Lab Seven :.

Polymerase Chain Reaction (PCR)

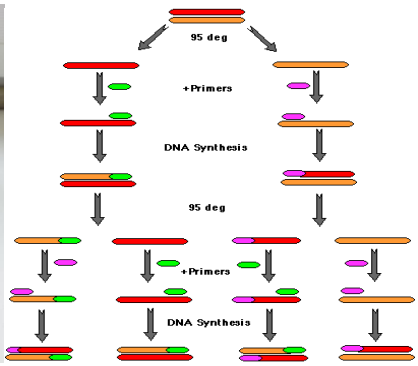
What is PCR?

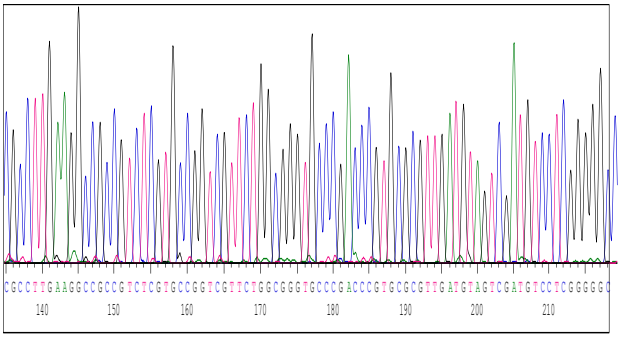
Is an ingenious technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence in vitro. This PCR process, invented by Kary Mullis in 1984 in California, allowed scientists 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. 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.

The rRNA is the most conserved (least variable) gene in all cells. Portions of the rDNA sequence from distantly-related organisms are remarkably similiar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been been used extensively to determine **taxonomy**, **phylogeny** (evolutionary relationships), and to estimate **rates of species divergence** among bacteria. Thus the comparison of 16s rDNA sequence can show evolutionary relatedness among microorganisms. This work was pioneered by **Carl Woese**, who proposed the **three Domain system** of classification - Archaea, Bacteria, and Eucarya - based on such sequence information.

In Bacteria, Archaea, Mitochondria, and Chloroplasts the small ribosomal subunit contains the 16S rRNA (where the S in 16S represents Svedberg units). The large ribosomal subunit contains two rRNA species (the 5S and 23S rRNAs). Bacterial 16S, 23S, and 5S rRNA genes are typically organized as a co-transcribed **operon**. There may be one or more copies of the operon dispersed in the genome (for example, *E coli* has seven). The Archaea contains either a single rDNA operon or multiple copies of the operon. To infer relationships that span the diversity of known life, it is necessary to look at genes conserved through the billions of years of evolutionary divergence. An example of genes in this category are those that define the ribosomal RNAs (rRNAs). Most prokaryotes have three rRNAs, called the 5S, 16S and 23S rRNA.

The Revolution: Polymerase Chain Reaction (PCR)





Why use Ribosomal RNA Genes?

1- All perform the same function--protein synthesis
2- High homology--good for probing or PCR.
3- Good for telling us big picture lineages.
4- Many new rapid molecular biological methods to detect.

Why we use Molecular detection?

1- Fastidious bacteria.

2- Hard to culture bacteria.
3- Slow growing agents.
4- Intracellular bacteria.

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods, while Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. 16S rRNA gene sequence analysis can better identify poorly described, rarely isolated, or phenotypically aberrant strains, can be routinely used for identification of mycobacteria, and can lead to the recognition of novel pathogens and noncultured bacteria. Problems remain in that the sequences in some databases are not accurate, there is no consensus quantitative definition of genus or species based on 16S rRNA gene sequence data

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.
- **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.
- 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.

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.