Lab Nine:.

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, allowed scientists to make millions of copies of a scarce sample of DNA. The technique has revolutionized many aspects of current research, including the diagnosis of genetic defects and the detection of the AIDS virus in human cells. The purpose of a PCR is to make a **huge number of copies of a gene**. As a result, it now becomes possible to analyze and characterize DNA fragments found in minute quantities in places like a drop of blood at a crime scene. PCR also affected evolutionary studies because large quantities of DNA can be manufactured from fossils containing but trace amounts. PCR has been automated for routine use in laboratories worldwide, the development of this technique resulted in an explosion of new techniques in molecular biology (and a Nobel Prize for Kary Mullins in 1993) as more and more applications of the method were published. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. 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. Since this technique involves amplification of DNA, the most obvious application of the method is in the detection of minuscule amounts of

specific DNAs. This important in the detection of low level bacterial infections or rapid changes in transcription at the single cell level, as well as the detection of a specific individual's DNA in forensic science . It can also be used in DNA sequencing, screening for genetic disorders, site specific mutation of DNA. The most important consideration in PCR is contamination. If the sample that is being tested has even the smallest contamination with DNA from the target, the reaction could amplify this DNA and report a falsely positive identification. For example, if a technician in a crime lab set up a test reaction (with blood from the crime scene) after setting up a positive control reaction (with blood from the suspect) cross contamination between the samples could result in an erroneous incrimination, even if the technician changed pipette tips between samples. A few blood cells could volatilize in the pipette, stick to the plastic of the pipette, and then get ejected into the test sample. The powerful amplification of PCR may be able to detect this cross contamination of samples. Modern labs take account of this fact and devote tremendous effort to avoiding this problem.

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA.

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. This could be DNA extracted from blood, skin, feathers, or old PCR products. We use a standard concentration at 25ng/µl.
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 thermo stable 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 30 sec- 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 :

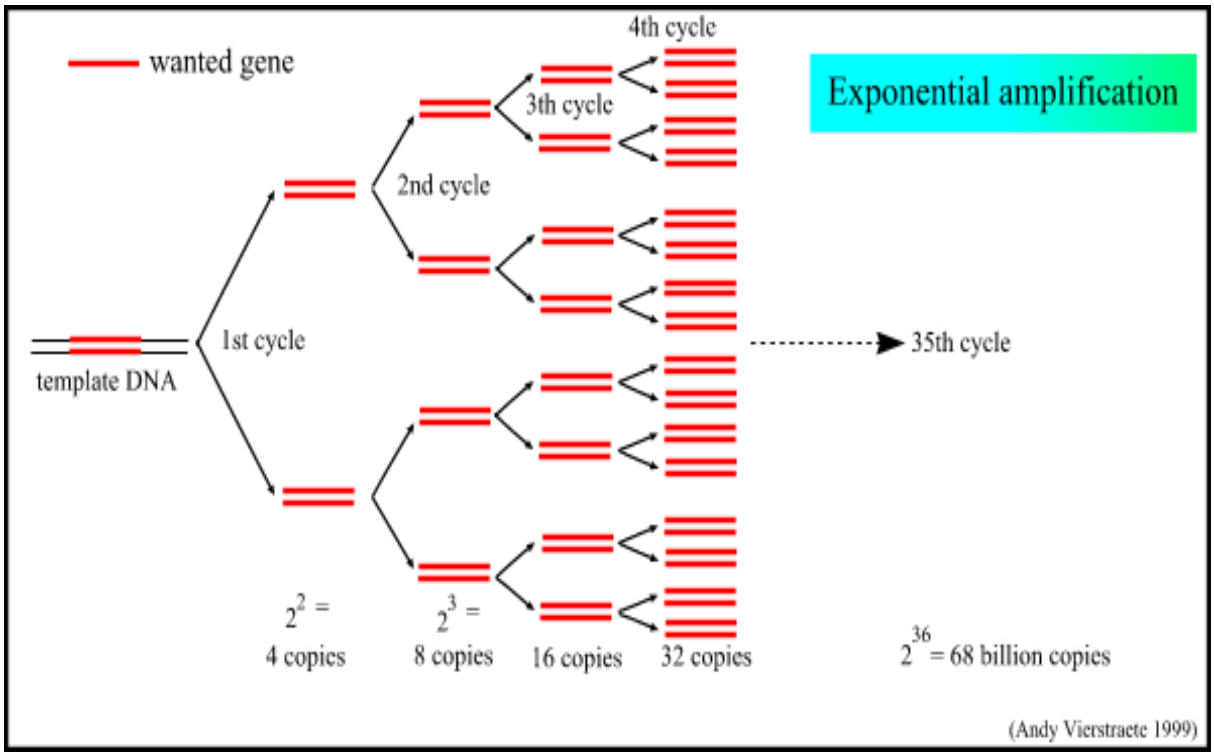
Denaturation step	94℃ 30s	1 cycle
Annealing step	55℃ 30s	
Extension step	72℃ 30s	

- Final elongation : 72℃ 10min

IMPORTANT REMINDERS:

- ALWAYS keep PCR reagents (master mix, primers, DNA template on ICE.
- Remember the centrifuge tubes are sterile so as soon as you are ready to start making the cell suspensions get your tubes and close them so that nothing contaminates your centrifuge tubes.
- Gloves are good to use to keep your tubes from getting contaminated.
- While making the cell suspension you should **NOT** flame the centrifuge tubes they might melt.

- Keep your tubes closed and make sure you do not cross contaminate with the tips of the micropipettes.
- Micropipettes come in different sizes and so do the pipette tips make sure you are using the correct pipette and tip for the amount you need to pick up.
- **VERY IMPORTANT** when using the micropipettes be careful and avoid contaminating the micropipettes by slowly releasing the plunger.
- Dirty pipette tips should all be autoclaved so they are to be discarded in the tin cans with red bags.
- Please balance the micro centrifuge machine by placing another tube across from your tube both tubes should have approximately the same amount of liquid, remember you are balancing.



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- It is also used in diagnosis of retroviral infection, bacterial infections, cancers, sex determination of embryos.
9- Evolutionary studies: It plays an important role in phylogenetic analysis.

How do we see our results after running a PCR?

The most common way of seeing the results of a PCR is by running a gel electrophoresis. The PCR product is pipetted into a special agar that will separate the DNA fragments according to their weight by using electricity

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. Nowadays, some specialized computer programs can do all calculations and determinations for proposed primers, e.g. Gene runner.