2. MODIFICATIONS OF PCR

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Though standard PCR assay is used for most of the diagnostic applications, in recent times, there have been a lot of modifications of PCR, which would suit different situations. For example, Reverse transcriptase PCR has been used when RNA is to be amplified; inverse PCR method is used when the border sequences of a DNA sequence that are not known; Nested PCR is used when the sensitivity and specificity of the reaction is to be increased; asymmetric PCR is used when the PCR product is to be sequenced; in situ PCR is used when amplification of DNA is to be done in fixed tissues or cells in microscope slides and multiplex PCR is used when more than one agent is to be detected in one reaction tube. So, depending upon the requirement, the standard PCR assay could be modified to suit the occasion. This paper describes few of the modifications of PCR with their principles and applications.

Inverse PCR

This is the amplification of unknown DNA sequence that flanks a core region of known sequence. In this method, amplification of that DNA sequence, which are away from the primers and not those, which are flanked by the primers, are carried out. In this technique, the primers are designed so that their 3' end face away from each other. This clever modification of the basic PCR technique allows amplification of unknown DNA sequences to one or the other or both sides of a known DNA segment. Inverse PCR has applications in producing probes of anonymous sequences or in determining the sequences of upstream and downstream flanking regions themselves.

Anchored PCR

When we have the knowledge about the sequence at only one of the two ends of the DNA sequence to be amplified, anchored PCR may be used, which would utilize only one primer instead of two primers. Thus, only one strand would be copied first, after which a poly G tail would be attached at the end of the newly synthesized strand utilizing an anchor primer with which a poly C sequence is linked to complement with poly G of the template. In the next cycle, both the original primer and anchored primer would be used for gene amplification. There are two anchored PCR methods for the DNA amplification: rapid amplification of cDNA ends (RACE-PCR) and ligation anchored PCR.

Reverse Transcriptase PCR

The RNA-PCR protocol requires two separate major reactions- a reverse transcription step, followed by PCR amplification. Oligo dT primer for mRNA that contains a poly(A) tail at the 3' end or random hexamers or a gene-specific primer can be used to prime cDNA synthesis. Reverse transcriptase is usually used to synthesize first-strand cDNA from RNA. Recently, a DNA polymerase known as Tth DNA polymerase, isolated from Thermus thermophilus which has got both the properties of reverse transcription and DNA amplification has been used. This enzyme, in the presence of manganese can reverse transcribe RNA and in the presence of magnesium can amplify DNA. Since, Tth DNA polymerase can utilize both DNA and RNA as templates, the whole procedure can be carried out in a single tube.

Nested PCR

In nested PCR, the larger fragment produced by the first round of PCR is used as the template for the second PCR. Nested PCR can also be performed with one of the first primer pair and a single nested primer. Using the nested PCR method one can dramatically increase the sensitivity and specificity of both DNA and RNA.
amplification. The specificity is particularly enhanced because this technique almost always eliminates any spurious non-specific amplification products.

Asymmetric PCR

In this method, the addition of one primer in vast excess over the other during the PCR results in the generation of an excess of one amplified strand relative to the other. Asymmetric PCR can be performed directly on any given template, although reproducible high yields of single-stranded products are often difficult to obtain. Alternatively, we could make a double-stranded PCR product by conventional PCR and reamplify this by asymmetric PCR, using only one primer for approximately 20 cycles. The single-stranded product is usually used for dideoxy DNA sequencing.

In situ PCR

This method involves performing PCR on fixed cells or tissues after semi-permeabilizing the cell membranes to allow diffusion of the PCR reagents into the cells. There are three major advantages of this method. First and foremost, in situ PCR combines the high sensitivity of PCR with the cell-localizing ability of in situ hybridization. Secondly, the possibility of contamination is greatly reduced, because the positive signal is localized to specific areas within the cell. Third advantage is that the in situ PCR provides enormous amount of information relative to the histological distribution of the amplified PCR product.

Multiplex PCR

Multiplex PCR is the simultaneous detection of more than one target sequence in one reaction tube using more than one primer pair. This co-amplification of two or more targets in a single reaction is dependent on the compatibility of the PCR primers used in the reaction. All primers in the reaction must have similar melting temperature (Tm) so that they anneal to and dissociate from complementary DNA sequences at approximately at the same temperature, allowing each amplification to proceed at the selected temperature. Primers should also be chosen, so that amplicons are of same size range. Majority of M-PCR studies have been involved with the detection of mutations.

Real Time PCR

Fairly recently, a new method of PCR quantification has been invented. This is called "real-time PCR" because it allows the scientist to actually view the increase in the amount of DNA as it is amplified. Several different types of real-time PCR are being marketed to the scientific community at this time, each with their advantages. TaqMan® real-time PCR is the one used more commonly, and fewer applications use other two types of real-time PCR, molecular beacon and SYBR® Green. The TaqMan® probe consists of two types of fluorophores, which are the fluorescent parts of reporter proteins (Green Fluorescent Protein (GFP) has an often-used fluorophore). While the probe is attached or unattached to the template DNA and before the polymerase acts, the quencher (Q) fluorophore reduces the fluorescence from the reporter (R) fluorophore. It does this by the use of Fluorescence (or Förster) Resonance Energy Transfer (FRET), which is the inhibition of one dye caused by another without emission of a proton. The reporter dye is found on the 5′ end of the probe and the quencher at the 3′ end. Once the TaqMan® probe has bound to its specific piece of the template DNA after denaturation (high temperature) and the reaction cools, the primers anneal to the DNA. Taq polymerase then adds nucleotides and removes the Taqman® probe from the template DNA. This separates the quencher from the reporter, and allows the reporter to give off its emit its energy. This is then quantified using a computer. The more times the denaturing and annealing takes place, the more opportunities there are for the Taqman® probe to bind and, in turn, the more emitted light is detected.

There are two other types of real-time PCR methods, the molecular beacon method and the SYBR® Green method. The molecular beacon method utilizes a reporter probe that is wrapped around into a hairpin. It also has a quencher dye that must be in close contact to the reporter to work. An important difference of the molecular beacon method in comparison to the TaqMan® method is that the probe remains intact
throughout the PCR product, and is rebound to the target at every cycle. The SYBR® Green probe was the first to be used in real-time PCR. It binds to double-stranded DNA and emits light when excited. Unfortunately, it binds to any double-stranded DNA which could result in inaccurate data, especially compared with the specificity found in the other two methods.

**Single-strand Conformational Polymorphism PCR (SSCP-PCR)**

This is a simple and powerful technique for identifying sequence changes in amplified DNA. Here the target sequence is first labeled and amplified by the PCR. The PCR product is then denatured and resolved by PAGE and mutations are detected as altered mobility of separated single strands in the autoradiogram. Thus, the overall procedures are rapid and simple. SSCP-PCR has been used most extensively to screen for inherited mutations or to detect somatic mutations in cancer cells. SSCP in microbiology has been used for classification of virus strains. The amount of product required in PCR-SSCP for detection is much less than that in some other PCR-based techniques. One obvious use of PCR-SSCP is detection of DNA polymorphisms.

**Ligase chain reaction**

The LCR has evolved as a very promising diagnostic technique that can be utilized in conjunction with a primary PCR amplification. LCR employs a thermostable ligase and allows the discrimination of DNA sequences differing in only a single base pair. PCR requires two oligonucleotide primers, a thermostable DNA polymerase and deoxynucleotide triphosphates. In contrast, LCR uses four nucleotide probes, a thermostable DNA ligase and a high-energy dinucleotide, NAD+, to catalyze target sequence amplification.

In the first step in LCR, a clinical sample is added to an LCR reaction mixture containing four oligonucleotide probes (two complementary pairs), the thermostable enzyme DNA ligase and NAD+. The oligonucleotides are chosen so that same-sense probes lie adjacent to one another when hybridized to a target DNA sequence. The reaction mixture is heated to 85-95°C to ensure strand separation of double-stranded DNA (both the target and the complementary oligonucleotide pairs). The reaction mixture is then cooled to allow hybridization of the oligonucleotides to their respective target DNA strands. The adjacent probes are then covalently linked by a thermostable DNA ligase. The ligated products, or amplicons, are complementary to the target nucleic acid sequence and function as targets in the next cycle of amplification. Thus, exponential amplification of the specific target DNA sequences is achieved through repeated cycles of denaturation, hybridization and ligation in the presence of excess oligonucleotide probes. Exponential amplification allows detection of minute levels of target using LCR.

LCR assays have been developed for the detection of genetic diseases as well as for the detection of bacteria and viruses.

**PCR-ELISA**

Recently, ELISA method has been clubbed with PCR methods to increase the sensitivity of both methods. Morato et al. (2003) developed a PCR-ELISA method for diagnosis of brucellosis. After amplification of a 223 bp sequence of a gene that codes for the synthesis of an immunogenetic membrane protein specific for the Brucella genus (BCSP31), the Digoxigenin-labeled amplified product was hybridized with a biotinylated capture probe which was complementary to the inner part of the amplicon. The hybrid was captured on streptavidin-coated microtitre plate and detected by using an antidigoxigenin Fab-peroxidase conjugate. Hong et al. (2003) used PCR-ELISA for detection of Campylobacter coli, C. jejuni and Salmonella enterica from poultry carcass.

**References**