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Highly Sensitive PNA Array Platform Technology for Single Nucleotide Mismatch Discrimination

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Reliable discrimination of a single nucleotide mismatch was demonstrated using arrays with peptide nucleic acid (PNA) probes. The newly developed PNA probes immobilization method and hybridization conditions for PNA arrays gave excellent specificity and sensitivity. In addition we compared the specificity, sensitivity, and stability obtained with the PNA and DNA arrays in discriminating single nucleotide mismatches. The PNA arrays had superior perfect match-to-mismatch signal ratios and sensitivities. The relative signal intensities of mismatch PNA probes ranged from 1.6% to 12.1% of the perfect-match PNA probes. These results demonstrated that the PNA arrays were 2.0 to 37.3 times more specific and about 10 times more sensitive than DNA arrays. The PNA array showed the same specificity and sensitivity after 12-month storage at room temperature.

Keywords: Peptide nucleic acid (PNA), PNA array, single nucleotide mismatch

Discrimination of single nucleotide mismatches in mutations and SNPs are important to molecular biology and clinical diagnostic methods. Since the majority of sequence variations in antibiotic-resistant pathogens and genetic diseases are associated with single nucleotide variations, reliable diagnostic methods capable of discriminating mutations and single nucleotide polymorphisms (SNPs) are highly desired [28].

DNA microarrays are becoming increasingly useful for clinical detection of sequence variants because they allow simultaneous detection of nucleic acid sequences [11, 19, 27]. Although the technology has many advantages and several working systems have been established, reliability and reproducibility are still issues and single nucleotide mismatch discrimination is difficult [8, 20]. DNA microarray technology needs to be improved in order to be widely adopted for the reliable and accurate analysis of single nucleotide variations [16, 18, 24, 26, 29].

Unnatural nucleic acid analogs have been made in order to overcome the limitations of natural nucleic acids of specificity, sensitivity, hybridization kinetics, thermodynamic properties, and stability [2, 3, 14, 29]. Among them, peptide nucleic acids (PNAs) are notable owing to their exceptional biological/chemical stability because they are not degraded by nuclease or protease.

In PNA, the negatively charged ribose-phosphate backbone of nucleic acids is replaced by an uncharged *N*-(2-aminoethyl)-glycine scaffold, to which the nucleobases are attached *via* a methylene carbonyl linker. Because all intramolecular distances and the configuration of the nucleobases are similar to those in natural DNA molecules, specific hybridization occurs between PNA and DNA or RNA sequences. The uncharged PNA is responsible for better thermal stability in the PNA–DNA duplexes as compared with the DNA–DNA duplexes. Its particular biochemical properties make it suitable for use in many biological applications. An increasing number of applications for PNA technology have been described, confirming the high potential of PNAs as a probe for antisense, PCR clamping, FISH, and microarrays [3, 7, 14, 18].

It was anticipated that use of PNA probes overcomes many limitations of DNA microarray [3], but this has not been demonstrated yet. There is no systematic PNA fabrication and performance technology for PNA probe design, linkers, spacers, PNA probe immobilization methods, hybridization, and washing conditions.

The purpose of this study was to establish PNA array fabrication technology as well as to compare the performances of the PNA array and DNA array in discriminating single

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nucleotide mismatches, especially with regard to specificity, sensitivity, and stability.

Materials and Methods

PNA Probes Synthesis

PNA probes were synthesized by Panagene's technology using benzothiazole-2-sulfonyl (Bts) as an amine protecting group [15]. A high-performance liquid chromatograph (Agilent 1100, Agilent Technologies, Wilmington, U.S.A.) purified the PNA probes. The PNA probe quality was assessed by MALDI–TOF (AXIMA-CFR, SHIMADZ Co., Kyoto, Japan). Concentrations of PNA probes were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, U.S.A.).

To select the optimum PNA probes, several candidate probes for each target were designed. PNA probes have a terminal amine group, which is required for the covalent attachment to the epoxy surface, and have spacers that enable the target to approach immobilized probes. A total of 12 PNA probes were designed based on one of the authors' previous works [12].

Target DNA Preparation

Nine clone DNAs containing one wild type and 8 mutant types associated with lamivudin-resistant HBV were used as the targets for the array test. These clone DNAs were made by site-directed mutagenesis. DNAs were aliquoted and stored at -20°C until they were used. PCR primers are typically designed to amplify the 206 base pairs of the target DNA containing the mutation sites of clones, and one primer of each pair is biotinylated at the 5' terminus to label the target strand of the PCR fragments [12]. The biotin-labeled primers HBF3 (forward, 5-CTTGTATTCCCATCATCCCATCATC-3') and HBR2 (reverse, 5-CAAAAGAAAATTGGTAACAGCGGTA-3) were used for PCR amplification. Briefly, the 25-μl reaction mixture contained 1 U of *Taq* DNA polymerase (Solgent, Daejeon, South Korea) and PCR buffer with 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate mixture, and 10 pmol of each primer. Four μl of clone DNA sample was added to the PCR mixture. The PCR conditions included 4 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C; and 10 min at 72°C. PCR was performed with a PCR Thermal Cycler Dice (Takara Bio Inc., Shiga, Japan).

Fabrication of the PNA Array

The sodalime glass slides were precleaned in a mixture of H_2SO_4 and H_2O_2 [4:1 (v/v)] for 30 min, rinsed with deionized water (DW) and ethanol, and dried. Then, silanization of the slide glass was performed according to a typical protocol [5, 21]. The PNA probes were dissolved in DW. Each well in the microplate contained a mixture of which concentrations range from 20 to 80 μM of probes in the proprietary spotting buffer. The spotting mixture was printed onto epoxy slides by using a Qarray Mini Microarrayer equipped with aQu solid pins (Genetix, New Milton, U.K.). The printed slides were incubated for at least 2 h at RT in a humidified microarryer (Humidity was set up to about 75–85%). The printed slides were immersed in succinic anhydride (SA) and DMF, each slide being sonicated for 2 h at 40°C for blocking. The slides were washed twice with DMF, ethanol, and DW, respectively. After being washed with DW, the slides were immersed in boiling water and incubated for 5 min. After drying with compressed air, they were ready for hybridization.

Hybridization and Scanning

The biotin-labeled PCR fragments were denatured for 5 min at 95°C and then chilled on ice for 2 min. Five μl of biotin-labeled target DNA was mixed with 95 μl of the PNA hybridization buffer containing Cy5-streptavidin (Amersham Pharmaia Biotech U.K. Limited, Buckinghamshire, England), and then applied to the PNA array. The PNA array was incubated for 1 h at 45°C. After hybridization, the slides were washed twice for 5 min with proprietary wash buffer. They were ready for scanning after being dried. The array images were taken by a nonconfocal fluorescent scanner (GenePix 4000B; Axon Instruments, Union City, U.S.A.) with a typical laser power of 33% and a PMT gain of 1035. Fluorescence signal intensities represent the hybridization signal of probe–target duplexes. The P/M ratio is defined as the ratio of the signal intensity of perfect-matched duplex (PM) to the respective intensities of the mismatched duplex (MM). Relative intensity (%) is defined as the % ratio of the signal intensity of mismatched duplex to the signal intensity of the perfect-matched duplex (PM).

Fabrication and Hybridization of DNA Array

DNA array was fabricated according to one of the authors' previous works [12]. The 5' end of 12 DNA probes was aminated to enable covalent attachment on the glass slide. Each well in a microplate contained a mixture of 50 μM of a target-specific probe in 3× SSC (450 mM NaCl plus 45 mM sodium citrate, pH 7.0). The arrays were spotted on silylated glass slides (Cell Associated, Texas, U.S.A.). The spotted slides were dried for 3 h at 50°C and treated with a freshly prepared 0.25% $NaBH_4$ solution for 5 min. The slides were washed once with 0.2% SDS for 5 min and five times with distilled water for 1 min to remove the unbound probes. The DNA array was fabricated and hybridized according to a typical protocol [12, 17]. The signal intensity of DNA arrays was measured after 2 h hybridization.

Results

Fabrication of PNA Arrays

Design of PNA probes. Several PNA probes for the wild type and mutant types were designed based on point mutation located in the center of the probe. Their lengths and Tm values were taken into consideration. As with DNA probes, the PNA probes were also designed to maximize their hybridization efficiency and to minimize any non-target hybridization.

Each type of the designed PNA probes was immobilized on a glass slide and hybridized with PCR fragments of DNA extracted from the HBV clones of the wild type and mutant types. To select suitable PNA probes, the high hybridization signal intensity and P/M ratio of single nucleotide mismatches were taken into consideration. Twelve 13- to 17-mer-sized PNA probes of four wild-type-specific probes (PL1, PM1, PI1, and PV1) and eight mutant-

Table 1. PNA probe sequences used in detection for wild type and mutant types of HBV.

Probe no.	Probe name	Peptide nucleic acid sequence (N→C)
1	PL1	NH_2-Spacer-AGCCA**G**GATAAAC
2	PL2	NH_2-Spacer-AGCCA**A**GATAAAC
3	PL3	NH_2-Spacer-TGAGCCA**T**GATAAAC
4	PM1	NH_2-Spacer-TCATCCA**T**ATAACTG
5	PM2	NH_2-Spacer-CATCCA**C**ATAACT
6	PI1	NH_2-Spacer-ATCATC**C**ATATAAC
7	PI2	NH_2-Spacer-CATCATC**T**ATATAACTG
8	PI3	NH_2-Spacer-CATCATC**G**ATATAACTG
9	PI4	NH_2-Spacer-CATCATC**A**ATATAAC
10	PV1	NH_2-Spacer-AATAC**C**A**C**ATCAT
11	PV2	NH_2-Spacer-CAATAC**A**A**T**ATCATC
12	PV3	NH_2-Spacer-CAATAC**T**A**T**ATCATC

In each sequence, the underlined letter is the position of the potential mismatch.

specific probes (PL2, PL3, PM2, PI2, PI3, PI4, PV2, and PV3) were selected to detect wild type and mutant types (Table 1). The optimized PNA probes for PNA arrays differed in length and sequence from optimized DNA probes for DNA arrays, which was developed by one of the authors [12]. Optimized PNA probes were designed at the complementary sequence to the DNA probes.

Optimal concentration of PNA probes. The concentration of probe is expected to be an important parameter for obtaining optimal hybridization. In order to determine the optimal PNA probe concentration for hybridization, different concentrations (20, 40, 60, and 80 μM) of PNA probes were printed on epoxy slides and hybridized with PCR fragments. Fig. 1 shows the results obtained for the hybridization of the target with different amounts of PNA probes. The highest signal intensity and P/M ratio of PNA probe–target hybridization occurred at concentrations greater than 40 μM (pmol/μl). Similar results were obtained for the other two target genes of *E. coli* and HPV (data not shown). The signal intensity changed a little at concentrations ranging 40–80 μM. The signal intensity and P/M ratio decreased slightly at 80 μM compared with 60 μM, probably owing to steric reasons for higher concentrations of the immobilized probes. These observations indicate that the optimal concentration of the PNA probe at printing is between 40 and 60 μM.

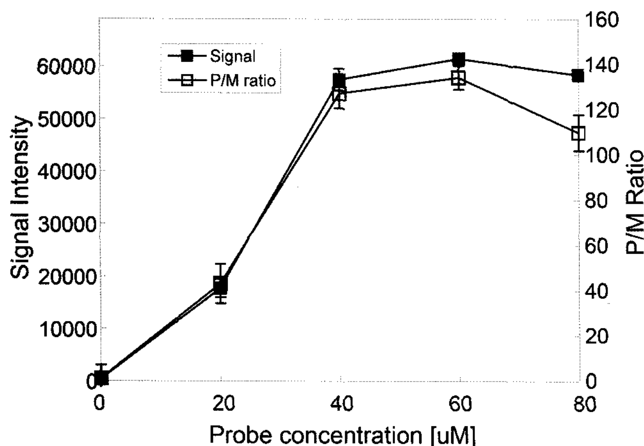

Fig. 1. Determining optimal concentrations for PNA probes.
The concentrations of the PNA probes in the spotting buffer ranged from 20 to 80 μM. The signal intensity was measured after hybridization of the PNA probes and target PCR fragments. The error bars represent one standard deviation from the mean based on three experiments.

Optimum hybridization buffer conditions. To optimize the hybridization conditions for PNA arrays, the effects of phosphate (0–200 mM) and salt (0–1,000 mM) concentrations on signal intensity and P/M ratio were examined. Fig. 2A shows a slight decrease in signal intensity and P/M ratio when a phosphate buffer greater than 100 mM is used. For the PNA–DNA hybridization, there seemed to be an optimal 50–100 mM phosphate concentration that resulted in the best compromise of signal intensity and P/M ratio of the PNA array.

The signal intensity remained nearly constant for salt concentrations from 50 to 200 mM, and decreased at

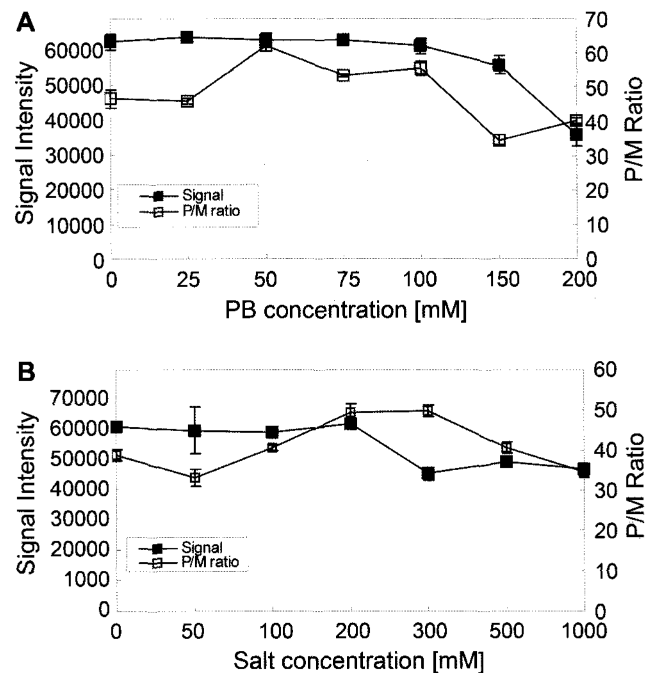

Fig. 2. Effects of phosphate and salt concentrations on signal intensity and P/M ratio of hybridization of the PNA probes and DNA targets.
This was followed by hybridization for 2 h with 50 μM PNA probes and PCR fragments. The effects of (A) phosphate concentration (0–200 mM). and (B) salt concentration (0–1,000 mM) on signal intensities and P/M ratios of the PNA probes and DNA targets. The P/M ratio is defined as the ratio of the signal intensity of perfect-matched duplex (PM) to the respective intensities of the mismatched duplex (MM).

concentrations greater than 200 mM. As the salt concentration increased from 50 to 300 mM, the P/M ratio generally increased (Fig. 2B). The optimal phosphate and salt concentrations seems to be 50 mM and 200 mM, respectively. These hybridization buffer conditions were used to discriminate single nucleotide mismatches on PNA arrays.

Fifty mM phosphate and 200 mM salt concentrations were chosen as the standard hybridization buffer for the PNA arrays in this study. Other phosphate and salt concentrations may be used because PNA provides a wider range of hybridization conditions.

Single Nucleotide Mismatch Discrimination

A comparison was made of the ability of the PNA and DNA arrays to discriminate single nucleotide mismatches. This ability was defined as the relative signal intensity (%) for a perfect-match duplex (PM) to the average of the respective intensities obtained with mismatch duplex (MM). It should be noted that, in all experiments, the hybridization signal intensity and single nucleotide mismatch discrimination (P/M ratio or relative signal intensity) was compared based on their respective optimized conditions for the PNA and DNA arrays. The single nucleotide mismatch discrimination of the array was tested against the PCR fragments of the 12 types. Perfect-match PNA probes and one to three mismatch probes were used (Table 1).

The representative fluorescence images of the probes after hybridization with their respective targets are shown in Fig. 3A. The uniqueness of the hybridization pattern of the PNA probes was more significant than that of the DNA probes. The PNA probes exhibited high P/M ratio between the perfect-match and mismatch duplexes. As shown in Fig. 3B, the relative signal intensity of mismatch PNA probes ranged from 1.6% to 12.1% of the perfect-match PNA probes, whereas that of DNA probes ranged from 7.0% to 82.9% of the perfect-match DNA probes. These results demonstrated that the PNA arrays were 2.0 to 37.3 times more specific than DNA arrays. It clearly shows that PNA arrays can discriminate single nucleotide mismatches unambiguously.

Sensitivity

The sensitivities of the PNA and DNA arrays were evaluated by 10-fold serially diluted PCR fragments of HBV clones. The results are given for signal intensities and P/M ratios with 1, 10-, 100-, 1,000-, 10,000-fold serially diluted

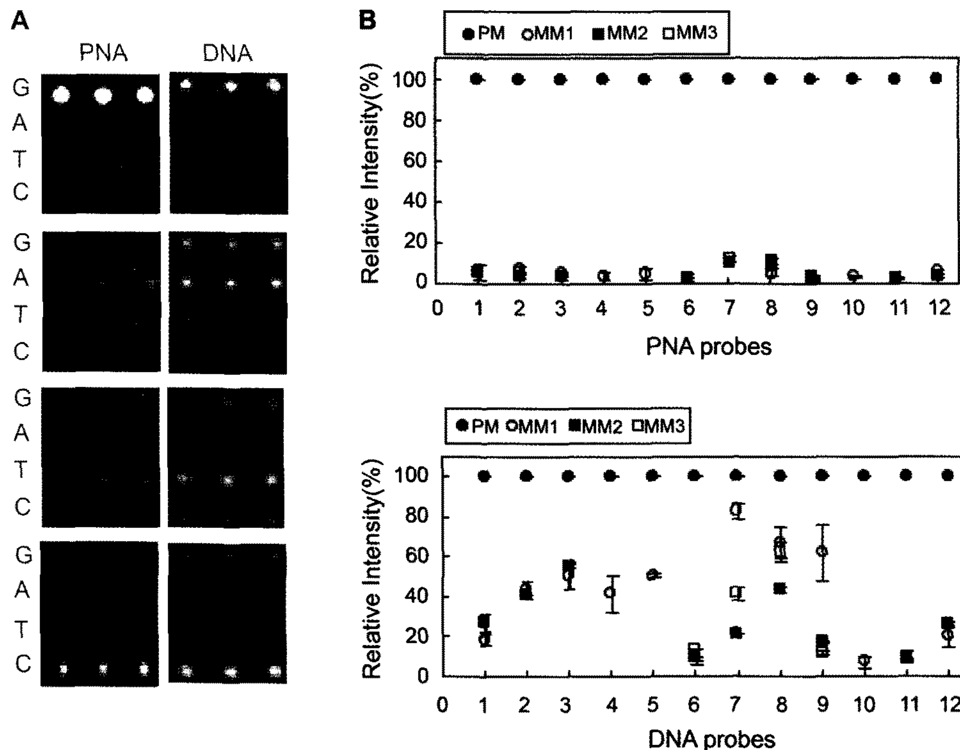

Fig. 3. Discrimination of single nucleotide mismatches.

A. The representative hybridization images of PNA and DNA probes after hybridization with their respective targets. **B.** The ability of single nucleotide mismatches discrimination is defined as the relative signal intensity (%) of perfect match (PM) and mismatch (MM). The relative signal intensity is the average of 20 independents. Relative intensity (%) is defined as the % ratio of the signal intensity of mismatched duplex to the signal intensity of the perfect-match duplex (PM).

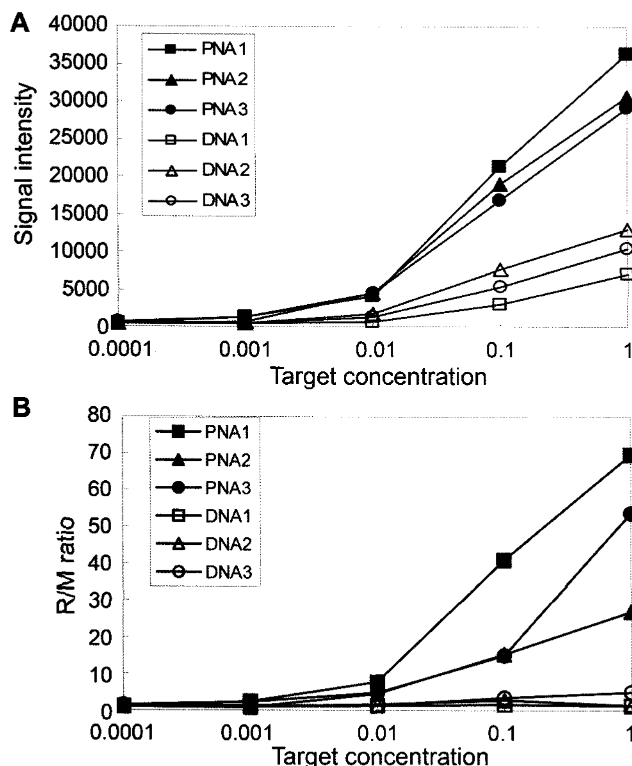

Fig. 4. The sensitivity of an array obtained from hybridization of target DNA with 10-fold serially diluted PCR fragments.
The signal intensity (**A**) and P/M ratio (**B**) of three representative PNA and DNA probes after hybridization with their respective targets. The concentration of 50 μM probes are printed on the slide. Each point represents the average signal intensity from three independent hybridization experiments.

PCR fragments. Ten separated PCRs were performed, and the products were hybridized to the PNA arrays.

At relatively high target concentrations of 1- and 10-fold dilution, the signal intensity of the PNA probes was 3 to 4 times stronger than the signal of DNA probes (Fig. 4A). Even at the low target concentration of 1,000-fold dilution, the signals of the PNA probes were higher than the signals of the three DNA probes. These data show similar results for each representative of the three PNA and DNA probes. These results demonstrated that the PNA arrays could detect target DNA with great sensitivity for a broad range of target DNA concentrations. Fig. 4B shows 18–70 P/M ratios of PNA array at PCR fragments of 1- to 100-fold dilutions. The detection limit of the PNA arrays was 10 times lower than that of the DNA arrays.

Stability

For practical applications, arrays should maintain P/M ratio (specificity) and signal intensity (sensitivity) to provide reliable results while stored. PNAs are completely resistant to nucleases and other degradation factors in the biological system. Hence, it was expected that they would be effective after long-term storage of the PNA array. To evaluate the shelf-life of the arrays, stability tests were performed on the PNA and DNA arrays after being stored. The arrays were stored in a plastic slide box wrapped in aluminum foil bags at room temperature. As shown in Fig. 5, the PNA array showed the same level of intensity of fresh fabricate (0 month) PNA array after 3-month storage, whereas that of the DNA arrays decreased rapidly after 3-month storage. The PNA arrays still showed identical signal intensities and P/M ratios after 1-year storage at room temperature.

Discussion

Microarray fabrication and performance can be considered as the composite of probe design, spacer, solid substrate (functionalized glass slide), probe immobilization, labeling, hybridization, and washing [1, 9, 25]. Fabricating the PNA arrays requires a unique protocol because the PNA probe has unique biochemical and physicochemical properties. Covalent coupling is most commonly achieved using amine-modified probes and either aldehyde- or epoxide-functionalized glass. Terminal covalent attachment allows the entire short oligonucleotide probe to be available for hybridization and to withstand the high temperatures and salt concentrations often required under the stringent washing conditions in subsequent steps of microarray processing [6, 30]. Amine-modified probes were also used in the PNA arrays. Spacers are reported to increase the efficiency of hybridization in the detection using PCR-amplified target DNAs because spacers keep the probes away from the solid surface and thereby minimize the influence of the surface [16]. We also found that optimized spacers are important for PNA probes to achieve specificity and sensitivity (data not shown).

Owing to their uncharged nature, PNA probes permit the hybridization of target DNA under low or no salt conditions, because no interstrand repulsions need to be counteracted, which occur between two negatively charged DNA strands. As a consequence, the target DNA has less secondary structure and is more accessible to the PNA probes, and thus resulting in fast hybridization. PNA is particularly advantageous for use as a microarray in discriminating single nucleotide mismatches because it enables highly specific binding at low ionic strength [4, 23].

The uncharged backbone PNA probes are independent on ionic strength. As expected, PNA–DNA duplex stabilities were found to be reduced as dependent on salt concentrations. As shown in Fig. 2, as the ionic strength changed, the signal intensity and P/M ratio of the PNA–DNA duplex were not effected significantly. These results suggest that concentrations of 50 mM phosphate and 200 mM salt are an adequate compromise between specificity (P/M ratio) and signal intensity (sensitivity) for PNA arrays to discriminate single nucleotide mismatches.

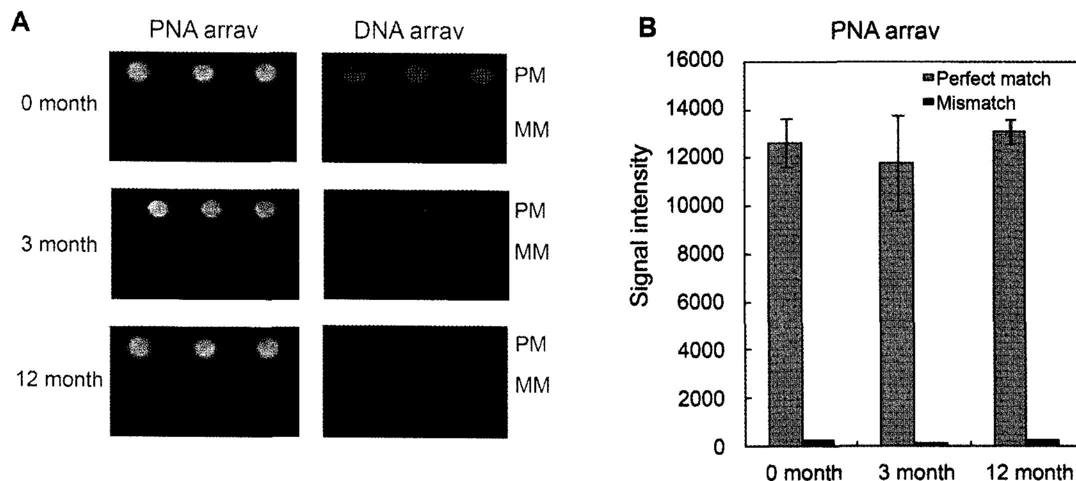

Fig. 5. The stability of the PNA and DNA arrays when stored at room temperature.
A. The hybridization images of the PNA and DNA arrays after 0-month (fresh fabrication), 3-month, and 12-month storage. Among 12 independent hybridization experiments, we represented the representative single experiment result. **B.** The change of signal intensity of PNA array compared between 0-month (fresh fabrication) and 12-month storage. Results were obtained through hybridization with PCR fragments of the HBV clone. PM and MM mean perfect-match and mismatch probes.

In general, more emphasis has been placed on specificity than on sensitivity in single nucleotide mismatch analysis [10]. This mismatch discrimination can be estimated from differences in melting temperature (Tm) between perfect-match and mismatch duplexes [22, 29]. The greater the difference between the melting temperature of the perfect-match versus mismatch duplexes (ΔTm), the better the probe is. Since the difference of stability between a perfect-match and a mismatch duplex can be as small as 0.5°C, the discrimination between two DNA sequences that differ by only a single nucleotide is difficult. This ΔTm is dependent upon the length of the probe, the type of mismatch, and the neighboring nucleotides. The differential stability of a perfect-match versus mismatch duplex is the main limitation to the use of oligonucleotide hybridization for detecting SNPs [22]. Because PNAs increase melting temperatures by approximately 1.0°C per base pair for a PNA–DNA duplex and ΔTm of PNA–DNA duplexes are 2.5–8.5°C higher than those of the corresponding DNA–DNA duplexes, PNA probes are expected to be more accurate. The increase in ΔTm is enough to allow single base mismatches to be accurately discriminated. This accurate discrimination at a single nucleotide level indicates that a PNA probe could be highly specific and has thus allowed the further possibility of a PNA-based array for molecular investigations and diagnosis [13, 18].

The unnatural backbone of PNAs also means that PNAs are not degraded by nuclease and proteases. Because of this resistance to enzyme degradation, the lifetime of PNAs is extended both *in vivo* and *in vitro* [18]. The PNA arrays were found to have a longer shelf-life than 12 months when stored in a plastic slide box wrapped in aluminum foil bag at room temperature (Fig. 5). Evaluation of a much longer shelf-life is currently in progress. Because the shelf-life of an array depends on storage conditions such as humidity, temperature, and wrapping methods, the shelf-lives of the arrays were experimentally investigated using the same storage conditions. PNA arrays had a greatly extended shelf-life relative to DNA arrays. The long shelf-life of an array is of great importance in diagnostic applications.

Our studies have established a PNA array platform technology to allow single nucleotide mismatches to be reliably discriminated. The accuracy and reliability of the PNA array in discrimination of single nucleotide mismatches has been demonstrated, especially with regard to sensitivity, specificity, and stability. We believe these result, especially the long shelf-life of PNA arrays at room temperature, can enable many practical application of microarray-based assays.

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