Gingras lab protocol for FLAG AP-MS in mammalian cells using agarose beads

2010/05/18 – updated by ACG
(adapted from Chen and Gingras, Methods, 2007)

Protocol type: Affinity Purification
Protocol name: Human cell lysis and FLAG IP – GC protocol
Protocol number: 139

Cells were lysed by passive lysis assisted by freeze-thaw. Briefly, to the frozen cell pellet, 1:4 or 1:5 pellet weight:volume ratio of lysis buffer was added. Lysis buffer was 50 mM Hepes-NaOH pH 8.0, 100 mM KCl, 2 mM EDTA, 0.1% NP40, 10% glycerol, 1 mM PMSF, 1 mM DTT and Sigma protease inhibitor cocktail, P8340, 1:500. No phosphatase inhibitors were added.

Resuspended pellets were incubated on ice (or on a nutator at 4°C) for 10 min to assist lysis, then pipetted up and down to break up the pellet. Tubes were frozen and thawed once (liquid nitrogen or dry ice ~5min, 37°C with agitation), then put on ice, and the lysate transferred to 2 ml Eppendorf tubes. An aliquot (20ul) was taken to monitor solubility (This aliquot was spun down, the supernatant transferred to a fresh tube, and 6 µl 4X Laemmli sample buffer added to the supernatant. The pellet was resuspendended in 26 µl 2X Laemmli sample buffer).

The 2 ml tubes were centrifuged at 14000 rpm for 20 min at 4°C, and the supernatant transferred to fresh 15 ml conical tubes. The protein concentration was measured (using BSA as a control). To the rest of the lysate, 25-30 µl packed FLAG M2 agarose beads (SIGMA) pre-washed 4X in lysis buffer were added, and the mixture was incubated 2 hours at 4°C with gentle agitation. Beads were pelleted by centrifugation (1000 rpm for 1 min), and a 20 µl aliquot of the lysate post-IP was taken for analysis.

Most of the supernatant was aspirated, and the beads were transferred with 1 ml of lysis buffer to a fresh eppendorf tube, and centrifuged at 3000 rpm for 5 sec (quick washes were performed to avoid losing interactors). Beads were let to settle for 10-15 sec and the supernatant was removed. The wash step was repeated three more times with lysis buffer (note that for some projects, only one additional wash is performed), followed by two washes with ammonium bicarbonate rinsing buffer pH 8 (50 mM ammonium bicarbonate, 75 mM KCl).

After the last wash, most of the liquid was removed and the samples were centrifuged again at 3000 rpm for 5 sec (a pipet with a narrow tip was to remove most of the remaining liquid).

Fresh ammonium hydroxide elution buffer pH 11-12 was prepared by diluting NH4OH (around 200-500 µl) in 5ml HPLC-grade H2O (pH was monitored). Elution buffer (150 µl) was added to each sample, and elution was performed by incubating for 15 min at 4°C (supernatant was transferred to a fresh tube after centrifugation). This elution step was repeated twice more. Elutions were pooled and centrifuged at 3000 rpm for 1 min. An aliquot of the elution (30 µl) was transferred to a fresh tube while the remainder of the elution (420 µl) was placed into a different fresh tube. Both tubes were evaporated in the speed vac until dryness. HPLC-grade water (100 µl) was added to each tube, and lyophilization was repeated. Samples were frozen until needed.