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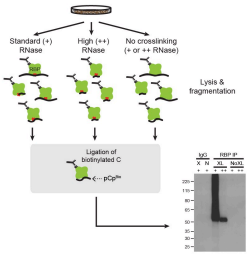
Biotin-Labelling of Immunoprecipitated RNA (v1pre) V.1



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Protocol status: Working
We use this protocol and it's working

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Abstract

RNA binding proteins (RBPs) regulate a diverse array of RNA processing steps by binding RNAs through sequence and structural elements. The development of crosslinking and immunoprecipitation (CLIP) enabled identification of RBP targets transcriptome-wide in a robust manner. During CLIP, it is often desired to visualize RBP:RNA complexes after denaturing SDS-PAGE electrophoresis and transfer to nitrocellulose membranes. However, standard methods used radiolabeling of RNA followed by autoradiography, introducing significant challenges for usability and scaling experiments to profile many RBPs. Here we describe an alternative approach to visualize RBP:RNA complexes using ligation of biotinylated nucleotides, followed by standard chemiluminescent imaging. This approach retains the advantages of visualization while decreasing handling complexity, enabling large-scale experiments to verify the presence and sizing of immunoprecipitated RBP:RNA complexes.

Guidelines

For best results, include the following experimental conditions:

1. UV-crosslinked cell lysate (4M cells worth), treated with 8U RNase I
2. UV-crosslinked cell lysate (4M cells worth), treated with 66.6U RNase I
3. Non-crosslinked cell lysate (4M cells worth), treated with 8U RNase I

Materials

MATERIALS

RNase Inhibitor, Murine - 15,000 units **New England Biolabs Catalog #**M0314L

T4 RNA Ligase 1 (ssRNA Ligase) (30,000 units/ml) - 5,000 units **Catalog #**M0437M

T4 Polynucleotide Kinase - 2,500 units **New England Biolabs Catalog #**M0201L

Dynabeads™ M-280 Sheep Anti-Mouse IgG **Thermo Fisher Catalog #**11201D

Dynabeads™ M-280 Sheep Anti-Rabbit IgG **Thermo Fisher Catalog #**11204D

Chemiluminescent Nucleic Acid Detection Module Kit **Thermo Fisher Catalog #**89880

TURBO™ DNase (2 U/µL) **Thermo Fisher Catalog #**AM2239

Ambion™ RNase I, cloned, 100 U/µL **Thermo Fisher Catalog #**AM2295

FastAP Thermosensitive Alkaline Phosphatase (1 U/µL) **Thermo Fisher Catalog #**EF0652

NuPAGE™ MOPS SDS Running Buffer (20X) **Thermo Fisher Catalog #**NP000102

NuPAGE™ Transfer Buffer (20X) **Thermo Fisher Catalog #**NP00061

NuPAGE™ LDS Sample Buffer (4X) **Thermo Fisher Catalog #**NP0008

NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm, 12-well **Thermo Fisher Catalog #**NP0322BOX

pCp-Biotin **Jena Bioscience Catalog #**NU-1706-BIO

- **Lysis buffer:**50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% NP-40 (Igepal CA630), 0.1% SDS, 0.5% sodium deoxycholate (protect from light), (add fresh) 1:200 Protease Inhibitor Cocktail III, in RNase/DNase free H_2O.
- **High salt wash buffer:**50 mM Tris-HCl pH 7.4**,** 1 M NaCl**,** 1 mM EDTA**,** 1% NP-40**,** 0.1% SDS**,** 0.5% sodium deoxycholate (protect from light), in RNase/DNase free H_2O.
- **Wash buffer**:20 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 0.2% Tween-20, in RNase/DNase free H_2O.
- Protease Inhibitor Cocktail III EMD Millipore 539134
- 1M DTT

Conjugate Antibody to Beads

1 For each 4M cell lysate, aliquot 25 µL of species-specific Dynabeads (e.g. ThermoFisher catalog # 11203D, 11202D, or equivalent)

2 Wash the beads twice in 500 µL cold Lysis Buffer.

3 Resuspend in 100 µL of Lysis Buffer.

4 Add 2 µg of RBP-specific antibody to each tube.

5 Rotate at room temperature for 45 min. Proceed to step 7 (Cell Lysis) while rotating.

6 Wash twice with 500 µL cold Lysis Buffer and reserve on ice until step 14.

Cell Lysis

7 Lyse cells in cold eCLIP lysis buffer containing 1X protease inhibitor.
- 5.5 µL protease inhibitor cocktail III per 1 mL Lysis Buffer.
- Lyse pellets at similar ratio to that used in CLIP experiments (200 µL Lysis Buffer per 4M cells)
- A typical experiment includes 2 (4 million cell) crosslinked samples and 1 (4 million cell) non-crosslinked sample

8 Lyse on ice for 15 min.

9 Sonicate on Low setting for 5 min, 30 sec on/30 sec off.

10 Make 2 dilutions of RNase I in PBS:
1. 1:25 (4 U/µL)
2. 1:3 (33.3 U/µL)

11 Add 1 µL Turbo DNase to each 4M cell lysate.

12 Add diluted RNase to lysate:

2 µL of 1:25 dilution to the 1st crosslinked lysate = Crosslink Standard RNase
2 µL of 1:3 dilution to the 2nd crosslinked lysate = Crosslink High RNase
2 µL of 1:25 dilution to the non-crosslinked lysate = Non-crosslinked Standard RNase

13 Immediately incubate lysates in a Thermomixer set to 37°C, shaking at 1200 rpm, for 5 min.

14 Centrifuge at 15,000 x *g* for 10 min at 4°C. Transfer cleared lysate to washed beads.

15 Rotate at 4°C overnight.

Post-IP Washes

16 For each sample, wash twice with 500 µL cold High Salt Wash Buffer.

17 To each sample, add 500 µL cold High Salt Wash Buffer, mix, add 500 µL cold Wash Buffer.

18 For each sample, wash 3X with cold Wash Buffer, keep on ice after last wash.

Dephosphorylation of IP Samples

19 Briefly spin tubes and remove residual wash buffer. Resuspend the beads in the following mix, volumes per sample:

	H2O	38.0 µL
	10X FastAP Buffer	5.0 µL
	Murine RNase	2.0 µL

	Inhibitor	
	TurboDNase	2.0 µL
	FastAP Enzyme	3.0 µL

20 Incubate the FastAP reaction in a Thermomixer at 37°C, mixing at 1200 rpm, for 10 min.

21 While incubating the FastAP reaction, prepare the following mix, volumes per sample:

	H2O	116.0 µL
	5X PNK pH 6.5 Buffer	30.0 µL
	T4 PNK Enzyme	4.0 µL

22 Without removing the FastAP mix, add 150 µL of the PNK mix to each sample and place back on Thermomixer, incubating at 37°C, 1200 rpm, for 20 min.

Post-PNK Washes

23 To each sample, add 200 µL High Salt Wash Buffer, mix, separate on magnet, and remove supernatant.

24 Add 500 µL High Salt Wash Buffer, mix, then add 500 µL Wash Buffer. Remove supernatant.

25 Wash 3X with 500 µL Wash Buffer.

Biotinylated Cytidine Ligation

26 In an RNase-free 1.5 mL microcentrifuge tube, mix the following mix, volumes per sample:

	H2O	9.6 µL
	10X RNA Ligase Buffer (no DTT)	3.0 µL
	0.1 M ATP	0.3 µL
	100% DMSO	0.9 µL
	1% Tween-20	0.6 µL
	50% PEG 8000	9.0 µL
	Murine RNase Inhibitor	0.4 µL
	RNA Ligase High Conc. Enzyme	2.4 µL
	Biotinylated Cytidine (Bis)phosphate	0.5 µL

27 Magnetically separate each IP sample, remove Wash Buffer, and resuspend the beads in 26 µL of master mix.

28 Incubate samples at 16°C with gentle shaking for 2 hrs or overnight (recommended).

Post-Ligation Cleanup

29 Add 500 µL cold High Salt Wash Buffer, mix, magnetically separate, and remove supernatant.

30 Add 500 µL cold High Salt Wash Buffer, move on magnet, add 500 µL cold Wash Buffer, magnetically separate, remove supernatant.

31 Wash 3X with 500 µL cold Wash Buffer.

32 Resuspend in 20 µL Wash Buffer.

Gel Electrophoresis & Transfer

33 Incubate at 70°C, mixing at 1200 rpm for 10 min.

34 Add 10.5 µL of denaturing mix for SDS-PAGE (7.5 µL 4X LDS buffer, 3 µL 1M DTT)

35 Place tubes on ice for > 1 min.

36 Magnetically separate samples and load 15 µL on gel, reserving the other half at -20°C as backup.

37 Run the gel at 150V for 75 min.

38 Transfer to nitrocellulose membrane at 30V overnight.

Membrane Development

39 Develop membrane as follows using the Chemiluminescent Nucleic Acid Detection Module kit (cat. no. 89880)

39.1 Slowly warm the Blocking Buffer and the 4X Wash Buffer to 37-50°C in a water bath until all particulates are dissolved.

39.2 Block membrane by adding 10 mL Blocking Buffer and incubate for 15 min with gentle shaking at room temperature (all further steps done at room temperature).

39.3 Prepare conjugate/blocking buffer solution by adding 31.25 µL of the Stabilized Streptavidin-Horseradish Peroxidase Conjugate to 10mL Blocking Buffer.

39.4 Decant blocking buffer from the membrane and add 10 mL to the conjugate/blocking solution. Incubate membrane in the conjugate/blocking buffer solution for 15 min with gentle shaking.

39.5 Prepare 1X wash solution by adding 40 mL of 4X Wash Buffer to 120 mL water.

39.6 Transfer membrane to a new container and rinse briefly with 20 mL of 1X wash solution.

39.7 Wash membrane four times for 5 min each in 20 mL of 1X wash solution with gentle shaking.

39.8 Transfer membrane to a new container and add 30 mL of Substrate Equilibration Buffer. Incubate membrane for 5 min with gentle shaking.

39.9 Prepare Chemiluminescent Substrate Working Solution by adding 2 mL Luminol/Enhancer Solution to 6 mL Stable Peroxide Solution. Note: Working solution is susceptible damage via prolonged light exposure. Keep solution in an amber bottle or keep away from light.

39.10 Remove membrane from the Substrate Equilibration Buffer and remove excess buffer. Place membrane in a clean container or clean sheet of plastic wrap.

39.11 Pour the Substrate Working Solution onto the membrane so that it completely covers the surface. Incubate membrane in the substrate solution for 5 min without shaking.

39.12 Remove membrane from the Working Solution and remove excess buffer. Do not allow the membrane to dry out.

39.13 Wrap the membrane in plastic wrap, avoiding bubbles, and place in a film cassette. Obtain optimal signal by adjusting film exposure time or by exposing membrane to multiple films simultaneously.