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Endogenous G-quadruplex-forming RNAs inhibit activity of SARS-CoV-2 RNA polymerase

Tamaki Endoh,[a] Shuntaro Takahashi[a] and Naoki Sugimoto *[b]

[a]Frontier Institute for Biomolecular Engineering Research (FIBER), Konan University, 7-1-20 Minatojima-minamimachi, Kobe, 650-0047, Japan
[b]Graduate School of Frontiers of Innovative Research in Science and Technology (FIRST), Konan University, 7-1-20 Minatojima-minamimachi, Kobe, 650-0047, Japan

*To whom correspondence should be addressed. Tel: +81-78-303-1416; Fax: +81-78-303-1495; Email: sugimoto@konan-u.ac.jp

Materials

DNA and RNA oligonucleotides used in this study were purchased from Fasmac Co., Ltd. or Japan Bio Services Co., Ltd. Chemical reagents not specifically mentioned are those appropriate for biochemical experiments.

Preparation of recombinant proteins

The plasmid vector pRSFDuet-1(nsp8-nsp7)(nsp12) for the expression of RdRp of SARS-CoV-2, which consists of a complex of Nsp7, Nsp8, and Nsp12, was a gift from Marc Delarue (Addgene plasmid #165451; http://n2t.net/addgene:165451; RRID: Addgene_165451).[1] In the construct, the hexa-histidine tag was fused to the N-terminus of the Nsp8 subunit. *E.coli* BL21(DE3) was transformed with the expression vector. The cells were cultured in LB medium to an A_{600} of around 0.5 and chilled on ice for 15 min. The cell culture was restarted by transfer to 18 °C, followed by addition of isopropyl β-D-1-thiogalactopyranoside. After overnight culture by shaking, the cells were harvested and lysed by sonication. The soluble fraction was loaded sequentially on His-trap HP, Hitrap Q HP, and Hiload Superdex 200 (GE Healthcare). Finally, the solution containing RdRp was passed through a MonoQ column (GE Healthcare) to eliminate residual RNase. The purified RdRp was concentrated using an Amicon Ultra centrifugal filter units (Millipore) and dialyzed against the storage buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 0,1 mM EDTA, 1 mM DTT, and 50%(v/v) glycerol). The purified RdRp was stored at -30°C until use.

Construction of fragmented cDNA library

Human lung carcinoma A549 cells were cultured on collagen-coated dishes (AGC Techno Glass) in Dulbecco's Modified Eagle Medium with 10% foetal bovine serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C under 5% CO_2. Total RNA was purified by using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Concentration of the purified RNAs was

determined from absorbance at 260 nm measured using a Nano Drop 1000 (Thermo Fisher Scientific). A fragmented cDNA library consisting of sequences derived from the total RNA was prepared from 10 ng total RNA using SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Takara Bio) according to the manufacturer's protocol. The concentration of the cDNA library was determined from absorbance at 260 nm measured using the Nano Drop 1000. Adapter sequences, A-T7 and trP1, for preparation of R-CAMPs were incorporated by PCR amplification using sense (A-T7-read2) and antisense (trP1-read1) primers (Table S4). Approximately 1.8 ng template cDNA library was amplified in the presence of 30 pmol primers by 7-cycle PCR using KOD-Plus-Ver.2 (Toyobo) in a 100-μL reaction volume. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) followed by size selection using an E-Gel SizeSelect II Agarose Gel (Thermo Fisher Scientific). Fragments of approximately 300 to 350 base pairs were extracted. Purified DNA fragments were quantified using the Mx3000P qPCR system (Agilent) using A-primer and B-trP1 primer (Table S4) by comparison to known concentrations of a standard DNA fragment.

Preparation of RNA-capturing microsphere particles (R-CAMPs)

Preparation of R-CAMPs from the cDNA library was performed according to the previously established protocol with some modifications.[2] Briefly, the fragmented cDNA library was mixed with the microsphere particles from the Ion PGM Hi-Q View OT2 Kit (Thermo Fisher Scientific). Single copies of DNA derived from the library were amplified and immobilized on the particles through emulsion PCR using the Ion OneTouch 2 system (Thermo Fisher Scientific) according to the manufacturer's protocol. The particles, which immobilize single-stranded clones derived from the fragmented cDNA library, were purified by the Ion OneTouch 2 system. The double-stranded template DNAs for RNA transcription were synthesized on the particles by primer extension using a capture primer shown in Table S4. The reaction product of the primer extension was diluted 15 fold into a transcription buffer containing 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, 1 mM rNTPs, 0.01% Tween-20, and 1 U/μL T7 RNA polymerase (Takara Bio). During the transcription at 25 °C for 20 min, R-CAMPs were constructed by capturing nascent RNA transcripts on the single-stranded tail remaining on the capture primer.[3] The buffer solution, in which R-CAMPs were dispersed, was replaced with 50 mM MES-LiOH (pH 7.0), 100 mM KCl, 0.5 mM $MgCl_2$, and 0.01% Tween-20 after termination of transcription by addition 800 nM competitive T7 promoter duplex consisting of 5′-GAAATTAATACGACTCACTATA-3′ and 5′-TATAGTGAGTCGTATTAATTTC-3′..

Selection of R-CAMPs

R-CAMPs were mixed with 100 nM NTA-ATTO647N (Sigma) in the presence or absence of 10 nM RdRp in a buffer containing 50 mM MES-LiOH (pH 7.0), 100 mM KCl, 0.5 mM $MgCl_2$, 100 ng/μL yeast tRNA (Thermo Fisher Scientific), 0.01% Tween 20 and kept at room temperature for at least 30 min. Fluorescence signals of R-CAMPs at 660 nm after excitation with a 633-nm laser were analysed using a FACS Aria II (BD Biosciences). Single particles were distinguished by the fluorescence signal of the

particle itself at 585 nm upon 488-nm excitation. R-CAMPs in a sorting gate with strong fluorescence of NTA-ATTO647N were sorted one-by-one into the wells of a 96-well plate (Ina-optika) containing 10 μL of H_2O.

Sequence analysis of sorted RNAs

DNAs immobilized on R-CAMPs were directly amplified by addition of 10 μL of reaction solution of KOD-Plus-Ver.2 containing A-primer and B-trP1 primers. After 20-cycle PCR reactions, aliquots of the samples were diluted 10 fold with H_2O to make DNA stocks. DNAs in the remaining reaction solutions were further amplified up to 35 cycles, and crude PCR products were sequenced by a contract sequencing service (Fasmac) with an A-primer.

Preparation of sorted RNAs

DNA templates for RNA transcription were prepared from 1 μL DNA stocks by 15-cycle PCR amplification using KOD-Plus-Ver.2 with A-primer and B-trP1 primers in 10 μL reaction volumes. The PCR products were directly diluted 3 fold into the transcription buffer containing 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, 1 mM rNTPs, 0.01% Tween-20, and 1 U/μL T7 RNA polymerase. After a 2-h transcription reaction at 37 ˚C, samples were mixed with 2 U of TURBO DNase (Thermo Fisher Scientific), and further incubated at 37 ˚C for 30 min. Transcribed RNAs were purified using AMPure XP (Beckman Coulter). Concentrations of the purified RNAs were measured using the Nano Drop 1000. To prepare highly purified RNAs, RNAs were transcribed using ScriptMAX Thermo T7 Transcription Kit (Toyobo) and purified using a denaturing polyacrylamide gel. The RNA concentrations were determined by measuring the absorbance at 260 nm at 90 °C using a UV/Vis spectrophotometer connected to a thermoprogrammer (UV-1700; Shimadzu). To transcribe G10 RNA variants, DNA templates were prepared by primer extension or PCR amplification using oligonucleotides shown in Table S4.

Primer extension assay

Substrate RNA for the reaction was prepared by annealing FAM-labelled 20-nt primer RNA (2 μM) and 40-nt template RNA (2 μM) (Table S4) by cooling from 95 to 25 ˚C at 1 ˚C min^{-1} in a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT. RdRp was pre-incubated at 37 ˚C with sorted RNAs or G10 RNA variants in a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 312.5 μM rNTPs and subsequently mixed with the annealed substrate RNA. Final concentrations of RdRp, rNTPs, and substrate RNA were 2 μM, 250 μM, and 200 nM, respectively. Sorted RNAs crudely purified using magnetic beads were added at 250 ng/μL, and RNAs highly purified by denaturing polyacrylamide gel electrophoresis were added at 3 μM. Samples were incubated at 37 ˚C for 60 min. Reactions were stopped by addition 4 volumes of a stop solution containing 10 mM EDTA, 80 vol% formamide, and 0.2 wt% blue dextran (SIGMA). Aliquots of the

samples were then separated on a 20% denaturing polyacrylamide gel containing 8 M urea. After gel electrophoresis, FAM-labelled RNAs were imaged using an FLA5100 fluorescence image scanner (Fuji Film) with 532 nm excitation and 575 nm emission. Signal intensities of initial and extended primers were quantified using ImageQuant TL software (GE Healthcare). Percent of extended primer to total primer were calculated using equation 1.

$$Percent\ of\ extended\ primer = \frac{I_{\mathrm{EP}}}{I_{\mathrm{IP}}+I_{\mathrm{EP}}} \times 100 \qquad (1)$$

where I_{IP} is signal intensity of initial primer and I_{EP} is signal intensity of extended primer.

When reaction was performed in the presence of LiCl, RdRp dialysed into a buffer containing LiCl (50 mM Tris-HCl pH 7.5, 50 mM LiCl, 0,1 mM EDTA, 1 mM DTT, and 50%(v/v) glycerol) was used, and reaction buffer contained 100 mM LiCl instead of 100 mM KCl.

Fluorescence assay of NMM

RNAs (100 nM) were mixed with NMM (1 μM) in a buffer containing 50 mM MES-LiOH (pH 7.0), 100 mM KCl, 0.5 mM $MgCl_2$, 0.1% DMSO, and 0.01% Tween20. The RNAs were refolded by cooling from 70 to 25 ˚C at 1 ˚C min^{-1} in the buffer before addition of NMM. Fluorescence signals of NMM were measured using a microwell plate reader (Infinite M200 pro, TECAN) at 400 nm excitation and 615 nm emission wavelengths after incubation at 25 °C for 60 min.

1. C. Madru, A. D. Tekpinar, S. Rosario, D. Czernecki, S. Brûlé, L. Sauguet and M. Delarue, *PLoS One*, 2021, **16**, e0250610.
2. S. Satpathi, T. Endoh, P. Podbevšek, J. Plavec and N. Sugimoto, *Nucleic Acids Res.*, 2021, DOI: 10.1093/nar/gkab189.
3. T. Endoh, T. Ohyama and N. Sugimoto, *Small*, 2019, **15**, 1805062.

Table S1. RNA sequences on selected R-CAMPs

Sample	Sequence[a]
A01	GGGGCGTATCAACCCTGCCCACAGGAAGCTGCAGGCCCGCCTTGACCAGATGAGAAAATTTAGACGCCAGCATGAACAGCTAAGAGCTGTTATCGT CAGGGTCCTGAGGCCACAGGTCACGGCAGTTGCACAACAGAATCAAGGAGAGGTCCCTGAAACTAA
A02	GGGGAAGATTATTTAGTCAAGAAAAAAAGAAAGACAAATCAACAATATGTCAAAAAATTCAGGTCCAATTATAGAGCAAAATAAAATGAGGCATGA TTTTGAGTTATTCATGAAGAATAAGAAGAGGCTTGATAGGTACATTTCCTTTTCTATGGCACAG
A03	GGCACTGTACAAGGGAAGCTTTTCCTCTCTCTAATTAGCTTTCCCAGTATACTTCTTAGAAAGTCCAAGTGTTCAGGACTTTTATACCTGTTATAC TTTGGCTTGGTTTCCATGATTCTTACTTTATTAGCCTAGTTTATCACCAATAATACTTA
A04	GGGTGGAAGAGGATTTGACAGTTATTAATATCAACAGCAGTGATGAAGAGGAGGAGGAAGAGGAAGAAGAGGAAGAAGAAGAGGAAGAAGAGG AAGAGGAGGAAGACTTTGAGGAAGAGGAAGAGGATGAAGAGGAATATTTTGAAGGGGAAG
A05	CGCCCTGAGGGGAGATGCACAAACCCCAGGACCCAAAAAATCAGGGGATTTGTGAGCAAATGATTTAACCCAAGACCCCGCAGGGTAGCACACCCC AAGTGGGTCTTGGTGCTGATTCTTACTGCAGCTCTCCTACTCCACACCCACGATAAGCCAC
A06	N.D.[b]
A07	N.D.[b]
A08	GGGAGGCACCCGGGTCTCGGGGGCAGCAACGCCGGGGCCCTTGCAGAACGGGGACTGTTTGGGGAAGTGGTTCCCGCACAGTGTCCGGAGAGGGGC CCTCCCTCTGTGCCCCCTTGGGAAACACTGCTTGGTACAATTCCAATACCCAGCTGTACCCTGCCCACG
A09	GGCTGTACAAATGACAATAGCTGTTGGAACTCGAATGCCACCGAAGAACTTCAGCCGTGGCTCTCTAGTCTTCGTGTCAATATCCTTTATTGTTTT GATGATTATTTCTTCAGCATGGCTCATATTCTACTTCATTCAGAAGATCAGGTACACAAATGCACGCAACGTC
A10	GGGGCGCATGAGCTGGAGTCCTAGGCACAGCTCTAAGCCTCCTTATTCGAGCCGAGCTGGGCCAGCCAGGCAACCTTCTAGGTAACGACCACATCT ACAACGTTATCGTCACAGCCCATGCATTTGTAATAATCTTCTTCATAGTAATAC
A11	GGGGGAAGAATTTGAGGAAGAAGAAGGTGAGTTAGAGGAAGAGGAAGAAGAGGAAGAAGAAGAAGAGGAAGAAGAGGAAGAGGAGGAAGACTTTGA GGAAGAGGAAGAGGATGAAGAGGATTTGACAGTTATTAATATCAACAGCAGTGATGAAGAGGGGGAGG
A12	GGGTGCAGCAGCAGAAGACGGCTCGAAGCAACATGGACAACATGTTCGAGAGCTACATCAACAACCTTAGGCGGCAGCTGGAGACTCTGGGCCAGG AGAAGCTGAAGCTGGAGGCGGAGCTTGGCAACATGCAGGGGCTGGTGGAGGACTTCAAGAACAAGTATGAGGA
B01	GGGGATGACTGTGGCGACAGCTCGGACGAGCGTGGCTGCCACATCAATGAGTGTCTCAGCCGCAAGCTCAGTGGCTGCAGCCAGGACTGTGAGGAC CTCAAGATCGGCTTCAAGTGCCGCTGTCGCCCTGGCTTCCGGCTGAAGGATGACGGCG
B02	GGGGACGAAGAAGAGGAAGAAGGTGGGGAGGAAGAGGAGGAGGAAGAAGAAGGTGATGGTGAGGAAGAGGATGGAGATGAAGATGAGGAAGCTGAG TCAGCTACGGGCAAGCGGGCAGCTGAAGATGATGAGGATGACGATGTCGATACCAAGAAGCAGA
B03	CGCCCACAGTGCAAGGCTTTGTATGCCTATGACGCTCAGGACACAGACGAACTCAGCTTTAATGCCAATGACATTATTGATATTATCAAAGAAGAT CCTTCTGGCTGGTGGACGGGTCGACTACGAGGCAAGCAGGGCCTGTTCCCCAACAACTATGTTACCA
B04	GGGGGGACCGGGGTCCGGTGCGGAGTGCCCTTCGTCCTGGGAAACGGGGCGCGGCCGGAAAGGCGGCCGCCCCCCTCGCCCGTCACGCACCGCACG TTCGTGGGGAACCTGGCGCTAAACCATTCGTAGACAAACCCTTGTGTCGAGGGCTGACTTTCAAT
B05	CGGCCTGCTGAGCCCCCTTGTCCTGGAGGTGGACCCCAACATCCAGGCCGTGCGCACCCAGGAGAAGGAGCAGATCAAGACCCTCAACAACAAGTT TGCCTCCTTCATAGACAAGGTACGGTTCCTGGAGCAGCAGAACAAGATGCTGGAGACCA
B06	GGGTGGGCTGTAGTGCGCTATGCCGATCGGGTGTCCGCACCAAGTTCGGCATCAATATGGTGACCTCCCGGGAGCGGGGGACCACCAGGTTGCCTA AGGAGGGGTGAACCGGCCCAGGTCGGAAACGGAGCAGGTCAAAACTCCAGTGC
B07	GGGTGAATCTACAGGATCAGGACAGAGACCTCCAACCATAGTCCTTGATCTTCAAGTAAGAAGAGTTCGTCCTGGAGAATATCGGCAGAGAGATAG CATAGCCAGCAGAACTCGGTCTAGGTCTCAGACACCAAACAACACTGTCACCTATGAAAGTGAACGAGA
B08	GGGGGGAAAATGTAATCAAACTACATAACAGTGTCATTCATTTTCATTTTTATTTTTGCTCAATTGCTATTTAAACCAATGGAAAGTTAGTATCAT TGCAACATTTACTGTAATATGAGTGTATTGTATATGAAGGTTTCAGGAGGTGGTCATTATTGTGTAGCC
B09	GGGGGGTAGCTGCCTATTTTGGGATGGACCACAATGTTGATCAAACTGGGAAAGCTGTCATCATCAACAAAACTAGTAACACAAGAATCCCTGAAC AGAGGTTCTCAGAACATATAAAGGATG
B10	GGGAGAGCGATTACATTAAGCTCTATGTGCGCCGTGTATTCATCACAGACGACTTCCATGATATGATGCCTAAATACCTCAATTTTGTCAAGGGTG TGGTGGACTCAGATGATCTCCCCTTGAATGTTTCCCGCGAGACTCTTCAGCAAC
B11	N.D.[b]
B12	GGGCTGGCAGCTTTGGGTAGAAGAGAACTAGATGACAGAGATCACTATGGAAACAAGAGATTGGATCTTGCTGGGCCGCTGCTTGCATTCTTATTT AGAGGTATGTTTAAGAATTTGCTTAAAGAAGTGCGGATCTATGCACAGAAATTTATTGATCGAGGAA
C01	N.D.[b]
C02	CGGATGGAGACTACACCAGCCAAAGGAAAGAAAGCTGCAAAAGTTGTTCCTGTGAAAGCCAAGAACGTGGCTGAGGATGAAGATGAAGAAGAGGAT GATGAGGACGAGGATGACGACGACGACGAAGATGATGAAGATGATGATGATGAAGATGAGCAGG
C03	GGGAGGGGAGGGAAAGGGGGAAAGCGGGCAACCACTTTTCCCTAGCTTTTCCAGAAGCCTGTTAAAAGCAAGGTCTCCCCACAAGCAACTTCTCTG CCACATCGCCACCCCGTGCCTTTTGATCTAGCACAGACCCTTCACCCCTCACCTCTATGCAGCCAGAACG
C04	N.D.[b]
C05	CGGAGTTGAAAGACACACAGAGGGTCACAGTCTGGAATTGACAGTGGTGGGATTCGAACCTATTTCACTCCAGAACTGCGTAGCGCCTTATACGCT GCGGCAAGTGTGACGTCATTCGAACAAACCATAGGCCCCGCCCCCGGACTAGCCACGCCCAC
C06	GGGTCGTGCCAGCAAGCAGTGGTTTATAAACATCACGGATATTAAGACTGCAGCCAAGGAATTGTTAAAAAAGGTGAAATTTATTCCTGGATCAGC ACTGAATGGCATGGTTGAAATGATGGACAGGCGGCCATATTGGTGTATATCAAGGCAAAG
C07	GGGGACATGAACGAGGCCAAGCGGAGGGTGCGCGAGCTCTACCGCGCCTGGTATCGGGAGGTGCCGAACACTGTGCACCAATTCCAGCTGGACATC ACTGTGAAAATGGGACGGGATAAAGTCCGAGAAATGTTTATGAAGAATGCCCATGTCACAGACCCCAGGGTGGTTGATCTTCTGGTCATTAAGGGA AAGATCGAACTGGAAGAAACAATG
C08	GGGCGTGCATACGCACGTAGACATTCCCCGCTTCCCACTCCAAAGTCCGCCAAGAAGCGTATCCCGCTGAGCGGCGTGGCGCGGGGGCGTCATCCG TCAGCTCCCTCTAGTTACGCAGGCAGTGCGTGTCCGCGCACCAACCACAGTGGC

C09	GGGCATCGATGAGGCCATGAGCTATGCCGAGGTCATGAGGCTGGTGAAGGGCATGAACTTCTCGGTGGTGGTATTTGACACGGCACCCACGGGCCA CACCCTGAGGCTGCTCAACTTCCCCACCATCGTGGAGCGGGGCCTGGGCCGGCTTATGCAGATCG
C10	GGGGAGCCAAGATCGTACCACTGCACTCCAGCCTGGGTGACAGAGTGAGACTCTGTCTCCAAAAAAAAACTTTGCTTGTATATTATTTTTGCCTTA CAGTGGATCATTCTAGTAGGAAAGGACAATAAGATTTTTTATCAAAATGTGTCATGCCAGG
C11	GGGTGGGGGTTAATCTGATGCCTACTCCCGCCTATAATGTCAATTCCATGAATATGAACACCTTGAATGCCATGAACAGCTATCGAATGACACAGC CCATGATGAACAGCAGTTACCATAGTAACCCTGCCTACATGAACCAGAAAGCGA
C12	GGGGGATGAGGCAGGAATCAAAGACAGATACTCCGACATAGGGTGCTCCGGCTCCAGCGTCTCGCAATGCTATCGCGTGCACACCCCCCAGACGAA AATACCAAATGCATGGAGAGCTCCCGTGAGTGGTTAATAGGGTGATAGACCTGTGATCCATCGTGATGTCTTATTTAAGGGGAACGTGTGGGCTAT TTAGGCTTTATGACCCTGAAGTAGGAACCAGATGTCGGATACAGTTCACTTTAGCTACCCCCAAGTGTTATGGACCC
D01	N.D.[b]
D02	GCCTCAGTGGACATATACATGAGCAACCCCAGAGAAATTCAGCTTGTCTGTTGTCTATCTCTGCATGGATCAGAGACTTACTTAGGGAAAGAAAAT ATGTGGGGAGATTATGATGAAGACCTCCCAAAATGTCCCGCATGGACATCCTAGCATCATATGGCC
D03	GGGTGCTCGGGGGAATAGGTTATGTGATTAGGAGTAGGGTTAGGATGAGTGGGAAGAAGAAAGAGAGGAAGTAAAGTTTAATTATGCCTTTTTGGG TTGAGGTGATGATGGAGGTGGAGATTTGGTGCTGTGAAATTGTTTTAGGTAATAGCTTTTCTAGTCAGG
D04	N.D.[b]
D05	N.D.[b]
D06	CGGAGCTACTCGGGAGGCTGAGGCTGGAGGATCGCTTGAGTCCAGGAGTTCTGGGCTGTAGTGCGCTATGCCGATCGGGTGTCCGCACTAAGTTCG GCATCAATATGGTGACCTCCCGGGAGCGGGGGACCACCAGGTTGCCTAAGGAGGGGTGAACCGGGCCC
D07	GCGGGAAGAGGACGAGGACGACGAGGAGGAGGAGGAGGAGGAAAAAGAAAAGAGTCTCATCGTGGAAGGCAAGAGGGAAAAGAAAAAAGTAGAGAG GTTGACAATGCAAGTCTCTTCCTTACAGAGAGAGCCATTTACAATTGCACAAGGAAAGGGGC
D08	GGGACTGTGCCTTTAGCCTTTTCATGTTTCTGTCTCAAAACACTTTGACAAAGCTCACCAACTTTAAAATATTAAGTTTTTAAAAAATCAAATATG AAGTATGTTCAGCAAAGGCATAATTTTCTAAAAACACACACAGAAATCGCTTGAGTGAGGAAAAGTCC
D09	N.D.[b]
D10	CGCTGACCCGAGATGACTCAAGTGGTGGATGGTTACCACTTGGAGGGAGTGGACTAAGCAGCGTCACTGTCTTCAAAGTCCCTCATCAGGAAGAGA ATGGCTGTGCTGACTTTTTTATCCGTGGAGAGCGACTCAGGGGCA
D11	CGGCAGAATCCTAGTACCCTAGCCCTCCAAAGATAACTCATACAAAAGAGTAAAGCAGAAAAAGTGGATGTGTTGAGGAAAAAAACCATTGCACTC TAAAGAGCATCTGATAATACAGTTTTGAAAATGAACTTAGCGCTGTGTAAACAGTGTTTAGCCAAT
D12	GGGGCAATTCAGGGAGATAGAATAATTGTTTAACTGAAAGCATGGGTGGGATGTGTTAAAAGCAATAGCTGGGCATGGTAACGTGCACCTGTAGTC TCAGCTACTTGGGAGGTGGAGGCAGGAGGATCCCATGAGCTAGGGAGTTGGAGTCC
E01	GGCCAACCCTCCTGCTGCTGTGAAGCTGGCGCTGGAGTCCATCTGCCTGCTGCTGGGGGAAAGCACCACAGACTGGAAGCAGATCCGCTCCATCAT CATGCGGGAGAACTTCATCCCCACCATCGTCAACTCCTCTGCAGAGGAGATCAGTGACGCCATAAGGA
E02	CGGGCTAAGTGAAGATCCCCCAGCATATGACCATGCTATCAAACTTGTAGGAGAAATCAAAGAGACTCTCTTATCTTTCTTGCTGCCTGGTCATAC TAGACTGAGAAACCAGATAACAGAAGTCTTGGATCTGGATCTGATAAAGCAGGAAGCAGTA
E03	GGGGGATGTGAGGGCGATCTGGCTGCGACATCTGTCACCCCATTGATCGCCAGGGTTGATTCGGCTGATCTGGCTGGCTAGGCGGGTGTCCCCTTC CTCCCTCACCGCTCCATGTGCGTCCCTCCCGAAGCTGCGCGCTCGGTCGAAGAGGACGACCATCCCCGATAGA
E04	GGGGAGTTGTTTTCTGGAGATAAGTTAACATGTGAAAACCATACTGCAAATAATTTTAGCCAGAAAGTTGTAGAATATTTGAAACCAGTGTCTGCT CTTTTGAAATAAAGAATTAGAGGGCGTGGTGGTGGGCACCTGTAGTCACAGCTACGTGGGAGGCTGAGACAGGAGAA
E05	GGCGACATCAGCACATGCCGGGGCAGAGATGGCCAGATCTGCAGCGAGCGTGGGCACTGTCTCTGTGGGCAGTGCCAATGCACGGAGCCGGGGGCC TTTGGGGAGATGTGTGAGAAGTGCCCCACCTGCCCGGATGCATGCGACAACAA
E06	GGGAACGAGACGTGGGGCGTAACAAAGGCAGCAGAGAAACAAATGAAGGACAAACAGGACGAGGAGCAGAGGCTTAAGGAGGAGGAAGAAGACAAG AAACGCAAAGAGGAGGAGGAGGCAGAGGACAAGGAGGATGATGAGGACAAAGATGAGGATGAGGAGGATGAGGAGGACAAGGAGGAAGACG
E07	N.D.[b]
E08	CGGATATCACAATCACCAGAGAACAGTAAGGGAGAAAGAAAGGAAATTGGTAGACTGTCATCGTGAACTGGAAAAACTAAATAAAGAATCTAGGCT TCTCAATCAGGAAAAATCAGAACTGCTTGTTGAACAGGGTCGTCTACAGCTGCAAGCAGATCGCAATC
E09	GCCAGGATTGTGGGGAGGAGGGGCTTTGGGTTCCTGTGTTTGTGCATATGAAGGGAGATGGGGGTTAGGTGGAGGAGGAGAGCAGCGTGGTTAGCT AAGGTTATTGCTTTTTGTGGCAAATCTAAAAAA
E10	GGCCTCGGCTCGTGCGTCGATGAAGAACGCAGCTAGCTGCGAGAATTAATGTGAATTGCAGGACACATTGATCATCGACACTTCGAACGCACTTGC GGCCCCGGGTTCCTCCCGGGGCTACGCCTGTCTGAGCGTCGCTAAGAGTCGAGTGATCCACCTA
E11	GGGCAGTGGTGCAGCCTTATGTGGAATCAAATCTTTTAGTTCCTATGGGTGGTGTAGGAGGACAGGTTCAAGTGTCCCAGCCAGGAGGGAGTTTAG CACAAGCCCCCACTACATCCTCCCAGCAAGCAGTTTTGGAGAGTACACACTT
E12	GGGGAGCGTCTTCGCTGGCAGGCCAGCTCCTTGCCTGCTGATGACCTTTGCACAGAAAATGCCATCATGCTGAAACGATTCAATAGGTATCCGCTG ATCATTGACCCCTCTGGACAGGCCACAGAATTCATTATGAATGAATATAAGGATCGTAAGATCACACGGACCTA
F01	GGGGGTGCTCCCTGGACGCACGGGCGGCGGAGCAACGTTCGCGTCATCACGGACGAGAACTGGAGAGAACTGCTGGAAGGAGACTGGATGATAGAA TTTTATGCCCCGTGGTGCCCTGCTTGTCAAAATCTTCAACCGGAATGGGAAAGTTTTGCTGAATGGGG
F02	CGCCATGCCTGAATTTGAAACCAAGTGCAACAATTCAAAGCCCAAAAAGAGTTACATTGCCACACAAGGCTGCCTGCAAAACACGGTGAATGACTT TTGGCGGATGGTGTTCCAAGAAAACTCCCGAGTGATTGTCATGACAACGAAAGATATAGA
F03	N.D.[b]
F04	GGCCCGCGCTGGAGTCCCAGCTGCAGGACACTCAGGAGCTGCTGCAGGAGGAGAACCGGCAGAAGCTGAGCCTGAGCACCAAGCTCAAGCAGGTGG AGGACGAGAAGAATTCCTTCCGGGAGCAGCTGGAGGAGGAGGAGGAGGCCAAGCACAACCTGGAGA
F05	GGGCGAAGAGGACGACCATCCCCGATAGAGGAGGACCGGTCTTCGGTCAAGGGTATACGAGTAGCTGCGCTCCCCTGCTAGAACCTCCAAACAAGC TCTCAAGGTCCATTTGTAGGAGAACGTAGGGTAGTCAAGCTTCCAAGACATTG
F06	GGGGTATACATTAGGGATGAGAAGGATGAGTATATATGTAGGGATGAAGGAGGATGAGTATATATGTAAGATGAAGGAAGATGAATATATATGTAG GAATGAAGGAGGATGAGTACATATGTAGAAATAAAGAAAGATGAGTGTACATGTAGGTATGTGA

F07	N.D.[b]
F08	N.D.[b]
F09	CGGCACATTGGGTCACGCCATTACTGGGGGCTTCAGTGGAGGAAGTAATGCTGAGCCTGCGAGGCCTGACATCACTTACCAGGAGCCTCAGGGAAC CCAGCCAGCACAGCAGCAGCAGCCTTGCCTCTATGAGATCAAACAGTTTCTGGAGTGTGCCCAGAACCAGGGA
F10	GGGGGGCAGAGAGTGCCACGTGCATACGCACGTAGACATTCCCCGCTTCCCACTCCAAAGTCCGCCAAGAAGCGTATCCCGCTGAGCGGCGTGGCG CGGGGGCGTCATCCGTCAGCTCCCTCTAGTTACGCAGGCAGTGCGTGTCCGCGCACCAACCACACGGGAC
F11	GGGGCTTAGGAAGCTGATTTCTTTACCTCATCGACTTCCACCCATTAGCGGTGCTAAGGAAAACAAATGGGATTGAAAAGGCAAGTTGCCAGATAC ATAAAAATAATCTGGGTGAGGACCAAGAGTGAGACTGTCTGTATTCAGCCTGTATGGAGAGATGTTCTAAAGCTATAAAATCATGGC
F12	GGGTCGCTTGAGCCCAGGAGTTCTGGGCTGTAGTGCGCTATGCCGATCGGGTGTCCGCACTAAGTTCGGCATCAATATGGTGACCTCCCGGGAGCG GGGGACCACCAGGTTGCCTAAGGAGGGGTGAACCGGCCCAGGTCGGAAACGGAGCAGGTCAAAACTCCCGGC
G01	GGGGAAGCTGGACCTGAATGGCAACACCCTGGGAGAAGAAGGCTGTGAACAGCTTCAGGAGGTGCTGGAGGGCTTCAACATGGCCAAGGTGCTGGC GTCCCTCAGTGATGACGAGGACGAGGAGGAGGAGGAGGAAGGAGAAGAGGAAGAAGAGGAAGCGAAGA
G02	GCGGGCCCAGGTCGGAAACGGAGCAGGTCAAAACTCCCGTGCTGATCAGTAGTGGGATCGCGCCTGTGAATAGCCACTGCACTCCAGCCTGGGCAA CATAGCGAGACCCCGTCTCTAGGCAACCTGGTGGTCCCCCGCTCCCGGGAGGTCACCATATTGATGCCGAACTTAGTGCGGACACCCC
G03	GGGCTTGACCGTCTACTGGAAATCTGAATGCTGTTATCACTGCTTGTTTCAGGTTCTGGTAAACGTTCCTCAGAGTCCAAAAGCAGGGAAGCCTAG TGCTGCAGCTGCCTCTGTCAGCACCCAGCACGGATCTATCCTGCAGCTGAACGACACCAAGGA
G04	GGGCTGGTCAAAGTTACCGGGCAGTGCTGCGAGGAGTGGGTCTGTGACGAGGATAGTATCAAGGACCCCATGGAGGACCAGGACGGCCTCCTTGGC AAGGAGCTGGGATTCGATGCCTCCGAGGTGGAGTTGACGAGAAACAATGAATTGATTGCAGTTGGAA
G05	GGGCATTGCCCTCATCACCCTGACACCCCCACATTCTCTCCTCTGGACCCCCAGGAGGCTGGTGCCAGGGAGACAGGCCCAACCCACCCCCATTTG CACTGAGAAGAGAAGTTTTGGAGCGTTGCCTCCTAGAATAAAGATACAGAGAGTCAAATAGGAG
G06	GCCGAGAACTTAAAATCTTAGAATGGAAAAAGTAAAGAAATATCAACTTCCAAGTTGGCAAGTAACTCCCAATGATTTAGTTTTTTTCCCCCCAGT TTGAATTGGGAAGCTGGGGGAAGTTAAATATGAGCCACTGGGTGTACCAGTGCATTAATTTGGGCA
G07	GGGAGCGCGAGGGCTACGAGCGTCTCCTGAAGATGCAAAACCAGCGTGGCGGCCGCGCTCTCTTCCAGGACATCAAGAAGCCAGCTGAAGATGAGT GGGGTAAAACCCCAGACGCCATGAAAGCTGCCATGGCCCTGGAGAAAAAGCTGAACCAGGCCC
G08	GGGAGATACCTTGTCAATAGCAACTTCTAATGAGCCAAGAATTTCCAGTGATACAGCTGATGGAAAATGTGTCCAGGAAGGGAATAAGTCTTCAGT CCAGAAACAATATAGATGTGATGTGTGTGATTATACAAGTACAACATATGTTGGTGTCAGAAACCACAGGCGA
G09	N.D.[b]
G10	CGGAAGGAGGGAGAGGAAAGGAAGGAAGGAGAGGGAAGGAAGGAGGGAGAGGAAAGGAAGGAAGGAGGGAGAGGAAAGGAAGGAAGGAGAGGAAAG GAAGGAAGGAGAGAGAGGAAAGAAAAGCAAA
G11	CGCCAGCCTCGGCCACCAAGGTGTCAGCTTCCTCTGGTGCAACCAGCAAGTCTTCCAGTATGAATCCCACAGAAACCAAGGCTGTAAAAACAGAAC CTGAGAAGAAGTCACAGTCAACCAAGCTGTCTGTGGTTCATGAGAAAAAATCCCA
G12	CGGAGATCCGGGTCGGCGGCTGCACTGCGGATGAGACCGGTGCGACTCATGAAGGTGTTCGTCACCCGCAGGATACCCGCCGAGGGTAGGGTCGCG CTCGCCCGGGCGGCAGACTGTGAGGTGGAGCAGTGGGACTCGGATGAGCCCATCCCTGCCAAGGAGGT
H01	GGCATCTTTAAACTTTTAAATGTGAAATTTCTATCATGTACCGTTAGCCTAACAAGATTTTCTTTCCTATTTCTGACTGGTGCCTTTCCCCTTTTT AGGAGCAACGAAAGCTACTCTCTTAGTTATGTTCTTGTGATGTGACAAAATGTCAAGA
H02	CGGGGAGGCTTGGCGGAGCTAGGGGAAGTAATGCGAGGCTCTGGGCGCCGAGCGAGGGCTCGCGGCGGCGAGGCGAAAGAAGAGAGAAGATAGGAA GAAGGGAAGTAGGGCGAGGTGGAGGAAGAAGAGGAAAGCGAGCGTGGGTGTCTGAGGC
H03	CGGCCAGCAACAAAATTTTAATAGGATATGTTTAAGACTAGTACTTCCAGCTCTATAATGATGTGTATATCAGGAAACAATAAAGCTTGAGCACAG TGGCCATATATATAAACGAAATTAACTCTTAAATCAGTGGTTCTCAACCACTGGCAATTTTAGCCCCC
H04	GGCAGCATAAGAAATTCGCCGAGCAGACGCTGCGGCAGAAGGCGCAGGTGGAGCAGGAGCTGACAACACTGCGGCTGCAGCTGGAGGAGACCGACC ACCAGAAGAACCTGCTGGACGAGGAGCTGCAGCGGCTGAAGGCGGAGGCCACGGAGGCCGCACGCCAGCGCAGCC
H05	GCGGAAGGGCTACGATTTCGACACGGTCAACAAACAGACGTGCCAGACCTACAGCTTCGGCAAGACTAGCTCCTGCCACCTGTCCATCGACGCCTC GCTCACCAAGCTCTTCGAGTGCATGACTTTGGCCTACAGTGGGAAGTTGGTGTCTCCAAAGTGGA
H06	GGGGAAGAGGAAGAAGGTGGGGAGGAAGAGGAGAAGGAAGAAGAAGGTGATGGTGAGGAAGAGGATGGAGATGAAGATGAGGAAGCTGAGTCAGCT ACGGGCAAGCGGGCAGCTGAAGATGATGAGGATGACGATGTCGATACCAAGAAGCAGA
H07	GGGTGCCAACGTTTCCCGGGAGCTCAGTTTGTGGCAGGCTCTGCCAAGCACTTTATGTATCTTGTATTTCCTCCGCCTCCTCCCAGCTCTGTGAGG TAGATACAATCTTTAGTCCCATTTTGTTATACATATCTGTTATTCAGCGACCCG
H08	GGCTTCAGATGGTGGGCCACGCCAACCGCCCTTTGCAGGACGATGAGGGGCGCTGTGTCATCATGTGTCAGGGCTCCAAGAAGGATTTCTTCAAGA AGTTCTTATATGAGCCATTGCCAGTAGAATCTCACCTGGACCACTGTATGCATGACCACTTCAATGCTG
H09	GGGGTCAGTCATATATGTTGCAAAAACCCACTGTGCTTTTTGTTTTGGGGGCTGACGGGAGGGATATGAGGACATTTTTAATGCTTGTATAAACAA ACATACCAATCTTCTTGATGACTTTGGTGTCTACCTGCAAGGGCTTTACTCTAGTTCCTAGTCACATTCCT
H10	GGGGTAGACTACATCAGTGTTTCTGGGAAGGGGAGGAGAGTATGCACTAGAGAAGCAAGTGCAGTTGGGGAGGGATGAAGAGGTGGCAGAAAGAAT ACTTAGGAGAGTTAAAAGAAGCTAGG
H11	GGGATAATAAACAAAGCTGGATTTACTATAACCAAACTCAAAATGATGATGCTTTCAAGGAAAGAAGCATTGGATTTTTATGTAGATCACCAGTCA AGACCCTTTTTCAATGAGCTGATCCAGTTTATTACAACTGGTCCTATTATTGCCATGGA
H12	CGGGAGACTGGAGCCATTACTTCAAGATCATCGAGGACCTGAGGGCTCAGATCTTCGCAAATACTGTGGACAATGCCCGCATCGTTCTGCAGATTG ACAATGCCCGTCTTGCTGCTGATGACTTTAGAGTCAAGTATGAGACAGAGCT

a) Sequences inserted between 5′ and 3′ consensus regions used for preparing fragmented cDNA library are shown.

b) N. D. indicates sequence could not be determined due to noise.

Table S2. Percent of extended primer in the presence of indicated RNAs

			with RNA selected using R-CAMPs							
	Sample	Percent extension	Sample [c]	Percent extension	Sample [c]	Percent extension	Sample [c]	Percent extension	Sample [c]	Percent extension
No RNA	0 min	2.0 ± 0.5 [a]	A1	63.5	C1		E1	60.2	G1	44.4
No RNA	60 min	70.2 ± 9.6 [a]	A2	60.3	C2	66.6	E2	68.7	G2	55.7
With tRNA		68.2 ± 2.9 [b]	A3	53.5	C3	77.5	E3	66.3	G3	39.2
With MS2RNA		76.8 ± 8.4 [b]	A4	22.1	C4	57.4	E4	54.4	G4	44.4
			A5	51.7	C5	55.8	E5	59.6	G5	51.2
			A6		C6	69.9	E6	43.7	G6	57.5
			A7	53.1	C7	68.6	E7	44.2	G7	63.4
			A8	60.4	C8	60.0	E8	62.9	G8	62.4
			A9	55.7	C9	59.2	E9	54.4	G9	
			A10	58.4	C10	57.9	E10	65.9	G10	31.9
			A11	33.5	C11	77.5	E11	67.7	G11	66.8
			A12	37.9	C12	79.6	E12	55.4	G12	48.5
			B1	63.5	D1	59.5	F1	69.3	H1	73.9
			B2	63.9	D2	66.8	F2	65.8	H2	58.8
			B3	71.6	D3	53.1	F3	56.8	H3	60.2
			B4	68.4	D4	58.2	F4	46.1	H4	45.7
			B5	58.5	D5	69.6	F5	60.1	H5	60.6
			B6	59.9	D6	72.3	F6	54.7	H6	59.9
			B7	63.2	D7	65.5	F7	70.2	H7	64.6
			B8	70.3	D8	68.6	F8		H8	67.9
			B9	66.2	D9		F9	46.6	H9	79.0
			B10	82.6	D10	75.9	F10	64.0	H10	66.1
			B11	55.9	D11	67.9	F11	49.4	H11	71.5
			B12	76.4	D12	63.8	F12	43.4	H12	51.4

a) Values are average ± s.d. obtained from 8 replicates.

b) Values are average ± s.d. obtained from 3 replicates.

c) For samples without percent extension given, sufficient RNA was not obtained to enable the assay to be performed. Samples highlighted in red had high levels of inhibitory activity of RdRp.

Table S4. Sequences of oligonucleotides used for experiments

Oligonucleotide primers for construction of fragmented cDNA library		
A-T7-read2		CCATCTCATCCCTGCGTGTCTCCGACTCAGTAATACGACTCACTATAGGGTTCAGA CGTGTGCTCTTCCGAT
trP1-read1		CCTCTCTATGGGCAGTCGGTGATACACTCTTTCCCTACACGACGC
Oligonucleotide primers for amplification of template DNAs		
A-primer		CCATCTCATCCCTGCGTGTCTCCGACTCAG
B-trP1 primer		CTATCCCCTGTGTGCCTTGGCAGTCTCAGCCTCTCTATGGGCAGTCGGTGAT
Oligonucleotide for preparing R-CAMPs		
capture primer [a]		ATCGGAAGAGCACACGTCTGAACCC-link-CAGTAATACGACTCACTATAGGG
Oligonucleotides for preparation of template DNAs of G10 RNA variants		
G10-core (primer extension)	sense	TAATACGACTCACTATAGG
	antisense	TTTGCTTTTCTTTCCTCTCTCTCCTTCCTTCCTTTCCTCTCCTTCCTTCCTTTCCT CTCCCTCCTTCCTTCCTTTCCTCTCCCTCCTTCCTTCCCTCTCCTTCCTTCCTTTC CTCTCCCTCCTTCCTATAGTGAGTCGTATTA
G10-short (primer extension)	sense	TAATACGACTCACTATAGG
	antisense	TTTGCTTTTCTTTCCTCTCTCTCCTTCCTTCCTTTCCTCTCCTTCCTTCCTTTCCT CTCCCTCCTTCCTTCCTTTCCTCTCCCTCCTTCCTTCCCTATAGTGAGTCGTATTA
G10-stem (PCR)	sense	TAATACGACTCACTATAGG
	template [b]	–
	antisense	CCTTTTACACGACGCTCTTCCGATCT
Oligonucleotides for primer extension assay		
FAM-labelled primer [c]		FAM-GUCAUUCUCCUAAGAAGCUA
Template RNA		CUAUCCCCAUGUGAUUUUAAUAGCUUCUUAGGAGAAUGAC

a) "-link-" indicates oligo ethylene glycol linker.
b) Template is a PCR product for transcription of G10 RNA amplified by A-T7 primer and B-trP1 primer.
c) "FAM" indicates carboxyfluorescein.

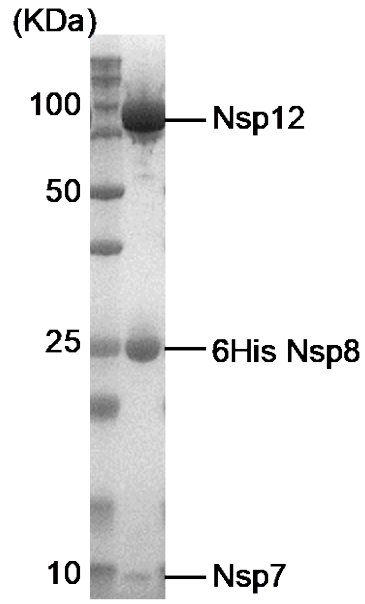

Figure S1. SDS-PAGE image of purified, recombinant, His-tagged SARS-CoV-2 RdRp

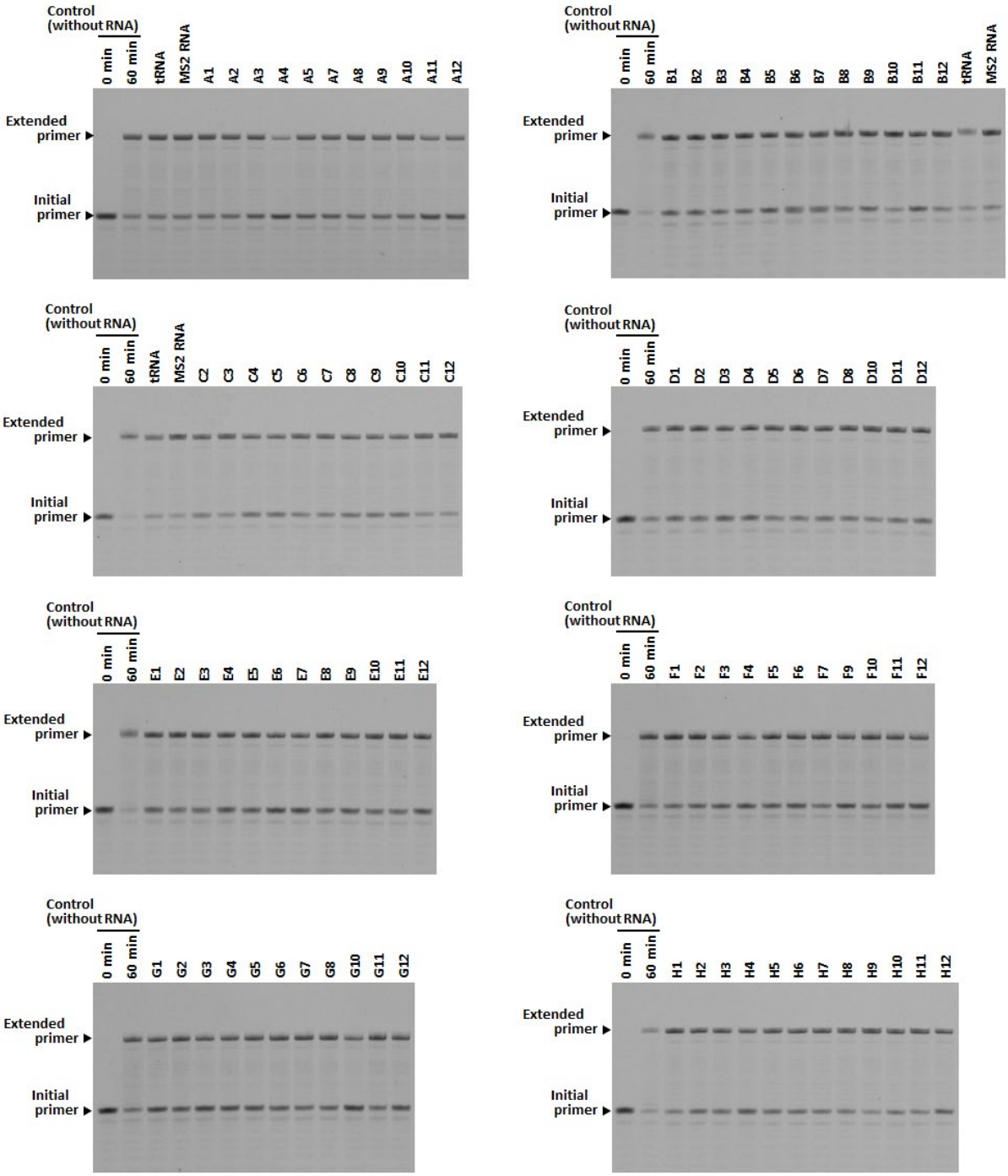

Figure S2. Polymerization products in the presence of indicated RNAs. Polymerization reaction by RdRp was performed in the presence of selected RNAs, tRNA, or MS2 RNA at 250 ng/μL for 60 min. Polymerization products were electrophoresed in denaturing polyacrylamide gels and imaged using a fluorescence image scanner. Control samples are products without additional RNA after 0- and 60-min reaction times.

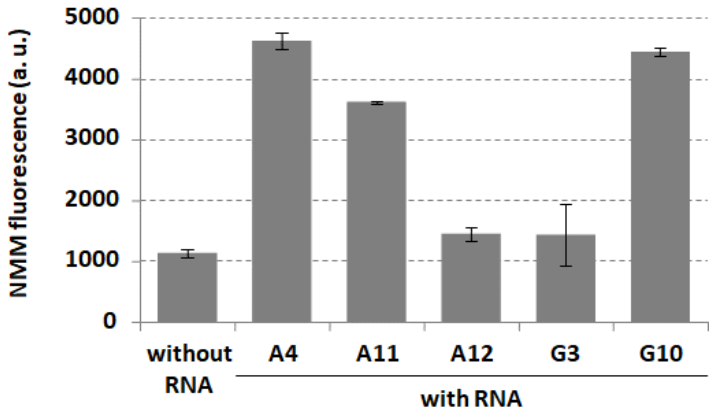

Figure S3. Fluorescence intensity of NMM mixed with selected RNAs. RNAs (100 nM) were mixed with 1 μM NMM in a buffer containing 50 mM MES-LiOH (pH 7.0), 100 mM KCl, 0.5 mM $MgCl_2$, 0.1% DMSO, and 0.01% Tween20. Fluorescence intensity of NMM at 615 nm was measured after 400-nm excitation. Values are average ± s.d. obtained from individually prepared triplicates.

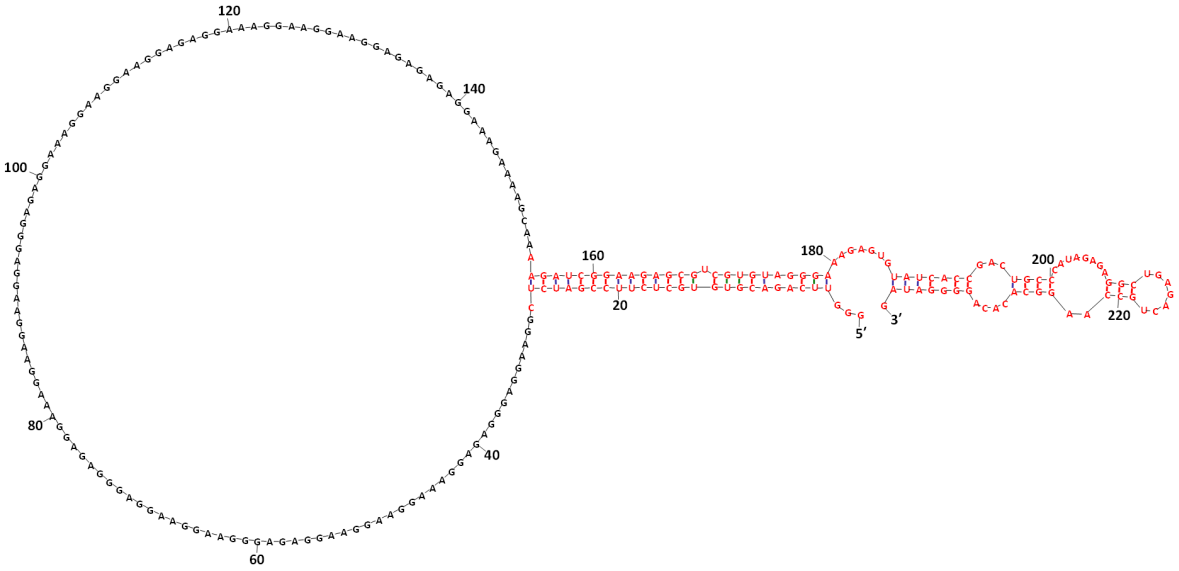

Figure S4. Predicted secondary structure of G10 RNA. Regions highlighted in red are sequences required for preparation of R-CAMPs.

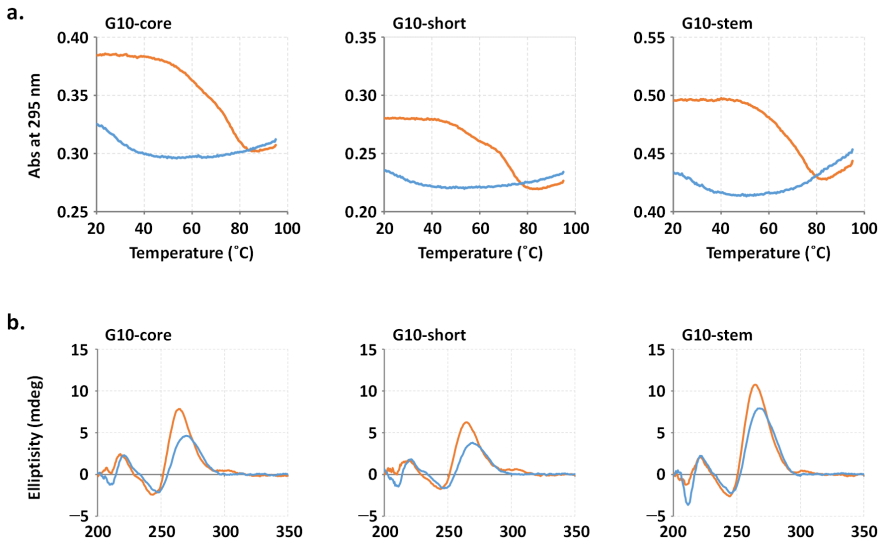

Figure S5. Spectroscopically analyzed G4 formation on RNA. Melting profiles of absorbance at 295 nm (a) and CD spectra at 37 ˚C (b) of 3 µM RNAs were measured in a buffer containing 50 mM MES-LiOH (pH 7.0), and 0.5 mM $MgCl_2$ in the presence of 100 mM KCl (orange) or 100 mM LiCl (blue). Buffer for CD spectra analyses also contains 0.01% Tween 20.

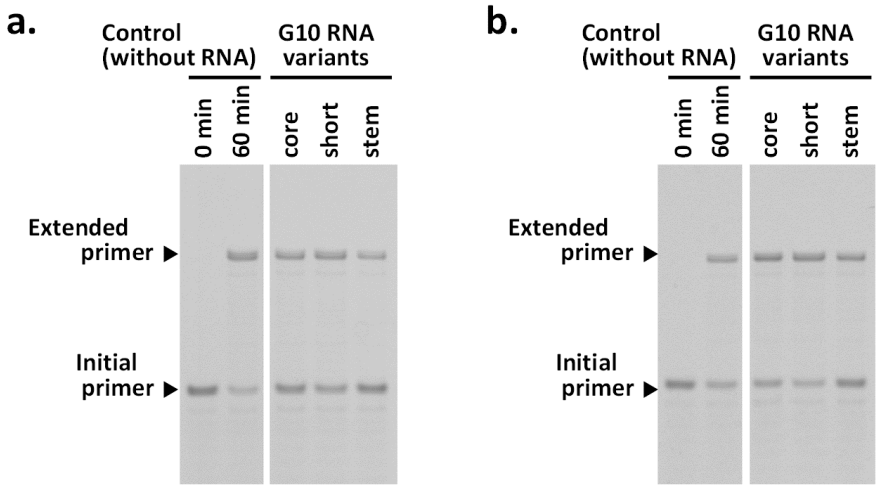

Figure S6. Gel analysis of primer extension reactions to evaluate effects of G-quadruplex formation on inhibition of RdRp. Polymerization reaction was performed in the presence of 2 μM RNA variants in a reaction buffer containing (a) 100 mM KCl or (b) 100 mM LiCl. Polymerized products were electrophoresed in denaturing polyacrylamide gels and imaged using a fluorescence image scanner. Control samples are products without additional RNA at 0- and 60-min reaction times.

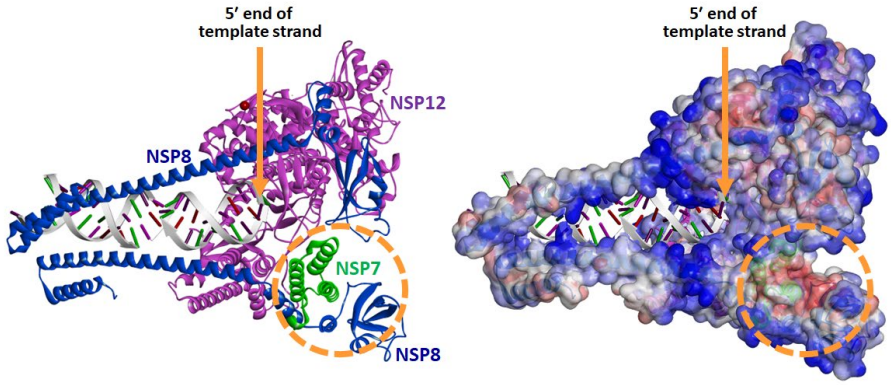

Figure S7. Hydrophobic regions in RdRp complexed with substrate RNA duplex (PDB ID: 6YYT). Views from side, at which template RNA strand is incorporated, are shown. At the image on right, hydrophilic and hydrophobic regions on the protein surface were visualized by blue and red, respectively, using Discovery Studio Visualizer. Hydrophobic region between Nsp7 and Nsp8 subunits is shown by orange circle.