Electronic Supplementary Material (ESI) for Chemical Science

Supporting Information

Quencher-free linear probe with multiple fluorophores on acyclic scaffold

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Experimental Section

Materials. Oligonucleotides containing only natural bases were obtained from Integrated DNA Technologies, Inc. The DNA with FAM and dabcyl moeties (**MB**) was purchased from Nihon Techno Service Co., Ltd. Other modified DNAs were synthesized in our lab.

Synthesis of modified DNAs: Schemes for synthesis of phosphoramidite monomers of $\mathbf{E_2}$ were reported previously.[1] Phosphoramidite monomers of $\mathbf{E_0}$ and $\mathbf{E_4}$ were synthesized as described in Supporting Information (Schemes S1 and S2). Linear probes were first purified by PAGE and then further purified by reversed phase HPLC. All the modified DNAs were characterized by MALDI-TOF mass spectrometry.

MALDI-TOF mass spectrometry: **1E$_4$**: Obsd. m/z 5397 (Calcd. for [**1E$_4$**+H$^+$]: m/z 5396); **2E$_4$-2**: Obsd. m/z 5897 (Calcd. for [**2E$_4$-2**+H$^+$]: m/z 5897); **3E$_4$-2**: Obsd. m/z 6399 (Calcd. for **3E$_4$-2**+H$^+$]: m/z 6398); **4E$_4$-2**: Obsd. m/z 6901 (Calcd. for [**4E$_4$-2**+H$^+$]: m/z 6900); **5E$_4$-2**: Obsd. m/z 7401 (Calcd. for [**5E$_4$-2**+H$^+$]: m/z 7401); **6E$_4$-2**: Obsd. m/z 7901 (Calcd. for [**6E$_4$-2**+H$^+$]: m/z 7902); **3E$_4$-1**: Obsd. m/z 6399 (Calcd. for [**3E$_4$-1**+H$^+$]: m/z 6398); **3E$_4$-3**: Obsd. m/z 6399 (Calcd. for [**3E$_4$-3** +H$^+$]: m/z 6398); **3E$_4$-4**: Obsd. m/z 6398 (Calcd. for [**3E$_4$-4** +H$^+$]: m/z 6397); **3E$_0$-2**: Obsd. m/z 6230 (Calcd. for [**3E$_0$-2** +H$^+$]: m/z 6230); **3E$_2$-2**: Obsd. m/z 6316 (Calcd. for [**3E$_2$-2** +H$^+$]: m/z 6315).

Spectroscopic measurements: The UV/Vis spectra were measured on a Shimadzu UV-1800 instrument with 10-mm quartz cells. Fluorescence spectra were measured on a JASCO model FP-6500 with a microcell (Figs. 1, 3, and 4) or 10-mm cell (Figs. 2 and 5). Both were equipped with programmed temperature controllers. Excitation wavelength was 425 nm. In order to monitor emission at equilibrium (Figs. 1 to 4), the fluorescence spectrum was measured after heating to 80 ^{o}C followed by a gradual decrease (5 ^{o}C min^{-1}) of temperature to 20 ^{o}C. For response-measurements in Fig. 5, probe (final concentration of 0.2 μM) was dissolved in buffered solution (100 mM NaCl, 10 mM phosphate buffer, pH 7.0) in the cell (10 mm x 10 mm) by magnetically stirring at 20 ^{o}C, followed by the addition of **b3a2** (to a final concentration of 0.4 μM) to start the reaction. For tracing the response of **MB**, emission at 512 nm from FAM was monitored by excitation at 495 nm.

Measurement of melting temperature: Melting curves of duplex DNA were obtained with a Shimadzu UV-1800 by measuring the change of absorbance at 260 nm versus temperature. The melting temperature (T_m) was determined from the maximum in the first derivative of the melting curve. Both the heating and cooling curves were measured, and the T_m measurements obtained from them coincided with each other within 2.0 °C. The temperature ramp was 0.5 ^{o}C min^{-1}. Solution conditions were as follows: 1.0 μM probe, 1.2 μM **b3a2**, 100 mM NaCl, 10 mM phosphate buffer, pH 7.0.

1 H. Kashida, T. Takatsu, H. Asanuma. *Tetrahedron Lett*., 2007, **48**, 6759-6762

Scheme S1. Synthesis of phosphoramidite monomer bearing perylene having a butylene linker (**E_4**) between chromophore and D-threoninol. i) $Pd(OH)_2$, dry THF, r.t., 7 hr, 61%, ii) $(i\text{Pr})_2\text{NP(Cl)(OCH}_2\text{CH}_2\text{CN)}$, Et_3N, CH_2Cl_2, 0 °C ,0.5 hr, 76 %.

Compound 2. Compound **1** (738 mg, 1.00 mmol) synthesized according to the previous report[1] and $Pd(OH)_2$ (250 mg) were put in a round-bottom flask and 60 mL of dry THF was added under nitrogen. Reduction was initiated by replacing nitrogen gas in the flask with hydrogen. The reaction was allowed to proceed at room temperature overnight with vigorous stirring. After removing $Pd(OH)_2$ by filtration, the filtrate was evaporated and remaining solid was subjected to silica gel column chromatography ($CHCl_3$:hexane:EtOAc:Et_3N, 30: 30:30:2.7) to afford compound **2** (452 mg, 0.61 mmol, yield 61%).

^{1}H NMR (500 MHz, $CDCl_3$): 8.20 - 7.31 (m, 11H, aromatic protons of perylene), 7.36 – 6.79 (m, 13H, aromatic protons of DMT), 6.07 (d, *J* = 9.0 Hz, 1H, -N*H*CO-), 4.08 (m, 1H, -C*H*NHCO), 3.94 (m, 1H, -C*H*OH), 3.74 and 3.73 (s, 6H, -OCH_3), 3.41 (dd, *J* = 9.5 and 4.5 Hz, 1H, -OCH_2-), 3.27 (dd, *J* = 9.5 and 3.5 Hz, 1H, -OCH_2-), 3.05 (t, *J* = 7.0 Hz, 2H, -CH_2-CH_2-CH_2-CH_2-perylene), 2.30 (m, 2H, -CH_2-CH_2-CH_2-CH_2-perylene), 1.83 (m, 4H, -CH_2-CH_2-CH_2-CH_2-perylene), 1.11 (d, *J* = 6.0 Hz, 3H, CH_3-). ^{13}C-NMR [$CDCl_3$, 126 MHz] δ = 173.4, 150.9, 144.6, 138.3, 135.8, 135.5, 134.9, 133.2, 132.0, 131.7, 130.2, 130.1, 129.8, 129.3, 128.7, 128.2, 128.1, 127.9, 127.6, 127.3, 127.1, 126.8, 126.7, 126.6, 123.9, 120.4, 120.3, 113.5, 87.0, 68.9, 65.5, 55.4, 53.6, 39.0, 37.0, 33.4, 30.4, 26.2, 20.2. HRMS (ESI) Calcd for $C_{50}H_{47}NNaO_5$ (M^+) 764.3352. Found 764.3205.

Compound 3. Et_3N (0.32 mL) and 2-cyanoethyldiisopropylchlorophosphoramidite (0.20 mL, 0.92 mmol) were added to a solution of compound **2** (340 mg, 0.46 mmol) in dry CH_2Cl_2 (3.0 mL) and the solution was stirred for 20 min at 0 °C, and then 10 min at room temperature. $CHCl_3$ was added to the reaction mixture. The mixture was washed with a saturated aqueous solution of $NaHCO_3$ and then with a saturated solution of NaCl. After drying over $MgSO_4$, the solvent was removed by evaporation. Silica gel column chromatography ($CHCl_3$:hexane:Et_3N, 50:50:3, R_f = 0.58) afforded **3** (325 mg, 0.345 mmol, yield 76%).

^{1}H NMR (500 MHz, $CDCl_3$): 8.19 - 7.39 (m, 11H, aromatic protons of perylene), 7.46 – 6.76 (m, 13H, aromatic protons of DMT),5.79 and 5.61(d, *J* = 9.0 Hz, 1H, -N*H*CO-), 4.32 (m, 1H, -C*H*NHCO),4.20 (m, 1H, -C*H*OP), 3.72 and 3.72(s, 6H, -OCH_3), 3.40-3.62(m, 4H, -OC$\underline{H}_2$$CH_2$CN, -N(C$\underline{H}$$(CH_3)_2)_2$,),3.15 (m, 2H, -OC$H_2$-),3.10(m, 2H, -C$H_2$-$CH_2$-$CH_2$-$CH_2$-perylene), 2.51 -2.27 (m, 2H, -OCH_2C$\underline{H}_2$CN),2.25 (m, 2H, -CH_2-CH_2-CH_2-CH_2-perylene), 1.80 (m, 4H, -CH_2-CH_2-CH_2-CH_2-perylene), 1.25-0.92 (m, 12H, -N(CH(CH_3)$_2$)$_2$ and -CH(C$\underline{H}_3$)OP and CH_3-). ^{13}C-NMR [$CDCl_3$, 126 MHz] δ =172.9, 172.7, 158.6, 158.5, 145.0, 145.0, 138.4, 138.4, 136.2, 136.1, 134.8, 133.1, 131.9, 131.6, 130.0, 130.2, 129.6, 129.2, 128.6, 128.4, 128.3, 127.9, 127.8, 127.4, 127.0, 126.9, 126.8, 126.7, 126.6, 126.4, 123.9, 120.2, 119.8, 118.0, 117.9, 113.2, 113.1, 86.2, 86.1, 69.8, 69.7, 69.0, 68.9, 63.2, 62.9, 58.4, 58.3, 58.0, 57.8, 54.1, 53.9, 53.8, 45.1, 43.3, 43.2, 43.1, 37.0, 36.9, 33.3, 30.3, 26.1, 24.8, 24.7, 24.6, 24.4, 20.5, 20.4, 19.8, 19.7. ^{31}P-NMR [$CDCl_3$, 500 MHz] δ = 148.7, 148.6. HRMS(ESI) Calcd for $C_{59}H_{64}N_3ClO_6P$ (M^-) 976.4227. Found 976.4235.

1 H. Kashida, N. Kondo, K. Sekiguchi, and H. Asanuma, *Chem. Commun.*, 2011, **47**, 6404-6406.

Scheme S2. Synthesis of phosphoramidite monomer bearing perylene (**E_0**) directly conjugated on D-threoninol through an amide bond. i) DCC, HOBt, DMF, r.t., overnight, 71%; ii) (iPr)$_2$NP(Cl)(OCH$_2$CH$_2$CN), DIPEA, dry CH$_2$Cl$_2$, 0 °C , 1 h, 86%.

Compound 6. Compounds **4** (285 mg, 0.7 mmol)[2] and **5** (156 mg, 0.53 mmol)[3] synthesized as previously reported were condensed in the presence of DCC (124 mg, 0.6 mmol, 1.2 eq) and HOBt· H$_2$O (92 mg, 0.6 mmol, 1.2 eq) in 20 mL of DMF by stirring overnight at room temperature. EtOAc was added to the reaction mixture, and the resulting solution was washed with a saturated solution of NaHCO$_3$ and then with brine. After drying over MgSO$_4$, the solvent was removed by evaporation, followed by silica gel column chromatography (hexane:CHCl$_3$:Et$_3$N, 50:50:3, Rf = 0.14) to afford compound **6** (340 mg, 0.50 mmol, yield 71%).

^{1}H-NMR [CDCl$_3$, 500 MHz] δ = 8.27 – 7.51 (m, 11H, aromatic protons of perylene), 7.42 – 6.81 (m, 13H, aromatic protons of DMT), 6.72 (d, J = 9.0 Hz, 1H, -N*H*CO-), 4.26 (m, 1H, -CH$_2$C*H*NH), 3.78 (m, 1H, -C*H*OH), 3.77 and 3.76 (s and s, 6H, -OC*H*$_3$), 3.71 (dd, J = 10 and 3.5 Hz, 1H, -OC*H*$_2$-), 3.44 (dd, J = 10 and 3.5 Hz, 1H, -OC*H*$_2$-), 1.31 (d, J = 6.0 Hz, 3H, C*H*$_3$).^{13}C-NMR [CDCl$_3$, 126 MHz] δ = 170.0, 158.6, 144.5, 135.6, 135.5, 134.3 , 133.3, 131.5, 131.1, 130.7, 130.1, 130.0, 128.8, 128.5, 128.2, 128.0, 127.9, 127.32, 127.0, 126.5, 126.5, 126.4, 125.7, 125.2, 120.9, 120.6, 120.5, 118.8, 113.3, 86.8, 68.6, 65.2, 55.2, 54.4, 20.4. HRMS (FAB) Calcd for C$_{46}$H$_{39}$NO$_5$ (M$^+$) 685.2828. Found 685.2814.

Compound 7. DIPEA (0.42 mL, 2.48 mmol, 5 eq) and 2-cyanoethyldiisopropylchlorophosphoramidite (0.22 mL, 0.99 mmol, 2 eq) were added to a solution of compound **6** (340 mg, 0.50 mmol) in dry CH$_2$Cl$_2$ (5.0 mL), and the solution was stirred for 30 min at 0 °C, and then 30 min at room temperature. EtOAc was added to the reaction mixture and the resulting solution was washed with saturated solution of NaHCO$_3$ and then with brine. After drying over MgSO$_4$, the solvent was removed by evaporation, followed by silica gel column chromatography (hexane:CHCl$_3$:Et$_3$N, 50:25:3, R_f = 0.18) to afford compound **7** (380 mg, 0.43 mmol, yield 86 %).

^{1}H-NMR [CDCl$_3$, 500 MHz] δ = 8.26 – 7.43 (m, 11H, aromatic protons of perylene), 7.37 – 6.79 (m, 13H, aromatic protons of DMT), 6.37 and 6.19 (d and d, J = 9.0 Hz, 1H, -NHCO-), 4.54 (m, 1H, -CH2C*H*NH), 3.79 (m, 1H, -CHOH), 3.77 (s , 6H, -OCH3), 3.51 (m, 2H, NC-CH$_2$-C*H*$_2$-O-), 3.54-3.31 (m, 2H, -OCH2-),3.46 (m, 2H, N(CH$_3$C*H*CH$_3$)$_2$), 2.56-2.28 (m, 2H, NC-C*H*$_2$-CH$_2$-O-), 1.34-1.07 (m, 12H, N(C*H*$_3$CHC*H*$_3$)$_2$), 0.92 (d, J = 6.5 Hz, 3H, CH$_3$-). ^{13}C-NMR [CDCl$_3$, 126 MHz] δ = 169.5, 158.6, 145.0, 136.2, 134.7, 134.2, 133.6, 131.9, 131.4, 131.1, 130.6, 130.3, 129.2, 128.8, 128.6, 28.4, 128.3, 128.2, 128.0, 127.7, 127.6, 127.0, 126.9, 126.7, 125.8, 121.2, 120.9, 119.3, 113.3, 86.3, 70.3, 69.6, 63.5, 58.1, 55.3, 55.0, 54.6, 43.2, 24.6, 20.3, 19.9. ^{31}P-NMR [CDCl$_3$, 300 MHz] δ = 148.6, 148.7. HRMS(FAB) Calcd for C$_{55}$H$_{56}$N$_3$O$_6$P (M$^+$) 885.3907. Found 885.3918.

2 Y. Hara, T. Fujii, H. Kashida, K. Sekiguchi, X.G. Liang, K. Niwa, T. Takase, Y. Yoshida, H. Asanuma, *Angew. Chem. Int. Ed*. 2010, **49**, 5502-5506.

3 S.S. Bag, Y. Saito, K. Hanawa., S. Kodate, I. Suzuka and I. Saito, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 6338-6341.

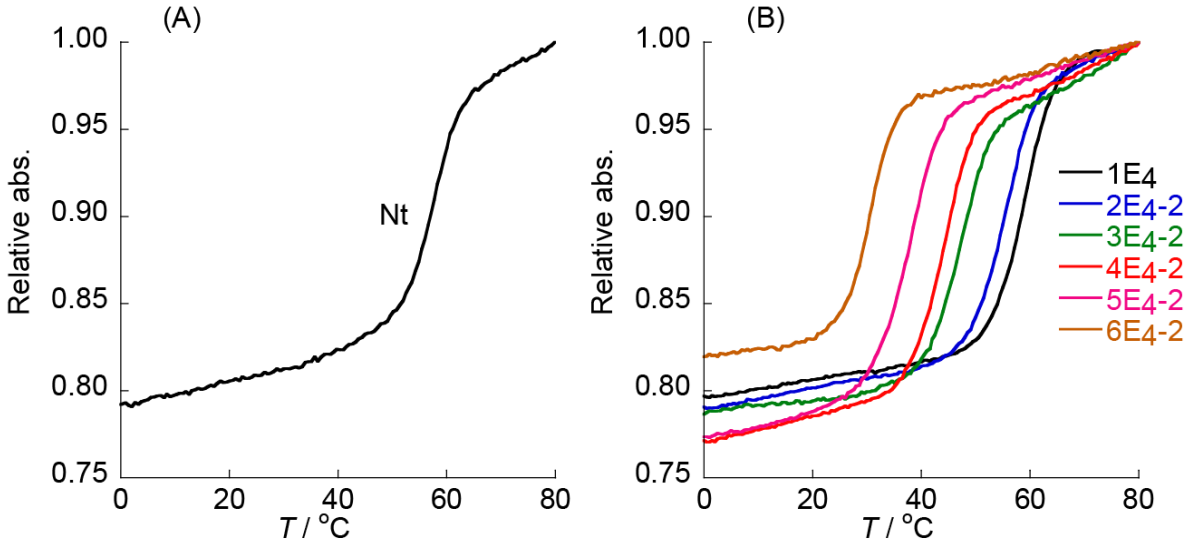

Figure S1. Actual melting curves of (A) **Nt** and (B) ***n*E$_4$-2** series hybridized with **b3a2**.
Solution conditions were 1.0 μM probe, 1.2 μM **b3a2**, 100 mM NaCl, 10 mM phosphate buffer (pH 7.0).

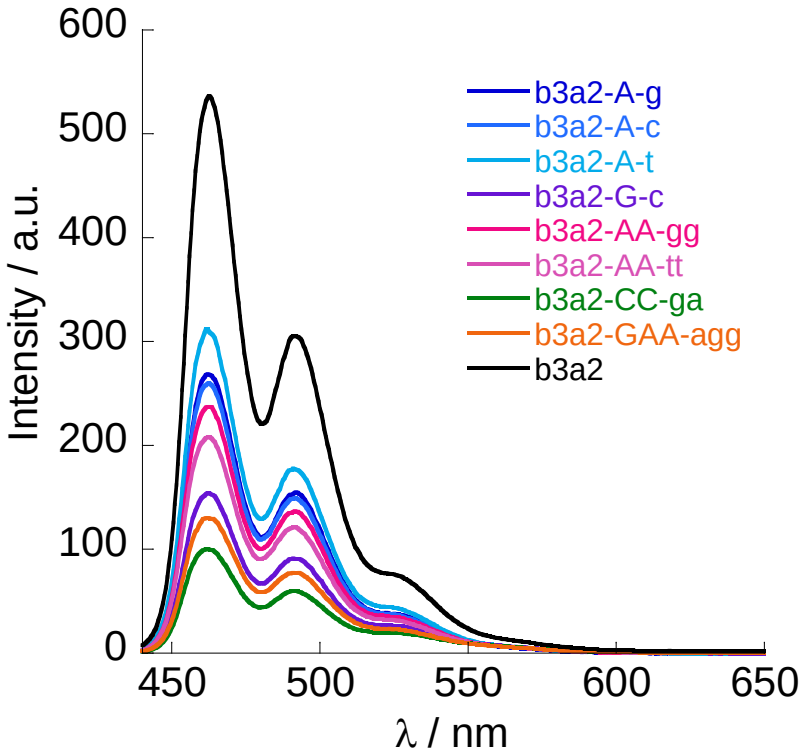

Figure S2. Emssion spectra of linear **5E$_4$-2** probe excited at 425 nm in the presence of fully matched **b3a2** (black line) and various mismatched target (colored lines). Sequences of the targets and the probe and the chemical structure of **E$_4$** are shown below. Solution conditions were 1.0 μM probes, 2.0 μM substrate, 100 mM NaCl, 10 mM phosphate buffer, pH 7.0, at 20 ^{o}C.

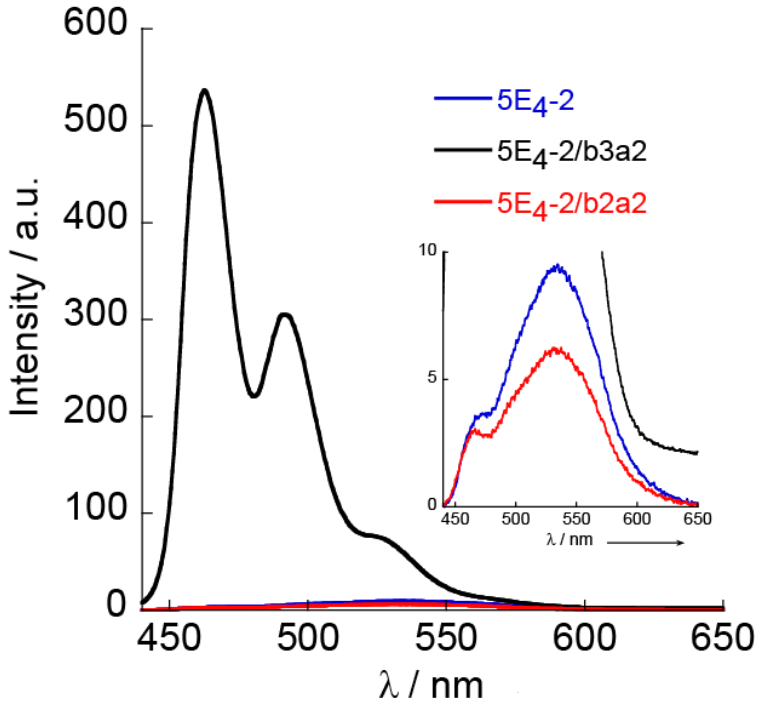

Figure S3. Emssion spectra of linear **5E$_4$-2** probe in the presence of fully matched **b3a2** (black line), half-matched **b2a2** (red line), and in the absence of the target (blue line). Sequences of the targets and the probe and the chemical structure of **E$_4$** are shown below. Underlined sequences in **b3a2** and **b2a2** are complementary to **5E$_4$-2**.Solution conditions were 1.0 μM probes, 2.0 μM **b3a2** or **b2a2**, 100 mM NaCl, 10 mM phosphate buffer, pH 7.0, at 20 ^{o}C.

(Underlined sequences in **b3a2** and **b2a2** are complementary to **5E$_4$-2**)

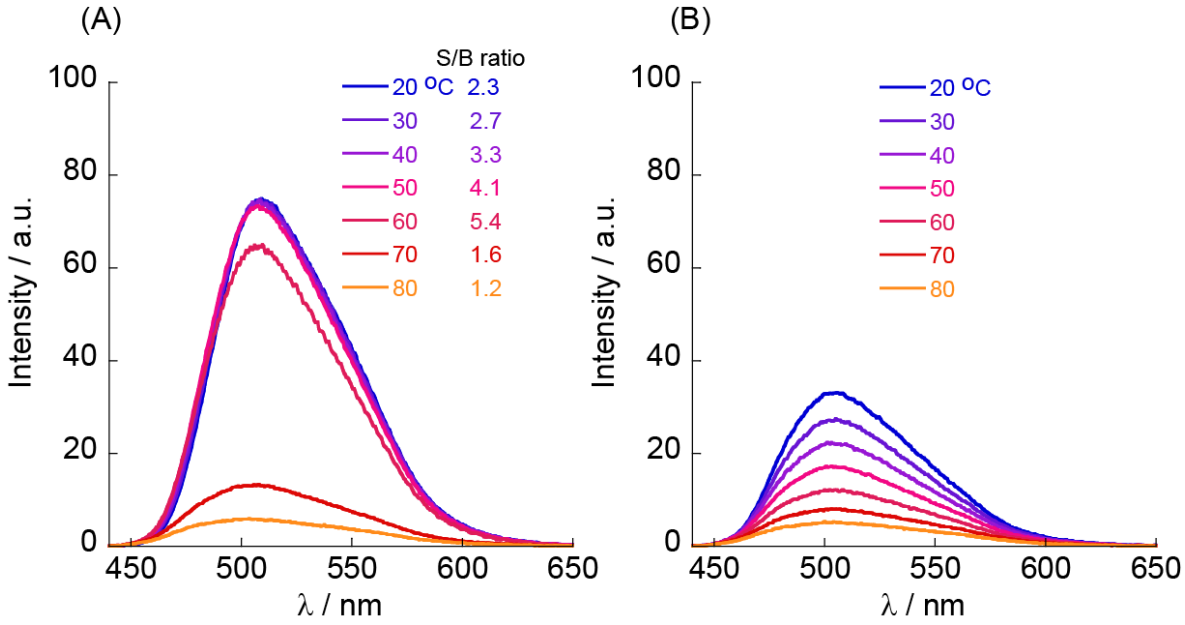

Figure S4. Emission spectra of **3E$_0$-2** in the (A) presence and (B) absence of b3a2. S/B ratios are superimposed in (A). Solution conditions were 1.0 μM probe, 2.0 μM **b3a2**, 100 mM NaCl, 10 mM phosphate buffer, pH 7.0, 20 °C.

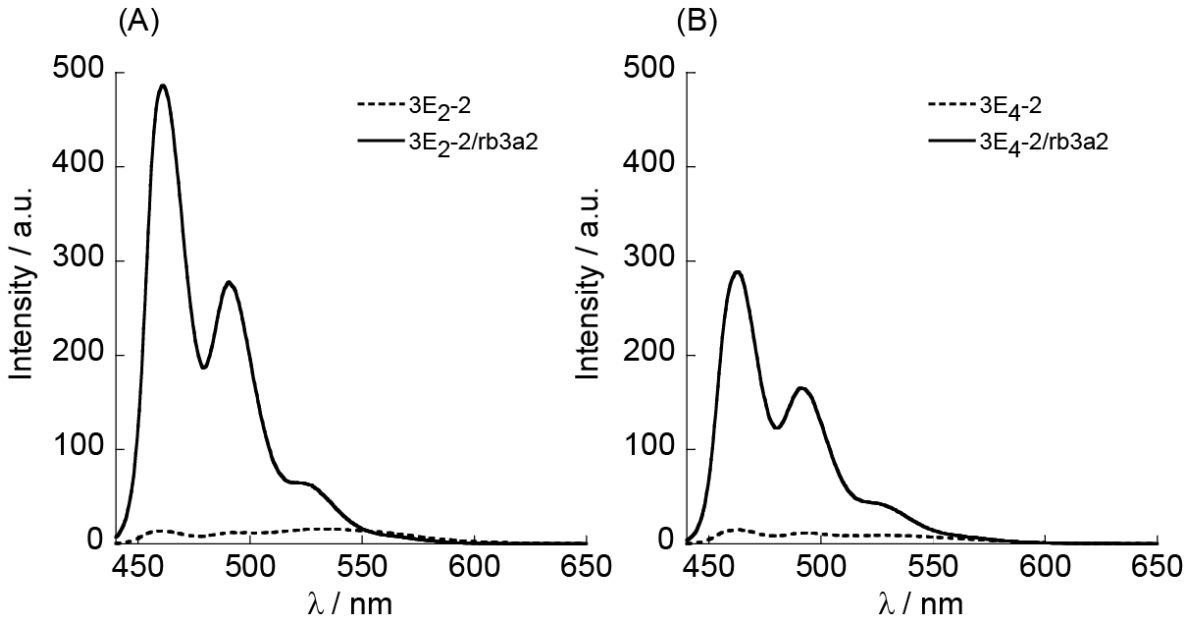

Figure S5. Fluorescence detection of RNA by (A) **3E$_2$-2** and (B) **3E$_4$-2** probes in the absence (dotted line) and presence (solid line) of **rb3a2**. Sequences of the probes and target and the chemical structures of **E$_4$** and **E$_2$** are shown below.
Conditions: excitation at 425 nm, 1 μM probe, 2.0 μM **rb3a2,** 100 mM NaCl, 10 mM phosphate buffer, pH 7.0, 20 ^{o}C.