BioID PULL-DOWN TO IDENTIFY CANDIDATE PROTEINS

Roux, K. J., Kim, D. I., Burke, B., & May, D. G. (2018). Current Protocols in Protein Science, 91, 19.23.1–19.23.15.

This protocol describes the utilization of cells stably expressing a BioID fusion protein (along with BioID-only control cells) to perform large-scale BioID pull-down experiments. The purpose of these experiments is to isolate sufficient amounts of proteins biotinylated by the BioID fusion protein to be identified by mass spectrometry. The starting material for these experiments may vary depending on a number of factors. These include the efficiency of biotinylation by the BioID fusion protein and the number of desired candidate proteins. This protocol describes the analysis of four confluent 10-cm plates of cells/condition (4×10^7 cells); however, comparable results can be obtained with two confluent 10-cm plates of cells/condition. In this case, the volume of Triton X-100 added (step 7), lysis diluent (step 10), and streptavidin beads (step 15) is halved. The protocol ends immediately prior to analysis by mass spectrometry, a service typically performed by a core facility.

Materials

-Four 10-cm dishes of cells for each experimental condition (cells expressing BioID constructs or control cells)

-Complete medium

-1 mM (20×) biotin (see recipe)

-Phosphate-buffered saline (PBS)

-Lysis buffer (see recipe)

-20% Triton X-100

-50 mM Tris·Cl, pH 7.4

-Streptavidin-Sepharose High Performance Beads (GE Healthcare)

-Wash buffer (see recipe)

-1 mM biotin in 20 mM ammonium bicarbonate (NH_4HCO_3), made freshly

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-1× SDS-PAGE sample buffer

-DNase/RNase-free tubes, screw cap 5- and 15-ml conical tubes and 1.5 ml lobind tubes

-Sonicator (Branson Sonifier-250 or equivalent)

-Rotator

Day 1: Perform cell lysis

Lysis and wash steps are performed at room temperature. To reduce keratin contamination, use DNase/RNase-free tubes that have not previously been opened, wear gloves.

1. Begin with four 10-cm dishes for each experimental condition (cells expressing BioID fusion protein or BioID-only constructs).

2. When cells reach approximately 80% confluency, change medium to fresh complete medium containing 50 µM biotin (1×).

3. Incubate cells for 24 hr.

Incubation time and conditions may vary depending on the goals of the experiment (e.g., cell cycle stage-specific labeling). In general, 16 to 18 hr of biotin results in a maximal labeling, whereas after 6 hr of biotin there is a considerably reduced level of biotinylated proteins (approximate 25%). Pilot studies with immunofluorescence and immunoblot analysis should be performed prior to large-scale experiments.

4. Remove medium completely by aspiration and rinse the cells 3 times at room temperature with 10 ml/dish of PBS.

This step is important in that it removes residual free-biotin from the medium.

5. Add 540 µl of lysis buffer/dish and scrape cells gently to harvest the cells. Perform this step at room temperature.

The purpose of this harsh lysis is to try to disrupt all protein interactions and completely denature/solubilize the proteins.

6. Transfer lysed cells to a 15-ml conical tube.

7. Add 120 µl of 20% Triton X-100 (final concentration 1%) and mix by trituration. Keep tube on ice during subsequent sonication.

8. Position the sonicator probe tip in the sample just above the tube bottom.

9. Apply sonication for two sessions with 60 pulses using a Branson Sonifier 250 (or equivalent) at 30% duty cycle and an output level of 4. Let the tube sit on ice for 2 min between each session to prevent overheating.

If the sample is still viscous and cloudy after sonication, apply an additional period of sonication. Sonication also functions to shear DNA.

10. Add 2.52 ml of prechilled lysis buffer, and mix well.

This dilution provides more favorable conditions for affinity capture.

11. Apply one session of sonication (60 pulses at 30% duty cycle and an output level of 4, using a Branson Sonifier 250 or equivalent).

This step helps solubilize any precipitated proteins that may be present due to reduced detergent concentrations from the previous step and assists in mixing the sample.

12. Aliquot the sample evenly into four prechilled 1.5 ml tubes (1.2 ml each).

13. Spin down 10 min at 16,500 × g, 4°C.

Perform affinity purification of biotinylated proteins

14. During the centrifugation in step 13, add 1 ml of room temperature lysis buffer to one 5 ml tube.

15. Mix the stock of streptavidin beads well with gentle tapping to resuspend and add 100 µl of beads to the tube prepared in step 14.

This step functions to equilibrate the beads in the binding buffer. Earlier protocols utilized paramagnetic streptavidin Dynabeads, which interfere with mass spectrometric analysis.

16. Centrifuge the equilibrated beads for 2 min at 1000 × g, then remove the supernatant gently by pipetting.

This step removes the buffer in which the beads equilibrated.

17. After sample centrifugation (step 13), carefully transfer supernatants to the tubes prepared in step 16. Do not disturb the small insoluble pellet on the tube wall when removing the supernatant.

Step 17 should be performed quickly to prevent the beads from drying out.

18. Resuspend the samples and beads with gentle pipetting.

19. Incubate the tube on a rotator at 4°C overnight.

Day 2: Bead washing

20. Spin the tubes for 5 min at 1000 × g to collect streptavidin beads.

21. Remove the supernatant gently by pipetting. Try not to disturb beads.

22. Add 1 ml of wash buffer to each tube, resuspend beads gently by flicking the tube, and transfer to a 1.5-ml microcentrifuge tube.

Avoid pipetting the beads excessively, as they can bind to the pipet tips, leading to sample loss.

23. Place tubes on a rotator for 8 min at room temperature.

24. Centrifuge for 2 min at 1000 × g and remove supernatant.

25. Repeat steps 21 to 24 three more times (four washes total).

26. Resuspend with 1 ml wash buffer.

27. Save 100 µl (10% of total) of resupended beads for further analysis by western blot. The remaining 900 µl are the beads destined for mass spectrometry analysis.

28. Centrifuge to collect beads as described in step 24 (both 900 µl and 100 µl tubes).

29. Remove the supernatant completely. Do not disrupt beads at the tube bottom.

30. Add 50 µl of 1 mM biotin in 20 mM ammonium bicarbonate to the tube that contained 900 µl of sample (destined for mass spectrometry analysis) and resuspend gently with flicking. Incubate at room temperature for 5 minutes. Proceed with 5 washes of 1 ml of 20 mM ammonium bicarbonate. Remove the supernatant completely. Store at -80°C for subsequent on-beads digestion.

Biotin binds all remaining unbound streptavidin to stabilize the protein and prevent tryptic-released peptides that are biotinylated from binding the beads. The final wash buffer may vary depending on analysis method. Before mass spectrometry analysis, freeze the sample quickly in liquid nitrogen. Frozen samples can be stored at −80°C until biotinylation has been confirmed by immunoblot.

31. For the 100-µl sample, after removing supernatant, resuspend beads in 50 mM Tris·Cl, pH 7.4 to remove urea.

32. Collect beads by centrifugation (2 min, 1000 × g) again and remove the supernatant.

33. Add 100 µl of 1× SDS-PAGE sample buffer. Heat samples at 98°C for 5 min. Samples can be stored at −20°C for further analysis by immunoblot.

Prior to sending samples for analysis by mass spectrometry, it is recommended to perform immunoblot analysis (Ni et al., 2017) of 15 µl from the protein in the SDS-PAGE sample buffer. This should follow the same protocol to check for evidence of biotinylated proteins from BioID fusion proteins. If immunoblot shows clear evidence of biotinylation in control and BioID fusion protein samples, proceed to mass spectrometry.

REAGENTS AND SOLUTIONS

Use MS-grade water in all recipes.

Biotin, 1 mM (20×)

Dissolve 12.2 mg biotin (Sigma, cat. no. B4501) in 50 ml of serum-free DMEM (or standard tissue culture medium). Pipetting may be required to dissolve biotin completely. Sterilize by passing through a 0.22-µm syringe-driven filter unit (Millex). Dispense into sterile 50-ml tube; cap tightly. Store up to 8 weeks at 4°C.

Lysis buffer

-8 M urea in 50 mM Tris·Cl, pH 7.4

-1× protease inhibitor (Halt Protease Inhibitor Cocktail, EDTA-Free, Thermo Scientific)

-1 mM dithiothreitol (DTT)

Prepare fresh

Wash buffer

-8 M urea in 50 mM Tris·Cl, pH 7.4

Prepare fresh