RNA modifications are a common occurrence across all domains of life. Several chemical modifications, including N⁶-methyladenosine, have also been found in viral transcripts and viral RNA genomes. Some of the modifications increase the viral replication efficiency while also helping the virus to evade the host immune system. Nonetheless, there are numerous examples in which the host’s RNA modification enzymes function as antiviral factors. Although established methods like MeRIP-seq and miCLIP can provide a transcriptome-wide overview of how viral RNA is modified, it is difficult to distinguish between the complex overlapping viral transcript isoforms using the short read-based techniques. Nanopore direct RNA sequencing (DRS) provides both long reads and direct signal readings, which may carry information about the modifications. Here, we describe a refined protocol for analyzing the RNA modifications in viral transcriptomes using nanopore technology.

**Keywords:** RNA virus, RNA modification, viral epitranscriptome, coronavirus, nanopore sequencing, direct RNA sequencing

**Overview**

The eukaryotic messenger RNAs often include chemically modified RNA bases in addition to the standard four ribonucleotides. N⁶-methyladenosine (m⁶A), which occurs as frequently as 2–3 in an average human mRNA molecule, is the most prevalent type of modification (Rottman et al., 1974). The m⁶A modification regulates a variety of cellular processes, including translation, RNA stability, splicing, and localization depending on the context (Zaccara et al., 2019). Another common RNA modification is pseudouridine, which makes up about 0.3% of all uridines in mRNA (Li et al., 2015). It is frequently associated with modulation of codon decoding and also has been utilized to avoid triggering the innate immune system initiated by cytoplasmic double-stranded RNAs in mRNA vaccines (Karikó et al., 2005; Cerneckis et al., 2022). Viral genomes constantly evolve in order to take advantage of new opportunities for host exploitation. RNA modifications are found in viral genomes and transcripts in several viral species, including HIV-1, HBV, SV40, Zika, and HCV (Baquero-Perez et al., 2021). Many of these enhance the replication efficiency of the viruses, yet anti-viral RNA modifications also exist that may be triggered by the host defense mechanisms (Williams et al., 2019; Courtney, 2021; Li and Rana, 2022).

Discovering, identifying, and quantifying RNA modifications is critical for understanding the dynamics and mechanisms of the viral epitranscriptome. Among the various approaches for measuring or detecting the RNA modifications (Table 1), the most fundamental tools for RNA modifications are small fragment analysis techniques, such as liquid chromatography-mass spectrometry (LC-MS) or nuclear magnetic resonance (NMR) (Wetzel and Limbach, 2016; Yoluç et al., 2021). These methods allow for the measurement of the stoichiometry between modified and unmodified nucleotides. Thus, these can be used to identify novel RNA modifications in isolated RNA samples as well as to identify global RNA modification changes brought on by perturbation of writers or readers.

When combined with high-throughput RNA sequencing, specific antibody purification or chemical reactions allow for a survey of the nucleotide sequences nearby the modified bases. For instance, MeRIP-seq identifies m⁶A-modified sites by sequencing RNA fragments that have been immunopurified with a modification-specific antibody (Meyer et al., 2012), whereas miCLIP improves resolution and specificity by adding additional UV-crosslinking between the antibody and modified bases (Hussain et al., 2013). Alternatively, bisulfite or N-cyclohexyl-N’-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMCT) treatments can reveal 5-methylcytidine (m⁵C) or pseudouridine (ψ) through the differential introduction of chemical-induced reverse-transcription errors (Schaefer et al., 2009; Carlile et al., 2014; Lovejoy et al., 1974).
 sweetheart sites. In 2019, Viehweger et al. (2019) published the first use of DRS for analyzing RNA modifications in the viral transcriptome. They employed DRS to sequence the human coronavirus 229E and sorted the full-length reads as per the subgenomic RNAs that they came from. By comparing the ion current signals to Tombo’s mC model (Stoiber et al., 2017), they were able to identify dozens of sites that are uniformly methylated irrespective of the sgRNA isofrom. Unsurprisingly, studies seeking mA in viral transcriptomes were quickly followed. Price et al. (2020) looked at the dynamics of mA modifications throughout the adenovirus life cycle using DRS. By using DRUMMER (Abebe et al., 2022a) to compare the signals from wild-type control and METTL3-knockout samples, they were able to spot the signal changes brought on by the depletion of the methyltransferase enzyme. Using the DRACH-motif as a guide, they were able to find modified RNA bases that works in all circumstances (Abebe et al., 2022b). We describe the approaches to try first, but other strategies might also be needed depending on the type of RNA modification, frequency, sequence contexts, and other variables. For more information on this issue, see a comprehensive review of various methods for viral epitranscriptome analysis using DRS (Abebe et al., 2022b).

Applications

In 2019, Viehweger et al. (2019) published the first use of DRS for analyzing RNA modifications in the viral transcriptome. They employed DRS to sequence the human coronavirus 229E and sorted the full-length reads as per the subgenomic RNAs that they came from. By comparing the ion current signals to Tombo’s mC model (Stoiber et al., 2017), they were able to identify dozens of sites that are uniformly methylated irrespective of the sgRNA isofrom. Unsurprisingly, studies seeking mA in viral transcriptomes were quickly followed. Price et al. (2020) looked at the dynamics of mA modifications throughout the adenovirus life cycle using DRS. By using DRUMMER (Abebe et al., 2022a) to compare the signals from wild-type control and METTL3-knockout samples, they were able to spot the signal changes brought on by the depletion of the methyltransferase enzyme. Using the DRACH-motif as a guide, they were able to find modified RNA bases that works in all circumstances (Abebe et al., 2022b). We describe the approaches to try first, but other strategies might also be needed depending on the type of RNA modification, frequency, sequence contexts, and other variables. For more information on this issue, see a comprehensive review of various methods for viral epitranscriptome analysis using DRS (Abebe et al., 2022b).
nucleus in fibroblasts. The authors used DRS to examine whether the methylation of host messenger RNAs is impacted by the expression of viral proteins. The data show that expression of the viral protein significantly lowers modification-induced basecall errors, thus suggesting the viral protein’s role in releasing m^6^A methylation factors from the nucleus.

With the start of the COVID-19 pandemic, SARS-CoV-2 has quickly risen as the most studied virus for research using nanopore DRS. Early in 2020, Kim et al. (2020b) used both short-read sequencing and nanopore DRS to map the overall transcriptome architecture and epitranscriptomic features, which include RNA modifications and poly(A) tails, of SARS-CoV-2 subgenomic RNAs. This study used in vitro transcribed (IVT) controls, which contain only the standard four bases and no modified bases. They used Tombo to estimate m^5^C methylation status, but the measured methylation rates between the viral RNAs and IVT controls were almost identical, reflecting the apparently high false positive rate. By comparing viral RNAs to IVT controls, they found 41 modified sites, many of which are differentially methylated based on the length of subgenomic RNAs. As the pandemic persisted, more research into specific modifications followed. Burgess et al. (2021) identified m^6^A modifications in SARS-CoV-2 and another human coronavirus OC43 by comparing control and METTL3 inhibitor treated cells. Chang et al. (2021) conducted a time-course DRS analysis throughout the SARS-CoV-2 infection timeline. The RNA modifications were found to remain unchanged throughout the infection. Two more studies confirmed that the SARS-CoV-2 RNAs’ m^6^A modification is controlled by the host factors METTL3 and FTO and that the extent of methylation varies among the virus variants (Campos et al., 2021; Zhang et al., 2021). Finally, a different approach led to the discovery of five new pseudouridine sites in the SARS-CoV-2 genome (Fleming et al., 2021). The study employed dwell-time analysis, which made it possible to find delayed translocation as well as a changed current signal around the modified sites.

### Methods

#### Viral RNA extraction and sequencing

**Preparing RNA extracts of virus-infected cells:** virus-infected cells are prepared using a cell line permissive for extensive viral growth. A high viral RNA fraction among total RNA is frequently preferred to achieve the required read coverage for viral transcripts with low expression. Vero and its derivatives are generally suitable for many mammalian viruses (Ammerman et al., 2008). Still, researchers should acknowledge that some cultured host cell types lack a specific component of the host machinery, resulting in inefficient or non-specific viral RNA modification. Infections with a high multiplicity of infection (MOI) generate more sequencing reads; however, in this case, the condition may diverge too far from the natural transcriptome due to insufficient host machinery for viral RNA processing or maturation. The highest purity and quality of RNA, essential for use in DRS, is typically produced when using cultured cells and TRIzol or comparable reagents. DNase I is applied to the extracted RNA to remove any DNA contamination that may interfere with the subsequent enzymatic reactions in library preparation.

**In vitro transcription:** it is highly recommended to use a negative control library that accurately reflects the nucleotide sequences of viral RNA to mitigate the high false positive rate in model-based classifiers without controls. Given that lengthy amplicons and templates are problematic in both PCR and in vitro transcription, it is preferable to split the genomic sequence into 2 kb tiles with small overlaps. Resquiggling is required for many types of signal analysis, but it frequently results in alignment errors at either end of the signals. Small tile overlaps can help solve such issues and make up for reads that have less coverage near their 5’ ends. DRS reads molecules in the sequencing library from 3’ end to 5’ end, and at a largely constant rate, the sequencing of a molecule terminates even in the middle of an RNA molecule. As a result, for large tiles, the read coverage near the 5’ ends of each tile

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**Fig. 1. Outline of modification analysis workflow.** Following the sequencing of the viral transcriptome, the reads are subjected to primary analysis, which involves basecalling, alignment to both viral and host genomes, and quality checks. After that, reads are grouped according to the viral RNA isoforms and subsampled for easier handling and more accurate statistics. The subsequent modification analysis consists of realigning the viral genome to increase alignment sensitivity, detecting modifications, and then performing comparative analyses to interpret the data biologically.
is often insufficient. In addition, for difficult-to-PCR sequences, such as those with high GC content, tiling frequency and primer positions may need to be changed. Some IVT libraries might need several iterations of design. IVT-sequencing because some problems are challenging to detect otherwise. The targeting sensitivity of the modification detection may be considered when determining the target coverage of IVT control. The coverage of 2000X in the shallowest region should deliver good enough performance while still being feasible with reasonable effort.

**Nanopore direct RNA sequencing:** the standard direct RNA sequencing kit (SQK-RNA002, Oxford Nanopore Technologies) employs a 3' adapter ligation that depends on poly(A) tails without any other nucleotides close to the 3' end. Viral transcripts lacking poly(A) tails are not picked up during library preparation, making them invisible in the sequencing data. *In vitro* polyadenylation should come before library preparation for such viruses. It is known that the poly(A) tails of many viral transcripts contain mixed tails, such as A/G or U tails (Kim et al., 2020a). It is necessary to perform a prior polyadenylation because mixed tails in the adapter ligation pose a challenge. Without a special adaptation for the mixed tails, data interpretation should be cautious due to their uneven distribution across the cellular RNAs and preference for a subset of cytoplasmic RNAs with long poly(A) tails. One million to two million reads are typically produced by a single DRS run. Because most viruses have small genomes, a single run usually produces more reads than is necessary. However, the sequencing reads of viral origin may not be sufficient for analysis depending on the characteristics of the viral life cycle. In this sort of situation, one should either pool data from multiple sequencing runs or attempt to enrich viral RNAs using an antisense oligo pull-down method.

**Post-processing and data analysis**

The primary analysis includes basecalling, sequence alignment, quality control, transcript sorting, and optional subsampling (Fig. 1). Comparisons between samples under two or more conditions or the IVT control are then made using sampling (Fig. 1). Comparisons between samples under two conditions or the IVT control are then made using sampling (Fig. 1). Comparisons between samples under two conditions or the IVT control are then made using sampling (Fig. 1). Comparisons between samples under two conditions or the IVT control are then made using sampling (Fig. 1). Comparisons between samples under two conditions or the IVT control are then made using sampling (Fig. 1). Comparisons between samples under two conditions or the IVT control are then made using sampling (Fig. 1).

**Basecalling and sequence alignment:** it is advised to perform basecall again using the high-accuracy model if the sequencing run was configured with fast basecalling or no basecalling. Minimap2 is commonly used for sequence alignment of nanopore reads (Li, 2018). The alignment programs are typically tuned against non-canonical junctions that are not likely produced by eukaryotic RNA splicing mechanisms by default. For viruses that produce transcripts as discontinuous copies of the genome, extensive parameter tuning is needed. Additionally, sequence alignment for viral genomes can be improved upon over the conventional sensitivity settings, taking into account the high error rate of both DRS and viral replication. To enable more sensitive alignment against the viral genome, align the sequences to both the host and viral genomes, then align the subset of reads that were aligned to the viral genome with more sensitive options. Most viral genomes can have minimap2's k-mer size and minimizer window size reduced to -k6 and -w2, respectively, without sacrificing scalability.

**Subsampling reads for RNA modification analysis:** most RNA modification detection methods look for differences between two populations at each transcript position. In the comparisons, uneven sample sizes for each transcript position in the control group lead to unevenly applied statistical power across the viral genome. The biological interpretation becomes challenging as a result. Since coverage continuously decreases from the 3' side to the 5' side in every tile of IVT controls, choosing the same number of full-length reads that span the 5' most positions of each tile should work (Fig. 2). Subsampling is also occasionally required for biological RNA reads, such as wild-type or knock-down sample reads. Because the distribution of viral transcript expression levels is extremely varied, transcripts with excessive abundance, such as nucleocapsid mRNAs in Nidovirales, occasionally consume a tremendous amount of computational time and memory and cause numerical data type overflows in analysis software. To reduce the computational workload, consider random subsampling of reads from transcripts with a high abundance for RNA modification detection.

**RNA modification detection:** modification detection can be performed at either the basecalled sequence or the ionic current signal levels. In most cases, it is practical to begin the analysis with an algorithm that uses differential error rates at the site level. The most popular programs for this approach are ELIGOS2 and DRUMMER (Jenjaroenpun et al., 2021; Abebe et al., 2022a). With this method, it is possible to compile a list of potential transcripts that are frequently or differently modified and analyze any correlations at the transcript level, such as those between modification rate and translation activity. Signal-level analyses are necessary for more complex analyses, such as single-molecule level analysis, enhancing sensitivity, and associations between regulatory elements within a molecule. The majority of analysis software in this approach employs “resquiggled” signals, which are scaled signals that alleviate uneven translocation speed through the nanopore by stretching and constricting them. Nanopolish and Tombo are the two most popular applications for resquiggling (Stoiber et al., 2017). In addition to ionic current...
level changes, dwell time can be considered when comparing samples. It is known that certain RNA modifications cause a significant slowdown in the translocation of the modified base near its -20 position (Stephenson et al., 2022).

Materials

Reagents

- TRIzol (Invitrogen, 15596018)
- Chloroform (Amresco, 0757-500ML)
- Isopropanol (Merck, 1.09634.1011)
- Ethanol (Fisher, A9951)
- Dithiothreitol (DTT) (Sigma-Aldrich, 43819-5F)
- Qubit RNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Q32852)
- Recombinant DNase I (RNase-free) (TaKaRa, 2270A)
- RNeasy MinElute Cleanup Kit (Qiagen, 74204)
- SuperScript IV Reverse Transcriptase (Invitrogen, 18090200)
- Q5 High-Fidelity 2X Master Mix (New England Biolabs, M0492L)
- 10X Loading Buffer (TaKaRa, 9157)
- Agarose (Seakem, 50004)
- Gel Extraction Kit (Labopass, CMG0112)
- MEGAscript T7 Transcription Kit (Invitrogen, AMB13345)
- TAE buffer (Dyne Bio, CBT3021)
- Gelgreen dye (Biotium, 41005)
- TURBO DNase (Ambion, AM2239)
- Oligo Clean & Concentrator (Zymo Research, D4061, optional)
- 1 kb Plus DNA Ladder (Invitrogen, 10787026)
- SUPERase·In RNase Inhibitor (Invitrogen, AM2696)
- Deionized Purified Water (obtained in house from Milli-Q Ultrapure Water System)
- DNA LoBind Microcentrifuge 1.5 ml tubes (Eppendorf, 022431021)
- Direct RNA Sequencing Kit (Oxford Nanopore Technologies, SQK-RNA002)
- MinION Flow Cell (R9.4.1) (Oxford Nanopore Technologies, FLO-MIN106D)

Equipment

- Thermocycler
- NanoDrop (Thermo Fisher Scientific)
- Safe Imager 2.0 Blue-Light Transilluminator (Thermo Fisher Scientific, G6600)
- MinION Mk1b or Mk1c Device (Oxford Nanopore Technologies, MIN-101B)
- Computer running Linux or an equivalent environment that is capable of running the software listed below. It is recommended to install a GPU card because basecalling with CPUs alone takes several days rather than one or two hours. At the time of writing, Guppy, the ONT production basecaller, only supports NVIDIA GPUs.

Computer software

- Guppy 6.1.2 (Oxford Nanopore Technologies)
- Minimap2 2.17 (Li, 2018)
- Bedtools 2.30.0 (Quinlan, 2014)
- Seqtk 1.3 (https://github.com/lh3/seqtk)
- Samtools 1.7 (Li et al., 2009)
- PycoQC 2.5.0.19 (Leger and Leonardi, 2019)
- ELIGOS2 2.0.1 (Jenjaroenpun et al., 2021)
- Nanopolish 0.13.2 (https://github.com/jts/nanopolish)
- Picard Toolkit 2.27.4 (https://github.com/broadinstitute/picard)
- m6Anet 1.1.0 (Hendra et al., 2021)

Protocols

Construction of sequencing libraries

A. RNA purification

1. This step begins with cell pellets in 1 ml TRIzol in a 1.5 ml tube. Pellets should be lysed completely.
2. Incubate the tube at 50°C for 15 min, occasionally inverting the tube.
3. Add 200 μl chloroform.
   ▶ NOTE: perform this step in a fume hood.
4. Vortex vigorously.
   ▶ NOTE: hold the tube cap tight to avoid leakage.
5. Incubate the tube at 25°C for 3 min.
6. Vortex vigorously.
   ▶ NOTE: hold the tube cap tight to avoid leakage.
7. Centrifuge the tube at 4°C for 10 min.
   ▶ NOTE: close the lid to avoid aerosols.
8. Carefully move the tube to the rack to avoid mixing two separate phases.
9. Set a P200 pipette to 150 μl, move all supernatants carefully (~600 μl) into a new tube. Discard the tube with the lower phase.
   ▶ NOTE: aqueous phase solution is prone to leakage.
10. Add 600 μl isopropanol.
11. Vortex vigorously.
   ▶ NOTE: hold the tube cap tight to avoid leakage.
12. Incubate the tube at 25°C for 10 min, occasionally vortexing or inverting the tube.
13. Centrifuge the tube at 4°C for 30 min.
14. Check if there is a white pellet. Discard the supernatant carefully.
   ▶ NOTE: always check if the supernatant does not contain the pellet.
15. Centrifuge the tube at 4°C for 10 sec.
16. Discard the supernatant carefully with a P20 pipette.
17. Add 1 ml pre-chilled 75% ethanol and vortex.
18. Centrifuge the tube at 4°C for 10 min.
19. Discard the supernatant carefully and repeat steps 17–18.
20. Centrifuge the tube at 4°C for 10 sec.
21. Discard the supernatant carefully with a P20 pipette.
   ▶ NOTE: remove ethanol as much as possible.
22. Open the tube cap and air-dry for 3 min. You can see a transparent pellet turn white.
23. Add 30 μl water and dissolve the pellet by pipetting.
B. DNase I treatment and purification
If the amount of RNA is low (< 10 μg) and the purity is good, DNase I treatment and purification step may be skipped to avoid loss of RNA.

24. Measure the RNA concentration by using the Qubit RNA HS Assay Kit or NanoDrop.

D. PCR and agarose gel purification for in vitro transcription template

25. Mix RNA (up to 45 μg), water up to 87 μl, 10 μl 10X DNase I buffer, 2 μl DNase I, and 1 μl SUPERase-In RNase Inhibitor in 1.5 ml tube by pipetting.

26. Incubate the tube at 37°C for > 30 min.
27. Add 350 μl RLT buffer and mix by pipetting.
28. Add 250 μl of 100% ethanol and mix by pipetting.
29. Move all solutions to a RNeasy MinElute spin column.
30. Centrifuge at 25°C for 1 min.
31. Discard the flow-through.
32. Add 500 μl of RPE buffer to the column.
33. Centrifuge at 25°C for 1 min.
34. Discard the flow-through.
35. Add 500 μl 80% ethanol into the column.
36. Centrifuge at 25°C for 1 min.
37. Discard the flow-through.
38. With a P20 pipette, remove residual ethanol from the ring around the cap.
39. Centrifuge at 25°C for 2 min.
40. Move the column to a new 1.5 ml tube.
41. Open the tube cap and air-dry at 25°C for 3 min.
42. Add > 20 μl water directly to the membrane.

C. Reverse transcription

43. Incubate at 25°C for 30 min.
44. Centrifuge at 25°C for 1 min.
45. Check the eluate and measure the RNA concentration by using Qubit RNA HS Assay Kit or NanoDrop.

46. Prepare 200 μl PCR tube and mix RNA (200 ng–1 μg), water up to 12.5 μl, 1 μl of 10 μM forward PCR primer, 1 μl of 10 μM reverse PCR primer, and 15 μl of Q5 High-Fidelity 2X Master Mix by pipetting.

47. Incubate at 50°C for > 30 min.
48. Add 4 μl of 5X SuperScript IV Buffer, 1 μl of 0.1 M di-thiothreitol (DTT), 1 μl of SUPERase-In RNase Inhibitor (or RNaseOUT Recombinant RNase Inhibitor, Invitrogen), and 1 μl of SuperScript IV Reverse Transcriptase.

49. Briefly spin down and mix by pipetting.
50. Incubate at 50°C for > 30 min.
51. Incubate at 80°C for 10 min and store at 4°C until the next step.

52. Prepare 200 μl PCR tube and mix cDNA (e.g. 0.5 μl), water up to 12.5 μl, 1 μl of 10 μM forward PCR primer, 1 μl of 10 μM reverse PCR primer, and 15 μl of Q5 High-Fidelity 2X Master Mix by pipetting.

53. Run PCR program: 98°C for 30 sec, (98°C for 20 sec, 60°C for 30 sec, 72°C for 1 min 30 sec) × 25–30 cycles, 72°C for 1 min, and hold at 16°C.

54. After the run, add 3.4 μl of 10X loading buffer.
55. Prepare 1% agarose gel by using a wide well enough to load ~35 μl PCR product, if applicable.
56. Load ~35 μl PCR product and 10–20 μl 1 kb plus ladder.
57. Run the gel at 130 V for 25 min using the 1X TAE buffer.
58. After the run, stain the gel with Gelgreen dye in a fresh 1X TAE buffer for 10 min.

E. In vitro transcription and RNA purification

59. Weigh the empty 1.5 ml tube.
60. Put the gel on Safe Imager 2.0 Blue-Light Transilluminator, cut the gel with a razor and move the gel into a new 1.5 ml tube.

61. Weigh the gel slice.
62. Add 3X volume of GB buffer from Gel extraction kit.
63. Vortex for 5 sec and incubate at 50°C for 10 min with occasional vortexing.

64. Load the solution onto a spin column.
65. Centrifuge at 25°C for 30 sec and discard the flow-through.
66. Load a 750 μl NW buffer on a spin column, centrifuge at 25°C for 30 sec and discard the flow-through.
67. Repeat step 66 once.
68. With a P20 pipette, remove the residual buffer at the ring around the cap.
69. Centrifuge at 25°C for 2 min.
70. Move the column to a new 1.5 ml tube.
71. Open the tube cap and air-dry at 25°C for 3 min.
72. Add > 20 μl water directly to the membrane.

73. Incubate at 25°C for 1 min.
74. Centrifuge at 25°C for 1 min.
75. Weigh the gel slice.
76. Add 3X volume of GB buffer from Gel extraction kit.
77. Vortex for 5 sec and incubate at 50°C for 10 min with occasional vortexing.

78. Load the solution onto a spin column.
79. Centrifuge at 25°C for 30 sec and discard the flow-through.
80. Load a 750 μl NW buffer on a spin column, centrifuge at 25°C for 30 sec and discard the flow-through.
81. Repeat step 66 once.
82. With a P20 pipette, remove the residual buffer at the ring around the cap.
83. Centrifuge at 25°C for 2 min.
2 μl, 10X Enzyme mix 2 μl, water 2 μl, 1 μl of SUPERase-In RNase Inhibitor by pipetting.

77. Incubate at 37°C overnight.
78. Add 1 μl of TURBO DNase and mix by pipetting.
79. Incubate at 37°C for > 15 min.
   • NOTE: if IVT RNA is longer than 200 nt, recommend using RNase Inhibitor by pipetting.
80. Add water 79 μl.
81. Add 350 μl RLT buffer and mix by pipetting.
82. Add 250 μl of 100% ethanol and mix by pipetting.
83. Move all solutions to a RNeasy MinElute spin column.
84. Centrifuge at 25°C for 1 min.
85. Discard the flow-through.
86. Add 500 μl of RPE buffer to the column.
87. Centrifuge at 25°C for 1 min.
88. Discard the flow-through.
89. Add 500 μl 80% ethanol into the column.
90. Centrifuge at 25°C for 1 min.
91. Discard the flow-through.
92. With a P20 pipette, remove residual ethanol from the ring around the membrane of the column and from the ring around the cap.
93. Centrifuge at 25°C for 2 min.
94. Move the column to a new 1.5 ml tube.
95. Open the tube cap and air-dry at 25°C for 3 min.
96. Add > 20 μl water directly to the membrane.
   • NOTE: be sure to put water into the membrane, not around the ring.
97. Incubate at 25°C for 1 min.
98. Centrifuge at 25°C for 1 min.
99. Check the eluate and measure the RNA concentration by using Qubit RNA HS Assay Kit or NanoDrop.

F. Nanopore direct RNA sequencing

Follow the manufacturer’s instructions with a few changes listed below (Direct RNA sequencing Kit, Oxford Nanopore Technologies). We used these procedures for SARS-CoV-2-infected Vero cells (Kim et al., 2020b), but other virus or host cells may require a modification due to the different RNA length, poly(A) tail length distribution, and also non-coding RNA composition.

100. For nanopore sequencing on non-infected and SARS-CoV-2-infected Vero cells, each 4 μg of DNase I-treated total RNA in 8 μl was used for library preparation with minor adaptations.
   a. Add 1 μl of SUPERase-In RNase inhibitor (20 U/μl) to both adapter ligation steps.
   b. SuperScript IV Reverse Transcriptase was adopted instead of SuperScript III.
   c. The reaction time of reverse transcription was lengthened by 2 h.
101. For nanopore sequencing on SARS-CoV-2 IVT RNA fragments, mix an equal amount of IVT RNA fragments for a total of 2 μg.
   a. Add 1 μl of SUPERase-In RNase inhibitor (20 U/μl) to both adapter ligation steps.
   b. SuperScript IV Reverse Transcriptase was adopted instead of SuperScript III for 30 min.

Data analysis

A. Preparing the reference sequences

Choose reference genome assemblies for the host cell line and the virus. Genomes from GENCODE or ENSEMBL are preferred because they provide a variety of preprocessed files and annotation files with extensive annotation. Because minimap2 only accepts a single file as a reference, the host genome sequences and viral genome must be combined into a single FASTA file. Pre-built index files can be made to avoid having to build index files from scratch each time a sample is processed.

1. Merge host and virus genome sequences into a single combined FASTA file.
   $ cat host-genome.fasta virus-genome.fasta > hostvirus-genomes.fasta
2. Create minimap2 indices for each FASTA file, both combined and virus-only.
   $ minimap2 -k 13 -w 6 -d hostvirus-genomes.mmidx host-virus-genomes.fasta
   $ minimap2 -k 6 -w 2 -d virus-genome.mmidx virus-genomefasta

B. Basecalling and quality check

The supplementary code contains an example analysis pipeline and notebooks (see Supplementary data 1). For the procedure in more detail, refer to the command lines and Jupyter notebooks in the repository.

1. Transfer the FAST5 files to a computer for data analysis from the sequencer or host computer. Use both "pass" and "fail" reads because re-analysis may improve the basecall quality.
2. Run basecalling using guppy with the high accuracy RNA model.
   $ guppy_basecaller -c rna_r9.4.1_70bps_hac.cfg --fast5_out --calib_detect --min_qscore 5 --num_callers 4 -i original-fast5 -s guppy-basecalls -x cuda:all
   The input FAST5 files should be located in a directory named "original-fast5" and outputs are produced in "guppy-basecalls/"
   • NOTE: on computers without an NVIDIA GPU, the "-x cuda:all" command line option should be removed.
3. Merge the FASTQ sequences from the output into a single compressed FASTQ file.
   $ zcat guppy-basecalls/pass/*.fastq | gzip -c - > pass-all.fastq.gz
4. Align sequences to the combined reference index.
   $ minimap2 -a --MD -a -x splice -N 32 -un -t 20 hostvirus-genomes.mmidx pass-all.fastq.gz | samtools sort -o pass-hostvirus.bam
5. Run pycoQC to generate a quality check report. Inspect each report carefully to ensure that the quality of each sequencing run is even and contains sufficient viral sequence reads.
   $ pycoQC -t guppy-basecalls/pass/sequencing_summary.txt -a pass-hostvirus.bam -o pycoQC.html
   • NOTE: verify the distribution of reads by length and
quality as well as any changes in these parameters over time (Fig. 3). Those library quality factors must be consistent across all samples, within the expected range of distributions. The majority of techniques for DRS-based RNA modification detection are particularly sensitive to the overall basecall quality shift between samples.

C. Processing viral sequence reads
RNA modification analysis can be done for either all reads of viral origin combined or separately for each viral transcript. In order to compare differentially modified sites based on viral transcript, the viral reads must be divided into separate folders containing sorted FASTQ and FAST5 files. The majority of the time, read coverage greater than 2000X has little to no bearing on the accuracy or sensitivity of the analysis; if necessary, one can randomly subsample highly abundant transcripts for modification analysis.

1. Extract viral RNA reads to a separate file. The command that follows presumes that the viral genome sequence has the ID “NC_045512.2.”

```bash
$ samtools view -F4 pass-hostvirus.bam | grep NC_045512.2 | cut -f1 | sort | uniq > pass-hostvirus.virus-id.txt
$ seqtk subseq pass-all.fastq.gz pass-hostvirus.virus-id.txt | gzip -c - > pass-virus.fastq.gz
```

2. Align the viral reads to the viral genome using more sensitive settings. To allow the virus-specific sequence recombination mechanisms in the alignments, additional parameter changes might be required.

```bash
$ minimap2 --splice -g 30000 -G 30000 -A1 -B2 -O 2,24 -E 1,0 -C0 -z 400,200 --no-end-flt -F 40000 -N 350 --splice-flank=no --max-chain-skip=40 -un --MD -a -p 0.7 -t 20 virus-genome.mmidx pass-virus.fastq.gz | samtools sort -o pass-virus.bam; samtools index pass-virus.bam
```

**NOTE:** add these options to give accepted recombination sites some preference in locating long deletions: --junc-bed viral-junction.bed --junc-bonus=50

2. Align the viral reads to the viral genome using more sensitive settings. To allow the virus-specific sequence recombination mechanisms in the alignments, additional parameter changes might be required.

3. Optionally, downsample reads for transcripts with an unusually high abundance. This procedure requires some programming that is specific to the structure of the viral transcriptome. See rules from “extract_junctions” to "get_balanced_fastq” in “Snakefile” in the Supplementary data 1 code for an example.

D. RNA modification analysis using ELIGOS2
1. Run ELIGOS2 with the sorted and index bam file as the input file.

```bash
$ eligos2 -pair_diff_mod -tbam pass-virus.bam -cbam pass-control.bam -reg viral-annotation.bed -ref viral-genome.fasta -t 5 --pval 1 --oddR 0 --esb 0 -o eligos2-output/
```

**NOTE:** select the sample with the greater expected modification rate as the “test” sample and the other as the “control” sample.

**NOTE:** the ELIGOS2 default threshold values are too restrictive. Try relaxing the threshold values for p-value, odd ratio, and error at specific base (ESB) values, and then filter in subsequent steps following inspection of global patterns.

2. Perform further visualization and inspection of RNA modification distributions across the viral genome in an exploratory data analysis environment, such as JupyterLab or RStudio. See the Supplementary data 1 code for an example.

E. RNA modification analysis using m6Anet
Ionic current signals are supported by m6Anet for single-molecule level measurements for RNA modification detection. Try using different programs as well because the outcomes of programs built on this methodology frequently do not coincide.

1. Prepare a resquiggled signal database using nanopolish.

```bash
$ nanopolish index -s guppy-basecalls/sequencing_summary.txt -d guppy-basecalls/workspace/ pass-virus.fastq.gz
$ nanopolish eventalign -t 5 --signal-index -r pass-virus.fastq.gz -b pass-virus.bam -g viral-genome.fasta =nanopolish-summary.txt --scale-events -n > nanopolish-eventalign.txt
```

2. Run m6Anet with the nanopolish eventalign outputs.

```bash
$ m6anet-dataprep -i nanopolish-eventalign.txt -o m6anet-dataprep-pdir/ $ m6anet-run_inference --input_dir m6anet-dataprep-dir/ --out_dir m6anet-outputdir/ --n_process 10
```

**NOTE:** because m6Anet assumes the user inputted the transcriptome alignment, the minus strand is not evaluated. The procedure should be modified accordingly if the gene is expressed from both strands of the viral genome.
3. Compare the outcomes from ELIGOS2 with the outcomes from m6Anet by performing additional statistical analysis and visualization. Explore the data and analyze the data based on hypotheses about the mechanisms and interactions of host factors that affect methylation.

**Expected Results**

Once RNA modification detection is complete, the reported sites can be analyzed in a number of ways to determine their reliability, whether or not they are subject to systematic bias, and whether they are associated with any particular biological mechanism. Statistics at the site level highlight potential heavily modified sites and their global distribution throughout the viral genome (Fig. 4A–B). This suggests modifications at particular locations within the viral genome, clustering of the modification sites, or general frequencies of the modifications. It should be noted that these types of statistics may not capture conditionally regulated modification that maintains low equilibrial stoichiometry at a site level. Such circumstances call for in-depth analysis, which frequently involves read sorting by RNA isoform, feature quantification (such as poly[A] length or local folding energy), and statistical association screening.

The various analytic approaches report different sites and scores, but each program frequently has a unique ability to detect particular types of modifications in terms of modification type and sequence context (Fig. 4C–D). In our previous study with SARS-CoV-2, ELIGOS2 found a relatively small number of sites with high rates of modification of any kind, whereas m6Anet found that m'A modification may be more widespread across the SARS-CoV-2 genome. Therefore, it should be easier to obtain potent statistical power in the result interpretations by preparing multiple conditions of viral RNA samples, such as latent and lytic phases.

Still in its very early stages, nanopore DRS anticipates years of continued growth and development. In late 2022, it is expected that new nanopore proteins with better homopolymer resolution will be made available for DRS. Analytical algorithms used in RNA modification are still underutilizing the information present in the squiggles. The accuracy and adaptability would be greatly enhanced if approaches similar to those used in state-of-art DNA basecallers were applied. Long and direct reads from DRS are the ideal features for high-throughput analysis of viral genomes and transcripts. Despite its limitations (low accuracy and a high rate of false positives), DRS-based transcriptome mapping is a cost-effective first step when analyzing a viral epitranscriptome.

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**Conflict of Interest**

The authors have no conflict of interest to report.

**Data Availability**

Source codes for the pipeline and interactive notebooks are available from https://github.com/ChangLabSNU/viral-epitranscriptome-pipeline and Supplementary data 1.

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