Definition:

The Polymerase Chain Reaction (PCR) : sometimes called (**molecular photocopying**) is an enzymatic method of synthesizing (amplifying) large quantities of a targeted region of DNA in vitro (extracellularly = in a test tube).

Polymerase Chain Reaction (PCR) was developed in 1983 by Kary Mullis (shared Nobel Prize in Chemistry for the work in 1993).

Note: PCR machine: Also called thermal cycler.

The Nobel Prize in Chemistry 1993

Nobel Prize Award Ceremony

Kary B. Mullis

Michael Smith

Kary B. Mullis Michael Smith

The Nobel Prize in Chemistry 1993 was awarded *"for contributions to the developments of methods within DNA-based chemistry"* jointly with one half to Kary B. Mullis *"for his invention of the polymerase chain reaction (PCR) method"* and with one half to Michael Smith *"for his fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies"*.

Source: http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1993/

Applications of PCR technique

1-This technique used to study the molecular pathogenesis and diagnosis of a variety of acquired, inherited, viral and bacterial diseases. PCR In diagnosis of disease; It permits early diagnosis of malignant diseases or can establish whether the person is at risk or not (by investigated the presence of a certain gene that is associated with a certain type of cancer).

2- PCR can be used for forensic analysis, when only a trace amount of DNA is available as evidence.

3-PCR-based techniques have been successfully used to analyse ancient DNA (tens of thousands of years old), such as a forty-thousand-year-old mammoth.

4- Also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of family tree of a Russian tsar.

Principles of PCR

PCR is based on ability of DNA polymerase to synthesize complementary strand to the template strand. As DNA polymerase can add a nucleotide only onto a 3'-OH group, it needs an artificial DNA strand (called DNA primer) of about 18 to 25 nucleotides complementary to 3' end of the DNA template. Each polynucleotide has a free 3' –OH group and 5' phosphate group. Moreover, a DNA strand has complimentary sequence, already paired by hydrogen bonding. Thus, primer can bind only when DNA strands are separated. This is generally done by heating. The primers anneal to the single-stranded DNA template at specific temperature (depends on primer sequence) and then DNA-Polymerase binds to this double stranded DNA produced. The again reaction mixture is heated to 72°C (extension); a temperature optimum for DNA- polymerase functions. This starts synthesis of the new DNA strand. Then reaction mixture is cooled to lower temperature for short term storage, if required. This completes one cycle. After first cycle, one DNA molecule has become two. After multiple cycle of the PCR reaction, the specific sequence will be accumulated in billions of copies.

The PCR Reaction Components

The PCR reaction requires the following components:

1- Water :
Water is present to provide the liquid environment for the reaction to take place. It is the matrix in which the other components interact. For most people and in most labs sterile, deionized water is the choice.

2- PCR Reaction Buffer:
This reagent is supplied with commercial polymerase and most often as a 10x concentrate. The primary purpose of this component is to provide an optimal pH and monovalent salt environment (potassium ions) for the final reaction volume.

3- Magnesium chloride :
Many commercially supplied PCR buffers already contain magnesium chloride (MgCl2). MgCl2 supplies the Mg++ divalent cations required as a cofactor for Type II enzymes, which include restriction endonucleases and the polymerases used in PCR. The standard final concentration of this reagent for polymerases used in PCR is 1.5mM. Sometimes it is necessary to change this concentration in order to optimize the PCR reaction. For this reason we choose to obtain PCR buffer without MgCl2 and to add it ourselves. 3.0ul of the standard 25mM MgCl2 provided commercially will yield a 1.5mM final concentration in a 50ul reaction volume.

4- Deoxynucleotide triphosphates (dNTPs):
The purpose of the deoxynucleotide triphosphates (dNTPs) is to supply the bricks= the building blocks. The PCR reaction requires energy. The only source of that energy is the β and γ phosphates of the individual dNTPs. It is best to obtain them commercially as a 10 mM dNTP mix and to immediately aliquot them into smaller working.

5- DNA template (target DNA) :
The quality and quantity of the target DNA is important. The DNA used as the PCR target should be as pure as possible but also it should be uncontaminated by any other DNA source. The PCR reaction does not discriminate between targets.

6- primers (Forward Primer & Reverse Primer) :

The primers (also called oligonucleotides—meaning short nucleotides) are short fragments of single stranded DNA (15-30 nucleotides in length) that are complementary to DNA sequences that flank the target region of interest. The purpose of PCR primers is to provide a “free” 3’-OH group to which the DNA polymerase can add dNTPs.

The primers easily synthesized in the laboratory and can be designed to be complementary to any known DNA sequence. They can range in size from 10 to 100 nucleotides in length, but typically they range from 15 to 30 bases.

The C and G nucleotides should be distributed uniformly throughout of the primer and comprise approximately 40-60% of the bases. More than three G or C nucleotides at the 3'-end of the primer should be avoided, as nonspecific priming may occur.

The primer should not be self-complementary or complementary to any other primer in the reaction mixture, in order to avoid primer-dimer and hairpin formation. All possible sites of complementarity between primers and the template DNA should be noted.

If primers are degenerate, at least 3 conservative nucleotides must be located at the primer's 3'-end.

The melting temperature of flanking primers should not differ by more than 5°C. Therefore, the GC content and length must be chosen accordingly.

If the primer is shorter than 25 nucleotides, the approx. melting temperature (Tm) is calculated using the following formula:

$$Tm = 4(G+C) + 2(A+T)$$

Where:G, C, A, and T, are the number of respective nucleotides in the primer.

If the primer is longer than 25 nucleotides, the melting temperature should be calculated using specialized computer programs where the interactions of adjacent bases, the influence of salt concentration, etc. are evaluated.

The PCR annealing temperature (TA) should be approximately 5°C lower than the primer melting temperature.

7- DNA polymerases (Taq DNA polymerase):

DNA polymerase sequentially adds nucleotides complimentary to template strand at 3'-OH of the bound primers and synthesizes new strands of DNA complementary to the target sequence. The most commonly used DNA polymerase is *Taq* DNA polymerase (from *Thermus aquaticus,* a thermophillic bacterium) because of high temperature stability (Figure 1). *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is also used widely because of its higher fidelity (accuracy of adding complimentary nucleotide).

Figure 1. Photograph of a thermal pool in Yellowstone National Park. The temperature of the water in the pool is at the boiling point for that altitude. As the water in the outflow on the right cools, an orange coloration begins to appear in the water (arrow) and continues downstream for several feet. At the mid-point of that coloration, the average temperature is 80oC. The organism responsible for the color is *Thermus aquaticus*. (Photo by Ric Devor).

Note:

In the very earliest days of the polymerase chain reaction amplifications were carried out using water baths and lab timers and the best available DNA polymerases of the time, T4 DNA polymerase. During the essential DNA denaturation step, 94oC or 95oC for up to a minute, the DNA target was rendered single stranded. It also destroyed the polymerase each time so that fresh enzyme had to be added just after each denaturation step. Since the average duration of a PCR cycle is about five minutes, this became a very labor-intensive bottleneck. The answer to this problem was, as are all good solutions, blindingly simple. There exists in nature organisms that are perfectly happy at very high temperatures. Such organisms, called *thermophiles* or "heat loving", The first of these thermophilic organisms to be exploited was the bacterium *Thermus aquaticus* which resides in the outflows of thermal pools in Yellowstone National Park .

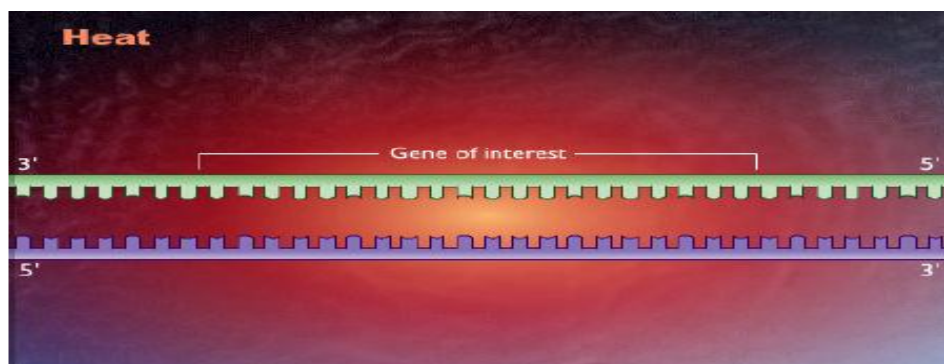
The DNA polymerase from *Thermus aquaticus* is stable at 95oC and allowed for automation of the PCR process. The nomenclature rule for enzymes derived from microorganisms is to use the first letter of the genus and the first two letters of the species. Thus, the DNA polymerase from ***T**hermus **aq**uaticus* is called **Taq**.

Since it was first isolated, Taq DNA polymerase has become the standard reagent for the PCR reaction. The gene has been cloned and used to produce the enzyme in non-thermophilic host bacteria so both native Taq, isolated from *Thermus aquaticus*, and cloned Taq, isolated from expression systems in other bacteria, are commercially available. In addition, a number of other thermal-stable DNA polymerases, isolated from other thermophilic species, have become available. Among these are enzymes from *Pyrococcus furiosus* (Pfu polymerase), *Thermus thermophilus* (Tth polymerase), *Thermus flavus* (Tfl polymerase), *Thermococcus litoralis* (Tli polymerase aka Vent polymerase), and *Pyrococcus species* GB-D (Deep Vent polymerase). Each of these, and other, polymerases has a specific set of attributes that can be selected depending upon the application.

PCR Steps:

PCR is a three-step process which is repeated in several cycles. Each synthesis cycle is composed of three steps: **Denaturation , Primer Annealing & Extension**

1. Denaturation step: This step consists of heating the reaction to 90–95 °C. It causes DNA separation by disrupting the hydrogen bonds between complementary bases, yielding single strands of DNA. The high temperature of the denaturing step has the added advantage of denaturing proteins (inactivating them) and disrupting cells so that you don’t have to always start with purified DNA as your amplification template. You can often amplify DNA directly from cell lysates or even whole cells.



2. Annealing step: During the second step of each cycle, the temperature is lowered to an annealing temperature, allowing binding (annealing) of the primers to their complementary targets on the DNA template (one for each DNA strand). These are designed to flank the desired target region of your DNA template and serve as the starting points for DNA synthesis by the Taq polymerase.

Each pair of primers will have a particular annealing temperature determined by the length of the primers and their G+C content. Using the proper annealing temperature for your primer set is essential for efficient and accurate amplification.

3. Extension/Elongation step: At this step, the Taq 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.
This process is repeated as many as 30 or 40 times, leading to more than one billion exact copies of the original DNA segment. The entire cycling process of PCR is automated and can be completed in just a few hours using a machine called a thermal cycler.