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Review on Polymerase Chain Reaction and its Applications in Genetic Engineering

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Abstract: Deoxyribonucleic acid (DNA) is a molecule that contains the biological instructions that make each species unique. DNA, along with the instructions it contains, is passed from adult organisms to their offspring during reproduction. Polymerase Chain Reaction (PCR) is a rapid procedure for *in vitro* enzymatic amplification of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands, heat stable DNA polymerase and thermal cycle. Repetitive cycles involving template denaturation, primer annealing and the extension of the annealed primers by DNA polymerase, result in the exponential accumulation of a specific fragment whose termini are defined by 5' end of the primers. PCR is a highly versatile technique developed by Kary Mullis in the 1980s and has been modified in different ways to suit specific applications. PCR is a fundamental method in cloning technique, which permits era of a lot of unadulterated DNA from minor measure of format strand and further investigation of a specific quality. Applications of the technique include DNA cloning for sequencing, gene cloning and manipulation, gene mutagenesis, construction of DNA-based phylogenies, diagnosis and monitoring of hereditary diseases, amplification of ancient DNA, analysis of genetic fingerprints for DNA profiling and detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases.In these review the different PCR technologies will be reviewed and the idea of PCR application in genetic engineering will behighlighted in this manuscript paper. Hence, the speed, ease of use, sensitivity, specificity and robustness of PCR has revolutionized molecular biology and made PCR the most widely used and powerful technique with great spectrum of research and diagnostic applications that must be available in every molecular biology laboratories with its fulfilled facilities as well as the idea of PCR with integration in genetic engineering must be expanded at the national level.

Key words: PCR · Amplification, Biotechnology · DNA · Genetic Engineering

INTRODUCTION

Biotechnology (commonly abbreviated as biotech) is the broad area of biology involving living systems and organisms to develop or make products, or "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use [1]. Biotechnology is the use of artificial methods to modify the genetic material of living organisms or cells to produce novel compounds or to perform new functions. Biotechnology has been used for improving livestock and crops through selective breeding. Since the discovery of the structure of DNA in 1953 and the development of methods to manipulate DNA in 1970s, biotechnology plays an important role with the manipulation of organisms' DNA at the molecular level. One example of modern biotechnology is genetic engineering. Genetic engineering is the process of transferring individual genes between organisms or modifying the genes in an organism to remove or add a desired trait or characteristic using biotechnology [2]. Alternatively, the development of molecular biology was one of the greatest achievements in biological science in the recent centuries. The discovery of Polymerase Chain Reaction (PCR) brought enormous benefits and scientific developments such as genome sequencing, gene expressions in recombinant systems and in the study of molecular genetics analyses

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[3]. PCR enables *in vitro* synthesis of nucleic acids through which a DNA segment can be specifically replicated in a semi-conservative way. Like gene expression and cloning, the idea of PCR was born only in the early 1970s [4].

PCR was developed in 1983 by Kary.B.Mullis, who won the Nobel Prize in chemistry in 1993 and is now acommontechnique used inmedical and biological research labs for a variety ofapplication such as; identification of the owner of a DNA sample left at a crime scene (paternity analysis), the comparison of small amounts of ancient DNA with modern organisms, DNA cloning for sequencing, diagnosis of hereditary and infectious diseases as well as for determining the sequence of nucleotides in a specific region [5]. Until K. Mullis success with this method, the only way biologists could make copies of whatever gene they were interested in was, by the relatively laborious and time-consuming process of identifying and isolating the gene through constructing a cDNA library and then inserting that gene into living cells that replicate the target DNA along with their own DNA. In contrast, PCR enables the production, or amplification, of billions of copies of an original piece of DNA in a test tube within minutes or hours [6]. The cloning of expressed genes and the PCR, are the two biotechnological breakthroughs of the 1970s and 1980s and continue to play significant roles in science today. PCR is a fundamental method in cloning technique that permits era of a lot of unadulterated DNA from minor measure of format strand and further investigation of a specific quality [7]. PCR is a set of technologies used to change the genetic makeup of cells, including the transfer of genes within and across species boundaries to produce improved or novel organisms [8]. In general, PCR is possibly, a single most important methodological invention in molecular biology to date. Since its conception in the mid-1980s, it has rapidly become a routine procedure in every molecular biology laboratory for identifying and manipulating genetic material, from cloning, sequencing, mutagenesis, to diagnostic research and genetic analysis [9]. With these facts in mind, the objectives of this paper are: to Review on the PCR technologies and to highlight the application of PCR in genetic engineering.

The DNA Molecule: All organisms are made up of cells that are programmed by the same basic genetic materials, called Nucleic acids. Nucleic acids are macromolecules made of nucleotides (a sugar, a phosphate and a nitrogenous base). The phosphate groups on these molecules each have a net negative charge. Deoxyribonucleicacid (DNA) is a large biomolecule that contains the complete genetic information for an organism [10]. DNA was first isolated by Friedrich Miescher in 1869, where as its molecular structure was first identified by Francis Crick and James Watson. Every cell of living organisms and many virusescontain DNA. The basic building block of a DNA molecule is called a nucleotide and a single strand of DNA may contain billions of nucleotides. Because DNA contains instructions for an organism to create several different proteins, it is useful to define another sub-unit of DNA called genes. Each gene is a small segment of DNA that contains a set of instructions for an organism to create a single protein; a single organism may have thousands of different genes [11]. An entire set of DNA molecules in organisms is called the genome. Each unit of DNA is made up of a combination of nucleotides like, adenine (A), guanine (G), thymine (T) and cytosine (C) as well as a sugar and a phosphate. The nucleotides are joined to one another in a chain by covalent bonds between the sugar of one nucleotide and the phosphate of the next, resulting in an alternating sugar-phosphate backbone. The nitrogenous bases of the two separate polynucleotide strands are bound together, according to base pairing rules (A with T and C with G), with hydrogen bonds to make double-stranded DNA. The, sequence of nucleotides in one strand is complementary to that in the other strand and this strands twist together into a spiral structure called a double helix (DNA). The complementary nitrogenous bases are divided into two groups, pyrimidines (thymine and cytosine) and purines (adenine and guanine). Each strand has a three prime (3') and a five prime (5') end. Their orientations oppose one another and the strands are therefore said to be anti-parallel. The sequence of these bases encodes the genetic information [10]. Within eukaryotic cells, DNA is organized into long structures called chromosomes. Before typical cell division, these chromosomes are duplicated in the process of DNA replication, providing a complete set of chromosomes for each daughter cell. Eukaryotic organisms store most of their DNA inside the cell nucleus as nuclear DNA and some in the mitochondria as mitochondrial DNA or in chloroplasts as chloroplast DNA. In contrast, prokaryotes (Bacteriaand Achaea) store their DNA only in the cytoplasm, in circular chromosomes. DNA usually occurs as linear chromosomes in eukaryotes and circular chromosomes in prokaryotes [12].

DNA Replication: DNA replication is the biological process of producing two identical replicas of DNA from one original DNA molecule. DNA replication occurs in all living organisms acting as the basis for biological inheritance. Cell division is essential for an organism to grow, but, when a cell divides, it must replicate the DNA in its genome so that the two daughter cells have the same genetic information as their parent. DNA ismade up of a double helix of two complementary strands. During replication, these strands are separated. Each strand of the original DNA molecule then serves as a template for the production of its counterpart, a process referred to as semi-conservative replication. Because of semi conservative replication, the new helix will be composed of an original DNA strand as well as a newly synthesized strand. Unwinding of DNA at the origin and synthesis of new strands, accommodated by an enzyme known as helicase, results in replication forks growing bi-directionally from the origin [13]. The separated strand's complementary DNA sequence is recreated by an enzyme DNA polymerase through the addition of the nucleotides. This enzyme makes the complementary strand by finding the correct base through complementary base pairing and bonding it onto the original strand. As DNA polymerases can only extend a DNA strand in a 5' to 3' direction, different mechanisms are used to copy the anti-parallel strands of the double helix [14]. DNA replication (DNA amplification) can also be performed *in vitro* (artificially, outside a cell). DNA polymerases isolated from cells and artificial DNA primers can be used to start DNA synthesis at known sequences in a template DNA molecule. Polymerase chain reaction (PCR) is an example of DNA replication outside a cell. The PCR process mimics the natural process of DNA replication occurring in all cellular organisms in which the DNA molecules of a cell are duplicated prior to cell division. Polymerase chain reaction (PCR) is a technique used toincrease rapidly, the number of copies of specific regions of DNA for further analyses. PCR uses a special form of DNA polymerase, the enzyme that replicates DNA and other short nucleotide sequences called primers that base pair to a specific portion of the DNA being replicated [5].

Basic Concepts of PCR and Principles: Polymerase chain reaction (PCR) is a technique widely used in molecular biology to make many copies of a specific DNA segment. PCR is an exponentially progressing synthesis of the defined target DNA sequences *invitro*.It derives its name from one of its key components, DNA polymerase enzyme, used to amplify a piece of DNA by *in-vitro* enzymatic replication [15]. It is called chain because the products of the first reaction become substrates of the following one. The repeated cycles result in an exponential reaction in which the original target sequence is amplified a million fold or more within a few hours [16]. In technical terms however, PCR may be defined as a rapid procedure for *in vitro* enzymatic amplification of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA.As PCR progresses the DNA generated is used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. The template DNA contains the target sequence, which may be tens or thousands of nucleotides in length [5]. A thermostableDNA polymerase, *Taq* DNApolymerse, an enzyme originally isolated from the thermophilic bacterium *Thermusaquaticus*, catalyzes the buffered reaction in which an excess of an oligonucleotide primer pair and four deoxynucleoside triphosphates (dNTPs) are used to make millions of copies of the target sequence [17]. The vast majority of PCR methods rely on thermal cycling. Thermal cycling exposes reactants to repeated cycles of heating and cooling to permit different temperature-dependent reactions specifically, DNA melting and enzyme-driven DNA replication [5]. Most PCR methods amplify DNA fragments, between 0.1 and 10-kilo base pairs (kbp) in length.Although, some techniques allow for amplification of fragments up to 40 kbp.The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses.PCR employs two main primers, which are short single stranded DNA(oligonucleotide) that are a complementary sequence to the target DNA region and a DNA polymerase. In the first step of PCR, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting [18]. In the second step, the temperature is lowered and the primers bind to the complementary sequences of DNA. The two DNA strands then become templates for DNA polymerase to enzymatically assemble a new DNA strand from free nucleotides, the building blocks of DNA. As PCR progresses, the DNA generated itselfis used as a template for replication, in a chain reaction in which the original DNA template is exponentially amplified [19]. The reaction is commonly carried out in a volume of 10–200 µL in small reaction tubes (0.2–0.5 mL volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at

each step of the reaction. Most thermal cyclers have heating lids to prevent condensation at the top of the reaction tube. Older thermal cyclers lacking a heating lids and require a layer of oil on top of the reaction mixture or a ball of wax inside the tube [18]. The Principles ofPCR process involves breaking down DNA by heating and unwinds the DNA double helix into separate single strands. Primer is needed because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group to add the first nucleotide. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. DNA polymerase then elongates its 3' end by adding more nucleotides to generate an extended region of double stranded DNA.Once these strands are separated, the DNA polymerase reads the nucleic acid sequence and produces a duplicate strand of DNA. This process is repeated again and again, doubling the amount of DNA each cycle and increasing the DNA exponentially until millions of copies of the original DNA are created (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) [15].

Historical Background of the PCR Technologies: A 1971 paper in the *Journal of Molecular Biology* by Kjell Kleppeand co-workers in the laboratory of H.Gobind Khorana first described a method of using an enzymatic assay to replicate a short DNA template with primers *in vitro*. However, this early manifestation of the basic PCR principle did not receive much attention at the time and the invention of the polymerase chain reaction in 1983 is generally credited to Kary Mullis [20]. The first practical demonstration of PCR was achieved by Kary Mullis in 1983, who tested the technique as part of a project to understand the genetic cause of sickle cell anemia at Cetus Corporation. Mullis, however, struggled to replicate his initial success, because the polymerase he chose for the operation kept being destroyed by high temperatures (95°C) required to split apart (denature) the DNA strands at the start of each replication cycle. He resolved the matter by the adoption of another polymerase in 1985 [6]. This was a polymerase isolated from a species of bacterium *Thermusaquaticus* (*Taq*) discovered at a hot spring in Yellowstone National Park by Thomas D Brock in 1969, paved the way for dramatic improvements of the PCR method. Importantly *Taq* polymerase proved able to withstand the high temperatures required to break apart the DNA strands at the start of the PCR cycle [21]. The DNA polymerase isolated from *T. aquaticus* is stable at high temperatures remaining active even after DNA denaturation, thus obtaining the need to add new DNA polymerase after each cycle. This allowed an automated thermo-cycler based process for DNA amplification [22]. Hence, it was only in 1983 that Kary Mullis synthesized this existing information into one of the most powerful tools of molecular biology, called the Polymerase Chain Reaction (PCR) or '*in vitro* cloning. PCR is a method of making unlimited copies of DNA using a test tube, a heat source and few simple reagents [template, primers, DNA polymerase, deoxyribonucleoside triphosphate (dNTPs) anda buffer) [9].

Components of PCR

Target DNA (Template DNA): It consists of the DNA to be amplified (the original paper to be copied). DNA is used as a template molecule for DNA replication, DNA repair, as well as for transcription. The segment represents a small part of a large and complex mixture of a specific DNA of a genome. The shape of DNA is a double helical structure, which consists of nucleotides that wind around each other in a helical shape.PCR requires a template DNA molecule and DNA polymerases use template DNA by covalently linking the dNTPs that base pair with template DNA to form a new, complementary DNA strand. The polymerase readsthe template in the 3'→5' direction, while synthesizing DNA in the 5'→3' direction to form the ant- parallel double-stranded DNA product [23].

Two Primers: The specific primers that are complementary to the DNA target region are selected beforehand and are often custom-made in a laboratory or purchased from commercial biochemical suppliers. They are forward and reverse primers, which are usually 16-30 nucleotidesin length. Primers limit the DNA sequence to be replicated and results in the amplification of a particular DNA sequence[24]. Primers are short, artificial DNA strands not more than 50 nucleotides, which determine the beginning and the end of the region to be amplified. The polymerase synthesizes the complementary sequence from each primer. If template contains "A" nucleotide, enzyme adds on "T" nucleotide to the primer and if template contains "G" nucleotide, enzyme adds on" C" nucleotide to the primer [25].

Deoxynucleotide Triphosphates (dNTPs): Deoxynucleosidetriphosphatesord NTPs are the building blocks from which the DNA polymerase synthesizes a new DNA strand. The deoxynucleotide triphosphates

(dATP, dCTP, dGTP and dTTP) are usually present at 50 to 200µl each. dNTPs are usually purchased either individually or as an (equimolar) mix from commercial suppliers and are chemically stable when stored in slightly alkaline aqueous solutions at -20°C. Higher concentration may tend to promote miss incorporations by the polymerase. At 50 and 200µl, there are sufficient precursors to synthesize approximately 6.5 and 25µg of DNA respectively. As deoxynucleotide triphosphate appear to quantitatively bind Mg2+, the amount of dNTPs present in a reaction will determine the amount of free magnesium available [26]. It should be noted that dNTPs are naturally acidic in solution; hence, working and stock solutions may have to be neutralized with alkaline compounds prior to long-term storage.NeutralizeddNTP solutions are normally adjusted to 10mM stock solutions by spectrophotometry, or by adding the correct volume of sterile water to the lyophilized product directly after its chemical synthesis [27].

The Buffer: It consists of Magnesium chloride which supplies Mg divalent cations required as a co-factor. It is needed in 1-5 mM concentration and Mg joins to nucleotides to be recognized by the polymerase enzyme. Magnesium (Mg2+) is an important cofactor for DNA polymerase. Magnesium assists phosphodiester bond formation and is required for successful PCR amplification. The buffer in a PCR reaction serves to facilitate amplification by stabilizing the polymerase. The primary purpose of this component is to provide an optimal pH and monovalent salt environment for the final reaction volume. The most commonly used buffer is 10mM Tris at pH 8.3, 50 mMkcl, 1.5-2.5 mMMgcl, DMSO, PEG 6000, formamide etc.Changes to the PCR reaction of buffer will usually affect the outcome of the amplification. In particular, the concentration of $MgCl_2$can have a profound effect on the specificity and yield of amplification. $MgCl_2$ supplies the Mg^{++} divalent cations required as a cofactor for Type II enzymes, which include restriction endonucleases and the polymerases used in PCR. A concentration of about 1.5mM is usually optimal (with 200µm of eachdNTPs) [26].

DNA Polymerase (Taq polymerase): Many microorganisms can live in inhospitable conditions or in the presence of salt\acidic concentrations. The bacteria synthesizes at the rate of 35-100 nucleotides\sec. The introduction of the thermostable DNA polymerase (*Taq* polymerase) isolated from the hot spring bacteria, *thermusaquaticus* transformed the PCR into a simple and robust reaction, which could now be automated by a thermal cycling device. The concentration of the enzyme typically used in PCR is about 2.5 units per 100µl reaction [23]. For amplification reactions involving DNA samples with high sequence complexity such as genomic DNA, there is an optimum concentration of *Taq*polymerase, usually 1 - 4 units per 100µl. Increasing the amount of enzyme beyond this level can result in greater production of non-specific PCR products and reduced yield of the desired target fragment. One of *Taq'*s drawbacks is its lack of 3' to 5' exonuclease proofreading activity resulting in relatively low replication fidelity. Originally, its error rate was measured at about 1 in 9, 000 nucleotides [28].

Steps in PCR (The Basics of PCR Cycling): Typically, PCR consists of a series of 20–40 repeated temperature changes, called thermal cycles, with each cycle commonly consisting of two or three discrete temperature steps. The cycling is often preceded by a single temperature step at a very high temperature (>90°C) and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters, including the enzyme used for DNA synthesis, the concentration of bivalent ions and dNTPs in the reaction and the melting temperature (Tm) of the primers. The first step of PCR simply entails mixing the template DNA, two appropriate oligonucleotide primers, DNA polymerase, deoxyribonucleoside triphosphates (dNTPs) and a buffer [29].

Denaturation\ Melting: This step is the first regular cycling event and consists of heating the reaction chamber to 94- 98°C (201–208 °F) for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA, which will act as templates for the production of the new strands of DNA molecules. It is important that the temperature is maintained at this stage for long enough to ensure that the DNA strands have separated completely [29].

Annealing: In the annealing step, the reaction temperature is lowered to 50-65°C (122-149 °F) for 20-40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates. Two different primers are typically included in the reaction mixture; one for each of the two single-stranded complements containing the target region. The primers are single-stranded sequences

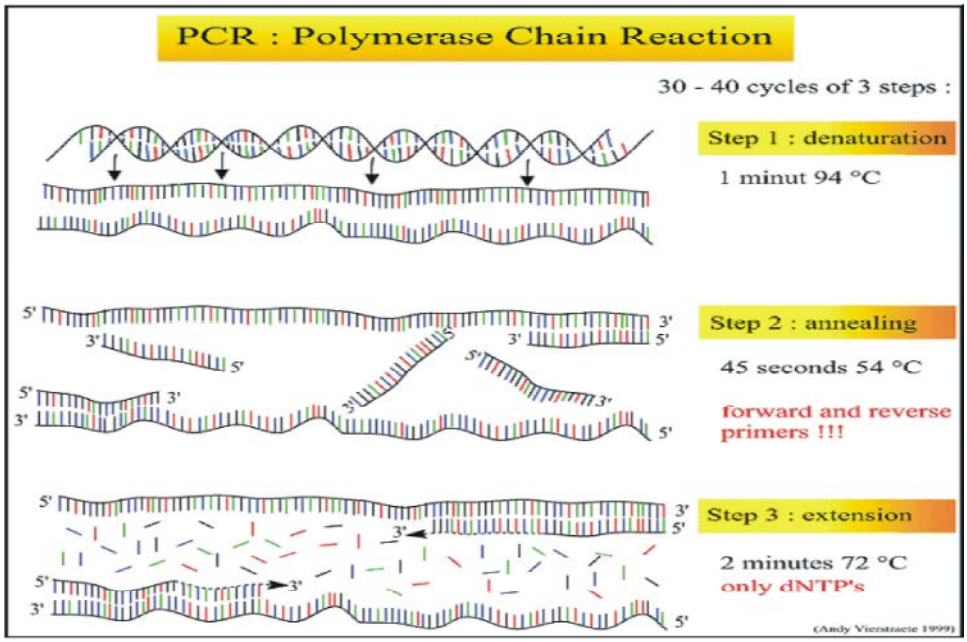

Fig. 1: The different steps in PCR:Source: [29]

themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand. It is critical to determine a proper temperature for the annealing step because efficiency and specificity are strongly affected by the annealing temperature. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should bind only to a perfectly complementary part of the strand and nowhere else. If the temperature is too low, the primer may bind imperfectly. If it is too high, the primer may not bind at all. A typical annealing temperature is about 3-5°C below the Tm of the primers used [30].

Extension/Elongation: Here the DNA polymerase fills the missing strands; the temperature involved will be dependent upon the stability of *Taq*polymerase. The temperature involved is 72°C for 5-15min and the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction by condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxy group at the end of the nascent (elongating) DNA strand. The amplification is done on logarithmic scale and amplified product of PCR process called as amplicon. The precise time required for elongation depends both on the DNA polymerase used and on the length of the DNA target region to amplify [31]. With each successive cycle, the original template strands plus all newly generated strands becomes template strands for the next round of elongation, leading to exponential (geometric) amplification of the specific DNA target region. The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies. The formula used to calculate the number of DNA copies formed after a given number of cycles is 2^n, where n is the number of cycles. Thus, a reaction set for 30 cycle's results in 2^{30}, or 1073741824, copies of the original double-stranded DNA target region [10].

Analysis of PCR Product: The analysis of PCR amplification products is an essential step in determining the quality and quantity of the DNA target that has been amplified. Two main methods are available forvisualizing PCR amplification products, namely, (1) staining of the amplimer double stranded DNA using chemical dyes which insert (intercalate) between the two strands of the duplex and (2) labeling of the PCR primer or dNTP nucleotides with fluorescent dyes (fluorophores) prior to PCR amplification, which results in the labels becoming directly incorporated into the PCR product during thermocycling [26].

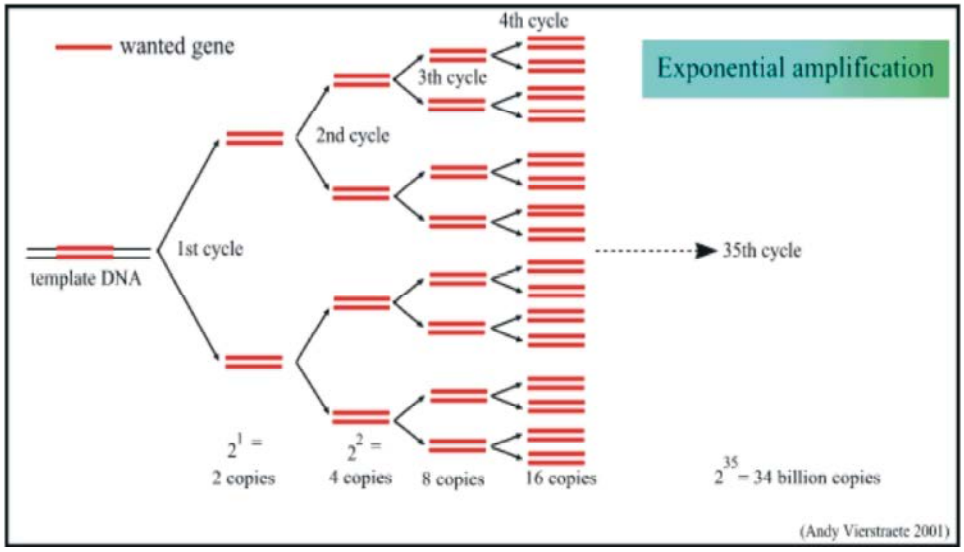

Fig. 2: Number of DNA copies formed after a given number of cycles (2n) Source: [10]

Intercalating Chemical Dyes: Intercalating chemical dyes insert between the organic bases within the duplex strands of DNA facilitating a large increase (up to 1, 000 times) in their capacity to fluoresce and as such are useful for determining DNA concentrations ranging between 0.01 and 20 µg per ml. Many intercalating dyes useful in PCRassay are currently commercially available, e.g. SYBR Green and Vista Green (Molecular Dynamics). However, probably the best-known and most widely used intercalating dye is 2, 7-diamino-9-phenyl-10-ethyl phenanthridiumbromide, better known as ethidium bromide (EthBr) [32]. Ethidium Bromide is an intercalating agent, which resembles a DNA base pair. Due to its unique structure, it can easily intercalate into DNA strand. Therefore, it is commonly used as nucleic acid fluorescent tag in various techniques of the life science field [33]. Chemical agents that intercalate within the DNA double helix disturb its regular structure and are usually potent carcinogens. These compounds need to be treated with care and the necessary precautions taken when handling such hazardous chemicals [34].

Agarose Gel Electrophoresis: The visualization of a specific amplification product by electrophoresis on an agarose or polyacrylamide gel is diagnostic for most amplification purposes. However, PCR amplification is sometimes combined with a hybridization assay to confirm the specificity of the reaction [35]. Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNAand proteins) and their fragments, based on their size and charge. DNA Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via polymerase chain reaction. Agarose gel consists of 0.9% agarose in 40mM Tris-base at a pH-8.3, 20mM acetic acid, 1mM purified form of 0.9% agarose gels using quick gel extraction kit. If the bands are present, it indicates the target sequence of original DNA sample and absences of any bands indicate the absence of original DNA sample. Gel electrophoresis helps visualize the size and type of DNA extracted using PCR and restriction enzymes [36].

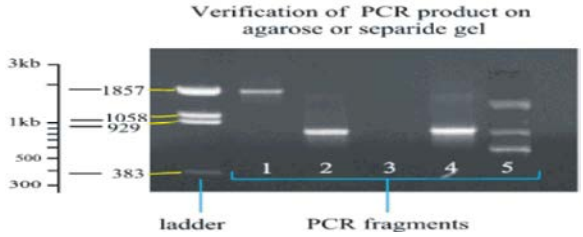

Fig. 3: Verification of the PCR product on gel; Source: [35]

where; Lane 1: PCR fragment is approximately 1850 bases long, Lane 2 and 4: the fragments are approximately 800 bases long, Lane 3: no product is formed, so the PCR failed, Lane 5: multiple bands are formed because one of the primers fits on different places.

Varieties of PCR

Real-Time PCR (qPCR): A real-time polymerase chain reaction, also known as quantitative polymerase chain

reaction (qPCR), is a laboratory technique of molecular biology which is used to measure the quantity of a target sequence (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA, or RNA. Quantitative PCRis commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. Quantitative PCR has a very high degree of precision. Quantitative PCR methods use fluorescent dyes, such as SYBR Green, EvaGreen or fluorophorecontaining DNA probes, such as TaqMan, to measure the amount of amplified product in real time. Not only is a real-time PCR, it is the method of choice for quantification of gene expression and the preferred method of obtaining results from array analyses in gene expressions at a global scale [37]. Unlike conventional PCR, this method avoids the previous use of electrophoresis techniques to demonstrate the results of all the samples. This is because, despite being a kinetic technique, quantitative PCR is usually evaluated at a distinct end. The technique therefore usually provides more rapid results and/or uses fewer reactants than electrophoresis. It monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time and not at its end, as in conventional PCR. Two common methods for the detection of PCR products in real-time PCR are: (1) non-specific fluorescent dyes that intercalatewith any double-stranded DNA and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence [19].

Real-time PCR can be used to quantify nucleic acids by two common methods: relative quantification and absolute quantification. Absolute quantification gives the exact number of target DNA molecules by comparison with DNA standards using a calibration curve. It is therefore essential that the PCR of the sample and the standard have the same amplification efficiency. Relative quantification is based on internal reference genes, to determine fold differences in expression of the target gene [38]. The quantification is expressed as the change in expression levels of mRNA interpreted as complementary DNA (cDNA, generated by reverse transcription of mRNA). Relative quantification is easier to carry out, as it does not require a calibration curve as the amount of the studied gene is compared to the amount of a control reference gene [39]. In quantitative PCR (real time PCR), the DNA or RNA molecules are tagged using fluorescent probes, so that the concentration of amplified products can be monitored and quantified in a real-time by tracking the level of fluorescence. Currently, there are four different fluorescent DNA probes available for the real-time PCR detection of PCR products: SYBR Green, TaqMan, Molecular Beacons and Scorpions. All of these probes allow the detection of PCR products by generating a fluorescent signal. While the SYBR Green dye emits its fluorescent signal simply by binding to the double-stranded DNA in solution, the TaqMan probes, Molecular Beacons and Scorpions generation of fluorescence depend on FlorescenceResonance Energy Transfer (FRET) coupling of the dye molecule and a quencher moiety to the oligonucleotide substrates [40].

SYBR Green: When the SYBR Green binds to the double-stranded DNA of the PCR products, it will emit light upon excitation. The intensity of the fluorescence increases as the PCR products accumulate. This technique is easy to use since designing of probes is not necessary given lack of specificity of its binding [41]. However, since the dye does not discriminate the double-stranded DNA from the PCR products and those from the primer-dimers, overestimation of the target concentration is a common problem. Nevertheless, among the real time (qT-PCR) product detection methods, SYBR Green is the most economical and easiest to use [42].

Taq Man Probes: TaqMan probes are oligonucleotides that have a fluorescent probe attached to the 5' end and a quencher to the 3' end.As the *Taq* polymerase extends the primer and synthesizes the nascent strand, the 5' to 3' exonuclease activity of the *Taq* polymerase degrades the probe that has annealed to the template. Degradation of the probe releases the fluorophore from it and breaks the proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR [43].

Molecular Beacon Probes: Similar to the TaqMan probes, Molecular Beacons also make use of FRET detection with fluorescent probes attached to the 5' end and a quencher attached to the 3' end of an oligonucleotide substrate. However, TaqMan fluorescent probes are cleaved during amplification, whereas, Molecular Beacon probes remain intact and rebind to a new target during each reaction cycle. When free in solution, the close proximity

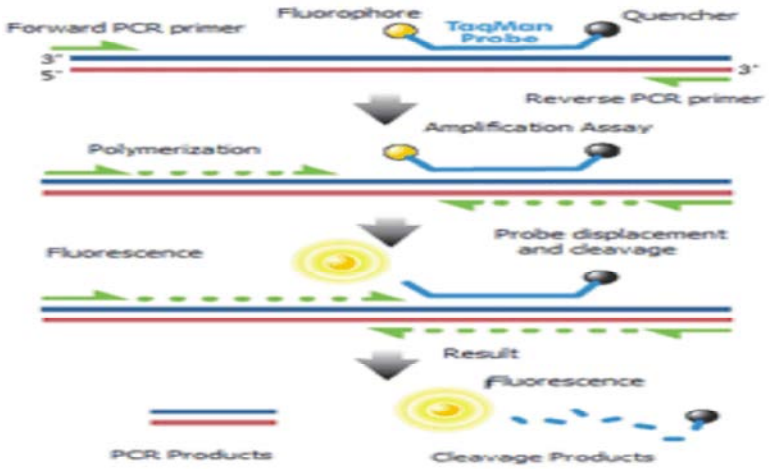

Fig. 4: Taqman DNA Probes; source: [44]

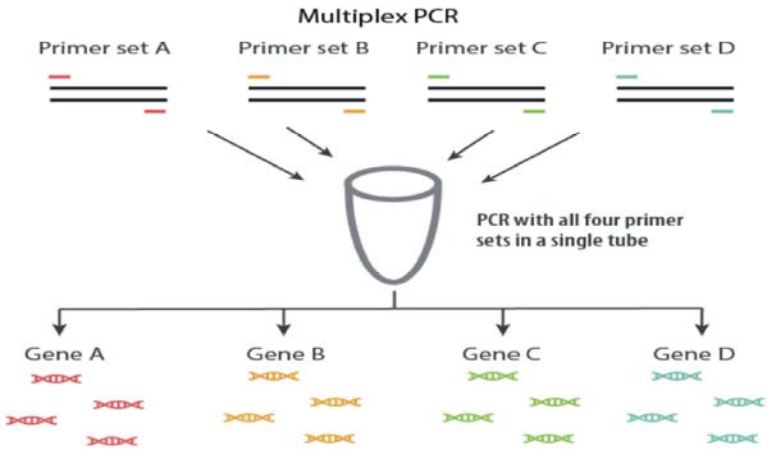

Fig. 5: Multiplex PCR;Source: [49]

of the fluorescent probe and the quencher molecule prevents fluorescence through FRET. However, when Molecular Beacon probes hybridize to a target, the fluorescent dye and the quencher are separated resulting in the emittance of light upon excitation. As is with the TaqMan probes, Molecular Beacons are expensive to synthesize and require separate probes for each RNA target [45].

Scorpion Probes: The Scorpion probes, like Molecular Beacon, will not be fluorescent active in an unhybridized state, again, due to the fluorescent probe on the 5' end being quenched by the moiety on the 3' end of an oligonucleotide. With Scorpions, however, the 3' end also contains sequence that is complementary to the extension product of the primer on the 5' end. When the Scorpion extension binds to its complement on the amplicon, the Scorpion structure opens, prevents FRET and enables the fluorescent signal to be measured [46].

Multiplex-PCR: Multiplex PCR is a widely used molecular biology technique for amplification of multiple targets in a single PCR experiment. Multiplex-PCR was first described in 1988 as a method to detect deletions in the dystrophic gene. In 2008, multiplex-PCR was used for analysis of microsatellites and SNPs [47]. It consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.In a multiplexing assay, more than one target sequence can be amplified by using multiple primer pairs in a reaction mixture. As an extension to the practical use of PCR, this technique has the potential to produce considerable savings in time and effort within the laboratory without compromising on the utility of the experiment [27].

The primer design for all primers pairs has to be optimizedso that all primer pairs can work at the same annealing temperature during PCR. Amplicon sizes of different genes such as their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis. Alternatively, if amplicon sizes overlap, the different amplicons may be differentiated and visualized using primers that have been dyed with different colour fluorescent dyes. Commercial multiplexing kits for PCR are available and used by many forensic laboratories to amplify degraded DNA samples [48].

Reverse Transcription Polymerase Chain Reaction (RT-PCR): Reverse transcription PCR, or RT-PCR, is used to create an amplicon from a sample of RNA rather than DNA. RT-PCR uses RNA as starting material for in vitro nucleic acid amplification. Reverse transcriptaseis an RNA-dependent DNA polymerase, catalyzing DNA synthesisusing RNA as the template.It is a laboratory technique combining reverse transcription of RNA into DNA to produce a complementary DNA (cDNA). In RT-PCR, the starting RNA is subsequently degraded, dsDNA is produced and PCR amplification proceeds in the usual manner. It is primarily used to measure the amount of a specific RNA and the RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification in aPCR [50]. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start sites and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be used to map the location of exons and introns in the gene. The 5' end of a gene (corresponding to the transcription start site) is typically identified by RACE-PCR (*Rapid* Amplification of cDNA Ends) [51].

Application of Pcr in Genetic Engineering

Genetic Engineering: Genetic engineering, also called genetic modification or genetic manipulation, is the direct manipulation of an organism's genes using biotechnology. It is a set of technologies used to change the genetic makeup of cells, including the transfer of genes within and across species boundaries to produce improved or novel organisms [52]. New DNA is obtained by eitherisolating and copying the genetic material of interest using recombinant DNA methods or by artificially synthesizing the DNA. A construct is usually created and used to insert this DNA into the host organism. The first recombinant DNA molecule was made by Paul Berg in 1972 by combining DNA from the monkey virus SV40 with the lambda virus.An organism that is generated through genetic engineering is considered to be genetically modified (GM) and the resulting entity is a genetically modified organism (GMO). The first GMO was a bacterium generated by Herbert Boyer and Stanley Cohen in 1973 [2]. The exact definition of a genetically modified organism and what constitutes genetic engineering varies, with the most common being an organism altered in a way that does not occur naturally by mating and/or natural recombination. Genes have been transferred within the same species, across species (creating transgenic organisms) and even across kingdoms. New genes can be introduced, or endogenous genes can be enhanced, altered or knocked out. The first company to focus on genetic engineering, Genentech, was founded in 1976 and started the production of human proteins. Genetically engineered human insulin was produced in 1978 and insulin-producing bacteria were commercialized in 1982, Jackson [53].

Detecting Genetically Modified Organisms: The polymerase chain reaction (PCR) is a biochemistry and molecular biology technique for isolating and exponentially amplifying a fragment of DNA, via enzymatic replication, without using a living organism. It enables the detection of specific strands of DNA by making millions of copies of a target genetic sequence. The target sequence is essentially photocopied at an exponential rate and simple visualisation techniques can make the millions of copies easy to see. PCR can also be used to detect the presence and quantity of known genetically modified organisms (GMOs) in the environment, by detecting the sections of DNA that are known to be modified. The technique can also be used to find unknown GMOs by targeting sequences that are common to many genetic modifications [54]. The method works by pairing the targeted genetic sequence with custom designed complementary bits of DNA called primers. In the presence of the target sequence, the primers match with it and trigger a chain reaction. DNA replication enzymes use the primers as docking points and start doubling the target sequences. The process is repeated over and over again by sequential heating and cooling until doubling and redoubling has multiplied the target sequence several million-fold. The millions of identical fragments are then purified in a slab of gel, dyed and can be seen with UV light. Irrespective of the variety of methods used for DNA analysis, only PCR in its

different formats has been widely applied in GMO detection/analysis and generally accepted for regulatory compliance purposes. Detection methods based on DNA rely on the complementarity of two strands of DNA double helix that hybridize in a sequence-specific manner. The DNA of GMO consists of several elements that govern its functioning. The elements are promoter sequence, structural gene and stop sequence for the gene [55].

The role of PCR in Genetic Engineering: All organisms are made up of cells that are programmed by the same basic genetic material, called DNA (deoxyribonucleic acid). Each unit of DNA is made up of a combination of the nucleotides such as, adenine (A), guanine (G), thymine (T) and cytosine (D) as well as a sugar and a phosphate. By definition, genetic engineering is the direct altering of an organism's genome. This is achieved through manipulation of the DNA. Doing this is possible because DNA is like a universal language; all DNA for all organisms is made up of the same nucleotide building blocks [56]. The advent of polymerase chain reaction (PCR) has greatly accelerated the progress of studies on the genomic structure of various organisms. Virtually any DNA sequence can also be engineered by "copying and pasting" with PCR as a replacement for conventional recombinant DNA technology where DNA is manipulated by "cutting and pasting" using restriction endonucleases and ligase enzyme. PCR therefore overcomes problems associated with the often limited availability of sites for the restriction endonucleases [57]. For the reason that, all organisms are made up of the same type of genetic material (nucleotides A, T, G and C), biotechnologists use enzymes to cut and remove DNA segments from one organism and recombine it with DNA of another organism. This is called recombinant DNA (rDNA) technology and it is one of the basic tools of modern biotechnology. Recombinant DNA technology is the laboratory manipulation of DNA in which DNA, or fragments of DNA from different sources, are cut and recombined using enzymes. This recombinant DNA is then inserted into a living organism. Recombinant DNA technology allows researchers to move genetic information between unrelated organisms to produce desired products or characteristics or to eliminate undesirable characteristics [54]. PCR is used to create millions or billions of copies of DNA through repeated cycles of denaturing, which separates the DNA into its two strands; annealing, which attaches specific primers that mar the beginning and end of the DNA to be copied; and extension/elongation, where the DNA strands are used as templates to build two new strands of DNA. These cloned DNA fragments can then be inserted into the target organism, including microorganisms or animals, using vectors such as bacteria and viruses [58]. Cloning with PCR differs from traditional cloning in that the DNA fragment ofinterest and even the vector can be amplified by the Polymerase Chain Reaction (PCR) and ligated together, without the use of restriction enzymes. PCR cloning is a rapid method for cloning genes and is often used for projects that require higher throughput than traditional cloning methods can accommodate. It allows for the cloning of DNA fragments that are not available in large amounts. Typically, a PCR reaction is performed to amplify the sequence of interest and then it is joined to the vector via a blunt or single-base overhang ligation prior to transformation [59].

PCR supplies a rapid alternatives to gene cloning protocols provided that some sequence information of the desired clone is available, either directly from previously sequenced genes or highly conserved regions of gene families, or indirectly as degenerate DNA sequences derived from the terminal sequencing of proteins. In general cloning is simply making one living organism from another, creating two organisms with the same exact genes. PCR enables scientists to produce billions of copies of a piece of DNA within hours. Application of the PCRto yield enough amounts of DNA for cloning and analysis is highly recommended, especially for those specimens that are in a minute amount. A number of PCRbasedtechniques, however, have been developed to amplify the unknown cellularDNA flanking sequence from the foreign DNA [60].

CONCLUSION AND RECOMMENDATIONS

PCR is a highly versatile technique developed by Kary Mullis in the 1980s and has been modified in different ways to suit specific applications. PCR is used to create millions or billions of copies of DNA through repeated cycles of denaturing, which separates the DNA into its two strands; annealing, which attaches specific primers that mark the beginning and end of the DNA to be copied; and extension/elongation, where the DNA strands are used as templates to build two new strands of DNA. A PCR master mix is a premixed solution that contains most of the components necessary to run a PCR assay. The mix contains Taq DNA polymerase, dNTPs, as well as enhancers and stabilizers in a buffer that is optimized for DNA amplification by PCR. There are different types

of PCR with their working principles and applications. PCR is a fundamental method in cloning technique, which permits era of a lot of unadulterated DNA from minor measure of format strand and further investigation of a specific quality. Applications of the PCR technique include DNA cloning for sequencing, gene cloning and manipulation, gene mutagenesis, construction of DNA-based phylogenies, DNA profiling, etc. Therefore, based on the above conclusions the following recommendations are suggested:

PCR must be commerciallyavailable in every research center and universities withthe appropriate PCR technologies.

All molecular biology laboratories (biotechnology lab) must be fulfilled with different varieties of PCR techniques with the skilled technicians.

Due consideration should be given on the expansion of biotechnology laboratories with its facilities at national level.

The idea of genetic engineering must be developed at the national level and application of PCR in genetic engineering must be practiced in every biotechnology lab and universities

List of Abriviations and Acronym

µg	Micro gram
µM	Micro Meter
cDNA	Complementary DNA
dATP,	Deoxyadenosine Triphosphate
dCTP	Deoxycytosine Triphosphate
dGTP	Deoxyguanosine Triphosphate,
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
dsDNA	Double Stranded DNA
dTTP)	Deoxythymidine Triphosphate
FRET	Florescence Resonance Energy Transfer
mM	Milli Moles
Mn	Manganese
PCR	polymerase chain reaction
PEG	Polyethylene Glycol
qPCR,	Quantitative PCR
RACE-PCR	Rapid Amplification of cDNA Ends
RT-PCR	Reverse transcription polymerase chain reaction
SNPs	Single Nucleotide Polymorphism
Taq	Thermusaquaticus
Tm	Melting Temperature

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