Nucleotide/nucleoside analogs (NAs) are important compounds used in antiviral drug development. To understand the action mode of NA drugs, we present an enzymology protocol to initially evaluate the intervention mechanism of the NTP forms of NAs on a coronaval RNA-dependent RNA polymerase (RdRP). We describe the preparation of SARS-CoV-2 RdRP proteins and RNA constructs, followed by a primer-dependent RdRP assay to assess NTP forms of NAs. Two representative NA drugs, sofosbuvir and remdesivir, are used for demonstration of this protocol.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol
Assessment of nucleotide/nucleoside analog intervention in primer-dependent viral RNA-dependent RNA polymerases

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SUMMARY
Nucleotide/nucleoside analogs (NAs) are important compounds used in antiviral drug development. To understand the action mode of NA drugs, we present an enzymology protocol to initially evaluate the intervention mechanism of the NTP forms of NAs on a coronaviral RNA-dependent RNA polymerase (RdRP). We describe the preparation of SARS-CoV-2 RdRP proteins and RNA constructs, followed by a primer-dependent RdRP assay to assess NTP forms of NAs. Two representative NA drugs, sofosbuvir and remdesivir, are used for demonstration of this protocol. For complete details on the use and execution of this protocol, please refer to Wu et al. (2021).

BEFORE YOU BEGIN
This protocol is mainly to initially investigate NA (sofosbuvir, remdesivir, etc.) (Gane et al., 2013, 2014; Gordon et al., 2020) intervention mechanisms against viral RdRPs, using severe acute respiratory syndrome virus 2 (SARS-CoV-2) RdRP complex nsp12-nsp7-nsp8 as the model system (Gao et al., 2020). Stock solutions of NTPs, NTP form of NA drugs, and buffer components were prepared accordingly. 20% (w/v) polyacrylamide (19:1 acrylamide/bisacrylamide)/7 M urea gel electrophoresis (denaturing PAGE) was used to resolve the RNA species in the quenched reaction solution.

Preparation of SARS-CoV-2 nsp12, nsp7, nsp8 proteins and RNA constructs

© Timing: 5 days

1. Transform the plasmid containing SARS-CoV-2 nsp12, nsp7, or nsp8 gene into Escherichia coli (E. coli) BL21(DE3) competent cells.
2. Grow cells at 37°C at 220 rpm for about 9 h in LB medium with 100 μg/mL ampicillin (AMP) for nsp12 and 50 μg/mL kanamycin (KAN) for nsp7 and nsp8, respectively, until the OD\textsubscript{600} is 1.0.
3. Transfer a 20-mL 9-h culture to 1 L of LB medium with 100 μg/mL AMP for nsp12 and 50 μg/mL KAN for nsp7 and nsp8, respectively, to reach an initial OD\textsubscript{600} around 0.02.
4. Grow cells at 37°C at 220 rpm for about 3 h to an OD\textsubscript{600} of 0.8 and cool to 16°C, and supplement with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for induction.
5. Harvest cells after a 16-h induction through centrifugation at 6,740 g for 15 min in an F10S×1000 rotor (Thermo Scientific), and resuspend the pellets in a Lysis buffer.

Note: It is recommended to resuspend the pellets of 1 L cell culture with 50 mL Lysis buffer.
6. Lyse the pellets by passage through an AH-2010 homogenizer at 14,500 psi (ATS Engineering).
7. Add IGEPAL CA-630 to a final concentration of 0.1% (v/v), and then add polyethlenimine (PEI) slowly to 0.05% (v/v) over a 35-min period by 5-min intervals to precipitate nucleic acid.
8. Centrifuge the lyzate at 34,310 g for 60 min in an F21-8x50y rotor (Thermo Scientific).
9. Load the clarified lysate onto a nickel-charged HisTrap HP column, followed by a step elution with 300 mM imidazole in a buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10% (v/v) glycerol.
10. Pool fractions containing nsp12, nsp7, or nsp8 and dilute by a Q low-salt buffer to reduce the NaCl concentration to approximately 70 mM, to ensure binding of target protein to the HiTrap Q column in the next step.
11. Load the diluted fractions onto a 5-mL HiTrap Q column and elute with a linear gradient to 1 M NaCl in 10 column volumes.
12. Concentrate the pooled fractions by Amicon Ultra centrifugal concentrators (100 kDa MWCO for nsp12, 30 kDa MWCO for nsp8, and 10 kDa MWCO for nsp7) to approximately 0.8 mL and run over a Superdex200 gel filtration column pre-equilibrated in a GF buffer.
13. Supplement pooled fractions with tris-(2-carboxyethyl) phosphine (TCEP) to a final concentration of 5 mM.
14. Concentrate proteins to approximately 30 mg/mL and dilute to 240 μM, 1000 μM and 1000 μM for nsp12, nsp7, and nsp8, respectively. The typical yield of pure protein per liter of bacterial culture is 0.5 mg, 30 mg, 30 mg for nsp12, nsp7, nsp8, respectively.
15. Flash freeze aliquots of 5–20 μL purified proteins in liquid nitrogen, and store at −80°C for single use.
16. Prepare the RNA constructs T33-1/P10 and T33-8/P10.
   a. Mix the template strand RNA (T33-1 or T33-8, with 5′-triphosphate and 2′,3′-cyclic phosphate at the 3′ end) to a final concentration of 40 μM with the primer strand RNA P10 (Integrated DNA Technologies) at a molar ratio of 1:1.1 with an RNA Annealing Buffer (RAB). The component concentrations of stock solution and final reaction mixture, volumes of each stock solution used to make the final reaction mixture are listed as follows.

| RNA construct | Stock concentration | Final concentration | Volume (μL) |
|---------------|---------------------|---------------------|-------------|
| T33-1/ T33-8  | 800 μM              | 40 μM               | 5           |
| P10           | 1000 μM             | 44 μM               | 4.4         |
| RAB           | n/a                 | n/a                 | 90.6        |
| Final volume  | n/a                 | n/a                 | 100         |

b. Incubate the mixture at 45°C for 3 min.
c. Transfer the mixture from 45°C to 25°C (room temperature, r.m.) for slow cooling (about 10 min).

▲ CRITICAL: It is important to anneal the template and primer prior to usage.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| *E. coli* BL21(DE3) | Beijing ComWin Biotech | Cat# CW0809S |
| **Chemicals, peptides, and recombinant proteins** | | |
| ATP                | Sigma  | Cat# A2383 |
| GTP                | Sigma  | Cat# G8877 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| CTP                 | Sigma  | Cat# C1506 |
| UTP                 | Sigma  | Cat# U6750 |
| DEPC-treated H₂O    | Thermo Fisher Scientific | Cat# 4387937 |
| Boric acid          | Sinopharm Chemical Reagent | Cat# 10004818 |
| Yeast extract       | Oxoid  | Cat# LP0021B |
| Tryptone            | Oxoid  | Cat# LP0042B |
| Sodium chloride     | Sinopharm Chemical Reagent | Cat# 10019318 |
| Imidazole           | Sinopharm Chemical Reagent | Cat# 30104916 |
| Ammonium persulfate (APS) | Sangon Biotech | Cat# A600072 |
| HEPES               | Shanghai Aladdin Biochemical Technology Co., Ltd | Cat# H109406 |
| Urea                | Sinopharm Chemical Reagent | Cat# 10023218 |
| Tris                | Sangon Biotech | Cat# A600194 |
| EDTA                | Sinopharm Chemical Reagent | Cat# 10004618 |
| Ampicillin          | Sangon Biotech | Cat# A100339 |
| Kanamycin           | Sangon Biotech | Cat# A600266 |
| Isopropyl-β-D-thiogalactopyranoside (IPTG) | BioFroxx | Cat# 1122GR100 |
| N, N’, N”, N’’ – Tetrathiomethylene diamine (TEMED) | Sigma | Cat# A9926 |
| 19:1 acrylamide/ bisacrylamide 40% (w/v) Solution | Sigma | Cat# 13021 |
| IGEPAL CA-630       | Sigma  | Cat# 408727 |
| Polyethyleneimine (PEI) | Sigma | Cat# V900064 |
| Formamide           | Vetec   | E9379 |
| Stains-All          | Sigma   | Cat# BS064 |
| Bromophenol blue    | Biosharp | Cat# C4706 |
| Tris(2-chloroethyl) phosphate (TCEP) | Sigma | Cat# 3483-12-3 |
| Dithiothreitol (DTT) | Sinopharm Chemical Reagent | Cat# 7791-18-6 |
| Magnesium chloride hexahydrate | Sigma | N/A |
| Remdesivir NTP form (RDV-TP) | SeNTInall BioTechnologies | N/A |
| Sofosbuvir NTP form (SOF-TP) | SeNTInall Bio Technologies | N/A |

### Oligonucleotides

| Oligonucleotide | Source | identifier |
|-----------------|--------|------------|
| T33-1: 5’-GGGAGAUGAAAGUCUCAC CUGUGUGCGGAAA-3’ | This paper | N/A |
| T33-8: 5’-GGGAGAUGAAAGUCUCCA UUAGAGUCGUCGAAA-3’ | This paper | N/A |
| DNA complementary to T33-1: 5’-TT TCGAGCACACAGGTGGA GACTTTICATCTCCC-3’ | Sangon Biotech | N/A |
| P10: UGUUCGACGA | Integrated DNA Technologies (IDT) | N/A |

### Recombinant DNA

| Recombinant DNA | Source | identifier |
|-----------------|--------|------------|
| pET22b-SARS-CoV-2-nsp12 | Zhihe Rao and Quan Wang laboratories, ShanghaiTech University | N/A |
| pET28a-SARS-CoV-2-nsp7 | Zhihe Rao and Quan Wang laboratories, ShanghaiTech University | N/A |
| pET28a-SARS-CoV-2-nsp8 | Zhihe Rao and Quan Wang laboratories, ShanghaiTech University | N/A |

### Software and algorithms

| Software and algorithms | Details | identifier |
|-------------------------|---------|------------|
| ImagJ Fiji distribution | (Schindelin et al., 2012) | https://imagej.net/Fiji |
## MATERIALS AND EQUIPMENT

### LB medium

| Reagent                                | Final concentration | Amount |
|----------------------------------------|---------------------|--------|
| Yeast extract                          | 0.5% (w/v)          | 5 g    |
| Tryptone                               | 1% (w/v)            | 10 g   |
| NaCl                                   | 1% (w/v)            | 10 g   |
| H₂O (ultrapure water with resistivity of 18.2 MΩ·cm) | n/a                 | To 1 L |

**Total**: n/a 1 L

*Note: Prepare freshly.*

### RAB

| Reagent                  | Final concentration | Amount |
|--------------------------|---------------------|--------|
| NaCl (5 M)               | 50 mM               | 50 µL  |
| Tris-HCl (pH 7.5) (1 M)  | 5 mM                | 25 µL  |
| MgCl₂ (1 M)              | 5 mM                | 25 µL  |
| DEPC-treated H₂O         | n/a                 | 4.9 mL |

**Total**: n/a 5 mL

*Note: Prepare freshly.*

### Lysis buffer

| Reagent                  | Final concentration | Amount |
|--------------------------|---------------------|--------|
| Tris-HCl (pH 8.0) (1 M)  | 50 mM               | 50 mL  |
| NaCl                     | 300 mM              | 17.54 g|
| Imidazole                | 10 mM               | 0.68 g |
| Glycerol                 | 10% (v/v)           | 100 mL |
| H₂O                      | n/a                 | To 1 L |

**Total**: n/a 1 L

*Note: Prepare freshly.*
### Q low-salt buffer

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Tris-HCl (pH 8.5) (1 M)  | 25 mM               | 25 mL    |
| NaCl                     | 50 mM               | 2.92 g   |
| EDTA (pH 8.0) (0.5 M)    | 0.1 mM              | 200 μL   |
| Glycerol                 | 10% (v/v)           | 100 mL   |
| H₂O                      | n/a                 | To 1 L   |
| **Total**                | n/a                 | 1 L      |

Note: Prepare freshly.

### Q high-salt buffer

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Tris-HCl (pH 8.5) (1 M)  | 25 mM               | 25 mL    |
| NaCl                     | 1 M                 | 58.44 g  |
| EDTA (pH 8.0) (0.5 M)    | 0.1 mM              | 200 μL   |
| Glycerol                 | 10% (v/v)           | 100 mL   |
| H₂O                      | n/a                 | To 1 L   |
| **Total**                | n/a                 | 1 L      |

Note: Prepare freshly.

### GF buffer for nsp12

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Tris-HCl (pH 8.0) (1 M)  | 20 mM               | 20 mL    |
| NaCl                     | 400 mM              | 23.38 g  |
| MgCl₂ (1 M)              | 4 mM                | 4 mL     |
| Glycerol                 | 10% (v/v)           | 100 mL   |
| H₂O                      | n/a                 | To 1 L   |
| **Total**                | n/a                 | 1 L      |

Note: Prepare freshly.

### GF buffer for nsp8/7

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Tris-HCl (pH 8.0) (1 M)  | 20 mM               | 20 mL    |
| NaCl                     | 200 mM              | 11.69 g  |
| MgCl₂ (1 M)              | 4 mM                | 4 mL     |
| H₂O                      | n/a                 | To 1 L   |
| **Total**                | n/a                 | 1 L      |

Note: Prepare freshly.

### 2 × stop solution

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Formamide                | 95% (v/v)           | 9.5 mL   |
| EDTA (pH 8.0) (0.5 M)    | 20 mM               | 400 μL   |
| Bromophenol blue         | 0.02% (w/v)         | 0.002 g  |
| DEPC-treated H₂O        | n/a                 | To 10 mL |
| **Total**                | n/a                 | 10 mL    |

Note: Store at –20°C within 6 months.
### 5 × TBE buffer

| Reagent                        | Final concentration | Amount  |
|-------------------------------|---------------------|---------|
| Tris                          | 450 mM              | 54.5 g  |
| Boric acid                    | 450 mM              | 27.8 g  |
| EDTA (pH 8.0) (0.5 M)         | 10 mM               | 20 mL   |
| H₂O                           | n/a                 | To 1 L  |
| Total                         | n/a                 | 1 L     |

Note: Store at 25°C within 6 months.

### 20% (w/v) polyacrylamide/7 M urea gel

| Reagent                        | Final concentration | Amount  |
|-------------------------------|---------------------|---------|
| Urea                          | 7 M                 | 2.1 g   |
| 5 × TBE buffer                | 1 ×                 | 1 mL    |
| 19:1 Acrylamide/bisacrylamide| 20% (w/v)           | 2.5 mL  |
| 10% (w/v) APS                 | 0.04% (w/v)         | 20 μL   |
| TEMED                         | 0.05% (v/v)         | 2.5 μL  |
| Total                         | n/a                 | 5 mL    |

Note: Prepare freshly.

### Stains-All solution

| Reagent                        | Final concentration | Amount  |
|-------------------------------|---------------------|---------|
| 0.1% (w/v) Stains-All in formamide| 5% (v/v)         | 10 mL   |
| Formamide                     | 5% (v/v)            | 10 mL   |
| Isopropanol                   | 25% (v/v)           | 50 mL   |
| Tris-HCl (pH 8.5) (1 M)       | 15 mM               | 3 mL    |
| H₂O                           | n/a                 | To 200 mL|
| Total                         | n/a                 | 200 mL  |

Note: Prepare freshly.

### Solution Contents Table

| Solution                  | Contents                                   | Final volume | Storage                   |
|---------------------------|--------------------------------------------|--------------|----------------------------|
| 1 × TBE buffer            | Dilute from 5 × TBE buffer to 1 × by H₂O  | 1 L          | 25°C, within 6 months     |
| 0.5 × TBE buffer          | Dilute from 5 × TBE buffer to 0.5 × by H₂O| 1 L          | 25°C, within 6 months     |
| 10% (w/v) APS             | 1 g APS, solvent: H₂O                      | 10 mL        | −20°C, within 6 months    |
| 0.1% (w/v) Stains-All in formamide | 0.1 g Stains-All, solvent: formamide     | 100 mL       | 4°C, within 6 months      |
| 100 mg/mL ampicillin      | 1 g ampicillin, solvent: H₂O              | 10 mL        | −20°C, within 6 months    |
| 100 mg/mL kanamycin       | 1 g kanamycin, solvent: H₂O               | 10 mL        | −20°C, within 6 months    |
| 1 M HEPES (pH 7.0)        | 2.6 g HEPES, solvent: DEPC-treated H₂O, adjusted to pH 7.0 by NaOH | 10 mL | −20°C, within 6 months |
| 1 M Tris-HCl (pH 7.5)     | 121.1 g Tris, solvent: H₂O, adjusted to pH 7.5 by HCl | 1 L | 4°C, within 6 months |
| 1 M Tris-HCl (pH 8.0)     | 121.1 g Tris, solvent: H₂O, adjusted to pH 8.0 by HCl | 1 L | 4°C, within 6 months |
| 1 M Tris-HCl (pH 8.5)     | 121.1 g Tris, solvent: H₂O, adjusted to pH 8.5 by HCl | 1 L | 4°C, within 6 months |
| 0.5 M EDTA (pH 8.0)       | 146.1 g EDTA, solvent: H₂O, adjusted to pH 8.0 by NaOH | 1 L | 4°C, within 6 months |
| 5 M NaCl                  | 2.9 g NaCl, solvent: DEPC-treated H₂O     | 10 mL        | −20°C, within 6 months    |
| 1 M NaCl                  | Dilute from 5 M NaCl to 1 M by DEPC-treated H₂O | 1 mL | −20°C, within 6 months |
| 100 mM NaCl               | Dilute from 5 M NaCl to 100 mM by DEPC-treated H₂O | 1 mL | −20°C, within 6 months |
| 1 M MgCl₂                 | 2 g MgCl₂, 6H₂O, solvent: DEPC-treated H₂O| 10 mL | −20°C, within 6 months |

(Continued on next page)
STEP-BY-STEP METHOD DETAILS
SARS-CoV-2 in vitro primer-dependent polymerase assays

© Timing: 90 min

In this section, we set up a primer-dependent RdRP assay for assessment of NTP forms of NAs.

1. An RdRP assay to characterize the NTP form of sofosbuvir (SOF-TP) intervention.
   a. Thaw and/or keep all the reagents on ice.
   b. Prepare a reaction pre-mix containing HEPES (pH 7.0), MgCl₂, DTT, and NaCl in a single tube. Mix thoroughly on ice.
   c. Prepare an enzyme mix of nsp12, nsp7, and nsp8 with a molar ratio of 1:1:2 in a single tube. Mix thoroughly on ice.
   d. Prepare an NTP mix (CTP, UTP/SOF-TP, and ATP, 2 mM each) by adding an equal volume of each NTP/NA solution (6 mM stock) in a single tube. Mix thoroughly on ice.
   e. Add 2 μL T33-8/P10 RNA construct to the 14 μL reaction pre-mix. Mix thoroughly on ice.
   f. Add 1 μL enzyme mix to “e”. Mix thoroughly on ice.
   g. Add 3 μL NTP mix to “f” to make a total volume of 20 μL for 1 reaction. Mix thoroughly on ice.
   h. The component concentrations of stock solution and final reaction mixture, volumes of each stock solution used to make the final reaction mixture are listed as follows with 1 and 3 reaction time point(s) setups as examples.

### Reaction pre-mix*

| Reagent                                  | Stock concentration | Final concentration | Volume (μL) required for 1 time point | Volume (μL) required for 3 time points |
|------------------------------------------|---------------------|---------------------|--------------------------------------|---------------------------------------|
| HEPES (pH 7.0)                           | 1 M                 | 71.4 mM             | 1                                    | 3                                     |
| NaCl                                     | 100 mM              | 43 mM               | 6                                    | 18                                    |
| MgCl₂                                    | 100 mM              | 6.4 mM              | 0.9                                  | 2.7                                   |
| DTT                                      | 100 mM              | 5.7 mM              | 0.8                                  | 2.4                                   |
| DEPC-treated H₂O                         | n/a                 | n/a                 | 5.3                                  | 15.9                                  |
| Final volume                             | n/a                 | n/a                 | 14                                   | 42                                    |

*Always freshly prepared.
**Note:** It is recommended to prepare the reaction pre-mix for 1–2 additional reactions.

### Enzyme mix*

| Reagent           | Stock concentration | Final concentration | Volume (µL) required for 1 time point | Volume (µL) required for 3 time points |
|-------------------|---------------------|---------------------|--------------------------------------|--------------------------------------|
| nsp12             | 240 µM              | 120 µM              | 0.5                                  | 1.5                                  |
| nsp8              | 1000 µM             | 240 µM              | 0.24                                 | 0.72                                 |
| nsp7              | 1000 µM             | 120 µM              | 0.12                                 | 0.36                                 |
| GF buffer for nsp8/7 | n/a                | n/a                 | 0.14                                 | 0.42                                 |
| Final volume      | n/a                 | n/a                 | 1                                    | 3                                    |

*Always freshly prepared.

**Note:** It is recommended to prepare 10 µL of enzyme mix due to pipetting error of small volumes.

### NTP mix*

| Reagent | Stock concentration | Final concentration | Volume (µL) required for 1 time point | Volume (µL) required for 3 time points |
|---------|---------------------|---------------------|--------------------------------------|--------------------------------------|
| CTP     | 6 mM                | 2 mM                | 1                                    | 3                                    |
| UTP/ SOF-TP | 6 mM              | 2 mM                | 1                                    | 3                                    |
| ATP     | 6 mM                | 2 mM                | 1                                    | 3                                    |
| Final volume | n/a                | n/a                 | 3                                    | 9                                    |

*Always freshly prepared.

**Note:** It is recommended to prepare the NTP mix for 1–2 additional reactions.

### Reaction solution

| Reagent            | Stock concentration | Final concentration | Volume (µL) required for 1 time point | Volume (µL) required for 3 time point |
|--------------------|---------------------|---------------------|--------------------------------------|--------------------------------------|
| Reaction pre-mix   | HEPES (pH 7.0)      | 71.4 mM             | 50 mM                                | 14                                   | 42                                   |
|                    | NaCl                | 43 mM               | 50 mM                                |                                       |                                       |
|                    | MgCl₂               | 6.4 mM              | 5 mM                                 |                                       |                                       |
|                    | DTT                 | 5.7 mM              | 4 mM                                 |                                       |                                       |
|                    | DEPC-treated H₂O    | n/a                 | n/a                                  |                                       |                                       |
| NTP mix            |                     |                     |                                       |                                       |                                       |
| T33-8/P10 b        | 40 µM               | 4 µM                | 2                                    | 6                                    |
| Enzyme mix b       | 120 µM c            | 6 µM                |                                       |                                       |
| Final volume       | n/a                 | n/a                 | 20                                   | 60                                   |

*T33-8/P10 was stored in RNA annealing buffer (RAB: 50 mM NaCl, 5 mM Tris-HCl (pH 7.5), 5 mM MgCl₂).

*Enzyme mix contained 300 mM NaCl and 4 mM MgCl₂, respectively.

*120 µM was the concentration of nsp12.

**Note:** A minimal setup is for three time points and the total volume of reaction mixture include counts the volume of an additional time point.

i. Incubate the reaction at 25°C for different time (5, 40, or 90 min in the case of SOF-TP assessment). For each reaction time point, a 20-µL aliquot is withdrawn from the reaction mixture and immediately quenched with equal volume of 2x stop solution. Mix thoroughly on ice.
j. Store the quenched samples at 4°C for 60 min or at –20°C overnight prior to denaturing PAGE analysis.

2. An RdRP assay to characterize the NTP form of remdesivir (RDV-TP) intervention.
   a. Thaw and/or keep all the reagents on ice.
   b. Prepare a reaction pre-mix containing HEPES (pH 7.0), MgCl₂, DTT, and NaCl in a single tube same as the step 1 “b”. Mix thoroughly on ice.
   c. Prepare an enzyme mix of nsp12, nsp7, and nsp8 with a molar ratio of 1:1:2 in a single tube same as the step 1 “c”. Mix thoroughly on ice.
   d. Prepare an NTP mix (CTP, ATP or RDV-TP, and GTP, 2 mM each) by adding an equal volume of each NTP/NA solution (6 mM stock) in a single tube. Mix thoroughly on ice.
   e. Add 2 µL T33-1/P10 RNA construct to the 14 µL reaction pre-mix. Mix thoroughly on ice.
   f. Add the enzyme mix 1 µL to “e”. Mix thoroughly on ice.
   g. Add the NTP mix 3 µL to “f” to make a total volume of 20 µL for 1 reaction. Mix thoroughly on ice.
   h. The component concentrations of stock solution and final reaction mixture, volumes of each stock solution used to make the final reaction mixture are listed as follows with 1 and 3 reaction time point(s) setups as examples. The reaction pre-mix and enzyme mix is same as in step 1 “h”.

---

**NTP mix**

| Reagent     | Stock concentration | Final concentration | Volume (µL) required for 1 time point | Volume (µL) required for 3 time points |
|-------------|---------------------|---------------------|--------------------------------------|---------------------------------------|
| CTP         | 6 mM                | 2 mM                | 1                                   | 3                                     |
| ATP/ RDV-TP | 6 mM                | 2 mM                | 1                                   | 3                                     |
| GTP         | 6 mM                | 2 mM                | 1                                   | 3                                     |
| Final volume| –                   | –                   | 3                                   | 9                                     |

*Always freshly prepared.

**Note:** It is recommended to prepare the reaction pre-mix for 1–2 additional reactions.

---

| Reagent        | Stock concentration | Final concentration | Volume (µL) required for 1 time point |
|----------------|---------------------|---------------------|--------------------------------------|
| Reaction pre-mix | –                   | –                   | 14                                   |
| NTP mix        | 2 mM                | 300 µM              | 3                                    |
| T33-1/P10      | 40 µM               | 4 µM                | 2                                    |
| Enzyme mix     | 120 µM              | 6 µM                | 1                                    |
| Final volume   | –                   | –                   | 20                                   |

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i. Incubate the reaction at 25°C for different time (20, 60, or 90 min in the case of RDV-TP assessment). For each reaction time point, a 20-µl aliquot is withdrawn from the reaction mixture and immediately quenched with equal volume of 2 × stop solution. Mix thoroughly on ice.

j. Store the quenched samples at 4°C for 60 min or at –20°C for overnight prior to denaturing PAGE analysis.

⚠️ CRITICAL: Take every possible consideration to avoiding RNase contamination (wear gloves, use RNase-free tips and tubes, use DEPC-treated H₂O when necessary.).

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**Preparation of 20% (w/v) polyacrylamide/7 M urea gel**

⏰ Timing: 1 h
In this section, we set up a method for preparation of 20% (w/v) polyacrylamide/7 M urea denaturing gel.

3. Weigh 2.1 g urea and transfer it into a 15 mL centrifuge tube, and then add 1 mL 5 × TBE buffer and 2.5 mL 19:1 acrylamide/bisacrylamide 40% (w/v) solution to the tube to make a 5-mL polyacrylamide solution.
4. Mix the polyacrylamide solution thoroughly by a multi-purpose rotary shaker until urea is dissolved completely.
5. Add 20 μL 10% (w/v) APS and 2.5 μL TEMED to step “4”. Mix thoroughly.
6. Pour the mixture immediately into the glass sandwich plates.
7. Insert the comb and let the gel polymerize for at least 30 min.

Note: It is recommended to use 0.75-mm thick gel. Pre-cast gel can also be used.

Denaturing PAGE analysis

@ Timing: 4 h

In this section, we set up a method for resolving RNA species by denaturing PAGE.

8. Incubate the quenched samples at 95°C for 45 s.

Note: If the quenched samples were stored at −20°C, thaw the samples on ice prior to 95°C incubation.

9. Cool the samples on ice.

Note: A DNA completely complementary to the RNA template T33-1 at a molar ratio of 3:1 was added to the 20 μl quenched sample to help resolve the RNA product well. The mixture was heated at 95°C for 45 s and slowly cooled to r.t. (25°C for about 30 min) to facilitate the annealing of T33-1 template RNA and the complementary DNA.

10. Resolve the RNA products through 20% (w/v) polyacrylamide/7 M urea gel electrophoresis under constant voltage (200 V) at 25°C with 0.5 × TBE buffer in the upper chamber and 1 × TBE buffer in the lower chamber for about 120 min until bromophenol blue just migrates out of the vertical gel.

△ CRITICAL: It is strongly recommend to prerun the gel for about 30 min until the electric current was steady, and then rinse the wells with 0.5 × TBE thoroughly just before sample loading.

11. Rinse the gel twice with H2O.
12. Stain the gel with Stains-All solution (Sigma-Aldrich) for 45 min using a horizontal rotator.
13. Rinse the gel twice with H2O. Destain the gel in H2O for 15 min using a horizontal rotator.
14. Scan the gel using a scanner (Epson Perfection V850 Pro) under the following settings for preview. And use the tone correction icon to adjust tone levels individually when necessary.
   a. Mode: Professional Mode.
   b. Document type: Film (with Film Area Guide).
   c. Film type: Positive Film.
   d. Image type: 48-bit Color.
   e. Resolution: 300 dpi.
   f. Target Size: Original.
g. Adjustments: Descreening, Backlight Correction, Dust Removal.

15. Analyze the gel images of expective RNA products qualitatively (see Figures 2 and 3). We can also analyze the gel images quantitatively using ImageJ Fiji distribution after converting them to gray-scale images.

EXPECTED OUTCOMES

The expected outcomes are shown in Figures 1, 2, and 3. We compared the properties of SOF-TP with UTP in a primer-dependent RdRP assay using SARS-CoV-2 nsp12-nsp7-nsp8 and a T33-8/P10 RNA construct comprising a 33-mer template (T33) and a 10-mer primer (P10) (Figure 2). In the presence of C, U, and ATP (C/U/A), the P10 can be readily converted to a 17-mer product (P17) within 90 min (Figure 2C, lanes 1–3). When UTP was replaced by SOF-TP (C/S/A), P10 was mainly converted to a 12-mer product (P12) after the incorporation of SOF (Figure 2C, lanes 5–6), suggesting that SOF may act as a chain terminator in SARS-CoV-2 RdRP replication. Incorporation of RDV led to appearance of the “i+3” 15-mer product (P15) on a T33-1/P10 RNA construct (Figure 3C, lanes 4–6). However, “i+3” product was not pronounced in the ATP comparison set (Figure 3C, lanes 1–3), suggesting that RDV may lead to delayed intervention in SARS-CoV-2 RdRP replication.

Figure 2. SOF-TP exhibits chain-terminating feature in a SARS-CoV-2 in vitro RdRP assay
(A) Structural formula of the NTP form of sofosbuvir (SOF or S). Parts that differ from regular nucleotide are shown in red.
(B) RNA construct used in the primer-dependent RdRP assay and the expected product species obtained through different NTP combinations.
(C) Denaturing PAGE analysis of the RNA species in quenched reaction mixtures.

Figure 1. Purified SARS-CoV-2 nsp12, nsp7, and nsp8 proteins
A 10% (w/v) SDS (sodium dodecyl sulfate)-PAGE analysis of SARS-CoV-2 nsp12, nsp7, and nsp8 proteins. M: Molecular weight markers.
LIMITATIONS
When the product RNA is extended to 16 nucleotide (nt) or longer, it could not be resolved perfectly through the 7M Urea denaturing PAGE. Adding a DNA completely complementary to the RNA template to help resolve the product RNA is necessary.

TROUBLESHOOTING
Problem 1
Low expression level of nsp12 (before you begin, step 4).

Potential solution
Use highly active E. coli BL21 (DE3) (nsp12) competent cells (before you begin, steps 1–4). Freshly prepare the ampicillin solution.

Problem 2
RNA product species are not detected (step-by-step method details, step 15).

Potential solution
Ensure that the RNA product species do not migrate out of the gel by continuously monitoring the migration of bromophenol blue (step-by-step method details, step 10).
Potential solution
Use fresh Stains-All solution (step-by-step method details, step 12).

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Peng Gong (gongpeng@wh.iov.cn).

Materials availability
All materials in this study can be obtained from sources given in the key resources table.

Data and code availability
The published article includes all datasets generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS
Q.L. and J.W. performed the experiments. Q.L., J.W., and P.G. analyzed the data. Q.L., J.W., and P.G. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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