NBCC Proteomics Protocol 4 (see Notes 1 and 2)
Version – January 4, 2019
Adapted from Protocol by Payman Samavarchi-Tehrani (Gingras lab)

Reference: Samavarchi-Tehrani, P. *et al.* Mol Cell Proteomics 2018 Nov;17(11):2256-2269

Lentiviral Production

Day 1:

1. Plate HEK293T cells in 6 well dishes so they are 85% confluent on day of transfection.

Day 2 (or Transfection Day):

2. Transfect cells with 1.3 µg of psPAX2, 0.8 µg of pCMV-VSV-G and 1.3 µg of BirA*-FLAG transfer vector encoding the gene of interest using the jetPRIME reagent as per the manufacturer's recommendations (see Note 3).

Day 3:

3. Ten hours after transfection, replace the media with 3 ml of virus production media (DMEM supplemented with 5% FBS and 50 U/ml Penicillin-Streptomycin solution).

Day 5:

4. Harvest media containing virus 36 to 40 hours post-media change.
5. Clarify by centrifugation (500x g, 5 min) and filter through a 0.45 µm filter.
6. Virus can be stored up to 1 week at 4°C or for several months at -80°C (see Note 4).

Lentiviral Titering (see Note 5)

Day 1:

7. Plate cells (such as HeLa cells) in a 24-well plate so they are 40% confluent on day of infection.

Day 2 (or Transduction Date):

8. Infect cells with a range of 25 to 100 µl of the BirA*-Flag tagged bait containing lentivirus and if required, the rtTA viral supernatant (see Note 6).

Day 3:

9. Replace media with media containing 1 µg/ml doxycycline and 40 µM biotin (See Notes 7 and 8).
10. Incubate for 24 hours for transgene induction and biotin labeling.

Day 4:

11. Wash the cells once in PBS++ (PBS with Calcium and Magnesium).
12. Fix in 4% paraformaldehyde in PBS++ for 10 min.
13. Wash cells in PBS and permeabilize with 0.25% NP-40 in PBS for 10 min.
14. Block in 2.5% bovine serum albumin (BSA) in PBS for 0.5-1 hour.
15. Stain cells with 1:2000 mouse anti-FLAG antibody in blocking buffer for 1 hour.

16. Wash 3 x PBS for 5 min each.
17. Incubate cells for 1 hour with 1:1000 goat anti-mouse Alexa FluorTM 488 and Alexa 1:2500 FluorTM 594 streptavidin conjugate to localize the sites of *in vivo* biotinylation and 1:20,000 of 20 mg/ml DAPI as a nuclear counterstain.
18. Determine the amount of viral supernatant that yields 75-85% infection rate as evidenced by positive FLAG and streptavidin staining.

Lentiviral Transduction for BioID and *In Vivo* Labelling

Day 1:

19. Plate cells in a single 10 cm dish so they will be 35-40% confluent on date of transduction.

Day 2 (or Transduction Date) and onwards:

20. Infect cells with the BirA*-tagged bait lentiviral supernatant, and rtTA if required, with the appropriate amount of virus to yield 75-85% infection.
21. When cells are ready to split, scale up the culture dish to one 15 cm plate for each biological replicate (see Note 9).
22. When cells reach 75% confluency, induce bait expression with 1 µg/ml doxycycline and add 40 µM biotin for 24 hours for labeling (See Note 10).

Cell harvest:

23. For harvesting, wash each plate with 2 x 10 ml ice-cold PBS. Keep plates cold and harvest a manageable number of plates to minimize harvesting time.
24. Using a cell scraper, scrape the cells in 1 ml of PBS and transfer to a pre-weighed 2 ml microfuge tube (See Note 11).
25. Using an additional 500 µl of PBS, collect the residual cells and pool and add to the microfuge tube.
26. Centrifuge the cells at 500x g for 5 minutes to collect the cells. Carefully remove all supernatant by aspiration. Weigh cell pellets and record weight on tube (see Note 12).
27. Flash freeze the tubes on dry ice and store at -80°C.

For streptavidin enrichment and preparation of samples for mass spectrometry, proceed to NBCC Protocol PR5.

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
Lentiviral Bir*-Flag parent vectors	Network Biology Collaborative Centre/Gingras Lab	nbcc.lunenfeld.ca/resources for vector list
Alexa FluorTM 594 streptavidin conjugate	ThermoFisher Scientific	S11227
Biotin	BioBasic	58-85-5
DAPI	Roche	10236276001
Doxycycline	Sigma-Aldrich	D9891

Goat anti-mouse Alexa FluorTM 488	ThermoFisher Scientific	A11001
HEK293T cells	ATCC	CRL-3216
HeLa cells	ATCC	CCL-2
jetPRIME reagent	Polyplus-transfection SA	114-01
Monoclonal anti-FLAG M2 antibody	Sigma-Aldrich	F3165
pCMV-VSV-G	Addgene	8454
psPAX2	Addgene	12260
rtTA-N144	Addgene	66810

Notes

[1]This protocol has been optimized for use with lentiviral BirA* vectors developed by the Gingras lab for BioID with the described reagents. Vector descriptions and ordering information are provided at nbcc.lunenfeld.ca/resources. The protocol will need to be optimized if other reagents are used.

[2]All experiments must be conducted in accordance with proper health and safety guidelines. Please consult your Institute's biosafety officer to ensure that you are properly trained to work with lentiviruses.

[3]Different transfection reagents can be used, however, the quantity of DNA used may need to be optimized.

[4]The titer will decrease with time and with freeze/thawing.

[5]Here we describe assessing viral titer using staining of infected cells in 24-well plates. However, other techniques such as flow-cytometry may be employed.
Due to differences in transduction efficiency of various cell types, it is recommended to assess viral titer in the cell types that will be used for the BioID experiment.

[6]If your vector does not contain the rtTA transactivator required to induce expression of the BirA*-tagged gene, you will need to infect cells with an rtTA viral supernatant (see reagent list for rtTA-N144). 50-57 µl of the rtTA viral supernatant is generally sufficient for testing of the viral titer for the bait genes in a 24-well plate.

[7]Prepare 10 mg/mL doxycycline stock and add 2 µL per 20 mL media. Precipitation of stock 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. Tetracycline can be substituted for doxycycline at the same concentration.

[8]Biotin stock solution (for 20 mL of 20 mM which is a 400× stock): add 2 mL of 30% NH_4OH to 100 mg of biotin and place on ice. Slowly add 5 mL of 1 N HCl, wait 5 min, and repeat for a total of 18 mL added. Store at 4°C protected from light. Extreme care must be taken in preparing biotin solutions, as it can easily precipitate from the solution if the pH is raised too rapidly or if it is not kept adequately chilled during preparation (this may be reversed by the re-addition of a small amount of ammonium hydroxide). In our experience, the biotin stock solution is very stable and can be used for approximately 6

months to a year without any change in potency when prepared and stored appropriately.

[9]Experiments should be performed with a minimum of 2 biological replicates. The following controls are recommended: 1) No BirA* control that will identify endogenously modified proteins and background binding to streptavidin beads; 2) BirA* expressed alone or fused to a fluorescent protein (e.g. EGFP) to identify non-specific biotinylation; and 3) if a specific cell compartment is under investigation, it may be appropriate to include a compartment specific control (e.g. BirA* fused to a Nuclear Localization Signal sequence).

[10]The recommended transgene induction period is 24 hours. Biotin can be added simultaneously for a full 24 hour biotin labeling. However if shorter biotinylation duration is desired, biotin can be added into the media at a later time point.

[11]Autoclaving tips and tubes may result in a residue being deposited on to plasticware, which may result in contamination during MS analysis. As sterility is not essential for the protocols (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.

[12]Following freezing of the cell pellets, the tubes can we reweighed to obtain the pellet weight. Pellet weight should be a minimum of 0.1 to 0.15 g (after PBS is fully aspirated) to ensure sufficient starting material. As tube weights vary, it is recommended to weigh the tube prior to adding cells to get a more accurate weight. Comparable starting material (based on pellet weight) will contribute to improved reproducibility and comparative data analysis.