50% ACN
- 50mM NH_4HCO_3
    - 200mg/50mL
- 10mM DTT
    - 1.54mg in 1.0mL of 50mM NH_4HCO_3
- 55mM IAA
    - 10mg in 1.0mL 50mM NH_4HCO_3
- Trypsin
    - stock=20ug vial is resuspended in 40ul
    - working solution= add 8ul of stock to 136ul of 50mM ABC
- Extraction solution: 5% formic acid (FA) in 50% ACN
    - 9.5mL water + 9.5ml ACN + 1mL FA

For silver stained gels only:

- Solution A
    - 20mM potassium ferricyanide in H20
    - 9.878mg in 1.0mL H20
    - single use only, store at 4C, protect from light
- Solution B
    - 100mM sodium thiosulfate in H20
    - 24.82mg in 1.0mL H20
    - single use only, store at 4C
- Solution C
    - 1:1 of solution A:solution B
- Solution D
    - ddH20

From intact gel: excise the bands	1. Take care to avoid keratin contamination by wear gloves, using clean tools, dishes, and reagents. Before starting, clean surfaces with ethanol or IPA 2. Rinse gel with milli-Q on the shaker (3-5min) 3. Excise the bands of interest with a clean scalpel. Minimize gel volume by cutting as close to band as possible 4. Excise a slice of gel from a non-protein containing region for a negative control 5. Slice bands into smaller pieces (~1mm2) and place in a microtube

From gel bands: destain the bands, Coomassie Blue:	1. If gel pieces have been shipped or have been in storage, rehydrate with 100ul of 50mM ABC. Vortex periodically for 10min, centrifuge briefly to collect liquid, then discard supernatant 2. Add 100ul (or enough to cover gel) of 25mM ABC (NH_4HCO_3) in 50% ACN and vortex periodically for 10min 3. centrifuge briefly to collect liquid 4. discard supernatant 5. repeat 1-2x 6. gels should be transparent before continuing
From gel bands: detain the bands, Silver stain:	1. Add 200ul of solution C to the gel slices and incubate in the dark for 20min 2. remove the supernatant and add 100ul of H2O for 15min, vortex periodically 3. remove the supernatant and repeat until gel pieces are colourless and transparent
Dry with ACN	1. Add 100ul of 100% ACN to gel piece and incubate for 5min with occasional mixing 2. centrifuge briefly and discard supernatant 3. repeat until gel pieces are white and shrunken
Reduce	1. Add 100ul 10mM DTT 2. incubate 30min at 50C 3. centrifuge briefly and discard supernatant
Dry with ACN	1. Add 100ul of 100% ACN to gel piece and incubate for 5min with occasional mixing 2. centrifuge briefly and discard supernatant
Alkylate	1. Add 100ul 55mM IAA 2. incubate 30min in the dark at RT 3. centrifuge briefly and discard supernatant
Wash	1. Add 200ul 50mM ABC 2. incubate 15min with occasional mixing 3. centrifuge briefly and discard supernatant

Dry with ACN	1. Add 100ul of 100% ACN to gel piece and incubate for 5min with occasional mixing 2. centrifuge briefly and discard supernatant
Dry with Speed Vac	1. Dry in speed vac ~20min
Digest	1. Add 10ul of working solution 2. incubate for 1hr at RT 3. Add 50ul 50mM ABC 4. incubate overnight at 37C
Stop digestion and extract peptides	1. Vortex, and then centrifuge briefly to collect 2. Add 50ul H20, vortex 2min, then centrifuge briefly to collect 3. Sonicate 10min in bath sonicator 4. Vortex briefly then centrifuge 30 seconds 5. Label a new tubes per sample: S=supernatant 6. Carefully transfer the supernatant to tube “S” 7. Add 75ul of 5% FA in 50% ACN to the original tube containing the gel piece. Vortex 2min, then centrifuge briefly to collect 8. Sonicate 5min in bath sonicator 9. Carefully transfer the supernatant to tube “S” (i.e. combined with supernatant from step 6) 10. Add 75ul of 5% FA in 50% ACN to the original tube containing the gel piece. Vortex 2min, then centrifuge briefly to collect. Sonication is NOT completed at this step 11. Carefully transfer the supernatant to tube “S” (i.e. combined with supernatant from step 6 and 9) 12. Dry the supernatant using the vacuum centrifuge
Prepare for MS	1. Peptides can be resuspended in 0.1% FA (formic acid) in water