Supporting Information

RdRp activity test using CRISPR/Cas13a enzyme (RACE) for screening of SARS-CoV-2 inhibitors

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METHODS

Expression and purification of nsp7, nsp8, nsp12, and LwaCas13a proteins. Full length SARS-CoV-2 nsp7 and nsp8 proteins were obtained by expression and purification according to protocols. Briefly, the nsp7 and nsp8 genes were inserted into a pET28a vector for expression as 6×histidine-tagged C-terminal fusion proteins (Addgene, Watertown, MA, USA) in BL21 (DE3) cells (Invitrogen, Waltham, MA, USA). Protein expression was induced by adding 1 mM isopropyl thiogalactoside (IPTG, Sigma-Aldrich, St. Louis, MO, USA) and incubating the culture at 20 °C for 16 h. Bacterial cells were harvested by centrifugation at 11,290 × g for 10 min, and the cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 5% glycerol, and 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich), pH 8.0). Cells were lysed by sonication (probe frequency of 20 kHz, Sonifier 450, Branson, Danbury, CT, USA), and the lysate was clarified by centrifugation at 11,290 × g for 15 min. The target proteins were then purified using Ni-NTA agarose (Qiagen, Hilden, Germany), and eluted using lysis buffer supplemented with 300 mM imidazole (Sigma-Aldrich).

To express the nsp12 protein, the gene was inserted into a pAcGP67A plasmid with a C-terminal 6×histidine tag (Addgene), and the proteins were produced in a eukaryotic expression system using *Spodoptera frugiperda* insect cells (Thermo Fisher Scientific, Waltham, MA, USA) at 28 °C for 48 h after infection. The culture medium was collected by centrifugation at 1,960 × g for 20 min at 4 °C. The expressed protein was subsequently purified to a high degree of purity (90%) using immobilized metal affinity chromatography (Sigma-Aldrich).

To assemble a stable nsp12-nsp7-nsp8 complex, purified nsp12 protein at a concentration of 5 μM was incubated with nsp7 and nsp8 proteins at concentrations of 10 μM each. Incubation was performed at 4 °C for 4 h, maintaining a molar ratio of 1:2:2, in a buffer containing 20 mM Tris-HCl, 250 mM NaCl, and 4 mM $MgCl_2$ (pH 7.5). Unbound proteins

were removed from the complexes by centrifugal ultrafiltration (Amicon Ultra Filters, Millipore Sigma, Burlington, MA, USA). The final complex was concentrated and stored at -80 °C for further use.

LwaCas13a was obtained by purification according to previously established protocols [1]. Briefly, BL21 (DE3) cells transformed with the Lwcas13a-Spytag expression vector (#90097, Addgene) were cultured in Terrific Broth medium (Sigma-Aldrich) at 37 °C until an optical density of 0.6 was reached. Protein expression was then induced by adding 0.5 mM IPTG and incubating the culture at 18 °C for 16 h. The resulting cell pellet was collected and resuspended in lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 5% glycerol, and 1 mM PMSF, pH 8.0). The cells were lysed by sonication (probe frequency of 20 kHz), and the lysate was clarified by centrifugation at 4 °C and 11,290 × g for 15 min. The supernatant obtained from bacterial cell lysis was purified in sequential steps using a HiTrap SP HP cation exchange column (GE Healthcare Life Sciences, Boston, MA, USA) and a gel filtration column (SuperdexTM 200 Incris 10/300 GL, GE Healthcare Life Sciences). The purified proteins were then dialyzed against storage buffer (600 mM NaCl, 50 mM Tris-HCl pH 7.5, 5% glycerol, and 5 mM β-mercaptoethanol (Sigma-Aldrich)) and stored at -80 °C for long-term storage.

SDS-PAGE. Recombinant proteins were prepared by mixing with 4× LDS sample buffer (Thermo Fisher Scientific) containing 10× dithiothreitol (DTT, Thermo Fisher Scientific). Protein samples were loaded onto 15% Bolt Bis-Tris Plus gels (Thermo Fisher Scientific) and separated using MOPS running buffer (Thermo Fisher Scientific) at a constant voltage of 120 V for 1 h according to the manufacturer's instructions. After electrophoresis, the gels were stained with Sun Gel staining solution (LPS solution, Daejeon, Korea) for 2 h. This staining step allowed visualization and detection of the separated proteins.

Western blot. Proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific) using a standard western blotting protocol. The PVDF membrane was then blocked with a solution of phosphate-buffered saline and Tween 20 (PBST, Sigma-Aldrich) containing 5% skim milk (Difco, Franklin Lakes, NJ, USA) at 25 °C for 1 h. After blocking, the membrane was washed five times with PBST to remove unbound substances. The primary antibodies specific for the proteins of interest (anti-nsp12, anti-nsp8, anti-nsp7, R&D Systems, Minneapolis, MN, USA) were then added to the membrane and incubated at 25 °C for 1 h. After another round of washing with PBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) for 1 h. The HRP-conjugated secondary antibodies bind to the primary antibodies and allow the detection of the target proteins [2]. To visualize the proteins, the membranes were treated with a chemiluminescent substrate (Thermo Fisher Scientific), which reacts with the HRP enzyme to produce a luminescent signal. The emitted light was captured using a Geldoc Go imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), allowing visualization and analysis of the proteins of interest.

Preparation of RNA. CRISPR RNA (crRNA) and ssRNA were synthesized using synthetic DNA templates (Bioneer, Daejeon, Korea) containing the T7 promoter sequence (Table S1). RNA transcription was performed using the MEGAshortscript T7 transcription kit (Thermo Fisher Scientific) at 37 °C for 4 h. To remove the DNA template, DNase (Invitrogen) was added and the mixture was incubated at 37 °C for 20 min. RNA transcripts were purified using the QIAamp Viral RNA Mini Kit (Qiagen). The concentration of RNA was determined by measuring the absorbance at 260 nm (ND-1000 spectrophotometer, NanoDrop, Wilmington, DE, USA), and the RNA was stored as concentrated stocks at -80 °C.

Gel electrophoresis for RdRp activity test. To analyze RdRp activity, we followed a previously described protocol [3]. Briefly, the experiment involved the annealing of RNA template and RNA primer (Bioneer) by heating them at 65 °C for 2 min followed by cooling to 25 °C. The primer-annealed RNA template contains a 40 mer RNA sequence representing the 3' end of the viral genome and a 20 mer primer labeled with FAM at its 5' end. The assay setup consisted of 400 nM primer-annealed RNA template, 200 nM purified RdRp complex, 400 μM rNTP (Thermo Fisher Scientific), and reaction buffer (20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 50 mM KCl, and 5 mM $MgCl_2$, pH 7.4). The reaction mixture was prepared in a 50 μL solution and incubated at 37 °C for 1 h. After incubation, the reaction products were resolved on a Novex 15% TBE-urea gel (Thermo Fisher Scientific) and images were captured on an Amersham Typhoon Biomolecular Imager (Cytiva, Waltham, MA, USA).

Fluorescence measurement for Cas13a activity test. To validate the activity of Cas13a, we used a target ssRNA with a sequence complementary to the RNA template. The Cas13a reaction was set up with the following components of 400 nM target ssRNA, 200 nM Cas13a, 600 nM crRNA, 300 nM FAM-U5-BHQ1 probe (Bioneer), reaction buffer (20 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$, and 1% glycerol, pH 7.4), and RNase inhibitor (#M0314, New England Biolabs, Ipswich, MA, USA). These components were mixed and transferred to a 96-well plate, which was then placed in a plate reader (Infinite 200 PRO, Tecan, Zurich, Switzerland) and incubated at 37 °C for 20 min.

RACE reaction. For the RACE reaction, the reaction mixture was prepared in the following order: diethyl pyrocarbonate (DEPC) water, RACE buffer (20 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$, and 1% glycerol, pH 7.4), RNase inhibitor, 400 nM primer-annealed RNA template,

200 nM Cas13a, 600 nM crRNA, 200 nM RdRp complex, and 300 nM FAM-U5-BHQ1 probe. The reaction was initiated by adding 100 μM rNTPs to the master mix, followed by incubation at 37 °C for 30 min. Fluorescence measurements were performed at a wavelength of 490/525 nm (excitation/emission) using the Infinite 200 PRO plate reader (Tecan) pre-heated to 37 °C with the FAM fluorophore. Fluorescence imaging was performed using the Amersham Typhoon Biomolecular Imager (Cytiva).

For the RACE reaction involving the nsp13, the reaction mixture was prepared by sequentially adding the following components: DEPC water, reaction buffer (20 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 1% glycerol, pH 7.4), RNase inhibitor, 400 nM primer-annealed RNA template, 200 nM Cas13a, 600 nM crRNA, 200 nM RdRp complex, 300 nM FAM-U5-BHQ1 probe, and 3 nM nsp13 (R&D Systems). The reaction was initiated by adding 100 μM rNTPs to the master mix, followed by incubation at 37 °C for 30 min. Fluorescence measurements were performed with the plate reader set to an excitation/emission wavelength of 490/525 nm. The plate reader was pre-heated to 37 °C and equipped with the FAM fluorophore. Fluorescence imaging was performed with the Amersham Typhoon Biomolecular Imager (Cytiva).

RACE inhibition assay. For the inhibition assays, we prepared RACE reaction mixtures without Cas13a, crRNA, FAM-U5-BHQ1 probe, rNTP, and RNase inhibitor. To assess the inhibitory effect, we added one of the inhibitory compounds at final concentrations of 1, 3, 6, 9, 12, 15, 18, 21, 50, or 100 μM. The mixture with the small molecule inhibitor was pre-incubated at 37 °C for 30 min, and the inhibitory effect was observed at 37 °C for another 30 min after the addition of 100 μM rNTP, 200 nM Cas13a, 600 nM crRNA, 300 nM FAM-U5-BHQ1 probe, and RNase inhibitor.

RACE-LFA. To develop the RACE-based lateral flow assay (RACE-LFA) test strip, we initiated the process by attaching a nitrocellulose membrane (MDI, Ambala Cantt, India) to an adhesive backing card. We then applied NeutrAvidin (2 mg/mL, Thermo Fisher Scientific) and goat anti-mouse IgG (1 mg/mL, Sigma-Aldrich) to create the test (T) and control (C) lines, respectively. Next, a conjugate pad containing colloidal Au-labeled mouse anti-FAM antibody (Bore Da Biotech, Siheung, Korea) was attached by overlapping the nitrocellulose membrane by 2 mm. The sample pad (Bore Da Biotech) was also adhered to the conjugate pad with an overlap of 4 mm. An absorbent pad (Bora Da Biotech) was placed on the opposite side of the backing card. Before assembling the components, the conjugate pad was treated with a combination of 1% polyvinylpyrrolidone (PVP, Sigmal-Aldrich) and 0.5% Surfactant 10G (Fitzgerald Industries, MA, USA), while the sample pad was treated with 1% bovine serum albumin (Sigma-Aldrich) and 0.2% Tween20, and then dried at 37 °C.

To evaluate the efficacy of the RACE-LFA strips in detecting RdRp inhibitors, 100 μL of diluted RACE reaction sample (40 μL sample mixed with 60 μL running buffer (0.1M Tis-HCl, 0.2 M NaCl, 0.2% Tween20, 0.02% NaN_3 (Sigma-Aldrich), pH 8.5)) was carefully added dropwise to the sample pad. The sample then migrated through the strip by capillary action towards the absorbent pad. Within 15 min, a visible test signal appeared, indicating the presence of an RdRp inhibitor.

Statistical analysis. The fluorescence signals obtained from each experiment were determined using the following equations:

$$\Delta FL = F - F_0 - N \qquad (1)$$

$$\text{Inhibition rate (\%)} = \{1 - (F - F_0 - N) / (P - P_0 - N)\} \times 100 \qquad (2)$$

$$\text{RdRp activity (\%)} = \{(F - F_0 - N) / (P - P_0 - N)\} \times 100 \qquad (3)$$

Where ΔFL is the subtracted fluorescence value, F is the fluorescence value of the RACE

reaction for each experiment, F_0 is F at start time, N is the fluorescence value of the RACE reaction without RdRp, P is the fluorescence value of the RACE reaction without inhibitor, P_0 is P at start time.

All values were determined by nonlinear regression analysis performed using GraphPad Prism software (version 5.0; GraphPad Software, San Diego, CA, USA).

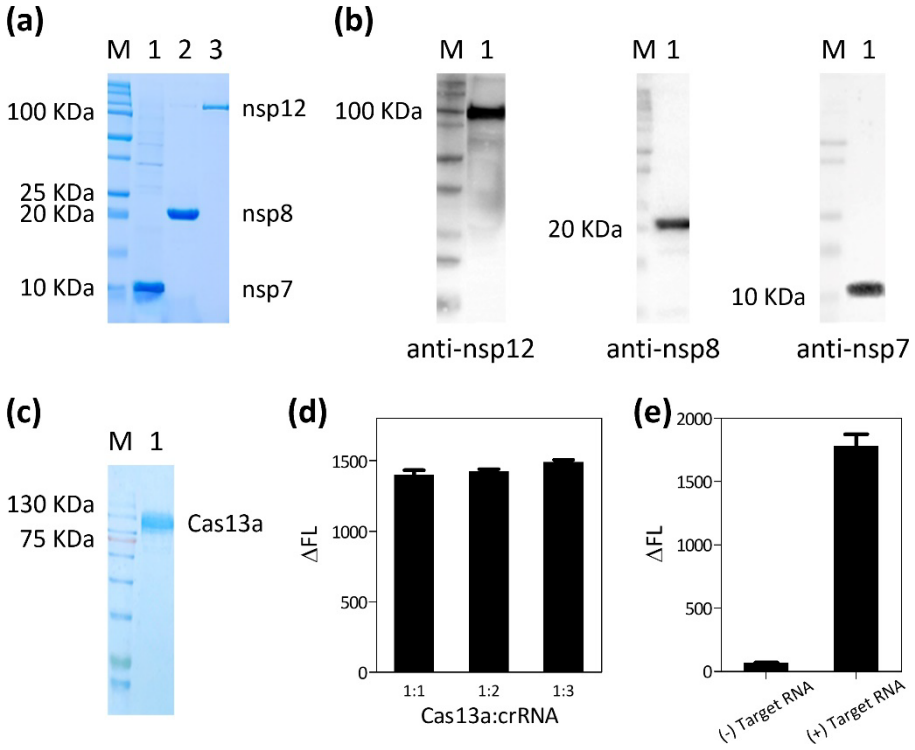
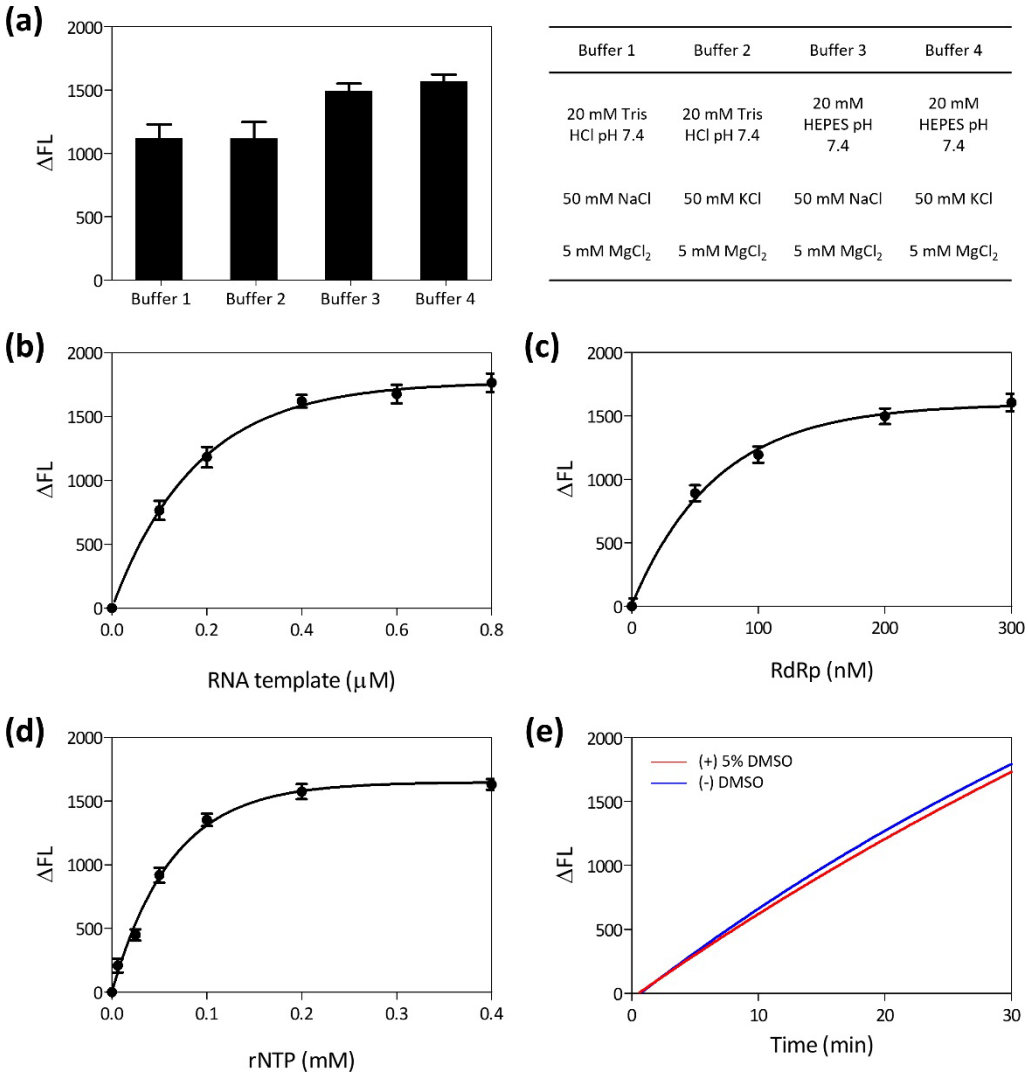


Figure S1. (a) SDS-PAGE result of nsp12, nsp7, and nsp8 proteins. (b) Western blot result of nsp12, nsp7, and nsp8 proteins with their respective antibodies. The successful recognition of the proteins by their specific antibodies indicates that the proteins are correctly folded. (c) SDS-PAGE results of Cas13a protein. (d) Plot of ΔFL after Cas13a/crRNA reaction with various ratio (1:1, 1:2, 1:3) for 30 min (n = 3, error bar = standard deviation). (e) Plot of ΔFL after Cas13a/crRNA reaction for 30 min (n = 3, error bar = standard deviation).



Buffer 1	Buffer 2	Buffer 3	Buffer 4
20 mM Tris HCl pH 7.4	20 mM Tris HCl pH 7.4	20 mM HEPES pH 7.4	20 mM HEPES pH 7.4
50 mM NaCl	50 mM KCl	50 mM NaCl	50 mM KCl
5 mM $MgCl_2$	5 mM $MgCl_2$	5 mM $MgCl_2$	5 mM $MgCl_2$

Figure S2. (a) Optimization of RACE buffer (n = 3, error bar = standard deviation). The optimal RACE buffer compositions were determined to be 20 mM HEPES (pH 7.4), 50 mM KCl, and 5 mM $MgCl_2$. (b-d) Optimization of RACE reaction components (n = 3, error bar = standard deviation). The most effective concentrations for the RACE system were 0.4 μM RNA template, 0.2 μM RdRp complex, and 0.1 μM rNTP. (e) Real-time monitoring of RdRp activity using the RACE system in the presence or absence of DMSO.

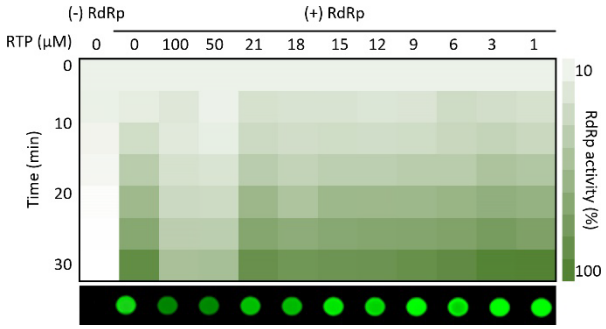


Figure S3. Fluorescence heat map of the RACE system during 30 min with RTP. The inhibitor concentration ranged from 1 μM to 100 μM. RTP blocked 50% of the RdRp activity at 100 μM. The bottom panel shows fluorescence images of RACE reaction wells after 30 min.

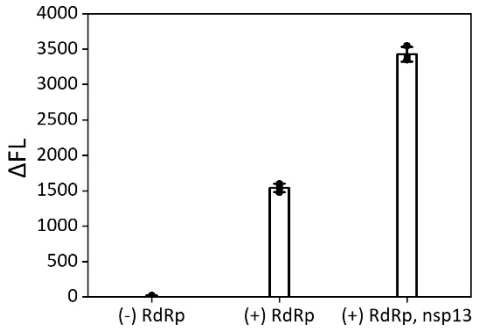


Figure S4. Plot of ΔFL after RACE reaction for 30 min under different component conditions (n = 3, error bar = standard deviation). The presence of nsp13 leads to an approximately 2-fold increase in fluorescence compared to its absence, indicating the production of both dsRNA and ssRNA during the RdRp reaction.

Table S1. Oligonucleotides used in this study.

Name	Sequence (5' → 3')
RNA template	AAAGACGUUCUUGAGUGUAAUGUGAAAACUACCGAAGUUGU AGGAGACAUUAUACUUAAACCAGCAAAUAAUAGUUUAAAAA UUACAGAAGAGGUUGGCCACACAGAUCUAAUGGCUGCUUAU GUAGACAAUUCUAGUCUUACUAUUAAGAAACCUAAUGAAUU AUCUAGAGUAUUAGGUUUGAAAAC
RNA primer	UUUAUUUAUUUAUUAUUGAGUGUAAUGUGAAAA
crRNA	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACCCAACC UCUUCUGUAAUUUUUAAACUAU

Table S2. Comparison of RACE system with previous RdRp activity assays.

Method	Analysis	Time	Scale	Template (cost)	Ref
Primer extension assay	Urea-PAGE using primer with fluorescent label	2~3 h (End point)	No description for scale up	Labeled primer (~$160)	[4]
Fluorescence based assay	Fluorescence (Ribogreen)	1 h (End point)	Well-plate	Label free RNA template and primer (~$430)	[5]
Strand displacement assay	Fluorescence (Primer with fluorescence label)	40 min (Real-time)	Well-plate	Labeled RNA template and primer (~$300)	[6]
RACE system	Fluorescence (Labeled RNA reporter probe)	5 min (Real-time)	Well-plate	Label free RNA template and primer, Labeled RNA reporter probe (~$230)	This study

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