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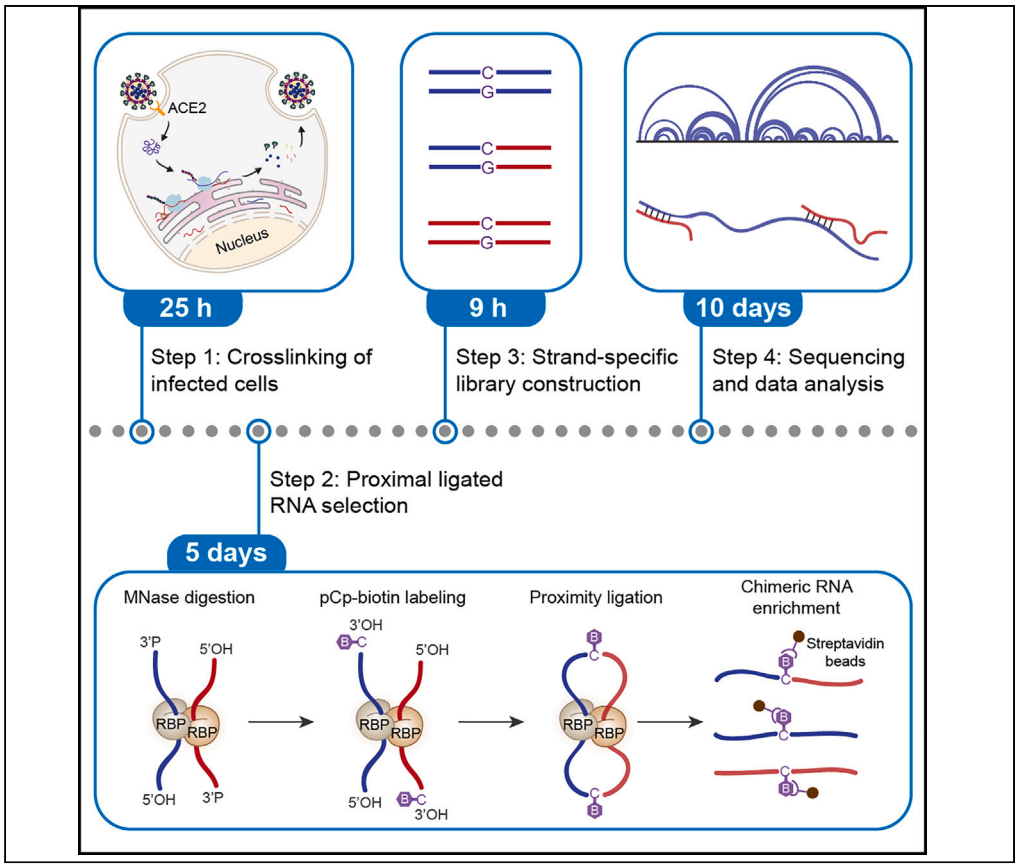
Protocol for profiling virus-to-host RNA-RNA interactions in infected cells by RIC-seq



Virus-to-host RNA-RNA interactions directly regulate host mRNA stability and viral replication. However, globally profiling virus-to-host *in situ* RNA-RNA interactions remains challenging. Here, we present an RNA *in situ* conformation sequencing (RIC-seq)-based protocol for mapping high-confidence virus-to-host *in situ* RNA-RNA interactions in infected cells. We detail steps for formaldehyde crosslinking, pCp-biotin labeling, *in situ* proximity ligation, chimeric RNA enrichment, strand-specific library construction, and data analysis. This protocol allows unbiased identification of virus-to-host RNA-RNA interactions for various RNA viruses and is potentially applicable to DNA virus-derived transcripts.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

RIC-seq for mapping virus-to-host *in situ* RNA-RNA interactions

Step-by-step protocol for constructing RIC-seq libraries

Pipelines of RIC-seq data analysis

Identification of high-confidence virus-to-host RNA-RNA interactions

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Protocol for profiling virus-to-host RNA-RNA interactions in infected cells by RIC-seq

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SUMMARY

Virus-to-host RNA-RNA interactions directly regulate host mRNA stability and viral replication. However, globally profiling virus-to-host *in situ* RNA-RNA interactions remains challenging. Here, we present an RNA *in situ* conformation sequencing (RIC-seq)-based protocol for mapping high-confidence virus-to-host *in situ* RNA-RNA interactions in infected cells. We detail steps for formaldehyde crosslinking, pCp-biotin labeling, *in situ* proximity ligation, chimeric RNA enrichment, strand-specific library construction, and data analysis. This protocol allows unbiased identification of virus-to-host RNA-RNA interactions for various RNA viruses and is potentially applicable to DNA virus-derived transcripts.
For complete details on the use and execution of this protocol, please refer to Zhao et al.[1]

BEFORE YOU BEGIN

RNA viruses can infect various host cells and interact with the cellular RNAs.[1–4] The interactions between viral RNA and host RNA have been demonstrated to regulate many biological processes, such as viral replication,[2,5,6] host immune evasion,[7,8] and viral packaging.[9] Identifying high-confidence virus-to-host RNA-RNA interactions is crucial for understanding the molecular mechanisms of viral replication and infection, which can be further employed for developing targeted antiviral therapies. We previously developed a RIC-seq (RNA *in situ* conformation sequencing) technology for profiling *in situ* RNA-RNA interactions in HeLa cells.[10] In this protocol, we further implemented RIC-seq in profiling SARS-CoV-2-to-host RNA-RNA interactions in infected cells and provided a step-by-step protocol. Of note, RIC-seq also identified intra-molecular RNA-RNA interactions of host or viral RNAs that can be used to deduce their secondary structures and tertiary interactions. In addition to SARS-CoV-2, this protocol can also be applicable for studying RNA-RNA interactions in other RNA or DNA virus-infected cells.

We routinely prepared three biological replicates for constructing RIC-seq libraries, each utilizing 1×10^7 infected cells. Considering the high abundance of SARS-CoV-2 RNA within the infected cells, which may promiscuously contact with host RNAs, we further introduced an *in vitro* random ligation library by extracting the whole complement of RNA fragments protected by proteins and performing random ligations in a diluted solution. To eliminate any potential false-positive signals and pinpoint reliable virus-to-host RNA-RNA interactions, we only used uniquely mapped inter-molecular chimeric reads that did not overlap with random ligated chimeric reads for downstream

Monte Carlo simulation.[11] The observed pairwise interactions were scrutinized against simulated interactions to identify significant SARS-CoV-2-to-host RNA-RNA interactions.

Adapter preparation

Timing: ~2 h

⚠ CRITICAL: Adapters should be prepared before the RIC-seq experiment.

1. Dissolve the paired-end illumina (PEI) adapter oligo A and B with DEPC-treated water to a final concentration of 100 μM, respectively.
2. Add 10 μL of PEI adapter oligo A (100 μM) and 10 μL of PEI adapter oligo B (100 μM) to a 200-μL PCR tube.
3. Mix thoroughly and incubate the solution at 70°C for 10 min in a preheated thermal cycler.
4. Remove the PCR tube and place it on the laboratory bench. Let the solution cool down to 20°C–25°C.

Note: The annealed adapters should be aliquot and stored at −20°C for several years. The final concentration of annealed adapters is 50 μM.

5. Before the RIC-seq experiment, dilute the 50 μM annealed adapters to 2 μM by mixing 2 μL of 50 μM annealed adapters with 48 μL of DEPC-treated water. Mix well and store at −20°C for no more than one year.

Infected cells preparation

Timing: ~25 h

6. The SARS-CoV-2 strain used in this protocol, IPBCAMS-YL01/2020, was isolated from a clinical sample by the Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College.
7. SARS-CoV-2 viruses were amplified in Vero cells. SARS-CoV-2 infectious titers were determined through plaque assay in Vero cells.
8. Cultured A549-ACE2 cells with a density of 80% in 10-cm dishes were brought to enhanced biosafety level 3 (BSL-3) facilities and infected with SARS-CoV-2 (MOI = 0.1) for 1 h in Opti-MEM medium. The infected cells were cultured in a maintenance medium (OPTI-MEM medium containing 1% penicillin/streptomycin and 1% BSA) for 24 h.
9. Remove the culture medium, and wash infected cells three times with ice-cold PBS.
10. Add 10 mL 1% (w/v) formaldehyde to the dish, and incubate for 20 min at 20°C–25°C on a horizontal shaker.

Note: The 20-min formaldehyde fixation in this step is necessary because the fixed samples must be heated at 65°C for 30 min to inactivate viruses following the strict P3 laboratory rules. This process may result in partial de-crosslinking. If your sample does not require heat treatment, the formaldehyde treatment time can be reduced to 10 min.

11. Add 500 μL of 2.5 M glycine to the dish to quench the crosslinking, mix gently, and incubate for 10 min at 20°C–25°C on a horizontal shaker.
12. Remove the supernatant and wash infected cells three times with ice-cold PBS.
13. Scrape infected cells from the culture dish and transfer them to a 50-mL centrifuge tube.
14. Centrifuge at 1,200 *g* at 4°C for 10 min to collect the cell pellet.

Note: This protocol is also applicable to tissue samples and suspension cells. For tissue samples, such as lung tissue, we typically take ~ 0.5 cm^3 of lung tissue from COVID-19 patients and cut it into ~1 mm^3 pieces with a sterile blade. Minced tissue samples or suspension cells were treated with the same conditions as adherent cells, but the minced tissue samples or cell pellets were washed and collected by centrifugation. See troubleshooting, problem 1.

15. Discard the supernatant completely. Resuspend the cell pellet with 1 mL ice-cold PBS and transfer to a 1.5-mL Eppendorf LoBind tube.
16. Centrifuge at 600 *g* at 4°C for 10 min, and discard the supernatant.
17. The fixed infected cells were heated at 65°C for 30 min to inactivate the virus.

⚠ CRITICAL: All the experiments related to live SARS-CoV-2 virus were conducted in enhanced biosafety level 3 (P3+) facilities authorized by the National Health Commission of the People's Republic of China.

Note: It should be conducted in a biosafety laboratory with the appropriate level if working with other RNA viruses.

Pause point: The fixed cell pellet can be stored at −80°C for no more than 1 month.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Tris	Amresco	Cat# 0497-1KG
Boric acid	Amresco	Cat# 0588-1KG
Glycine	Amresco	Cat# 0167-1KG
Hydrochloric acid (HCl)	Sinopharm	Cat# 10011008
Sodium hydroxide (NaOH)	Sigma-Aldrich	Cat# S8045-500G
Formaldehyde solution	Sigma-Aldrich	Cat# F8775-500ML
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	Sigma-Aldrich	Cat# M0250-500G
Sodium chloride (NaCl)	Sigma-Aldrich	Cat# S3014-1KG
IGEPAL CA-630 (NP-40)	Sigma-Aldrich	Cat# I8896-100ML
Triton X-100	Sigma-Aldrich	Cat# T8787-250ML
Tween 20	Sigma-Aldrich	Cat# P9416-100ML
Calcium chloride ($CaCl_2$)	Sigma-Aldrich	Cat# 793639-500G
Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)	Sigma-Aldrich	Cat# E3889-100G
Ethylenediaminetetraacetic acid disodium salt dehydrate ($(Na_2EDTA \cdot 2H_2O)$)	Sigma-Aldrich	Cat# E5134-1KG
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	Cat# L3771-500G
Ethanol	Sinopharm	Cat# 10009218
Isopropanol	Sinopharm	Cat# 80109218
Acid phenol: chloroform	Amresco	Cat# E277-400ML
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich	Cat# D5758-100ML
GelRed	Biotium	Cat# 41003
Agarose	Biowest	Cat# 111860
Yeast RNA	Roche	Cat# 10109223001
10× PBS	Invitrogen	Cat# AM9624
SuperScript II reverse transcriptase	Thermo Fisher Scientific	Cat# 18064-014
DNA polymerase I	Enzymatics	Cat# P7050L
5× second strand buffer	Thermo Fisher Scientific	Cat# 10812-014
T4 polynucleotide kinase	Enzymatics	Cat# Y9040L
T4 DNA polymerase	Enzymatics	Cat# P7080L
Klenow fragment	Enzymatics	Cat# P7060L

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Klenow exo- (3′ to 5′ exo minus)	Enzymatics	Cat# P7010-LC-L
T4 DNA ligase (rapid)	Enzymatics	Cat# L6030-HC-L
Platinum SuperFi DNA polymerase	Thermo Fisher Scientific	Cat# 12351050
T4 polynucleotide kinase	Thermo Fisher Scientific	Cat# EK0032
RQ1 RNase-free DNase	Promega	Cat# M6101
RiboLock RNase inhibitor	Thermo Fisher Scientific	Cat# EO0381
Proteinase K	Takara	Cat# 9034
SUPERase·In RNase inhibitor	Thermo Fisher Scientific	Cat# AM2694
FastAP thermosensitive alkaline phosphatase	Thermo Fisher Scientific	Cat# EF0651
T4 RNA ligase	Thermo Fisher Scientific	Cat# EL0021
Micrococcal nuclease	Thermo Fisher Scientific	Cat# EN0181
USER enzyme	New England Biolabs	Cat# M5505S
Protease inhibitor cocktail	Sigma-Aldrich	Cat# P8340-5ML
RNase H	Thermo Fisher Scientific	Cat# EN0202
TURBO DNase	Thermo Fisher Scientific	Cat# AM2238
Dynabeads MyOne streptavidin C1	Thermo Fisher Scientific	Cat# 65002
Agencourt AMPure XP beads	Beckman Coulter	Cat# A63881
GlycoBlue coprecipitant	Thermo Fisher Scientific	Cat# AM9515
Sodium acetate (3 M, pH 5.5)	Thermo Fisher Scientific	Cat# AM9740
TRIzol reagent LS	Thermo Fisher Scientific	Cat# 10296028
Chloroform	Sinopharm	Cat# 10006818
Adenosine 5′-triphosphate	New England Biolabs	Cat# P0756S
Deoxynucleotide (dNTP) solution mix	New England Biolabs	Cat# N0447S
Deoxynucleotide (dNTP) solution set	New England Biolabs	Cat# N0446S
dUTP solution	Thermo Fisher Scientific	Cat# R0133
Penicillin-streptomycin	Thermo Fisher Scientific	Cat# 15140163
DMEM	Thermo Fisher Scientific	Cat# C11965500BT
Opti-MEM	Thermo Fisher Scientific	Cat# 31985070
Trypsin-EDTA (0.25%)	Thermo Fisher Scientific	Cat# 25200072
Fetal bovine serum	Thermo Fisher Scientific	Cat# 10091-148
Critical commercial assays		
Qubit dsDNA HS assay kit	Thermo Fisher Scientific	Cat# Q32854
Qubit RNA HS assay kit	Thermo Fisher Scientific	Cat# Q32855
MinElute gel extraction kit	QIAGEN	Cat# 28606
RNA 3′ end biotinylation kit	Thermo Fisher Scientific	Cat# 20160
RNA clean and concentrator-5	Zymo Research	Cat# R1016
Agilent DNA high sensitivity kit	Agilent	Cat# 5067-4626
Experimental models: Cell lines		
Human: A549-ACE2	Dr. Jianwei Wang laboratory	N/A
Deposited data		
Raw and analyzed data	Zhao et al.[1]	GSA-Human: HRA005709
Oligonucleotides		
PEI adapter oligo A	Cao et al.[12]	/5Phos/GATCGGAAGAGCACACGTCT (5Phos: 50 phosphorylation)
PEI adapter oligo B	Cao et al.[12]	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
P5 primer	Cao et al.[12]	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT
P7 index primer	Cao et al.[12]	CAAGCAGAAGACGGCATACGAGAT NNNNNNGTGACTGGAGTTCAGACGT GTGCTCTTCCGATCT (N: random nucleotide)
Software and algorithms		
RICpipe	Cao et al.[12]	https://github.com/caochch/RICpipe
Trimmomatic version 0.36	Bolger et al.[13]	RRID:SCR_011848; http://www.usadellab.org/cms/index.php?page=trimmomatic

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
cutadapt version 2.6	Martin[14]	RRID: SCR_011841; http://code.google.com/p/cutadapt/
BLASTN version 2.5.0	Altschul et al.[15]	RRID: SCR_001598; https://github.com/JacobLondon/Blastn
STAR aligner version 2.5.2b	Dobin et al.[16]	RRID:SCR_004463;https://github.com/alexdobin/STAR
Bwa aligner version 0.7.17	Li[17]	RRID:SCR_010910;https://biobwa.sourceforge.net/
Bowtie2 version 2.3.4.3	Langmead et al.[18]	RRID: SCR_016368; https://github.com/BenLangmead/bowtie2
HISAT2 aligner version 2.1.0	Kim et al.[19]	RRID: SCR_015530; http://daehwankimlab.github.io/hisat2/
BEDTools version 2.30.0	Quinlan and Hall[20]	RRID:SCR_006646; https://bedtools.readthedocs.io/en/latest/
Piranha version 1.2.1	Andrew D. Smith	RRID:SCR_010903; http://smithlab.usc.edu/plone/software/piranha
Circos version 0.65	Krzywinski et al.[21]	RRID: SCR_011798; http://circos.ca/
The script for data analysis	This paper	Zenodo: https://doi.org/10.5281/zenodo.10609902 Github: https://github.com/Zhaohailian/SARS-CoV-2_STAR_protocols
Others		
Filter unit (0.22 μm)	Merck	Cat# SLGV033RB
Cell culture dishes (10 cm)	NEST	Cat# 704001
15 mL centrifuge tubes	NEST	Cat# 601002
50 mL centrifuge tubes	NEST	Cat# 602002
5 mL serological pipette	NEST	Cat# 326001
10 mL serological pipette	NEST	Cat# 327001
Qubit assay tubes	Thermo Fisher Scientific	Cat# Q32856
Cell lifter	Corning	Cat# 3008
1.5 mL LoBind microcentrifuge tubes	Eppendorf	Cat# 022431021
0.2 mL PCR tubes	Axygen	Cat# PCR-02-C
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
NanoDrop 2000c spectrophotometer	Thermo Fisher Scientific	Cat# ND-2000
Qubit 3.0 fluorometer	Thermo Fisher Scientific	Cat# Q33216
Agarose gel electrophoresis (Mini-Sub Cell GT)	Bio-Rad	Cat# 170-4486
Gel tray	Bio-Rad	Cat# 170-4435
Safe Imager2.0 blue-light transilluminator	Invitrogen	Cat# G6600
Razor blade	Personna	Cat# 84-0701
Universal power supply	Bio-Rad	Cat# 1645070
DynaMag-2 magnet for 1.5 mL tubes	Thermo Fisher Scientific	Cat# 12321D
Vortex mixer	Thermo Fisher Scientific	Cat# 88880018
Tube rotator	Crystal	Cat# TR-02U
CO2 incubators for cell culture	Thermo Fisher Scientific	Cat# 3111

MATERIALS AND EQUIPMENT

All the solutions and buffers used to construct RIC-seq libraries were prepared with DEPC-treated water.

- 1 × PBS. Dilute 100 mL of 10 × PBS with 900 mL of DEPC-treated water. The solution could be stored at 4°C for several months.
- 1% (w/v) formaldehyde solution. Mix 270 μL of 37% (w/v) formaldehyde with 9.73 mL of 1 × PBS. The solution should be prepared freshly before use.

⚠ CRITICAL: Formaldehyde is toxic. Wear the laboratory coat and gloves and handle it carefully.

- 2.5 M glycine. Add 3.7535 g of glycine to 15 mL of DEPC-treated water and adjust the volume to 20 mL. The solution should be sterilized with a 0.22-μm filter and could be stored at 20°C–25°C for several months.

- 1 M Tris-HCl, pH 7.0, pH 7.4, pH 7.5, and pH 8.0. Add 6.057 g of Tris base to 40 mL of DEPC-treated water. Use HCl to adjust the pH values in a fume hood and supply the total volume to 50 mL with DEPC-treated water. The solution should be sterilized with a 0.22-μm filter and could be stored at 4°C for several months.

⚠ CRITICAL: HCl is highly corrosive and can easily cause severe burns to the skin. To prevent skin or eye contact, wear a laboratory coat, gloves, and goggles when preparing the solution.

- 5 M NaCl. Add 14.61 g of NaCl to 45 mL of DEPC-treated water and adjust the volume to 50 mL. The solution should be sterilized with a 0.22-μm filter and could be stored at 20°C–25°C for several months.
- 1 M $CaCl_2$. Add 5.549 g of $CaCl_2$ to 45 mL of DEPC-treated water and adjust the volume to 50 mL. The solution should be sterilized with a 0.22-μm filter and could be stored at 20°C–25°C for several months.
- 1 M $MgCl_2$. Add 10.165 g of $MgCl_2 \cdot 6H_2O$ to 45 mL of DEPC-treated water and adjust the volume to 50 mL. The solution should be sterilized with a 0.22-μm filter and could be stored at 20°C–25°C for several months.
- 5 M NaOH. Add 2 g of NaOH to 8 mL of DEPC-treated water and adjust the volume to 10 mL. The solution could be stored at 20°C–25°C for several months.

⚠ CRITICAL: NaOH is highly corrosive. To prevent skin or eye contact, wear a laboratory coat, gloves, and goggles when preparing the solution.

- 0.5 M EGTA, pH 7.4. Add 9.51 g of EGTA to 40 mL of DEPC-treated water. Use NaOH to adjust the pH values and supply the volume to 50 mL with DEPC-treated water. The solution should be sterilized with a 0.22-μm filter and could be stored at 4°C for several months.
- 0.5 M EDTA, pH 8.0. Add 9.306 g of $Na_2EDTA \cdot 2H_2O$ to 40 mL of DEPC-treated water. Use NaOH to adjust the pH values and supply the volume to 50 mL with DEPC-treated water. The solution should be sterilized with a 0.22-μm filter and could be stored at 4°C for several months.
- 10% (w/v) SDS. Add 5 g of SDS to 40 mL of DEPC-treated water and adjust the volume to 50 mL. The solution should be sterilized with a 0.22-μm filter and could be stored at 20°C–25°C for several months.

⚠ CRITICAL: SDS can cause skin irritation and severe eye damage. Wear the laboratory coat, gloves, and goggles when preparing the solution.

10 × TBE buffer

Reagent	Final concentration	Amount
Tris	890 mM	108 g
$Na_2EDTA \cdot 2H_2O$	20 mM	7.44 g
Boric acid	890 mM	55 g
Total	N/A	N/A

Note: Dissolve the Tris base, $Na_2EDTA \cdot 2H_2O$, and Boric acid with 800 mL of DEPC-treated water and adjust the volume to 1 L. The solution could be stored at 20°C–25°C for up to 6 months.

Vero and A549-ACE2 cell culture medium

Reagent	Final concentration	Amount
DMEM basic medium	N/A	445 mL
FBS	10%	50 mL
Penicillin-streptomycin	100 U/mL	5 mL
Total	N/A	500 mL

Note: The ready-prepared medium could be stored at 4°C for several months.

Permeabilization buffer

Reagent	Final concentration	Amount
1 M Tris-HCl (pH 7.5)	10 mM	100 μL
5 M NaCl	10 mM	20 μL
NP-40	0.5% (v/v)	25 μL
Triton X-100	0.3% (v/v)	15 μL
Tween 20	0.1% (v/v)	5 μL
DEPC-treated water	N/A	9.835 mL
Total	N/A	10 mL

Note: The permeabilization buffer without protease inhibitor and RNase inhibitor should be sterilized with a 0.22-μm filter and could be stored at 4°C for several months. Add protease inhibitor (final concentration of 1×) and SUPERase·In RNase inhibitor (final concentration of 2 U/μL) before use.

1 × PNK buffer (0.2% NP-40)

Reagent	Final concentration	Amount
1 M Tris-HCl (pH 7.4)	50 mM	2.5 mL
1 M $MgCl_2$	10 mM	500 μL
NP-40	0.2% (v/v)	100 μL
DEPC-treated water	N/A	46.9 mL
Total	N/A	50 mL

Note: 1 × PNK buffer (0.2% NP-40) should be sterilized with a 0.22-μm filter after NP-40 was completely dissolved. The solution could be stored at 4°C for several months.

1 × MN buffer

Reagent	Final concentration	Amount
1 M Tris-HCl (pH 8.0)	50 mM	500 μL
1 M $CaCl_2$	5 mM	50 μL
DEPC-treated water	N/A	9.45 mL
Total	N/A	10 mL

Note: 1 × MN buffer should be sterilized with a 0.22-μm filter and could be stored at 4°C for several months.

1 × PNK buffer (0.05% NP-40)

Reagent	Final concentration	Amount
1 M Tris-HCl (pH 7.4)	50 mM	2.5 mL
1 M $MgCl_2$	10 mM	500 μL
NP-40	0.05% (v/v)	25 μL
DEPC-treated water	N/A	46.975 mL
Total	N/A	50 mL

Note: 1 × PNK buffer (0.05% NP-40) should be sterilized with a 0.22-μm filter after NP-40 was completely dissolved. The solution could be stored at 4°C for several months.

1 × PNK+EGTA buffer

Reagent	Final concentration	Amount
1 M Tris-HCl (pH 7.4)	50 mM	2.5 mL
0.5 M EGTA	20 mM	2 mL
NP-40	0.5% (v/v)	250 μL
DEPC-treated water	N/A	45.25 mL
Total	N/A	50 mL

Note: 1 × PNK+EGTA buffer should be sterilized with a 0.22-μm filter after NP-40 was completely dissolved. The solution could be stored at 4°C for several months.

High-salt buffer

Reagent	Final concentration	Amount
10 × PBS	5 ×	25 mL
NP-40	0.5% (v/v)	250 μL

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Continued		
Reagent	Final concentration	Amount
DEPC-treated water	N/A	24.75 mL
Total	N/A	50 mL

Note: High-salt buffer should be sterilized with a 0.22-μm filter after NP-40 was completely dissolved. The solution could be stored at 4°C for several months.

Proteinase K buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl (pH 7.5)	10 mM	100 μL
0.5 M EDTA	10 mM	200 μL
10% SDS (wt/vol)	0.5% (w/v)	500 μL
DEPC-treated water	N/A	9.2 mL
Total	N/A	10 mL

Note: Proteinase K buffer should be sterilized with a 0.22-μm filter and could be stored at 20°C–25°C for several months.

5× hybridization buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl (pH 7.4)	500 mM	500 μL
5 M NaCl	1 M	200 μL
DEPC-treated water	N/A	300 μL
Total	N/A	1 mL

Note: 5× hybridization buffer could be stored at −20°C for several years.

Solution A		
Reagent	Final concentration	Amount
5 M NaCl	50 mM	100 μL
5 M NaOH	100 mM	200 μL
DEPC-treated water	N/A	9.7 mL
Total	N/A	10 mL

Note: Solution A should be sterilized with a 0.22-μm filter and could be stored at 4°C for several months.

Solution B		
Reagent	Final concentration	Amount
5 M NaCl	100 mM	200 μL
DEPC-treated water	N/A	9.8 mL
Total	N/A	10 mL

Note: Solution B should be sterilized with a 0.22-μm filter and could be stored at 4°C for several months.

2× Tween washing and binding buffer (2× TWB buffer)		
Reagent	Final concentration	Amount
1 M Tris-HCl (pH 7.5)	10 mM	100 μL
0.5 M EDTA	1 mM	20 μL
5 M NaCl	2 M	4 mL
Tween 20	0.02% (v/v)	2 μL
DEPC-treated water	N/A	5.878 mL
Total	N/A	10 mL

Note: 2× TWB buffer should be sterilized with a 0.22-μm filter after Tween 20 was completely dissolved. The solution could be stored at 4°C for several months.

1× TWB buffer		
Reagent	Final concentration	Amount
2× TWB buffer	1×	5 mL
DEPC-treated water	N/A	5
Total	N/A	10 mL

Note: 1× TWB buffer should be sterilized with a 0.22-μm filter and could be stored at 4°C for several months.

PK buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl (pH 7.0)	10 mM	100 μL
5 M NaCl	100 mM	200 μL
0.5 M EDTA	10 mM	20 μL
10% SDS (wt/vol)	0.5% (w/v)	500 μL
DEPC-treated water	N/A	9.18 mL
Total	N/A	10 mL

Note: PK buffer should be sterilized with a 0.22-μm filter and could be stored at 20°C–25°C for several months.

STEP-BY-STEP METHOD DETAILS

Permeabilization and micrococcal nuclease digestion

Timing: ~1.5 h

During this procedure, fixed cells are treated with permeabilization buffer to punch holes in cellular membranes for subsequent enzymatic reactions while maintaining the cells intact. Micrococcal nuclease treatment can remove free RNAs not protected by proteins (Figure 1).

1. Permeabilize cells by resuspending the cell pellet with 1 mL of permeabilization buffer.
2. Mix well by gently pipetting 10 times and incubating the mixture at 4°C for 15 min on the rotator at 20 rpm.
3. Collect the cell pellet by centrifuging at 4°C for 5 min at 1,200 *g*, and remove the supernatant.
4. Wash the cell pellet with ice-cold 1× PNK buffer (0.2% (v/v) NP-40) three times.
   a. Add 600 μL of ice-cold 1× PNK buffer (0.2% (v/v) NP-40) to resuspend cell pellet by gently pipetting 10 times.
   b. Incubate the mixture at 4°C for 5 min on the rotator at 20 rpm.
   c. Collect the cell pellet by centrifuging the tube at 4°C for 5 min at 1,200 *g*, then remove the supernatant.
   d. Wash the cell pellet twice more by repeating steps a-c.
5. Prepare micrococcal nuclease (MNase) working solution.
   a. Add 0.2 μL of MNase and 199.8 μL of 1× MN buffer to a 0.2-mL PCR tube, and mix well by gently pipetting 20 times (1:1000 dilution).
   b. Add 45 μL of diluted MNase and 405 μL of 1× MN buffer to a 1.5 mL Eppendorf LoBind tube and mix well by gently pipetting 20 times (1:10,000 dilution).
6. Resuspend cell pellet from step 4 with MNase working solution from step 5b.
7. Mix well by gently pipetting 10 times, and incubate the sample at 37°C for 10 min in a ThermoMixer with 15 s of intermittent mixing at 1,000 rpm every 2 min.
8. Collect the cell pellet by centrifuging the tube at 4°C for 5 min at 1,200 *g*.
9. Wash cell pellet twice with ice-cold 1× PNK + EGTA buffer, then twice with ice-cold 1× PNK buffer (0.2% (v/v) NP-40) as described in step 4.

pCp-biotin labeling

Timing: ~18 h

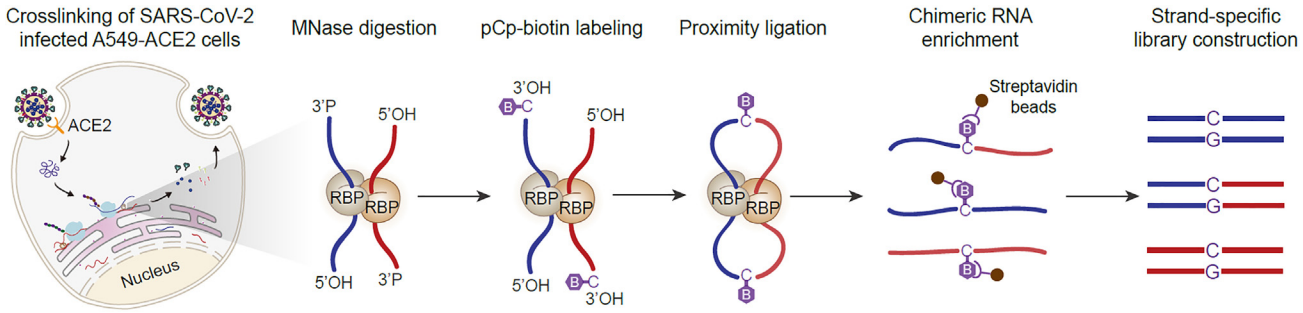

Figure 1. Diagram RIC-seq on profiling virus-to-host RNA-RNA interactions
Adapted from Zhao et al., Mol Cell, 2024.[1]

During this procedure, the 3′ end of proximal RNA fragments were pCp-biotin labeled for enriching chimeric RNA subsequently (Figure 1).

10. Fast AP treatment:
    a. Resuspend cell pellet with Fast AP reaction mixture containing 10 μL of 10× Fast AP buffer, 10 μL of fast alkaline phosphatase, and 80 μL of DEPC-treated water.
    b. Mix well by gently pipetting 10 times, and incubate the sample at 37°C for 15 min in a ThermoMixer with 15 s of intermittent mixing at 1,000 rpm every 2 min.

    Note: Please cut off the first 3 mm of the 200-μL tip to prevent cell damage.

    c. Collect the cell pellet by centrifuging the tube at 4°C for 5 min at 1,200 *g*.
    d. Wash cell pellet twice with ice-cold 1× PNK + EGTA buffer, twice with ice-cold high-salt buffer, and then three times with ice-cold 1× PNK buffer (0.05% (v/v) NP-40) as described in step 4.
11. pCp-biotin labeling:
    a. Resuspend cell pellet with labeling reaction mixture containing 10 μL of 10× T4 RNA ligase reaction buffer, 6 μL of Ribolock RNase inhibitor, 4 μL of Biotinylated cytidine (Bis) phosphate (pCp-biotin), 10 μL of T4 RNA ligase (10 U/μL), and 20 μL of DEPC-treated water.
    b. Mix well by gently pipetting 10 times, then add 50 μL of 30% (w/v) PEG 20000 to the sample with a cut-off 200-μL tip.
    c. Mix well by gently pipetting 10 times, and incubate the sample at 16°C for 12–16 h in a ThermoMixer with 15 s of intermittent mixing at 1,200 rpm every 3 min.

    Note: PEG 20000 is sticky. Homogenize the reaction mixture gently, and do not vortex.

12. Add 2 μL of T4 RNA ligase and 4 μL of 10 mM ATP to the sample on the next day. Mix well by gently pipetting 10 times, and incubate the sample at 16°C for 3 h in a ThermoMixer with 15 s of intermittent mixing at 1,200 rpm every 3 min.
13. Collect the cell pellet by centrifuging the tube at 4°C for 5 min at 1,200 *g*.
14. Wash cell pellet three times with ice-cold 1× PNK buffer (0.2% (v/v) NP-40) as described in step 4.

Proximity ligation

Timing: ~19 h

During this procedure, proximal RNAs are ligated with T4 RNA ligase (Figure 1).

15. Fast AP treatment:
    a. Resuspend cell pellet with Fast AP reaction mixture containing 10 μL of 10× Fast AP buffer, 10 μL of fast alkaline phosphatase, and 80 μL of DEPC-treated water.
    b. Mix well by gently pipetting 10 times, and incubate the sample at 37°C for 15 min in a ThermoMixer with 15 s of intermittent mixing at 1,000 rpm every 2 min.

    Note: To prevent cell damage, please cut off the first 3 mm of the 200-μL tip.

    c. Collect the cell pellet by centrifuging the tube at 4°C for 5 min at 1,200 *g*.
    d. Wash cell pellet twice with ice-cold 1× PNK + EGTA buffer, twice with ice-cold high-salt buffer, and then three times with ice-cold 1× PNK buffer (0.2% (v/v) NP-40) as described in step 4.
16. T4 PNK treatment:
    a. Resuspend cell pellet with T4 PNK reaction 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 water.
    b. Mix well by gently pipetting 10 times, and incubate the sample at 37°C for 45 min in a ThermoMixer with 15 s of intermittent mixing at 1,000 rpm every 3 min.
    c. Collect the cell pellet by centrifuging the tube at 4°C for 5 min at 1,200 *g*.
    d. Wash cell pellet twice with ice-cold 1 × PNK + EGTA buffer and twice with ice-cold 1 × PNK buffer (0.05% (v/v) NP-40) as described in step 4.
17. Proximal RNA ligation:
    a. Resuspend cell pellet with ligation mixture containing 20 μL of 10× T4 RNA ligase reaction buffer, 8 μL of Ribolock RNase inhibitor, 20 μL of 1 mg/mL BSA, 10 μL of T4 RNA ligase (10 U/μL), and 142 μL of DEPC-treated water.
    b. Mix well by gently pipetting 10 times, and incubate the sample at 16°C for 12–16 h in a ThermoMixer with 15 s of intermittent mixing at 1,000 rpm every 3 min.
18. Add 2 μL of T4 RNA ligase and 4 μL of 10 mM ATP to the sample on the next day. Mix well by gently pipetting 10 times, and incubate the sample at 16°C for 3 h in a ThermoMixer with 15 s of intermittent mixing at 1,000 rpm every 3 min.
19. Collect the cell pellet by centrifuging the tube at 4°C for 5 min at 1,200 *g*.
20. Wash cell pellet three times with ice-cold 1× PNK buffer (0.2% (v/v) NP-40) as described in step 4.

RNA purification

Timing: ~15 h

During this procedure, total RNAs are isolated and purified from protein-RNA complexes.

21. Proteinase K treatment:
    a. Resuspend cell pellet with 200 μL of proteinase K buffer, then add 50 μL of proteinase K to the sample. Mix well by gently pipetting 10 times.
    b. Incubate the sample at 37°C for 60 min and then 56°C for 15 min in a ThermoMixer with 15 s of intermittent mixing at 1,000 rpm every 3 min.
22. Add 750 μL of Trizol LS to the sample. Mix well by gently pipetting 20 times and incubate at 20°C–25°C for 5 min.
23. Add 200 μL of chloroform to the sample and shake vigorously on the vortex for 15 s.
24. Incubate the sample at 20°C–25°C for 3 min, then centrifuge the tube at 4°C for 10 min at 16,000 *g*.
25. Collect the aqueous phase carefully and transfer it to a new 1.5-mL Eppendorf tube. Precipitate total RNA by adding 500 μL of isopropanol and 1 μL of GlycoBlue to the tube.
26. Mix well by gently pipetting 20 times and placing the sample at −20°C for 12–16 h.
27. Next day, centrifuge the tube at 4°C for 15 min at 16,000 *g* and remove the supernatant.

28. Wash the RNA pellet twice with 600 μL of 75% (v/v) ethanol stored at −20°C. Centrifuge the tube at 4°C for 5 min at 16,000 *g*.
29. Remove the supernatant completely, and air-dry the RNA pellet for 2 min.

Note: Avoid over-drying the RNA.

30. Add 20 μL of DEPC-treated water to the tube and dissolve the pellet completely by gently pipetting 50 times. Take 1 μL of sample and measure the concentration using NanoDrop 2000c.

Note: The expected RNA yield in this step is 50 μg–80 μg. The amount of total RNA depends on the host cell type, and different cells may yield various amounts of total RNA.

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

DNase digestion

Timing: ~13 h

RNA-DNA ligation products produced by T4 RNA ligase are removed from total RNA with RQ1 DNase treatment during this procedure.

31. RQ1 DNase treatment:
    a. Take 20 μg of total RNA to a new 1.5-mL Eppendorf tube, and add 10 μL of 10× RQ1 DNase buffer, 3 μL of Ribolock RNase inhibitor, 5 μL of RQ1 DNase to the tube. Adjust the total volume to 100 μL with DEPC-treated water.
    b. Incubate the sample at 37°C for 20 min in a ThermoMixer C.
32. Add 100 μL of DEPC-treated water and 200 μL of acid phenol: chloroform to the sample sequentially. Mix well by shaking.
33. Centrifuge the tube at 4°C for 10 min at 16,000 *g*.
34. Collect the aqueous phase carefully and transfer it to a new 1.5-mL Eppendorf tube. Precipitate RNA by sequentially adding 20 μL of 3 M sodium acetate, 1 μL of GlycoBlue, and 500 μL of 100% (v/v) ethanol to the tube.
35. Mix well by gently pipetting 20 times and placing the sample at −20°C for 12–16 h.
36. Next day, centrifuge the tube at 4°C for 20 min at 16,000 *g* and remove the supernatant.
37. Wash the RNA pellet twice with 600 μL of 75% (v/v) ethanol stored at −20°C. Centrifuge the tube at 4°C for 5 min at 16,000 *g*.
38. Remove the supernatant completely, and air-dry the RNA pellet for 2 min.

Note: Avoid over-drying the RNA.

39. Add 6 μL of DEPC-treated water to the tube and dissolve the pellet completely by gently pipetting 50 times.

Pause point: The DNase-treated RNA can be stored at −80°C for several weeks.

rRNA depletion

Timing: ~2 h

This procedure removes rRNAs in total RNAs using the published RNase H-based rRNA depletion method.[22] We synthesized 195 DNA probes with 50 nt-length that were reverse complementary to each rRNA and mixed them with equal molar masses.

40. Transfer the RNA sample from step 39 to a new 0.2-mL PCR tube. Add 10 μL of rRNA probes (2 μg/μL) mixture and 4 μL of 5× hybridization buffer to the tube. Mix well by gently pipetting 20 times.
41. Incubate the sample for 2 min at 95°C in a thermal cycler, then gradually slow down to 22°C at −0.1°C/s, hold at 22°C for 5 min, and immediately place the tube on ice.
42. Add 10 μL of RNase H digestion mixture containing 3 μL of 10× RNase H buffer, 2 μL of DEPC-treated water, and 5 μL of RNase H to the tube.
43. Mix well by gently pipetting 20 times, and incubate the sample for 30 min at 37°C in the thermal cycler.
44. Add 10 μL of Turbo DNase digestion mixture containing 4 μL of 10× TURBO DNase buffer, 1 μL of DEPC-treated water, and 5 μL of TURBO DNase to the tube.
45. Mix well by gently pipetting 20 times, and incubate the sample for 30 min at 37°C in the thermal cycler.
46. Purify the rRNA-depletion RNA using an RNA Clean & Concentrator-5 kit following the manufacturer's instructions. Finally, RNA was eluted with 18.5 μL of DEPC-treated water, and we could get 17 μL product. Take 1 μL of RNA and quantify it using a Qubit RNA HS assay kit and Qubit fluorometer following the manufacturer's instructions.

Note: The expected RNA yield in this step is 2 μg–4 μg. See troubleshooting, problem 2.

Pause point: The rRNA-depletion RNA can be stored at −80°C for several weeks.

pCp-biotin selection

Timing: ~16 h

Biotin-labeled RNAs are selected with Dynabeads MyOne Streptavidin C1 beads during this procedure (Figure 1).

Note: To save time, Dynabeads MyOne Streptavidin C1 beads could be prepared during rRNA removal.

47. Blocked C1 beads preparation:
    a. Homogenize the C1 beads in the vial by vortexing for 30 s at least. Transfer the required volume (20 μL for each sample) of C1 beads to a 1.5-mL Eppendorf tube.
    b. Place the tube on the magnetic rack for 1 min and remove the supernatant.
    c. Add an equal volume of solution A to the tube, resuspend the C1 beads by gently pipetting 10 times, and incubate at 20°C–25°C for 2 min.
    d. Place the tube on the magnetic rack for 1 min and remove the supernatant.
    e. Wash C1 beads once more by repeating steps c and d.
    f. Add an equal volume of solution B to the tube, and resuspend the C1 beads by gently pipetting 10 times.
    g. Place the tube on the magnetic rack for 1 min and remove the supernatant. Add blocking mixture containing 100 μL of 2× TWB buffer, 32 μL of yeast RNA (~50 μg), and 68 μL of DEPC water to the tube.
    h. Mix well by gently pipetting 20 times and incubate the sample on a rotator at 20°C–25°C for 60 min at the speed of 20 rpm.
    i. Place the tube on the magnetic rack for 1 min and remove the supernatant. Resuspend the beads with 600 μL of ice-cold 1× TWB buffer and mix well by gently pipetting 10 times.
    j. Wash C1 beads twice more by repeating step i.
    k. Place the tube on the magnetic rack for 1 min and remove the supernatant.
48. RNA fragmentation:
    a. Transfer the RNA sample from step 46 to a 0.2-mL PCR tube, add 4 μL of 5× first-strand buffer to the tube, and mix well by gently pipetting 20 times.

b. Incubate the sample for 5 min at 94°C in the thermal cycler, and immediately place the tube on ice.

49. pCp-biotin selection:
    a. Add 50 μL of 2× TWB buffer, 20 μL of fragmented RNA from step 48, and 30 μL of DEPC-treated water to the blocked beads from step 47 and mix well by gently pipetting 20 times.
    b. Incubate the sample on a rotator at 20°C–25°C for 30 min at 20 rpm.
    c. Wash C1 beads four times with 600 μL of ice-cold 1× TWB buffer by gently pipetting up and down.
    d. Place the tube on the magnetic rack for 1 min and remove the supernatant.
50. RNA elution:
    a. Resuspend the beads with 100 μL of PK buffer and mix well by gently pipetting 10 times.
    b. Place the tube in a ThermoMixer C and incubate at 95°C for 10 min with continuous mixing at 1,000 rpm.
    c. Place the tube on the magnetic rack for 1 min and transfer the supernatant to a new 1.5-mL Eppendorf tube.
    d. Repeat steps a and b once more to elute RNA. At last, rinse C1 beads with 100 μL of PK buffer.
    e. Place the tube on the magnetic rack for 1 min and transfer the supernatant to the 1.5-mL Eppendorf tube. The total eluted volume is 300 μL.
51. Purify RNA with acid phenol: chloroform:
    a. Mix the eluted product with 300 μL of acid phenol: chloroform.
    b. Mix well by shaking and centrifuge the tube at 4°C for 10 min at 16,000 *g*.
    c. Collect the aqueous phase carefully and transfer it to a new 1.5-mL Eppendorf tube. Precipitate RNA by adding 18 μL of 5 M NaCl, 1 μL of GlycoBlue, and 900 μL of 100% (v/v) ethanol to the tube.
    d. Mix well by gently pipetting 20 times and placing the sample at −20°C for 12–16 h.
52. Next day, centrifuge the tube at 4°C for 20 min at 16,000 *g* and remove the supernatant.
53. Wash the RNA pellet twice with 600 μL of 75% (v/v) ethanol stored at −20°C. Centrifuge the tube at 4°C for 5 min at 16,000 *g*.
54. Remove the supernatant completely, and air-dry the RNA pellet for 2 min.

Note: Avoid over-drying the RNA.

55. Add 10 μL of DEPC-treated water to the tube and dissolve the pellet completely by gently pipetting 50 times.

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

Strand-specific library construction

Timing: ~9 h

This procedure converts RNAs selected by streptavidin beads into a strand-specific library for paired-end deep sequencing.

56. First-strand cDNA synthesis:
    a. Transfer the RNA from step 55 to a 0.2-mL PCR tube, and add 0.5 μL of 100 ng/μL N6 primer to the sample.
    b. Incubate the sample for 5 min at 65°C in the thermal cycler, and immediately place the tube on ice for at least 2 min.
    c. Add the first-strand cDNA synthesis reaction mixture containing 3 μL of 5× first-strand buffer, 0.5 μL of Ribolock RNase inhibitor, 0.5 μL of 0.1 M DTT, 1 μL of 10 mM dNTPs, and 0.5 μL of SuperScript II reverse transcriptase to the tube.

d. Mix well by gently pipetting 20 times. Incubate the sample at 25°C for 10 min, 42°C for 40 min, 70°C for 15 min, and hold at 12°C in the thermal cycler.

57. Second-strand DNA synthesis:
    a. Transfer the reverse transcription product to a new 1.5-mL Eppendorf tube. Add the second-strand DNA synthesis mixture containing 10 μL of 5× second-strand buffer, 0.2 μL of RNase H, 0.8 μL of 25 mM dNTPs (dUTP), 20.5 μL of Ultra-pure water, and 2.5 μL of Escherichia coli DNA polymerase I to the tube.

Note: Prepare 25 mM dNTPs (dUTP) by mixing 10 μL of 100 mM dATP, 10 μL of 100 mM CTP, 10 μL of 100 mM dGTP, 2 μL of 100 mM dTTP, and 8 μL of 100 mM dUTP. The final concentrations of dATP, dCTP, dGTP, dTTP, and dUTP are 25 mM, 25 mM, 25 mM, 5 mM, and 20 mM respectively.

    b. Mix well by gently pipetting 20 times, and incubate the sample at 16°C for 2 h in the ThermoMixer C with 15 s of intermittent mixing at 300 rpm every 3 min.

58. Purify DNA product with AMPure XP beads:
    a. Equilibrate the AMPure XP beads for 30 min at 20°C–25°C and homogenize the XP beads by vortexing for 30 s at least.

Note: AMPure XP beads could be equilibrated at 20°C–25°C during second-strand DNA synthesis to save time.

    b. Transfer 90 μL of XP beads to the sample from step 57, and mix well by gently pipetting 10 times.
    c. Incubate the sample for 5 min at 20°C–25°C, then place the Eppendorf tube on the magnetic rack until the solution becomes clear.
    d. Remove the supernatant. Add 200 μL of freshly prepared 80% (v/v) ethanol to the Eppendorf tube. Gently spin the tube for two revolutions on the magnetic rack.
    e. Repeat step d to wash the XP beads once more.
    f. Discard the ethanol completely. Leave the open Eppendorf tube on a magnetic stand and air-dry for 1–3 min.
    g. Add 44 μL of Qiagen elution buffer and resuspend XP beads by gently pipetting 10 times.
    h. Incubate the sample for 5 min at 20°C–25°C. Place the Eppendorf tube on the magnetic rack until the solution becomes clear.
    i. Transfer the supernatant (43 μL) to a new 1.5-mL Eppendorf tube carefully.

59. Take 1 μL of DNA product and quantify it using a Qubit dsDNA HS assay kit and Qubit fluorometer following the manufacturer's instructions.

Note: The expected DNA yield in this step is 10 ng–25 ng.

Pause point: The purified DNA product can be stored at −20°C for several weeks.

60. End repair:
    a. Add end repair reaction mixture containing 5 μL of 10× T4 polynucleotide kinase buffer, 0.4 μL of 25 mM dNTPs, 1.2 μL of T4 DNA polymerase, 0.2 μL of Klenow fragment, and 1.2 μL of T4 polynucleotide kinase to the sample from step 58.
    b. Mix well by gently pipetting 20 times, and incubate the sample at 20°C for 30 min in the ThermoMixer C.

61. Purify the end-repaired product with 90 μL of AMPure XP beads and elute DNA with 20.5 μL of Qiagen elution buffer following step 58.
62. Transfer the supernatant (19.7 μL) to a new 1.5-mL Eppendorf tube carefully.

63. dA-tailing:
    a. Add dA-tailing reaction mixture containing 2.3 μL of 10× Blue buffer, 0.5 μL of 5 mM dATP, and 0.5 μL of Klenow (3′–5′ exo-) to the sample.
    b. Mix well by gently pipetting 20 times, and incubate the sample at 37°C for 30 min in the ThermoMixer C.
64. Adapter ligation:
    a. Add adapter ligation reaction mixture containing 1.4 μL of 2× rapid ligation buffer, 0.1 μL of 10 mM ATP, 1 μL of 2 μM Adapter, and 1 μL of T4 DNA ligase (Rapid) to the sample from step 63.

⚠ CRITICAL: T4 DNA ligase (Rapid) should last be added to the sample to prevent adapter self-ligation.

    b. Mix well by gently pipetting 20 times, and incubate the sample at 20°C for 15 min in the ThermoMixer C.

⚠ CRITICAL: The reaction time of this step should be strictly limited to prevent excessive adapters from self-ligating.

65. Purify the DNA product with 47.7 μL of AMPure XP beads and elute DNA with 25.5 μL of Qiagen elution buffer following step 58.
66. Transfer the supernatant (25 μL) to a new 1.5-mL Eppendorf tube carefully.
67. Purify the DNA product once more with 45 μL of AMPure XP beads and elute DNA with 17 μL of Qiagen elution buffer following step 58.
68. Transfer the supernatant (16.2 μL) to a new 1.5-mL Eppendorf tube carefully.

Pause point: The purified DNA product can be stored at −20°C for several weeks.

69. Test the optical PCR cycle number to avoid overamplification:
    a. Prepare the following PCR reaction mixture in 0.2-mL tubes, and mix well by gently pipetting 20 times.

Reagent	Final concentration	Amount
P5 primer (10 μM)	0.4 μM	1 μL
P7 index primer (10 μM)	0.4 μM	1 μL
5× SuperFi buffer	1×	5 μL
25 mM dNTPs	0.4 mM	0.4 μL
Platinum SuperFi DNA Polymerase	0.008 U/μL	0.1 μL
USER enzyme	0.02 U/μL	0.5 μL
DNA template	N/A	1 μL
ddH_2O	N/A	16 μL
Total	N/A	25 μL

    b. Perform the PCR amplification using the following conditions.

Steps	Temperature	Time	Cycles
Pretreatment	37°C	15 min	1
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	10 and 14
Annealing	62°C	30 s	
Extension	72°C	30 s	

(*Continued on next page*)

Continued

Steps	Temperature	Time	Cycles
Final extension	72°C	5 min	1
Hold	12°C	∞	

Note: Pretreatment at 37°C for 15 min enables the USER enzyme to degrade the dUTP-containing strand before the PCR reaction, allowing us to obtain strand-specific libraries.

c. Add 5 μL of 6× DNA loading buffer to the sample and mix well by gently pipetting 10 times. Detect the signal strength of the PCR product by performing 1.5% (w/v) agarose gel electrophoresis for ~1 h at 120 V in 1 × TBE buffer.

d. Visualize the DNA band in the gel using a blue-light transilluminator. See troubleshooting for problems 3 and 4.

70. Final PCR with optimal PCR cycles:

Note: The intensity of the DNA signal determines the optimal number of PCR cycles in step 69.

a. Prepare the following PCR reaction mixture in 0.2-mL tubes, and mix well by gently pipetting 20 times.

Reagent	Final concentration	Amount
P5 primer (10 μM)	0.4 μM	1 μL
P7 index primer (10 μM)	0.4 μM	1 μL
5 × SuperFi buffer	1 ×	5 μL
25 mM dNTPs	0.4 mM	0.4 μL
Platinum SuperFi DNA Polymerase	0.032 U/μL	0.4 μL
USER enzyme	0.12 U/μL	3 μL
DNA template	N/A	14.2 μL
Total	N/A	25 μL

Note: Different samples require different index primers for pooling sequencing and demultiplexing sequencing reads.

b. Perform the PCR amplification using the following conditions.

Steps	Temperature	Time	Cycles
Pretreatment	37°C	15 min	1
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	11
Annealing	62°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Hold	12°C	∞	

Note: We typically use 11 cycles for final libraries PCR. Users may adjust the PCR cycles based on the signal intensity of the product in step 69. Do not use PCR cycle numbers that are too high to avoid high PCR duplicates and low library complexity.

c. Add 5 μL of 6× DNA loading buffer to the sample and mix well by gently pipetting 10 times. Run the electrophoresis on 1.5% (w/v) agarose gel for ~1 h at 120 V in 1 × TBE buffer.

d. Visualize the DNA band in the gel using a blue-light transilluminator.

Note: UV transilluminator should be forbidden as it may damage DNA.

e. Cut the smeared DNA band from 200 bp to 450 bp, and purify the DNA product with a Qiagen MinElute gel extraction kit following the manufacturer's instructions.

f. Elute the purified DNA in 15 μL of elution buffer. Take 1 μL of product and quantify it using a Qubit dsDNA HS assay kit and Qubit fluorometer following the manufacturer's instruction.

Note: The expected DNA yield in this step is 10 ng–100 ng.

Pause point: The libraries can be stored at −20°C for several months.

71. *In vitro* random ligation library construction.

Note: To identify high-confidence RNA-RNA interactions, we construct *in vitro* random ligation libraries to remove the potential random RNA-RNA contact background.

a. Prepare equal numbers of infected cells for *in vitro* random ligation library construction.
b. Perform the 'Permeabilization and micrococcal nuclease digestion' and 'pCp-biotin labeling' following steps 1–14 in the main procedure.
c. Perform the 'Fast AP treatment' and 'T4 PNK treatment' following steps 15 and 16 in the main procedure.
d. Perform the 'Proteinase K treatment' following step 21 in the main procedure.
e. Purify total RNA following steps 22–29 in the main procedure.
f. Add 20 μL of DEPC-treated water to the tube and dissolve the pellet completely by gently pipetting 50 times.
g. *In vitro* random ligation:
    i. Add the ligation mixture containing 10 μL of T4 RNA ligase (10 U/μL), 200 μL of 10× T4 RNA ligase reaction buffer, 200 μL of 1 mg/mL BSA, 8 μL of RNAsin, and 1,562 μL of DEPC-treated water to the RNA sample.
    ii. Mix well by gently pipetting 20 times. Place the tube on a rotator and incubate the sample at 16°C for 16 h at 20 rpm.
h. Purify RNA with acid phenol: chloroform:
    i. Aliquot the sample into four 1.5-mL Eppendorf tubes, and add 500 μL of acid phenol: chloroform to each tube.
    ii. Mix well by shaking, then centrifuge the tube at 4°C for 10 min at 16,000 *g*.
    iii. Collect the aqueous phase carefully and transfer it to a new 1.5-mL Eppendorf tube. Precipitate total RNA by adding 1 μL of GlycoBlue and 500 μL of isopropanol to the tube.
    iv. Mix well by gently pipetting 20 times and placing the sample at −20°C for 12–16 h.
i. Next day, centrifuge the tube at 4°C for 20 min at 16,000 *g* and remove the supernatant.
j. Wash the RNA pellet twice with 600 μL of 75% (v/v) ethanol stored at −20°C. Centrifuge the tube at 4°C for 5 min at 16,000 *g*.
k. Remove the supernatant completely, and air-dry the RNA pellet for 2 min.
l. Add 5 μL of DEPC-treated water to the tube, dissolve the pellet completely by gently pipetting 50 times, then combine the RNA samples. Take 1 μL of sample and measure the concentration using NanoDrop 2000c.

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

m. Take 20 μg of RNA for 'Genomic DNA digestion', 'rRNA depletion', 'pCp-biotin selection', and 'Strand-specific library construction' following steps 31–70 in the main procedure.

Deep sequencing and data processing

Timing: ~10 days

RIC-seq libraries are sequenced with the Illumina NovaSeq 6000 platform during this procedure. *In situ* and *in vitro* random ligation libraries should yield comparable levels of raw reads.

72. The RIC-seq libraries typically require ~100 million raw reads per sample.
73. Process the RIC-seq paired-end raw reads in FASTQ files using the RICpipe program (https://github.com/caochch/RICpipe)[12] to obtain intra- and inter-molecular chimeric reads in sam files. See troubleshooting for problems 5 and 6.
    a. Trim the adapters using the Trimmomatic program[13] and low-complexity fragments for each end of reads using the cutadapt program.[14]
    b. Align the clean reads to the rRNA using the STAR program[16] and collect the unmapped reads.
    c. Assemble the virus and host reference genome into a reference genome and align the unmapped to the reference genome using STAR and BWA[17] programs.
    d. Classify the chimeric reads as intra-molecular if both arms are mapped to the same host or virus reference transcript, while those with two arms mapped to different transcripts are classified as inter-molecular.

    Note: Chimeric reads whose left and right arms separately map to the virus and the host reference genome are also classified as inter-molecular, representing virus-to-host RNA-RNA interactions.

    e. Combine the chimeric reads from multiple replicates for downstream analysis if they are highly correlated.

    Note: The percentage of chimeric reads is a parameter to assess the library quality. In our hands, the successful RIC-seq library in infected A549-ACE2 cells contains a chimeric reads ratio of approximately 17%, with more than 20% of the chimeric reads representing virus-to-host interactions. The high percentage of virus-to-host chimeric reads increases the detection power for virus RNA targets. Moreover, the Pearson correlation coefficient calculated by the count of chimeric reads or interaction frequency greater than 0.9 indicates high reproducibility across the replicates.

74. Remove the false-positive virus-to-host RNA-RNA interactions:
    a. Save the inter-molecular chimeric reads as a SAM format file named "intergene.sam".
    b. Extract the sequences of the pairwise arms for the virus-to-host chimeric reads and save them as two FASTA format files named "part_A.fa" and "part_B.fa".
    c. Discard the virus-to-host chimeric reads with both arms aligned to the virus genome by BLASTN[15] with an identity higher than 80%.
    d. Save the remaining inter-molecular chimeric reads as a SAM format file named "intergene.rm_FalsePosi.sam" in the output directory.

```
> perl extract_seq.pl intergene.sam

> blastn -query part_A.fa -task megablast -db virus.fa -out part_A.out -evalue 1 -word_size 5 -outfmt 7 -num_threads 16

> blastn -query part_B.fa -task megablast -db virus.fa -out part_B.out -evalue 1 -word_size 5 -outfmt 7 -num_threads 16

> perl filter_according_to_BLASTN.pl part_A.out part_B.out intergene.sam 0.8 > intergene.rm_FalsePosi.sam
```

75. Collect the high-confidence chimeric reads with both arms uniquely mapped to the genome.
    a. Extract the sequences of the pairwise arms for the chimeric reads and save them as a FASTQ format file named "rm_FalsePosi.fq".

b. Align the chimeric reads to the reference genome using HISAT2,[19] Bowtie2,[18] and STAR programs.
c. Retain reads with alignment quality greater than 20 using at least one of the three mapping softwares for two aligned arms, and save them as a SAM format file named " rm_FalsePosi.-bothUniq.sam" in the output directory.

```
> perl step1.prepare_reads_fragment.pl rm_FalsePosi.sam

> hisat2 -p 12 -k 2 -x human_virus.pan.fa -U rm_FalsePosi.fq -S rm_FalsePosi.reMapHisat2.sam
--un rm_FalsePosi.unMapHisat2.fq 2> rm_FalsePosi.hisat2.log

> bowtie2 -un rm_FalsePosi.unMapBowtie.fq --sensitive -N 1 -L 17 --end-to-end -p 12 -x human_virus.-
pan.fa -U rm_FalsePosi.fq -S rm_FalsePosi.reMapBowtie.sam 2> rm_FalsePosi.reMapBowtie2.log

> STAR --runMode alignReads --genomeDir Pangenome --readFilesIn rm_FalsePosi.fq --outFileName
Prefix rm_FalsePosi.toGenome_ --outReadsUnmapped Fastx --outFilterMatchNminOverLread 0.9
--outFilterScoreMinOverLread 0.9 --outSAMattributes All --runThreadN 16

> perl step2.separatBothUniq_and_Other.requireLocitoo.pl rm_FalsePosi.fq intergene.rm_
FalsePosi.sam rm_FalsePosi.reMapHisat2.sam rm_FalsePosi.reMapBowtie.sam rm_FalsePosi.
toGenome_Aligned.out.sam
```

76. Remove the background inter-molecular chimeric reads from the *in vitro* random ligation library.
    a. Extract the coordinates of the pairwise arms of the inter-molecular chimeric reads obtained from both *in situ* and *in vitro* random ligation libraries and save them as two BEDPE format files named "intergene.rm_FalsePosi.bothUniq.bedpair" and "random.intergene.rm_FalsePosi.bothUniq.bedpair".
    b. Remove the inter-molecular RNA-RNA interaction fragments from the *in situ* ligation library that overlapped with the *in vitro* random ligation library and save them as a SAM format file named "intergene.rm_FalsePosi.bothUniq.rm_Random.sam" in the output directory.

```
> perl from_sam_to_pair_reads_bed.pairs.pl intergene.rm_FalsePosi.bothUniq.sam

> bedtools pairtopair -a intergene.rm_FalsePosi.bothUniq.bedpair -b random.intergene.rm_
FalsePosi.bothUniq.bedpair -type notboth -f 0.05 > intergene.rm_FalsePosi.bothUniq.rm_
Random.bedpair

> python obtain_sam_file.py intergene.rm_FalsePosi.bothUniq.rm_Random.bed intergene.rm_
FalsePosi.bothUniq.sam > intergene.rm_FalsePosi.bothUniq.rm_Random.sam
```

Note: In our hands, only 7% of the inter-molecular chimeric reads in the *in situ* libraries that overlapped with the *in vitro* random ligation libraries were discarded. The low overlapping percentage indicates less background resulting from randomly ligating.

77. Identify high-confidence virus RNA targets:
    a. Adopt the Monte Carlo simulation strategy[11] to detect significant inter-molecular RNA-RNA interactions by comparing observed with the simulated random interactions.
    b. Consider the virus-to-host RNA-RNA interactions supported by at least two chimeric reads with a *P*-value lower than 0.05 as the high-confidence virus-to-host interactions.

```
> perl MonteCarlo_simulation_use_fragment.pl whole_gene_region.bed 100000 0.05 20 inter-
gene_simulation intergene.rm_FalsePosi.bothUniq.rm_Random.sam > run.sh

> bash run.sh
```

```
> awk -F '\t' 'BEGIN{OFS="\t"}{if(($1=="NC_045512.2")&&($13>=2)) print $7,$8,$9,$10,$11,$12;else if(($7=="NC_045512.2")&&($13>=2)) print $1,$2,$3,$4,$5,$6 }' intergene_simulation.significant.interMolecular.interaction.list > virus_target.txt
```

Note: We set a minimum cutoff for two chimeric reads to support the inter-molecular RNA-RNA interactions. The thresholds for the count of chimeric reads and *P*-value can be adjusted by the users according to their requirements.

78. Identify virus binding peaks in the host genome:
    a. Extract the 50-nt fragments around the juncture of virus-to-host chimeric reads in the host transcripts from step 76 and save them as a BED format file named "fragment50nt.bed".
    b. Retain the fragments located in the mature RNAs using the Bedtools program.[20]
    c. Identify the peaks using the Piranha software and save them as a BED format file named "peaks.bed" in the output directory.

```
> perl find_targets_related_to_a_region_plus.pl intergene.rm_FalsePosi.bothUniq.rm_Random.sam NC_045512.2 0 30000 > virus.TargetAndSource.sam

> perl extract_junction.pl virus.TargetAndSource.sam

> python junction_fragment_region.py out1.arms.info 50 > fragment50nt.bed

> sort -k1,1 -k2,2n fragment50nt.bed |awk -F '\t' 'BEGIN{OFS="\t"{if($2>=0) print $0 }' > fragment50nt.sort.bed

> grep -E 'PC5UTR|PCCDS|PC3UTR' chlSab2.gene_element.bed |bedtools intersect -a fragment50nt.sort.bed -b - -s -u > fragment50nt.exon.bed

> Piranha -s -o peaks.bed -p 0.05 -b 20 -a 0.96 fragment50nt.exon.bed
```

79. Capture U3 snoRNA secondary structure:
    a. Assemble the U3 snoRNA sequence (GenBank: NR_006880.1) into a mini-genome and map sequencing reads to the mini-genome using the RICpipe program.[12]
    b. Collect the chimeric reads and save them as a SAM format file named "U3_chimeric_reads.sam".
    c. Extract the junctures of chimeric reads to construct the RNA interaction map with 2-nt resolution and discard the pairwise 2-nt interaction windows with a connection score less than 0.01.
    d. Cluster the pairwise 2-nt interaction pairs with both ends overlapping within 2-windows distance.
    e. Visualize the resulting chimeric reads in the representative clusters using the Integrative Genomics Viewer (IGV) tool.[23]

```
> perl sam_to_bedpe.pl U3_chimeric_reads.sam 0.01 U3_chimeric_reads.list U3_chimeric_reads.2nt.bedpe

> perl cluster_pixels.pl U3_chimeric_reads.2nt.bedpe > U3_chimeric_reads.cluster.bedpe

> perl creat_artifical_bam.pl U3_chimeric_reads.list U3_chimeric_reads.cluster.bedpe U3.fasta

> awk -F '\t' 'BEIGN{OFS="\t"}{if($12=="RG:Z:ManualLoops_1") print $0 }' U3.read_of_cluster.sam > U3.read_of_cluster1.sam

> samtools view -t chr.sizes -bSo U3.read_of_cluster1.bam U3.read_of_cluster1.sam
```

```
> samtools sort -o U3.read_of_cluster1.sort.bam U3.read_of_cluster1.bam

> samtools index U3.read_of_cluster1.sort.bam
```

Note: We set a minimum cutoff for interacting windows with a connection score above 0.01 to retain the high-confidence interacting windows, which can be adjusted by the users according to their requirements. The window size of 2 nucleotides could provide high nucleotide resolution, and this parameter can be adjusted according to the length of RNA and the users' requirements.

80. Predict the 3′ UTR secondary structure of the virus:
    a. Collect the chimeric reads that were only mapped to the virus reference genome and save them as a SAM format file named "virus_chimeric_reads.sam".
    b. Extract the junctures of chimeric reads within the virus genome to construct the RNA interaction matrix with 2-nt resolution.
    c. Predict 10 candidate secondary structures for the 3′ UTR sequence with a maximum pairing distance of 250 nt using the Fold program from the RNAstructure software suite.[24]
    d. Compare the RIC-seq signal of paired and unpaired pairwise 2-nt windows for each candidate secondary structure using a one-tailed t-test and sort 10 candidate secondary structure models by *P*-values.
    e. Select the secondary structure model with the lowest minimum free energy (MFE) ranked by *P*-values among the top 5 structure models.

```
> perl sam_to_loops.pl virus_chimeric_reads.sam | sort -k3,3 -k7,7 > virus.sort.list

> java -jar juicebox_tools.jar pre -r 1,2,5,10,25,50,100 virus.sort.list virus.hic hs.size

> java -jar juicebox_tools.jar dump observed VC_SQRT virus.hic hs1 hs1 BP 2
virus.2nt.vcrt.matrix

> perl format_hic_to_matrix.pl virus.2nt.vcrt.matrix > virus.2nt.vcrt.format.matrix

> Fold 3UTR.fa 3UTR.ct --maxdistance 250

> perl select_local_structure.pl 3UTR.ct virus.2nt.vcrt.format.matrix > log.3UTR.txt

> python3 select_structure.py log.3UTR.txt 3UTR.ct > best_structure.ct
```

EXPECTED OUTCOMES

Chimeric reads and virus targets of RIC-seq libraries in SARS-CoV-2 infected A549-ACE2 cells

In our hands, the *in situ* RIC-seq libraries in SARS-CoV-2 infected A549-ACE2 cells yielded 13,827,107 intra-molecular chimeric reads and 13,386,942 inter-molecular chimeric reads, with 5,888,407 representing virus-to-host chimeric reads. After removing the potential false-positive inter-molecular chimeric reads from *in vitro* random ligation libraries, we obtained 12,448,253 inter-molecular chimeric reads, with 5,548,860 high-confidence virus-to-host chimeric reads for identifying 5,754 virus targets.

PCR products of RIC-seq libraries

Figure 2 displays the expected length distribution of RIC-seq DNA libraries on the agarose gel.

The length distribution of RIC-seq libraries

Figure 3 displays the length distribution of RIC-seq DNA libraries analyzed by Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit.

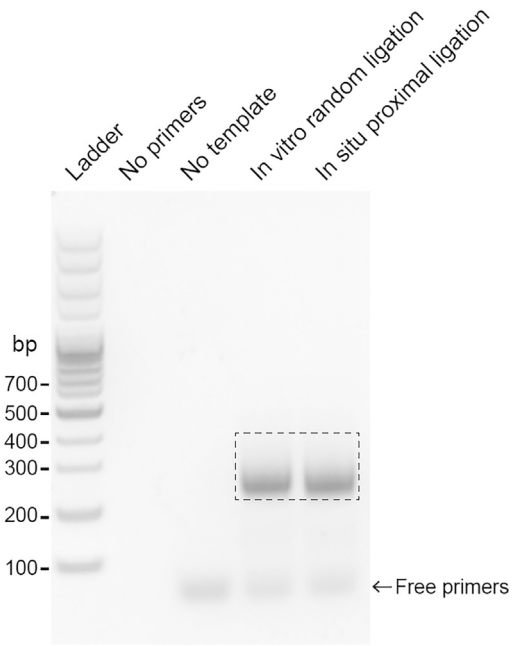

Figure 2. Size distribution of the RIC-seq DNA libraries

The secondary structure of SARS-CoV-2 3′ UTR

Figure 4 displays the RNA interaction map and predicted secondary structure of 3′ UTR of SARS-CoV-2.

SARS-CoV-2 and host RNA-RNA interactions revealed by RIC-seq

Figure 5 displays the SARS-CoV-2 targets along the host genome (left) and inter-molecular chimeric reads for SARS-CoV-2 *ORF-M* and host RNA *NFKBIZ* (right). Pairwise interacting RNA fragments are illustrated as arc lines. The red outer circle of the circos plot represents SARS-CoV-2-to-host chimeric reads in the virus genome. The purple and green histograms represent SARS-CoV-2-to-host chimeric reads and peaks in the host genome. The inner circle (blue arcs) represents high-confidence SARS-CoV-2 targets. *NFKBIZ* is marked with a red arrow.

Host U3 snoRNA secondary structure captured by RIC-seq

Figure 6 displays the known U3 snoRNA secondary structure as blue arc lines. Different clusters of intra-molecular chimeric reads mapped to U3 snoRNA are shown in distinct colors. Black lines mark the junctures of chimeric reads.

LIMITATIONS

RIC-seq can capture the whole complement of protein-mediated intra- and inter-molecular RNA-RNA interactions. If the RNA-RNA interactions are merely mediated by base pairing rather than proteins, these interactions will be largely lost at MNase digestion and the subsequent washing steps.

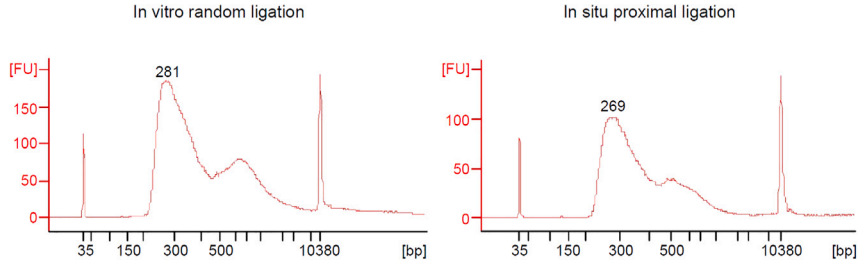

Figure 3. Bioanalyzer analyzing the length of RIC-seq libraries

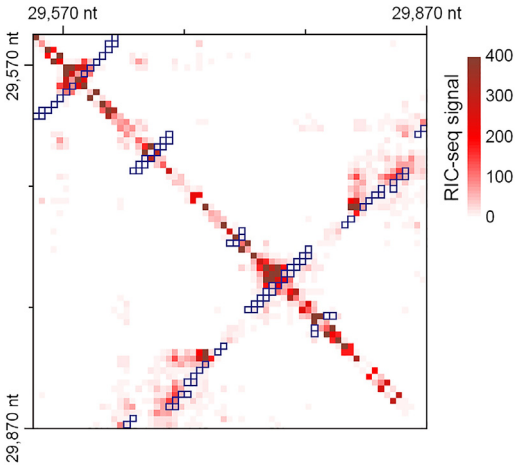

Figure 4. The secondary structure of SARS-CoV-2 3′ UTR

However, some RNA duplexes that protrude from a protein complex can also be captured by RIC-seq,[10] though it is inefficient compared with interactions directly mediated by proteins.

Single-stranded and positive-sense RNA viruses, such as SARS-CoV-2, will generate genomic RNA and subgenomic RNA after entering host cells. RIC-seq cannot distinguish whether the virus-to-host chimeric reads are derived from genomic RNA or subgenomic RNA. In addition, RIC-seq may be inefficient in capturing the genomic RNA interacted host RNAs for those double-stranded RNA viruses. However, these double-stranded RNA viruses transcribed positive-strand RNAs can be efficiently captured by RIC-seq technology.

The percentage of virus-to-host chimeric reads primarily depends on the abundance of viral RNA in infected host cells. RIC-seq application in RNA viruses replicating weakly in host cells may be unsuitable due to the lower percentage of virus-to-host chimeric reads that can be detected. However, designing the modified probes targeted viral RNAs to enrich those virus-to-host chimeric reads may address this limitation.

TROUBLESHOOTING

Problem 1

Cells remain in the culture dish or float in the supernatant after centrifugation (steps 13 and 14 in the infected cells preparation section).

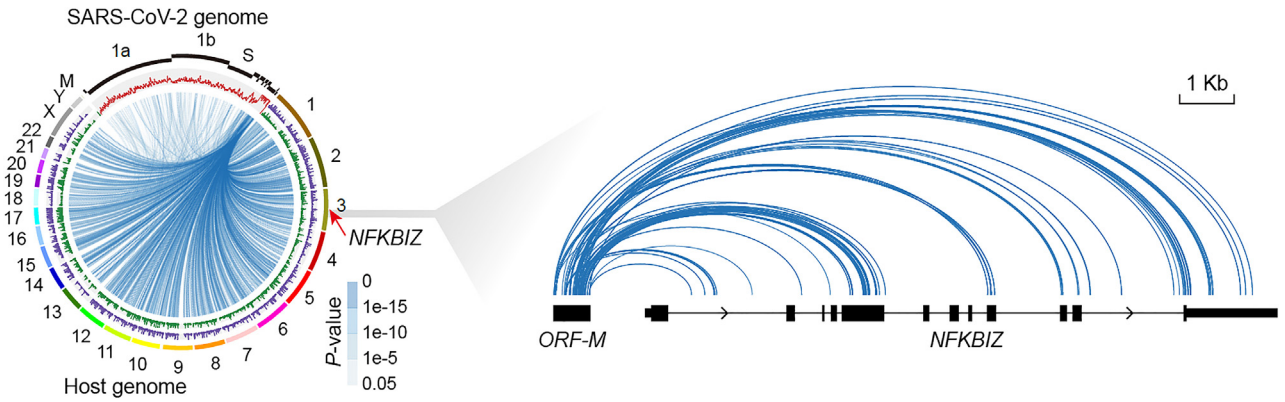

Figure 5. SARS-CoV-2 and *NFKBIZ* RNA-RNA interactions revealed by RIC-seq

P-values are adjusted using Benjamini–Hochberg multiple testing correction.
Adapted from Zhao et al., Mol Cell, 2024.[1]

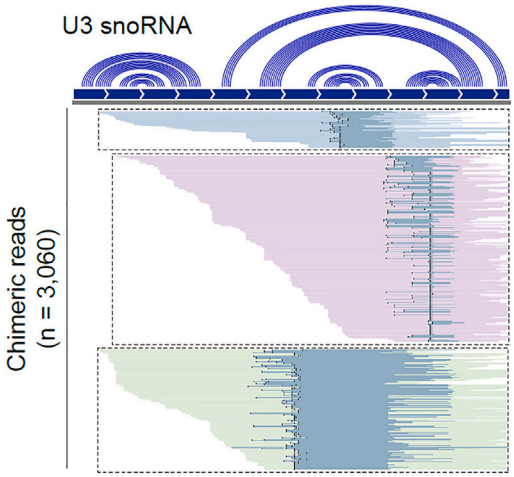

Figure 6. U3 snoRNA secondary structure captured by RIC-seq

Potential solution

- Add 0.01% (v/v) of NP-40 to the PBS before scraping cells from the culture dish.
- Increase the centrifugation speed to 2,400 *g*.

Problem 2

Excessive RNA yield after rRNA depletion (step 46).

Potential solution

The potential reason is insufficient rRNA removal. Two solutions may solve this problem. 1) You can repeat rRNA depletion following steps 40–46 directly. 2) You can take another 20 μg total RNA. Perform genomic DNA digestion and rRNA depletion following steps 31–46. The amounts of rRNA probes in step 40, RNase H in step 42, and TURBO DNase in step 44 can be doubled.

Problem 3

Prominent DNA band between 100 and 200 bp (step 69).

Potential solution

The potential reason is adapter self-ligation in step 64. You should reduce the amount of adapters and control the reaction time strictly to 15 min in step 64.

Problem 4

There is no visible DNA smear band on the gel between 200 and 450 bp (step 69).

Potential solution

The potential reason is low input total RNA. You can increase the PCR cycles in step 69 or increase the total RNA in step 31.

Problem 5

The chimeric reads ratio is too low (step 73).

Potential solution

The potential reason is low proximal ligation efficiency. You can increase the amounts of T4 RNA ligase and the reaction time in step 17.

Problem 6

The percentage of virus-to-host chimeric reads is too low (step 73).

Potential solution

The potential reason is the low abundance of viral RNA in infected cells. You can increase the MOI usage and virus infection time in step 8 of infected cells preparation.

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).

Technical contact

Further information and requests for the details of protocol should be directed to and will be fulfilled by the technical contact, Zhaokui Cai (caizhaokui@ibp.ac.cn)

Materials availability

This study did not generate new unique reagents.

Data and code availability

RIC-seq data for infected A549-ACE2 cells (recently published in Zhao et al., Mol cell[1]) are available in the Genome Sequence Archive for Human (GSA-Human) under accession number HRA005709. The scripts for RIC-seq data analysis can be found at https://github.com/Zhaohailian/SARS-CoV-2_STAR_protocols and https://doi.org/10.5281/zenodo.10609902.

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AUTHOR CONTRIBUTIONS

Y.X. conceived and supervised the project. Z.C. performed the experiments and prepared the experimental parts of this protocol, and H.Z. performed bioinformatics analysis and prepared the bioinformatic part.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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