DNA Sensors

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Secondary-Structure-Inducible Ligand Fluorescence Coupled with PCR**

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Single-stranded DNA can change its structure dynamically in response to the presence of a complementary strand. The large structural change from the hairpin secondary structure to the double-stranded form leads to the chemistry of molecular beacons (MBs), or hairpin DNA oligomers having both a fluorescent and a quenching chromophore in the hairpin stem.[1] MBs can be used to report the presence of complementary strands by measuring the increasing fluorescence because of the decreasing energy transfer efficiency. Most DNA probes for fluorescent detection are covalently bound to fluorophores to make energy transfer effective.[1,2]

We have focused on an alternative way to link the fluorescence change with the structural changes of DNA that are induced during polymerase chain reaction (PCR) amplification.[3] We describe herein the chemical concept of noncovalent fluorescent DNA labeling by ligand binding to the secondary structure, which allows us to monitor PCR progress by measuring the change in fluorescence emitted from ligand–primer complexes in a homogeneous solution. This concept is not only complementary to that using fluorescent dye bound selectively to PCR product duplexes,[4] but it also expands the possibility of real-time PCR.

The concept of DNA labeling by secondary-structure-inducible ligand fluorescence is shown in Figure 1. PCR primers are labeled with a hairpin tag containing cytosine bulges (C-bulges). The molecule 2,7-diamino-1,8-naphthyridine (DANP) binds to a C-bulge in its protonated form ($DANPH^+$) and emits fluorescence at 430 nm with a 30 nm bathochromic shift from the fluorescence of free, unbound DANP.[5] We hypothesized that, as the PCR progresses, the hairpin tag will dissolve and be transformed into a duplex, thus resulting in the loss of the DANP binding site and a decrease in the fluorescence at 430 nm. Toward this end, we investigated the hairpin tags. These tags should identify the DANP binding site without disturbing the fluorescence efficiency, should not interfere with the PCR, should be transformed effectively into the duplex during PCR, and should be applicable to diverse primers.

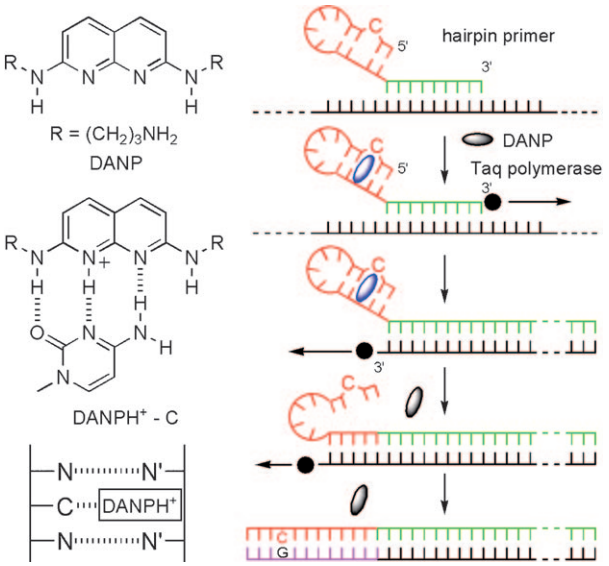

Figure 1. An illustration of the concept of DNA labeling by secondary-structure-inducible ligand fluorescence.

First, the sequence flanking the C-bulge producing the highest fluorescence intensity was investigated by measuring DANP fluorescence with duplexes having a C-bulge flanked by A–T and T–A base pairs. The G–C and C–G base pairs were omitted from the flanking base pairs because of their quenching of DANP fluorescence.[5b] The characteristic spectrum with a broad single peak at 430 nm was obtained for the A_A/TCT sequence. The relative fluorescence intensity at 450 nm for the four C-bulge duplexes and a fully matched duplex are shown in Figure S1 in the Supporting Information. The C-bulge in the A_A/TCT sequence enhanced DANP fluorescence by 7.6-fold compared with the fully matched duplex and by 82-fold compared with free, unbound DANP.

We then designed hairpin tags comprising one to three A_A/TCT units separated by three to five base pairs and a T4 hairpin loop (Table 1). The intensity of DANP fluorescence (F_O) was markedly dependent on the number of base pairs separating the C-bulge sites in the hairpin tags. HP-2, which

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Table 1: Sequence, T_m, and fluorescence intensity of hairpin tags.

Tag name	Tag sequence	T_m[a]	F_O[b]	F_N
HP-1	5′-CATCCAA_ACAACCA *—GTAGGTTCTGTTGGT T_4	71.7	150	150
HP-2	5′-ATCATCTCA_AC *—TAGTA_AGTCTG T_4	39.3	102	51
HP-3	5′-ATCATCTACA_AC *—TAGTA_ATGTCTG T_4	41.8	260	130
HP-4	5′-ATCATCTACTA_AC *—TAGTA_ATGATCTG T_4	42.9	290	145
HP-5	5′-ATCAA_ACATCTCA_AC *—TAGTTCTGTA_AGTCTG T_4	38.3	147	49
HP-6	5′-TAGTA_ACATCTACA_AC *—ATCATCTGTA_ATGTCTG T_4	39.3	356	119

[a] T_m of hairpin tags (5 μM) with the primer sequence 3′-d(CAG TAT CGA CAA AGG AC)- at the 3′ end (shown as an asterisk) was measured in 10 mM sodium cacodylate buffer (pH 7.0) with 100 mM sodium chloride. T_m is the temperature at which 50% of the duplex DNA dissociates into single strands. [b] Fluorescence spectra were measured with DANP (50 μM).

has two C-bulge sites with three base pairs inserted in between, showed only one-third of the normalized fluorescence intensity per unit (F_N) of HP-1, which contains one C-bulge unit. As the number of base pairs between C-bulges increased, F_N increased. The F_N value for HP-4, with an insert of five base pairs, was almost the same as that of HP-1. Similar results were obtained for HP-5 and HP-6, which each have three C-bulge units.

The feasibility of using the designed hairpin tags in PCR was then examined with primers labeled with an HP-3 tag. PCR was performed with the plasmid pUC18 as a template with primers M13M3 and M13RV in the presence of DANP (Figure 2A). The reverse primer M13RV was used with or without the HP-3 tag. The PCR product with M13RV is 106 nucleotides in length, whereas that of M13RV with the HP-3 tag is 134 nucleotides. Native polyacrylamide gel electrophoresis (PAGE) analysis of PCR products with HP-3-labeled M13RV showed one product band with the expected nucleotide length (Figure 2B and Figure S3 in the Supporting Information). The bands at 200 bp were most likely due to polymorphic structure of the hairpin sequence, because the bands were not observed when analyzed by denaturing PAGE. The change in the fluorescence intensity during PCR was measured after the indicated number of PCR cycles at 25 °C (Figure 2C). The fluorescence intensity of the PCR samples started to decrease after 10 cycles and was 64% of the initial intensity after 35 cycles. The increase in PCR product bands on PAGE analysis correlated well with the decrease in the fluorescence at 450 nm.

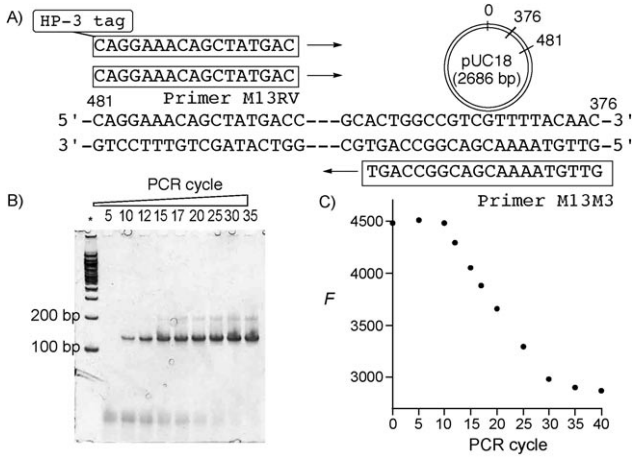

Figure 2. A) Alignment of primers on pUC18. B) Native PAGE analysis of PCR products after indicated PCR cycles. The lane (*) is 100 bp ladder markers. C) Fluorescence intensity of PCR products.

We investigated the single-nucleotide polymorphism (SNP) of the cytochrome P450 gene 2C9*3 with the chemistry described above. The SNP site at the 1075th position is adenine for the wild-type allele (A allele) and cytosine for the mutant allele (C allele). According to the allele-specific PCR reported for the 2C9*3 alleles,[6] two HP-3-labeled forward primers, FW-1075T and FW-1075G, which had thymine and guanine in the second position from the 3′ end, respectively, were used for the PCR (Figure 3A). To increase the allele specificity, an additional T–T mismatch was introduced at the

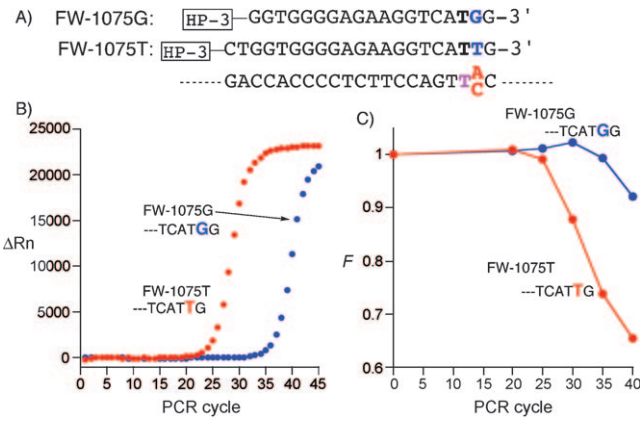

Figure 3. A) Primers used for P450 2C9*3. B) Real-time PCR amplification plot obtained from FW-1075G (blue) and FW-1075T (red) with the A allele template. C) Relative fluorescence intensity of PCR products.

third position. As shown in the real-time PCR amplification plot (Figure 3B), the PCR with the A allele template proceeded with high allele specificity to produce PCR products with FW-1075T after 25 PCR cycles. The fluorescence intensity of the PCR solution showed clearly the allele-specific consumption of the FW-1075T. The fluorescence intensity started to decrease after 25 cycles, whereas that of FW-1075G changed after 35 cycles (Figure 3C). A similar result was obtained for PCR with the C allele (Figure S8 in the Supporting Information). These experiments confirmed that the HP-3 hairpin tag was not limited to the M13RV primer but applicable to primers for other templates. We have confirmed that the HP-3 hairpin tag works with more than ten different primers without any interference in PCR.

The changes in DANP fluorescence from before to after PCR with primers labeled with the C-bulge hairpin tag were well correlated to the PCR progress and can report the allele type when combined with an allele-specific PCR. The concept of secondary-structure-inducible ligand fluorescence de-

scribed herein would expand the chemistry of molecules binding to specific DNA structures and emitting characteristic fluorescence.

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