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Multiplex–microsphere–quantitative polymerase chain reaction: Nucleic acid amplification and detection on microspheres

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ABSTRACT

We report the development of a new system to monitor the amplification of nucleic acids on microspheres. This was realized by the design of (i) a "universal" oligonucleotide "tagged" polymerase chain reaction (PCR) forward primer, (ii) a sensor sequence complementary to the universal sequence on the forward PCR primer modified with a fluorescent dye, and (iii) a universal oligonucleotide coupled to Luminex microspheres. The PCR takes place with the microspheres present in the reaction tube. With the consumption of the universal oligonucleotide tagged forward primer, the fluorescently labeled sequences can bind to the universal oligonucleotide on the microspheres. We tested the microsphere quantitative PCR system with up to three different target genes (*Neisseria meningitides porA* and *ctrA* and influenza A M gene segment) as templates in a single PCR tube. The analytical sensitivity of this quantitative PCR system was tested and compared with the TaqMan system. The multiplex–microsphere–quantitative PCR system does not require design of unique internal probes for each target and has potential for a high degree of multiplexing, greater than the limited multiplexing achievable with current real-time protocols.

Polymerase chain reaction (PCR)[1] has become an essential tool in molecular biology research and clinical diagnostics [1]. During its early development phase, PCR amplification could be analyzed only at the end of a reaction in a qualitative or semiquantitative manner. In that format, determination of the relative initial quantities of nucleic acid templates was difficult because DNA replication could reach a plateau before endpoint measurements were undertaken [2]. Roughly 25 years ago, the invention of real-time PCR addressed these problems, providing a means to monitor the generation of PCR products dynamically [3].

Real-time PCR is most commonly implemented using fluorescence detection methods. There are two main types of real-time fluorescence that can be described as "universal binding" and "specific binding". Universal binding refers to a fluorescent dye binding to any nucleic acid. The best known of these is the SYBR Green system, targeting to double-stranded DNA [3,4]; however, this system is not multiplexable. Specific binding refers to the use of fluorogenic hybridization probes, in some cases in combination with the 5′ nuclease activity of Taq DNA polymerase. The availability of these fluorogenic probes enabled the development of a method for detection of only specific amplification products [5]. The development of fluorogenic probes also made it possible to eliminate post-PCR processing. The TaqMan system (Fig. 1A) is the commercial version of this format used in many analysis areas [6]; however, multiplexing potential is limited by current detection platforms.

In the TaqMan system, the fluorescence detected is directly proportional to the fluorophore released and the amount of DNA template present in the PCR (Fig. 1A) [6,7]. The key feature of the TaqMan system is that, in contrast to the SYBR Green system, it has high specificity for amplification product and is amenable to multiplex detection in a single tube up to approximately five targets (and sometimes more if reactions are split or combined with melt analysis). However, the expense of dual-labeled probe and the complexity of probe design are drawbacks. Clearly, there is a need for a method integrating lower expense and the capability for a high degree of multiplexing, which will enable quantitative PCR technology to advance to another level.

Recently, a new probe-based real-time PCR system, PrimRglo, was described [8]. In the current article, the PrimRglo system is further developed and integrated with microsphere-mediated

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[1] *Abbreviations used:* PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

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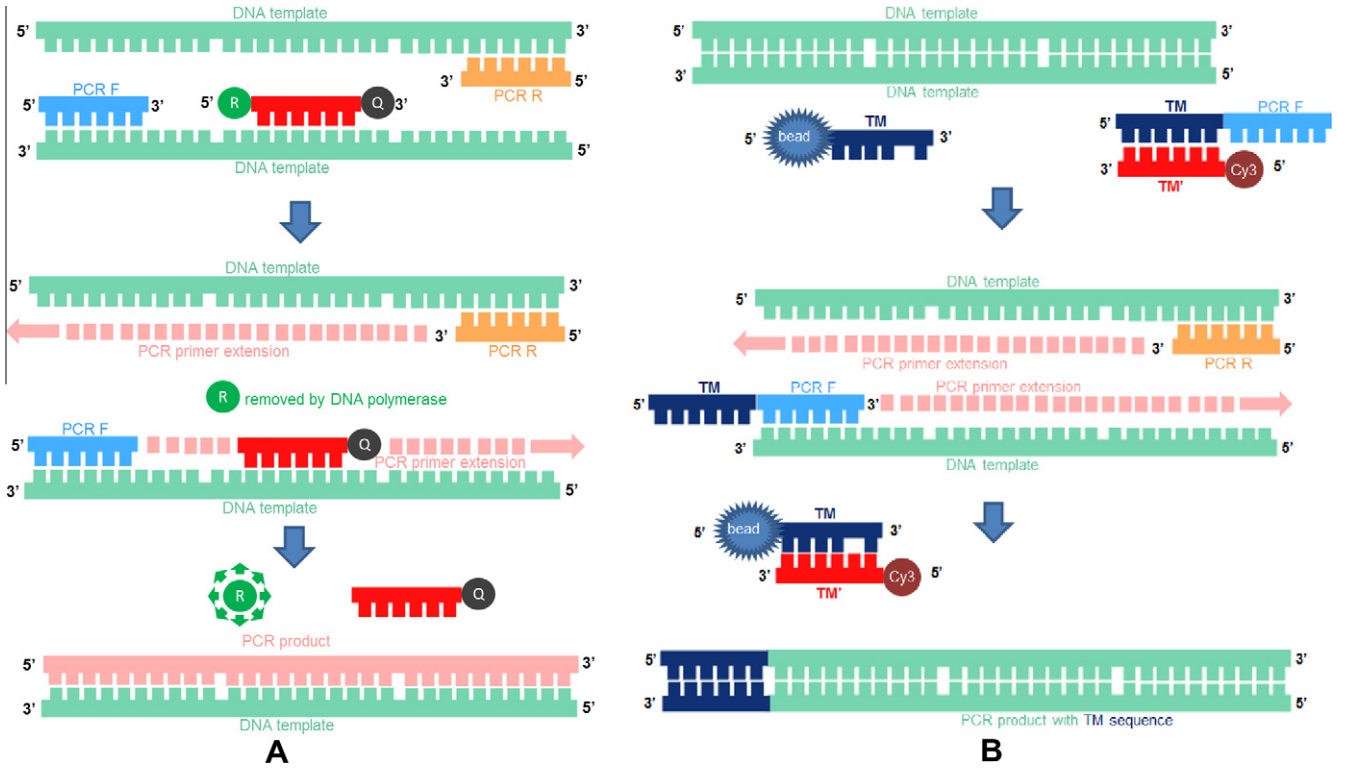

Fig.1. (A) Schematic representation of the TaqMan probe real-time PCR system. This system depends on fluorophore-labeled probes and the 5′ nuclease activity of Taq DNA polymerase. A target product-specific oligonucleotide (shown in red) is designed with a reporter fluorescent dye on the 5′ end (labeled R and shown in dark green) and a quencher dye on the 3′ end (labeled Q and shown in black). When the probe is intact, the fluorescence of the reporter dye is repressed by the absorbing effect of quencher next to it. When the target DNA template (shown in light green) is denatured to single strand, the probe can anneal to the sequence and is cleaved by Taq DNA polymerase along with extension of primer. The reporter dye is freed from quencher, which increases the fluorescent signal. (B) Schematic representation of the multiplex–microsphere–quantitative PCR system. In this system, fluorescent signal detection relies on the hybridization between the microsphere-linked probe and a reporter reverse probe (labeled Tm′, in red). Complementary binding between the PCR forward primer (consisting of a Tm sequence and sequence complementary to the target nucleic acid) and the reporter reverse probe is favored because of the existence of a single base pair mutation in the microsphere-immobilized probe. With PCR progress, the PCR forward primers are sequestered into the replicated PCR product. As the PCR forward primers are consumed, annealing between the microsphere-immobilized probe and reverse reporter probe is favored. The microspheres with localized fluorescent signal of the reporter dye can be detected in the Luminex machine.

fluorescence detection, conferring the potential to analyze up to 100 different genes in one reaction (corresponding to the 100 spectral regions available for the Luminex microspheres), in comparison with 5 channels in the TaqMan system (Fig. 1A) currently. In the new system, an oligonucleotide probe is linked to Luminex microspheres (microsphere-TM in Fig. 1B). The oligonucleotide attached to the microsphere is complementary to a fluorescent reporter-labeled probe (TM′), which hybridizes with the probe linked to the microspheres (Fig. 1B). The localized fluorescence signal can be detected by the Luminex machine, scanning the microspheres as they flow through a capillary. At the commencement of the PCR, the forward PCR primer (consisting of conjoined TM and forward primer sequences, "TM–F") binds to the fluorescent reporter-labeled probe (TM′), blocking the formation of a fluorescent complex on the microsphere surface (Fig. 1B). As PCR progresses, the consumption of forward primer during the generation of amplification products allows formation of reporter complexes on the microsphere surface. In this way, the fluorescent signal on microspheres increases in each cycle. This increase of fluorescence signal can be detected by dispensing the reaction mix into a Luminex machine. The currently used microsphere sets include 100 distinguishable individual spheres, which in theory allows for up to 100 multiplexing channels in one reaction. As a quantitative PCR system with more channels to choose in one reaction, and minimal effort in adapting existing primers, PrimRglo on microspheres provides more efficiency and flexibility.

In this article, the integration of PrimRglo with the Luminex system is evaluated in "singleplex" and "triplex" assays, and its analytical sensitivity is compared with the TaqMan system.

Materials and methods

Primer and functional oligonucleotide design

The PCR forward primer (FluA-MF) and reverse primer (FluA-MR) were designed to amplify the gene encoding the matrix protein of influenza A virus. The use of this set of primers in a diagnostic setting has been published previously [9]. The PCR forward primer designed for the PrimRglo system (TM3-FluA-MF) has two segments: the TM3 sequence followed by the FluA-MF sequence. The TaqMan 5′ endonuclease probe (FAM-FluA-M-BHQ) was designed to target the matrix protein gene of influenza virus A. The probe has been modified with FAM attached at the 5′ end and BHQ1 attached at the 3′ end. This probe was described in a previous study [9]. For PrimRglo, the reporter complex consists of two single strands of complementary oligonucleotide known as Cy3-TM3′ and microsphere-TM3. To favor the interaction between the TM3-FluA-MF PCR forward primer and the TM3′ reporter probe at the initiation of PCR, a single base mismatch was introduced within the microsphere-TM3 sequence. For all three reactions (*Neisseria meningitides porA* and *ctrA* genes and influenza A M gene segment), we found that a single base mismatch close to the center of the immobilized probe sequences