Supplementary Protocol – RaPID-Western

A. Generate pMotif construct
Clone BoxB stem loop (GCCCTGAAAAAGGGC) adjacent to RNA of interest

B. Transfection/Infection
Depending on the cell type, transfect or infect BoxB. In this protocol, we will use HEK293T and RaPID labeler plasmid (E.coli BirA*)
- Day 0 – Plate 300K HEK293T cells per well in a 6 well plate
- Day 1 – Transfect 2ug of pMotif plasmid with 0.3ug of RaPID labeler plasmid
- Day 2 – Make labeling media. Dilute 20x Biotin (4mM Biotin) with DMEM to make labeling media. Aspirate out existing media and add labeling media to each well.
- Day 3 – Processing Samples

C. Processing Samples
Make Lysis buffer fresh. Wash Buffer 1 can be made and stored at room temperature. Wash Buffers 2-4 can be made in bulk and stored at 4C.

1. Aspirate out labeling media. Add cold 1xPBS and gently mix. Aspirate out 1xPBS
2. Add 500uL cold 1xPBS to each well and scrape cells. Collect in individual eppendorf tubes
3. Spin cells down at 500g at 4C
4. Asplirate out PBS

STOP Point: Eppendorf tubes can be stored at -80C for processing later

5. Lyse cells in each tube with 200uL Lysis buffer at room temperature.
6. Add 20uL 25% Triton X-100. Mix thoroughly
7. Add 220uL cold Wash Buffer 4
8. Sonicate samples throughly – 10% for 10s. Ensure lysate is clear after sonication. If lysate is not clear, cool for 5s and repeat sonication
9. Spin lysate at 15,000 g at 4C for 10 minutes
10. Carefully transfer supernatant to 3K MWCO filter tube

11. Spin filter tubes at 1500g at 4C for 1hour
12. Pipette retente into new eppendorf tubes.
13. Estimate protein concentration in each tube and use Wash Buffer 4 to equalize concentration across all samples
14. After equalizing concentration, pipette 20uL into a new tube and label as Lysate_Sample
15. Calculate amount of protein in each sample. Calculate the amount of beads required for each sample.

Use 50uL of MyOne C1 Streptavidin beads per mg of protein. Ensure a minimum of 30uL of beads is used per sample to capture biotinylated proteins and account for loss of beads during processing.
16. Wash the MyOne C1 beads in Wash Buffer 4×2
17. Prepare fresh set of tubes and label them Pulldown_Sample. Add equal volume of sample and beads to each tube.
18. End-over-end rotation for 1hr at room temperature OR overnight at 4C

Washing
19. Place tubes on magnetic stand and aspirate out lysate
20. Add 1mL of Wash Buffer 1 to each tube and perform end-over-end rotation for 5 mins
21. Place tubes on magnetic stand and aspirate out Wash Buffer 1
22. Add 1mL of Wash Buffer 1 to each tube and perform end-over-end rotation for 5 mins
23. Place tubes on magnetic stand and aspirate out Wash Buffer 1
22. Add 1mL of Wash Buffer 2 to each tube and perform end-over-end rotation for 5 mins
23. Place tubes on magnetic stand and aspirate out Wash Buffer 2
24. Add 1mL of Wash Buffer 3 to each tube and perform end-over-end rotation for 5 mins
25. Place tubes on magnetic stand and aspirate out Wash Buffer 3
26. Add 1mL of Wash Buffer 4 to each tube and perform end-over-end rotation for 5 mins
27. Place tubes on magnetic stand and aspirate out Wash Buffer 4

28. Add 40uL of Elution Buffer to each tube and heat at 98C for 20 minutes.

29. Run Western Blot for Pulldown & Lysate for samples

Reagents

• Biotin (Sigma B4501)
• Macrosep Advance Spin Filter 3K MWCO 20 mL Capacity 24/pack (VWR 89131-974)
• Dynabeads® MyOne™ Streptavidin C1 (Life Technologies #65002)
• UltraPure™ SDS Solution, 10% (ThermoFisher #24730020)
• DTT, 1M (ThermoFisher P2325)
• Sodium Deoxycholate, 10% Solution (bioworld #40430018)
• UltraPure™ 1 M Tris-HCl Buffer, pH 7.5 (ThermoFisher #15567027)
• 10% Nonidet P40 (Sigma #11332473001)
• LiCl 7.5 M Solution
(ThermoFisher AM9480)
• NaCl 5M Solution (ThermoFisher AM9759)
• HEPES, 0.5M buffer solution, pH 7.5 (Alfa Aesar J61275)
• Triton X-100 (Sigma T8787)
• UltraPure™ 0.5M EDTA, pH 8.0 (ThermoFisher #15575020)

Reagent Setup

4mM Biotin Labeling Solution (20x)
- Dissolve 50mg of biotin in 50mL of serum-free media
- Pipette to completely dissolve biotin
- Store in sterile 50mL tube and store at 4C. Can be stored for 2 months.

Lysis Buffer (0.5M NaCl, 50mM Tris-HCl, 0.2% SDS, 1mM DTT)
Wash Buffer 1 (2% SDS)
Wash Buffer 2 (0.1% Na-DOC, 1% Triton X-100, 0.5M NaCl, 50mM HEPES pH 7.5, 1uM EDTA)
Wash Buffer 3 (0.5% Na-DOC, 250uM LiCl, 0.5% NP-40, 10mM Tris-HCl, 1uM EDTA)
Wash Buffer 4 (50mM Tris-HCl)
Elution Buffer (1x LDS, 0.02M DTT, 4mM Biotin)