1 *Affinity tagging kinases and phosphatases*

The first step in our AP-MS procedure is to generate an epitope tagged version of a protein of interest. We utilize the Flp-In vector pcDNA5-FRT-TO (Invitrogen) to inducibly express fusion proteins with a single N-terminal FLAG epitope. The single epitope tag was engineered by subcloning the FLAG and multiple cloning sites from the vector pcDNA3-FLAG [74] into the Flp-In parent vector pcDNA5-FRT-TO. N- and C-terminal triple FLAG tag vectors in the Gateway system (pDEST 5' Triple FLAG pcDNA5-FRT-TO; pDEST 3' Triple FLAG pcDNA5-FRT-TO) are also available from K. Colwill and T. Pawson. Standard cloning procedures are employed, and we recommend that the final construct be sequenced.

2 *Creation of stably transfected, tetracycline-inducible, Flp-In 293 T-REx pools*

The transfection and cell selection protocol outlined here has been optimized for use with Flp-In 293 T-REx cells. Alternate methods for transfection can be used. Note that while we find pools to be adequate for most AP-MS experiments using the Flp-In T-REx cells, individual clones may easily be picked during step 5 below, and expanded individually.

1) Day 1: Seed low passage Flp-In 293 T-REx cells into a 6 well dish at 50% confluency for transfection on Day 2.
2) Day 2: Transfect Flp-In 293 T-REx cells with 0.2 μg of pcDNA5-FRT-FLAG-protein and 2 μg of the Flp recombinase vector pOG44 per well in a 6 well dish. Lipofectamine PLUS (Invitrogen) or jetPRIME (Polyplus) is used for transfection, according to the manufacturer's instructions.
3) Day 3: Passage the cells into a 10 cm plate in complete medium (DMEM with 10% FBS and 100 units/ml of pen-step).
4) Day 4: Replace the medium with selection medium (complete medium supplemented with 200 μg/ml hygromycin). Selection medium is then replaced every 2 – 4 days until non-transfected cells die and isolated clones are ~1-2 mm in diameter (this takes approximately 13-15 days).
5) ~Day 17: Re-plate the cells from this 10 cm plate into fresh selection medium (the size of the new plate is dictated by the number and size of initial colonies). Re-plating these clones into one new mixture generates a stably transfected Flp-In 293 T-REx "pool". This pool will eventually be transferred to a 15 cm plate.
6) When the new 15 cm plate reaches 75-80% confluency, split this plate as follows: 1 plate for freezing down four tubes of pools (for re-culture as needed); 2 plates for AP-MS biological replicate #1 and 2 plates for AP-MS biological replicate #2.

3 *Induction of Flp-In 293 T-REx pools and cell collection*

Two 15 cm plates are used for each biological replicates to be analyzed by AP-MS (we suggest that at least two such replicates be analyzed). Cells are induced to express the FLAG-tagged protein of interest and collected following the procedure outlined below.

1) Culture two 15 cm plates of cells in complete medium to ~65% confluency and induce with 1μg/ml tetracycline for 24 hours.

2) After 24 hours of induction cells should be subconfluent (~85-95% confluent). Collect these cells by draining medium from the plate, adding 0.5 ml ice-cold PBS, and scraping the cells from the plate (using a silicon cake spatula or cell scraper). Transfer the cells and PBS from each pair of plates to a 2 ml microfuge or 15 ml conical tube, and place on ice.
3) Collect the cells by centrifugation (5 min at 500 g 4°C). Aspirate the PBS, and wash the cells by resuspending in 1 ml ice-cold PBS prior to another centrifugation (5 min at 500 g, 4°C). Repeat this washing step once more, aspirate the remaining PBS and determine the weight of the cell pellet.

Freeze these cell pellets on dry ice, and transfer them to -80°C for storage until ready for cell lysis and affinity purification.

4 *Cell lysis*

This protocol was developed to perform affinity purification from 2 x 15 cm plates prepared in the step above. This normally corresponds to a dry cell pellet weighing ~250 – 500mg. In this procedure cells are lysed by passive lysis assisted by freeze-thaw.

Lysis Buffer

- 50 mM Hepes-KOH pH 8.0, 100 mM KCl, 2 mM EDTA, 0.1% NP40 and 10% glycerol, supplemented with 1 mM PMSF, 1 mM DTT and 1X protease inhibitor cocktail (Sigma-Aldrich)

1) Resuspend the frozen cell pellet in ice-cold lysis buffer at a 1:4 pellet weight:volume ratio. Phosphatase inhibitors may also be added to the lysis buffer (0.25 mM sodium orthovanadate, 50 mM β-glycerolphosphate and 10 mM NaF) or freshly supplemented (5 nM okadaic acid and 5 nM calyculin A) depending on the type of interactions being studied. While phosphorylation-dependent interactions clearly benefit from the inclusion of such inhibitors, we have also noticed that some of the interactions with the catalytic subunits of phosphatases are lost upon addition of phosphatase inhibitors.
2) Perform one to two freeze-thaw cycles by incubating the tube on dry ice ~5-10min, then transfer it to a 37°C water bath with agitation until only a small amount of ice remains and then transfer the tube to an ice bucket. Keep lysate on ice or at or below 4°C during lysis and affinity purification.
3) At this time a 20µl aliquot should be taken to monitor expression, lysis efficiency and solubility. Spin this aliquot down, transfer the supernatant to a fresh tube, add Laemmli sample buffer to the supernatant. Resuspend the pellet in 2X Laemmli sample buffer. These samples should be saved for western blot analysis.
4) Remove cell debris from the sample by centrifugation (20 min at >16, 000 g, 4°C). Transfer the supernatant to a fresh tube (remove or avoid the lipid layer on top of the lysate if present).

5 *Affinity purification using anti-FLAG magnetic beads*

FLAG magnetic rinsing buffer

- 20mM Tris-HCl (pH 8.0) and 2mM $CaCl_2$

1) Prepare a master mix of anti-FLAG M2 magnetic beads (M8823, Sigma-Aldrich), using 25µl of a 50% slurry for each sample. Add the total required volume of beads to a microfuge tube and resuspend in 1ml of lysis buffer. Magnetize the beads and aspirate the supernatant, remove the beads from the magnet and resuspend beads by pipetting up and down in 1ml lysis buffer. Magnetize and aspirate the lysis buffer. This process should be completed three times. Following the last wash, resuspend the beads in enough lysis buffer to distribute 25µl into each sample.
2) Distribute 25µl of the 50% bead slurry to the clarified lysate, and incubate this mixture for 2 hours at 4°C with gentle agitation on a nutator.
3) Magnetize the sample and remove a15µl aliquot of the post-IP lysate. Save this for western blot analysis (with lysate and pellet portions from cell lysis step).
4) Aspirate the remaining buffer and proceed to wash beads once with 1ml lysis buffer, followed by one wash with FLAG magnetic rinsing buffer. For each of these washes, demagnetize the sample, resuspend the beads by pipetting up and down 4 times in the wash buffer, magnetize for 30 seconds and then remove the supernatant. The washing steps should be done as quickly as possible, where a complete wash cycle takes between 1-2 minutes. (Alternatively, the stringency of the washes can be increased by washing the beads three times with 1ml lysis buffer, followed by two times with FLAG magnetic rinsing buffer)
5) After the last wash, remove most of the liquid, centrifuge the sample briefly to pellet the beads at the bottom of the tube (500 g for 1 min), magnetize and remove any remaining liquid with a fine pipette.

6 *On bead Trypsin digestion (optional protocol)*

The elution with ammonium hydroxide (as presented in the protocol Anti-FLAG Agarose 2012) may be performed efficiently on the FLAG M2 magnetic beads, and an in-solution digest done as also described. However, we have found that – as opposed to the agarose beads – the use of magnetic beads enabled us to also perform on bead tryptic digestion which is outlined here:

1) Resuspend the beads in 5µl of 20mM Tris-HCl (pH 8.0). Add 500ng of trypsin (T6567, Sigma-Aldrich) to the mixture and incubate at 37°C on a rotator for 4 hours.
2) Magnetize the sample for 30 seconds and transfer the supernatant to a fresh tube, add an additional 500ng of trypsin and incubate overnight at 37°C (no agitation required). (Alternatively, the first trypsin incubation can be performed overnight with 750ng trypsin, and the 'spike' or second incubation is then done with 250ng trypsin the following day for an additional 3 hours).
3) Following the second trypsin incubation, add formic acid to the sample to a final concentration of 2% (from 50% formic acid stock solution). The sample can then be stored at -20°C or directly analyzed by mass spectrometry.

To load the sample onto a column, centrifuge the sample at 16, 000 g for 5 min to remove any debris that may otherwise clog the HPLC column, transfer the supernatant to a fresh tube and load it directly onto a C18 reversed phase column for subsequent LC/MS analysis. (Alternatively, the sample may be loaded to an HPLC column via an autosampler and a pre-column system).