NBCC Proteomics Protocol 5
Version – January 8, 2019
Adapted from Protocols in the Gingras lab (see Notes 1 to 3)
Preceding NBCC Protocols – Protocol 3 (Flp-IN T-REx Cell Preparation) or Protocol 4 (Lentiviral Cell Preparation)

Cell Lysis and Streptavidin Purification

Cell Lysis:

1. Resuspend each frozen cell pellet in ice-cold modified RIPA lysis buffer at a 1:4 pellet weight:volume ratio.
2. Sonicate cells for 15 sec (5 sec on, 3 sec off for three cycles) at 30% amplitude on a Sonicator with 1/8" microtip (see Note 4). Keep samples on ice.
3. Add 250 U of TurboNuclease and 10 µg of RNase and rotate (end-over-end) at 4°C for 15 min (see Note 5).
4. Increase the SDS concentration to 0.4% (by the addition of 10% SDS) and rotate at 4°C for 5 min.
5. Centrifuge at 20,817x g for 20 min at 4°C and transfer supernatant to a 2 ml centrifuge tube (see Note 6).

Streptavidin Purification:

6. Prepare a master mix of streptavidin beads using 35 µl of slurry (20 µl bed volume) for each sample plus 10% to account for any losses. Wash streptavidin agarose beads 3 times with 1 ml lysis buffer (minus proteinase inhibitors, PMSF and deoxycholate). To wash beads, mix by inversion, centrifuge 400x g, 1 min and aspirate supernatant. Following the last wash, resuspend beads as a 50% slurry.
7. Add 40 µl of the 50% slurry of streptavidin beads to the clarified supernatant and rotate using gentle end-over-end rotation for 3 hours at 4^{o}C (see Note 7).
8. Pellet beads by centrifugation at 400x g for 1 min at 4^{o}C. Option to take post-IP aliquot for IP efficiency (see Note 6). Aspirate supernatant. Transfer beads in 1 ml fresh RIPA-wash buffer to a new microcentrifuge tube (this removes 'sticky' proteins bound to side of tube).
9. Wash beads once with SDS-Wash buffer, twice with RIPA-wash buffer, once with TNNE buffer and three times with 50 mM ammonium bicarbonate pH8.0 (ABC) buffer. For each wash step, mix by inversion, centrifuge 400x g, 1 min and aspirate supernatant.

Trypsin Digest:

10. Remove residual ABC buffer by pipette.
11. Resuspend beads in 70 µl of 50 mM ABC buffer and add 1 µg trypsin dissolved in 50 mM ABC buffer. Incubate at 37^{o}C overnight with agitation (see Note 8).
12. Add an additional 0.5 µg of trypsin and incubate for a further 3 hours.
13. Centrifuge beads (400x g, 2 min) and collect supernatant in a new 1.5 ml tube.
14. Wash beads twice with 150 µl mass spectrometry grade H_20 (pelleting beads in between) and pool the wash supernatant with the peptide supernatant.
15. Centrifuge supernatant at 16,100x g for 10 min and transfer most of the supernatant (leaving ~30 µl residual so as not to transfer beads) to a new tube.
16. Lyophilize the pooled supernatant using vacuum centrifugation without heat.
17. Store dried peptides at -80^{o}C until ready for mass spectrometry analysis.

Buffers

Modified RIPA Lysis Buffer

50 mM Tris-HCl, pH 7.4
150 mM NaCl
1 mM EGTA
0.5 mM EDTA
1 mM $MgCl_2$
1% NP40
0.1% SDS
0.4% sodium deoxycholate
1 mM PMSF (add fresh)
1x Protease Inhibitor cocktail (add fresh)

SDS-Wash buffer

25 mM Tris-HCl, pH 7.4
2% SDS

RIPA-wash buffer

50 mM Tris-HCl, pH 7.4
150 mM NaCl
1 mM EDTA
1% NP40
0.1% SDS
0.4% sodium deoxycholate

TNNE-wash buffer

25 mM Tris-HCl, pH 7.4
150 mM NaCl
0.1% NP40
1 mM EDTA

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
Bir*-FLAG and BioID2-FLAG parent vectors	Gingras Lab	Available upon request
Ammonium Bicarbonate	Bioshop	05-402-7
Monoclonal anti-FLAG M2 antibody	Sigma-Aldrich	F3165
Protease Inhibitor Cocktail	Sigma-Aldrich	P8340

RNase	Bio Basic	RB0473
Streptavidin Agarose beads	GE Healthcare Life Science	17511301
Trypsin	Sigma-Aldrich	T6567
Turbonuclease	BioVision Inc.	9207

Notes

[1]This protocol is designed to work with BirA*-FLAG and BioID2-FLAG-tagged constructs that have been stably transfected into a Flp-IN T-REx cell line (NBCC Protocol 3) or transduced by lentiviral infection (NBCC Protocol 4). More notes on the BioID method are discussed in Hesketh et al., Methods Mol Biol. 2017;1550:115-136.

[2]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, 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.

[3]Pellet weight should be a minimum of 0.1 to 0.15g (after wash is fully aspirated) to ensure sufficient starting material. In HEK293 cells, this translates to 3 to 5 mg total protein in the lysate.

[4]Protocol has been optimized using a Q500 Sonicator (QSonica, Cat# 4422).

[5]To simply this protocol, steps 3 to 4 can be merged by including nucleases in lysis buffer and starting with 0.4% SDS in the lysis buffer. As written, this protocol is optimal for nuclease action. 250U of Benzonase may be substituted for TurboNuclease.

[6]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 5. 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.

[7]The biotinylated protein capture can be extended to overnight. If the scale of cell material used or biotin labeling times are altered, the amount of beads may need to be re-optimized.

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