Mass Spectrometric Analyses for anti-FLAG Immuno-Precipitated Proteins

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Wear powder-free nitrile gloves and lab coat, and perform as many manipulations as possible under the hood.

1. Rinse 100 ml Pyrex bottle with mass spec-gread H_2O.
2. Make up (50 ml for four pull-downs) Lysis buffer in 100 mL Pyrex bottle.
3. Take 50 mL buffer (50 mM HEPES, pH 7.5; 70 mM KOAc; 5 mM $Mg(OAc)_2$).
4. Add 0.1 g n-dodecyl-D-Maltoside (Thermo Scientific; Cat # 89903).
5. Add 1 Protease Tablet (Roche; Cat # 1117700).

■ **Harvest** (should start with one 90–100% confluent 14 cm dish).

1. Wash cells with in 5 ml Hank's dissociation buffer (Gibco; Cat #13150-016).
2. Add 2 ml Hank's dissociation buffer and incubate in 37 °C incubator for 5 min.
3. Detach cells from the dish by vigorous tapping.
4. Add 13 ml cold PBS and transfer cells to a 50 ml conical tube.
5. Centrifuge.
6. Remove supernatant and add 1 ml cold PBS.
7. Gently resuspend cells and transfer to non-stick microcentrifuge tube (VWR; cat # 20170-650).
8. Spin down and aspirate off media using a syringe needle.
9. Store cells in –80 °C freezer should you want to stop.

■ Lysis and FLAG Immuno-Precipitation

1. Add 1 mL lysis buffer to the cell pellet and resuspend cells.
2. Incubate on rotator in cold room for 30 min.
3. Spin in cold microcentrifuge for 15 min at 16,600×g.
4. Prepare beads (anti-FLAG M2 affinity gel; sigma A2220).
   ① 50 µL beads per sample (~5 mg) in each tube.
   ② Wash 3 times with 1 ml Lysis buffer.
5. Save 50 µl of the supernatant for Western blot analysis and protein quantitation.
   · For Protein quantification
   ① Take 5 µl and 95 µL water.
   ② Add 20 µl to Quick Bradford Buffer.

③ Measure OD.

④ Calculate concentration—take 30 μg for western blotting and freeze.

6. Add the supernatant corresponding to 5 mg of protein plus lysis buffer to 1 ml to tubes containing beads.
   ① Incubate 1 hr on rotator at 4 °C.
   ② Wash 5 times with 1 ml Lysis buffer.
   ③ Wash 2 times with 1 ml 0.1 M Tris–HCl, pH 8.5 (Mass Spec Buffer).
   ④ Spin and aspirate off any residual buffer.
   ⑤ Resuspend in 100 μl MS Buffer.
   ⑥ Save 5 μl for western blotting.
   ⑦ Spin and aspirate.

■ Digest

1. Make up 10 M urea.
2. Elute from the beads with 64 μl, place on Thermomixer for 15 minutes.
3. Add to Bio-rad micro bio-spin chromatography column (cat # 732-6204) place in microcentrifuge tube.
4. Spin 2 min at 6,000 X g in microcentrifuge.
5. Add 16 μl MS buffer to dilute to 8 M Urea.
6. Add 0.5 μl 500 mM TCEP.
7. Incubate for 20 min at room temperature.
8. Make up 500 mM 2-chloro-acetamide (18.7 mg in 400 μl MS buffer).
9. Add 1.8 μl.
10. Incubate for 15 min at room temperature.
11. Add 2 μl Lys-C endoprotease (100 ng/ μl).
12. Incubate for 4 hr at 37 °C.
13. Add 240 μl MS Buffer to dilute to 2 M urea.
14. Add 3.2 μl 100 mM $CaCl_2$.
15. Add 2 μl Trypsin (100 ng/ μl) (Promega; VS11A).
16. Incubate for up to 14 hr at 37 °C and desalt using StageTips (Thermo; SP301) for LC injection.