Polymerase Chain Reaction (PCR)

What is PCR?

Is an ingenious technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence in vitro. This PCR process, invented by Kary Mullis in 1984 in California. The purpose of a PCR is to make a **huge number of copies of a gene**. The invention of the PCR (Polymerase Chain Reaction) technique has resulted in a revolution for evolutionary biologists interested in genetic questions. Suddenly there was a **fast, robust** and relatively **inexpensive** technique to get hold of genetic information from small samples of e.g. skin, blood or faeces. An advantage with PCR based molecular studies of DNA is that, once DNA is extracted and purified, the techniques are very similar regardless of the taxonomy of the study organisms.

The technique was made possible by the discovery of Taq polymerase, the DNA polymerase that is used by the bacterium **(*Thermus aquaticus*)** that was discovered from Yellow Stone National Park hot springs. This DNA polymerase is stable at the high temperatures need to perform the amplification, whereas other DNA polymerases become denatured.

The method relies on **thermal cycling instrument(thermal cycler)**, which provide program consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA.

Applications of PCR:

1- Medicine: The PCR technique enables early diagnosis of malignant diseases.
2- Classification of organisms .
3- Mutation detection .
4- Detection of pathogens .
5- Gene therapy.
6- Finger print .
7- Forensic science: PCR is very important for the identification of criminal

8-Evolutionary studies: It plays an important role in phylogenetic analysis.

Requirements of PCR Technology

1- **The thermal cycler** heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction.
2- **DNA template** that contains the DNA region (target) to be amplified, the source of DNA for the PCR amplification. We use a standard concentration at 25ng/µl. DNA purity is very important, because template contaminants (i.e. excesses of phenolic compounds, EDTA) may leads to PCR inhibition and give false-negative results.
3- **Pair of primers:** short artificial DNA fragments containing sequences complementary to the target region, that are complementary to the 3' (three prime) ends of each of the sense(forward) and the 5' end of the anti-sense (reveres) strand of the DNA target, usually the length of 18-30 nt.
4- **Master Mix** Which Contains:

- **Taq DNA polymerase** the enzyme that puts the free nucleotides together. It starts at the 3'end of the primer, and uses the complementary DNA strain as a template.
- **Deoxynucleoside triphosphates** (dNTPs, sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), Free nucleotides (G, A, T,C) of which the artificial DNA copies are made, the building-blocks from which the DNA polymerase synthesizes a new DNA strand.
- **Buffer solution**, maintains pH and ionic strength of the reaction solution suitable for the activity of the enzyme.
- **Mg^{++} ions -** cofactor of the enzyme.

5- free nuclease water: must be present for the reaction to work**.**

Procedure:

Typically, PCR consists of a series of 25-40 repeated temperature changes, called cycles, each cycle of PCR includes steps for template denaturation, primer annealing and primer extension:

- **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 30sec.-1min. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.
- **Annealing step**: The reaction temperature is lowered to 50–65 °C for 30 sec-1min allowing annealing of the primers to the single-stranded DNA template. Typically, the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.
- **Extension/elongation step**: 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, condensing 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 rule-of-thumb, 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.

PCR Steps :

- Initialization step : 94℃ 10min
- 35 Cycles :

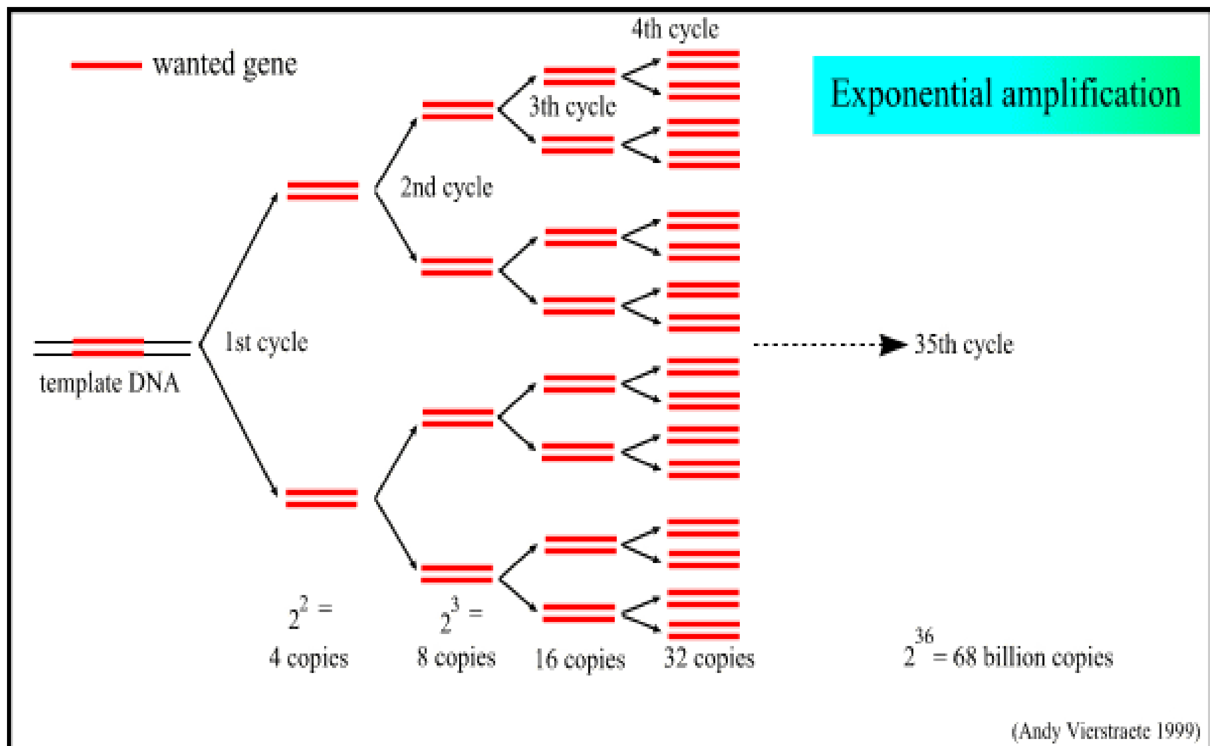
Denaturation step 94℃ 30s
Annealing step 55℃ 30s
Extension step 72℃ 30s

} **cycle**

- Final elongation : 72℃ 10min

IMPORTANT REMINDERS:

- ALWAYS keep PCR reagents (master mix, primers, DNA template on ICE.
- Gloves are good to use to keep your tubes from getting contaminated.
- Keep your tubes closed and make sure you do not cross contaminate with the tips of the micropipettes.
- **VERY IMPORTANT** when using the micropipettes be careful and avoid contaminating the micropipettes by slowly releasing the plunger.
- PCR product(amplicon) is separated in agarose and the result is examined via gel electrophoresis



Why "Polymerase"? It is called "polymerase" because the only enzyme used in this reaction is DNA polymerase.

Calculating annealing temperature (Tm) of a primer:

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

where G,C,A,T are the number of respective nucleotides. This formula can be used for primers that are no longer than 25 nt long

Preparation of primers

Primers were provided in lyophilized form, dissolved in de ionized distilled water as recommended by provider to give a final concentration of 100 pmol/µl, this is stock solution. Working solution was prepared by diluting the stock to 1/10 to obtain 10 pmol/µl, and stored in a deep freezer until used in PCR amplification.