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Analysis of solid-phase allele-specific primer extension characteristics on biochip in combination with modified primers and PicoGreen staining method

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DNA microarray technology offers potential for future high-throughput variation genotyping. Allele-specific primer extension procedure on microarray has been considered as an efficient method for single nucleotide polymorphism (SNP) genotyping. However, the high cost of the fluorescent-labeled dNTP used for signal detection in this method limits its application. In the present study, we evaluated the characteristics of solid-phase allele-specific primer extension, in terms of specificity and efficiency and demonstrated that compared to liquid-phase reaction, it requires lower annealing temperature, and higher template and Mg^{2+}concentrations. The extension efficiency and specificity were though linked, behave diametric during the gradient change of template and Mg^{2+}concentrations or annealing temperature. To obtain both optimal signal intensity and specificity, we introduced an artificial mismatched base at the third position from the primer 3′end, which enhanced the specificity significantly. The PicoGreen staining method, which could decrease the cost greatly, was then introduced to replace the fluorescent-labeled dNTP for signal detection.

Keywords: Single nucleotide polymorphism, allele-specific primer extension, DNA microarray, extension efficiency and specificity, genotyping, PicoGreen staining

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Single nucleotide polymorphisms (SNPs) are the most common source of human genetic variation, which occur at a frequency of at least 1% in the population[1-3]. High abundance, low mutation rate and easy automation of typing make them ideal genetic markers. Identification and dense mapping of SNPs have considerable significance in the studies of complex diseases[4-7], pharmacogenetics[8,9], population genetics[10,11] and physical mapping[12]. Therefore, there is an urgent need for specific and cost-efficient methods to detect SNPs on large-scale. Among various techniques of SNP typing, microarray is one of the most powerful approaches for high-parallel, large-scale SNPs analysis[13,14].

Although many approaches have been applied in DNA microarray for SNP genotyping, the most commonly used procedure is the differential hybridization with allele-specific oligonucleotide. However, its specificity depends on the nucleotide sequence context of the SNPs and the hybridization conditions[15,16]. The power of genotyping SNPs using high-density allele-specific oligonucleotide arrays was shown to be limited; in a mapping study, where 400-500 SNPs were analyzed, correct genotype could be assigned to only 60-70%[17]. For enhancing the discriminating power of hybridization, the enzymatic reactions on microarray were explored, which included minisequencing[18,19], allele-specific ligation[20,21] and allele-specific primer extension[22-25].

As allele-specific extension required only a single fluorophore and two immobilized primers, thus was considered cost-efficient and easy-designed strategy for future high-throughput SNP genotyping. It is based on the extension of primers that differs at their 3'nucleotide defining the allelic variant of the SNPs. Unlike liquid phase reactions, the primers immobilized on slides could not be hybridized efficiently with DNA template in solution, thus the extension efficiency was lower and unstable. Besides, some mismatches such as G:T, C:A or C:T could be well extended, in some cases[26-28]. Thus, to obtain superior results, it is important to optimize the efficiency and specificity, which rely on detailed characteristics of this detection procedure. Although some characteristics of the solid-phase amplification

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Abbreviations: ASO, allele-specific oligonucleotide; SNP, single nucleotide polymorphism; SSB, single-stranded DNA-binding protein; SDS, sodium dodecyl sulphate; SSC, sodium chloride-sodium citrate

process[29,30] have been reported, studies on the extension specificity and the discriminatory efficiency are lacking. Moreover, approaches are lacking, except apyrase[31], to avoid the mismatch primer extension for the solid-phase allele-specific extension on chip.

In this paper, effects of the template and Mg^{2+} concentrations as well as annealing temperature on the efficiency and specificity of allele-specific primer extension on biochip were analyzed. We also introduced an artificial mismatched base at the third position from the primer 3′end on the microarray detection procedure to improve extension specificity, and also the PicoGreen staining method to replace the fluorescent-labeled dNTP.

Materials and Methods

DNA samples

Genomic DNA samples from voluntary donors isolated from whole blood using a genomic DNA extraction kit (SBS, China) were used as templates for PCR amplification of target. The PCR products were purified by Watson's PCR purification kit (Watson, China). Informed consents were obtained from the participants.

Oligonucleotides

The target site was selected on the X chromosome and the primers for amplifying the target area and four traditional allele-specific primers were designed with the primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi)[32]. Each primer was designed with a 3′ G, A, T or C terminal base and was synthesized with a 5′terminal $(T)_{10}$-NH_2 modification. Furthermore, four modified allele-specific primers were designed with the SNAPER program (http://patho.mgh.harvard.edu/ausubelweb)[33]. In each primer, an additional base change was introduced at the third position from the primer 3′end. The oligonucleotides used in the study are listed in Table 1.

Preparation of microarray

The standard microscope glass slides, precleaned with 1 *M* NaOH overnight and washed several times with distilled water, were neutralized with 1% HCl, washed extensively with distilled water, and immersed in 2% 3-aminopropyl-trimethoxysilane (Sigma, USA) for 15 min, followed by five washings in acetone. Subsequently, the washed slides were baked at 110°C for 45 min and activated with 1,4-phenylene diisothiocyanate (Sigma, USA) as described[34]. Prior to spotting, the oligonucleotides were dissolved in a 400 m*M* Na_2CO_3 buffer (*p*H 9.0) to a final concentration of 20 μ*M*. The resultant solutions (0.3 μl) were applied directly to the activated slides in the designed grid pattern and immediately after spotting, the slides were deactivated in 10% NH_4OH solution for 30 min. Finally, they were rinsed five times in distilled water and centrifuged at 1000 rpm.

Characteristics analysis of allele specific primer extension on microarray

Effect of annealing temperature

For evaluating the influence of annealing temperature, the reaction was performed in 25 μl of

Table 1—Allele-specific primers and PCR primers

No.	Sequences (5'-3')[a]	Experimental role
1	NH_2-TTTTTTTTTTTTTTTTTTCCCAGGTGATACTTGAAAG	Traditional allele-specific primers
2	NH_2-TTTTTTTTTTTTTTTTTTCCCAGGTGATACTTGAAAC	
3	NH_2-TTTTTTTTTTTTTTTTTTCCCAGGTGATACTTGAAAT	
4	NH_2-TTTTTTTTTTTTTTTTTTCCCAGGTGATACTTGAAAA	
5	NH_2-TTTTTTTTTTTTTTTTTTCCCAGGTGATACTTGAÇAG	Modified allele-specific primers
6	NH_2-TTTTTTTTTTTTTTTTTTCCCAGGTGATACTTGAÇAC	
7	NH_2-TTTTTTTTTTTTTTTTTTCCCAGGTGATACTTGAÇAT	
8	NH_2-TTTTTTTTTTTTTTTTTTCCCAGGTGATACTTGAÇAA	
9	CCCCTCTACAAGGAAATAGAC	PCR primers
10	AGAAAAGGGTCACAAGTTGCC	

[a]Underlined letters represent discriminating nucleotides and double underlined wild type reference sequences. Underdotted letters were modified nucleotides to improve the extension specificity

an extension mixture containing 10 m*M* Tris-HCl (*p*H 8.3), 50 m*M* KCl, 3 m*M* $MgCl_2$, 80 μ*M* of each dGTP, dCTP, and dTTP, 40 μ*M* [α-^{32}p]-dATP (Yahui, China), 3 U of a thermostable *Taq* DNA polymerase (Takara, Japan) and 1 n*M* DNA target. The area containing the oligonucleotide array was sealed with FrameSeal slide chambers and the PCR mix was placed into it. The glass slides were then put into Amplitron II *in situ* slide thermocycler (Thermolyne, USA) and cycling was carried out according to the following scheme: 6 min at 95°C, followed by 40 cycles of denaturation (30 sec at 95°C), annealing (1 min separately at 40, 45, 50, 55 and 60°C) and extension (1 min at 72°C). For each annealing temperature, a reaction without DNA template was also designed to ensure that there is no self-priming between the oligonucleotide primers.

Effect of template and Mg^{2+} concentration

The template concentration greatly influences base pairing efficiency of attached primer and the template. To determine the effect of template, a set of gradient template concentrations (0.001, 0.01, 0.1, 1 and 10 n*M*) was designed. The templates were diluted from asymmetric PCR amplified human genomic DNA as described[20].

As the absorption of Mg^{2+} may vary with different template concentrations and the surface of the chip may also absorb some Mg^{2+} as described for *in situ* PCR[35], the effect of Mg^{2+} concentration was analyzed at the same time. For each template concentration, a set of gradient Mg^{2+} concentrations (0.75, 1.5, 3.0, 4.5 and 6.0 m*M*) was designed. All other conditions were same as the above, except that the annealing temperature was set at the optimum, 50°C. In addition, reaction without DNA template was also designed for each Mg^{2+} concentration, to ensure no self-priming between the oligonucleotide primers. In fluorescence detection method, 40 μ*M* Cy3-dUTP (Pharmacia, USA) was used to replace the dTTP, whereas in PicoGreen staining, the common dNTP was used.

Modified allele specific primer extension on chip

It was difficult to optimize conditions for both efficiency and specificity for traditional allele-specific primer extension on chip, in some cases[33,36]. To overcome this problem, the modified allele-specific primer extension was performed. We used both [α-^{32}p]-dATP and Cy3-dUTP to be incorporated during the extension process. Then, the PicoGreen staining method was also combined. All the conditions were same as the above. Following the extension reaction during which [α-^{32}p]-dATP and Cy3-dUTP were incorporated, slides were washed with MilliQ water 3 times for 5 min each at room temperature. After centrifugation at 1000 rpm for 3 min, the slides were ready for signal detection. However, for PicoGreen dye staining, the slides were washed for 6 min in pre-warmed (65°C) 2 × SSC, 0.1% SDS, and then for 3 min in 1 × SSC by shaking gently. The slides were ready for PicoGreen dye staining, after centrifugation at 1000 rpm for 3 min.

PicoGreen dye staining of slides

PicoGreen is a dsDNA-specific intercalating fluorescent dye used to detect different allele after allele-specific primer extension on chip[37]. PicoGreen dye staining method was introduced as a cost-efficient procedure to replace the Cy3-dUTP-incorporated method. After the thermocycling reaction, the slides were stained with the dye solution [5% PicoGreen (Molecular Probes, USA) in 2 × SSC] for 10 min protected from light, washed in 2 × SSC gently for 1 min after taking out of the staining solution, and then centrifuged at 1000 rpm for 3 min.

Scanning and data analysis

Differentially treated slides were detected by different methods. Incorporation of [α-^{32}p]-dATP was detected by fluorescent image analyzer FLA-3000 series (FujiFilm, Japan) and images were analyzed by ImageJ (NIH, USA). Incorporation of Cy3-dUTP and the PicoGreen signal were detected by GMS418 scanner (Affymetrix, USA) and images were analyzed using the Genepix 2.0 software (Axon, USA). The signal intensity corresponding to each allele-primer was an average of three repeated experiments after subtraction of local background. The difference between signal intensity of the matched primer and maximal signal intensity of the mismatched primers was calculated. Specificity of the procedure was evaluated by the ratio of this difference value to the signal intensity of the matched primer and was defined as diversity percent.

Results

Characteristics analysis of allele-specific primer extension on microarray

Effect of annealing temperature

The effect of annealing temperature was evaluated first as it influences base pairing efficiency greatly and unsuitable temperature could cause failure of primer extension[30]. As shown in Fig. 1 A and B, the

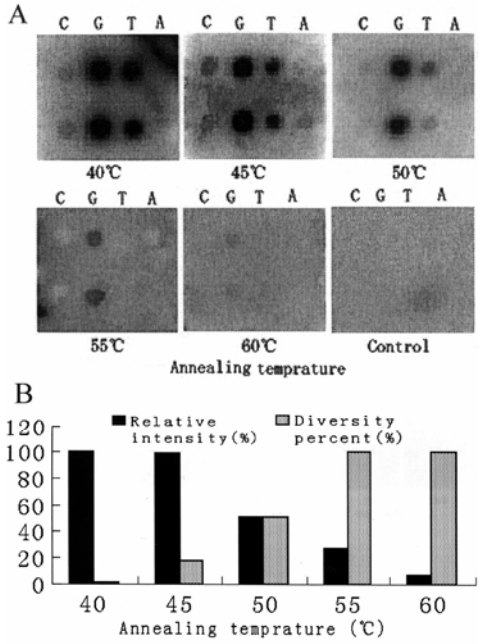

Fig. 1—Effects of annealing temperature to the primer extension procedure on chip [(A): Images obtained from the analysis of traditional primer extension at different annealing temperatures and no template control reaction. The 3′ terminal nucleotide of arrayed primers are indicated at the top of each image. Each allele-specific primer has two repeated spots. All the results of controls are the same and only one is shown; (B): Comparison of signal intensities and diversity per cents at different annealing temperature. Signal intensities are obtained for the spots corresponding to the template matched primer. Intensities shown are normalized so that the maximum value in this image is 100]

extension signal changed markedly with the variation of annealing temperature, indicating that such variation significantly influences the efficiency of the solid-phase reaction. At the temperature above 50°C, though the specificity was high, the extension efficiency became very low and the signal was very weak. As the annealing temperature became lower than 50°C, the detection signal strengthened, however, the level of mismatch extension was high and the diversity per cent fell below 50%. Considering efficiency and specificity, 50°C was selected as the optimal annealing temperature for the following experiment, which was about 5°C lower than that of liquid-phase reaction determined through separate experiment (data not shown).

Effect of template and Mg^{2+} concentrations

The effects of template and Mg^{2+} concentration are shown in Fig. 2 (A-C). As the template or Mg^{2+} concentration was increased gradually, extension signal was enhanced and the specificity of the procedure decreased (Fig. 2 B and C). The result was consistent with the liquid-phase reaction[26], though the optimal concentrations were higher. Considering the extension efficiency and specificity, 1 n*M* of template and 3.0 m*M* of Mg^{2+} were selected as optimal conditions. When the template was higher than 1 n*M* at the 0.75 m*M* Mg^{2+} concentration, no signal was detected, indicating that at low Mg^{2+} concentration, the Mg^{2+} absorption by the templates or the surface of chip may cause extension failure.

Modified allele-specific primer extension with [α-32p]-dATP and Cy3-dUTP on chip

Modified allele-specific primer extension with [α-^{32}p]-dATP and Cy3-dUTP gave similar results with improved specificity (Fig. 3 A and B). The diversity per cent, which was 60-70% for traditional allele-specific primer extension with [α-^{32}p]-dATP and Cy3-dUTP, increased to more than 90% for modified allele-specific primer extension.

PicoGreen dye staining method replacing the Cy3-dUTP incorporated method

With the traditional primers, the diversity percent of PicoGreen dye staining method was very low (27%). However, with modified primers, the PicoGreen dye staining method gave similar results as that of Cy3-dUTP-incorporated fluorescent method with a diversity per cent of more than 90% (Fig. 3 C).

Discussion

In the liquid phase, the allele-specific extension procedure on chip appears to be highly promising for high throughput SNP genotyping[26,33,36,39] and is widely used for SNP typing. However, the solid-phase reaction has different mechanism and its characteristics still remain unclear. Hence, we evaluated the systematic characteristics of the solid-phase allele-specific extension procedure, in terms of specificity and efficiency. Our results showed the influence trend of the three main factors viz., annealing temperature, template and Mg^{2+} concentrations, useful for optimization of the procedure.

The effect of annealing temperature, Mg^{2+} and template concentrations in this procedure was different from the liquid-phase reaction, mainly because the annealing of immobilized primers with templates was less efficient than free primers. The specificity and efficiency of the procedure though linked, behave in opposite fashion; higher specificity is usually accompanied with lower efficiency and *vice versa*. Annealing at 50°C (i.e. at 5°C lower than the optimal annealing temperature of liquid-phase

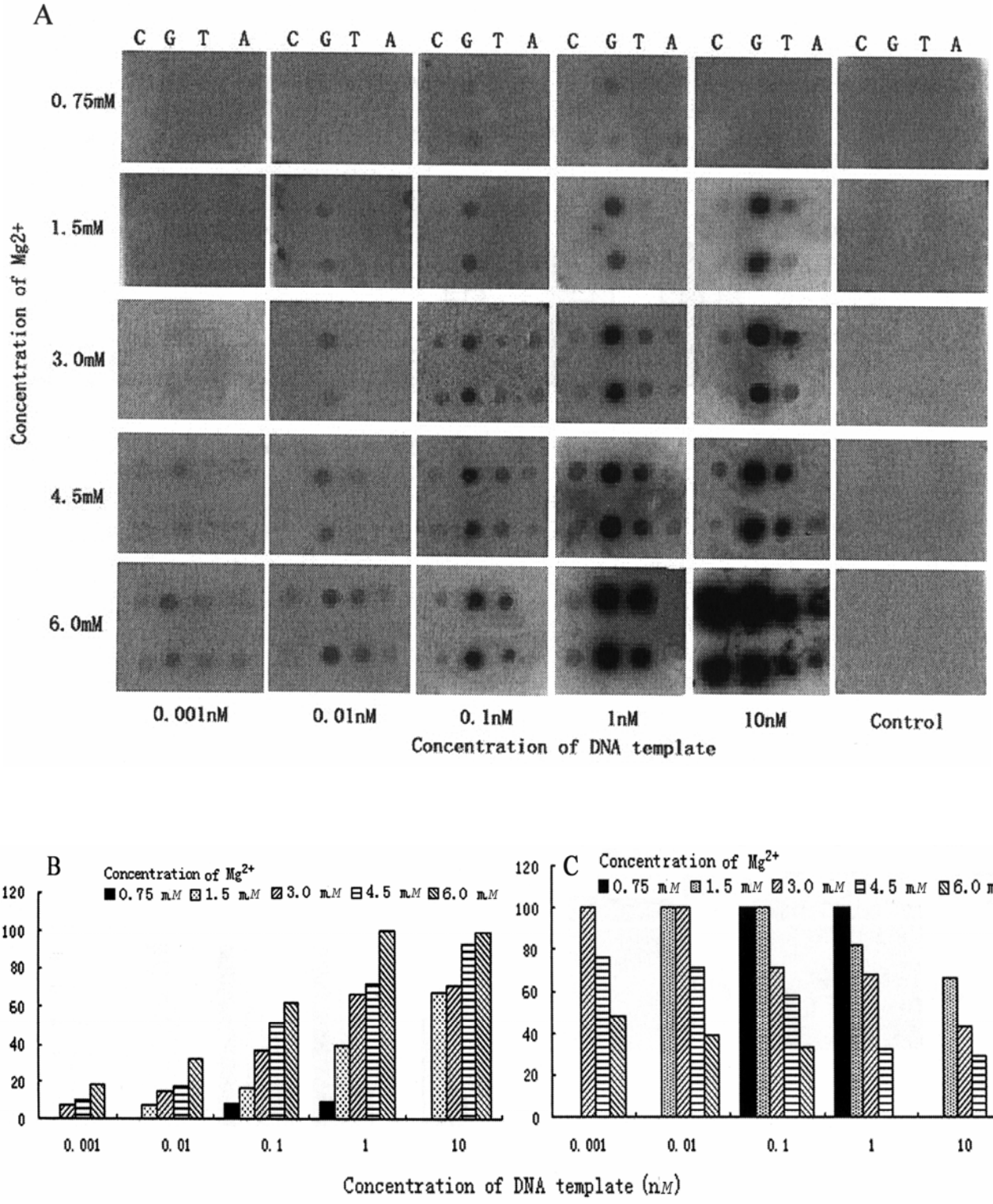

Fig. 2—Effects of template and Mg^{2+} concentrations in primer extension procedure on chip [(A): Images obtained from the analysis of traditional primer extension at different template and Mg^{2+}concentrations and at no template control reaction; (B): Comparison of signal intensities obtained for the spots corresponding to the template matched primer at different template and Mg^{2+} concentrations. Intensities shown are normalized so that the maximum value in this image is 100; and (C): Comparison of signal (diversity per cent) at different template and Mg^{2+} concentrations]

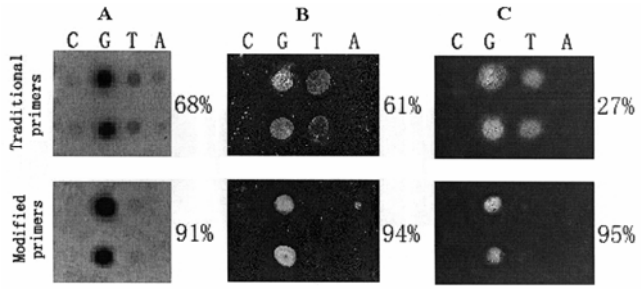

Fig. 3—Comparison of primer extension with traditional primers and modified primers for three different signal detection methods [(A): Images for primer extension with [α-^{32}p]-dATP; (B): Images for primer extension with Cy3-dUTP; and (C) Images for the slides stained with PicoGreen solution. Top and bottom images show extension with traditional and modified primers, respectively. The diversity per cent for each image is shown to the right]

reaction), 1 n*M* templates and 3.0 m*M* Mg^{2+} were found to be the optimal conditions for immobilized primer extension (Fig. 1 and 2).

In some cases, however, for the traditional allele-specific primer extension, optimization is difficult due to the extension of some mismatched primers. To avoid false extension, different approaches were proposed for soluble allele-specific extension, such as introduction of an extra mismatch in the primer[33,36], and adding a single-stranded DNA-binding protein (SSB)[40] or the apyrase[41], a nucleotide-degrading enzyme to the extension solution. However, for solid-phase primer extension on chip, only apyrase[31] was applied. We employed modified primers to the primer extension procedure on chip and found a significant increase in the specificity. Furthermore, this made it easier to obtain optimal conditions for both the efficiency and specificity.

Nucleic acid stains are mainly used for quantification of DNA or RNA samples. In the liquid-phase reaction, for allelic discrimination, Cybr-Gold staining method coupled with thermostable ligation and rolling circle amplification is used[42]. In addition, some nucleic acid stains have also been applied to microarray slides for quality control, and have been used to determine the hybridized and non-hybridized probes in the channels of chip[43]. However, combination of nucleic acid stains and the primer extension procedure on chip has not been reported earlier. In the present study, after modified primer extension, the matched primers were converted into dsDNA and the mismatched primers were still single stranded. Under optimized washing condition, the dsDNA-specific PicoGreen dye achieved allelic discrimination easily; moreover, the staining solution can be reused. Thus, the PicoGreen staining method could significantly reduce the cost by replacing the incorporation of fluorescent-labeled dNTP.

The genotyping power of modified allele-specific primer extension coupled with PicoGreen staining method on chip, evaluated in more than 40 assays demonstrated an accuracy of nearly 100%. In comparison, the allele-specific oligonucleotide (ASO) arrays gave a success rate of 71% only[38]. Minisequencing method yielded >10-fold better discrimination power than the simple ASO hybridization[22]. The traditional allele-specific primer extension on chip gave similar specificity as minisequencing method[18,22,23].

In conclusion, the use of modified primers to the allele-specific primer extension procedure on chip resulted in a significant increase in the specificity. The combination of modified primers and PicoGreen staining methods may provide a promising strategy for SNP analysis on microarray.

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