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Supplementary Information for

Kinetic analysis for highly effective triplex formation between small molecule-peptide nucleic acid conjugate probe and Influenza A virus RNA promoter region at neutral pH

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Experimental

General Information

Rink Amide Resin was purchased from Merck Millipore (Billercia, MA). Fmoc/Bhoc protected PNA monomers were purchased from Panagene (Daejeon, South Korea). DPQ was synthesized according to previous literature[S1] and thiazole orange (TO) according to the literature.[S2] Other reagents were commercially available analytical grade and were used without further purification. All the RNAs used were custom synthesized and HPLC purified (99%) by GeneDesign Inc. (Osaka, Japan). The concentration of the RNAs were determined from the molar extinction coefficients at 260 nm at 85°C, according to the literature.[S3] Ultrapure water (18.2 MΩ cm specific resistance) with an Elix 5 UV water purification system and a Milli-Q synthesis A10 system (Millipore Co., Bedford, MA), followed by filtration through a Biopak filter (Millipore Co.) to remove RNase.

Unless otherwise mentioned, all measurements were performed in 10 mM sodium acetate buffer solutions (pH 5.5) or sodium phosphate buffer solutions (pH 7.0) containing 100 mM NaCl and 1.0 mM EDTA. Before measurements, RNA-containing samples were annealed as follows: heating at 95°C for 10 min, and cooling at 1°C for 1 min all the way down to 25°C, where it is held until the next step. Typically, the probe was added to the annealed-RNA containing solutions, and measurements were performed after an incubation period of 30 min.

UV-Vis and Fluorescence Spectroscopic Measurements

UV-Vis experiments were recorded with a JASCO model V-570 UV-Vis spectrophotometer (Japan spectroscopic Co. Ltd., Tokyo, Japan) at 25°C. Two 2 x 10 mm quartz cell (optical path length: 10 mm) was used- one for the sample, and the other for the reference (ultrapure water). A baseline correction was first done using only the buffer before actual UV measurement of the samples. All fluorescence experiments were done using a JASCO FP-6500 spectrofluorophotometer (Japan spectroscopic Co. Ltd., Tokyo, Japan) with a 3 x 3 mm quartz cell at 25°C. Both instruments were equipped with thermoelectrically temperature-controlled cell holders. Excitation wavelength for cyanine dyes in the absence of target RNAs was set as the absorption maximum wavelength. Fluorescence quantum yield (Φ) was determined relative to fluorescein (Φ = 0.93)[S4] in which the probes in free or target bound state was examined ([Probe] = 0.10 μM, [target RNA] = 1.0 μM).

Determination of Binding Constant by Fluorescence Titration Experiments

Fluorescence titration experiments were performed as previously described.[S5,S6] The probe concentration was fixed at 100 nM and the concentration of the target RNA was varied from 0 to 1.0 μM. The changes in fluorescence intensity of the probe at the maximum emission wavelength were monitored as a function of the target RNA. The resulting titration curves were analyzed by a nonlinear least-square regression based on a 1:1 binding fitting model.[S6] All data analyses were done using Kaleida Graph software 4.5.3 (Synergy software, Reading, PA).

UV Melting Temperature Experiments

UV melting experiments were conducted using a UV-Vis Spectrophotometer Model UV-2450 (Shimadzu Co. Ltd., Kyoto, Japan) equipped with a thermoelectrically temperature controlled micro-multicell holder (8 cells, optical length: 10 mm). Before each experiment, the sample solutions containing annealed RNA and the probe were put into the quartz cuvettes and covered with paraffin oil (to prevent solvent loss) and silicon caps, and then the cuvettes were incubated for 3h at 10°C. The total sample volume was 120 μl with a final concentration of 3.0 μM for both the RNA and probe buffer solutions. In the experiment, absorbance was monitored at 260 nm or 520 nm while increasing the temperature from 10 to 100°C with a temperature ramp of 0.5°C / min to give the resultant absorption spectra. The melting points were determined from a differential method.

Stopped-flow Measurements

Experiments were performed under second-order conditions using a stopped-flow spectrophotometer system, RSP-2000 (Unisok, Co. Ltd., Osaka, Japan) equipped with a temperature-controlled reaction chamber connected with an F12 refrigerated/heating circulator (Julabo, Seelbach, Germany) as previously reported.[S7,S8] Light was collected from a 75W xenon arc lamp housing equipped with a MD200 monochromator (Unisok) to the reaction chamber through a bundled optical fiber and the optical path length was 10 mm. For the measurements, a total volume of 600 μL for the 3.0 μM RNA and probe solutions were prepared. Before each experiment, the annealed RNA and probe solutions were set in each chamber and thermally equilibrated at 25°C. After equilibration, an equal volume of the two solutions were mixed in the reaction chamber, followed by monitoring the progress of the reactions by observing the decrease in the absorbance at 260 nm. Prior to the actual measurements, baseline correction was performed by measuring the absorbance of the buffer. The second-order association rate constant (k_{on} / $M^{-1}s^{-1}$) was determined from a nonlinear least-square regression by Unispek (Unisok) and the dissociation rate constant (k_{off}/s^{-1}) was calculated from $k_{off} = K_d \times k_{on}$.

Synthesis

Solid-phase synthesis of the probes

The probes were synthesized by a combination of help from the Biotage Initiator (Biotage, Uppsala, Sweden) inside a corresponding microwave peptide vial and manual synthesis by a divergent solid-phase using commercially available Fmoc/Bhoc PNA monomers and Fmoc-aeg(alloc)-OH.[S9,S10] Rink amide resin was used as a support. Coupling reagents for PNA monomers were COMU and DIEA. For the coupling of the dyes, the alloc protecting group of the PNA backbone was deprotected, followed by coupling of carboxylated TO derivatives (TO-C1-COOH and TO-C3-COOH) by using COMU/PPTS/DIEA coupling chemistry.[S5] The assembly on the resin was performed by repetitive cycles of Fmoc deprotecting with 20% piperidine in DMF and DBU, coupling with DIEA/COMU in DMF and capping with 5% acetic anhydride / 6% lutidine in DMF. The completion of the coupling reaction stages done manually was confirmed using kaiser tests. Fmoc-aeg(alloc)-OH was used for introducing the TO base surrogates and the use of Lys (Alloc)-OH was utilized for introducing the DPQ small molecule.[S10] To increase solubility, a lysine was added to the C-termini of the probes in addition to the Lys (alloc) used for introducing the DPQ small molecule.

Alloc deprotection of the first Lys was done with 8 equimolar of dimethylamine borane (DMAB) and an equimolar tetrakis(triphenylphosphine)palladium (0) (Pd $(Phh_3)_4$ in dichloromethane to introduce the DPQ small molecule which was coupled with the resulting free secondary amine by using COMU/NMP/DIEA. After successfully coupling the monomer units, the alloc group of the PNA backbone was deprotected with 8 equimolar DMAB and an equimolar (Pd $(Phh_3)_4$ in DCM. Then, TO-Cx-COOH dyes (X = 1 or 3) were coupled with the resulting free secondary amine by using COMU/PPTS/DIEA coupling chemistry. After deprotection of the terminal Fmoc group, deprotection of Bhoc groups in the PNA monomers and cleavage from the resin were carried out using a mixture of trifluoroacetic acid (TFA)/ m-cresol (85:15). The solution was added to the resin and the resultant mixture was shaken for a total of 3 hours. Afterwards, the solution was filtered, and the filtrate collected in PE centrifuge tubes (GE Healthcare UK Limited, Buckinhamshire, UK) for removal of the resin. The resin was washed with a small amount of TFA/ m-cresol mixture. Cold diethyl ether was added to the combined filtrate to precipitate the crude product. The tubes were left in the freezer overnight and the probes were collected by centrifugation the following day.

Purification of Probes

The crude product was purified by a reverse-phase HPLC system with an Inertsil ODS-3.5 µm (20 x 250 mm) column (GL sciences, Torrance, CA), with JASCO UV 2070-plus spectrometer (Japan spectroscopic Co. Ltd., Tokyo, Japan) using a gradient of water/acetonitrile containing ≥ 0.1 % TFA. The wavelengths were set to 510 nm (for TO) and 260 nm (for PNA), and the oven temperature was set to 55°C. The separated fractions were checked using MALDI-TOF mass spectrometry (matrix: CHCA) (Bruker Daltonics autoflex speed-S1, Bruker, Billerica, MA) to confirm the presence of the purified probes. The concentration of the probe solutions was determined via UV-Vis spectrophotometer at 25°C by measuring the absorbance at 260 nm in MilliQ water, with the extinction coefficients of 8800 $M^{-1}cm^{-1}$ for thymine, 7300 $M^{-1}cm^{-1}$ for cytosine and 9400 $M^{-1}cm^{-1}$ for TO.[S10]

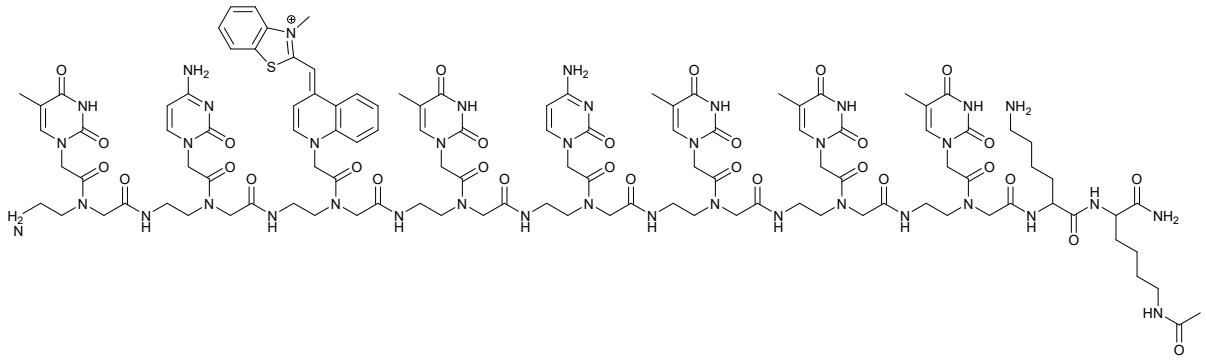

Fig. S1 Chemical structure of the Control probe used in this study.

Purification and characterization of the probes

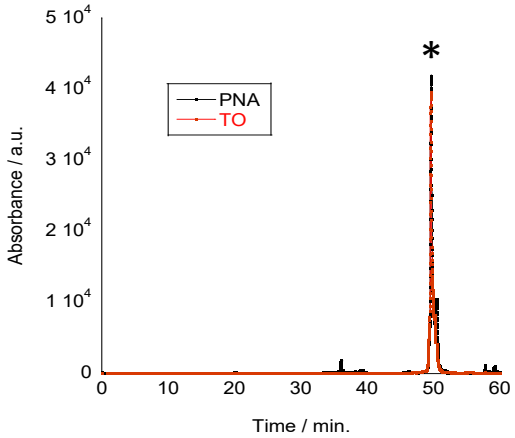

Fig. S2 HPLC profile for the purification of Probe **2**. Gradient condition: Solvent A is TFA/H_2O and Solvent B is TFA/CH_3CN during 50 min. Column temperature 55ºC, flow rate, 8 mL / min. The peak (*) was collected and identified as the purified probe by MALDI-TOF-MS.

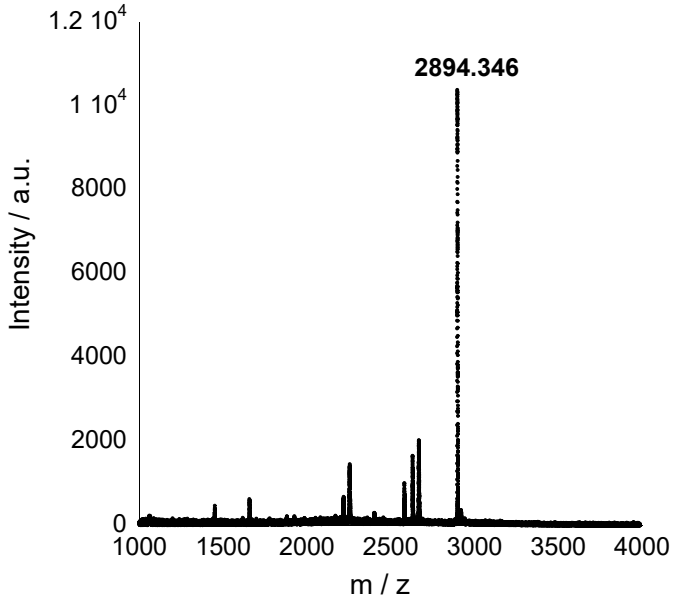

Fig. S3 MALDI-TOF-MS spectrum of purified Probe **2**.

Table S1. MALDI-TOF-MS data for the probes under study

	Molecular Formular	Calculated MW ([M]	Observed MW
Probe **1** [a]	$C_{127}H_{165}N_{44}O_{33}S^+$	2866.23	2868.715
Probe **2**	$C_{129}H_{169}N_{44}O_{33}S^+$	2894.26	2894.346
Control probe [a]	$C_{113}H_{148}N_{39}O_{31}S^+$	2579.09	2580.468

[a] Values from the literature.[S10]

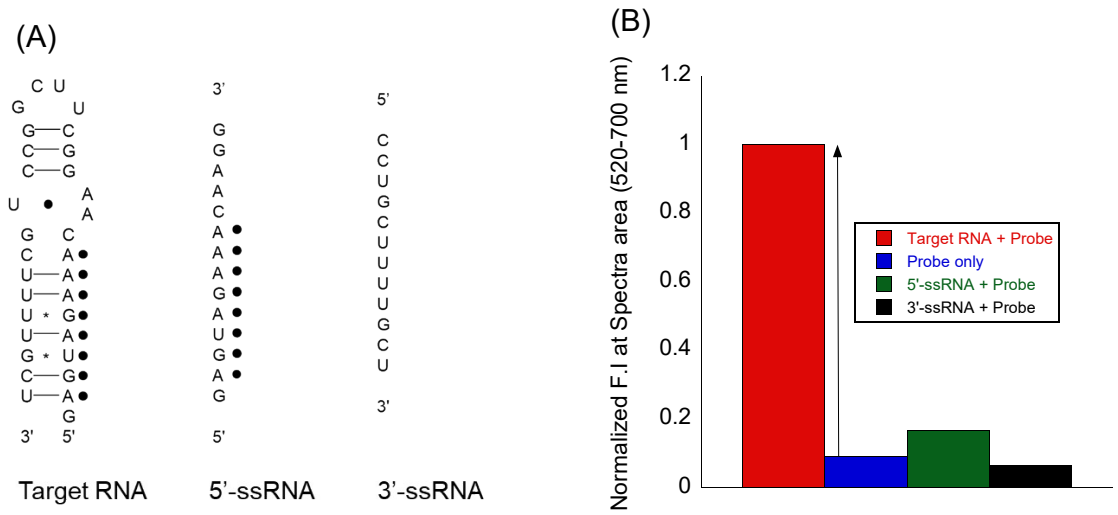

Fig. S4 (A) Sequence of the target RNA and control RNAs used in this study. Target purine rich sequences and loop are indicated by dots. (B) Normalized fluorescence intensity (spectral area) of Probe **2** in the absence or presence of RNAs. [Probe] = 100 nM, [RNA] = 100 nM, pH = 7.0, *T* = 25 °C, Excitation wavelength = 512 nm.

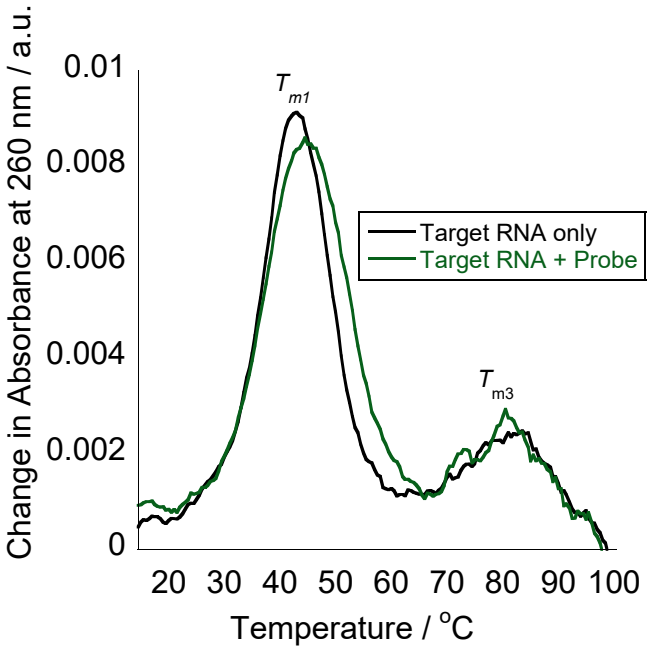

Fig. S5 UV melting curves of the target IAV RNA (3.0 µM) recorded at 260 nm in the presence and absence of the control probe (3.0 µM) under study at pH 7.0.

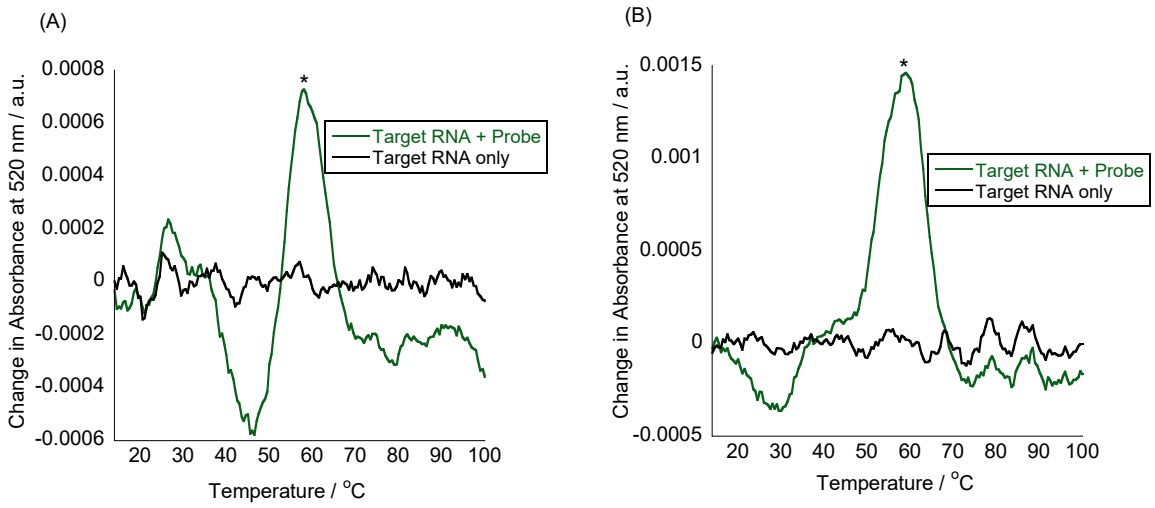

Fig. S6 UV melting curves of the target IAV RNA (3.0 μM) in the presence and absence of the tFIT-DPQ probes (3.0 μM) under study at pH 7.0. (A) Probe **1** and (B) Probe **2**. The peak (*) of 520 nm absorbance change for tFIT-DPQ probes under discussion.

Here, the change in absorbance of the tFIT-DPQ probes was observed at 520 nm. This is a longer wavelength where we assumed we can utilize the change in absorbance at the TO site when bound to the probe. From the results of the T_m experiments (Fig. S6), we observed a peak at 57.6 °C for Probe **1** which is similar to the peak observed at 58.7 °C for Probe **2**, suggesting that this peak is coming from a similar melt which is the dissociation of the entire probe coupled with TO that is bonded to the target RNA. This implies that after the initial dissociation of the duplex and triplex into individual strands (T_{m1} in main text: 41-43 °C), the DPQ small molecule was still bonded to the internal loop region, and this facilitated some sort of simple hybridization that followed next between the probes and the complementary single strand of the target RNA opposite it.

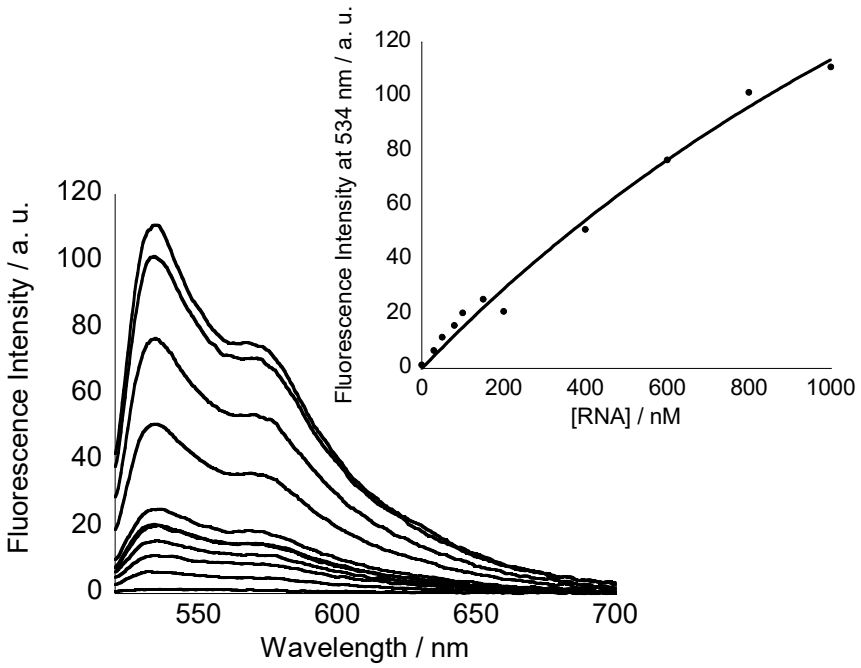

Fig. S7 Fluorescence response of the control probe (100 nM) for target RNA (0-1000 nM) at pH 7.0. Inset: Fluorescence titration curve for the binding of the control probe (100 nM) to target RNA (0-1000 nM) at pH 7.0. Measurements were performed in 10 mM sodium phosphate buffer solution containing 100 mM NaCl and 1.0 mM EDTA. Excitation, 515 nm. Analysis, 534 nm. Temperature, 25°C.

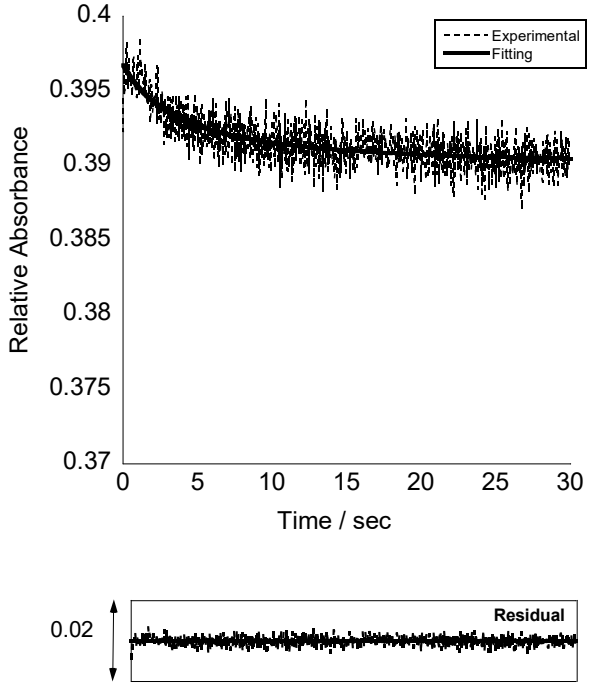

Fig. S8 Stopped-flow kinetics trace of absorbance at 260 nm for the control probe (1.5 μM) binding to equimolar target RNA at pH 7.0. Temperature, 25°C. The fitting curve is the bold line, and the corresponding residual plot is presented below the kinetics trace. k_{on} value was determined as 0.21 ± 0.15 x 10^6 $M^{-1}s^{-1}$. k_{off} was calculated as 0.32 s^{-1}.

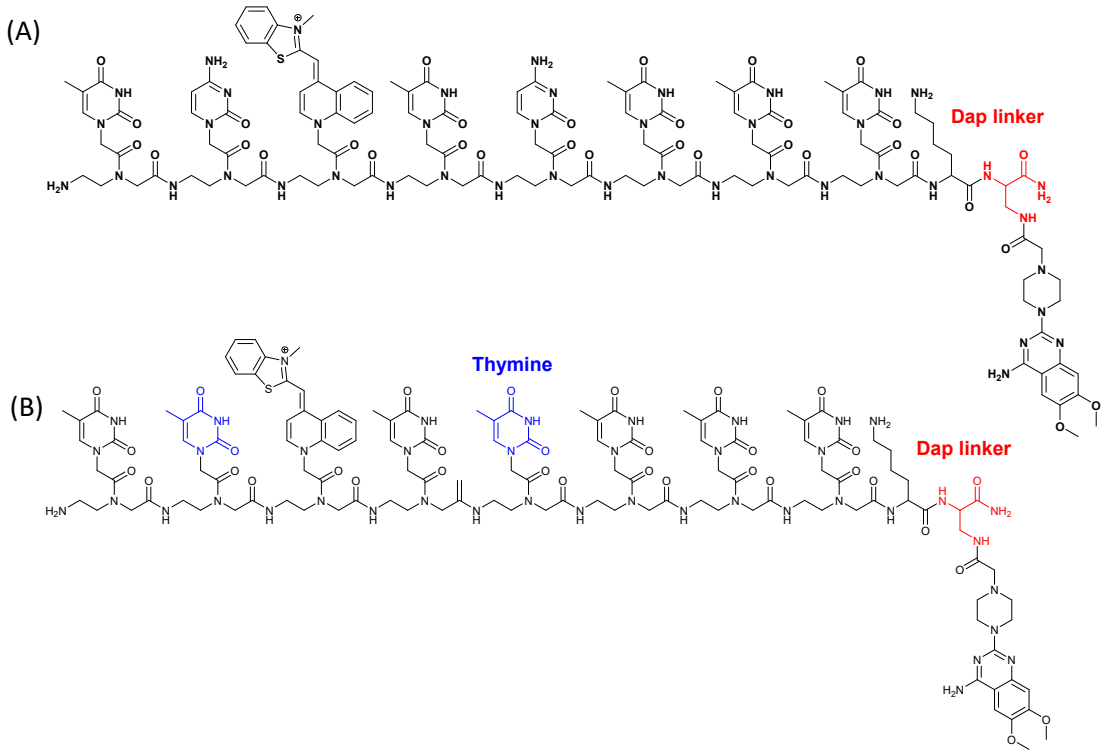

Fig. S9 Chemical structures of the tFIT-DPQ probes replacing the Lysine with Dap linker for attaching the DPQ small molecule into the tFIT unit. (A) DPQ-Dap probe: H_2N-TC(TO-C1)TCTTT-Lys-Dap(DPQ)-$CONH_2$, (B) DPQ-Dap(pT) probe: H_2N-TT(TO-C1)TTTTT-Lys-Dap(DPQ)-$CONH_2$.

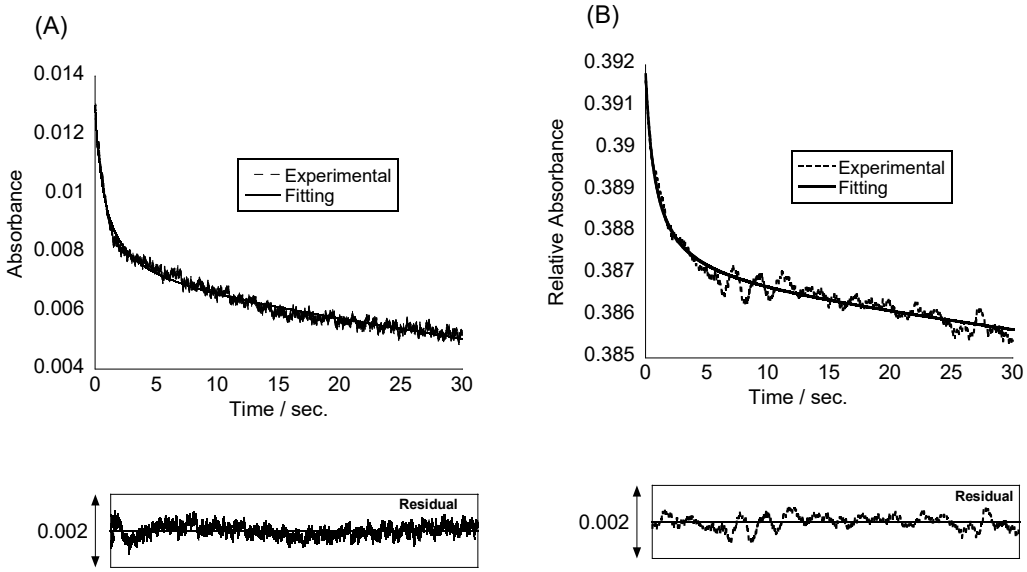

Fig. S10 Stopped-flow kinetics trace of absorbance at 260 nm for (A) DPQ-Dap probe and (B) DPQ-Dap(pT) probe (1.5 μM), binding to equimolar IAV target RNA at pH 7.0. Temperature, 25°C. The fitting curve is the bold line, and the corresponding residual plot is presented below the kinetics trace.

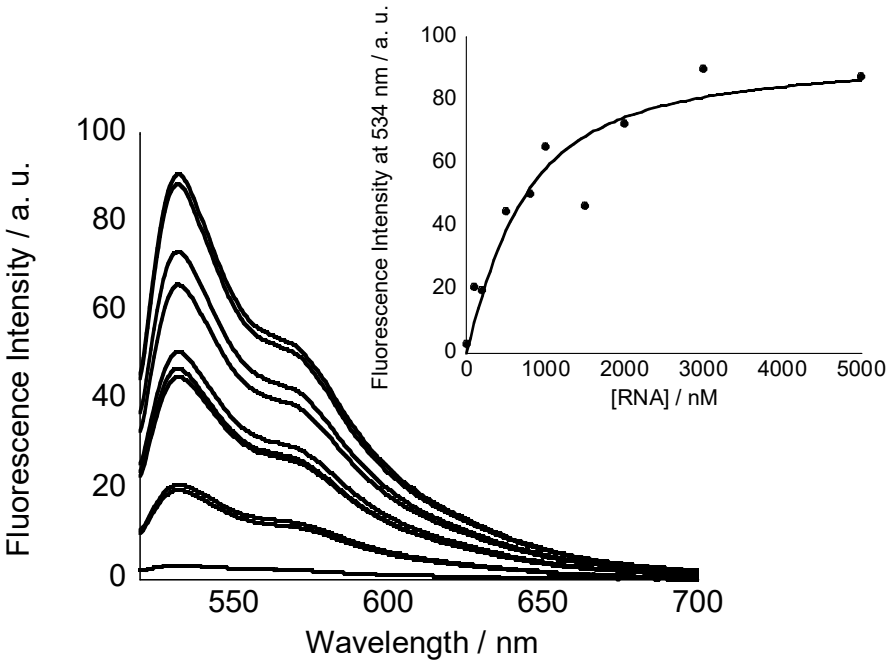

Fig. S11 Fluorescence response of DPQ-Dap(pT) probe (500 nM) for target RNA (0-5000 nM) at pH 7.0. Inset: Fluorescence titration curve for the binding of DPQ-Dap(pT) probe (500 nM) to target RNA (0-5000 nM) at pH 7.0. Measurements were performed in 10 mM sodium phosphate buffer solution containing 100 mM NaCl and 1.0 mM EDTA. Excitation, 515 nm. Analysis, 534 nm. Temperature, 25°C.

Supporting References

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