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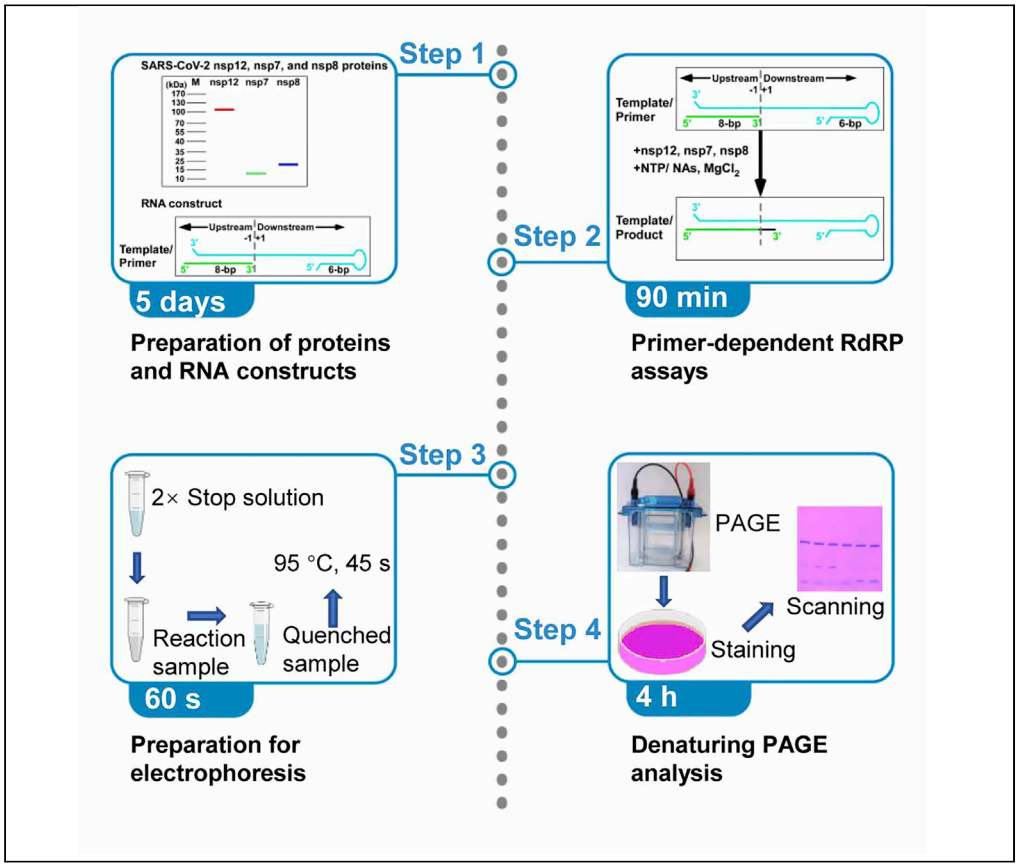
Protocol

Assessment of nucleotide/nucleoside analog intervention in primer-dependent viral RNA-dependent RNA polymerases



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.

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

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Highlights

Enzymology protocols for nucleotide analog assessment in primer-dependent viral RdRPs

Detailed steps to prepare SARS-CoV-2 RdRP proteins and RNA constructs

A primer-dependent RdRP assay to assess NTP forms of nucleotide analogs

Assessment of two representative NA drugs, sofosbuvir and remdesivir

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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_{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_{600} around 0.02.
4. Grow cells at 37°C at 220 rpm for about 3 h to an OD_{600} of 0.8 and cool to 16°C, and supplement with 0.5 mM isopropyl-ß-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 lysate at 34,310 g for 60 min in an F21-8×50y 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			
Reagent	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

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CTP	Sigma	Cat# C1506
UTP	Sigma	Cat# U6750
DEPC-treated H_2O	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# A600286
Isopropyl-ß-D-thiogalactopyranoside (IPTG)	BioFroxx	Cat# 1122GR100
N, N, N′, N′ – Tetramethylethylenediamine (TEMED)	Sinopharm Chemical Reagent	Cat# 80125336
19:1 acrylamide/ bisacrylamide 40% (w/v) Solution	Sigma	Cat# A9926
IGEPAL CA-630	Sigma	Cat# 13021
Polyethlenimine (PEI)	Sigma	Cat# 408727
Formamide	Vetec	Cat# V900064
Stains-All	Sigma	E9379
Bromophenol blue	Biosharp	Cat# BS064
Tris(2-chloroethyl) phosphate (TCEP)	Sigma	Cat# C4706
Dithiothreitol (DTT)	Sinopharm Chemical Reagent	Cat# 3483-12-3
Magnesium chloride hexahydrate	Sigma	Cat# 7791-18-6
Remdesivir NTP form (RDV-TP)	SeNtInall BioTechnologies	N/A
Sofosbuvir NTP form (SOF-TP)	SeNTInall Bio Technologies	N/A
Oligonucleotides		
T33-1: 5′-GGGAGAUGAAAGUCUCCAC CUGUGUCGUCGAAA-3′	This paper	N/A
T33-8: 5′-GGGAGAUGAAAGUCUCCA UUAGAGUCGUCGAAA-3′	This paper	N/A
DNA complementary to T33-1: 5′-TT TCGACGACACAGGTGGA GACTTTCATCTCCC-3′	Sangon Biotech	N/A
P10: UGUUCGACGA	Integrated DNA Technologies (IDT)	N/A
Recombinant DNA		
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		
ImagJ Fiji distribution	(Schindelin et al., 2012)	https://imagej.net/Fiji

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
HisTrap HP column	GE Healthcare	17524802
HiTrap Q column	GE Healthcare	17115401
Superdex200 gel filtration column	GE Healthcare	28989335
Amicon 100 kDa MWCO centrifugal concentrators	MilliporeSigma	Cat# UFC910096
Amicon 30 kDa MWCO centrifugal concentrators	MilliporeSigma	Cat# UFC903096
Amicon 10 kDa MWCO centrifugal concentrators	MilliporeSigma	Cat# UFC901096
Homogenizer	ATS Engineering Ltd.	AH-2010
Rotor	Thermo Scientific	F10S×1000
Rotor	Thermo Scientific	F21-8×50y
PAGE apparatus	Tanon	VE-180B
Multi-purpose rotary shaker	Haimen Kylin-Bell Lab Instruments Co., Ltd.	QB-206
Horizontal rotator	Zhicheng	ZWY-304
Scanner	Epson	Perfection V850 Pro

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
H_2O (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
$MgCl_2$ (1 M)	5 mM	25 μL
DEPC-treated H_2O	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
H_2O	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
H_2O	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
H_2O	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
$MgCl_2$ (1 M)	4 mM	4 mL
Glycerol	10% (v/v)	100 mL
H_2O	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
$MgCl_2$ (1 M)	4 mM	4 mL
H_2O	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 H_2O	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
H_2O	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 40% (w/v) Solution	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
H_2O	n/a	To 200 mL
Total	**n/a**	**200 mL**

Note: Prepare freshly.

Solution	Contents	Final volume	Storage
1 × TBE buffer	Dilute from 5 × TBE buffer to 1 × by H_2O	1 L	25°C, within 6 months
0.5 × TBE buffer	Dilute from 5 × TBE buffer to 0.5 × by H_2O	1 L	25°C, within 6 months
10% (w/v) APS	1 g APS, solvent: H_2O	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: H_2O	10 mL	−20°C, within 6 months
100 mg/mL kanamycin	1 g kanamycin, solvent: H_2O	10 mL	−20°C, within 6 months
1 M HEPES (pH 7.0)	2.6 g HEPES, solvent: DEPC-treated H_2O, 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: H_2O, 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: H_2O, 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: H_2O, 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: H_2O, adjusted to pH 8.0 by NaOH	1 L	4°C, within 6 months
5 M NaCl	2.9 g NaCl, solvent: DEPC-treated H_2O	10 mL	−20°C, within 6 months
1 M NaCl	Dilute from 5 M NaCl to 1 M by DEPC-treated H_2O	1 mL	−20°C, within 6 months
100 mM NaCl	Dilute from 5 M NaCl to 100 mM by DEPC-treated H_2O	1 mL	−20°C, within 6 months
1 M $MgCl_2$	2 g $MgCl_2 \bullet 6H_2O$, solvent: DEPC-treated H_2O	10 mL	−20°C, within 6 months

(*Continued on next page*)

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Solution	Contents	Final volume	Storage
100 mM $MgCl_2$	Dilute from 1 M $MgCl_2$ to 100 mM by DEPC-treated H_2O	1 mL	−20°C, within 6 months
1 M DTT	1.54 g DTT, solvent: DEPC-treated H_2O	10 mL	−20°C, within 6 months
100 mM DTT	Dilute from 1 M DTT to 100 mM by DEPC-treated H_2O	1 mL	−20°C, within 6 months
100 mM NTPs	1.015 g, 0.95 g, 0.952 g, 1.006 g of GTP, ATP, CTP and UTP, respectively, solvent: DEPC-treated H_2O	15 mL	−80°C, within 6 months
6 mM NTPs	Dilute from 100 mM NTPs to 6 mM by DEPC-treated H_2O	1 mL	−80°C, within 6 months
100 mM NTP form of NA drugs	0.03 g, 0.032 g of SOF-TP and RDV-TP, solvent: DMSO	0.6 mL	−80°C, within 6 months
6 mM NTP form of NA drugs	Dilute from 100 mM NTP form of NA drugs to 6 mM by DEPC-treated H_2O	0.1 mL	−80°C, within 6 months
100 mM DNA complementary to T33-1	Add 0.325 mL RAB to 10 OD of the DNA	0.325 mL	−20°C, within 6 months

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), $MgCl_2$, 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
$MgCl_2$	100 mM	6.4 mM	0.9	2.7
DTT	100 mM	5.7 mM	0.8	2.4
DEPC-treated H_2O	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		
	$MgCl_2$	6.4 mM	5 mM		
	DTT	5.7 mM	4 mM		
	DEPC-treated H_2O	n/a	n/a		
NTP mix		2 mM	300 μM	3	9
T33-8/P10 [a]		40 μM	4 μM	2	6
Enzyme mix [b]		120 μM [c]	6 μM	1	3
Final volume		n/a	n/a	**20**	**60**

[a]T33-8/P10 was stored in RNA annealing buffer (RAB: 50 mM NaCl, 5 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$).
[b]Enzyme mix contained 300 mM NaCl and 4 mM $MgCl_2$, respectively.
[c]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 2 × 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), $MgCl_2$, 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**

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 H_2O when necessary.).

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 H_2O.
12. Stain the gel with Stains-All solution (Sigma-Aldrich) for 45 min using a horizontal rotator.
13. Rinse the gel twice with H_2O. Destain the gel in H_2O 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.

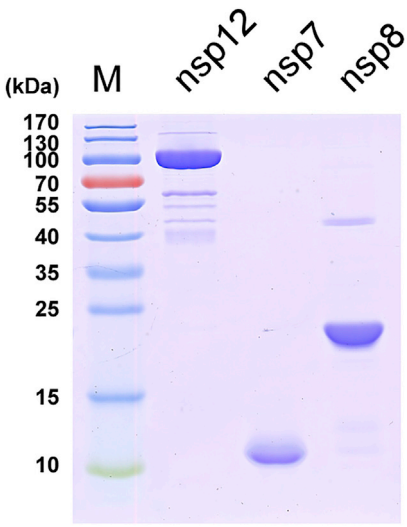

Figure 1. Purified SARS-CoV-2 nsp12, nsp7, and nsp8 proteins
A 10% (w/v) SDS (sodium dodecyl sulfate)-PAGE analysis of SARS-CoV-2 nsp12, nsp7, and nsp8 proteins. M: Molecular weight markers.

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.

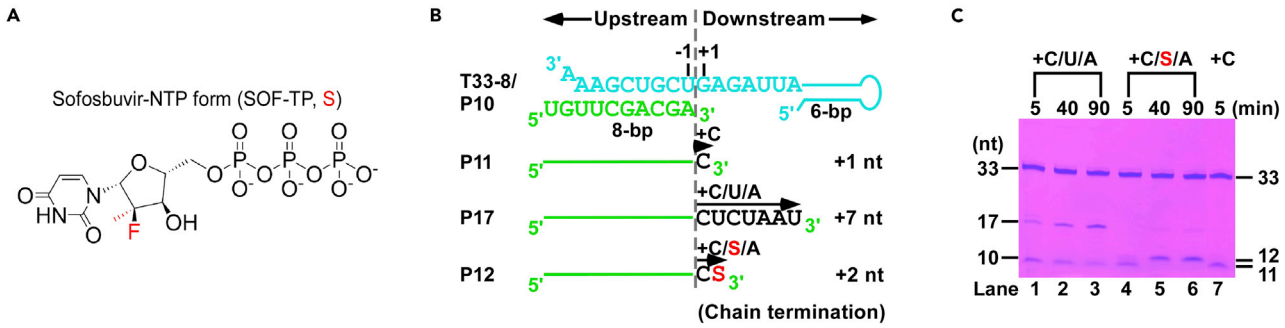

Figure 2. SOF-TP exhibits chain-terminating feature in a SARS-CoV-2 *in vitro* RdRP assay
(A) Structural formula of the NTP form of sofosbuvir (SOF or S). Parts that differ from regular nucleotide are shown in red.
(B) RNA construct used in the primer-dependent RdRP assay and the expected product species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

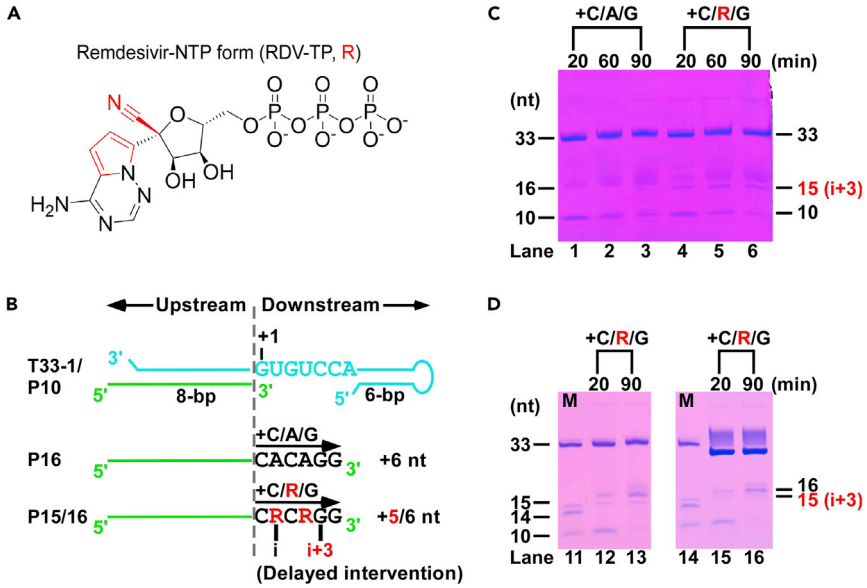

Figure 3. RDV-TP exhibits delayed intervention feature in SARS-CoV-2 *in vitro* RdRP assay

(A) Structural formula of the NTP form of remdesivir (RDV or R). Parts that differ from regular nucleotide are shown in red.

(B) RNA construct used in primer-dependent RdRP assays and the expected product species. obtained through different NTP combinations.

(C) Denaturing PAGE analysis of the RNA species in quenched reaction mixtures.

(D) A comparison of RNA product species resolved in the absence or presence of a DNA complementary to the template RNA. Left: The complementary DNA was not provided. Right: The complementary DNA was added to help resolve relatively long RNA product species. For ''i'' and ''i+x'', ''i'' represents the product length (in nt) corresponding to the first RDV incorporation event, and ''x'' denotes the number of nucleotide additions following the first RDV incorporation event.

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).

Problem 3

The background of the stained gel is dirty (see an example in Figure 4, step-by-step method details, step 15).

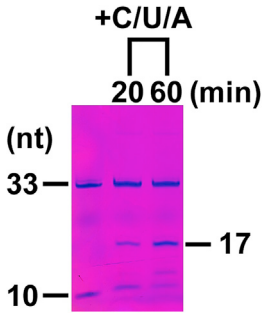

Figure 4. An example of stained gel with dirty background

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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