Flp-In BioID Streptavidin sepharose - July 2013
Developed by Amber Couzens
Anne-Claude Gingras Laboratory

Prohits Protocol ID 701: Induction of Flp-In 293 T-REx pools and cell collection

Four 15 cm plates are used for each biological replicate to be analyzed by BioID-MS. Cells are induced to express the FLAG-tagged protein of interest and collected following the procedure outlined below.

1) Culture four 15 cm plates of cells to ~70% confluency and induce with 1µg/ml tetracycline and media supplementation with 50uM biotin (for protein labeling) for 24 hours.
2) After 24 hours of induction cells should be subconfluent (~85-95% confluent). Collect these cells by draining medium from each plate, wash once with 5ml PBS per 150mm plate and then harvest by scraping in 1ml of ice cold PBS (using a silicon cake spatula or cell scraper). Transfer the cells and PBS from each plate to a 2 ml microfuge or 15 ml conical tube, and place on ice.
3) Collect the cells by centrifugation (5 mins at 233 g 4°C). Aspirate the 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 407:Cell lysis and affinity purification using anti-Streptavidin sepharose beads

RIPA Lysis Buffer

- 50 mM Tris-HCL pH 7.5, 150 mM NaCl, 1% NP-40, 1mM EDTA, 1 mM EGTA, 0.1% SDS, Sigma protease inhibitors P8340 1:500, and 0.5% Sodium deoxycholate), supplemented with 250U of benzonase

1) Resuspend frozen cell pellets corresponding to 4 x 150mm plates in 2mL of ice-cold RIPA lysis buffer, then add 1uL of benzonase and incubate with agitation for 1 hour at 4°C.
2) Sonicate lysates on ice at 30% amplitude (3 x 10 second bursts with 2 seconds rest in between). 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) Centrifuge lysates for 30 min at 20,817g at 4°C. During this time, wash streptavidin agarose beads (GE Cat# 17-5113-01) 3 times with 1mL RIPA buffer (minus protease inhibitors and sodium deoxycholate). Beads are pelleted at 400 x g, 1 minute in

between washes. Transfer cleared supernatants to a 2 mL microfuge tubes and add a 30 µL bed volume of pre-washed streptavidin sepharose beads to each sample.

5) Perform affinity purification at 4°C on a nutator for 3 hours. Then pellet beads (400 x g, 1 min), remove supernatant and transfer the beads to a 1.5mL eppendorf in 1 mL RIPA buffer.
6) Wash beads by pipetting up and down (4x per wash step) first with an additional 1mL RIPA buffer followed by two washes in TAP lysis buffer (50 mM HEPES-KOH pH 8.0, 100 mM KCl, 10% glycerol, 2 mM EDTA, 0.1% NP-40), and three washes in 50mM ammonium bicarbonate pH 8.0 (ABC). Beads are pelleted by centrifugation (400 x g, 1 min) and the supernatant aspirated in between wash steps. After the last wash all residual 50mM ABC is pipetted off and proteins are digested on beads.

Prohits Protocol ID 408: On bead Trypsin digestion

1) After affinity purification and removal of all washing buffer, re-suspended beads in 200 µL of 50 mM ammonium bicarbonate (ABC) pH 8, add 1 µg trypsin (T6567, Sigma-Aldrich) and incubate at 37°C overnight with agitation.
2) The next day, an additional 0.5 µg of trypsin was added to each sample (in 10 µL 50 mM ABC) and the samples incubated an additional 2 hours at 37°C.
3) Beads are then pelleted (400g, 2 min) and the supernatant transferred to a fresh 1.5 mL eppendorf tube. The beads are then rinsed 2 times with 150 µL of mass spec-grade H_2O (pelleting beads at 400 x g, 2 min in between), and these rinses are combined with the original supernatant. The pooled fractions are centrifuged at 16,100g for 10 minutes and most of the supernatant (minus 30uL to get rid of all beads) is transferred to a new 1.5 mL eppendorf and lyophilized in a speed-vac. The samples are resuspended in 10 µl of 5% formic acid and 3 µl is used per analysis.