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Polymerase Chain Reaction (PCR): Back to Basics

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ABSTRACT

Advanced molecular technology has become a crucial tool for identifying new genes with importance in medicine, agriculture, animal production, health, environment, industry other related areas. Among the applications of molecular techniques is important to highlight the use of the Polymerase Chain Reaction (PCR) in the identification and characterization of viral, bacterial, parasitic and fungal agents. PCR is a process used in molecular biology to amplify a single copy 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. Mechanisms involved in this methodology are similar to those occurring in vivo during DNA replication. Through this paper we will review procedure, advantages, types & applications of PCR.

Keywords: *PCR, DNA Hybridization, Denaturation, Annealing, RT-PCR*

INTRODUCTION

Modern days of the oral pathologist resting entirely on the examination & reporting of tissue sections stained by histochemical methods is slowly being replaced by advanced immunologic and molecular techniques of *DNA, RNA, Protein structure or function* extend the process by which complicated infectious, metabolic, inflammatory and neoplastic diseases are diagnosed and treated[1].

Contemporary molecular technology has become a crucial tool for identifying new genes with importance in medicine, agriculture, animal production, health, environment, industry related to these areas. Among the applications of molecular techniques is important to highlight the use of the ***Polymerase Chain Reaction (PCR)*** in the identification and characterization of viral, bacterial, parasitic and fungal agents. It has been considered as an essential tool in molecular biology which allows *amplification of nucleic acid sequences* (DNA and RNA) through repetitive cycles *in vitro*. Mechanisms involved in this methodology are similar to those occurring *in vivo during DNA replication*[2].

This technique was developed by Kary Mullis in the mid 80's.

"*Beginning with a single molecule of the genetic material DNA, the PCR can generate 100 billion similar molecules in an afternoon. The reaction is easy to execute. It requires no more than a test tube, a few simple reagents, and a source of heat.....*" Kary B. Mullis[3].

DEFINITION OF PCR

It is a genetic technique that occurs *in vitro* which allows the enzymatic synthesis of large quantities *(amplification)* of a targeted region of DNA in *exponential manner.* DNA is synthesized in the same manner as that seen *in vivo* (in the cells) using a *DNA polymerase* (enzymes that cells use to replicate their DNA)[3].

PRINCIPLE OF PCR

Polymerase chain reaction (PCR) is a powerful and widely used technique that has greatly advanced our ability to analyze genes. Genomic DNA present in cells contains many thousands of genes. This makes it difficult to isolate and analyze any individual gene[4].

PCR allows *specific DNA sequences*,__usually corresponding to genes or parts of genes, to be copied from genomic DNA in a simple enzyme reaction. Only requirement is that some of the DNA sequence at either end of the region to be copied is known. DNA corresponding to the sequence of interest is copied or *amplified by PCR more than one million fold* and becomes the predominant DNA molecule in the reaction. Sufficient DNA is obtained for detailed analysis or manipulation of the amplified gene[4].

ESSENTIAL COMPONENTS OF REACTION (PCR)

1. Template DNA: Template DNA containing genomic DNA sample from patient can be used in *single or double–stranded form.* Closed circular DNA templates are amplified slightly less efficiently than linear DNAs. deally PCR requires only a single copy of target sequence as template[4].

2. Oligonucleotide Primers: A pair of synthetic oligonucleotides should be *short, single stranded, and complementary to opposite strands* of the flanking regions of the fragment of interest. Standard reactions contain 0.1 - 0.5µM of each primer, which is sufficient for 30 cycles of amplification of a 1-kb segment of DNA. Higher concentrations of primer favour mispriming and may lead to nonspecific amplification. Oligonucleotide primers synthesized on an automated DNA synthesizer can be used for standard PCR[4].

3. Thermostable DNA polymerase: A thermostable DNA polymerase, which can withstand the denaturation temperatures (94-95 ºC), is essential to catalyse the template dependent synthesis of DNA. Originally the Klenow fragment of Escherichia coli pol I was used (Saiki et al., 1985). *This enzyme was inactivated at high temperatures* required for separation of two strands of DNA and hence had to be added freshly after every denaturation step. This hurdle was overcome with the introduction of thermostable DNA polymerase, *Taq DNA polymerase_isolated from thermophilic bacterium Thermus aquatics.* For a standard 25-50µl reaction, 0.5-0.25 units of Taq polymerase are used[4].

4. Deoxynucleoside triphoshates (dNTPs): Standard PCRs contain equimolar concentrations of …

- dCTP, Deoxycytidine triphosphate
- dTTP, Deoxythymidine triphosphate
- dATP, Deoxyadenosine triphosphate
- dGTP, Deoxyguanosine triphosphate (200-250 µM each).

These dNTPs are available commercially and supplied as pyrophosphate free mixtures. dNTPs must be stored at -200 ºC. During long term storage, small amounts of water evaporate and freeze over the walls of the vial. Hence in order to minimize the change in concentration, vials should be centrifuged before use[4].

5. Divalent Cations: Free divalent cations are needed for the activity of thermostable polymerases, usually Mg^{+2} is used. Mg^{+2} binds to dNTPs and oligonucleotides. Molar concentration of cation should exceed the molar concentration of phosphate groups in the dNTPs and primers together. Hence the optimal concentration of the cations should be calculated empirically for each reaction. A concentration of 1.5 mM of Mg^{+2} is routinely used. Excess Mg^{+2} will result in accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce the yield[5].

6. Buffer to maintain pH: pH of reaction mixture is adjusted to 8.3 - 8.8 at room temperature for standard PCR[6].

7. Monovalent cations: 50 mM of KCl is used in standard PCR for amplification of DNA segments more than 500 base pairs in length[7].

8. Others: Some researchers have reported that the efficiency of reaction is increased by inclusion of 10% dimethyl sulfoxide (DMSO) in the Taq polymerase buffer[8,9].

PROCEDURE

DNA or RNA suitable for PCR analysis can be obtained from most of tissue sources, including…

- *Fresh or frozen unfixed tissue,*
- *Cytology smears,*
- *Stained sections,*
- *Blood films and*
- *Body fluids like saliva also*[10].

DNA extracted from formalin fixed paraffin wax embedded tissues can also be used for amplification by PCR. Such DNA is highly degraded as compared to that obtained from fresh or frozen samples and cannot be used

for analysis by techniques like southern blot analysis[11].

Fixatives such as Bouin's solution should be avoided to fix tissues meant for PCR amplification; whereas buffered formalin fixative can be used. If the tissue remains in formalin for more than a few days, the extracted DNA may not be successfully amplified. Quality of DNA extracted from tissues embedded in paraffin wax is not affected even though stored for long duration of time[12].

Amplification can be conveniently performed in a DNA thermal cycler.

The reaction mixture contains..

- A target sequence of 100-500 base pair length
- 50 mM KCl
- 10 mM Tris.HCI (pH of 8.4 at room temp)
- 1.5 mM $MgCl_2$
- 100 µg/ml Gelatine
- 0.25 µM of each Primer
- 200 µM of each Deoxynucleotide Triphoshates (dATP, dCTP, dGTP, and dTTP)
- 2.5' units of Taq Polymerase[13]

Amplification of the target sequence is achieved by a repetitive series of cycles involving three steps:

1. ***Denaturation*** of the template by heat.
2. ***Annealing*** of the oligonucleotide primers to single stranded target sequences.
3. ***Extension*** of the annealed primers by thermostable DNA polymerase[14].

1. DENATURATION (Fig 1)

In PCRs catalysed by Taq polymerase, *denaturation is carried out at 94-95 ºC*, which is the highest temperature the enzyme can withstand for 30 or more cycles without being damaged. During the first cycle, denaturation is carried out for 5 minutes to ensure complete denaturation of the long molecules of template DNA. But at times such longer duration of denaturation may be deleterious. Denaturation for 45 seconds at a temperature of 94-95 ºC is recommended for routine amplification of linear DNA templates containing 55% or lesser amount of G+C. Higher temperatures may be required to denaturate templates containing higher amounts of G+C. In addition, longer the DNA templates, greater is the denaturation time required. *If denaturation temperature is too low or if time is too short,* only the A–T rich regions of template DNA will be denatured. Such DNA will re-anneal back when denaturation temperature is reduced later during PCR cycle[15].

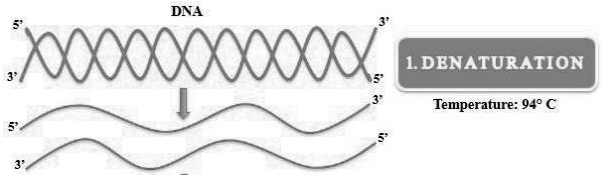


Figure 1: Denaturation of DNA

2. ANNEALING (Fig 2)

Annealing temperatures range from 55-65 ºC depending on the primer sequence and length.

Annealing temperature is critical.*If annealing temperature is too high,* the oligonucleotide primers anneal poorly and the amplified DNA is too low. *If annealing temperature is too low,* nonspecific annealing of primers may occur, resulting in the amplification of unwanted segments of DNA. *Annealing temperature can be optimized* by performing series of trial PCRs at room temperatures ranging from 2-100 ºC below the melting temperatures of oligonucleotide primers. Also a sequential series of annealing temperatures can be used in a routine PCR[15].

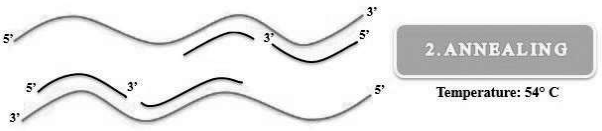


Figure 2. Annealing

EXTENSION (Fig 3)

DNA polymerase catalyses the extension of oligonucleotide primers and there by a new strand, having sequences complementary to template strand is synthesised. Optimal temperature for DNA synthesis may vary slightly depending on the DNA polymerase used. When Taq polymerase is used, the ideal temperature for DNA synthesis is about 72-78 ºC. Taq polymerase can insert about 2000 nucleotides every minute at this temperature.

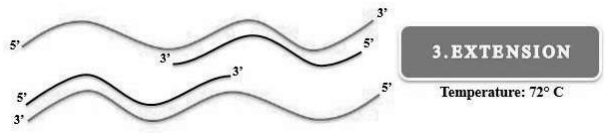


Figure 3: Extension

Extension of from one primer proceeds beyond the sequence complementary to the binding site of the other primer during the first two cycles. In the next cycle, the length of the DNA molecules produced is equal to the

segment of DNA delimited by binding sites of primers. From the third cycle onwards, this segment of DNA is amplified geometrically while the longer amplification products accumulate arithmetically[15]. **(Fig 4)**

Figure 4: Polymerase Chain Reaction

NUMBER OF CYCLES (Fig 5)

Number of cycles needed for amplification depends on…

- Number of template DNA sequences present in the reaction mixture
- Efficiency of primer extension.

Amplified products accumulate *exponentially*[16].

Figure 5: Number of cycles

PCR PHASES (Fig 6)

A basic PCR run can be broken up into three phases:

1. EXPONENTIAL:

Exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). Reaction is very specific and precise.

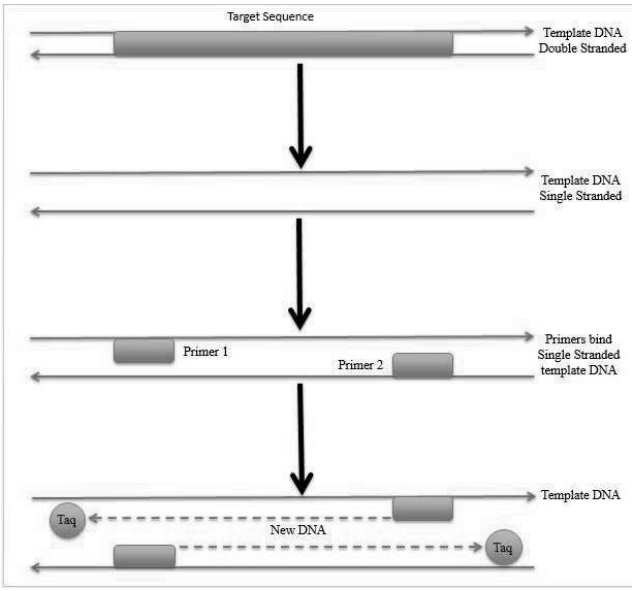


Figure 6: PCR PHASES

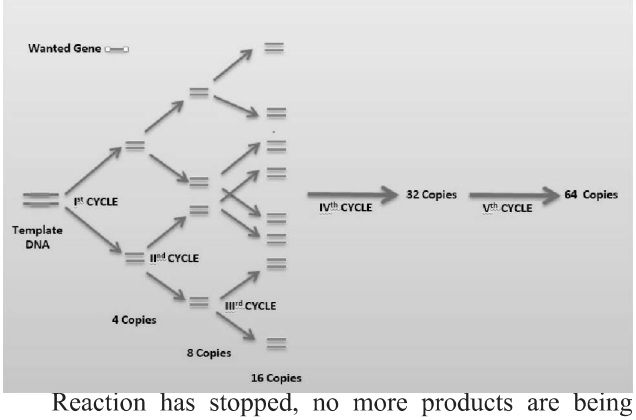
2. LINEAR:

(HIGH VARIABILITY):

Reaction components are being consumed, the reaction is slowing, and products are starting to degrade.

3. PLATEAU:

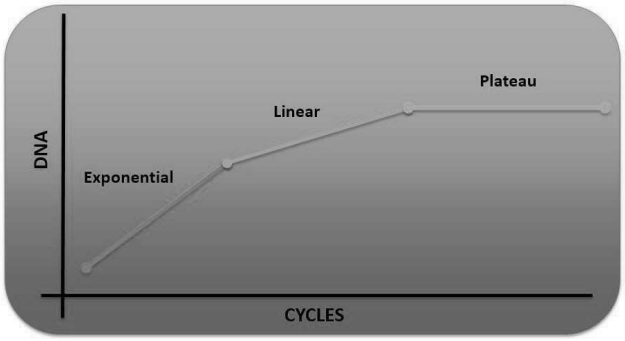
(END-POINT: GEL DETECTION FOR TRADITIONAL METHODS):



Reaction has stopped, no more products are being made and if left long enough, the PCR products will begin to degrade**.** In most cases the plateau is unavoidable but by the time it occurs, adequate amounts of product will have accumulated. If more material is required, multiple reactions can be easily set up[17].

ADVANTAGES

1. SIMPLE TECHNIQUE: PCR is a relatively



simple technique that can detect a nucleic acid fragment and amplify this sequence.

2. SENSITIVITY: This technique offers sensitivity because from small amounts of genetic material can be detected target sequences in a sample.

3. SPECIFICITY: Also this offers specificity due to a specific sequence of DNA is amplified through strict conditions.

4. FAST TECHNIQUE: It is considered a fast technique compared with other methods to detect microorganisms such as bacteria, fungus or virus, which require isolation and culture using culture media or cell lines.

5. VERSATILITY: Versatility due to the genetic sequences from various microorganisms can be identified with the same reaction conditions for diagnosis of different pathologies[18].

TYPES OF PCR

In recent years, modifications or variants have been developed from the basic PCR method to improve performance and specificity, and to achieve the amplification of other molecules of interest in research as RNA. Some of these variants are:

i. Multiplex PCR whichsimultaneously amplified several DNA sequences (usually exonic sequences).

ii. Nested PCR increases the specificity of the amplified product for a second PCR with new primers that hybridize within the amplified fragment in the first PCR.

iii. Semiquantitative PCR which allows an approximation to the relative amount of nucleic acids present in a sample.

iv. RT-PCR which generates amplification of RNA by synthesis of cDNA (DNA complementary to RNA) that is then amplified by PCR; and,

v. Real time PCR which performs absolute or relative quantification of nucleic acid copies obtained by PCR.

APPLICATIONS OF PCR

1. Detecting pathogens using genome-specific primer pairs in clinical samples – All organisms have DNA sequences (rDNA) which code for ribosomal RNA. There are regions in the rDNA which vary between genera and species. These variable regions are amplified and then sequenced to determine the identity of the unknown organisms

2. Detection of viral pathogens and other micro-organisms which persist in low levels in infected cells and are difficult to be identified by routine methods. Quantitative Real-Time can be used to detect viral genomes such as HIV or HPV.

3. Diagnosis of genetic disorders such as phenylketonuria, haemophilia, sickle cell anaemia, thalassemia.

4. Identification of genetic mutations like deletions, insertions and point mutations.

5. Screening specific genes for unknown mutations.

6. Identification and analysis of mutations in eukaryotic DNA.

7. Gene polymorphisms.

8. Gene expression

9. Forensic Odontology[20]

CONCLUSION

DNA evidence is also a powerful tool that has been used to ultimately prove the innocence of previously convicted individuals. This technique makes possible the specific in vitro amplification of extremely small numbers of a relevant DNA sequence up to amounts which allow for its study by conventional sequencing techniques. However, tests of the use of PCR in forensic analyses have largely proved these concerns to be exaggerated, with even degraded samples giving repeatable and reliable results.

Ethical Clearance: Approved & Obtained.

Prior publication	NIL
Support	NIL
Conflicts of interest	NIL
Permissions	NIL

REFERENCES

1. Valones MA, Guimarães RL, Brandão LA, Souza PR, Carvalho AD, Crovela S. Principles and applications of polymerase chain reaction in medical diagnostic fields: a review. Brazilian Journal of Microbiology. 2009 Mar;40(1):1-1.
2. Millar BC, Xu J, Moore JE. Molecular diagnostics of medically important bacterial infections. Molecular Diagnostics: Current Technology and Applications. 2007;9(1):21-39.
3. Mullis KB. The unusual origin of the polymerase chain reaction. Scientific American. 1990 Apr 1;262(4):56-61.
4. Metzker, M. L. & Caskey, C. T. (2009) Polymerase Chain Reaction (PCR). Encyclopedia of Life Sciences (ELS), John Wiley & Sons, Ltd: Chichester.
5. Rittié L, Perbal B. Enzymes used in molecular biology: a useful guide. Journal of cell communication and signaling. 2008 Jun 1;2(1-2): 25-45.
6. Innis MA, Gelfand DH. Optimization of PCRs. Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. Academic Press: San Diego, CA, USA; 2012 Dec 2.
7. Ruano G, Kidd KK. Coupled amplification and sequencing of genomic DNA. Proceedings of the National Academy of Sciences. 1991 Apr 1;88(7):