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Universal Molecular Beacon-Based Tracer System for Real-Time Polymerase Chain Reaction

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DNA diagnostic has been moving from expensive, low-throughput, multistep methods to inexpensive, higher throughput, closed-tube, and automated methods. Fluorescence is the favored signaling technology for such assays. In this method, we describe a universal molecular beacon (U-MB) as the fluorescent tracer in the real-time PCR technique. A 5′-universal template primer (5′-UT primer) has been designed with a tail in complementary to the loop and 5′-side arm sequence of U-MB at the 5′-end of forward target specific primer. As PCR cycles increase, a new DNA fragment with a 5′-UT primer tail is synthesized, which is used as the template for next PCR cycle. As the reverse primer extends to the 5′-UT primer tail, the U-MB hybridized is displaced and the fluorescence from the fluorophore of the U-MB is quenched, indicating that the allele-specific PCR is in progress. This tracing system combined with an allele-specific reverse primer and vent (exo-) DNA polymerase, a polymerase that lacks 3′- to 5′-exonuclease activity, was used for the detection of point mutations of base G in codon 259 (AGA) of exon 7 of *p53* gene on a panel of breast cancer individuals.

Nucleic acids analysis has become increasingly important in a variety of applications, such as the genotyping of individuals, the diagnostics of heredity diseases, the detection of infectious diseases, and disease-related genetic mutations.[1−3] Techniques based on PCR provide a powerful tool for the amplification of a minute amount of initial target DNA sequences.[4,5] High-throughput, cost-effective genotyping methods are essential for making the most advantageous and immediate use of these mutation data.[6] To date, a variety of probe-based, real-time PCR genotyping strategies have been developed.[7,8] Most of these methods involve PCR amplification of a short DNA fragment containing the mutation sites to be detected in the presence of specific detection probe. These methods all depend on sequence-specific hybridization of fluorescent oligonucleotide probes. Currently, widely used probes include TaqMan (5′-exonuclease assay) probe,[9,10] molecular beacon,[11,12] and Scorpion primer probe.[13,14] Though more or less satisfied in practical use, it is relatively expensive and troublesome in practical use. Recently, several simple and less expensive methods that use universal probes have been developed. One method combines a universal forward primer (Uniprimer) with a 5′-tailed forward primer.[15,16] The second universal tracing system reported previously is based on the use of a 5′-tailed forward template primer (UT primer)[17] and TaqMan probe, which is hydrolyzed due to the 5′-exonuclease activities of DNA polymerase. This leads to the separation of the fluorophore and the quencher at the two ends of TaqMan probe and the generation of fluorescent signal. The 5′−3′-hydrolysis requires that the fluorophore and the quencher moieties in the TaqMan probe are not too close to each other.[18] However, the efficiency of energy transfer decreases with the inverse sixth power of the distance between the fluorophore and quencher.[19] Compared with a molecular beacon, the TaqMan probes generally show high background fluorescence due to relatively low quenching efficiency, which compromised the assay sensitivity.

The principle of the present real-time PCR method is presented in Figure 1, which is based on allele-specific amplification.[20−22] A

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(1) Kruglyak, L. *Nat. Genet.* **1997**, *17*, 21−24.
(2) Weiss, K. M. *Genome Res.* **1998**, *8*, 691−697.
(3) Risch, N.; Merikangas, K. *Science* **1996**, *273*, 1516−1517.
(4) Schweitzer, B.; Kingsmore, S. *Curr. Opin. Biotechnol.* **2001**, *12*, 21−27.
(5) Heid, C. A.; Stevens, J.; Williams, P. M.; Livak, K. J. *Genome Res.* **1996**, *6*, 986−994.
(6) Kwok, P. Y. *Pharmacogenomics* **2000,** *1*, 95−100.
(7) Ranasinghe, T. R.; Brown, T. *Chem. Commun.* **2005**, *44*, 5487−5502.
(8) Salvatore, A. E.; Marras, Tyagi, S.; Kramer, F. R. *Clin. Chim. Acta* **2006**, *363*, 48−60.
(9) Livak, K. J.; Marmaro, J.; Todd, J. A. *Nat. Genet.* **1995**, *9*, 341−342.
(10) Holland, P. M.; Abramson, R. D.; Watson, R.; Gelfand, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7276−7280.
(11) Kostrikis, L. G.; Tyagi, S.; Musa, M. M.; Ho, D. D.; Kramer F. R. *Science* **1998**, *279*, 1228−1229.
(12) Tyagi, S.; Bratu, D. P.; Kramer, F. R. *Nat. Biotechnol.* **1998**, *16*, 49−53.
(13) Nazarenko, I. A.; Bhatnagar, S. K.; Hohman, R. J. *Nucleic Acids Res.* **1997**, *25*, 2516−2521.
(14) David, W.; Jane, T.; Simon, P. G.; Tom, B.; Steve, L. *Nat. Biotechnol.* **1999**, *17*, 804−807.
(15) Nuovo, G. J.; Hohman, R. J.; Nardone, G. A.; Nazarenko, I. A. *J. Histochem. Cytochem.* **1999**, *7*, 273−279.
(16) Bengra, C.; Mifflin, T. E.; Khripin, Y.; Manunta, P.; Williams, S. C.; Jose, P. A.; Felder, R. A. *Clin. Chem.* **2002**, *48*, 2131−2140.
(17) Zhang, Y. L.; Zhang, D. B.; Li, W. Q.; Chen, J. Q.; Peng, Y. F.; Cao, W. *Nucleic Acids Res.* **2003**, *31*, e123.
(18) Lyamichev, V.; Brow, M. A. D.; Dahlberg, J. E. *Science* **1993**, *260*, 778−783.
(19) Selvin, P. R. *Methods Enzymol.* **1995**, *246*, 300−340.
(20) Newton, C. R.; Graham, A.; Heptinstall, L. E.; Powell, S. J.; Summers, C.; Kalsheker, N.; Smitj, J. C.; Markham, A. F. *Nucleic Acids Res.* **1989**, *17*, 2503−2516.
(21) Wu, D. Y.; Ugozzoli, L.; Pal, B. K.; Wallace, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2757−2760.
(22) Myakishev, M. V.; Khripin, Y.; Hu, S.; Hamer, D. H. *Genome Res.* **2001**, *11*, 163−169.

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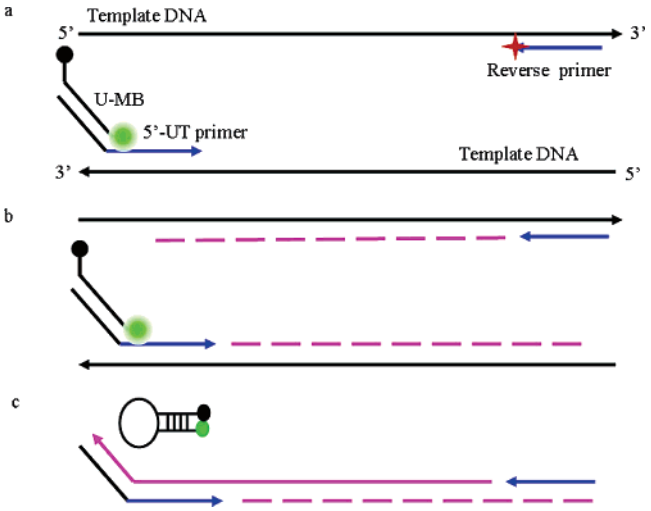

Figure 1. Schematic presentation of signal generation of the real-time PCR amplification. (a) Annealing, 5′-UT−U-MB hybrid and reverse primer specifically hybridized to the target DNA. (b) Extension, UT primer and reverse primer are extended and new DNA fragments with the 5′-UT-U-MB hybrid are synthesized, which are used as the template for next PCR cycles, (c) Reverse primer, hybridized with the synthesized DNA fragments, extends and replaces the prehybridized U-MB, which returns to the closed form and the fluorescence from the fluorophore of the U-MB is quenched, indicating that the PCR is in progress.

5′-universal template primer (5′-UT primer) is designed with a 5′-tail complementary to the loop and 5′-side arm sequence of the universal molecular beacon (U-MB). The 3′-end of the 5′-UT primer has a target-specific primer sequence. As PCR progresses, new DNA fragments with the 5′-UT primer tail are synthesized, which are used as the template for next PCR cycles. As a target-specific reverse primer extends to the 5′-UT primer tail, the prehybridized U-MB is displaced and the fluorescence from the fluorophore of the U-MB is quenched, indicating that the PCR is in progress. Combined with an allele-specific reverse primer and a vent (exo-) DNA polymerase that lacks 3′- to 5′-exonuclease activity, this PCR tracing system was used for the detection of a G to A point mutation in codon 259 of exon 7 on *p53* gene in breast cancer individuals. Theoretically, the tracing system proposed can be extended to any kind of real-time PCR systems, which substantially reduces the cost and simplifies experiment design.

EXPERIMENTAL SECTION

DNA Extraction. 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 Co. (Beijing, China). According to manufacturer's instructions, 100 μL of genome DNA (10−30 μg) extracted was obtained from 30 mg of breast cancer tissue. The genome DNA solution was kept at −80 °C for further use.

Design of the U-MB and Primers. The U-MB has a universal sequence of poly(AG)$_{10}$ in its loop part, which is designed to avoid significant sequence similarity with any known human genomic sequence especially those of the *p53* gene. The loop and one arm of the stem of the U-MB are complementary to the 5′-end of the 5′-UT primer. The U-MB is labeled with 6-carboxyfluorescein (FAM) on the 5′-end and 4-(4′-dimethylaminophenylazo)benzoic acid (DABCYL) on the 3′-end. The sequence of U-MB is 5′-FAM-CCCGG(AG)$_{10}$CCGGG-DABCYL-3′. And the sequence of the 5′-UT primer is 5′- (CT)$_{10}$CCGGGGTGTTGTCTCCTAGGTTGGC-3′. The 3′-end of the reverse primer is specific for the G variant of the codon 259 in exon 7 of the *p53* gene. The sequences of the reverse primers 1 to 4 are 5′-GTGGCTCCTGACCTGGAGTN-3′, where N at the 3′-end represents C, T, A, and G, respectively. Primers and U-MB were synthesized and purified by Sangon Co. (Shanghai, China)

Optimization of Assay Conditions. The amplified fragment (103 bp) was within exon 7 of the *p53* gene of the wild-type homozygote samples. G allele was used as reverse primer. Optimization of 5′-UT primer versus reverse primer concentrations was carried out in 50 μL of reaction mixture with the concentration of U-MB fixed at 600 nM. With the concentration of the reverse primer at 50, 100, 300, or 600 nM, the concentration of 5′-UT primer at 50, 100, or 300 nM, was studied respectively. Each of the 50 μL of reaction solution also contains 0.4 mM dNTPs each (Tiangen Biotechnology Co., Beijing, China), 1 unit of Vent$_R$ (exo-) DNA polymerase (NEB), and 1× allele-specific buffer (20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100). Concentrations of reverse primer versus U-MB were further optimized with an optimal concentration of 5′-UT primer at 100 nM. The [reverse primer] (nM)/[U-MB] (nM) ratios used were 100:100, 128:100, 160:100, 100:128, 128:128, 160:128, 100:160,128:160, and 160:160. The compositions of all other reagents in the reaction mixture were the same as those described above.

Allele-specificity PCR discrimination efficiencies were detected using a different quantity of Vent$_R$ (exo-) DNA polymerase. Each reaction system (50 μL) contained 1× allele-specific buffer, 0.4 mM dNTPs each, 100 nM 5′-UT primer, 160 nM reverse primer, and 100 nM U-MB. The units of Vent$_R$ (exo-) DNA polymerase studied included 1, 1.2, 1.4, 1.6, 1.8, and 2.0 for each tube.

The total cycles for the real-time PCR were set at 35−50. The PCR amplification was carried out in a sealed tube in a Stratagene Mx3000*p* real-time PCR instrument with a program of 94 °C denaturation for 50 s, 50 °C annealing for 1 min, 72 °C extension for 1 min, and finally 25 °C for 1 min to cool down for high fluorescence quenching efficiency. Fluorescence measurements were taken at the end of the cooling-down segment.

Point Mutation Genotyping. The reaction mixture (50 μL) is composed of 1× allele-specific buffer, 100 nM 5′-UT primer, 160 nM reverse primer, 100 nM U-MB, 1 unit of Vent$_R$ (exo-) DNA polymerase (NEB), and 0.4 mM dNTPs each. The human genomic DNA extract was thawed, and 6 μL from it was directly used for each reaction. For no template controls, 6 μL of ddH_2O was used. Real-time PCR was run with the following program: initial denaturation at 94 °C for 10 min, followed by 40−50 cycles, which are the same as that described above.

DNA sequencing was performed on an Applied Biosystem 377 Genetic Analyzer by AuGCT Biotechnology Co. (Beijing, China).

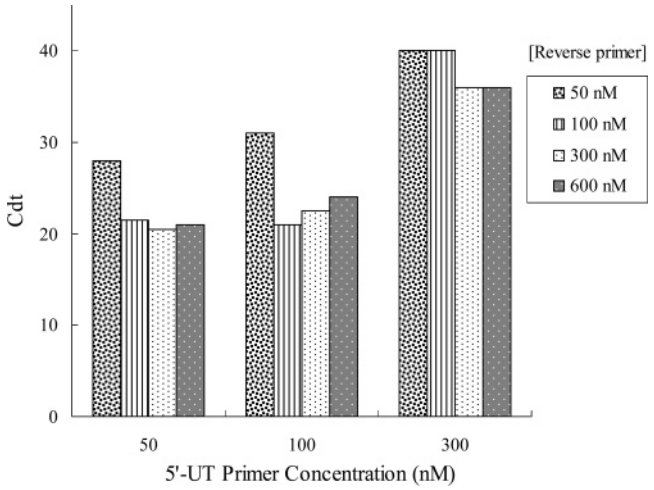

Figure 2. Optimization of 5′-UT primer concentration at different reverse primer concentrations. The concentration of the U-MB is 600 nM.

RESULTS

Optimization of Assay Conditions. A variety of experiments were performed to establish optimal assay conditions. The major influencing factors for the assay are concentrations for primers and U-MB as well as the quantity of the $Vent_R$ (exo-) DNA polymerase.[23] Different concentration combinations of the 5′-UT primer, reverse primer, and U-MB were tested to establish the conditions that gave both the greatest amplification efficiency and the highest fluorescence quenching rate.

According to the concentrations of the primers currently used, 5′-UT primer and reverse primer in the range of 50−600 nM with the U-MB concentration fixed at 600 nM were studied. As shown in Figure 2, all primer concentration combinations give comparable Cdt values. Cdt is defined as the PCR cycle at which the fluorescence quenching rate (Q_f) equals a threshold value of 15% or relative fluorescence intensity (R_f) equals 85%. It is best to get the Cdt values as low as possible, and it is also important to reduce the overall primer concentration to reduce the reagent cost. The 100 nM 5′-UT primer and the 50 nM reverse primer system give low-end Q_f. After analyzing the Cdt, Q_f, and experimental cost, 100 nM 5′-UT primer and 100 nM reverse primer system were chosen for further optimization.

With the 5′-UT primer concentration at 100 nM, the concentrations of reverse primer and U-MB were further optimized. Apparently, the concentration of U-MB should be close to the concentration of the 5′-UT primer while that for the reverse primer should be higher than both the U-MB and 5′-UT primer. This will ensure complete displacement of U-MB during amplification. Concentrations of the reverse primer and U-MB ranging from 100 to 160 nM were studied. Optimal U-MB and reverse primer concentrations were also selected on the criterion of the lowest Cdt and the highest Q_f. As shown in Figure 3, 160 nM reverse primer was selected. U-MB should be equal molar or a bit more than the 5′-UT primer. This is quite understandable since U-MB must hybridize with all the 5′-UT primer to indicate the PCR amplification, while less U-MB could not denote all extension of the reverse primer. The lower the overall concentration, the less chance that primers will interfere with one another, and more U-MB might bring a high background. Thus, 100 nM U-MB was selected as optimal.

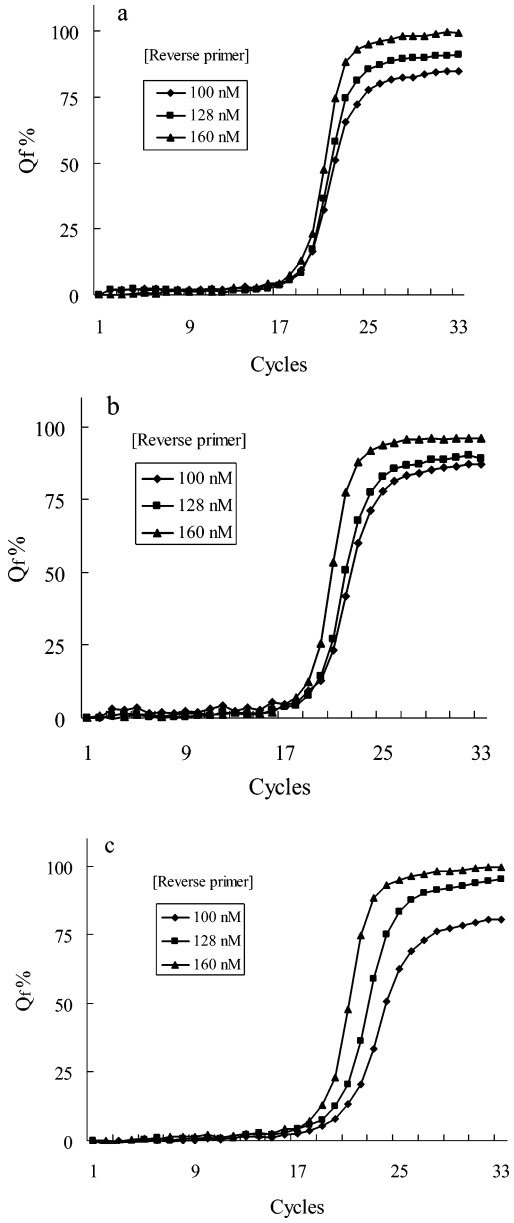

Figure 3. Optimization of reverse primer (the wild type) and U-MB concentrations under optimized 5′-UT primer concentration (100 nM). The concentrations of U-MB are 100 (a), 128 (b), and 160 nM (c).

Minimizing of nonspecific amplification is also very important since the reverse primers also amplify template DNA with low efficiency when 3′-terminal mismatches. The specificity is highly dependent on the type and also the quantity of the DNA polymerase used. For the polymerase selected, the influence of its quantity in each reaction tube on amplification specificity was evaluated. The quantities of the $Vent_R$ (exo-) DNA polymerase used include 1, 1.2, 1.4, 1.6, 1.8, and 2.0 units. It is evident that the discriminating power (Cdt differences) between matching and mismatching systems are polymerase quantity dependent (Figure 4). High Cdt differences indicate high differentiation capability at the polymerase quantity used. Thus, the polymerase with 1 unit per tube was selected and used for further study.

(23) New England Biolabs, U.S. Patent No. 5,322,785.

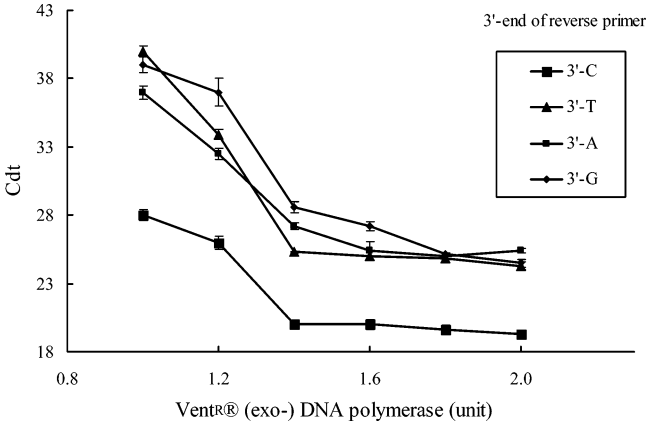

Figure 4. Dependence of PCR discrimination power on the quantity of Vent$_R$ (exo-) DNA polymerase. The concentrations of 5′-UT primer, U-MB, and reverse primer are 100, 100, and 160 nM, respectively.

Point Mutation Genotyping. The U-MB system was applied for the analysis of point mutation of G in codon 259 (AGA) of exon 7 in the *p53* gene of breast cancer samples. 5′-UT primer and four reverse primers with the only difference at their 3′-end were used to amplify the 103-bp fragment. The fluorescence output was monitored at the end of each cool-down segment (25 °C for 1 min) and relative fluorescence intensity (R_f) was calculated, which was plotted against cycle number. R_f of PCR reaction without 5′-UT primer and template did not show any appreciable change over the cycles. The genotyping of codon 259 was presented in Figure 5 and the statistical results were summarized in Table 1. The amplification efficiencies or Cdts of different reverse primers for the same sample differed considerably. The Cdt differences between the matching and mismatching primers of the allele-specific amplification are statistically significant ($P < 0.01$) by the Student's *t*-test. Therefore, the parameter Cdt proposed can be used to discriminate the matched primers from the mismatched ones or to confirm the type of bases in the template samples. These results indicate that for all the samples studied they are either wild type or G to A mutated ones (including mutant homozygotes and heterozygotes). The genotypes of the samples elucidated by the present method were confirmed by DNA sequencing.

DISCUSSION

U-MB techniques rely on bimolecular reaction, but they are more efficient than conventional bimolecular reaction probes. In the reaction, U-MB specifically hybridizes with the 5′-universal tail of the 5-UT primer. With the amplification progressing, the U-MB is displaced and the complementary strand of DNA fragment containing the universal tail is synthesized, so the U-MB will compete with the complementary strand when hybridizing with the 5′-universal tail at the annealing temperature. But this does not matter, because in the cooling-down segment, both the unhybridized and the displaced U-MB return to the closed forms. The fluorescence decay induced by the two cases indicates the increase of amplification. In this point, conventional sequence-specific molecular beacon methods are less efficient because the formation of a probe−target hybrid is a bimolecular process whereas the reformation of the beacon to its stem−loop state is an intramolecular one that is kinetically and entropically favored.[24]

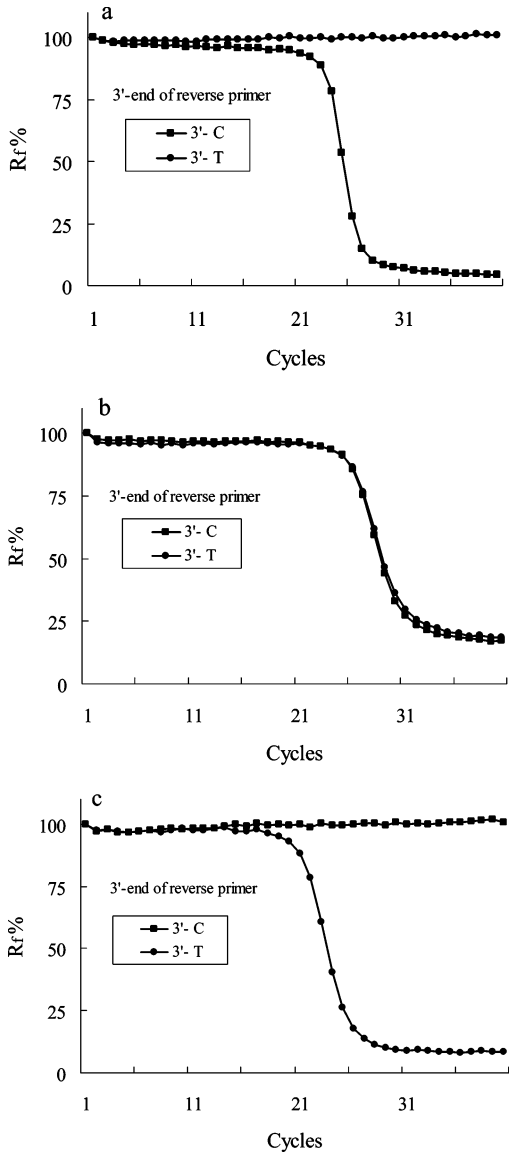

Figure 5. Codon 259 (AGA) mutation genotyping. (a) G/G homozygote. (b) G/A heterozygote. (c) A/A homozygote. The concentrations of 5′-UT primer, U-MB, and reverse primer are 100, 100, and 160 nM, respectively. The quantity of the Vent$_R$ (exo-) DNA polymerase is 1 unit.

A share−stem U-MB is designed that forms more stable duplexes with 5′-UT primer than conventional molecular beacons.[25] In contrast, the arms of a conventional molecular beacon do not bind to the target and are thus more likely to interact with each other as driven by thermal energy, increasing the tendency of forming a closed molecular beacon dissociating from the target. So the difference in thermodynamic behavior between conventional and share−stem molecular beacons can be understood in terms of the flanking arms to interact with each other.

In the U-MB system, the U-MB hybridizes to the external attachment, the 5′-universal tail of the UT-primer, while in the conventional MB real-time PCR techniques, the MB hybridizes

(24) Tsourkas, A.; Behlke, M. A.; Rose, S. D.; Bao, G. *Nucleic Acids Res.* **2003**, *31*, 1319−1330.
(25) Tsourkas, A.; Behlke, M. A.; Bao G. *Nucleic Acids Res.* **2002**, *19*, 4208−4215.

Table 1. Cdts Determined for Point Mutation in Codon 259 of Exon 7 on the *p53* Gene in Breast Cancer with Different Reverse Primers.

3′-end base type of the reverse primer	Cdt[a] ± SD (n = sample numbers) wild-type[b] homozygotes	mutant[c] heterozygotes	mutant[c] homozygotes
3′- C(wild-type)	26.0 ± 2.2(n = 12)	25.3 ± 2.1(n = 3)	39.0 ± 1.0 (n = 3)
3′- T(mutant type)	23.6 ± 1.5 (n = 3)	25.0 ± 2.6 (n = 3)	39.2 ± 2.6 (n = 12)
3′- A		40.0 ± 2.7 (n = 18)	
3′- G		39.2 ± 2.3 (n = 18)	

[a] The Cdt differences between the matching primers and mismatching ones of the allele-specific amplification are statistically significant (P < 0.01). [b] Wild-type homozygotes refer to G allele. [c] Mutant homozygotes refer to A allele.

to the sequence inside the amplified segment. This enable the design of short amplification fragments in contrast to conventional MB real-time PCR methods that require a longer target sequence.[17] In addition, the additional sequence at the 5′-end of the 5′-UT primer causes no decrease in priming specificity.

Allele specificity can be affected by the 3′-exonuclease activity of DNA polymerase.[26−28] The amplification of the mismatched template is usually delayed compared with the matched ones. Ct (cycles needed to surpass a threshold) delay of about four cycles is adequate to allow typing.[29] In general, the quantity of the vent (exo-) DNA polymerase affects the allele-specific PCR efficiency. For the codon 259 (AGA) detection in several breast cancer samples, Cdt values for the G:A/T/G mismatched systems were 10 cycles higher than those from G:C matched system when 1 unit of $Vent_R$ (exo-) DNA polymerase was used. With the increasing quantity of the $Vent_R$ (exo-) DNA polymerase, the allele-specific PCR discriminating efficiency became less distinct. When 2 units of $Vent_R$ (exo-) DNA polymerase were used, the differences of the Cdts between the matched and the mismatched primers were not obvious. Nevertheless, it still could distinguish the matched one (G:C) from the mismatched ones (G:A/T/G).

For any tracing systems used in real-time PCR, the specificity and sensitivity are two important factors to be considered. In the present system, the U-MB sequence contains a $poly(AG)_{10}$ segment that is designed with high competency to avoid significant sequence similarity with any human genomic sequence especially those of the *p53* gene. The hybridization of the U-MB with human genomic DNA is reduced to the lowest level that ensures the detection of fluorescence intensity at the end of each amplification cycle has a low background. Thus, the sensitivity of the method can be increased. U-MB shows higher sensitivity than TaqMan probes because of the presence of a stem structure.[30] Unlike TaqMan probes, the fluorescence produced in U-MB system at each amplification cycle is reversible since U-MB is not destroyed, resulting in a lower overall background. TaqMan assays particularly rely on the 5′-exonuclease activity of DNA polymerase for signal generation, and there is evidence that the 5′-exonuclease activity of DNA polymerase plays an important role in the TaqMan assay, but a recent study demonstrated that the 5′-exonuclease activity of a range of commercially available DNA polymerases varied significantly and some enzymes reported with 5′-exonuclease activity failed to produce any fluorescent signal.[31]

Our work of mutation genotyping employs a U-MB in combination with a 5′-UT primer and reverse primers that differ at the first base of their 3′-end. The U-MB is independent of the sequence of the target gene that is different from the conventional sequence-specific probes. Though it is only used for the study of point mutation in the present work, theoretically it can be extended to any real-time PCR systems where detection of amplification of a target gene is required. In all applications, the same U-MB could be used and the only thing one needs to do is to add the universal tail to the 5′-end of the forward primer. Therefore, the present U-MB system is cost-effective. Furthermore, with the same forward 5′-UT primer, any point mutation in the downstream of the primer but within the PCR extension capability can be detected using different reverse primers. Preliminary study using variable 3′-end at 5′-UT primer instead of at reverse primer gave the similar results. This approach is finally excluded since a short reverse primer with variable 3′-end is much easier to synthesize and purify. The use of a variable 3′-end reverse primer is not only economically favored but also advantageous in the view of selectivity and sensitivity. The present method can also to be extended to multicolor U-MB systems with the restriction by the PCR instrument or the availability of the number of interference-free fluorescence energy-transfer couples. Thus multiple un-overlapped DNA fragments within the human genome might be studied at the same time within a single tube.

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(26) Kwok, S.; Kellogg, D. E.; McKinney, N.; Spasic, D.; Groda, L.; Levenso, C.; Sninsky, J. J. *Nucleic Acids Res.* **1990**, *18*, 999−1005.
(27) Kyger, E. M.; Krevolin, M. D.; Powell, M. J. *Anal. Biochem.* **1998**, *260*, 142−148.
(28) Matsubara, Y.; et al. *Hum. Mutat.* **2000**, *15*, 189−196.
(29) Germer, S.; Higuchi, R. *Genome Res.* **1999**, *9*, 72−78.
(30) Tyagi, S.; Kramer, F. R. *Nat. Biotechnol.* **1996**, *14*, 303−308.
(31) Kreuzer, K-A.; Bohn, A.; Lass, U.; Peters, U. R.; Schmidt, C. A. *Mol. Cell Probes* **2000**, *14*, 57−60.