PCR and its Types

What is PCR?

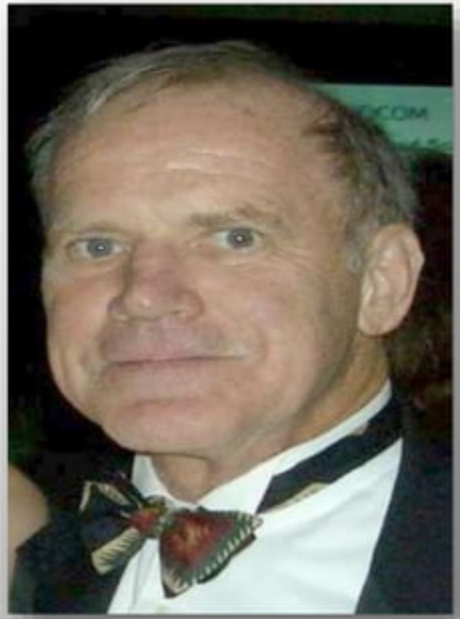
- Polymerase chain reaction is a technique that results in exponential amplification of a desired region of a DNA molecule *in vitro*.

- With this technique, small amounts the genetic material can be amplified (i.e., to make a huge number of copies of a DNA) to be able to identify and manipulate DNA.
- Detect infectious organisms, detect genetic variations including mutation in human genes and numerous other tasks.

History

- The of PCR technique was invented by **Kary Mullis**, a Research Scientist at a California Biotech Company, Cetus, in *1983*.

- For this work, Mullis received the *1993* Noble Prize in Chemistry.



PCR

- **Why "Polymerase"?**

Because the only enzyme used in the reaction is DNA polymerase.

- **Why "Chain" ?**

Because the products of the first reaction become the substrates of the following one and so on.

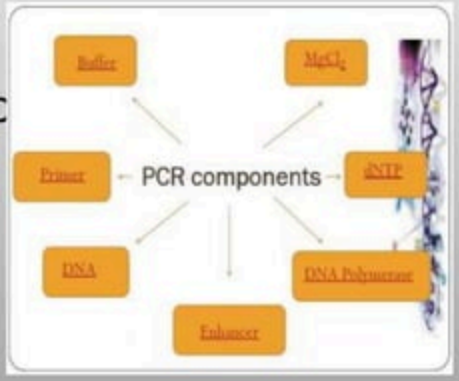
Setting up PCR Reaction

- Steps in PCR reaction :

1. Denaturation
2. Annealing
3. Extension

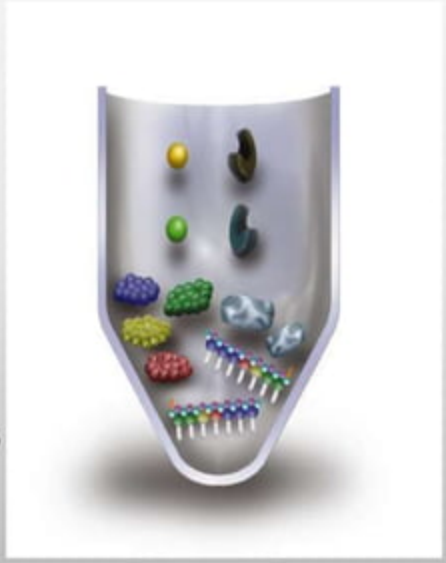
- Constituents of PCR reaction :

1. Target DNA
2. Pair of primers
3. dNTPs
4. Thermostable DNA Polymerase
5. Mg++ ions
6. Buffer solutic



PCR Requirements for 25 micro litres

- DNAse free water- 16.4 uL
- Magnesium chloride: 50mM-
- Buffer: pH 8.3-8.8 – 2uL
- dNTPs: 10mM – 0.4uL
- Primers: 1.5uL
- DNA Polymerase: 1-2.5 units
- Target DNA: ≤ 1 µg



The "Reaction" Components

1) Target DNA - contains the sequence to be amplified.

2) Pair of Primers - oligonucleotides that define the sequence to be amplified.

4) Thermostable DNA Polymerase - enzyme that catalyzes the reaction

5) Mg^{++} ions - cofactor of the enzyme

6) Buffer solution – maintains pH and ionic strength of the reaction solution suitable for the activity of the enzyme

PCR Machine

How does PCR work?

- Heat (94°C) to denature DNA strands
- Cool (54°C) to anneal primers to template
- Warm (72°C) to activate *Taq* Polymerase, which extends primers and replicates DNA
- Repeat multiple cycles

Factors for Optimal PCR:

❑ PCR Primers

-correctly designed pair of primers is required

-primer dimer, hairpin formation should be prevented

❑ DNA Polymerase

-Thermus aquaticus-170° F

-Taq polymerase is heat resistant

-It lacks proof reading exonuclease activity

-Other polymerases can be used .eg:

Tma DNA Polymerase from *Thermotoga maritama*,

Pfu DNA Polymerase from *Pyrococcus furiosus*.

❑ Annealing Temperature

- Very important since the success and specificity of PCR depend on it because DNA-DNA hybridization is a temperature dependent process.
- If annealing temperature is too high, pairing between primer and template DNA will not take place then PCR will fail.
- Ideal Annealing temperature must be low enough to enable hybridization between primer and template but high enough to prevent amplification of non target sites.
- Should be usually 1-2° C or 5° C lower than melting temperature of the template-primer duplex.

❑ Melting Temperature

- Temperature at which 2 strands of the duplex dissociate.

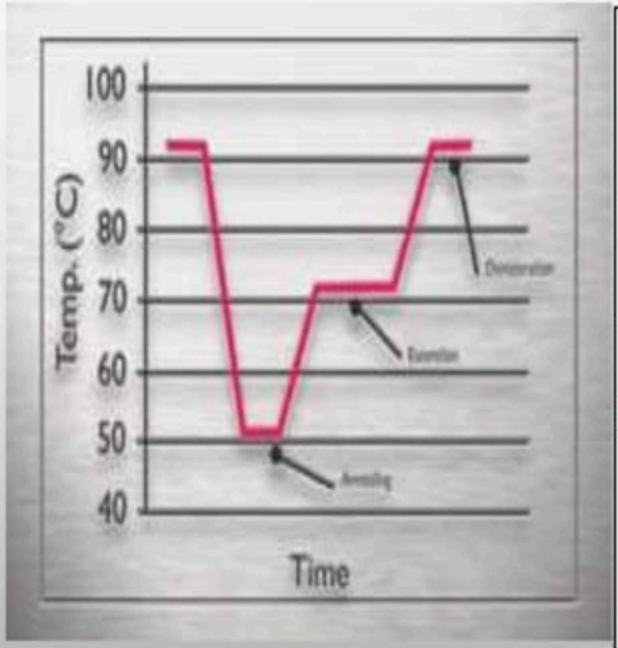
It can be determined experimentally or calculated from formula

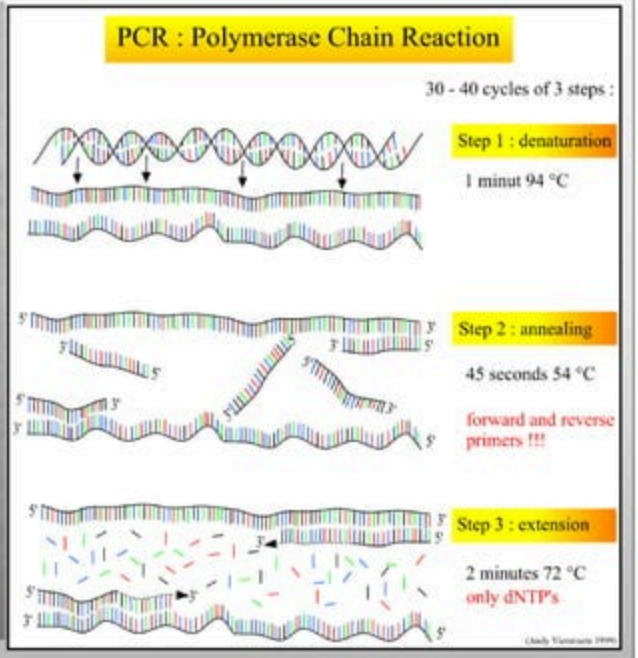
$$Tm = (4(G+C)) + (2(A+T))$$

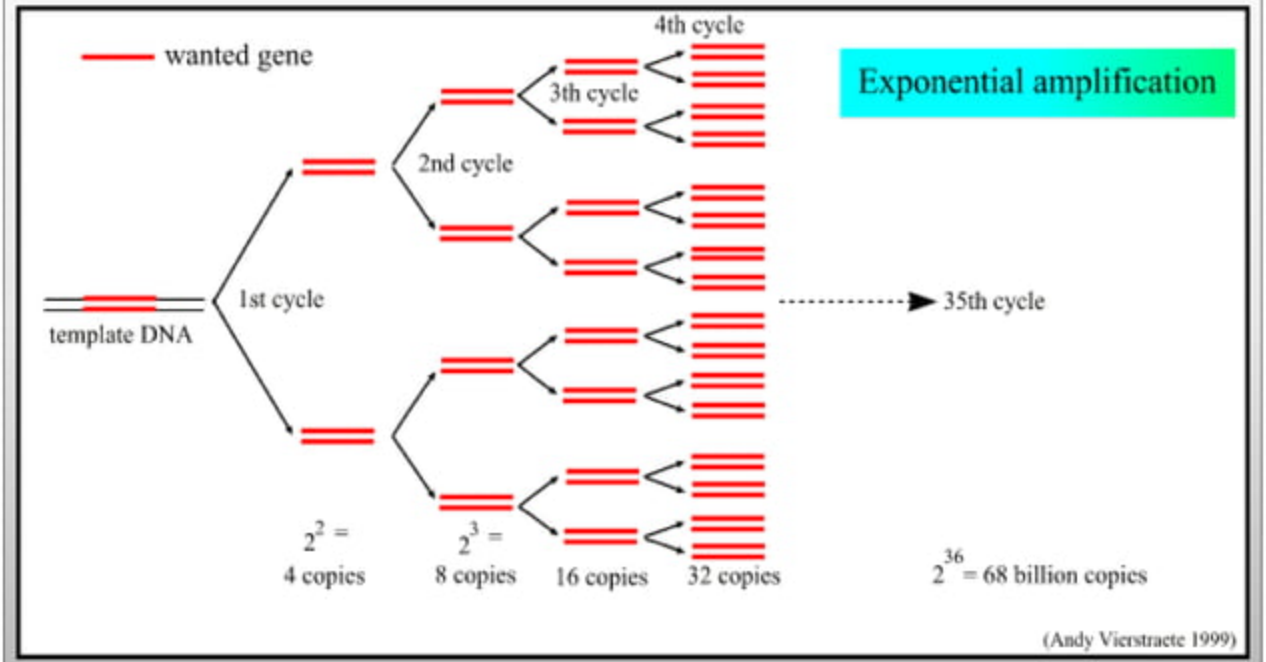
❑G/C content

- ideally a primer should have a near random mix of nucleotides, a 50% GC content
- there should be no PolyG or PolyC stretches that can promote non-specific annealing

Steps in PCR


100
90
80
70
60
50
40
Temp. (°C)
Time


PCR : Polymerase Chain Reaction
30 - 40 cycles of 3 steps :
Step 1 : denaturation
1 minut 94 °C
Step 2 : annealing
45 seconds 54 °C
forward and reverse primers !!!
Step 3 : extension
2 minutes 72 °C
only dNTP's


wanted gene
4th cycle
3th cycle
2nd cycle
1st cycle
template DNA
35th cycle
Exponential amplification
2^2 = 4 copies
2^3 = 8 copies
16 copies
32 copies
2^{36} = 68 billion copies
(Andy Vierstraete 1999)

Instrumentation

Advantages of PCR

- Small amount of DNA is required per test
- Result obtained more quickly - usually within 1 day for PCR
- Usually not necessary to use radioactive material (32P) for PCR.
- PCR is much more precise in determining the sizes of alleles - essential for some disorders.
- PCR can be used to detect point mutations.

Advantages of PCR over other DNA techniques:

1) Sensitive: Exponential amplification from trace amounts of DNA (pg – ng)
2) Fast: Can be done in less than one day
3) Safe: Non-radioactive method without toxic organic solvents

Disadvantages / Problems with PCR:

1) Contamination can be a serious problem, due to extreme sensitivity
2) Can sometimes be quite "fickle" and inconsistent

	Parameter	PCR	Gene cloning
1.	Final result	Selective amplification of specific sequence	Selective amplification of specific sequence
2.	Manipulation	In vitro	In vitro and in vivo
3.	Selectivity of the specific segment from complex DNA	First step	Last step
4.	Quantity of starting material	Nanogram (ng)	Microgram (m)
5.	Biological reagents required	DNA polymerase (Taq polymerase)	Restriction enzymes, Ligase, vector. bacteria
6.	Automation	Yes	No
7.	Labour intensive	No	Yes
8.	Error probability	Less	More
9.	Applications	More	Less
10.	Cost	Less	More
11.	User's skill	Not required	Required
12.	Time for a typical experiment	Four hours	Two to four days

Limitations of PCR

- Need for target DNA sequence information
  Primer Designing for unexplored ones.
  Boundary regions of DNA to be amplified must be known.
- Infidelity of DNA replication.
  Taq Pol – no Proof reading mechanism – Error 40% after 20 cycles
- Short size and limiting amounts of PCR product
  Up to 5kb can be easily amplified .
  Up to 40kb can be amplified with some modifications.
  Cannot amplify gene >100kb
  Cannot be used in genome sequencing projects.

Applications of PCR

Molecular Identification

- DNA fingerprinting
- Classification of organisms
- Genotyping
- Pre-natal diagnosis
- Mutation screening
- Drug discovery
- Genetic matching
- Detection of pathogens

Sequencing

- Bioinformatics
- Genomic cloning
- Human Genome Project

Genetic Engineering

- Site-directed mutagenesis
- Gene expression studies

Types of PCR

1. Inverse PCR
2. Multiplex PCR
3. Hot start PCR
4. Nested PCR
5. In situ PCR
6. Long PCR
7. Colony PCR
8. Real time PCR
9. Touch down PCR
10. Band stab PCR
11. Reverse transcriptase PCR
12. Degenerate PCR
13. Anchored PCR
14. Asymmetric PCR
15. Assembly PCR
16. Quantitative PCR
17. Methylation specific PCR
18. Ligation mediated PCR
19. Allele specific PCR
20. Digital PCR
21. Overlap Extension PCR
22. Solid phase PCR
23. Miniprimer PCR
24. Universal fast walking PCR
25. VNTR PCR
26. ISSR PCR
27. Semi quantitative PCR
28. Differential display reverse transcriptase PCR

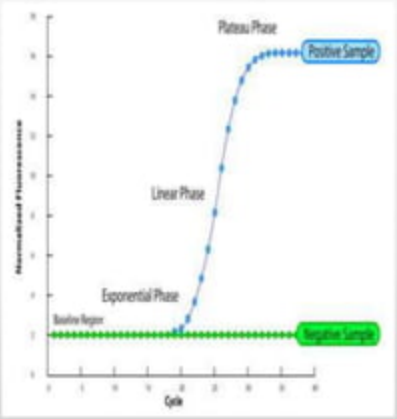
Real-Time PCR

- Real time PCR is adaptation of the PCR method to quantify the number of copies during PCR
- Quantification of gene expression, Diagnostic uses, Clinical quantification and genotyping

Steps

- Add DNA Sample
- Add desired Primer and Probe
- Probe is short sequence complementary to DNA Like Primer
- One Side of Probe is Fluorescent Molecule while on Other end Quencher is Present

- Run PCR
- Fluorescent Molecule emitting Fluorescent light with each copy Completing
- Probe is between Two Primers
- And this Fluorescent intensity detected by the Fluorescent detector in the PCR
- Graph developed to determine the copies at any point of PCR



Asymmetric PCR

- Direct sequencing and hybridization probing
- Amplifies just one strand of the target DNA
- First produce Double stranded DNA
- Then to produce single stranded DNA
- Unequal primer concentrations
- Amplification become slow after the one Primer used so Increase cycles

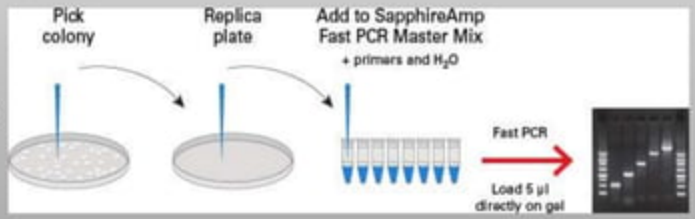
Steps

- DNA sample
- Add two primers of different concentration
- Run PCR
- Result on Gel Electrophoresis

Colony PCR

- Colony PCR for determining the presence or absence of insert DNA in plasmid of Bacteria
- Size of the DNA sequence
- Biotechnology Products
- No need for Extraction and culturing of DNA or plasmid purification steps

- **Steps**
- Small quantities of bacterial cells from bacterial colonies are directly added
- Add desired Primers for amplification
- Run PCR
- Results on Gel Electrophoresis

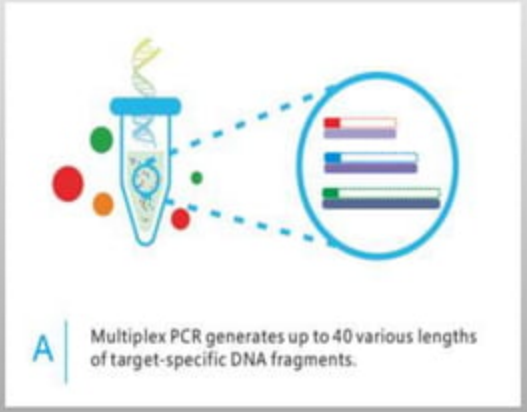


Assembly PCR

- It also known as Polymerase Cycling Assembly or PCA
- It is a method for the assembly of large DNA oligonucleotides from shorter fragments.
- It uses the same technology as PCR, but takes advantage of DNA hybridization and annealing
- as well as DNA polymerase to amplify a complete sequence of DNA in a precise order based on the single stranded oligonucleotides
- allows for the production of synthetic genes and even entire synthetic genomes

Multiplex polymerase chain reaction

- refers to the use of PCR to amplify several different DNA targets (genes) simultaneously
- amplifies genomic DNA samples using multiple primers and a temperature-mediated DNA polymerase in a thermal cycler.
- primer design for all primers pairs has to be optimized
- so that all primer pairs can work at the same annealing temperature during PCR.



Nested polymerase chain reaction

- is used to increase the specificity of DNA amplification
- Two sets of primers are used in two successive reactions
- In the first PCR, one pair of primers is used to generate DNA products, which may contain products amplified from non-target areas.
- products from the first PCR are then used as template in a second PCR
- using one ('hemi-nesting') or two different primers whose binding sites are located (nested) within the first set, thus increasing specificity.

In situ PCR

- It is a collective term used to describe amplification of DNA and RNA template by PCR and its subsequent detection within the histological tissue section or cell preparation.
- Detection of products is done by in situ hybridisation.
- It is somewhat difficult to detect the genes of low copy number by in situ PCR as it is below the detection limit.

Inverse PCR

- Inverse PCR uses standard PCR primers oriented in the reverse direction of the usual orientation.
- The template for the reverse primers is a restriction fragment that has been self ligated.
- Inverse PCR functions to clone sequences flanking a known sequence.
- Flanking DNA sequences are digested and then ligated to generate circular DNA.

Application :

Amplification and identification of flanking sequences such as transposable elements, and the identification of genomic inserts.

THANK YOU…