Molecular Pathology Techniques: Advances in 2018

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KEYWORDS

• Molecular pathology • Methodology • RT-PCR • FISH • RFLP • SNP
• Hybrid capture • Mass spectrometry

KEY POINTS

- Polymerase chain reaction (PCR) remains the cornerstone methodology for nucleic acid amplification. Improvements in nucleic acid detection methodologies (for example PCR) have increased the detection sensitivity by using fluorescent and bead array–based technologies.
- Single base-pair lesions can be detected via sequencing and related techniques to discern point mutations in disease pathogenesis.
- Novel technologies, such as high-resolution melting analysis, provide fast, high-throughput post-PCR analysis of genetic mutations or variance in nucleic acid sequences.
- Infectious disease can now be detected by fluorophore or chemiluminescent detection assays, such as hybrid capture hybridization technology, allowing for rapid diagnosis.

POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is a chemical reaction that facilitates the in vitro synthesis of potentially unlimited quantities of a targeted nucleic acid sequence. Basically, the reaction consists of a target DNA molecule, an excess of the forward and reverse oligonucleotide primers (typically 15–30 nucleotides long), a thermostable DNA polymerase (typically *Taq* or *Pfu*), an equimolar mixture of deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), Mg^{21} or Mn^{21} (depending on the type of polymerase used), KCl, and an appropriate Tris-HCl buffer.

The reaction consists of 3 steps: denaturation, annealing, and extension, which taken together are referred to as a *cycle*. To begin, the reaction mixture is heated (usually to 95°C) to separate the 2 strands of target DNA (denaturation) and then cooled to

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a temperature at which the primers bind to the target DNA in a sequence-specific manner (annealing). Immediately after primer annealing, the DNA polymerase binds (as the temperature is raised to 72°C) and initiates polymerization, resulting in the extension of each primer at its 3' end (extension). During the following cycle, the primer extension products are subsequently heated to dissociate from the target DNA. Each new extension product, as well as the original target, can serve as a template for subsequent rounds of primer annealing and extension. In doing so, at the end of each cycle, the PCR products are theoretically doubled.[1,2]

The whole procedure is carried out in a programmable thermocycler that precisely controls the temperature at which the steps occur, the length of time that the reaction is held at the different temperatures, and the number of cycles. Ideally, after 20 cycles of PCR, a million-fold amplification is achieved and, after 30 cycles, the replicons approach a billion-fold.

REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

As described previously, PCR is suitable for the amplification of DNA targets because DNA polymerase does not recognize DNA-primed RNA templates. Reverse transcription (RT)-PCR helps overcome this problem by using the enzyme reverse transcriptase to first synthesize a strand of complementary DNA (cDNA) using the RNA as a template. Because thermolabile RNA is often referred to as the message transcribed from the DNA template, this process provides a thermostable mirror image of the RNA transcript. Typically, recombinant RT is added to a reaction mixture identical to the one for PCR and is incubated at between 37°C and 42°C for 30 minutes, during which time the first-strand cDNA synthesis occurs. Subsequently, the reaction proceeds much like a regular PCR reaction for the appropriate number of cycles at the appropriate temperatures. This method can, however, present problems in terms of both the nonspecific primer annealing and inefficient primer extension due to formation of RNA secondary structures. A secondary RNA structure is a direct consequence of the low temperature at which the reaction is carried out, due to the heat labile nature of most RTs. These problems have been largely overcome by the development of a thermostable DNA polymerase derived from *Thermus thermophilus* (for example Taq polymerase), which, under the proper conditions, can function efficiently as both an RT and a DNA polymerase.[2]

REAL-TIME POLYMERASE CHAIN REACTION

Real-time PCR (also called quantitative PCR or quantitative Real-time PCR, or kinetic PCR or kinetic RT-PCR) is a closed-system assay that can be used to determine the relative quantity of gene expression as well as genotyping by detection of single-nucleotide polymorphisms (SNPs).

In principle, the method works much like the PCR discussed previously; however, real-time PCR also uses an additional oligonucleotide probe. This probe is target message specific and contains a fluorochrome at one end and a quencher molecule at the other. When unhybridized, the probe forms a hairpin structure that brings the fluorochrome in proximity with and binds the quencher, effectively muting its fluorescence. When hybridized, however, the quencher molecule is cleaved, and the bound fluorochrome is now unencumbered and can be detected by a fluorescence absorption assay. Single-nucleotide differences like SNPs can be detected in PCR products by the sequence-specific hybridization of the probe. Because it is possible to have different colored fluorochromes, the probes can be differentially labeled, allowing both alleles of an SNP to be typed in the same tube. These molecules can be used