

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 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 20 μ l 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 M2 agarose beads

FLAG agarose-rinsing buffer

- 50 mM NH_4HCO_3 , pH 8.0 and 75 mM KCl

Elution Buffer

- 0.5 M NH₄OH, pH 11.0–12.0. Note buffer should always be prepared fresh and the pH tested (pH paper is sufficient) as NH₄OH is very volatile. Using a freshly prepared solution ensures proper elution from beads.
- 1) Prepare a master mix of anti-FLAG M2 agarose beads (A2220, Sigma-Aldrich) using 15µl of a 50% slurry for each sample. Wash the beads three times by adding 1 ml of lysis buffer and inverting the tube. Next pellet the beads by centrifugation (500 g for 1 min).
 - 2) Distribute 15µl of the 50% bead slurry (equivalent of 7.5µl packed beads) to the clarified lysate, and incubate this mixture for 2 hours at 4°C with gentle agitation on a nutator.
 - 3) Pellet the beads by centrifugation (500 g for 1 min).
 - 4) At this time a 15µl aliquot of the lysate post-IP is removed. This aliquot should be analyzed by western blot with both the pellet and supernatant portion from 4.4 step 3.
 - 5) Remove most of the remainder of the supernatant, leaving enough so as not to disturb the beads (~100µl).
 - 6) Wash beads 3 times with 1ml of lysis buffer and three more times with FLAG agarose-rinsing buffer, by adding 1ml of buffer, mix by pipetting up and down 4 times, then pellet the beads by centrifugation (500 g for 1 min).
 - 7) Elute proteins 3 times with 50µl of freshly prepared elution buffer. Add 50µl of elution buffer, flick tube to mix and incubate for 2 min on bench. Pellet the beads by centrifugation (500 g for 1 min). Alternatively, 2% HCO₂H (formic acid) can be used for elution; For samples which will then be used for phospho-enrichment we recommend using acid elution, although this can increase the amount of FLAG antibody in the eluate.
 - 8) Lyophilize eluate in a centrifugal evaporator. To ensure removal of any remaining NH₄OH which may impede trypsin digestion, wash with 200µl of water (alternatively, use 50 mM NH₄HCO₃, pH 8.0) and lyophilize again in a centrifugal evaporator. At this point, the proteins may be stored at -20°C or immediately digested with trypsin.

6 In solution tryptic digestion

- 1) Resuspend protein with 750ng trypsin (T6567, Sigma-Aldrich) at 100ng/µl in 50 mM NH₄HCO₃, pH 8.0. Digest samples overnight at 37 °C. The next day, add 250ng trypsin, and continue the digestion for another 2–3 hours.
- 2) Add formic acid to the sample to a final concentration of 2% (from 50% stock solution). Lyophilize eluate in a centrifugal evaporator.
- 3) Resuspend the lyophilized peptides in 7µl of HPLC reversed-phase buffer A (2% acetonitrile, 0.1% formic acid). The sample can then be stored at -20°C or directly analyzed by mass spectrometry.
- 4) 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 the reversed-phase column via an autosampler and a precolumn system.