Reduction-triggered red fluorescent probes for dual-color detection of oligonucleotide sequences†

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We have developed a new red fluorogenic compound derived from naphthorhodamine for a reduction-triggered fluorescence probe to sense oligonucleotides. The fluorogenic reaction between naphthorhodamine azide derivatives and reducing reagents such as triphenylphosphine (TPP) on the DNA target does not use any enzyme or reagent, and fluoresces at 650 nm. The probes were used for dual color detection of a single nucleotide difference on the leukemia-related *bcr/abl* gene.

Introduction

Single nucleotide polymorphisms (SNPs) in human genes, which cause various genetic disorders, are important targets for diagnosis.[1] Fluorescence-based reactions based on nucleic acid templates are used for the detection of nucleic acids with single nucleotide specificity.[2–7] This strategy exploits the target strand as a template between two functionalized DNAs or PNAs. For example, native chemical ligation,[8,9] catalytic hydrolysis,[10–13] the Staudinger reaction,[14–16] transfer of reporter group,[17] DNAzyme,[18,19] an S_N2 quencher displacement reaction,[20–25] and organomercury-activated reaction[26] have been applied to fluorescent nucleic acid reactions. In addition to DNA, these methods could allow for the detection of RNAs in cells.[15,18,20,23,25] Designing stable fluorescent probes specific for the target under physiological conditions continues to be a challenge.

Recently, we designed a new fluorogenic molecule, rhodamine azide, that is activated by a specific reducing reagent on the oligonucleotide target and is very stable under biological conditions (Scheme 1A).[15] The reaction of rhodamine azide, triggered by reduction of the azide group, opens the lactone ring to activate the fluorescence. The reduction-triggered fluorescence (RETF) probe consists of two DNA strands, with one probe having a rhodamine azide and the other having reducing reagents such as dithiothreitol or triphenylphosphine. The RETF probe uses a reaction between the azide group of nonfluorescent rhodamine derivatives and reducing reagents on an oligonucleotide template. This reaction proceeds only in the presence of complementary oligonucleotide templates. The RETF probe was used to sense nucleic acids in vitro and endogenous RNA in bacterial cells. A single nucleotide difference is most easily distinguished by using two colors, generating a qualitative difference when the target is altered.[27,28] Imaging of cellular RNA requires a fluorogenic molecule with an emission wavelength toward the red end of the spectrum.[29,30] Here we synthesized a new red fluorogenic molecule derived from naphthorhodamine (Scheme 1B). Our dual-color RETF system can distinguish single nucleotide differences in a target.

Results and discussion

Design and synthesis of red colored naphthorhodamine azide

Previous studies showed that rhodamine fluorescence was controlled by the lactone ring, which is altered by reduction of the azide group (Scheme 1A).[15] After opening the lactone, the longer conjugated system emits fluorescence. This mechanism can help design other fluorogenic compounds.[31] For naphthorhodamine we expanded the two phenyl groups of rhodamine to naphthyl groups (Scheme 1B). The expanded π system of the naphthyl group red shifts emission compared with rhodamine. In addition, the conjugated system of naphthorhodamine should have a similar fluorogenic molecular mechanism to rhodamine. We designed a new fluorogenic molecule of naphthorhodamine bis-azide and N-hydroxysuccinimide (NHS)-activated carboxyl group for conjugation with a DNA probe. The synthesis of the naphthorhodamine azide derivative **3** is shown in Scheme 2. A solution of 6-amino-1-naphthol and 4-carboxyphthalic anhydride in trifluoromethane sulfonic acid was heated at 140 °C for 2 hours to give a mixture of two regioisomers of carboxynaphthorhodamine **1** with a yield of 89%. The two isomers were not clearly separated by silica gel chromatography. Finally, preparative reverse-phase HPLC was used to purify the two isomers. HPLC peak area analysis showed that the isomers existed in a 2:1 ratio, and the major isomer eluted slowly (Figure S1A). The major isomer was shown to be 5-carboxynaphthorhodamine by NMR analysis. The HMBC spectrum indicated long-range correlations from H-4 to both carbonyl carbons of the 3- and 5-carboxyl groups (Figure S2). We found that compound **1** consists of 5-carboxynaphthorhodamine and 6-carboxynaphthorhodamine in a 2:1 ratio. Bisamino-naphthorhodamine **1** was transformed to the diazo intermediate by treatment with $NaNO_2$, and the diazo group

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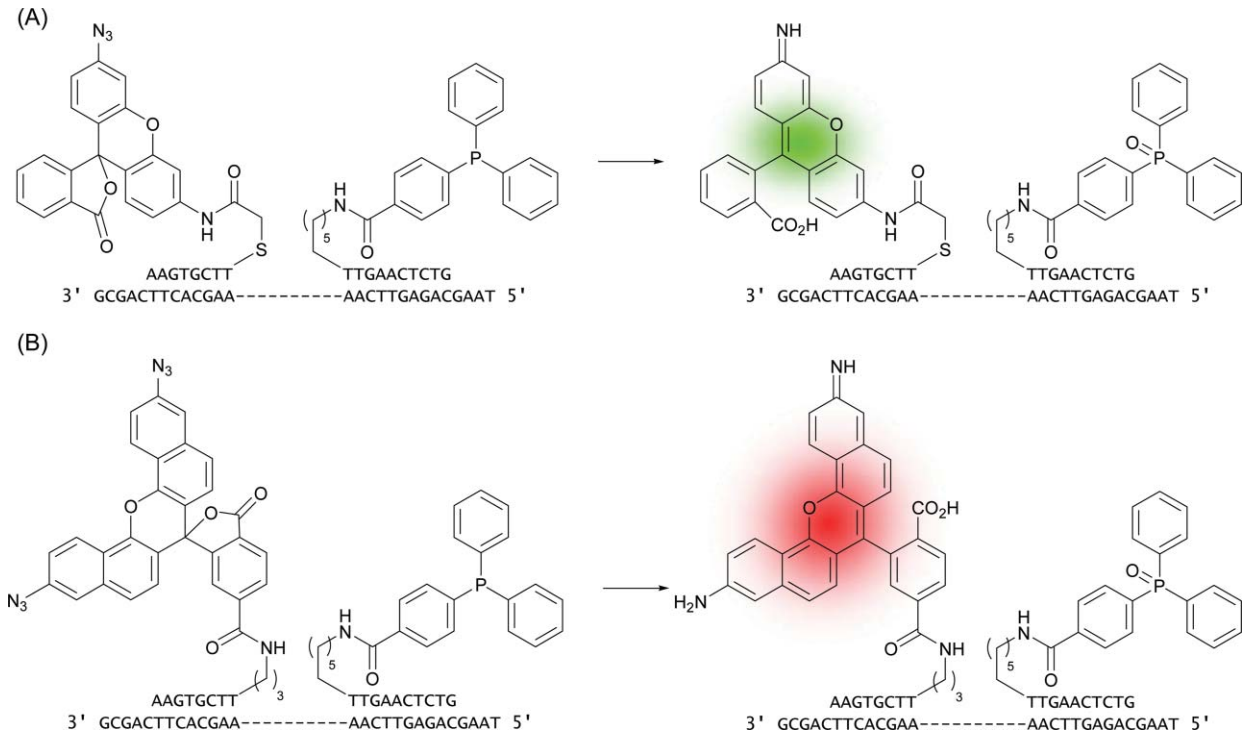
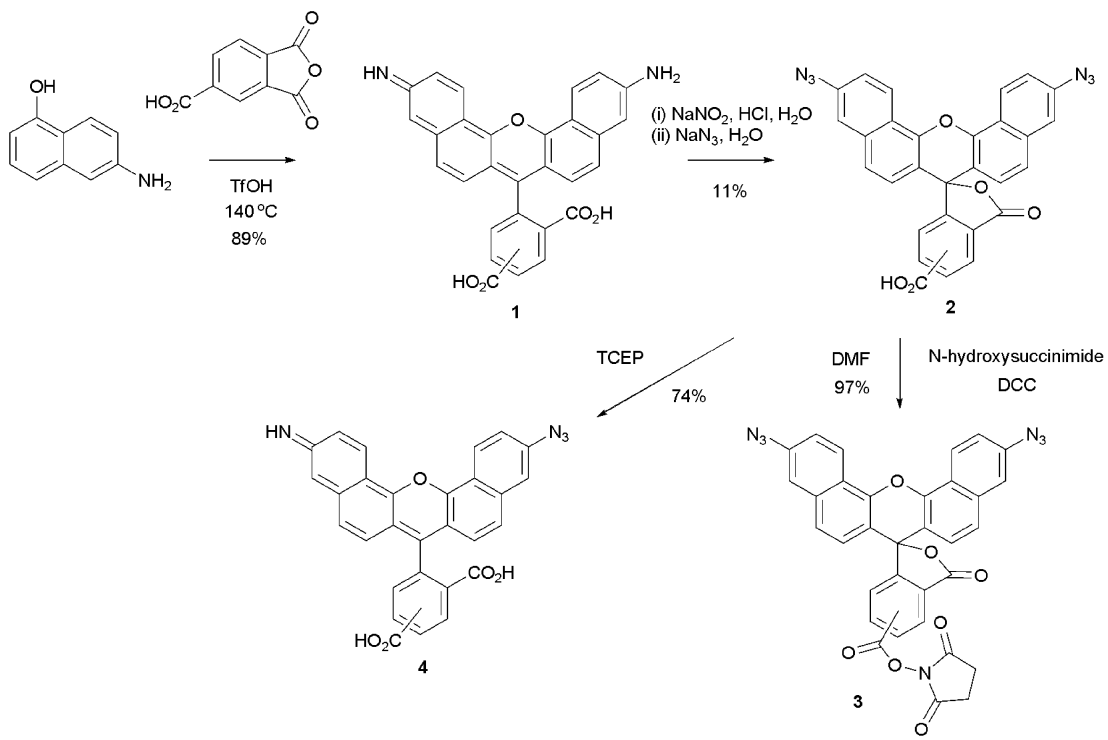
† Electronic supplementary information (ESI) available: HPLC analysis of isomer, HPLC and MALDI–TOF mass analysis of products in the reaction of the RETF probe, and NMR data. See DOI: 10.1039/b817228e



Scheme 1 Probe sequences and structures of (A) previous and (B) new RETF system.



Scheme 2 Synthesis of fluorogenic compound **3**.

was replaced with an azide group by addition of NaN_3 to give carboxynaphthorhodamine-azide **2** in a 2:1 mixture of 5-carboxy and 6-carboxy isomers (Figure S1B) with an 11% yield. The desired NHS ester **3** was prepared by dicyclohexylcarbodiimide-mediated coupling of N-hydroxysuccinimide with compound **2**. Compound **3** was introduced into the DNA probe.

Spectroscopic properties of naphthorhodamine derivatives

We tested the photochemical properties of naphthorhodamine derivatives. Naphthorhodamine azide **2** showed no absorbance at wavelengths longer than 550 nm in 20 mM Tris-HCl buffer (pH 7.2), but the corresponding naphthorhodamine **1** showed a maximum absorption at 585 nm (Fig. 1A). When a high concentration of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added as a reducing reagent to a solution of azide **2**, a peak with a maximum of 600 nm increased because of reduction of the azide group. Naphthorhodamine monoazide **4** (see ESI†) also showed absorption in the long-wavelength region (maximum at 560 nm), although it was weaker than that of bisamino **1** and TCEP-treated **2**.

We also examined the fluorescence properties of naphthorhodamine derivatives (Fig. 1B). No significant fluorescence with excitation at 595 nm was observed for bis-azide **2**. On the other hand, naphthorhodamine **1** showed strong fluorescence emission around 650 nm. After addition of TCEP to the solution of azide **2**, a strong emission appeared around 650 nm, where the emission was enhanced 550-fold. Naphthorhodamine monoazide **4** also had intense fluorescence in the red region. The fluorescence intensity of monoazide **4** was 27 times less than that of **1**. This is likely due to the different *pKa* values for the amino groups of **1** and **4**. Naphthorhodamine **1** and monoazide naphthorhodamine **4** showed high fluorescence quantum yields, 0.13 and 0.068, respectively, in contrast with the low yield of azide **2**, 0.0087 (Table 1). This indicates that the observed fluorescence comes from **1** or **4** when a DNA probe with naphthorhodamine bis-azide is reacted with the target sequence. We also measured the quantum yields of the two separated isomers of compound **1**. The quantum yields of 5-carboxy isomer and 6-carboxy isomer were 0.13 and 0.12, respectively (Table 1), indicating that the position of carboxylic group has negligible effects on the fluorescence emission. The mixture of isomers could be used for further experiments.

Table 1 The quantum yields of the compounds[a]

Compound	**1**	**2**	**4**	5-carboxy	6-carboxy
Φ_F[b]	0.13	0.0087	0.068	0.13	0.12

[a] All measurements were performed in Tris-HCl buffer (190 mM, pH 7.2). Compounds were excited at 595 nm. [b] Quantum yields were determined using naphthofluorescein in carbonate/bicarbonate buffer (pH 9.5) as standard ($\Phi_F = 0.14$).

Design of RETF probe with naphthorhodamine azide

Next, we synthesized three new DNA probes and two DNA targets (Fig. 2). Probe **5** was modified at the 3′ terminus with NHS ester-naphthorhodamine-azide **3**, by reaction of the NHS ester group of **3** with the amino linker at the 3′ end of the oligonucleotide. Similarly, probe **10** was modified with rhodamine azide by conjugation of the phosphorothioate group at the 3′ end of the oligonucleotide. Probe **6** was modified at the 5′ terminus with 2′-carboxytriphenylphosphine, which was conjugated with the 5′ amino linker of the DNA probe through an amide bond. DNA target sequences were derived from the human *bcr/abl* gene, which is related to chronic myelogenous leukemia. We designed a wild-type *bcr/abl* sequence (*bcr/abl-1*) as well as a single-base mismatched sequence (*bcr/abl-2*). Probes **5** and **10** were fully matched with targets *bcr/abl-1* and *bcr/abl-2*, respectively. As shown in Scheme 3, a reduction–oxidization reaction between

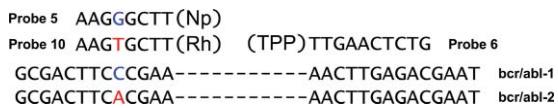


Fig. 2 Probe and target DNA sequences of dual color SNP detection. "Np", "Rh", and "TPP" indicate naphthorhodamine azide, rhodamine azide, and triphenylphosphine, respectively.

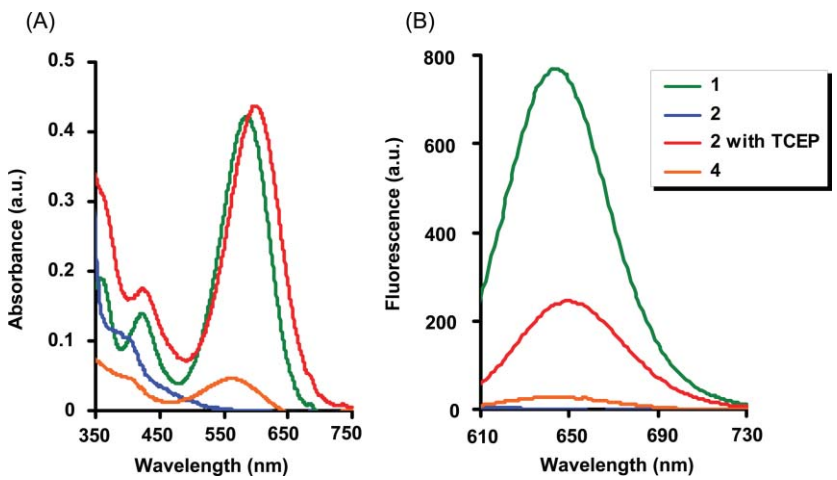
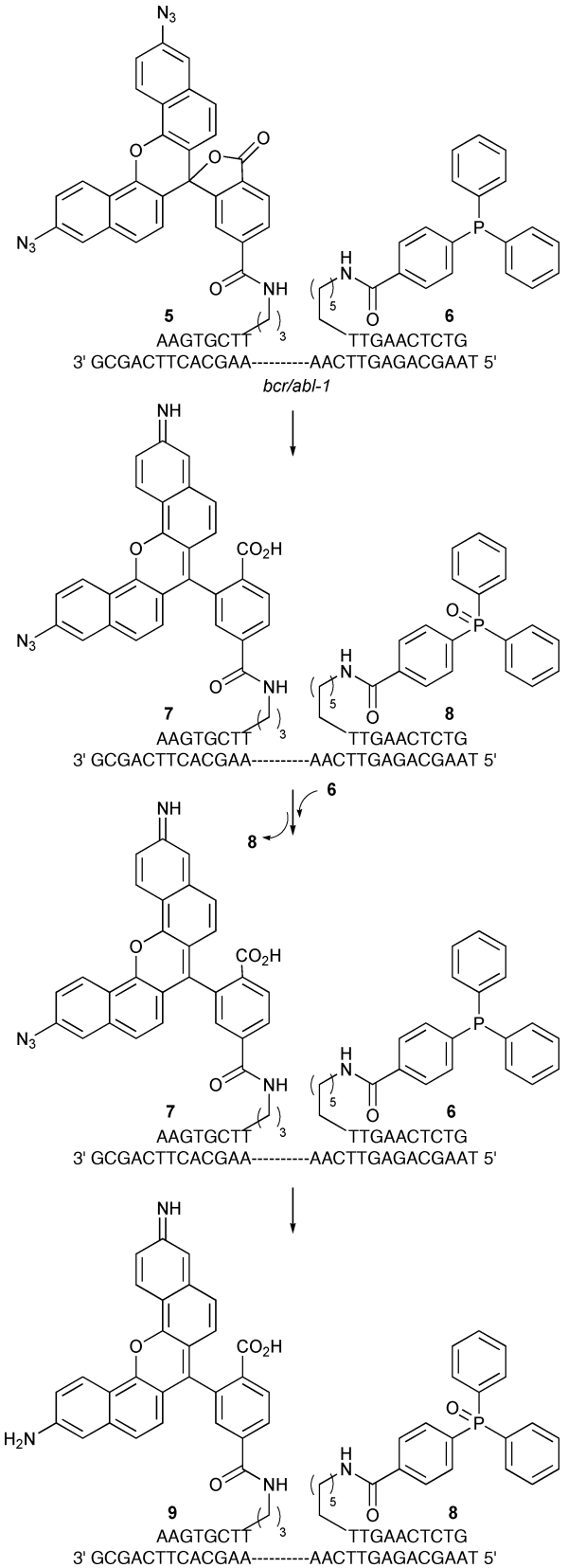


Fig. 1 Spectrum analysis. (A) Absorption spectra of 100 μM of **1** (green), **2** (blue), **2** treated with 10 mM of tris(2-carboxyethyl)phosphine (TCEP) at 25 °C for 1 hour (red), and **4** (orange) in 20 mM Tris-HCl (pH 7.2) buffer at 25 °C; (B) Fluorescent emission spectra with excitation at 595 nm of 10 μM of **1** (green), **2** (blue), **2** treated with 10 mM of TCEP at 25 °C for 1 hour (red), and **4** (orange) in 20 mM Tris-HCl (pH 7.2) buffer at 25 °C.



Scheme 3 Schematic presentation of a reduction–oxidization reaction between probe **5** and **6** on *bcr/abl-1.*

probe **5** and **6** on *bcr/abl-1* should produce moderately fluorescent product **7** and phosphine oxide **8** as the product of the first stage of the reaction. As there are reports of catalytic turnover for DNA-templated reactions, our probes could be involved in multiple chemical reactions on DNA templates.[4,11,22,32] We assumed that the bis-azide probe **5** reacted with two eq. of phosphine probe **6** and became bis-amino product **9** to yield a strong fluorescence signal. The mechanism is explained by phosphine oxide **8** from the first stage of the reaction falling off the target, and a second phosphine probe **6** binding to the target and reacting with monoazide probe **7** (Scheme 3).

Fluorescence detection of DNA sequence

To prove whether the probes could detect target DNA sequences and emit fluorescence, we tested our naphthorhodamine probe in solution (Fig. 3). When 250 nM of probes **5** and **6** were incubated in pH 7.2 Tris-HCl buffer, the fluorescence signal at 655 nm increased in the presence of target *bcr/abl-1* over 30 min by reduction of the azide group to the amino group of probe **5**, and no significant increase in fluorescence was observed in the absence of *bcr/abl-1* over 30 min. HPLC and MALDI–TOF mass analyses (Figure S3) showed that 80.8% of probe **5** reacted with probe **6** after 30 min to give the corresponding monoamino (probe **7**, 61.6%) and bisamino (probe **9**, 19.2%) products. When two eq. of probe **6** to 250 nM of probe **5** was used, the fluorescence signal increased more rapidly than in the case of one eq. of probe **6**. The reaction with two eq. of probe **6** offered fluorescence 3 times higher in intensity than that with one eq. of probe **6** after 30 min. HPLC analysis showed that the reaction of probe **5** (250 nM) and probe **6** (500 nM) gave bisamino probe **9** in a yield of 91% and only a trace amount of monoamino probe **7** after 24 h. Therefore, in the reaction of probes **5** and **6**, the increase in fluorescence at 655 nm was caused by the mixture of reduced monoamino product **7** and bisamino product **9**, where the bis-amino product was more fluorescent than the monoamino product. In addition, we confirmed that multiple chemical reactions occurred on the DNA target and that bis-azide naphthorhodamine was transformed to bis-amino naphthorhodamine by two eq. of phosphine (Scheme 3).

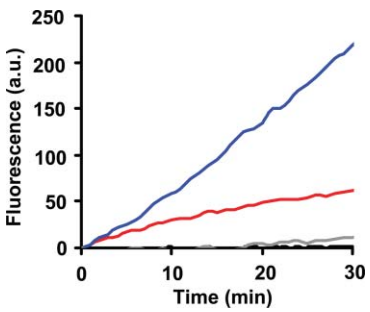


Fig. 3 Time course of the fluorescence intensity for the reaction between 250 nM of probe **5** and 250 nM of probe **6** in the presence (red) or absence (black) of 250 nM of *bcr/abl-1*, or between 250 nM of probe **5** and 500 nM of probe **6** in the presence (blue) or absence (gray) of 250 nM of *bcr/abl-1*.

Dual color discrimination of single base difference

To examine whether the RETF probe could distinguish DNA sequences at the single nucleotide level using dual colors, we

designed a red fluorescent probe **5** as well as a green fluorescent probe **10**, previously reported as a rhodamine azide probe. Fluorescence signals from the two probes were well separated in their excitation and emission wavelengths (naphthorhodamine ex/em 595/655 nm, rhodamine ex/em 490/550 nm). The target sequences *bcr/abl-1* or *-2* derived from the human *bcr/abl* gene are different at only a single base. In this dual-color probe strategy, the red fluorescent signal should appear when *bcr/abl-1* is added to the solution because only the red probe **5** binds to the target and reacts with the phosphine probe **6**. A green signal should appear when *bcr/abl-2* exists in solution. Reactions were carried out using one eq. of probe **6** to azide probe **5** or **10**, because two eq. of probe **6** increased undesired background green fluorescence signal.

The fluorescence intensities of each color probe were monitored as a function of time in the presence of *bcr/abl-1* or *-2* (Fig. 4). Fluorescence intensities of the red and green probes were normalized by dividing the initial intensity by the intensity after 30 min for the full-match target and multiplying by factor of 100. A strong fluorescence signal was observed, corresponding to the predicted, correctly matched color probes and targets.

For *bcr/abl-1*, the emission of the red probe **5** was 8.1 times higher than the emission of the green probe **10** after 30 min. In contrast, the emission of green probe **10** in the presence of *bcr/abl-2* was 30.3 times higher than the emission of red probe **5** after 30 min. To distinguish a single base difference clearly, the ratio of fluorescence intensity of R/G (red/green) must be significant. In this experiment, detection of the C/A difference in *bcr/abl* was achieved by an R/G factor of 8.1 or a G/R factor of 30.3. A lower R/G factor for *bcr/abl-1* is likely caused by a small amount of T-C mismatching between *bcr/abl-1* and green probe **10**, and by a difference in the chemical reaction rate between red probe **5** and green probe **10**. For *bcr/abl-2*, the signal intensity from green probe **10** increased quickly in the initial stage and reached saturation within 20 min, whereas that from red probe **5** increased continuously over 30 min for *bcr/abl-1*, where 19.2% of probe **5** remained after 30 min (Figure S3), indicating that the chemical reaction rate of green probe **10** was faster than that of red probe **5**. Our dual-color RETF system has the potential to detect SNPs rapidly with high sensitivity and selectivity.

Experimental

Carboxylic naphthorhodamine (1)

To a mixture of 6-amino-1-naphthol (95.8 mg, 0.602 mmol) and 4-carboxyphthalic anhydride (86.8 mg, 0.452 mmol) was added triflic acid (1.5 ml). The reaction mixture was stirred at 100 °C for 2 h and at 140 °C for 2 h, then was cooled to room temperature and poured into 40 ml brine (including 5 ml 5% H_2SO_4). The product was extracted into CH_2Cl_2/MeOH (4:1). The organic layer was combined and washed with brine, dried by Na_2SO_4, concentrated, and subjected to flash column chromatography (silica gel, 4:1 CH_2Cl_2:MeOH) to give a crude product (78.3 mg, 0.536 mmol, 89%) as black crystalline solid. ^{1}H NMR (δ_H)(400 MHz, CD_3OD) 6.74 (1/2H, d, J = 8.3 Hz), 6.89 (1H, d, J = 7.1 Hz), 6.91 (1H, d, J = 8.8 Hz), 7.28 (1/2H, d, J = 8.0 Hz), 7.57 (1H, d, J = 5.8 Hz), 7.59 (1H, d, J = 5.8 Hz), 7.70 (2H, m), 7.85 (2H, brd, J = 7.8 Hz), 8.32 (1/2H, dd, J = 1.4 Hz, 8.0 Hz), 8.45 (1/2H, d, J = 9.0 Hz), 8.69 (1/2H, s), 8.96 (2H, d, J = 9.0 Hz), 9.37 (1/2H, m). ^{13}C NMR (δ_C)(100 MHz, CD_3OD) 108.31, 120.02, 120.90, 121.89, 122.92, 123.22, 124.20, 124.21, 125.32, 125.68, 125.96, 126.10, 126.27, 126.42, 128.05, 128.21, 129.29, 129.78, 129.85, 129.90, 129.98, 131.26, 131.34, 131.40, 131.55, 131.96, 132.20, 132.43, 133.01, 133.86, 134.09, 134.25, 134.70, 135.07, 137.51, 138.41, 138.67, 140.78, 140.85, 144.37, 148.22, 155.61, 160.22, 160.31, 167.46, 167.77, 167.85, 167.91, 169.50. QSTAR (Applied Biosystems/MDS SCIEX) (ESI): [M + H^+]: 475.1294, found: 475.1260.

Carboxylic naphthorhodamine bis-azide (2)

Compound **1** was dissolved in water (5 ml) and 12 N HCl (1 ml). Then sodium nitrite (92.5 mg, 1.34 mmol) was added. The reaction mixture was stirred for 1 h and NaN_3 (104.5 mg, 1.61 mmol) was added slowly. After stirring for 1 h at ambient temperature the product was extracted into CH_2Cl_2. The organic layer was washed with brine, dried by Na_2SO_4, concentrated, and subjected to flash column chromatography (silica gel, 7:1, CH_2Cl_2:MeOH) to give bis-azide (16.7 mg, 31.7 μmol, 10.5% for two steps) as pale yellow crystalline solid. ^{1}H NMR (δ_H) (300 MHz, DMSO) 6.97 (2H, d, J = 6.0 Hz), 7.47–7.80 (7H, m), 8.30 (2H, d, J = 9.0 Hz), 8.52 (1H, s), 8.85 (2H, d, J = 9.0 Hz). ^{13}C NMR (δ_C) (75 MHz, DMSO) 82.56,

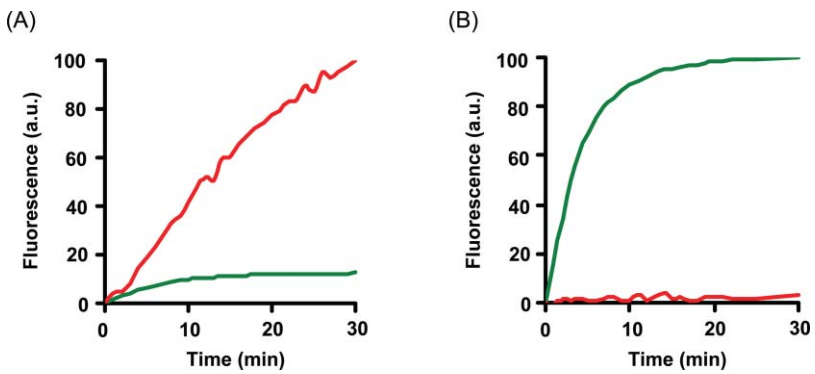


Fig. 4 Time course of the fluorescence intensity in the reaction with 250 nM of *bcr/abl-1* (A) or *-2* (B), probe **5** (250 nM), probe **6** (250 nM), and probe **10** (250 nM) at 37 °C. The fluorescence intensity was measured for 0.5 s at 1 min intervals: ex/em 490 nm/550 nm (for probe **10**: green line) and 595 nm/655 nm (for probe **5**: red line).

111.34, 116.16, 120.06, 120.55, 123.62, 124.33, 124.83, 125.05, 125.88, 126.27, 134.99, 136.41, 139.67, 145.69, 156.36, 166.01, 167.89. QSTAR (Applied Biosystems/MDS SCIEX) (ESI): [M + H^+]: 527.1104, found: 527.1089.

Naphthorhodamine bis-azide NHS ester (3)

To a solution of compound **2** (67.7 mg, 0.129 mmol) in DMF (3 ml) was added N-hydroxysuccinimide (16.9 mg, 0.146 mmol) and DCC (31 mg, 0.150 mmol). The reaction mixture was stirred at ambient temperature for 3 h and filtered. The filtrate was partitioned by AcOEt and H_2O (× 2). The organic layer was washed by brine, dried by Na_2SO_4, concentrated, and subjected to flash column chromatography (silica gel, 2:1 Hexane:AcOEt) to give the NHS ester (78.3 mg, 0.125 mmol, 97%) as pale yellow crystalline solid. ^{1}H NMR (δ_H)(400 MHz, $CDCl_3$) 2.84 (2H, brs), 2.95 (2H, brs), 6.81 (1H, d, J = 8.0 Hz), 6.81 (1H, d, J = 8.8 Hz), 7.29 (1/2H, d, J = 8.3 Hz), 7.35 (2H, m), 7.45 (4H, m), 7.85 (1/2H, s), 8.25 (1/2H, d, J = 8.0 Hz), 8.40 (1H, m), 8.64 (2H, d, J = 9.0 Hz), 8.90 (1/2H, d, J = 0.7 Hz). ^{13}C NMR (δ_C)(100 MHz, $CDCl_3$) 24.89, 25.51, 25.56, 25.64, 83.29, 83.50, 110.62, 115.66, 119.53, 119.57, 121.01, 121.05, 123.19, 123.25, 123.94, 124.50, 124.80, 125.63, 126.11, 127.05, 127.28, 127.81, 131.08, 131.27, 131.85, 135.11, 136.64, 139.91, 139.97, 146.17, 146.21, 153.58, 156.41, 158.57, 160.19, 160.21, 167.27, 167.50, 168.32, 168.49. QSTAR (Applied Biosystems/MDS SCIEX) (ESI): [M + Na^+]: 646.1087, found: 646.1076.

Carboxylic naphthorhodamine monoazide (4)

To a solution of **2** (10 mg, 0.02 mmol) in DMF (280 µl) was added tris(2-carboxyethyl)phosphine hydrochloride (5.44 mg, 0.02 mmol) in H_2O (100 µl) slowly. After the reaction mixture was stirred for 1 h, the product was extracted into $CHCl_3$. The organic layer was washed by brine, dried by Na_2SO_4, concentrated, and subjected to flash column chromatography (silica gel, 10:1 CH_2Cl_2:MeOH) to give the mono-azide (7.0 mg, 0.014 mmol, 74%) as pale blue crystalline solid. ^{1}H NMR (δ_H)(400 MHz, $CDCl_3$) 6.51–6.54 (1H, m), 6.69–6.73 (1H, m), 6.87 (1H, s), 7.08–7.39 (6H, m), 7.64 (1/2H, s), 8.07 (1/2H, d, J = 8.0 Hz), 8.23–8.35 (2H, m), 8.58 (1H, t, J = 16.0 Hz). ^{13}C NMR (δ_C)(100 MHz, $CDCl_3$) 108.98, 109.20, 109.25, 110.94, 112.85, 116.96, 118.04, 119.94, 120.08, 120.76, 112.64, 123.71, 124.02, 124.21, 124.49, 125.32, 125.47, 125.98, 127.25, 128.17, 132.40, 136.78, 137.71, 138.15, 141.49, 146.11, 147.96, 148.06, 148.13, 149.61, 158.14. QSTAR (Applied Biosystems/MDS SCIEX) (ESI): [M + H^+]: 501.1193, found: 501.1206.

Synthesis of unmodified oligonucleotides

All oligonucleotides were synthesized on a 0.2 µmol scale on a DNA synthesizer (H-8-SE; Gene World) using standard phosphoramidite coupling chemistry. Deprotection and cleavage from the CPG support was carried out by incubation in concentrated ammonia for 4 h at 55 °C. Following deprotection, the oligonucleotides were purified by reverse-phase column chromatography (MicroPure II column; Biosearch Technologies), and quantitated by UV absorbance using the nearest neighbor approximation to calculate molar absorptivities.

3′ Naphthorhodamine azide-conjugated oligonucleotide (probe 5)

Compound **3** was reacted with 3′ amino-modified oligonucleotide. 3′ PT-amino-modifier C3 CPG (Glen Research) was used to prepare 3′ amino-modified oligonucleotide. 50 nmol of the 3′ amino-modified oligonucleotide in 50 µl of 80 mM sodium tetraborate (pH 8.5) were shaken for 5 h at room temperature with 0.75 µmol of compound **3** in 200 µl of dimethylformamide. The reacted products were collected by ethanol precipitation. Next, the collected products were purified by reverse-phase HPLC (0–80% acetonitrile/50 mM triethylammonium acetate gradient). The probe structure was confirmed by ESI–TOF mass spectrometry. 5′-compound **3**-AAG**G**GCTT-3′: calculated mass, $C_{111}H_{119}N_{39}O_{53}P_8$ 3093.57; found 3093.77.

5′ Triphenylphosphine (TPP)-linked oligonucleotide (probe 6)

Carboxy-triphenylphosphine (TPP) NHS ester was reacted with 5′ amino-modified oligonucleotide. 5′ amino-modifier C6 (Glen Research) was used to prepare 5′ amino-modified oligonucleotide. 50 nmol of the 5′ amino-modified oligonucleotide in 135 µl of 93 mM sodium tetraborate (pH 8.5) were shaken for 5 h at room temperature with 2 µmol of TPP NHS ester in 115 µl of dimethylformamide. The reacted products were collected by ethanol precipitation. Next, the collected products were purified by reverse-phase HPLC (0–50% acetonitrile/50 mM triethylammonium acetate gradient). The probe structure was confirmed by ESI–TOF mass spectrometry. 5′-TPP-TTGAACTCTG-3′: calculated mass, $C_{123}H_{152}N_{35}O_{64}P_{11}$ 3483.68; found 3483.70. A peak corresponding to the oxidized product (+O) was also seen and presumed to arise from oxidation during purification.

3′ Rhodamine-azide-conjugated oligonucleotide (probe 10)

The bromoacetyl group of the rhodamine-azide was reacted with the phosphorothioate group on the ODNs. For 3′ phosphorothioate sequences, the 3′-phosphate CPG was sulfurized by the sulfurizing reagent (Glen Research) after the first nucleotide was added. 75 nmol of the 3′-phosphorothioate oligonucleotide in 50 µl of 400 mM triethylammonium bicarbonate buffer were shaken for 5 h at room temperature with 750 nmol of rhodamine-azide in 200 µl of dimethylformamide. The reacted products were collected by ethanol precipitation. Next, the products were purified by reverse-phase HPLC (0–80% acetonitrile/50 mM triethylammonium acetate gradient). The probe structure was confirmed by ESI–TOF mass spectrometry. 5′-AAG**T**GCTT-Rh_azide-3′: calculated mass, $C_{101}H_{113}N_{33}O_{53}P_8S$ 2915.48; found 2915.69.

Measurement of quantum yield

A 1 mM DMF stock solution of each compound was prepared. Absorption spectra were obtained with a 190 mM tris-HCl buffer (pH 7.2) solution of each compound at the desired concentration, adjusted by appropriate dilution of the 1 mM DMF stock solution. For determination of the quantum efficiency of fluorescence (Φ_{fl}), naphthofluorescein in carbonate/bicarbonate buffer (pH 9.5) was used as a fluorescence standard (Φ = 0.14). The quantum efficiency of fluorescence was obtained with the following equation

(F denotes fluorescence intensity at each wavelength and $\sum [F]$ was calculated by summation of fluorescence intensity).

$$\Phi_{fl}^{\ sample} = \Phi_{fl}^{\ standard} \mathrm{Abs}^{standard} \sum [F^{sample}] / \mathrm{Abs}^{sample} \sum [F^{standard}]$$

Detection of DNA sequence with red colored RETF probe

Reactions on the DNA template were performed in 1.2 ml of tris-HCl buffer (20 mM, pH 7.2) containing 100 mM $MgCl_2$ and 0.01 mg/ml BSA with target *bcr/abl-1* (250 nM), probe **5** (250 nM), and probe **6** (250 nM) at 37 °C. The increase in fluorescence intensity produced by reduction of compound **3** on probe **5** was continuously monitored at time intervals. Reactions were observed by fluorescence spectrometry (FP-6500; JASCO). For the time course of the azide reduction, the fluorescence intensity was measured for 0.5 s at 1 min intervals: excitation, 595 nm; emission, 655 nm.

Dual color SNP typing

The probe **5** (red) and probe **10** (green) were used for the dual-color SNPs typing. Reactions on the DNA template were performed in 1.2 ml of tris-HCl buffer (20 mM, pH 7.2) containing 100 mM $MgCl_2$ and 0.01 mg/ml BSA with 250 nM of *bcr/abl-1* (5′-TAA GCA GAG TTC AAA AGC **C**CT TCA GCG-3′; complementary to probe 5) or *bcr/abl-2* (5′-TAA GCA GAG TTC AAA AGC **A**CT TCA GCG-3′; complementary to probe 10), probe **5** (250 nM), probe **6** (250 nM) and probe **10** (250 nM) at 37 °C. The increase in the fluorescence intensity was continuously monitored at time intervals. For the time course of the azide reduction, the fluorescence intensity was measured for 0.5 s at 1 min intervals: ex/em 490 nm/550 nm (for probe **10**); 595 nm/655 nm (for probe **5**). For standardization, all fluorescence intensities derived from both probes were divided by the intensity obtained in the case of using each full-match target DNA at 30 min.

Conclusions

We developed a dual-color RETF probe for the detection of single nucleotide discrimination in vitro. A red-color fluorogenic compound, naphthorhodamine azide **3**, was designed and synthesized for this chemistry. Dual-color probes were used for rapid detection of SNPs with high sensitivity and selectivity.

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