Electronic Supplementary Information (ESI)

Enhancement of exon skipping activity by reduction in secondary structure content of LNA-based splice-switching oligonucleotides

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Experimental details

Synthesis of oligonucleotides

We used SSOs (Supplementary Table S1), native RNAs (Supplementary Table S2), and DNA oligonucleotides (Supplementary Table S3) for each assay. All SSOs and native RNAs were synthesized and purified by GeneDesign Inc. (Osaka, Japan). All SSOs have phosphorothioate backbone linkages. The DNA oligonucleotides for quantitative RT-PCR (qRT-PCR) were synthesized by Hokkaido System Science (Sapporo, Japan).

Polyacrylamide gel electrophoresis (PAGE) analysis

Twenty percent native polyacrylamide (19:1) gel electrophoresis in 1×Tris-borate EDTA (TBE) was carried out to detect secondary structures of SSOs. Annealed SSOs were dissolved in phosphate buffer (4 μM), and used for PAGE analysis. Before sample loading, gel equipment and SSO solutions were incubated for at least 1 h in temperature-controlled rooms at 25°C. Electrophoresis was performed for 6–18 h under constant low voltage conditions at 60 V to avoid temperature changes. Gel was stained by SYBR Gold (Thermo Fisher Scientific), followed by fluorescence imaging and quantitative analysis using FLA-5000 (Fuji Film, Tokyo, Japan). Each experiment was repeated three or six times to ensure the reproducibility of the results. The percentage of structured form was calculated using Image Gauge ver. 4.21 for Mac or ver. 2.2 for Win as the amount of upper band (structured form) relative to the total amount of upper and lower bands.

UV melting experiments using single stranded oligonucleotides

The UV melting experiments were performed according to our previous reports.[1] The experiments were conducted using a Shimadzu UV-1800 UV-Vis spectrophotometer equipped with a T_m analysis accessory TMSPC-8 (Shimadzu,Kyoto, Japan). All SSOs were dissolved in a buffer containing 100 mM NaCl, 10 mM phosphate (pH 7.2) and 1.0 mM EDTA to give a final strand concentration of 4 μM. Normalized melting curves were present in the data reported in Figure 3 and Supplementary Figure S7. Each experiment was repeated five times to ensure the reproducibility of the results.

Thioflavin T (ThT assay)

Fluorescence spectra of 4 μM oligonucleotides (ONs 1-3) and 1.0 μM ThT in 10 mM phosphate buffer (pH 7.2) containing 1.0 mM EDTA and 100 mM NaCl at 25°C were measured by Jasco FP-8200 (JASCO, Tokyo, Japan) after heating at 90°C for 5 min followed by annealing to 25°C with temperature change 0.5°C/min. Excitation wave length was 450.0 nm and emission between 460 to 660 nm was detected.

CD melting experiment

The CD spectra of ONs without complementary strand were measured by a JASCO J-820 spectrometer equipped with a Peltier temperature controller JASCO PTC-432L. Spectral data were collected via a software JASCO Spectra Manager ver. 1.55.00. All SSOs were dissolved in a 10 mM phosphate buffer containing 100 mM NaCl and 1.0

mM EDTA to give a final concentration of 4 μM. After heating at 90°C for 5 min followed by annealing to 20°C with temperature change 0.5°C/min, CD signal from 200 to 320 nm was measured with cumulative number of 6. After the measurement at 20°C, the sample was heated to 5°C higher temperature (25°C) and after 10 min incubation, the CD spectrum at 25°C was measured in the same way. The repetitive measurements were carried out until 85°C.

Cell culture

The stable reporter cell line that can evaluate the *DMD* exon 58 skipping activity was cultured according to our previous reports.[1]

SSO transfection into the stable reporter cell line

The stable reporter cells were seeded 24 h before transfection at a density of 2.0×10^5 cells/well on 24-well plates (Iwaki Techno Glass, Tokyo, Japan). After 24 h, the stable cells were transfected with 30 nM SSOs using Lipofectamine 2000 (Thermo Fisher Scientific, Pittsburgh, PA).

Quantitative RT-PCR analysis

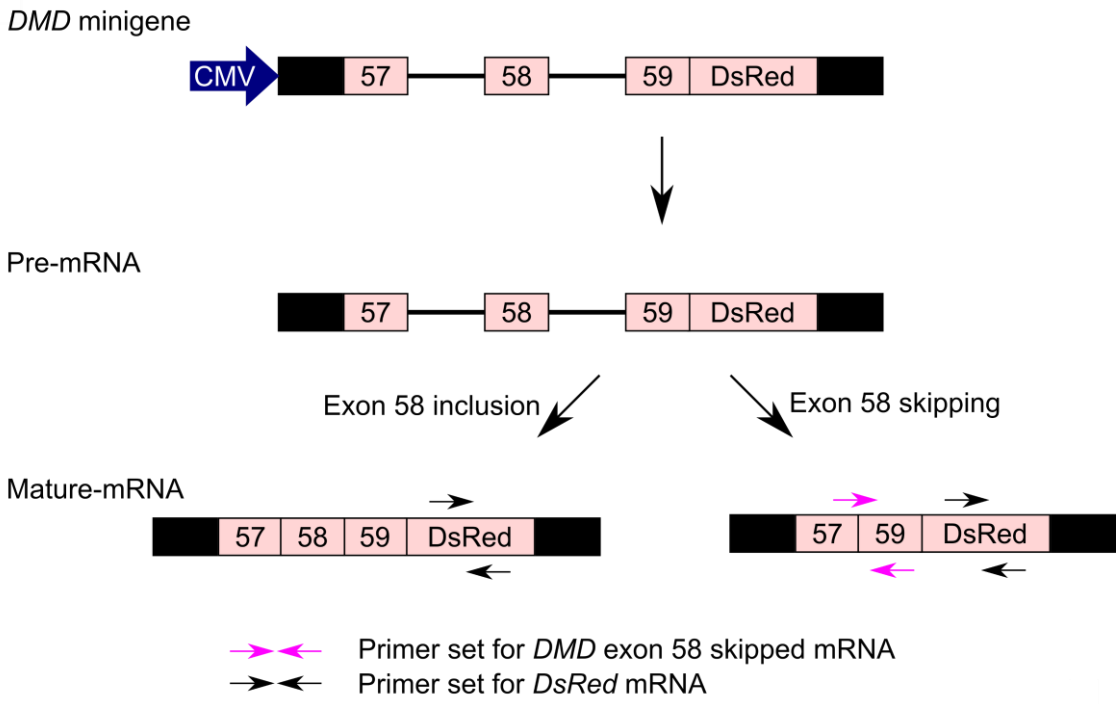
qRT-PCR was performed according to our previous report.[2] The analyses were performed using the SYBRGreen Real-time PCR Master Mix-Plus (Toyobo, Osaka, Japan) and the StepOnePlus device (Thermo Fisher Scientific) according to

manufacturer's protocols; however, the annealing time was reduced to 15 sec and the annealing temperature was set at 65˚C. The stable reporter cell line used in the study expresses the *DMD* minigene that codes for *DsRed* gene downstream of the cassette of *DMD* exon 57-58-59. For qRT-PCR data normalization, we analyzed the expression levels of *DsRed* mRNA (Schemes S1 and S2), relative to the value in the ON2 (Fig. 3C) or ON5 (Fig. 4B) set as 1. Each experiment was duplicated and repeated three times to ensure the reproducibility of the results.

$$Relative\ expression\ of\ DMD\ exon\ 58\ skipped\ mRNA = \frac{Expression\ level\ of\ DMD\ exon\ 58\ skipped\ mRNA}{Expression\ level\ of\ DsRed\ mRNA}$$

Scheme S1. Calculation of the relative expression of *DMD* exon 58 skipped mRNA.

The formula for the relative expression of *DMD* exon 58 skipped mRNA with normalization, using the expression levels of *DsRed* mRNA.



Scheme S2. Design of DNA primer sets for qRT-PCR.

UV melting experiments using double stranded oligonucleotides

The UV melting experiments were performed according to our previous reports.[1] The experiments were conducted using a Shimadzu UV-1800 UV-Vis spectrophotometer equipped with a T_m analysis accessory TMSPC-8 (Shimadzu,Kyoto, Japan). All SSOs and native RNA strands were dissolved in a buffer containing 10 mM NaCl and 10 mM phosphate (pH 7.2) to give a final strand concentration of 2 μM. Each experiment was repeated three times to ensure the reproducibility of the results.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using EZR software.[3] The data were analyzed by ANOVA, and if this was statistically significant, group differences were analyzed using Turkey's test or Dunnett's multiple comparison test corresponding to the purpose.

Supplementary Tables

Supplementary Table S1. SSOs used for the UV melting experiment and number of LNA analogues and nucleotides (g: 2′-deoxyguanine; ^{7C}g: 7-deaza-2′-deoxyguanosine; i: 2′-deoxyinosine) in the sequence.

Sequences are shown from 5' to 3'. A: LNA-adenosine; a: 2′-deoxyadenosine G: LNA-guanosine; g: 2′-deoxyguanosine; T: LNA-thymidine; t: thymidine; C: LNA-methylcytidine; c: 2′-deoxycytidine; ^{7C}g: 7-deaza-2′-deoxyguanosine; i: 2′-deoxyinosine.

Entry	Sequence (5’-3’)	LNA	Nucleotide		
		(LNA-G)	g	^{7C}g	i
ON1	cCtCtGgGcTcCt	6 (2)	3	0	0
ON2	cCtCtGgGcTcCtGgTa	8 (3)	5	0	0
ON3	cCTCTgggcTcCTggTa	8 (0)	5	0	0
ON4	cCTCT^{7C}g^{7C}g^{7C}gcTcCT^{7C}g^{7C}gTa	8 (0)	0	5	0
ON5	cCTCTiiicTcCTiiTa	8 (0)	0	0	5
ON6	cctctgggctcctggta	0 (0)	5	0	0
ON7	cCTCTiggcTcCTggTa	8 (0)	4	0	1
ON8	cCTCTgigcTcCTggTa	8 (0)	4	0	1
ON9	cCTCTggicTcCTggTa	8 (0)	4	0	1
ON10	cCTCTgggcTcCTigTa	8 (0)	4	0	1
ON11	cCTCTgggcTcCTgiTa	8 (0)	4	0	1
ON12	cCTCT^{7C}gggcTcCTggTa	8 (0)	4	1	0
ON13	cCTCTg^{7C}ggcTcCTggTa	8 (0)	4	1	0
ON14	cCTCTgg^{7C}gcTcCTggTa	8 (0)	4	1	0
ON15	cCTCTgggcTcCT^{7C}ggTa	8 (0)	4	1	0
ON16	cCTCTgggcTcCTg^{7C}gTa	8 (0)	4	1	0

Supplementary Table S2. RNA strands used for the UV melting experiment.

Sequences are shown from 5' to 3'. A: adenosine; G: guanosine; U: uridine; C: cytidine.

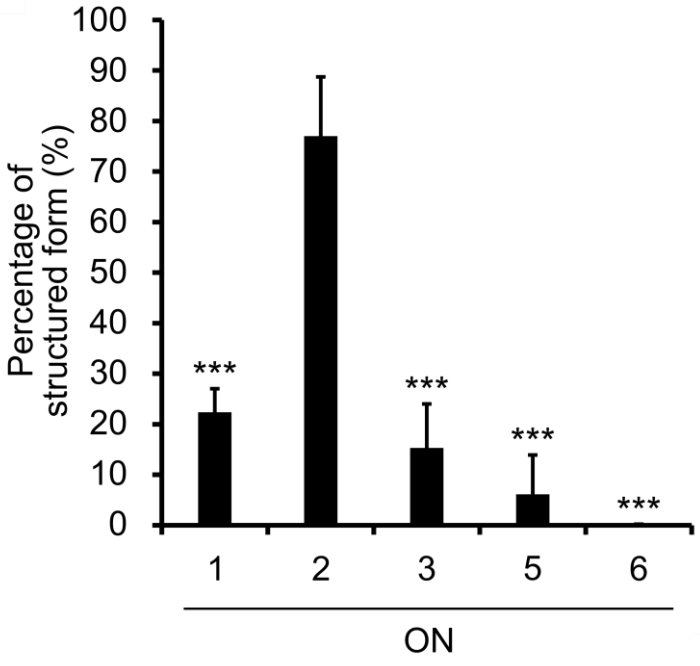
Entry	Sequence (5’-3’)
Complementary RNA 1	AGGAGCCCAGAGG
Complementary RNA 2	UACCAGGAGCCCAGAGG

Supplementary Table S3. DNA primers used for the qRT-PCR analysis.

Sequences are shown from 5' to 3'. a: 2′-deoxyadenosine; g: 2′-deoxyguanosine; t: 2′-deoxythymidine; c: 2′-deoxycytidine.

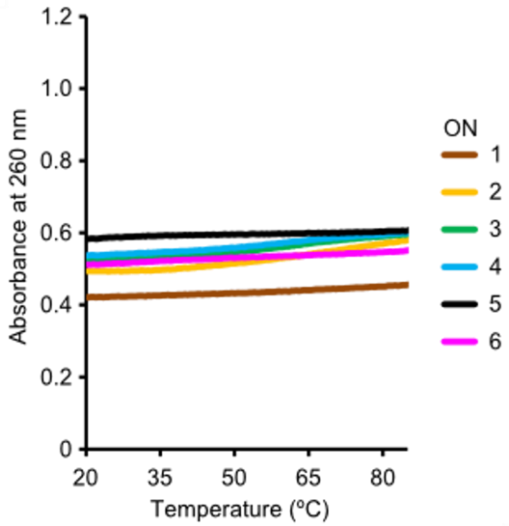
Gene	ID	Sequence (5’-3’)	Size
DMD	For. primer	agttctgaccagtggaagcg	156 bp
	Rev. primer	cctcaggaggcagctcctat	
DsRed	For. primer	tgatgaacttcgaggacggc	94 bp
	Rev. primer	acgccgatgaacttcacctt	

Supplementary Figures



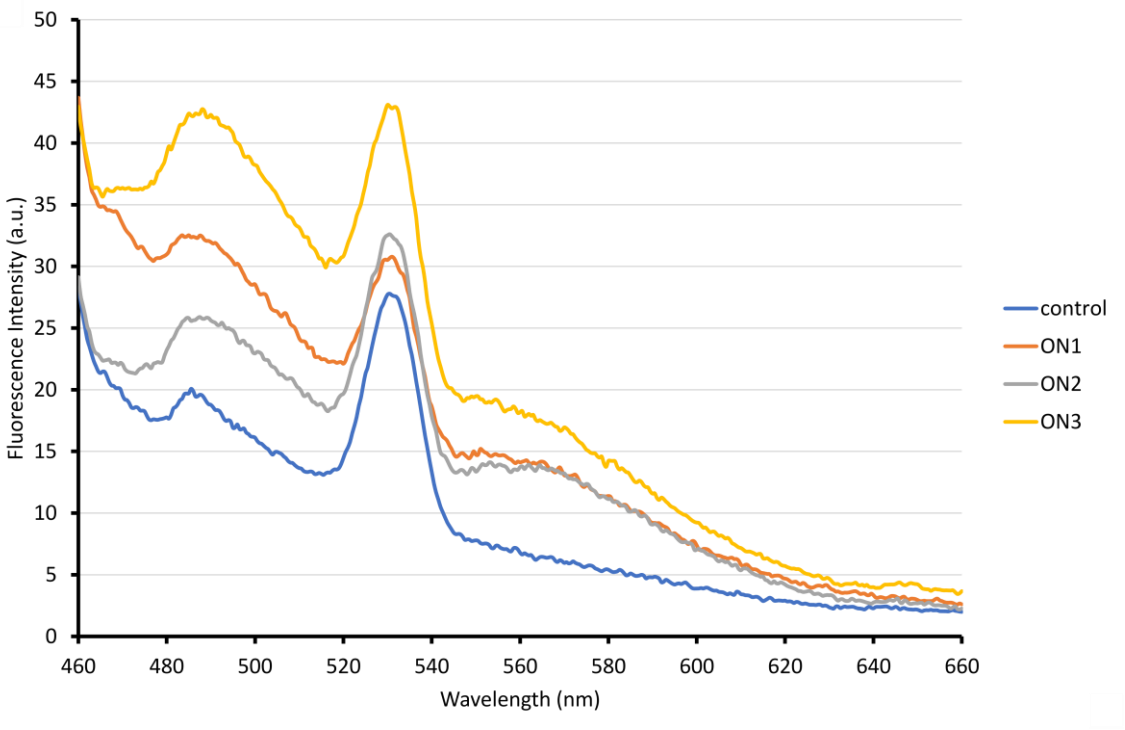
Supplementary Figure S1. Evaluation of secondary structures formed by LNA-based SSOs containing base modifications.

The percentage of structured form of each ON was calculated from Fig.3A as the amount of upper band (structured form) relative to the total amount of upper and lower bands. Values represent the mean ± standard deviation of three (ON1) or six (ONs 2-6) independent experiments. Data were analyzed by ANOVA (P<0.001). Significant differences compared to the ON2 were determined using Dunnett's test and are indicated by asterisks (***P<0.001).



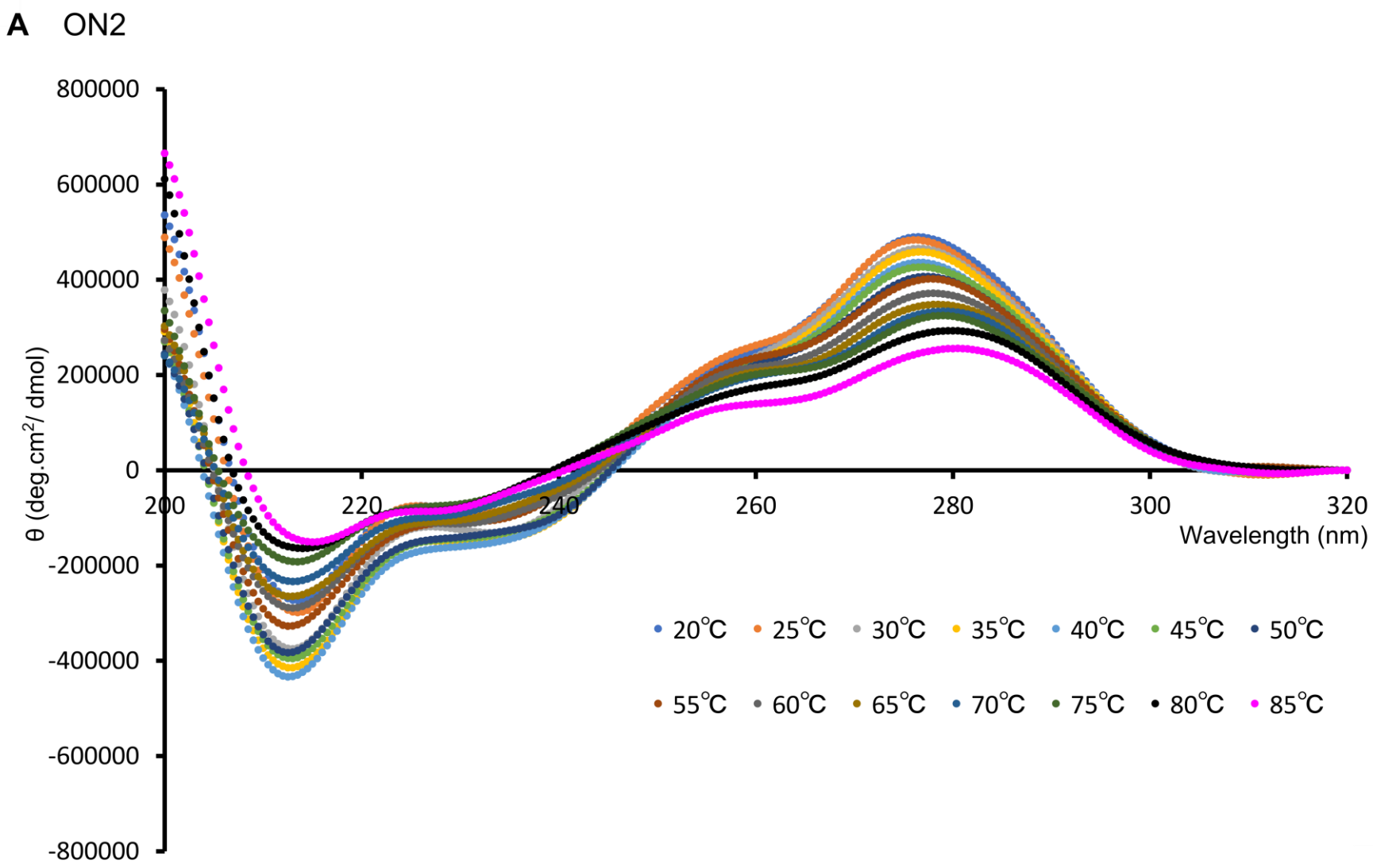
Supplementary Figure S2. UV melting experiments using LNA-based SSOs containing 7-deaza-2′-deoxyguanosine or 2′-deoxyinosine-modifications.

UV absorption spectra of six LNA based SSOs at 260 nm were measured from 20 to 85°C. The final concentration of single-strand SSOs was 4 μM in 100 mM NaCl, 10 mM phosphate buffer (pH7.2) and 1.0 mM EDTA. The experiments were repeated five times to ensure reproducibility of results. The raw data of Fig. 3B are shown.



Supplementary Figure S3. Thioflavin T (ThT) assay of structured form of ONs 1-3.

Fluorescence spectra of 4 µM oligonucleotides (ONs 1-3) and 1.0 µM ThT in 10 mM phosphate buffer (pH 7.2) containing 1.0 mM EDTA and 100 mM NaCl at 25°C were measured by Jasco FP-8200 after heating at 90°C for 5 min followed by annealing to 25°C with temperature change 0.5°C/min. Excitation wave length was 450.0 nm and emission between 460-660 nm was detected. The peak at 529 nm is Raman scattering of excitation light in water and the fluorescence of ThT-quadruplex structure should appear around 485 nm at least 300 a.u. under this condition.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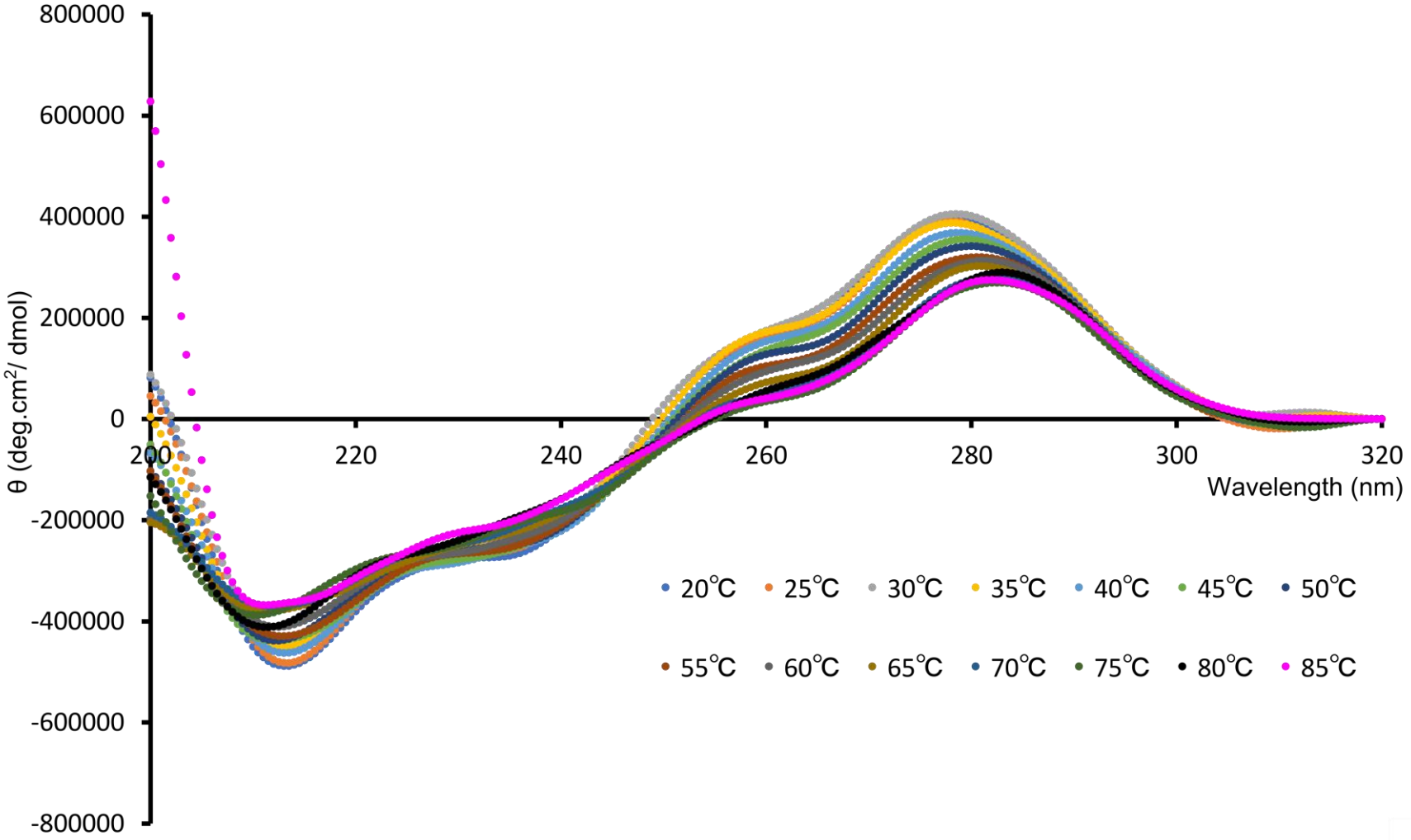
B ON3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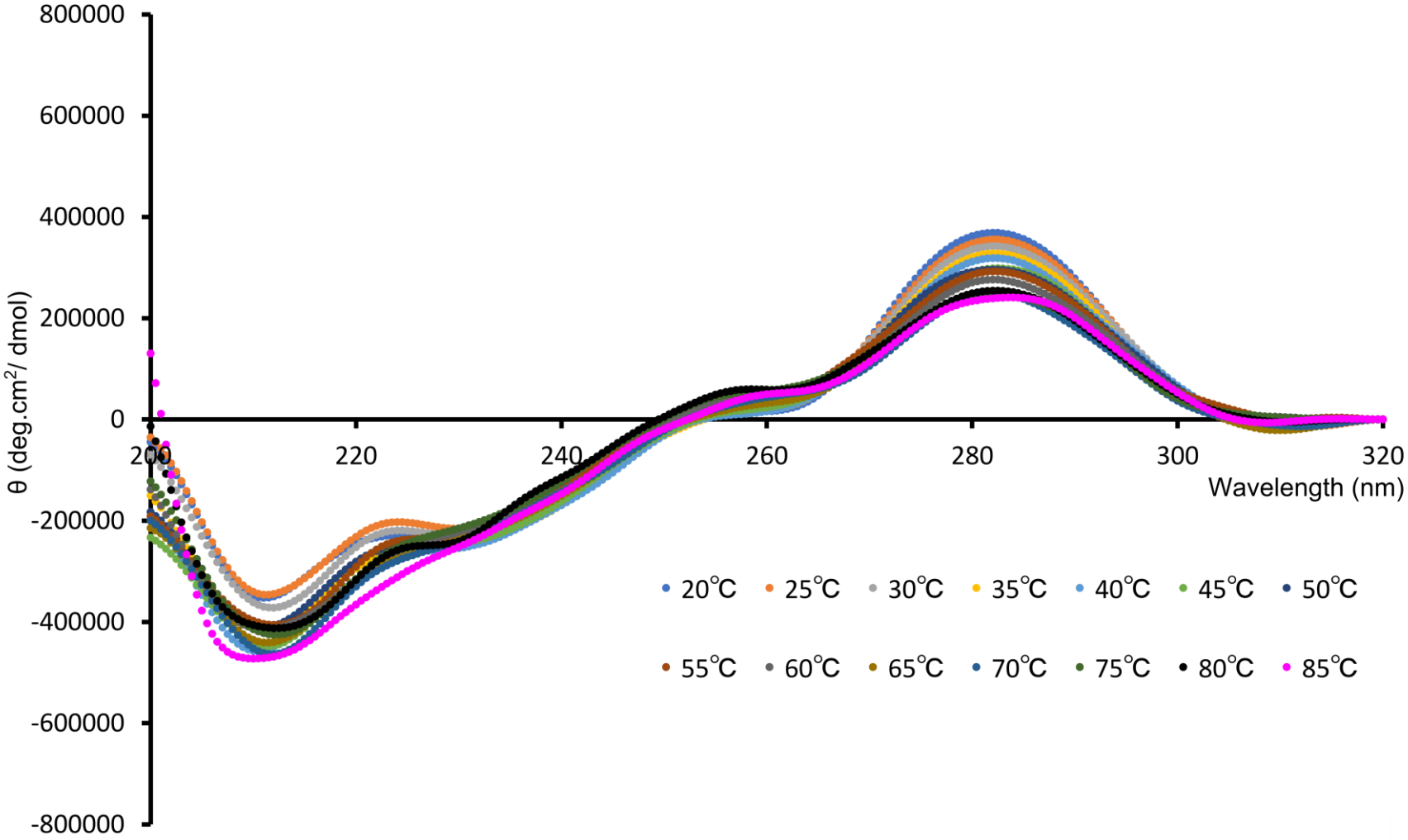
C ON5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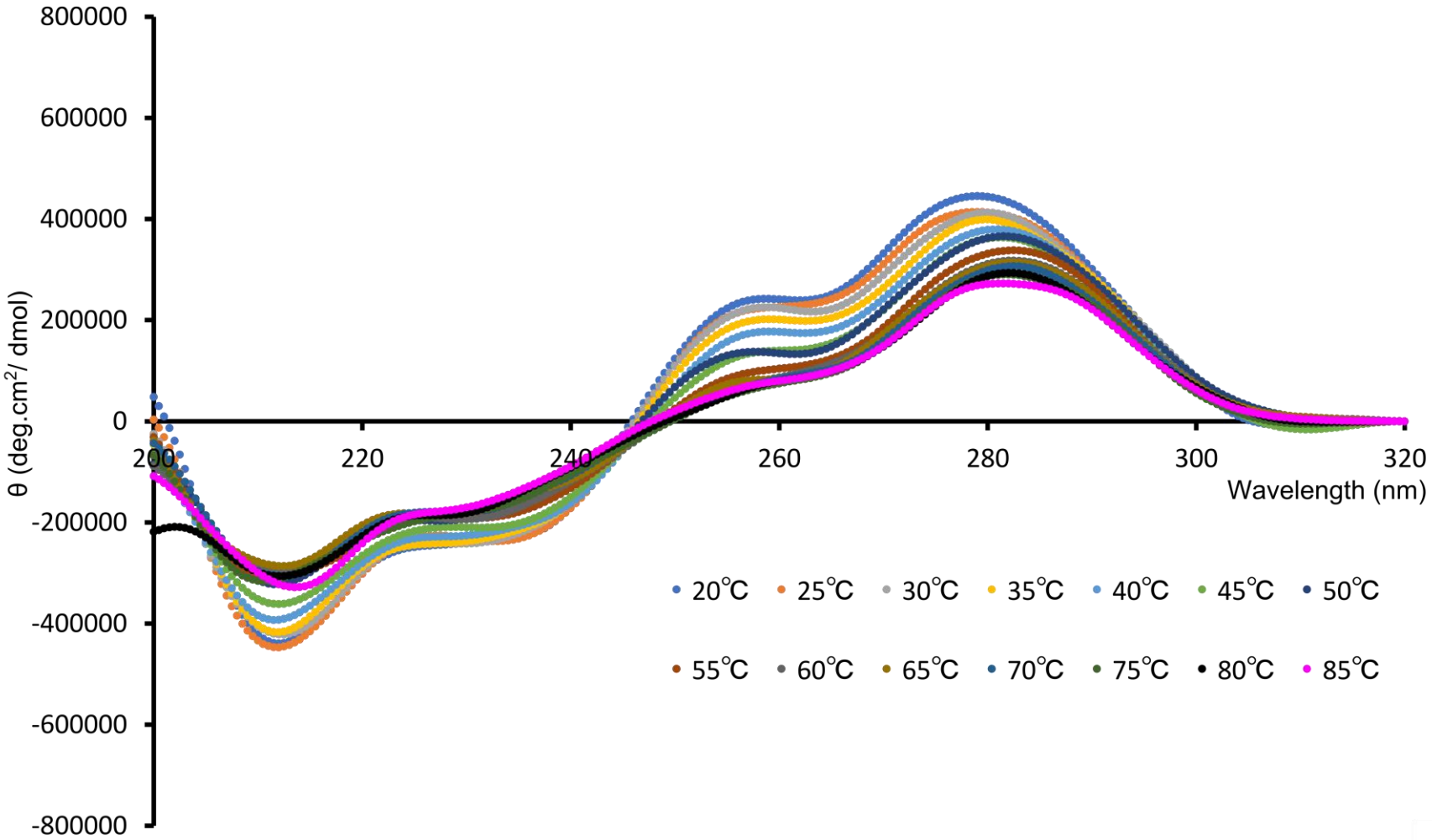
D ON7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Wavelength (nm)
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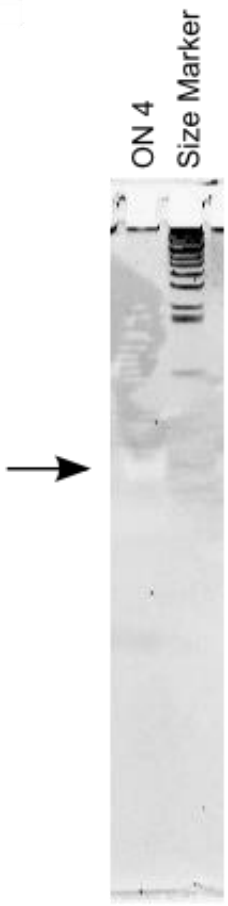
55℃ 60℃ 65℃ 70℃ 75℃ 80℃ 85℃

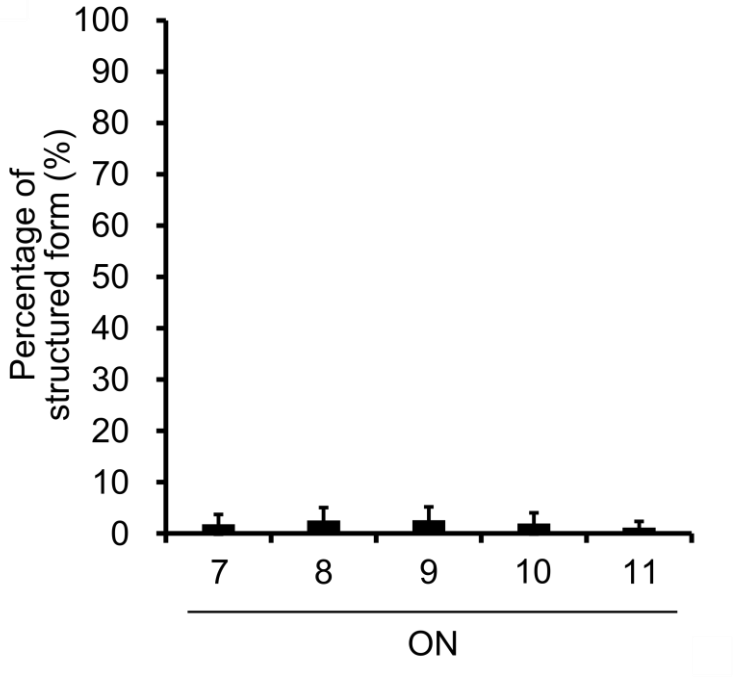
Supplementary Figure S4. CD melting experiments for analyzing structures of LNA-based SSOs.

The CD spectra of ONs without complementary strand were measured by a JASCO J-820 spectrometer equipped with a Peltier temperature controller JASCO PTC-432L. Spectral data were collected *via* a software JASCO Spectra Manager ver. 1.55.00. All SSOs were dissolved in a 10 mM phosphate buffer containing 100 mM NaCl and 1.0 mM EDTA to give a final concentration of 4 μM. After heating at 90°C for 5 min followed by annealing to 20°C with temperature change 0.5°C/min, CD signal from 200 to 320 nM was measured with cumulative number of 6. After the measurement at 20°C, the sample was heated to 5°C higher temperature (25°C) and after 10 min incubation, the CD spectrum at 25°C was measured in the same way. The repetitive measurements were carried out until 85°C. Data shown in the panel A, B, C and D indicate the result of ONs 2, 3, 5 and 7, respectively. The CD spectra of the all ONs indicate A-form like structures. However for the ONs 3, 5 and 7, the spectral change observed in CD melting experiment was rather small within wide temperature range. Additionally, native PAGE analysis shows that almost all ONs 3, 5, 7 exist in single stranded form. Especially for ONs 5 and 7, almost no structured form could be seen as shown in Figs 3A and 4A. Therefore, the melting behavior with isodichroic points in CD melting experiments of the ONs 3, 5, 7 may mainly reflect the increment of structural freedom of single stranded form, i.e. the collapse of base stacking in the single strand.



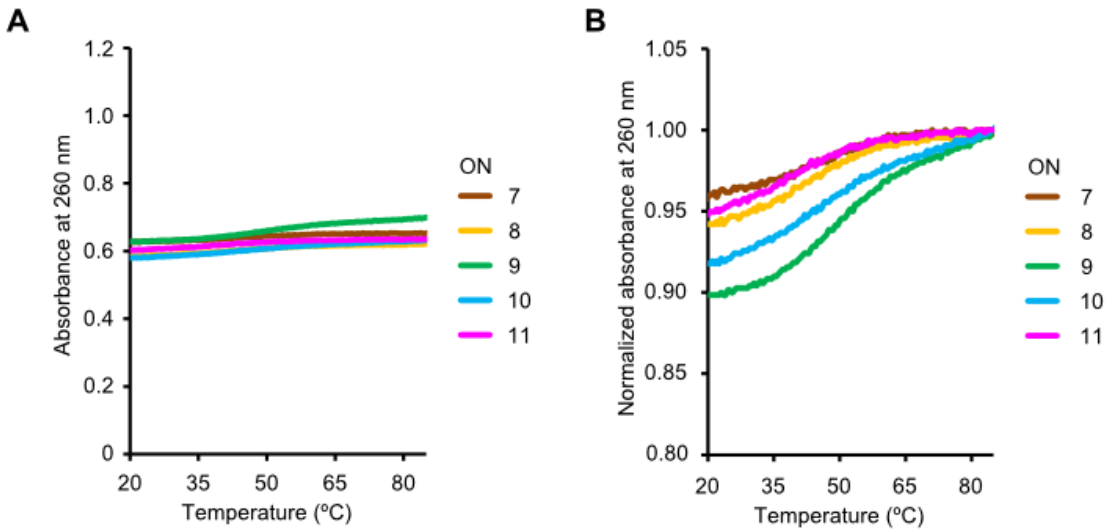
Supplementary Figure S5. Native PAGE analysis of LNA-based SSOs containing 7-deaza-2′-deoxyguanosine.

The PAGE analysis of ON4 was carried out at 25°C and the gel was stained with SYBR Gold. As previously described, the result showed that 7-deazaguanine quenched SYBR gold fluorescence.[4] Due to this property of 7-deazaguanine, quantitative analysis on the samples containing 7-deazaguanine could not be achieved. The experiment was repeated three times to ensure reproducibility of the results.



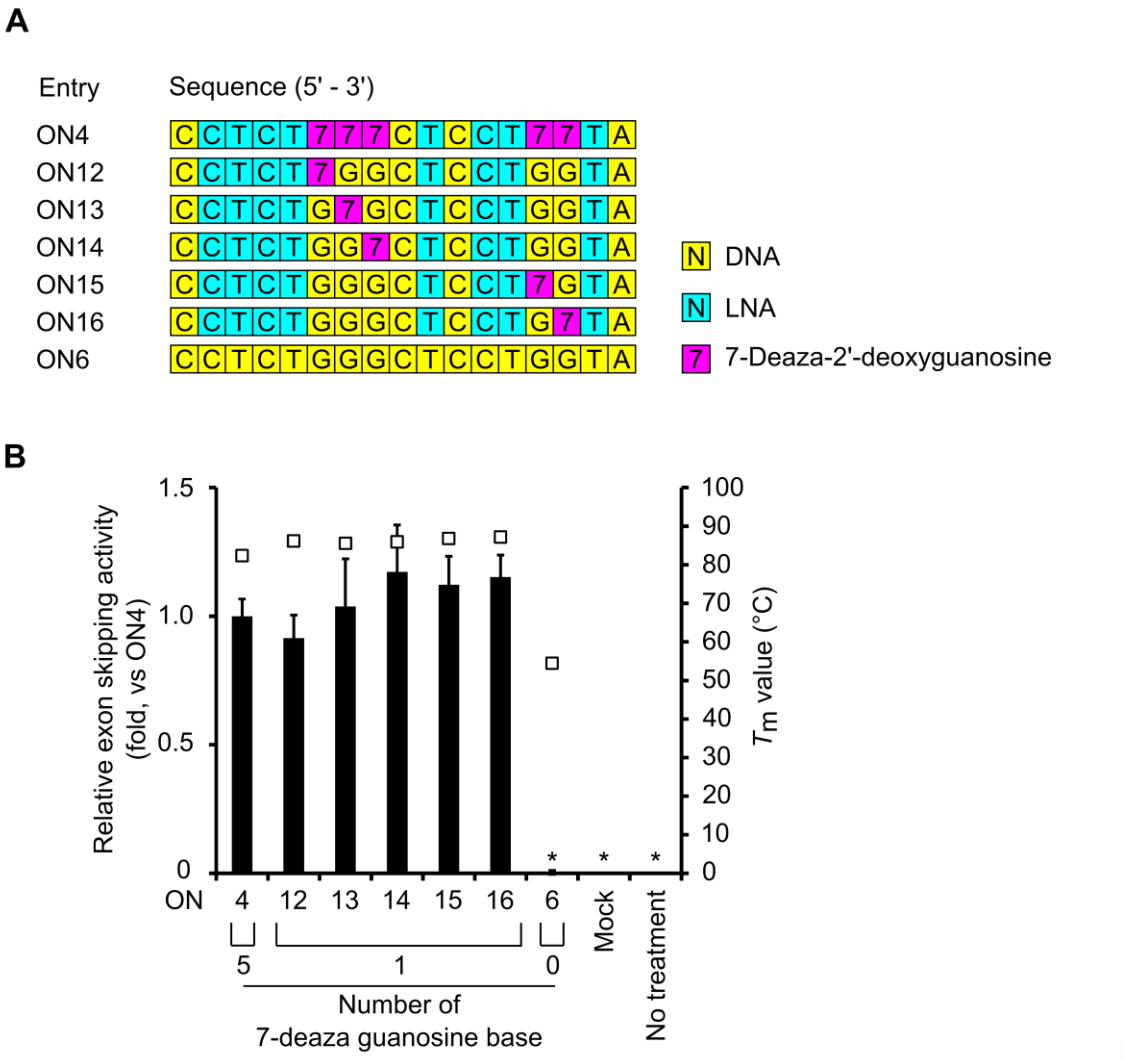
Supplementary Figure S6. Evaluation of secondary structures formed by LNA-based SSOs containing base modifications.

The percentage of structured form of each ON was calculated from Fig.4A as the amount of upper band (structured form) relative to the total amount of upper and lower bands. Values represent the mean ± standard deviation of six independent experiments.



Supplementary Figure S7. UV melting experiments using LNA-based SSOs containing partial 2'-deoxyinosine-modifications.

UV absorption spectra of five LNA-based SSOs at 260 nm were measured from 20 to 85°C. The final concentration of single-strand SSOs was 4 μM in 100 mM NaCl, 10 mM phosphate buffer (pH7.2), and 1.0 mM EDTA. The experiments were repeated five times to ensure reproducibility. A) The raw data and B) normalized data (normalized by the UV absorptions at 85°C) are shown. In the normalized data, every melting behavior seems to reach a plateau at 85°C.



Supplementary Figure S8. Evaluation of exon skipping activity of LNA-based SSOs containing a single 7-deaza-2′-deoxyguanosine *in vitro*.

A) LNA-based SSOs with 7-deaza-2′-deoxyguanosine used in the experiment. B) The relative exon skipping activities were measured by qRT-PCR (black bar). Mock: transfection reagent only. The level of expression of *DsRed* mRNA was used for normalizing the data, relative to the value in the ON4 set as 1. The T_m value of SSOs was measured from 5 to 95°C (open box). The final concentration of SSOs and complementary RNA was 2 μM in 10 mM NaCl and 10 mM phosphate buffer

(pH7.2). The experiment was duplicated and repeated three times to ensure the reproducibility of the results. Data were analyzed by ANOVA ($P<0.001$). Significant differences were determined using Turkey's test and are indicated by asterisks (*$P<0.05$ relative to ON4).

References

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3. Y. Kanda, *Bone Marrow Transplant*, 2013, **48**, 452-458.
4. H. Li, X. Peng and F. Seela, *Bioorg Med Chem Lett*, 2004, **14**, 6031-6034.