293 Flp-In FLAG magnetic chromatin optimized AP - July 2013

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Prohits Protocol ID 254: 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. 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 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 258: 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 steps above. This normally corresponds to a dry cell pellet weighing ~250 – 500mg for chromatin bound baits. 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 P8340)

1) Resuspend frozen cell pellets by pipetting up and down in 1:4 pellet (weight: volume; 1mL of lysis buffer for 250mg of cells) ratio of ice-cold lysis buffer.
2) Perform one to two freeze-thaw cycles by incubating the tubes on dry ice ~5-10min, and then transfer them to a 37°C water bath with agitation until only a small amount of ice remains. Return thawed samples on ice, and transfer lysates to 2 ml Eppendorf tubes (normally 1 or 2 tubes per sample).
3) Sonicate lysates on ice at 30% amplitude (3 x 10 second bursts with 2 seconds rest in between) using a QSONICA 125W sonicator equipped with 1/8" probe to shear DNA. Add 1uL of benzonase (Sigma product #E1014; 250 units/uL) to each sample and incubate at 4°C on a nutator for 1 hour to further digest chromatin. Keep lysate on ice or below 4°C during lysis and affinity purification.

4) 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.
5) Centrifuge lysates for 20 min at 20,817g at 4°C. Then transfer the supernatants to fresh 2mL tubes. During this time, prepare anti-FLAG M2 magnetic beads (Sigma product # M8823) as follow: aliquot 25ul of the 50% slurry for each IP (2 x 150mm plates), wash beads in batch mode by 3 x 1 ml of cold lysis buffer. Before removing the supernatant off the last wash, aliquot beads (volume equivalent of the original 25uL) in a fresh labelled tube.
6) Transfer centrifuged lysates to the appropriate tube containing the anti-FLAG magnetic beads taking care of not collecting insoluble material in doing so. Incubate mixture for 2 hours at 4°C with gentle agitation (nutator). Then pellet beads by centrifugation (1000 rpm for 1 min or using a small table-top microfuge) and take a 15ul aliquot of the lysate post-IP for analysis.
7) Perform washes by placing tubes on a cold magnetic rack (placed on ice itself) to collect the beads on the tubes side. Remove supernatant slowly with a pipette and resuspend beads in 1mL of cold lysis buffer. Transfer beads to a fresh 1.7mL tube and wash two more times with 1mL of cold lysis buffer and once more with 1mL of 20mM TrisHCl pH8 2mM $CaCl_2$. Following the last wash, centrifuge samples quickly and remove the last drops of liquid with a fine pipette.

Prohits Protocol ID 259: On bead Trypsin digestion

1) Resupend the now-dried beads in 7.5ul of 20mM Tris-HCl (pH 8.0) containing 500ng of trypsin (Sigma Trypsin Singles, T7575) and incubate at 37°C on a rotator overnight (~15hours).
2) After this first incubation, magnetize the sample and transfer the supernatant to a fresh tube, add an additional 500ng of trypsin (in 5uL of 20mM Tris-HCl (pH8) and incubate at 37°C for 3-4 hours (no agitation required).
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 at -40 or -80°C or directly analyzed by mass spectrometry.