NBCC Proteomics Protocol 2
Version – January 8, 2019
Adapted from Protocols in the Gingras Lab

Induction of Flp-IN T-REx Pools and Cell Collection

Cell culturing:

1. Culture stable Flp-IN T-REx cells (see Note 1) in 15 cm dishes (2 dishes per sample, see Note 2) so they are 70-75% confluent on day of induction.

Protein induction and biotin labeling:

2. Replace media with media containing 1 µg/ml doxycycline (See Note 3). Incubate for 24 hours.

Cell harvest:

3. Wash each plate with 2 x 10 ml ice-cold PBS. Keep plates cold and work as quickly as possible.
4. Aspirate PBS with plate on bed of sloped ice.
5. Using the left over PBS wash (~500 µl), scrape the cells and transfer to a 14 ml tube (see Note 4).
6. Centrifuge the cells at 500x g for 5 min at 4°C to collect the cells. Carefully remove all supernatant by aspiration.
7. Weigh cell pellets and record on tube (see Note 5).
8. Flash freeze the tubes on dry ice and place at -80^{o}C.

Cell Lysis and Immunopurification

Cell Lysis:

9. Resuspend each frozen cell pellet in ice-cold lysis buffer at a 1:4 pellet weight:volume ratio (e.g., 400 µL buffer to a 100 mg pellet).
10. 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.
11. Optional for baits in the nucleus or those binding nucleic acids particularly those associated with chromatin: sonicate cells for 15 sec (5 sec on, 3 sec off for three cycles) at 30% amplitude on a sonicator with 1/8" microtip. Keep samples on ice. Then, add 250 U of TurboNuclease and 10 µg of RNase and rotate (end-over-end) at 4°C for 15-20 min (see Note 6).
12. Centrifuge at 20,817x g for 20 min and transfer supernatant to a 2 ml centrifuge tube (see Note 7).

Immunopurification:

13. Prepare a master mix of anti-FLAG M2 magnetic beads using 25 µl of a 50% slurry for each sample plus 10% to account for any losses. Add the total required volume of beads to a microfuge tube and resuspend in 1ml of lysis buffer. Magnetize the beads on a cold magnetic rack (kept on ice) and aspirate the supernatant, remove the beads from the magnet and resuspend beads by pipetting up and down several times in 1 ml 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 25 µl into each sample.

14. Distribute 25 µl of the 50% bead slurry to the clarified lysate, and incubate this mixture for 2 to 3 hours at 4°C with gentle 'end-over-end' rotation (See Note 8).
15. Pellet beads by centrifugation at 500x g for 1 to 2 min at 4°C.
16. Magnetize the sample and remove a 15 µl aliquot of the post-IP lysate (see Note 7). Aspirate the remaining buffer and proceed to wash beads once with 1 ml lysis buffer, and transfer to fresh 1.5 ml microcentrifuge tube (this removes 'sticky' proteins bound to side of tube). Magnetize again, then perform 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 sec and then remove the supernatant. The washing steps should be done as quickly as possible where a complete wash cycle takes between 1 to 2 min (see Note 9).
17. After the last wash, remove most of the liquid, use a small volume of 20 mM Tris-HCl pH 8.0 to wash beads to the bottom of the tube, magnetize and remove any remaining liquid with a fine pipette.

Trypsin Digest

18. Resuspend the beads in 7.5 µl of trypsin digestion buffer (100 ng/µl trypsin in 20mM Tris-HCl (pH 8.0). Incubate at 37°C overnight with agitation (see Note 10).
19. Centrifuge the samples at 500x g for 1 min, magnetize the beads for 30 s and transfer the supernatant to a fresh tube, add an additional 2.5 µl trypsin digestion buffer and incubate for a further 3 hours (no agitation required).
20. Following the second trypsin incubation, add 1 µl of 50% formic acid (see Note 11) to the sample to a final concentration of ~5%. The sample can then be stored at -20°C or directly analyzed by mass spectrometry.

Buffers

Lysis Buffer

50 mM Hepes-NaOH pH 8.0,
100 mM KCl,
2 mM EDTA,
0.1% NP40 and
10% glycerol
1 mM PMSF (add fresh)
1 mM DTT (add fresh)
1X protease inhibitor cocktail (add fresh)

The lysis buffer may also be supplemented with freshly added phosphatase inhibitors (0.25 mM sodium orthovanadate, 50 mM β-glycerolphosphate, 10 mM NaF, 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.

FLAG magnetic rinsing buffer

20 mM Tris-HCl, pH 8.0
2 mM $CaCl_2$

Key Reagents

This protocol has been optimized using these suggested reagents. For chemicals, you should use these recommendations or reagents of equivalent grade:

Reagent	Company	Catalog Number
Parent Vectors	Gingras lab	Available upon request
Ammonium Bicarbonate	Bioshop	05-402-7
Doxycycline[1]	Sigma-Aldrich	D9891
FLAG M2 magnetic beads	Sigma-Aldrich	M8823
Formic Acid	ThermoFisher	14-955-319
Flp-IN T-REx HEK293 cells	ThermoFisher	R78007
Monoclonal anti-FLAG M2 antibody	Sigma-Aldrich	F3165
Protease Inhibitor Cocktail	Sigma-Aldrich	P8340
RNase	Bio Basic	RB0473
Tetracycline	Bioshop	TET701.25
Trypsin	Sigma-Aldrich	T6567
Turbonuclease	BioVision Inc.	9207

Notes

[1]This protocol is designed to work with single- or triple-FLAG-tagged constructs that have been stably transfected into a Flp-IN T-REx cell line. For baits where BioID (*in vivo* biotinylation) is also to be performed, FLAG-BirA* or FLAG-BioID2 vectors may also be used). This protocol has been optimized for HEK293 stable cell pools but other Flp-IN T-REx cell lines may be used. More notes on the FLAG IP method are discussed in Hesketh et al., Methods Mol Biol. 2017;1550:115-136.

[2]Experiments should be performed with a minimum of 2 biological replicates. The following negative controls are recommended: 1) empty vector and/or 2) FLAG epitope fused to a fluorescent protein (e.g. EGFP) to identify non-specific binders.

[3]Tetracycline can be substituted for doxycycline at the same concentration. Prepare 10 mg/mL stock and add 2 µL per 20 mL media. Precipitation may occur with long-term storage but should not affect your experiments. Prepare aliquots to avoid freeze-thawing and warm the solution to room temperature prior to use.

[4]Autoclaving may result in a residue being deposited on to plasticware, which may result in contamination during MS analysis. As sterility is not essential for this protocol (other than during cell culture), use tips and tubes directly as received from the manufacturer. Keep all plasticware and reagents protected from dust and other environmental contaminants as much as possible and use gloves for all preparation steps.

[5]Pellet weight should be a minimum of 0.25 g (after wash is fully aspirated) to ensure sufficient starting material. In HEK293 cells, this translates to 6 to 10 mg total protein in the lysate. As tube weights vary, it is recommended to weigh the tube prior to adding cells to get a more accurate weight.

[6]Protocol for sonication has been optimized using a Q500 Sonicator (QSonica, Cat# 4422). 250U of Benzonase may be substituted for TurboNuclease.

[7]An aliquot should be taken of the pellet and supernatant to monitor expression, lysis efficiency and solubility of the bait on a gel. To represent the same amount of sample in soluble and pellet fractions in western blot diagnostics, take an aliquot of total lysate and transfer to a microcentrifuge tube, then centrifuge this together with the rest of the lysate in step 12. After centrifugation, transfer the supernatant from this aliquot to a new tube, labeled 'sup' and freeze it along with the pellet at the bottom of the initial microcentrifuge tube that can be labeled as 'pellet'. For the supernatant, transfer 20 µl to a new tube and add 2x Laemmli sample buffer. Resuspend the pellet in 40 µl in 2x Laemmli sample buffer. Analyze both aliquots by immunoblotting using an M2 anti-FLAG antibody. Since the 'sup' and 'pellet' came from the same aliquot, analyzing this 1:1 ratio will yield normalized diagnostics on bait/prey solubility. An aliquot of post-IP supernatant (unbound fraction) should also be compared to pre-IP supernatant to monitor the efficiency of immunoprecipitation.

[8]If the scale of cell material used is altered, the amount of beads may need to be re-optimized.

[9]The stringency of the washes can be increased by washing the beads up to three times with 1 ml lysis buffer, followed by up to two times with FLAG magnetic rinsing buffer. Extra washes will affect the recovery of interactors so this should be carefully tested.

[10]For on-bead trypsin digestion, we use a "rotating drum" style rotator (Cel-Gro Tissue Culture Rotator, ThermoScientific) placed inside a 37°C incubator.

[11]50% stocks of Formic Acid can be prepared in HPLC-grade water and stored in clean glass vials (Cat #14-955-319, Fisher Scientific). 50% stocks should be replaced every 4 to 6 months.