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Simulation of TaqMan by two single-labelled probes

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A novel method for duplex probes is designed to simulate the TaqMan probe during polymerase chain reaction (PCR); two single-labelled probes are used for this method, which relies on the 5′-exonuclease activity of nucleic acid polymerase.

Recently, a significant advance in PCR has been the homogeneous 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 two such robust read-out probes. A few months ago we designed a modified molecular beacon (TaqMan-MB)[3] combining the properties of the TaqMan probe. These probes, which are based on fluorescence energy transfer, are characterized by relatively high signal-to-noise ratios and possess a good ability to discriminate between positive and negative reactions. However, for these methods the fluorophore and the fluorescence quencher are dual-labelled on the probes. This complicates the design and the synthesis of the probes[4] and consequently increases the expense of experiments.

We present a novel method for the detection of a specific nucleic acid sequence in a homogeneous solution with two single-labelled probes, relying on the 5′-exonuclease activity of nucleic acid polymerase (Fig. 1). Probe 1 is an oligonucleotide chain matched perfectly to the target sequence and is labelled with a fluorophore at the 5′-end, whereas probe 2 is a complementary chain to probe 1 with several bases mismatched and is labelled with a fluorescence quencher at the 3′-end. At lower temperatures these two probes form a mismatched duplex, which keeps the fluorophore and quencher in close proximity to each other. Therefore the fluorescence of the fluorophore is quenched. At the anneal-extend step of PCR, in which the temperature is higher than the melting temperature (T_m) of the mismatched duplex and lower than that of the perfectly complementary duplex formed by probe 1 with the target sequence, probe 1 is cleaved by 5′-3′ exonuclease activity of *Taq* DNA polymerase, producing an exponential increase in the emission intensity of the reporter dye. Thus, we can deduce the presence of the predetermined target by observing the change of fluorescence at a temperature lower than the T_m of the mismatched duplex. In this study, we use hepatitis B virus (HBV) DNA as a target gene and discuss the advantages of this method over conventional TaqMan probes.

Two single-labelled probes were designed. Probe 1: FAM (6-carboxyfluorescein)-5′-CCGTCTGTGCCTTCTCATCTGCCGG, a 3′-blocking phosphate group is contained to prevent probe extension during PCR cycling. Probe 2: CCGGCAGATGACAAGCCAGAGACGG-3-DABCYL (4-[4′-dimethylaminophenylazo]benzoic acid) (underlines identify mismatched bases), and a corresponding TaqMan probe: FAM-5′-CCGTCTGTGCCTTCTCATCTGCCGG-3′-TAMRA (tetramethylrhodamine).

For PCR, the primers (primer 1: 5′-ATCCTGCGCGGGACGTCCTT-3′, primer 2: 5′-CGTTCACGGTGGTCTCCATG-3′) were designed to amplify a 225 bp region within a HBV gene. PCR conditions were identical for the duplex probe and TaqMan probe, 25 μl reaction mixture consisted of 1 × PCR buffer, 3 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U of *Taq* DNA polymerase, 0.4 μM of each primer, 0.3 μM of either probe, 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, 64 °C for 1 min). Fluorescence emission was measured at E_x = 490 nm, E_m = 516 nm.

In the mismatched duplex, as in molecular beacons, two forms of energy transfer may exist: direct energy transfer, where the donor and acceptor moieties may be in contact, may be dominant,[5] and FRET may also occur over rather longer distances. This mechanism of energy transfer ensures maximal quenching efficiency and minimal background. The quenching efficiency of the quencher to light emission from the fluorophore was calculated according to the following formula: $E_{ff} = [1-(F_q-F_b)/(F_{uq}-F_b)] \times 100\%$. Here F_q and F_{uq} are the fluorescence intensities of the mismatched duplex and probe 1, respectively, F_b is the background fluorescence intensity of the buffer only. For the TaqMan probe, F_q is the fluorescence intensity of the TaqMan probe alone. For the duplex probe the quenching efficiency is 94.4%, which is compatible with that of molecular beacons,[6] and much higher than that of the TaqMan probe: 82.5%. That is to say, our duplex probe has a much better quenching efficiency than the TaqMan probe and ensures a low level of background signal.

Using HBV DNA extracted from an identical serum sample as templates, we monitored three different polymerase chain reactions (Fig. 2). In reaction 1, using our duplex probes, the probes were added directly to the PCR mixture before reaction. This reaction reflects the typical characteristics of PCR. At the initial stage, a small amount of PCR product is synthesized, and the fluorescence of the molecular beacon remains at a low level. With the increasing of cycling numbers, PCR product accumu-

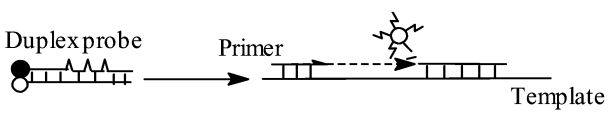

Fig. 1 Assay principle of the duplex probe.

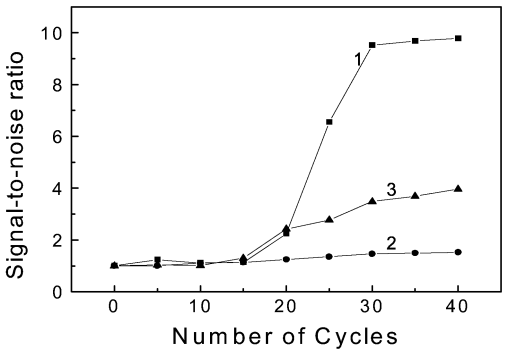

Fig. 2 Monitoring duplex probes assay during PCR: (1) Using the duplex probe, the probe was added directly to the PCR mixture before reaction. (2) Using the duplex probe, but the probe was added to the PCR mixture after reaction. (3) Using the TaqMan probe, the probe was added to the mixture before reaction.

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lates exponentially and the fluorescent signal increases rapidly simultaneously. At the last stage of amplification, the accumulation of PCR product stops due to depletion of reaction components, and the increasing of the fluorescent signal is slowing down. Here the magnitude of fluorescence correlates strongly with the amount of synthesized PCR product.

Our duplex probe possesses some properties of molecular beacons. The fraction of probe 1, which exactly hybridises to amplicon, can also provide a fluorescent signal before cleavage. In order to investigate the hybridisation contribution, we designed reaction 2. In this reaction, probe 1 and probe 2 were added to the PCR mixture after amplification. Thus the fluorescence signal only came from the probe 1 fraction that hybridised to the target. To ensure sufficient hybridisation between probe 1 and amplicon, the solutions were denatured at 95 °C for 5 min, annealed at 50 °C for 15 min, and allowed to cool to ambient temperature. Fluorescent data show that reaction 2 has a much weaker fluorescent signal change than reaction 1. This means that hybridisation only had a very small fluorescence contribution in the PCR/duplex probes assay, and the fluorescence signal mostly comes from the cleavage of probe 1.

As a comparison, we also monitored the fluorescence change of the TaqMan probe (reaction 3). TaqMan has a relatively higher background signal, its change of signal-to-noise ratio is not so remarkable as reaction 1.

From these three reactions, we can find that our duplex probe actually acts as a TaqMan probe during PCR amplification and, with its better quenching efficiency, it can work much better than conventional TaqMan probes.

With the competition of probe 2 our duplex probe is provided with much better specificity than conventional TaqMan probes. Four human serum specimens were analysed by PCR/duplex probe assay. The results show that only HBV-infected serum gives an intense fluorescent signal above background. For healthy serum, HCV-infected serum and HEV-infected serum the fluorescence intensities are very low and can be considered as negative.

Some patients may be simultaneously infected by several kinds of viruses such as HBV, HCV, and HEV. To determine whether our duplex probe can specifically recognize HBV DNA in mix-infected sera, four mix-infected sera were prepared by mixing the HBV, HCV or/and HEV-infected sera equivalently for PCR/duplex probe assay. As expected, HCV/HEV mix-infected serum has low fluorescent signal intensity, all the other three sera (HBV/HCV, HBV/HEV and HBV/HCV/HEV) give positive results and the fluorescence intensities are compatible with that of solely HBV-infected serum. This result shows that the duplex probe 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 (Fig. 3). The result shows that the intensity of the fluorescent signal of the duplex probe was significantly higher than that of the TaqMan probe for each dilution (up to 10^{-6} dilution). Thus, as a nucleic acid probe the optimised duplex probe is preferable to the conventional TaqMan probes with its increased signal intensity and its increased sensitivity in detection of fluorescence analysis by performing PCR.

Compared with dual-labelled probes, single-labelled ones have the advantages of low expense and ease of design, synthesis and purification. During the synthesis of molecular beacons and TaqMan probes, at least two times purification are performed step by step. This will greatly affect the productivity

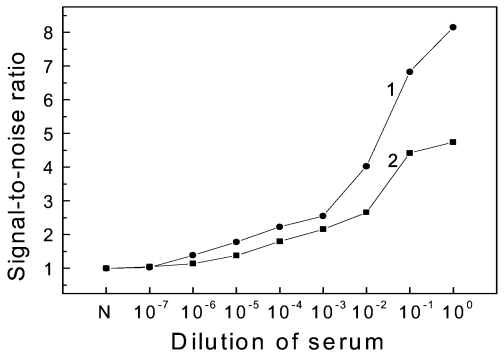

Fig. 3 Relative fluorescence signal from duplex probe (1) and TaqMan probe (2) in the presence of different concentrations of HBV.

of probes. In this paper, we introduce two single-labelled probes which only need one step of purification for each. The process of probe preparation is simplified and less costly. On the other hand, the presence of oligonucleotides, which are only labelled with fluorophore, will significantly increase the background signal and hence affect detective effects. This problem will not occur in our probes. In addition, we can acquire optimised quenching efficiency by adjusting the ratio of the two probes.

The most critical problem associated with TaqMan technology is the distance between the dye and the quencher.[7] If they are close together, the likelihood of cleaving the dye from the quencher drastically decreases, even though a lower background fluorescence is observed. On the other hand, placement of the quencher at the 3′- end of the probe increases the signal during PCR assays, but at the expense of a much higher background.[8] Our duplex probes have a similar energy transfer mechanism as molecular beacons and their quenching efficiency is much better than conventional TaqMan probes. Consequently, a better detective sensitivity can be acquired. Compared with TaqMan probes, another advantage of our duplex probes is that they possess better recognition specificity and this specificity can be adjusted by altering the numbers of mismatched bases between the two probes. For example, if a point mutation is to be detected, we can design a duplex probe with only one base mismatch between the two probes. Thus, in an appreciable temperature range, probe 1 can specifically hybridise to a perfectly matched target. The presence of larger amounts of probe 2 will prevent probe 1 from binding to even one base mismatched templates. If we want to detect a predetermined sequence which can tolerant one base mismatch, two bases mismatched duplex probes can be designed. This makes the experiment more flexible.

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