Crosslinked RNA-protein pulldown protocol
in vivo Interactions by Pulldown of RNA (vIPR)

- based on Rajewsky et al. 2019
- adapted by Ben McCarthy and Eli Waite in Ahmed lab, June 2024

Note

The main difference is that we avoid using liquid culture to grow worms, since this induces a period of starvation that does not reflect the native context of the worms in which we perform our studies (i.e. lifespan assays, environmental stress tests, etc.). Information about where to find reagents and equipment is also provided where useful.

Reagents and Equipment

M9 (MgSO4 added, see recipe in recipe box)
0.1M NaCl
Sterile serological pipets and pipet controller (or rubber bulb)
Large Glass Test Tubes (in Plasticware cabinet)
UV Crosslinker 254nm (Marzluff lab)
Mortar and pestle
Liquid Nitrogen
Pulldown Lysis Buffer (50 mM Tris–Cl, pH 7.0, 10 mM EDTA, 1% SDS, 1 mM DTT, 1 mM PMSF, 1 µg mL−1 Pepstatin A, 1 tablet Complete EDTA-free Protease Inhibitor (Roche), 0.1 U µL−1 RiboLock RNase Inhibitor (Thermo Fisher Scientific))
20 gauge needle
25 gauge needle
Centrifuge
Syringes and 0.2 µm Minisart syringe filters, Sartorius
Pierce BCA Protein Assay Kit (Thermo Fisher Scientific)
Lysis Buffer
Pulldown Hybridization Buffer (750 mM NaCl, 1% SDS, 50 mM Tris–Cl, pH 7.0, 1 mM EDTA, 15% formamide)
MyOne Streptavidin C1 magnetic beads (Thermo Fisher Scientific; 100 µL per 1 mL lysate with protein concentration 2 µg µL−1)
Hybridization Oven (under our microcentrifuge)
Magnet (DynaMag-15, Thermo Fisher Scientific)
Biotinylated DNA Probes
Wash Buffer (2× SSC, 0.5% SDS)

Protein LoBind tubes (Thermo Fisher Scientific)
A second magnet? (DynaMag-2, Thermo Fisher Scientific)
Benzonase elution buffer (10 mM Tris–Cl, pH 7.5, 1 mM MgCl2, 1 mM DTT, 0.625 U µL−1 Benzonase (Millipore, 71205-3)) **for protein elution**
Proteinase K buffer (100 mM NaCl, 10 mM Tris–Cl, pH 7.0, 1 mM EDTA, 0.5% SDS, 1 mg mL−1 Proteinase K (Roche)) **for RNA isolation**
1 µL GlycoBlue (ThermoFisher Scientific) **for RNA isolation**
Trizol RNA isolation (Thermo Fisher Scientific) **for RNA isolation**

Protocol

Probe design – Follow probe design guidelines given in Chang et al. 2012 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3460573/). The guidelines are repeated here:

1. Design anti-sense oligo probes using the online probe designer at singlemoleculefish.com

2. Use these parameters: number of probes = 1 probe /100 bp of RNA length; 2) Target GC% = 45; 3) Oligonucleotide length = 20; 4) Spacing length = 60-80. Break RNA into segments if too long for the designer. Omit regions of repeats or extensive homology.
3. Order anti-sense DNA probes with BiotinTEG at 3-prime end. Mix probes to obtain a final probe concentration of 100µM (if 10 probes, 10µM each) in nuclease-free TE pH 8.0.
4. **Label probes according to their positions along the RNA. Separate them into two pools so that the "even" pool contains all probes numbering 2, 4, 6, etc. and the "odd" pool contains probes numbering 1, 3, 5, etc. Dilute pool of probes to 100 µM concentration and store at -20 °C.
5. **All experiments are to be performed using both pools, which serve as internal controls for each other. Real RNA-dependent signal would be present from both pools, while probe-specific noises would be unique to each pool. This applies for both ChIRP-qPCR and ChIRP-seq.
   ** these steps can be omitted since we are not doing ChIRP

Preparation of worm pellets

1. Grow worms on 60mm NGM plates seeded with OP50 until plates have ample worms but are not starved. Strains differing in growth rate and/or fecundity must be grown to compensate for these differences (i.e. staging L4 parents on different days and/or using different numbers of parents). Plates will have mixed stages of worms when harvested (1 whole box of plates per strain?).

Get enough unseeded NGM 2% agarose plates from the cold room for crosslinking (about 4-5 per strain)

Do all the following steps for one strain at a time:

Add 1uL MgSO4 per 1 mL M9 to the M9 (should be 100 uL MgSO4 added per bottle)

2. Wash worms off plates using M9 (or 0.1M NaCl as in original protocol) and collect all worms from plates of the same strain in a single large glass test tube (may need to remove supernatant as worms are collected). Remove supernatant.
3. Perform 2-3 washes of the entire test tubes of worms using M9 or 0.1M NaCl. Remove supernatant, leaving about 1mL of worms.
4. Transfer worms to unseeded 60 mm 2% Agarose NGM plates (put about 250 uL of worms per plate) and UV crosslink at 254 nm. Set Power to 9999.
5. Wash worms twice more in M9 or 0.1M NaCl?
6. Pellet worms, remove buffer, and transfer into 2.5 mL tubes. Freeze cross-linked worms pellets in liquid nitrogen. Keep pellets at -80C until ready to continue with worm lysis.

Worm lysis

7. Make about 10 mL Lysis Buffer per sample. Set aside 5 mL of buffer for the protein assay. Make 5 mL pulldown buffer per sample. Wait to make the Proteinase K buffer and Benzonase buffer.
8. Grind worm pellets with mortar and pestle in liquid nitrogen. Resuspend worm powder in ~7X volume pulldown lysis buffer (1 mL of lysis buffer per sample) and incubate for 30min on ice.
9. During incubation, pass lysates through a 20 gauge needle 4X and through a 25 gauge needle 3X.
10. Clear lysates by centrifugation (28,900 × g, 4 °C, 30 min). Filter supernatant through 0.2µm syringe filters (to new tubes) and discard pellet from the centrifugation step.
11. For each sample, take two 12 µL samples into PCR tubes. Add 108 µL lysis buffer to each sample. These are the diluted samples that will be assayed.
12. Determine protein concentrations using the Pierce BCA Protein Assay Kit. Adjust concentrations using lysis buffer so final protein concentrations are equivalent across all samples (typically 2-3.5 µg/µL)

13. Dilute lysates by adding 2x volume of pulldown hybridization buffer.

Preparation of beads and preclearing

14. Wash MyOne Streptavidin C1 Magnetic Beads (100µL per 1mL lysate with protein concentration 2 µg/µL) three times in 1x original volume lysis buffer. Resuspend in 0.5x volume lysis buffer, and add to lysate.
15. Preclear by incubating tubes at 37C for 1-2 hours under constant rotation.
16. To remove beads from lysates, place tubes on a magnet (DynaMag-15) and transfer lysates to new tubes twice.

Probe hybridization and capture

17. Add probes (50 pmol per 1mL lysate with protein concentration 2 µg/µL) to lysates – remember probes are 100 µM in their stock tubes. Incubate probe + lysate samples at 37C for 2 hours or overnight.
18. To capture probes, add the MyOne C1 beads (prepared as above) to the samples (100 µL beads per 50 pmol probes). Incubate samples at 37C for an hour.
19. Separate beads from lysate (magnet? centrifugation?) and take supernatant samples.
20. Wash beads five times with ~13mL wash buffer (2× SSC, 0.5% SDS). Resuspend beads in 1mL wash buffer and transfer to Protein LoBind tubes.
21. Take 50-100 µL aliquots from each sample to be used for RNA isolation.
22. Remove wash buffer via magnet (DynaMag-2) and resuspend beads in Benzonase elution buffer for protein elution and Proteinase K buffer for RNA isolation.

RNA isolation

23. Beads, input and supernatant samples were resuspended in 100 µL Proteinase K buffer, and incubated at 50 °C for 45 min, shaking at 400 rpm. Proteinase K was inactivated by boiling at 95 °C for 10 min. Samples were chilled on ice, and 1 µL GlycoBlue (ThermoFisher Scientific) was added, followed by Trizol RNA isolation (Thermo Fisher Scientific).

Protein elution

24. Input and supernatant samples (10 µL) were resuspended in 100 µL and beads in 200 µL Benzonase elution buffer. Crosslinked proteins were eluted by incubation at 37 °C for 3 h, shaking at 400 rpm. Beads were separated from elution via magnet, and elution was transferred to new Protein LoBind tubes. Samples were snap-frozen in liquid nitrogen and kept at −80 °C until processed further.