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**Chapter**

# Polymerase Chain Reaction

*Shaheen Shahzad, Mohammad Afzal, Shomaila Sikandar and Imran Afzal*

## Abstract

Polymerase chain reaction (PCR) is an efficient and one of the most common methods used in biological sciences for in vitro multiplication of a target DNA molecule. The technique has significantly contributed in changing and developing different fields of biological sciences since 1980s. PCR has a vital role in supporting the processes involved in genetic engineering, particularly the cloning of DNA fragments used to modify the genomes of microorganisms, animals, and plants. Consequently, the technique has numerous applications in fundamental and applied research in medicine agriculture, environment, and bio-industry. The main focus of this chapter is to describe briefly the principles, methodology, various types, and applications of PCR in different fields. Besides, different components of PCR, trouble shooting during the execution, and limitations of the techniques are also outlined.

**Keywords:** PCR, primer, DNA template, nucleotides, sequence, polymerase

## 1. Introduction

Polymerase chain reaction (PCR) is one of the most commonly used method in modern molecular biology. The technique was developed by the Nobel laureate, Kary Mullis, in 1984. It is an in vitro process to multiply a target molecule of DNA with extreme precision, making it easy to be handled and examined by routine molecular biological methods [1–4]. Since its inception, PCR has significantly contributed in changing and developing biological sciences. The first PCR machine was introduced in market in 1988. The Human Genome Project has been result of PCR based approaches [5, 6]. Owing to its wide range of applications, numerous variants of PCR techniques have emerged over the past few decades [2–4].

PCR begins with the separation (denaturation) of the strands of a target DNA molecule (known as template) followed by annealing (hybridization) of oligonucleotide primers to the target template. The annealed primers provide a start for the DNA polymerase point to add new nucleotides (deoxynucleoside triphosphates or dNTPs). The sequence of nucleotides to be added is determined by the template. This entire process of the amplification of template, i.e., separation, annealing, and polymerization, is accomplished in vitro by cyclical alterations of temperature [2, 4, 7–10]. DNA polymerases used in PCR originate in thermophilic microorganisms, largely archaea, thriving temperature between 41 and 122°C. This ability to withstand high temperature is requited in PCR to melt or separate the double-stranded DNA. Today, PCR has become a main stay in biotechnology, genomics, diagnostics, systematics, and many more areas [2–6].

## 2. Principles of PCR

PCR typically involves a series of 20–40 repeated temperature changes, called cycles, with each cycle usually comprising three discrete temperature shifts (**Figure 3**) [2, 4, 7–10].

1. Denaturation: 94–96°C

2. Primer annealing (depending on the primer): 45–60°C

3. Primer extension: usually 72°C

The cycling steps often start and end with a temperature step called "hold" where product extension is performed at (>90) and (~72°C), respectively. The final product is kept at 4°C before its analysis or storage. The most of PCR methods typically amplify DNA fragments of up to ~10 kilo base pairs (kb). However, some techniques can amplify up to 40 kb.

## 3. The components of PCR

Setting up a basic PCR requires many ingredients, reagents, and conditions which are described below (**Figure 1**) [2, 4, 7–10].
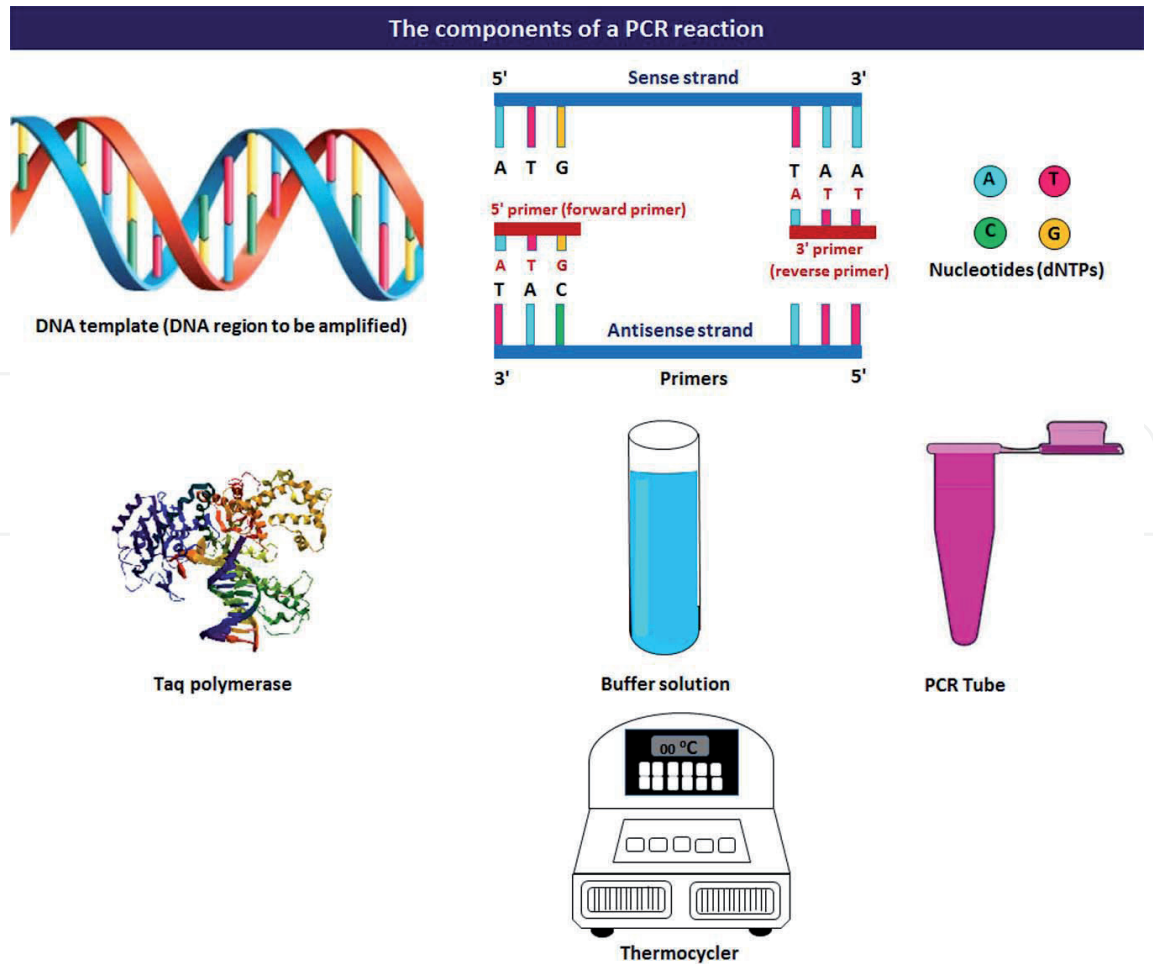


**Figure 1.**
*The component of PCR.*

### 3.1 DNA template

The double-stranded DNA molecule amplified by PCR is called the target or template DNA. The template defines the sequence in which new nucleotides are added during the PCR process [11]. This process is carried out in vitro by cyclically varying temperature, enabling separation of DNA strands, hybridization of primers, and polymerization.

DNA isolated from any source can be used as a template for PCR provided that it contains the target sequence. The DNA used in PCR can be isolated from blood, tissue, forensics specimens, paleontological samples, or microbial/tissue cells grown in the lab. Whatever the source, we need to have some information of the target DNA sequence, so that primers for PCR can be designed [2, 12]. The PCR primers can be designed very easily nowadays owing to the plethora of software tools that only requires target sequence information. If the sequence information is not known, the designing of primers becomes very challenging. This problem can be circumvented by using degenerate primers [2].

### 3.2 Primers

Primers are single-stranded DNA molecules usually synthesized commercially, i.e., polynucleotides of variable sizes [2, 7–10]. These short polynucleotide DNA strands have a free 3′ hydroxyl group, also called as 3′ end. The free 3′ hydroxyl group on the primer is needed by the DNA polymerase to add new nucleotides during the polymerization process, thereby synthesizing a new complementary strand [12, 13]. The binding of DNA primer to the target requires the separation of two complementary DNA strands (Denaturation) which is generally achieved by heating process. To perform PCR, two primers are needed to enhance both the strands of the template: a primer for one strand (or sense strand), called the "forward primer," which is the beginning of the template, and another primer for the complementary strand (or the antisense strand) called the "reverse primer." Thus, both the primers bind to 5′ ends of the sense and antisense strand.

The length of primers plays an important role in correctly identifying their designated target complementary regions. Increasing the length of primers improves their chances of matching the target (specificity). These primers are usually commercially synthesized with their size ranging between 18 and 25 nucleotides.

Primers should bind (anneal or hybridize) to the template with good specificity and strength to ensure amplification of the correct sequence. The specific temperature that is needed for primer annealing also depends on the primer sequences, e.g., the longer the primer, the higher the annealing temperature. Therefore, the maximum specificity and efficiency of PCR depends on optimal primer sequences and appropriate primer concentrations [2, 7, 8]. This in return depends on the way primers are designed and used. Improper primers may amplify undesired DNA segments (nonspecific products), lower the yield of specific products, or completely fail the results of PCR. These undesired outcomes can be circumvented by designing and validating primers that preferentially bind to their target sequences. The online IDT Sci Tools Software Oligo Analyzer 3.1 and Primer Quest are invaluable aids both in primer design and validation [14]. These software tools also ensure that the two primers do not contain sequences that are complementary to each other. If primers contain self-complementary sequences, then hybridization will occur to each other, and they form "primer-dimmers." Consequently, the primers will fail to bind to their target template, leading to a compromised PCR efficiency. In addition, presence of complementary sequences within a primer leads to the formation of hairpin loop structures [15].

As the bonding of guanine and cytosine bases (GC) is stronger than that between adenine and thymine (AT) bases, primers having GC at 3′ end should be preferred for a strong bonding with the template [16]. However, the primers should not contain runs of three or more C or G bases, as this may lead to nonspecific binding to G- or C-rich sequences (mispriming) in the DNA which is not the target sequence [17].

### 3.3 DNA polymerase

Discovery of DNA polymerase in 1955 was the onset of PCR technology, which exploits the ability of bacterial DNA polymerase to make a complementary strand of a target DNA [2, 4, 7, 8, 18]. DNA polymerase starts making a new DNA from the 3′ end of the template. The 3′ end of the two template stands is where the primers bind which are then extended by the DNA polymerase. The most commonly used DNA polymerase is Taq DNA polymerase isolated from *Thermus aquaticus*, a thermophilic bacterium. Taq polymerase extends the DNA chain by adding ~1.0 kb per min with the enzymatic half-life achieved at 95°C in 40 minutes. Alternatively, the DNA polymerase from *Pyrococcus furiosus*, called Pfu, is also used widely due to its 3′–5′ exonuclease activity (proofreading) which is not present in Taq DNA polymerase. Proofreading allows Pfu to remove incorrectly added nucleotide during polymerization and therefore to synthesize new DNA with minimum errors. A recombinant DNA polymerase, KOD DNA polymerase, derived from the thermophilic solfatara bacterium *Thermococcus kodakarensis* KOD1 type strain, functions optimally at 85°C with 3′–5′ exonuclease proofreading activity, resulting in blunt-ended DNA products [19, 20]. KOD DNA polymerase exhibits high fidelity and processivity for small amplicons. However, for the amplicons over 5 kb, the amplification is lowered due to strong 3′–5′ exonuclease activity of the enzyme [5]. This problem can be solved by mixing wild type with the mutant form of the enzyme (with lower 3′–5′ exonuclease activity), which can result in more correct amplification of the amplicons between 5 and 15 kb [21]. Other sources of DNA polymerases used in PCR include thermophilic species like *Thermus thermophilus* (Tth) and *Thermus flavus* (Tfl) [18].

### 3.4 Nucleotides

PCR requires four different deoxynucleoside triphosphates or dNTPs to synthesize new DNA strands: adenine(A), guanine(G), cytosine(C), thymine(T). The dNTPs are usually provided at a concentration of 200 μM in the reaction mixture [22]. The concentration of these four dNTPs must be equal in the reaction mixture, as unequal concentration of even a single dNTPs leads to misincorporation of nucleotides by the DNA polymerase.

### 3.5 Buffer solution

The function of PCR buffer solution is to provide suitable conditions and chemicals to the DNA polymerase for optimal activity and stability [23]. The buffers often contain Tris-Hcl, KCl, and sometimes $MgCl_2$. PCR buffers are often available in 10× concentration and are sometimes Taq formulation-specific including the compounds shown in **Table 1**.

### 3.6 Monovalent cations

Potassium chloride (KCl) is normally used in a PCR amplification of DNA fragments at a final concentration of 50 mM [24].

| Component | Function |
|---|---|
| 100 mM Tris-HCl (pH 8.8 at 25 °C) | Maintains reaction pH |
| 500 mM KCl | Stabilizes primer-template annealing |
| 15 mM MgCl$_2$ | Cofactor for DNA polymerase |
| 0.8% (v/v) Nonidet P40 (Optional) | Suppresses secondary structure formation |

*(Thermo Fisher Scientific™B16: https://www.thermofisher.com/order/catalog/product/B16)*

**Table 1.**
*Concentrations of PCR buffers.*

### 3.7 Divalent cations

Magnesium ions are needed by the DNA polymerase enzyme as a cofactor. The divalent cations may include 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 [25].

### 3.8 PCR tube

PCR is performed in a small, thin-walled plastic tube called PCR tube. The tube is specifically designed to permit favorable thermal conductivity equilibration during thermal cycling.

### 3.9 Thermal cycler

A thermal cycler or thermocycler is a device used to rapidly heat and cool the reaction mixtures and cycle them between the three PCR temperature steps [26]. Many modern thermocyclers employ the Peltier effect to achieve this temperature ramping, which is done by reversing the electric current [11]. Modern thermocyclers are also provided with heated lids to prevent condensation of reaction mixture during PCR operation. Older thermocyclers lacked this feature, and the evaporation was prevented by applying oil or wax balls on the surface of PCR mixture.

## 4. Procedure

Each cycle or round of PCR comprises three major steps, viz., denaturation, annealing, and extension, repeated for 30 or 40 cycles on a thermocycler (**Figure 2**) [2, 4, 7–10]. A number of parameters determine the range of temperature and the duration of each cycle step (**Figure 3**), e.g., the polymerase used for DNA synthesis; melting temperature (Tm) of the primers; and the concentration of reagents used, i.e., divalent ions and dNTPs. The melting temperature depends on the length and specific nucleotide sequence of a primer. At Tm, half of the DNA molecules are in the single-stranded form.

### 4.1 Denaturation

It is the first cycling step that involves heating the reaction mixture to 94–98°C for 20–30 seconds. Such higher temperature disrupts the hydrogen bonding of the two complementary strands to produce the single-stranded DNA templates. Thus, denaturation prepares the DNA template for the binding of primers.
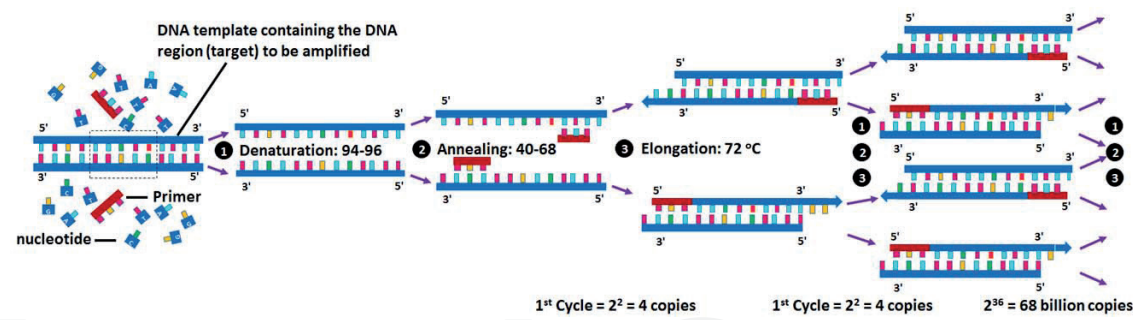
**Figure 2.**
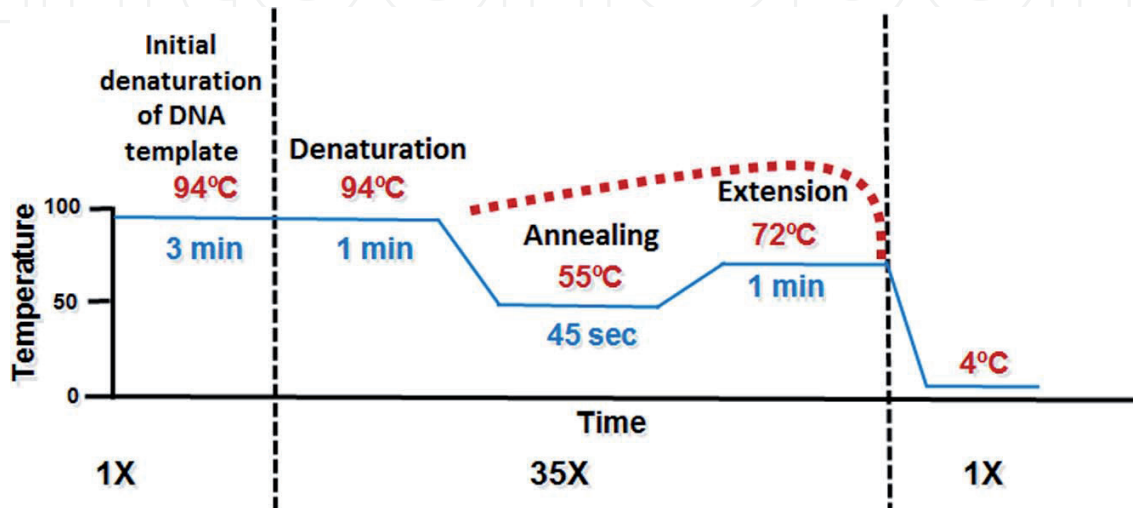*A basic PCR protocol—DNA synthesis cycle.*



**Figure 3.**
*PCR—a simple thermocycling protocol.*

## 4.2 Annealing

After denaturation, the next cycling step is annealing, in which the temperature of the PCR reaction is decreased to 50–65°C and kept for 20–60 seconds. This promotes hybridization between primers and single-stranded templates. Optimal annealing occurs at temperatures that are 3–5°C less than the primer Tm. The primers should have sufficient length and GC content to strongly bind to their target template during annealing.

## 4.3 Extension

DNA polymerase synthesizes (polymerizes) new DNA molecule by adding dNTPs complementary to the template bases in a 5′–3′ direction. The temperature and extension time depend on the type of DNA polymerase used: Taq polymerase performs optimally between 75 and 80°C. However, the enzyme is routinely used at 72°C. The extension time also depends on the length of the template.

A single cycle of PCR comprises the entire processes of denaturation, annealing, and extension/elongation. Under ideal conditions (optimal temperature ramping, presence of substrates/reagents, absence of inhibitors), the quantity of target DNA is doubled at the end of each cycle, resulting in exponential amplification of the specific DNA segment.

Final elongation: This single step is optional and performed at 70–74°C. The final elongation may take for 5–15 minutes after the last PCR cycle and allows any remaining single-stranded DNA to be fully extended.

Final hold: The final step may be employed for short-term storage of the reaction by cooling the reaction chamber to 4–15°C for an indefinite time.

## 5. Magnitude of amplification

Multiple cycling steps of a PCR can exponentially increase the copies of target DNA template to millions. The number of DNA copies produced by a PCR can be calculated using the formula "$2^n$", where n is the number of cycles [27, 28]. For example, a PCR set for 36 cycles results in 68 billion copies of the template. Under optimal conditions, even with minimal efficiency, a PCR in 50 ml volume may produce 0.2 mg of 150 bp DNA from 100 template molecules after 35–40 cycles, with the molar weight of the fragment equal to 99,000 Da.

## 6. Validating PCR

The degree of a PCR success can be determined in many ways [3, 29–36]:

1. Ethidium bromide (EtBr) can be used for the staining of amplified DNA product [31, 32]. It has UV absorbance maxima at 300 and 360 nm, and an emission maximum at 590 nm, and being a DNA intercalator, EtBr inserts itself between the base pairs in the double helix. The detection limit of DNA bound to ethidium bromide is 0.5–5.0 ng/band.

2. A three primer combination approach can provide a more cost-effective end-labeling of PCR products: (i) fluorescently labeled universal primer, (ii) modified locus-specific primers, and (iii) 5′ universal primer sequence tails [34].

3. Agarose gel electrophoresis: The most commonly employed validating method, gel electrophoresis, makes use of electric current to separate charged molecules like DNA using gel as molecular sieve. Gelling agents can be agarose (for DNA >500 bp) or polyacrylamide (<500 bp). Different DNA sequences are separated out based on their sizes. DNA staining dyes (like EtBr) are applied to the gel to help visualize the DNA bands using UV transilluminator [34, 36]. The presence of a correct size DNA band (confirmed using a DNA ladder) indicates that the target sequence was present and that the PCR has amplified a correct product. Absence of any DNA band indicates that the target DNA was absent, while the presence of incorrect size DNA band indicates production of a spurious product [37].

4. The direct sequencing is often not practiced due to in accessibility or cost of DNA sequencer or even the time needed to undertake such an analysis. However, restriction enzyme digestion can also be used to assess the sequence of an amplicon indirectly [29].

## 7. Types of PCR

Owing to ever-growing applications, a wide variety of PCR techniques have emerged over the past few decades [2–4]. Some of the variants are mere optimization close to the basic PCR to fulfill the specific needs. Others have undergone massive modifications to suit novel applications in different biological, biomedical, agricultural, and environmental fields [6].

### 7.1 Conventional PCR

This is a standard PCR in which a single-primer pair is used to bind to the two separated target strands. The primers also define the target sequences that will be copied. The PCR generates millions of copies of the target DNA sequences [2–4].

### 7.2 Multiplex-PCR

It is a special type of PCR for the detection of pathogenic microorganisms by using several pairs of primers annealing to different target sequences in a single sample [2–4, 38]. The multiplex-PCR is mainly used to identify exonic or intronic sequences to detect mutations, deletions, insertions, and rearrangements in pathogenic specimens.

### 7.3 Nested-PCR

It is used to increase the specificity of DNA amplification by reducing the nonspecific amplification [2–4, 39]. The two sets of primer pairs are used for a single locus point in two successive PCR reactions. The first round of PCR is performed with a primer pair that anneals to the sequence that flanks the target region. This generates a much larger DNA product that includes the target sequence. The second PCR is performed with a primer pair that precisely anneals to the target sequence, internal to the product of first PCR. This ensures that only the correct product is amplified in the second PCR [7]. Although Nested PCR improves specificity of amplification, it has disadvantage like primer-dimer formations [40].

### 7.4 Real-time PCR/quantitative PCR (qPCR)

A qPCR is a technique used to quantify the amplification of a template DNA in real time during the PCR reaction. This type of PCR is commonly employed to estimate the number of DNA targets present in a sample or to study and compare the gene expression [7, 37]. When real-time PCR is used quantitatively (qPCR), the amount of amplification is measured either by using a nonspecific fluorescent dyes or sequence-specific DNA oligonucleotide fluorescent probes [4, 41]. When quantitative PCR is used above/below a certain amount of DNA molecules, it is called semi quantitative real-time PCR. Although the quantitative real-time PCR has many applications, it is more frequently used in basic research and diagnostic purposes. There is a growing industrial use of the technique, e.g., quantification of microbial load in processed foods, detection of GMOs, quantification of pathogenic viruses, etc. [42–44].

### 7.5 Hot start/cold finish PCR

This technique reduces nonspecific amplification during the initial stages of a PCR [4, 7, 45]. To prevent nonspecific amplification at lower temperatures, hybrid polymerases are used which remain inactive at ambient temperature and is only activated at higher temperatures. Inhibition of the polymerase activity at ambient temperature is done by using an antibody or covalently bound inhibitors. Simply, in this technique the reaction components are heated to the DNA melting temperature (e.g., 95°C) before adding the polymerase.

### 7.6 Touchdown PCR (step-down PCR)

This type of PCR is designed to minimize nonspecific amplification by gradually decreasing the primer annealing temperature in the successive cycles. PCR is started with initial cycles having an annealing temperature 3–5°C higher than the primer Tm. The annealing temperature is then gradually decreased to 3–5°C lower below the Tm. The higher annealing temperature increases the specificity of the primers at initial stages of the reaction, while the lower temperature permits more efficient amplification later at the end [4, 7, 46].

### 7.7 Assembly PCR or polymerase cycling assembly (PCA)

This technique is used for the synthesis of long DNA molecules from long oligonucleotides with short overlapping segments, alternating between sense and antisense directions. The process begins with an initial PCR with primers that have an overlap, followed by a second PCR using the products of the first PCR as the template to generate the final full-length DNA structure [4, 7, 47].

### 7.8 Colony PCR

It is a convenient high-throughput technique used to confirm the addition of DNA insert in the recombinant clones and their uptake by the bacterial cell. A single set of insert specific primers are designed for the areas of the vector flanking the site where target DNA fragments are already inserted. This results in the amplification of the inserted sequences. The technique is used for the screening of bacterial colonies transformed with the recombinant vectors and to perform PCR without initially extracting the bacterial genomic DNA [3, 4, 7, 48].

### 7.9 Methylation-specific PCR (MSP)

It is a variant of PCR used to identify promoter hyper-methylation at CpG islands in cell lines and clinical samples, including fresh/frozen tissues. The target DNA is first treated with sodium bisulfite, which transforms the unmethylated cytosine bases in to uracil, which pair with adenosine of the PCR primers. The modified DNA is then amplified using two types of primers that only differ at their CpG islands. One primer set anneals to DNA with cytosine (corresponding to methylated cytosine), while the other anneals to DNA with uracil (corresponding to unmethylated cytosine). The MSP technique provides quantitative information about the methylation when used in quantitative PCR [3, 4, 7, 49, 50].

### 7.10 Inverse PCR

This type of PCR is used to detect the sequences that surround the target DNA (flanking sequences). It involves a series of restriction enzyme digestions and self-ligation. The primers amplify sequences at either end of the target by extending outward from the known DNA segment [4, 7, 51].

### 7.11 Reverse transcription-PCR (RTP)

In this technique, the PCR is preceded by a reaction converting RNA into cDNA using viral reverse transcriptase. The resulting cDNA is used as a template for a second conventional PCR. The technique is widely used in the detection of RNA viruses and to

study gene expression [7, 52, 53]. A variant of the RTP, called differential-display reverse transcription-PCR or RNA arbitrarily primed PCR (RAP-PCR), is used to study and compare the gene expression of organism grown under different conditions. The variant employs the use of short and random 10-mer or 11-mer radio-labeled primers that are annealed at low stringency conditions to promote the extension of random sequences during the first PCR cycle. This is followed by high-stringency cycles to extend the products of first cycle. The resulting products are analyzed using standard sequencing gels, and RAP-PCR fingerprints are visualized by autoradiography. The technique is extremely useful in studying tissue-specific and condition-specific gene expressions [54, 55].

## 8. Variants of PCR

In addition to the above mentioned techniques, numerous other variants of PCR are in use to serve a wide variety of research, diagnostic, and industrial needs, e.g., after exponential PCR, allele specific PCR, asymmetric PCR, arbitrary PCR, core sample PCR, degenerate PCR, dial-out PCR, digital PCR, high-fidelity PCR, hot start PCR, in silico PCR, inter-sequence PCR, ligation-mediated PCR, mini primer PCR, nanoparticle-PCR, overlap-extension PCR, solid-phase PCR, splicing by overlap/overhang extension PCR, suicide PCR, thermal asymmetric interlaced PCR, etc. Some of the important variants of PCR are described below:

### 8.1 Extreme PCR

In extreme PCR the concentration of primers and polymerase is increased 10–20 times; the amplification rate of instrument reaches about 0.4–2.0 s/. When the primers' concentration is more than 10 mol/L, the polymerase concentration is 1 mol/L, and the extreme PCR is suitable for rapid detection of virulent infectious and bioterrorism pathogens [56].

### 8.2 Photonic PCR

It is achieved by fast heating and based on energy conversion, thus shortening the PCR time. The specific process is carried out by using electronic resonance light emitting diode. The energy conversion process is more rapid than the conventional cooling process, causing amplification of target DNA within 5 min and thus making the PCR detection more convenient and fast [57, 58].

### 8.3 COLD-PCR

It is a low denatured temperature-PCR for enriching mutant genes by reducing the reactive temperature of PCR. The basic principle is founded on the base mismatch in any strand of DNA affecting the denaturation temperature. Therefore, the denaturation temperature of the mutant DNA is often lower than that of wild type DNA. The assay is often used for viral gene mutation [59] detection, cancer associated gene mutations (p53) [60, 61], EGFR, KRAS, etc.), beta globulin (HBB) mutations that cause beta thalassemia [62], etc.

### 8.4 Nanoparticle-PCR

Gold nanoparticles have superior electrical, optical, thermal, and catalytic activities and have the same properties as single-stranded binding proteins (ssb), which bind to single-stranded DNA and do not interact with double-stranded DNA. Therefore, the

amplification effect of high GC template can be significantly improved by adding gold nanoparticles as additives to slowdown or touchdown PCR reaction systems [63].

## 8.5 HPE-PCR

It is an amplification technique for templates with long DNA chains and large numbers of CTG repeats involving the increase in the denaturation temperature of PCR to solve the problem of high content of DNA (G+C) [64].

## 8.6 LATE-PCR

It generates high concentrations of single-stranded DNA that can be analyzed at the end point using probes which hybridize over a wide temperature range [65].

## 8.7 Digital PCR

Digital PCR (dPCR) enables precise and sensitive quantification of nucleic acids in a wide range of applications in both healthcare and environmental analysis. It is based on detection in two discrete optical channels, focused on the quantification of one or two targets within a single reaction [66]. The technique has become a promising quantification strategy that combines absolute quantification with high sensitivity.

## 9. Applications of PCR

The PCR technique and its several advanced variants act as powerful tools with specialized applications which were once impossible by the scientific world [67, 68]. This versatile technique brought enormous benefits and scientific developments such as genome sequencing, gene expressions in recombinant systems, and the study of molecular genetic analysis, including the rapid determination of both paternity and the diagnosis of infectious disease [69, 70]. It enables the in vitro synthesis of nucleic acids through which a DNA segment can be specifically replicated in a semiconservative way. It generally exhibits excellent detection limits [71, 72]. It has significantly transformed

| Application | Description | Reference |
|---|---|---|
| Diagnosis of infections | PCR approaches are used to specifically and sensitively diagnose infections (bacterial, viral, protozoan, fungal). They are routinely used in clinical laboratories to confirm and quantify these infectious agents | [71, 74] |
| Diagnosis of genetic defects | PCR-based detection systems are used to accurately detect (before disease onset) and confirm (after the onset) many genetic disorders | [74–76] |
| Diagnosis and prognosis of cancers | PCR-based approaches can identify cancer genes and analyze their expression to determine genetic predisposition to certain cancers, confirmation of cancer type, their prognosis, and treatment | [74, 77] |
| Phylogenetics | Phylogenetic analysis of organisms routinely relies on PCR amplification of phylogenetic markers to identify and classify them | [78, 79] |
| Archeology | Ancient DNA (aDNA) recovered from archeological remains are usually degraded and are in low amounts. Such miniscule quantities of aDNA are amplified using PCR techniques to improve their quality and quantity to make them analyzable for archeological study | [80–82] |
| Recombinant DNA technology | PCR techniques are used to generate hybrid DNA with ease and precision. The techniques are also employed to clone DNA in to specific vectors to get protein expression | [83, 84] |

| Application | Description | Reference |
|---|---|---|
| Metagenomics | Gene-targeted metagenomics combines PCR with metagenomics to identify rarest members of a sampled community and rare genes in the community members | [85, 86] |
| Site-directed mutagenesis | PCR-based approaches are commonly used to insert mutations (deletions, additions, and substitutions) at specific locations in a gene to study role of specific amino acids in the structure and function of proteins | [87, 88] |
| Personalized medicine | PCR technologies are employed in pharmacogenomics and pharmacogenetics to track genetic markers that determine the response of individuals to treatments and are used to design tailor-made drugs and to prescribe drugs in effective doses | [74, 89] |
| Forensics sciences | The power of PCR is employed to amplify poor quality and quantity DNA samples from crime scenes and make them reliably analyzable. | [67, 68, 90] |
| DNA profiling | DNA profiling methods utilize PCR-based approaches to exploit the polymorphic nature of DNA (SNPs, DNA repeats, etc.) to study the structure and diversity ecological communities, phylogeny, and population genetics | [90] |
| Gene expression profiling | Reverse-transcriptase PCR and qPCR are routinely employed to profile the expression of genes and to validate transcriptome profiles generated through techniques like microarray and RNA-seq | [91–93] |
| Identifying medicinal plants | PCR-based DNA barcoding is a tool that utilizes specific DNA sequences to rapidly and accurately identify medicinal plants species from other morphologically similar plants. This approach is also used by ecologists and conservation biologists to identifying endangered and new species | [94] |
| Detecting GMO | PCR techniques are used to quickly and reliably track the presence of genetically modified organism in food and feed to ensure their regulation and protection of consumer rights | [95, 96] |
| Meat traceability | PCR methods are used to identifying and quantifying adulteration of meat in raw and processed food. | [97–99] |

**Table 2.**
*The most important applications of PCR.*

the scientific research and diagnostic medicine. Over the years, it has become a vital of clinical and diagnostic research. It has a wide range of applications in almost every field of science, for example, clinicians widely use the technique for disease diagnosis. Biologists, including agriculturists, clone and sequence genes using PCR and rapidly carry out sophisticated quantitative and genomic studies. Now for criminal identification, PCR assays are commonly employed. DNA fingerprinting is also used in paternity testing, where the DNA from an individual is matched with that of his possible children, siblings, or parents [67, 68]. Besides, PCR has enormous role in diagnosing genetic disease, whether inherited genetic changes or as a result of spontaneous genetic mutations, is becoming more common. Diseases can be diagnosed even before birth. Even PCR can also be employed with significant precision to predict cure of diseases [73]. The most important applications of PCR are summarized in **Table 2**.

## 10. Limitations of PCR

Since it discovery in 1980s, the PCR technique has brought about significant changes in biological sciences. Huge scientific undertakings like the Human Genome Project have been possible due to PCR-based approaches [5, 6]. It is a very sensitive

and flexible technique to amplify DNA of interest. A very small amount of the target DNA can be used as a starting material. Even old or degraded DNA samples may yield successful amplification. However, there is also a long list of PCR limitations. High-quality DNA amplification needs information about target DNA sequence. The sensitivity of PCR is also its major disadvantage since the very end result of a PCR is highly susceptible to contamination or false amplification. Therefore, amplification of DNA by PCR may not be 100% specific. Moreover, the specificity of amplification is dependent on physicochemical parameter, such as temperature and Mg++ concentration. The PCR is also inhibited by the presence of certain chemicals such as ethanol, phenol, isopropanol, detergent compounds like sodium dodecyl sulfate (SDS), high salt concentration, chelators, etc. There is an upper limit to the size of DNA that can be synthesized by PCR. Additionally, analysis and product detection usually take much longer time than the PCR reaction itself.

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