In Gel fractionation and digest: sample prep for MS sequencing

Reagents:

100mM DTT (FW=154.25)
125mM iodoacetamide (FW=184.96) in H2O (make fresh)
SDS page gel
1ug/ul sequencing grade tripsin gold Promega in 50 mM acetic acid
Axogen maximum recovery (VWR# PCR-05-L-C) 0.6 mL eppendorf tubes
25 mM NH_4HCO_3 (100 mg/50 ml)
25 mM NH_4HCO_3 in 50% acetyl nitrile (ACN)
50% ACN/5% formic acid

Homogenize sample and measure total protein content in preparation for this protocol.

Gel Fractionation

0. Samples are ideal for MS with 1 mm gel with 40 uL well volume
1. Take 250 ug of total protien for reduction and alkylation, store remainder @ -80 for use later, concentrate protein sample down to a volume of approx... 12 ul.
2. add 15 uL of 4X sample loading buffer
3. add 3.5 uL DTT (11.4 mM final) and mix well
4. denature at 95 °C for 5 min
5. cool sample to rm temp.
6. add 9.5 uL iodoacetamide (30 mM final). Mix well
7. incubate 1 Hr in the dark at room temp
8. load entire sample onto gel and run long enough to ensure good MW separation
9. stain gel 1-2 Hr in Biosafe coomassie G-250
10. destain in ddH2O, 4X 30 min - use kimwipes to enhance destain
11. Scan Gel and measure densitometry of the individual MW regions.
12. Gel can be stored overnight at 4°C if necessary.

LCMS sample preparation

1. Wipe down Biosafety cabinet with EtOH, **use biosafety cabinet for steps B-J**
2. cut gel pieces according to MW map, and place pieces into a in low bind tube. Mince to ~1mm^2 pieces avoid making pieces to small. (Samples can be stored at -80° for extended period of time at this stage.)
3. Add ~100µL (or enough to cover) of 25mM NH_4HCO_3/50% ACN and vortex for 10 min.
4. Using pipet tip, extract the supernatant and discard supernatant.
5. Repeat steps D and E once or twice, dye should wash away.
6. Speed Vac the gel pieces to complete dryness (~ 20 min).
7. Dilute trypsin stock to 12.5 ng/µL trypsin in 25mM NH_4HCO_3
8. Add trypsin solution to just barely cover the gel pieces. Estimate the gel volume and add about 3x volume of trypsin solution. This volume will vary from sample to sample, but on average ~20-50 µL is sufficient.

9. Rehydrate the gel pieces for 10 min. Spin down. Add 25mM NH_4HCO_3 as needed to cover the gel pieces (~100 uL).

<u>Centrifuge any droplets to the bottom of the tube and incubate at 37°C overnight</u>.

Next Day

1. To the gel pieces, add 50-100 µL (enough to cover) of 50% ACN/5% formic acid, mix gel pieces vortexer ~20min at slow speed, sonicate in a low power bath sonicator for 5 min. Remove supernatant with a gel loading pipet tip and save supernatant in clean low bind tube. Repeat 1 time, pool supernatant.
2. Spin down the pooled supernatant.
3. Pipet the top ¾ of the supernatant volume into a clean mass spec vial.
4. Speed Vac to dryness, then resuspend in 15 ul of 0.1% formic acid 3% ACN solution
5. Store tubes 4 °C up to 10 days, at -80C for extended periods of time.