293 Flp-In FLAG magnetic AP - July 2013

Developed by Marilyn Goudreault
Anne-Claude Gingras Laboratory

Prohits Protocol ID 274: Induction of Flp-In 293 T-REx pools and cell collection

Two 15 cm plates are used for each biological replicate to be analyzed by AP-MS (we suggest that at least two such replicates are 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 to ~70% 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 mins 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 mins at 500 g, 4°C). Repeat this washing step once more, aspirate the remaining PBS and determine the weight of the cell pellet.

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

Prohits Protocol ID 274:Cell lysis and affinity purification using anti-FLAG magnetic beads

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-NaOH 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 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, by running both the pellet and supernatant portion of this aliquot on a gel. 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. Run both portions on a gel in separate lanes.
4) Remove cell insolubles by centrifugation (20 mins 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).

Gel free 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.

Prohits Protocol ID 283: On bead Trypsin digestion

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).

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.