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SCIENTIFIC CONCEPTS OF POLYMERASE CHAIN REACTION IN FORENSIC SCIENCE AND MOLECULAR BIOLOGY

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ABSTRACT

This paper is specifically designed to explain the important scientific points of Polymerase Chain Reaction (PCR) technology in forensic science and molecular biology. Scientific procedures presented in the paper are complex, simple were specifically designed to better explain and reinforce the key concepts of PCR. The polymerase chain reaction (PCR) is a laboratory technique for amplifying a specific sequence of a minute amount of Deoxyribonucleic acid even from a single hair root or a microscopic blood stain left at a crime scene. Today's PCR has found widespread and innumerable uses in forensics, genetics, DNA fingerprinting, bacteriology, virology, cloning and many other areas. Major research areas such as biomarker discovery, gene regulation and cancer research are challenging today's PCR technologies with more demanding requirements. Reproducibility of data and time to results are still major problems encountered by researchers.

KEYWORDS

Polymerase chain reaction, Deoxy ribo nucleic acid, Blood stain and DNA finger printing.

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INTRODUCTION

Molecular biology has expanded tremendously since its beginning in the early 1950s[1-3,] from a branch of the biological basic sciences to the point where DNA is now a household term. Early forensic detection systems relied on the quality and quantity of the DNA sample to be analyzed. The large amount of isolated DNA had to be relatively fresh or undegraded-essentially, unadulterated-for these detection systems to yield a sufficient profile. For samples considered too miniscule or determined to be degraded, the polymerase chain reaction or amplification process is now performed.

Polymerase Chain Reaction (PCR) (Figure No.1) is one of the method for the amplification of DNA that has varied applications in many and diverse areas of biological and clinical science[4,5]. PCR has allowed genetic analysis to become widely accessible, and its diverse applications continue to expand fields of scientific inquiry, both on conceptual and practical levels[6]. PCR is an enzymatic process[7], in which specific regions of DNA are duplicated repeatedly to yield millions of copies of a particular sequence in a matter of few hours[8-9]. This was first described in 1985 by Kary Mullis, has revolutionized molecular biology[10]. In 1993, Mullis was awarded the Nobel Prize in Chemistry for his work on PCR[11]. With this technique it is possible to make makes a huge number of copies of a piece of DNA even though it is initially present in a mixture containing many different DNA molecules.

Most PCR methods typically amplify DNA fragments of between 0.1 and 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size[12]. The PCR can amplify single molecules of a target nucleic acid sequence sufficiently to permit isotopic[13-16] or, if the test sample contains little background DNA, non-isotopic detection[17-22].

PCR requires specialized equipment that is customized to fluctuate between 36 specifically timed temperature variations. Before PCR is performed, DNA must be isolated from peripheral blood, hair follicles, cheek cells, or tissue samples. Isolated DNA is double stranded, meaning that there are two sequences of letters or nucleotide bases (A or adenine, G or guanine, C or cytosine, and T or thymine). The double stranded DNA is held together by complementary base pairings in that A binds to T, C binds to G makes the complementary strand of the molecule understood. So,

TTAACGGGGCCCTTTAAA.....TTTAAACCCG

GGTTT would pair with

AATTGCCCCGGGAAATTT.....AAATTTGGGC

CCAAA.

Therefore, knowing of the sequence of one strand will reveal the sequence of the complementary strand. Amplification is necessary because there are 3.9 billion bases, and although there is a lot of total DNA, there is not enough to properly analyze specific gene or gene segments. Amplification, therefore, makes it possible to obtain ample quantities of specific sequences of DNA to perform a variety of analyses. Further information can be obtained from a number of reference texts, which provide comprehensive introductions to PCR[23-32].

Principle

The basic principle of replicating a piece of DNA using two primers had already been described by Gobind Khorana in 1971[33]. The core principle of PCR is the use of an enzyme called DNA polymerase to make a copy of a DNA strand. Normally DNA exists as a double strand, but the enzyme can only work on a single strand. Therefore it is first necessary to separate the strands of DNA. Unknown DNA is heated, which causes the paired strands to separate. Then add primers relative to the amount of DNA being amplified, and cool the reaction mixture to allow double-strands to form again because of the large excess of primers, the two strands will always bind to the primers, instead of with each other. Then, to a mixture of all 4 individual letters (deoxyribonucleotides), add an enzyme which can read the opposing strand's sentence and extend the primer's sentence by hooking letters together in the order in which they pair across from one another - A:T and C:G. This particular enzyme is called a DNA polymerase. In 1986, Cetus scientists isolated the Taq polymerase from Thermus *aquaticus*, a bacterium found in hot springs. Because Taq could withstand high temperatures necessary for DNA strands separation, whereas other DNA polymerases become denatured it removed the need for human intervention during the reaction. Taq DNA polymerase.

Catalyzes the buffered reaction in which an excess of oligonucleotide primer pair and four deoxynucleoside triphosphates (dNTPs) are used to make millions of copies of the target sequence. A primer and dNTPs are added along with a DNA template and the DNA polymerase (Taq).

METHODS

Typically, PCR consists of a series of 25-35 repeated temperature changes cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three. The cycling is often preceded by a single temperature step at a high temperature (>90°C), and followed 73 by one hold at the end for final product extension or brief storage.

Initialization

This step consists of heating the reaction to a temperature of 94-96 °C, which is held for 2–8 minutes.

It is only required for DNA polymerases that require heat activation by hot-start PCR.

Denaturation

This step is the first regular cycling event and consists of heating the reaction to 94-98 °C for 20-30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules. Double stranded DNA templates denature at a temperature that is determined in part by their G-C content. Denaturation for 45 seconds at 94-95^{0}C is routinely used to amplify linear DNA molecules whose GC content is <55% and higher temperature for template or target DNAs whose G-C content is >55%.

Annealing

The reaction temperature is lowered to 55-70 °C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.

Extension/elongation

Extension of oligonucleotide primers is carried out at or near the optimal temperature for DNA synthesis catalyzed by the thermo stable polymerase. The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 72–78 °C. Heating at 720C for 45 seconds is the ideal working temperature for polymerase. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent DNA strand.

Number of cycles

The number of cycles required for amplification depends on the number of copies of template DNA present at the beginning of the reaction and the efficiency of primer extension and amplification. The completion of each cycle doubles the number of target DNA molecules. A 30 cycle amplification process generally amplifies the target between 100,000-10,000,000 fold. Subsequently target sequences from 100-2000 bp in length are most commonly used for PCR amplification.

Components of the PCR mixture

Buffers

Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase. Most buffers contain Tris at 10 mMand KCl at 50 mM concentrations; the pH varies from 8.2 to 9.0 (25°C). So pH 9.0 at 25°C translates to approximately pH 7.6 at 72°C, which is the optimal temperature for polymerase activity. pH 7.0-7.5 is considered optimal for Taq polymerase.

Magnesium Chloride

Magnesium is a cat ion that binds to the polymerase and is an essential cofactor for polymerase activity. The lower the magnesium concentration, the more stringent the conditions for primer annealing. A change of 0.25 mM can mean the difference between excellent yield of product of the expected length or no product at all.

Primers

When designing primers for a PCR assay, follow these guidelines:

1. Design primers that have a GC content of 50–60%.
2. Strive for a Tm between 50 and 65°C.
3. Avoid secondary structure; adjust primer locations so that they are located outside secondary structure in the target sequence, if required.

4. Avoid repeats of Gs or 121 Cs longer than 3 bases.
5. Place Gs and Cs on ends of primers.
6. Check the sequence of forward and reverse primers to ensure no 3' complementarity.
7. Avoid primer-dimmer formation.

Deoxynucleotide Triphosphates (dNTPs)

Deoxynucleoside triphosphates (dNTPs; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand. Equal amounts of each of the four dNTPs (dATP, dCTP, dGTP, dTTP) are included in the reaction, usually at a concentration of 0.2 mM(200 μM) each[34].

Thermostable Polymerase (Taq polymerase)

Taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C. Initially purified from Thermus aquaticus and used in its native form, it was soon replaced by a recombinant version made from the cloned T. aquaticus gene expressed in E. coli[35].

Stabilizers

In order to stabilize the polymerase, bovine serum albumin (BSA; 0.01%), gelatin (0.01%), or glycerol (1–2 %) are sometimes included in the reaction mix.

Enhancers

A number of chemicals can be used to optimize the performance and sensitivity of PCR, including dimethyl sulfoxide (DMSO), tetra-methyl ammonium chloride (TMAC) and betaine. In effect, this lowers the Tm of the annealing step.

DNA Template

However, the cleaner the template, the higher the sensitivity.

Monovalent cat ions

Monovalent cat ions, potassium ions.

Divalent cat ions

Divalent cat ions, magnesium or manganese ions; generally Mg2+ is used, but Mn2+ can be utilized for PCR-mediated DNA mutagenesis, as higher Mn2+ concentration increases the error rate during DNA synthesis.

RESULTS

Advantages of PCR

1. 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 (Table No.1 and 2)[36].
2. The polymerase chain reaction will undoubtedly prove to be the method of choice for the study of DNA retrieved from archaeological samples.
3. As it involves an amplification process it is more sensitive than the single-locus and multi-locus probe technologies and the protein and antigenic systems.
4. The DNA of interest can be amplified with the DNA from just one cell.
5. Old or degraded DNA very often yields enough starting material to amplify the DNA of interest
6. The technology is potentially very cheap, unless kit technology is used.
7. The PCR procedure is much faster.
8. The technology is simple to understand and much easier to perform.
9. The amplification process is largely automated and therefore should not be subject to human error.
10. The PCR products do not appear to be altered by degradation of the template DNA caused by decomposition of the sample.
11. The PCR process can be tailored for a particular locus.
12. Results can often be obtained from crude DNA preparations.

Disadvantages of PCR

Of course, some technical problems can arise with PCR. The most important is contamination of the sample with extraneous genetic material that could generate numerous copies of irrelevant DNA[37-38]. Due to extensive handling of the sample by researchers, PCR products in both cases were found to have high degree of contamination by modern human DNA[39]. PCR has a number of disadvantages that must be considered before adopting the PCR system:

- PCR has relatively low discriminating power in compared to RFLP technology.
- PCR cannot be used for the efficient amplification of DNA segments in excess of 2000 bp.
- The PCR process may be inhibited by various chemicals present in the DNA extract. For example haemoglobin is powerful inhibitor of the PCR process.
- False negatives may rarely occur due to genetic variations within individuals that prevent the binding of one or both of the primers.
- False positives can occur if a great deal of care is not taken to prevent the contamination of samples, DNA extracts or reagents from amplified DNA or other sources of foreign human DNA.

Parameters that affects PCR

Amplification of template DNA depends on factors like, Mg+ ion concentration in the buffer, annealing temperature of the primers, repeat sequence in the template, Secondary structure in template DNA, PCR inhibitors present in template etc (Table No.3 a and b). Table No.4 shows the causes related to cycling times and temperatures.

Types of PCR

PCR technique has been modified to suit various applications. Some types of PCR includes: nested PCR, multiplex PCR, inverse PCR, RT-PCR, Assembly PCR, Asymmetric PCR, Real time PCR, touchdown PCR, Hot-start PCR, colony PCR, etc.

Laboratory set-up

Due to the sensitivity of PCR based tests, certain precautions are necessary to avoid contamination of samples with other sources of DNA. To minimize the potential for contamination several aspects of the PCR process should be considered: DNA extraction, PCR setup and Amplified DNA analysis. Each aspects of PCR process should be separated by time and space. The following precautions must be taken to reduce the errors in the laboratory.

1. The work area for DNA extraction should include dedicated equipment and supplies.
2. Use disposable gloves at all times and change frequently.
3. DNA extraction and PCR setup should be conducted within self-contained hoods.
4. DNA extraction of questioned sample should be performed separately from the extraction of known samples.
5. Every sample to be analyzes should be properly labelled and recorded with a unique identification number.
6. Always change pipette tips between handling each sample.
7. Before and after setting up the DNA extractions, clean all work surfaces thoroughly with a 10% solution of bleach and prior to leaving the laboratory area, always remove the gloves and wash your 3 hands.

The future of PCR

Extraordinary miniaturization of the hardware is al so underway, as experimenters squeeze PCR onto chip-sized devices. Crisscrossed with the tiniest of troughs to hold the reagents and the DNA, the chips are heated electrically and cool down much faster than the present generation of machines, so amplification is even speedier than today's swift process. While such experimental chip-based devices are not yet ready for prime time, they are hastening the day when scientists can take them on the road, and patients will be able to get on-the-spot readouts of their DNA. PCR is doing for genetic material what the invention of the printing press did for written material making copying easy, inexpensive, and accessible. In principle, PCR can reproduce the genetic material of any organism in essentially unlimited quantities, so it can be used to analyze any cell 233 s containing that material.

Whether they are germs, rare medicinal plants, or human beings, eventually we can know whatever is recorded in their DNA.

DISCUSSION

The introduction of PCR technology has substantially increased our powers of biological discrimination particularly in rape cases. Amplification of DNA is critical in cases where the source of DNA is minimal or the integrity is compromised. DNA evidence is also a powerful tool

that has been used to ultimately prove the innocence of previously convicted individuals. Forensic science relies heavily on PCR technology to amplify specific sequences of DNA that will establish a connection between a specific suspect and a crime scene.

Table No.1: Standard Reaction conditions for PCR

S.No	Reagents	Amount (µl)
1	De-ionized water	37.5
2	Taq assay buffer	(10x) 5
3	Template DNA	1
4	dNTPs mix	2
5	Forward primer	2
6	Reverse primer	2
7	Taq DNA polymerase	5

Table No.2: Steps of PCR Process

S.No	Step	Temperature	Time (mints)
1	Initialization	96^0C	5
2	Denaturation	96^0C	30
3	Annealing	68^0C	30
4	Elongation	72^0C	45

Table No.3 (a): Causes related to PCR components

S.No	Causes related to PCR components
1	A thermo stable DNA polymerase to catalyse template-194 dependent synthesis of DNA
2	A pair of synthetic oligonucleotides to prime DNA synthesis
3	Deoxynucleoside triphosphates (dNTPs)
4	Divalent cat ions
5	Buffer to maintain Ph
6	Monovalent cat ions
7	Template DNA

Table No.3 (b): Causes related to PCR components

S.No	Causes related to PCR components
I	**dNTP concentration**
	If the dNTP concentration is too high, Mg2+ depletion occurs. Each dNTP should be present at 200 μM in the final reaction.
II	**Impure dNTPs**
	Contaminants in the dNTP mix can lead to incomplete or incorrect amplification or PCR inhibition. Use high-quality dNTPs.
III	**PCR product: GC content (>65%)**
1	G-C-rich PCR products are difficult to amplify. To improve amplification, increase the annealing temperature.
2	For greater accuracy, optimize the annealing temperature by using a thermal gradient.
3	Dimethyl sulfoxide (DMSO) or bovine serum albumin (BSA), and glycerol as de-stabilizer can be added (10%).
IV	**Template contained inhibitors**
1	Template may be sheared or may contain PCR inhibitors. If inhibitors are suspected dilute existing template or use fresh template and increase cycles.
2	Try a control reaction in which you use a pure plasmid with the addition of the template to determine if any inhibitory effects exist.
3	Contaminants in primers may inhibit PCR. Use desalted primers or more highly purified primers.
V	**Primers contained impurities**
1	Contaminants in primers may inhibit PCR. Use desalted primers or more highly purified primers.
2	You can try to dilute the primers to determine if inhibitory effects exist, but do not add less than 0.02 μM of each primer.
VI	**Template size**
	Insufficient amplification can result if the initial amount of template is too low. Increase the number of amplification cycles in increments of 5, or, if possible, increase the amount of template.
VII	**Primer concentration**
1	Excessive concentration of primers can increase the chance of primers binding non-specifically to undesired sites on the template or to each other.
2	Excessive concentration of primers can increase the chance of primers binding non-specifically to undesired sites on the template or to each other.
3	If the primer concentration is too low, annealing may be inefficient.
4	Use a concentration of 0.1–1.0 μM of each primer. For many applications, a primer concentration of 0.2 μM will be sufficient. Store all primer solutions at -20°C.
5	Use a concentration of 0.1-1.0 μM of each primer. For many applications, a primer concentration of 0.2 μM will be sufficient. Store all primer solutions at -20°C.
VIII	**Enzyme concentration**
	If the polymerase concentration is too low, not all PCR products will be fully replicated. The optimal enzyme concentration depends on the length and difficulty of the template.

IX	**Primers design**
1	Verify that primers have the correct sequence and are complementary to the template.
2	Avoid complementarity in the 2–3 bases at the 3' end of the primer pairs.
3	Avoid mismatches between the3' end of the primer and the template.
4	Avoid runs of 3 or more Cs or Gs at the 3' end of the primer.
5	Avoid complementarity within primers and between the primer pair.
6	Avoid a T as ultimate base at the 3' end.
7	Perform a BLAST search to avoid primers that could amplify pseudo genes or that might prime unintended regions.
8	Use the lowest Tm of the primers. **Tm calculation= 2°C x (A +T) + 4°C x (G+C).**
X	**Target length**
1	PCR component concentrations or cycling conditions may not be sufficient for longer target sequences.
2	Re-optimize your existing assay protocol or increase the duration of PCR steps, especially the extension step.
XI	**Water**
	Water could have been contaminated during prior pipetting events. Use fresh nuclease-free water.
XII	**Not enough Mg^{2+}**
1	Insufficient or omitted magnesium will result in no or reduced PCR product.
2	Use 1.5 mM in the final reaction

Table No.4: Causes related to cycling times and temperatures

S.No	Causes related to cycling times and temperatures
I	**Positive control**
1	Inclusion of control reactions is essential for monitoring the success of PCR reactions.
2	Wherever possible, a positive control should be included to check that the PCR conditions used can success fully amplify the target sequence.
II	**Negative control**
1	As PCR is extremely sensitive, requiring only a few copies of target template.
2	A negative control containing no template DNA should always be included to ensure that the solutions used for PCR have not become contaminated with the template DNA.

III	**PCR cycles**
1	Using too few PCR cycles can lead to insufficient amplification. Use 20-35 cycles.
2	Use fewer cycles when template concentration is high and use more cycles when template concentration is low.
IV	**Extension time**
1	If the extension time is too short, there will be insufficient time for complete replication of the target.
2	Generally, use an extension time of 1 min/kb.
V	**Annealing time**
1	If the annealing time is too short, primers do not have enough time to bind to the template.
2	Use an annealing time of at least 30 sec.
VI	**Annealing temperature**
1	If the annealing temperature is too high, the oligonucleotide primers anneal poorly and the yield of amplified DNA is very low.
2	If the annealing temperature is too low, nonspecific annealing of primers may occur, resulting in the amplification of unwanted segments of DNA.
3	The rule of thumb is to use an annealing temperature that is 5°C lower than the Tm of the primer. Use the lowest primer Tm when calculating the annealing temperature.
4	For greater accuracy, optimize the annealing temperature by using a thermal gradient.
5	If the primer Tm minus 5°C is close to the extension temperature (72°C), consider running a two-step PCR protocol.
6	The annealing temperature should not exceed the extension temperature.
VII	**Denaturation temperature**
	If the denaturation temperature is too low, the DNA will not completely denature and amplification efficiency will below. Use a denaturation temperature of 95°C.
VIII	**Denaturation time**
1	If the denaturation time is too long, DNA might be degraded.
2	If the denaturation time is too short, the DNA will not completely denature and amplification efficiency will be low
3	For the initial denaturation, use 3 min at 95°C.
4	For denaturation during cycling, use 30 sec at 95°C.

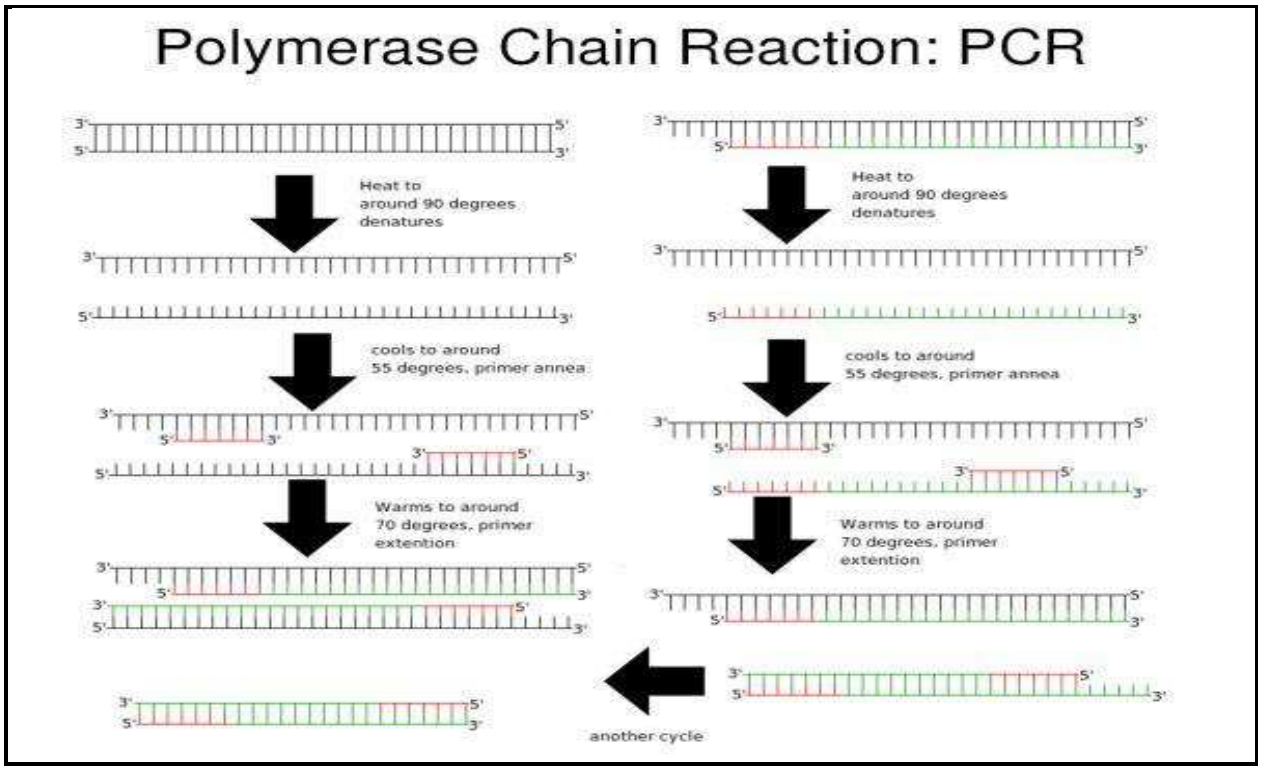

Figure No.1: Cleavage pattern of PCR

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[36]. 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[37-43].

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