Data File S1. Detailed Protocol

Lysis

1. Thaw cell pellet vials on ice.
2. Add 0.5 mL of SDS Lysis Buffer (see recipe) per vial and let set for 5 min. Each vial contains 10^7 cells ~0.5 mg of protein.
3. Take 21G syringe and pump up and down slowly 12X, then switch to 25G needle and pump 8X using a new syringe for each sample.
4. Transfer to protein-lo bind 1.5mL Eppendorf tube.

Reduction and Alkylation of Cysteines

1. After lysis, transfer tubes to heated 56 C plate for 25 min or 37C degree for 1hr.
2. Remove from the incubator and cool to room temp.
3. Add 15μL of 0.5M iodoacetamide (see recipe) to a final concentration of 15 mM.
4. Incubate for 30 minutes at room temp in the dark.

Protein Assay

1. Remove 8.33 μL per sample and add 16.67 μL of water to each well of a microplate.
2. Perform the BCA Assay from Pierce (Rockford, IL) in duplicate according to manufacturer's instructions. Use a reduced and alkylated vial of lysis buffer as the blank for the cellular samples.

Chloroform:Methanol Precipitation

1. Take 150 μg of sample and place in a 1.5 mL protein lo-bind Eppendorf tube.
2. Add 465 μL of cold Methanol and vortex.
3. Add 310 μL of Chloroform and vortex.
4. Add (543 μL - sample volume) of water and vortex thoroughly.
5. Place sample in a centrifuge and spin for 10 min at 10k rpm.
6. Remove and discard upper layer.
7. Add 465 μL of cold Methanol and vortex.
8. Spin for 5 min at 10k rpm.
9. Remove liquid without disrupting pellet.
10. Repeat steps 8-9 two times, removing the last bit of methanol after the last wash.
11. Redisolve the pellet in 50 μL of freshly prepared 8M urea digestion buffer (see recipe).
12. Incubate at 37C degree for 10 min to aid re-solubilizing.

Lys-C Digest

1. Dilute samples 2x by adding 50 μL of 20 mM HEPES pH 8.5 and vortex.
2. Add 2 μg of Lys-C for a 1:75 enzyme:substrate ratio and mix.
3. Incubate at room temperature overnight for 16 hr.

Trypsin Digest

1. Dilute samples by adding 150 μL of 20 mM HEPES pH 8.5 and vortex.
2. Add 2 μg of Trypsin for a 1:75 enzyme:substrate ratio and mix.
3. Incubate at 37C degree for 6hr.
4. Take 1 μg per sample and desalt to test digestion efficiency (missed cleavages should be <20%).

TMT Labeling

1. Vortex and spin samples at 10k rpm for 5min.
2. Remove 60 μg to new tubes, trying not to disturb the pellet if one is present.
3. Add ACN (acetonitrile) to 10% v/v.
4. Add 7μL of TMT (Pierce, Rockford, IL). Vortex and incubate 1hr at room temperature.
5. Remove 1 μg per sample, pool all samples in each 10-plex and desalt to test labeling efficiency (should be greater than 99%).
6. Add 1% final v/v of hydroxyl amine and let incubate for 10 min at room temperature to quench.
7. Combine samples and add formic acid to lower pH to <3.
8. Spin 12000 rpm x 10min.
9. Remove supernatant and dilute to lower ACN % to <5% with 1% formic acid (FA) in water.
10. Desalt and evaporate to dryness in a speed vac.
11. Optional - use a bridge sample (combination of all samples) or a known amount of target peptide, as optional additional reference channels for comparison.

Injecting samples

1. Reconstitute dried sample in 3% ACN, 5% FA in HPLC grade water.
2. Add trigger peptide to target sample, and load 5-8 μg of peptide onto the LC column.
3. See methods for instrument settings.

Recipes

<u>100 mL of the Lysis Buffer</u>

1. 2g SDS
2. 3mL of 5M NaCl
3. 5mL of 1M Tris pH 8.8
4. *500μL 1M DTT (1M stock made fresh dissolving 154.25 mg in 1mL H2O)
5. 91.7mL H2O (HPLC Grade)
6. 100uL of Sodium Vanadate 200mM
7. 10 tablets of Roche phosstop phosphatase inhibitor
8. 2 tablets of Roche Complete protease inhibitor (EDTA Free)
9. Freeze in 6 50 mL tubes containing 16.5 mL each.

*Added fresh on the day of use.

3mL of 0.5M Iodoacetamide

1. Dilute 277.44mg iodoacetamide in 3mL HPLC grade water and vortex.

10 mL of Digestion Buffer - made fresh before each use

1. Add 4.8 g Urea to 15 mL falcon tube.
2. Add 1mL of 200 mM HEPES pH = 8.5 (pH adjusted with amine-free acids and bases)
3. Add HPLC grade water up to 10 mL mark on tube and vortex thoroughly.