## Overview and Considerations:

A total cell lysate made with RIPA buffer, which contains three detergents, is rigorous. Note, that some protein complexes will not be stable in this buffer. Additionally, this technique is not compatible with many protein estimation methods. Use the BCA method for the best results.

## Reagents:

1. PBS (to harvest cells);
2. Labeled eppendorf tubes (the same number as you have samples)
3. Ice bucket/Ice
4. Dry ice in a foam container
5. Microcentrifuge, 4oC
6. **Extraction buffer:** RIPA: 50mM Tris HCl, 150mM NaCl, pH7.5, 1% v/v NP-40, 0.5% sodium deoxycholate, 0.1% SDS.
   * Add inhibitors to the desired volume of buffer immediately before use:
   * PMSF stock is 100mM or 1000x, final working concentration 0.1mM (*inhibit proteases*)
   * PIC2 & PIC3 are 1000x (*inhibit proteases*)
   * KF stock is 1M or 100x, final working concentraiton 10mM (*inhibit phosphatases*)
   * b-glycerophosphate stock is 1M or 100x, final working concentraiton 10mM (*inhibit phosphatases*)
   * TSA1 stock is 3mM or 1000x, final working concentration 3uM (*inhibits deacetylation*)
   * Nicotinamide (Stock 1M, 1000x) (*inhibits deacetylation*) \* this inhibitor is optional
   * PUGNAc (100mM stock, 10, 000x stock), NAGT (10mM, 1000x stock), Thiamet-G (2mM, 1000x stock)
   * Note many PUGNAc, NAGT, and ThiametG stocks are sterile filtered for use in the tissue culture room. Please keep inhibitor stocks, that are no longer sterile, separate from sterile stocks.

## Protocol:

1. Wash cells in PBS;
2. Harvest, and pellet. Aspirate supernatant;
3. Snap freeze cells on dry ice and store at -80oC until required;
4. Add inhibitors to extraction buffer\*, for 5mls:
   * 5ul PMSF (Stock 100mM in ETOH, final concentration 0.1mM) \*\*;
   * 5ul PIC3 (Stock 1000x, final concentration = 1x);
   * 5ul Pic2(Stock 1000x, final concentration = 1x);
   * 5ul NAG-T (Stock 10mM, final concentration = 10nM);
   * 5ul TSA1 (Stock 3.3mM, final concentration 3.3nM);
   * *50ul* nicotinamide (Stock 1M, final concentration 1mM).
   * 50ul Kf or NaF (Stock 1M, final concentration 1mM)
   * 50ul b-glycerophosphate (Stock 1M, final concentration 1mM);
   * The inhibitors “expire”. So we add inhibitors to the volume of buffer that we are going to use for our  experiment. For 10 samples, if we are extracting in 500ul, we will use 5000ul of extraction buffer. So we will make up 6000ul of extraction buffer with inhibitors
   * PMSF is inactivated within 30minutes of adding it to solutions. Add PMSF right before adding extraction buffer to the samples. PMSF is VERY toxic, especially as a powder. Take appropriate precautions.
5. Remove samples from the -80oC, transfer to an ice bucket. Do not do this before your extraction buffer is ready, we want our cells to defrost in extraction buffer so we can inhibit phosphatases, O-GlcNAcase, proteases etc as they warm up.
6. Add extraction buffer to each sample, the amount you add will depended on the number of cells, and the cell type.
7. Let the cells “melt” on ice for 10minutes. RSS the cells by vortexing.
8. Incubate the cells on ice for 30minutes. Vortex the cells for 5-10s every 10minutes.
9. Sonicate cells for ~5s on setting 2 (this is lower than the 1% NP-40 to avoid excess foaming of the sample)
10. Pellet at full speed in a microcentrifuge or 14,000RPM for 30minutes at 4oC.
11. Remove tubes to an ice bucket, and transfer the supernatant to a new labeled tube.
12. Discard the pellet.
13. Determine the protein concentration of the samples.
14. It is best to determine the protein concentrations, and make up gel samples, before you freeze the sample. The freeze thaw process can induce proteins to aggregate, which complicates the process of protein determinations. For protein extracts that have been frozen, you should ALWAYS centrifuge them (At least 10 000xg, for 10min) before use with the exception of SDS-PAGE gel samples.