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Supporting Information

Efficient Inhibition of SARS-CoV-2 Using Chimeric Antisense Oligonucleotides through RNase L Activation**

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Experimental Procedures

Design of oligonucleotide sequences. Secondary structures of SARS-CoV-2-E RNA and SARS-CoV-2-S-RBD RNA were predicted by RNAfold web server (Institute for Theoretical Chemistry, University of Vienna) based on minimum free energy (MFE) and partition function algorithms. Antisense oligonucleotide candidates targeting specific SARS-CoV-2 RNA fragments were given by Oligowalk (Mathews group, University of Rochester Medical Center) for the further selection of antisense oligonucleotides.

Preparation of oligonucleotides. Chimeric oligonucleotides (Chimera-E or Chimera-S), ASO-S control oligonucleotides and 3′-Cy3 labeled E-RNA segment were purchased from Biosyntech. Chimera-E-PO, ASO-E and $4A_{2\text{-}5}$ control oligonucleotide were synthesized on ABI DNA/RNA synthesizer based on standard phosphoramidite chemistry, and were purified through HPLC (Waters, Alliance e2695) after the cleavage and deprotection. All the oligonucleotides were confirmed by ESI-MS (Sangon Biotech). Each oligonucleotide was dissolved in nuclease-free water and quantified with NanoDrop 2000 (Thermo Fisher Scientific) at 260 nm before use.

Preparation of plasmids. The pCAG-FLAG vectors containing SARS-CoV-2-E gene (pCAG-nCoV-E-FLAG) or SARS-CoV-2-S gene (pCAG-nCoV-S-FLAG) were generously provided by Prof. Wang Pei-Hui's lab (Shandong University). Full length RNase L gene was synthesized and subcloned into pGEX-4T-3 vector (pGEX-4T-RNaseL-GST) by GENEWIZ as previously described [1].

Plasmid pcDNA 3.1-SARS-CoV-2-Spike, pLVX-hACE2-IRES-puro, pMD2G-VSVG, pspAX.2, pLenti-FLuc-GFP were constructed to generate SARS-CoV-2 pseudovirus and establish transgenic cell line HEK293T-hACE2. Briefly, gene segment containing spike protein of SARS-CoV-2 was synthesized by GenScript Inc. without codon optimization and was inserted into pcDNA 3.1 to get pcDNA 3.1-SARS-CoV-2-Spike using NEBuilder® HiFi DNA Assembly Master Mix (NEB) according to the manufacturer's instructions. In order to construct transfer plasmid pLVX-hACE2-IRES-puro, plasmid containing complete ORF of hACE29 (pMD18-T-hACE2) was purchased from Sino biological Inc. and hACE2 gene was sequenced by BGI Inc. Then hACE2 segment was amplified by primer F 5′-ATGTCAAGCTCTTCCTGG-3′ and primer R 5′-CTAAAAGGAGGTCTGAACATC-3′, then restriction enzyme cutting site XhoI and XbaI was added using primer forward: 5′- CTCGAGCTCGAGGCCGCCACCATGTCAAGCTCTTCCTGGC-3′ and reverse: 5′-TCTAGATCTAGACTAAAAGGAGGTCTGAACATCA-3′. Lentiviral transfer plasmid pLVX-IRES-puro was stored in our lab. Insertion of hACE2 into pLVX-IRES-puro was conducted by double digestion of XhoI and XbaI (Fermantas) and ligation of T4 ligase (NEB) according to manufacturer's instructions. Plasmid pMD2G-VSVG, pspAX.2, pLenti-FLuc-GFP was stored in our lab [2].

Mutant plasmids pCMV-hnCoV-S-N501Y (forward: 5′-CCAGCCTACATATGGCGTGGGCT-3′, reverse: 5′-AAGCCGTAAGACTGGAGTG-3′) and pCMV-hnCoV-S-ΔH69/ΔV70 (forward: 5′-TCCGGCACAAACGGCACA-3′, reverse: 5′-GATGGCGTGGAACCATGT C-3′) were obtained from the wild type plasmids pCMV-hnCoV-S via Q5 SiteDirected Mutagenesis Kit (NEB). pCMV-hnCoV-S-H501Y-Δ69/70 was obtained from pCMV-7.1-hnCoV-S-N501Y (forward: 5′-TCCGGCACAAACGGCACA-3′, reverse: 5′-GATGGCGTGGAACCATGTC-3′) via Q5 SiteDirected Mutagenesis Kit (NEB). All plasmids were confirmed by gene sequencing (BGI Beijing). All plasmids used for transfection were amplified using a Maxiprep kit (Promega), according to the manufacturer's instructions.

Preparation of RNase L-GST protein. The RNase L-GST fusion protein was expressed in *Escherichia coli* strain DH5α transformed with pGEX-4T-RNaseL-GST plasmid as previously described [1]. Briefly, cells were grown at 30 °C to A_{595} = 0.5, then 0.1 mM isopropylthio-galactoside was added and cells were grown for another 3 h at 30 °C before harvest. After centrifugation at 4000 rpm at 4 °C for 15 min, cells were washed with 0.8% NaCl and resuspended in 50 mL buffer A (10 mM NaH_2PO_4, pH7.4, 600 mM NaCl, 10% glycerol, 1 mM EDTA, 0.1 mM ATP, 5 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 1 μg/mL leupeptin) supplemented with 1% Triton X-100, 1 mM PMSF, 1 μg/mL lysozyme and 10 mM DTT. Then cells were sonicated on ice and cell lysates were centrifugated at 11, 000 rpm at 4 °C for 40 min to collect supernatants. RNase L-GST protein in supernatants was purified via GST affinity chromatography (HP, Cytiva) with buffer B (20 mM glutathione, 300 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 μg/mL leupeptin) as eluent. Fractions containing RNase L-GST protein were collected and the purity of the protein were analyzed by SDS-polyacrylamide gel electrophoresis.

***In vitro* Cleavage of E-mRNA by RNase L.** Conditions for RNase L cleavage of single strand RNA were formerly reported [3]. Briefly, Cy3-labeled E-RNA fragment as the substrate RNA was folded in 1× RNase L NM Buffer (25 mM Tris-HCl, pH7.4, 100 mM KCl) at 8 μM by heating the solution at 95 °C for 30 s and slowly cooling to 25 °C. Then the above solution was supplemented with 2×

Supplementary Buffer (25 mM Tris-HCl, pH7.4, 100 mM KCl, 20 mM $MgCl_2$, 14 mM β-mercaptoethanol, 100 μM ATP) and aliquots of $4A_{2-5}$ or Chimera-E-PO was then added, followed by incubation at 25 °C for 30 min. Both $4A_{2-5}$ and Chimera-O-E were diluted in 1× RNase L M Buffer (25 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 50 μM ATP). Then RNase L was added at an equimolar concentration of $4A_{2-5}$ or Chimera-O-E. Each sample was supplement to a final volume of 8 μL and was further incubated at 25 °C for 120 min. After quenching RNase L cleavage by adding 2× Loading Buffer (8 M urea, 20 mM EDTA, 2 mM Tris-base, 0.01% bromophenol blue and 0.01% xylene cyanol), samples were heated at 95 °C for 3 min and loaded in a denaturing 12.5% polyacrylamide gel. The gel was run at 250 V for 20 min and imaged using Chemiluminescence gel imaging system (ChemiDoc XRS).

Cell culture and Transfection Procedure. Vero cells and A549 cells were grown at 37 °C, 5% CO_2 in DMEM (M&C) supplemented with 10% fetal bovine serum (PAN), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were seeded and incubated for 24 h. Transfection of oligonucleotides and/or plasmids were performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. After 6 h incubation, cells were replaced with fresh medium and incubated at 37°C, 5% CO_2 in for another 18 h. HEK293T cells and transgenic cell line HEK293T-hACE2 were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin, and 100 μg/mL streptomycin. HEK293T cells were stored in our lab [4]. Transfection of oligonucleotides and plasmids in pseudovirus infection models were performed using using Lipofectamine™ 3000 (Invitrogen) according to the manufacturer's instructions. After 6 h incubation, cells were replaced with fresh medium and incubated at 37°C, 5% CO_2 in for another 42 h.

Real-time Polymerase Chain Reaction. Vero cells or A549 cells were seeded into 24-well plates with the density of 7.5×10^4 cells per well (for A549 cells, the density is 1×10^5 cells per well). Oligonucleotides and/or plasmids (250 ng per well) were transfected to each well according to the group setting. After 24 h incubation at 37 °C, total RNA was extracted using BioZol reagent (Bioer) according to the manufacturer's instructions. cDNAs were synthesized with HiScript III 1st cDNA Synthesis Kit (+gDNA wiper) (Vazyme Biotech). Real time-polymerase chain reactions were performed with GoTaq qPCR Master Mix (Promega) according to the manufacturer's instructions and completed on QuantStudio 6 Flex system (ABI). RNA expression levels were determined through the ΔΔCt method and normalized with GAPDH or 18S as a housekeeping gene.

SRB Assay. Oligonucleotides and plasmids (60 ng per well) were transfected into Vero cells (seeded in 96-well plates with the density of 2×10^4 cells per well). Cells were replaced with fresh medium after 6 h incubation. After another 18 h, culture medium was removed and cold 10% TCA was added (100 μL per well). The plate was incubated at 4 °C for 1 h and then washed with deionized water (200 μL per well) for four times. After naturally drying, 4 mg/mL Sulforhadamine B (SRB) dissolved in 1% aqueous acetic acid was added (100 μL per well) and the plate was incubated at room temperature for 30 min. Each well was rinsed with 1% acetic acid for five times and naturally dried. Finally, 10 mM unbuffered Tris base (pH 10.5) was added (100 μL per well). Read the optical density at 540 nm by a microplate reader (SYNERGY H1, BioTek).

Establishment of transgenic cell line HEK293T-hACE2. Procedure to establish a cell line expressing human angiotensin-converting enzyme 2 (hACE2) receptor was previously described [5] and introduced in brief. HEK293T cells were used for lentiviral vector packaging and transduction. The cells were cultured in DMEM supplemented with 10% FBS (Gibco) and 1 mM nonessential amino acids (Gibco). Sub confluent HEK293T cells in 6-well plates were co-transfected with 0.72 μg of pLVX-hACE2-IRES-puro transfer plasmid, 0.64 μg of pMD2G-VSVG and 0.64 μg of pspAX.2 using transfecting reagent Megatran 1.0 (Origene). Then, 6 h post transfection, the medium was replaced by DMEM supplemented with 3% FBS and 1 mM nonessential amino acids. Next, the lentiviral-containing supernatant was harvested at 48 h post transfection and filtered by a 0.45 μm filter (Pall). The resultant lentiviruses were used to integrate hACE2 gene into the genome of HEK293T cells. Procedure of stable lentiviral transduction was carried out as follows: HEK293T cells were seeded in a 6-well plate and transducted 24 h later with lentiviral filtrate in presence of 8 μg/mL polybrene (Macgene). Then, selection was performed under the pressure of 1 μg/mL puromycin (Invitrogen) until cells died completely. Then the cell line was verified by western blot.

Generation of SARS-CoV-2 pseudovirus. Construction of a VSV pseudovirus carrying the spike protein of SARS-CoV-2 was formerly reported [5] and introduced in brief. HEK293T cells were used for pseudovirus packaging. Subconfluent HEK293T cells in 6-well plates were co-transfected with 1.2 μg of pLenti-FLuc-GFP transfer plasmid, 0.4 μg of pcDNA 3.1-SARS-CoV-2-Spike plasmid, 0.4 μg of pspAX.2 plasmid and oligonucleotides (0.3~1.1 μg) per well. 6 h post transfection, the medium was replaced by DMEM supplemented with 10% FBS. Next, cell status and green fluorescence was captured by inverted fluorescence microscope (Olympus) 48 h post transfection, then the pseudovirus-containing supernatant was harvested and filtered by a 0.45 μm filter (Pall). The resultant pseudoviruses were further analyzed for viral tilter by flow cytometry and luciferase assay.

SUPPORTING INFORMATION

Pseudovirus infection and luciferase assay. In order to determine the titration of pseudovirus, expression of firefly luciferase was conducted as follows: HEK293T-hACE2 cells were seeded into 96-well black/clear bottom plates (Nunc) at 5×10^3 cells per well and cultured for 24 h. Then the medium was replaced by 100 μL pseudovirus pLenti-FLuc-GFP filtrate and cells were incubated for another 48 h. Expression of firefly luciferase was quantitated by Bright Glo™ luciferase assay system (Promega) and the plates were read using a plate reader (Tecan Infinite M2000 PRO).

Flow cytometry. The transfection efficiency during pseudovirus packaging was analyzed by flow cytometry. Briefly, HEK293T cells transfected with plasmid pcDNA pLenti-FLuc-GFP, pcDNA 3.1-SARS-CoV-2-Spike, pspAX.2 and oligonucleotides were incubated for 48 h and GFP expression level was analyzed by CytoFLEX flow cytometer (Beckman). In order to confirm the titration of pseudovirus, HEK293T-hACE2 cells were seeded into 6-well plates. After 24 h incubation, the medium was replaced by 1 mL fresh medium mixed with 1 mL pseudovirus pLenti-FLuc-GFP filtrate. Cells were incubated for 48 h and GFP expression level was analyzed by CytoFLEX flow cytometer (Beckman).

Statistical Analysis. GraphPad Prism 7.04 was used for statistical analysis and graphing. Two-tailed Student's t test was used to compare data of two experimental groups.

References

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Author Contributions

X.T. and X.S. conceived this study and designed experiments. X.S. and Q.W. prepared the protein. B.C. and Z.G. constructed pseudovirus mutants. W.M. and B.C. performed experiments related to pseudovirus. X.S. performed most of the experiments and analyzed data except those noted. X.S wrote the manuscript. X.T., Q.W., D.F., W.M. and D.Z. revised the manuscript.

Table S1. Oligonucleotides synthesized in this study.

Name	Sequence (5'→3')
Chimera-S4	p*-r(X*X*X*X)-p*-(PEG unit)$_6$-p*-m(U*A*U*G*A*U*U*G*U*A*A*A*G*G*A)
Chimera-S5	p*-r(X*X*X*X)-p*-(PEG unit)$_6$-p*-m(A*C*U*A*C*U*C*U*G*U*A*U*G*G*U)
Chimera-S6	p*-r(X*X*X*X)-p*-(PEG unit)$_6$-p*-m(A*U*C*A*G*U*A*G*U*G*U*C*A*G*C)
ASO-S4	(PEG unit)$_6$-p*-m(U*A*U*G*A*U*U*G*U*A*A*A*G*G*A)
ASO-S5	(PEG unit)$_6$-p*-m(A*C*U*A*C*U*C*U*G*U*A*U*G*G*U)
ASO-S6	(PEG unit)$_6$-p*-m(A*U*C*A*G*U*A*G*U*G*U*C*A*G*C)
Chimera-E	p*-r(X*X*X*X)-p*-(PEG unit)$_6$-p*-m(U*A*A*C*A*A*U*A*U*U*G*C*A*G*C)
Chimera-E-PO	p-XXXX-p-(PEG unit)$_6$-p*-m(U*A*A*C*A*A*U*A*U*U*G*C*A*G*C)
ASO-E	(PEG unit)$_6$-p*-m(U*A*A*C*A*A*U*A*U*U*G*C*A*G*C)
3'-Cy3 E-RNA	ACUGCUGCAAUAUUGUUAACGUGAGUCUUGUAAAACCUUCUUUUUACGUUUACUCUCGUGUU-Cy3

X = 2'-5'linked adenine nucleotide; *: Phosphorothioate; m: 2'-*O*-methyl.

Table S2. Primers used for RT-qPCR.

Primer	Sequence (5'→3')
GAPDH F	TGCACCACCAACTGCTTAGC
GAPDH R	GGCATGGACTGTGGTCATGAG
18S F	GTAACCCGTTGAACCCCATT
18S R	CCATCCAATCGGTAGTAGCG
RNase L F	GACACCTCTGCATAACGCAGT
RNase L R	AGGGCTTTGACCTTACCATACA
IFN-β F	GCCGCATTGACCATCTATGA
IFN-β R	GCCAGGAGGTTCTCAACAATAG
IL-6 F	ATCTAGATGCAATAACCACCCCT
IL-6 R	AGCTGCGCAGAATGAGATGA

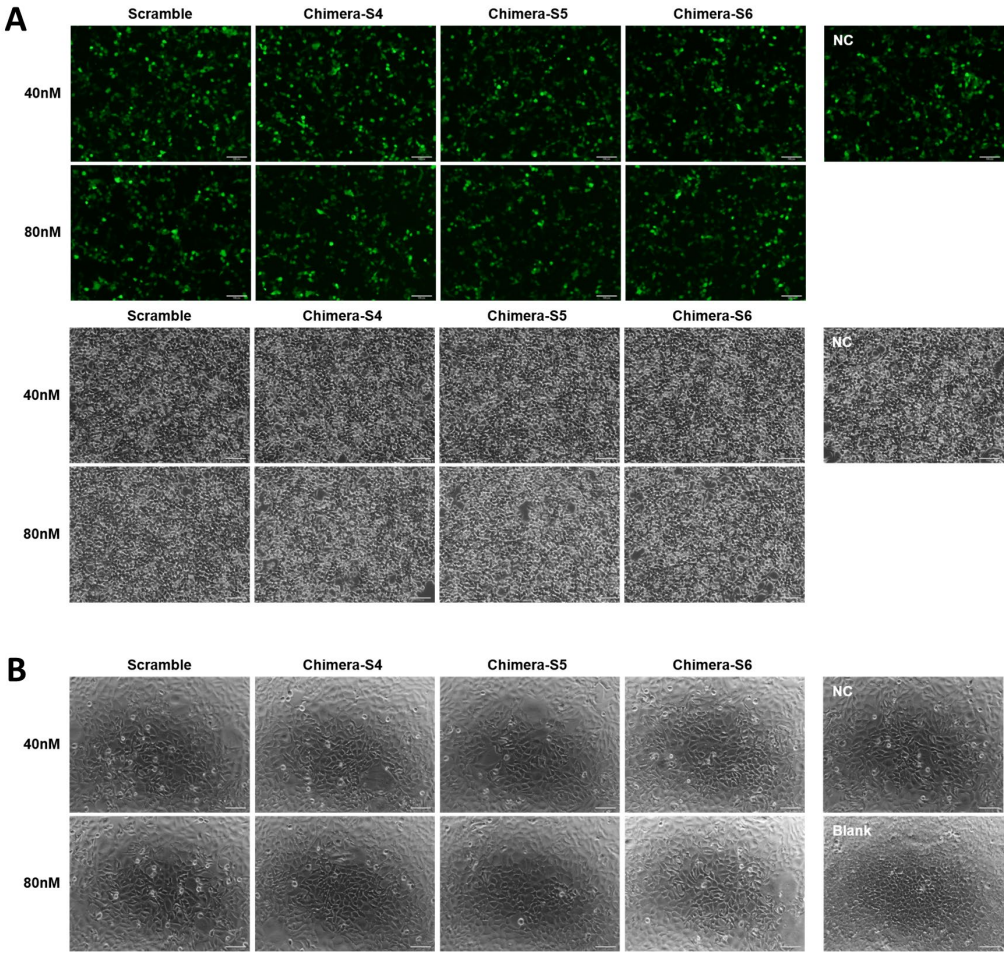


Fig. S1. Transfection efficiency and cell status in pseudotyped SARS-CoV-2 model during Chimera-S screening stage. **(A)** Fluorescent images and brightfield images of HEK293T cells incubated for 48 h after transfection of 40 nM or 80 nM Chimera-S4, Chimera-S5, Chimera-S6 or scrambled oligonucleotides. Similar green fluorescence of GFP between experimental groups and negative control (NC) group showed nearly equal transfection efficiency. **(B)** Brightfield images of HEK293T-hACE2 cells incubated for 48 h after infection. Scale bar = 100 μm.

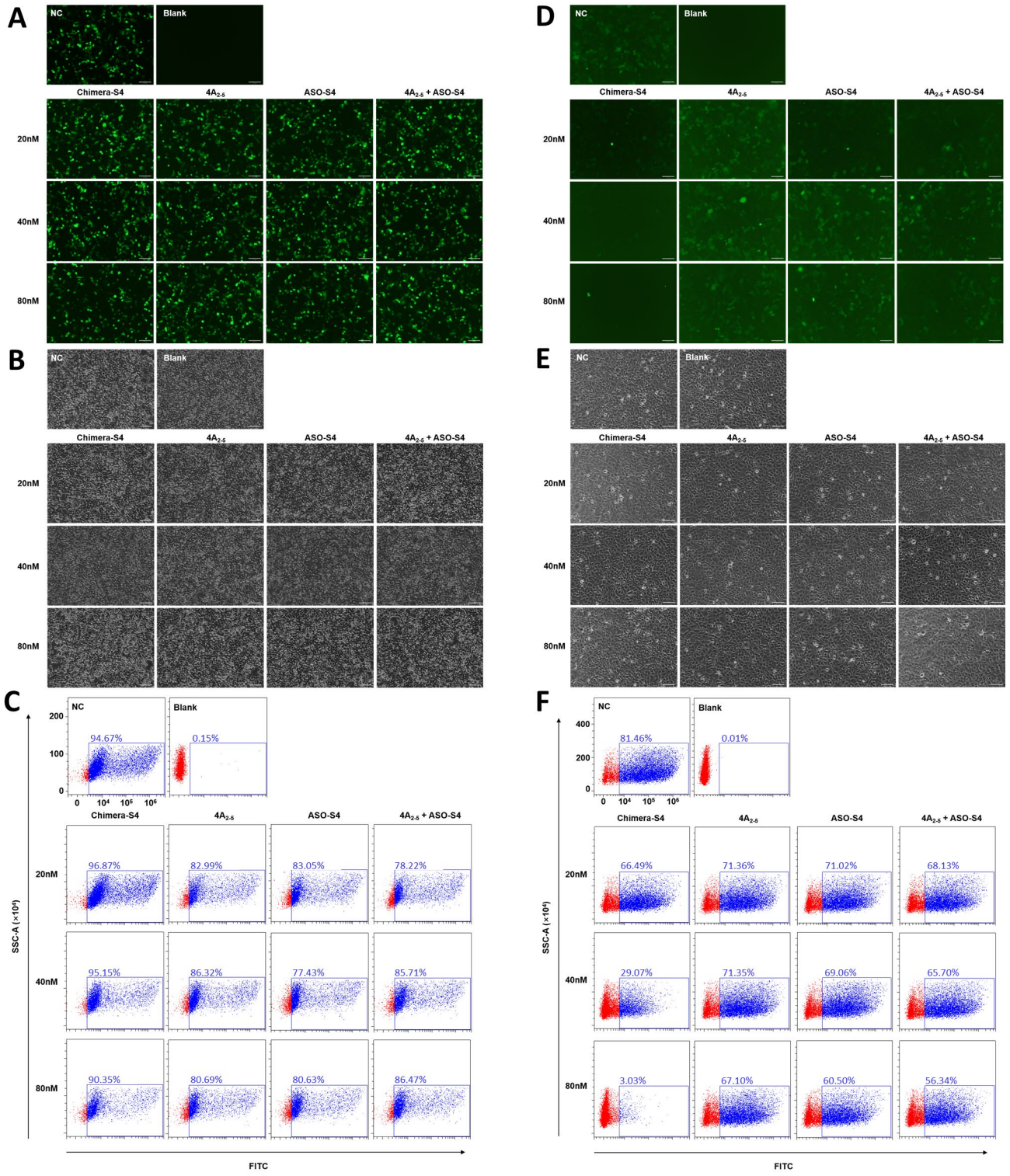


Fig. S2. Chimera-S4 inhibited assembly of SARS-CoV-2 in concentration dependent manner. Fluorescent images **(A)**, brightfield images **(B)** and quantified GFP signals **(C)** of HEK293T cells incubated for 48 h after transfection of different concentrations of Chimera-S4, 4A2-5, ASO-S4 and 4A2-5 + ASO-S4. For HEK293T-hACE2 cells post 48 h infection, fluorescent images **(D)**, brightfield images **(E)** were also captured and their GFP signals **(F)** were analyzed by flow cytometry.

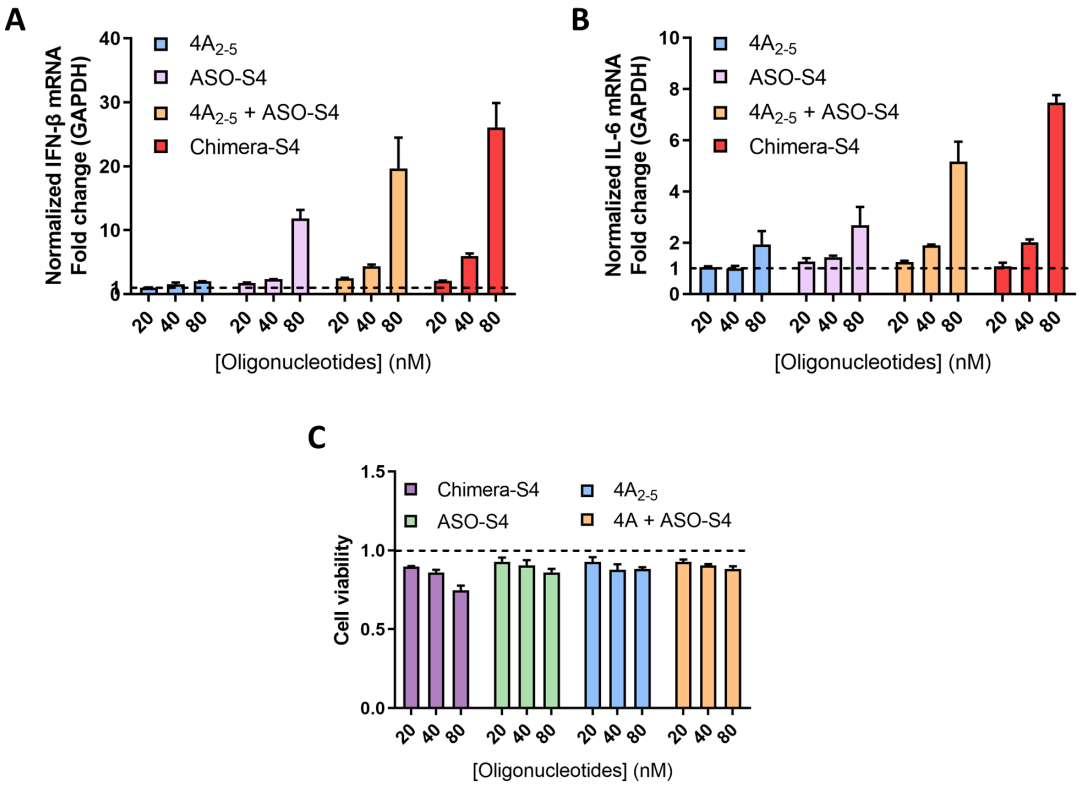


Fig. S3. Intracellular mRNA levels of IFN-β **(A)** and IL-6 **(B)** in Vero cells incubated for 24 h after transfection of different concentrations of Chimera-S4, 4A$_{2\text{-}5}$, ASO-S4 and 4A$_{2\text{-}5}$ + ASO-S4, as measured by RT-qPCR. **(C)** Cell cytotoxicity assay in Vero cells. Data represent mean ± s.e.m. (n ≥ 3).

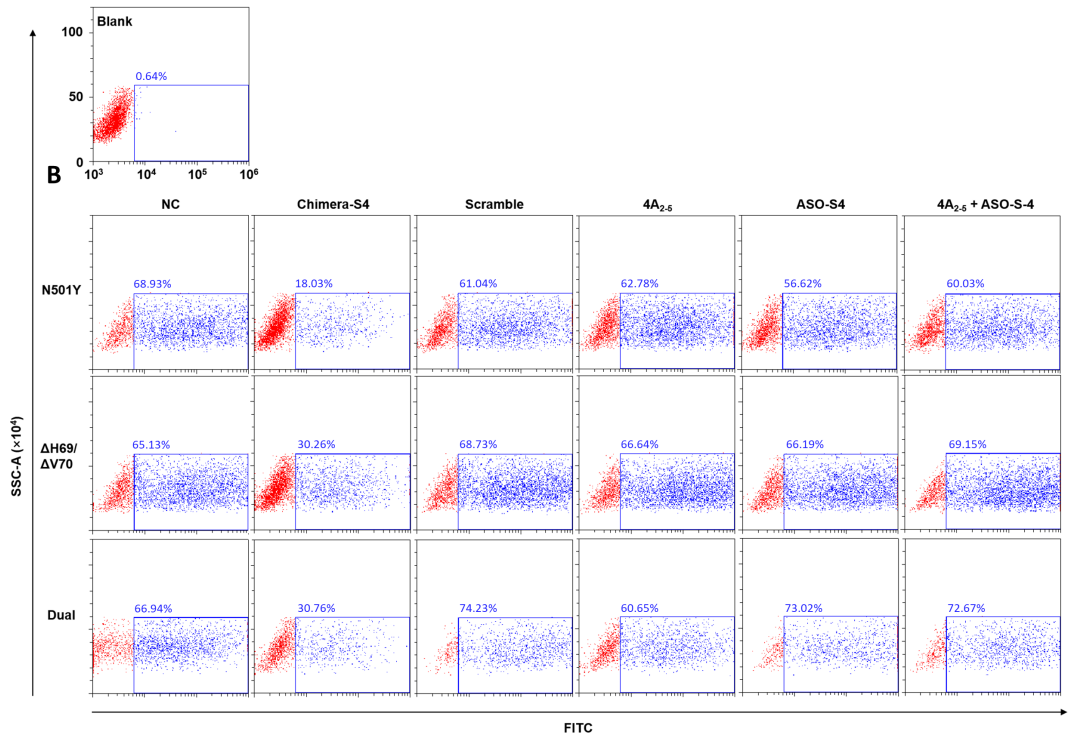


Fig. S4. Efficiently inhibited infection of three mutated SARS-CoV-2 pseudoviruses, N501Y, ΔH69/ΔV70 and their combined mutants (Dual) in HEK293T-hACE2 cells after Chimera-S4 treatment (40 nM, 48 h), as measured by GFP signal analysis.

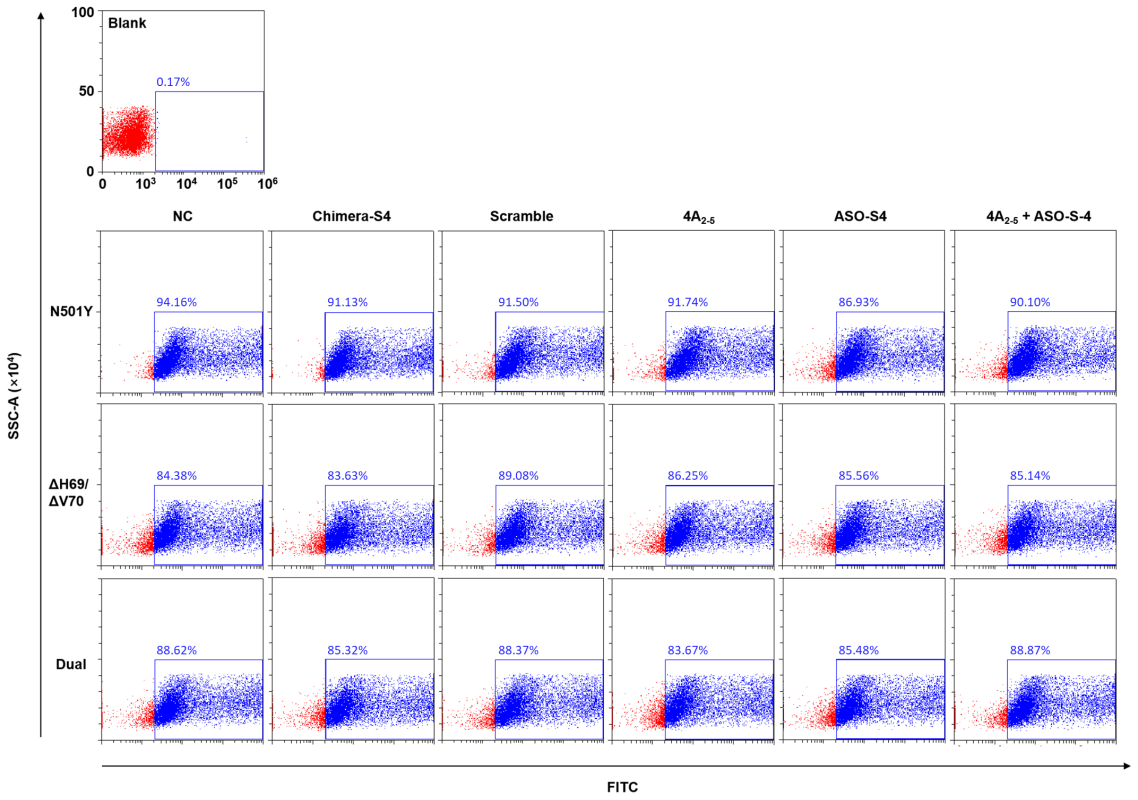
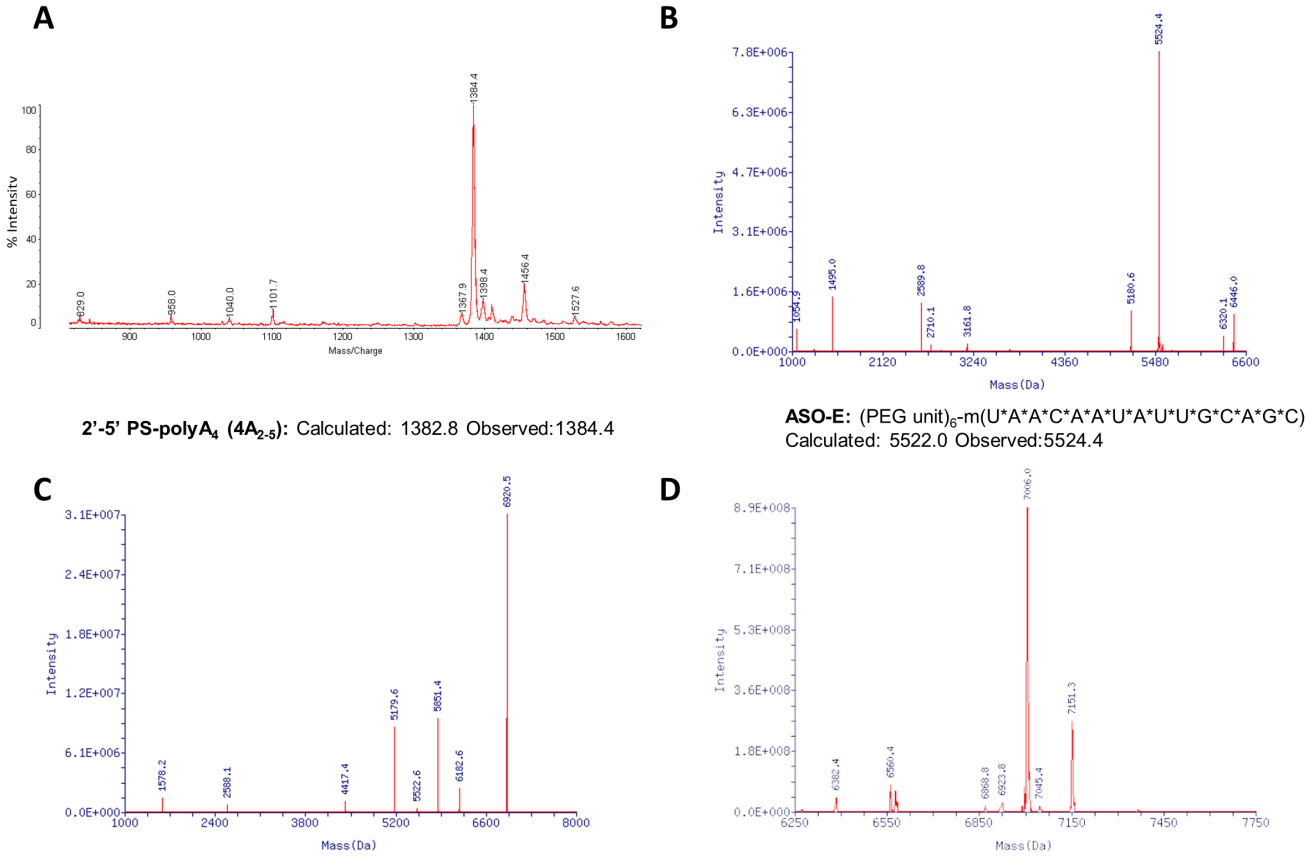


Fig. S5. Flow cytometry analysis of GFP expression in HEK293T cells to confirm the similar transfection efficiencies of different experimental groups in 48 h.



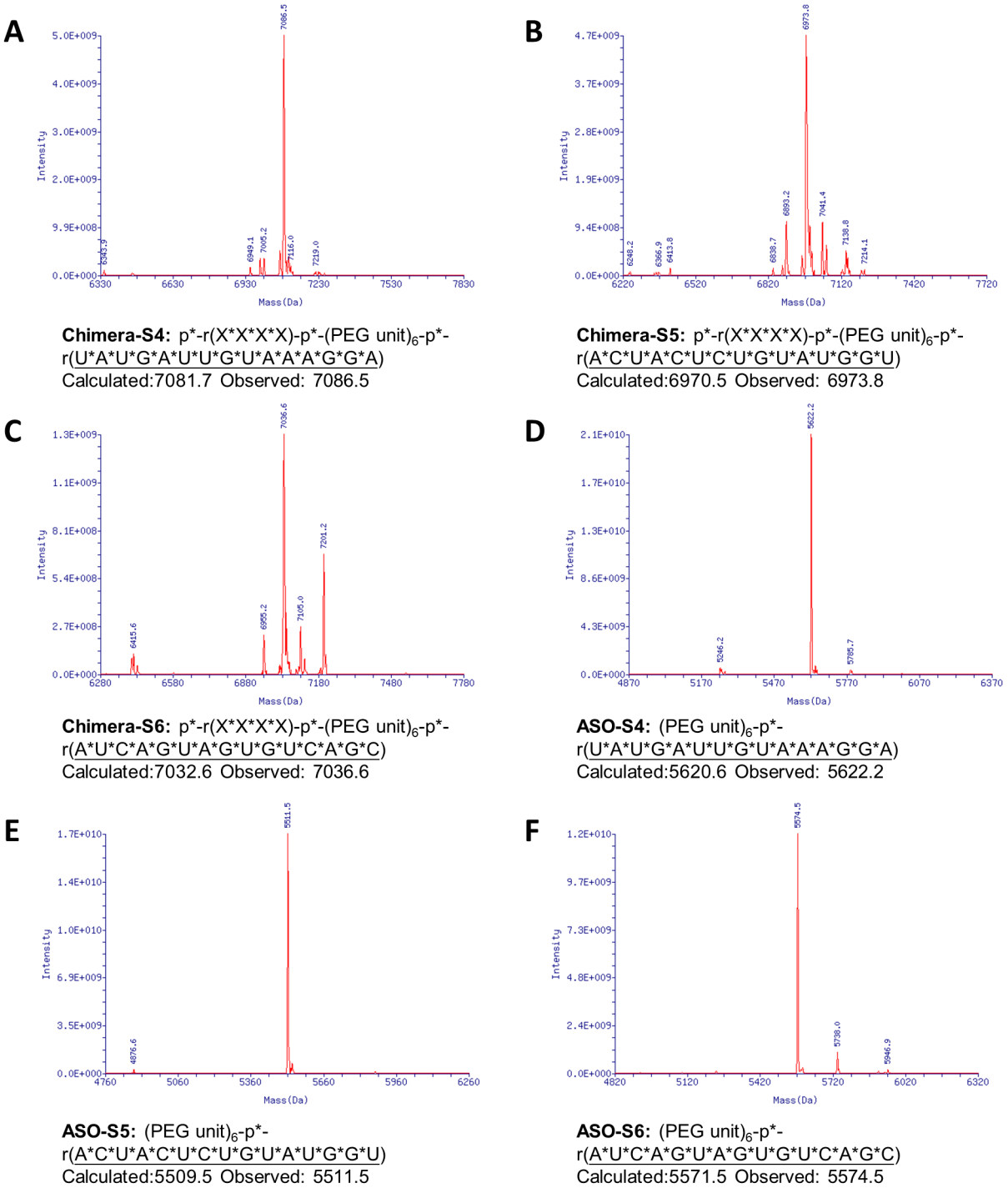
2'-5' PS-polyA_4 ($4A_{2\text{-}5}$): Calculated: 1382.8 Observed:1384.4

ASO-E: (PEG unit)$_6$-m(U*A*A*C*A*A*U*A*U*U*G*C*A*G*C)
Calculated: 5522.0 Observed:5524.4

Chi-E-PO: p-r(XXXX)-p-(PEG unit)$_6$-p*-m(U*A*A*C*A*A*U*A*U*U*G*C*A*G*C)
Calculated: 6918.9 Observed:6920.5

Chimera-E: p*-r(X*X*X*X)-p*-(PEG unit)$_6$-p*-m(U*A*A*C*A*A*U*A*U*U*G*C*A*G*C)
Calculated:7015.7 Observed:7006.0

Fig. S6. Mass spectra of 2'-5' polyA_4 (**A**), ASO-E (**B**), Chimera-E-PO (**C**) and Chimera-E (**D**). X = 2'-5'linked adenine nucleotide; *: phosphorothioate; m: 2'-*O*-methyl.



Chimera-S4: p*-r(X*X*X*X)-p*-(PEG unit)$_6$-p*-r(U*A*U*G*A*U*U*G*U*A*A*A*G*G*A)
Calculated:7081.7 Observed: 7086.5

Chimera-S5: p*-r(X*X*X*X)-p*-(PEG unit)$_6$-p*-r(A*C*U*A*C*U*C*U*G*U*A*U*G*G*U)
Calculated:6970.5 Observed: 6973.8

Chimera-S6: p*-r(X*X*X*X)-p*-(PEG unit)$_6$-p*-r(A*U*C*A*G*U*A*G*U*G*U*C*A*G*C)
Calculated:7032.6 Observed: 7036.6

ASO-S4: (PEG unit)$_6$-p*-r(U*A*U*G*A*U*U*G*U*A*A*A*G*G*A)
Calculated:5620.6 Observed: 5622.2

ASO-S5: (PEG unit)$_6$-p*-r(A*C*U*A*C*U*C*U*G*U*A*U*G*G*U)
Calculated:5509.5 Observed: 5511.5

ASO-S6: (PEG unit)$_6$-p*-r(A*U*C*A*G*U*A*G*U*G*U*C*A*G*C)
Calculated:5571.5 Observed: 5574.5

Fig. S7. Mass spectra of Chimera-S4 (**A**), Chimera-S5 (**B**), Chimera-S6 (**C**), ASO-S4 (**D**), ASO-S5 (**E**) and ASO-S6 (**F**). X = 2'-5'linked adenine nucleotide; *: phosphorothioate; m: 2'-*O*-methyl.