POLYMERASE CHAIN REACTION

PCR

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

- Developed in 1983 by Kary Mullis, PCR is now a common technique used in clinical and research laboratories for a broad variety of applications.
- In 1993, Mullis was awarded the Nobel Prize in Chemistry for his work on PCR.

Components of PCR

DNA template:

DNA template is DNA target sequence. DNA template is the DNA molecule that contains the DNA region (segment) to be amplified, the segment we are concerned which is the target sequence.

DNA polymerase:

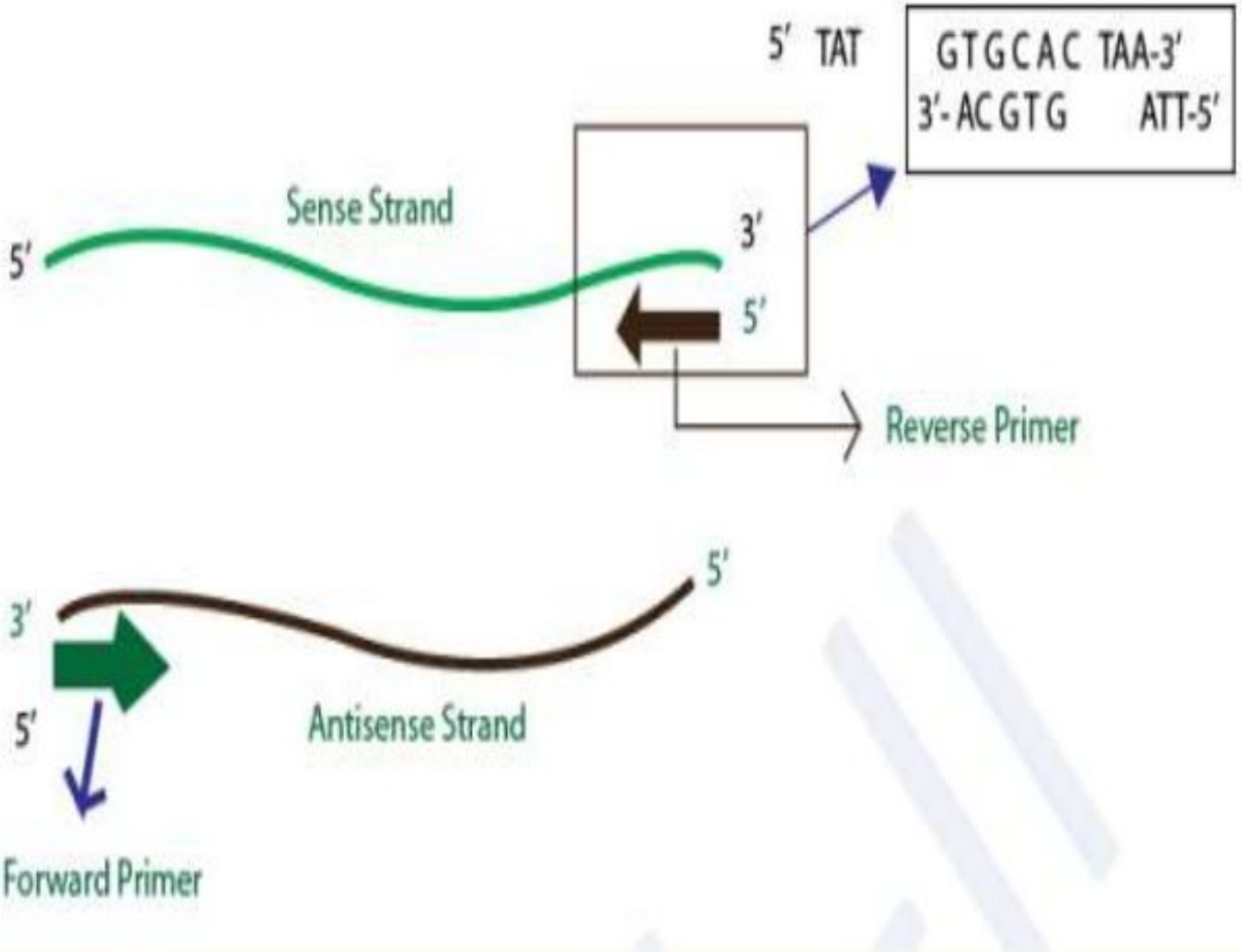
DNA polymerase sequentially adds nucleotides complimentary to template strand at 3'-OH of the bound primers and synthesizes new strands of DNA complementary to the target sequence. The most commonly used DNA polymerase is *Taq* DNA polymerase (from *Thermus aquaticus,* a thermophillic bacterium) because of high temperature stability.

Pfu DNA polymerase (from *Pyrococcus furiosus*) is also used widely because of its higher fidelity (accuracy of adding complimentary nucleotide). Mg2+ ions in the buffer act as co-factor for DNA polymerase enzyme and hence are required for the reaction.

Primers:

Primers are synthetic DNA strands of about 18 to 25 nucleotides complementary to 3' end of the template strand. DNA polymerase starts synthesizing new DNA from the 3' end of the primer .

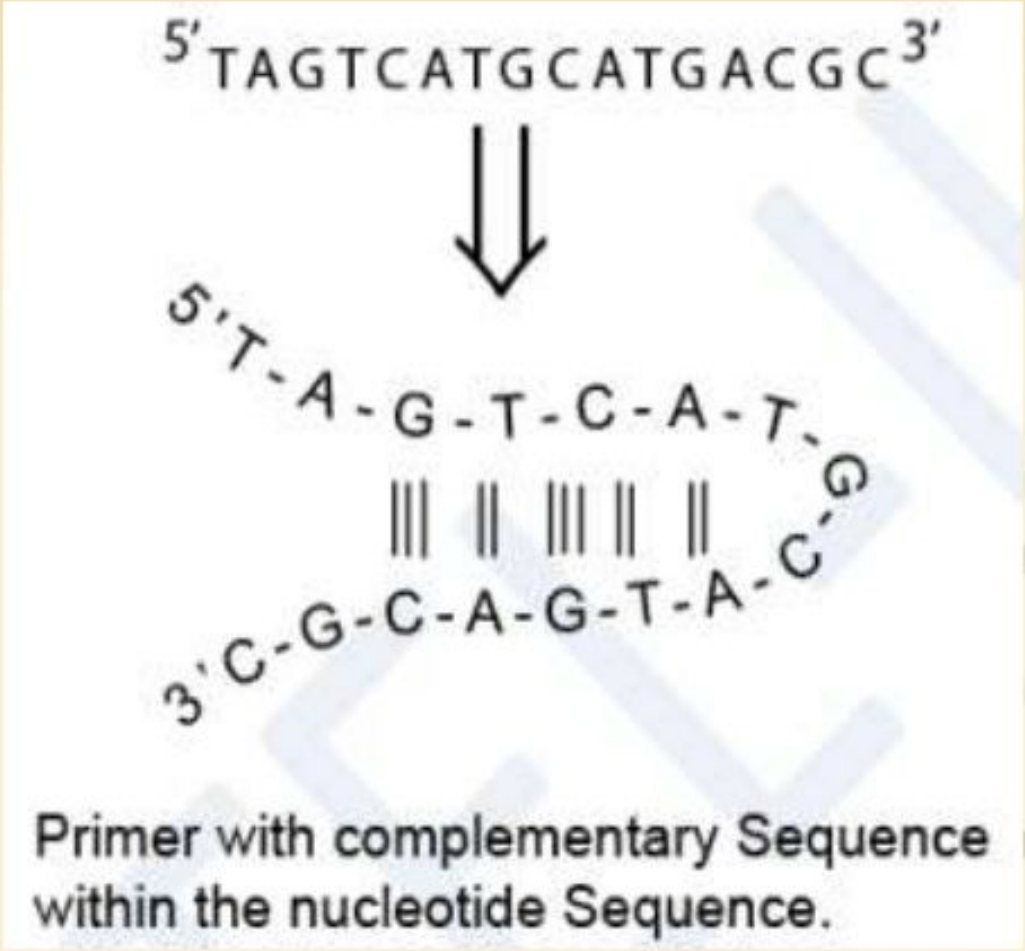
Two primers must be designed for PCR; the forward primer and the reverse primer. The forward primer is complimentary to the 3' end of antisense strand (3'-5') and the reverse primer is complimentary to the 3' end of sense strand (5'-3'). If we consider the sense strand (5'-3') of a gene, for designing primers, then forward primer is the beginning of the gene and the reverse primer is the reverse-compliment of the 3' end of the gene.


5' TAT
GTGCAC TAA-3'
3'-ACGTG ATT-5'
Sense Strand
5'
3'
5'
Reverse Primer
3'
5'
5'
Antisense Strand
Forward Primer

Primer Design

Primers should bind to template with good specificity and strength. If primers do not bind to correct template, wrong sequence will get amplified. Optimal primer sequences and appropriate primer concentrations are essential for maximal specificity and efficiency in PCR.

Complementary nucleotide sequences within a primer and between primers should be avoided. If there are complimentary sequences in two primers used (one primer for each DNA strand), the primers will hybridize with each other thus forming primer-dimmers and will not be available for binding with template. If there are complementary sequences within a primer, it will make hairpin loop structures as shown below.



Primer with complementary Sequence within the nucleotide Sequence.

- The primers should preferably end on a Guanine and Cytosine (GC) sequence so that it can attach with sufficient strength with template. This increases efficiency of priming due to stronger bonding of G and C bases.
- Run of three or more Cytosine (C) or Guanine (G) at the 3'-ends of primers should be avoided. This may promote mis-priming i.e non-specific binding to G or C rich sequences in the genome other than the target sequence.

- As Adenine and Thymine base pairs with a single H-bond so Thymine (T) or Adenine (A) residues should be avoided at the 3' end of primers as this weaken the primer's hold on the template DNA.

Nucleotides (dNTPs or deoxynucleotide triphosphates):

All types of nucleotides are "building blocks" for new DNA strands and essential for reaction. It includes Adenine(A), Guanine(G), Cytosine(C), Thymine(T) or Uracil(U).

Magnesium

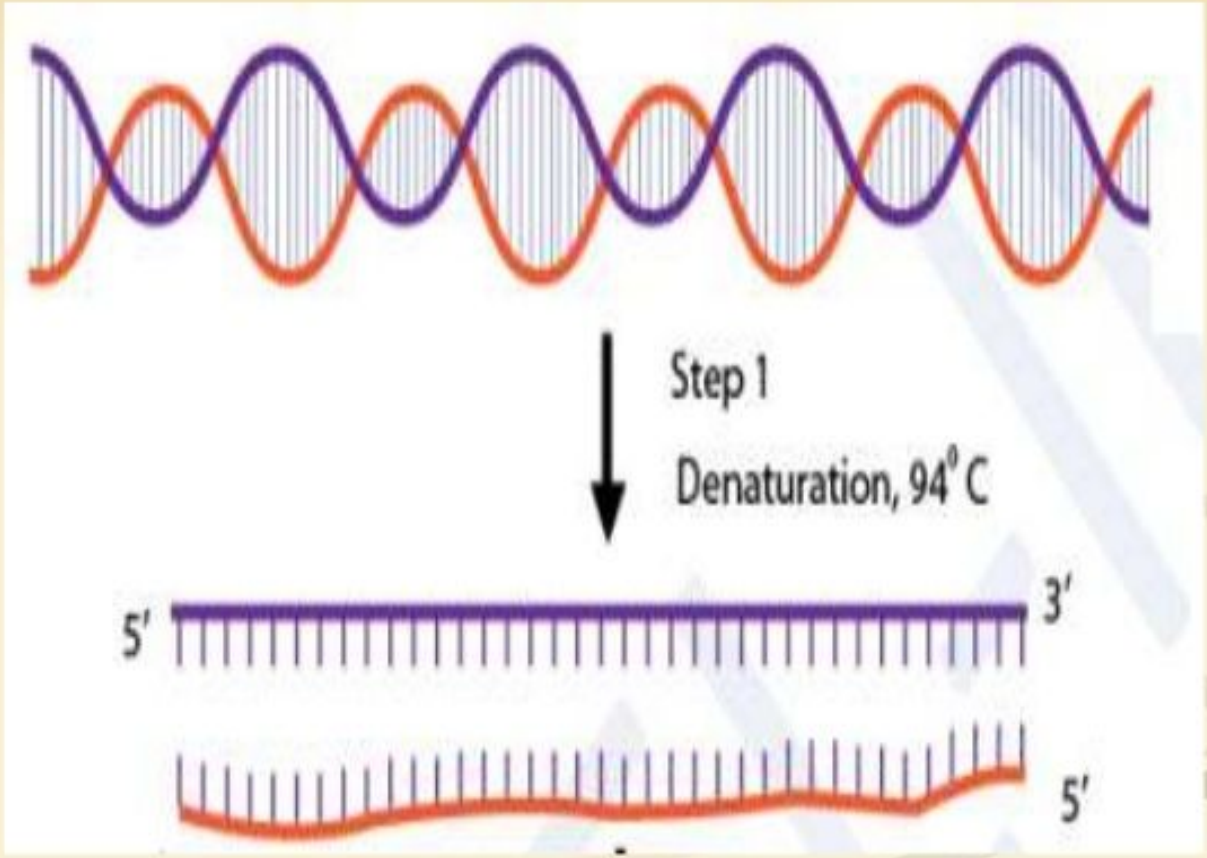
Magnesium affects primer annealing and template denaturation, as well as enzyme activity. An excess of magnesium gives non-specific amplification products, while low magnesium yields lesser amount of desired product.

Procedure

There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation at 94°C :

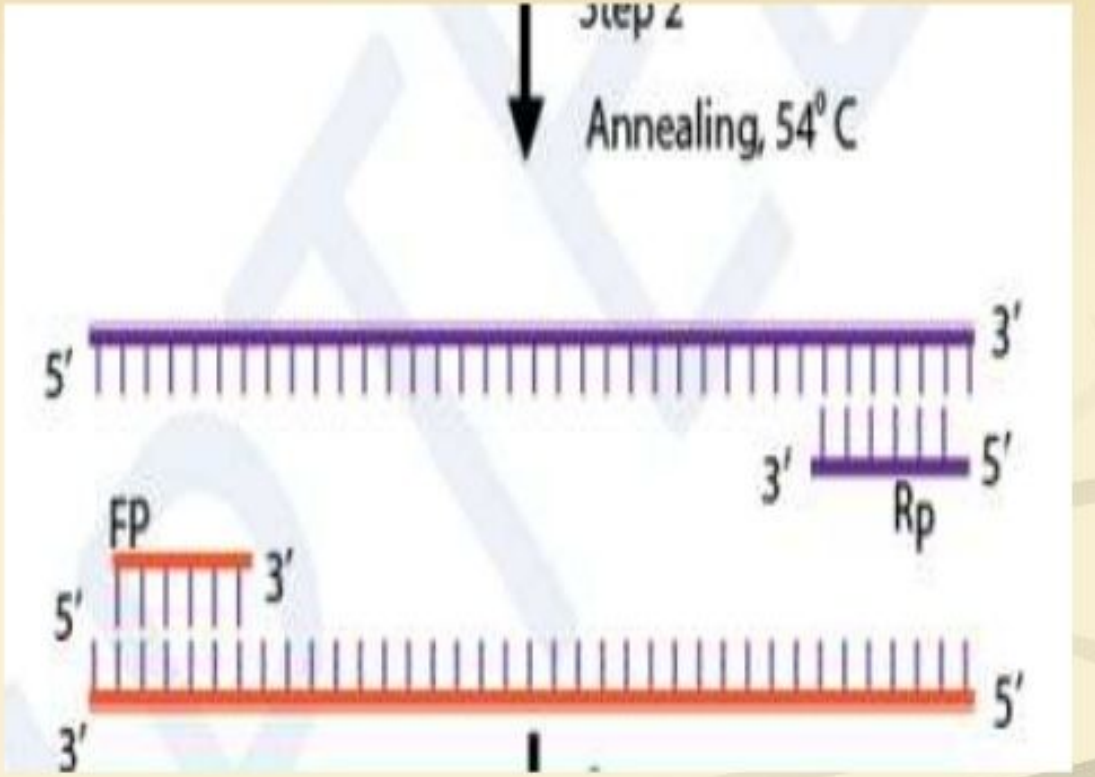
During the heating step (denaturation), the reaction mixture is heated to 94°C for 1 min, which causes separation of DNA double stranded. Now, each strand acts as template for synthesis of complimentary strand.


Step 1
Denaturation, 94° C
5′
3′
5′

Annealing at 54°C :

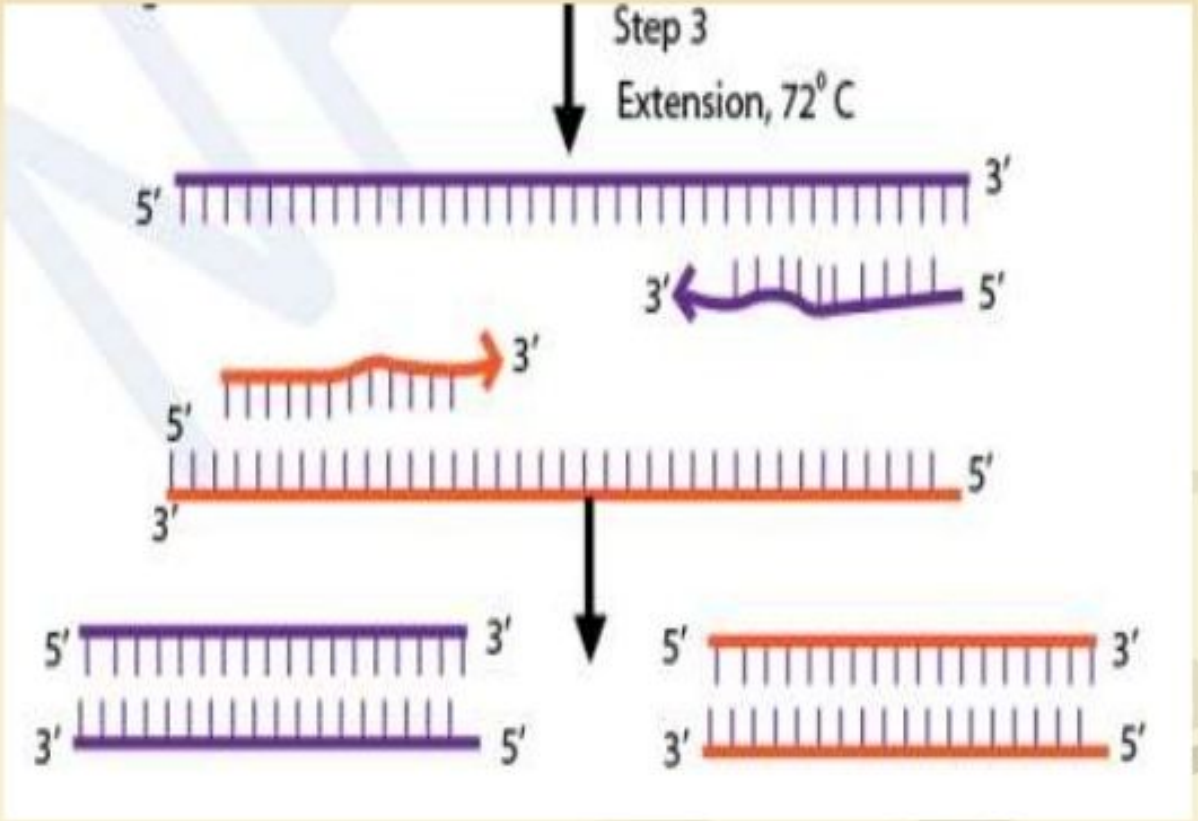
This step consist of cooling of reaction mixture after denaturation step to 54°C, which causes hybridization (annealing) of primers to separated strand of DNA (template). The length and GC-content (guanine-cytosine content) of the primer should be sufficient for stable binding with template.

Guanine pairs with cytosine with three hydrogen bonding adenine binds with thymine with two hydrogen bonds. Thus, higher GC content results in stronger binding. In case GC content is less, length may be increased to have stronger binding (more number of H bonding between primer and template).


Step 2
Annealing, 54° C
5′
3′
3′
5′
Rp
FP
5′
3′
5′
3′

Extension at 72°C :

The reaction mixture is heated to 72°C which is the ideal working temperature for the Taq polymerase. The polymerase adds nucleotide (dNTP's) complimentary to template on 3' –OH of primers thereby extending the new strand.

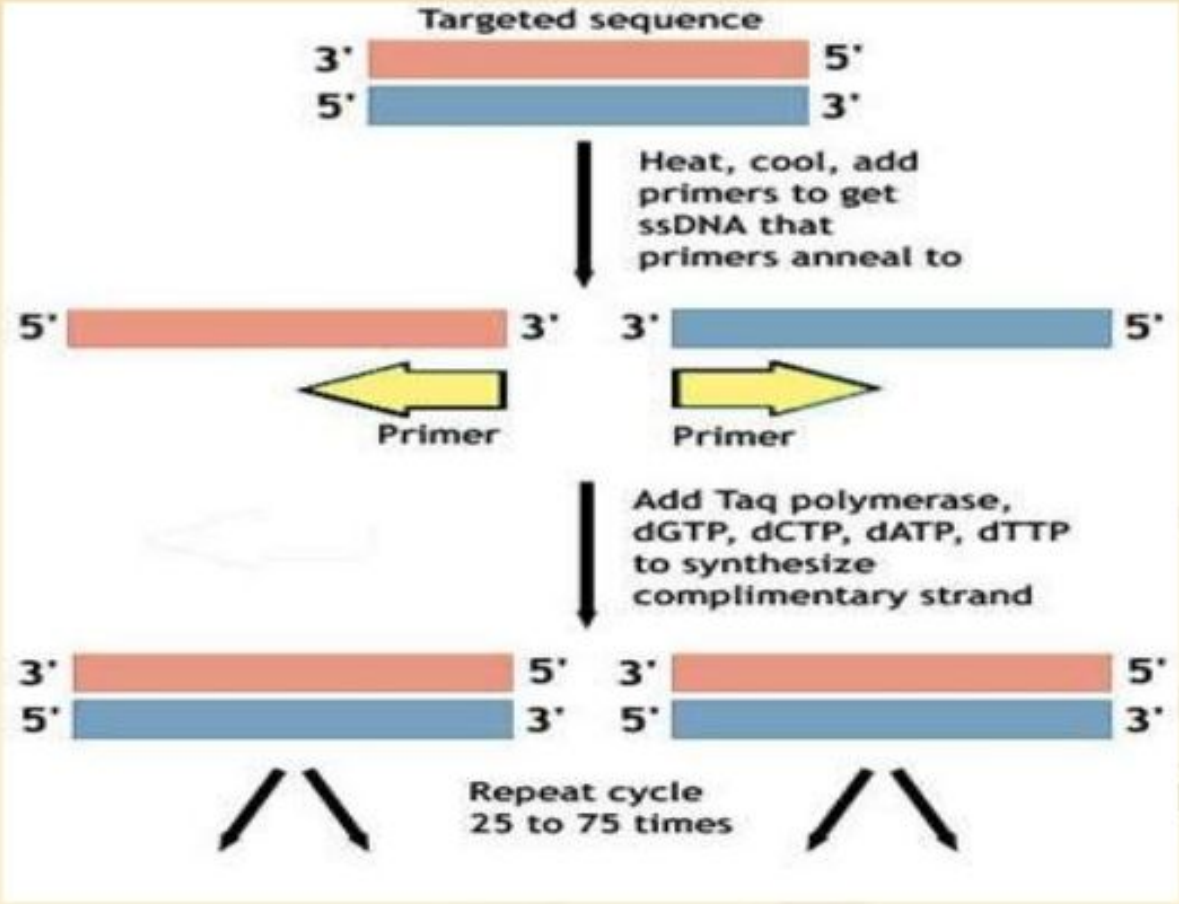

Step 3
Extension, 72° C
5′
3′
3′
5′
3′
5′
5′
3′
5′
3′
5′
3′
3′
5′
5′
3′
3′
5′

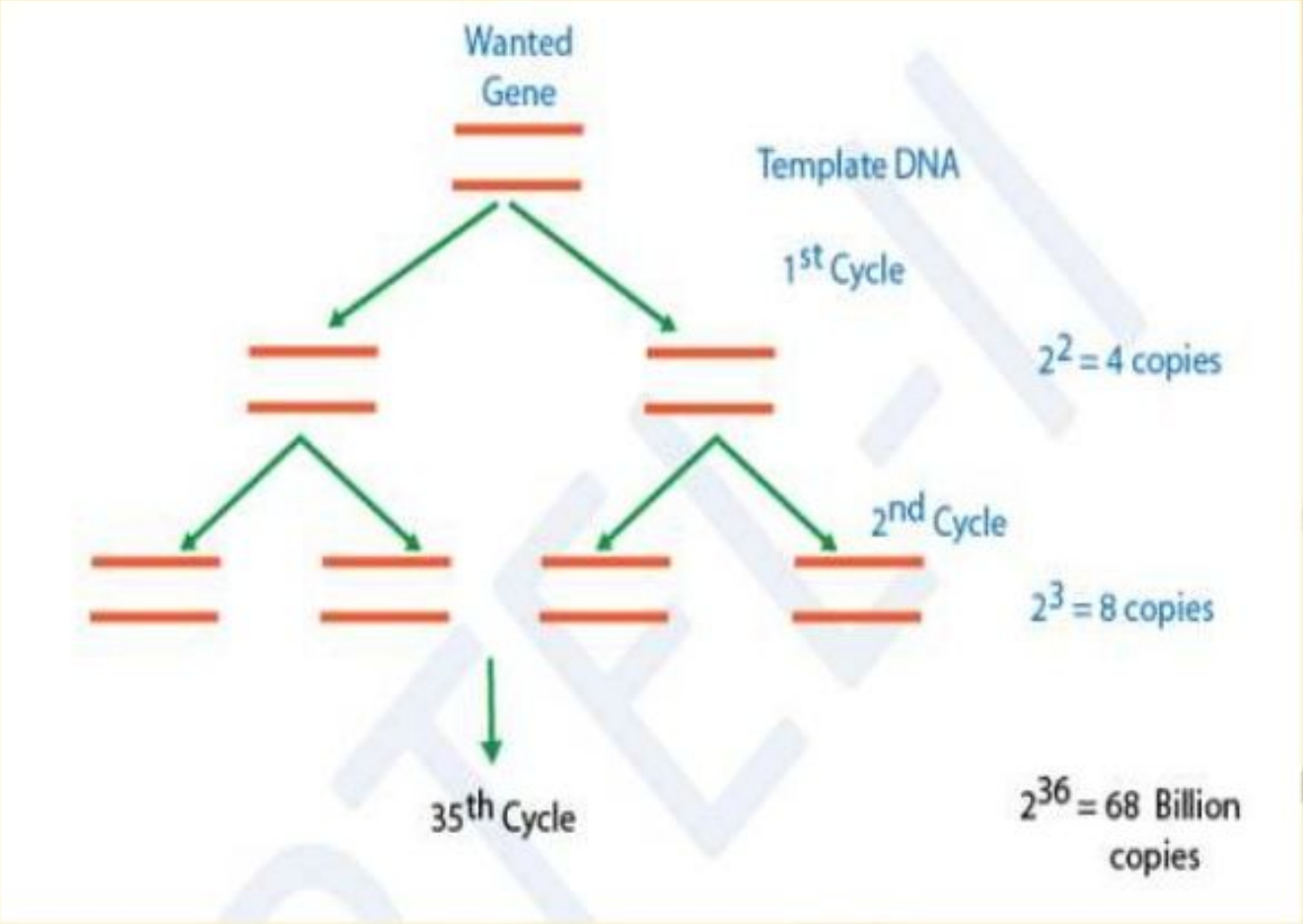
Final hold:

First three steps are repeated 35-40 times to produce millions of exact copies of the target DNA. Once several cycles are completed, during the hold step, 4–15 °C temperature is maintained for short-term storage of the amplified DNA sample.

PCR-an exponential cycle:

As both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. Suppose there is only one copy of the desired gene before the PCR starts, after one cycle of PCR, there will be 2 copies, after two cycles of PCR, there will be 4 copies. After three cycles there will be 8 copies and so on.


Targeted sequence
3'
5'
5'
3'
Heat, cool, add primers to get ssDNA that primers anneal to
5'
3'
3'
5'
Primer
Primer
Add Taq polymerase, dGTP, dCTP, dATP, dTTP to synthesize complimentary strand
3'
5'
5'
3'
3'
5'
5'
3'
Repeat cycle 25 to 75 times


Wanted
Gene
Template DNA
1st Cycle
2^2 = 4 copies
2nd Cycle
2^3 = 8 copies
35th Cycle
2^{36} = 68 Billion
copies

Reaction Condition & Experimental Protocol

- Denaturing conditions are best at 94-95°C for 30-60 seconds. Lower temperatures may result in incomplete denaturation of target template and PCR products. Higher temperatures and a longer amount of time can lead to enzyme activity loss.

General PCR Protocol

Prepare following mixture in appropriately sized eppendorf tube (0.5 mL or 0.2 mL):

ingredient	volume per rxn
H_2O	15-16 µl
Mg	2 µl
dNTP (2 mM each)	1 µl
forward primer (12.5 µM)	0.5 µl
reverse primer (12.5 µM)	0.5 µl
Taq polymerase (5u/µl)	0.2 µl
template DNA	0.1-1.0 µl
total	20 µl

PCR machine: Load the reactions into 0.2 ml PCR tubes. Close lid and turn knob until it stops.

Turn on PCR machine (switch on back). The menu should point at "START" (if not use arrows up and down).

Press "ENTER"

Use arrow keys to select the program you want to run. Press "ENTER". The program will ask you what kind of tube you're using and the reaction volume; select the tube with the "SELECT" button, enter the volume on the number keypad; hit "ENTER". -- While the program is running, you can use the "OPTION" key to check how much longer you have to wait.

Program Naming Convention: All programs use a 15s denaturation at 94°C, followed by 15s annealing, extension at 72°C. Programs are identified by their annealing temperature (A##), extension time (E##), and cycle number (letter code: A=20, B=25, C=30, D=35, E=40). For example, "A60E120C" means: Annealing at 60°C, Extension for 120 s, 30 cycles.

Applications

Used in molecular biology and genetic disease research to identify new genes; for example, the sample containing pathogenic DNA can be PCR amplified using different known specific primers. The amplification indicates presence of pathogenic DNA.

In fields such as anthropology and evolution, sequences of degraded ancient DNAs can be tracked after PCR amplification.

With its exquisite sensitivity and high selectivity, PCR has been used for wartime human identification and validated in crime labs for mixed-sample forensic casework.

PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas. PCR assays can be performed directly on genomic DNA samples to detect translocation specific malignant cells, infectious agents, like mycobacterium, anaerobic bacteria, or viruses.

PCR - Polymerase Chain Reaction for Site Directed Mutagenesis -This technique is used for introduction of mutations at the desired place in a DNA sequence.

Advantages of PCR

- Speed
- Ease of use
- Sensitivity
- Robustness

Limitations of PCR

- **Need for target DNA sequence information**
  - Primer Designing for unexplored ones.
  - Boundary regions of DNA to be amplified must be known.

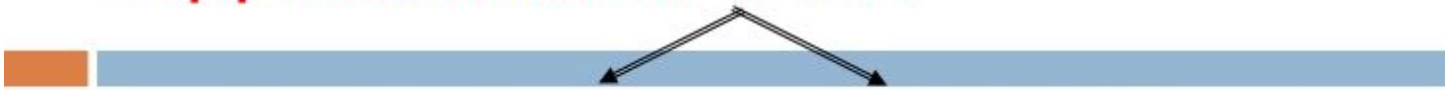
- **Infidelity of DNA replication.**
  - Taq Pol – no Proof reading mech – Error 40% after 20 cycles

- **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

- Medical applications
- Infectious disease applications
- Forensic applications
- Research applications

Applications of PCR



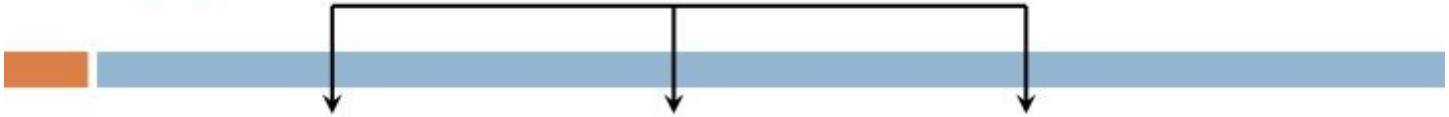
Basic Research

- Mutation screening
- Drug discovery
- Classification of organisms
- Genotyping
- Molecular Archaeology
- Molecular Epidemiology
- Molecular Ecology
- Bioinformatics
- Genomic cloning
- Site-directed mutagenesis
- Gene expression studies

Applied Research

- Genetic matching
- Detection of pathogens
- Pre-natal diagnosis
- DNA fingerprinting
- Gene therapy

Applications of PCR



Molecular Identification

- Molecular Archaeology
- Molecular Epidemiology
- Molecular Ecology
- 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 ??

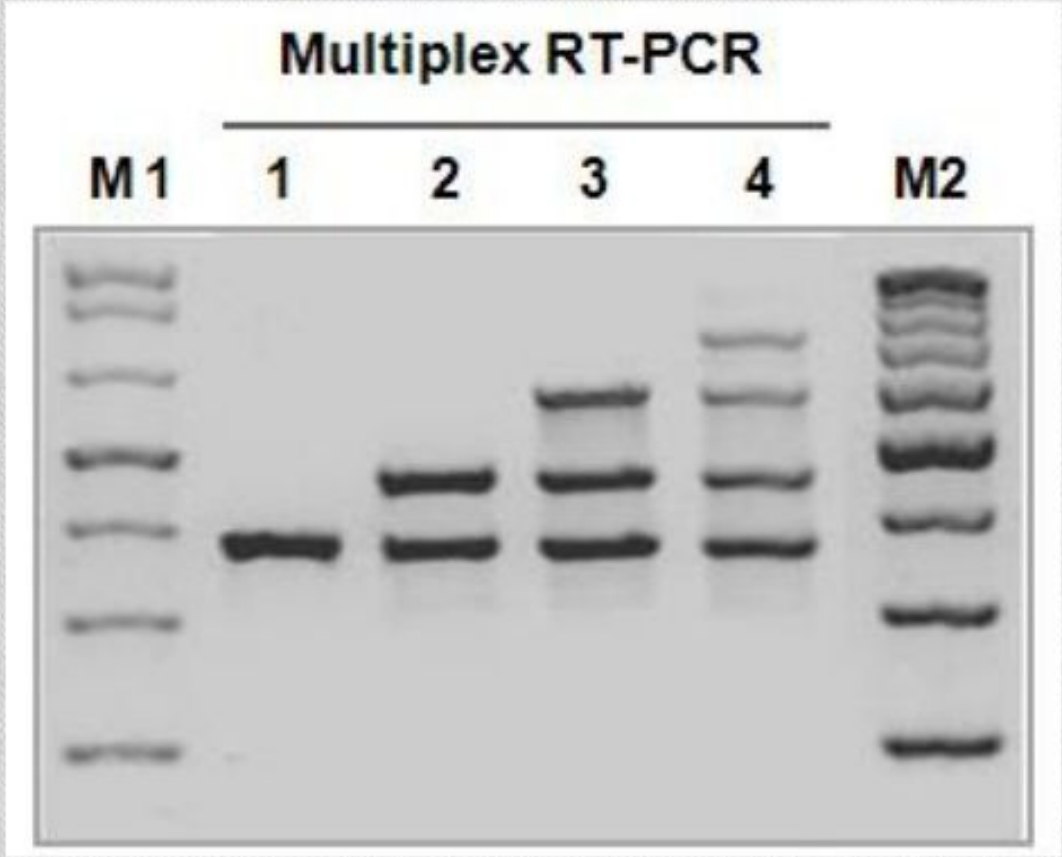
- Conventional (Qualitative)PCR.
- Multiplex PCR.
- Nested PCR.
- RT-PCR and qRT-PCR.
- Quantitative PCR.
- Hot-start PCR.
- Touchdown PCR.
- Assembly PCR.
- Colony PCR.
- Methylation-specific PCR.
- LAMP assay.

Multiplex-PCR:

It is a special type of the PCR used for detection of multiple pathogens by using Multiple primers sets each one targets a particular pathogen.

Uses:

This permits the simultaneous analysis of multiple targets in a single sample.


Multiplex RT-PCR
M1
1
2
3
4
M2

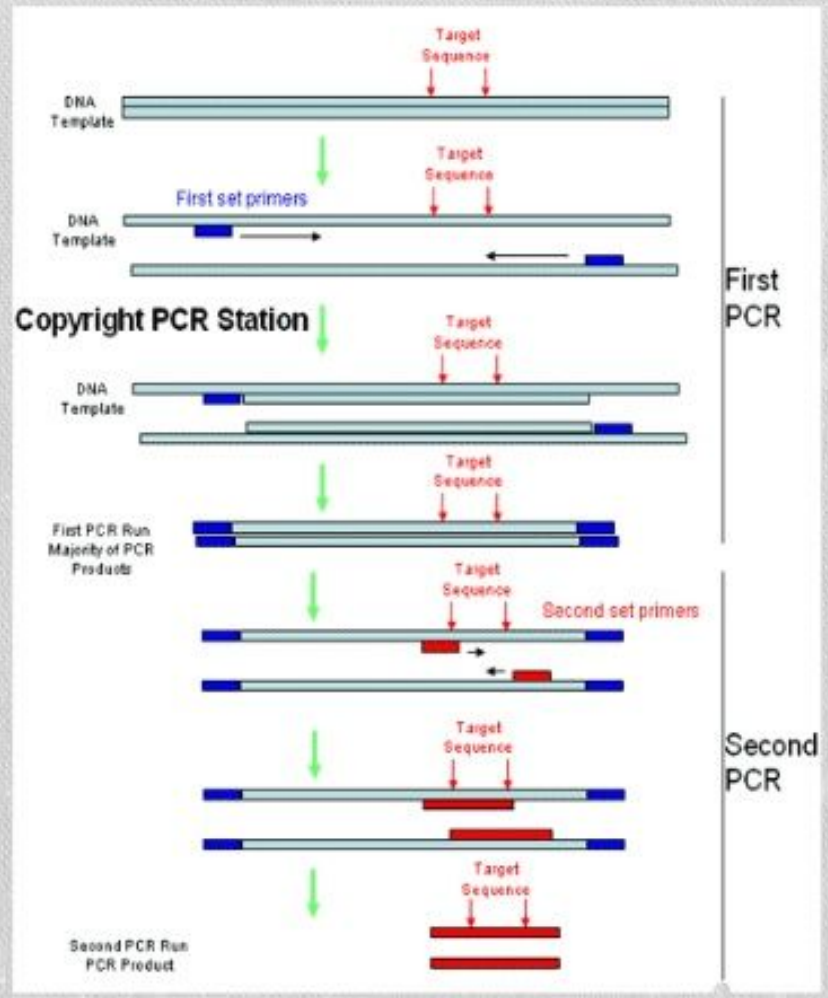
Nested-PCR:

- 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 will be the target for the second reaction.

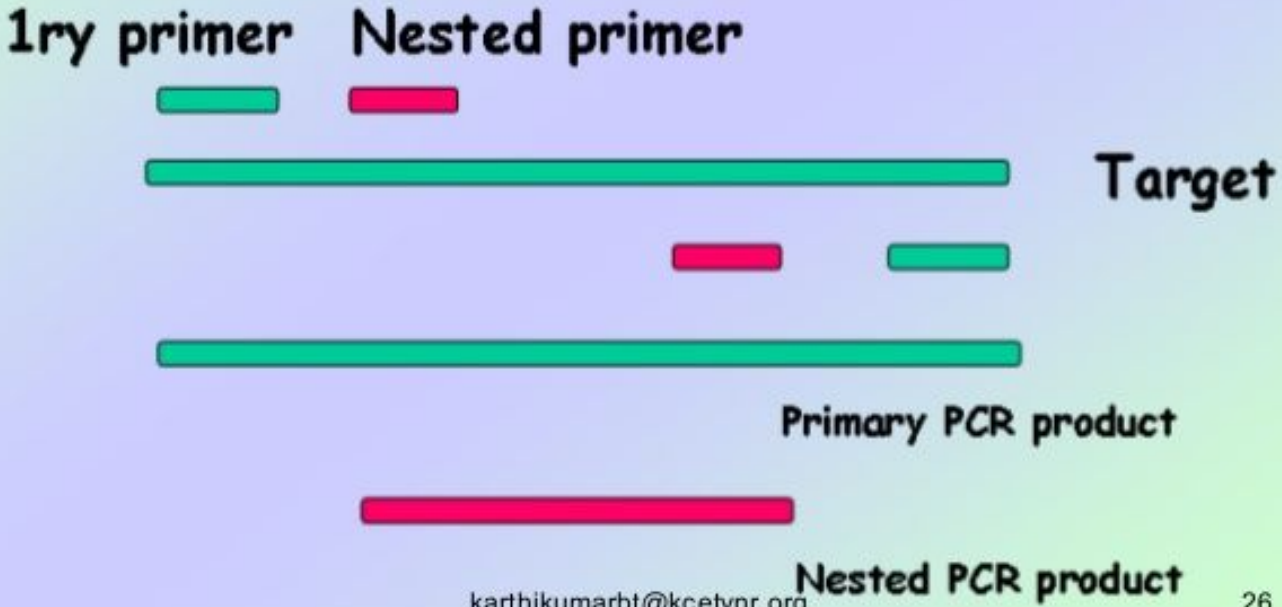
- Using one ('hemi-nesting') or two different primers whose binding sites are located (nested) within the first set, thus increasing specificity.

Uses:

Detection of pathogens that occur with very few amount.


Target Sequence
DNA Template
Target Sequence
First set primers
DNA Template
Copyright PCR Station
Target Sequence
DNA Template
Target Sequence
First PCR Run Majority of PCR Products
Target Sequence
Second set primers
Target Sequence
Target Sequence
Second PCR Run PCR Product
First PCR
Second PCR

Nested PCR


1ry primer
Nested primer
Target
Primary PCR product
Nested PCR product

RT-PCR (Reverse Transcription PCR, Real Time - PCR)

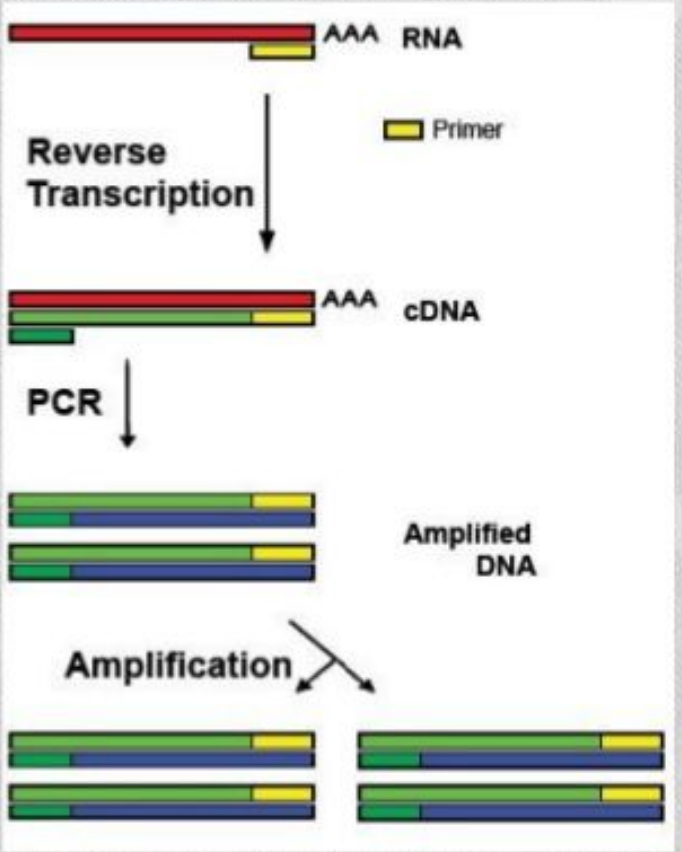
- Used to reverse-transcribe and amplify RNA to cDNA.
- PCR is preceded by a reaction using reverse transcriptase, an enzyme that converts RNA into cDNA.
- The two reactions may be combined in a tube.

Uses:

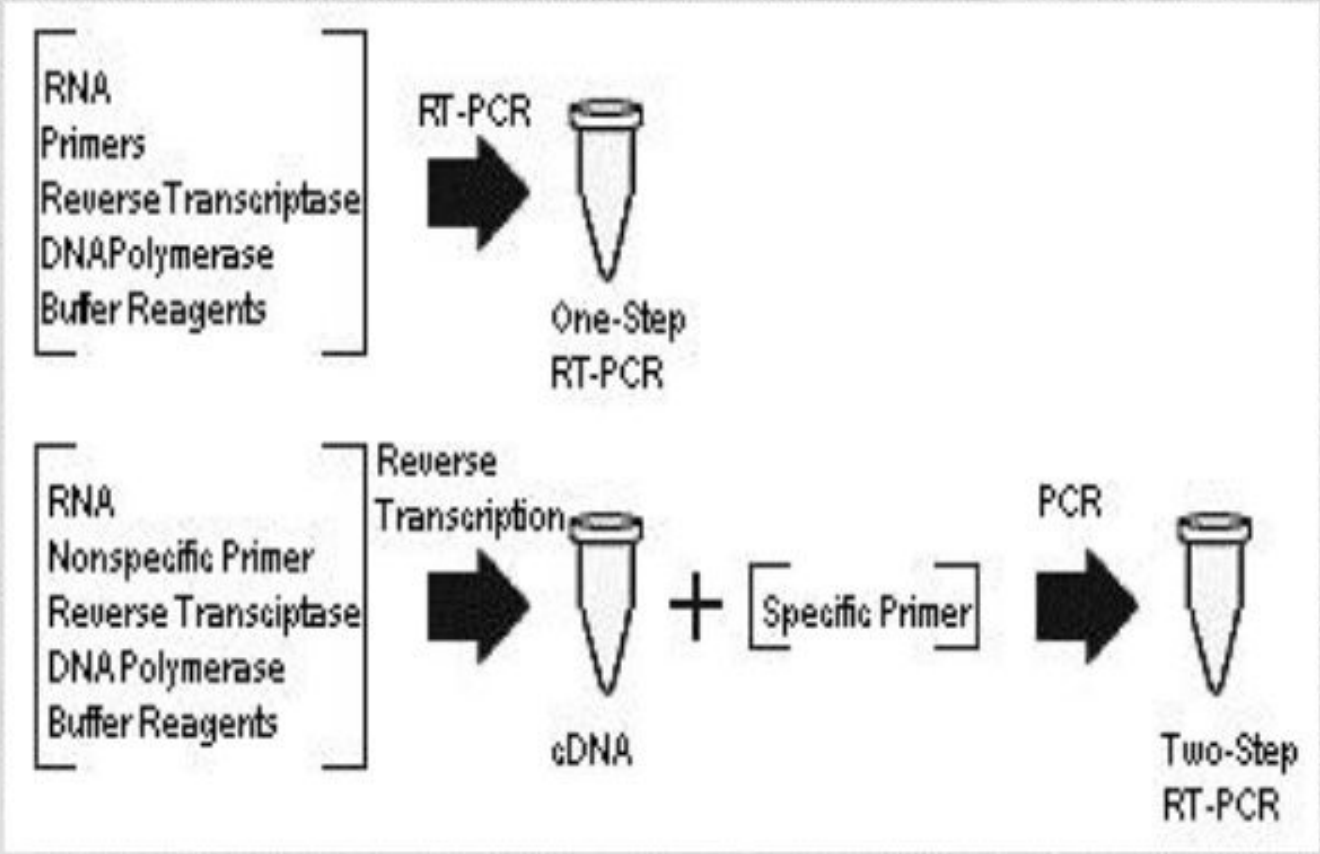
1-Detection of RNA virus like (HCV).

2-Detection of other M.O. through targeting of their Ribosomal RNA.

Reverse Transcription PCR, Real Time - PCR



Reverse Transcription *PCR, Real Time - PCR*



Quantitative – PCR:

- Used to measure the specific amount of target DNA (or RNA) in a sample.
- By measuring amplification only within the phase of true exponential increase, the amount of measured product more accurately reflects the initial amount of target.
- Special thermal cyclers are used that monitor the amount of product during the amplification.

Hot-start PCR:

- It is a technique performed manually by heating the reaction components to the DNA melting temperature (e.g. 95°C) before adding the polymerase.

Touchdown PCR:

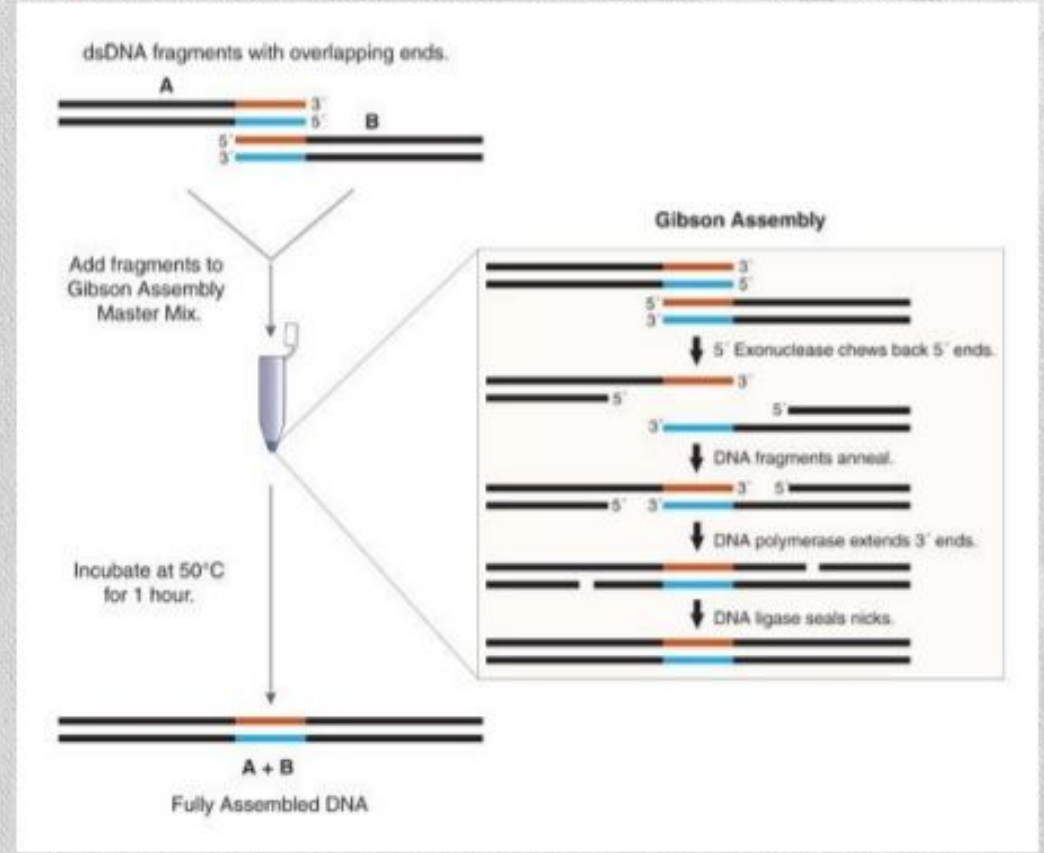
- In this type the annealing temperature is gradually decreased in later cycles.
- The annealing temperature in the early cycles is usually 3-5°C **above** the standard T_m of the primers used, while in the later cycles it is a similar amount **below** the T_m.
- The initial higher annealing temperature leads to greater specificity for primer binding, while the lower temperatures permit more efficient amplification at the end of the reaction.

Assembly-PCR

(also known as *Polymerase Cycling Assembly* or *PCA*)

- In this type synthesis of long DNA structures by performing PCR on a pool of long oligonucleotides with short overlapping segments, to assemble two or more pieces of DNA into one piece.
- It involves an initial PCR with primers that have an overlap and a second PCR using the products as the template that generates the final full-length product.
- This technique may substitute for Ligation-based assembly

Assembly PCR:



Colony PCR

- Bacterial colonies are screened directly by PCR, for example, the screen for correct DNA-vector constructs.
- Colonies are sampled with a sterile pipette tip and a small quantity of cells transferred into a PCR mix.

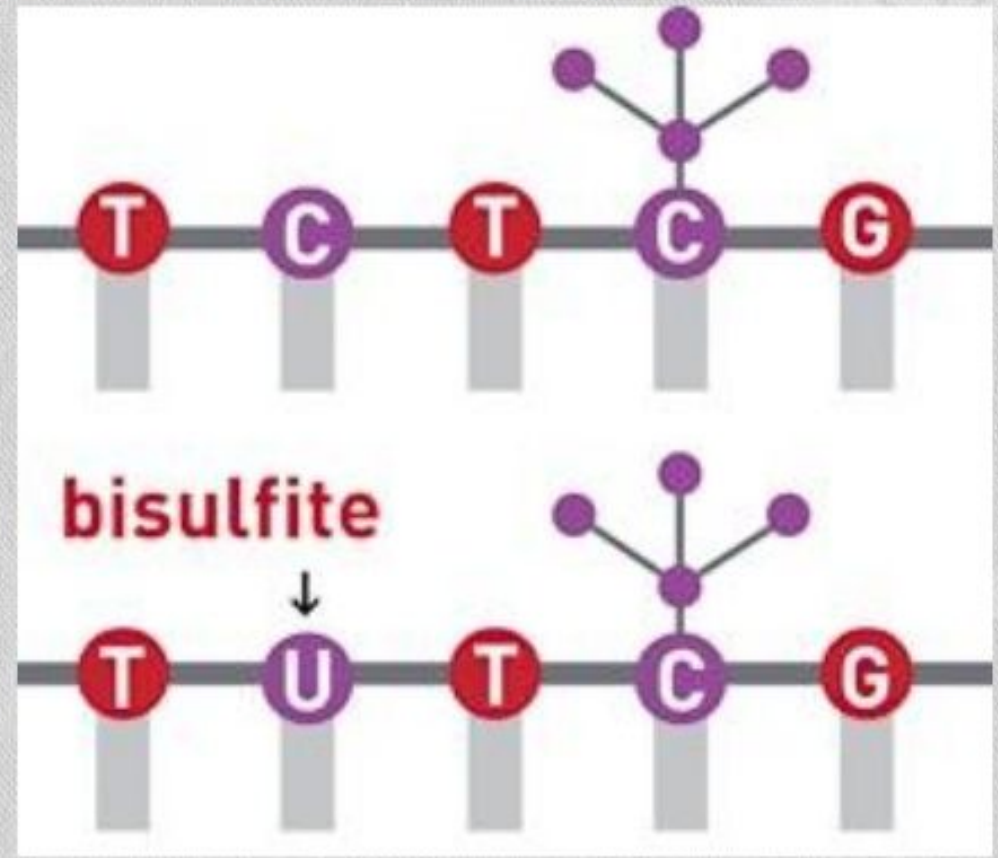
Methylation-specific PCR (MSP)

- Used to identify patterns of DNA methylation at cytosine guanine islands (C&G islands) in genomic DNA. CpG islands, are concerned in regulation of gene expression in mammalian cells.
- Target DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is complementary to adenosine in PCR primers.
- Two amplifications are then carried out on the bisulfite-treated DNA:

Methylation-specific PCR (MSP)

- One primer set anneals to DNA with cytosine (corresponding to methylated cytosine),
- The other set anneals to DNA with uracil (corresponding to unmethylated cytosine).
- MSP used in quantitative PCR provides quantitative information about the methylation state of a given CpG island.

Methylation-specific PCR (MSP)



LAMP assay:
(Loop-mediated isothermal amplification)

- It is a Modified type of the PCR using 3:6 primers sets one of them is loop like primer.
- This test use Bst- polymerase enzyme

(Bacillus stearothermophilus DNA Polymerase).

- Using only two temperatures (63°C for 45 min. then 85°C for 5 min.), may be carry out in water path.