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Polymerase Chain Reaction: Types and its Applications in the Field of Biology

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Abstract: For disease control and eradication and in countries trying to establish a disease free status, effective diagnostic is a paramount. Many diagnostics find its application throughout different levels of laboratory research processes. Polymerase Chain Reaction (PCR) is one of the most important molecular diagnostic tools which allow the detection of nucleic acid targets. Because of its excellent sensitivity, specificity and speed, PCR has rapidly become the widely used molecular biological techniques in scientific, medical and research fields. There are different types of PCRs which are used specifically for certain specific purposes. The types of PCR and their applications are discussed in this review article.

Key words: Polymerase chain reaction, research processes, molecular biology

INTRODUCTION

Molecular biology techniques for the direct detection of microbial genomes in the specimen will play an increasingly important role in the clinical microbiology laboratory. Molecular techniques can be divided into two categories: those that do not involve amplification and those that involve amplification (like, PCR). Dr. Kary B. Mullis who invented this technique was rewarded the Nobel 1993 Prize in Chemistry for his invention of the PCR method[1].

PCR allows the *in-vitro* amplification of specific target DNA sequences by a factor of 10^6 and making it an extremely sensitive technique. It is based on an enzymatic reaction involving the use of synthetic oligonucleotides flanking the target nucleic acid sequence of interest. These oligonucleotides act as primers for the thermo stable *Taq* polymerase enzyme. Repeated cycles of denaturation (25°C to 40°C) of the template DNA at 94°C; annealing of primers to their complementary sequences at 50°C and primer extension at 70°C result in the exponential production of the specific target fragment Fig. 1[1]. The advantages of PCR include,

- High sensitivity, which may detect even one viral genome per sample volume,
- Easy reaction setting and
- Fast turnaround time. Some of the disadvantages are,
- Extremely liable to contamination,
- High degree of operator skill required
- Not easy to quantitate results,
- A positive result may be difficult to interpret, especially with latent organisms, where any sero-positive sample will have causative agent present in their sample irrespective of the presence or absence of the disease.

Types of polymerase chain reaction

Amplified Fragment Length Polymorphisms (AFLP): The use of randomly amplified polymorphic DNA (RAPD) markers in systematic studies has been reviewed[2]. RAPD techniques were embraced due to the relatively high levels of polymorphism and their low cost compared to other techniques, such as allozymes and Restriction Fragment Length Polymorphisms (RFLP)[3]. Two new marker methodologies appear to be supplanting RAPD analyses [AFLPs and Simple Sequence Repeats (SSRs; microsatellites)]. Whilst the RAPD technique is fairly simple, both AFLP and SSR protocols are technically demanding[4].

Alu-PCR: This PCR is performed using the Alu primers designed to have recognition sequence of Alu restriction enzyme[5].

Asymmetric PCR: A PCR in which the predominant product is a single-stranded DNA, as a result of unequal primer concentrations. As asymmetric PCR proceeds, the lower concentration primer is quantitatively incorporated into double-stranded DNA and the higher concentration

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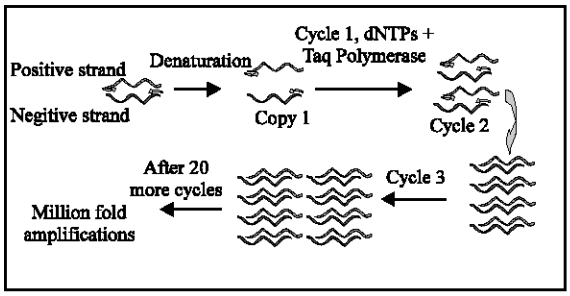

Fig. 1: Schematic diagram of sequences in a polymerase chain reaction

primer continues to primer synthesis, but only of its strand. This type of PCR has been used for the detection of a target gene of adenovirus[6].

Allele-Specific PCR (AS-PCR): This is a selective PCR amplification of one of the alleles to detect Single Nucleotide Polymorphism (SNP). Selective amplification is usually achieved by designing a primer such that the primer will match/mismatch one of the alleles at the 3'-end of the primer.

Balanced PCR: In which a balanced amplification of allelic fragments from two genomes will occur. Unique tags are used for each genome to distinguish sequences for the two genomes.

Box-PCR: Box elements are repetitive sequence elements in bacterial genomes such as Streptococcus genome. Single PCR primer targeting to the repeats can be used to fingerprint bacterial species.

Colony PCR: Colony PCR is useful in determining whether or not a specific colony on a plate has a desired sequence. Primers for the specific sequence (antibiotic resistance, or primers flanking a cloned region) should be used when preparing the reaction mixture and it allows rapid detection of transformants containing the desired sequence.

Competitive PCR (cPCR): This is a method used for quantifying DNA using real-time PCR. A competitor internal standard is co-amplified with the target DNA and the target is quantified from the melting curves of the target and the competitor.

Consensus-PCR: This PCR is carried out with flanking primers to amplify repeat regions from a number of species. In this case, degenerate/consensus primers may be used for amplifying the flanking sequences.

Degenerate PCR: Degenerate PCR is in most respects identical to ordinary PCR, but with one major difference i.e., instead of using specific PCR primers with a given sequence, mixed PCR primers will be used. That is, "wobbles" are inserted into the primers in case if the exact the sequence of the gene is not known so that there will be more than one possibility for exact amplifications. Degenerate PCR has proven to be a very powerful tool to find "new" genes or gene families. By aligning the sequences from a number of related proteins the conserved and variable parts can be determined. Based on this information one can use conserved protein motifs for starting points for designing degenerate PCR primers.

Degenerate Oligonucleotide-primed PCR (DOR PCR): A PCR amplification of limited sample using degenerate primers is called the DOR-PCR.

Differential Display PCR (DD-PCR): DD-PCR is used for cloning purpose; it combines the comparative analysis of several samples with the sensitivity of PCR. Recent studies shows that by modifying the primer design, sampling of differentially expressed genes can be greatly enhanced and relevant genes can be isolated[7].

Digital PCR (Dig-PCR): A technique for detecting low levels of mutations present in the samples to be analyzed. The Samples are diluted into 96 wells to low DNA copy number. After PCR is performed, the samples are analyzed for the presence of mutant and wild type sequences.

Forensic PCR: The VNTR (i.e., variable and frequently observed tandem repeats in human individual genome) locus is PCR amplified to compare DNA samples from different sources.

Hairpin PCR: A method of error-free DNA amplification for mutation detection. It first converts a DNA sequence to a hairpin. True mutations will maintain the hairpin structure during amplification while PCR errors will disrupt the hairpin structure.

Hot-start: It is a method designed to prevent the non-specific binding of primer to template and PCR amplification during sample preparation. Samples are prepared at a hot temperature or one component is added only after the PCR mix is hot.

***In-Situ* PCR (IS-PCR):** IS-PCR is performed on fixed cells. DNA or RNA is immobilized in their sub-cellular locations. *In-Situ* Hybridization (ISH) or IS-PCR has proven to be a very important molecular tool in diagnostic and research

and has significantly advanced the study of gene structure and expression at the level of individual cells. This technique has resulted in an increased understanding of infectious and neoplastic diseases and improvements in diagnosis of disease. More recently, an intracellular reverse transcription step to generate complementary DNA from mRNA templates prior to *in-situ* PCR has been used for the detection of low copy mRNA sequences. This modification of *in-situ* PCR has been termed as '*in-situ* RT-PCR' or 'RT *in-situ* PCR' or '*in-situ* cDNA PCR'. Utility of immuno-histochemistry, *in-situ* hybridization and *in-situ* PCR amplification in the surgical and cytopathology of viral infections has been reported[8]. Applications of ISH or *In-situ* PCR includes,

- Detection and diagnosis of viruses and other infectious agents in specific cell types within tissues
- Detection and characterization of tumor cells within a tissue
- Detection and diagnosis of genetic mutations in inherited diseases
- Detection of genes and gene expression in tissue
- Detection of the presence of cancer-causing viruses in cells obtained from PAP smears and
- Any assay in which identification of cells expressing a target gene is required. Main Advantages are its Low background, High specificity, Fast assay with shorter turn-around time and No need of radioactive chemicals.

Inverse PCR: Is a type of standard PCR that is used to amplify the segments of DNA that lie between two inward-pointing primers. Inverse PCR (also known as inverted or inside-out) is used to amplify and clone unknown DNA that flanks one end of a known DNA sequence and for which no primers are available. The technique involves digestion by a restriction enzyme of a preparation of DNA containing the known sequence and its flanking region. The individual restriction fragments are converted into circles by intramolecular ligation and the circularized DNA is then used as a template in PCR. The unknown sequence is amplified by two primers that bind specifically to the known sequence and point in opposite direction. Like other polymerase chain reaction processes, inverse polymerase chain reaction is used to amplify DNA samples, via the temperature-mediation.

Long-PCR (LA PCR): Used for the amplification of long target DNA sequences. The key to LA PCR is an enzyme, a thermostable DNA polymerase, which possesses 3' to 5' exonuclease activity, or proofreading activity. The efficiency drastically declines when incorrect bases are incorporated. The 3' to 5' exonuclease activity removes these misincorporated bases and makes the further reaction proceed smoothly. Therefore, the amplification of long DNA fragments can be achieved.

Multiplex PCR: Multiplex PCR is the term used when more than one pair of primers is used in a PCR. The goal of multiplex PCR is to amplify several segments of target DNA simultaneously. This PCR technique is used for genetic screening, micro-satellite analysis and other applications where it is necessary to amplify several products in a single reaction. This technique often requires extensive optimization because having multiple primer pairs in a single reaction increases the likelihood of primer-dimers and other non-specific products that may interfere with the amplification of specific products. In addition, the concentrations of individual primer pairs often need to be optimized since different multiplex amplicons are often amplified with differing efficiencies and multiple primer pairs can compete with each other in the reaction.

Multiplex RT-PCR: Multiplex RT-PCR (also referred to as relative RT-PCR) is commonly used for the semi-quantitative analysis of gene expression levels when defining tissue-restricted gene expression patterns. Typically, multiplex RT-PCR is performed to determine the changes in expression level of a gene in a series of tissue types, throughout stages of development or cellular differentiation, or after specific experimental treatments. Multiplex RT-PCR is also commonly used to examine the expression patterns of a series of related genes and to look at various regions of a large message for mutation analysis.

Nested PCR: Nested PCR refers to a pair of PCRs run in series each with a pair of primers flanking the same sequence. The first PCR amplifies a sequence as seen in any PCR experiment. The second pair of primers (nested primers) for the second PCR binds within the first PCR product and produces a second PCR product that is shorter than the first one. The technique, because it uses four specific primers, rather than two, has greater specificity than regular PCR. It can also yield detectable product in cases where simple PCR fails to do so. Nested PCR is a modification of PCR intended to reduce the contaminations in products due to the amplification of unexpected primer binding sites. Semi-nested PCR using degenerate primers for the generic detection of small ruminant lentivirus proviral DNA had been developed[9].

Nested RT-PCR: This term refers to a nested PCR reaction that is initiated with cDNA that has been reverse transcribed from RNA.

PCR-ELISA: The PCR products are labeled (digoxigenin) during amplification. A capture probe specific to the PCR amplicon is used to immobilize the amplicon to immuno-well plate. ELISA is then used against the label (anti-digoxigenin) to quantitate PCR products.

PCR-RFLP: Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another. By designing primers that will introduce or destroy a restriction site for one of the alleles, the PCR products for SNP alleles can be distinguished by restriction fragment lengths.

PCR-Single Strand Conformational Polymorphism (PCR-SSCP): This is a type of PCR followed by SSCP detection of point mutations.

Quantitative Polymerase Chain Reaction (Q-PCR): This is a modification of normal PCR used to rapidly measure the quantity of a product of the reaction. It is preferably done in real-time, thus is an indirect method for quantitatively measuring starting amounts of DNA, cDNA or RNA. This technique is commonly used for the purpose of determining the presence and the copy number of the genetic sequence. The three commonly used methods of quantitative PCR are

- through agarose gel electrophoresis,
- the use of SYBR Green,
- a double stranded DNA dye and the flourescent reporter probe.

Q-RT-PCR: An early method of Q RT-PCR involves comparing the amount of specific product generated in different samples from a particular target sequence. In this technique, the amounts of amplified product were measured at several time points during the exponential phase of the reaction and were analyzed by linear regression.

RACE-PCR: Rapid Amplification of cDNA Ends (RACE) PCR. This technique is used to obtain the 3'end of a cDNA; it requires some sequence information internal to the mRNA under study. The sequence information obtained from this technique can be utilized to obtain full length cDNA clones using the 5'RACE technique.

RAPD-PCR: RAPD stands for Random Amplification of Polymorphic DNA. RAPD reactions are PCR reactions, but they amplify segments of DNA which are essentially unknown. Primers are designed based on the chosen sequence which will anneal to sequences flanking the sequence of interest. Thus, PCR leads to the amplification of a particular segment of DNA.

Real-time PCR: The real-time PCR system is based on the detection and quantitation of a fluorescent reporter as signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template[10]. In Taqman PCR, the fluorescent moiety and a quencher are near one end of the molecule. Hydrolysis by the advancing polymerase releases the fluorescent nucleotide from the effect of the quencher. In Molecular Beacons, the fluorophore and the quencher, attached to opposite ends of the oligonucleotide, are held together by a base paired stem that becomes disrupted on hybridization of the loop to a target nucleic acid[11,12].

Types of real-time PCR includes: Real-time RT-PCR: This term refers to a real-time PCR that is initiated with cDNA that has been reverse transcribed from RNA.

Real-time RT-asymmetric PCR: This term refers to an asymmetric PCR that is initiated with cDNA that has been reverse transcribed from RNA.

Real-time RT semi-nested PCR: This term refers to a semi-nested PCR that is initiated with cDNA that has been reverse transcribed from RNA.

Real-time RT-nested multiplex PCR: This term refers to a nested PCR that is initiated with cDNA that has been reverse transcribed from RNA and includes multiple primer pairs at one or both of the consecutive PCRs.

Rep-PCR: Is used for Genomic Fingerprinting of Plant-Associated Bacteria and Computer-Assisted Phylogenetic Analyses. A recently developed method to classify

bacteria on the basis of their genomic fingerprint patterns, using collections of both symbiotic and pathogenic plant-associated bacteria. The genomic fingerprinting method employed is based on the use of DNA primers corresponding to naturally occurring interspersed repetitive elements in bacteria, such as the REP, ERIC and BOX elements[13].

RT-PCR: The enzyme used is reverse transcriptase to produce cDNA from RNA. Together with all 4 deoxynucleoside triphosphates, magnesium ions and at neutral pH, the reverse transcriptase synthesizes a complementary DNA on the mRNA template. Then the cDNA will be utilized in PCR for amplification of the specific sequence.

Touchdown PCR: Touchdown PCR is a method by which degenerate primers are used to avoid amplifying nonspecific sequence. The temperature at which primers anneal during a cycle of PCR determines the specificity of annealing. The melting point of the primer sets the upper limit on annealing temperature. At temperatures just below this point, only very specific base pairing between the primer and the template will occur. At lower temperatures, the primers bind less specifically. Nonspecific primer binding obscures polymerase chain reaction results, as the nonspecific sequences to which primers anneal in early steps of amplification.

Vectorette PCR: Vectorette PCR is a method that enables the amplification of specific DNA fragments *in-situ*ations where the sequence of only one primer is known. Thus, it extends the application of PCR to stretches of DNA where the sequence information is only available at one end[14].

CONCLUSION

Polymerase Chain Reaction is one of the most important molecular diagnostic tools. It is used in all the fields of biology as a diagnostic because of its high sensitivity and specificity. Theoretical considerations and practical applications indicate that PCR and RT-PCR assay systems share several advantages over other quantitative molecular methodologies, thus suggesting that these techniques are the methods of choice for the absolute quantitation of viral nucleic acids. The PCR is very promising to elucidate the etiological agent of which is present in too small numbers to be detected by traditional techniques, agents difficult or impossible to cultivate and in making the distinction between infection and rejection in transplant recipients. Thus, the advent of nucleic acid amplification techniques for the clinical laboratory provides not only new diagnostic opportunities but new challenges as well.

REFERENCES

1. Saiki, R.K., T.L. Bugawan, G.T. Horn, K.B. Mullis and H.A. Erlich, 1986. Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. Nature, 324: 163-166.
2. Harris, S.A., 1999. RAPDs in Systematics-a Useful Methodology? In: Hollingsworth, P.M., R.M. Bateman and R.J. Gornall (Edn.), Molecular Systematics, Plant and Evolution. Taylor and Francis, London, pp: 221-228.
3. Francisco-Ortega, J., H.J. Newbury and B.V. Ford-Lloyd, 1993. Numerical-analyses of RAPD data highlight the origin of cultivated tagasaste (Chamaecytisus proliferus ssp palmensis) in the Canary Islands. Theoretical and Applied Genetics, 87: 264-270.
4. Karp, A., S. Kresovich, K.V. Bhat, W.G. Ayand and T. Hodgkin, 1997. Molecular tools in plant genetic resources conservation: A guide to the technologies. IPGRI Technical Bulletin No. 2, International Plant Genetic Resources Institute, Rome, Italy.
5. Liu, P., J. Siciliano, D. Seong, J. Craig, Y. Zhao, P.J. de Jong and M.J. Siciliano, 1993. Dual Alu PCR primers and conditions for isolation of human chromosome painting probes from hybrid cell. Cancer Genet. Cytogenet., 65: 93-99.
6. Poddar, S.K., 2000. Symmetric vs asymmetric PCR and Mol. beacon probe in the detection of a target gene of adenovirus. Mol. Cell Probes., 14: 25-32.
7. Daniel Graf, A.G., Fisher and M. Merkenschlager, 2005. Rational primer design greatly improves differential display-PCR (DD-PCR). Nucleic Acid Res., 25: 2239-2240.
8. Nuovo, G.J., 2006. The surgical and cytopathology of viral infections: Utility of immunohistochemistry, *in-situ* hybridization and *in-situ* polymerase chain reaction amplification. Ann. Diagn. Pathol., 10: 117-131.
9. Eltahir, Y.M., C.I. Dovas, M. Papanastassopoulou, M. Koumbati, N. Giadinis, S. Verghese-Nikolakaki and G. Koptopoulos, 2006. Development of semi-nested PCR using degenerate primers for the generic detection of small ruminant lentivirus proviral DNA. J. Virol. Methods (In press).

10. Kubista, M., J.M. Andrade, M. Bengtsson, A. Forootan and J. Jonak *et al.*, 2006. The real-time polymerase chain reaction. Mol. Aspects Med., 27: 95-125.
11. Higuchi, R., G. Dollinger, P.S. Walsh and R. Griffith, 1992. Simultaneous amplification and detection of specific DNA sequences. Biotechnol., 10: 413-417.
12. Higuchi, R., C. Fockler, G. Dollinger and R. Watson, 1993. Kinetic PCR: Real time monitoring of DNA amplification reactions. Biotechnol., 11: 1026-1030.
13. De Bruijn, F.J., J. Rademaker, M. Schneider, U. Rossbach and F.J. Louws, 1996. Rep-PCR Genomic Fingerprinting of Plant-Associated Bacteria and Computer-Assisted Phylogenetic Analyses. In: G. Stacey, B. Mullin and P. Gresshoff (Edn.), Biology of Plant-Microbe Interaction; Proceedings of the 8th International Congress of Molecular Plant-Microbe Interactions, APS Press, pp: 497-502.
14. Arnold, C. and I.J. Hodgson, 1991. A novel approach to genomic walking. PCR. Methods Appl. 1: 39-42.