

*Supporting Information to*  
**Topological capture of mRNA for silencing gene expression**

**Table of Contents**

- **General**
- **Oligonucleotides Synthesis**
- **Organic Synthesis**
- **NMR Charts**
- **Chemical Modification of Oligonucleotides**
- **TCF Reactions**
- **MALDI-TOF MS Measurements**
- **Design of Reactive ODNs**
- **TCF Reaction on RNA Template**
- **RNase H Assay**
- **Knocking Down Experiments in Cells**
- **References**

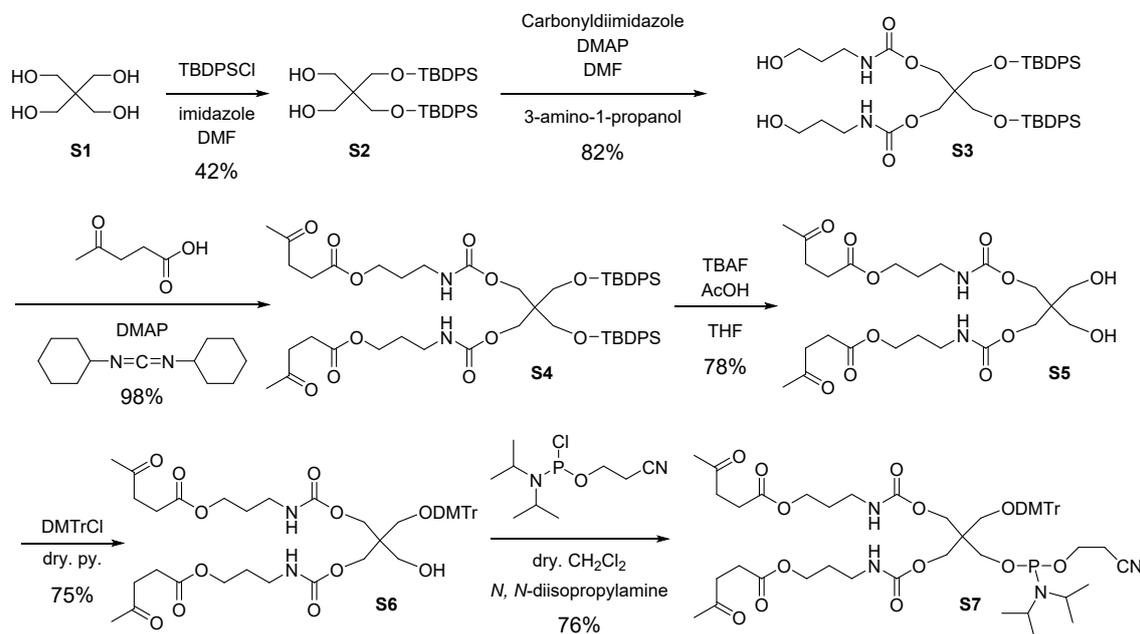
## ■ General

General reagents were purchased from Wako Pure Chemical Industries, Ltd. (Japan), Kanto Chemical Co., Ltd. (Japan), and Tokyo Chemical Industry Co., Ltd (Japan). The phosphoramidite and CPG used for DNA solid phase synthesis was purchased from Glen Research (USA) or ChemGenes (USA). NMR spectra were recorded on ECS-400 (JEOL, Japan). Chemical shifts were reported in the scale relative to the solvent used as an internal reference for  $^1\text{H}$  ( $\delta = 7.26$  ppm for  $\text{CDCl}_3$ ) and  $^{13}\text{C}$  NMR ( $\delta = 77.00$  ppm for  $\text{CDCl}_3$ ). Multiplicity of signals was indicated using the abbreviations s: singlet, d: doublet, t: triplet, m: multiplet, br: broad, respectively. ESI-mass spectra were measured using micrOTOF-QII (ESI) (Bruker). ESI Tuning Mix (Agilent Technologies) was used for calibration. Analytical and preparative HPLC was carried out using LaChrom Elite HPLC System (L-2455, L-2130, L-2485, HITACHI Co., Japan) and reversed-phase C18 columns; HS12S05-2546WT or HS12S05-2510WT (YMC, Japan). The concentration of oligonucleotide solution was measured by NanoDrop 2000 (Thermo Scientific, USA). MALDI-TOF MS measurement was performed using Bruker Ultraflex III-MALDI TOF / TOF Mass Spectrometer, and 3-hydroxy picolinic acid and diammonium hydrogen citrate were used as matrixes. For desalting of MALDI-MS samples, Zip Tip U-C18 (Merck Millipore Co., USA) was used, if necessary. Photographing and quantitative analysis of the polyacrylamide gels after electrophoresis were carried out with ChemiDoc XRS system (Bio-Rad, USA).

## ■ Oligonucleotides Synthesis

Synthesis of oligo DNA was synthesized at 0.2  $\mu\text{mol}$  or 1.0  $\mu\text{mol}$  scale by DNA / RNA synthesizer MR-2A 7 MX (Nihon Techno Service Co., Japan) or H-8 SE (Gene World, Tokyo, Japan). For phosphorothioate DNA synthesis, sulfurizing reagent (ChemGenes, RN-1535) or sulfurizing reagent II (Glen Research, 40-4037) was used. For hexaethylene glycol unit, DMT Hexaethylene Glycol phosphoramidite (ChemGenes, CLP-9765) was used. For introduction of fluorescein unit at 5' terminus and 3' terminus, 6-FAM phosphoramidite (ChemGenes, CLP-9777) and 6-Fluorescein Phosphoramidite (Glen Research, 10-1964) were used respectively. Synthesis of the branched DNAs was carried out using Lev-protected branching phosphoramidites; Asymmetrical branching CED phosphoramidite (ChemGenes, CLP-7169) for single branching, and **S7** for double branching, which was synthesized from hexaglycerol. Removal of Lev group was performed by treating the CPGs with a 0.5 M hydrazine in pyridine: AcOH = 4 : 3 solution at room temperature for 15 minutes. For ODNs having a primary amino group at the terminus, CPGs were treated with 10% diethylamine/acetonitrile solution for 5 minutes before the cleavage of ODNs from the CPG. For the deprotection and cleavage of ONs, CPG was extruded into a 1.5 mL loop-attached screw cap (Sarstedt, Germany) and was treated with the UltraFast method ( $\text{NH}_3$ : 40% methylamine = 1: 1, 65 ° C, 15 min). Deprotection was performed under conditions recommended by the manufacture (Glen Research), if there was restriction due to chemically modified phosphoramidites. For purification of the ON whose synthesis was terminated with 5'-DMTr group on, purification and deprotection of DMTr group were carried out using MicroPure II columns (BioResearch Technologies, USA). After the deprotection, the sample was concentrated by a centrifugal evaporator (Centrifugal Evaporator CVE-3100, EYELA Co. Japan), then purified by a reversed phase HPLC.

## Organic Synthesis



**Scheme S1.** Synthetic route of the branching phosphoramidite **S7**

**S3** was synthesized based on the reported protocols.<sup>1</sup>

### 8,8-bis(((*tert*-butyldiphenylsilyl)oxy)methyl)-5,11-dioxo-6,10-dioxo-4,12-diazapentadecane-1,15-diyl bis(4-oxopentanoate) (**S4**)

Levulinic acid (1.96 mL, 19.2 mmol), DCC (4.86 g, 20.6 mmol) and DMAP (308 mg, 2.50 mmol) were added to dehydrated dioxane (50.0 mL) and stirred at room temperature. After 15 minutes, a dehydrated dioxane solution (40.0 mL) of compound **S3** (4.86 g, 5.96 mmol) was added. After 17 hours, removal of the insoluble material by Celite filtration, concentration under reduced pressure were performed and the resulting mixture was dissolved in AcOEt. The residue was purified by neutral flash column chromatography (SiO<sub>2</sub>, Hexane/AcOEt = 1/1-1/3). Partially purified compound **S4** was obtained in an amount of 5.88 g, ca. 5.81 mmol (ca. 97% yield), which was used for the next reaction without further purification.

### 8,8-bis(hydroxymethyl)-5,11-dioxo-6,10-dioxo-4,12-diazapentadecane-1,15-diyl bis(4-oxopentanoate) (**S5**)

Compound **S4** (5.88 g, ca. 5.81 mmol) was dissolved in THF (50.0 mL). A mixed solution of 1 M TBAF THF solution (14.0 mL, 14.0 mmol) and AcOH (1.60 mL, 27.9 mmol) was added, and the mixture was stirred at room temperature overnight. It was concentrated under reduced pressure and dissolved in AcOEt. The residue was purified by neutral flash column chromatography (SiO<sub>2</sub>, DCM/MeOH = 20/1-10/1) to afford compound **S5** (1.91 g, 3.57 mmol, 78%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.58-5.52 (m, 2H) 4.16-4.00 (m, 8H), 3.56-3.36 (brs, 4H), 3.24-3.15 (m, 4H), 2.74-2.69 (t, *J* = 6.6 Hz, 4H), 2.52-2.46 (t, *J* = 6.6 Hz, 4H), 2.14 (s, 6H), 1.82-1.72 (m, 4H), <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>) δ 207.20,

173.06, 157.40, 62.84, 62.00, 61.82, 45.85, 38.04, 37.92, 29.88, 28.84, 28.00, HRMS (ESI+)  $m/z$  calcd. for  $C_{23}H_{39}N_2O_{12}$ ,  $[M+H]^+$  535.2498, found 535.1740.

**8-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-8-(hydroxymethyl)-5,11-dioxo-6,10-dioxo-4,12-diazapentadecane-1,15-diyl bis(4-oxopentanoate) (S6)**

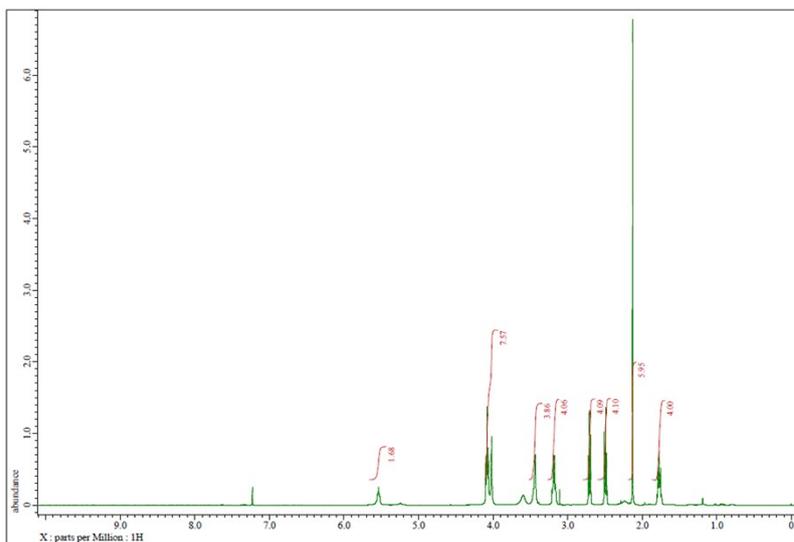
Compound **S5** (1.71 g, 3.21 mmol) was dissolved in dehydrated pyridine, to which was added 856  $\mu$ L (4.28 mmol, 1.2 eq.) of 0.5 M DMTrCl in dehydrated pyridine, and the mixture was stirred overnight. The mixture was concentrated *in vacuo*, dissolved in AcOEt and washed with saturated aqueous  $NaHCO_3$  and saturated brine. The separated organic phase was dried over  $Na_2SO_4$  and concentrated to dryness. The residue was purified by neutral flash column chromatography ( $SiO_2$ , Hexane/AcOEt = 4/1 (with 0.5% TEA) to give compound **S6** in 2.01 g, 2.40 mmol, 75% yield.  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  7.51-7.15 (m, 9H), 6.74-6.81 (m, 4H), 5.10-4.90 (m, 2H), 4.22-4.06 (m, 8H), 3.77 (s, 6H), 3.44 (brs, 2H), 3.27-3.05 (m, 6H), 2.74 (t,  $J = 6.4$  Hz, 4H), 2.54 (t,  $J = 6.4$  Hz, 4H), 2.16 (s, 6H), 1.85-1.75 (m, 4H),  $^{13}C$ -NMR (100 MHz,  $CDCl_3$ )  $\delta$  206.93, 172.97, 158.51, 156.78, 144.72, 135.88, 130.17, 128.25, 127.87, 126.84, 113.15, 86.15, 63.47, 61.98, 61.88, 61.40, 55.27, 45.15, 38.03, 37.89, 29.88, 29.00, 28.00. HRMS (ESI+) calcd. for  $C_{44}H_{56}N_2O_{14}$ ,  $[M+Na]^+$  859.3624, found 859.3053

**8-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-8-(((2-cyanoethoxy)(diisopropylamino)phosphanyl)oxy)methyl)-5,11-dioxo-6,10-dioxo-4,12-diazapentadecane-1,15-diylbis(4-oxopentanoate) (S7)**

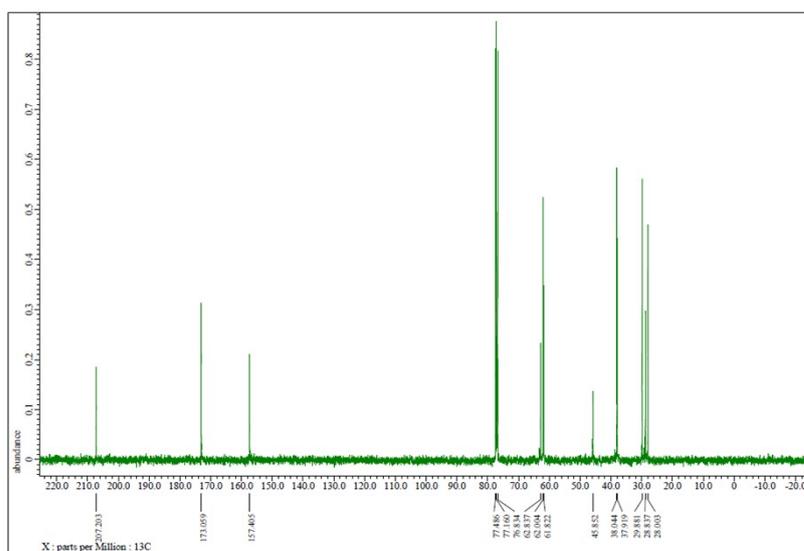
Compound **S6** (0.77 g, 0.93 mmol) was dissolved in toluene and azeotroped three times and then dissolved in dehydrated dichloromethane (20.0 mL). *N,N*-diethylisopropylamine (1.00 mL, 7.10 mmol) and CEP-Cl (383  $\mu$ L, 1.73 mmol) were added and the mixture was stirred at room temperature for 1.5 h. The reaction solution was washed twice with saturated aqueous  $NaHCO_3$  solution and the separated organic phase was dried over  $Na_2SO_4$  and concentrated to dryness. The residue was purified by neutral flash column chromatography ( $SiO_2$ , Hexane / AcOEt = 1/3 (with 0.5% TEA)) to afford 0.73 g of compound **S7** (0.70 mmol, 76% yield).  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  7.45-7.15 (m, 9H), 6.94-6.58 (d,  $J = 8.8$  Hz 4H), 5.05-4.92 (brs, 2H), 4.20-4.05 (m, 8H), 3.87 (s, 6H) 3.78-3.60 (m, 2H), 3.58-3.45 (m, 2H), 3.25-3.10 (m, 4H), 2.79-2.70 (t,  $J = 6.8$  Hz, 4H), 2.60-2.52 (m, 6H), 2.18 (s, 6H), 1.85-1.75 (m, 4H), 1.19-1.11 (d,  $J = 6.8$  Hz, 6H), 1.11-1.05 (d,  $J = 6.4$  Hz, 6H),  $^{31}P$ -NMR (161 MHz,  $CDCl_3$ )  $\delta$  148.32,  $^{13}C$ -NMR (100 MHz,  $CDCl_3$ )  $\delta$  206.82, 172.96, 158.47, 158.38, 136.07, 130.28, 128.36, 127.82, 126.78, 113.09, 85.95, 63.76, 62.07, 55.30, 43.26, 43.14, 38.07, 37.82, 29.92, 29.1, 28.04, 24.73, 24.66, 20.49, HRMS (ESI+) calcd. for  $C_{53}H_{73}N_4O_{15}P$   $[M+Na]^+$  1059.4702, found 1059.4877  $[M+Na]^+$ .

## ■ NMR Charts

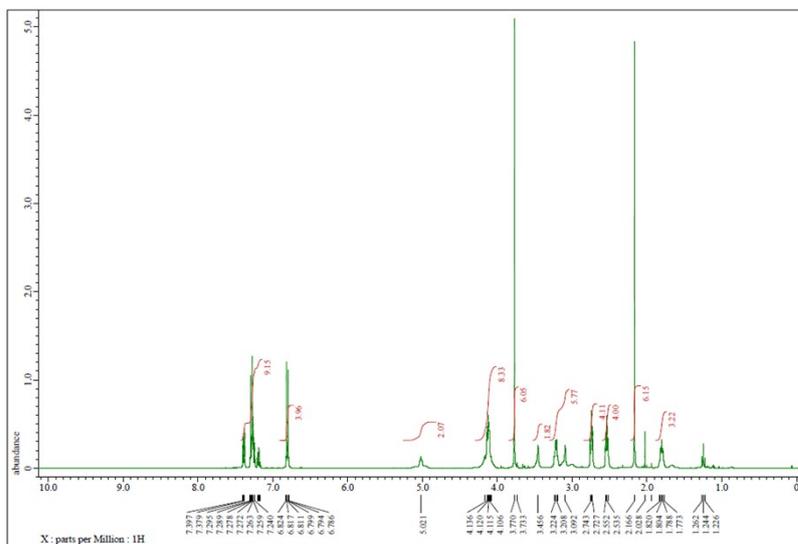
### S5 $^1\text{H}$ NMR



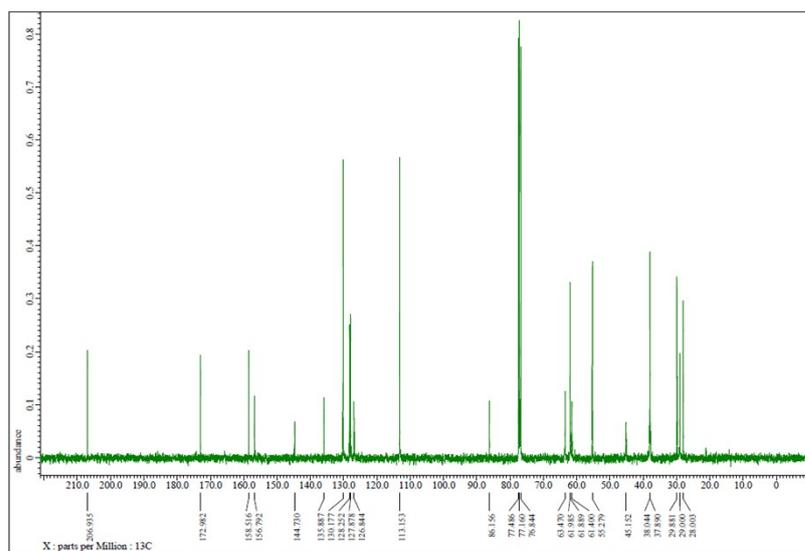
### S5 $^{13}\text{C}$ NMR



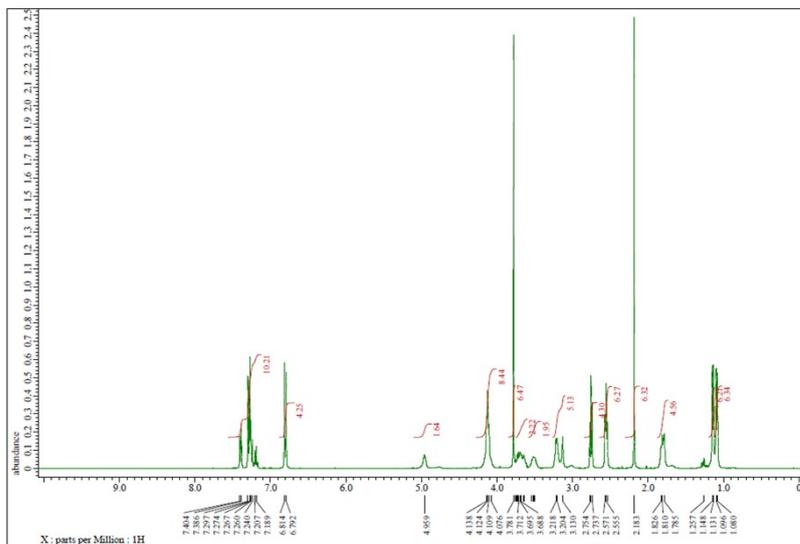
### S6 <sup>1</sup>H NMR



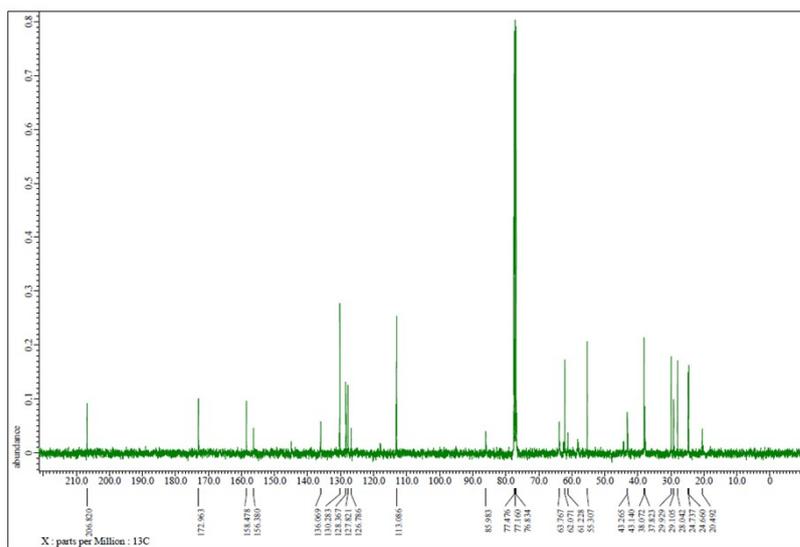
### S6 <sup>13</sup>C NMR

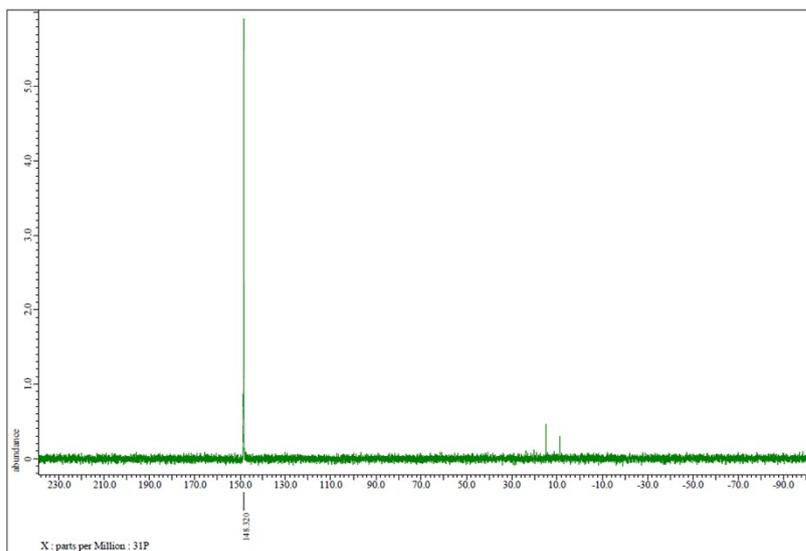


### S7 <sup>1</sup>H NMR



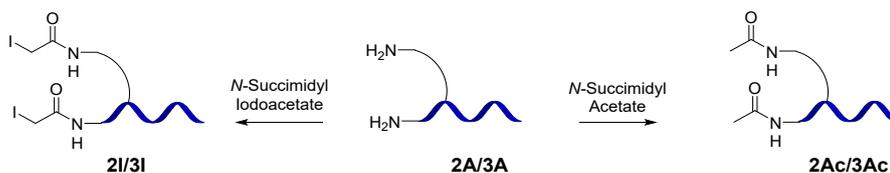
### S7 <sup>13</sup>C NMR





S7  $^{31}\text{P}$  NMR

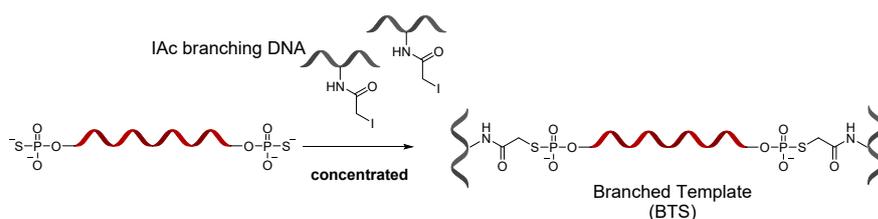
## ■ Chemical Modification of Oligonucleotides



### ● Preparation of Acetyl and IAc-modified amino-ONs

**IAc-ONs;** To a solution containing NH<sub>2</sub>-DNA (final conc.; 100 μM) in sodium borate buffer (final conc.; 50 mM, pH 8.5), IAc NHS ester DMF solution (final conc.; 10 mM) was added and the mixture was incubated at 37 ° C for 2 hours (total in 20 μL volume). HPLC purification with a reversed phase column was performed and acetonitrile was removed under the reduced pressure. Desalting was performed with Amicon Ultra 3K - 0.5 mL (Merck Millipore).

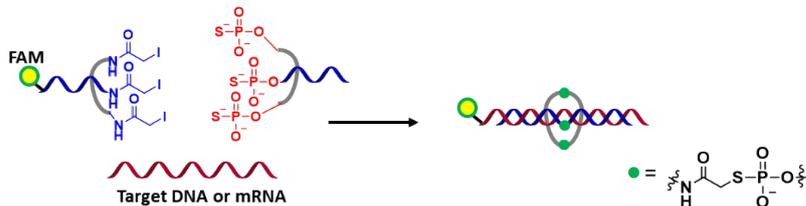
**Ac-ONs;** To a solution containing NH<sub>2</sub>-DNA (final conc.; 100 μM) in sodium borate buffer (final conc.; 50 mM, pH 8.5), acetyl NHS ester in DMF (final conc.; 10 mM) was added and the mixture was incubated at 37 ° C for 2 hours (total in 20 μL volume). HPLC purification with a reversed phase column was performed and acetonitrile was removed under the reduced pressure. Desalting was performed with Amicon Ultra 3K - 0.5 mL (Merck Millipore).



### ● Preparation of Branched Template Strand

Doubly PS-terminated template DNA (final conc.; 200 μM) was mixed with IAc-branching DNA (final conc.; 500 μM), in sodium borate buffer (final conc.; 50 mM, pH 8.5) containing DTT (final conc.; 0.05 mM). The reaction was carried out in 500 μL solution. The volatiles were removed under the reduced pressures to dryness with a centrifugal evaporator and the resulting mixture was dissolved in Milli-Q (500 μL). This operation was repeated 5 times, and the target branching template was isolated with 15% denaturing polyacrylamide gel electrophoresis (25% formamide and 7.5 M urea). The target ON band was cut out and the target oligonucleotide was extracted by the crush & soak method overnight (Elution; MQ). Desalting and concentration were performed with Amicon Ultra 10K-0.5 mL (Merck Millipore).

## ■ TCF Reactions



### ● TCF reaction targeting DNA, RNA or mRNA *in vitro*

The target ON (DNA or RNA template (final conc.; 1.5  $\mu\text{M}$ ) or AcGFP mRNA (0.45  $\mu\text{g}/\mu\text{L}$ )) and PS-DNA (final conc. 3.0  $\mu\text{M}$ ), were added to a phosphate buffer (final conc.; 20 mM, pH 7.0) containing NaCl (final conc.; 100 mM),  $\text{MgCl}_2$  (final conc.; 20 mM), DTT (final conc.; 200  $\mu\text{M}$ ). After heating at 90 °C for 3 minutes, the mixture was slowly cooled down to the room temperature. To this was added fluorescein-labelled IAc-ON (final conc.; 1.0  $\mu\text{M}$ ) and the mixture (total 20  $\mu\text{L}$  in volume) was incubated at 37 °C for 1 hour. After the reaction, an equivalent amount of 2 × loading buffer was added and 10% denaturing polyacrylamide gel electrophoresis containing 6.5 M urea, or 8% denaturing polyacrylamide gel electrophoresis was carried out at 4 °C. Detection of the ONs was carried out based on fluorescence signal or SYBR Green II staining (Lonza, Cat #: 50523). For targeting DNA, MALDI-TOF MS measurement of the reaction solution was carried out and the structure was confirmed.

### ● Preparation of AcGFP mRNA

Using AcGFP1 vector (Z2468N, Takara, Japan) and primers (forward, CCCGGATCC TAATACGACTCACTATAGGGAGACGTACCGGTCGCCACCAT, reverse; GCCGCTCA CTTGTACAGCTC), PCR was performed to obtain template DNA for AcGFP mRNA (PrimeSTAR HS DNA polymerase, R010A, Takara, Japan; 94 °C, 2.5 min; 30 cycles of “94 °C, 30 sec; 55 °C, 30 sec; 72 °C, 60 sec”, 72 °C, 7min). The DNA samples was roughly isolated by phenol-chloroform extraction and isopropanol precipitation. After separated with 1.5% agarose gel electrophoresis, the corresponding DNA band was cut out and the DNA was isolated with QIAquick Gel Extraction Kit (28704, Qiagen, USA) by following the manufacture’s protocols. Using the template DNA, transcription was performed with MEGascript T7 High Yield Transcription Kit (Ambion, P/N AM1334) by following the manufacture’s protocols. After phenol-chloroform extraction, the RNA samples were isolated by isopropanol precipitation.

### ● RNase H assay use recombinant Human RNase H1 (ab153634)

**Natural antisense (NA):** AcGFP1 mRNA (final conc.; 25 ng/ $\mu\text{L}$ ) and ASO (NA; final conc.; 3.0  $\mu\text{M}$ ) were added to the tris-HCl buffer (final conc.; 60 mM, pH 7.5) containing KCl (final conc.; 60 mM),  $\text{MgCl}_2$  (final conc.; 2.5 mM) and DTT (final conc.; 2 mM). After heating at 90 °C for 3 minutes, the mixture was slowly cooled down to room temperature. To the mixture, human RNase H1 (abcam, ab153634, final conc.; 3.5 ng/ $\mu\text{L}$ ) was added and the solution was incubated at 37°C for 30 minutes. The reaction was quenched by adding 5  $\mu\text{L}$  of 0.5 M EDTA. After the reaction, an equivalent amount of 2× loading buffer was added and After the reaction, an equivalent amount of 2× loading buffer was added, and the mRNA was analyzed by 10% dPAGE (6.5 M urea, stained with SYBR Gold and Low Range ssRNA

Ladder as a marker).

**TCF system:** AcGFP1 mRNA (final conc.; 25 ng/ $\mu$ L) and PS-DNA (final conc.; 3.0  $\mu$ M) were added to the tris-HCl buffer (final conc.; 60 mM, pH 7.5) containing KCl (final conc.; 60 mM),  $MgCl_2$  (final conc.; 2.5 mM) and DTT (final conc.; 2 mM). After heating at 90 °C for 3 minutes, the mixture was slowly cooled down to room temperature. To this was added IAc-ON (final conc.; 3.0  $\mu$ M) and the mixture was incubated at 37 °C for 1 hour. Then, human RNase H1 (abcam, ab153634, final conc.; 3.5 ng/ $\mu$ L) was added and the mixture was incubated at 37°C for 30 minutes. The reaction was quenched by adding 5  $\mu$ L of 0.5 M EDTA. After the reaction, an equivalent amount of 2 $\times$  loading buffer was added, and the mRNA was analyzed by 10% dPAGE (6.5 M urea, stained with SYBR Gold and Low Range ssRNA Ladder as a marker).

- **Evaluation of thermal stability of the topological complexes**

AcGFP1 mRNA (final conc.; 0.50  $\mu$ g/ $\mu$ L) and PS-DNA (final conc.; 3.0  $\mu$ M) were added to the phosphate buffer (final conc.; 20 mM, pH 7.0) containing NaCl (final conc.; 100 mM). After heating at 90 °C for 3 minutes, the mixture was cooled down to room temperature. Solution of fluorescein-labelled IAc-DNA (final conc.; 1.5  $\mu$ M) was added and the mixture was incubated at 37 °C for 1 hour (total 20  $\mu$ L in volume). The mixture was mixed with 10  $\times$  loading buffer, and the sample was applied to 5% polyacrylamide gel. Electrophoresis was carried out using a perpendicular temperature gradient gel electrophoresis apparatus (Micro TG, TAITEC) at a constant current of 12 mA for 10 minutes with a linear temperature gradient from 20 °C to 60 °C. ONs were detected based on fluorescence signal.

- **Cellular experiments for evaluation of antisense effects**

HeLa cells (RIKEN Cell Bank, Japan) were cultured in Dulbecco's Eagle's medium (D-MEM) supplemented with inactivated 10% fetal bovine serum (GE Healthcare, USA) and 1% penicillin-streptomycin solution (Wako, Japan) at 37°C under a humidified atmosphere of 5% CO<sub>2</sub>. Cells were detached from culture dish by a treatment with 0.05% trypsin-EDTA solution (Wako, Japan). Trypsin reaction was terminated by D-MEM and cells were seeded on 96 well or 48 well plate. The cell seeding density was approximately 2.0 $\times$ 10<sup>4</sup> cm<sup>-2</sup>. Plasmid DNA, pAcGFP-C1 and pLLx-mCherry, were utilized as expression plasmid for AcGFP and mCherry, respectively. (For microscopic analysis, pLLx-mCherry was not administrated.) Plasmid DNAs and antisense fragments were mixed with Lipofectamine P3000 reagent (Invitrogen USA), separately and induced into HeLa cells on 96 well or 48 well plate without time interval. The final concentrations of plasmid DNA and antisense fragments were 1 ng/ $\mu$ L and 1  $\mu$ M, respectively. Culture media was replaced with fresh D-MEM after a 4 hr culture period. Fluorescence in cells were analyzed at 24 hr post-transfection. For fluorescence observation, cells were observed with ECLIPSE Ti2 microscope (Nikon, Japan) equipped with iXon+ EMCCD camera (ANDOR, Japan). GFP and mCherry fluorescence was imaged with Nikon FITC filter set (480/30 nm bandpass excitation filter, 505 nm long-path dichromatic filter, and 535/40 nm bandpass emission filter) and Nikon Texas-red filter (542-582 nm bandpass excitation filter, 593 nm long-path dichromatic filter, and 604-644 nm bandpass emission filter), respectively. For quantitative analysis, cells were collected by trypsin treatment and

analyzed with EC800 flow-cytometer (Sony, Japan). GFP and mCherry fluorescence were excited by 488 nm bandpass laser and recorded with 525 nm filter and 640 nm bandpass filter, respectively. Anti-sense effects are determined by measuring fluorescence intensity of GFP in cells expressing mCherry protein. The silencing effect was calculated by the equation below;

$$\text{Silencing Effect (\%)} = (1 - F_{\text{sample}}/F_{\text{noDNA}}) \times 100$$

where  $F_{\text{sample}}$  and  $F_{\text{noDNA}}$  are GFP fluorescence intensity with fragment ONs and that without DNA, respectively.

For the experiment in Fig. S7, the protocols were slightly changed as follows, because the different type of spectral analyzer was used;

Cells were seeded on 24 well plate and the cell seeding density was approximately  $5\sim 6 \times 10^4$  per well. After same transfection procedures and trypsin treatment, the collected cells were analyzed with SA-3800 spectral cell analyzer (Sony, Japan), where at least 30,000 events of live cells were counted. Similarly, antisense effects are determined by measuring fluorescence intensity of GFP in mCherry positive cells.

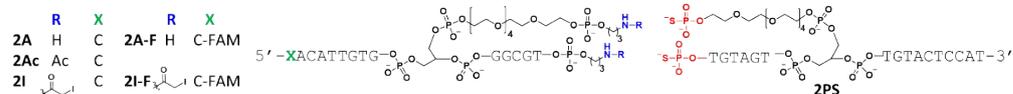
The silencing effect was calculated by the equation below:

$$\text{Silencing effect (\%)} = ((F_{\text{no DNA}} - F_{\text{sample}}) / (F_{\text{no DNA}} - F_{\text{neg}})) \times 100\%$$

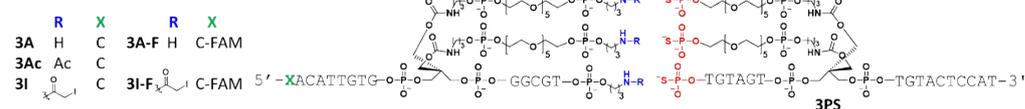
where  $F_{\text{sample}}$  and  $F_{\text{no DNA}}$  are mean GFP fluorescence intensity (MFI) with fragment ONs and that without DNA respectively.

## MALDI-TOF MS Measurements

### (1) Bifurcated strands pair



### (2) Trifurcated strands pair



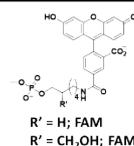
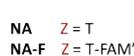
### (3) Template strands

5'-YTATGGAGTACAACCTACAACGCCCAATGTGTY-3'



### (4) Natural antisense strands

5'-CACATTGTGGCGTTGTAGTTGTACTCCAZ-3'

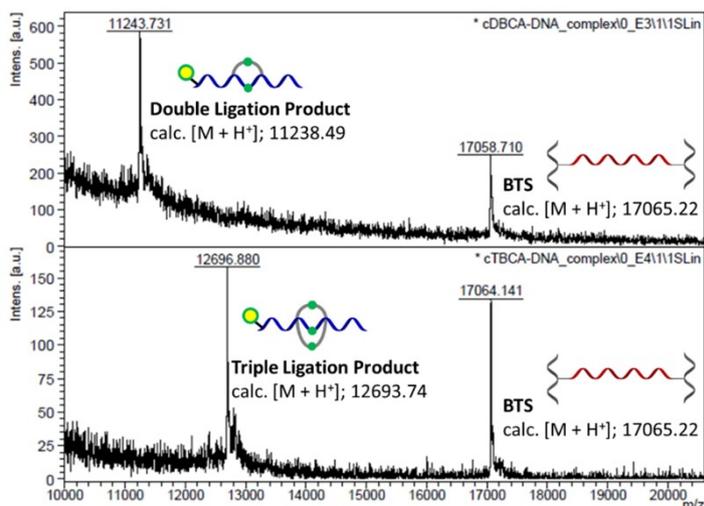


Sample	Calcd. [M+H] <sup>+</sup>	found
2PS	5552.65	5554.364
2A	5068.33	5069.303
2Ac	5152.41	5150.264
2I	5404.20	5405.290
2A-F	5605.79	5606.716
2I-F	5941.66	5943.388
3PS	6240.24	6240.848
3A	5795.98	5798.078
3Ac	5922.08	5921.521

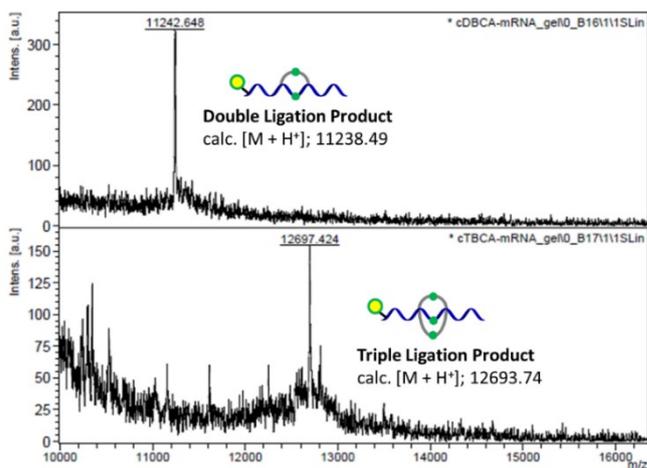
Sample	Calcd. [M+H] <sup>+</sup>	found
3I	6257.69	6298.08
3A-F	6333.43	6333.737
3I-F	6837.23	6837.311
TS	10408.80	10408.853
BTS	17065.22	17062.989
TS-RNA	10827.54	10826.86
NA-F	9785.68	9786.576
NA	9218.55	9218.970
NA-MOE	10481.67	10480.98

**Fig. S1.** MALDI-TOF-MS analysis of ONs used in this study

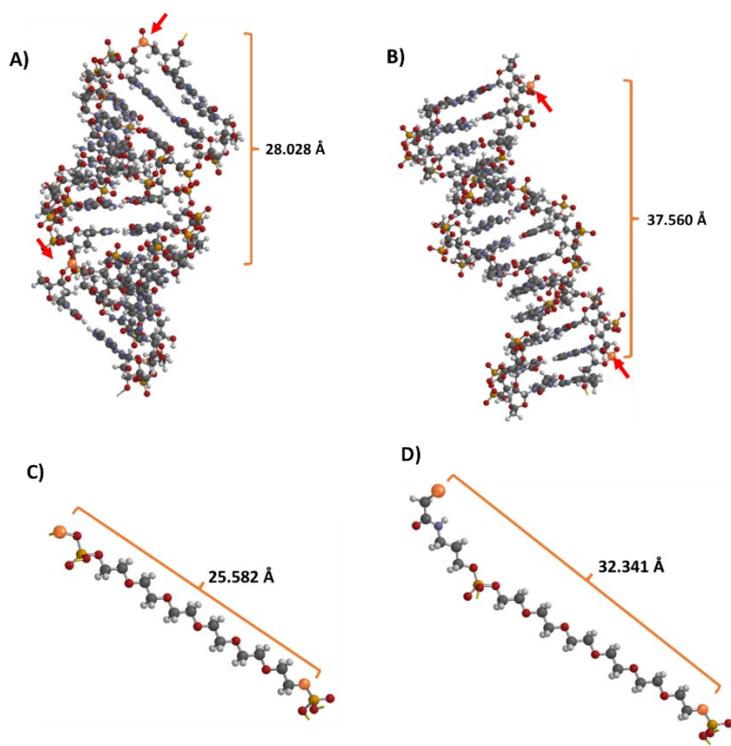
MALDI-TOF MS measurement was performed using Bruker Ultraflex III-MALDI TOF / TOF Mass Spectrometer, and 3-hydroxy picolinic acid and diammonium hydrogen citrate were used as matrixes. (TS-RNA: 5'- UUAUG GAGUA CAACU ACAAC GCCCA CAAUG UGUU - 3'; NA-MOE; 5'- CACAT TGTGG GCGTT GTAGT TGTAC TCCAT - 3'; underline: 2'-MOE; all phosphorothioate linkage)



**Fig. S2.** MALDI-TOF MS analysis of the samples extracted from the complex bands in dPAGE (Reaction with DNA template). MALDI-TOF MS measurement was performed using Bruker Ultraflex III-MALDI TOF / TOF Mass Spectrometer, and 3-hydroxy picolinic acid and diammonium hydrogen citrate were used as matrixes. If the MALDI-TOF MS measurement was performed with the TCF reaction crude without the desalting trying to observe the pseudorotaxane complex, no MS peak was observed. High salt concentration in the sample might probably prevent the proper ionization of the oligonucleotides.

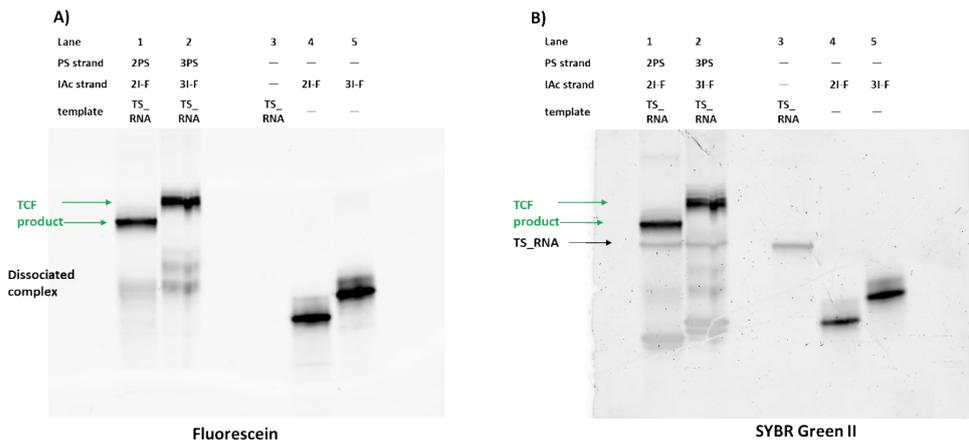


**Fig. S3.** MALDI-TOF MS analysis of the samples extracted from the complex bands in dPAGE (Reaction with mRNA template). MALDI-TOF MS measurement was performed using Bruker Ultraflex III-MALDI TOF / TOF Mass Spectrometer, and 3-hydroxy picolinic acid and diammonium hydrogen citrate were used as matrixes

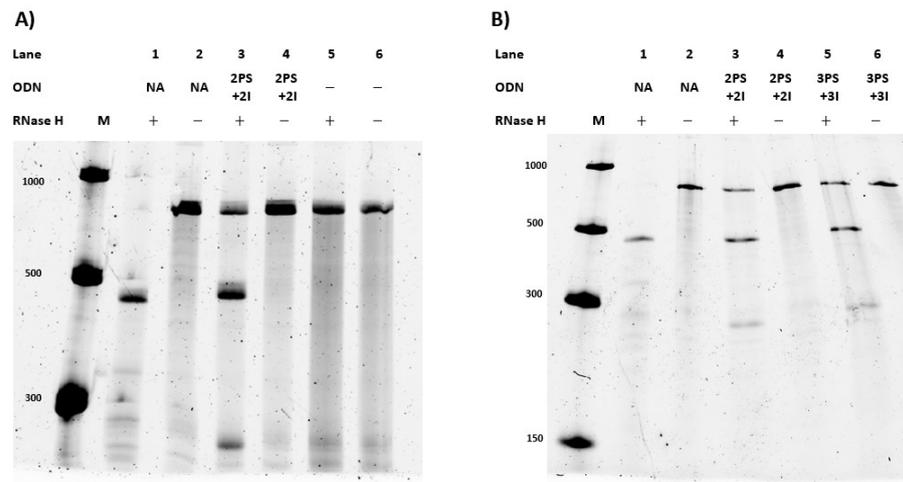


**Fig. S4.** Molecular modeling of the duplex and linker (spartan' 14)

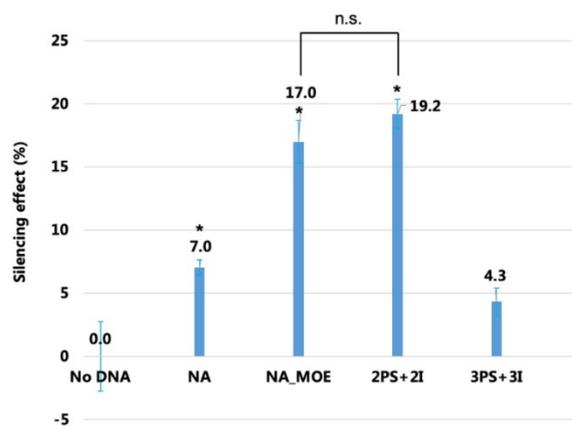
A) A type duplex, B) B type duplex, C) Hexaethylene Glycol linker and phosphorothioate group, D) Hexaethylene Glycol linker and C3 amino linker and iodoacetyl group



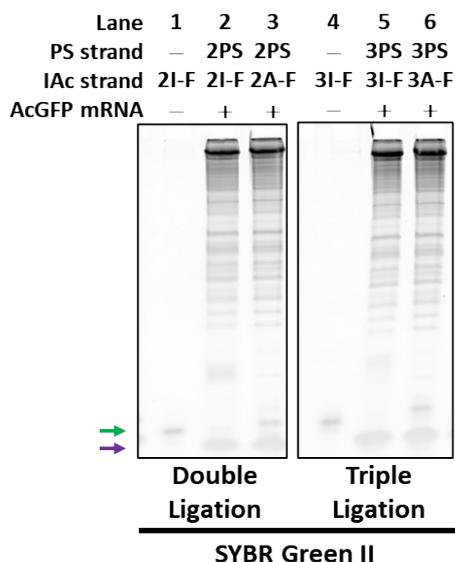
**Fig. S5.** TCF on 30 nt template RNA. (A); Detection with fluorescein signal. (B); Stained with SYBR Green II. Bands indicated by green arrows are complexes of the ligated strands on template RNA.



**Fig. S6.** RNase H cleavage assay. (A) Comparison of natural antisense (NA) and the bifurcated ODNs (2PS + 2I), (B) Comparison of the bifurcated ODNs (2PS + 2I) and the trifurcated ODNs (3PS + 3I).



**Fig. S7.** Gene silencing of AcGFP in HeLa cells based on flow cytometry analysis. Data represent the mean  $\pm$  s.d. for 3 replicates. \* $p < 0.05$  versus no DNA (positive control), n.s.: not significant.



**Fig. S8.** TCF on AcGFP mRNA. dPAGE results with lower detection threshold corresponding Fig. 4C. The reaction was carried out with fluorescein-labeled IAc-DNA (2I-F or 3I-F, 1.0  $\mu\text{M}$ ), PS-DNA (2PS or 3PS, 3.0  $\mu\text{M}$ ), AcGFP1 mRNA (0.45  $\mu\text{g}/\mu\text{L}$ ) in phosphate buffer (pH 7.0, 20 mM) containing NaCl (100 mM), DTT (0.2 mM) at 37°C for 1 h. 10% denaturing PAGE, 6.5 M urea, at 4°C. Stained with SYBR Green II. Bands indicated by green arrows corresponded to IAc-DNA and those indicated by purple arrows corresponded to PS-DNA.

#### ■ Reference

- 1) Ueno, Y.; Takeba, M.; Mikawa, M.; Matsuda, A. Nucleosides and Nucleotides. 182. Synthesis of Branched Oligodeoxynucleotides with Pentaerythritol at the Branch Point and Their Thermal Stabilization of Triplex Formation1. *The Journal of Organic Chemistry*, **1999**, *64*, 1211.