SUPPLIMENTARY

Enhancements in the Utilization of Antigene Oligonucleotides in the Nucleus by Booster Oligonucleotides

Lei Wang, Hidenori Okamura, Shigeki Sasaki and Yosuke Taniguchi

Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, 812-8582 Fukuoka, Japan
taniguch@phar.kyushu-u.ac.jp, sasaki@phar.kyushu-u.ac.jp

1. Preparation of the AGOs incorporating ΨdC derivatives

The AGOs or FAM-labeled AGOs incorporating pseudo-dC derivatives were synthesized at 1 μmol scale using 3'-PT-amino modifier C3 CPG (Glen Research) and DNA automated synthesizer (Nihon Techno Service Co., Ltd.) by the standard phosphoramidite chemistry. Cleavage from the resin was accomplished by treating with 28% ammonium hydroxide for overnight at 55°C, followed by reverse-phase HPLC purification (column: Nacalai tesque COSMOSIL 5C18-ARII, 10 x 250 mm, solvents: A: 50 mM NH_4HCO_3 buffer, B: CH_3CN, Gradient: B for 10% to 40%/20 min, flow rate: 3.0 mL/min, UV: 254 nm, column oven: 55°C). The DMTr group was deprotected in 5% aqueous acetic acid at room temperature for 30 min, followed by reverse-phase HPLC purification (HPLC conditions: column: Nacalai tesque COSMOSIL 5C18-ARII, 10 x 250 mm, solvents: A: 50 mM NH_4HCO_3 buffer, B: CH_3CN, B: 5% to 20%/10 min, flow rate: 3.0 mL/min, UV: 254 nm, column oven: 55°C). The concentration of the oligonucleotides were determined by UV measurement. The structural integrity of the synthesized oligonucleotides were analyzed by MALDI-TOF MS measurement using solution of hydroxypicolinic acid and diammonium hydrogen citrate as a matrix.

2. MALDI-TOF Mass analysis of the all oligonucleotides

Table S1. MALDI-TOF mass analysis of the all ODNs (measured in negative mode)

	Calculated $[M-H]^-$	Found (*m*/*z*)
AGO-1 [a]	8433.38	8433.01
FAM-AGO1 [a]	9000.56	8999.76
AGO2 [b]	8853.66	8854.12
FAM-AGO2 [b]	9420.84	9424.02
AGO3 [c]	8917.69	8918.22
FAM-AGO3	9484.82	9487.70
Bst-ODN1 [a]	7462.08	7461.05
Bst-ODN1-Q [a]	8015.26	8015.62
Bst-ODN2 [a]	7546.18	7548.62
Bst-ODN3 [a]	7490.11	7489.51
Bst-ODN4 [a]	7518.15	7519.49
Bst-ODN5 [a]	7546.18	7547.96
ODN(A) [a]	7638.16	7638.05
ODN(A)-Q [a]	8192.33	8190.83

[a] These ODNs were purchased from Japan Bio Services Co.,LTD. (Saitama, JAPAN).

[b] ref. H. Okamura, Y. Taniguchi and S. Sasaki, *Angew. Chem. Int. Ed.* 55, 12445 (2016).

[c] ref. L. Wang, Y. Taniguchi, H. Okamura and S. Sasaki, *Nucleic Acids Res.*, 46, 8679 (2018).

3. T_m values mesurment of duplex DNA between AGOs and Bst-ODNs

One micromolar of each AGO and Bst-ODN was heated to 90 °C for 5 min and annealed by slow cooling in buffer containing 20 mM Tris-HCl, 2.5 mM MgCl2, and 2.5 mM spermidine at pH 7.5. UV melting behavior was monitored at 260 nm between 20 and 90 °C at a rate of 1.0 °C/min using a DU 800 spectrometer (Beckman Coulter), and melting data were analyzed by the program MeltWin (v3.5). The T_m values are summarized in Table S2.

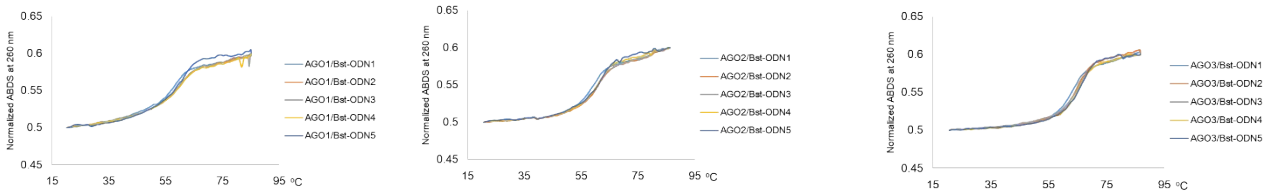


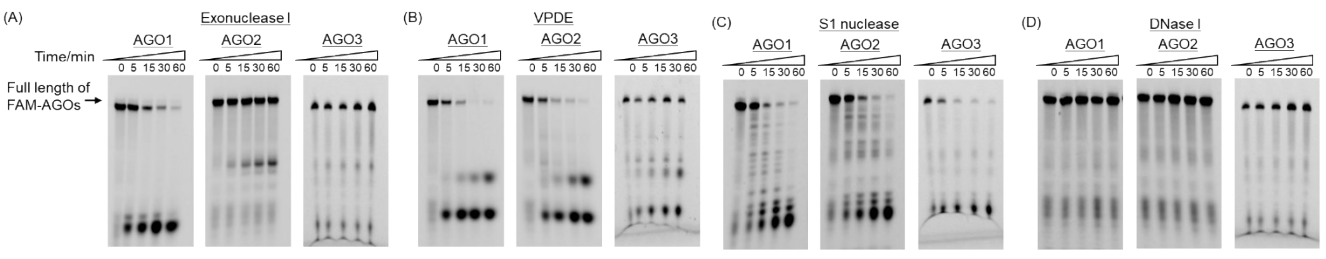
Table S2. T_m values (°C) of duplex formation between AGOs and Bst-ODNs.

	AGO1	AGO2	AGO3
Bst-ODN1	56.6	58.5	62.9
Bst-ODN2	58.6	60.5	64.5
Bst-ODN3	58.2	60.2	64.5
Bst-ODN4	58.5	60.5	64.9
Bst-ODN5	60.7	61.0	65.2

One micromolar of each AGO and Bst-ODN was heated to 90 °C for 5 min and annealed by slow cooling in buffer containing 20 mM Tris-HCl, 2.5 mM MgCl2, and 2.5 mM spermidine at pH 7.5. UV melting behavior was monitored at 260 nm between 20 and 90 °C at a rate of 1.0 °C/min using a DU 800 spectrometer (Beckman Coulter), and melting data were analyzed by the program MeltWin (v3.5). These values are within 0.5 °C as an average value obtained at least three times.

4. Evaluation of enzymatic resistance of single-stranded AGOs and duplex DNA between AGOs and Bst-ODN1 or Bst-ODN5

The single-stranded FAM-labeled AGOs (12 μL, 200 nM) and Bst-ODN1 (12 μL, 200 nM) or Bst-ODN5 (12 μL, 200 nM) were annealed by heating at 90°C for 3 min in the buffer containing 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 2.5 mM spermidine, pH 7.5 and gradually cooling to room temperature. To this solution, nuclease (6.0 μL, Exonuclease, 3 U; VPDE, 0.006 U; S1 nuclease, 0.6 U; DNase I, 0.6 U) was added, and the total volume was adjusted to 60 μL with MilliQ and incubated at 37 °C. The reaction mixture (10 μL) was picked up at the indicated time (0, 5, 15, 30, 60 min) and was terminated by adding formamide containing 20 mM EDTA and heating at 95°C for 5 min. The products were separated by 15% denatured polyacrylamide gel, and electrophoresed for 1 hr at a voltage of 250 V. The bands of FAM-labeled AGOs were visualized and those intensities were quantified using LAS-4000 luminoimage analyzer (FUJIFILM).



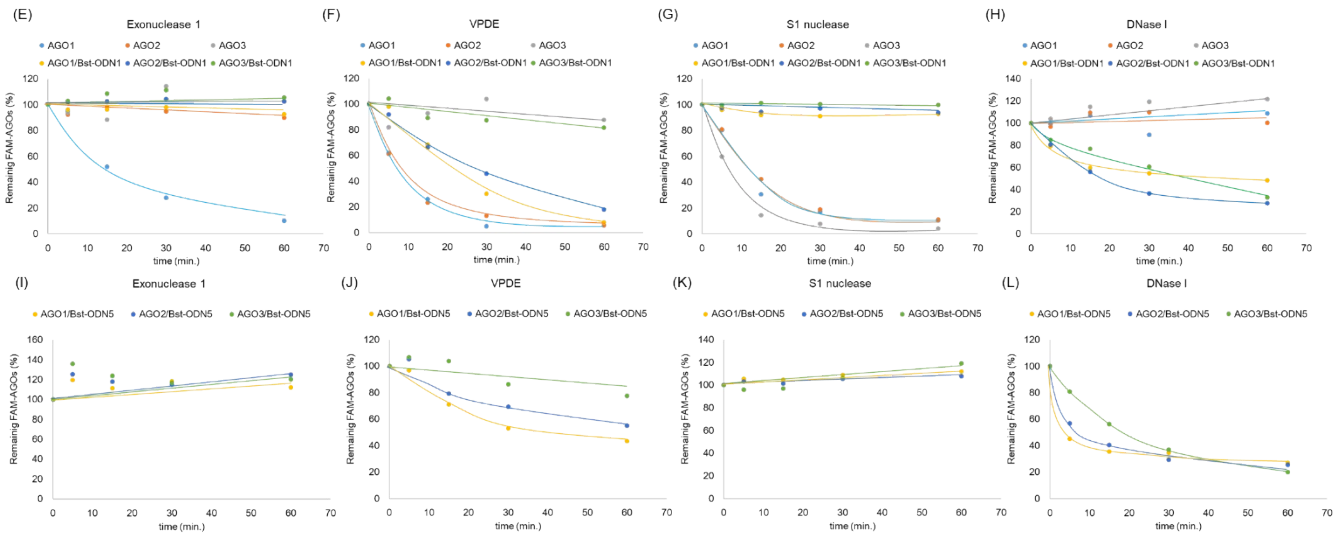


Figure S1. Each FAM-AGOs (200 nM), FAM-AGOs/Bst-ODN1 (200 nM) and FAM-AGOs/Bst-ODN5 (200 nM) were treated with (A) exonuclease I (0.5 U), (B) VPDE (0.001U), (C) S1 nuclease (0.1U), and (D) DNase I (0.1 U) in buffer containing 20 mM Tris-HCl , 2.5 mM $MgCl_2$, and 2.5 mM spermidine at 37 °C and pH 7.5 for 0-60 min. The reaction was stopped by the addition of formamide containing 20 mM EDTA and heating at 95 °C for 5 min, and the mixture was then added to a 15% denaturing polyacrylamide gel. Visualization of the gel was performed using the LAS-4000 luminoimage analyzer (FUJIFILM). The remaining FAM-AGOs were plotted as graphs (E-H) FAM-AGOs and FAM-AGOs/Bst-ODN1, (I-L) FAM-AGOs/Bst-ODN5.

5. Evaluation of free AGOs release assay from the duplex DNA between AGOs and Bst-ODN1 with the treatment of UDG

The single-stranded FAM-labeled AGOs (1.0 μL, 100 nM) and Bst-ODN1 (1.0 μL, 100 nM) was annealed by heating at 90°C for 3 min in the buffer containing 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 2.5 mM spermidine, pH 7.5 and gradually cooling to room temperature. To this solution, UDG enzyme (0.5 U/μL, 1 μL) was added and the total volume was adjusted to 10.0 μL with MilliQ and incubated 5 min and 30 min at 37°C. UDG inhibitor (1 U/μL, 1 μL) was added to each solution, followed by loading buffer (9.0 μL) and applied to a 10% non-denatured polyacrylamide gel and electrophoresed for 2 hrs at a voltage of 130 V. The bands of FAM-labeled AGOs were visualized and their intensities were quantified using LAS-4000 luminoimage analyzer (FUJIFILM).

6. Evaluation of triplex formation in the mixture of the AGOs/Bst-ODNs complex and target hTERT gene in the presence of UDG enzyme

The duplex formed by single-stranded AGOs (1.0 μL, 100 nM) and Bst-ODNs (1.0 μL, 100 nM) was annealed by heating at 90°C for 3 min in the buffer containing 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 2.5 mM spermidine, pH 7.5 and gradually cooling to room temperature. The target hTERT duplex formed by Pu strand (1.0 μL, 100 nM) and FAM-labeled Py strand (1.0 μL, 100 nM) was annealed by heating at 90 °C for 3 min in the buffer containing 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 2.5 mM spermidine, pH 7.5 and gradually cooling to room temperature. After mixing duplex DNA solution and target hTERT duplex, UDG enzyme (1.0 μL, 0.5 U, 1.0 U and 2.5 U) was added to the mixture and the total volume was adjusted to 10.0 μL with MilliQ. The UDG enzyme was replaced with water (1.0 μL) as the control. The reaction mixture was incubated for the indicated time (2 hrs or 12 hrs). Loading buffer (10 μL) was added to each solution and applied to a 10% non-denatured polyacrylamide gel and electrophoresed at 4°C for 4 hrs at a voltage of 130 V. The bands of FAM-labeled Py strand of target hTERT duplex were visualized using LAS-4000 luminoimage analyzer (FUJIFILM).

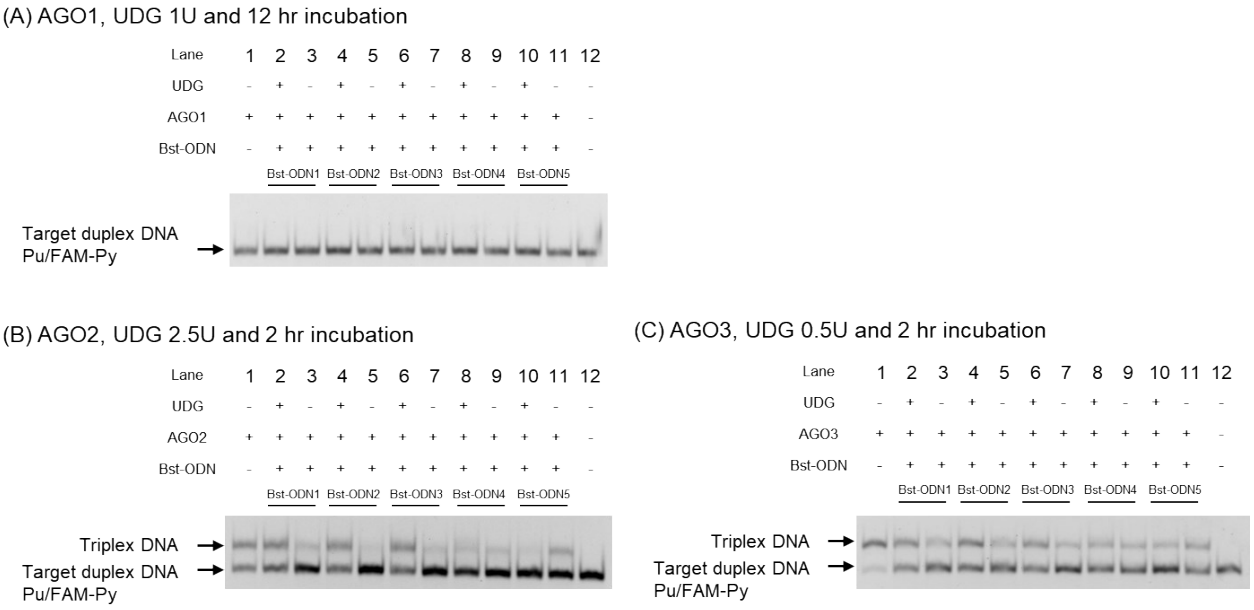


Figure S2. Conformation of triplex DNA formation in the presence or absence of UDG for (A) AGO1/Bst-ODNs, (B) AGO2/Bst/ODNs, and (C) AGO3/Bst-ODNs. AGOs/Bst-ODNs (100 nM) and the FAM-labeled target

hTERT duplex (100 nM) were mixed in buffer containing 20 mM Tris-HCl , 2.5 mM $MgCl_2$, and 2.5 mM spermidine at pH 7.5. The UDG enzyme was added to the solution, and the UDG enzyme was replaced with water as the control. The reaction mixture was incubated at 37 °C for the indicated time and analyzed on a 10% non-denatured polyacrylamide gel. Visualization of the gel was performed using the LAS-4000 luminoimage analyzer (FUJIFILM).

7. Cell Culture

HeLa cells were grown in the complete medium (DMEM containing 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin). Cells were cultured in a 5% CO_2 humidified incubator at 37˚C. Cells were harvested by treating with 0.1% trypsin (Gibco, diluted with PBS) and passaged once in 4 days.

8. Fluorescence microscopy measurement of the HeLa cells transfected with the FAM-labeled AGOs or FAM-AGOs/Bst-ODN1-Q complex

HeLa cells (1 x 10^4 cells) were seeded onto 35 mm glass bottom culture dishes (IWAKI) and grown in the complete medium 24 hrs before transfection. The fresh medium (DMEM containing 20 % FBS, without antibiotics, 100 μL) was replaced and cells were transfected with 200 nM of AGOs or 200 nM of duplex between AGOs and Bst-ODN1 using Calcium phosphate transfection reagent (CalPhos™ Mammalian Transfection Kit, Clontech, 100 μL). After 6 hrs, the cells were washed with PBS and incubated in a complete medium containing 1.0 μg of Hoechst 33258 for 10 min. The medium was substituted with DMEM without Phenol Red, and cells were immediately analyzed using a fluorescence microscope (EVOS, FL). Hoechst 33258: λ_{ex} = 360nm, λ_{em} = 447 nm; FAM: λ_{ex} = 470 nm, λ_{em} = 525 nm. Image data was processed and analyzed using ImageJ.

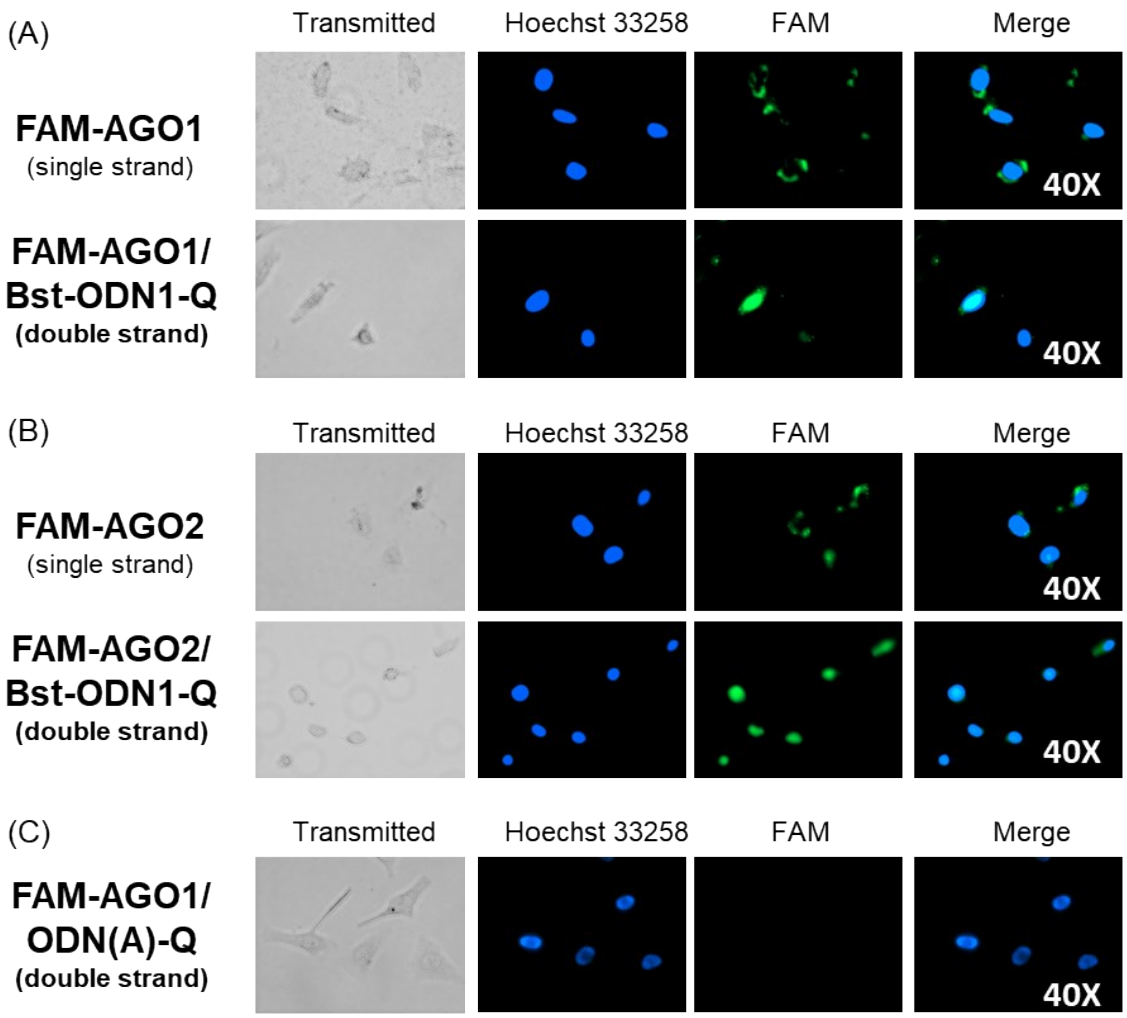


Figure S3. Confirmation of the uptake of (A) FAM-AGO1 and FAM-AGO1/Bst-ODN1-Q, (B) FAM-AGO2 and FAM-AGO2/Bst-ODN1-Q, and (C) FAM-AGO1/ODN(A)-Q into cultured cells by fluorescence microscopy. HeLa cells (1×10^4 cells) were seeded on 35-mm glass-bottomed culture dishes (IWAKI) and grown in medium 24 hr before transfection. After the removal of medium, FAM-AGOs (200 nM) or FAM-AGOs/Bst-ODN1-Q (200 nM) was transfected with a calcium phosphate transfection reagent at 37°C for 6 hr in a 5% CO_2 humidified incubator. After the treatment with Hoechst 33258, cells were immediately analyzed using a fluorescence microscope. The green color represents single-stranded AGO3 labeled with FAM, and the blue color nucleus staining with Hoechst 33258. Hoechst 33258: λ_{ex} = 360 nm, λ_{em} = 447 nm; FAM: λ_{ex} = 470 nm, λ_{em} = 525 nm.

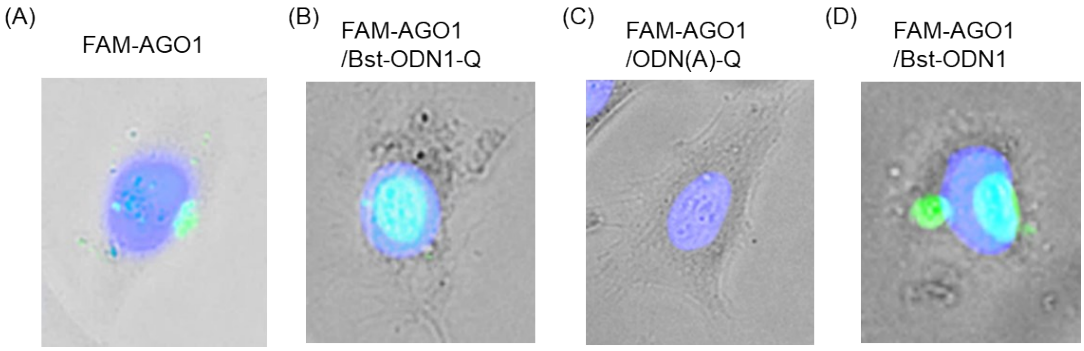


Figure S4. Confirmation of the uptake of (A) FAM-AGO1, (B) FAM-AGO1/Bst-ODN1-Q, (C) FAM-AGO1/ODN(A)-Q, and (D) FAM-AGO1/Bst-ODN1 into cultured cells by fluorescence microscopy. See the footnote of Figure S3 for details.

9. Intracellular inhibition of *hTERT* gene expression using AGOs, Bst-ODNs and the AGOs/Bst-ODNs complex

HeLa cells (1 x 10^4 cells/well) were seeded onto 24-well plate and grown in the complete medium 24 hrs before transfection. The fresh medium (DMEM containing 20% FBS, without antibiotics, 100 μL) was replaced and HeLa cells were transfected with single-stranded AGOs (100 nM), single-stranded Bst-ODNs (100 nM) or duplex DNA between AGOs and Bst-ODNs (100 nM) using Calcium phosphate transfection reagent (CalPhos™ Mammalian Transfection Kit, Clontech, 100 μL). For the control, HeLa cells were incubated with transfection reagent without the any oligonucleotide. After 6 hrs incubation at 37°C, the complete medium (DMEM containing 10 % FBS and antibiotics, 1.0 mL) was replaced and further incubated at 37°C in a 5 % CO_2 humidified incubator for additional 24 hrs. Total RNA was extracted from cells by RNeasy Mini Kit (Qiagen), and reverse transcribed to cDNA using Verso cDNA Synthesis Kit (Thermo Fisher Scientific). The cDNA (20 ng), a pair of primers (500 nM), TaqMan probe (250 nM) and TaqMan Fast Advanced Master Mix (ABI) were mixed together and subjected to real-time PCR using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The sequences of primers and TaqMan probes are previously reported (H. Okamura, Y. Taniguchi and S. Sasaki, *Angew. Chem. Int. Ed*. 2016, 55, 12445-12449). The PCR cycles are as follows: polymerase activation: 95°C for 20 sec, annealing: 95°C for 5 sec, extension: 60°C for 30 sec, and the reaction was performed for 45 cycles. The threshold cycles (Ct) of each samples were normalized to that of GAPDH gene. The gene expression level was calculated by the *ΔΔ*Ct method by comparing the *Δ*Ct of each sample with the *Δ*Ct of the control, and normalized against the gene expression level of the cells treated with transfection reagent only. All experiments are performed in triplicate. The relative gene expression inhibition rate were obtained by normalizing that of inhibition efficiency of AGO1.