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Supplemental Information

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Dual Mechanisms of Action of Self-Delivering Anti-HIV-1 FANA Oligonucleotides as a Potential New Approach to HIV Therapy

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Materials and Methods

Cell lines and cell culture

All cells were cultured in a humidified 5% CO_2 incubator at 37°C. HEK293T cells were purchased from ATCC and cultured in 90% DMEM supplemented with 10% fetal bovine serum. HEK293T cells are an adherent cell line and were split 1:10 or 1:5 twice per week upon reaching confluence by washing with PBS and detaching cells using Cell stripper (Cellgro, Mediatech Inc) in order to minimize the damage to the cellular surface receptors. The cells were stained with Trypan Blue to detect viability; >95% live cells were used for seeding. Cells were carried for no more than 15 passages. CCRF-CEM cells were purchased from ATCC and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). The CCRF-CEM cell line is a suspension cell line and was split 1:10 once per week upon reaching confluence.

Internalization and intercellular localization studies (live-cell confocal microscopy) (Figure S1)

On the day of experiments, PBMCs were washed twice with pre-warmed PBS and seed in a polylysine-coated 4-compartment 35/10-mm plate with seeding at 0.5×10^6 cells in 500 μL pre-warmed RPMI-1640 medium supplemented with 10% FBS. Cells were incubated for 30-60 min in a humidified 5% CO_2 incubator at 37°C for attaching on the dish surface. Cy3-labeled Cy3-FANA ASOs (100 nM) were added to media and incubated on a 5% CO_2 microscopy incubator at 37°C for 4 h. The cells were stained by treatment with 0.15 mg/ml Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The z-stack images were collected with the step size of 1 μm using a Zeiss LSM 510 Meta Inverted two-photon confocal microscopy system (City of Hope Confocal Microscopy Core, Duarte, CA) under water immersion at 40× magnification.

Internalization studies of CD8-depleted PBMCs (live-cell confocal microscopy) (Figure S2)

On the day of experiments, human PBMCs were freshly isolated from healthy donors and CD8 cells were depleted from the PBMCs using Dynabeads CD8 (Thermo Fisher Scientific) as described in the main text. CD8-depleted PBMCs were washed twice with pre-warmed PBS and seed in a polylysine-coated 4-compartment 35/10-mm plate with seeding at 0.5×10^6 cells in 500 μL pre-warmed RPMI-1640 medium supplemented with 10% FBS. Cells were incubated for 30-60 min in a humidified 5% CO_2 incubator at 37°C for attaching on the dish surface. Cy3-labeled ASOs (100 nM, 300 nM, 500 nM) were added to media and incubated on a 5% CO_2 microscopy incubator at 37°C for 4 h. The cells were stained by treatment with 0.15 mg/ml Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The images were collected as described in the main text.

Internalization and intercellular localization studies of CCRF-CEM cells (live-cell confocal microscopy) (Figure S3A)

On the day of experiments, PBMCs were washed twice with pre-warmed PBS and seed in a polylysine-coated 4-compartment 35/10-mm plate with seeding at 2.5×10^5 cells in 500 μL pre-warmed RPMI-1640 medium supplemented with 10% FBS. Cells were incubated for 30-60 min in a humidified 5% CO_2 incubator at 37°C for attaching on the dish surface. Cy3-FANA ASOs (50 nM, 100 nM, 300 nM) were added to media and incubated on a 5% CO_2 microscopy incubator at 37°C for 4 h. The cells were stained by treatment with 0.15 mg/ml Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The images were collected as described in the main text.

Cellular uptake analysis of CCRF-CEM cells (flow cytometry) (Figure S3B)

CCRF-CEM cells were seeded in a 48-well plate at 1×10^5 cells per well in 200 μL pre-warmed RPMI-1640 medium supplemented with 10% FBS. Cy3-FANA was added to the cells at a final concentration of 100 nM. After 4 h incubation, cells were washed with PBS and treated with trypsin for 5 min at 37°C. Cells were washed again and stained with DAPI (Thermo Fisher Scientific, Waltham, MA). Cells were immediately analyzed using flow cytometry as described in the main text.

HIV-1 challenge assay (dose-response study) (Figure S4)

Human PBMCs were freshly isolated from healthy donors and CD8 cells were depleted from the PBMCs using Dynabeads CD8 (Thermo Fisher Scientific) as described in the main text. After cultured for 3 days in the activated T-cell culture medium, cells were washed with pre-warmed PBS and infected with HIV-1_{NL4-3} at MOI of 0.01 for four days. The infected cells were gently washed with pre-warmed PBS three times to remove free virus and resuspended with RPMI-1640 medium containing 60 units/mL IL-2. Infected cells and uninfected cells were mixed at 1:1 ratio and the mixed cells were seeded in a 48-well plate at 5×10^5 cells per well. ASOs were added to the cells at final concentrations of 100 nM, 200 nM, 400 nM, 800 nM or 1600 nM. Culture supernatants were collected at 7-day post-treatment for HIV-1 p24 ELISA (PerkinElmer, Waltham, MA). The % viral production was calculated by the formula: p24 expression amount in the samples/p24 expression amount in the ASO non-treated sample (NT).

Cytotoxicity assay (Figure S5)

Human PBMCs were freshly isolated from healthy donors and CD8 cells were depleted from the PBMCs using Dynabeads CD8 (Thermo Fisher Scientific) as described in the main text. Cells were infected with HIV-1_{NL4-3} as described above. Infected cells and uninfected cells were mixed at a 1:1 ratio and the mixed cells were seeded in a 96-well plate at 2×10^5 cells per well. ASOs were added to the cells at a final concentration of 3 μM for 10 days. Cytotoxicity was measured using CellTiter 96® AQueous One Solution Cell Proliferation Assay System (Promega, Madison, WI) according to the manufacturer's protocol.

5'-RACE assays to detect target RNA cleavage in HEK293T cells (Figure S6)

Twenty-four h before transfection, HEK293T cells were seeded in 6-well plates at $3x10^5$ cells per well in 1 mL pre-warmed DMEM medium supplemented with 10% FBS. Tat/rev siRNA (sense: 5'-GCG GAG ACA GCG ACG AAG AGC UCA UCA-3'; antisense: 5'-UGA UGA GCU CUU CGU CGC UGU CUC CGC dTdT-3') or tat/rev-FANA (final concentration of each at 50 nM) was co-transfected with a Rev-EGFP fusion construct harboring the tat/rev target using Lipofectamine2000 (Thermo Fisher Scientific, Waltham, MA) according to the manufacture's protocol. After a 48-h incubation, total RNA was isolated from cells using STAT-60 (TEL-TEST, Friendswood, TX). Residual DNA was digested using the DNA-free kit (Thermo Fisher Scientific, Waltham, MA) per the manufacturer's instructions. Subsequently, total RNA (10 µg) was ligated to a GeneRacer adaptor (5'-CGA CTG GAG CAC GAG GAC ACT GAC ATG GAC TGA AGG AGT AGA AA-3'**,** Thermo Fisher Scientific, Waltham, MA) without prior treatment. Ligated RNA was reverse transcribed using a gene-specific primer 1 (Rev-GFP RACE GSP1: 5'-TCA CCC TCT CCA CTG ACA GAG AAC TT-3'). To detect cleavage products, nested PCR was performed using primers complementary to the RNA adaptor (5'-cDNA primer: 5'-GGA CAC TGA CAT GGA CTG AAG GAG TA-3') and gene-specific primer 2 (Rev-GFP RACE GSP2: 5'-TAA CCT CTC AAG CGG TGG TAG CTG AA-3'). Amplification products were resolved using agarose gel electrophoresis and visualized using ethidium bromide staining. Specific PCR products were recovered using a QIAquick Gel purification Kit (Qiagen, Germantown, MD), then were cloned into the TOPO TA cloning vector pCR4-TOPO vector (Thermo Fisher Scientific, Waltham, MA). Individual clones were identified using DNA sequencing.

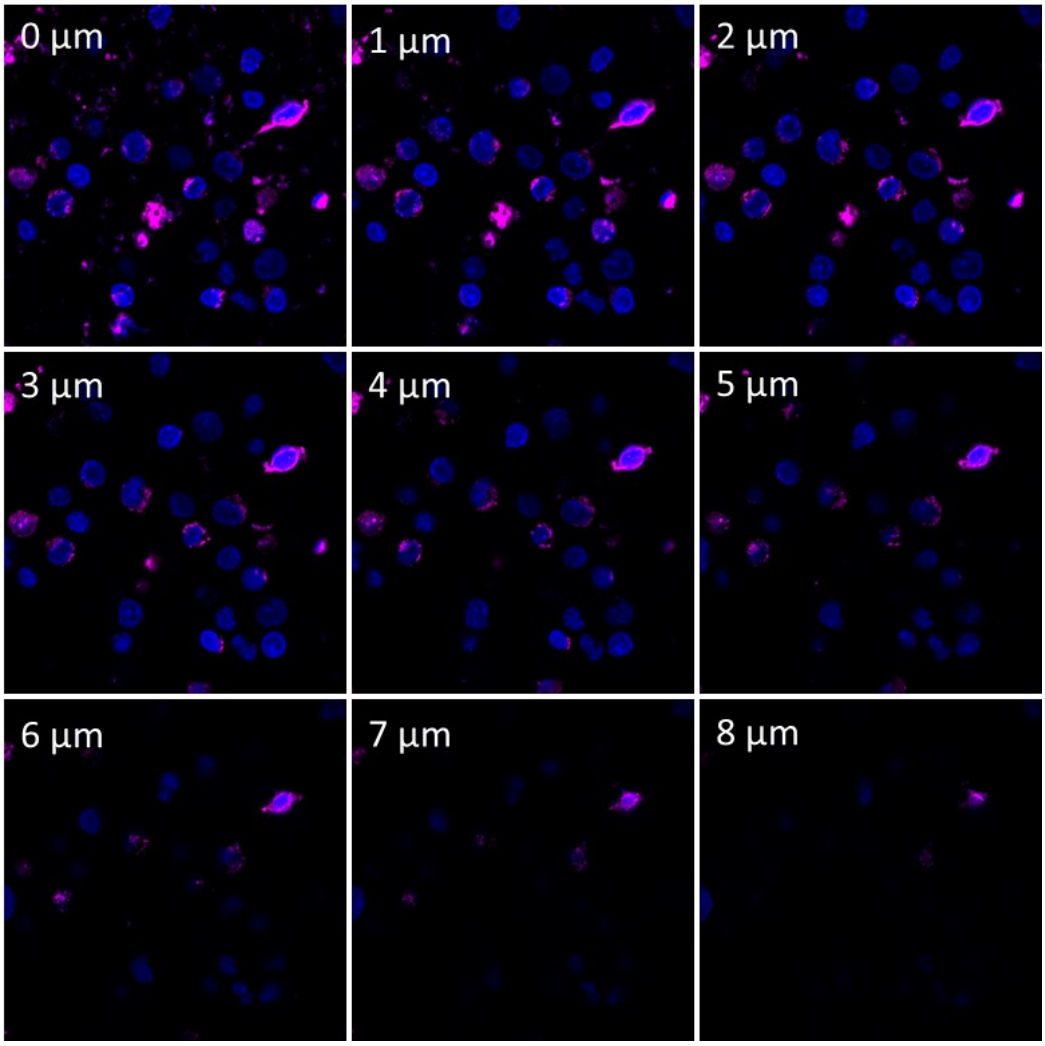


Figure S1. Real-time live-cell confocal microscopy analysis. PBMCs were incubated with Cy3-FANA at a final concentration of 100 nM for 4 h. Z-stack images were collected with a step size of 1 µm, as indicated, using 40x magnification. Magenta: Cy3-FANA; Blue: Hoechst 33342.

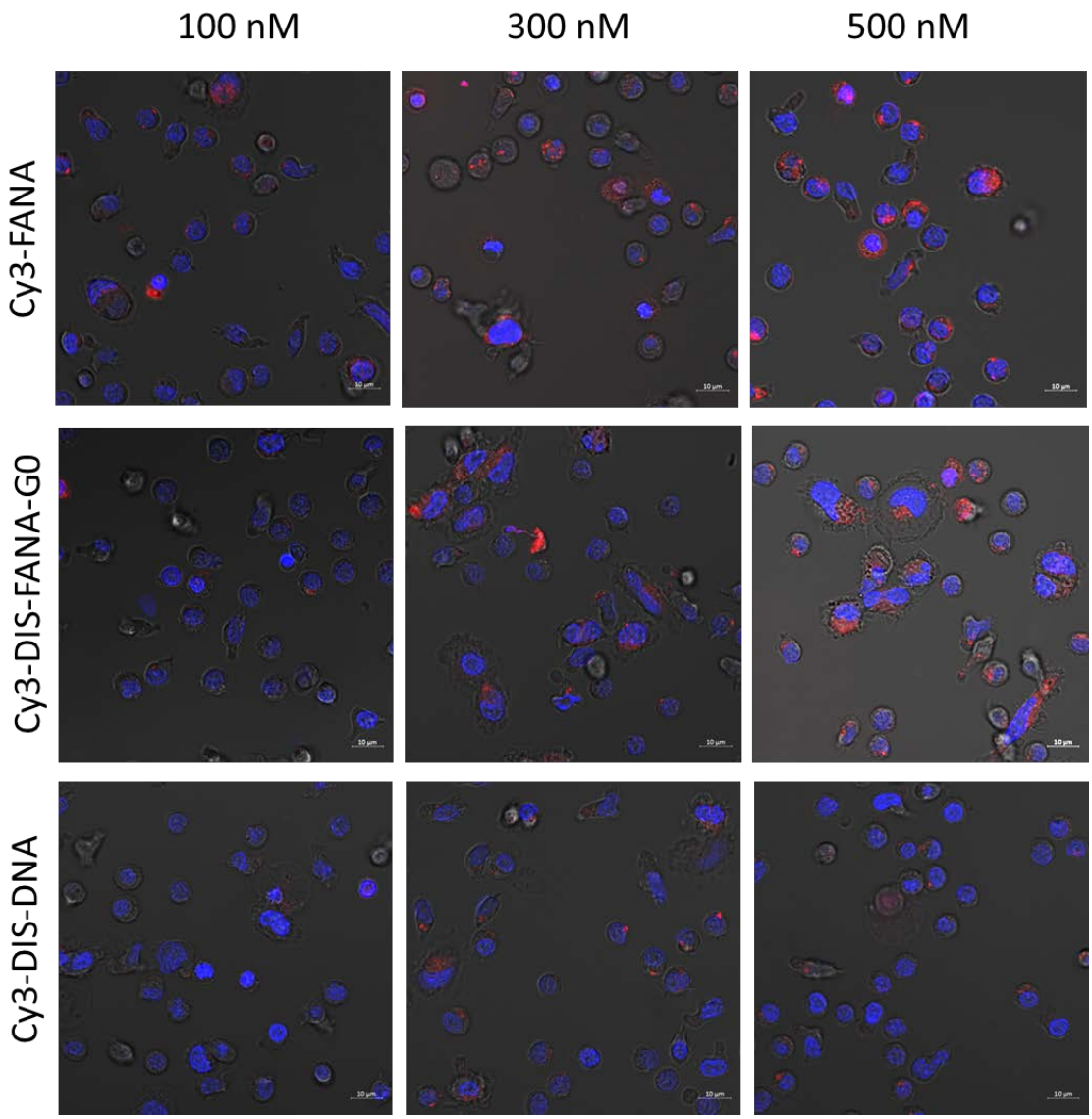


Figure S2. Real-time live-cell confocal microscopy analysis of carrier-free cellular internalization of ASOs in CD8-depleted PBMCs. CD8-depleted PBMCs were incubated with Cy3-labeled ASOs at indicated concentrations for 4 h. Representative images were collected using 40x magnification. Red: Cy3-FANA, Cy3-DIS-FANA-G0, or Cy3-DIS-DNA; Blue: Hoechst 33342.

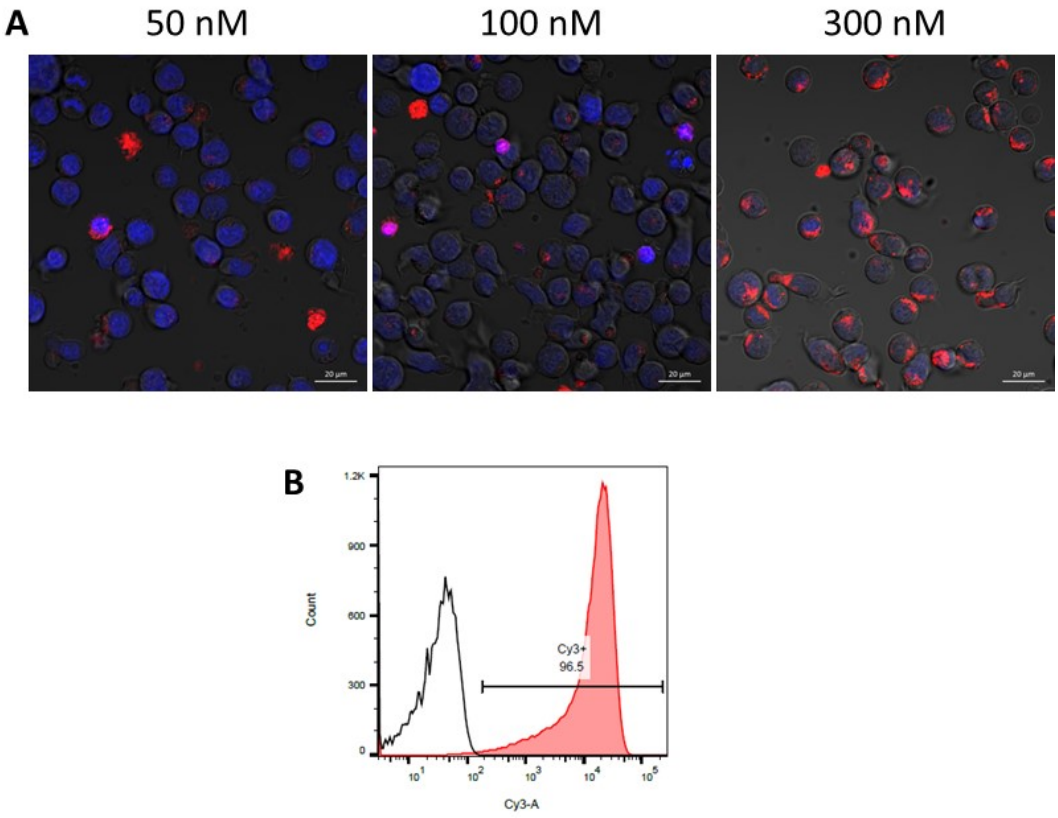


Figure S3. Carrier-free cellular internalization studies of FANA ASO in CCRF-CEM cells. (A) Real-time live-cell confocal microscopy analysis. CCRF-CEM cells were incubated with Cy3-FANA at indicated concentrations for 4 h. Representative images were collected using 40x magnification. Red: Cy3-FANA; Blue: Hoechst 33342. (B) Cellular uptake level of Cy3-FANA assessed by flow cytometry. CEM-CCRF cells were incubated with Cy3-FANA at a concentration of 100 nM in complete media for 4 h. After being washed and stained with DAPI, cells were immediately analyzed by flow cytometry.

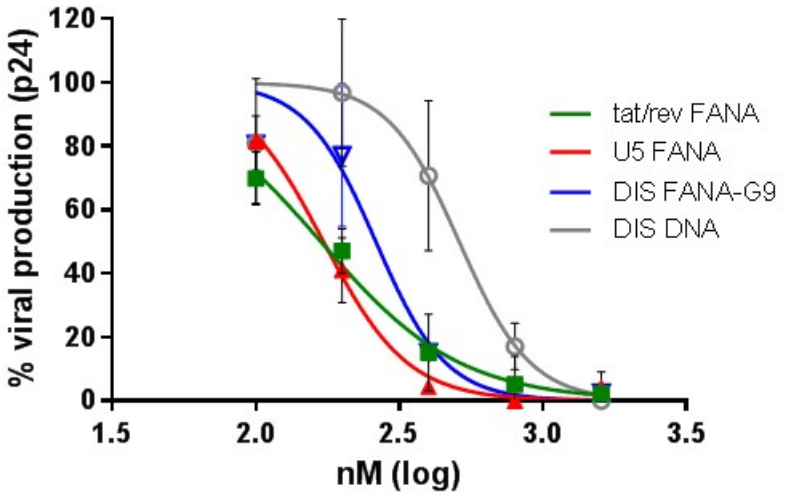


Figure S4. Dose-response curves for anti-HIV ASOs. HIV-1_{NL4-3}-infected PBMC-$CD4^+$ T cells were treated with indicated FANA ASOs at final concentrations of 100 nM, 200 nM, 400 nM, 800 nM, or 1600 nM. Data represent the relative viral production level calculated based on HIV-1 p24 expression. All experiments were performed in triplicate and data show mean values from at least three assays, each of which used cells isolated from different donors. Error bars indicate SD.

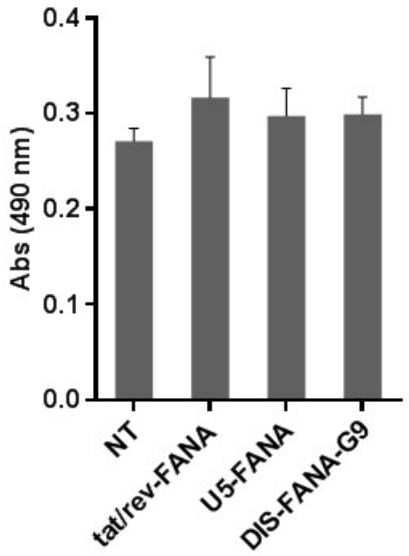
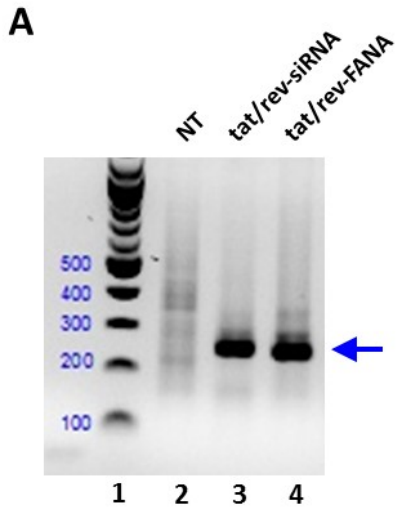


Figure S5. Effect of FANA ASOs on cell proliferation of PBMCs. HIV-1_{NL4-3}-infected PBMC-$CD4^+$ T cells were incubated with indicated FANA ASOs at final concentrations of 3 μM for 10 days. Cytotoxicity was measured using CellTiter 96® AQueous One Solution Cell Proliferation Assay System. All experiments were performed in triplicate and data show mean values and standard deviation from at least three assays, each of which used cells isolated from different donors. Error bars indicate SD.



B

Nucleotide	# of clones	% of clones
A	0	0
G	0	0
A	0	0
C	0	0
A	0	0
G	0	0
C	0	0
G	0	0
A	0	0
C	0	0
G	0	0
A	0	0
A	0	0
G	1	5.56
A	2	11.11
G	2	11.11
C	9	50.00
T	2	11.11
C	1	5.56
A	1	5.56
Total	18	100.0

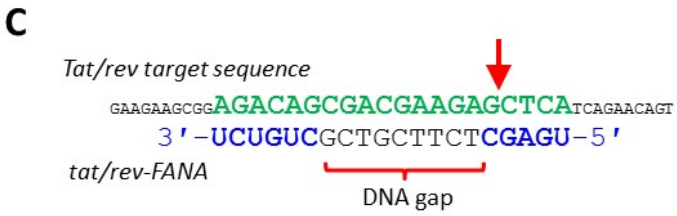


Figure S6. 5'-RACE PCR analysis of RNase H-mediated cleavage of target RNA. Tat/rev siRNA or tat/rev-FANA was co-transfected into HEK293T cells with a Rev-EGFP fusion construct harboring the tat/rev target using Lipofectamine2000. After a 48-h incubation, total RNA was isolated from cells, ligated to a GeneRacer adaptor, and reverse transcribed using a gene-specific primer 1. Nested PCR was performed using primers complementary to the RNA adaptor and gene-specific primer 2. (A) Nested PCR products were resolved in an agarose gel. Lane 1: 100 bp DNA ladder; lane 2: NT; lane 3: tat/rev-siRNA; lane 4: tat/rev-FANA. RNase H-mediated cleavage RNA RACE PCR products are marked with a blue arrow. (B and C) DNA sequence analyses of cloned RACE PCR products. The position of the major site of RNase H-mediated cleavage in the tat/rev target RNA is indicated with a red arrow.

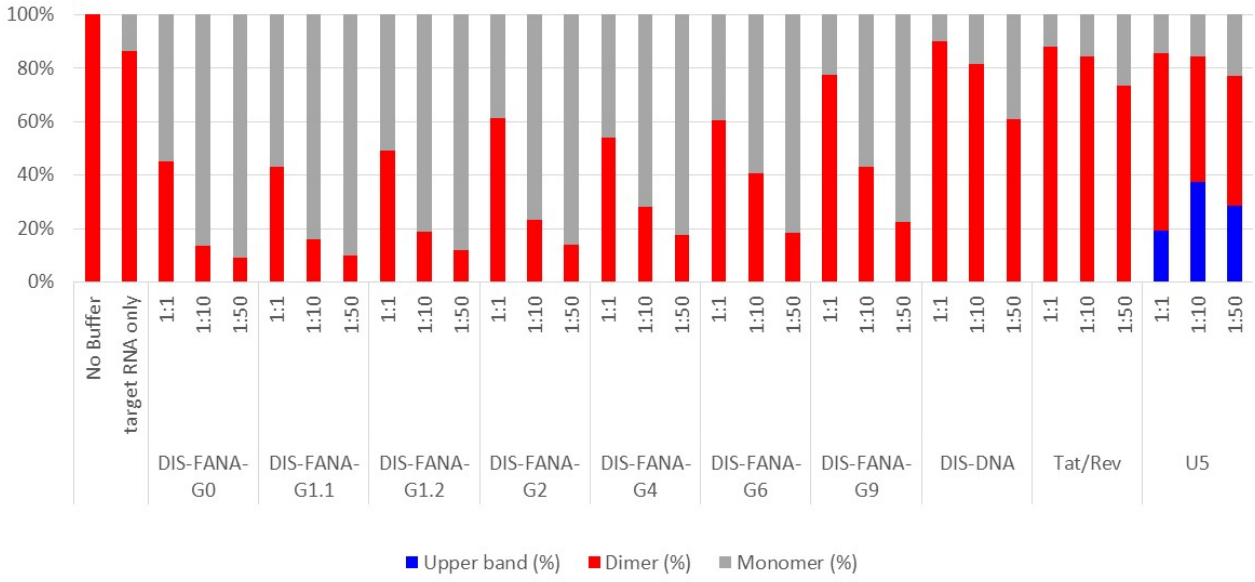


Figure S7. Inhibition of viral RNA dimerization by DIS-FANA ASOs. Dimers and monomers were separated by agarose gel electrophoresis (Figure 6). The bands were quantified and plotted as a ratio of the amount of dimer (red), monomer (grey), and unknown product (upper band; blue) to the total amount of the three products (100%).