POLYMERASE CHAIN REACTION (PCR)

In molecular biology, the **polymerase chain reaction** (**PCR**) is basically a DNA amplification process. It is a technique to amplify a single or few copies of a piece of DNA by several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on **thermal cycling**, consisting of cycles of repeated heating (for DNA melting) and cooling (for primer annealing) followed by enzymatic replication of the DNA. Primers (short oligonucleotide fragments) containing sequences complementary to a part of target region along with a DNA polymerase (after which the method is named) are key components to enable **selective and repeated amplification**. As PCR progresses, the DNA generated is itself used as a template for the next round of replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide variety of genetic manipulations.

Thermal cycling & Thermostable Polymerase: PCR applications use a heat-stable DNA polymerase such as **Taq polymerase**, an enzyme originally isolated by Chien and colleagues in 1976 from the bacterium ***Thermus aquaticus***, a thermophilic bacterium found in a hot spring in Yellowstone National Park. This polymerase was found to synthesize DNA at an optimal temperature of 75-80°C and can survive temperatures up to 97°C. Other thermostable polymerases eventually became prominent. Among these are **Pfu Polymerase** and **Pfx Polymerase** which found uses in DNA amplifications requiring higher fidelity or accuracy. **Tth polymerase** with its intrinsic reverse transcriptase activity became ideal for reverse transcription and RT-PCR.

Heat stable DNA polymerases like Taq polymerase enzymatically assemble new DNA strand from DNA building blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, *i.e*., alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary –

- first to physically separate two strands in a DNA double helix at a high temperature in a process called DNA melting.
- At a lower temperature, each strand is annealed to primer and
- then used as the template in DNA synthesis by the DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

Developed in 1983 by **Kary Mullis**, PCR is now a common and often indispensable technique used in medical and biological research laboratories for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogenetic studies or functional analysis of genes; diagnosis of hereditary diseases; identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded Nobel Prize in Chemistry for his work on PCR.

REQUIREMENTS

PCR is used to amplify a specific region of a DNA strand or the DNA target. A basic PCR set up requires several components and reagents. These components include:

- ***DNA template*** that contains the DNA region (target) to be amplified. Most PCR methods typically **amplify DNA fragments** of up to **~10 kb**, although some techniques allow for amplification of fragments **up to 40 kb** in size.

- **Two *primers*** (Forward primer and reverse primer) that are complementary to the 5′ (three prime) ends of each of the sense and anti-sense strand of the DNA target.
- ***Taq polymerase*** or any other DNA polymerase with a temperature optimum at ~ 70 °C.
- ***Deoxynucleoside triphosphates*** (dNTP) which are building blocks from which the DNA polymerases synthesizes a new DNA strand.
- ***Buffer solution***, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- ***Divalent cations***, generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis
- ***Monovalent cation*** (K^{+}).

PCR is commonly carried out in a reaction volume of 10–200 µl in small reaction tubes (0.2–0.5 ml) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Many modern thermal cyclers make use of the Peltier effect which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favourable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid required a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

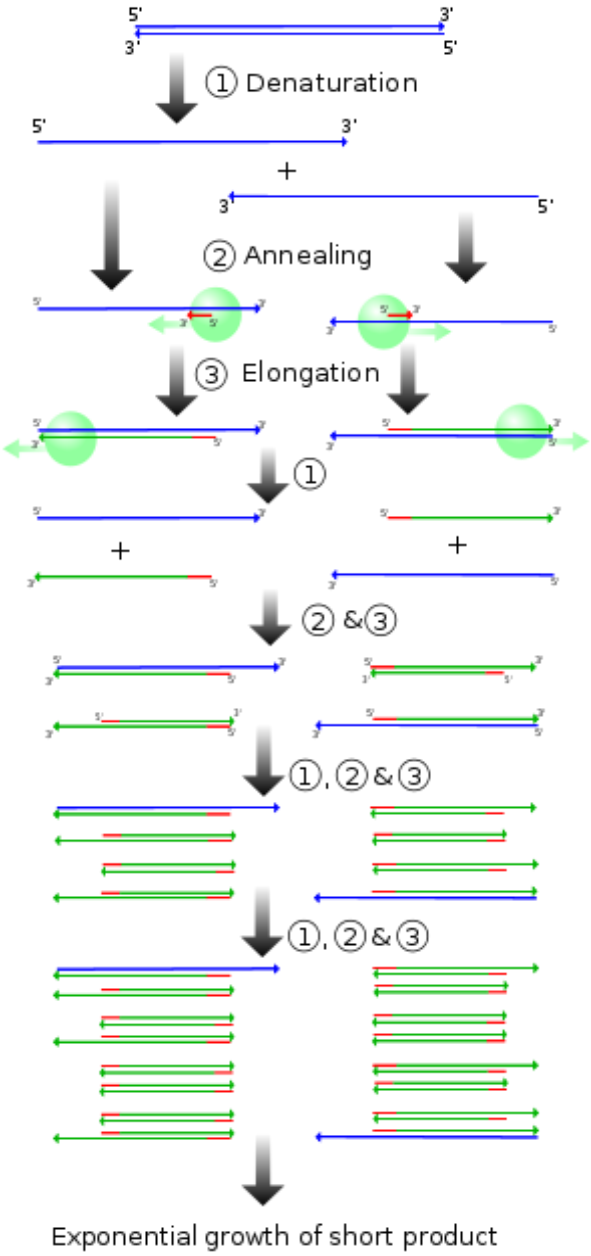

Figure 1: **Schematic drawing of the PCR cycle.** (1) Denaturing at 94–96 °C. (2) Annealing at ~65 °C (3) Elongation at 72 °C.
Four cycles are shown here. The blue lines represent the DNA template to which primers (red arrows) anneal that are extended by the DNA polymerase (light green circles), to give shorter DNA products (green lines), which themselves are used as templates as PCR progresses.

PROCEDURE

The PCR usually consists of a series of 20-40 repeated temperature changes called cycles. Most commonly PCR is carried out with cycles that have three temperature steps (Fig. 1). The process starts with a single temperature step (called ***hold***) at a high temperature (>90°C), and ends by one hold after the 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. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (T_m) of the primers.

Initialization step: This step consists of heating the reaction to a temperature of 94–96°C (or 98°C if extremely thermostable polymerases are used), which is held for 1–9 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 94–98°C for 20–30 seconds. It causes *melting of the DNA template* by disrupting the hydrogen bonds between complementary bases, yielding single strands of DNA.

Annealing step: The reaction temperature is lowered to 50–65°C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5°C below the T_m of the primers used. Stable 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 synthesis.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80°C, and commonly a temperature of 72°C is used with this enzyme. 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, joining the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand.

The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a general rule, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, *i.e.*, if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

Final elongation: This single step is occasionally performed at a temperature of 70–74°C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended in this step.

Final hold: This step at 4–15°C for an indefinite time may be employed for short-term storage of the reaction.

To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the **amplimer** or **amplicon**), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products.

PCR OPTIMIZATION

In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions. Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants. This usually involves spatial separation of PCR-setup areas from areas for analysis or purification of PCR products, use of disposable plasticware, and thoroughly cleaning the work surface between reaction setups. Primer-design techniques are important in improving PCR product yield and in avoiding the formation of spurious products, and the usage of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA.

APPLICATION OF PCR

1. Selective DNA isolation:

- PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR helped in developing many methods, such as generating hybridization probes for Southern or Northern hybridization and DNA cloning, which require larger amounts of DNA, representing a specific DNA region. PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material.

2. Use in DNA Sequencing:

- One important application of PCR is in DNA sequencing to determine unknown PCR-amplified sequences.

3 Amplification and quantification of DNA:

- PCR may also be used for DNA fingerprinting, a forensic technique used to identify a person or organism. Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence.
- Some PCR fingerprints methods have high discriminative power and can be used to identify genetic relationships between individuals, such as parent-child or between siblings, and are used in paternity testing.
- PCR followed by DNA fingerprinting may also be used to determine evolutionary relationships among organisms. PCR can be effectively used in the analysis of ancient DNA. These PCR-based techniques have been successfully used on animals, such as a 40,000-year-old mammoth, and also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian Tsar.

4. Quantification of DNA:

- Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample – a technique often applied to quantitatively determine levels of gene expression. Real-time PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

5. PCR in diagnosis of diseases:

- PCR allows early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity which is at least 10,000 fold higher than other methods.
- PCR also permits identification of non-cultivatable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.
- Viral DNA can likewise be detected by PCR. The primers used need to be specific to the targeted sequences in the DNA of a virus, and the PCR can be used for diagnostic analyses or DNA sequencing of the viral genome. The high sensitivity of PCR permits virus detection soon after infection and even before the onset of disease. Such early detection may give physicians a significant lead in treatment. The amount of virus (viral load) in a patient can also be quantified by PCR-based DNA quantitation techniques.

SOME VARIATIONS OF THE BASIC PCR TECHNIQUE

1. ***Hot-start PCR*:**
   A technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the melting temperature (*e.g.*, 95°C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that only dissociate after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.

2. ***Multiplex-PCR*:**
   Consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, *i.e.*, their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis.

3. ***Multiplex Ligation-dependent Probe Amplification* (*MLPA*):**
   Permits multiple targets to be amplified with only a single primer pair, thus avoiding the resolution limitations of multiplex PCR.

4. ***Reverse Transcription PCR (RT-PCR):***
   Here reverse transcriptase first forms cDNA from RNA, which is then amplified by PCR. 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 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.

5. ***Real Time PCR*:**
   Real-time PCR (RT-PCR) is also called **quantitative PCR** or **qPCR**. The key feature in RT-PCR is that amplification of DNA is detected in real time as PCR is in progress by the use of fluorescent reporter. The fluorescent reporter signal strength is directly proportional to the number of amplified DNA molecules. Real-time PCR uses an increase in the intensity of a fluorescent signal generated by an intercalating dye or from the breakdown of a dye-labeled probe during amplification of a target sequence to detect nucleic acids either for their presence or absence or for their amount. The PCR cycle number where fluorescent signal becomes discernable above background noise is called the C_T value.
   RT-PCR can be divided into four stages: linear ground phase, early exponential phase, linear exponential phase (log phase) and plateau phase. In the first phase, PCR is just starting, fluorescent signal has not risen above background. The second phase is where fluorescent signal just rise significantly above background, the cycle at which occurs is called *cycle threshold* (C_T). In linear exponential phase, PCR is in its optimal amplification stage with doubling PCR products in every cycle. The last phase is when substrates are exhausted and Taq DNA polymerase is in its end of life, fluorescent signal will no long increase.

VARIATIONS OF DNA POLYMERASE

DNA polymerases are enzymes responsible for assembling nucleotides to create new DNA molecules. Polymerase Chain Reaction (PCR), one of the most common and fundamental technology in genetic and molecular research, utilizes the ability of DNA polymerase to replicate DNA strands in order to amplify large amount of DNA from a small amount of nucleic acids.
PCR originally utilized the Klenow fragment, a proteolytic product of DNA polymerase I isolated from *E. coli*. While it has high fidelity, having retained both polymerase activity and 3′-5′ exonuclease activity, it is irreversibly denatured from the high temperatures used to separate the new strands of DNA. New enzyme must be manually added after every cycle – for the typical 30-40 cycles in PCR, this poses a contamination risk and is labour- and time- consuming. The Klenow fragment has since been replaced by thermostable enzymes from thermophilic bacteria species listed below. These enzymes do not require addition of fresh polymerase between cycles.

1. Taq DNA Polymerase

- Taq DNA polymerase is a thermostable DNA polymerase used in PCR, originally isolated from *Thermus aquaticus*, a thermophilic bacteria found in hot springs and hydrothermal vents.
- PCR takes advantage of Taq's ability to withstand the high temperatures required during the denaturation step for strand separation. With an optimum activity temperature of 75-80°C, Taq polymerase can be reused through several cycles of PCR without being denatured by the heat itself.
- Taq polymerase also displays high processivity replicating 1 kb of DNA within 30-60 seconds during PCR.
- Taq DNA polymerases also display 5'-3' exonuclease activity, allowing for the excision of nucleotides from the 5' end of the DNA strand in a nick translation reaction. This activity is important as it cleaves labelled oligonucleotide probes from the 5' end of the DNA to generate a detectable signal in real-time PCR application.
- One of the major drawbacks to Taq is its inability to proofread as it lacks 3'-5' exonuclease activity, therefore giving low replication fidelity (1 error in 9000 base pairs).
- For routine PCR with short amplicons, or applications in which incorporation of non-standard nucelotides such as deoxyuridine and inosine are necessary, Taq's high processivity and low fidelity is more advantageous. The lack of 3'-5' proofreading activity also results in a single adenine overhang at the 3' ends of both strands, producing DNA with sticky ends. A potential use for these products is in TA cloning, where the they can be directly ligated into a plasmid vector containing thymine 3' overhangs.

2. Pfu DNA Polymerase

- Pfu DNA polymerase is a thermostable enzyme originally isolated from *Pyrococcus furiosus*, a hyperthermophilic species of Archaea.
- Similarly to Taq, Pfu DNA polymerase can be reused throughout several PCR cycles as it operates optimally at 90°C and is not denatured by the heating steps.
- In addition, Pfu DNA polymerase displays 3'-5' exonuclease activity, and therefore has the ability to proofread by excising mis-incorporated nucleotides, giving it very high replication fidelity (1 error in 1.3 million base pairs). Pfu DNA polymerase's superior fidelity is due to its slower speed also (it requires up to 2 minutes to amplify 1 kb of DNA during a PCR cycle.
- Pfu DNA polymerase also produces DNA products with blunt ends, requiring the use of blunt-ended vectors for cloning applications.

3. KOD DNA Polymerase

- KOD DNA polymerase is a recombinant form of DNA polymerase derived from the thermophilic bacterium *Thermococcus kodakaraensis* KOD1 type strain.
- KOD DNA polymerase functions optimally at 85°C and displays 3'-5' exonuclease proofreading activity, producing blunt-ended DNA products.
- While KOD DNA polymerase displays high fidelity and processivity for small amplicons, long-distance amplification of amplicons over 5 kb tends to lower product yield due to its strong 3'-5' exonuclease activity.
- This can be avoided by mixing wildtype KOD polymerase with mutant forms with lower 3'-5' exonuclease activity, allowing for accurate amplification of amplicons up to 15 kb (23).

4. Bst DNA Polymerase

- *Bacillus stearothermophilus* is a thermophilic species of bacteria common to soil, hot spring, and ocean sediment environments.
- Bst DNA polymerase displays helicase-like activity to unwind DNA strands in addition to its polymerase activity.
- Bst polymerase functions optimally at 60-65°C, but denatures above 70°C, making it more suitable for loop-mediated isothermal amplification (LAMP) which does not undergo the high-temperature denaturation steps and thermocycling used in PCR.
- While Bst polymerase has 5'-3' exonuclease activity, it cannot proofread as it lacks 3'-5' exonuclease activity.

5. Tth polymerase

- Tth polymerase is derived from *Thermus thermophilus*, a thermophilic thermal vent bacterium.
- Tth DNA polymerase functions optimally at 75°C with high processivity, but lacks proofreading 3'-5' exonuclease activity.
- Tth DNA polymerase displays efficient intrinsic reverse transcriptase (RT) activity in the presence of Mn^{++} ions, allowing it to assemble cDNA from RNA. Because of this property, Tth polymerase can be used for RT-PCR, followed by subsequent amplification of the cDNA product in the presence of Mg^{++} ions.
- Tth polymerase produces sticky-ended DNA products.

6. Pwo DNA polymerase

- Pwo DNA polymerase is derived from the ultra-thermophilic archea *Pyrococcus woesei* found in deep marine environments.
- Pwo polymerase functions optimally at 100-103°C, and displays high proofreading 3'-5' exonuclease activity, giving it 18-fold higher fidelity than Taq polymerase.
- Pwo polymerase creates blunt-ended products.

7. Long-Range DNA polymerase

- While conventional PCR can be used on amplicons up to 3-4 kb, Taq DNA polymerase is best optimized for amplicons smaller than 2 kb. Taq DNA polymerase lacks 3'-5' exonuclease activity renders it unable to remove misincorporated bases, causing it to stall

and dissociate without completing the entire sequence. On larger amplicons, accumulation of enough mismatches can inhibit PCR, leading to truncated products.

- Long-range DNA polymerase is optimized for DNA segments of up to 20 kb. These polymerases combine a thermostable DNA polymerase, usually Taq polymerase, for its high processivity, with a proofreading enzyme containing 3'-5' exonuclease activity to increase fidelity. The proofreading polymerase is often derived from a recombinant source and works to remove Taq polymerase's 3′ mismatches during primer extension.

8. Hot-start DNA Polymerase

- Hot-start DNA polymerases are used to increase product yield by reducing nonspecific amplification during PCR setup. Since most DNA polymerases can be active even at room temperature, the combination of reaction components during PCR setup can lead to nonspecific primers annealing to each other or to the template. These nonspecifically annealed primers compete for Taq polymerase binding and extension to create undesirable PCR products.
- In hot-start PCR, the DNA polymerase, usually Taq or Pfu DNA polymerase, is chemically modified or antibody bound to remain inactive during the lower annealing temperature. When heated in the initial denaturation step, the chemical or antibody inhibitors become inactive or dissociate from the DNA polymerase, therefore making the DNA polymerase become active again and is free to start incorporating nucleotides.
- Hot-start PCR is advantageous for amplifying low amounts of DNA template, highly complex DNA templates or in multiplex PCR when multiple pairs of primers are used, as it can significantly improve the specificity and yield of the product. However, as they lack proofreading activity, the fidelity of hot-start polymerases is limited and these are not suitable for applications such as subsequent cloning or mutagenesis.

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