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A Probe Containing Two Base-discriminating Fluorescent (BDF) Nucleosides for SNP Typing

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We have developed a new probe containing two base-discriminating fluorescent (BDF) nucleosides for single nucleotide polymorphism (SNP) typing. It is possible to determine the SNP type using just one probe. We confirmed suitability of the probe from hybridization assay results obtained for the ALDH2 gene.

SNP (single nucleotide polymorphism) appears at an incidence of 1% or more in a specific population. SNP exists in the whole genome, it exists not only in an exon site but in an intron site, and splicing control may change with the variation. The identification of SNP type is likely to prove extremely useful in clinical diagnostics.[1]

Since two homologs alleles which have the major and/or minor polymorphisms, exist in a cell, there are three possible genotypes: major homozygote, minor homozygote, and heterozygote. Usually, SNP type is determined by the ratio of the major allele and minor allele. For conventional SNP typing, the hybridization assay is carried out with the complementary fluorophore-labeled DNA (major and minor probes) in order to identify the DNA extracted from analyte. The major and minor probes are fluorescently labeled in different colors.[2] From the ratio of fluorescence intensity of the major and minor probes, the genotypes can be determined, i.e., major homozygous, heterozygous and minor homozygous types correspond to 2:0, 1:1, and 0:2. Thus, the conventional SNP typing needs to two different probes in order to identify SNP type.

Recently, a new concept using base-discriminating fluorescent (BDF) nucleosides has been proposed.[3] These are molecular fluorescent devices which emit a strong fluorescence when they form a stable base pair with a target base. A new SNP typing assay was successfully demonstrated using these BDF probes.[4] Due to the hybridization of BDF probes with a target DNA strand containing an SNP site, the type of the SNP base was easily determined fluorometrically and furthermore, to deal with false positives caused by mismatched hybrids or nonhybridized probes, a number of SNP samples were typed simultaneously without time-consuming or troublesome processes. Although this method is a very useful tool for SNP typing, two different probes are required for the identification of SNP type in the same manner as the conventional SNP typing.

In the present communication, we report for the first time a new probe for SNP typing in which two different BDF nucleosides were incorporated into a single-strand DNA (Figure 1). This probe has two different adenine-discriminating fluorescent nucleosides, AMPyU and PyU (Figure 2). Both AMPyU and PyU selectively emit fluorescence only when the base opposite BDF nucleoside is adenine, as shown in Figure 1. AMPyU and PyU have different fluorescence spectra, and they were incorporated into a SNP site and a non-SNP site in the DNA, respectively. After the hybridization, the genotype is identified easily from the ratio of the fluorescence intensity of the SNP and non-SNP sites using a single probe. This method is a very powerful tool for high-throughput SNP typing, especially for many SNP sites in a DNA because a SNP site requires only a single probe.

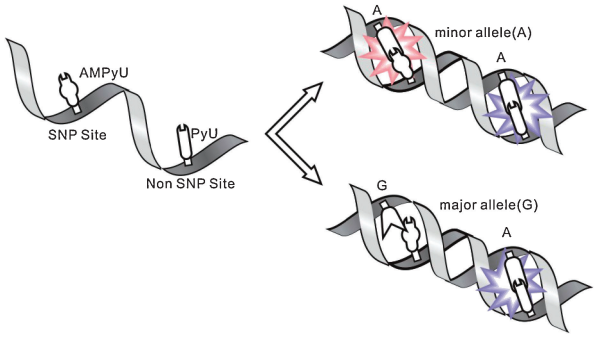

Figure 1. Schematic illustration of the probe containing two base-discriminating fluorescent (BDF) nucleosides for SNP typing.

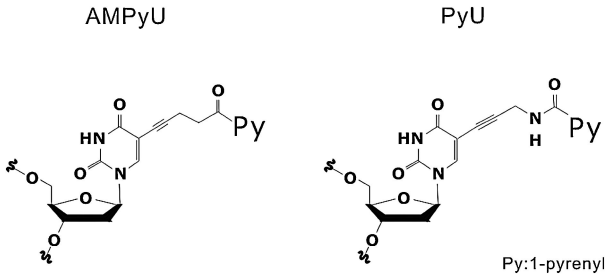

Figure 2. Structure of the BDF nucleosides, AMPyU and PyU.

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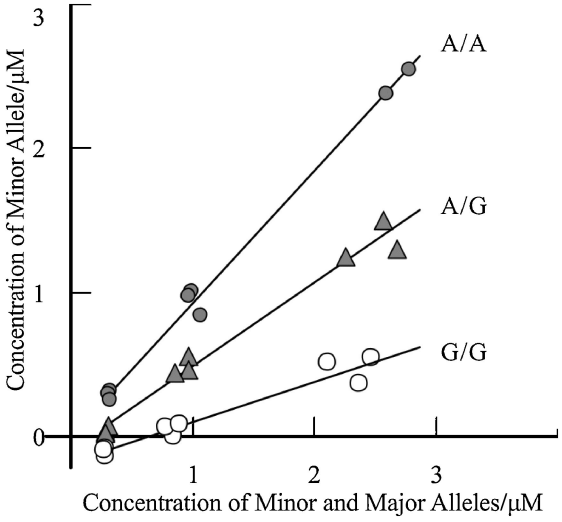

Figure 3. Relationship between the concentration of minor allele and the total concentration of minor and major alleles. Shaded circles: A/A (minor homozygous), shaded triangles: heterozygous (A/G), and open circles: major homozygote (G/G).

The probe containing AMPyU and PyU was synthesized by using the previously mentioned protocol.[3,4] In order to evaluate the performance of the probe developed here, we tested the SNP typing of samples obtained by amplification of ALDH2 (aldehyde dehydrogenase 2) gene sequence via hybridization assay. PCR amplification was performed using a GeneAmp PCR System 9700 (Applied Biosystems) with Takara Ex Taq (Takara Bio). We determined the A/G SNP at the nucleotide position 114 of exon 12 in the sequence of ALDH2 gene, 5′-(GGCTGCAGGC **Y** TACACT **X** AAGTGAAAA)-3′ (**X** = an A/G SNP site, **Y** = a non-SNP site: A). In the case of this gene, **X** = A and **X** = G are minor and major alleles, respectively. Two kinds of PCR products (SNP site: A or G), which were prepared using commercial clones (Invitrogen CS0DM00Y123 and Open Biosystems 4849418) as templates, and a mixture of them in equal amounts were used as the samples [A/A (minor homozygous), G/G (major homozygous), and A/G (heterozygous)]. The 28-mer probe, 5′-(TTTTCACTT $\mathbf{B_1}$ AGTGTA $\mathbf{B_2}$ GCCTGCAGCC)-3′ ($\mathbf{B_1}$ = AMpyU, $\mathbf{B_2}$ = PyU), was mixed with the samples in the PBS buffer (pH 7.0) containing 100 mM NaCl. After incubation at 37 °C for 30 min, the fluorescence was measured with a spectrofluorometer (Shimadzu RF-5300PC). As was previously reported, the excitation and detection wavelength for an AMPyU/A base pair were 374 and 460 nm, respectively.[4] On the other hand, those for a PyU/A base pair were 345 and 400 nm, respectively.[3] In melting temperature measurements of the duplex, a high duplex stability was observed for AMPyU and PyU containing duplexes.[4]

Since both AMPyU and PyU are emitted by the recognition of adenine (A) in the complementary DNA, the fluorescence intensities of AMPyU and PyU correspond to the concentration of minor allele and the total concentration of minor and major alleles, respectively. Therefore, in the present experiment, the concentration ratios obtained from the fluorescence intensities were basically 2:2 for A/A, 1:2 for A/G, and 0:2 for G/G, respectively.

Figure 3 shows the relationship between the concentration of minor allele and the total concentration of minor and major alleles. The concentration of minor allele linearly increased with increasing total concentration of minor and major alleles for three samples (A/A, A/G, and G/G). The (linear) regression equations for these data are as follows: $y = 0.03 + 0.90x$ ($R^2 = 0.99$) for A/A, $y = 0.07 + 0.57x$ ($R^2 = 0.98$) for A/G, and $y = -0.16 + 0.28x$ ($R^2 = 0.94$) for G/G. From these results, the concentration ratios were obtained as 1.8:2 for A/A, 1.14:2 for A/G, and 0.56:2 for G/G. Although the concentration ratios of A/A and A/G were in good agreement with the expected ratios, the ratio of G/G was a slight difference between the obtained and the expected ratio. We attributed the difference to the background fluorescence of AMPyU; AMPyU will emit fluorescence by absorbing the fluorescence of PyU excited at 345 nm. However, this does not become a problem in the actual SNP typing, because G/G can be clearly distinguished from A/A and A/G.

In summary, we have developed a new probe containing two base-discriminating fluorescent nucleosides for SNP typing. By using only this probe, easy SNP typing with high accuracy can be realized. Moreover, since SNP typing using the probe is rapid and low cost, and can assay a lot of samples simultaneously, it should be extremely useful in the clinical field. The probe can also be applied to DNA microarray and has great potential for gene expression profiling.

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