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A review on types of polymerase chain reaction for diagnosis and cloning technique

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Abstract
PCR is a simple, ingenious, enzymatic assay, which allows specific DNA fragment to amplify from a complex pool of DNA. In earlier days for the first it allowed to detect specific region and large amounts of DNA from this method. These strategies in PCR have propelled huge scientific endeavors such as the Human Genome Project (HGP). The technique is currently widely used by clinicians and researchers for disease diagnose, clone and sequence genes, and carry out refined quantitative and genomic studies in a hasty and very receptive manner. PCR technique is not only used for diagnosing disease but also used in identifying inhibitor or enhancers in food samples. It is also used in the detection of Apolipoprotein E Genotype by DNA electrochemical biosensor coupled with PCR. This article describes the process involved in PCR and its types which is majorly used in diagnose and cloning technique.

Keywords: Enzymatic assay, DNA fragments, Human Genome Project (HGP), disease diagnoses

1. Introduction
Polymerase Chain Reaction is a technique used to create several copies of a certain DNA segment. This method is an efficient and cost-effective way to amplify small segments of DNA or RNA. Dr. Kary Mullis, who discovered the PCR assay, stated it "lets you pick the piece of DNA you're interested in and have as much of it as you want" said by Mullis in 1990[1]. This simple method allows clinicians to diagnose and monitor diseases using a minimal amount of sample, such as blood or tissue applied laboratory test for the diagnosis of a wide variety of central nervous system (CNS) diseases which includes genetic and autoimmune diseases, malignant neoplasms, and infections. This technique was developed in 1983 by an American biochemist Kary Mullis. PCR has made it possible for a small segment of DNA to generate millions of copies. This tool is commonly used in molecular biology and biotechnology labs. DNA segment of interest, specific primers heat-resistant DNA polymerase enzyme are the Standard ingredients in the mixture and the four different types of DNA nucleotides, the salts needed to create a suitable environment for the enzyme to act [2]. The methods used to amplify and for generate copies of the recombinant DNA fragments were time-consuming and labor-intensive before the development of PCR. In contrast to it, a machine designed to carry out PCR reactions can complete many rounds of replication and producing billions of copies of a DNA fragment, in only a few hours[41]. In this technique, a cell uses to replicate a new DNA strand itself [3]. The integral component is the template DNA was the DNA that contains the region to be copied, such as a gene. Only one DNA molecule can serve as a template. The only information needed for this fragment to be replicated is the sequence of two short regions of nucleotides at either end of the region of interest. These two short template sequences must be well-known. So that two primers, short stretches of nucleotides that correspond to the template sequences in the region can be synthesized. The primer binds to the template at their complementary sites. It serves as the starting point for copying it whereas DNA synthesis at one primer is directed toward the other, resulting in replication of the intervening sequence [42].

2. Principle
The principle of PCR is based on the enzymatic replication of nucleic acids. Primer mediated enzymes are used for the amplification of DNA. PCR amplify stretches of DNA up to 10kb, though some can work with DNA of up to 40kb [4].

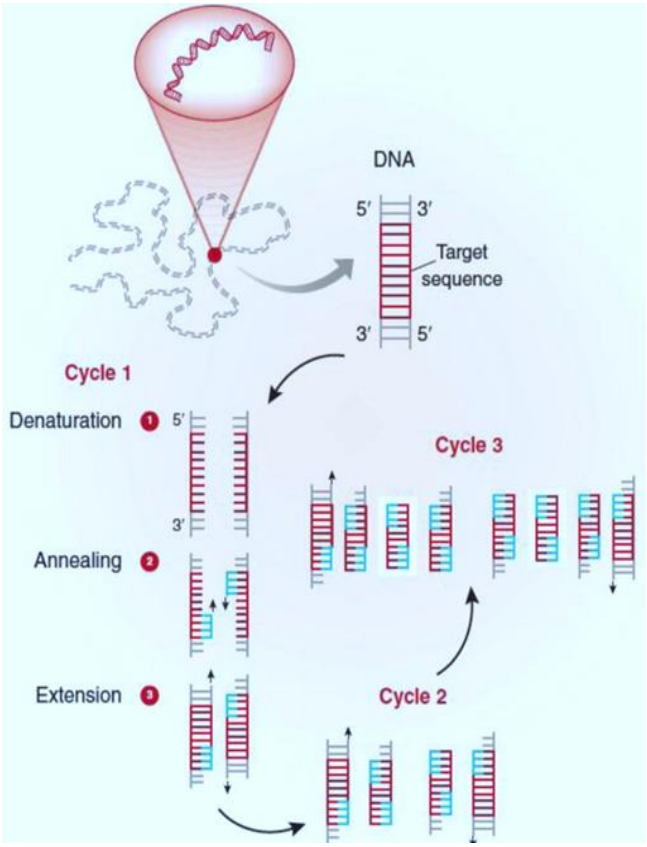

Fig 2: Polymerase Chain Reaction Cycle

3. *Taq* polymerase

Taq polymerase is a thermostable DNA polymerase I. This is named after the thermophilic bacterium *Thermus aquaticus* from which it was first isolated by Chien in 1976 [6]. Its name is habitually abbreviated to *Taq* pol [43]. It is used in the PCR as a method for significantly amplifying the quantity of short segments of DNA, for example, *T. aquaticus*. *Taq* polymerase was identified as an enzyme which is able to withstand the protein-denaturing conditions [7].

4. Sample preparation

DNA must be isolated from a sample before initiating PCR. DNA extraction is a multi-step process that is done manually. And also it is done with an instrument like the COBAS AmpliPrep Instrument, the primary instrument that prepared samples automatically. Following sample preparation, the three-step PCR process is initiated in it [5].

4.1 Separating the target DNA - denaturation

The first step is called denaturing, where is carry out in 94-98degrees C for 20-30 seconds, hydrogen bonds in between the complementary bases and double-stranded DNA is converted in to single-stranded DNA The high temperature breaks the relatively weak bonds between the nucleotides which form the DNA code. In many PCR thermocyclers, the solutions are held in small plastic tubes in metal blocks and are heated and cooled by reversing the electrical currents through the block which is known as the Peltier effect [35].

4.2 Binding primers to the DNA sequence—annealing

All DNA in the sample is not copied. It copies only a specific sequence of genetic code, targeted by the PCR primers. For example, Chlamydia has a unique pattern of nucleotides which is specific to the bacteria so the PCR will copy specific DNA sequences only that is present in Chlamydia. For doing this, PCR uses primers, man-made oligonucleotides (short pieces of synthetic DNA) that bind, or anneal, only to sequences on either side of the target DNA region. Two primers are used in step two 1) each of the newly separated single DNA strands[40]. The primers bind to the beginning of the sequence that will be copied, marking off the sequence for third step [8]. During second step, the tube is cooled and primer binding occurs between 40 and 60 degrees Celsius (104 – 140 degrees Fahrenheit). Step two yields two separate strands of DNA, with sequences marked off by the primers. The two strands are ready to be copied using PCR.

4.3 Making a copy—extension

In the third phase of the reaction, called extension, the temperature is increased to approximately 72 degrees Celsius which mean 161.5 degrees Fahrenhein. Starting at the regions noticeable by the primers, nucleotides in the solution are added to the annealed primers by the DNA polymerase to create a new strand of DNA complementary to each of the single template strands of DNA. After completing the extension, two identical copies of the original DNA have been produced [34].

After making two copies of the DNA through PCR, the cycle begins again but this time using the new duplicated DNA. Each duplicate creates two new copies through it. Approximately 30 or 40 PCR cycles, more than one billion copies of the original DNA segment have been made through this technique [9].

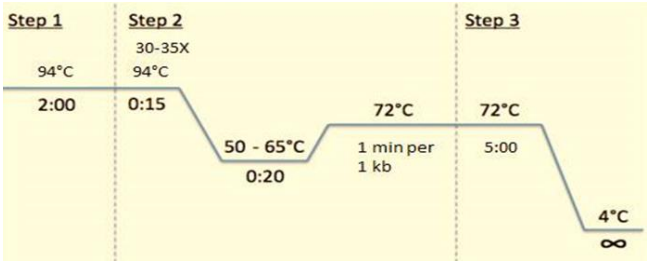

Fig 3: Diagrammatric representation of PCR cycle

5. Visualization of result of PCR

Gel electrophoresis is the common method for visualizing the result of the PCR reaction. This technique is undergoes in which fragments of DNA are pulled through the matrix of gel by a electric current [10].

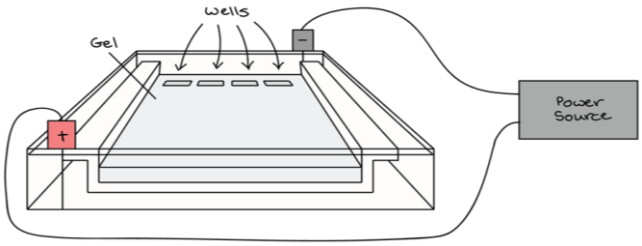

Fig 4: Instrumental structure of Gel electrophoresis

According to its size, it separates DNA fragments. DNA ladder is included for the determination of fragment Sixe in the PCR sample. 'Band' is formed in the gel. It can be seen by our naked eye by using the DNA binding dye.

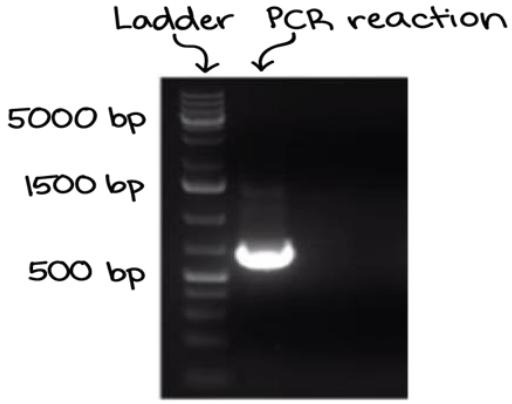

Fig 5: Sample banding pattern

Each band contains a large number of the DNA fragments, all of the same size. They all have traveled as a group in the same position [50]. The bands in the sample are undergoes a comparison so we can determine their sizes. For instance the bright band on the gel above is roughly 700700700 base pairs in size.

6. Types of PCR

6.1 Multiplex PCR

Multiplex polymerase chain reaction: is used to detect multiple pathogens using multiple primer. and it is used to amplify several different DNA sequences simultaneously. Using multiple primers, it amplifies DNA in samples and a temperature-mediated DNA polymerase in a thermal cycler

of the reaction. The primer design for all primers pairs has to be optimized definitely. So that all primer pairs can work at the same annealing temperature during PCR reaction. Multiplex-PCR was first described in the year 1988 [11]. This method is to detect deletions in the dystrophine gene. This is also used with the steroid sulfatase gene. In 2008, microsatellites and SNPs used for the analysis of multiplex PCR [39].

Within a single PCR mixture, multiplex-PCR consists of multiple primer sets to produce amplicons of varying sizes which may be specific to different DNA sequences. The additional information may be gained from a single test run by targeting multiple sequences at once, that otherwise would require several reagents and more time to perform [12].

Annealing temperatures must be optimized for each of the primer sets to work correctly within a single reaction. It is divided into two categories: Single template PCR Reaction and Multiple template PCR reaction

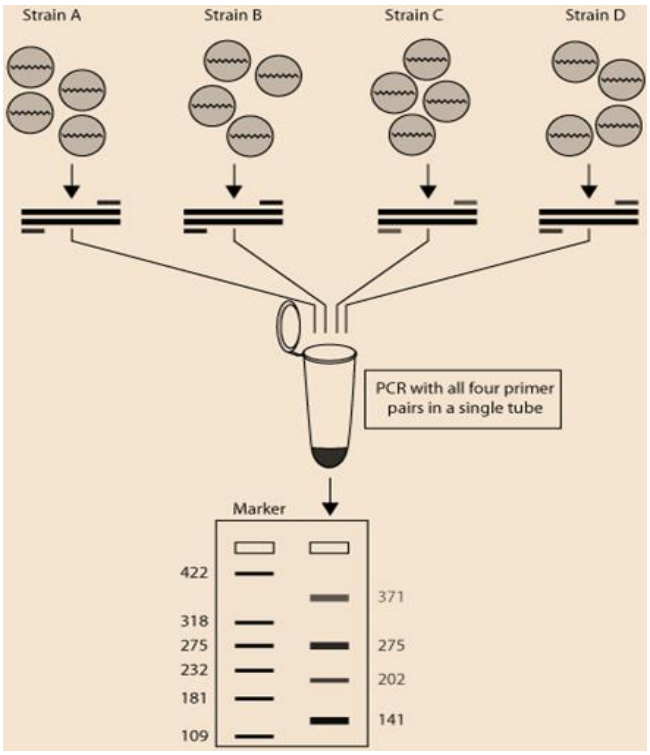

Fig: 6 Multiple PCR

6.2 Nested polymerase chain reaction (Nested PCR)

For improving sensitivity and specificity it is designed. Two primer sets and two successive PCR reactions are involved. In the initial PCR reaction, the first set of primer is used to anneal to sequence upstream by the second set of primer.

Green color indicates the target DNA which undergoes the first run of PCR with the first set of primers [13]. The selection of alternative and similar primer binding sites gives a selection of products, only one containing the projected sequence.

Red color in the (figure 7) indicates the product from the first reaction which undergoes a second run with the second set of primer. It is inprobable that any of the not needed PCR products contain binding sites for both the new primers, ensuring the product from the second PCR has little contamination from unnecessary products of primer dimers, hairpins, and alternative primer target sequences [14].

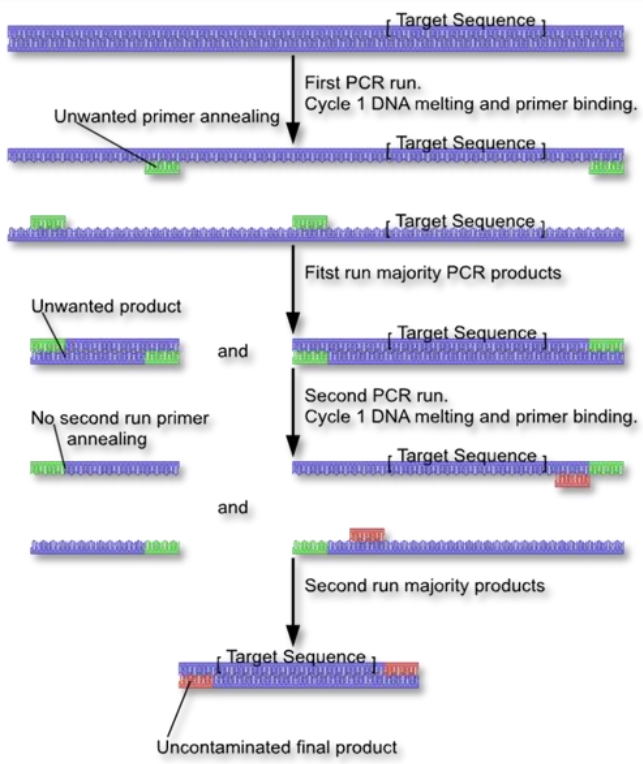

Fig 7: Nested PCR

6.3 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (Q-PCR) is a method by which the amount of the PCR product can be determined, in real-time, and is very useful for investigating gene expression [50]. This method is sometimes also referred to as real-time PCR or often abbreviated to Q-PCR or depending on the application, quantitative reverse-transcriptase PCR [15]. We will refer to all quantitative PCR methods as Q-PCR in this chapter, as this will distinguish between the two types of RT-PCR as necessary[44]. Q-PCR does not rely on any downstream analysis such as electrophoresis or densitometry and is extremely versatile, enabling multiple PCR targets to be assessed simultaneously but can sometimes be a little trickier to set up than "standard" PCR; however, if you are sufficiently familiar with "standard" PCR then you are in a good position to successfully undertake Q-PCR [16].

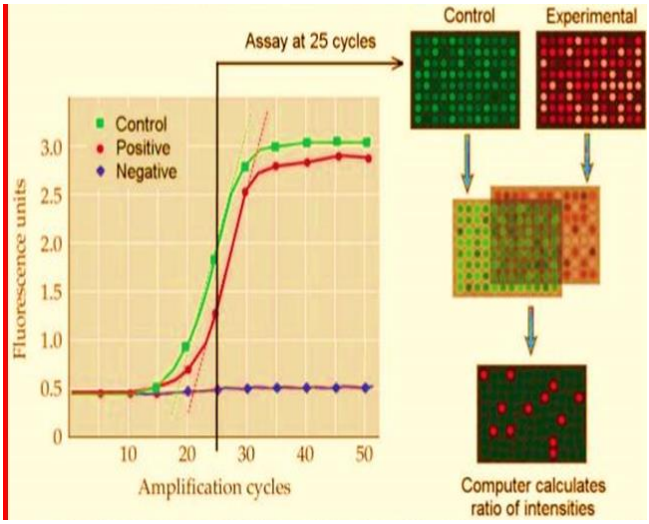

Fig 8: Graphical representation of Quantitative PCR

6.4 Colony PCR

Colony PCR is a suitable high-throughput method for determining the being there or nonappearance of insert DNA in plasmid constructs. Individual transformants can either be lyses in water with a short heating step or added directly to the PCR reaction and lyses during the initial heating step. This initial heating step causes the release of the plasmid DNA from the cell, so it can serve as template for the amplification reaction. Primers designed to specifically target the insert DNA that can be used to identify if the construct contains the DNA fragment of interest. Alternatively, primers targeting vector DNA flanking the insert can be used to determine whether or not the insert is the correct molecular size [17]. Insert specific primers can provide information on both the specificity and size of the insert DNA while the use of vector specific primers allows screening of multiple constructs simultaneously. Colony PCR can also be used to determine insert orientation. PCR amplification of the plasmid using an insert specific primer paired with a vector specific primer can be designed to produce an amp icon of a specific size only if the insert is in the correct orientation [18]. In all experimental designs, presence or absence of a PCR amp icon and size of the product are determined by electrophoresis alongside a DNA size marker on an agars gel.

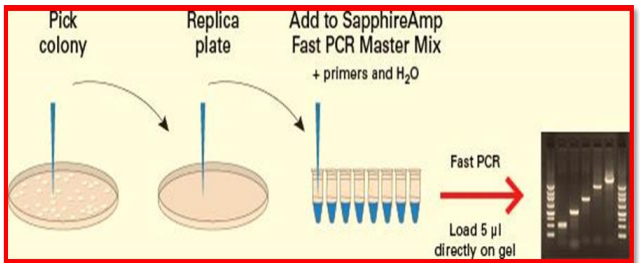

Fig 9: Colony PCR

7. Advantages and Limitations

There are multiple advantages to PCR. First, it is a simple technique to understand and it produces results rapidly. It is an extremely sensitive technique with high potential to produce millions to billions of copies of a specific product for sequencing, cloning, and analysis [19]. This is true of art-PCR as well, but art-PCR has the advantage of quantification of the synthesized product. In tumors, microbes, or other disease states can be used to analyze alterations of gene expression levels [38].

While PCR is an expensive technique, it does have limitations. Because PCR is a highly sensitive technique, any form of contamination of the sample by even trace amounts of DNA can produce misleading results [47]. Additionally, in order to design primers for PCR, some prior sequence data is required [20]. Therefore, PCR can only be used to identify the presence or absence of a known pathogen or gene. Another limitation is that the primers used for PCR can anneal non-specifically to sequences that are similar, but not completely identical to target DNA. In addition, incorrect nucleotides can be incorporated into the PCR sequence by the DNA polymerase, albeit at a very low rate.

8. Diagnostic application of PCR

8.1 Neurologic Diseases

Viruses are frequent causative agents in certain neurologic diseases. Examples are meningitis, encephalitis, and mellitus. It is difficult, a lot impossible, to isolate many viruses by standard cell culture techniques. PCR is attractive because it overcomes most of these limitations [21]. PCR is a mostly applied laboratory test for the diagnosis of a wide variety of genetic and autoimmune diseases central nervous system (CNS) diseases, malignant neoplasm, and infections [22]. It have an ability to detect minute amounts of DNA or RNA contained in tissues or fluids, PCR enhanced understanding of pathogenesis, the rapidity and accuracy of diagnosis is improved, and also helped identify infectious causes for diseases previously considered idiopathic. In addition, PCR can be performed on a variety of tissues preserved in different ways where even archival specimens can be used to provide important epidemiological information. By making quick and precise diagnoses, appropriate treatments can be instituted and redundant or persistent investigations can be avoided [23].

8.2 Dentistry

8.2.1 Periodontal Diseases

A clinical diagnosis of periodontal disease is made by measuring the loss of connective tissue attachment on the root surface, which means clinical attachment loss and loss of alveolar bone ways radiographic bone loss [24]. But the clinical diagnosis does not indicate the cause, pathogenesis, clinical course, progress, and prognosis of the disease. In addition to the conventional examination, various diagnostic methods play a vital role in the confirmation of the clinical diagnosis [48]. The PCR technique is a more accurate, sensitive, and rapid technique for the detection, identification, and quantification of periodontal bacteria - PCR or real-time PCR with species-specific primers provide accurate quantification of individual microbial species and total bacterial count in dental plaque samples [25]. This precise and sensitive method serves as a useful tool for studies on etiology of periodontal diseases [49].

Various putative perio-pathogens such as, *Tannerella forsythia* (Tf), *Prevotella intermedia* (Pi), *Prevotella nigrescens*, *Parvimonas micra* (Pm), Pg, *Aggregatibacter actinomycetecomitans* (Aa) *Eubacteria*, *Campylobacter rectus* (Cr), *Capnocytophaga sputigena*, *Capnocytophaga ochracea*, and *Capnocytophag gingivalis* have been detected in subgingival plaque sample [26].

8.2.2 Oral Cancer

One of the uses of PCR in dentistry is the detection of markers in the diagnosis and prognosis of some types of oral cancer [46]. For this purpose, PCR is a fast, easy method with a relatively low cost [27]. Diagnoses, prognoses and treatment can be improved through the study and use of genetic markers identified by means of immune histochemistry, PCR and other molecular biology procedures [28].

Squamous cell carcinoma of the oral cavity is generally accompanied by other types of aerodigestive tract carcinomas, such as oropharyngeal and esophageal carcinoma. *Streptococcus anginosus* is a bacterium that is

isolated in different parts of the body and has been particularly isolated in squamous cell carcinoma of the head and neck. Through real-time PCR, *S. anginosus* can be detected with greater sensitivity and specific approximation in squamous cell carcinomas of the oral cavity [29].

8.3 Forensic Science

Genetic fingerprinting can uniquely discriminate any one person from the total population of the world. Minute samples of DNA can be isolated from a crime scene and compared to that from suspects, or from a DNA database of earlier evidence or convicts [51]. Simpler versions of these tests are often used to hurriedly rule out suspects during a criminal investigation. Evidence is compiled or collected from the old decades crimes will be tested and exonerating the people originally convicted [30]. Less discriminating forms can help in DNA paternity testing where a relevant is matched with their close relatives. DNA from unidentified human remains can be tested, and compared with that from possible parents, children or siblings. Alike testing can be used to confirm the biological parents of an adopted or kidnapped child [31]. The real biological father of a newborn can also be confirmed.

8.4 Medical Application

Prospective parents can be tested for being genetic carriers and their children might be tested for actually being affected by a disease.DNA samples for prenatal testing can be obtained by chorionic villus sampling, amniocentesis or even by the analysis of rare fetal cells circulating in the mother's bloodstream [45]. PCR analysis is also vital to pre implantation genetic diagnosis, where developing embryo of a individual cells are tested for mutations [32].PCR can also be very important to organ transplantation, used as part of a sensitive test for tissue testing. As of 2008, there is even a proposal to replace the traditional antibody-based tests for blood type with PCR-based tests.PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already be used routinely [33].

9. Conclusion

PCR is the simple technique that used to produce large amplified number of copies. Larger Fragment of the plasmid DNA is produced by PCR.PCR is moreover complicated with high specificity, sensitivity. It is used in inhibitory activities of the metal ions. These techniques have a vast application in disease detection. Recombinant clones are produced by the PCR technique. PCR can be applied in many fields like, in diagnosis therapy, in agricultural science, in dentistry, in cancer therapy, in PCR fingerprinting. This technique is also used in the accurate detection of the microorganisms.

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