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Review

Nucleic acid amplification-based techniques for pathogen detection and identification

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Abstract

Nucleic acid amplification techniques have revolutionised diagnostic and research industries. Current technologies that allow the detection of amplification in real-time are fast becoming industry standards, particularly in a diagnostic context. In this review, we describe and explore the application of numerous real-time detection chemistries and amplification techniques for pathogen detection and identification, including the polymerase chain reaction, nucleic acid sequence-based amplification, strand displacement amplification and the ligase chain reaction. The emergence of newer technologies, such as lab-on-a-chip devices and photo-cleavable linkers, is also discussed.

Keywords: Nucleic acid; Amplification; Detection; Real-time PCR; PCR; NASBA; Biosensor; LCR

Contents

1. Introduction

A variety of nucleic acid amplification techniques were developed in the mid to late 1980's. These include the polymerase chain reaction (PCR) (Mullis and Faloona, 1987), ligation-mediated amplification (Wu and Wallace, 1989) and transcription-based amplification (Kwoh et al., 1989). Since then, these techniques have been refined and alternative approaches have been developed for amplification (e.g. transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), ligase chain reaction (LCR), strand displacement amplification (SDA), linear linked amplification, see Monis et al., 2002 for an overview). None of these techniques have achieved the same widespread research application as PCR, most likely due to the simplicity and cost-effectiveness of PCR. However, some of these techniques have been incorporated into clinical diagnostic assays (e.g. SDA is a platform technology used by Becton Dickinson for *Mycobacteria* and

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Chlamydia detection, NASBA is used by Biomérieux for HIV-1, CMV and Enteroviruses, TMA is used by Gen-Probe for the detection of *Mycobacteria*, *Neisseria* and *Chlamydia*). As an adjunct to the emerging amplification technologies that are rapidly being developed, conventional amplification techniques continue to play an integral role in characterising and genotyping parasites for medical, environmental and epidemiological investigations. Techniques such as amplified restriction fragment length polymorphism (AFLP), PCR-restriction fragment length polymorphism (PCR-RFLP) and random amplified polymorphic DNA (RAPD) are currently employed to answer specific biological and evolutionary questions and these techniques have been the subject of numerous reviews (e.g. Masiga et al., 2000; Monis et al., 2002; Singh, 1997). The greatest recent advancement in amplification technology has been the development of systems that allow monitoring of amplification in real-time. This paper will provide an overview of recent developments in amplification and detection technologies, with a focus on real-time detection chemistries.

1.1. Real-time amplification

The first real-time amplification system used ethidium bromide and a mounted CCD camera to monitor PCR amplification in a closed reaction tube (Higuchi et al., 1992). Since then, significant advancements have been made in technology and software exploiting Higuchi's initial principle of monitoring changes in amplification signal with time. As a result, real-time PCR now provides researchers and diagnostic laboratories with additional tools for disease diagnosis, identification of species, quantifying gene expression, single nucleotide polymorphism (SNP) detection and monitoring infection loads during therapy. There are many fluorescent detection chemistries currently employed in real-time PCR assays and while there may be superficial similarities between some chemistries, specific assay design criteria need to be adhered to for each chemistry application. (Readers are directed to a recently published text book for design criteria (Edwards et al., 2004).)

The advent of real-time PCR has overcome a number of short-comings of conventional PCR. Real-time PCR readily allows quantitation of DNA over a broad dynamic range and it is a closed-tube format that requires no post PCR handling for identification of amplicons, reducing the potential for sample contamination and making the entire process more amenable to high throughput analysis. For quantitation, real-time PCR exploits the proportional relationship between the cycle where exponential amplification is detected (the threshold cycle or C_t) and the starting number of copies of the target nucleic acid fragment. In order to do this, standards with defined numbers of copies of the target fragment are used to generate a standard curve (based on C_t value versus copy number) and the gene copy number in an unknown sample is estimated by comparison to this standard curve (Saunders, 2004). Closed tube verification of the amplification of the correct fragment can be achieved by DNA melting curve analysis, which is analogous to the detection of a band by conventional gel electrophoresis. In the case where intercalating dyes are used, the dissociation kinetics of the entire amplified fragment is measured, and plotting the first derivative of the melting curve versus temperature allows determination of the melting temperature of the product. Probe:amplicon hybrids can be analysed in a similar fashion, except the melting temperature is determined by measuring the dissociation of the probe from target DNA, rather than measuring the melting temperature of the whole of the amplified fragment. In both cases, the melting temperature is affected by the GC content of the DNA duplex (the higher the GC, the higher the melting temperature), the absolute order of the bases in the sequence and the size of the amplicon or probe:target hybrid.

1.2. Real-time detection chemistries

The initial development of real-time PCR made use of the double-stranded DNA (dsDNA)-specific intercalating dye ethidium bromide (Higuchi et al., 1992). dsDNA-specific intercalating dyes exhibit little or no fluorescence when free in solution (Fig. 1a) but produce a large quantum yield increase in fluorescence when bound to dsDNA and exposed to the appropriate wavelength of light (Fig. 1b). SYBR Green I (Becker et al., 1996) is currently the industry standard, although the use of other dyes has been described (e.g. BEBO (Bengtsson et al., 2003), LC Green (Wittwer et al., 2003), SYTO9 (Monis et al., 2005)). Intercalating dyes are the most cost effective chemistry and are possibly now the most widely used detection chemistry, particularly for gene expression studies. Caution must be taken when using SYBR Green I, however, as there are disadvantages that limit its ease of use. In general, reaction conditions require further optimisation by including additional reagents to improve reaction efficiency, such as DMSO (Jung et al., 2001), BSA and Triton X-100 (Karsai et al., 2002). Depending on the reaction conditions, SYBR Green I also appears to be inhibitory to PCR in a concentration dependent manner (Monis et al., 2005; Nath et al., 2000; Wittwer et al., 2003) and the degradation products of the dye have also been reported to be inhibitory to PCR (Karsai et al., 2002). In addition, the preferential binding of SYBR Green to specific amplicons during multiplex PCR limits its use for this application (Giglio et al., 2003). Recent work describing the evaluation of SYTO9 for real-time PCR has found that this new dye is much less inhibitory to PCR compared with SYBR Green I and that it does not appear to exhibit preferential binding to specific amplicons, allowing it to be used to analyse multiplex PCRs by DNA melting curve analysis (Monis et al., 2005). In addition, SYTO9 appears to be much more robust and reliable for DNA melting curve analysis compared to SYBR Green I, making melting curve analysis a more reliable tool for genetic discrimination/