

## Molecular Beacon-Based Homogeneous Fluorescence PCR Assay for the Diagnosis of Infectious Diseases

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A rapid and simple homogeneous fluorescence PCR assay was developed for the clinical diagnosis of infectious diseases based on a molecular beacon. The established method could reproducibly detect *Mycobacterium tuberculosis* at the 10 bacteria/mL level. The analytical specificity was tested with 14 strains of mycobacterium, four unrelated bacteria and 220 negative samples; no false positive results were obtained. A blind test was also performed to evaluate its performance in *Mycobacterium tuberculosis* diagnosis. The results showed that both the clinical sensitivity and the specificity were 100%, and that the detection limit was in the range of 1–10 bacteria/ml. A clinical study with 466 patient samples demonstrated that fluorescence PCR assay correlated well with smear (93.6%) and culture (98.4%) methods for positive samples. However, fluorescence PCR could detect positive samples (62.9%) more than smear (30.3%) and culture (31.4%), indicating a higher sensitivity of the present method than the traditional ones. The feasibility of this method was further approved by successful detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis*.

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The importance of nucleic acid amplification in the detection of a target sequence derives from its ability to increase the concentration of specific target sequences to levels that are detectable by various traditional techniques. A multitude of schemes for the detection of the products of nucleic acid amplification have been adapted to the polymerase chain reaction (PCR) since its introduction.<sup>1</sup> These detection formats can be divided into two major categories: heterogeneous and homogeneous. Heterogeneous methods are distinguished by the separation of nucleic acid amplification products from amplification reagents before detection. Homogeneous methods allow for the specific detection of amplification products without a separation or a wash step. The current widely used heterogeneous gel electrophoresis method, though simple and cheap, is time-consuming, labor intensive and prone to carry-over contamination. A homogeneous method that can identify the specific amplicon is obviously preferable due to its simplicity, speed and no risk of contamination.

Different kinds of homogeneous detection methods have been designed. The simplest formats are those without a probe, e.g., the use of fluorescent dyes,<sup>2</sup> fluorescence polarization measurement,<sup>3</sup> combination of a fluorogenic primer,<sup>4</sup> etc. However, all of these methods are unable to identify the specific amplicon, and are limited to “clear” PCR reactions. The introduction of a probe can overcome these drawbacks. A variety of probes based on fluorescence energy transfer have been widely used, e.g. double strand probes,<sup>5,6</sup> a double single-strand probe,<sup>7</sup> a cleavable TaqMan probe<sup>8</sup> and a molecular

beacon<sup>9</sup> have been developed. Among them, molecular beacon possesses distinguished advantages regarding specificity, sensitivity and simplicity.

A molecular beacon has a special loop-stem structure and is non-fluorescent in the absence of its target sequence. When in the presence of its target, the molecular beacon's stem will spontaneously open and become fluorescent. Figure 1 is a schematic diagram of the molecular beacon and its role in the

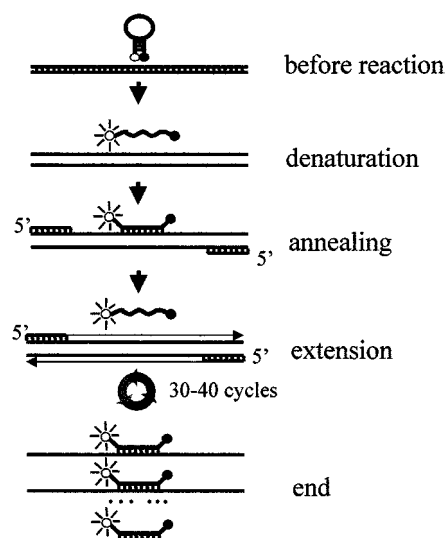


Fig. 1 Schematic diagram of a molecular beacon and its principle for PCR detection.

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PCR reaction. Molecular beacons are non-fluorescent before a reaction. During the denaturation step, they assume a random coil configuration and fluoresce. As the temperature is lowered to allow annealing of the primers, a stem hybrid forms rapidly, preventing fluorescence. However, the molecular beacons also bind to the amplicons and generate fluorescence. When the temperature is raised to allow primer extension, the molecular beacons dissociate from their targets and do not interfere with polymerization. A new hybridization takes place in the annealing step of every cycle, and the intensity of the resulting fluorescence thus indicates the amount of accumulated amplicons which is directly relevant to the original template quantity.

In this work, we established a general homogeneous fluorescence PCR assay based on a molecular beacon for the diagnosis of infectious diseases. With the novel design of the molecular beacon and a simple PCR vial holder on fluorometer, we could detect *Mycobacterium tuberculosis* (TB) in real samples with high sensitivity and specificity. Similar results were also obtained with the detection of *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT). This study shows the great potential of the present method in routine clinical diagnosis of infectious diseases.

## Experimental

### *Bacterial strains, samples and clinical specimens*

TB samples with a known number of bacteria per milliliter ten mycobacterium strains involving *M. kansasii*, *M. scrofulaceum*, *M. smegmatis*, *M. avium*, *M. marinum*, *M. fortuitum*, *M. intracellulare*, *M. vaccae*, *M. gastri*, and *M. phlei*, and four unrelated bacterial strains including *Streptococcus pneumoniae*, *Brucella*, *Escherichia coli* and *Bordetella pertussis* were all from the National Institute for the Control of Pharmaceutical Biological Products (NICBPB, China).

Negative reference samples included normal human sera, sputa and urea. Negative control reagents included physiological saline, distilled water, human DNA, Tris-EDTA buffer and extraction buffer. Positive reference samples were those that had been repeatedly validated by both smear and culture methods. Positive standard samples were BCG vaccine serially diluted with normal human sputum containing  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ ,  $10^1$  bacteria/mL, and TB serially diluted with physiological saline containing  $10^3$ ,  $10^2$ ,  $10^1$  and 1 bacteria/mL. Certified TB standard 98-A containing 22 blind samples was from NICBPB. Totally, 466 specimens from tuberculosis of lungs, kidney and bones and joints were collected and were all tested with acid-fast staining, while 204 of them were cultured according to routine procedures.

### *DNA extraction*

Sputa were mechanically homogenized and liquefied with two volumes of 1 M NaOH in about 20 min. Five hundred microliters of liquefied sample was transferred to a 500- $\mu$ L vial and centrifuged for 10 min at 14000 rpm; the sediment was collected. Five hundred microliters of sterile distilled water was added to the vial and centrifuged for another 10 min; the upper liquid was aspirated and discarded. The addition of water and the centrifugation procedure were repeated once. Subsequently, 50  $\mu$ L of the extraction buffer was added to resuspend the residual by vortex. The vial was then placed in a boiling water bath for 15 min, and centrifuged for 5 min at 14000 rpm. The upper layer was collected and used for a PCR reaction. Urea, blood, joint fluid, cerebrospinal fluid, and hydrothorax were first mechanically homogenized with vortex and then centrifuged for

10 min at 14000 rpm, followed by the same treatment as for sputa. Bacterial samples were first dissolved in physiological water, centrifuged for 10 min at 14000 rpm. Then, 50  $\mu$ L of the extraction buffer was added to the residual and mixed by vortex. The vial was subsequently placed in a boiling-water bath for 15 min, followed by 5 min centrifugation at 14000 rpm. The supernatant was collected and used as a PCR template. Standard samples were treated according to the procedures for sputa; negative references were processed similarly, but omitting the homogenization step.

### *Construction of molecular beacon*

A sequence in the middle of the two primers specific for *M. tuberculosis* complex was used to construct a molecular beacon, fluorescein-5'-**GCG AGG AAC GGC TGA TGA CCA AAC TCT CGC**-3'-dabcyl (synthesized by Synthegen, AG, USA). The nucleotides shown in bold letters constitute the arm of molecular beacon, while those in italic form the loop portion. One distinct feature of this molecular beacon is that one of its arms (the first five nucleotides) is complementary to the target. This design is different from the original idea of a molecular beacon, which requires the arm sequence to be an unrelated one. A thermal denaturation profile of the molecular beacon was obtained by measuring its fluorescence intensity in the temperature range of 30 – 80°C with 2.5 – 5°C step in a 10 mM Tris-HCl buffer containing 3.5 mM MgCl<sub>2</sub>, pH 8.0. The fluorescence was measured with a Hitachi F-4010 spectrofluorometer (Hitachi, Japan) connected to a water bath. Each step was held for ca. 2 min to obtain a constant fluorescence. In order to avoid over exposure of the sample to the excitation light, the shutter of the fluorometer had to be closed when no measurement was being made. Hybridization curve of the molecular beacon was obtained by measuring its fluorescence intensity vs. time in the presence of the excess of its target sequence.

### *Fluorescence PCR detection*

IS986 DNA was amplified with an MJ/PTC-100 thermal cyclor (Gene, USA). A 245 bp region of IS986 was amplified with primers INS1 (5'-CGT GAG GGC ATC GAG GTG GC-3') and INS2 (5'-GCG TAG GCG TCG GTG ACA AA-3'), which correspond to nucleotides 641 to 660 and 866 to 885, respectively, of the insertion sequence.<sup>10</sup>

The reaction volume was 25  $\mu$ L and contained a mixture of the deoxynucleotide triphosphates (dNTPs) at a concentration of 200  $\mu$ M each, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 1.0 U Taq DNA polymerase (Promega), 0.4  $\mu$ M each primer and 0.4  $\mu$ M molecular probe in a 20  $\mu$ L buffer of 10 mM Tris-HCl. Five microliters of DNA were added to the reaction mixture, and the mixture was covered with 50  $\mu$ L of mineral oil. Amplification reactions were conducted with an initial 5-min denaturation step at 94°C coupled to a repeating cycle of 30 s at 94°C, 1 min at 65°C and 1 min at 72°C for 40 cycles, followed by final denaturation (30 s at 94°C) and extension (1 min at 65°C). The fluorescence of the samples in the reaction tubes was measured directly on a Fluorat-02-2M fluorometer (Lumex, Russia) equipped with a PCR vial holder. The holder, which was self-designed and customer manufactured by Lumex, had the same dimension and form as a standard cuvette, with two small holes on both the excitation and emission sides. The size and position of the holes were precisely adjusted for measuring a 25  $\mu$ L solution with the highest sensitivity and least light scattering.

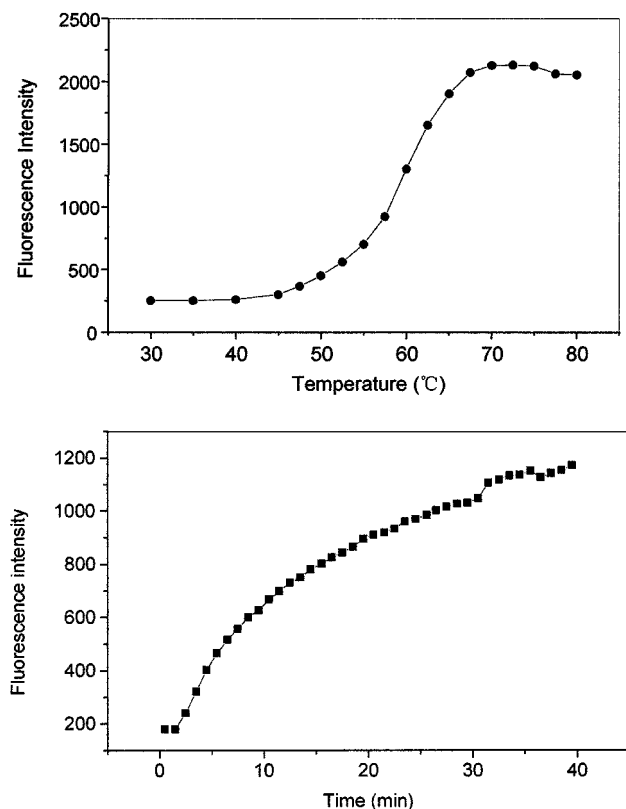


Fig. 2 Thermal denaturation profiles (a) and hybridization curve (b) of the designed molecular beacon.

## Results and Discussion

### Evaluation of the molecular beacon

A thermal-denaturation profile and a hybridization curve of the designed molecular beacon are shown in Figs. 2(a) and (b), respectively, which reveal the distinct features of the molecular beacon. From Fig. 2(a), we could obtain a loop melting temperature ( $T_m$ ) of 62°C, at which point the stem becomes open. When combined with PCR, the loop  $T_m$  of the molecular beacon should be close to the annealing temperature (65°C in this PCR). From Fig. 2(b) we could calculate the  $S/N$  ratio of this beacon to assess its efficiency by measuring the fluorescence intensity of the molecular beacon before and after hybridization and the background fluorescence of the buffer. A value of 55 was obtained, which is in the range of 30–200, as required for an effective beacon.<sup>9</sup>

It should be noted that the design of the above molecular beacon was not in accordance with the suggested guidelines,<sup>9</sup> which require that the arm sequences should be unrelated to the target. In our case, one arm of the beacon was complementary to the target, while the basic hairpin structure was retained. Our reason is that the molecular beacon will bind more firmly with an additional arm sequence, and, moreover, the fluorophor at 5' end of the molecular beacon will be in a more restrictive surrounding compared with that with a free and dissociative length of 5 nucleotides. Because the later is supposed to increase the distance between the fluorophor and the quencher, fluorescence would be restored to a greater extent. Though no detailed study was performed in this work, the experimental results showed that the new design worked well. Since we kept the arm sequence to be mainly composed of G-C pairs, for other

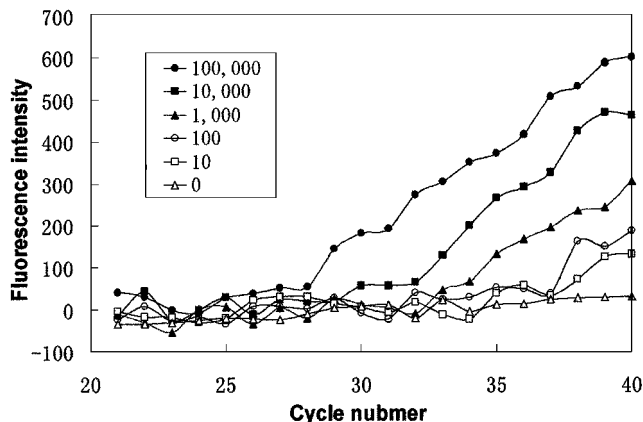


Fig. 3 Homogeneous fluorescence PCR detection of BCG vaccine at different cycle numbers.

detection, *e.g.*, NG and CT in this paper, not all bases on the stem were complementary to the target. A further study is now being undertaken to make the arm complementary to the target without considering its composition.

### Fluorescence PCR detection

Homogeneous fluorescence PCR has become popular since the emergence of real-time detection systems. However, the necessary expensive and complicated instruments have limited their wide application in routine diagnosis. End-point assay is comparatively simple, and can be well adapted to any model of currently used thermal cyclers for PCR. Nevertheless, in order to measure the fluorescence from the small volume of a PCR solution of 25–50  $\mu$ L, people had to transfer the solution to a microcell of a fluorometer; usually dilution was needed to reach the minimal measurable volume.<sup>9–11</sup> The post manipulations involved greatly affected the advantages of homogeneous PCR. To deal with these problems, we designed a simple PCR vial holder that can be mounted easily to the cell holder of a fluorometer. In this way, we can directly and reproducibly measure the fluorescence from the PCR vials. It needs only to put the PCR vial into the holder that already mounted in fluorometer and then measure the fluorescence. The measurement time is negligible during the course of homogeneous fluorescence PCR. Furthermore, end-point detection can be easily developed into a quantitative assay by competitive PCR. From sample extraction, amplification to fluorescence detection, it takes less than 4–5 h. Serially diluted BCG vaccine samples were detected with the homogeneous fluorescence PCR. Fluorescence at different cycle numbers was recorded, as shown in Fig. 3. In the range of 0–10000 bacteria/mL, fluorescence intensity is proportional to the bacteria concentration. Even one copy shows a clear difference from the blank. It can be seen from Fig. 3 that before 25 cycles, no obvious fluorescence increase was observed for all samples. Also, the PCR profiles show a nearly linear, rather than exponential, tendency, as in a standard PCR. These facts imply that the presence of a molecular beacon may hinder the PCR to some extent. Though we have no data to show the influence of a molecular beacon on PCR, it is likely that hybridization of the molecular beacon with the amplicon in PCR might decrease the reaction efficiency. If the lower amplification efficiency changes PCR from an exponential to a linear format, accurate end-point detection could be established. We also observed that, with the introduction of a molecular beacon, the PCR conditions were not changed very much, which greatly

simplified the condition optimization of homogeneous fluorescence detection.

#### Evaluation for TB detection

In order to investigate the performance of the present method in real clinical sample detection, we first established the cut-off value for negative-positive discrimination. Two hundred and twenty negative samples including normal human sera, sputa, urea, and 30 negative controls including physiological saline, distilled water, human DNA, Tris-EDTA buffer and extraction buffer were detected. The obtained average fluorescence intensity was 20.6, and the standard deviation was 23.3. According to the 3SD method, we obtained a cut-off value of 100. If the measured fluorescence intensity was greater than 100, the sample was considered to be positive; otherwise, it was negative.

The specificity of the method was assessed by measuring 10 mycobacterium strains, four unrelated bacteria strains and 220 negative samples. None of these specimens reached the cut-off value. Assuming a zero prevalence of TB of these samples, the specificity was 100%. By measuring TB standards prepared by being serially diluted with physiological saline containing  $10^3$ ,  $10^2$ ,  $10^1$  and 1 bacteria/mL, the detection limit was found to be 10 bacteria/mL, which had a fluorescence intensity of 142 with a standard deviation of 11.4% ( $n = 10$ ).

The performance of this method was also evaluated by a technician from NICPBP, China, using the Certified TB standards 98-A (blind samples) and self-prepared controls. The evaluation was carried out in triplicate within three days. 98-A contained 17 negative samples and five positive samples. Self-prepared controls included 10 negative patient samples, 5 negative reference samples, 10 positive patient samples and 5 positive standard samples. Also, one positive standard was used for a precision assessment. The evaluation could correctly identify all 17 negative and 5 positive samples in 98-A. The analytical sensitivity was 1 - 10 bacteria/mL. For all of the self-prepared controls, either positive or negative, the results were in 100% accordance. The standard deviation obtained from the three evaluations (10 tests each time) was 10.6%, 14.1% and 7.8%, respectively.

Clinical tests were conducted with a total of 466 samples in 4 hospitals. All samples were tested with the smear method, while 204 of them were cultured. The results are listed as follows:

		Smear		Culture	
		+	-	+	-
P	+	132	161	63	56
C					
R	-	9	164	1	84

It can be seen that the fluorescence PCR assay correlates well with both the smear (93.6%) and the culture (98.4%) for the positive samples, while the former could detect more positive samples (62.9%) than the smear (30.3%) and culture (31.4%) methods, displaying its sensitivity to be higher than the two traditional methods.

#### Fluorescence PCR detection of NG and CT

Similar assays were also developed for NG and CT, respectively. The results showed that NG could be detected in

the range of 0 - 100000 copies/mL; the detection limit was 10 copies/mL. When this method was tested with NICPBP's certified NG standards (blind testing), all 19 positive and 10 negative samples could be correctly revealed. For CT, a fluorescence PCR assay could obtain a good dose-response curve in the range of 1 - 10000 copies/mL; and the detection limit was 1 copy/mL. A semi-quantitative assay could be established for CT when combined with standard samples.

This preliminary study showed that molecular beacon based fluorescence PCR detection had great potential in the clinical diagnosis of TB and other infectious diseases. When compared with other homogeneous PCR formats, the development of a molecular beacon based assay is relatively simple, cheap and has no tedious conditions optimization.<sup>12</sup> Fluorescence detection can be easily accomplished with a PCR vial holder, which could be adapted to any common fluorometer. The distinguishing characteristics of a molecular beacon make the assay highly specific and sensitive. Furthermore, its ability in multiplex PCR could find more flexible applications in clinical diagnosis.

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