Chemicals:

- 200 mM sodium carbonate pH 11
- Methanol
- Chloroform
- RapiGest (Waters Corporation)
- 50 mM ammonium bicarbonate pH 7.8
- 500 mM DTT
- 500 mM IAA (light sensitive)
- 100 mM calcium chloride
- Trypsin – modified sequencing grade (Roche)
- 5 M HCl
- Buffer A (95% water, 5% acetonitrile, 0.1% formic acid)

High pH Carbonate Wash

- Add 200 mM sodium carbonate pH 11 to your pellet. I add 100 µl for a small pellet…basically you want to completely cover the pellet.
- Vortex the pellet to death for about 5 minutes.
- Spin sample at 14K, 4 °C, 10 minutes.
- Remove supernatant (you can keep it and digest it if you think there might be something of interest here. Otherwise throw it out).
- Take your pellet and methanol-chloroform precipitate (see below).

Methanol-Chloroform Precipitation

- For standard samples you want to add 4 parts methanol, 3 parts protein sample resuspended in water and 1 part chloroform.
- If sample has a high concentration of phosholipids, you want to add 2 parts chloroform
- To 300 µl protein sample, add 400 µl methanol and vortex.
- Then add 100 µl (or 200 µl if high phospholipid content) chloroform in the hood to your sample and vortex vigourously.
- Centrifuge sample at 14K, 4 °C, 5 minutes.
- You should see three phases: the clear upper aqueous phase, the cloudy precipitated protein interphase and the clear lower chloroform phase.
- Carefully remove and discard the top aqueous phase without disrupting the protein interphase.
- Add 300 µl methanol to the remaining phases and vortex.
- Centrifuge sample at 14K, 4 °C, 5 minutes to pellet protein.
- Remove supernatant from pellet and speed vacuum pellet for 10 minutes with no heat.

- Pellet is now ready for digestion or protein quantitation.

Pellet Resuspension and Digestion

- Add 50 µl of 0.4% RapiGest in 50mM Ammonium Bicarbonate pH 7.8 to pellet. If pellet is larger add more volume.
- Vortex for ~ 2 minutes.
- Boil sample at 99 °C for 5 minutes with intermittent vortexing to resuspend as much of the pellet as you can.
- Allow sample to cool at room temperature for 2 minutes.
- Add 0.5 µl 500mM DTT and incubate at 60 °C for 30 minutes.
- Allow sample to cool and add 1.5 µl 500mM IAA.
- Incubate at room temperature IN DARK for 30 minutes.
- Add 0.6 µl 100mM CaCl2.
- Add 12 µl 750 ng/µl Trypsin and incubate overnight at 37 °C with shaking.
- Next day, add 50 µl Buffer A (95% water, 5% acetonitrile, 0.1% formic acid) and then add ~3 µl 5M HCl.
- Incubate at 37 °C for 45 minutes.
- Spin at 14K, 4 °C, 10 minutes and remove supernatant into a fresh tube.
- Your sample is ready to load onto a column.