

Supplementary Information

***In Vitro* Selection of DNA-based Aptamers that Exhibit RNA-like Conformations Using a Chimeric Oligonucleotide Library that Contains Two Different Xeno-Nucleic Acids**

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EXPERIMENTAL DETAILS

1. Materials

Sample solutions and buffers were prepared using ultrapure water obtained using the Ultrapure Water system CPW-100 (Advantec Co. Ltd., Tokyo, Japan). The initial DNA library, DNA primers and templates, known 29-mer oligodeoxyribonucleotides (ODNs) of thrombin-binding aptamer (TBA)¹ labeled with 6-carboxyfluorescein (6-FAM) at the 5'-end (*i.e.*, 29TBA) were purchased from Japan Bio Services Co. Ltd. (Saitama, Japan) or Hokkaido System Science Co. Ltd. (Hokkaido, JAPAN). The 2',4'-bridged/locked nucleic acid (2',4'-BNA/LNA) primer (P1fb; 5'-/6-FAM/TCG CCT TGC^{me} CGG ATC^{me} GCA GA-3'), which contained 2'-O,4'-C-methylene-bridged/linked bicyclic ribonucleotides (B/L nucleotides) bearing adenine (A), guanine (G), 5-methylcytosine (C^{me}), and thymine (T) as shown in bold capitals, was purchased from GeneDesign Inc. (Osaka, Japan). The B/L nucleotide bearing C^{me} not cytosine (C) was used because the C analog was not in the supplier's regular assortment. Primers P1fb was labeled with 6-FAM at the 5'-end to facilitate fluorescence detection. The initial DNA library T1 comprised random regions (30 bases) flanked by the primer sequence (20 bases each) 5'-/6-FAM/TCG CCT TGC CGG ATC GCA GA-(30 random bases)-TGG TCC GTG AGC CTG ACA CC-3'. Primer P1p (5'-/phosphate/TCG CCT TGC CGG ATC GCA GA -3') and Primer P2p (5'-/phosphate/GGT GTC AGG CTC ACG GAC CA-3') were labeled with monophosphate at the 5'-end to prepare single stranded DNA using lambda exonuclease after PCR amplification of selected Chimeric ODNs. 2'-O,4'-C-Methylene-bridged/linked bicyclic guanosine-5'-triphosphate (g^bTP) was synthesized in compliance with previously reported procedures.^{2,3}

Thrombin from human plasma, thrombin from rat plasma, tris(hydroxymethyl)aminomethane (Trizma[®] base), chicken egg lysozyme were purchased from Sigma-Aldrich, Inc. (MO, USA). Lambda exonuclease, soybean lectin, streptavidin type II, ethanol, aqueous HCl, boric acid, sodium tetraborate, NaCl and MgCl₂·6H₂O were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fused-silica

capillary (eCAPTM capillary tubing, 75 μ m I.D., 375 μ m O.D.) was purchased from Beckman Coulter, Inc. (CA, USA). The 4 dNTPs (dATP, dGTP, dCTP, and TTP) were purchased from Roche Diagnostics K.K. The 4 n^fTPs (a^fTP, g^fTP, c^fTP, and t^fTP) were purchased from TriLink BioTechnologies. (CA, USA). *KOD Dash* DNA polymerase was purchased from Toyobo Co. Ltd. (Osaka, Japan) and *Taq* DNA polymerase from Invitrogen Co. Ltd. (CA, USA). The plasmid DNA purification kit was purchased from Cosmo Genetech Co. Ltd. (Seoul, Korea). The binding buffer contained 20 mM Tris-HCl buffer (pH 7.40), MgCl₂ (1 mM) and NaCl (10 mM). The running buffer for separation contained 100 mM sodium borate (pH 8.35). Binding and running buffers were filtered through a 0.45- μ m membrane filter (EMD Milipore Co., MA, USA).

2. Enzymatic synthesis of FNA strands using 2'-deoxy-2'-fluoro-nucleoside-5'-triphosphates

In Figure S1, each reaction mixture (20 μ L) except for lane 1 contains the primer (5'-/6-FAM/GGA TTA GCG AAC AGG CCA TAC CTT T -3') at 0.5 μ M, the template (5'-/phosphate/CCG ATA AAA GGT ATG GCC TGT TCG CTA ATC C-3') at 0.625 μ M and the reaction buffer supplied with the enzyme (at 1 \times concentration). A mixture in lane 1 contains the primer only. A reaction with natural dNTPs (dATP, dGTP, dCTP and TTP) was used as a positive control (lane 2), and a reaction in absence of four natural dNTPs was used as a negative control (lane 3). The primer extension reactions were performed with an n^fTP analog (a^fTP, g^fTP, c^fTP or t^fTP) in place of the corresponding dNTP (dATP, dGTP, dCTP or TTP) and the other three dNTPs (lanes 4, 6, 8, 10). The primer extension reaction in absence of a dNTP (dATP, dGTP, dCTP or TTP) was used as each negative control (lanes 5, 7, 9, 11). The reactions were denatured at 95°C for 1 min with a TC-312 thermal cycler (Techne, Staffordshire, UK), and then annealed at room temperature for 1 h. Subsequently, 2 μ L of *KOD Dash* DNA polymerase (0.25 U/ μ L) was added to the mixture, and the reaction tube was quickly placed in a thermoregulated bath and incubated at 74°C during the reaction. Reactions were monitored at 5 min. After the reactions were started, the reaction tubes were removed from the bath sequentially, and immediately quenched by freezing in liquid nitrogen. The frozen reaction mixtures were

then mixed with 40 mM EDTA (2 μ L) containing 0.1% bromophenol blue and 7 M urea (12 μ L) containing 3 mM EDTA, and then were melted into a homogeneous solution by vortexing. The sample solutions were resolved by denaturing PAGE, and gel images were recorded on the imager. The amount of reactant and products was measured from the intensity of each band with excitation at 488 nm to visualize the 5'-labelled fluorophore.

3. CE-SELEX using 2',4'-BNA/FNA chimeric ODN library

3.1. Preparation of 2',4'-BNA/FNA chimeric ODN library

For preparation of initial chimeric ODN library, symmetric PCR (20 PCR cycles at 94°C for 30 s, 54°C for 30 s and 74°C for 60 s) in the thermal cycler was performed using T1, the forward primer P1fb and the reverse primer P2p. The reaction mixture (800 μ L) contained the template T1 (80 nM), forward primer P1fb and reverse primer P2p (0.4 μ M each), dATP, TTP, dCTP, and g^fTP in place of dGTP (0.2 mM each), *KOD Dash* DNA polymerase (0.1 U/ μ L), and 1 \times polymerase reaction buffer (step 1; Figure 1). The reaction mixture was then subjected to ethanol precipitation to remove dNTPs and yield roughly purified modified dsODN. To degrade the complementary strand (*i.e.* P2p-elongating product), dsODN (ca. 320 pmol) was incubated at 37°C for 30 min with 1 \times lambda exonuclease reaction buffer (760 μ L) containing lambda exonuclease (0.005 U/ μ L). Single-stranded modified ODN were purified by denaturing polyacrylamide gel electrophoresis (PAGE) and used as the chimeric ODN library for the following round (step 2).

After affinity separation using CE (step 3),⁴ a fraction containing the chimeric ODN–thrombin complex was subjected to symmetric PCR amplification (16–20 PCR cycles at the abovementioned conditions) using the forward primer P1p (0.4 μ M) and the reverse primer P2p (0.4 μ M each) (step 4). The reaction mixture was then subjected to ethanol precipitation to remove dNTPs to yield roughly purified natural double stranded DNA (dsDNA). Subsequently, PCR was performed using natural dsDNA as a template, dATP, dGTP, dCTP, g^fTP in place of dGTP (0.2 mM each), the forward primer P1fb and the reverse primer P2p

(0.4 μM), *KOD Dash* DNA polymerase (0.1 U/ μL) and the reaction buffer. The reaction mixture (800 μL) was subjected to 20 PCR cycles at the abovementioned conditions (step 5). The reaction mixture was then subjected to ethanol precipitation to remove dNTPs and yield roughly purified modified dsODN. To degrade the P1p-elongating product and P2p-elongating product, dsDNA was treated with lambda exonuclease under the same conditions as used above. Single-stranded modified ODNs were purified by denaturing PAGE (step 6) and used as the chimeric ODN library for the following round.

3.2. Affinity separation using capillary electrophoresis (CE)

All CE separations were performed using the P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Inc., Fullerton, CA, USA) equipped with 32 Karat software. A laser-induced fluorescence (LIF) detector was used to monitor the separation. Excitation was generated using the 488 nm line of an Ar⁺ laser (Beckman Coulter) and emission was collected at 520 nm. At the start of daily use, the separation capillary (bare fused silica, 75 μm I.D., 375 μm O.D., total length = 80 cm, length to detector = 70 cm) was rinsed with 0.1 M aqueous NaOH for 10 min and with the running buffer for another 10 min. Before each separation, the capillary was flushed with 0.1 M aqueous NaOH for 10 min followed by a 10 min rinse with the running buffer. Human thrombin (200 nM) was incubated with the chimeric ODN library (500 nM) in the binding buffer (20 μL total volume) for 30 min at 37°C before loading onto the CE instrument for separation. All samples were housed in the P/ACE MDQ sample chamber which was held at 15°C. The incubation mixture (ca. 60 nL) was injected into the separation capillary using pressure injection (16 s, 0.5 psi). A voltage of +18 kV was applied across the capillary for 12.0–12.6 min to migrate ODNs that bind to human thrombin into a collection vial containing 20 μL of the running buffer. Unbound ODNs remaining in the capillary were later washed into a waste vial using pressure. The temperature of the capillary was automatically maintained at 25°C for all separations. CE was performed 3 times for every round of separation to obtain sufficient active species. Five rounds of selection saturated the enrichment of active species (Figure 2).

3.3. Isolation and sequencing of 2',4'-BNA/FNA chimeric aptamers

After five cycles of separation and amplification, enriched chimeric ODN library were amplified by PCR using *KOD Dash* DNA polymerase with non-labeled forward/reverse primers P1/P2, and the four natural dNTPs. The resulting dsDNA was further amplified by PCR using *Taq* DNA polymerase. Subsequently, amplified dsDNA was cloned into a TA vector by the TA cloning method⁵ according to the manufacturer's protocol. Plasmid DNAs were selected from different clones and isolated using the plasmid DNA purification kit. Sequences inserted into the prepared plasmids were analyzed using DNA sequencing services at Operon Biotechnologies Co. Ltd. (Tokyo, Japan) and Takara Bio Inc. (Shiga, Japan). Forty-two aptamers were isolated from chimeric ODN library.

4. Preparation of isolated TBAs, their chemical variants

TBAs used for affinity analysis (*i.e.*, A#1, A#3, A#4, A#7, A#11, A#15, and A#25) were enzymatically prepared using the respective isolated plasmid DNAs as templates in compliance with the protocol for preparation of the chimeric ODN library. As described in the main text, chemical variants, A#1B, A#15B, A#1N, and A#15N, were prepared in compliance with the protocol for preparing 2',4'-BNA/FNA chimeric aptamers using g^bTP instead of g^fTP for A#1B and A#15B and using dGTP instead of g^fTP for A#1N and A#15N.

5. Affinity and specificity analyses by CE

TBAs were dissolved in binding buffer, and refolded by denaturing at 94°C for 0.5 min and cooling to 25°C at a rate of 0.5°C/min using the thermal cycler. Sample solutions (40 µL) containing TBA and human thrombin (1 nM each in the binding buffer) were prepared for K_d value determination. Similarly, those containing TBA (10 nM) and human thrombin (40 nM) were prepared for activity assays (Capillary

electrograms in Figure S2). These solutions were incubated at 37 °C for 30 min prior to CE analysis. For specificity analyses, sample solutions (40 µL) containing TBA and a protein, *e.g.* rat thrombin, lysozyme, soybean lectin or streptavidin (1 nM each in the binding buffer) were prepared (Capillary electrograms in Figure S3).

Analysis was performed using the P/ACE MDQ CE instrument equipped with a LIF detector ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 488/520$ nm). Fused-silica capillaries having a length of 30.2 cm (20 cm to the detector), an internal diameter of 75 µm and an external diameter of 375 µm were used for the experiment. These were pretreated with running buffer before daily use and rinsed between runs. The premixed TBA–protein samples were introduced by hydrodynamic injection at 0.5 psi for 7.8 s. Separations were performed at 25°C for approximately 5 min by applying a voltage of +12 kV. Electrograms were recorded at 4 Hz and analyzed with 32 Karat software ver. 8.0.

$$K_d = \frac{[P]_0 \left\{ 1 + \frac{A_3}{A_1 + A_2} \right\} - [D]_0}{1 + (A_1 + A_2)/A_3} \quad \text{eq. 1}$$

The apparent dissociation constants (K_d) of the thrombin–TBA complex were determined according to the literature.⁶ These K_d values were calculated using equation 1, where A_1 , A_2 and A_3 are peak areas of DNA in the protein–DNA complex, DNA dissociated from the complex during electrophoretic separation, and free DNA in a non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) electropherogram, respectively. Initial concentrations of protein and DNA are represented by $[P]_0$ and $[D]_0$, respectively.

Precision of determination of K_d values (% relative standard deviation) was <10% for all analytes measured.

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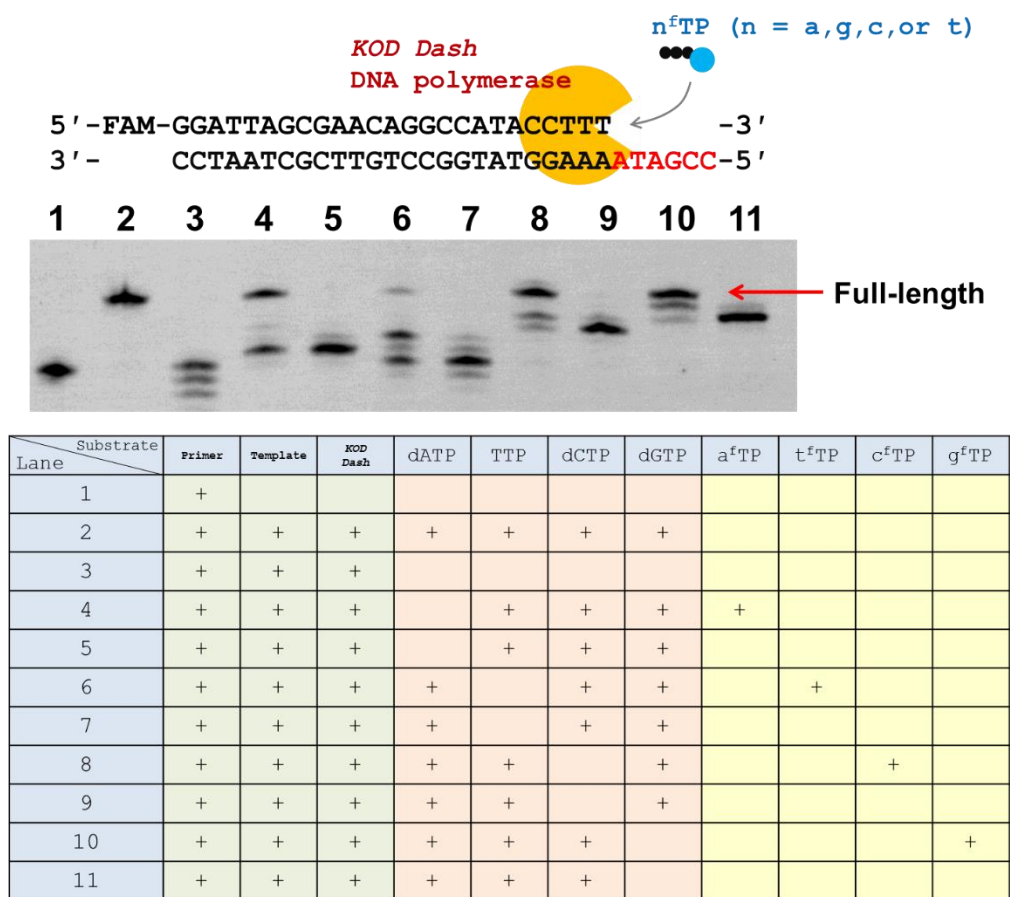


Figure S1. Primer extension reactions using 2'-deoxy-2'-fluoro-nucleoside-5'-triphosphates ($n^f\text{TPs}$).

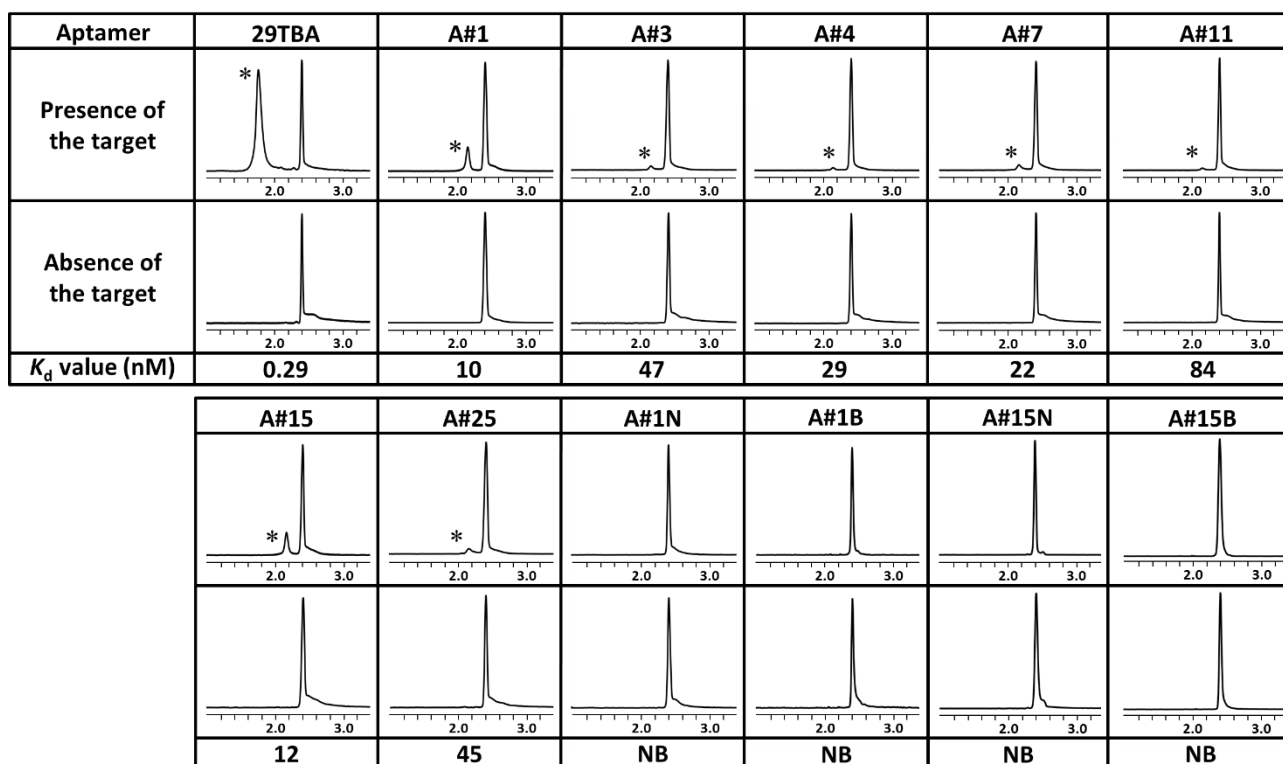


Figure S2. Capillary electrograms for 29TBA, 2',4'-BNA/FNA chimeric aptamers, and chemical variants of A#1 and A#15 with or without human thrombin. All electrograms recorded fluorescent intensity of 5'-labelled 6-FAM versus migration time (min). The asterisk indicates the peak of the thrombin–aptamer complex. K_d values were determined by NECEEM. Precision of determination of K_d values (% relative standard deviation) was <10% for all analytes measured. NB, no binding.

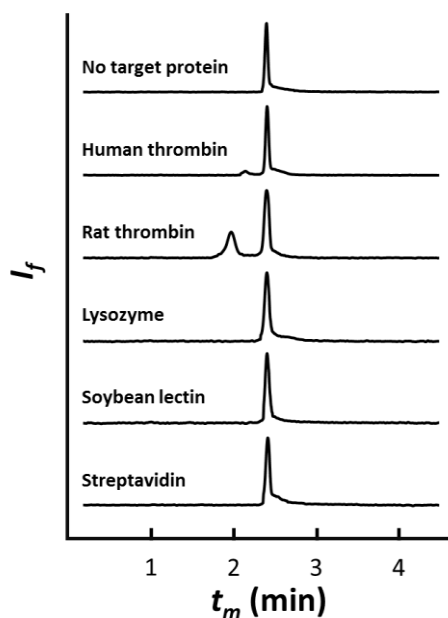


Figure S3. Binding specificity of A#1 for protein targets. The aptamer–target complex was observed when human thrombin and rat thrombin were used as targets. The peak of the aptamer–target complex was observed prior to that of the free aptamer in NECEEM. t_m = migration time, I_f = fluorescence intensity at 520 nm.

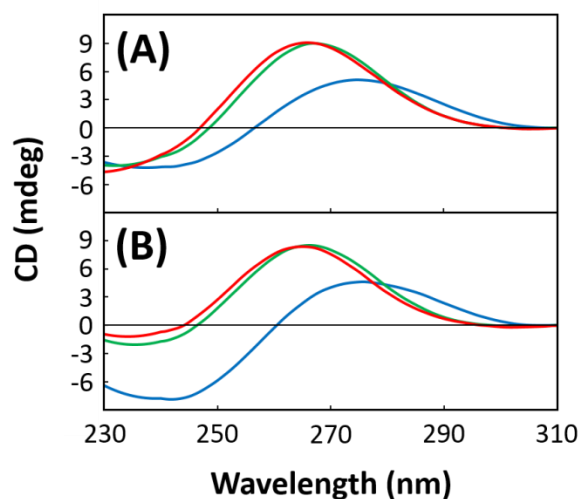


Figure S4. CD spectra for (A) A#1, A#1N, and A#1B and (B) A#15, A#15N, and A#15B in 20 mM Tris-HCl buffer containing 1 mM MgCl₂ and 100 mM NaCl, pH 7.4: A#1 and A#15 (green), A#1N and A#15N (blue), and A#1B and A#15B (red).

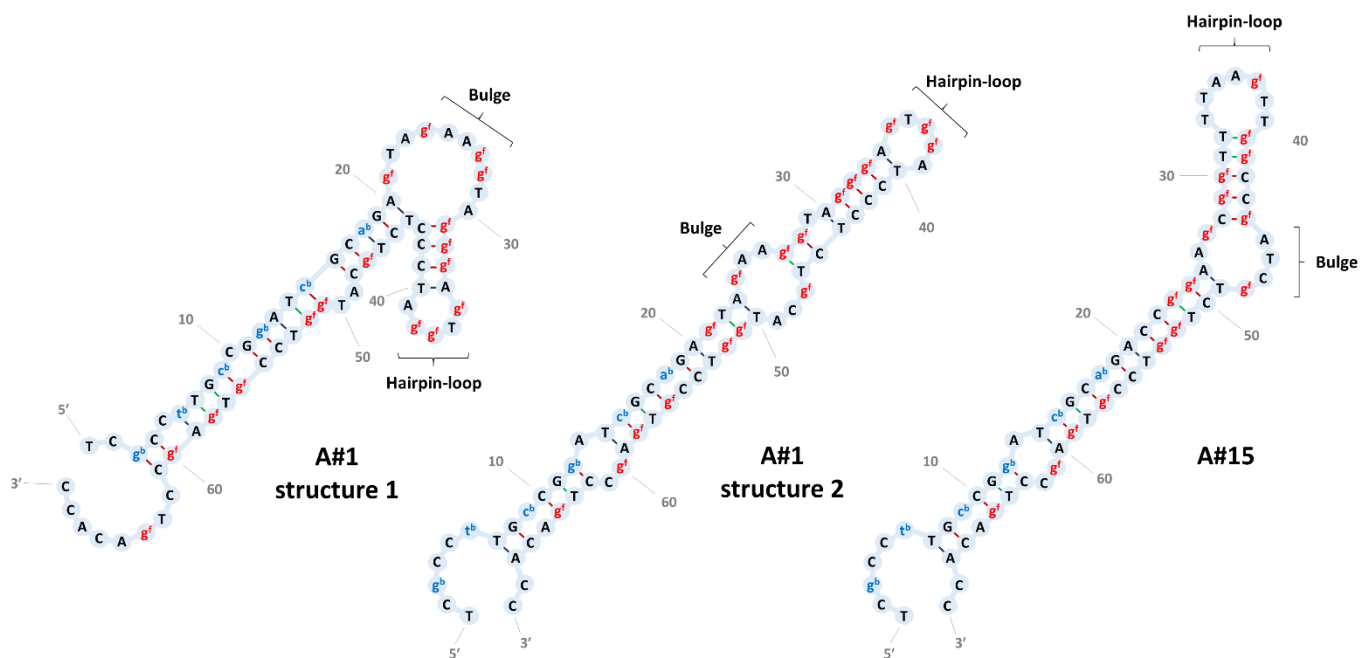


Figure S5. Predicted secondary structures of A#1 and A#15 using mfold-RNA folding form. 2'-Deoxy-2'-fluoro-guanosine and B/L nucleotides bearing adenine, guanine, 5-methylcytosine, and thymine are shown in g', a', g', c', and t', respectively.