

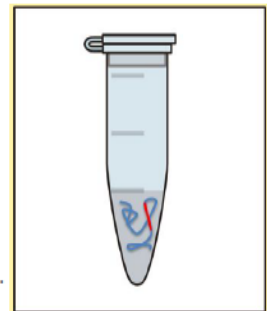
# Polymerase Chain Reaction

## 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.

## PCR-Polymerase Chain Reaction:

- PCR is a means to **amplify a particular piece of DNA** .
  - **Amplify**= making numerous copies of a segment of DNA.
- PCR can make billions of copies of a **target sequence of DNA** in short time.



## Polymerase Chain Reaction (PCR)=DNA Photocopier



- 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.

## Amplification of a specific target sequence:

- PCR **does not copy all of the DNA** in the sample. It copies only a **very specific sequence** of genetic code from a template DNA, targeted by **PCR primers**.
- It does require the knowledge of some DNA sequence information which flanks the fragment of DNA to be amplified (**target DNA**).



### DNA polymerase:

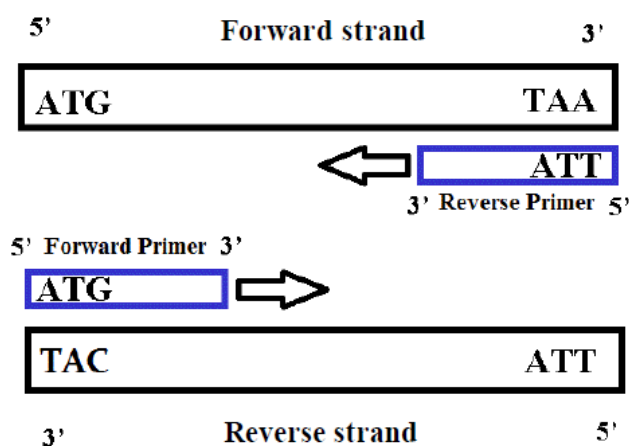
DNA polymerase sequentially adds nucleotides complimentary to template strand at 3'-OH of the bound primers and synthesizes new strands of DNA complementary to the target sequence. The most commonly used DNA polymerase is *Taq* DNA polymerase (from *Thermus aquaticus*, a thermophilic bacterium) because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

*Pfu* DNA polymerase (from *Pyrococcus furiosus*) is also used widely because of its higher fidelity (accuracy of adding complimentary nucleotide). Mg<sup>2+</sup> 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 .

## Primers



- In a PCR reaction you need **two primers** to amplify the target sequence:

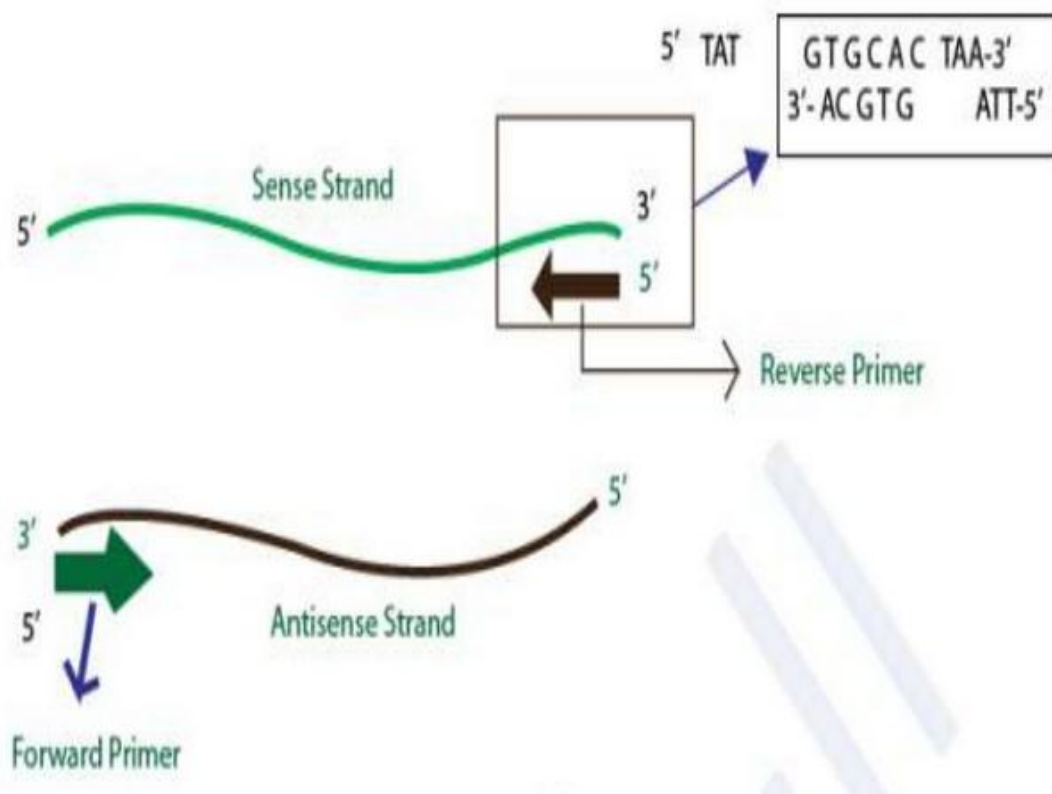
→ One called: **Forward primer**, which have the same sequence of forward DNA strand and bind to the complementary reverse strand.

→ The second called: **Reverse primer**, which have the same sequence of reverse DNA strand and bind to the complementary forward strand.

\*If there is only one primer, only one strand of the double stranded DNA will be amplified in the PCR reaction.



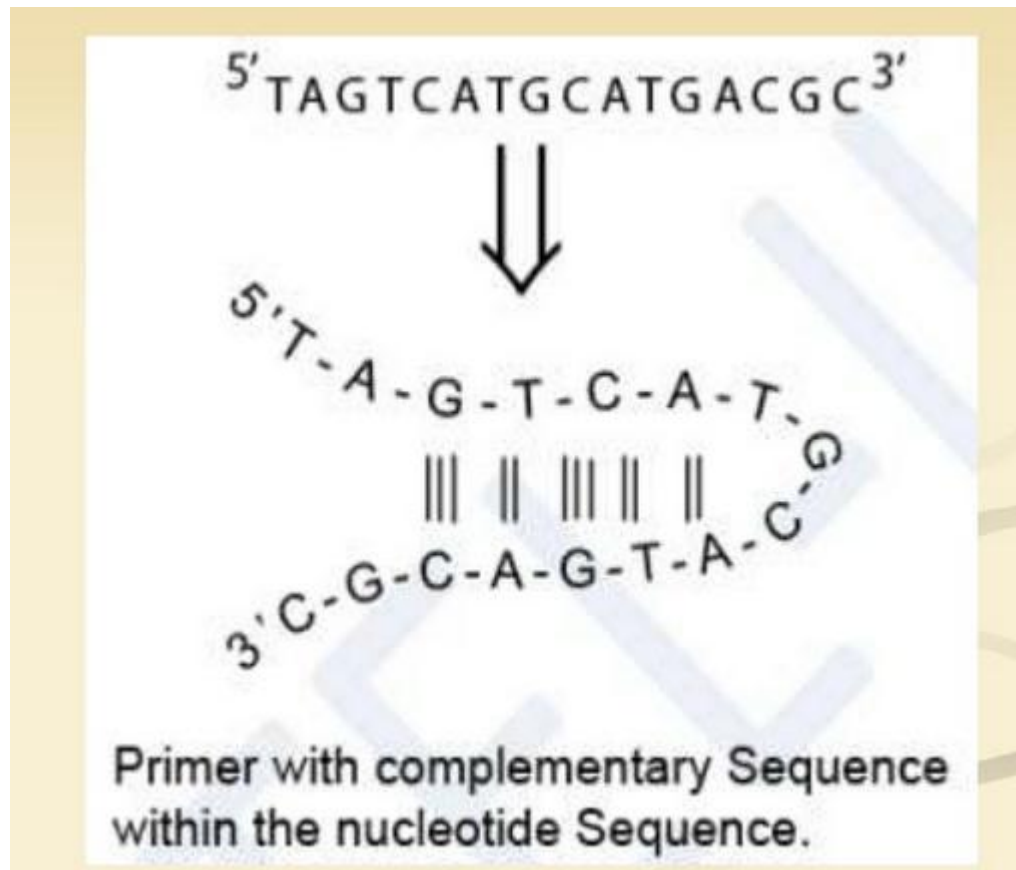
Two primers must be designed for PCR; the forward primer and the reverse primer. The forward primer is complementary to the 3' end of antisense strand (3'-5') and the reverse primer is complementary to the 3' end of sense strand (5'-3'). If we consider the sense strand (5'-3') of a gene, for designing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



## 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-dimers 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.



- 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.



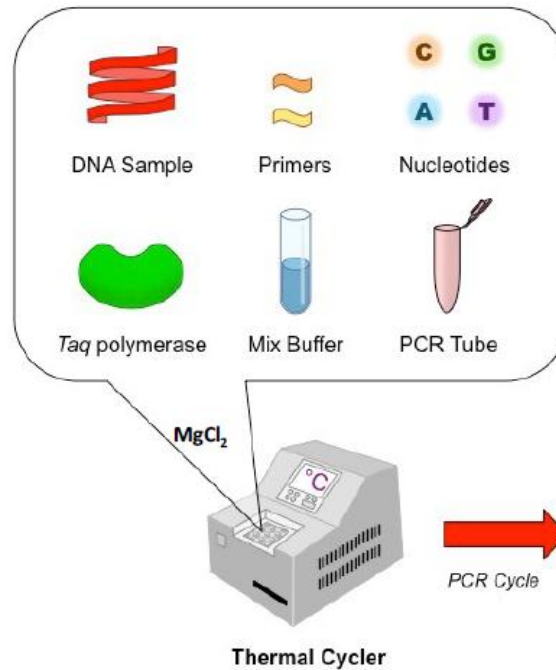
- As Adenine and Thymine base pairs with a single H-bond so Thymine (T) or Adenine (A) residues should be avoided at the 3' end of primers as this weakens the primer's hold on the template DNA.

### **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).



# Components of PCR



Additional reagents may included

## 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

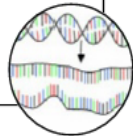
There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

## The PCR Cycle :

- PCR proceeds in **THREE** distinct steps Governed by **Temperature:**

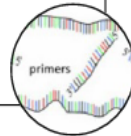
• The double-stranded template DNA is denatured by heating, typically to 95°C, to separate the double stranded DNA.

**Denaturation:**  
(95°C)



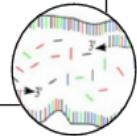
• The reaction is rapidly cooled to an annealing temperature to allow the oligonucleotide primers to hybridize to the template.

**\*\* Annealing:**  
(50-65°C)



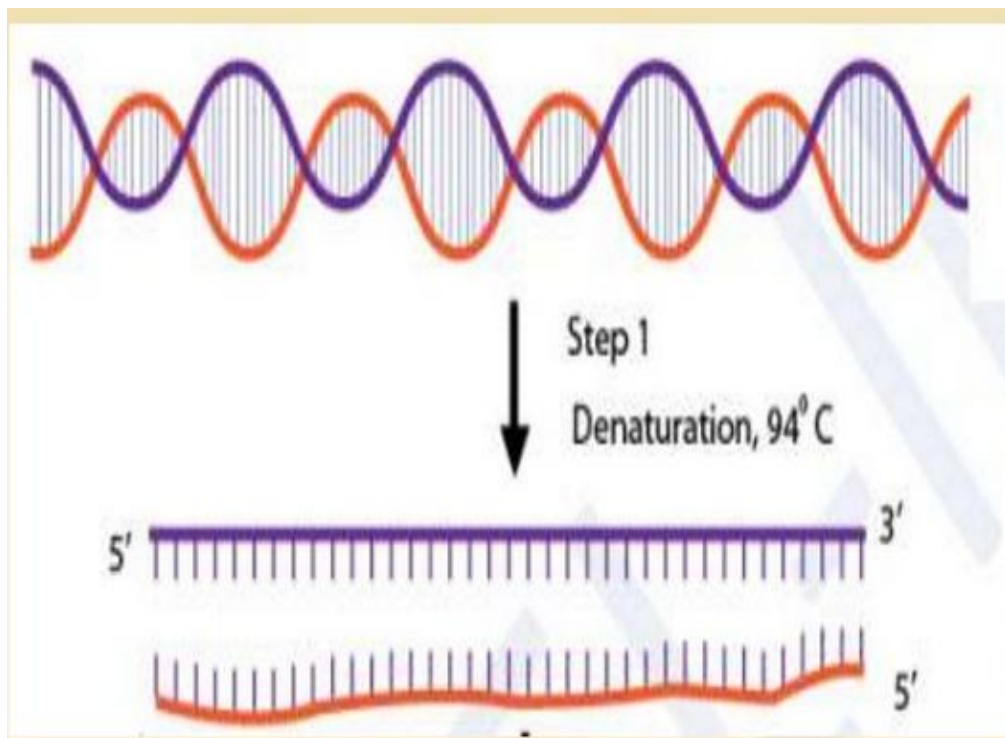
• The reaction is heated to a temperature, typically 72°C for efficient DNA synthesis by the thermostable DNA polymerase.

**Extension:**  
(72°C)



## 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.

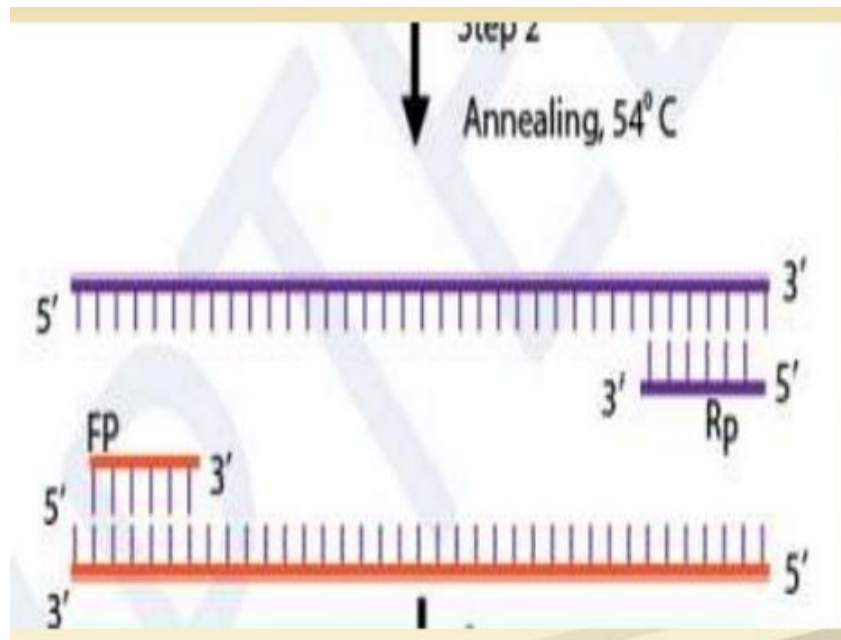




## **Annealing at 54°C :**

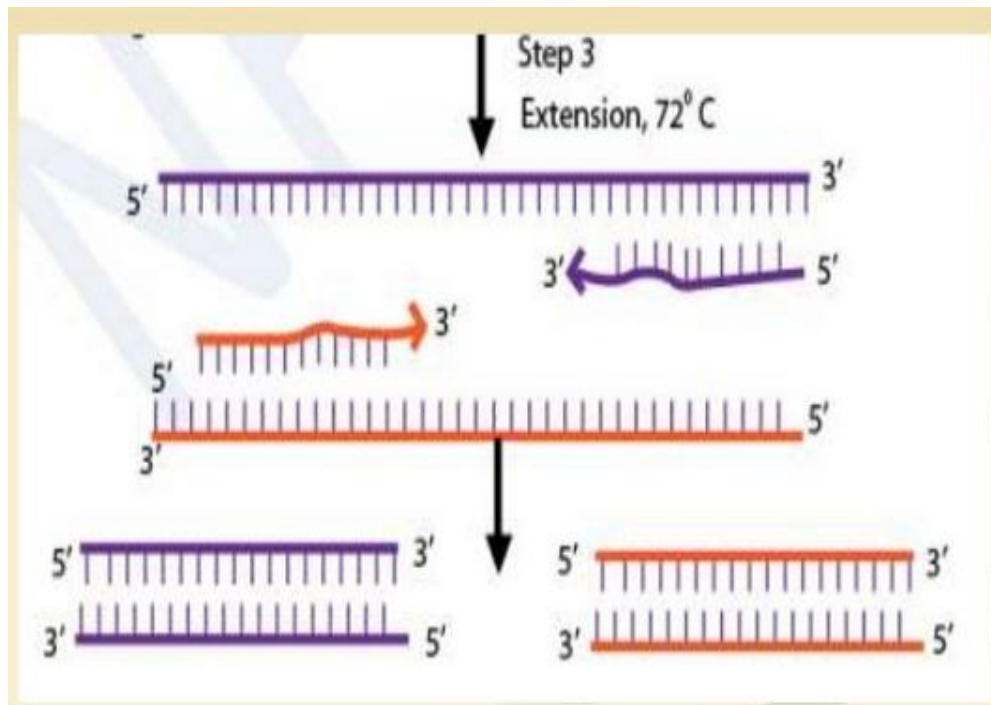
This step consist of cooling of reaction mixture after denaturation step to 54°C, which causes hybridization (annealing) of primers to separated strand of DNA (template). The length and GC-content (guanine-cytosine content) of the primer should be sufficient for stable binding with template.

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).



### **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.



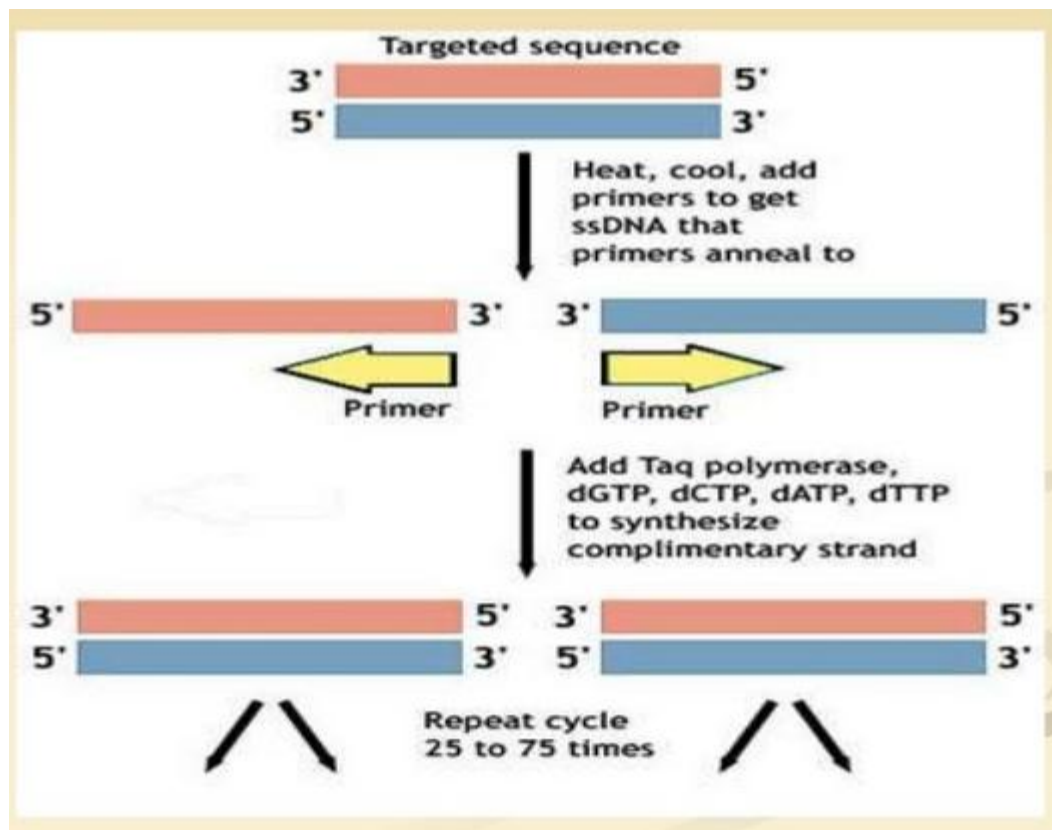
### **Final hold:**

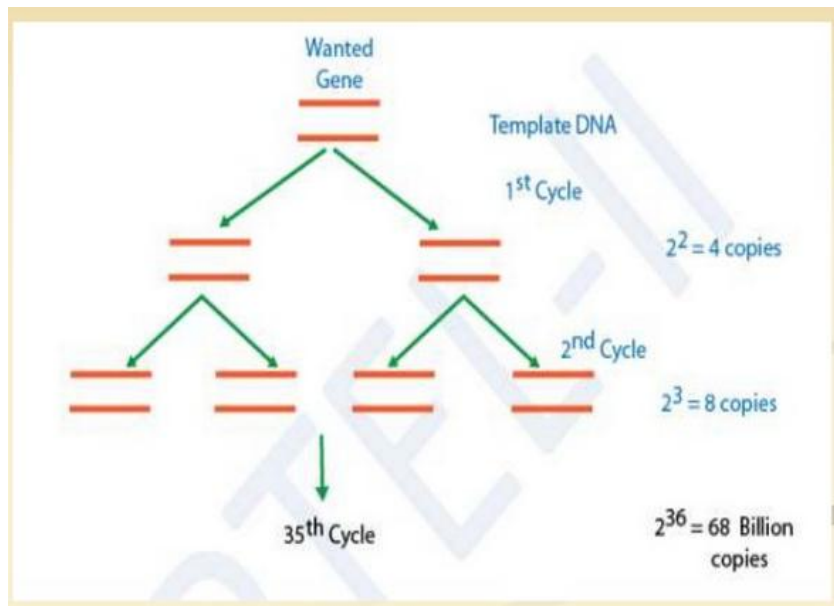
First three steps are repeated 35-40 times to produce millions of exact copies of the target DNA. Once several cycles are completed, during the hold step, 4–15 °C temperature is maintained for short-term storage of the amplified DNA sample.



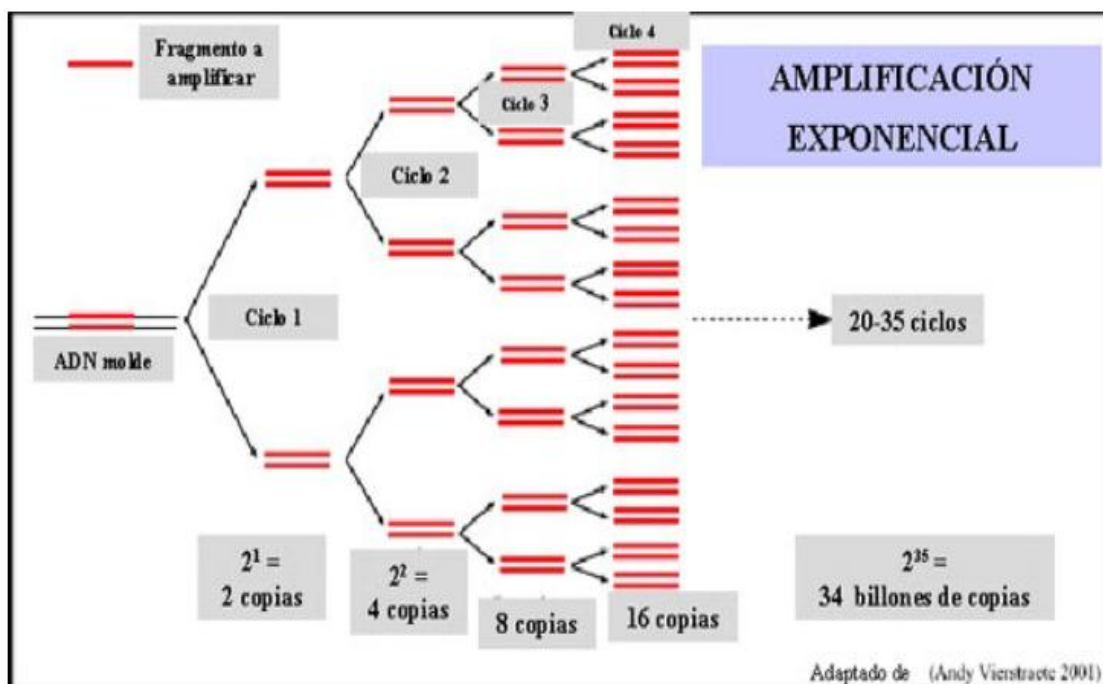
## PCR-an exponential cycle:

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.





- At the end of the PCR reaction, the **specific sequence** will be accumulated in billions of copies (**amplicons**).
- In only 20 cycles, PCR can product about a million ( $2^{20}$ ) copies of the target.



## 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.

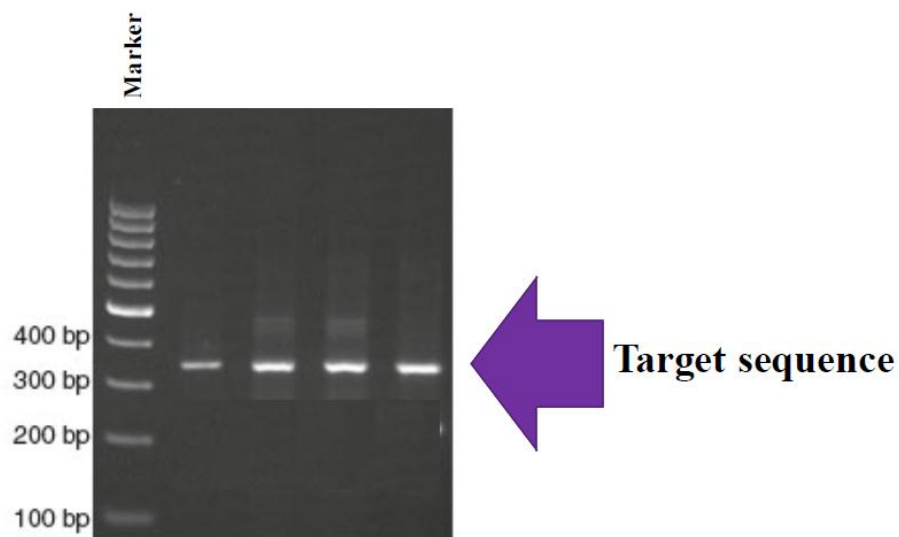
PCR machine: Load the reactions into 0.2 ml PCR tubes. Close lid and turn knob until it stops. Turn on PCR machine (switch on back). The menu should point at “START” (if not use arrows up and down). Press “ENTER”





**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.

## AGE results



## 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.

## PCR advantages:

- Simplicity, easier methodology, sensitive, extensively validated standard operating procedure and availability of reagents and equipment



# Primer Design Guidelines:

## 1. Primer sequence:

- Must be complementary to flanking sequences of target region.
- Avoid:
  - Complementary sequences between primers.
  - Repeat (ex: ATATATAT) → **misprime**.
  - Runs (ex: AGCGGGGGAT) → **misprime**.
  - Mismatch at 3' end.

## 2. Primer length:

- It is generally accepted that the optimal length of primers is **18-25 bp**.
- Not too long nor too short



**Thanks**