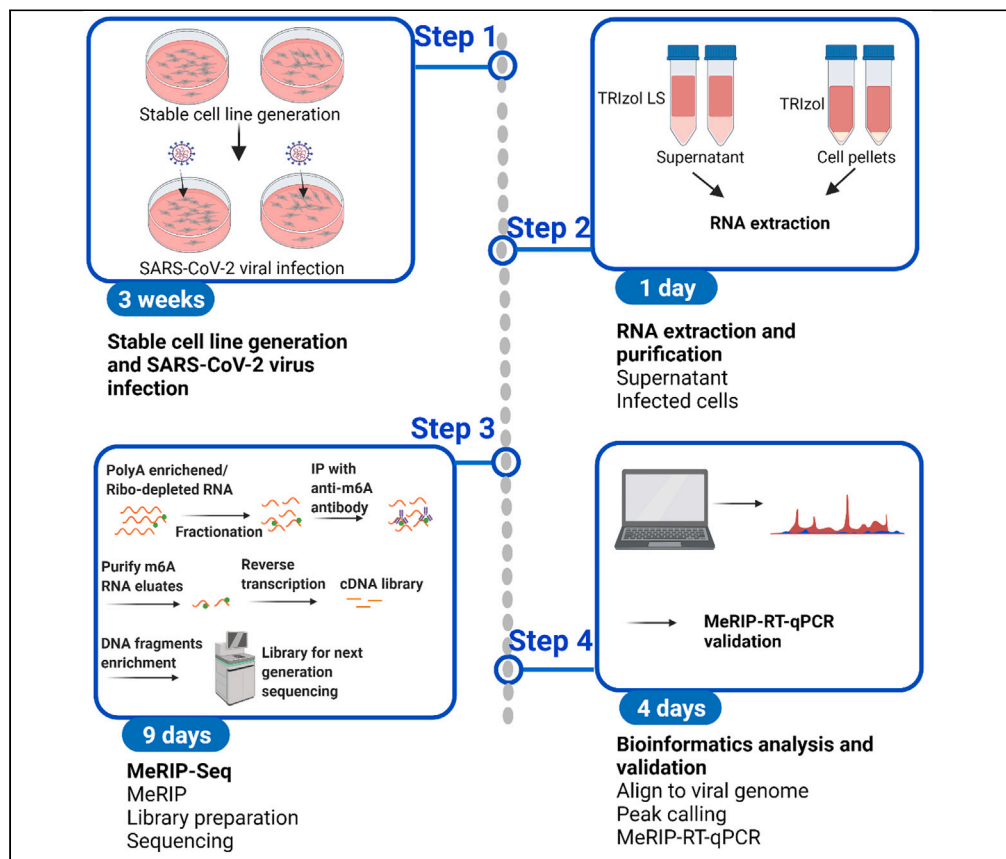


Protocol

Detection of N⁶-methyladenosine in SARS-CoV-2 RNA by methylated RNA immunoprecipitation sequencing



*N*⁶-methylation of adenosine (m⁶A) is the most abundant internal mRNA modification and is an important post-transcriptional regulator of gene expression. Here, we describe a protocol for methylated RNA immunoprecipitation sequencing (MeRIP-Seq) to detect and quantify m⁶A modifications in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA. The protocol is optimized for low viral RNA levels and is readily adaptable for other applications.

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Highlights
Procedure to generate stable cell lines for SARS-CoV-2 viral infection

Protocol for profiling m⁶A modifications in SARS-CoV-2 viral RNA by MeRIP-Seq

Procedures for bioinformatics analysis and validation by MeRIP-RT-qPCR

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Protocol

Detection of N⁶-methyladenosine in SARS-CoV-2 RNA by methylated RNA immunoprecipitation sequencingNa Li^{1,3} and Tariq M. Rana^{1,2,4,*}¹Division of Genetics, Department of Pediatrics, Program in Immunology, University of California San Diego, 9500 Gilman Drive MC 0762, La Jolla, CA 92093, USA²Institute for Genomic Medicine, University of California San Diego, 9500 Gilman Drive MC 0762, La Jolla, CA 92093, USA³Technical contact⁴Lead contact*Correspondence: trana@ucsd.edu<https://doi.org/10.1016/j.xpro.2021.101067>

SUMMARY

N⁶-methylation of adenosine (m⁶A) is the most abundant internal mRNA modification and is an important post-transcriptional regulator of gene expression. Here, we describe a protocol for methylated RNA immunoprecipitation sequencing (MeRIP-Seq) to detect and quantify m⁶A modifications in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA. The protocol is optimized for low viral RNA levels and is readily adaptable for other applications. For complete details on the use and execution of this protocol, please refer to Li et al. (2021).

BEFORE YOU BEGIN

We describe here the protocol for MeRIP-Seq analysis of SARS-CoV-2 viral RNA extracted from infected Caco-2, a human colorectal adenocarcinoma cell line, and culture supernatants. However, we have also used this protocol for analysis of viral RNA extracted from infected Vero cells, and for analysis of mammalian RNA extracted from mouse tumor tissues (Li et al., 2020). The protocol is applied here to SARS-CoV-2 infected Caco-2 cells that harbor shRNA targeting the methyltransferase METTL3 (shMETTL3) or control shRNA (shControl), thus providing an example of normal and experimentally reduced m⁶A-modified RNA. We describe the selection of stable shRNA-expressing Caco-2 cell lines and SARS-CoV-2 infection first, followed by a detailed step-by-step description of MeRIP-Seq and analysis. All the lentivirus and SARS-CoV-2 infection experiments should be performed after obtaining approval from the Institutional Biosafety Committee.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-METTL3	Proteintech	Cat# 15073-1-AP, RRID:AB_2142033
Rabbit monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Cell Signaling Technology	Cat# 2118, RRID:AB_561053
Rabbit polyclonal anti-N ⁶ -methyladenosine (m ⁶ A)	Abcam	Cat# ab151230, RRID:AB_2753144
Bacterial and virus strains		
SARS-CoV-2 (Isolate USA-WA1/2020)	BEI Resources	Cat# NR-52281

(Continued on next page)



Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Lipofectamine 3000	Thermo Fisher Scientific	Cat# L3000015
Puromycin	Alfa Aesar	Cat# 53-79-2
Ambion RNA Fragmentation Reagent	Thermo Fisher Scientific	Cat# AM8740
N6-Methyladenosine (m6A)	Abcam	Cat# ab145715
TRlzol LS Reagent	Thermo Fisher Scientific	Cat# 10296010
TRlzol Reagent	Thermo Fisher Scientific	Cat# 15596026
Pierce Protein A/G Magnetic Beads	Thermo Fisher Scientific	Cat# 88802
Ethyl alcohol, Pure	Sigma-Aldrich	Cat# E7023
Isopropanol	Sigma-Aldrich	Cat# I9516
GlycoBlue	Thermo Fisher Scientific	Cat# AM9515
Sodium Acetate	Invitrogen	Cat# AM9740
Chloroform	Sigma-Aldrich	Cat# 288306
high-glucose DMEM	Thermo Fisher Scientific	Cat# 11995073
high-glucose MEM	Thermo Fisher Scientific	Cat# A1451801
MEM NEAA	Thermo Fisher Scientific	Cat# 11140050
Penicillin-Streptomycin	Thermo Fisher Scientific	Cat# 15140122
Fetal Bovine Serum	Thermo Fisher Scientific	Cat# 10438018
Dulbecco's Phosphate Buffered Saline (DPBS)	Thermo Fisher Scientific	Cat# 14190144
Polybrene	Sigma-Aldrich	Cat# TR-1003-G
Critical commercial assays		
Direct-zol RNA Kit	ZYMO RESEARCH	Cat# R2052
RNA Clean & Concentrator-5	ZYMO RESEARCH	Cat# R1013
Lenti-X qRT-PCR Titration Kit	Takara Bio	Cat# 631235
Magnetic mRNA Isolation kit	New England BioLabs	Cat# S1550S
MEGAscript T7 Transcription kit	Thermo Fisher Scientific	Cat# AM1334
iScript cDNA Synthesis kit	Bio-Rad Laboratories	Cat# 1708891
iTaq Universal SYBR Green on-step Kit	Bio-Rad Laboratories	Cat# 1725150
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad Laboratories	Cat# 1725272
TruSeq mRNA library preparation kit	Illumina	Cat# 20020595
Deposited data		
Raw and analyzed data	This paper	GEO: GSE167075
Experimental models: Cell lines		
Caco-2 cells	ATCC	Cat# HTB-37
Vero E6 cells	ATCC	Cat# CRL-1586
293T cells	ATCC	Cat# CRL-3216
Recombinant DNA		
pLKO.1-shMETTL3-1	MilliporeSigma	TRC Clone ID TRCN0000034714
pLKO.1-shMETTL3-2	MilliporeSigma	TRC Clone ID TRCN0000034717
pLKO.1-Control	MilliporeSigma	Cat# SHC016
Software and algorithms		
GraphPad Prism 8	Software	https://www.graphpad.com/scientific-software/prism/
Bowtie2	Langmead and Salzberg (2012)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Samtools	Li et al. (2009)	http://samtools.sourceforge.net/
STAR	Dobin et al. (2013)	https://github.com/alexdobin/STAR
MACS2	Zhang et al. (2008)	https://github.com/mac3-project/MACS
m6A Viewer	Antanaviciute et al. (2017)	http://dna2.leeds.ac.uk/m6a/
Cutadapt	Martin (2011)	https://cutadapt.readthedocs.io/en/stable/
Integrative Genomics Viewer	Robinson et al. (2011, 2017, 2020) and Thorvaldsdottir et al. (2013)	https://software.broadinstitute.org/software/igv/

MATERIALS AND EQUIPMENT

Cell Culture Media

Medium/supplement	Final concentration	Source	Identifier
DMEM	n/a	Thermo Fisher Scientific	Cat# 11995073
Fetal bovine serum (FBS)	10% v/v	Thermo Fisher Scientific	Cat# 10438018
Penicillin/streptomycin	50 U/mL	Thermo Fisher Scientific	Cat# 15140122
MEM	n/a	Thermo Fisher Scientific	Cat# A1451801
Fetal bovine serum (FBS)	20% v/v	Thermo Fisher Scientific	Cat# 10438018
MEM NEAA	n/a	Thermo Fisher Scientific	Cat# 11140050
Penicillin/streptomycin	50 U/mL	Thermo Fisher Scientific	Cat# 15140122

n/a, not applicable.

TE Buffer

Reagent/stock concentration	Final concentration	Amount/volume
Tris-Cl (pH 8.0) 1 M	10 mM	0.5 mL
EDTA (pH 8.0) 0.5 M	1 mM	0.1 mL
ddH ₂ O	n/a	49.4 mL
Total		50 mL

Stocks can be kept at 25°C for 6 months. n/a, not applicable.

MeRIP Binding Buffer

Reagent/Stock concentration	Final concentration	Amount/Volume
Tris-HCl (pH 7.4) 1 M	10 mM	0.5 mL
NaCl, 5 M	150 mM	1.5 mL
NP-40	0.1%	50 μL
ddH ₂ O	n/a	47.95 mL
Total	n/a	50 mL

Stocks can be kept at 4°C for 6 months. n/a, not applicable.

MeRIP Wash Buffer

Reagent/stock concentration	Final concentration	Amount/volume
Tris-HCl (pH 7.4) 1 M	10 mM	0.5 mL
NaCl, 5 M	1 M	10 mL
NP-40	0.1%	50 μL
ddH ₂ O	n/a	39.45 mL
Total	n/a	50 mL

Stocks can be kept at 4°C for 6 months. n/a, not applicable.

MeRIP Elution Buffer

Reagent/Stock concentration	Final concentration	Amount/Volume
N ⁶ -Methyladenosine (m ⁶ A)	25 mM	7.031 mg
MeRIP Wash buffer	n/a	1 mL
Total	n/a	1 mL

The elution buffer must be made fresh and kept on ice. n/a, not applicable.

STEP-BY-STEP METHOD DETAILS

Stable cell line generation

⌚ Timing: 2 weeks

Note: All steps involving lentivirus vectors and experiments should be performed in accordance with institute policies and obtained approval from the Institutional Biosafety Committee.

1. Package shRNA-encoding lentiviruses in 293T cells
 - a. Purchase lentiviral vectors containing the appropriate gene-targeting or control shRNAs, or clone the shRNA sequences into the lentiviral vector PLKO.1 or lentiCRISPR-v2.
 - b. Produce lentivirus in 293T cells.
 - i. The day before transfection, resuspend 5×10^5 293T cells in DMEM medium supplemented with 10% fetal bovine serum and 50 U/mL penicillin-streptomycin and seed in a 6-well plate at a density sufficient to reach ~80% confluence ~24 h later.
 - ii. Mix the shRNA plasmids with packaging plasmids (as indicated in the table below) in 100 μ L Opti-MEM medium and then add 5 μ L P3000 reagent and mix well.

Plasmid	DNA amount
pMD2.G	0.5 μ g
PsPAX	0.75 μ g
Lentiviral plasmid (pLKO.1-shMETTL3-1, pLKO.1-shMETTL3-2, or pLKO.1-shControl)	1 μ g

- iii. Dilute 6.5 μ L Lipofectamine 3000 in 100 μ L Opti-MEM, mix with the plasmid/P3000 mixture, incubate for 10 min at 25°C, and then gently add dropwise to the plated cells in 2 mL medium.

Alternatives: Lipofectamine 2000 or polyethylenimine (PEI) reagent can also be used as transfection reagents.

- iv. Incubate the plate at 37°C. After 12 h, exchange the medium for fresh DMEM medium and continue the incubation at 37°C.
 - v. At 48 h and 72 h incubation, collect and pool the supernatants and centrifuge at 500 \times g for 10 min to pellet residual 239T cells.
 - vi. Remove the virus-containing supernatants and either transduce cells freshly or freeze at –80°C until use. Physical and functional titers can be tested before freezing the virus. For the physical titer, it can be easily determined by Lenti-X qRT-PCR Titration Kit or other RT-qPCR based method to detect lentiviral backbone components. For functional titer, if the vector contains fluorescence markers, use fluorescence-activated single cell sorting (FACS) to count the infected cells number after being infected with serial dilution of the viral supernatant. If the vector contains antibiotic resistance genes (e.g., Puromycin), colony-forming units can be quantified after the target cells were transduced with serial dilution of the viral stock and selected with the antibiotic. The Multiplicity of Infection (MOI) should be >1 for transduction.
2. Prepare Caco-2 cells and transduce them with shControl or shMETTL3 lentiviruses.
 - a. The day before transduction, seed 5×10^5 Caco-2 cells in high-glucose MEM supplemented with 20% fetal bovine serum, MEM NEAA and 50 U/mL penicillin-streptomycin and seed in a 6-well plate at a density sufficient to reach ~50% confluence ~24 h later.

- b. Remove the supernatant from the seeded Caco-2 cells, add 800 μ L shControl or shMETTL3 lentivirus in 1.2 mL medium, and then add Polybrene to a final concentration of 8 μ g/mL.

Note: The precise volume for added virus will be based on the lentiviral titer (see [troubleshooting problem 1](#)).

- c. Spin the plates at 485 \times g for 2 h at 25°C.
- d. Incubate the plates at 37°C for 12 h, exchange the transduction medium for fresh MEM medium, incubate at 37°C for 48 h, and then start selection of stably transduced cell lines by changing to medium containing puromycin at 2 μ g/mL. The exchange media must be treated with 10% bleach in the hood for at least 30 min before disposal.
- e. Passage and expand the cells in puromycin (2 μ g/mL-containing MEM medium for ~7 days, and then measure METTL3 mRNA and protein expression to determine silencing efficiency ([Figure 1](#)). If necessary, continue the selection until high knockdown efficiency is achieved. Usually, the high knockdown efficiency indicates that the gene expression in the shRNA transduced cells is below 20% compared to control cells by RT-qPCR or Western blotting (see [troubleshooting problem 1](#)).

Infect cells with SARS-CoV-2

⌚ Timing: 1 week

Note: All steps involving SARS-CoV-2 live virus should be performed in a BSL3 laboratory.

3. Prepare SARS-CoV-2 for infection using standard procedures for virus propagation and quantification of infectious units by plaque assay with Vero E6 cells ([Harcourt et al., 2020](#)). We obtained SARS-CoV-2 isolate USA-WA1/2020 from BEI Resources.

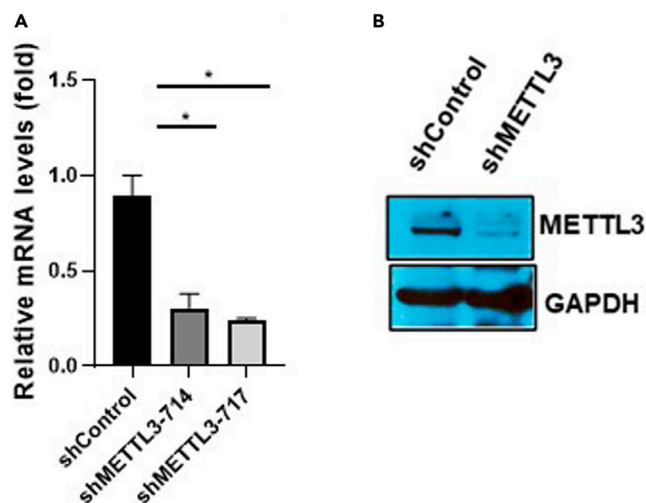


Figure 1. Expression of METTL3 in control and METTL3-knockdown Caco-2 cells

(A) RT-qPCR analysis of METTL3 mRNA in Caco-2 cells expressing control or two METTL3-targeting shRNAs. Expression levels were normalized to GAPDH mRNA. Data are expressed as the mean \pm SEM (n = 3). *p < 0.05 by Student's t test.

(B) Western blot analysis of METTL3 protein in Caco-2 cells expressing control or METTL3-targeting shRNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was probed as a loading control. These data are from the original [Figures 1D and 4F](#) in [Li et al. \(2021\)](#).

4. Resuspend the control and METTL3-knockdown Caco-2 cells in complete MEM medium and seed at $\sim 3 \times 10^6$ cells in one 10-cm dish or one full 6-well plate. Prepare at least triplicate dishes/wells per experimental condition.
5. Infect Caco-2 cells with SARS-CoV-2 at a multiplicity of infection (MOI) of 2 diluted in serum-free MEM medium, one hour after infection, the virus-containing medium is disposed, and infected cells are washed by DPBS once, add fresh complete MEM medium and incubate the cells at 37°C for 24 h. Collect the supernatants and then harvest the cells by addition of TRIzol.
6. Obtain the viral copy number in the supernatant by RT-qPCR analysis by using iTaq Universal SYBR Green One-Step Kit and comparison with a standard curve generated from *in vitro*-transcribed viral nucleocapsid (N) region (Figure 2).
7. Proceed to the following [step-by-step method details](#) section for extraction and quantification of viral RNA and cellular mRNA.

Extract and purify viral RNA from cell supernatants and mRNA from infected cells

⌚ Timing: 1 day

This section describes the steps for RNA extraction from supernatants and infected Caco-2 cells collected in the preceding step.

Note: All steps with SARS-CoV-2 live virus-infected cells and supernatants should be performed in a BSL3 laboratory.

8. Extract viral RNA from Caco-2 culture supernatants.
 - a. Add 30 mL TRIzol LS per 10 mL supernatant, mix by pipetting, and incubate for 5 min at 25°C.

Note: Samples can be transferred from the BSL3 laboratory after completion of this step.

⏸ **Pause Point:** The supernatant-TRIzol LS mixture can be frozen at -80°C for ≤ 7 days before moving on to RNA extraction.

- b. Aliquot the culture supernatant/TRIzol LS mixture into 50 mL tubes at 20 mL per tube. Add 4 mL of chloroform to each tube and shake for 20 s.
 - c. Incubate the tubes for 10 min at 25°C.

Log ₁₀ (Viral copy numbers)	11.25234124	10.95131125	9.252341244	7.252341244	5.252341	3.252341
Ct value	8.673333333	8.976666667	15.263333333	20.263333333	29.2	36.05333

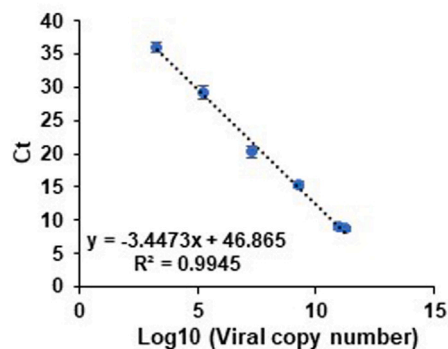


Figure 2. Standard curve for calculation of viral copy number by RT-qPCR

The equation and R^2 are shown. N_Sarbeco primer sets were used for qPCR (Corman et al., 2020). Data are presented as the mean \pm SEM (n=3).

- d. Centrifuge the tubes at 12,000×g for 15 min at 4°C.
- e. Transfer the ~7 mL upper phase to a fresh 50 mL tube, add 2 μL GlycoBlue, and mix.
- f. Add 10 mL isopropanol per tube, mix, and incubate for 10 min at 25°C.

△ **CRITICAL:** The isopropanol step precipitates the RNA.

- g. Centrifuge the 50 mL tubes at 12,000×g for 15 min at 4°C.
- h. Remove and discard the supernatant and gently wash the RNA pellet by adding 1 mL of 75% ethanol.
- i. Aspirate the ethanol, air dry the RNA pellet for 5–10 min, and dissolve in 20 μL nuclease-free water.
- j. Perform in-column DNase I treatment at 25°C for 15 min and concentrate it using an RNA Clean & Concentrator-5 kit according to the manufacturer's instructions https://files.zymoresearch.com/protocols/_r1013_r1014_r1015_r1016_rna_clean_concentrator-5.pdf
- k. Aliquot the RNA and store at –80°C until use.

Alternatives: RNA in the supernatants can also be extracted using a Direct-zol RNA Purification kit.

9. Extract cellular mRNA from infected Caco-2 cells.
 - a. Add TRIzol directly to the adherent Caco-2 cells at a 10cm dish or 6-well plate, aspirate the lysate, and mix by pipetting five times.
 - b. Extract total RNA from the sample and perform on-column DNase I treatment using a Direct-zol Miniprep Plus RNA kit according to the manufacturer's instructions https://files.zymoresearch.com/protocols/_r2070t_r2070_r2071_r2072_r2073_direct-zol_rna_miniprep_plus_kit.pdf

Note: If fewer than 5×10^6 cells are extracted, use the Direct-zol RNA Microprep/Miniprep kit.

- c. Isolate mRNA from the 100 μg purified total RNA sample using a Magnetic mRNA Isolation kit according to the manufacturer's instructions <https://www.neb.com/-/media/nebus/files/manuals/manuals1550.pdf?rev=bf3077d4994b492fb81dc09702571e84&hash=EC035147BC5AE92258976BFD95143341>. Perform two rounds of polyA selection to ensure the mRNA is of high purity.
- d. Check the mRNA quality (e.g., Agilent TapeStation) to be sure that ribosomal RNA (rRNA) is depleted (Figure 3).

Alternatives: rRNA depletion can also be performed using an rRNA depletion kit such as the RiboMinus Eukaryote System v2.

Fragment the purified RNA and perform MeRIP

⌚ **Timing:** 2 days

This section describes the steps for performing immunoprecipitation of m6A (MeRIP) from the isolated viral RNA and cellular mRNA. The steps are identical for both RNA types.

Note: During optimization experiments, we determined that this fragmentation step can be performed on as little as 300 ng of cellular mRNA (see [troubleshooting problems 2 and 3](#)).

10. RNA fragmentation
 - a. Dilute equal amounts of supernatant RNA or cellular mRNA in 9 μL nuclease-free water.
 - b. Add 1 μL of 10X Fragmentation Buffer (Ambion RNA Fragmentation Reagent) to the RNA samples, mix well by pipetting, and incubate at 70°C for 15 min.

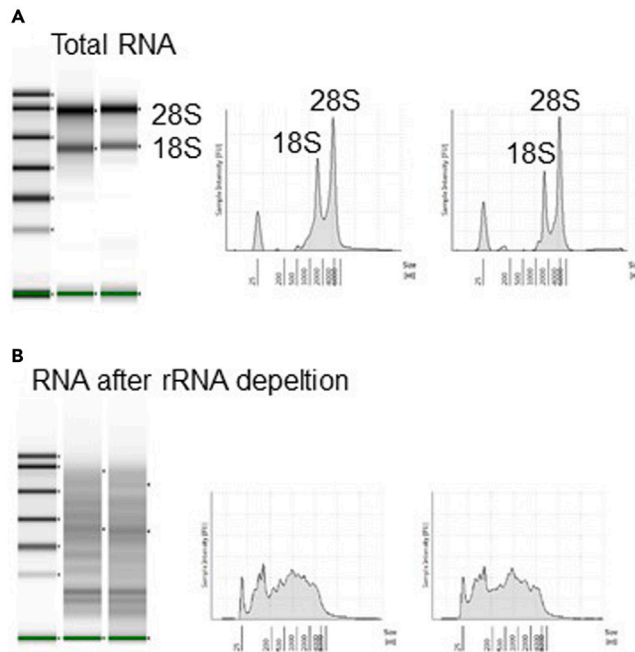


Figure 3. Examination of Caco-2 mRNA purity using Agilent TapeStation

(A and B) Gel image (left) and electropherogram (right) of two samples of total cellular RNA (A) and RNA after depletion of ribosomal RNA (rRNA) (B). Samples were analyzed using an Agilent RNA Screen TapeStation.

- c. Add 1 μL of Stop Solution (Ambion RNA Fragmentation Reagent) per sample and place on ice.

△ CRITICAL: Incubate for *exactly* 15 min after adding the fragmentation buffer and place *immediately* on ice after adding the stop solution. These steps are important to ensure the optimal fragment size (100–200 bp).

- d. Add a 350 μL TE buffer to the fragmented RNA.
- e. Add 40 μL 3 M sodium acetate, 1.5 μL GlycoBlue, and 1 mL 100% ethanol to the solution, mix gently, and incubate at -80°C for ~ 14 h.
- f. Place the mixture on ice and then centrifuge at $12,000\times g$ at 4°C for 30 min. Aspirate the supernatant without touching the RNA pellet.
- g. Wash the RNA pellet by adding 1 mL of 75% ethanol and centrifuging at $12,000\times g$ at 4°C for 5 min. Aspirate the supernatant without touching the RNA pellet.
- h. Air dry the RNA pellet for 5 min and then dissolve in 20 μL nuclease-free water.

▯▯ Pause Point: The RNA can be stored in -80°C for ≤ 7 days.

- i. Measure the concentration of fragmented RNA using a Nanodrop or Qubit.
- j. Remove 10% of the fragmented RNA solution (2 μL) and place at -80°C for use as the “input RNA” sample. The remaining ~ 18 μL of fragmented RNA is used for MeRIP.

Note: The recovery rate of fragmented RNA should be $>90\%$.

11. MeRIP of fragmented RNA

- a. Add 500 μL MeRIP binding buffer to the ~ 18 μL fragmented RNA solution and mix gently.
- b. Add 5 μL of anti-m6A antibody and 2.5 μL of RNase inhibitor to the sample.

- c. Rotate the sample at 4°C for 2 h to allow binding of the antibody to m6A-modified RNA fragments.
- d. For each immunoprecipitation (IP) sample, prepare 50 µL of protein A/G magnetic beads by washing twice with a MeRIP binding buffer.
- e. Add the IP sample solution to the pre-washed magnetic beads and rotate at 4°C for 2 h.
- f. Collect the beads using a magnetic stand and discard the supernatant.
- g. Add 500 µL of MeRIP binding buffer to the magnetic beads, mix well, collect the beads using the magnetic stand, and discard the supernatant.
- h. Add 500 µL of MeRIP wash buffer to the tube, mix well, and collect the beads with a magnetic stand, discard the supernatant. Repeat the wash twice.
- i. Add 50 µL of freshly prepared MeRIP elution buffer to the beads and incubate in a Thermo-Mixer at 25°C for 20 min. Collect the eluates.
- j. Repeat step i for a second elution of the beads and combine the two eluates.
- k. Purify RNA from the eluates using an RNA Clean & Concentrator kit according to the manufacturer's instructions https://files.zymoresearch.com/protocols/_r1013_r1014_r1015_r1016_rna_clean_concentrator-5.pdf. Eluate the MeRIP RNA in 6 µL RNase-free water.

△ **CRITICAL:** The MeRIP elution buffer must be prepared fresh for each experiment to ensure efficient elution (see [troubleshooting problems 2 and 3](#)).

Library preparation for next-generation sequencing (NGS) and bioinformatics analysis of the sequencing results

⌚ **Timing:** 3–7 days

This section describes the steps to prepare the library for NGS from the input RNA and MeRIP RNA samples and the bioinformatics analysis of the sequencing results.

12. Prepare input and MeRIP sample libraries for NGS.
 - a. Take out the purified input and MeRIP RNA and equalize the volume to 6 µL by adding 4 µL RNase-free water to 2 µL input RNA.
 - b. Prepare the libraries using an Illumina TruSeq Stranded mRNA kit with the following modifications.

Note: Locate and thaw the reagents before starting the preparation.

- c. Instead of the kit instructions, bring the volume of the RNA solutions up to 18 µL with FPF mix, which contains the random hexamer primers.
- d. Instead of repeating the fragmentation, heat the samples at 65°C for 5 min, which disrupts RNA structure and facilitates priming, and then proceed to the first-strand synthesis step. Thereafter, follow the manufacturer's instructions. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/truseq-stranded-mrna-workflow/truseq-stranded-mrna-workflow-checklist-100000040600-00.pdf
- e. Check the library quality, normalize, and pool the libraries for NGS. The sequencing is performed in the Illumina NovaSeq 6000 instrument. The read length is 101 bp pair end and the reading depth is ~50 M/sample.

Alternatives: The library can also be prepared using an Illumina TruSeq Stranded Total RNA kit (or similar kit) with the following modifications. Instead of the kit instructions, add 8.5 µL EPH buffer to 8.5 µL of RNA samples. Instead of repeating the fragmentation, heat the samples at 65°C for 5 min to disrupt the RNA structure and facilitate priming, and then proceed to first-strand synthesis. Thereafter, follow the manufacturer's instructions <https://>

support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/illumina_prep/RNA/illumina-stranded-total-rna-checklist-1000000124515-01.pdf (see [troubleshooting problem 3](#)).

13. Bioinformatic analysis of sequencing data (please also refer to [Li et al. \(2021\)](#))
 - a. Remove sequencing adaptors using Cutadapt (parameters: `cutadapt -a ADAPTER_FWD -A ADAPTER_REV -o out.1.fastq -p out.2.fastq reads.1.fastq reads.2.fastq`; where assuming the sequencing file is in `reads.1.fastq` and `reads.2.fastq`). The target reads for each sample is ~50 M. Align the sequencing reads to the SARS-CoV-2 genome using Bowtie2 or STAR (parameters for Bowtie2: `bowtie2 -x SARS_CoV_2 genome -1 1.fastq.gz -2 2.fastq.gz -S 1_map_and_unmap.sam`; parameters for STAR: `star --runThreadN 4 --genomeDir SARS_CoV_2 --readFilesIn reads.1.fastq, reads.2.fastq --outFileNamePrefix 1/1_ --outSAMtype BAM SortedByCoordinate`; where assuming the sequencing file is in `reads.1.fastq` and `reads.2.fastq`)
 - b. Use Samtools to generate and sort bam files for aligned reads (parameters: `samtools sort 1_map.bam -o 1_map.sorted.bam`; `samtools index 1_map.sorted.bam`; where assuming the 1 is the bam file generated from Bowtie2 or STAR)
 - c. Call m6A peaks with m6A viewer or MACS2 based on the paired m6A-RIP/input data from the aligned reads (parameters: `macs2 callpeak -t MeRIP_map.sorted.bam -c input_map.sorted.bam -f BAMPE -g 2.9903e+4 -n MeRIP-CoV2 -p 0.05 --out 3 --nomodel --extsize 200 --call-summits`).
 - d. For the m6A viewer, we used the following parameters: expected peak length 200, enrichment fold >3, false discovery rate <0.01, minimum peak height 10,000. For MACS2, we used the following parameters: p value < 0.05, call-summit no model, BAMPE, maximum duplicate fragments = 1 or keep all duplicates.
 - e. Upload the BedGraph file and the bed file to view the called peaks in Integrative Genomics Viewer ([Figure 4](#)) (see [troubleshooting problem 4](#)).
14. Validate the Me-RIP-Seq findings by performing MeRIP-RT-qPCR.

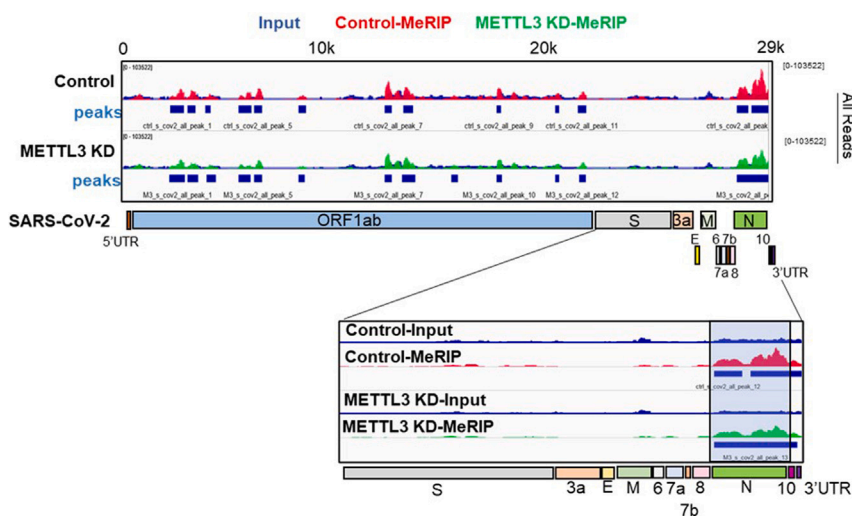


Figure 4. Genome browser tracks for input total RNA and MeRIP-Seq RNA from SARS-CoV-2
SARS-CoV-2 reads were aligned with STAR and peaks were called by MACS2 without removing duplicates. Blue, red, and green colors indicate input viral RNA, and viral RNA extracted from supernatants of control and METTL3-knockdown cells. Bed files of the so-called peak regions are shown under the MeRIP track of each group. The scale of the peak density is the same for all groups. The enlarged view shows m6A signals in the 3' end of the SARS-CoV-2 genome. These data are from the original [Figure 1F](#) in [Li et al. \(2021\)](#).

- a. Design 4 sets of qPCR primers for the m6A-enriched nucleocapsid (N) region in SARS-CoV-2 (primers for N1–N4 are listed here: MeRIP-N1: nCoV_N1-F: 5'-GACCCCAAATCAGC GAAAT-3', nCoV_N1-R : 5'-TCTGGTACTGCCAGTTGAATCTG-3'; MeRIP-N2: nCoV_N2-F: CACATTGGCACCCGCAATC, nCoV_N2-R: GAGGAACGAGAAGAGGCTTG; MeRIP-N3: nCoV_N3-F: GGAACTAATCAGACAAGGAAC, nCoV_N3-R: GAAATTTGGATCTTTGT CATC; MeRIP-N4: nCoV_N4-F: ACATTCCCACCAACAGAGCC, nCoV_N4-R: CAGCACT GCTCATGGATTG).
- b. For negative controls, design primers to amplify SARS-CoV-2 envelope (E) gene and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which represent unmethylated viral and Caco-2 genes, respectively. For the present analysis, the primers were designed based on the location of the m6A peaks in the SARS-CoV-2 viral genome (Figure 5A).
- c. Perform MeRIP on the RNA samples as described in steps 10–11 above. In the IP step, include samples incubated with IgG control antibodies equal to the amount of anti-m6A antibody (5 µg). Equalize the final volume of purified 10% input and MeRIP RNA to 6 µL in RNase-free water.
- d. Take 1 µL of the final eluates from the MeRIP samples and 1 µL of the reserved input RNA and perform reverse transcription with an iScript cDNA synthesis kit using the following reaction conditions:

Step	Temperature	Time
Priming	25°C	5 min
Reverse transcription	46°C	40 min
RT inactivation	95°C	1 min

- e. Dilute the cDNA 10-fold in Nuclease-free water, and prepare 10 µL PCR reaction samples as indicated below and run the samples on a LightCycler 480.

Material	Volume
Diluted cDNA	4 µL
2X PCR Mix	5 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL

- f. Calculate the enrichment of m6A-modified RNA as a percentage of input RNA for each independent replicate using the following equation.

$$\text{Enrichment (as \% of input)} = 2^{\text{Ct (Input)} - \text{Ct (MeRIP)}} \times 10$$

- g. Average the values for the three biological replicates per group (Figure 5B) (See [troubleshooting problem 5](#)).

Note: Multiplication by 10 corrects for the percentage of input (10%). The absolute input % value of the positive region should be >0.5 and the negative control should be <0.1. The relative value of the positive m6A modified region is compared with IgG. The value of fold enrichment from the positive region should be more than 5 over IgG, and the value of the negative region should be about 1.

△ CRITICAL: At least three biological replicates should be included for each group.

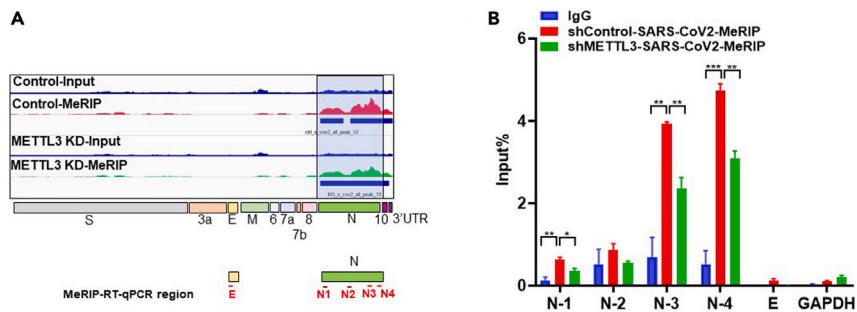


Figure 5. Validation of MeRIP-Seq data by MeRIP-RT-qPCR

(A) Expanded view of the m6A signals in the SARS-CoV-2 genome (as in Figure 4) with the qPCR primer design of the N gene (N1–4) and E gene shown below.

(B) MeRIP-RT-qPCR of viral RNA extracted from SARS-CoV-2-infected Caco-2 cells expressing shControl or shMETTL3. Equal amounts of viral RNA were immunoprecipitated with control IgG antibody or anti-m6A antibody. Amplification of E and GAPDH RNA served as negative viral and Caco-2 controls, respectively. Data are presented as the mean \pm SEM (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001 by Student's t test. These data are adapted from the original Figures 1F and 1G in Li et al. (2021).

EXPECTED OUTCOMES

The human Caco-2 stable cell lines of control and METTL3 knockdown are generated and validated by both RT-qPCR and Western blotting (Figure 1). The RNA from SARS-CoV-2 infected cells and supernatant are collected by adding Trizol/LS reagents. The isolated RNAs are treated with DNase I, concentrated, and rRNA are depleted from the total RNAs. rRNAs are greatly depleted after rRNA depletion treatment (Figure 3). After MeRIP-Seq, the reads are aligned to SARS-CoV-2 viral genome, and m6A-modified RNA peaks will be determined and visualized. When knockdown of m6A methyltransferase METTL3 is achieved, the fold enrichment of m6A signals in the C-terminal of the virus is decreased in the METTL3 knockdown cells (Figure 4). MeRIP-RT-qPCR is used to validate the data from MeRIP-Seq, and the RT-qPCR results showed the m6A positive (N-1, N-3, N-4) and negative modified region (E, N2) of SARS-CoV-2 genome, which is consistent with sequencing results. And in METTL3 knockdown cells, m6A signals decrease in the modified regions of SARS-CoV-2. These results confirm and validate the MeRIP-Seq results (Figure 5). In summary, the expected results will show the existence of m6A modifications in SARS-CoV-2 genome, which is positively regulated by the host m6A methyltransferase METTL3.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as the mean \pm standard error (SEM) of the number of biological replicates indicated in the legends. Student's t test was used to compare group means. p < 0.05 indicates statistical significance.

LIMITATIONS

If the amount of RNA and mRNA required for MeRIP-Seq or MeRIP-RT-qPCR is limiting, samples may be pooled for sequencing. We performed MeRIP of both supernatant RNA and cellular mRNA, and virions were not purified from the viral supernatant. Therefore, the results of viral RNA m6A modification should ideally be confirmed using purified virions. To validate the results of the experiment shown here, we compared the m6A signals from SARS-CoV-2 viral RNA isolated from cultures of control and METTL3-knockdown cells by both MeRIP-Seq and MeRIP-RT-qPCR. Other methods can also be applied to validate the results, including m6A mapping at individual nucleotide resolution using cross-linking and immunoprecipitation (miCLIP) (Linder et al., 2015).

TROUBLESHOOTING

Problem 1

Extensive cell death and poor knockdown efficiency in transduced Caco-2 cells after puromycin selection for stable cell line generation.

Transduction and/or selection may result in >50% cell death or knockdown failure in the surviving cells, as demonstrated by little to no change in RNA and protein expression (steps 1–2).

Potential solution

Low viral titers and poor cell condition during transduction are the main reasons for failure of knockdown and poor cell yield. When generating the virus, ensure that the 293T cells are seeded 24 h before transfection and are in logarithmic growth phase with normal morphology before transduction. When changing the medium and collecting viral supernatants, aspirate the medium gently and avoid touching the cell layer. The optimal MOI for the target cells is best determined in pilot studies using various dilutions of the virus.

Problem 2

Low MeRIP RNA amounts after m6A IP and RNA purification.

The amount of purified RNA is too low after MeRIP for library preparation for next-generation sequencing (NGS). The minimum RNA for library preparation should be at least 1 ng (steps 8–11).

Potential solution

Use at least 300 ng of cellular mRNA or rRNA-depleted RNA to perform the MeRIP experiments by increasing viral infected cell numbers and total RNA amount (steps 8–11). Recover the RNA as much as possible in each purification steps by following the instructions (steps 8–11). Combine two m6A-modified RNA eluates by using a freshly prepared MeRIP elution buffer (step 11).

Problem 3

Low number of reads to align to the viral genome.

When constructing the library, the amount of cDNA and final amplified DNA may be too low for sequencing (step 12).

Potential solution

Increase the number of cells and/or volume of supernatant from which RNA is extracted (steps 8–10) and thus the amount of RNA available for MeRIP (step 11). When preparing the library, determine the number of PCR cycles required for a given amount of cDNA template. The number of required PCR cycles is dependent on the cDNA amount, primer-dimer ratio and other factors, usually minimum PCR cycles should be used to achieve enough libraries for sequencing. Target about 5×10^7 sequencing reads per sample (step 12).

Problem 4

MeRIP-Seq data has high background noise

High background noise and low signal-to-background ratio leads to poor peak calling (step 13).

Potential solution

For MeRIP, it is essential that the elution buffer is freshly prepared. If problems remain even with a fresh elution buffer, also use a freshly prepared binding buffer and wash buffer. For the immunoprecipitation step, titrate the amount of antibody required in pilot experiments and use the minimum amount to obtain a low background signal.

Problem 5

Poor enrichment of m6A-modified RNA sequences in confirmatory MeRIP-RT-qPCR

Poor enrichment of sequences identified as having positive m6A peaks in the MeRIP-RT-qPCR confirmation experiments (step 14).

Potential solution

Design primers that are specific for RNA regions that are positive and negative for m6A peaks according to the MeRIP-Seq results (e.g., N region and E region, respectively, in [Figure 5](#)). Use the same amount of IgG as the anti-m6A antibody in the IP step. Prepare all buffers fresh. Increase the input RNA amount in MeRIP experiments (step 14c) or decrease the cDNA dilution (step 14e).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tariq Rana (trana@health.ucsd.edu).

Materials availability

This study did not generate any new or unique reagents.

Data and code availability

The datasets generated during this study are available at GEO: GSE167075.

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AUTHOR CONTRIBUTIONS

N.L. designed and performed the experiments, analyzed the data, performed the bioinformatics analysis, and wrote the manuscript. T.M.R. conceived the overall project and participated in the experimental design, data analysis, data interpretation, and manuscript writing.

DECLARATION OF INTERESTS

T.M.R. is a founder of ViRx Pharmaceuticals and has an equity interest in the company. The terms of this arrangement have been reviewed and approved by the University of California San Diego in accordance with its conflict of interest policies.

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