Please cite this article as: Bailey, A. S. *et al.* (2022). Identification of Protein-RNA Interactions in Mouse Testis Tissue Using fRIP. *Bio-protocol* 12(01): e4286. DOI: 10.21769/BioProtoc.4286.

bio-protocol www.bio-protocol.org/e4286 Bio-protocol 12(01): e4286. DOI:10.21769/BioProtoc.4286

Identification of Protein-RNA Interactions in Mouse Testis Tissue Using fRIP

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[Abstract] During development, cells must quickly switch from one cell state to the next to execute precise and timely differentiation. One method to ensure fast transitions in cell states is by controlling gene expression at the post-transcriptional level through action of RNA-binding proteins on mRNAs. The ability to accurately identify the RNA targets of RNA-binding proteins at specific stages is key to understanding the functional role of RNA-binding proteins during development. Here we describe an adapted formaldehyde RNA immunoprecipitation (fRIP) protocol to identify the *in vivo* RNA targets of a cytoplasmic RNA-binding protein, YTHDC2, from testis, during the first wave of spermatogenesis, at the stage when germ cells are shutting off the proliferative program and initiating terminal differentiation (Bailey *et al*., 2017). This protocol enables quick and efficient identification of endogenous RNAs bound to an RNA-binding protein, and facilitates the monitoring of stage-specific changes during development.
Keywords: fRIP, RNA-binding proteins, Protein-RNA interactions, Post-transcriptional regulation, Immunoprecipitation, RNA targets

[Background] RNA-binding proteins (RBPs) play pivotal roles in controlling gene expression programs during development through both co- and post-transcriptional regulation of RNAs, including splicing, export from the nucleus, mRNA stability, subcellular localization, and translation (Glisovic *et al*., 2008). During cell differentiation, RNA-binding proteins can help facilitate sharp and accurate cell state transitions by rapidly promoting translation of a new set of RNAs or by degrading RNAs from the previous cell state. RNA-protein interactions can change rapidly during differentiation. Therefore, to uncover the functional role of RBPs in the dynamic state changes that take place in development and tissue restoration or repair, it is critical to be able to efficiently capture RNA-protein interactions as cells within a tissue proceed through the differentiation process.

Several methodologies have been developed to identify RNA-protein interactions, including RNA immunoprecipitation (RIP) and cross-linking immunoprecipitation (CLIP) protocols, which both facilitate identification of RNAs bound to a protein of interest (Ramanathan *et al*., 2019; Majumder and Palanisamy, 2021). The CLIP method stabilizes RNA-protein complexes through ultraviolet (UV) radiation cross-linking to create an irreversible covalent bond between RNA and protein. However, while

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CLIP-seq allows precise detection of RBP-RNA interaction sites, poor UV cross-linking efficiency and adverse effects of UV cross-linking on the ability of an antibody to detect the protein can sometimes prevent sufficient UV cross-linked complexes from being purified using CLIP protocols. As CLIP may not work for all RNA-binding proteins and in all tissue settings, alternative methods to detect endogenous RNA-protein interactions are needed.

The formaldehyde RNA immunoprecipitation (fRIP) method described here, adapted from a protocol developed by the Rinn laboratory, utilizes a light formaldehyde cross-linking step to preserve endogenous RNA-protein interactions and allows efficient RNA and protein recovery that is specific, reproducible, and quantitative (Hendrickson *et al.*, 2016) (Figure 1). The cross-linking step used in the fRIP protocol provides several advantages over previous RIP-seq protocols, by allowing more stringent washes, enhancing protein-RNA specificity, and preventing post-lysis reassociation between proteins and RNA transcripts. Our modified fRIP protocol enabled us to identify both mRNA and long non-coding RNA (lncRNA) targets of the cytoplasmic RNA binding protein YTHDC2 in the mouse testis during the first wave of germ cell development (Bailey *et al.*, 2017). The fRIP protocol is excellent for cell-type-specific analysis in developing tissues, as it requires less material than CLIP methods and the light formaldehyde cross-linking reduces post-lysis contamination from RNAs expressed in other cell types.

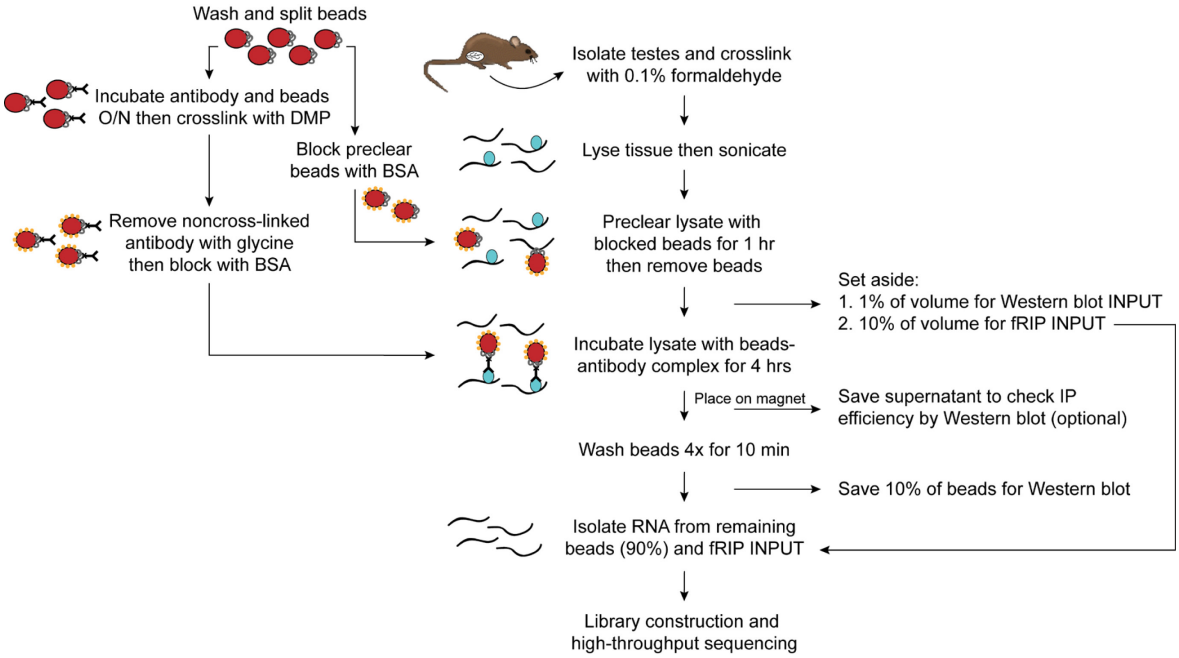

Figure 1. Flowchart of fRIP procedure.

Materials and Reagents

1. 25 G × 5/8 inch needle (Becton Dickinson, catalog number: 305122)
2. 1 mL syringe (Becton Dickinson, catalog number: 309659)
3. 1.7 mL microcentrifuge tubes (Thomas Scientific, catalog number: C2170)
4. Avant 1.7 mL low binding tubes (Midland Scientific, catalog number: AVSC1510)

5. Mice (wild type and mutant for gene of interest)
6. Formaldehyde, 16%, methanol free, Ultra Pure (Polysciences Inc., catalog number: 18814-10)
7. Glycine (Fisher Scientific, catalog number: BP381-500)
8. Phosphate buffer saline, 10× (PBS) (Gibco, catalog number: 70011044)
9. Dynabeads protein A (Invitrogen, catalog number: 10001D)
10. Ythdc2 antibody, 0.2 µg/µL (Bethyl Laboratories, catalog number: A303-025A)
11. Bovine serum albumin (BSA) (Gemini, catalog number: 700-100P)
12. Triethanolamine (Sigma, catalog number: 90279)
13. Dimethyl pimelimidate dihydrochloride (DMP) (Sigma, catalog number: D8388)
14. OmniPur TRIS hydrochloride (Millipore Sigma, catalog number: 9310-OP)
15. Potassium chloride (Fisher Chemical, catalog number: P333-500)
16. Triton X-100 (Sigma, catalog number: T8787)
17. OmniPur glycerol (Millipore Sigma, catalog number: 4750-OP)
18. OmniPur sodium dodecyl sulfate (SDS) (Millipore Sigma, catalog number: 7910-OP)
19. Sodium deoxycholate (Sigma, catalog number: D6750)
20. UltraPure 0.5 M EDTA (Thermo Fisher Scientific, catalog number: 15575020)
21. Sodium chloride (Fisher Chemical, catalog number: S271-500)
22. DTT (Thermo Fisher Scientific, catalog number: P2325)
23. Complete protease inhibitor tablets (CPI) (Roche, catalog number: 05892791001)
24. IGEPAL CA-630 (NP-40) (Sigma, catalog number: I8896)
25. Phenylmethanesulfonyl fluoride (PMSF) (Sigma, catalog number: P7626)
26. RNaseOUT recombinant ribonuclease inhibitor (Invitrogen, catalog number: 10777019)
27. Bradford reagent (Bio-Rad, catalog number: 500-0205)
28. 5× protein loading buffer (Fermentas, catalog number: R0891)
29. 10% Mini-PROTEAN TGX precast protein gels, 50 µL (Bio-Rad, catalog number: 4561034)
30. Proteinase K (Invitrogen, catalog number: AM2546)
31. TRIzoL (Invitrogen, catalog number: 15596026)
32. Chloroform (Millipore Sigma, catalog number: 3150)
33. GlycoBlue coprecipitant (Invitrogen, catalog number: AM9515)
34. Isopropanol (EMD Millipore, catalog number: PX1835)
35. RiboMinus eukaryote system v2 kit (Ambion, catalog number: A15026)
36. TruSeq RNA sample preparation kit (Illumina, catalog number: FC-122-1001)
37. UltraPure DNase/RNase-free water (Thermo Fisher Scientific, catalog number: 10977015)
38. PBST (see Recipes)
39. 0.1% formaldehyde (see Recipes)
40. DMP cross-linking solution (see Recipes)
41. Bead coupling buffer (see Recipes)
42. Lysis buffer (see Recipes)
43. Binding/Wash buffer (see Recipes)

44. Elution buffer (see Recipes)
45. PK buffer (see Recipes)

Equipment

1. P200 pipette (Rainin, catalog number: 17008652)
2. -80°C freezer (Thermo Scientific)
3. Forceps (Electron Microscopy Sciences, catalog number: 72705-01)
4. Bioruptor for sonication (Diagenode Bioruptor, model: UCD-300)
5. 6-tube magnetic stand (Ambion, catalog number: AM10055)
6. Tube rotator, Labquake Shaker (Lab Industries Inc., catalog number: 400-110)
7. Centrifuge (Eppendorf, model: 5415C)
8. Heat block (VWR Scientific Products)
9. Spectrophotometer (Beckman Coulter, model: DU 640)

Software

1. Spliced Transcripts Alignment to a Reference (STAR) (Dobin *et al*., 2013, http://code.google.com/p/rna-star/)
2. HTSeq (Anders *et al.*, 2015, RRID: SCR_005514)
3. DESeq2 (Love *et al.*, 2015, http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html)

Procedure

A. Cross-linking mouse testes
   1. Dissect mouse testes, remove the outer sheath, and carefully tease apart seminiferous tubules using fine forceps (Figure 2).

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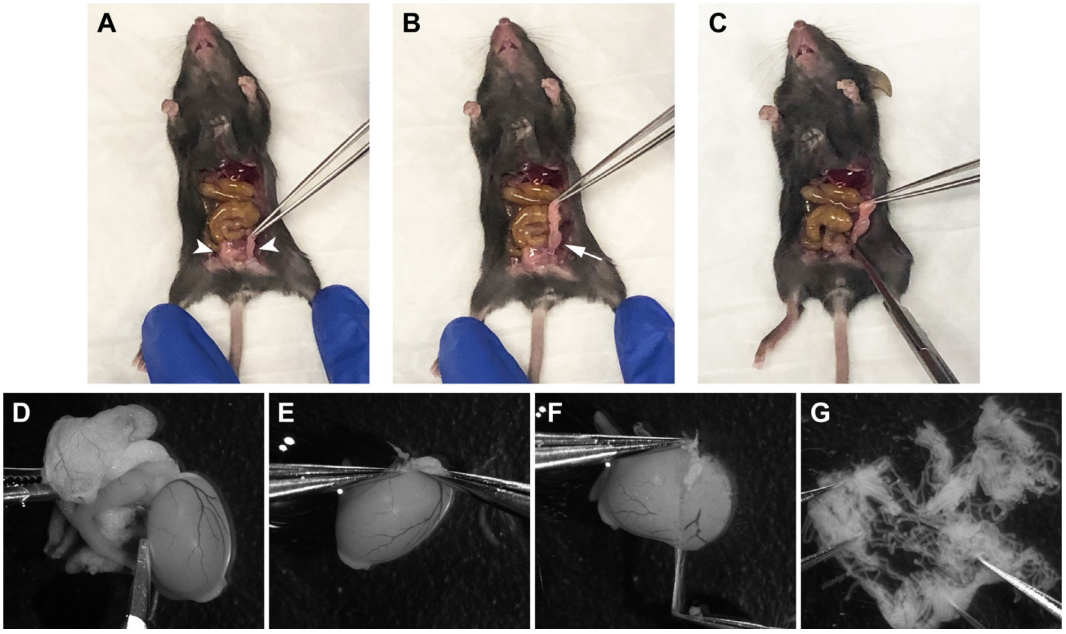

Figure 2. Dissection of mouse testis tubules.

A. After euthanizing the mouse and sanitizing the abdomen with 70% ethanol, use scissors and forceps to open the abdomen and expose the fat pads in the lower peritoneal cavity (white arrowheads). B. Gently pull on the fat pad until the testis is exposed (white arrow). C. Cut the fat below the testis. D. While working under a microscope, cut the fat and epididymis away from the testis. E. Grab the tunica surrounding the testis with fine forceps. F. Gently pull the tunica away from the testis tubules. G. Carefully tease apart the seminiferous tubules using fine forceps.

2. Place separated seminiferous tubules in a 1.7 mL microcentrifuge tube and incubate in 0.1% formaldehyde in PBS at room temperature for 10 min while rotating.
3. Halt cross-linking by adding glycine drop-wise using a P200 pipette to a final concentration of 125 mM. Incubate at room temperature for 5 min while rotating.
4. Pellet the tubules by spinning for 5 min at 500 × *g*. Remove the supernatant using a pipette and wash twice with 4°C PBS.
5. Re-pellet the tubules, remove the PBS, and flash-freeze in liquid nitrogen. Store at -80°C.

B. Cross-linking magnetic Dynabeads to antibody

Day 1

1. Resuspend the Protein A magnetic Dynabeads by pipetting.
2. Transfer 100 µL of Protein A Dynabeads per IP to separate 1.7 mL microcentrifuge tubes.
3. Place the tubes on the magnet, remove the supernatant, and wash the beads twice with 1 mL of 3% BSA in PBST for 5 min at room temperature while rotating. After each wash, use the magnet to capture the beads, then remove and discard the supernatant.
4. Resuspend the beads in 500 µL of 3% BSA in PBST.
5. Set aside 200 µL for the preclear step and store at 4°C.

6. Place the remaining 300 µL on the magnet, remove the supernatant, and resuspend in 200 µL of bead coupling buffer.
7. Add 2 µg YTHDC2 antibody and incubate at 4°C overnight while rotating.
   Note: When new antibodies are used, the optimal amount of antibody should be determined by testing the immunoprecipitation efficiency on cross-linked tissue using multiple antibody concentrations (typically between 1-10 µg per immunoprecipitation).

Day 2

8. Place the beads-antibody complex on the magnet and discard the supernatant. Wash three times with 1 mL of 0.2 M triethanolamine pH 8.2 for 5 min at room temperature while rotating. After each wash, use the magnet to capture the beads, then remove and discard the supernatant.
9. Resuspend the beads-antibody complex in 1 mL of dimethyl pimelimidate (DMP) cross-linking solution, and incubate at room temperature for 30 min while rotating.
10. Place the tubes on the magnet to capture the beads, then remove and discard the supernatant.
11. Resuspend the beads-antibody complex in 1 mL of 50 mM Tris, pH 7.5. Incubate at room temperature for 15 min while rotating.
12. Wash three times with 1 mL of PBST for 5 min at room temperature while rotating. After each wash, use the magnet to capture the beads, then remove and discard the supernatant.
13. Resuspend the beads-antibody complex in 200 µL of PBST and store at 4°C.

C. Tissue lysis

1. Remove the testis tubules from the freezer and quickly add 275 µL of lysis buffer with 100 U/mL RNaseOUT to each sample.
   Note: We used testis tubules from two postnatal day 12 (P12) mice for each immunoprecipitation.
2. Mechanically break up the testis tubules by pipetting up and down with first a 1,000 µL and then a 200 µL pipette tip until visibly disrupted. Follow with a 25 G × 5/8 inch needle until the lysate can easily pass through the needle (Figure 3).

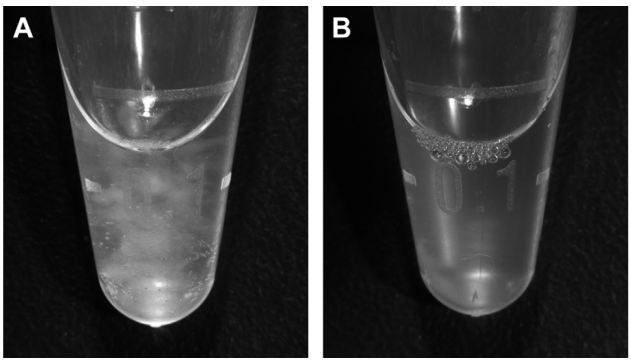

Figure 3. Mechanical disruption of testis tubules.
Cross-linked testis tubules (A) before and (B) after mechanical disruption in lysis buffer.

3. Incubate the lysate at 4°C for 15 min while rotating.
4. Sonicate the lysate in a 4°C water bath using a Bioruptor (1 cycle, 30 s pulse on Medium).
   Note: May need to test multiple sonication cycle numbers prior to fRIP. The 1 cycle sonication step used in this experiment was gentle enough to not degrade the RNA and protein while lightly fragmenting the RNA. Should be able to easily pipette the lysate following sonication.
5. Spin down debris at 16,000 × *g* for 15 min at 4°C.
6. Collect the supernatant and transfer it to a new 1.7 mL microcentrifuge tube, then dilute by adding an equal volume of binding/wash buffer with 100 U/mL RNaseOUT.
7. Calculate protein concentration using the Bradford assay.
   Note: We typically obtain around 1.5-2 mg total protein from each lysate (testis tubules from two P12 mice).
8. Use between 1-2 mg total protein per IP.

D. Preclear lysate
1. Place the beads previously set aside in section B for the preclear step on the magnet and remove the supernatant. Wash the beads twice with 1 mL of 3% BSA in PBST for 15 min at 4°C while rotating. Use the magnet to capture the beads, then remove and discard the supernatant.
2. For each IP, add ~1 mg of total protein to the preclear beads and incubate the lysate for 1 h at 4°C while rotating.
3. Use the magnet to capture the preclear beads, then collect the lysate and transfer it to a new 1.7 mL microcentrifuge tube. Keep the precleared lysate at 4°C.
4. Set aside 1% of the volume of the precleared lysate for western blot analysis. Add protein loading buffer, boil for 5 min at 100°C, and freeze.
5. Set aside 10% of the volume of the precleared lysate for fRIP INPUT. Keep the fRIP INPUT at 4°C.

E. Beads-antibody preparation
1. To remove any non cross-linked antibody, place the cross-linked beads-antibody complex from section B on the magnet, discard the supernatant, then quickly resuspend the beads in 1 mL of 100 mM glycine pH 2.5 by inverting the tube. Immediately place the beads back on the magnet, discard the supernatant, and repeat the glycine wash.
2. Wash the beads-antibody complex three times with 1 mL of PBST for 5 min at room temperature while rotating. After each wash, use the magnet to capture the beads, then remove and discard the supernatant.
3. Resuspend the beads-antibody complex in 1 mL of 3% BSA in PBST and incubate for 30 min at 4°C while rotating.
4. Wash the beads-antibody complex three times quickly in 1 mL of PBST at room temperature. After each resuspension in PBST, use the magnet to capture the beads, then remove and discard the supernatant.

5. Resuspend the beads-antibody complex in PBST and transfer to 1.7 mL low binding tubes for immunoprecipitation.

F. Immunoprecipitation (IP)
1. Place the beads-antibody complex from section E on the magnet, discard the supernatant, and add the precleared lysate from section D.
2. Incubate for 4 h at 4°C while rotating.
3. Capture the beads with bound protein of interest using the magnet, collect the supernatant, and store at -80°C to check IP efficiency by western blot (optional). Keep the beads for the next step.
4. Wash the beads four times with 1 mL of binding/wash buffer, rotating at 4°C for 10 min each time. After each wash, use the magnet to capture the beads, then remove and discard the supernatant. Transfer the suspended beads to new 1.7 mL low binding tubes before the final wash.
5. After the final wash, resuspend the beads in 1 mL of binding/wash buffer and transfer to new 1.7 mL low binding tubes.
6. Save 10% of each bead suspension for western blot to confirm successful IP and use 90% for RNA isolation.
7. For western blot samples (10% of each IP) (Figure 4):
   a. Capture the beads using the magnet and remove the supernatant. Resuspend the beads in 100 µL of lysis buffer and transfer to new 1.7 mL low binding tubes.
   b. Place the beads on the magnet and remove the supernatant. Add 40 µL of fresh elution buffer to the beads and incubate on a heat block at 70°C for 30 min. Briefly vortex the samples every 5 min during the elution step.
   c. Spin the tubes at room temperature for 10 s at 16,000 × *g*.
   d. Capture the beads using the magnet and transfer the 40 µL of supernatant to new tubes. Add 10 µL of 5× protein loading buffer, boil 5 min at 100°C, and freeze.
   e. Run samples on a Mini-PROTEAN precast gel.

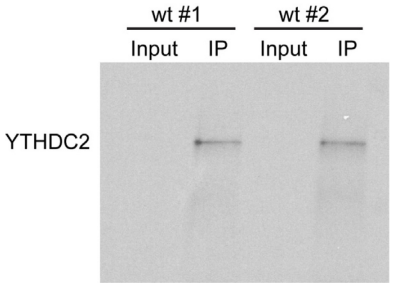

Figure 4. Western blot to confirm success of immunoprecipitation.
Input and 10% of the immunoprecipitation (IP) samples from wild type (wt) were separated by polyacrylamide gel electrophoresis (PAGE) then transferred to a polyvinylidene difluoride (PVDF) membrane for analysis by western blot. IPs were performed in duplicate. The blot was probed with anti-YTHDC2 antibody.

Note: Proceed with the RNA release step immediately following the immunoprecipitation.

G. Release of RNA

Day 1

1. Place the 90% of beads set aside for RNA isolation in step F6 on the magnet and remove the supernatant. Resuspend the beads in 93 µL of PK buffer, then add 5 µL of 20 mg/mL Proteinase K, 1 µL of 40 U/µL RNaseOUT, and 1.5 µL of 1 M DTT (100 µL total volume).
2. To the fRIP INPUT samples from step D5, add 43 µL of PK buffer, followed by 5 µL of 20 mg/mL Proteinase K, 1 µL of 40 U/µL RNaseOUT, and 1.5 µL of 1 M DTT (100 µL total volume).
3. Incubate samples for 1 h at 42°C, then another hour at 55°C.
4. Add 1 mL of TRIzol, mix by inversion, and let sit ~5 min at room temperature.
5. Add 200 µL of chloroform and shake vigorously for 15 s.
6. Centrifuge 20 min at 4°C at 16,000 × *g*.
7. Collect aqueous layer and add 1 µL of GlycoBlue, and 750 µL of isopropanol.
8. Incubate overnight at -20°C.

Day 2

9. To isolate the RNA pellet, centrifuge 40 min at 4°C at 16,000 × *g*.
10. Wash the RNA pellet three times with fresh 75% cold ethanol, spinning 15 min at ~7,000 × *g* between washes.
11. Spin an extra 5 min at ~7,000 × *g* and air-dry pellet.
12. Resuspend pellet in 10 µL of UltraPure DNase/RNase-free water.

H. Library construction

1. Remove ribosomal RNA from both the INPUT and fRIP samples using the RiboMinus Eukaryote System v2 Kit, according to the manufacturer's instructions.
2. Prepare libraries using a TruSeq RNA Sample Preparation Kit per the manufacturer's instructions, with the following modification: do not fragment the RNA with FPF solution.

I. High-throughput sequencing

Sequence libraries using the Illumina NextSeq for 2 × 75 bp cycle run.

Note: All work must be performed in an RNase-free environment throughout the fRIP-seq protocol. Only RNase-free solutions should be used.

Data analysis

Analyze data as described in the Methods section of Bailey *et al.* (2017). Briefly, filter reads for all samples for quality score and map to rRNA to remove rRNA reads. Map the remaining reads to the

appropriate genome assembly using STAR. Extract reads mapping to each transcript with HTseq, using a custom annotation that includes piRNA precursors, in addition to coding and long-noncoding transcripts. Determine transcripts enriched by IP with DESeq2 using multifactor analysis. Inclusion of the null mutant samples allows for the identification of non-specific interactions.

Recipes

1. PBST
   PBS + 0.1% Tween-20 diluted from 20% stock solution.
   Store at room temperature.
2. 0.1% formaldehyde
   5 mL of PBS + 31.25 µL of 16% formaldehyde stock solution.
   Make fresh and use immediately.
3. DMP cross-linking solution
   5.4 mg dimethyl pimelimidate (DMP) in 1 mL of 0.2 M triethanolamine pH 8.2.
   Make fresh and use immediately.
4. Bead Coupling Buffer

Stock Reagent	Amount for 1 mL	Final concentration
1 M Tris-HCl pH 8	20 µL	20 mM
5 M NaCl	27 µL	135 mM
Glycerol	100 µL	10%
10% NP-40	100 µL	10%
0.5 M EDTA	20 µL	5 mM
UltraPure DNase/RNase-free water	733 µL	n/a

5. Lysis Buffer

Stock Reagent	Amount for 1 mL	Final concentration
1 M Tris-HCl pH 8	50 µL	50 mM
2 M KCl	75 µL	150 mM
10% SDS	10 µL	0.1%
10% Triton X-100	100 µL	1%
0.5 M EDTA	10 µL	5 mM
10% Sodium Deoxycholate	50 µL	0.5%
1 M DTT	0.5 µL	0.5 mM
Complete Protease Inhibitor (CPI) 7×	150 µL	1×
UltraPure DNase/RNase-free water	555 µL	n/a

   **Add 100 Units/mL RNaseOUT fresh.

6. Binding/Wash Buffer

Stock Reagent	Amount for 1 mL	Final concentration
1 M Tris-HCl pH 7.5	25 µL	25 mM

2 M KCL	75 µL	150 mM
0.5 M EDTA	10 µL	5 mM
10% NP-40	50 µL	0.5%
1 M DTT	0.5 µL	0.5 mM
Complete Protease Inhibitor (CPI) 7×	150 µL	1×
UltraPure DNase/RNase-free water	690 µL	n/a

**Add 100 Units/mL RNaseOUT fresh.

7. Elution Buffer

Stock Reagent	**Amount for 1 mL**	**Final concentration**
10% SDS	100 µL	1%
0.5 M EDTA	20 µL	10 mM
1 M Tris-HCL, pH 8.0	50 µL	50 mM
100 mM PMSF	10 µL	1 mM
Complete Protease Inhibitor (CPI) 7×	150 µL	1×
UltraPure DNase/RNase-free water	670 µL	n/a

8. PK Buffer

Stock reagent	**Amount for 25 mL**	**Final concentration**
1 M Tris pH 7.0	250 µL	10 mM
5 M NaCl	500 µL	100 mM
0.5 M EDTA	50 µL	1 mM
10% SDS	1,250 µL	0.5%
UltraPure DNase/RNase-free water	22,950 µL	n/a

Acknowledgments

This protocol was adapted from Hendrickson *et al.* (2016). We thank the Stanford Functional Genomics Facility for high-throughput sequencing. This work was supported by the National Institutes of Health (NIH T32 AR007422) to A.S.B., the National Cancer Institute, Intramural Research Program of the NIH to P.J.B., the National Institutes of Health (NIH R01 HG004361) to H.Y.C., and the National Institutes of Health (R01 GM122951 and R35 GM136433) and the Reed-Hodgson Professorship in Human Biology to M.T.F. This adapted protocol was originally published in our previous manuscript (Bailey *et al.*, 2017; Doi: 10.7554/eLife.26116).

Competing interests

The authors declare no competing interests.

Ethics

The experiment was approved by the Stanford University Animal Care and Use Committee (IACUC), protocol (#21656).

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