Supporting Information

for

Ratiometric Sandwich-type assays for RNAs with a point mutation using benzo[a]pyrene-modified probes.

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Materials and Instrumentation

Materials and instrumentation Reagents, solvents, and oligonucleotides were purchased from commercial sources. Flush column chromatography was performed using silica gel (200 mesh and 300 mesh).^{1}H NMR spectra were recorded on a 300 MHz NMR spectrometer. All benzo[a]pyrene-modified probes were purified with a reversed-phase HPLC system with a C18 reversed-phase column (HPLC conditions: 0.8 mL/min; solvent A = 0.1 M triethylammonium acetate (TEAA); solvent B = 50% acetonitrile/0.1 M TEAA linear gradient, monitored at 260, 342, and 400 nm). A UV-Vis spectrometer (Shimadzu: UV-1800) was used to measure the absorption spectra of benzo[a]pyrene-modified probes. A fluorescence spectrometer (HITACHI: F2700) was used to measure the fluorescence spectra and the fluorescence quantum yields.

Fluorescence spectroscopy

Fluorescence spectra were measured using a fluorescence spectrometer (HITACHI: F2700) and 10 mm quartz cuvettes in phosphate buffer (pH 7.0) containing 2 mM $MgCl_2$ and 150 mM NaCl. The final concentrations of the oligonucleotides were adjusted to 1 μM. The fluorescence quantum yield was determined using perylene in dichloromethane as a reference with a known Φ_{em} value of 0.97. The quantum yield was calculated according to the following equation: $\Phi_{em(S)}/\ \Phi_{em(R)} = (E_{(S)}/E_{(R)}) \times (A_{(R)}/A_{(S)}) \times (n_{(S)}^2/n_{(R)}^2)$. Here, Φ is fluorescence quantum yield of fluorescence emission, A is the magnitude of absorbance, E is the integrated fluorescence intensity and n is the refractive index of the solvent. R and S denote values for the reference and sample, respectively.

UV-vis spectroscopy

Absorption spectra were measured using the UV-vis spectrometer and 10 mm quartz cuvettes in phosphate buffer (pH 7.0) containing 2 mM $MgCl_2$ and 150 mM NaCl. The final concentration of the oligonucleotides was adjusted to 1–10 μM. Melting temperatures were measured using the UV-vis spectrometer and 10 mm quartz cuvettes in phosphate buffer (pH 7.0) containing 2 mM $MgCl_2$ and 150 mM NaCl. The final concentrations of the oligonucleotides were adjusted to 2 μM. Absorbance at 260 nm was monitored during a thermal cycle. Melting temperatures were the average values of triplicate measurements.

Sandwich-type assay

Preparation of capture beads.

500 μL of NHS-activated Sepharose 4Fast flow (GE healthcare) was added to the 1.5 mL tube. After centrifugating at 8,000 rpm at room temperature for 2 min, the supernatant was removed. The residue was washed with distilled water three times, 2 M NaOH aq. (500 μL) and EtOH (500 μL) was added. After stirring at room temperature for 24 h, the residue was washed with distilled water and

suspended in 1 mL of Dulbecco′s PBS (pH 7.4) (carboxylic modified beads). the carboxylic modified beads solution (200 μL) was added to 1.5 mL tube. After centrifugating at 8,000 rpm at room temperature for 90 sec, the supernatant was removed, and the residue was washed with anhydrous DMF (800 μL) three times. To the residue, DMF (600 μL) containing 200 mM *N*-hydroxyl succinimide (NHS) and 80 mM 1-ethyl-3-(3-dimethylamiinopropyl)carbodiimide (EDC) was added. After stirring at 65 °C for 2 h, the supernatant was removed. The residue was washed with 100 mM HCl (600 μL) once and 10 mM phosphate buffer (pH 7.0, 600 μL) once and the resulting residue was added to reaction buffer (30 μL) containing 50 μM **ODN12**, 20 mM phosphate buffer (pH 7.0), and 0.2 mM thymidine. The mixture was stirred at 25 °C for 30 min and the supernatant was collected for the analysis of the amount of **ODN12** immobilized on the agarose beads. The residues were washed with 10 mM phosphate buffer (pH 7.0) three times to yield capture beads solution. Reverse-phased HPLC analysis of the supernatant of the reaction solution revealed that **ODN12** of 22 fmol was immobilized on one agarose bead (**Figure S6**).

Sandwich-type detection of RNAs with a point mutation.

To a solution (90 μL) of capture beads (10 particles), 1 μM benzo[a]pyrene-modified in phosphate buffer containing 2 mM $MgCl_2$ and 300 mM NaCl, 10 pM–1 nM target RNAs (10 μL) was added. After stirring at room temperature for 24 h, the solution was transferred to glass bottom dish and covered with cover slip. Then, the fluorescence images of capture beads were obtained with confocal laser microscopy (Nikon; A1R HD25). The microscope setting as follows: excitation, 405 nm; observed range: 415-607 nm (Δ=6 nm); objective lens, 60×3; DM, 405/488 . Fluorescence intensities were quantified using Image J software (NIH: version 1.53p) and NIS-elements AR (Nikon).

Synthesis

Synthesis of 2’/3’-*O*-[1-(benzo[a]pyrenyl)methyl]uridine (**2a** and **2b**).

To a solution of uridine (1.71 g, 7 mmol) in anhydrous methanol (350 mL), dibutyltin oxide (1.79 g, 7.2 mmol) was added at room temperature. After refluxing at 110 °C for 30 min, the solution was cooled to room temperature and concentrated under reduced pressure. The residue was co-evaporated with anhydrous DMF (15 mL) three times. The resulting residue was dissolved in DMF (35 mL) and then was added 6-chloromethylbenzo[a]pyrene (601.5 mg, 2 mmol). After stirring at 90 °C for 2 h, the mixture was cooled to room temperature. After the addition of ice, the solution was concentrated to near dryness under reduced pressure. The insoluble solid was suspended in EtOAc and filtrated, washed with EtOAc, and dried *in vacuo*. The residue was purified by chromatography on a column of silica gel (C200) with DCM:MeOH (100:0–19:1, v/v) to give **2a** and **2b** (849.0 mg, 84%). HRMS (ESI-TOF) m/z: $[M+Na]^+$ calcd for $C_{30}H_{24}N_2NaO_6$ 531.1527; found 531.1555.

Synthesis of 5'-*O*-(4,4'-dimethoxyltrityl)-2'-*O*-[1-(benzo[a]pyrenyl)methyl]uridine (**3**).

To a solution of **2a** (509.0 mg, 1 mmol) in anhydrous pyridine (20 mL), DMTrCl (508.4 mg, 1.5 mmol) was added at room temperature. After stirring at room temperature for 3 h, the mixture was diluted with EtOAc, washed 5% $NaHCO_3$ aq., and dried with anhydrous Na_2SO_4. The mixture was filtrated and concentrated under reduced pressure. The residue was purified by chromatography on a column of silica gel (C300) with Hexane:EtOAc (4:1–1:1, v/v) containing 0.5% pyridine to give **3** (520.6 mg, 64%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.38 (s, 1H), 9.30–9.27 (m, 2H), 8.77 (d, J = 8.1 Hz, 1H), 8.57 (d, J = 9.6 Hz, 1H), 8.48 (d, J = 9.0 Hz, 1H), 8.38 (d, J = 7.8 Hz, 1H), 8.23 (d, J = 7.5 Hz, 1H), 8.12–8.07 (m, 2H), 7.92–7.81 (m, 2H), 7.63 (d, J = 8.1, Hz), 7.32–7.16 (m, 9H), 6.84 (t, J = 6.9 Hz, 4H), 6.01 (d, J = 3.9 Hz, 1H), 5.86 (d, J = 11.4 Hz, 1H), 5.75 (d, J = 11.4 Hz, 1H), 5.61 (d, J = 6 Hz, 1H), 5.18 (d, J = 7.8 Hz, 1H), 4.46–4.37 (m, 2H), 4.03 (d, J = 3.0 Hz, 1H), 3.73 (d, J = 3.6 Hz, 6H), 3.32–3.23 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 162.9, 158.1, 150.4, 144.5, 140.0, 135.4, 135.0, 130.9, 130.3, 130.2, 129.7, 129.7, 128.7, 128.6, 128.2, 127.8, 127.7, 127.6, 127.6, 126.9, 126.7, 126.4, 126.2, 126.1, 125.3, 124.4, 124.2, 123.6, 122.5, 122.4, 113.2, 101.5, 87.3, 85.9, 83.2, 80.8, 68.9, 64.8, 62.9, 55.0. HRMS (ESI-TOF) m/z: $[M+Na]^+$ calcd for $C_{51}H_{42}N_2NaO_8$ 833.2833; found 833.2866.

Synthesis of 5'-*O*-(4,4'-dimethyltrityl)-2'-*O*-[1-(benzo[a]pyrenyl)methyl]uridine-3'-(2-cyanoethyl-*N*,*N*-diisopropylphophoramidite) (**4**).

To a solution of **3** (182.8 mg, 0.23 mmol) in anhydrous DCM (2 mL), *N*,*N*-diisopropylethylamine (52 μL, 0.3 mmol) was added under N_2 atmosphere. Then, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (53 μL, 0.24 mmol) was added to the solution. After stirring at room temperature for 2 h, the mixture was washed with saturated $NaHCO_3$ aq. and brine, and dried with anhydrous Na_2SO_4. The mixture was filtrated and concentrated under reduced pressure. The residue was purified by chromatography on a column of silica gel (C200) with hexane:EtOAc (2:1–1:4,v/v) containing 1% triethylamine to give **4** (162.2 mg, 70%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.06–9.03 (m, 2H), 8.77–8.71 (m, 1H), 8.513 (dd, J = 4.2, 9.6 Hz, 1H), 8.34 (dd, J = 3.0, 9.3 Hz, 1H), 8.24 (d, J = 7.5 Hz, 1H), 8.06 (d, J = 7.5, 1H), 7.98–7.91 (m, 2H), 7.82 (dd, J = 3.6, 6.3 Hz, 2H), 7.58 (dd, J = 8.1, 13.5 Hz, 1H), 7.25–7.11 (m, 8H), 6.75 (ddd, J = 3.6, 8.3 Hz, 4H), 6.26–6.15 (dd, J = 3.3, 28.5 Hz, 1H), 5.98–5.83 (m, 2H), 4.94 (dd, J = 8.1, 9.3 Hz, 1H), 4.70–4.51 (m, 1H), 4.36–4.21 (m, 2H), 3.75 (d, J = 1.2 Hz, 3H), 3.74 (s, 3H), 3.71–3.30 (m, 7H), 2.43 (t, J = 6.3 Hz, 1H), 2.32–2.07 (m, 1H), 1.15–1.05 (m, 9H), 0.96 (d, J = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.0, 158.6, 150.3, 150.3, 144.3, 144.2, 139.6, 135.3, 135.2, 135.1, 134.9, 131.2, 130.7, 130.7, 130.6, 130.5, 130.2, 130.1, 129.4, 129.3, 128.9, 128.2, 128.2, 128.1, 128.0, 127.8, 127.8, 127.0, 126.6, 126.5, 126.2, 126.2, 126.1, 125.8, 125.8, 125.4, 125.3, 125.2, 125.1, 124.0, 123.3, 123.3, 123.2, 122.0, 117.7, 117.5, 113.2, 102.1, 101.9, 88.3, 87.9, 87.1, 86.9, 82.9, 82.7, 81.4, 80.7, 70.4, 70.2, 70.0, 65.3, 61.9, 61.3, 58.6, 58.4, 58.1, 55.2,

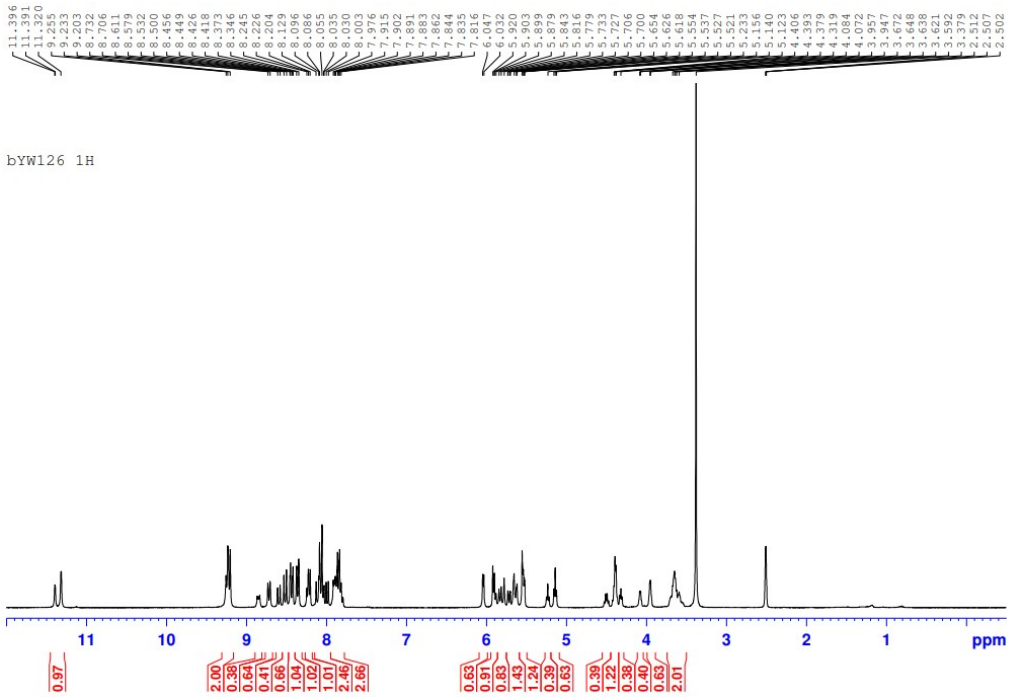
43.4, 43.2, 43.2, 43.0, 24.8, 24.7, 24.6, 24.5, 24.4, 20.3, 20.2, 20.1, 20.1. ^{31}P NMR (121 MHz $CDCl_3$) δ 149.7, 149.6. HRMS (ESI-TOF) m/z: $[M+Na]^+$ calcd for $C_{60}H_{59}N_4NaO_9P$ 1033.3912; found 1033.3905.

Synthesis of the benzo[a]pyrene-modified probes.

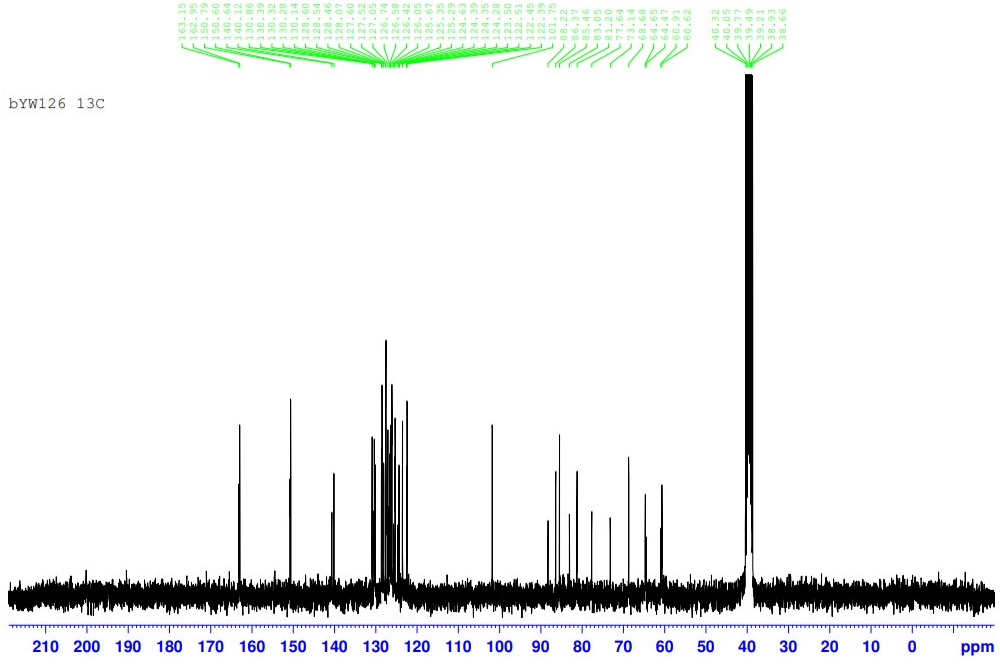
All the benzo[a]pyrene-modified probes were synthesized by the standard solid-phase synthesis method. The coupling time for the Ubpy phosphoramidite unit **4** was extended to 15 min. All the benzo[a]pyrene-modified probed were deprotected by 28% NH_3 aqueous solution at 55 °C for 16 h and purified with reversed-phase HPLC (**Figure S2**). All the benzo[a]pyrene-modified probes were characterized with ESI-TOF-MS spectrometry. ESI-TOF-MS m/z: $[M-5H]^{5-}$ calcd for **Probe1** 1063.88 found for 1063.89; $[M-5H]^{5-}$ calcd for **Probe2** 1397.26 found for 1397.25; $[M-5H]^{5-}$ calcd for **Probe3** 1064.80 found for 1064.80; $[M-5H]^{5-}$ calcd for **Probe4** 1413.25 found for 1413.27; [M-5H]5- calcd for **Probe5** 1411.04 found for 1411.02; $[M-5H]^{5-}$ calcd for **Probe6** 1347.75 found for 1347.70; $[M-6H]^{6-}$ calcd for **Probe7** 1114.71 found for 1114.63; $[M-4H]^{4-}$ calcd for **Probe8** 1586.85 found for 1586.79; $[M-5H]^{5-}$ calcd for **Probe9** 1255.93 found for 1255.93; $[M-5H]^{5-}$ calcd for **Probe10** 1272.63 found for 1272.60; [M-5H]5- calcd for **Porbe11** 1280.60 found for 1280.60.

^{1}H, ^{13}C, and ^{31}P NMR spectra

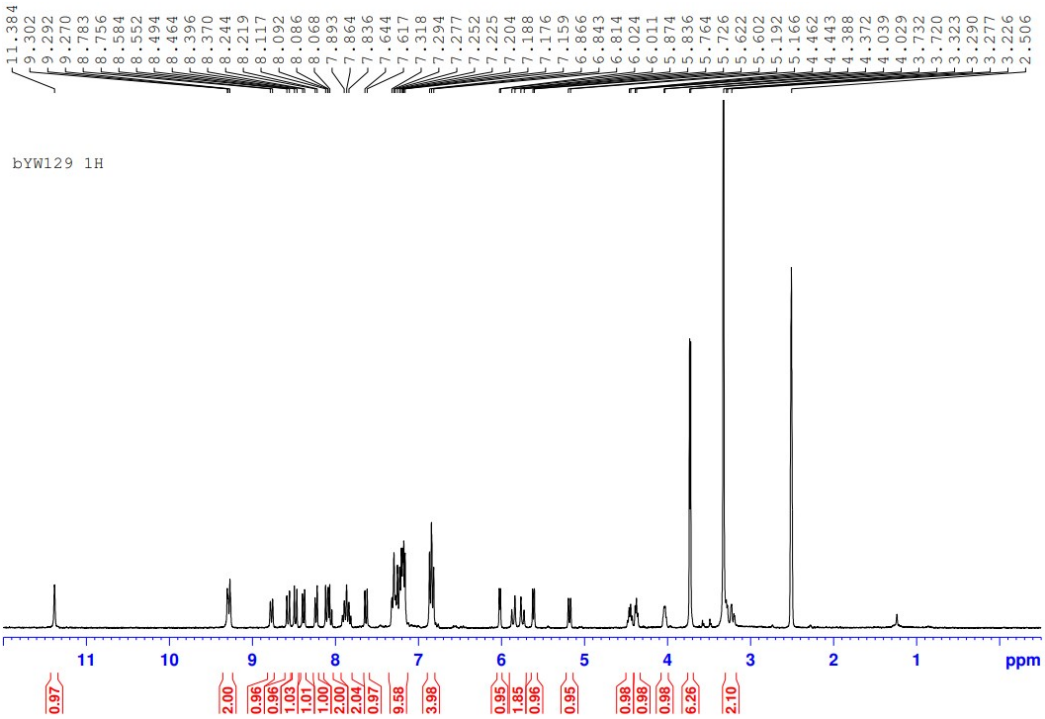
^{1}H NMR spectra of **2a** and **2b**



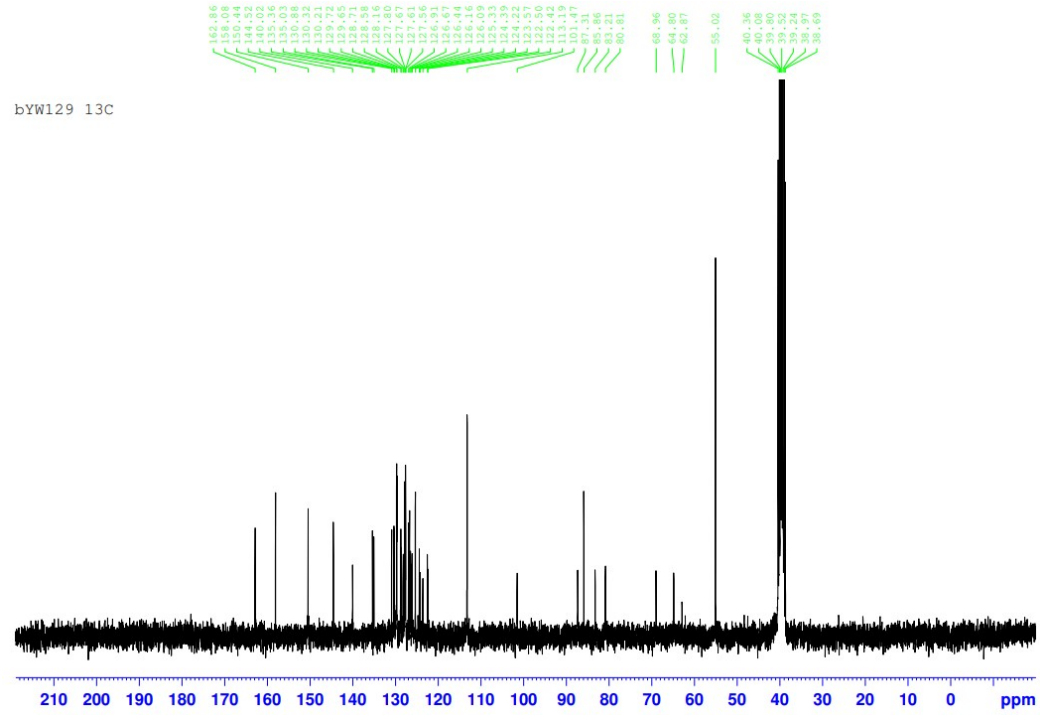
^{13}C NMR spectra of **2a** and **2b**



[1]H NMR spectra of **3**



[13]C NMR spectra of **3**



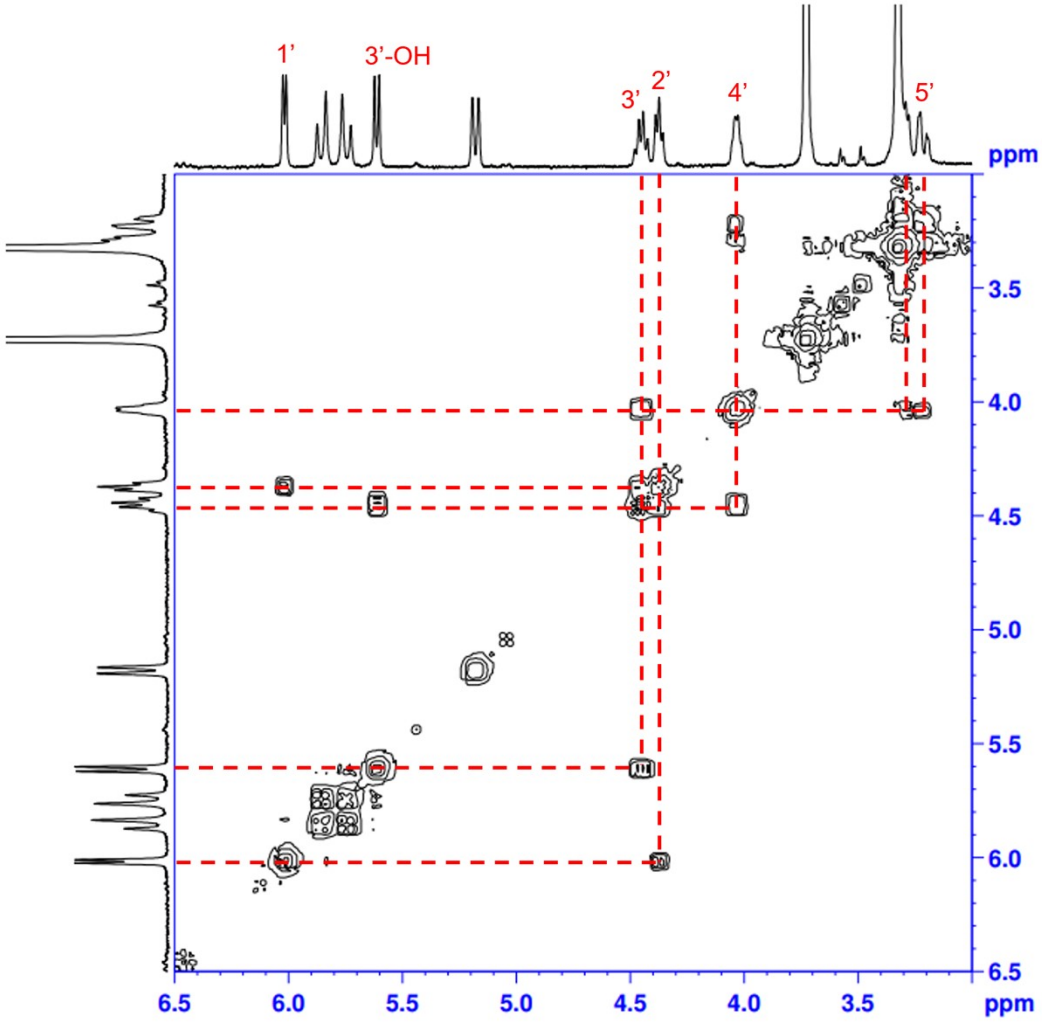
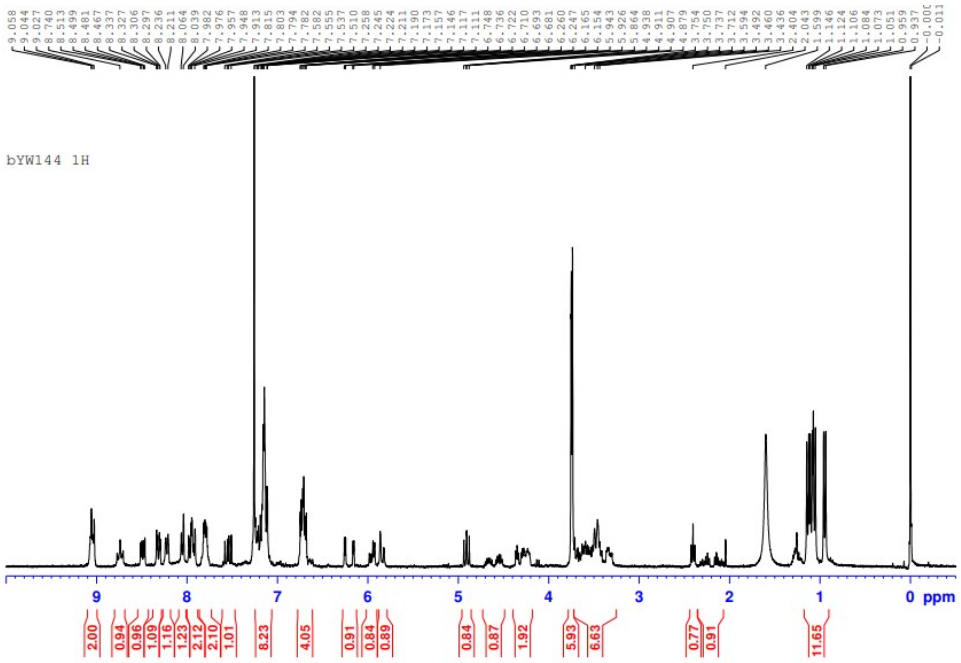
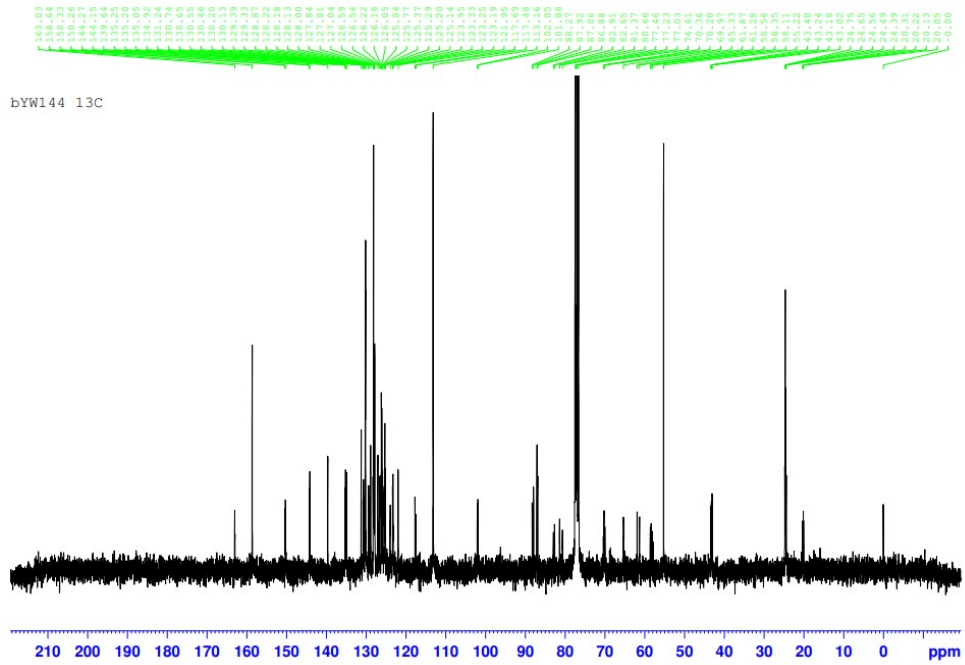


Figure S1. ^{1}H-^{1}H COSY spectra of **3**

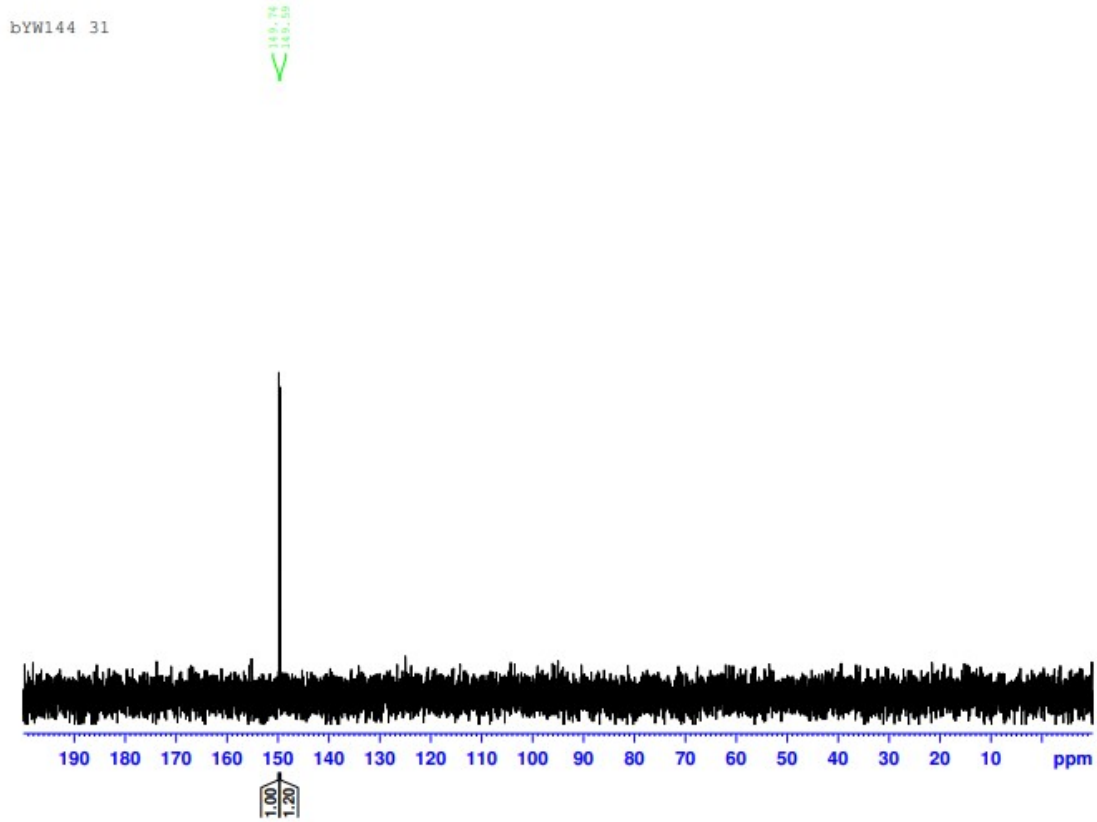
^{1}H NMR spectra of **4**



13C NMR spectra of **4**



^{31}P NMR spectra of **4**



Reversed-phase HPLC profiles of benzo[a]pyrene-modified probes.

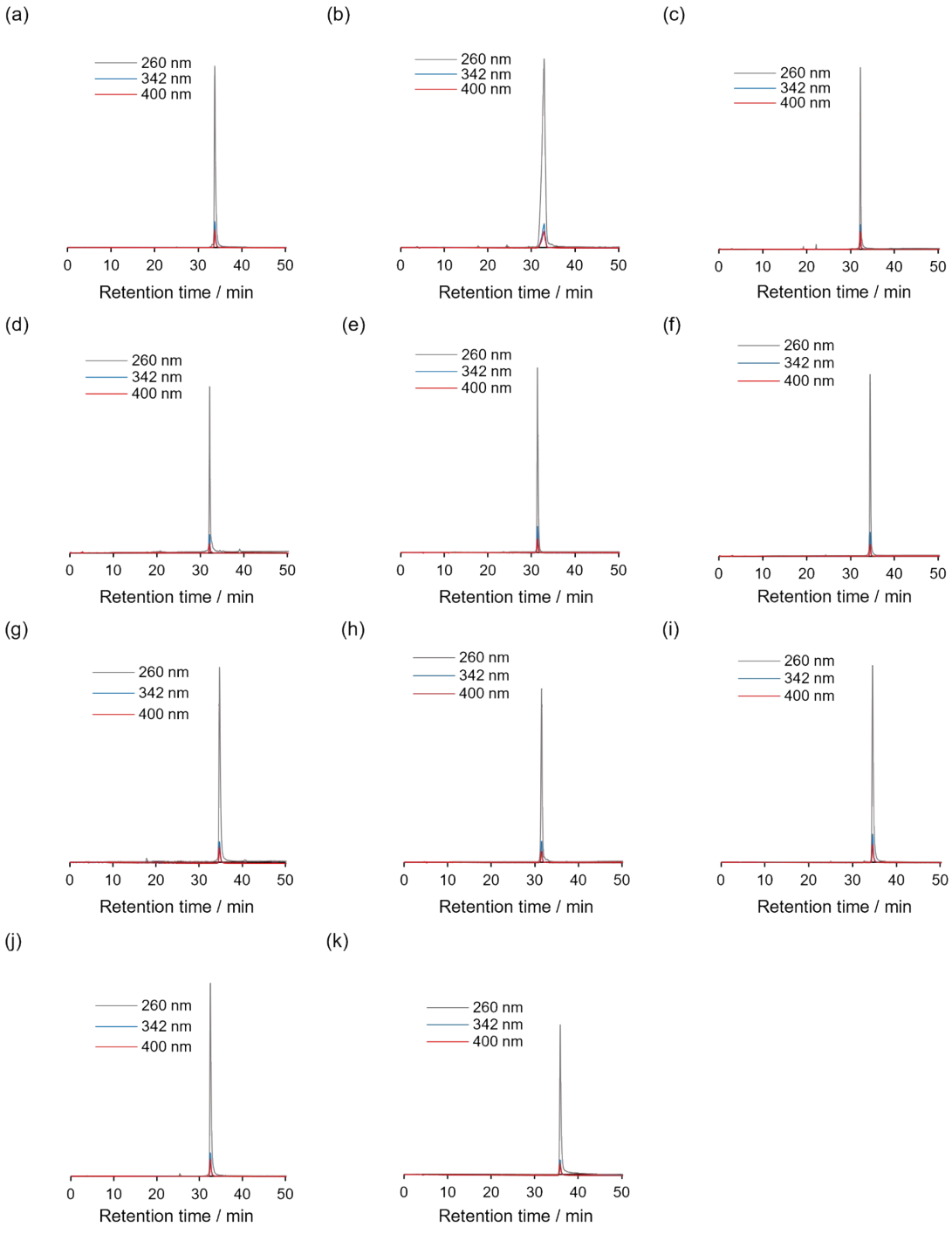


Figure S2. Reversed-phase HPLC profiles of benzo[a]pyrene-modified probes. (a) **Probe1**, (b) **Probe2**, (c) **Probe3**, (d) **Probe4**, (e) **Probe5**, (f) **Probe6**, (g) **Probe7**, (h) **Probe8**, (i) **Probe9**, (j) **Probe10**, (k) **Probe11**. HPLC Conditions: Solvent A = 0.1 M TEAA; Solvent B = 50% CH_3CN/0.1 M TEAA linear gradient from 0% to 100% over 50 min, monitored at 260, 342, and 400 nm; Temp 40 °C.

Melting profiles of the probe–RNA duplexes and the probe–DNA duplexes.

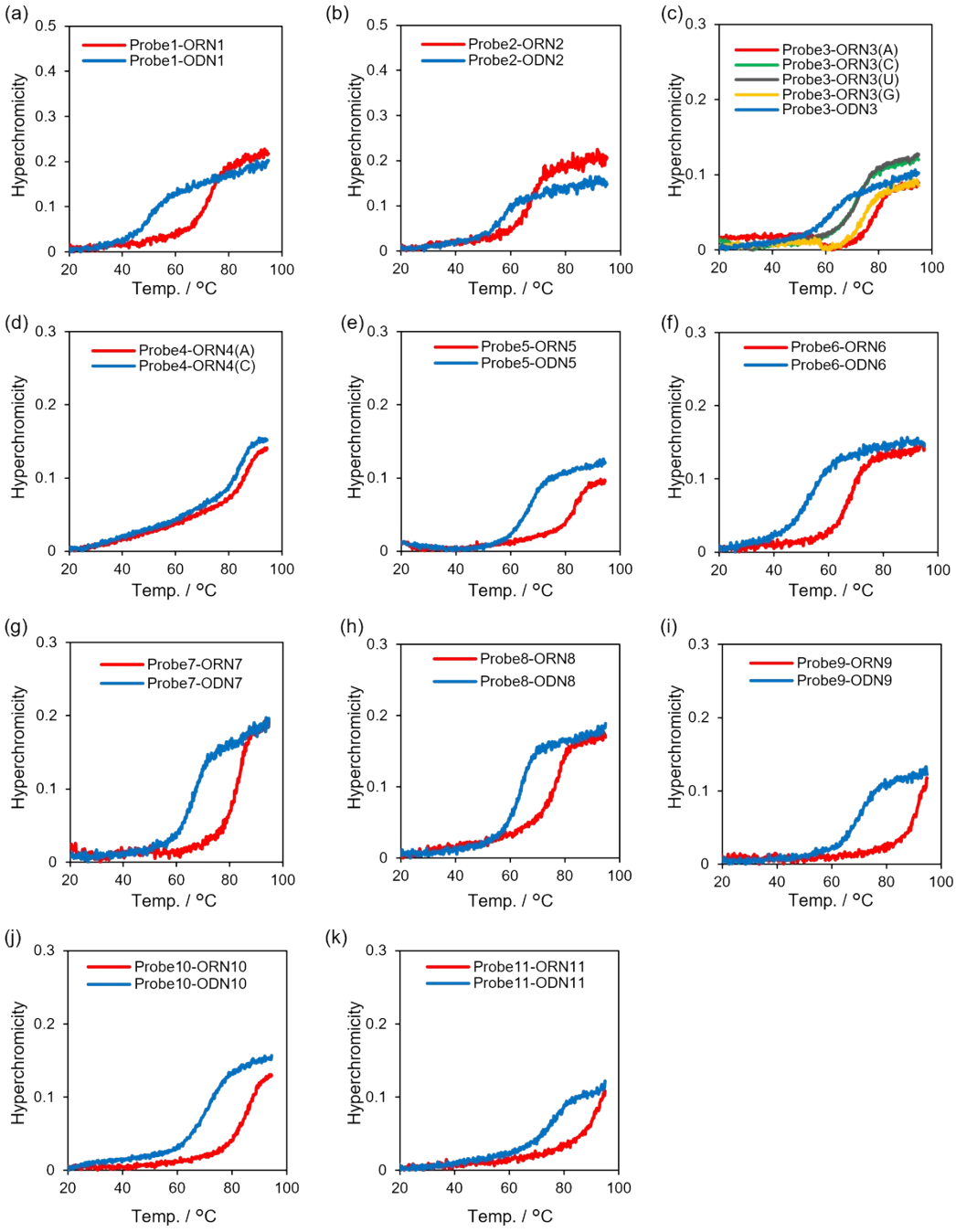


Figure S3. Melting profiles of the duplexes between the benzo[a]pyrene-modified probes and complementary RNAs and DNAs. (a) **Probe1**, (b) **Probe2**, (c) **Probe3**, (d) **Probe4**, (e) **Probe5**, (f) **Probe6**, (g) **Probe7**, (h) **Probe8**, (i) **Probe9**, (j) **Probe10**, (k) **Probe11**. Conditions: 2.0 μM oligonucleotides, 150 mM NaCl, 2 mM $MgCl_2$, 10 mM phosphate buffer (pH 7.0).

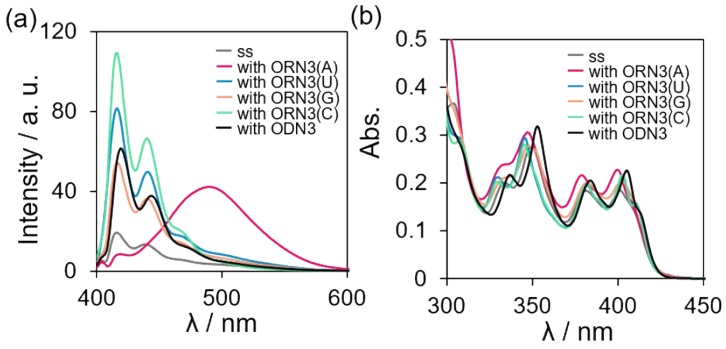


Figure S4. (a) Fluorescence and (b) absorption spectra of **Probe3** in the absence (gray) and presence of **ORN3(A)** (red), **ORN3(U)** (blue), **ORN3(G)** (orange), **ORN3(C)** (green), and **ODN3** (black). Conditions for the fluorescence measurement: Ex, 405 nm; Temp., 25 °C; band width, 5 nm; slit width, 5 nm; [Probe] = [ODN] = [ORN] = 1μM. Conditions for the UV-bis measurement: Temp., 25 °C, [Probe] = [ODN] = [ORN] = 1 μM.

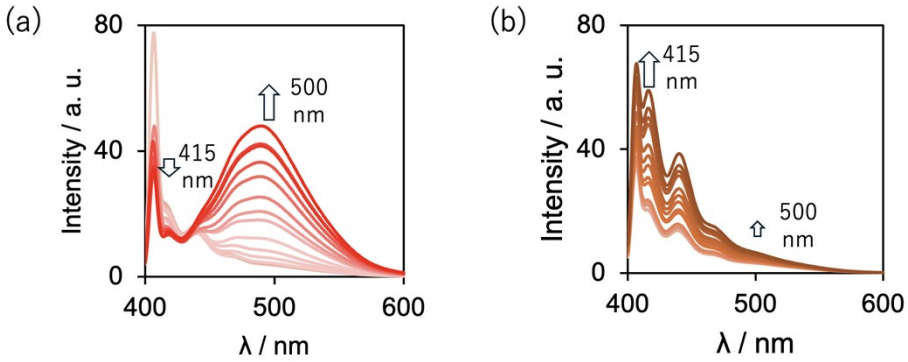


Figure S5. Fluorescent titration spectra of **Probe 3** with (a)**ORN(A)** and (b)**ORN(G)**. Concentration of **Probe 3** was 1000 nM, and that of **RNA(A)** and **RNA(G)** was gradually increased in 100 nM increments from 0 nM to 1000 nM. The ratios of the fluorescence intensities at 500 and 415 nm was used for the ratiometric approach.

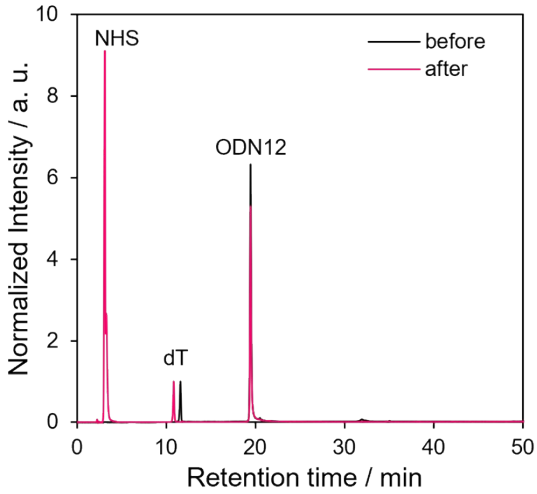


Figure S6. Reversed-phase HPLC profiles of amounts of **ODN12** in the reaction buffer before (black) and after (red) NHS coupling. HPLC Conditions: Solvent A = 0.1 M TEAA; Solvent B = 50% CH_3CN/0.1 M TEAA linear gradient from 0% to 100% over 50 min, monitored at 260 nm; Temp 40 °C.

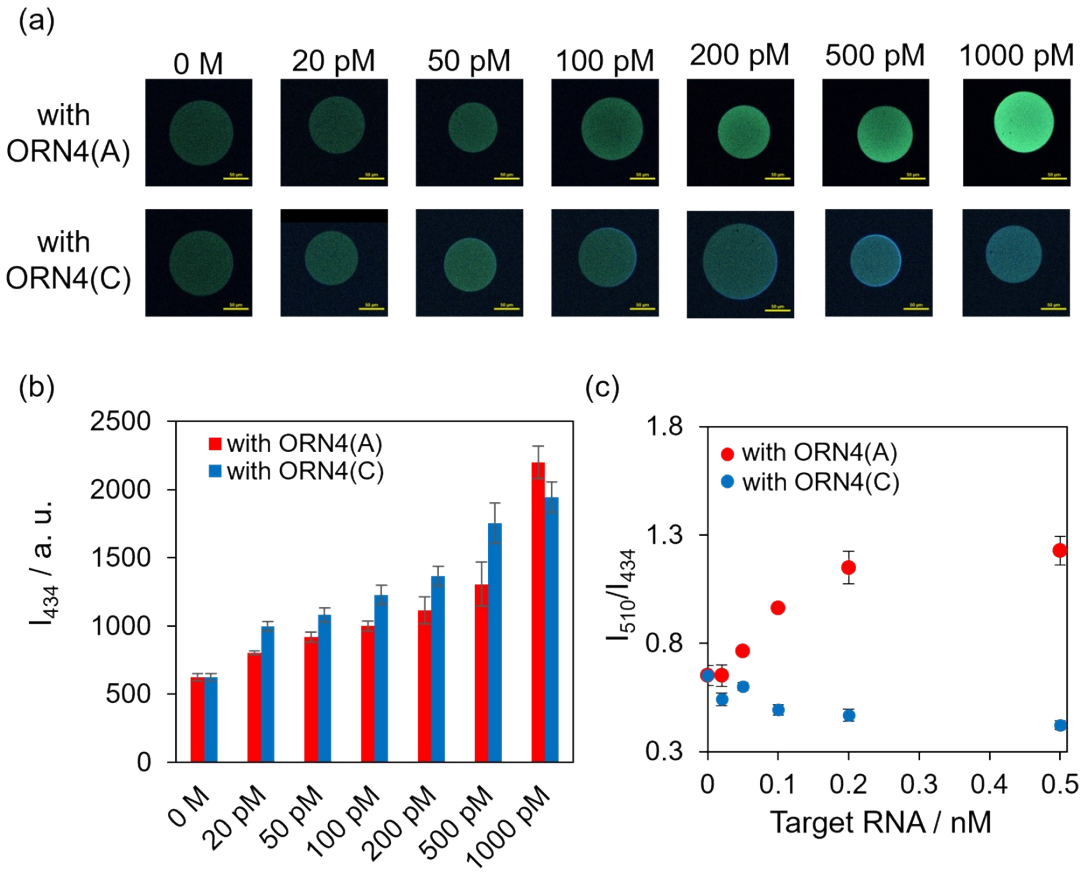


Figure S7. The ratiometric sandwich-type assay of various concentrations of **ORN4(A)** and **ORN4(C)**. (a) Fluorescence images of the capture beads. Conditions: λ_{ex} = 410 nm, [**Probe4**] = 100 nM, [**ORN4(N)**] = 1 nM, [Na_2HPO_4] = 10 mM, [NaCl] = 150 mM, [$MgCl_2$] = 2 mM, pH 7.0. Scale bar: 50 μm (b) Intensity at 434 nm of the capture beads. (c) The I_{510}/I_{434} values of the capture beads.

Table S1 Sequences of target RNAs and DNAs. Captal and small letters denote RNA and DNA, respectively.

Name	Sequences (5' →3')
ORN1	GAGGAGAAACACAUC
ORN2	AUGGUGUUAAAGGUUUUAAU
ORN3	GGAGCUGAUGGCGUA
ORN4	GCCGGUAGCAAACCUUGUAA
ORN5	GGAGGGCAAGUCUGGU
ORN6	AUCAACACUGGAUGU
ORN7	UGGUAUCGUGGAAGGACUC
ORN8	GGUGCAAGCGUUAAUCGG
ORN9	GCGAAAGCGUGGGGAGCA
ORN10	AGUACGGCCGCAAGGUUA
ORN11	UAAGUCGACCGCCUGGGG
ODN1	gaggagaaacacauc
ODN2	atggtgttaaaggttttaat
ODN3	ggagctgatggcgta
ODN4	gccggtagcaaaccttgtaa
ODN5	ggagggcaagtctggt
ODN6	atcaacactggatgt
ODN7	tggtatcgtggaaggactc
ODN8	ggtgcaagcgttaatcgg
ODN9	gcgaaagcgtggggagca
ODN10	agtacggccgcaaggtta
ODN11	taagtcgaccgcctgggg

Table S2 The T_m values of the probe–RNA duplexes and the probe–DNA duplexes.

Name	Sequences (5'→3')	Tm (℃)	
		with ORN	with ODN
Probe1	*GAUGUGU*$U_{bpy}U_{py}$*CUCCUC*	71.8 ± 0.3	50.6 ± 0.3
Probe2	*AUUAAAACCU*$U_{bpy}U_{py}$*AACACCAU*	66.2 ± 0.4	56.7 ± 0.3
Probe3	*UACGCCAU*$_{bpy}C_{py}$*AGCUCC*	77.5 ± 0.1	61.4 ± 0.8
Probe4	*UUACAAGGU*UbpyUpy*GCUACCGGC*	85.5 ± 0.3	63.5 ± 0.2
Probe5	*ACCAGAC*$U_{bpy}U_{py}$*GCCCUCC*	83.4 ± 0.7	65.4 ± 0.5
Probe6	*ACAU*$_{bpy}C_{py}$*CAGUGUUGAU*	66.4 ± 0.4	52.6 ± 0.2
Probe7	*GAGUCCU*$_{bpy}U_{py}$*CCACGAUACCA*	82.2 ± 0.3	66.2 ± 0.2
Probe8	*CCGAUUAACGC*$U_{bpy}U_{py}$*GCACC*	74.2 ± 0.2	61.8 ± 0.2
Probe9	*UGCUCCCCACGCUU*$U_{bpy}C_{py}$*GC*	85<	68.1 ± 0.8
Probe10	*UAACC*$U_{bpy}U_{py}$*GCGGCCGUACU*	82.6 ± 0.5	70.1 ± 0.3
Probe11	*CCCCAGGCGG*$U_{bpy}C_{py}$*GACUUA*	85<	72.8 ± 0.2

Table S3 Photophysical and thermodynamic properties of **Probe3** in the absence and presence of complementary strands.

Oligonucleotides	Φ_{em}	I/I_0 at 500 nm	I/I_0 at 415 nm	I_{500}/I_{415}	λ_{max}	T_m (℃)
Probe3	0.14	1.0	1.0	1.0	349, 401	-
with **ORN3(A)**	0.51	12	0.41	30	347, 399	77.5±0.1
with **ORN3(U)**	0.44	2.6	4.2	0.61	345, 403	71.9±0.4
with **ORN3(G)**	0.31	2.0	2.8	0.72	347, 402	73.1±0.9
with **ORN3(C)**	0.51	1.4	5.7	0.25	345, 403	71.5±0.6
with **ODN3**	0.27	1.5	2.7	0.55	353, 405	61.4±0.8

Table S4 Photophysical and thermodynamic properties of **Probe3** and **ODN12** in the absence and presence of complementary strands.

Oligonucleotides	Φ_{em}	I/I_0 at 500 nm	I/I_0 at 415 nm	I_{500}/I_{415}	λ_{max}	T_m (℃)
Probe4	0.67	1.0	1.0	1.0	345, 401	-
with ORN4(A)	0.63	6.6	0.28	24	347, 400	85.5 ± 0.3
with ORN4(C)	0.42	0.93	0.63	1.5	346, 403	84.0 ± 0.2
with ODN4	0.76	2.0	1.2	1.7	350, 403	-
ODN12	-	-	-	-	-	-
with ORN4(A)	-	-	-	-	-	54.6 ± 0.3
with ORN4(C)	-	-	-	-	-	54.8 ± 0.1