

POLYMERASE CHAIN REACTION: METHODS, PRINCIPLES AND APPLICATION

Dr.Mohini Joshi^{1*}, Dr.Deshpande J.D².

¹Department of Anatomy, Rural Medical College, Pravara Institute of Medical Sciences, Loni, Maharashtra, India

²Department of Community Medicine, Rural Medical College, Pravara Institute of Medical Sciences, Loni, Maharashtra, India

Corresponding author*: atharvamohini@gmail.com

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ABSTRACT

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. There are three major steps involved in the PCR technique: denaturation, annealing, and extension. PCR is useful in the investigation and diagnosis of a growing number of diseases. Qualitative PCR can be used to detect not only human genes but also genes of bacteria and viruses. PCR is also used in forensics laboratories and is especially useful because only a tiny amount of original DNA is required. PCR can identify genes that have been implicated in the development of cancer. Molecular cloning has benefited from the emergence of PCR as a technique. The present paper is an attempt to review basics of PCR.

KEY WORDS: PCR, Principles, Application

INTRODUCTION

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Polymerase Chain Reaction was developed in 1984 by the American biochemist, Kary Mullis. Mullis received the Nobel Prize and the Japan Prize for developing PCR in 1993.¹ However the basic principle of replicating a piece of DNA using two primers had already been described by Gobind Khorana in 1971. Progress was limited by primer synthesis

and polymerase purification issues.² PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications.³ The polymerase chain reaction is a powerful technique that has rapidly become one of the most widely used techniques in molecular biology because it is quick, inexpensive and simple. The technique amplifies specific DNA fragments from minute quantities of source DNA material, even when that source DNA is of relatively poor quality.⁴ PCR; the quick, easy method for generating unlimited copies of any

fragment of DNA, is one of those scientific developments that actually deserve timeworn superlatives like "revolutionary" and "breakthrough. From the daily practicalities of medical diagnosis to the theoretical framework of systematics, from courts of law to field studies of animal behavior, PCR takes analysis of tiny amounts of genetic material—even damaged genetic material—to a new level of precision and reliability. Furthermore, many important contributions to the development and application of PCR technology have been made; however the present paper is an attempt to review basics of PCR.

Basic concept of PCR

The basic PCR principle is simple. As the name implies, it is a chain reaction: One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e. the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates. PCR is a method used to acquire many copies of any particular strand of nucleic acids. It's a means of selectively amplifying a particular segment of DNA. The segment may represent a small part of a large and complex mixture of DNAs *e.g.* a specific exon of a human gene. It can be thought

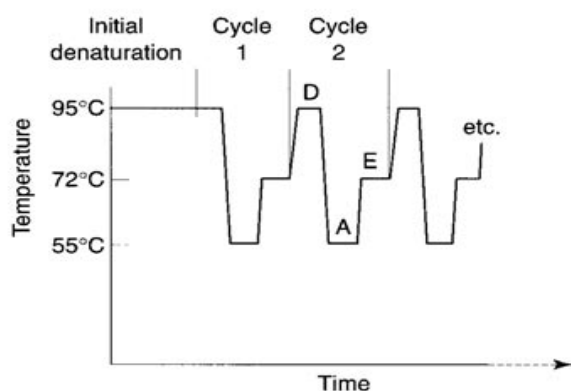
of as a molecular photocopier. PCR can amplify a usable amount of DNA (visible by gel electrophoresis) in ~2 hours. The template DNA need not be highly purified — a boiled bacterial colony. The PCR product can be digested with restriction enzymes, sequenced or cloned. PCR can amplify a single DNA molecule, *e.g.* from a single sperm. The polymerase chain reaction relies on the ability of DNA-copying enzymes to remain stable at high temperatures. PCR has transformed the way that almost all studies requiring the manipulation of DNA fragments may be performed as a result of its simplicity and usefulness.⁵ In Mullis's original PCR process, the enzyme was used *in vitro*. The double-stranded DNA was separated into two single strands of DNA by heating it to 96°C. At this temperature, however, the *E.Coli* DNA polymerase was destroyed, so that the enzyme had to be replenished with new fresh enzyme after the heating stage of each cycle. Mullis's original PCR process was very inefficient since it required a great deal of time, vast amounts of DNA-Polymerase, and continual attention throughout the PCR process.⁶

Steps in PCR

There are three major steps involved in the PCR technique: denaturation, annealing, and extension. In step one; the DNA is denatured at high temperatures (from 90 - 97 degrees Celsius). In step two, primers anneal to the DNA template strands to prime extension. In step three, extension occurs at the end of the annealed primers to create a complementary copy strand of DNA. This effectively doubles the DNA quantity through the third steps in the PCR cycle. To amplify a segment of DNA using PCR, the sample is first heated so the DNA denatures, or separates into two pieces of

single-stranded DNA. Next, an enzyme called "Taq polymerase" synthesizes - builds - two new strands of DNA, using the original strands as templates. This process results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Then each of these strands can be used to create two new copies, and so on, and so on.⁷ The annealing phase happens at a lower temperature, 50-60°C. This allows the primers to hybridize to their respective complementary template strands, a very useful tool to forensic chemistry. The newly-formed DNA strand of primer attached to template is then used to create identical copies off the original template strands desired. Taq polymerase adds available nucleotides to the end of the annealed primers. The extension of the primers by Taq polymerase occurs at approx 72°C for 2-5 minutes. DNA polymerase I cannot be used to elongate the primers as one would expect because it is not stable at the high temperatures required for PCR. The beauty of the PCR cycle and process is that it is very fast compared to other techniques and each cycle doubles the number of copies of the desired DNA strand. After 25-30 cycles, whoever is performing the PCR process on a sample of DNA will have plenty of copies of the original DNA sample to

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conduct experimentation. Assuming the maximum amount of time for each step, 30 cycles would only take 6 hours to complete.

As the process of denaturation, annealing, and polymerase extension is continued the primers repeatedly bind to both the original DNA template and complementary sites in the newly synthesized strands and are extended to produce new copies of DNA. The end result is an exponential increase in the total number of DNA fragments that include the sequences between the PCR primers, which are finally represented at a theoretical abundance of 2^n , where n , is the number of cycles.^{5,8}

Due to the introduction of a thermostable DNA polymerase, the Taq DNA polymerase once, at the beginning of the PCR reaction.⁹ The thermostable properties of the DNA polymerase activity were isolated from *Thermus aquaticus* (Taq) that grow in geysers of over 110°C, and have contributed greatly to the yield, specificity, automation, and utility of the polymerase chain reaction. The Taq enzyme can withstand repeated heating to 94°C and so each time the mixture is cooled to allow the oligonucleotide primers to bind the catalyst for the extension is already present.¹⁰ After the last cycle, samples are usually incubated at 72°C for 5 minutes to fill in the protruding ends of newly synthesized PCR products. To ensure success, care should be taken both in preparing the reaction mixture and setting up the cycling conditions. Increasing the cycle number above ~35 has little positive effect because the plateau occurs when the reagents are depleted; accumulate. The specificity of amplification depends on the extent to which the primers can recognize and bind to sequences other than the intended target DNA sequences.

METHODS

In molecular biology, real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. Traditionally, PCR is performed in a tube and when the reaction is complete the products of the reaction (the amplified DNA fragments) are analyzed and visualized by gel electrophoresis. However, Real-Time PCR permits the analysis of the products while the reaction is actually in progress. This is achieved by using various fluorescent dyes which react with the amplified product and can be measured by an instrument. This also facilitates the quantitation of the DNA. Quantitative PCR (Q-PCR), as this technique is known, is used to measure the quantity of a PCR product (usually in a real-time PCR procedure). It is the method of choice to quantitatively measure starting amounts of DNA, cDNA or RNA. PCR is therefore often used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. Another advantage of Real-Time PCR is rapidity of the assay, since it is not necessary to perform electrophoresis or other procedure after the DNA amplification reaction.^{11, 12} Digital PCR concept was conceived in 1992 by Sykes et al.¹³ It is a refinement of conventional polymerase chain reaction methods that can be used to directly quantify and clonally amplify nucleic acids including DNA, cDNA or RNA. The key difference between dPCR and traditional PCR lies in the method of measuring nucleic acids amounts, with the former being a more precise method than PCR. PCR carries out one reaction per single sample. dPCR also carries out a single reaction within a

sample, however the sample is separated into a large number of partitions and the reaction is carried out in each partition individually. This separation allows a more reliable collection and sensitive measurement of nucleic acid amounts.¹⁴ Inverse PCR is a variant of the polymerase chain reaction that is used to amplify DNA with only one known sequence. One limitation of conventional PCR is that it requires primers complementary to both termini of the target DNA, but this method allows PCR to be carried out even if only one sequence is available from which primers may be designed.¹⁵ Nested polymerase chain reaction is a modification of polymerase chain reaction intended to reduce the contamination in products due to the amplification of unexpected primer binding sites.¹⁶ Touchdown polymerase chain reaction is a method of polymerase chain reaction by which primers will avoid amplifying nonspecific sequences. The earliest steps of a touchdown polymerase chain reaction cycle have high annealing temperatures. The annealing temperature is decreased in increments for every subsequent set of cycles.¹⁷

Applications of PCR

PCR is helping in the investigation and diagnosis of a growing number of diseases. It has also long been a standard method in all laboratories that carry out research on or with nucleic acids. Even competing techniques such as DNA chips often require amplification of DNA by means of PCR as an essential preliminary step. The polymerase chain reaction is used by a wide spectrum of scientists in an ever-increasing range of scientific disciplines. The use of reverse transcriptase's to evaluate RNA levels and the extension of PCR technology to quantify DNA amplification in real time

has brought major advances to the application of PCR. By allowing the determination and quantification of changes in gene expression, these techniques have provided a greater understanding of disease processes and now serve as a foundation for diagnostics and basic science research.¹⁸ In microbiology and molecular biology, for example, PCR is used in research laboratories in DNA cloning procedures, Southern blotting, DNA sequencing, recombinant DNA technology, to name but a few. In clinical microbiology laboratories PCR is invaluable for the diagnosis of microbial infections and epidemiological studies. PCR is also used in forensics laboratories and is especially useful because only a tiny amount of original DNA is required, for example, sufficient DNA can be obtained from a droplet of blood or a single hair. In fact, a number of trials using PCR for detection of a broad range of bacteria in CSF specimens have been reported.¹⁹⁻²¹ Since the culture of *C. pneumoniae* is difficult in most clinical laboratories, determination of this bacterium in clinical specimens has been widely performed using the PCR technique even though there is no standardized PCR method for detection of this organism. Nested PCR is one of these protocols for detection of only a few bacteria in clinical specimens.²²

Qualitative PCR can of course be used to detect not only human genes but also genes of bacteria and viruses. One of the most important medical applications of the classical PCR method is therefore the detection of pathogens. Many viruses contain RNA rather than DNA. In such cases the viral genome has to be transcribed before PCR is performed, and RTPCR is therefore used. Sometimes it is also necessary to detect pathogens outside the body. Fortunately, the PCR method can detect the DNA of microorganisms in

any sample, whether of body fluids, foodstuffs or drinking water. Quantitative PCR provides additional information beyond mere detection of DNA. It indicates not just whether a specific DNA segment is present in a sample, but also how much of it is there. This information is required in a number of applications ranging from medical diagnostic testing through target searches to basic research. Another important application of quantitative PCR is in molecular diagnosis, i.e. the diagnosis of diseases based on molecular findings rather than on physiological symptoms. In this connection the diagnosis of viral diseases is an area that is gaining increasing importance. PCR is the most sensitive test for herpes simplex virus, varicella-zoster virus, and human papillomavirus infections. Other diagnostic uses, including tests for genetic diseases, cancers, and other infectious diseases, are evolving.²³ Another important application in which quantitative PCR is used in the field of infectious diseases is AIDS. It can detect the AIDS virus sooner during the first few weeks after infection than the standard ELISA test.

Genetic factors are always involved in the development of cancer. Their contribution varies greatly depending on the type of cancer. Genes not only help to determine progression of the disease but can also have a substantial influence on the effectiveness of the available treatments. Identifying the genes that play a role in the development of cancer is therefore an important step towards improving treatment. Both qualitative and quantitative PCR play a crucial role in the fight against cancer. PCR can identify genes that have been implicated in the development of cancer. There are numerous applications for real-time polymerase chain reaction in the laboratory. It is commonly used for both

basic research and is deployed as a tool to detect newly emerging diseases, such as flu, in diagnostic tests. Digital PCR has many potential applications, including the detection and quantification of low-level pathogens, rare genetic sequences, copy number variations, and relative gene expression in single cells. Clonal amplification enabled by single-step digital PCR is a key factor in reducing the time and cost of many of the "next-generation sequencing" methods and hence enabling personal genomics.¹⁴ Inverse PCR is especially useful for the determination of insert locations. For example, various retroviruses and transposons randomly integrate into genomic DNA. To identify the sites where they have entered, the known, "internal" viral or transposon sequences can be used to design primers that will amplify a small portion of the flanking, "external" genomic DNA.¹⁵ Nested polymerase chain reaction is a key part of many genetics research laboratories, along with uses in DNA fingerprinting for forensics and other human genetic cases. Conventional PCR requires primers complementary to the termini of the target DNA. A commonly occurring problem is primers' binding to incorrect regions of the DNA, giving unexpected products.¹⁶ RT-PCR is commonly used in studying the genomes of viruses whose genomes are composed of RNA, such as Influenza virus A and retroviruses like HIV. PCR can be used for the diagnosis of Indian visceral leishmaniasis with great accuracy. PCR can also be employed with significant precision to predict cure of the disease.²⁴ Molecular cloning has benefited from the emergence of PCR as a technique. Direct cloning was first conducted using a DNA fragment amplified by PCR and oligonucleotide primers which contained restriction

endonuclease recognition sites added to their 5' ends.²⁵

CONCLUSION

The advancement of science has transformed our lives in ways that would have been unpredictable just a half-century ago. Molecular methods have shown a promise in this aspect. PCR and its applications hold scientific and medical promise. PCR has very quickly become an essential tool for improving human health and human life. PCR has completely revolutionized the detection of RNA and DNA viruses. PCR is valuable as a confirmatory test. PCR is a rapid technique with high sensitivity and specificity. PCR has also been credited to have been able to detect mixed infections with ease in many studies. PCR, a more sophisticated technique, requires infrastructural support, is expensive but nevertheless, one cannot discount its utilitarian advantages which are many compared to the existing conventional diagnostic methods.

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