IJPSR (2014), Vol. 5, Issue 8 **(Review Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 08 February, 2014; received in revised form, 08 May, 2014; accepted, 28 June, 2014; published 01 August, 2014

SCIENTIFIC CONCEPTS OF POLYMERASE CHAIN REACTION (PCR)

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Keywords:

Polymerase Chain Reaction, Deoxyribonucleic acid (DNA), crime scene, DNA fingerprinting

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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 PCR is a laboratory technique for amplifying a specific sequence of a minute amount of Deoxyribonucleic acid (DNA) even from a single hair root or a microscopic blood stain left at a crime scene. Todays, 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.

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.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(8).3086-95

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(8).3086-95

Polymerase Chain Reaction (PCR) 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]. Since its initial development in the early 1980s, the basic PCR has been adapted to a wide variety of tasks in molecular cloning, in vitro mutagenesis, mutation genetics, cloning of cDNA and genomic DNA, and allotyping [4, 10]. 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 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.....TTTAAACCC
GGGTTT

Would pair with;

AATTGCCCCGGGAAATTT.....AAATTTGGG
CCCAAA.

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 [5]. 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).

The basics of PCR: 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 by one hold at the end for final product extension or brief storage. **Figure 1 and Table 1** show the basic steps of PCR process.

Initialization step: 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 step: This step is the first regular cycling event and consists of heating the reaction to 90–98°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^{o}C is routinely used to amplify linear DNA molecules whose GC content is <55% and higher temperature for template or target DNAs whose GC content is >55%. Extended period of denaturation is unnecessary for linear DNA molecules and may sometimes be deleterious [40]

Annealing step: Annealing is usually performed 3^{o}C-5^{o}C lower than the calculated melting temperature at which the oligonucleotide primers dissociate from their templates. 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 step: Extension of oligonucleotide primers is carried out at or near the optimal temperature for DNA synthesis catalyzed by the thermostable polymerase. The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 70–78°C. Heating at 72^{o}C 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.

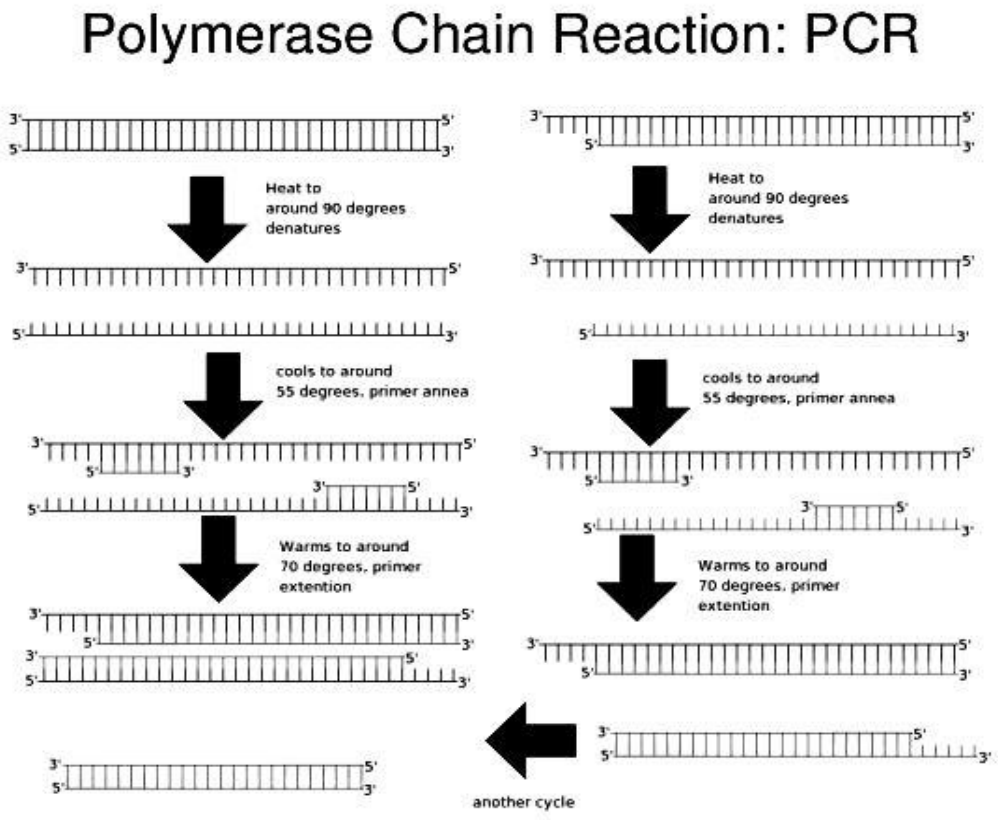

Figure 1: Diagram shows the steps involved in the first few rounds of a PCR, consisting of three elements: Denaturation of the template by heat, annealing of the oligonucleotide primers to the single-stranded target sequence(s), and the extension of the annealed primers by a thermostable DNA polymerase. The original template at the top is double stranded DNA, and the left and right oligonucleotide primers.

TABLE 1: SHOWS THE STEPS OF PCR PROCESS

Steps	Temperature	Time
Initialization	96^0C	5 minutes
Denaturation	96^0C	30 seconds
Annealing	68^0C	30 seconds
Elongation	72^0C	45 seconds

Numbers 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:

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 [5], it was soon replaced by a recombinant version made from the cloned *T. aquaticus* gene expressed in *E. coli* [35].

Primers: The following equation can be used to calculate the probability that a sequence exactly complementary to a string of nucleotides will occur by chance within a DNA sequence space that consist of a random sequence of nucleotides [41].

$$K= [g/2]^{G+C} \times [(1-g)/2]^{A+T},$$

Where K is the expected frequency of occurrence within the sequence space, g is the relative G+C content of the sequence space, and G, C, A and T are the number of specific nucleotides in the oligonucleotide. When designing primers for a PCR assay, follow these guidelines:

Design primers that have a GC content of 50–60%. Strive for a T_m between 55 and 60°C [42]. Avoid secondary structure; adjust primer locations so that they are located outside secondary structure in the target sequence, if required. Avoid repeats of Gs or Cs longer than 3 bp in length and place Gs and Cs on ends of primers. Check the sequence of forward and reverse primers to ensure no 3' complementarity. Avoid primer-dimer formation.

Buffers to maintain pH: Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase. Most buffers contain Tris at 10 mM and 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 cation 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.

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), tetramethyl 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 cations: Monovalent cations, potassium ions. A standard PCR buffer contains 50mM Kcl and work well for the amplification of segment of DNA 500bp in length.

Divalent cations: All thermostabe DNA Polymerases require free divalent cations-usually Mg2+- for activity. Calcium ions are quite ineffective [43]. 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. Although a concentration of 1.5mM Mg2+ is routinely used, increasing the concentration of Mg2+ to 4.5mM or 6mM has been reportrd to decrease nonspecific priming in some cases [see 44, 45] and to increase it in others [see 46].

Inhibitors: Almost anything will inhibit PCRs if present in excess. This includes proteinase k, phenol and EDTA. Other substances that can cause problems are ionic detergents [47], Heparin [48], polyanions such as spermidine [49], hemoglobin and gel loading dyes such as bromophenol blue and xylene cyanol FF [50]. **Table 2** shows the standard reaction conditions for PCR.

TABLE 2: SHOWS THE STANDARD REACTION CONDITIONS FOR PCR

Reagents	Amount(µl)
Deionized water	37.5 µl
Taq assay buffer	(10x) 5 µl
Template DNA	1µl
dNTPs mix	2 µl
Forward primer	2 µl
Reverse primer	2 µl
Taq DNA polymerase	5 µl

Advantages of PCR:

- 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].
- Sometimes used as a method of choice for the study of DNA retrieved from archaeological samples.
- The DNA of interest can be amplified from just one cell.
- Even Old or degraded DNA yields enough starting material to amplify the DNA of interest.
- The technology is potentially very cheap, unless kit technology is used.
- The PCR procedure is much faster.
- The technology is easier to understand and simple to perform.
- The amplification process is largely automated.
- The PCR products do not appear to be altered by degradation of the template DNA caused by decomposition.
- The PCR process can be tailored for a particular locus.
- 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 e.g. proteins, hemoglobin etc.
- 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 affect 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 3** shows the causes related to cycling times and temperatures. **Table 4** shows the causes related to PCR components

- A thermostable DNA polymerase to catalyse template-dependent synthesis of DNA
- A pair of synthetic oligonucleotides to prime DNA synthesis:
- Deoxynucleoside triphosphates (dNTPs)
- Divalent cations
- Buffer to maintain pH
- Monovalent cations
- Template DNA

TABLE 3: SHOWS THE CAUSES RELATED TO CYCLING TIMES AND TEMPERATURES

Positive control	✓ Inclusion of control reactions is essential for monitoring the success of PCR reactions. ✓ Wherever possible, a positive control should be included to check that the PCR conditions used can success fully amplify the target sequence.
Negative control	✓ As PCR is extremely sensitive, requiring only a few copies of target template. ✓ 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.
PCR cycles	✓ Using too few PCR cycles can lead to insufficient amplification. Use 20–35 cycles. ✓ Use fewer cycles when template concentration is high and use more cycles when template concentration is low.
Extension time	✓ If the extension time is too short, there will be insufficient time for complete replication of the target. ✓ Generally, use an extension time of 1 min/kb.
Annealing time	✓ If the annealing time is too short, primers do not have enough time to bind to the template. ✓ Use an annealing time of at least 30 sec.
Annealing temperature	✓ If the annealing temperature is too high, the oligonucleotide primers anneal poorly and the yield of amplified DNA is very low. ✓ If the annealing temperature is too low, nonspecific annealing of primers may occur, resulting in the amplification of unwanted segments of DNA. ✓ 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. ✓ For greater accuracy, optimize the annealing temperature by using a thermal gradient. ✓ If the primer T_m minus 5°C is close to the extension temperature (72°C), consider running a two-step PCR protocol. ✓ The annealing temperature should not exceed the extension temperature.
Denaturation temperature	✓ If the denaturation temperature is too low, the DNA will not completely denature and amplification efficiency will be low. Use a denaturation temperature of 95°C.
Denaturation time	✓ If the denaturation time is too long, DNA might be degraded. ✓ If the denaturation time is too short, the DNA will not completely denature and amplification efficiency will be low ✓ For the initial denaturation, use 3 min at 95°C. ✓ For denaturation during cycling, use 30 sec at 95°C.

TABLE 4: SHOWS THE CAUSES RELATED TO PCR COMPONENTS

dNTP concentration	✓ If the dNTP concentration is too high, Mg2+ depletion occurs. Each dNTP should be present at 200 μM in the final reaction.
Impure dNTPs	✓ Contaminants in the dNTP mix can lead to incomplete or incorrect amplification or PCR inhibition. Use high-quality dNTPs
PCR product: GC content (>65%)	✓ GC-rich PCR products are difficult to amplify. To improve amplification, increase the annealing temperature. ✓ For greater accuracy, optimize the annealing temperature by using a thermal gradient. ✓ Dimethyl sulfoxide (DMSO) or bovine serum albumin (BSA), and glycerol as destabilizer can be added (10%).
Template contained inhibitors	✓ Template may be sheared or may contain PCR inhibitors. If inhibitors are suspected dilute existing template or use fresh template and increase cycles. ✓ Try a control reaction in which you use a pure plasmid with the addition of the template to determine if any inhibitory effects exist.
Primers contained impurities	✓ Contaminants in primers may inhibit PCR. Use desalted primers or more highly purified primers. ✓ 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.
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.
Primer concentration	✓ Excessive concentration of primers can increase the chance of primers binding nonspecifically to undesired sites on the template or to each other. ✓ If the primer concentration is too low, annealing may be inefficient. ✓ 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.
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.
Primers design	✓ Verify that primers have the correct sequence and are complementary to the template. ✓ Avoid complementarity in the 2–3 bases at the 3' end of the primer pairs. ✓ Avoid mismatches between the 3' end of the primer and the template. ✓ Avoid runs of 3 or more Cs or Gs at the 3' end of the primer. ✓ Avoid complementarity within primers and between the primer pair ✓ Avoid a T as ultimate base at the 3' end. ✓ Perform a BLAST search to avoid primers that could amplify pseudo genes or that might prime unintended regions. ✓ Use the lowest T_m of the primers. T_m calculation: 2°C x (A +T) + 4°C x (G+C).
Target length	✓ PCR component concentrations or cycling conditions may not be sufficient for longer target sequences. ✓ Re-optimize your existing assay protocol or increase the duration of PCR steps, especially the extension step.
Water	✓ Water could have been contaminated during prior pipetting events. Use fresh nuclease-free water.
Not enough Mg2+	✓ Insufficient or omitted magnesium will result in no or reduced PCR product. ✓ Use 1.5 mM in the final reaction.

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 Setup: Due to the sensitivity of PCR based tests, certain precautions are necessary to avoid contamination of samples with other source 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.

- The work area for DNA extraction should include dedicated equipment and supplies.
- Use disposable gloves at all times and change frequently.
- DNA extraction and PCR setup should be conducted within self-contained hoods.
- DNA extraction of questioned sample should be performed separately from the extraction of known samples.
- Every sample to be analyzes should be properly labeled and recorded with a unique identification number.
- Always change pipet tips between handling each sample.
- 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 hands.

The future of PCR: Extraordinary miniaturization of the hardware is also 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 can reproduce the genetic material of any organism in essentially unlimited quantities, so it can be used to analyze any cells containing that material, whether they are germs, rare medicinal plants, or human beings, eventually we can know whatever is recorded in their DNA.

DISCUSSION AND CONCLUSION: The introduction of PCR technology has substantially increased our powers of biological discrimination particularly in rape cases. However, the use of PCR in forensic analyses has largely proved to be exaggerated, with even degraded samples giving repeatable and reliable results [51-54]. 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. 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.

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How to cite this article:
Verma K, Dalal J and Sharma S: Scientific concepts of Polymerase Chain Reaction (PCR). Int J Pharm Sci Res 2014; 5(8): 3086-95.doi: 10.13040/IJPSR.0975-8232.5(8).3086-95