

A novel one cycle allele specific primer extension—Molecular beacon displacement method for DNA point mutation detection with improved specificity

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Abstract

We report here a new method for the real-time detection of DNA point mutations with molecular beacon as the fluorescence tracer and 3' (exo-) *Bst* DNA polymerase large fragment as the polymerase. The method is based on the mechanism of allele specific primer extension-strand displacement (ASPE-SD). To improve the specificity of the method only one cycle of the allele specific polymerase chain reaction (PCR) was used that could largely eliminate the non-specific reactions between the primers and template of the “wrong” genotype. At first, the primer and molecular beacon both hybridize to the DNA template, and the molecular beacon emits intensive fluorescence. The role of 3' exonuclease excision of *Bst* DNA polymerase large fragment is utilized for primer extension. When 3'-termini matches its corresponding template, the primer would efficiently extend and replace the molecular beacon that would simultaneously return to its closed form leading to the quenching of the fluorescence. However, when 3'-termini of the primer mismatches its corresponding template primer extension and molecular beacon displacement would not happen and fluorescence of the hybridized molecular beacon holds the line without fluorescence quenching. This approach was fully demonstrated in synthetic template systems and applied to detect point mutation at codon 259, a possible point mutation site in exon 7 of *p53* gene, obtained from human genomic DNA samples with unambiguous differentiation power.

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1. Introduction

The ability to determine efficiently and unambiguously the mutational status or genotype of an organism has great application in molecular diagnostics, drug discovery, and genetics [1–4]. Commonly human disease and genetic disorder are associated with point mutations and the most common polymorphisms in human genome [5,6]. So it is meaningful to obtain a reliable tool for detecting DNA point mutations. There are a host of chemistries available for allelic discrimination. Broadly speaking, methods based on polymerase chain reaction (PCR) provide a powerful tool for the amplification of minute amounts of initial target sequences. A number of techniques are based on real-time PCR assay such as 5' nuclease (Taqman) assay [7–9],

scorpions assay [10–12], and molecular beacon assay [13–17]. These examples rely on the close proximity of donor fluorophore and quenchers in the unhybridized probe conformation such that little or no signal is generated. Upon hybridization to the target, the physical separation of the fluorophore and quencher moieties produces an increase in signal. These reactions are done in a closed tube and the signal is monitored in real-time PCR which is convenient and free of contamination. Other techniques developed were done by opening the tube because they required multiple sample processing such as dynamic hybridization [18], flap endonuclease discrimination (Invader assay) [19], and primer extension [20–23]. Oligonucleotide ligation (OLA) [24] has also been applied to point mutations. DNA sequencing is also largely used in point mutation detection, it could detect large fragment at one time, but it requires expensive experiment. The development of amplification refractory mutation system (ARMS) or allele specific PCR [25–27] allows simple and reliable allele detection. Allele specific PCR is limited by

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non-specific reactions between the mismatched primers and template with the cycles increasing. To improve the specificity of the method we proposed a one cycle allele specific PCR with pre-amplification of a DNA fragment with the possible mutant point included by normal PCR.

Here, we have developed an efficient detection strategy that allows the precise analysis of DNA point mutation. The method combined the advantages of PCR technique and molecular beacon [28]. It has entailed a two-main-step, involving amplification of target sequences and allele specific primer extension with strand displacement. Allele specific primer extension [25] is a powerful technique for detecting mutations and polymorphisms in DNA. Strand displacement activity is the ability of a certain DNA polymerase displacing downstream DNA encountered during synthesis [29]. The basic idea of this work is that, given a sufficient quantity of DNA (obtained by pre-amplification), the identity of a particular nucleotide at a particular position can be determined by hybridizing a primer to the DNA at a position in which the nucleotide being examined is complementary (or not complementary) to the 3' nucleotide of the primer, hybridizing a molecular beacon to a sequence in the DNA downstream from the primer, and incubating with DNA polymerase to see whether the primer is extended, resulting in the displacement of the molecular beacon and a consequent significant reduction in its fluorescence. Thus, using 3' (exo-) *Bst* DNA polymerase large fragment [30,31] discriminates whether the extension occurs, and this is dependent on whether the 3' nucleotide of the primer

is complementary to the nucleotide in the DNA that is being examined. The chief mechanism of our method is shown in Fig. 1. Separation of the DNA amplification and point mutation detection into two steps has two advantages: (1) from limited tissue sample enough DNA fragments in question can be prepared for unlimited number of experiments and (2) the discrimination power of allele specific extension reaction is the highest for the first circle of the PCR reaction.

In practice, this technique would be used for genotyping human alleles, as follows: (1) the region of the DNA being queried is first amplified by a polymerase chain reaction, (2) the primers used for this amplification and dNTPs are destroyed by incubation with an exonuclease and Antarctic phosphatase, which are later destroyed by heating, (3) the amplified DNA is then divided into two tubes, each containing one of two different primers (call them “wild-type primer” and “mutant-type primer”) and each containing the same molecular beacon, and (4) each tube is placed in spectrofluorometer and incubated with DNA polymerase and dNTPs to observe whether the fluorescence of the molecular beacon is diminished by virtue of primer extension occurring. There are three possible outcomes: (A) fluorescence is reduced significantly in the tube containing wild-type primer, but is not reduced in the tube containing mutant-type primer, indicating that the DNA in the sample is homozygous for the nucleotide complementary to the 3' nucleotide of wild-type primer, (B) fluorescence is reduced significantly in the tube containing mutant-type primer, but is not reduced in the

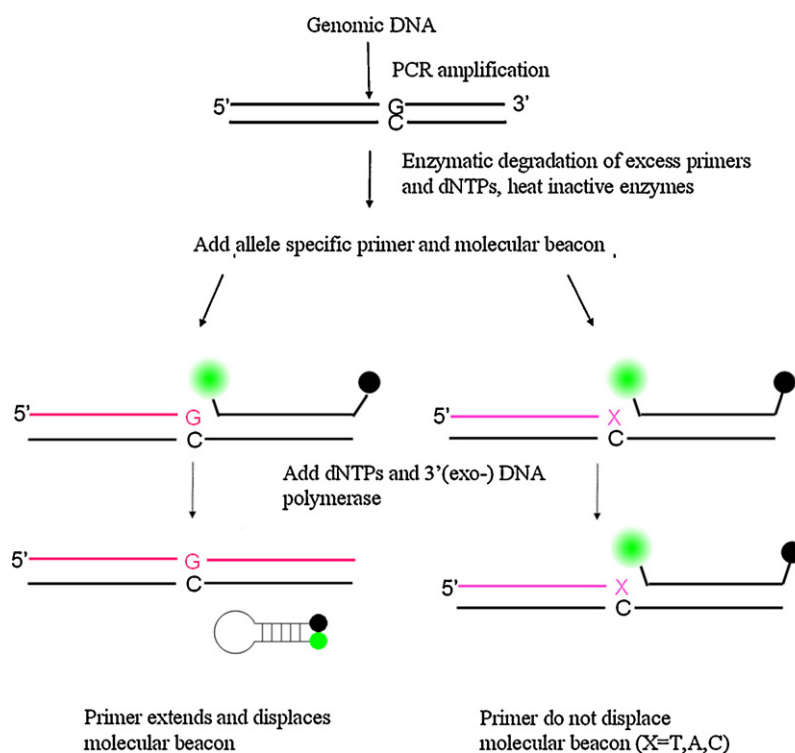


Fig. 1. Principle of allele specific primer extension-strand displacement method: primers and molecular beacon hybridize to the template results in fluorescence emission. When 3'-termini of the primer matches the template the primer extension will displace the molecular beacon that will return to its stem-loop form and fluorescence will be quenched; while primer with 3'-termini mismatches the template will not lead to primer extension and thereby the following molecular beacon displacement, thus the fluorescence remains unchanged.

tube containing wild-type primer, indicating that the DNA in the sample is homozygous for the nucleotide complementary to the 3' nucleotide of mutant-type primer, and (C) fluorescence is reduced to half in each tube, indicating that the DNA in the sample is heterozygous for the nucleotides complementary to the 3' nucleotides of both wild-type primer and mutant-type primer.

We have applied this method to the detection of possible point mutation in human tumor suppressor *p53* gene. About 20–60% tumor patients have mutation in *p53* gene and 95% of the mutations are single-base mutations rather than deletions, insertions, or multi-base mutations (database, 2002; IARC, 2002) [32,33]. Mutation at codon 259 in exon 7 of the gene has high probability [34]. In present study, the second base of codon 259 (AGA) of *p53* gene from human breast cancer was chosen as the targeted analyte to demonstrate the efficiency of the newly developed method.

2. Experimental

2.1. Reagents

The sequence of synthetic templates (T1, T2) is shown in Fig. 2, representing the wild and mutant types in the IGH gene with the variant base located at position 20. Primers (alleles 1 and 2) and molecular beacon 1 (MB1, 10 base pairs in the stem and 10 bases in the loop) for the study of T1 and T2 are also shown in Fig. 2. PCR primers 5'-GTGTTGTCTCCTAGGTTGGC-3' and 5'-CAAGTGGCTCCTGACCTGGA-3' are used for the amplification of a fragment (110 bp, position 14,000–14,109) from exon 7 of *p53* gene, in which the possible mutation point at codon 259 is included. The sequence of two 20-mer primers with only differences at 3'-termini are shown in Fig. 4, which are used for the discrimination of possible mutation at the first base of codon 259 (wild-type and mutant-type primer) within the PCR pre-amplified DNA fragment. The sequence of the downstream 30-mer molecular beacon (MB2, 10 base pairs in the stem part and 10 bases in the loop part) is also included in Fig. 4. All the primers and templates were synthesized and purified by AuGCT Biotechnology (Beijing, China). The fluorophore of the molecular beacon is FAM (6-carboxyfluorescein), and the quencher is DABCYL (4-[4'-dimethylaminophenylazo] benzoic acid). Molecular beacons were synthesized and purified by Sangon (Shanghai, China). Enzymes including 3' (exo-) *Bst*

DNA large fragment, Vent_R[®] (exo-) DNA polymerase, *E. coli* exonuclease I and Antarctic phosphatase were purchased from NEB (United Kingdom), dNTPs were purchased from Tiangen Biotechnology (Beijing, China). All chemicals employed were of analytical grade and commercially available.

2.2. Instrumentation

Fluorescence measurement was performed on a LS-50B spectrofluorometer (Perkin-Elmer) at room temperature. The molecular beacons were excited at 488 nm and fluorescence was measured at 515 nm. PCR amplification was performed on a TC312 mini PCR instrument from Techne (United Kingdom). DNA sequencing was performed on an Applied Biosystem 377 Genetic Analyzer by AuGCT Biotechnology.

2.3. Sample

Fresh breast cancer tissue samples from a cohort of patients were provided by Beijing Cancer Hospital with informed consent of the patients. The patients had not undergone any treatment before surgery. The samples were stored at -80°C and without any pretreatment before extraction of DNA. DNA was extracted from the tissue using a genomic DNA purification kit purchased from Tiangen Biotechnology Company. According to manufacturer's instructions, 100 μL of genome DNA (10–30 μg) extracted was obtained from 30 mg breast cancer tissue. The genome DNA solution was kept at -80°C for further use.

2.4. PCR for real sample

DNA amplification was done in 0.5 mL microtubes (Axygen, USA) in PCR instrument. Six-microlitre human genomic DNA extracted from each individual was amplified in a 50 μL reaction mixture containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 0.24 mM dNTPs, 0.8 μM of the exon 7 primers, and one unit Vent_R[®] (exo-) DNA polymerase. Thermal cycling conditions to amplify exon 7 DNA fragment were as follows: initial denaturalization at 94°C for 5 min, followed by 50 cycles at 94°C for 30 s, 60°C for 15 s, and 72°C for 15 s, final extension at 72°C for 10 min. The PCR products (110 bp) resulting from amplifying the exon 7 of *p53* gene were gel identified by running the products on a 1.5% agarose gel in $1\times$ TAE.

2.5. Primers and dNTPs degradation

To remove the remaining primers and dNTPs in the above PCR reaction mixture, added 6 μL $10\times$ exonuclease I buffer and 5 unit *E. coli* exonuclease I to the 50 μL of the above PCR reaction mixture and incubated with at 37°C for 15 min; then, 7 μL $10\times$ Antarctic phosphatase reaction buffer and 10 units of Antarctic phosphatase were added and incubated at 37°C for another 15 min. Finally, the enzymes were inactivated by heating at 80°C for 20 min. Each reaction tube got 80 μL mixture with amplified target DNA fragment kept at 4°C for further use.

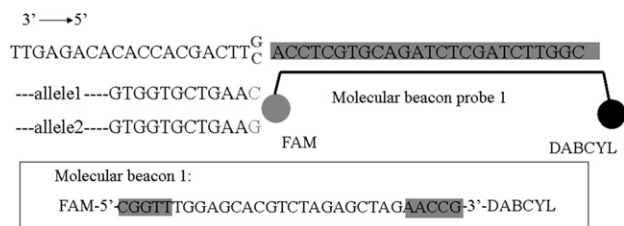


Fig. 2. Sequence of synthetic templates (gloomy marked is the part that is complementary to the beacon), primers together with allele positions, and sequence of molecular beacon (MB1, gloomy marked is the stem part) in allele specific extension-strand displacement method for detecting synthetic templates.

2.6. Genotyping analysis with molecular beacon by fluorescence spectrometry

2.6.1. Synthetic templates analysis

In each test tube, a 600 μL Tris–HCl (10 mM, pH 7.9) buffered reaction mixture containing 100 nM template, 120 nM primer, 120 nM MB1, 50 mM NaCl, and 10 mM MgCl_2 was prepared. The solution was heated at 94 $^\circ\text{C}$ for 4 min for denaturation, and then it was cooled to room temperature for 20 min to allow the primer and the molecular beacon to hybridize with the template. Then, the mixture was transferred to a quartz cuvette (10 mm \times 2 mm in cross-section). The dynamic molecular beacon displacement by primer extension indicated by fluorescence quenching was recorded at room temperature immediately after addition of 10 μL 10 mM dNTPs containing five units 3' (exo-) *Bst* DNA polymerase large fragment. The excitation and emission wavelengths were set at 488 and 515 nm, respectively.

2.6.2. Human genomic DNA samples analysis

Three tubes of the enzyme digested PCR mixture (240 μL), 6 μL 20 μM MB2, and 6 μL 20 μM 3'-allelic specific primer were added to 360 μL 10 mM Tris–HCl buffer solution (pH 7.9) containing 50 mM NaCl and 10 mM MgCl_2 . The mixture was heated at 94 $^\circ\text{C}$ for 4 min for denaturation, and then it was cooled to room temperature for 20 min to allow the primer and the molecular beacon to hybridize with the template. Then, the mixture was transferred to the quartz cuvette. The fluorescence spectroscopy and reaction kinetics of the displacement of the molecular beacon by primer extension were taken by the same procedure as described for synthetic templates system.

3. Results and discussion

3.1. Assay of synthetic templates system

To demonstrate the feasibility of the designed method a model system with synthetic primer, template, and molecular beacon was used at the beginning. Two synthetic 45-mer templates (T1 and T2) have identical sequence except for the position 20 where the 3'-termini of the primer hybridizes. Based on the templates sequences two synthetic 20-mer primers (alleles 1 and 2) were used, which have identical sequence except for 3'-termini positions where each of the two bases is represented. MB1 is a 30-mer polynucleotide with 25 continuous bases hybridizes to the 45-mer template. All sequences are shown in Fig. 2.

One set of experiments was carried out using T1 as the only template: each primer was dissolved in two separate reaction buffers containing appropriate concentrations of T1 (100 nM), MB1, and dNTPs. The reaction dynamics triggered by adding 3' (exo-) *Bst* DNA polymerase large fragment were recorded directly by fluorescence spectrometry. $Q_f\%$ refers to the fluorescence quenching rate. The results are shown in Fig. 3(a). It can be seen that the 3'-termini base of the primer 1 (allele 1) which is perfectly complementary to T1 displayed a significant fluorescence quenching rate; the primer 2 (allele 2) with 3'-termini base mismatch to T1 displayed no obvious fluorescence changes. The other set of experiment was designed for the mixed synthetic

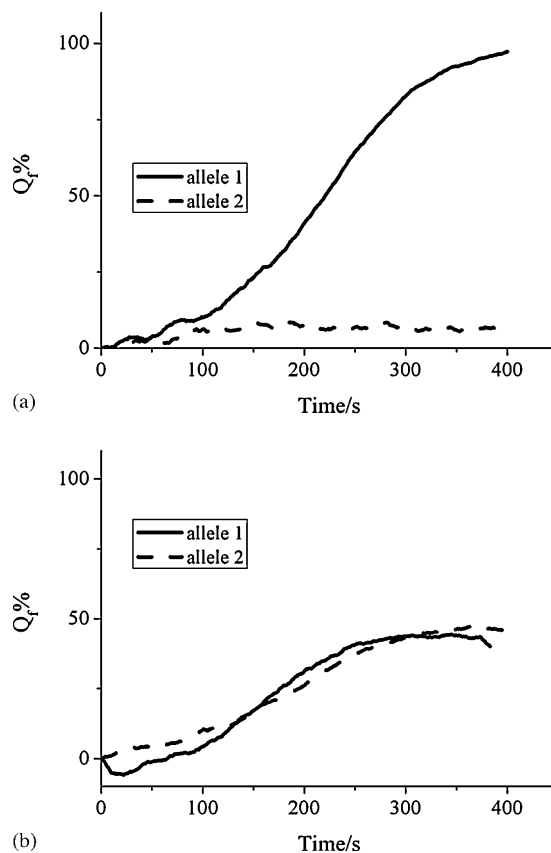


Fig. 3. Time dependence of the fluorescence intensities (expressed by $Q_f\%$) during the primer extension and molecular beacon displacement reaction catalyzed by 3' (exo-) *Bst* DNA polymerase large fragment. Two primers annealed to the template T1 (a) or T1 + T2 (b), only the primers (allele 1 in (a) and alleles 1 and 2 in (b)) with 3'-termini perfectly match the templates could extend and displace the molecular beacon with fluorescence quenching.

templates (T1 + T2). Each primer was dissolved in two separate reaction buffers containing equimolar T1 and T2 (50 nM each). The results are shown in Fig. 3(b). It was found that the allele 1 and allele 2 reactions all displayed fluorescence quenching, but not as significant as that of primer 1 reaction in Fig. 3(a) where 100 nM single template T1 was used. The fluorescence quenching rate $Q_f\%$ in the mixed templates reactions is 43–50%, about half of that from the single template (T1) reaction that shows a change of 87%. Both sets of the experiments showed that $Q_f\%$ of the 3'-mismatched primer systems are very low, ranging from 1 to 8% approximate to the instrumental fluctuation. So, the 3' (exo-) *Bst* DNA polymerase large fragment almost does not catalyze the extension of the mismatching primers. In general, the results revealed that with proper molecular beacon synthesized, we could detect each mutation base by designing proper 3'-termini base specific primer.

3.2. Assay of PCR products from human genomic DNA samples

To confirm that the method described above can be used in detection of real samples, we collected a panel of unrelated tissue samples of breast cancer individuals. Their specific

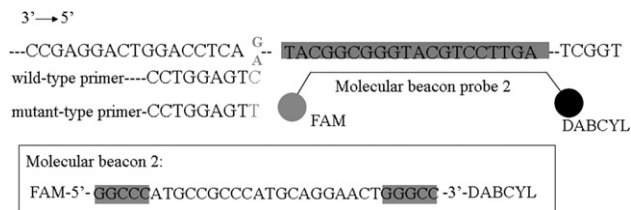


Fig. 4. Sequence of target DNA (gloomy marked is the part that is complementary to the beacon), primers together with allele positions, and molecular beacon 2 (MB2, gloomy marked is the stem part) in extension-strand displacement method for detecting breast cancer samples.

fragment of genomic DNA was amplified and detected. These samples were studied to determine if the second base of codon 259 (AGA) in exon 7 of *p53* gene is mutated or not. Sequences of allele primers and MB2 are shown in Fig. 4. The genotypes are depicted in Fig. 5.

The first is wild-type primer with the base of C at its 3'-end. For the wild-type homozygotes, mutant heterozygotes (G to A mutation), and mutant homozygotes (G to A mutation, n refers to the number of samples) the $Q_f\%$ are 38.8 ± 5.8 (mean \pm S.D., $n=6$), 18.6 ± 2.8 ($n=3$), and 7.6 ± 1.3 ($n=3$), respectively. The second is the mutant-type primer with the base of T at its 3'-end. The $Q_f\%$ for mutant homozygotes (G to A mutation), mutant heterozygotes (G to A mutation), and wild-type homozygotes are 35.8 ± 8.4 ($n=3$), 15.7 ± 3.2 ($n=3$), and 7.0 ± 2.6 ($n=6$), respectively. Therefore, the parameter $Q_f\%$ proposed can be used to discriminate the matched primers from the mismatched ones or to confirm the type of bases in the template samples. The genotypes of the samples elucidated by present method were confirmed by DNA sequencing. The $Q_f\%$ of the matched primer systems distinguish obviously from those mismatched ones. For the mutant heterozygotes both wild-type primer and mutant primer systems give the close $Q_f\%$ values which suggest that the alleles in the mutant heterozygotes were half mutated.

It is noticeable that the results from the real samples are not as good as those for the demonstrating template systems in the fluorescence quenching rate. This might be due to the complex matrix effect of the real sample systems where a number of steps for target DNA fragment preparation are involved before the final assay. The multiple sample processing may destroy the target template which would affect the hybridization with the molecular beacon. More importantly, for the much complex genomic DNA system, the efficiency and specificity of the PCR reaction cannot be compared with the synthetic single-strand DNA system, because the competitive hybridization of the molecular beacon with the double strand target template is in a relatively low efficiency compared with synthetic ssDNA. So the reliability of the method is largely dependent on the efficiency and selectivity of the PCR, also the sample preparation and primers design. Nevertheless, the differences are still large enough that allow us to have an unambiguous differentiation of the wild type from the mutant ones or the vice versa.

The results indicated that 3' (exo-) *Bst* DNA polymerase large fragment that can well discriminate the match and mismatch of the 3'-termini bases of the primers could be used to detect wild type and mutant target alleles. 3' (exo-) *Bst* DNA polymerase

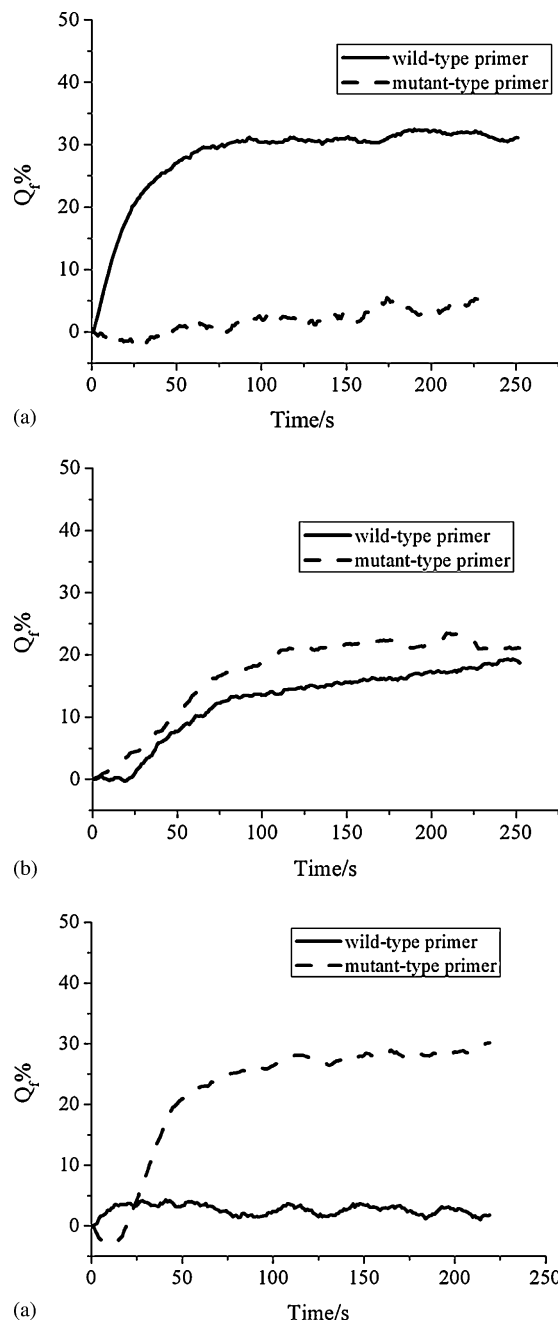


Fig. 5. Time dependence of the fluorescence intensities (expressed by $Q_f\%$) in the allele specific extension-strand displacement method for codon 259 (AGA) mutation genotyping: (a) G/G homozygote, (b) G/A heterozygote, and (c) A/A homozygote.

large fragment exhibits 5'-3' polymerase activity at room temperature. Lacking of 3' and 5' exonuclease activities of DNA polymerase are important in the assay. *Bst* DNA polymerase lacks 3'-5' proofreading exonuclease activity and 5' exonuclease activity. Whether the 3'-termini of the primer is complementary to the corresponding template or not is crucial for the point mutation analysis. Because *Bst* DNA polymerase is in defect of 3'-5' proofreading exonuclease activity it can only catalyze the extension of the matched primers but not the mismatched ones. The 5' exonuclease activity deficient of *Bst* DNA polymerase also ensures the molecular beacon stay in its intact form during the

experiment. So comparing with reported methods in which more than one molecular beacon hybridizing to the mutation point [35,36], the discernment of the 3'-termini bases of the primers is by the 3' (exo-) *Bst* DNA polymerase large fragment which is the key to detect the point mutation. In this method, Vent_R[®] (exo-) DNA polymerase was used in the amplification of exon 7 of the breast cancer sample. The Vent_R[®] (exo-) DNA polymerase lacks 3'-5' proofreading exonuclease activity and 5' exonuclease activity. Without adding the 3' (exo-) *Bst* DNA polymerase large fragment, the Vent_R[®] (exo-) DNA polymerase would also extend the matched allele specific primer and replace the molecular beacon, but the fluorescence quenching dynamic process was very slow. When adding the 3' (exo-) *Bst* DNA polymerase large fragment, the fluorescence quenching dynamic process became much faster, so the 3' (exo-) *Bst* DNA polymerase large fragment displays higher polymerase activity at room temperature. Due to the inherent fluorescence signal transduction mechanism, a molecular beacon functions as a sensitive probe for real-time monitoring, so in this method molecular beacon was chosen as the sensitive signal tracer.

The concentration of molecular beacon used is higher than PCR product, so the competition from the annealing of double-strand PCR product becomes less significant that ensured a relatively high start fluorescence signal. In addition, less restriction on the sequence of the molecular beacon is needed. In principle, any sequence downstream the primer within the chain extension range can be used though a sequence near the primer might be favored. In practice, the length of the probe sequence is chosen so that it will form a stable hybrid with its target sequence at assay temperature, whereas the arm sequences are chosen so that they will form a stable stem hybrid when there is no target present. Traditional allele primer extension PCR system need to design more primers and optimization experiments might require additional primers with mismatches at the ($n - 1$) base adjacent to the 3'-termini mismatch to decrease the amplification of the wild type [37]. The method present in this paper exhibits a much greater specificity, only one mismatch at the 3'-termini is enough, it gives a +/- switch and elicit the accurate base type of the possible mutation point. When the 3'-termini of the primer matches the template, molecular beacon can be replaced and fluorescence would quench (+). On the other hand, when the 3'-termini of the primer mismatches the template, molecular beacon cannot be replaced and fluorescence remains (-). The base type of the mutation point becomes obvious from the +/- switch. Clearly, the fidelity of the polymerase used ensures a false positive-free result obtained at least at the qualitative level.

A question might arise that why use two step reactions (first step to amplify a DNA fragment containing possible mutation sites and a second step to detect if the queried site within the amplified fragment is mutated or not) rather than using a real-time PCR with a Taqman assay (configured in a allele specific format) since the latter one is expected to give the similar result. The idea is that the efficiency in discriminating the matched from the mismatched is the highest in the first cycle of reaction and will decrease as the allele specific PCR reaction circles increase. In the present method, the allele specific reaction is carried out for only one circle and the result was obtained. Therefore, the one

cycle reaction could reduce the non-specific reactions between the primers and template of the "wrong" genotype and improves the specificity of the allele specific PCR. Furthermore, when the sample questioned is very limited and the multiple sites with a great number of experiments are required, the present method provide an opportunity to amplify and even re-amplify the target DNA fragments to enough quantity before the detection step. And it is also possible to amplify different fragments within the human genome at the same time in the first PCR step and then the sites being queried within a same fragment or distributed in different DNA fragments can be studied separately. The major disadvantage of the present method is that it cannot be multiplexed in its current format meaning that four different reactions will be required to determine the allelic composition at one locus unless the possible mutation types are known that may reduce the number of primers or reactions.

Demands for genetic point mutation testing are expected to increase dramatically in the areas of diagnostic, forensics, and population studies, but such studies are currently difficult and expensive even for well-equipped laboratories. A simple, standardized DNA diagnostic test will make it possible for any clinical laboratory design and perform carrier testing within a kindred. The presented PCR/hybridization assay offers a simple, fast, and false-free method for single point mutation with simple instruments and low cost. In general, this method can be applied for the diagnostic of known mutation in disease, e.g., in cancer, but the high throughput of this method need further developing to set up large scale studies designed to establish the risk associated with certain genetic defects.

4. Conclusion

In conclusion, the present work using molecular beacon demonstrates allele specific primer-strand displacement method is capable of functioning as a highly specific, sensitive recognition, and signaling assay for the possible point mutation analysis. Although the design of allelic primers and molecular beacon are required, the design of allelic primers, molecular beacon, and PCR optimization are easy to realize. With the genotyping of the possible mutation in the *p53* gene using this method, we are going to increase the number of allele specific primer-strand displacement reactions that can be multiplexed and analyze other possible mutation hotspots within the *p53* gene. Although in this report the method is only applied for point mutation analysis, SNP can also be detected by this method. Furthermore, the use of several molecular beacons each coupling to a different fluorophore should enable simultaneous detection of multiple possible mutation sites. If a real-time PCR instrument is used instead of using a spectrofluorometer, the sensitivity will be increased and the quantity of DNA required can be highly reduced. With enough pre-amplified DNA fragments up to 96 experiments can be carried out within a same plate.

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