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Fluorescence detection of single nucleotide polymorphisms using nucleic acid probe containing tricyclic base-linked acyclonucleoside

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ABSTRACT

A reliable and simple method for detecting nucleobase mutations is very important clinically because sequence variations in human DNA cause genetic diseases and genetically influenced traits. A majority of sequence variations are attributed to single nucleotide polymorphisms (SNPs). Here, we developed a method for SNP detection using DNA probes that contained a fluorescent tricyclic base-linked acyclonucleoside **N**. The type of nucleobases involved in the SNP sites in an RNA target could be determined using four DNA probes containing **N**. Further, we found that the SNP in the RNA target could be detected by a visible color. Thus, this system would provide a novel and simple method for detecting SNPs in an RNA target.

The development of a reliable and simple method for detecting single nucleotide polymorphisms (SNPs), a common source of genetic variations in the human genome, is currently an important research area because SNPs are important for identifying disease-causing genes and for pharmacogenetic studies.[1–7] Recently, we have developed a novel and simple method for SNP detection using a nucleic acid probe that contains a fluorescent tricyclic base-linked acyclonucleoside **Q** (Fig. 1).[8] The design principle of SNP detection using the nucleoside surrogate **Q** is outlined in Figure 2. We designed DNAs containing **Q** at the 5′ (5′-Q-probes) or 3′ sides (3′-Q-probes) of discriminating bases **D** to produce bulges. The fluorescence intensity of the nucleoside surrogate **Q** changes depending on solvent polarity. When the **D**s match the sequences of opposite bases, **D**s form base pairs with complementary bases, causing the nucleoside surrogate **Q** to flip outside the DNA helix and strengthening the fluorescence intensity of **Q**. On the other hand, when the mismatched bases have sites opposite the **D**s, that is, when the **D**s cannot form base pairs with the opposite bases, **Q** intercalates into the DNA helix because the tricyclic base-linked nucleoside surrogate **Q** is more intercalative than natural mono- or bicyclic natural nucleobases. This weakens the **Q** fluorescence intensity. We can determine the type of nucleobase involved in the SNP site by comparing the fluorescence intensities of duplexes composed of each probe containing **Q**. However, since the fluorescence emission maximum of **Q** is around 390 nm, we could not

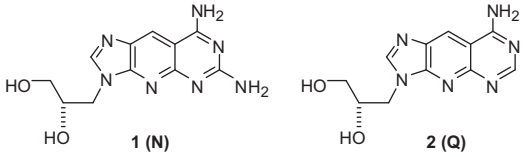

Figure 1. Structures of tricyclic base-linked nucleoside surrogates.

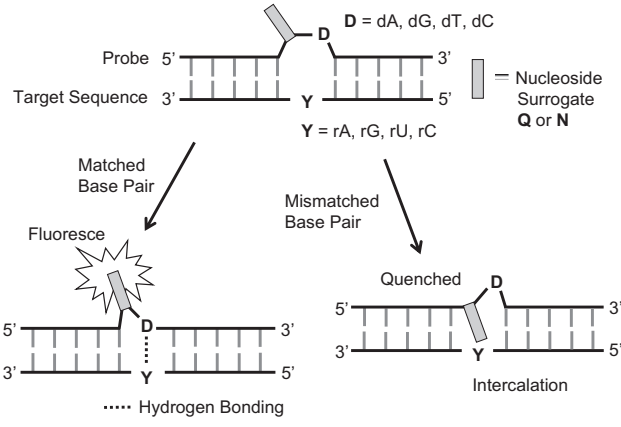

Figure 2. Design principle of SNP detection using the nucleoside surrogate **Q** or **N**. **D** denotes discriminating bases. **Y** indicates SNP sites.

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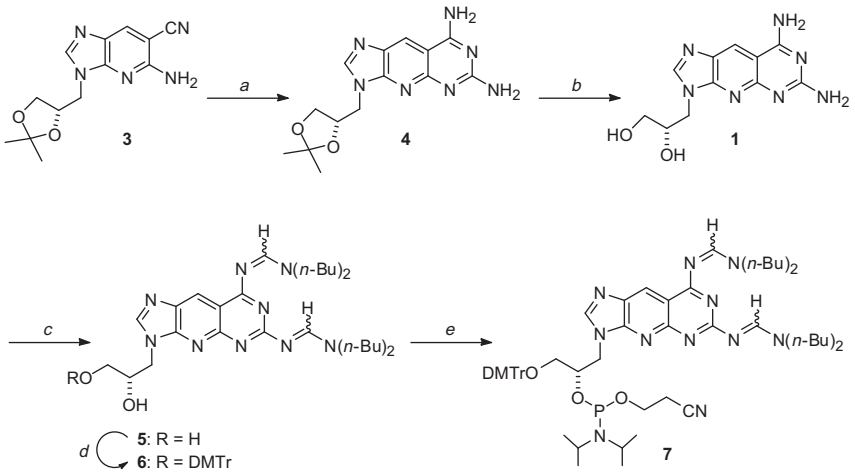

Scheme 1. Reagents and conditions: (a) $NH{=}C(NH_2)_2$, EtOH, reflux, 23 h, 76%; (b) 80% CH_3CO_2H, 60 °C, 8 h, 97%; (c) $(MeO)_2CHN(n\text{-}Bu)_2$, DMF, 60 °C, 24 h; (d) DMTrCl, pyridine, rt, 3 h, 23% (two steps); and (e) chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, *i*-Pr_2NEt, THF, rt, 3 h, 93%.

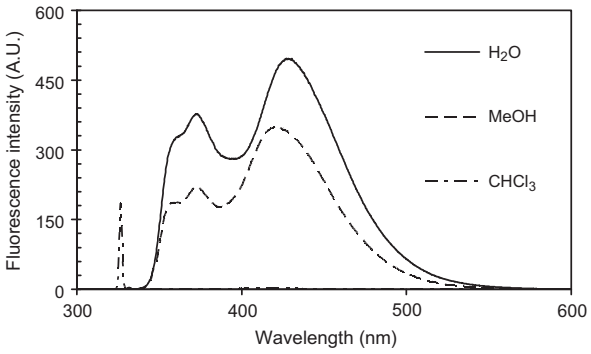

Figure 3. Fluorescence emission spectra of **N** in various solvents. Spectra were obtained on a spectrofluorophotometer in quartz cuvettes with a path length of 1.0 cm and a 30 μM **N** concentration in an appropriate solvent. Spectra were recorded by using an excitation slit of 1.5 nm and an emission slit of 1.5 nm.

detect the SNPs in the targets by visible colors. Here, we report SNP detection by a visible color using DNAs that contain a fluorescent tricyclic base-linked acyclonucleoside, 6,8-diamino-3-(2,3-dihydroxypropyl)imidazo[4′,5′:5,6]pyrido[2,3-*d*]pyrimidine (**N**).

In general, amplified DNAs are used for the SNP typing. However, the first product of gene expression is RNA, which is a transcription product. Therefore, it is advantageous to use RNA in SNP diagnosis rather than as DNA after RT-PCR. Furthermore, in relation to discovery of a large number of noncoding RNAs, a method to detect intracellular RNAs in real-time is required. Thus, in this study, we focused on detection of SNPs on RNA strands.

Table 1
Sequences of oligonucleotides

Abbreviation	Sequence
NA	5′-d(GAA GGT CAA **N**A**N**G TAT CTC T)-3′
NG	5′-d(GAA GGT CAA **N**G**N**G TAT CTC T)-3′
NC	5′-d(GAA GGT CAA **N**C**N**G TAT CTC T)-3′
NT	5′-d(GAA GGT CAA **N**T**N**G TAT CTC T)-3′
AN	5′-d(GAA GGT CAA A**N**G TAT CTC T)-3′
GN	5′-d(GAA GGT CAA G**N**G TAT CTC T)-3′
CN	5′-d(GAA GGT CAA C**N**G TAT CTC T)-3′
TN	5′-d(GAA GGT CAA T**N**G TAT CTC T)-3′
SrA	3′-r(CUU CCA GUU *a*CA UAG AGA)-5′
SrG	3′-r(CUU CCA GUU *g*CA UAG AGA)-5′
SrC	3′-r(CUU CCA GUU *c*CA UAG AGA)-5′
SrU	3′-r(CUU CCA GUU *u*CA UAG AGA)-5′

The underlined letters indicate discriminating bases. The small italic letters represent target bases.

The synthesis of the tricyclic base-linked nucleoside surrogate **N** is shown in Scheme 1. 5-Amino-6-cyano-3-[(2,2-dimethyl-1,3-dioxolan-4-yl)methyl]imidazo[4,5-*b*]pyridine (**3**), which was synthesized by a method reported previously,[8] was treated with guanidine to produce an isopropylidene derivative of **N** in 76% yield. Deprotection of the isopropylidene group, protection of the amino groups with a di(*n*-butyl)formamidine group,[9] and protection of the primary hydroxy group with a 4,4′-dimethoxytrityl (DMTr) group afforded the properly protected nucleoside surrogate **6**. Phosphitylation of the secondary hydroxy group of **6** gave phosphoramidite unit **7** in 93% yield.

Typical emission spectra of the tricyclic base-linked nucleoside surrogate **N** are shown in Figure 3. The nucleoside surrogate **N** showed emission maxima at 427 nm under 334-nm excitation in H_2O, which was a wavelength approximately 35 nm longer than that of **Q**. The fluorescence intensity of **N** was dependent on solvent polarity; that is, the fluorescence intensity of **N** was greater in more polar solvents such as methanol and water than in less polar solvents such as chloroform (Table S1).

The sequences of oligodeoxynucleotides (ODNs) containing **N** are listed in Table 1. CYP2C9 is one of the predominant cytochrome P450 (CYP) enzymes expressed constitutively in the human liver.[10,11] It metabolizes a variety of therapeutically important drugs.[10,11] In the present study, we chose the sequence containing an A1075(C) mutation in the *CYP2C9* gene as a model sequence. The probe ODNs were composed of sequences complementary to the *CYP2C9* gene. The probes (5′-N-probes) termed **NA**, **NG**, **NC**, and **NT** contained **N** at the 5′ sides of **D**s, whereas the probes (3′-N-probes) termed **AN**, **GN**, **CN**, and **TN** possessed **N** at the 3′ sides of **D**s. Target sequences are abbreviated as S. All the ODNs containing **N** were synthesized using a DNA synthesizer. The obtained ODNs were analyzed by MALDI-TOF/MS, and the observed molecular weights were in agreement with their structures.

The T_m values of the duplexes between the probes and the RNA targets are listed in Table S2. When the discriminating bases and the target bases matched, the T_m values of the duplexes were the greatest in all sequences except for the **TN** probe for the SrU target. Thus, the discriminating bases seemed to have base selectivities in the DNA/RNA duplexes containing the tricyclic nucleoside surrogate **N**.

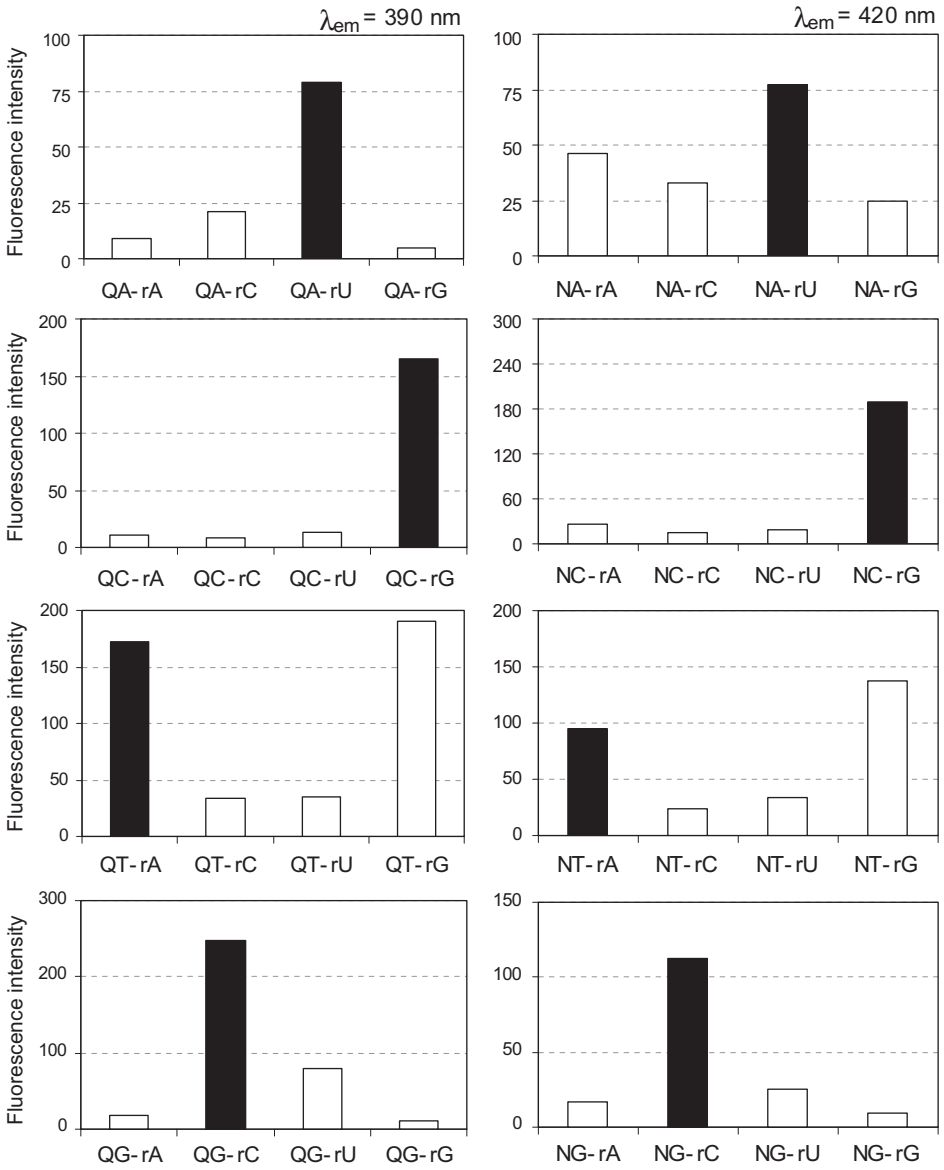

Figure 4. Fluorescence intensities of duplexes. Left column: 5′-Q-probe/RNA target. Right column: 5′-N-probe/RNA target. Data of 5′-Q-probe/RNA target were quoted from a previous paper.[8] Spectra were measured at 20 °C.

Fluorescence emission spectra of the duplexes between the probes and the target RNAs are shown in Fig. S1. Fluorescence spectra intensity of the duplexes correlated with the T_m values of the duplexes. The fluorescence intensities were the greatest when the discriminating bases were complementary to the target bases except for the duplex comprising the **NT** probe and the SrG target. Next, we compared the fluorescence intensities of the duplexes containing **N** at 420 nm (Figs. 4 and 5, right columns) with those containing **Q** at 390 nm (Figs. 4 and 5, left columns). The pattern of the fluorescence intensities for the 5′-N-probes containing the nucleoside surrogate **N** was similar to that for the 5′-Q-probes containing **Q** (Fig. 4). The fluorescence intensities of the duplexes were the greatest when the discriminating bases were complementary to the target bases except for the QT/SrG and NT/SrG duplexes. The fluorescence of the mismatched QT/SrG and NT/SrG duplexes were attributed to the wobble-type base pairings between dT and rG bases.[12] The fluorescence intensities of the single-stranded N-probes were almost equal to those of the mismatched duplexes (data not shown).

The results for the probes containing the surrogate **N** or **Q** at the 3′ side of the discriminating bases (3′-N-probes and 3′-Q-probes) are shown in Figure 5. Fluorescence intensities of the duplexes were the greatest when the discriminating bases were complementary to the target bases except for the **CQ** probe. No fluorescence was observed with the **CQ** probe for the target sequences. On the other hand, when the 3′-N-probes were used, fluorescence intensities of the duplexes were the greatest when the discriminating bases were complementary to the target bases: the **AN** probe for the SrU target, **CN** probe for the SrG target, **TN** probe for the SrA target, and **GN** probe for the SrC target. The fluorescence intensities of the duplexes increased 7–20-fold compared to those with mismatched sequences. Thus, it was found that we could determine the type of nucleobases involved in the SNP site in the RNA target using the 3′-N-probes.

Finally, we assessed whether or not we could detect the SNP in the RNA target by a visible color. Photographs of solutions containing the TN/SrU or TN/SrA duplexes, which were irradiated with a handy-type UV lamp at 254, 302, or 365 nm, are shown in Figure

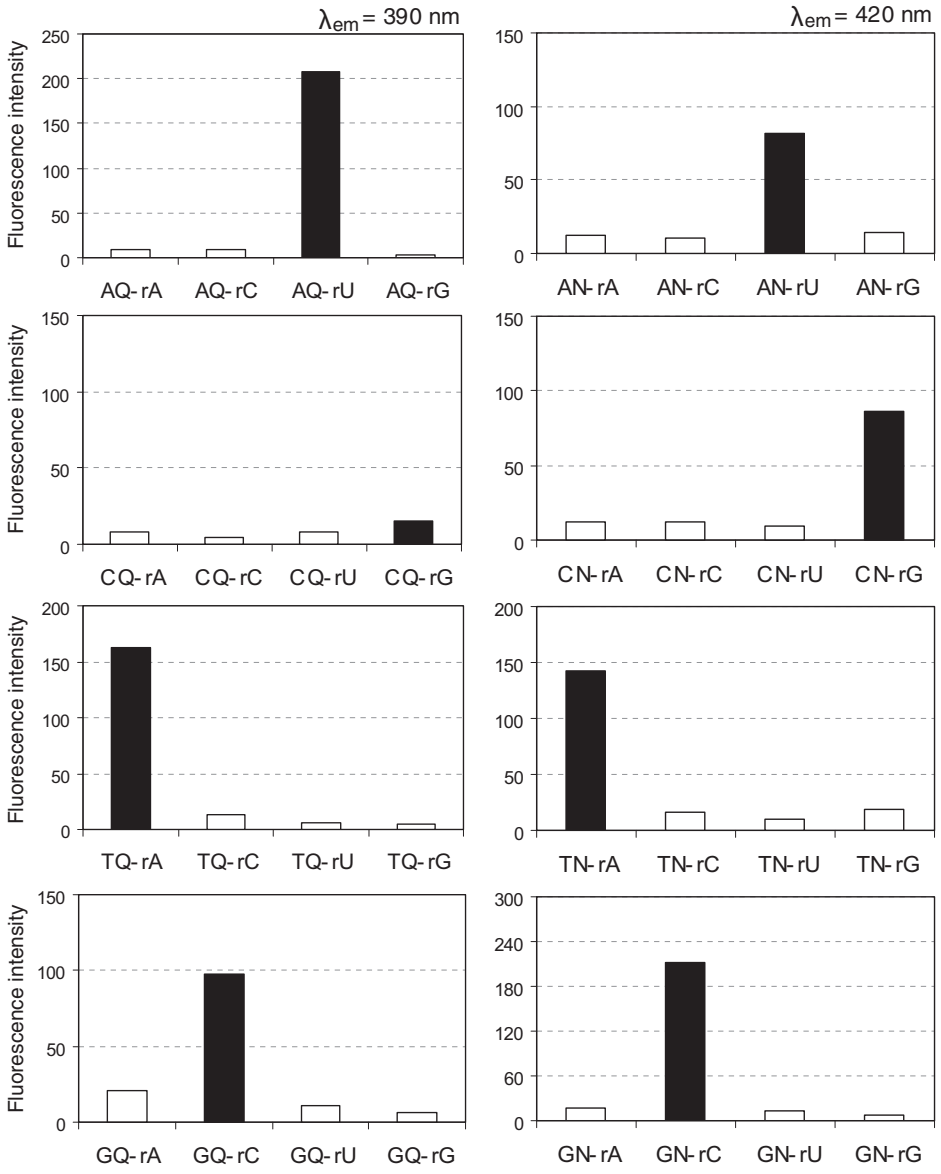

Figure 5. Fluorescence intensities of duplexes. Left column: 3′-Q-probe/RNA target. Right column: 3′-N-probe/RNA target. Data of 3′-Q-probe/RNA target were quoted from a previous paper.[8] Spectra were measured at 20 °C.

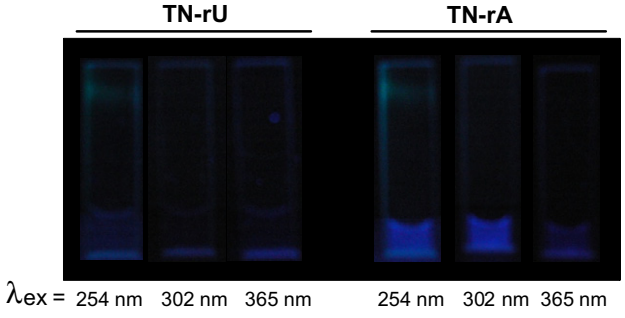

Figure 6. Photographs of solutions containing the TN/SrU or TN/SrA duplexes irradiated with a handy-type UV lamp at 254, 302, or 365 nm. Duplex concentrations: 3.0 μM in a T_m buffer.

6. No fluorescence was observed for the TN/SrU duplex, whereas blue colors were observed for the TN/SrA duplex when the solutions were irradiated with UV light of 254 or 302 nm.

In conclusion, we demonstrated the synthesis of DNA containing a fluorescent tricyclic base-linked acyclonucleoside **N**. We examined properties of the DNA containing **N** as an SNP-detecting probe. It was found that we can determine the type of nucleobases involved in the SNP sites in an RNA target with high sensitivities using four probes containing **N**. Further, it turned out that the SNP in the RNA target could be detected by a visible color. Thus, this system would provide a reliable and simple method for detecting SNPs in an RNA target.

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Supplementary data

Supplementary data (experimental procedure, T_m values, fluorescence emission spectra) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.11.022.

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