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A modified molecular beacon combining the properties of TaqMan probe

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A modified molecular beacon that possesses a stem-hairpin structure as seen in conventional molecular beacons and can be cleaved during PCR is designed, and it can specifically recognize the presence of the target and was obviously more sensitive than conventional molecular beacons.

Recently a significant advance in PCR (polymerase chain reaction) is the development of homogenous assay for real-time fluorescence detection of PCR-amplified products in a closed-tube. In general, homogeneous detection provides advantages of high sensitivity, specificity, ease of use, and multiplexing ability. So far, TaqMan[1] and molecular beacons[2] are the two most commonly used read-out probes in homogeneous detection.

TaqMan are linear probes with quite high background signals. The stem-hairpin structure of molecular beacons ensures maximal quenching efficiency and minimal background signals. But for this technology a critical problem is its sensitivity. The molecular beacon is an internal probe, which is complementary only to its target strand of double-stranded amplicom. Only the fraction of the probe which successfully competes against the complementary strand of the target in the hybridization reaction will fluoresce.

In this work we have designed a modified molecular beacon probe (TaqMan–MB) that combines the advantages of both TaqMan probes and molecular beacons. This probe consists of a stem-loop structure to ensure a low background signal and good specificity, as for conventional molecular beacons, and, besides the loop sequence, also the 5′-end arm sequence of the probe is complementary to a predetermined target DNA. On other hand the probe hybridizes to the PCR product and is hydrolyzed between the fluorophore and the quencher by the 5′–3′ exonulcease activity of Taq poymerase during PCR, as seen for TaqMan. Thus, not only the probes which successfully hybridize to amplicom but also the fraction which is cleaved by Taq DNA polymerase provide the fluorescent signal. Therefore, the intensity of the signal will be greatly enhanced. In this study, we use hepatitis B virus (HBV) DNA as a target gene and discuss the advantages of this system over a conventional molecular beacon system.

HBV DNA was extracted from patients serum by the proteinase K-phenol method, the DNA pellet was dissolved in 20 μl Tris-HCl (pH 8.0)–1 mM EDTA (TE), 2 μl was used in PCR amplification.

For PCR the primers (primer1: 5′-CACCAAATGCCCCTATCTTA-3′, primer2: 5′-GTTTCCCACCTTATGAGTCC-3′) were designed to amplify a 180 bp region within a HBV gene. PCR was performed with 1 × PCR buffer, 2 mM $MgCl_2$, 0.4 mM each dNTP, 2.5 U of Taq DNA polymerase, 0.4 μM of each primer, 0.3 μM of TaqMan–MB (FAM-5′-CCCGAGATTGAGATCTTCTGCGACTCGGG-3′-DABCYL) or molecular beacon A (FAM-5′-CGAGCATCTTCTGCGACGCGGGCTCG-3′-DABCYL) and 2 μl template. Cycling is designed with a pre-cycle (95 °C for 8 min), 40 cycles for amplification (94 °C for 30 s, 63 °C for 15 s and 59 °C for 1 min) and one cycle of post-amplification (95 °C for 5 min and 59 °C for 15 min). Fluorescence was measured at E_x = 490 nm, E_m = 516 nm.

Our aim is to design a modified molecular beacon that can work as a TaqMan probe during PCR amplification. So the probe must bind to the target before the primer extension occludes the probe-binding site. This can be achieved by manipulating the sequence and length of the probe. In this study, we designed a TaqMan–MB probe with 23 nucleotides (nt), a 6-nt arm sequence and a 17-nt loop sequence. Unlike conventional molecular beacons, not only the loop sequences but also the 5′-end arm sequence can bind to the target. John Santalucia's 'Nucleic Acid Hybridization' software was used to calculate the melting temperatures (T_ms) of the TaqMan–MB's probe sequence and the two primers. In 100 mM NaCl and 2 mM $MgCl_2$ the predicted T_m of TaqMan–MB is 67.4 °C, of primer1 is 60.7 °C and of primer2 is 61.2 °C. Therefore TaqMan–MB can bind to the target at higher temperatures than primers. So we added a probe hybridization step between the PCR denaturation step and the anneal–extend step. At this step only TaqMan–MB could bind to the target, whereas the primers could not bind to the target and no extension occurs. We performed a series of PCR amplifications with different anneal–extend temperatures. It was found that the PCR product could be generated by using 57, 59 or 61 °C as the anneal–extend temperature, but the band intensity for 61 °C was much lower than that for 57 and 59 °C. By using 63 °C as the anneal–extend temperature no products were generated. Thus, we selected 63 °C as the probe hybridization temperature and 59 °C as the anneal–extend temperature.

The quenching efficiency of light emitted from the fluorophore by the quencher was calculated according to the following formula: $\text{Eff} = [1 - (F_{uh} - F_b)/(F_{ch} - F_b)] \times 100\%$. Here F_{uh} and F_{ch} are the fluorescence intensities of the unhybridized and the completely hybridized probe, respectively, F_b is the background fluorescent intensity of buffer only. For the two probes, the quenching efficiencies are 91.0% for TaqMan–MB and 93.1% for molecular beacon A, which are lower than that reported previously: 99.9%[3] and 96%,[4] but much higher than normal TaqMan probes. Therefore, it ensures a low level of background for signals.

We monitored three different polymerase chain reactions in real time (Fig. 1). For all three reactions, the strength of the fluorescent signal increased as the number of cycles increased. By comparison of these three reactions we found that reactions 2 and 3 had a similar fluorescent signal change, and reaction 1 had a much stronger fluorescence change.

For reaction 2 the TaqMan–MB probe was added to the PCR mixture after amplification and the fluorescent signal only came from the probe fraction that successfully hybridized to the target. For reaction 3, molecular beacon A with a sequence of 15-nt was used. Its T_m was much lower than the primers. Therefore, it could not bind to the target at the anneal–extend temperature and could not be cleaved by 5′–3′ exonuclease activity of Taq DNA polymerase. So using reaction 2 only the probe hybridization gave a fluorescent contribution. By reaction 1 the probe hybridization step in PCR amplification ensured that TaqMan–MB binding to the target occurred before primer extension. Therefore, it could be hydrolysed between fluorophore and quencher by Taq DNA polymerase, as seen for a TaqMan probe. Thus, not only the probes that successfully

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hybridized to amplicom but also the fraction that was cleaved by Taq DNA polymerase contributed to the fluorescent signal. Therefore, the result was a much stronger fluorescence change.

The structure of TaqMan–MB is a stem-loop just as for conventional molecular beacons. The structural constraints on probe conformation lead to enhanced specificity. Four human serum specimens were analysed by PCR/TaqMan–MB assay. The results showed that only HBV-infected serum gave an intense fluorescent signal over the background. For healthy serum, HCV-infected serum and HEV-infected serum, the fluorescent intensities were very low and could be considered negative.

Some patients may be simultaneously infected by several kinds of viruses such as HBV, HCV, and HEV. To determine whether the TaqMan–MB probe could specifically recognize HBV DNA in mix-infected sera, four mix-infected sera were prepared by mixing HBV-infected, HCV-infected or (and) HEV-infected serum for PCR/TaqMan–MB assay. As expected HCV/HEV mix-infected serum had a low fluorescent signal intensity, all the other three sera gave positive results and the fluorescent intensities were compatible with that of HBV-infected serum. This result shows that TaqMan–MB can specifically detect HBV in mix-infected sera, other concurrent viruses did not interfere with the detection.

HBV DNAs were extracted from healthy serum and serially diluted HBV-infected serum by using the protease K-phenol method. We performed PCR with a template of these HBV DNAs as described above. From Fig. 2 we found that reaction 2 and 3 had a similar detection efficiency, whereas, the intensity of the fluorescent signal by reaction 1 was significantly higher than that by reaction 2 and 3 for each dilution (up to 10^{-6} dilution). These results demonstrated further that degradation of the probe by TaqMan–MB led to enhance the intensity of the signals. Thus, as a nucleic acid probe the optimized TaqMan–MB probe is preferable over conventional molecular beacons for its increased signal intensity and hence for its increased sensitivity in detection of fluorescence analysis by performing PCR.

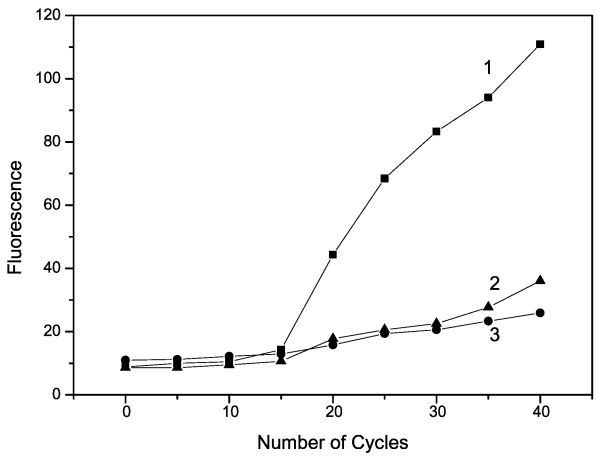

Fig. 1 Monitoring polymerase chain reactions in real time: (1), using TaqMan–MB with the probe added directly to the PCR mixture before the reaction; (2), using TaqMan–MB with the probe added to the PCR mixture after the reaction; (3), using molecular beacon A with the probe added to the mixture before the reaction.

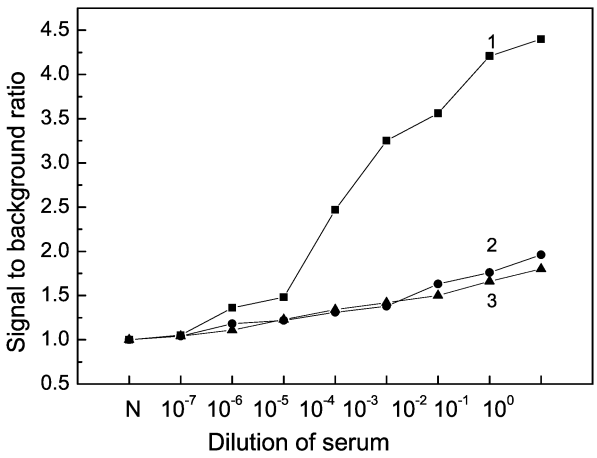

Fig. 2 Relative fluorescence signal from TaqMan–MB and molecular beacon A in the presence of different concentrations of HBV: (1), using TaqMan–MB with the probe added directly to the PCR mixtures before the reaction; (2), using TaqMan–MB with the probe added to the PCR mixture after the reaction; (3), using molecular beacon A with the probe added to the mixture before the reaction.

In this study, we designed a modified molecular beacon probe (TaqMan–MB) which combines the functions of TaqMan probes and molecular beacons. We also performed a 3-step PCR, but it is different from normal 3-step PCR. A probe hybridization step was introduced between the denaturation and the anneal–extend steps. This step ensures that the probe binds to the target before the primer extension occludes the probe binding site and causes probe degradation. Our results shows that TaqMan–MB can specifically recognize the presence of a target and possesses a much higher sensitivity for detection than conventional molecular beacons. The emergence of TaqMan–MB may provide a very effective nucleic acid probe which can be used in homogenous assay of PCR-amplified products in a closed-tube format.

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