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PNA for One-Base Differentiating Protection of DNA from Nuclease and Its Use for SNPs Detection

Makoto Komiyama,* Sheng Ye, Xingguo Liang, Yoji Yamamoto, Takafumi Tomita, Jing-Min Zhou, and Hiroyuki Aburatani

Contribution from the Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan

Received November 29, 2002; E-mail: komiyama@mkomi.rcast.u-tokyo.ac.jp

Abstract: By the combination of peptide nucleic acid (PNA) with single-stranded DNA specific nucleases, alteration of a single base to another in DNA has been detected with high accuracy. Only the DNAs in DNA/PNA duplexes involving a mismatch are efficiently hydrolyzed by these enzymes, whereas fully matching sequences are kept intact. This difference is visually scored by adding 3,3′-diethylthiadicarbocyanine, which changes its color from blue to purple upon binding to DNA/PNA duplexes. These findings are applied to the convenient and straightforward detection of single nucleotide polymorphisms (SNPs). When the target site in the sample DNA is completely complementary with the PNA, a notable amount of DNA/PNA duplex remains and thus the solution exhibits purple color. In the presence of even one mismatch between PNA and DNA, however, the DNA is completely digested by the enzyme and therefore the dye shows its intrinsic blue color. The SNPs in the apolipoprotein E gene of human DNA have been successfully genotyped by this method.

Introduction

Single nucleotide polymorphisms (SNPs), single-base alterations in the human genome, change both the structure and the function of encoded protein and are strongly related to various medical and physiological features of human beings. With the information from them, for example, the possibility of hereditary diseases of individuals and their responses toward administered medicines can be predicted. Thus, high-through-put, economical, and precise detection of SNPs is a key issue in today's chemical biology, biotechnology, medicine, and pharmacogenomics.[1−13] However, few of the previously proposed methods fulfills all the requirements for versatile application (promptness, preciseness, reproducibility, cheapness, easiness, and others). Here we report a new method which is based on our new finding that the combination of peptide nucleic acid (PNA)[14] and single-stranded DNA specific nucleases strictly recognizes a one-base alteration in DNA with remarkable specificity. Even a one-base

* To whom correspondence should be addressed. Tel: +81-3-5452-5200. Fax: +81-3-5452-5209.

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Scheme 1. Strategy of SNP Detection by the PNA/Nuclease/Dye System

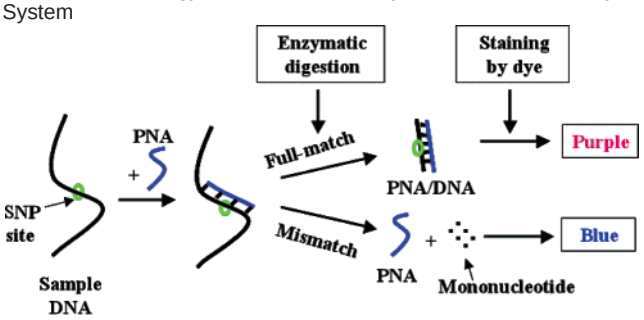
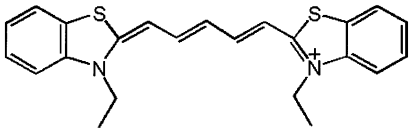


Chart 1. Structure of 3,3′-Diethylthiadicarbocyanine Dye ($DiSC_2(5)$)



mismatch in DNA to PNA makes this DNA susceptible to the enzymatic digestion. When the mixtures are stained with a dye after the enzymatic digestion, SNP alleles are easily and precisely determined by absorption spectroscopy or even with our naked eyes (see Scheme 1).

Peptide nucleic acid (PNA), elegantly developed by Nielsen's group,[14] is one of the most widely used DNA analogues. Its application both *in vivo* and *in vitro* is highly promising, mainly because of the following three characteristics: (1) The binding of PNA to its complementary DNA is satisfactorily strict. (2) The DNA/PNA duplex is more stable than is the corresponding DNA/DNA duplex due to the absence of electrostatic repulsion between negative charges. (3) PNA is strongly resistant to nucleases. About 10 years ago,[15] it was reported that oligonucleotides are efficiently protected from nuclease S1 when they are bound to the complementary PNA. The DNA in these fully matching DNA/PNA duplexes cannot be sufficiently recognized by this single-stranded DNA specific nuclease. This pioneering work prompted us to study on the effects of a mismatch in DNA/PNA duplexes on their digestion by nucleases. Our expectation was that conformational changes, molecular flexibility, and the resultant physicochemical perturbations at the DNA/PNA mismatch site should facilitate the access of enzymes to this site, resulting in the digestion of DNA there. If the differentiation of the fully matching site and mismatching site is sufficiently explicit, a one-base alteration in DNA sequence should be able to be detected in terms of the difference in digestion efficiency. As described in this paper, the required one-base differentiating DNA digestion, at the site dictated by the PNA, is successfully achieved by nuclease S1 and Mung Bean nuclease. Only when there exists a mismatch in DNA/PNA duplexes, the DNA is efficiently hydrolyzed, and otherwise it is kept intact. The difference in DNA sequence of only one nucleotide is concretely recognized. Furthermore, one-base alteration at potential SNPs sites is visually detected by using the unique dye 3,3′-diethylthiadicarbocyanine ($DiSC_2(5)$ in Chart 1), which changes its color upon binding to DNA/PNA duplexes.[16,17] The color

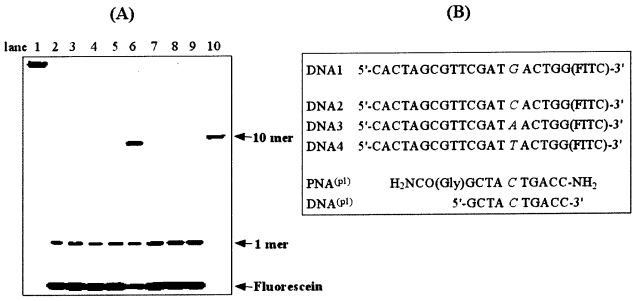


Figure 1. (A) Polyacrylamide gel electrophoresis patterns for DNA digestion by nuclease S1 in the presence of PNA: lane 1, control (DNA1 + $PNA^{(p1)}$, prior to the enzymatic reaction); lane 2, DNA1 + $DNA^{(p1)}$; lane 3, DNA2 + $DNA^{(p1)}$; lane 4, DNA3 + $DNA^{(p1)}$; lane 5, DNA4 + $DNA^{(p1)}$; lane 6, DNA1 + $PNA^{(p1)}$; lane 7, DNA2 + $PNA^{(p1)}$; lane 8, DNA3 + $PNA^{(p1)}$; lane 9, DNA4 + $PNA^{(p1)}$; lane 10, the marker (10-mer DNA). Reaction conditions: [DNA substrate (3′-end labeled by fluorescein)]$_0$ = 10 μM, [$PNA^{(p1)}$ or $DNA^{(p1)}$]$_0$ = 12 μM; [nuclease S1]$_0$ = 0.5 U/μL; at pH 4.6 and 20 °C for 20 min. As presented in (B), one of the internal bases of DNA (in italics) is systematically changed. The DNA/DNA and DNA/PNA duplexes are completely matching in lanes 2 and 6, whereas they involve a mismatch in lanes 3−5 and 7−9. Almost the same results were obtained even when NaCl (280 mM) was added to the reaction mixtures.

difference is vivid in the visible-light region so that SNPs are detectable even with our naked eyes. No labeling of samples or probes is required. Coexisting DNA/DNA duplexes, if any, do not disturb the detection because of the difference in absorption spectra. The present method has been applied to the detection of SNPs in the apolipoprotein E (*apoE*) gene of human genomic DNA, which is related to serious diseases involving Alzheimer's disease.

Results and Discussion

Effect of Single-Base Mismatch in a DNA/PNA Duplex on the DNA Digestion by Nuclease S1 and Mung Bean Nuclease. The mismatch-detecting activity of PNA/nuclease S1 combination was first investigated by using gel electrophoresis (Figure 1). In the presence of $PNA^{(p1)}$ probe, the DNA substrates in Figure 1B (20-mers labeled by fluorescein at the 3′-end) were treated with nuclease S1. The 10-mer strand in the 3′-side of DNA1 is completely complementary with $PNA^{(p1)}$. As shown in lane 6 in Figure 1A, a notable amount of oligonucleotide remained undigested in the reaction mixture. According to matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS), this oligonucleotide was the 9-mer (with 5′-phosphate and 3′-fluorescein) spanning from G12 to G20 in DNA1 (the observed mass number was 3145.8, whereas the theoretical value is 3145.6). This shows that the complementary DNA sequence of DNA1 was protected by $PNA^{(p1)}$ from the digestion by nuclease S1 and thus DNA1 was digested at the 5′-end down to the 9-mer. Under milder digestion conditions, the 10-mer oligonucleotide (from C11 to G20) was also obtained (see Supporting Information Figure S1). In the enzymatic digestion, the 10-mer was first formed and then converted to the 9-mer due to the enzymatic removal of its 5′-terminal nucleotide. Apparently, the DNA portion in this fully matching DNA/PNA duplex is strongly resistant to the enzymatic hydrolysis, producing the oligonucleotides of predeter-

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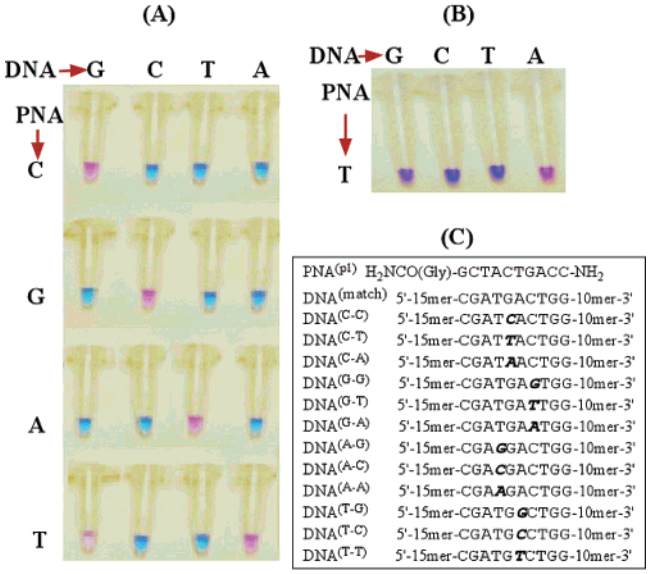


Figure 2. (A) Visual detection of mismatches by using 20 μM $DiSC_2(5)$. The DNA/$PNA^{(p1)}$ duplexes in (C) involving 13 kinds of nucleobase pairs were treated by nuclease S1 under the conditions described in Figure 1, and the dye $DiSC_2(5)$ was added. All the DNAs are named in terms of the nucleobase in PNA first and then the one in DNA. For example, the blue solution second from the right in the top row was obtained from $DNA^{(C-T)}$. The pattern B shows the results for the T−G, T−C, T−T, and T−A pairs (bottom row of **(**A)) that are stained with 50 μM $DiSC_2(5)$ to magnify the color difference (see the text for details). In (C), the mismatching sites are designated in bold italics. The 10-mer of the 3′-side is CACTTGCGTT, whereas the 15-mer of the 5′-side is CACTAGCGTTTGAGA.

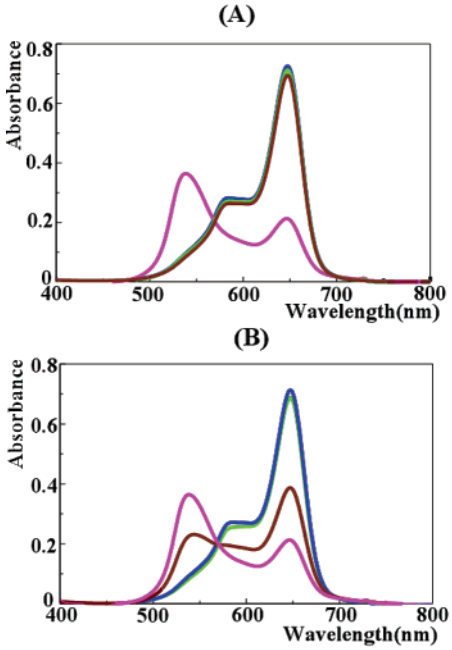


Figure 3. UV/visible absorption spectra of enzymatic digests stained with 20 μM $DiSC_2(5)$. (A): purple, C (in PNA)−G (in DNA); blue, C−C; green, C−A; brown, C−T. (B): purple, T−A; blue, T−C; green, T−T; brown, T−G. The purple curve in (A), for example, refers to the combination of $PNA^{(p1)}$ and $DNA^{(match)}$ (C−G pair) and is for the purple solution in the top left of Figure 2A.

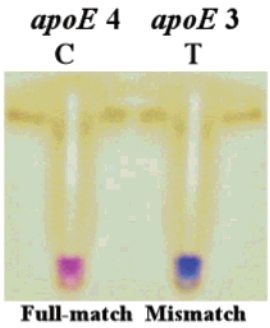
mined sequence and length. This result is consistent with the previous finding by Nielsen and Frank-Kamenetskii et al.[15]

In contrast with this partial and sequence-selective digestion of fully matching DNA1 by nuclease S1 to the oligonucleotides, the other DNA substrates (DNA2, DNA3, and DNA4), which contain a mismatch toward $PNA^{(p1)}$, were completely digested down to mononucleotides under identical conditions (lanes 7−9). Even a single-base mismatch made the DNA/PNA duplex portion susceptible to the enzymatic hydrolysis. Virtually the same results were obtained when other PNAs (e.g., $H_2NCO(Gly)-ACTCTGCTACTGAC-NH_2$) were used for the cleavage of the corresponding DNAs which have either a fully complementary sequence or one involving a one-base mismatch (data not shown). The remarkable discrimination between matching site and mismatching site by the PNA/nuclease S1 combinations was evident. Mung Bean nuclease, another single-stranded DNA specific nuclease, showed similar activity for the mismatch detection in DNA/PNA duplexes (see Supporting Information Figure S2).

Visual Detection of DNA/PNA Mismatch by Using $DiSC_2(5)$ Dye. These strict discriminations between full match and mismatch by the PNA/nuclease S1 (and PNA/Mung Bean nuclease) combinations can be easily and clearly visualized to naked eyes, by adding 3,3′-diethylthiadicarbocyanine ($DiSC_2(5)$)[16] to the enzymatic digests (see Figure 2). This dye changes its color from blue to purple when it is bound to DNA/PNA duplexes. As shown at the top left in Figure 2A, the solution of enzymatic digest of $DNA^{(match)}$ containing this dye took on a purple color. The central 10-mer portion of this DNA is complementary with $PNA^{(p1)}$ and thus was sufficiently protected from the enzymatic digestion. The DNA/PNA duplexes left in this solution bound the $DiSC_2(5)$ dye, giving rise to the purple color (the absorption maximum λ_{max} is 534 nm, as presented by the purple line in Figure 3 A). This color change occurred immediately on the addition of the dye and lasted for more than 1 month as long as the specimen was shielded from ambient light (otherwise, the color gradually faded due to photodegradation of the dye). On the other hand, $DNA^{(C-C)}$, $DNA^{(C-T)}$, and $DNA^{(C-A)}$ have a mismatch to the PNA and, thus, were easily digested down to mononucleotides by the nuclease. There were no DNA/PNA duplexes left in the reaction mixtures, and therefore, all of the solutions remained blue (the three tubes in the top row in Figure 2A). The absorption spectra of all these three mixtures almost completely superimposed each other (λ_{max} = 652 nm) and were identical with that of the dye solution without any additive (the blue, the brown, and the green lines in Figure 3A). Other mismatches (G (in PNA)−G (in DNA), G−T, G−A, A−G, A−C, A−A, T−C, and T−T) also provided blue solutions and were clearly discriminated by naked eyes from the fully matching pairs. Advantageously, contaminating DNA/DNA duplexes, if any, caused no significant problem, since this dye remains blue even in the presence of these duplexes.[16] The blue → purple change is specific to DNA/PNA duplexes and, thus, precisely reflects the perfect matching of sample DNA to the PNA probe.

Improvement of Discrimination between T−G Mismatch and T−A Full Match. In Figure 2A, only the T (in PNA)−G (in DNA) mismatch (the bottom left) took a pale-pink color. According to the reversed-phase HPLC, the enzymatic digestion is not so efficient in this mixture and some amount of DNA/PNA duplex still remained in the final digestion mixture (data not presented).[18] However, this T−G mismatch was explicitly distinguishable from the T−A full match by visible-absorption spectroscopy (compare the purple line and the brown one in Figure 3B).[19] Furthermore, simply by increase of the concentra-

(A)



(B)

Substrate DNA:

apoE 4: 5′-aggcccggctgggcgcggacatggagg ACGTG *C* GCGG
ccgcctggtgcagtaccgcggcgaggtgcaggccatgctcggc-3′

apoE 3: 5′-aggcccggctgggcgcggacatggagg ACGTG *T* GCGG
ccgcctggtgcagtaccgcggcgaggtgcaggccatgctcggc-3′

$PNA^{(p2)}$: $H_2NCO(Gly)$-TGCAC *G* CGCC-NH_2

Figure 4. (A) Detection of SNPs of human *apoE* gene by the PNA/nuclease S1/$DiSC_2(5)$ system. As shown in (B), $PNA^{(p2)}$ is complementary with the underlined part of *apoE*4 but has a G−T mismatch to *apoE*3. Conditions for enzymatic digestion: $[DNA\ substrate]_0 = 10\ \mu M$, $[PNA^{(P2)}]_0 = 20\ \mu M$; $[nuclease\ S1]_0 = 6.0\ U/\mu L$ at pH 4.6 and 20 °C for 30 min. The staining was made by 50 μM $DiSC_2(5)$.

tion of $DiSC_2(5)$ from 20 to 50 μM, the color difference could be greatly magnified to allow naked-eye detection (Figure 2C). The T−G mismatching solution was now blue (left), since the small amount of DNA/PNA duplex therein was almost completely saturated by the $DiSC_2(5)$ dye and unbound dye was now the dominant species. The color of the solution containing the fully matching duplex was less dependent on the $DiSC_2(5)$ concentration. Thus, all kinds of SNPs are easily detectable.

Detection of SNPs of the Human Gene. The method described above has been applied to the detection of SNPs in human DNA. The target factor chosen is the *apoE* gene, which has been reported to be associated with lipid disorders[6] and be related to Alzheimer's disease.[7] The 80 mer DNA between the position 120 and 199 **(**containing the codon 112 SNP (TGC → CGC) in *apoE* gene) was used (Figure 4 B). The SNPs site is the nucleotide 152, which is T in *apoE*3 but C in *apoE*4. After amplification by asymmetric PCR, the single-stranded DNA containing this SNP site was treated at 20 °C for 30 min with nuclease S1 in the presence of $PNA^{(p2)}$ ($H_2NCO(Gly)$−TGCAC G CGCC−NH_2), which is complementary to the underlined sequence in *apoE*4, and then the reaction mixture was stained with $DiSC_2(5)$.

As shown in Figure 4A, the solution from the allele of *apoE*4 (completely complementary with $PNA^{(p2)}$) was purple in color, whereas the solution from the allele of *apoE*3 (containing a G−T mismatch) was blue. The absorption spectra of these two solutions are notably different from each other. In another series of experiments, these two DNAs were treated with nuclease S1 in the presence of PNA which is complementary with *apoE*3. Exactly as expected, the solution from the allele of *apoE*3 was purple, and the solution from the allele of *apoE*4 was blue (data not shown). The type of allele of the patient has been unambiguously pinned down.

Origin of the Remarkable Mismatch Discrimination by the Combination of PNA and Nuclease S1 (or Mung Bean Nuclease). It is noteworthy that the use of PNA is essential for these experiments. When the DNA probe ($DNA^{(p1)}$) was used instead, all the substrate DNAs were digested to mononucleotides and no oligonucleotides remained irrespective of the presence or the absence of a mismatching site (lanes 2−5 in Figure 1). No color change occurred even upon the addition of $DiSC_2(5)$. All the attempts to find appropriate reaction conditions for limited enzymatic digestion with DNA probes were unsuccessful. These mismatch-discriminating experiments could be achieved most favorably at 20−25 °C. At higher temperatures, the digestion of fully matching DNA concurrently proceeded, diminishing the amount of PNA/DNA duplex left in the solution. A purple solution was also obtained, but the color was less deep. On the other hand, the digestion of mismatching DNAs was incomplete at the lower temperatures.

The overwhelming superiority of PNAs in the mismatch detection is associated with the fact that a mismatch in DNA/PNA duplexes destabilizes the duplexes to a far greater extent than does a mismatch in DNA/DNA duplexes.[20] The melting temperature T_m of the mismatching DNA4/$PNA^{(p1)}$ duplex, for example, is 35 °C, which is by 21 °C lower than the corresponding value of the fully matching DNA1/$PNA^{(p1)}$ duplex. On the other hand, the difference in T_m between the DNA4/$DNA^{(p1)}$ duplex and the DNA1/$DNA^{(p1)}$ duplex is only 10 °C (the T_m values are 17 and 27 °C, respectively). It is indicated that the structure of the DNA/PNA duplex is notably perturbed at the position of a mismatch,[21] and accordingly, nuclease S1 and Mung Bean nuclease can competitively bind the phosphodiester linkage at this site and hydrolyze it. The small oligonucleotides formed by this primary digestion are removed from the PNA because of thermodynamic instability of their duplexes and are further digested to small oligonucleotides. Consistently, only mono- and dinucleotides were detected even when the enzymatic digestion at 20 °C was stopped in the early stage of the reaction.[22] In addition to this match−mismatch discriminating activity of PNA, its significant resistance against enzymatic digestion should be also essential to avoid the deterioration of this sequence-selective protecting agent and make the present SNPs detection successful.

(18) Probably this combination forms a rather stable non-Watson-Crick base pair. It is noteworthy that the G(PNA)−T(DNA) mismatch provided a blue color, as also shown in Figure 2. Furthermore, it was reported that G−T and T−T mismatches do not much destabilize PNA/DNA duplexes ((a) Igloi, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 8562−8567. (b) Jensen, K. K.; Ørum, H.; Nielsen, P. E.; Nordén, B. *Biochemistry* **1997**, *36*, 5072−5077. (c) Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S. M.; Driver, D. A.; Berg, R. H.; Kim, S. K.; Nordén, B.; Nielsen, P. E. *Nature* **1993**, *365*, 566−568). Apparently, the stability of the transition state for this enzymatic digestion does not directly reflect the stability of the duplex in the ground state.

(19) The ratio of the absorbances at the maxima of the two bands (534 and 652 nm) is 1.8 for the T−A full match, while the value for the T−G mismatch is 0.7. The differences are far greater for other full match and mismatch combinations.

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(21) The structure of fully matching DNA/PNA duplex was determined by NMR: Eriksson, M.; Nielsen, P. E. *Nat. Struct. Biol.* **1996**, *3*, 410−413.

(22) Together with these small oligomers, 10-mer and 9-mer were also perceived as expected. When the 10-mer portion of DNA was fully matching with the PNA, however, only 10-mer and 9-mer were formed.

Conclusions

The present molecular recognition of a one-base alteration in DNA sequence by PNA takes advantage of notable structural and physicochemical differences between the fully matching site and mismatching site in DNA/PNA duplexes. Dictated by this difference, single-stranded DNA specific enzymes selectively choose these mismatching sites and hydrolyze the DNA there. This recognition is so precise and strict that the DNA is efficiently cleaved even when there exists only one mismatch between DNA and PNA. In contrast, the fully matching DNA/PNA duplex is sufficiently stable against the enzymatic digestion. To achieve this one-base differentiating protection and digestion, PNA must be used because (1) a mismatch in DNA/PNA duplex notably destabilizes the duplex, facilitating the enzymatic reaction at this site, and (2) PNA is resistant to the enzymatic digestions and functions as a DNA-protecting agent throughout the reaction time. Accordingly, SNPs typing is easily and straightforwardly accomplishable by adding $DiSC_2(5)$ dye after digesting the DNA by nuclease S1 or Mung Bean nuclease in the presence of appropriate PNA. One of the most important advantages of this method is its simplicity. Without any complicated equipment, SNPs are visually and conveniently detectable. When the solutions are purple, the DNA is completely matching with the PNA. Even with only one mismatch, however, blue solutions are obtained. The sensitivity of detection should be further improved by designing still more effective dyes and/or by modifying the PNA appropriately. The present method should be also applicable to high-through-put genotyping of multiple SNPs. In combination with array or beads technology, amplified DNA fragments in multiplex reactions can be captured on specific PNA probes and then subjected to the enzymatic digestion. Undigested DNA/PNA duplex will be detected with $DiSC_2(5)$ dye by absorption spectroscopy. The ratio of absorbance in the purple region to that in the blue region can be a reliable measure of mismatch.[19] Such PNA probes on a solid surface are resistant to nuclease digestion and can be utilized repeatedly. These attempts are currently under way in our laboratory.

Experimental Section

Materials. The PNAs were manually prepared and characterized according to the literature.[23] To increase the solubility, a glycine residue was attached to their C-termini. The oligonucleotides were prepared on an ABI 394 DNA synthesizer. For the gel electrophoresis experiments, 3′-(6-FAM) CPG columns (Glen Research Co.) were used to label the oligonucleotides with fluorescein at their 3′-termini. All the PNAs and DNAs were purified by high-quality prepacked column (Kanto Chemical Co., Inc., Tokyo) and characterized by mass analysis. Their purities were greater than 99% according to the HPLC. The concentrations of DNA and PNA were measured at 260 nm. The human DNA containing the sequence of *apoE* genome was isolated from the whole blood from individuals with known sequences by use of the Puregene DNA extraction kit (vide infra). Nuclease S1 (*Aspergillus orzyae*) and Mung Bean nuclease were purchased from Life Technologies (Carlsbad, CA) and Promega (Tokyo), respectively. The dye $DiSC_2(5)$ was from Sigma-Aldrich (Tokyo).

Enzymatic Digestion. In 1 mM acetate buffer (pH 6.5), DNA substrate (10 μM) and PNA (12 μM) were mixed and heated to 90 °C for 3 min and then slowly cooled to room temperature in 1 h. The digestion by nuclease S1 (or Mung Bean nuclease) was carried out at pH 4.6 in the buffer containing 30 mM sodium acetate, 1.0 mM zinc acetate, and 5% (v/v) glycerol. After a predetermined time (10−30 min) at 20 °C, EDTA solution was added to stop the reaction (its final concentration was 10 mM). The 20% polyacrylamide gel electrophoresis of the fragments was achieved under denaturing conditions and quantified with a Fuji Film FLA-3000G imaging analyzer.

Staining of Enzymatic Digests with $DiSC_2(5)$. After the enzymatic digestion, stock solution of $DiSC_2(5)$ in methanol (0.5 mM) was added at room temperature, and the color change was analyzed by either a spectrophotometer or our naked eyes. Before the absorption spectra were recorded, the sample solutions were incubated at 20 °C for 5 min to complete the equilibria in the solutions (it turned out later that all the equilibria were attained rapidly so that this incubation procedure was not necessarily required). The photographs were taken by use of a digital camera (Caplio RDC-i500 from Ricoh).

Spectroscopy. After the PAGE electrophoresis, the DNA fragments were isolated from the gel according to the conventional method. The mass spectra were obtained on a Kratos Kompact MALDI 2 TOF-MS spectrometer. Prior to the analysis, the mixtures were desalted by using an ODS column and then mixed with MALDI matrix solution containing 3-hydroxy-2-picolinic acid and diammonium hydrogen citrate. Visible-absorption spectroscopy was done on a Jasco V-530 UV/vis spectrophotometer. The T_m values were determined under the reaction conditions for enzymatic digestion by monitoring the absorbance at 260 nm with the temperature ramp 1.0 °C/min.

PCR Amplification of ApoE DNA. The DNA used in Figure 4 was prepared by the following procedure to avoid the induction of any mutation during PCR and obtain unambiguous results. The *apoE* DNA fragments were amplified by PCR from human genome DNA of the E3/E4 genotype and then inserted into a pGEM(R)*-T Easy vector (Promega, Tokyo). After the structures of constructs were confirmed by restriction mapping and the genotype by DNA sequencing on an ABI PRISM 310NT genetic analyzer, these plasmids were used as templates for PCR to produce the 80-bp double-stranded DNA between the positions 120 and 199. The double-stranded DNA was then used as a template to produce single-stranded DNA by asymmetric PCR involving only the forward primer.[24] The products were purified by a PCR purification kit (Qiagen, Hilden, Germany). For practical application, however, this rather complicated procedure was not necessary. The same genotyping results were obtained when human genome DNA was directly amplified from the human genome DNA simply by asymmetric PCR.

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Supporting Information Available: Electrophoresis patterns for short treatment with nuclease S1 and for the treatment with Mung Bean nuclease. This material is available free of charge via the Internet at http://pubs.acs.org.

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