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TaqMan probe array for quantitative detection of DNA targets

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ABSTRACT

To date real-time quantitative PCR and gene expression microarrays are the methods of choice for quantification of nucleic acids. Herein, we described a unique fluorescence resonance energy transfer-based microarray platform for real-time quantification of nucleic acid targets that combines advantages of both and reduces their limitations. A set of 3′ amino-modified TaqMan probes were designed and immobilized on a glass slide composing a regular microarray pattern, and used as probes in the consecutive PCR carried out on the surface. During the extension step of the PCR, 5′ nuclease activity of DNA polymerase will cleave quencher dyes of the immobilized probe in the presence of nucleic acids targets. The increase of fluorescence intensities generated by the change in physical distance between reporter fluorophore and quencher moiety of the probes were collected by a confocal scanner. Using this new approach we successfully monitored five different pathogenic genomic DNAs and analyzed the dynamic characteristics of fluorescence intensity changes on the TaqMan probe array. The results indicate that the TaqMan probe array on a planar glass slide monitors DNA targets with excellent specificity as well as high sensitivity. This set-up offers the great advantage of real-time quantitative detection of DNA targets in a parallel array format.

INTRODUCTION

Microarry-based analyses have been well established and are currently used in a wide range of biological assays (1–5). Extrapolation of their use for infectious diagnostics and detection of biodefense related agents provides an attractive alternative to conventional analytical approaches. Hence the critical need for advanced diagnostic systems in microbiology is to detect rapidly genetic information within the known and unanticipated pathogenic microrganisms associated with the human health (i.e. viruses, bacteria and fungi). With the parallelism offered by DNA microarray technology we are able to pursue and develop an approach to large-scale analyses of such abundance of genetic information in these organisms. The main technical challenge in this field arises from the difficulty in labeling sufficient copies of pathogenic biomarkers. However, when integrated in one portable diagnostic device label-free detection has great potential for addressing this neckbottle and speeds up practical application of microarray technology.

Real-time PCR technologies as label-free methods have been used widely for gene expression, allelic discrimination and pathogen detection in solution (6–10). Their principle is fluorescence resonance energy transfer, where fluorescence is detected as a result of a change in physical distance between a reporter fluorophore and a quencher molecule. Molecular beacons firstly introduced by Tyagi and Kramer (9) and TaqMan probes originated from 5′ nuclease cleavage activity (11) and subsequently refined by Lee *et al*. (12) are the two main probe types in the real-time PCR systems for detecting the accumulation of specific PCR product and discriminating alleles. Their application is usually limited detecting only a few DNA targets, although several efforts have been made to improve the throughput performance of TaqMan probes in

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a single tube. For example, Lee *et al*. (13) have used some TaqMan probes labeled with different fluorophores to detect simultaneously seven various DNA targets. Tong *et al*. (14) have also described a combinatorial design of distinct fluorescence emission signatures for higher throughput assays. Also, several groups have immobilized molecular beacons on a solid surface to construct a molecular beacon array to resolve the target DNA sequences spatially (15–18). These pioneering works provide a new opportunity in quantitative, label-free and high-throughout detection of nucleic acid analysis. However, a high fluorescent background because of incomplete quenching of the molecular beacons and the interfacial effect induced by the electrostatic properties at the solid–liquid interface, greatly decreases the signal-to-background ratio. Previously, our research group had fabricated molecular beacon arrays on an agarose film to detect single nucleotide polymorphisms of unlabeled PCR products. Because of a solution-like hybridization environment and high binding capacity provided by the agarose film, an improved signal-to-background ratio for single nucleotide mismatch were observed (19). But this approach is not suitable for real-time monitoring of the PCR and hybridization process since the agarose film is not capable of withstanding high temperature during PCR. Therefore, we need more robust microarray technology to resolve this problem.

Here, we introduce a more robust microarray set-up using TaqMan probes for high-throughput quantification of nucleic acids. The conventional TaqMan probe is a double-labeled fluorogenic probe consisting of an oligonucleotide with a reporter fluorescent dye attached to the 5′ end and a quencher dye attached to the 3′ end (8). Owing to these chemical properties such probes can not be directly used in the immobilizaton on a glass slide because the reporter dyes of these probes are cleaved and released into the solution during the PCR. In this paper, we report firstly a set of innovative amino-modified TaqMan probes immobilized on a glass slide to form a regular array pattern for quantitative nucleic acid detection. The structure of this unique TaqMan probe consists of an amino group at the 3′ end for immobilization, poly$(T)_{20}$ as a linker arm, 6-FAM-labeled phosphoramidite at the middle as the reporter dye and a dabcyl group at 5′ end as the quencher. In contrast with conventional TaqMan probe where the fluorophore is located at 5′ end of the probe, our 3′ amino-modified TaqMan probe is designed to have a quencher located at the 5′ end of the probe so that the quencher can be cleaved and the fluorescent signal on the array can be restored during the PCR. These features ensure that the TaqMan probes can be used to quantify nucleic acid targets on a solid–liquid surface. We used this approach to demonstrate the feasibility of real-time nucleic acid analysis in parallelism directly from genomic DNA, e.g. from a mip gene of *Legionella pneumophila* (**DNA1**), from a hexon gene of human adenovirus (**DNA2**), from a rfbE gene of *Escherichia coli* O157:H7 (**DNA3**), as negative control one from a non-related human ESR1 gene (**DNA4**), and lastly from a specific nucleic acid fragment of severe acute respiratory syndrome coronavirus (**DNA5**). We also investigated the specificity, sensitivity and cleavage dynamics of TaqMan probes immobilized on a glutaraldehyde-modified glass slide. Like the cleavage in solution, we find the cleavage of the quencher dye occurs on the array and causes an increase in reporter fluorescence intensity because the reporter is no longer quenched. TaqMan probe arrays have a great potential to provide a fresh and attractive scheme that has the ability to high-throughput, real-time quantitative detection of the unlabeled DNA targets.

MATERIALS AND METHODS

Amino-TaqMan probes

3′-Amino-modified TaqMan probes and other oligonucleotides used in this study were synthesized and purified with double reversed-phase high-performance liquid chromatography by the Department of chemistry, University of Florida. Fmoc (fluorenylmethoxycarbonyl) protected 3′-amino-modifier C7 CPG (Glen Research: 20-2957) was used as the solid support for synthesis. The probes were synthesized with UltraMild CE phosphoramidites [Pac-dA-CE (10-1601), Ac-dC-CE (10-1015), iPr-Pac-dG-CE (10-1621) and dT-CE (10-1030)]. 5′-Amino-modifier C6 (10-1906) was introduced at 5′ end for Dabcyl-NHS ester coupling. Finally the Fmoc amino protection was removed with 20% poperidine/DMF and the TaqMan probes were cleaved from the CPG with 50 mM K_2CO_3 in anhydrous MeOH. These TaqMan probes are specially designed for prefect complement with the corresponding specific nucleic acid fragment of a severe acute respiratory syndrome coronavirus, *E.coli* O157:H7, a human adenovirus, *L.pneumophila* and a human ESR1 gene. Their genomic DNAs were extracted from the reference strains of five different microorganisms by a commercial Kit (Qiagen, Inc.).

Data acquisition

The fluorescence images were collected with the standard FITC filter by laser scanning confocal microscope (Leica TCS SP) employing a 488 nm Ar ion laser. A low-powerful objective lens (10×) was used in all the experiments. The system control program is Leica TCS NT software version 1.6.587. The laser moves from point to point to produce the scanned images at medium speed and in unidirectional scan mode. The slide is imaged with a zoom factor of 1 and the image size of the scanning field area is 1 mm × 1 mm. Adjust the gain and offset of PMT1 until the image is bright enough and shows a good contrast. All relevant parameters of the Leica TCS system were not adjusted again in all the scanning process. Images were analyzed by ImageJ version 1.3 (NIH) (http://rsb.info.nih.gov/nih-image/index.html).

Manufacture of TaqMan probe arrays

The amino-silane derived glass slides (DAKO, Catalog no. S3003) were cleaned with deionized distilled water and incubated in 5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) buffer (pH = 7.4) for 2 h. Then the slides were thoroughly washed twice with methanol, acetone and deionized distilled water, and dried. Spotting solutions were obtained by dissolving TaqMan probes in sodium carbonate buffer (0.1 M, pH = 9.0) at the concentration of 10 μM. Pin-based spotting robot PixSys5500 (Cartesian Tech. Inc.) with SMP3 pin was used to perform TaqMan probe array spotting. About 500 pl spotting solution was spotted

on the glutaraldehyde derived glass slide with 120 μm diameter and 200 μm spacer. After spotting, glutaraldehyde derived glass slides were incubated at room temperature for 2 h and at 37°C for 2 h. Next, the slides were soaked twice in 0.1% SDS for 2 min at room temperature with vigorous agitation and then transferred the slides into a sodium borohydride solution (Dissolve 1.5 g NaBH4 in 450 ml PBS, and then add 133 ml 100% ethanol) for 5 min at room temperature to reduce free aldehydes. Finally, the slides were washed thoroughly in 0.1% Tween, rinsed in distilled water and dried by a flow of nitrogen gas. The TaqMan probe array can be used for immediately 5′ nuclease PCR assays on a solid surface or stored at 4°C for future use.

TaqMan assay in solution

The PCRs were performed in a total volume of 25 μl containing 1× TaqMan Universal PCR Master Mix, which is an optimized real-time PCR buffer supplied by Applied Biosystems for TaqMan reactions, 200 nM of each primer for different fragments, 200 nM amino-modified TaqMan probes and 10 ng different genomic DNAs in a PCR tube. The amplification condition consists of an initial 2 min at 50°C for optimizing the UNG enzyme, and 10 min denaturation at 95°C followed by 40 cycles of 30 s of denaturing at 95°C, 30 s of annealing at 52°C, and 60 s of extension at 65°C. The instrument is the Rotor-Gene 3000 (Corbett Research Ltd, Australia).

Real-time amplification on TaqMan probe array

As for the heterogeneous 5′ nuclease PCR assay, glass slides on which 3′ amino-modified TaqMan probes were covalently bonded, were subjected to a 1 h blocking step using 5× SSC buffer containing 0.1% Tween 20, 0.1% BSA, washed in 5× SSC buffer and finally washed in water. DNA amplification was initiated on the glass slides with the same PCR mixture as above except without the amino-modified TaqMan probes in a chamber sealed by Gene-frame (Catalog no. AB-0576). The microscope glass slides were put into the 16 × 16 twin tower block thermocycler (PTC 225; MJ Research). Thermocycling was carried out as follows: an initial 2 min at 50°C for optimal UNG enzyme, and 10 min denaturation at 95°C and various cycle numbers from 20 to 38 cycles (95°C for 30 s, 52°C for 45 s and 65°C for 2 min).

Single mismatch nucleotide detection on TaqMan probe array

In order to investigate the capability of single nucleotide polymorphisms of the TaqMan probe array, a 193 base oligomer of the hexon gene fragment (with an A/G wild type/mutation type mismatch at position 37) was designed to be complementary to the 3′ amino-modified TaqMan probe TM2 and this was obtained via gene synthesis method (20) service provided by Shanghai Sangon Co., China and verified by sequencing. During the amplification 1 ng DNA targets of the wild-type hexon gene fragment and the synthesized oligomer with the PCR mixture descried above were respectively injected to the chamber sealed by Gene-frame at each annealing temperature (at 50, 52, 54, 56 and 58°C, respectively), the instrument, reaction condition and the acquisition of fluorescence images are identical to those described above.

RESULTS AND DISCUSSION

Design of TaqMan probes

The conventional TaqMan probe is a double-labeled fluorogenic probe consisting of an oligonucleotide with a reporter fluorescent dye attached to the 5′ end and a quencher dye attached to the 3′ end (8). When the reporter dye is excited by irradiation, its fluorescent emission will be quenched through the process of fluorescence energy transfer. During the annealing/extension step of PCR amplification, the probe will be recognized by DNA polymerase so that the cleavage will occur between the reporter and the quencher dyes by 5′–3′ nuclease activity of the *Taq* DNA polymerase. Then the reporter dye is no longer quenched and the increase in reporter fluorescence intensity will be observed. In this process, a surface immobilized TaqMan probes was designed and synthesized with an amino group at the 3′ end, fluorescein-dT at the middle as the reporter dye and Dybcyl at the 5′ end as a quencher. A CPG with Fmoc protected amino group and Ultra-Mild CE phosphoramidites were used for synthesis. The Fmoc group is quite stable and base liable. After detritylation in 3% dichloroacetic acid (DCA)/dichloromethane (DCM), the monomethoxytritylamino (MMT) was removed from 5′ end amino group, which was then used to couple with Dabcyl-NHS ester on the CPG while the 3′ end amino group was still protected by Fmoc. After removal of Fmoc amino protection and cleavage, the probes were released from the CPG and 3′ end amino group was used for immobilization. The 20 base T was used as a linker to minimize the space hindrance for enzyme cleavage. When the 5′–3′ nuclease activity of *Taq* DNA polymerase cleave the 5′ dabcyl molecule into solutions during PCR on slides, the FAM reporter dye at 3′ end is still bound to the solid surface by covalent bond. Then the fluorescence increment of the FAM dye on the specific array can be directly acquired by a scanner. Dabcyl is non-fluorescent and can quench FAM dye well. Furthermore, dabcyl is compatible with the amino group and economical in the probes synthetic process. In addition, a 20 base thymine spacer is used to minimize destabilization caused by 3′ end immobilization.

Owing to the synthesis of the 3′-amino-modified TaqMan probes with three different chemical modifications on an oligo probe is relative complex and not popular in commercial synthesis, we designed 10 3′ amino-modified TaqMan probes and obtained five good probes. The 3′ amino-modified TaqMan probes and the corresponding primer pairs are summarized in Table 1. We used the laser scanning confocal microscope (Leica TCS SP) to acquire the experiment data and to produce the scanned images at stable speed. Under the certain objective (10×) and eye lens (10×), the image size of the scanning field area is 1 mm × 1 mm. The area of the probe array of 3 × 5 almost covers the size of the scanning field area. Of course, we can assemble a series of different frames of the collected images together if you want to expand the scanning area for immobilizing more probes. The physical distance between the reporter and quencher are extremely important in the design of this type of fluorogenic probe. According to the previous studies, the melting temperature (T_m) value and the distance of the two dyes of the conventional TaqMan probe, and amplion length are all critical factors in a successful real-time PCR. For example, a conventional probe system will

provide an appropriate ratio of the fluorescence intensity of the reporter dye over the quencher dye, typically both in solution at 60–67°C of T_m and 75–150 bp of amplion length (8,21). If too large physical distance between the two dyes in solution will directly result in low quench efficiency. We choose 20–24 bases between the two dyes in all the amino-modified TaqMan probes (TM1–TM5) and their amplion sizes are no more than 250 bp. Increasing the length of amplicons may result in a longer annealing/polymerization step or a higher Mg^{2+} concentration, which decreases the specificity of the PCR. It might also make the system less efficient and inflexible.

TaqMan probes array

The scheme of TaqMan probe arrays before and after cleavage is illustrated in Figure 1. The 3′ end amino-modified TaqMan probes TM1 and TM2 immobilized on the glass slide maintain a close physical proximity between the reporter and the quencher (Figure 1a) and emit little fluorescence. When PCR mixtures including a template **DNA1** are added, **DNA1** and its complementary probe TM1 form a rigid probe–target duplex (Figure 1b). Then *Taq* DNA polymerase will recognize this hybrid and cleave the quencher of TM1 because of its inherent 5′ nuclease activity. The quencher of TM1 is released into solution and the reporter dye at 3′ end is still bound on surface so that the fluorescence of TM1 is restored, while TM2 is still quenched (Figure 1c). Then reporter fluorescence increments from different sites on surface can be directly acquired by a fluorescence confocal microscope. These fluorescence signals are spatially resolved to determine the DNA target's quantity (Figure 1d).

Table 1. The sequences of primers and TaqMan probes used in this study

CovF	5′-AAATGAATTACCAAGTCAATGG-3′
CovR	5′-TACCCACAGCATCTCTAGT-3′
EF	5′-GTGGGAACATTTGGAGATATTT-3′
ER	5′-CATAAAGTGTTTTGTCATTCGT-3′
AdVF	5′-ATTATCCATACCCGCTCATC-3′
AdVR	5′-ATCCACCTCAAAAGTCATGT-3′
LpF	5′-AACCGATGCCACATCATTAG-3′
LpR	5′-CGGACTAACATCAATGCCTT-3′
TM5 (Cov)	5′-Dabcyl-ACAGCCCTCTACATCAAAGCCAA–FAM-$(T)_{20}$-3′-NH_2
TM4 (Control)	5′-Dabcyl-CCAAAGCATCCGGGATGGCC-$(T)_{20}$-3′-NH_2
TM3 (*E.coli*)	5′-Dabcyl-ACAACCATTCCACCTTCACCTGT-FAM-$(T)_{20}$-3′-NH_2
TM2 (Adv)	5′-Dabcyl-ACTACTGCCGTTAAGAGTGTCACC-FAM-$(T)_{20}$-3′-NH_2
TM1 (L.pp)	5′-Dabcyl-AGTTCTTTCCCAAATCGGCACCAA-FAM-$(T)_{20}$-3′-NH_2

Amino-modified TaqMan assay in solution

Initially, we examined the quantitative performance of the 3′ end amino-modified TaqMan probes during real-time PCR system in solution. To further evaluate the sensitivity of these probes, we cloned a 191 bp fragment of the hexon gene of human adenovirus and prepared serial dilutions ranging from 1.2×10^7 to 1.2×10^1 copies of the target. The consecutive real-time PCR provide well-shaped curves of the increase in fluorescence intensity of the amino-modified TaqMan probe for the specific hexon gene over this broad dynamic detection range (Figure 2). From left to right, the curves denoted the 1.2×10^7, 1.2×10^6, 1.2×10^5, 1.2×10^4, 1.2×10^3, 1.2×10^2 and 1.2×10^1 copies, respectively. The fluorescence signal increase above the instrument threshold during PCR for the specific fragment occurred at distinct cycles. High fluorescence intensities and well-shaped standard curves with relatively identical distances for the serial dilutions led to clear and interpretable results (shown in Figure 2 inset). The results suggest that 5′ end of the dabcyl quencher and 3′ end of the FAM reporter in our amino-modified TaqMan probes can be used in quantitative analysis of nucleic acid targets in solutions.

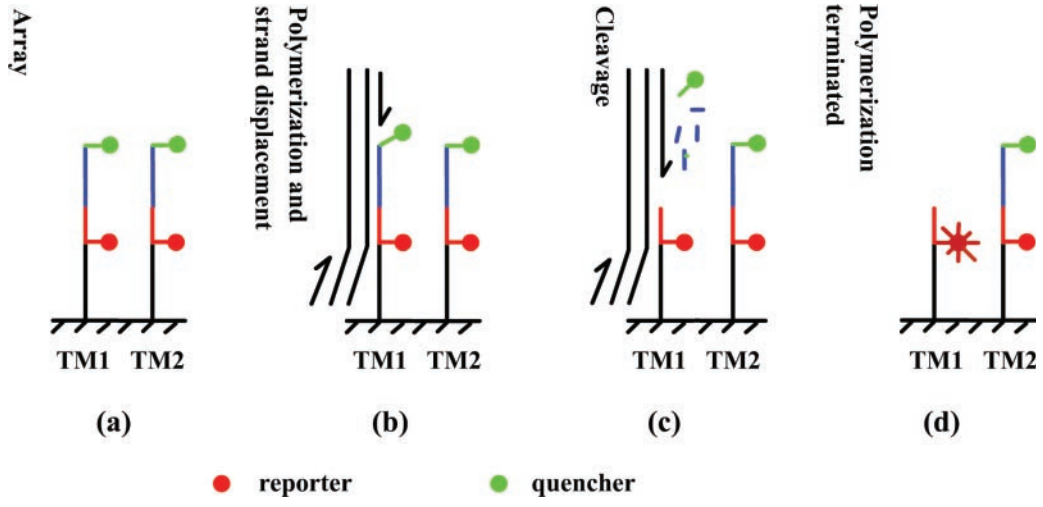

Figure 1. Schematic representation of 5′ nuclease PCR assay on the TaqMan probe array. (**a**) A set of TaqMan probes are immobilized on the glutaraldehyde derived glass slide. Two dyes, one fluorescent reporter and one quencher, are attached to the TaqMan probes. (**b**) The polymerization-associated, 5′–3′ nuclease activity of *Taq* DNA polymerase acts on double-labeled TaqMan probes during one annealing/polymerization step of a PCR cycle. (**c**) When the probe is intact, reporter dye emission is quenched by the quencher. During each extension cycle of the PCR, the *Taq* DNA polymerase cleaves the quencher from the probe. (**d**) Once quencher is released, the reporter dye still immobilized on the glass surface emits its characteristic fluorescence that can then be acquired by CCD or PMT.

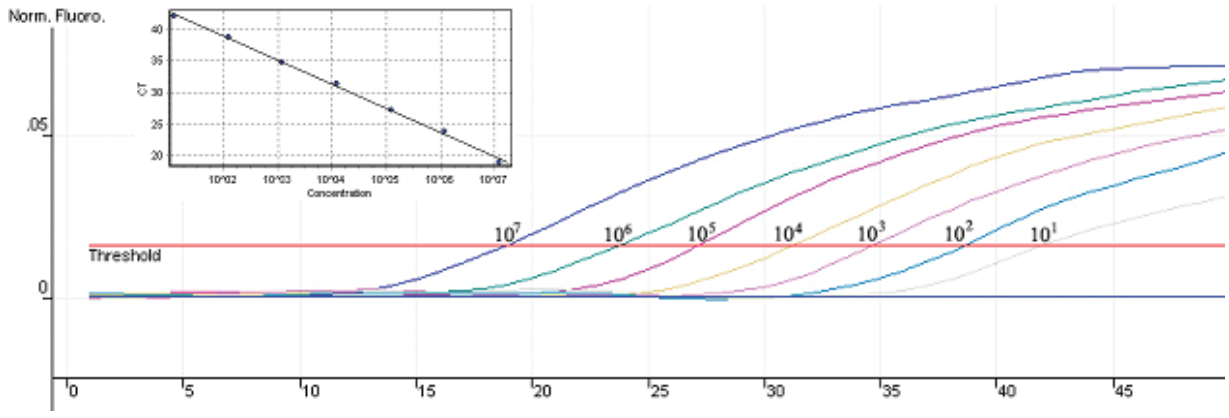

Figure 2. A quantitative curve of an amino-modified TaqMan probe (TM2) in solution and the stand curve of CT versus concentration (inset).

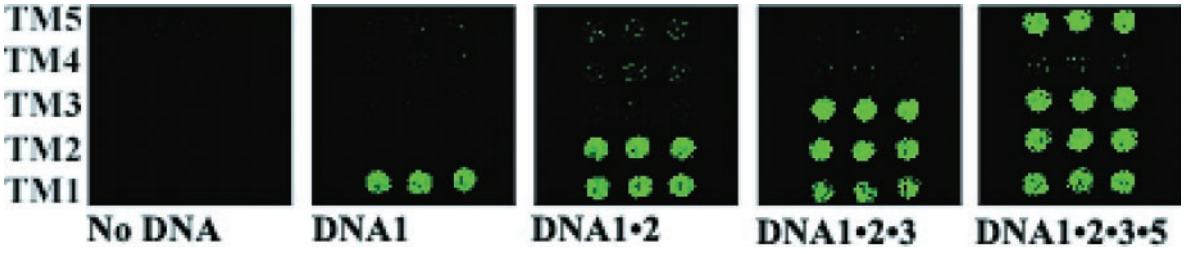

Figure 3. We show the specificity of detecting directly various genomic DNA targets with the TaqMan probe array. In the array, from bottom to top, the probes are TM1 (complementary to **DNA1**), TM2 (complementary **DNA2**), TM3 (complementary to **DNA3**), TM4 (complementary to **DNA4**) and TM5 (complementary to **DNA5**), respectively. From left to right the fluorescence images are without any DNA target, with only **DNA1**, with **DNA1·2**, with **DNA1·2·3** and with **DNA1·2·3·5**, respectively.

Specificity of TaqMan probes array

Figure 3 shows the specificity of detecting unlabeled nucleic acid targets by a 5′ nuclease PCR assay using the amino-modified TaqMan probe glass array. The PCR mixtures contain 1× TaqMan Universal PCR Master Mix, different concentrations of each primer, a final 1 ng concentration of different genomic DNA templates, a no **DNA** template control, and **DNA1**, **DNA12**, **DNA123** and **DNA1235**, respectively. Then the PCR mixtures were injected to a chamber sealed by the gene-frame on the five different slides, respectively, and amplified in the 16 × 16 twin tower block thermocycler (PTC 225; MJ Research). After the reaction, the slides were washed in 2× SSC, 0.1% Tween-20 (2 × 5 min), once in 0.2× SSC, 0.1% Tween-20 and rinsed in distilled water. The fluorescence images were collected by a confocal fluorescence microscope (Materials and Methods). We then found that the fluorescence intensities of various amino-modified TaqMan probes are different and that the fluorescence intensity increments of the different DNA template are clearly located at corresponding sites. The results indicate the high specificity of the 5′ nuclease PCR using the amnio-modified TaqMan probes arrayed on surface and demonstrate how our TaqMan probe arrays can successfully detect the presence of specific gene fragments.

Fluorescence changes in real-time PCR

We also investigated the dynamic characteristics of fluorescence intensity changes on the amino-modified TaqMan probe arrays. A mixture of PCR described in Materials and Methods containing 1 ng **DNA2** template was injected into a chamber sealed by the gene-frame and amplified. The reaction condition is also same as described above except that the PCR cycle numbers are different. To evaluate the dynamic changes of fluorescence intensities, we acquired serial fluorescence images at various cycle numbers i.e. 1, 20, 23, 26, 29, 32, 35 and 38 (shown in Figure 4A). The scatter diagram in Figure 4B plots the fluorescence signal data versus PCR cycle number. This result with a slightly diminished signal-background-ratio is similar to that reported above in solutions. There are two possible interpretation of the signal-background-ratio reduction for the immobilized TaqMan probe arrays. First, the interfacial effect makes their inherent structure different from that in the solution. This structure change influences negatively on quenching efficiency and increases their fluorescence backgrounds on solid–liquid surfaces. Second, the steric effects caused by high immobilization densities decrease the rate of the duplex formation of the immobilized probe and DNA target, which might also decrease the cleavage efficiency. Therefore, the T_m value of the amino-modified TaqMan probe should be set about 10°C higher than that of the primer. This ensure that each TaqMan probe is coupled with a DNA target via base pairing before the forward and inverse primers are attached, and that the free 5′ end of the immobilized TaqMan probe is accessible for nuclease activity by DNA polymerase. This also ensures that the large amount of the probes participates in the cleavage process and it is easier to get the high signal-background-ratio.

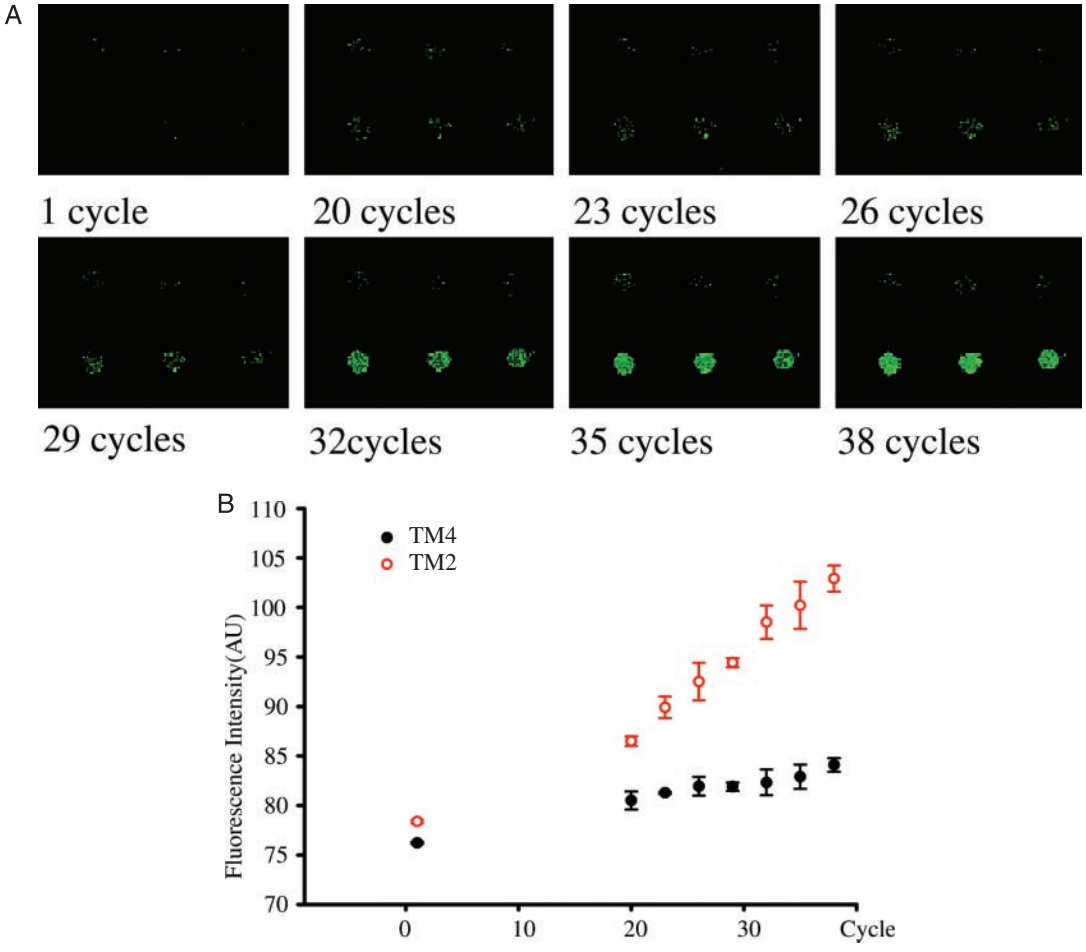

Figure 4. (**A**) The dynamic fluorescence images of a TaqMan probe array on a glutaraldehyde derived glass slide with various cycle numbers during a real-time PCR. **DNA2** as the only template strand was added to this PCR. In the array, the bottom row is the probe TM2 for the hexon gene of a human adenovirus (complementary **DNA2**) and the top row as a control is the probe TM4 for the human ESR1 gene (complementary to **DNA4**) in triplicates. (**B**) The plot of fluorescence signal versus cycle number of the TM2 probe (red) and the TM4 probe (black). The error bars show the SDs of triplicates.

Sensitivity of TaqMan probe array

In order to investigate the sensitivity of an immobilized 3′ end amino-modified TaqMan probe array, the same PCR mixture containing 1× TaqMan Universal PCR Master Mix and different copies of the hexon gene (prepared as described above) in a PCR tube was used. After the amplification, each image of different concentration DNA targets was collected. In these images, the TaqMan probes were spotted on the slides in triplet format. From top to bottom, the probes are TM5, TM4, TM3, TM1 and TM2, respectively. Figure 5 showed the fluorescence intensity changes of the amino-modified TaqMan probe (TM2) on the surface with a broad range of 1.2×10^7 to 1.2×10^1 target DNA copies of the hexon gene. The results indicate that the amino-modified TaqMan probe array has good sensitivity.

Single nucleotide mismatches detection of TaqMan probe array

Finally, we also evaluated the ability of detecting a single nucleotide mismatch in DNA targets on a TaqMan probe array. The two different DNA targets, the 193 base synthesized oligomer (with an A/G wild type/mutation type mismatch at position 37) and the wild type of the hexon gene fragment, were applied to the amplification on a TaqMan probe array. During the amplification corresponding images at different annealing temperatures were collected at 50, 52, 54, 56 and 58°C, respectively, and these are shown in Figure 6. The fluorescence signals of the two targets on the TaqMan probe array are obviously different. Hence, the results clearly discriminate between a perfectly matched target and single nucleotide mismatched target.

CONCLUSIONS

In this paper, we have designed a set of amino-modified dual-labeled fluorescence probes with pretty high quenching efficiency and high specificity for TaqMan assays on glass slides. We have also immobilized successfully 3′ amino-modified TaqMan probes to aldhyde-modified glass slides for quantitative PCRs in real-time by the poly$(T)_{20}$ linker molecule and 3′-amino group. The poly$(T)_{20}$ linker molecule is chemically robust easily enabling a decrease both in the steric effect and also in the surface effect. The thermal stability and the retention of 3′ amino-modified TaqMan probes bound to the functionalized glass surface are very suitable for the PCR

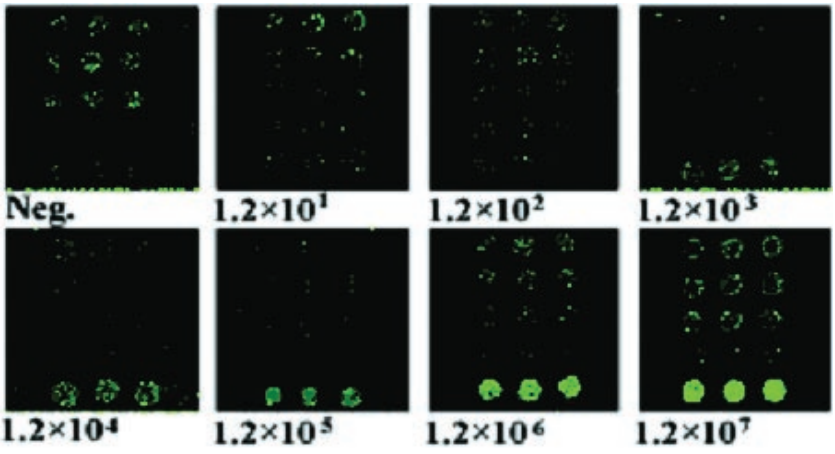

Figure 5. The sensitivity of a TaqMan probes array. In the array from bottom to top, the probes are: TM2 (complementary to **DNA2**), TM1 (complementary **DNA1**), TM3 (complementary to **DNA3**), TM4 (complementary to **DNA4**) and TM5 (complementary to **DNA5**), respectively. From left to right and then from top to bottom, the fluorescence images are firstly without any DNA target as negative control (Neg.), and then subsequently with different concentrations of DNA targets from 1.2×10^1 to 1.2×10^7copies, respectively.

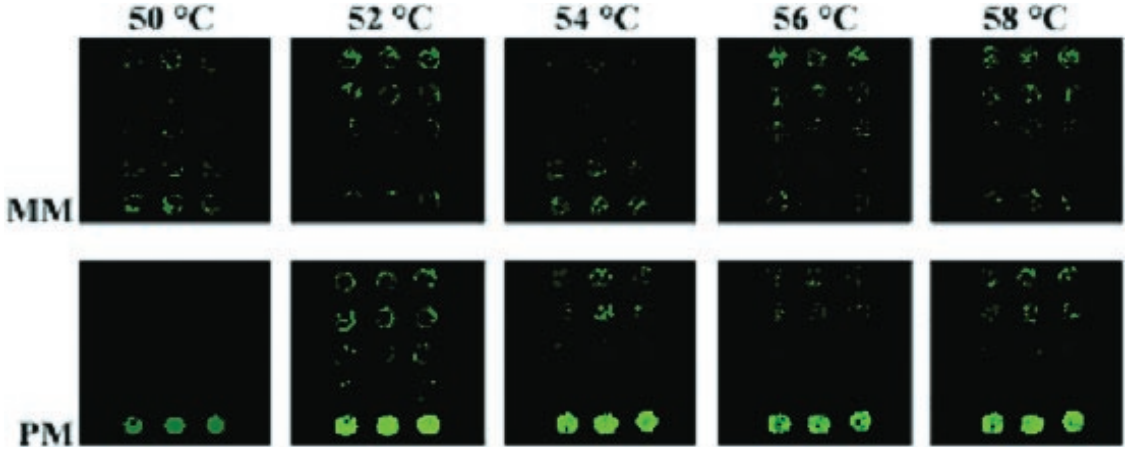

Figure 6. Fluorescence images of an amino-modified TaqMan probe (TM2) during the real-time PCR amplification on glass slides with single mismatch (top) and perfect match target (bottom) and at different annealing temperature (shown from left to right). In the array from bottom to top, the probes are TM2 (complementary to **DNA2**), TM1 (complementary **DNA1**), TM3 (complementary to **DNA3**), TM4 (complementary to **DNA4**) and TM5 (complementary to **DNA5**), respectively.

thermocycling reaction using the covalent bond linkage. Finally, a TaqMan probe array on an aldhyde derived glass slide was fabricated and we investigated its specificity, its sensitivity and the dynamics change of fluorescence intensity at various PCR cycle numbers.

This study presents a first experimental demonstration of direct real-time quantitative nucleic acid analysis from genomic DNA by 5′ nuclease cleavage assays on a surface array format. In all previous studies of real-time PCR in solution, freely-diffusing TaqMan probes were used, which have limited parallel analysis capability. Our results indicate that TaqMan probe arrays have potentially a great advantage in real-time quantification of multiple unlabeled nucleic acid targets, which promises a great opportunity for clinical applications such as gene expression and single nucleotide polymorphism analysis. In the future, advances in chemistry for instance in reporter and quencher dyes technology resulting in greater quench efficiency will even further improved the performance of our TaqMan probe arrays.

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Conflict of interest statement. None declared.

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