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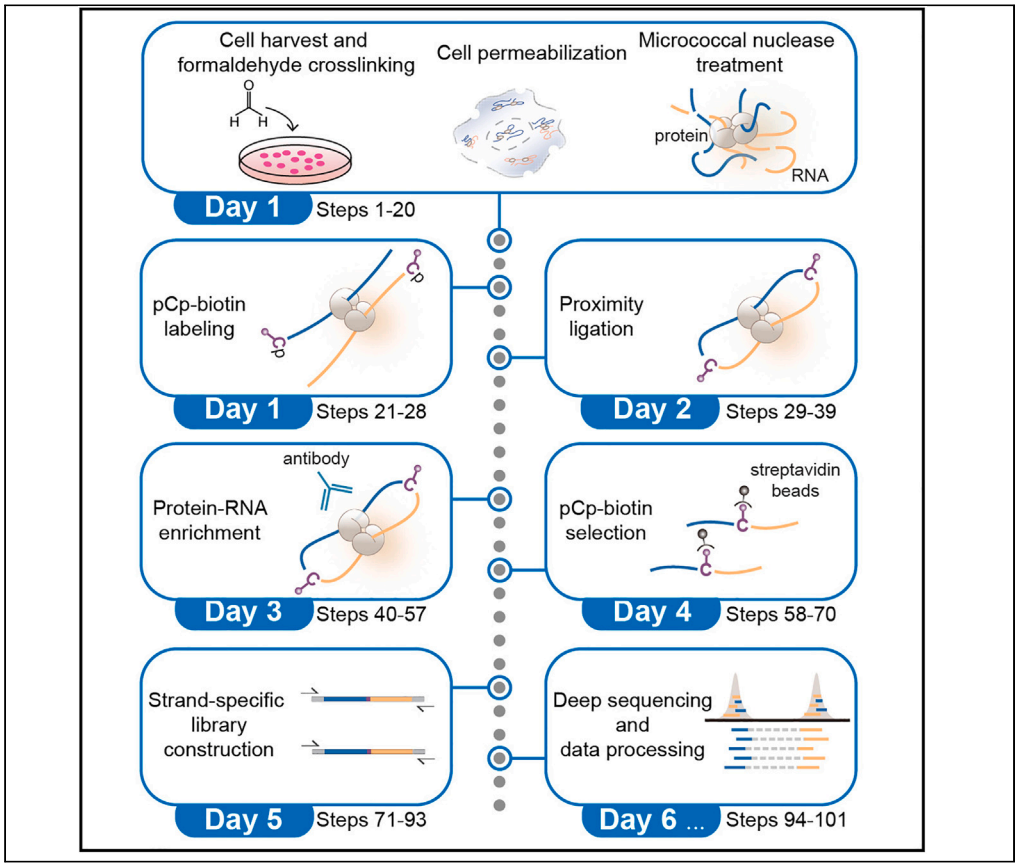
# CRIC-seq protocol for *in situ* profiling of proximal RNA-RNA contacts associated with RNA-binding proteins




RNA-binding proteins (RBPs) can bind and mediate RNA-RNA contacts. However, identifying specific RBP-organized RNA-RNA contacts remains challenging. Here, we present a capture RIC-seq (CRIC-seq) technique to map specific RBP-associated RNA-RNA contacts globally. We describe steps for formaldehyde cross-linking to fix RNA *in situ* conformation, pCp-biotin labeling to mark RNA juncture, and *in situ* proximity ligation to join proximal RNAs. We then detail immunoprecipitation to isolate specific RBP-associated RNA-RNA contacts, biotin-streptavidin selection to enrich chimeric RNAs, and library construction for paired-end sequencing.



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## Highlights

CRIC-seq for mapping specific RBP-associated *in situ* RNA-RNA contacts

Step-by-step protocol for PTBP1 CRIC-seq library construction

CRIC-seq data analysis pipelines

Model RNA secondary structures using PTBP1-associated RNA-RNA contacts

Ye et al., STAR Protocols 4, 102401
September 15, 2023 
https://doi.org/10.1016/j.xpro.2023.102401

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# CRIC-seq protocol for *in situ* profiling of proximal RNA-RNA contacts associated with RNA-binding proteins

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https://doi.org/10.1016/j.xpro.2023.102401

## SUMMARY

**RNA-binding proteins (RBPs) can bind and mediate RNA-RNA contacts. However, identifying specific RBP-organized RNA-RNA contacts remains challenging. Here, we present a capture RIC-seq (CRIC-seq) technique to map specific RBP-associated RNA-RNA contacts globally. We describe steps for formaldehyde cross-linking to fix RNA *in situ* conformation, pCp-biotin labeling to mark RNA juncture, and *in situ* proximity ligation to join proximal RNAs. We then detail immunoprecipitation to isolate specific RBP-associated RNA-RNA contacts, biotin-streptavidin selection to enrich chimeric RNAs, and library construction for paired-end sequencing. For complete information on the generation and use of this protocol, please refer to Ye et al.[1]**

## BEFORE YOU BEGIN

We previously reported an RNA *in situ* conformation sequencing (RIC-seq) technology to profile all expressing RBP-associated RNA-RNA contacts.[2] However, most researchers focus on a specific RBP at one time. We, thus, invented CRIC-seq by combining RIC-seq with immunoprecipitation for global profiling of *in situ* RNA conformation associated with a specific RBP. As an example, this protocol describes the steps for mapping PTBP1-associated proximal RNA-RNA contacts in HeLa cells with CRIC-seq. PTBP1 prefers to bind CU-rich motifs and was initially characterized as a splicing repressor.[3,4] Aligning the PTBP1 binding landscape around its regulated exons later reveals that PTBP1 can repress or enhance the usage of cassette exons by binding at different positions.[5] Several models propose that PTBP1-mediated exon-spanning RNA loops or RNA loops around 3′ splice sites and the branch point may repress cassette exon splicing.[6] However, direct experimental evidence is lacking. Moreover, how PTBP1-associated RNA loops contribute to exon inclusion remains unclear. Thus, mapping the RNA-RNA contacts associated with PTBP1 helps decipher how PTBP1-associated RNA conformation regulates alternative splicing.

In addition to PTBP1, our CRIC-seq method has been successfully used to profile RNA-RNA contacts organized by other RBPs, such as SRSF1 and hnRNPA1, and should be applicable to other cell lines and tissue samples. While the three RBPs we examined are primarily localized to the nucleus, our method can also be adapted for assaying cytoplasmic proteins. This is supported by the success of RIC-seq, which utilized a similar principle and captured 78.7% of cytoplasmic RNAs in HeLa cells.[2]

For CRIC-seq experiments, we suggest using two biological replicates and starting with $1 \times 10^7$ HeLa cells for each replicate. However, users may need to adjust the initial cell number or tissue volume based on their target protein's RNA affinity and the average yield of total RNA. Ideally, the extracted RBP-bound RNA yield should be at least 100 ng.

To control for background contacts during the pulldown steps, we generated two sets of control CRIC-seq libraries: IgG IP with pCp-biotin labeling (IgG IP, +pCp) to exclude non-specific background during the antibody-protein immunoprecipitation process; PTBP1 antibody IP without pCp-biotin labeling (PTBP1 IP, −pCp) to exclude non-specific background during biotin-streptavidin enrichment.

⚠ CRITICAL: The whole experimental procedure should be performed under RNase- and DNase-free conditions.

### Culture cell lines

Timing: ~2–3 days

1. Seed and culture HeLa cells in 15 cm dishes with DMEM medium containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin at 37°C in a 5% $CO_2$ incubator.
2. Ensure the cells are free of Mycoplasma contamination.
3. Harvest cell at around 80% confluency.

### Adapter preparation

Timing: ~2 h

4. Mix 10 μL of 100 μM PEI Adapter oligo A and 10 μL of 100 μM PEI Adapter oligo B in a 200 μL PCR tube.
5. Adapter annealing.
   a. Incubate the PCR tube in a thermal cycler at 70°C for 10 min.
   b. Take out the PCR tube and let it naturally cool down to room temperature (20°C–22°C) on the PCR tube rack to allow annealing.

   *Note:* The final concentration of adapters is 50 μM.

   c. Aliquot the annealed adapters.
   d. Store the adapters at −20°C for up to several years.
6. Before use, dilute the 50 μM annealed adapters to a final concentration of 2 μM using DEPC-treated $H_2O$. Store the 2 μM adapters at −20°C for up to 12 months.

### dNTP (dUTP) mixture preparation

Timing: ~10 min

7. To prepare a dNTP mixture containing dUTP, mix 50 μL of 100 mM dATP, 50 μL of 100 mM dGTP, 50 μL of 100 mM dCTP, 10 μL of 100 mM dTTP, and 40 μL of 100 mM dUTP solutions.

   *Note:* The final concentrations of dATP, dGTP, and dCTP are 25 mM, and the final concentrations of dTTP and dUTP are 5 mM and 20 mM. Store the dNTP (dUTP) mixture at −20°C.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse monoclonal anti-PTBP1 (BB7) (15 μg per 1 mL cell lysate) | Chou et al.,[7] | https://doi.org/10.1016/S1097-2765(00)80260-9 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Mouse anti-IgG (15 μg per 1 mL cell lysate) | Santa Cruz Biotechnology | Cat# sc-2025, RRID: AB_737182 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Adenosine 5′-triphosphate (ATP) | New England Biolabs | Cat# R1016 |
| Agencourt AMPure XP | Beckman Coulter | Cat# A63881 |
| Deoxynucleotide (dNTP) Solution Mix (10 mM) | New England Biolabs | Cat# N0447L |
| Deoxynucleotide (dNTP) Solution Mix (25 mM) | Enzymatics | Cat# N2050L |
| Dynabeads MyOne Streptavidin C1 | Thermo Fisher Scientific | Cat# 65002 |
| dUTP solution | Thermo Fisher Scientific | Cat# R0133 |
| dATP | Thermo Fisher Scientific | Cat# 10216018 |
| FastAP Alkaline Phosphatase | Thermo Fisher Scientific | Cat# EF0651 |
| Formaldehyde solution (37%, w/v) | Sigma-Aldrich | Cat# F8775 |
| GlycoBlue™ Coprecipitant | Thermo Fisher Scientific | Cat# AM9515 |
| Klenow (3′ to 5′ exo-) | Enzymatics | Cat# P7010-LC-L |
| Klenow Fragment | Enzymatics | Cat# P7060L |
| Micrococcal nuclease | Thermo Fisher Scientific | Cat# EN0181 |
| PBS (10×) pH 7.4, RNase-free | Thermo Fisher Scientific | Cat# AM9624 |
| Platinum Pfx DNA Polymerase Kit | Thermo Fisher Scientific | Cat# 11708-013 |
| Proteinase K | Takara | Cat# 9034 |
| Protein A/G magnetic beads | Thermo Fisher Scientific | Cat# 88802 |
| Protease Inhibitor Cocktail | Sigma-Aldrich | Cat# P8340-5ML |
| RiboLock RNase Inhibitor | Thermo Fisher Scientific | Cat# EO0381 |
| RNase H | Thermo Fisher Scientific | Cat# EN0202 |
| RQ1 RNase-Free DNase | Promega | Cat# M6101 |
| TURBO™ DNase | Thermo Fisher Scientific | Cat# AM2238 |
| Sodium acetate (3 M, pH 5.5) | Thermo Fisher Scientific | Cat# AM9740 |
| SUPERase· In™ RNase Inhibitor | Thermo Fisher Scientific | Cat# AM2694 |
| SuperScript II Reverse Transcriptase | Thermo Fisher Scientific | Cat# 18064-014 |
| T4 DNA Polymerase | Enzymatics | Cat# P7080L |
| T4 DNA Ligase (Rapid) | Enzymatics | Cat# L6030-HC-L |
| T4 RNA Ligase | Thermo Fisher Scientific | Cat# EL0021 |
| T4 Polynucleotide Kinase | Enzymatics | Cat# Y9040L |
| T4 Polynucleotide Kinase | Thermo Fisher Scientific | Cat# EK0032 |
| TRIzol™ LS Reagent | Thermo Fisher Scientific | Cat# 10296028 |
| USER Enzyme | New England Biolabs | Cat# M5505S |
| Tris | Amresco | Cat# 0497-1KG |
| Glycine | Amresco | Cat# 0167-5KG |
| Hydrochloric acid (HCl) | Sigma-Aldrich | Cat# H1758 |
| Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) | Sigma-Aldrich | Cat# M0250-500G |
| Ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) | Sigma-Aldrich | Cat# E3889-100G |
| Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA \cdot 2H_2O$) | Sigma-Aldrich | Cat# E5134-1KG |
| IGEPAL® CA-630 (NP-40) | Sigma-Aldrich | Cat# I8896-100ML |
| Agarose | Biowest | Cat# 111860 |
| Triton X-100 | Sigma-Aldrich | Cat# T8787-250ML |
| Tween 20 | Sigma-Aldrich | Cat# P9416-100ML |
| Calcium chloride ($CaCl_2$) | Sigma-Aldrich | Cat# 793639-500G |
| UltraPure™ DEPC-Treated Water | Thermo Fisher Scientific | Cat# 750023 |
| Sodium deoxycholate | Sigma-Aldrich | Cat# 30970 |
| Sodium dodecyl sulfate (SDS) | Sigma-Aldrich | Cat# L3771-500G |
| Chloroform | Sinopharm | Cat# 10006818 |
| Sodium hydroxide (NaOH) | Sigma-Aldrich | Cat# S8045-1KG |
| Sodium chloride (NaCl) | Sigma-Aldrich | Cat# S7653-1KG |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Yeast RNA | Roche | Cat# 10109223001 |
| Ethanol | Sigma-Aldrich | Cat# E7023-500ML |
| 2-Propanol | Sigma-Aldrich | Cat# I9516-500ML |
| DMEM | Thermo Fisher Scientific | Cat# C11965500BT |
| Penicillin-Streptomycin | Thermo Fisher Scientific | Cat# 15140-122 |
| Fetal bovine serum | PAN-Seratech | Cat# ST30-3302 |
| GelRed | Biotium | Cat# 41003 |
| Acid phenol: chloroform | Thermo Fisher Scientific | Cat# AM9722 |
| **Critical commercial assays** | | |
| RNA 3′ end biotinylation kit | Thermo Fisher Scientific | Cat# 20160 |
| RNA Clean and Concentrator-5 | Zymo Research | Cat# R1016 |
| Qubit dsDNA HS Assay Kit | Thermo Fisher Scientific | Cat# Q32854 |
| MinElute Gel Extraction Kit | Qiagen | Cat# 28606 |
| Agilent DNA High Sensitivity kit | Agilent | Cat# 5067-4626 |
| **Deposited data** | | |
| Raw and analyzed data | Ye et al.,[1] | GEO: GSE210583 |
| **Experimental models: Cell lines** | | |
| Human: HeLa (< 50 passages) | ATCC | Cat# CCL-2, RRID: CVCL_0030 |
| **Oligonucleotides** | | |
| PEI Adapter oligo A | Cao et al.,[8] | /5Phos/GATCGGAAGAGCACACGTCT (5Phos: 5′ phosphorylation) |
| PEI Adapter oligo B | Cao et al.,[8] | ACACTCTTTCCCTACACGACG CTCTTCCGATCT |
| Illumina PE 1.0 primer | Cao et al.,[8] | AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTC TTCCGATCT |
| Index primer | Cao et al.,[8] | CAAGCAGAAGACGGCATACGAGAT NNNNNNGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT (N: random nucleotide) |
| **Software and algorithms** | | |
| RICpipe | Cao et al.,[8] | https://github.com/caochch/RICpipe |
| bedtools | Quinlan and Hall.,[9] | RRID: SCR_006646; https://github.com/arq5x/bedtools2 |
| FastQC | Simon Andrews | RRID:SCR_014583; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ |
| Trimmomatic v0.36 | Bolger et al.[10] | RRID:SCR_011848; http://www.usadellab.org/cms/index.php?page=trimmomatic |
| cutadapt v2.6 | Martin[11] | RRID:SCR_011841; http://code.google.com/p/cutadapt/ |
| STAR v2.5.2 | Dobin et al.[12] | RRID:SCR_004463; http://code.google.com/p/rna-star/ |
| SAMtools v1.14 | Danecek et al.[13] | RRID:SCR_002105; http://htslib.org/ |
| IGV | Broad Institute and the Regents of the University of California | RRID:SCR_011793; http://www.broadinstitute.org/igv/ |
| FIMO v5.0.4 | Grant et al.[14] | RRID:SCR_001783; http://meme-suite.org/ |
| The scripts for CRIC-seq data analysis | This paper | Zenodo: https://doi.org/10.5281/zenodo.7668477 Github:https://github.com/HuNaijing/CRIC-seq |
| **Other** | | |
| T100 Thermal Cycler | Bio-Rad | Cat# 1861096 |
| ThermoMixer C | Eppendorf | Cat# 5382000074 |
| Refrigerated centrifuge | Thermo Fisher Scientific | Cat# 75004380 |
| Refrigerated microcentrifuge | Thermo Fisher Scientific | Cat# 75002445 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Universal power supply | Bio-Rad | Cat# 1645070 |
| NanoDrop 2000c Spectrophotometer | Thermo Fisher Scientific | Cat# ND-2000 |
| 1.5 mL LoBind microcentrifuge tubes | Eppendorf | Cat# 022431021 |
| 0.2 mL PCR tubes | Axygen | Cat# PCR-02-C |
| Cell lifter | Corning | Cat# 3008 |
| 10 mL serological pipette | NEST | Cat# 327001 |
| 5 mL serological pipette | NEST | Cat# 326001 |
| 0.1–2 μL single-channel manual pipettes | Rainin | Cat# 17014393 |
| 2–20 μL single-channel manual pipettes | Rainin | Cat# 17014392 |
| 20–200 μL single-channel manual pipettes | Rainin | Cat# 17014391 |
| 100–1,000 μL single-channel manual pipettes | Rainin | Cat# 17014382 |
| 15 mL centrifuge tubes | NEST | Cat# 601002 |
| 50 mL centrifuge tubes | NEST | Cat# 602002 |
| Cell culture dishes (15 cm) | NEST | Cat# 715001 |
| Agarose Gel Electrophoresis (Mini-Sub Cell GT) | Bio-Rad | Cat# 170-4486 |
| Gel tray | Bio-Rad | Cat# 170-4435 |
| Filter unit (0.22 μm) | Merck | Cat# SLGV033RB |
| DynaMag™-2 Magnet– Magnetic rack for 1.5 mL tubes | Thermo Fisher Scientific | Cat# 12321D |
| Safe ImagerTM2.0 blue-light transilluminator | Invitrogen | Cat# G6600 |
| Razor blade | Personna | Cat# 84-0701 |
| Vortex mixer | Thermo Fisher Scientific | Cat# 88880018 |
| Tube rotator | CRYSTAL | Cat# TR-02U |
| Qubit Assay Tubes | Thermo Fisher Scientific | Cat# Q32856 |
| Qubit 3.0 Fluorometer | Thermo Fisher Scientific | Cat# Q33216 |
| Agilent 2100 Bioanalyzer | Agilent | Cat# G2939A |
| Cell culture incubator | Thermo Fisher Scientific | Cat# 3111 |

***Alternatives:*** T4 RNA ligase and RNase inhibitor contained in the RNA 3′ end biotinylation kit can be replaced with T4 RNA ligase (Thermo Fisher Scientific, Cat# EL0021) and RiboLock RNase inhibitor (Thermo Fisher Scientific, Cat# EO0381), respectively.

## MATERIALS AND EQUIPMENT

Recipes for the solutions and buffers used in this protocol are described and listed below. Ensure that all the buffers, reagents, and equipment are RNase- and DNase-free.

- 1 × PBS: dilute 50 mL of 10 × PBS with 450 mL DEPC-treated $H_2O$ for preparing 1 × PBS solution. This solution could be stored at 4°C for up to 12 months.
- 1% (w/v) Formaldehyde solution: dilute 270 μL of 37% formaldehyde solution (w/v) with 9.73 mL of 1 × PBS to prepare 1% (w/v) formaldehyde solution. This solution should be freshly prepared before use.

⚠ CRITICAL: Formaldehyde is harmful and toxic. Carefully handle it in a fume hood and wear a laboratory coat, goggles, and gloves.

- 2.5 M Glycine solution: dissolve 9.3838 g of glycine in 40 mL of DEPC-treated $H_2O$ and adjust the volume with DEPC-treated $H_2O$ to 50 mL for preparing 2.5 M glycine solution. This solution should be sterilized with a 0.22-μm filter and could be stored at room temperature (20°C–22°C) for up to 12 months.
- 1 M Tris-HCl (pH 8.0, pH 7.5, pH 7.4, and pH 7.0): dissolve 6.057 g of Tris base in 40 mL of DEPC-treated $H_2O$ and adjust the volume with DEPC-treated $H_2O$ to 50 mL for preparing 1 M Tris-HCl. Adjust to the needed pH values with HCl. This solution should be sterilized with a 0.22-μm filter and could be stored at 4°C for up to 12 months.

⚠ CRITICAL: HCl can cause severe burns once contact with skin and eye damage, even blindness. Carefully handle it in a fume hood and wear a laboratory coat, goggles, and gloves.

- 1 M $MgCl_2$: dissolve 10.165 g of $MgCl_2 \cdot 6H_2O$ in 40 mL of DEPC-treated $H_2O$ and adjust the volume with DEPC-treated $H_2O$ to 50 mL for preparing of 1 M $MgCl_2$. This solution should be sterilized with a 0.22-μm filter and could be stored at room temperature (20°C–22°C) for up to 12 months.
- 1 M $CaCl_2$: dissolve 5.549 g of $CaCl_2$ in 40 mL of DEPC-treated $H_2O$ and adjust the volume with DEPC-treated $H_2O$ to 50 mL for preparing of 1 M $CaCl_2$. This solution should be sterilized with a 0.22-μm filter and could be stored at room temperature (20°C–22°C) for up to 12 months.
- 5 M NaCl: dissolve 14.61 g of NaCl in 40 mL of DEPC-treated $H_2O$ and adjust the volume with DEPC-treated $H_2O$ to 50 mL for preparing 1 M NaCl. This solution should be sterilized with a 0.22-μm filter and could be stored at room temperature (20°C–22°C) for up to 12 months.
- 5 M NaOH: dissolve 2 g of NaOH in 7 mL of DEPC-treated $H_2O$ and adjust the volume with DEPC-treated $H_2O$ to 10 mL for preparing 5 M NaOH. This solution could be stored at room temperature (20°C–22°C) for up to 12 months.

⚠ CRITICAL: The NaOH solution can cause skin irritation and severe eye damage. Wear a lab coat, goggles, and gloves when handling the NaOH solution.

- 0.5 M EDTA (pH 8.0): dissolve 9.306 g of $Na_2EDTA \cdot 2H_2O$ in 40 mL of DEPC-treated $H_2O$, adjust the pH to 8.0 with NaOH, and adjust the volume with DEPC-treated $H_2O$ to 50 mL for preparing 0.5 M EDTA (pH 8.0). This solution should be sterilized with a 0.22-μm filter and could be stored at 4°C for up to 12 months.
- 0.5 M EGTA (pH 7.4): dissolve 9.51 g of EGTA in 40 mL of DEPC-treated $H_2O$, adjust the pH to 7.4 with NaOH, and adjust the volume with DEPC-treated $H_2O$ to 50 mL for preparing 0.5 M EGTA (pH 7.4). This solution should be sterilized with a 0.22-μm filter and could be stored at 4°C for up to 12 months.
- 10% (v/v) Triton X-100: dilute 5 mL of Triton X-100 with 45 mL DEPC-treated $H_2O$ for preparing 10% (v/v) Triton X-100. This solution should be sterilized with a 0.22-μm filter and could be stored at room temperature (20°C–22°C) for up to 12 months.
- 10% (v/v) Tween 20: dilute 5 mL of Tween 20 with 45 mL DEPC-treated $H_2O$ for preparing 10% (v/v) Tween 20. This solution should be sterilized with a 0.22-μm filter and could be stored at room temperature (20°C–22°C) for up to 12 months.
- 10% (v/v) NP-40: dilute 5 mL of NP-40 with 45 mL DEPC-treated $H_2O$ for preparing 10% (v/v) NP-40. This solution should be sterilized with a 0.22-μm filter and could be stored at room temperature (20°C–22°C) for up to 12 months.
- 10% (w/v) sodium deoxycholate: dissolve 5 g of sodium deoxycholate with 40 mL DEPC-treated $H_2O$ and adjust the volume with DEPC-treated $H_2O$ to 50 mL for preparing 10% (w/v) sodium deoxycholate. This solution should be sterilized with a 0.22-μm filter and could be stored at room temperature (20°C–22°C) for up to 12 months.
- 10% (w/v) SDS: dissolve 5 g of SDS with 40 mL DEPC-treated $H_2O$ and adjust the volume with DEPC-treated $H_2O$ to 50 mL for preparing 10% (w/v) SDS. This solution should be sterilized with a 0.22-μm filter and could be stored at room temperature (20°C–22°C) for up to 12 months.

⚠ CRITICAL: The SDS solution can cause skin irritation and severe eye damage. Wear a lab coat, goggles, and gloves when handling SDS.

- Solution A: dilute 100 μL of 5 M NaCl and 200 μL of 5 M NaOH in 9.7 mL DEPC-treated $H_2O$ to prepare solution A. This solution should be sterilized with a 0.22-μm filter and could be stored at 4°C for up to 6 months.
- Solution B: dilute 200 μL of 5 M NaCl in 9.8 mL DEPC-treated $H_2O$ for preparing solution B. This solution should be sterilized with a 0.22-μm filter and could be stored at 4°C for up to 6 months.

- 1 M $NaH_2PO_4$: dissolve 6.9 g of $NaH_2PO_4 \cdot H_2O$ in DEPC-treated $H_2O$ and adjust the volume with DEPC-treated $H_2O$ to 50 mL for preparing 1 M $NaH_2PO_4$. This solution could be stored at 4°C for up to 6 months.
- 1 M $Na_2HPO_4$: dissolve 7.1 g of $Na_2HPO_4 \cdot H_2O$ in DEPC-treated $H_2O$ and adjust the volume with DEPC-treated $H_2O$ to 50 mL for preparing 1 M $Na_2HPO_4$. This solution could be stored at 4°C for up to 6 months.
- 10 × TBE buffer: dissolve 108 g of Tris base, 7.44 g of $Na_2EDTA \cdot 2H_2O$, and 55 g of Boric acid in 800 mL of ultra-pure $H_2O$ and adjust the volume with DEPC-treated $H_2O$ to 1 L for preparing 10 × TBE buffer. This solution could be stored at room temperature (20°C–22°C) for up to 6 months.

| **Permeabilization buffer** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris-HCl (1 M, pH 7.5) | 10 mM | 50 μL |
| NaCl (5 M) | 10 mM | 10 μL |
| NP-40 (10%, v/v) | 0.5% (v/v) | 250 μL |
| Triton X-100 (10%, v/v) | 0.3% (v/v) | 150 μL |
| Tween 20 (10%, v/v) | 0.1% (v/v) | 50 μL |
| DEPC-treated $H_2O$ | N/A | 4.49 mL |
| **Total** | **N/A** | **5 mL** |

*Note:* Permeabilization buffer can be stored at 4°C for up to 6 months. Freshly add the RNase Inhibitor (final concentration of 2 U/mL) and the protease inhibitor cocktail (1:100 dilution) to the buffer before use.

| **1 × PNK buffer (0.2% NP-40, v/v)** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris-HCl (1 M, pH 7.4) | 50 mM | 5 mL |
| $MgCl_2$ (1 M) | 10 mM | 1 mL |
| NP-40 (10%, v/v) | 0.2% (v/v) | 2 mL |
| DEPC-treated $H_2O$ | N/A | 92 mL |
| **Total** | **N/A** | **100 mL** |

*Note:* 1 × PNK buffer (0.2% NP-40, v/v) can be stored at 4°C for up to 12 months.

| **1 × PNK buffer (0.05% NP-40, v/v)** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris-HCl (1 M, pH 7.4) | 50 mM | 5 mL |
| $MgCl_2$ (1 M) | 10 mM | 1 mL |
| NP-40 (10%, v/v) | 0.05% (v/v) | 500 μL |
| DEPC-treated $H_2O$ | N/A | 93.5 mL |
| **Total** | **N/A** | **100 mL** |

*Note:* 1 × PNK buffer (0.05% NP-40, v/v) can be stored at 4°C for up to 12 months.

| **1 × PNK+EGTA buffer** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris-HCl (1 M, pH 7.4) | 50 mM | 5 mL |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| EGTA (0.5 M) | 20 mM | 4 mL |
| NP-40 (10%, v/v) | 0.5% (v/v) | 5 mL |
| DEPC-treated $H_2O$ | N/A | 86 mL |
| Total | N/A | 100 mL |

*Note:* 1 × PNK+EGTA buffer can be stored at 4°C for up to 12 months.

| **High-salt wash buffer** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| PBS (10 ×) | 1 × | 50 mL |
| NP-40 (10%, v/v) | 0.5% (v/v) | 5 mL |
| DEPC-treated $H_2O$ | N/A | 45 mL |
| Total | N/A | 100 mL |

*Note:* High-salt wash buffer can be stored at 4°C for up to 12 months.

| **1 × Micrococcal Nuclease reaction buffer (1 × MN buffer)** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris-HCl (1 M, pH 8.0) | 50 mM | 500 μL |
| $CaCl_2$ (1 M) | 5 mM | 50 μL |
| DEPC-treated $H_2O$ | N/A | 9.45 mL |
| Total | N/A | 10 mL |

*Note:* 1 × MN buffer can be stored at 4°C for up to 6 months.

| **0.1 M sodium phosphate buffer (pH 8.0; 0.05% Tween 20, v/v)** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| $Na_2HPO_4$ (1 M) | 93.2 mM | 932 μL |
| $NaH_2PO_4$ (1 M) | 6.8 mM | 68 μL |
| Tween 20 (10%, v/v) | 0.05% (v/v) | 500 μL |
| DEPC-treated $H_2O$ | N/A | 8.5 mL |
| Total | N/A | 10 mL |

*Note:* Adjust pH to 8.0 and store at 4°C for up to 1 month.

| **Wash buffer** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| PBS (10 ×) | 1 × | 10 mL |
| NP-40 (10%, v/v) | 0.5% (v/v) | 5 mL |
| SDS (10%, w/v) | 0.1% (w/v) | 1 mL |
| Sodium deoxycholate (10%, w/v) | 0.5% (w/v) | 5 mL |
| DEPC-treated $H_2O$ | N/A | 79 mL |
| Total | N/A | 100 mL |

*Note:* Wash buffer can be stored at 4°C for up to 12 months.

| 1 × PNK buffer (0.5% NP-40, v/v) | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris-HCl (1 M, pH 7.4) | 50 mM | 5 mL |
| $MgCl_2$ (1 M) | 10 mM | 1 mL |
| NP-40 (10%, v/v) | 0.5% (v/v) | 5 mL |
| DEPC-treated $H_2O$ | N/A | 89 mL |
| **Total** | **N/A** | **100 mL** |

*Note:* 1 × PNK buffer (0.5% NP-40, v/v) can be stored at 4°C for up to 12 months.

| High-salt wash buffer II | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| PBS (10 ×) | 5 × | 50 mL |
| NP-40 (10%, v/v) | 0.5% (v/v) | 5 mL |
| SDS (10%, w/v) | 0.1% (w/v) | 1 mL |
| Sodium deoxycholate (10%, w/v) | 0.5% (w/v) | 5 mL |
| DEPC-treated $H_2O$ | N/A | 39 mL |
| **Total** | **N/A** | **100 mL** |

*Note:* High-salt wash buffer II can be stored at 4°C for up to 12 months.

| 2 × Tween washing and binding buffer (2 × TWB buffer) | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris-HCl (1 M, pH 7.5) | 10 mM | 100 μL |
| EDTA (0.5 M) | 1 mM | 20 μL |
| Tween 20 (10%, v/v) | 0.02% (v/v) | 20 μL |
| NaCl (5M) | 2 M | 4 mL |
| DEPC-treated $H_2O$ | N/A | 5.86 mL |
| **Total** | **N/A** | **10 mL** |

*Note:* 2 × TWB buffer can be stored at 4°C for up to 12 months.

| Protease K buffer | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris-HCl (1 M, pH 7.5) | 10 mM | 100 μL |
| EDTA (0.5 M) | 10 mM | 200 μL |
| SDS (10%, w/v) | 0.5% (w/v) | 500 μL |
| DEPC-treated $H_2O$ | N/A | 9.2 mL |
| **Total** | **N/A** | **10 mL** |

*Note:* PK buffer can be stored at room temperature (20°C–22°C) for up to 6 months.

| PK buffer | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris-HCl (1 M, pH 7.0) | 10 mM | 100 μL |

(*Continued on next page*)

*Continued*

| Reagent | Final concentration | Amount |
|---|---|---|
| EDTA (0.5 M) | 1 mM | 20 μL |
| NaCl (5M) | 100 mM | 200 μL |
| SDS (10%, w/v) | 0.5% (w/v) | 500 μL |
| DEPC-treated $H_2O$ | N/A | 9.18 mL |
| **Total** | **N/A** | **10 mL** |

***Note:*** PK buffer can be stored at room temperature (20°C–22°C) for up to 6 months.

## STEP-BY-STEP METHOD DETAILS

### Cell harvest and formaldehyde crosslinking

**Timing: ~1 h**

The following steps describe how to harvest adherent cells like HeLa cells for CRIC-seq. Suspension cells can be collected and washed by centrifugation.

1. Aspirate and discard the medium from the culture dish.
2. Rinse HeLa cells with 10 mL of pre-chilled 1 × PBS buffer three times.
3. Fix cells with 10 mL freshly prepared 1% (w/v) formaldehyde solution by shaking the dish on a platform rotator at low speed at room temperature (20°C–22°C) for 10 min.
4. Quench the fixation by adding 500 μL of 2.5 M glycine solution and shaking the dish on an orbital shaker at low speed at room temperature (20°C–22°C) for 10 min.
5. Discard the medium from the dish and rinse cells with 10 mL of pre-chilled 1 × PBS buffer three times.
6. Scrape off the cells from the culture dish and transfer the cell suspension to a new 50 mL centrifuge tube.
7. Centrifuge the cell suspension at 1,200 g for 10 min at 4°C and discard the supernatant. See troubleshooting, problem 1.
8. Resuspend the cell pellet with 1 mL of pre-chilled 1 × PBS buffer and transfer the cell suspension to a new 1.5 mL Eppendorf LoBind tube.
9. Centrifuge the cell suspension at 600 g for 10 min at 4°C and discard the supernatant.
10. Store the cell pellet at −80°C for up to one month or directly go to the following steps.

### Cell permeabilization and micrococcal nuclease treatment

**Timing: ~1.5 h**

In this step, cell permeabilization with detergents allows us to "punch holes" in cellular membranes for subsequent enzyme treatment while maintaining the cell intact. Micrococcal nuclease displays both endo- and exonuclease activity to digest free RNA unbound by proteins (Figure 1).

11. Permeabilize the cell pellet with 1 mL of permeabilization buffer and mix well by gently pipetting.
12. Incubate the cell suspension at 4°C for 15 min on a tube rotator at 20 rpm.
13. Centrifuge the cell suspension at 1,200 g for 5 min at 4°C and discard the supernatant.
14. Wash the cell pellet three times with pre-chilled 1 × PNK buffer (0.2% NP-40, v/v):
    a. Resuspend the cell pellet with 600 μL of pre-chilled 1 × PNK buffer (0.2% NP-40, v/v) and incubate the cell suspension at 4°C for 5 min on a tube rotator at 20 rpm.
    b. Centrifuge the cell suspension at 1,200 g for 5 min at 4°C and discard the supernatant.
    c. Repeat steps a and b twice.

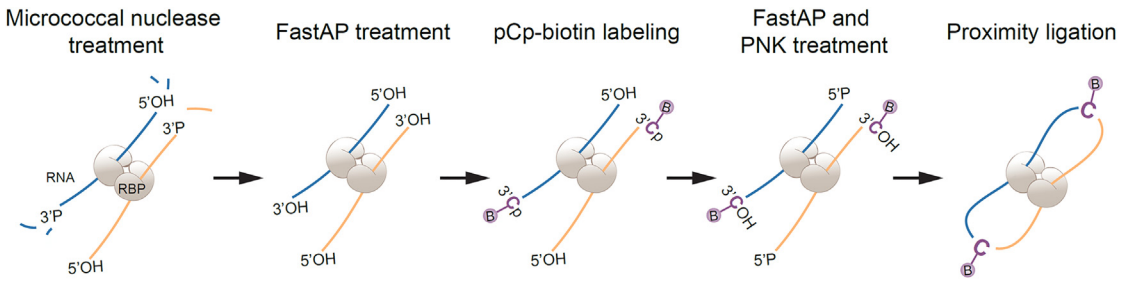


**Figure 1. Schematic diagram showing the *in situ* part of the CRIC-seq method**

15. Dilute the micrococcal nuclease to the final concentration of 0.03 U/μL (Here, we take the micrococcal nuclease from Thermo Fisher, Cat# EN0181, as an example):
    a. Dilute 0.5 μL of micrococcal nuclease with 499.5 μL of 1 × MN buffer (1:1000).
    b. Dilute 50 μL of 1:1000 dilution of micrococcal nuclease with 450 μL of 1 × MN buffer (1:10).
16. Resuspend the cell pellet from Step 14 with 200 μL of 1:10,000 dilution of micrococcal nuclease and mix well by gently pipetting.
17. Incubate the cell suspension at 37°C for 10 min in a ThermoMixer programmed with 15 s of intermittent mixing at 1,000 rpm every 3 min.
18. Centrifuge the cell suspension at 1,200 g for 5 min at 4°C and discard the supernatant.
19. Wash the cell pellet twice with pre-chilled 1 × PNK + EGTA buffer as described in Step 14.
20. Wash the cell pellet twice with pre-chilled 1 × PNK buffer (0.2% NP-40, v/v) as described in Step 14.

## pCp-biotin labeling

Timing: ~18 h

In this step, the 3′ends of fragmented RNAs are labeled with pCp-biotin to mark the junction of proximally ligated RNAs and allow the subsequent enrichment of chimeric RNAs (Figure 1).

21. Fast AP treatment:
    a. Set up the Fast AP reaction mixture containing 10 μL of 10 × Fast AP buffer, 10 μL of Fast Alkaline Phosphatase, and 80 μL of DEPC-treated $H_2O$ for each sample.
    b. Resuspend the cell pellet with the Fast AP reaction mixture and mix well by gently pipetting.
    c. Incubate the sample at 37°C for 15 min in a ThermoMixer programmed with 15 s of intermittent mixing at 1,000 rpm every 3 min.
22. Centrifuge the cell suspension at 1,200 g for 5 min at 4°C and discard the supernatant.
23. Wash the cell pellet twice with pre-chilled 1 × PNK + EGTA buffer as described in Step 14.
24. Wash the cell pellet twice with pre-chilled High-salt buffer as described in Step 14
25. Wash the cell pellet three times with pre-chilled 1 × PNK buffer (0.05% NP-40, v/v) as described in Step 14.
26. pCp-biotin labeling:
    a. Set up the labeling mixture containing 10 μL of 10 × RNA ligase reaction buffer, 6 μL of Ribolock RNase inhibitor, 4 μL of Biotinylated Cytidine (Bis) phosphate, 10 μL of T4 RNA ligase, and 20 μL of DEPC-treated $H_2O$ for each sample.

    *Note:* For PTBP1 IP (−pCp) libraries, exclude the Biotinylated Cytidine (Bis) phosphate.

    b. Resuspend the cell pellet with the labeling mixture and mix well by gently pipetting.
    c. Add 50 μL of 30% (w/v) PEG 20000 to the cell suspension and mix well by gently pipetting.

d. Incubate the sample at 16°C overnight (12–16 h) in a ThermoMixer programmed with 15 s of intermittent mixing at 1,000 rpm every 3 min.

*Note:* Cut off the pipette tip before aspirating the 30% (w/v) PEG 20000, and do not vortex when homogenizing the suspension.

e. Add 4 μL of 10 mM ATP and 2 μL of T4 RNA ligase to the sample and mix well by gently pipetting up and down on the next day.
f. Incubate the sample at 16°C for another 3 h in a ThermoMixer programmed with 15 s of intermittent mixing at 1,000 rpm every 3 min.

27. Centrifuge the sample at 1,200 g for 5 min at 4°C and discard the supernatant.
28. Wash the cell pellet three times with pre-chilled 1 × PNK buffer (0.2% NP-40, v/v) as described in Step 14.

### Proximity ligation

Timing: ~19 h

In this step, protein-mediated proximal RNA-RNA contacts are ligated (Figure 1).

29. Fast AP treatment:
    a. Set up the Fast AP mixture containing 10 μL of 10 × Fast AP buffer, 10 μL of Fast Alkaline Phosphatase, and 80 μL of DEPC-treated $H_2O$ for each sample.
    b. Resuspend the cell pellet with the Fast AP mixture and mix well by gently pipetting.
    c. Incubate the sample at 37°C for 15 min in a ThermoMixer programmed with 15 s of intermittent mixing at 1,000 rpm every 3 min.
30. Centrifuge the cell suspension at 1,200 g for 5 min at 4°C and discard the supernatant.
31. Wash the cell pellet twice with pre-chilled 1 × PNK + EGTA buffer as described in Step 14.
32. Wash the cell pellet twice with pre-chilled High-salt buffer as described in Step 14
33. Wash the cell pellet three times with pre-chilled 1 × PNK buffer (0.2% NP-40, v/v) as described in Step 14.
34. PNK treatment:
    a. Set up the PNK mixture containing 10 μL of 10 × reaction buffer A, 10 μL of T4 Polynucleotide Kinase, 15 μL of 10 mM ATP, and 65 μL of DEPC-treated $H_2O$ for each sample.
    b. Resuspend the cell pellet with the PNK mixture and mix well by gently pipetting.
    c. Incubate the sample at 37°C for 45 min in a ThermoMixer programmed with 15 s of intermittent mixing at 1,000 rpm every 3 min.
35. Wash the cell pellet twice with pre-chilled 1 × PNK + EGTA buffer as described in Step 14.
36. Wash the cell pellet three times with pre-chilled 1 × PNK buffer (0.05% NP-40, v/v) as described in Step 14.
37. Proximal RNA ligation:
    a. Set up the ligation mixture containing 20 μL of 10 × RNA ligase reaction buffer, 8 μL of Ribolock RNase inhibitor, 20 μL of 1 mg/mL BSA, 10 μL of T4 RNA ligase, and 142 μL of DEPC-treated $H_2O$ for each sample.
    b. Resuspend the cell pellet with the ligation mixture and mix well by gently pipetting.
    c. Incubate the sample at 16°C overnight (12–16 h) in a ThermoMixer programmed with 15 s of intermittent mixing at 1,000 rpm every 3 min.
    d. On the next day, add 4 μL of 10 mM ATP and 2 μL of T4 RNA ligase to the sample and mix well by gently pipetting.
    e. Incubate the sample at 16°C for another 3 h in a ThermoMixer programmed with 15 s of intermittent mixing at 1,000 rpm every 3 min.
38. Centrifuge the sample at 1,200 g for 5 min at 4°C and discard the supernatant.

39. Wash the cell pellet three times with pre-chilled 1 × PNK buffer (0.2% NP-40, v/v) as described in Step 14.

### Protein-RNA enrichment

Timing: ~7 h

In this step, the complex of PTBP1 and its associated RNA-RNA contacts is immunoprecipitated with a PTBP1-specific antibody.

40. Beads preparation:
    a. Prepare 50 μL of protein A/G magnetic beads per sample. Expel the beads into a new 1.5 mL Eppendorf LoBind tube and place it on a magnetic rack for 1 min.
    b. Discard the supernatant and rinse the beads three times with 500 μL of 0.1 M sodium phosphate buffer (pH 8.0) containing 0.05% Tween 20 by gently pipetting up and down.
    c. Resuspend beads with 150 μL 0.1 M sodium phosphate buffer (pH 8.0, 0.05% Tween20) containing 15 μg PTBP1-specific antibody.

    *Note:* Ensure that the volume of 0.1 M sodium phosphate buffer (pH 8.0) is triple the volume of the antibody. For IgG IP (+pCp) libraries, use IgG-specific antibodies.

    d. Incubate the beads with PTBP1 antibody at room temperature (20°C–22°C) for 1 h on a tube rotator at 20 rpm.
    e. Place the tube on a magnetic rack for 1 min and discard the supernatant.
    f. Wash beads three times with 500 μL of pre-chilled wash buffer by gently pipetting up and down.

    *Note:* To prevent over-drying the beads, prepare the cell lysis as described in Step 41 during the incubation in "d" and directly resuspend the PTBP1 antibody-coupled beads with the cell lysate after washing beads.

41. Cell lysis and immunoprecipitation:
    a. Resuspend the cell pellet from Step 39 with 500 μL of wash buffer and sonicate on ice with BRANSON SLPe at 40% amplitude for 10 s on/ 30 s off for 3 cycles. See troubleshooting, problem 2.
    b. Incubate the cell suspension at 4°C for 15 min on a tube rotator at 20 rpm.
    c. Centrifuge the sample at 16,000 g for 15 min at 4°C and collect the supernatant into a new 1.5 mL Eppendorf LoBind tube.
    d. Resuspend the PTBP1 antibody-coupled beads from Step 40 with the cell lysate and mix well by gently pipetting.
    e. Incubate the sample at 4°C for 4 h on a tube rotator at 20 rpm.
    f. Place the tube on a magnetic rack for 1 min and discard the supernatant.
42. Wash beads three times with 500 μL of pre-chilled wash buffer by gently pipetting up and down.
43. Wash beads once with 500 μL of pre-chilled High-salt wash buffer II by gently pipetting up and down.
44. Wash beads twice with 500 μL of pre-chilled 1 × PNK buffer (0.5% NP-40, v/v) by gently pipetting up and down.
45. Place the tube on a magnetic rack for 1 min and discard the supernatant.

### RNA purification

Timing: ~15 h

In this step, RNA-RNA contacts associated with PTBP1 are isolated from the protein-RNA complexes.

46. Resuspend the beads from Step 45 with 200 μL of proteinase K buffer and 50 μL of proteinase K and mix well by gently pipetting.
47. Incubate the sample at 37°C for 60 min, and then 56°C for 15 min in a ThermoMixer programmed with 15 s of intermittent mixing at 1,000 rpm every 3 min.
48. Place the tube on a magnetic rack for 1 min and transfer the supernatant to a new 1.5 mL Eppendorf LoBind tube.
49. Add 750 μL of Trizol LS to the supernatant, homogenize it by pipetting up and down 20 times, and incubate the sample at room temperature (20°C–22°C) for 5 min.
50. Add 220 μL of chloroform to the sample, mix well by shaking vigorously for 15 s, and incubate at room temperature (20°C–22°C) for 3 min.
51. Centrifuge the sample at 16,000 g for 15 min at 4°C and carefully pipette the aqueous phase to a new 1.5 mL Eppendorf LoBind tube.
52. Add 1 μL of GlycoBlue and 500 μL of isopropanol to the sample and mix well by pipetting up and down.
53. Precipitate the RNA at −20°C overnight (12–16 h).
54. Centrifuge the sample at 16,000 g for 15 min at 4°C and discard the supernatant.
55. Wash the RNA pellet twice with 600 μL of 75% (v/v) ethanol and centrifuge the sample at 16,000 g for 5 min at 4°C.
56. Discard the supernatant thoroughly and air-dry the pellet for 2 min.

*Note:* Do not over-dry the RNA.

57. Dissolve the pellet in 15 μL of DEPC-treated water.

**Pause point:** The RNA sample can be stored at −80°C for several weeks.

### pCp-biotin selection

**Timing: ~16 h**

In this step, chimeric RNAs are selected with streptavidin beads through biotinylated cytidine at the junction of ligated RNA fragments.

58. Blocking beads:
    a. Vortex the Dynabeads MyOne Streptavidin C1 beads in the vial for at least 30 s and transfer 20 μL of beads per sample to a new 1.5 mL Eppendorf LoBind tube.
    b. Place the tube on a magnetic rack for 1 min and discard the supernatant.
    c. Resuspend the beads with an equal volume of solution A and mix well by gently pipetting up and down.
    d. Incubate the beads at room temperature (20°C–22°C) for 2 min, place the tube on a magnetic rack for 1 min, and discard the supernatant.
    e. Repeat Steps a-d once.
    f. Wash the beads with an equal volume of solution B by gently pipetting up and down.
    g. Place the tube on a magnetic rack for 1 min and discard the supernatant.
    h. Resuspend the beads with 200 μL of the blocking mixture containing 50 μg of yeast RNA and 100 μL of 2 × TWB buffer. Mix well by gently pipetting up and down.
    i. Incubate the beads at room temperature (20°C–22°C) for 1 h on a tube rotator at 20 rpm.

    *Note:* To save time, perform Steps 59 and 60 during this 1-h incubation.

    j. Wash the beads three times with 600 μL of 1 × TWB buffer by gently pipetting up and down.

k. Place the tube on a magnetic rack for 1 min and discard the supernatant.

59. DNase I treatment:
    a. Set up the DNase I mixture containing 15 μL of RNA from Step 57, 10 μL of 10 × RQ1 DNase I buffer, 3 μL of RiboLock RNase Inhibitor, 5 μL of DNase I, and 67 μL of DEPC-treated $H_2O$.
    b. Incubate the DNase I mixture at 37°C for 20 min in a ThermoMixer programmed with 15 s of intermittent mixing at 1,000 rpm every 3 min.
    c. Purified RNA with ZYMO RESEARCH RNA Clean and Concentrator-5 according to the manufacturer's instructions. Elute the RNA with 17.5 μL DEPC-treated water and transfer the 16 μL of eluted RNA to a new 200 μL PCR tube.

**Pause point:** The resultant RNA can be stored at −80°C for several weeks.

60. RNA fragmentation:
    a. Add 4 μL of 5 × first-strand buffer to the RNA and mix well by pipetting up and down.
    b. Incubate the sample at 94°C for 5 min and put it on ice immediately.
61. Biotin-streptavidin selection:
    a. Add the 20 μL of fragmented RNA, 30 μL of DEPC-treated water, and 50 μL of 2 × TWB buffer to the beads from Step 58.
    b. Incubate the beads at room temperature (20°C–22°C) for 0.5 h on a tube rotator at 20 rpm.
    c. Place the tube on a magnetic rack for 1 min and discard the supernatant.
    d. Wash the beads four times with 600 μL of 1 × TWB buffer by gently pipetting up and down.
    e. Place the tube on a magnetic rack for 1 min and thoroughly discard the supernatant.
62. RNA elution:
    a. Resuspend the beads with 100 μL of PK buffer and mix well by pipetting up and down.
    b. Incubate the sample at 95°C for 10 min at 1000 rpm in a ThermoMixer.
    c. Place the tube on a magnetic rack for 1 min and transfer the supernatant to a new 1.5 mL Eppendorf LoBind tube.
    d. Repeat Steps a and b once. Place the tube on a magnetic rack for 1 min and transfer the supernatant to the same 1.5 mL Eppendorf LoBind tube from Step c.
    e. Resuspend the beads with 100 μL of PK buffer and mix well by pipetting up and down.
    f. Place the tube on a magnetic rack for 1 min and transfer the supernatant to the same 1.5 mL Eppendorf LoBind tube from Step c.
63. Add 300 μL of Acid Phenol: Chloroform to the 300 μL of sample and mix well by shaking vigorously.
64. Centrifuge the sample at 16,000 g for 10 min at 4°C and transfer the aqueous phase carefully to a new 1.5 mL Eppendorf LoBind tube.
65. Add 18 μL of 5 M NaCl, 1 μL of GlycoBlue, and 900 μL of 100% (v/v) ethanol to the sample sequentially. Mix well by pipetting up and down.
66. Precipitate the RNA at −20°C overnight (12–16 h).
67. On the next day, centrifuge the sample at 16,000 g for 20 min at 4°C and discard the supernatant.
68. Wash the RNA pellet twice with 600 μL of 75% (v/v) ethanol and centrifuge the sample at 16,000 g for 5 min at 4°C.
69. Discard the supernatant thoroughly and air-dry the pellet for 2 min.

*Note:* Do not over-dry the RNA.

70. Dissolve the RNA pellet in 10 μL of DEPC-treated water.

**Pause point:** The resultant RNA can be stored at −80°C for several weeks.

### Strand-specific library construction

**Timing:** ~9 h

In this step, RNAs enriched by PTBP1 antibody and streptavidin beads are converted into a strand-specific library with defined adapters for paired-end deep sequencing.

71. First-strand synthesis:
    a. Transfer the RNA solution from Step 70 to a new 200 μL PCR tube, add 0.5 μL of 100 ng/μL N6 primer to the solution and mix well by pipetting up and down.
    b. Incubate the sample at 65°C for 5 min in a thermal cycler, and immediately put it on ice for at least 2 min.
    c. Set up the reaction mixture containing 3 μL of 5 × first-strand buffer, 0.5 μL of Ribolock RNase inhibitor, 1 μL of 10 mM dNTPs, 0.5 μL of 0.1 M DDT, and 0.5 μL of SuperScript II Reverse Transcriptase.
    d. Add the reaction mixture to the RNA sample from Step b and mix well by pipetting up and down.
    e. Incubate the sample at 25°C for 10 min, 42°C for 40 min, and 70°C for 15 min in a thermal cycler.
    f. Transfer the sample to a new 1.5 mL Eppendorf LoBind tube.
72. Second-strand synthesis:
    a. Set up the second-strand mixture containing 10 μL of 5 × second-strand buffer, 0.8 μL of 25 mM dNTPs (dUTP), 0.2 μL of RNase H, 2.5 μL of *E. Coli* DNA Polymerase I and 20.5 μL of DEPC-treated $H_2O$ and mix well by pipetting up and down.
    b. Add the second-strand mixture to the 16 μL sample from Step 71 and mix well by pipetting up and down.
    c. Incubate the sample at 16°C for 2 h in a ThermoMixer programmed with 15 s of intermittent mixing at 300 rpm every 3 min.

*Note:* During the incubation, take out the AMPure XP beads from 4°C and equilibrate the beads for 30 min at room temperature (20°C–22°C).

73. Vortex the AMPure XP beads for 30 s, aspirate 90 μL of beads to the sample from Step 72 and mix well by pipetting.
74. Incubate the sample at room temperature (20°C–22°C) for 5 min, and then place the tube on a magnetic rack.
75. Discard the supernatant when the solution is clear from the beads.
76. Wash the beads with 200 μL of freshly prepared 80% (v/v) ethanol twice by spinning the tube on the magnetic rack.
77. Thoroughly discard the ethanol and let the beads dry in the air.
78. Resuspend the beads with 44 μL of Qiagen Elution Buffer and mix well by pipetting up and down.
79. Incubate the sample at room temperature (20°C–22°C) for 5 min, and then place the tube on a magnetic rack.
80. Transfer the 43 μL supernatant to a new 1.5 mL Eppendorf LoBind tube. Quantify the concentration of the cDNA with 1 μL of the sample with Qubit dsDNA HS Assay Kit according to the manufacturer's instruction.

*Note:* The expected yield is about 0.1–0.3 ng/μL.

81. End repair:
    a. Set up the end repair mixture containing 42 μL of cDNA from Step 80, 0.4 μL of 25 mM dNTPs, 1.2 μL of T4 DNA polymerase, 0.2 μL of Klenow Fragment, 1.2 μL of T4 Polynucleotide Kinase and 5 μL of 10 × Polynucleotide Kinase buffer and mix well by pipetting up and down.
    b. Incubate the sample at 20°C for 30 min in a ThermoMixer.
    c. Purify the sample with 90 μL of AMPure XP beads as described in Steps 73–77.

d. Resuspend the beads with 20.5 μL of Qiagen Elution Buffer and mix well by pipetting up and down.
e. Incubate the sample at room temperature (20°C–22°C) for 5 min, and then place the tube on a magnetic rack.
f. Transfer the 19.7 μL supernatant to a new 1.5 mL Eppendorf LoBind tube.

82. dA-tailing:
    a. Set up the dA-tailing mixture containing 19.7 μL of the sample, 2.3 μL of 10 × Blue buffer, 0.5 μL of 5 mM dATP, and 0.5 μL of Klenow (3′ to 5′ exo-).
    b. Incubate the sample at 37°C for 30 min in a ThermoMixer.
83. Adapter ligation:
    a. Set up the adapter mixture containing 1.4 μL of 2 × rapid ligation buffer, 0.1 μL of 10 mM ATP, and 1 μL of 2 μM adapter.
    b. Add the adapter mixture to 23 μL of the sample from Step 82 and mix well by pipetting up and down.
    c. Add 1 μL of T4 quick DNA ligase to the mixture from the last step and mix well by pipetting up and down.

    *Note:* Adding T4 quick DNA ligase last can prevent potential adapter-adapter ligation.

    d. Incubate the sample at 20°C for 15 min in a ThermoMixer.
84. Purify the sample with 47.7 μL of AMPure XP beads as described in Steps 73–77.
85. Resuspend the beads with 25 μL of Qiagen Elution Buffer and mix well by pipetting up and down.
86. Incubate the sample at room temperature (20°C–22°C) for 5 min, and then place the tube on a magnetic rack.
87. Transfer the 25 μL of supernatant to a new 1.5 mL Eppendorf LoBind tube.
88. Purify the sample with 45 μL of AMPure XP beads as described in Steps 73–77.
89. Resuspend the beads with 18.5 μL of Qiagen Elution Buffer and mix well by pipetting up and down.
90. Incubate the sample at room temperature (20°C–22°C) for 5 min, and then place the tube on a magnetic rack.
91. Transfer the 17.7 μL of supernatant to a new 200 μL PCR tube.

**Pause point:** The resultant DNA can be stored at −20°C for up to 1 month.

92. Test optimal PCR cycle numbers:
    a. Take 1 μL of the sample from Step 91 as a template and set up the PCR reaction master mix as follows:

| Reagent | Amount |
|---|---|
| DNA template | 1 μL |
| Platinum Pfx DNA Polymerase | 0.1 μL |
| User Enzyme | 0.5 μL |
| Illumina PE primer 1.0 (10 μM) | 1 μL |
| Index primer (10 μM) | 1 μL |
| MgSO4 (50 mM) | 1 μL |
| 10 × Pfx buffer | 2.5 μL |
| dNTPs (25 mM) | 0.4 μL |
| $ddH_2O$ | 17.5 μL |

    b. Perform the PCR amplification using the PCR cycling conditions:

| Steps | Temperature | Time | Cycles |
|---|---|---|---|
| Pretreatment | 37°C | 15 min | 1 |
| Initial Denaturation | 94°C | 2 min | 1 |
| Denaturation | 94°C | 15 s | 14 and 18 cycles |
| Annealing | 62°C | 30 s | |
| Extension | 72°C | 30 s | |
| Final extension | 72°C | 10 min | 1 |
| Hold | 4°C | forever | |

*Note:* Pretreatment at 37°C allows the digestion of dUTP-containing strand by USER enzyme. Typically test two different PCR cycle numbers for PTBP1 IP (+pCp) samples but not PTBP1 IP (−pCp) and IgG IP (+pCp) samples.

c. Determine the amount of PCR products by running the samples in 1.5% agarose gel in 1 × TBE at 120 V for ~1 h and examine the DNA bands with blue-light transilluminator. See troubleshooting for problems 3 and 4.

93. PCR with optimal PCR cycle number:

a. Set up the PCR reaction master mix as follows:

| Reagent | Amount |
|---|---|
| DNA template | 15.7 μL |
| Platinum Pfx DNA Polymerase | 0.4 μL |
| User Enzyme | 3 μL |
| Illumina PE primer 1.0 (10 μM) | 1 μL |
| Index primer (10 μM) | 1 μL |
| MgSO4 (50 mM) | 1 μL |
| 10 × Pfx buffer | 2.5 μL |
| dNTPs (25 mM) | 0.4 μL |

*Note:* Use different index primers for different samples. Amplifying all the samples with the same cycle numbers.

b. Perform the PCR amplification using the PCR cycling conditions:

| Steps | Temperature | Time | Cycles |
|---|---|---|---|
| Pretreatment | 37°C | 15 min | 1 |
| Initial Denaturation | 94°C | 2 min | 1 |
| Denaturation | 94°C | 15 s | the optimal cycle |
| Annealing | 62°C | 30 s | |
| Extension | 72°C | 30 s | |
| Final extension | 72°C | 10 min | 1 |
| Hold | 4°C | forever | |

⚠ CRITICAL: Excessive PCR amplification will increase the duplicate rate and decrease the complexity of libraries.

c. Run the samples in 1.5% agarose gel in 1 × TBE at 120 V for ~1 h and examine the DNA bands with blue-light transilluminator.

*Note:* Avoid using the UV transilluminator to prevent DNA damage.

d. Cut the smeared DNA bands between 200–450 bp from the agarose gel.
e. Purify the DNA with a Qiagen MinElute Gel Extraction kit according to the manufacturer's instruction and elute with 15 μL of Elution Buffer.
f. Quantify the concentration of the DNA with 1 μL of the sample with Qubit dsDNA HS Assay Kit according to the manufacturer's instruction.
g. Detect DNA length distribution of libraries with Agilent 2100 Bioanalyzer according to the manufacturer's instruction.

*Note:* The libraries can be stored at −20°C for 6 months or directly sequenced with the Illumina HiSeq X Ten platform using paired-end mode.

### Deep sequencing and data processing

Timing: ~12 days

In this step, strand-specific CRIC-seq libraries are sequenced with the Illumina NovaSeq 6000 platform in paired-end mode.

94. The RBP IP (+pCp) CRIC-seq libraries typically require ~200 million raw reads per sample, whereas the equal volume of IgG IP (+pCp) and RBP IP (−pCp) CRIC-seq libraries only can produce ~0.15 million raw reads per sample in our hands.
95. Process the CRIC-seq paired-end raw reads in FASTQ files using the RICpipe program (https://github.com/caochch/RICpipe)[8] to obtain intra- and inter-molecular chimeric reads in sam files. See troubleshooting for problems 5.
    a. Trim the adapters using the Trimmomatic software and remove PCR duplicates with identical sequences, but keep only one read.
    b. Align the remaining reads sequentially to the human genome using the STAR and BWA softwares to identify chimeric reads that are chiastically mapped.
    c. Classify the chimeric reads as intra-molecular if both arms are mapped to the same transcript while classifying those with two arms mapped to different transcripts as inter-molecular.
    d. Remove the intra-molecular chimeric reads that are mapped to splicing junctions.
    e. Combine the two replicates for downstream analysis if they exhibit a high correlation of chimeric reads per transcript.

*Note:* A high percentage of PCR duplicates in a sequencing dataset can indicate poor sequencing quality or unsuccessful library construction. The low percentage of PCR duplicates (~20%) in the case of PTBP1 CRIC-seq libraries suggests that the library construction was successful. Chimeric ratio measurement is another parameter to assess library quality. For the PTBP1 CRIC-seq library, we observed a chimeric read ratio of approximately 7%, with 90% of the chimeric reads being intra-molecular, consistent with our expectations. Additionally, we evaluated the reproducibility of the CRIC-seq library across biological replicates by calculating their Pearson correlation coefficient. The correlation coefficient was greater than 0.9, indicating high reproducibility between the replicates.

96. Identify background contacts during CRIC-seq library construction:
    a. Merge the chimeric reads from IgG IP (+pCp) and RBP IP (−pCp) and save them as a SAM format file named "background_Chimeric.sam".
    b. Extract the coordinates of the pairwise junction sites for the background chimeric reads and save them to a BEDPE format file named "background_Chimeric_junction.bedpair".
    c. Cluster the chimeric reads in the background group if their junction sites are in a pairwise 10-nt genomic window.

```
>cat IgG_(+pCp)_CRIC_Chimeric.sam RBP_(-pCp)_CRIC_Chimeric.sam > background_Chimeric.sam

>perl from_sam_to_bedOfJunctionPair.pl background_Chimeric.sam > background_Chimeric_
junction.bedpair

>perl merge_IgG_pCp_minus_to_background.pl background_Chimeric_junction.bedpair
```

97. Calculate the chimeric read counts for RBP IP (+pCp) and background CRIC-seq:
    a. Extract the coordinates of pairwise junction sites from RBP IP (+pCp) CRIC-seq chimeric reads and store them in a BEDPE format file named "RBP_(+pCp)_Chimeric_junction.bedpair".
    b. Collect the read counts for RBP IP (+pCp) CRIC-seq chimeric reads and background chimeric reads residing in background clusters and save them as files named "RBP_(+pCp)_Chimeric.in_background_cluster.list" and "background_Chimeric.in_background_cluster.list".

```
> perl from_sam_to_bedOfJunctionPair.pl RBP_(+pCp)_CRIC_Chimeric.sam > RBP_(+pCp)
_Chimeric_junction.bedpair

>bedtools pairtopair -a background.raw_cluster.bed -b RBP_(+pCp)_Chimeric_junction.bed-
pair -is -rdn > RBP_(+pCp)_Chimeric.in_background_cluster.list

>bedtools pairtopair -a background.raw_cluster.bed -b background_Chimeric_junction.bed-
pair -is -rdn > background_ Chimeric.in_ background_cluster.list
```

98. Remove background from RBP IP (+pCp) CRIC-seq libraries:
    a. Identify RBP IP (+pCp) CRIC-seq chimeric reads with read counts that are at least 5 times higher than the background chimeric read counts obtained in step 97, and extract them.
    b. Save the extracted chimeric reads as a SAM format file named "RBP_(+pCp)_CRIC_removed_background_Chimeric.sam" in the output directory.

```
> python remove_background_from_RBP_IP_chimeric_reads.py background.raw_cluster.bed
background_Chimeric.in_background_cluster.list RBP_(+pCp)_Chimeric.in_background_clus-
ter.list RBP_(+pCp)_CRIC_Chimeric.sam
```

*Note:* To determine the fold change item in "a", we have tested different values ranging from 1 to 100 for the removal of background chimeric reads. We found that a 5-fold change is an optimal threshold, as it effectively removes the background chimeric reads while preserving more of the RBP IP CRIC-seq chimeric reads. This parameter is independent of RNA expression and can be customized to suit the user's project requirements.

99. Identify the high-confidence clusters:
    a. Extract the pairwise junction site coordinates from the RBP IP (+pCp) CRIC-seq chimeric reads from step 98 and save them to a BEDPE file named "RBP_(+pCp)_CRIC_removed_background_Chimeric.list".
    b. Cluster the junction sites that are located within the same pairwise 10-nt genomic windows, and save the clustered sites to a file named "RBP_(+pCp)_CRIC_removed_background_Chimeric.cluster".

```
> perl from_sam_to_listOfJunctionPair.pl RBP_(+pCp)_CRIC_removed_background_Chimeric.
sam > RBP_(+pCp)_CRIC_removed_background_Chimeric.list
```

```
>less RBP_(+pCp)_CRIC_removed_background_Chimeric.list | sort -k3,3 -k8,8 > RBP_(+pCp)
_CRIC_removed_background_Chimeric.sort.list

> perl cluster_RBP_IP_chimeric_reads.pl RBP_(+pCp)_CRIC_removed_background_Chimeric.sort.
list > RBP_(+pCp)_CRIC_removed_background_Chimeric.cluster
```

*Note:* The determination of window sizes in "b" did not involve the use of statistical methods but is based on laboratory experience and published articles.[2,15] We found that a window size of 10 nucleotides provides high nucleotide resolution for RNA-RNA contact information. This parameter can be customized to meet the specific requirements of individual projects.

c. Select clusters that are supported by at least 2 chimeric reads.
d. Save the genome coordinates of these clusters as a BEDPE file named "removed_background_RBP_merged.Chimeric.cluster.2.bedpair".

```
> python make_RBP_IP_cluster_bedpair.py RBP_(+pCp)_CRIC_removed_background_Chimer-
ic.cluster read_count_cutoff
```

*Note:* To identify a cluster assembly, we set a minimum requirement of two chimeric reads and did not try a more stringent cutoff for the number of chimeric reads. The threshold for the number of chimeric reads can be customized by the user according to their requirements.

100. Predict the structure of U1 snRNAs:
    a. Assemble U1 (NR_004430.2) sequences into a mini-genome.
    b. Map sequencing reads to the U1 mini-genome using the RICpipe program.[8]
    c. Visualize the resulting chimeric reads mapped to U1 in the BAM format file using the Integrative Genomics Viewer (IGV) tool.

```
> samtools view -bS -o U1_chimeric_reads.bam U1_chimeric_reads.sam

> samtools sort -o U1_chimeric_reads.sort.bam U1_chimeric_reads.bam

> samtools index U1_chimeric_reads.sort.bam
```

*Note:* RNA-RNA interactions can sometimes lead to RNA duplex formation. Combining the RNA proximity information provided by CRIC-seq with RNA structure prediction software can model those RNA duplexes. Here, we took U1 snRNA as an example.

101. Detect the distribution of the PTBP1 binding motif in chimeric reads with the FIMO package from MEME suites[14]:
    a. Randomly shuffle the locations of chimeric reads of the same size to establish control.
    b. Calculate the average motif density in 100 windows around $\pm$ 1 kb of chimeric reads.

```
> fastaFromBed -fi human_reference_genome.fa -fo chimeric_reads.fa -bed chimeric_reads.bed
-name+ -s

> fasta-get-markov chimeric.reads.fa chimeric_reads.markov.b -norc -m 1

> fimo -no-qvalue -norc -thresh 0.01 -motif-pseudo 0.1 -max-stored-scores 100000000 -bgfile
chimeric_reads.markov.b -oc ./output PTBP1_binding_motif_pwms.txt chimeric_reads.fa

> bedtools shuffle -i chimeric_reads.bed -g human_reference_genome_size.txt -excl
chimeric_reads.bed > chimeric_reads_shuffled.bed
```

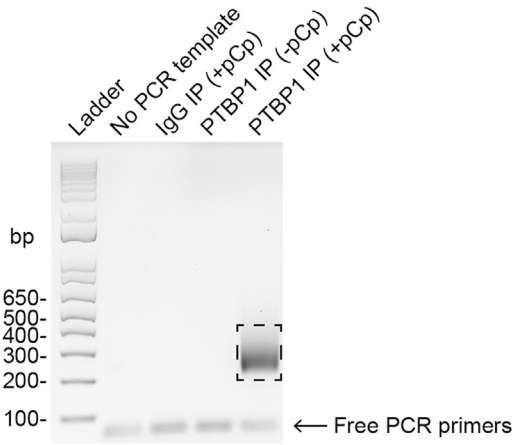


**Figure 2. Agarose gel showing the PCR products of CRIC-seq libraries**

*Note:* The position weight matrix of the PTBP1 binding motif, named "PTBP1_binding_motif_pwms.txt", is generated using the uniprobe2meme package of the MEME suite.[14]

## EXPECTED OUTCOMES

### PCR results for CRIC-seq libraries

The agarose gel image for expected CRIC-seq DNA libraries is shown in Figure 2. In contrast to no template PCR control and the two control libraries, smear bands can only be observed for the PTBP1 IP (+pCp) sample.

### Length distribution of CRIC-seq libraries

Before deep sequencing, users can analyze the size distribution of CRIC-seq libraries using the Agilent High Sensitivity DNA Kit with Agilent 2100 Bioanalyzer. The expected distribution of different libraries is shown in Figure 3.

### Chimeric read and cluster numbers of PTBP1 CRIC-seq libraries

The PTBP1 CRIC-seq libraries yielded 23,095,421 intra-molecular and 14,687,742 inter-molecular chimeric reads. After background removal, we obtained 23,076,006 intra-molecular and 14,683,054 inter-molecular chimeric reads, among which 3,983,560 and 718,602 high-confidence intra- and inter-molecular clusters were identified.

### U1 secondary structure captured by CRIC-seq

Figure 4 displays the U1 secondary structure as blue arc lines. Intra-molecular chimeric reads (n = 7380) mapped to U1 are illustrated as lines, and a black dot indicates the junction site.

### PTBP1 binding motif distribution in chimeric reads

PTBP1 binding motif (CCUCUCC) is enriched in chimeric reads compared with the shuffle group, as shown in Figure 5.

## LIMITATIONS

This protocol employs extensive buffer washing to inactivate enzymes instead of heat denaturation to preserve *in situ* RNA-RNA contacts. However, the extensive washing steps may result in the loss of many cells during centrifugation, leading to an inadequate cell lysate for efficient immunoprecipitation.

The target protein's expression level and the antibody's quality are limiting factors for this protocol. Low protein expression levels and inefficient antibodies would decrease the yield of protein-bound RNAs.

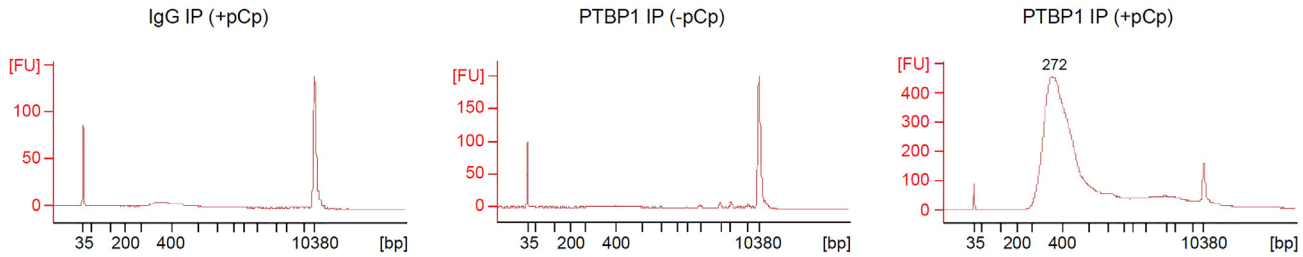


**Figure 3. Agilent 2100 Bioanalyzer showing length distribution of CRIC-seq libraries**

CRIC-seq cannot distinguish between direct and indirect RNA-RNA contacts associated with the RBP. However, sequencing data from CLIP-seq and its variants can be used to identify direct RBP binding sites. Integration with the RBP binding information can address this limitation.

Of note, it is common to combine replicates if they show a high correlation of chimeric reads per transcript to simplify the analysis process of various deep sequencing libraries, just like we did in this protocol. However, such an arbitrary data processing method does not explicitly address the variability of single events among replicates. Analyzing different replicates combinedly or separately can be determined by the user's research purpose.

## TROUBLESHOOTING

### Problem 1

Cells floating in the supernatant (cell harvest and formaldehyde crosslinking step 7).

### Potential solution

- Prolong the centrifugation time to 15 min and increase the speed to 2,400 g.
- Before centrifugation, add NP-40 to the cell suspension at the final concentration of 0.01% (v/v) to reduce cell surface tension.

### Problem 2

Cell suspension remains turbid after sonication (protein-RNA enrichment step 41).

### Potential solution

- Double the amount of wash buffer.
- Perform sonication one more time.

### Problem 3

DNA band between 100 and 200 bp (strand-specific library construction step 92).

### Potential solution

This DNA band is likely to be the self-ligation adapters from Step 83, which can be prevented by reducing the amount of adapters used in Step 83.

### Problem 4

No evident DNA band on the gel (strand-specific library construction step 92).

### Potential solution

- Increase the PCR cycle number.
- Increase the cell inputs at the beginning of CRIC-seq.

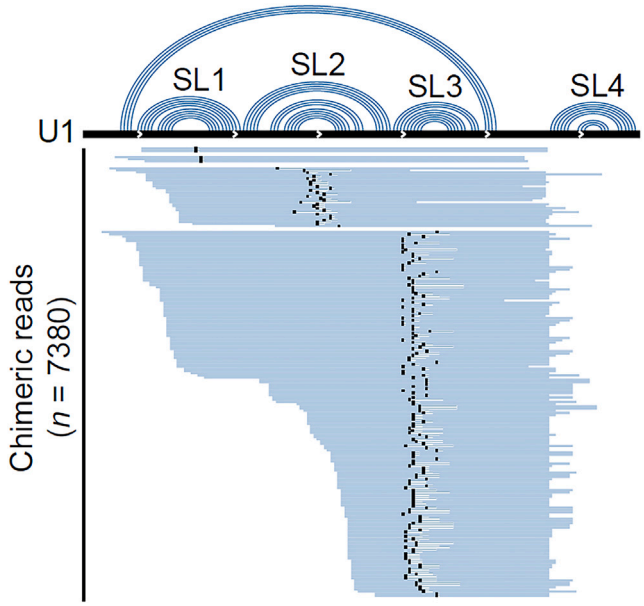


**Figure 4. U1 secondary structure captured by PTBP1 CRIC-seq chimeric reads**

### Problem 5

The chimeric read ratio is too low (deep sequencing and data processing step 95).

### Potential solution

- Increase the T4 RNA ligase concentration, ligation time, and sequencing depth.
- Confirm the cells are not contaminated.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yuanchao Xue (ycxue@ibp.ac.cn).

### Materials availability

This study did not generate new unique reagents.

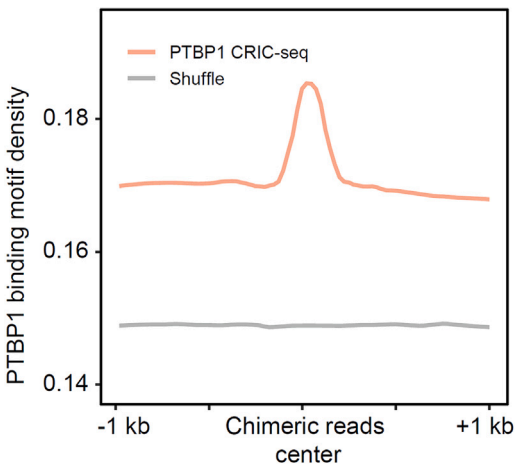


**Figure 5. The PTBP1 binding motif is enriched around CRIC-seq chimeric reads**

### Data and code availability

The accession number for the PTBP1 CRIC-seq data for HeLa cells (originally published in Ye et al.[1]) reported in this paper is GEO: GSE210583. The scripts for CRIC-seq data analysis can be found at Github: https://github.com/HuNaijing/CRIC-seq and Zenodo: https://doi.org/10.5281/zenodo.7668477.

## ACKNOWLEDGMENTS

This work was supported by the Strategic Priority Program of CAS (XDB37000000), the National Natural Science Foundation of China (32130064, 32025008, 91940306, and 81921003), the National Key R&D Program (2022YFA1303300), and the K.C. Wong Education Foundation (GJTD-2020-06) to Y.X.

## AUTHOR CONTRIBUTIONS

Y.X. conceived and supervised the project. R.Y. developed the CRIC-seq method and prepared the experimental parts of this protocol, and N.H. performed bioinformatics analysis and prepared the bioinformatics part.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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