POLYMERASE CHAIN REACTION

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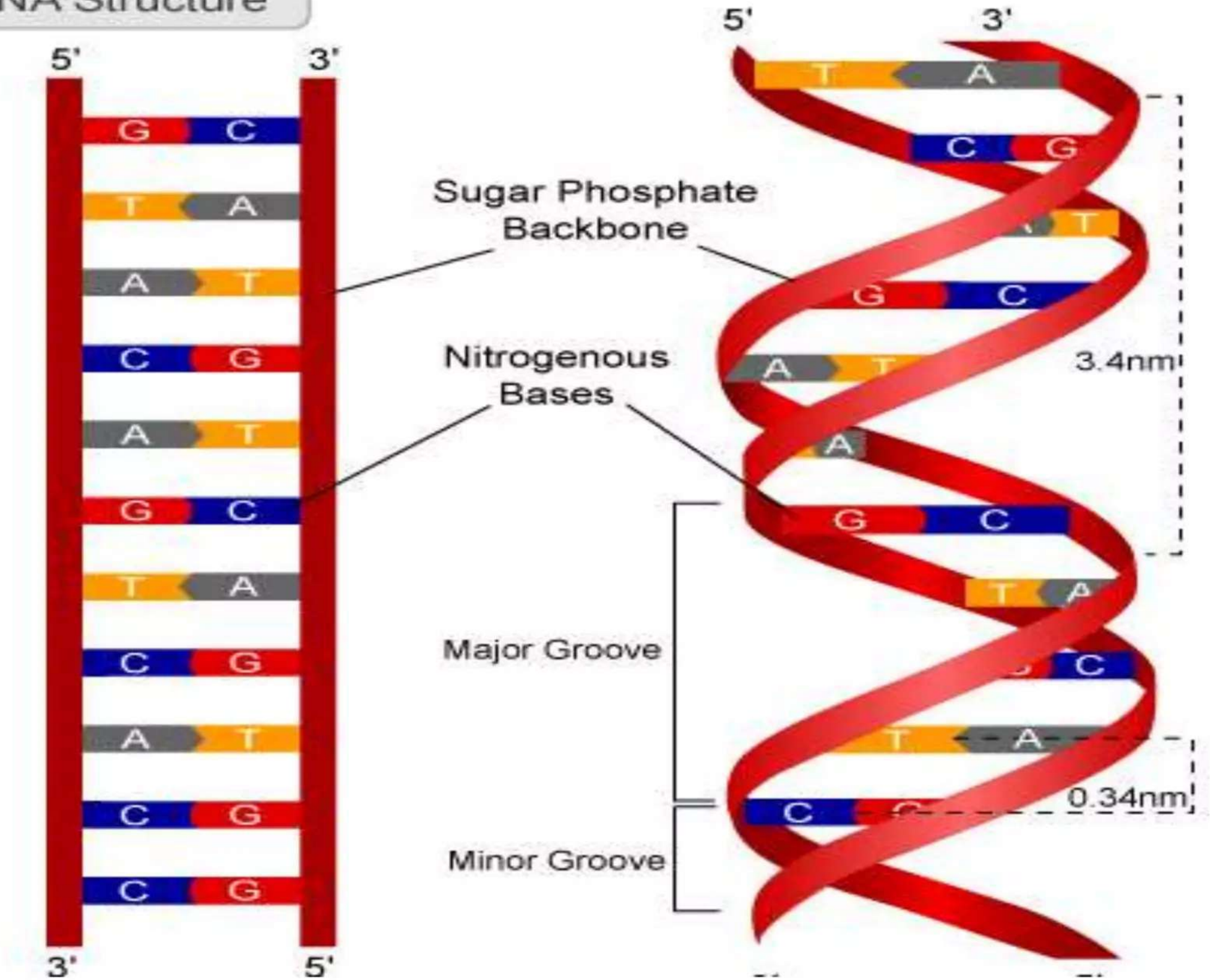
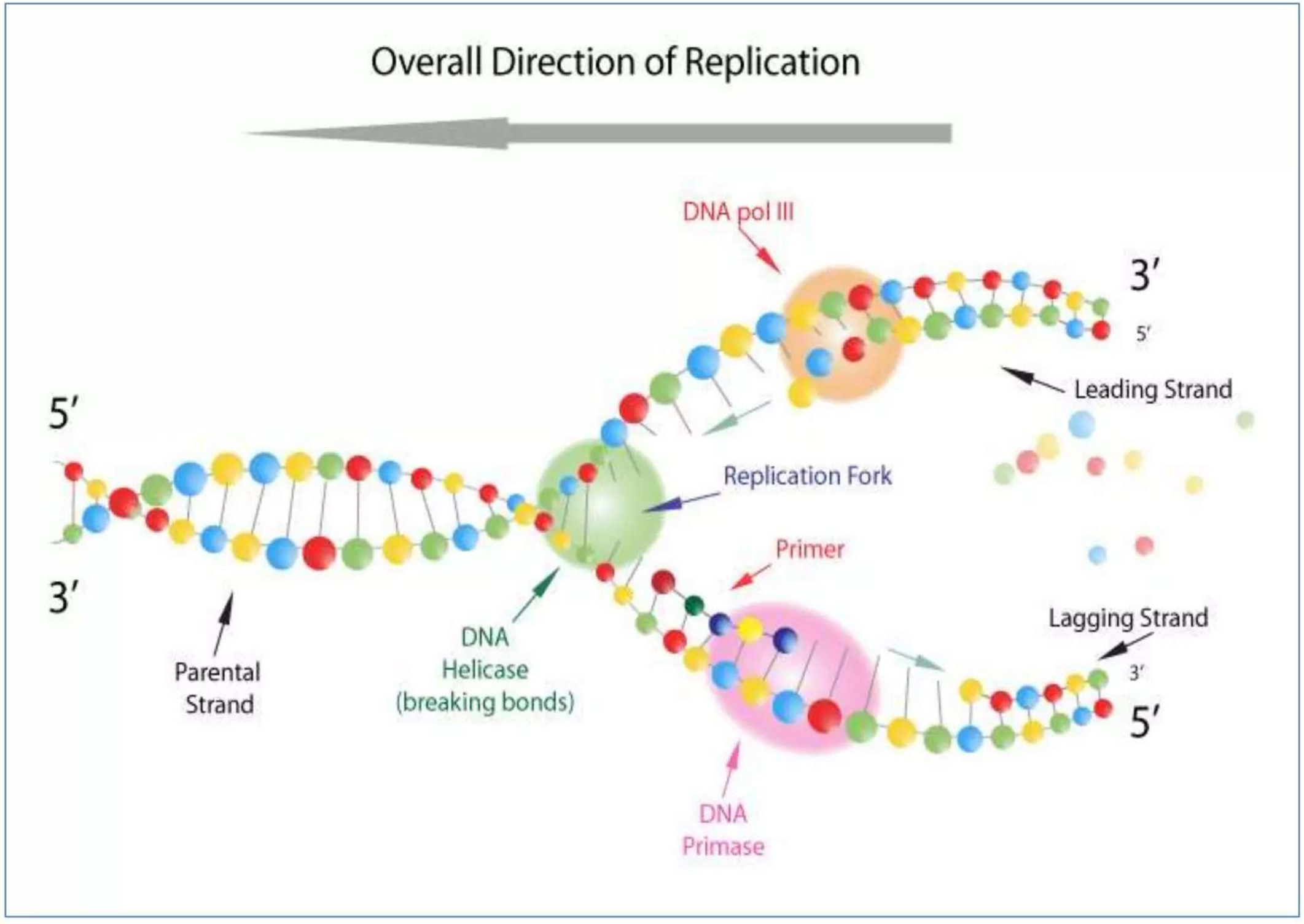
DNA Structure




Overall Direction of Replication
DNA pol III
3′
5′
Leading Strand
5′
Replication Fork
Primer
3′
Lagging Strand
3′
5′
Parental
Strand
DNA
Helicase
(breaking bonds)
DNA
Primase

INTRODUCTION-PCR

- In genetic engineering , the availability of sufficient amount of DNA of interest is indispensable in order to study its structure , function and manipulation.

- Until recently the micro-organisms are the chief source to obtain multiple copies of DNA of interest.

- Now a new and novel cell free technique are available to obtain millions of copies, known as polymerase chain reaction (PCR technique)

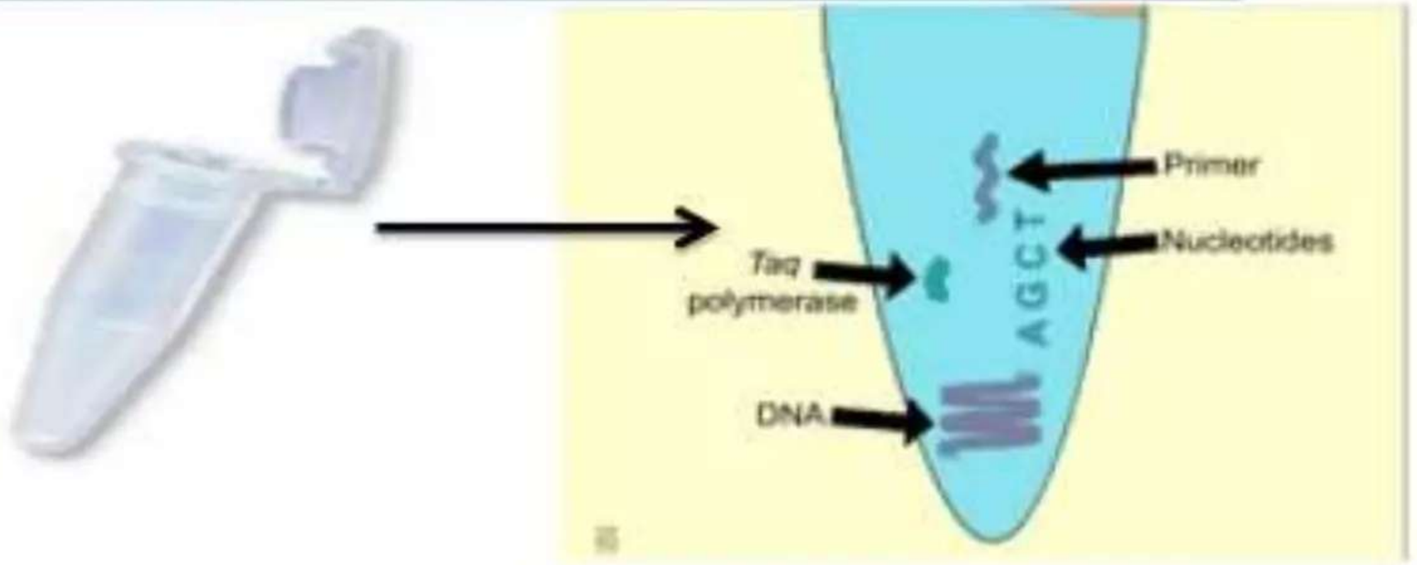
- PCR, polymerase chain reaction, is an in-vitro technique that allows amplification of a particular DNA sequence and is able to generate innumerable copies of specific DNA sequence.

- The technique is so precise and powerful, that even a single gene sequence can be amplified into a detectable amount within a short time.

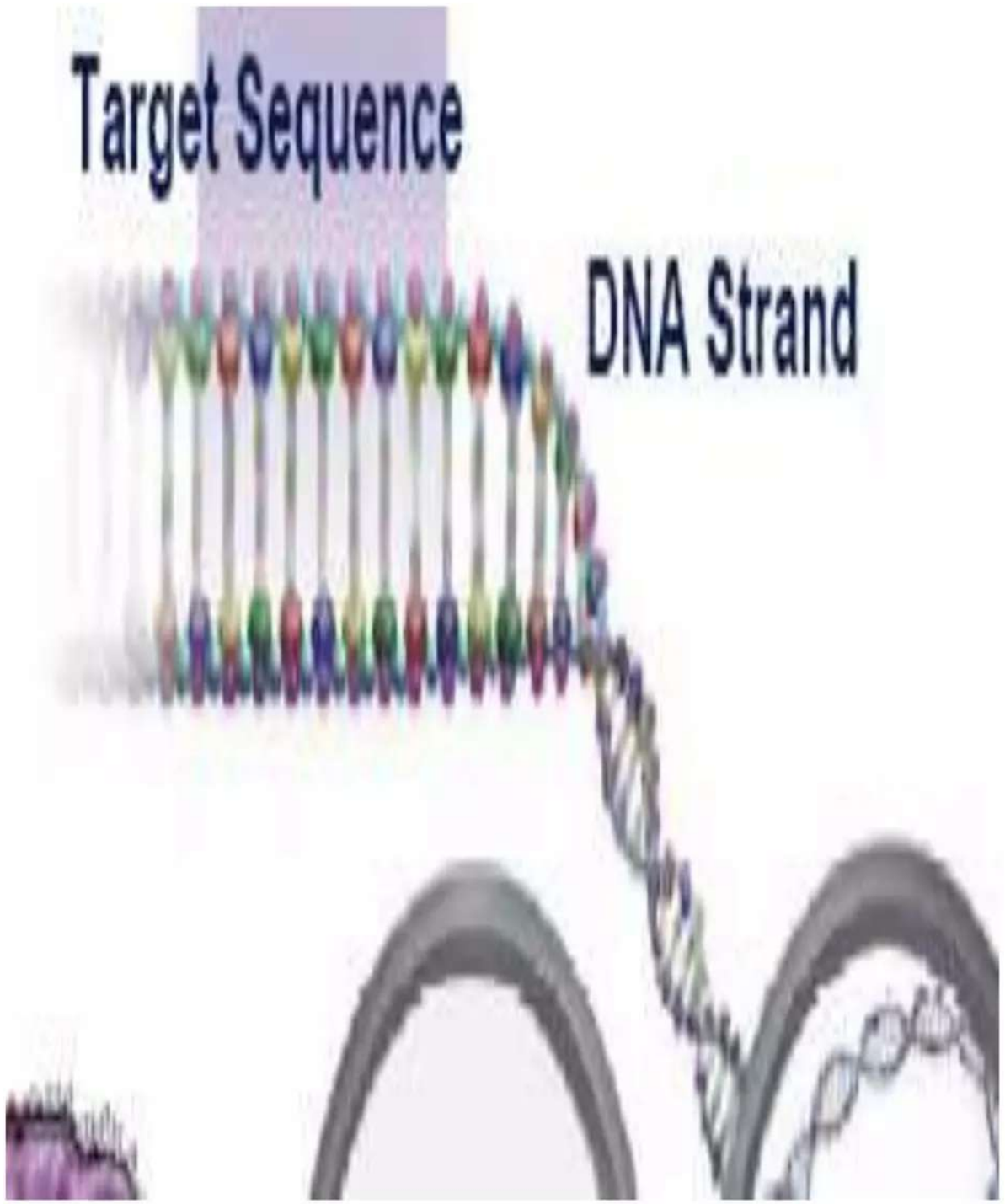
- The technique was independently designed and developed by Karymullis in 1980.

REQUIREMENTS FOR PCR

- DNA Template
- Primers
- Taq polymerase
- Deoxynucleoside - triphosphates(dNTPs)
- Buffer solution
- Divalent cations(eg.Mg^{2+})



1- DNA TEMPLATE

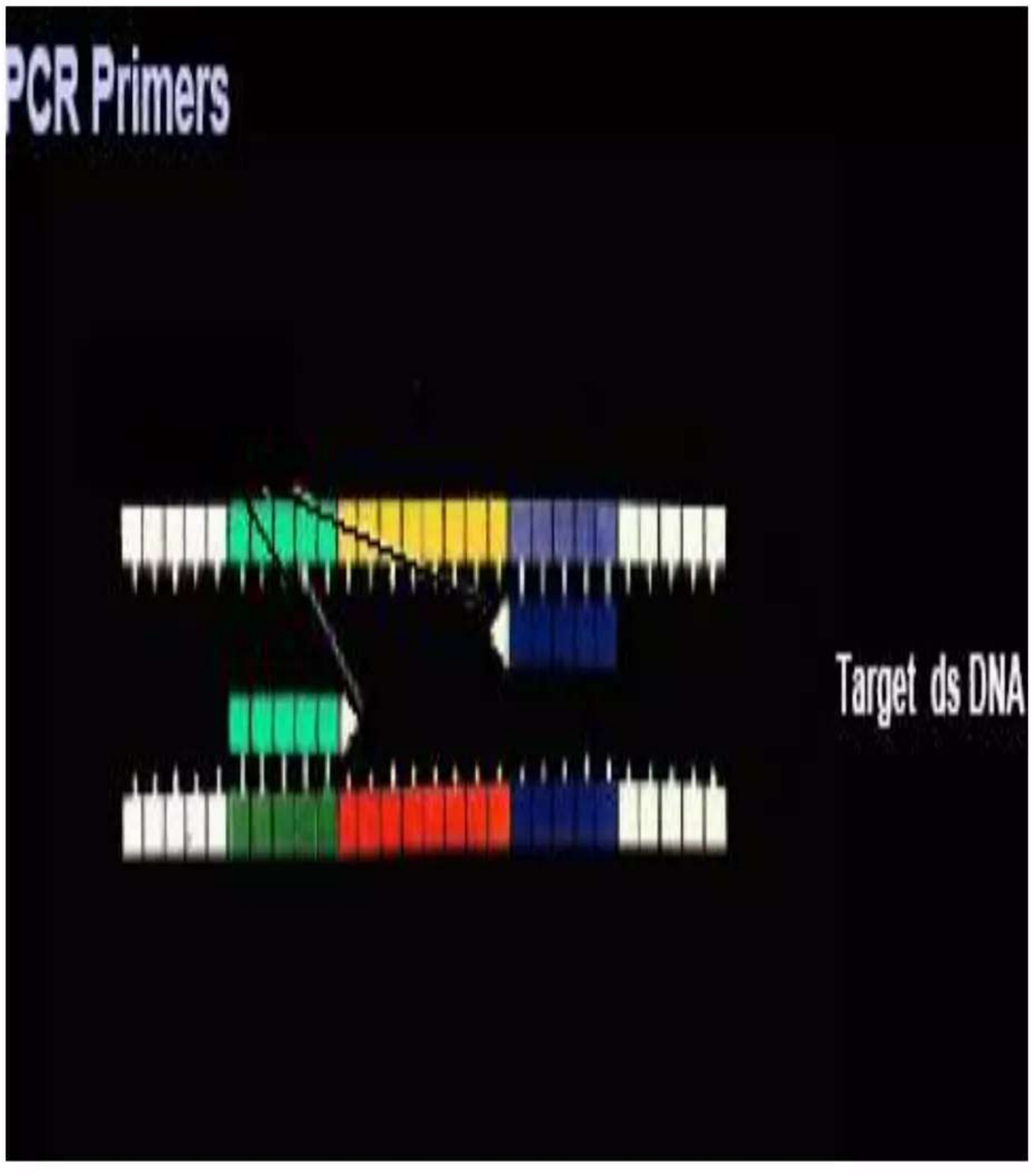


- DNA containing region (target) to be sequenced or amplified

- Size of target DNA to be amplified : up to 3 Kb

2- PRIMERS

- 1 sets of primers
- Generally 20-30 nucleotides long
- Synthetically produced
- complimentary to the 3’ ends of target DNA
- not complimentary to each other



3-ENZYME

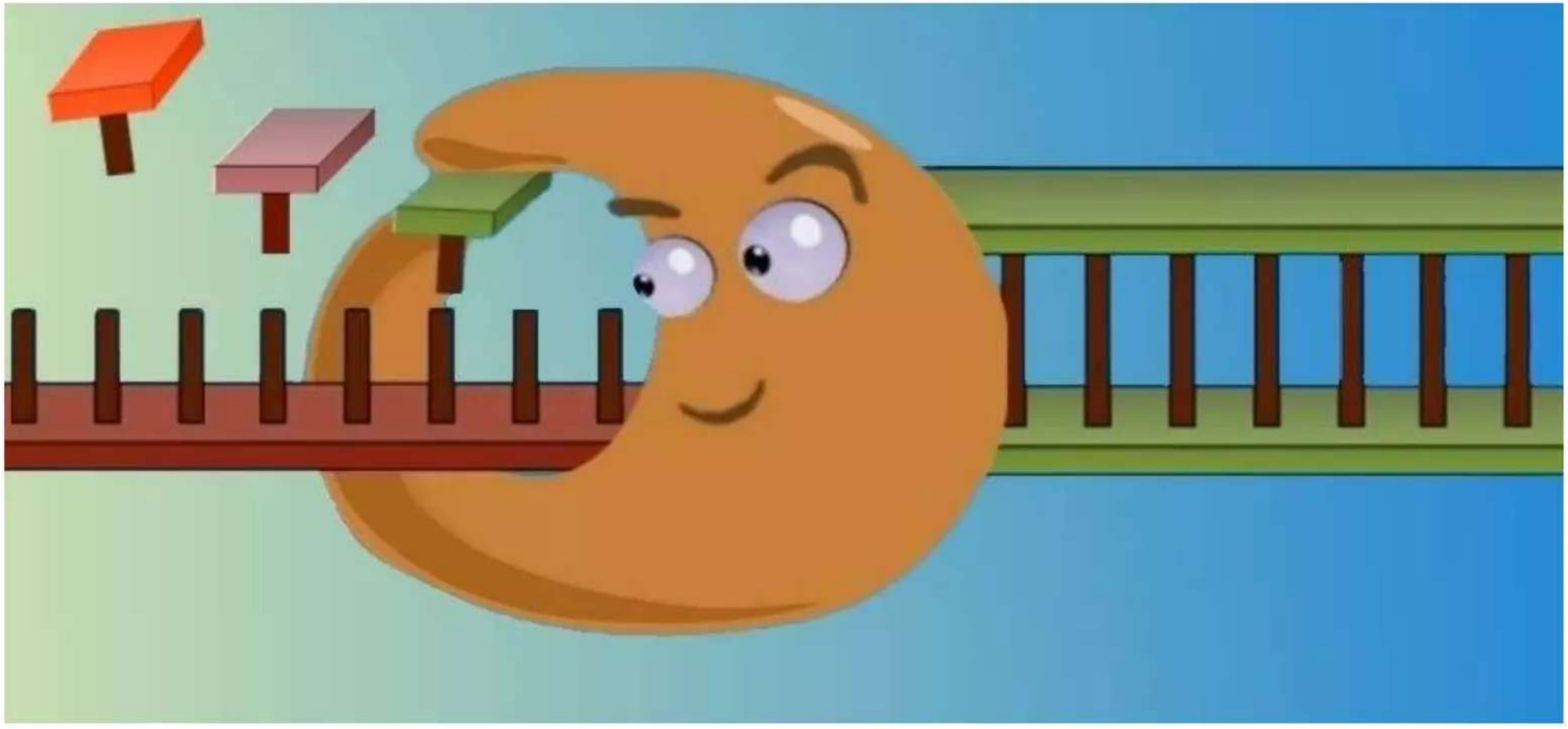
- Usually Taq Polymerase or anyone of the natural or Recombinant thermostable polymerases

- Taq polymerase – obtained from Thermus aquaticus

- This enzyme,Taq polymerase has an optimum temperature 72°C and the enzyme remains stable at 95^0 C

➢Another reason for using thermostable DNA polymerase is that :

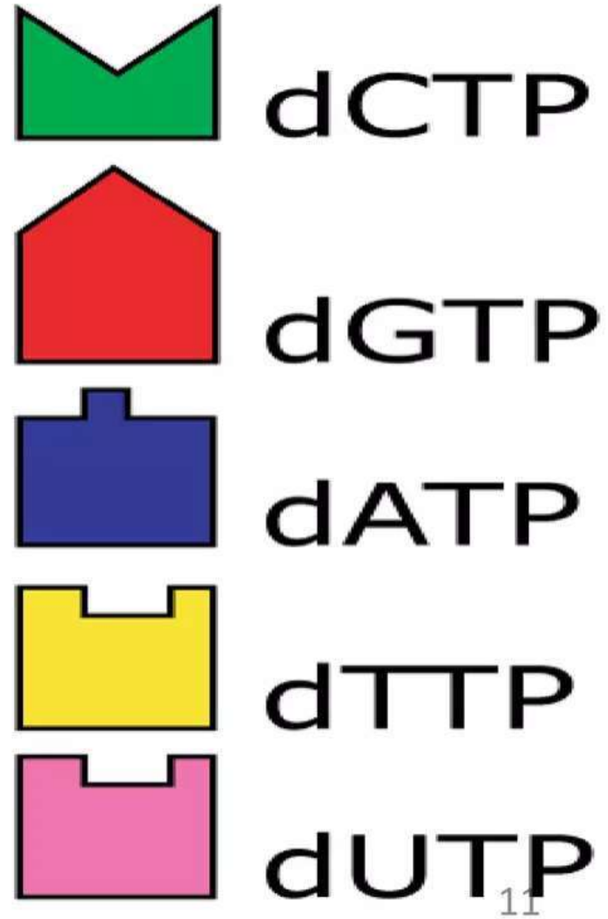
❑ DNA synthesised at high temperature is relatively more error free than the DNA synthesised at ordinary temperature like 25° to 30°C.



DEOXYNUCLEOTIDE TRIPHOSPHATES

➢ The four nucleosides containing triphosphate groups; dNTPs

➢They are the building-blocks from which the DNA polymerase synthesizes a new DNA strand.



BUFFER SOLUTION

- Providing a suitable chemical environment for optimum activity and stability of the DNA polymerase

- Tris HCl , KCl , MgCl2 , Taq buffer etc can be used.

DIVALENT CATIONS

- Magnesium or manganese ions

- Generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR mediated DNA mutagenesis

- As higher Mn^{2+} concentration increases the error rate during DNA synthesis

- PCR, POLYMERASE CHAIN REACTION, is an in-vitro technique that allows amplification of a particular DNA sequence and is able to generate innumerable copies of specific DNA sequence.

- DNA Template
- Primers
- Taq polymerase
- Deoxynucleoside
- triphosphates(dNTPs)
- Buffer solution
- Divalent cations(eg.Mg^{2+})

PRINCIPLE

A characteristic feature of DNA is that bringing it to a certain high temperature known as melting temperature (Tm)

↓

The double strands fall apart into single strands by dissolution of the H-bonds

↓

When the temperature is lowered , the two strands again anneal to restore the double helix structure (annealing temperature)

↓

PCR technique utilizes this property of DNA for replication

THE PCR MACHINE

- The PCR machine is a thermal cycler in which the temperature , time and number of cycles required for amplification of the sample DNA can be preset for automatic operation.

- Normal DNA polymerase is thermo labile → a thermo stable DNA polymerase obtained from a thermophilic organism is used.

- This avoids the addition of the enzyme in each cyclic operation.

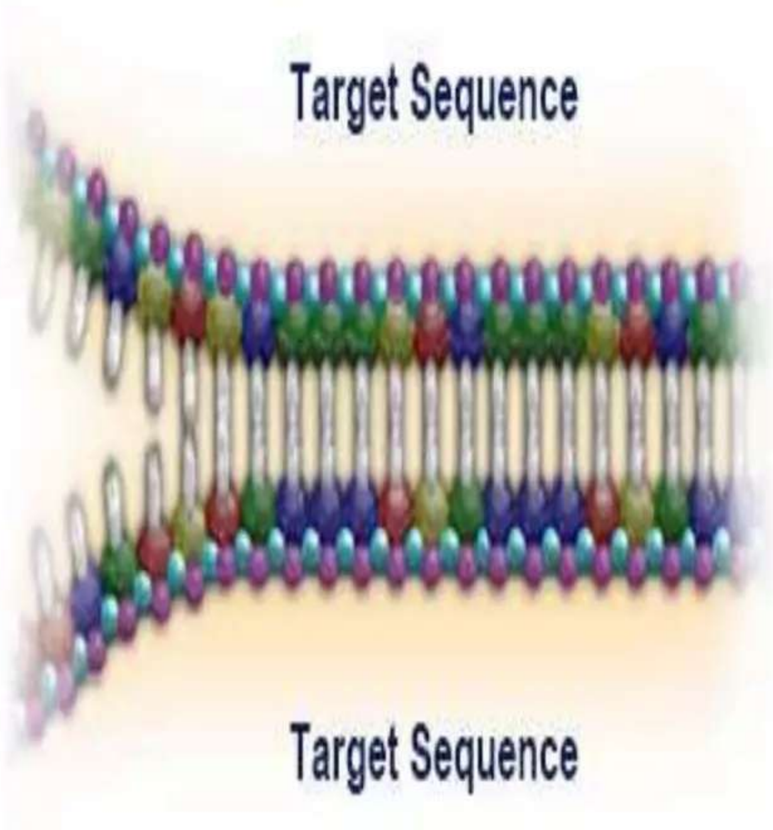
PCR INSTRUMENT

THE PCR CYCLE

Comprised of 3 steps:

- Denaturation of DNA at 95^0C
- Primer hybridization (annealing) at $40\text{-}50^0C$
- DNA synthesis (Primer extension) at 72^0C

PCR Cycle - Step 1 - Denaturation Template DNA by Heat (95°C)

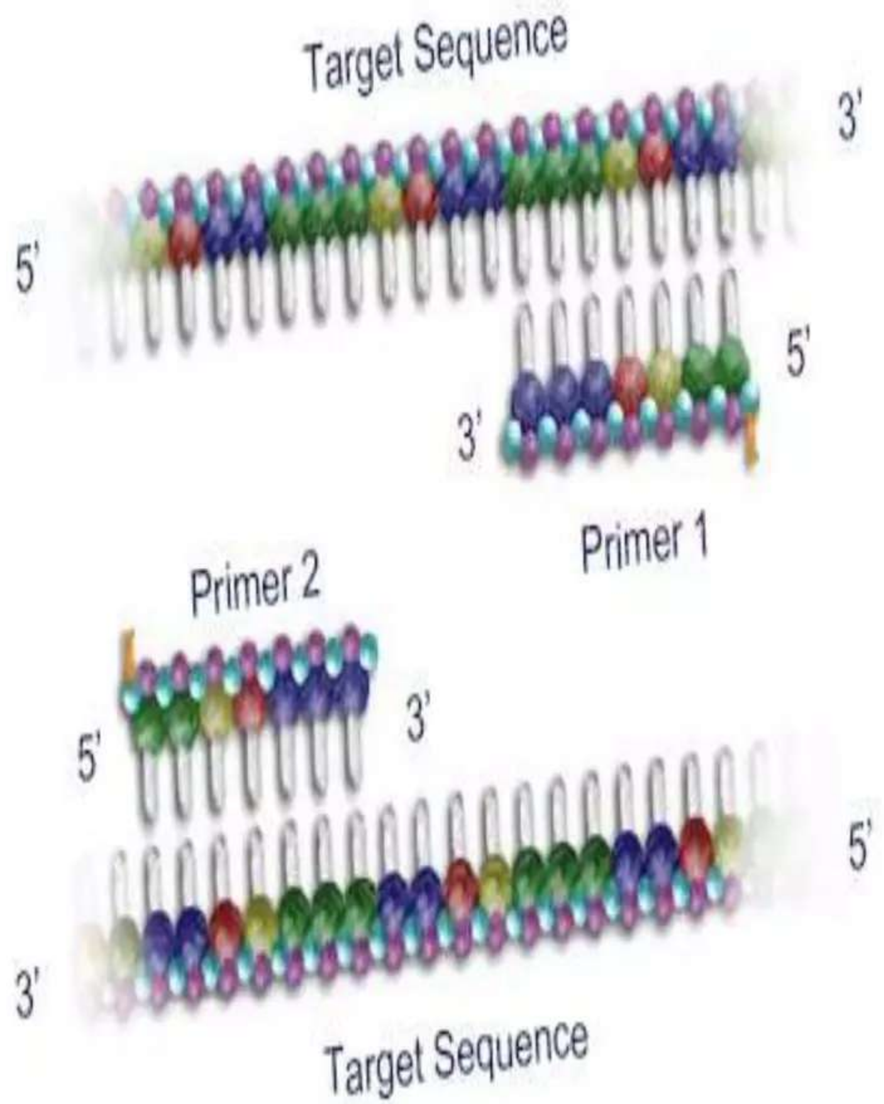


DENATURATION

- The first step is the denaturation of DNA sample in a reaction mixture to 95 °C .
- At this thermal condition DNA strand gets separated.
- The temperature is maintained up to 5 minutes.

PCR Cycle - Step 2 –

Temperature is lowered (T_m) and primers anneal to target sequences

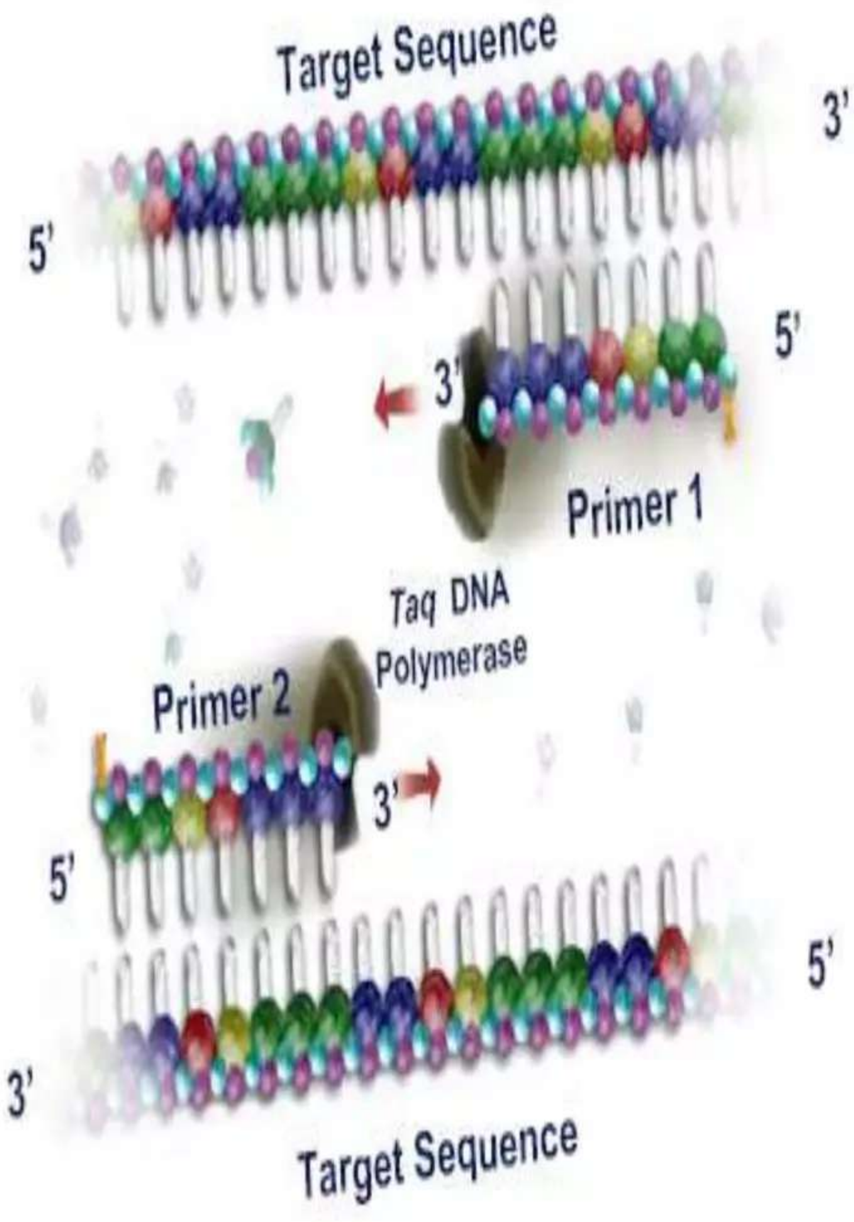


RENATURATION

- The temperature is lowered to 50 °C to allow the 2 oligonucleotide primers to anneal (bind) to the complementary sequence in the DNA molecule.
- The time duration of 30 seconds is maintained for renaturation.

PCR Cycle - Step 3 -

At 72 °C *Taq* DNA polymerase catalyses primer extension as complementary nucleotides are incorporated

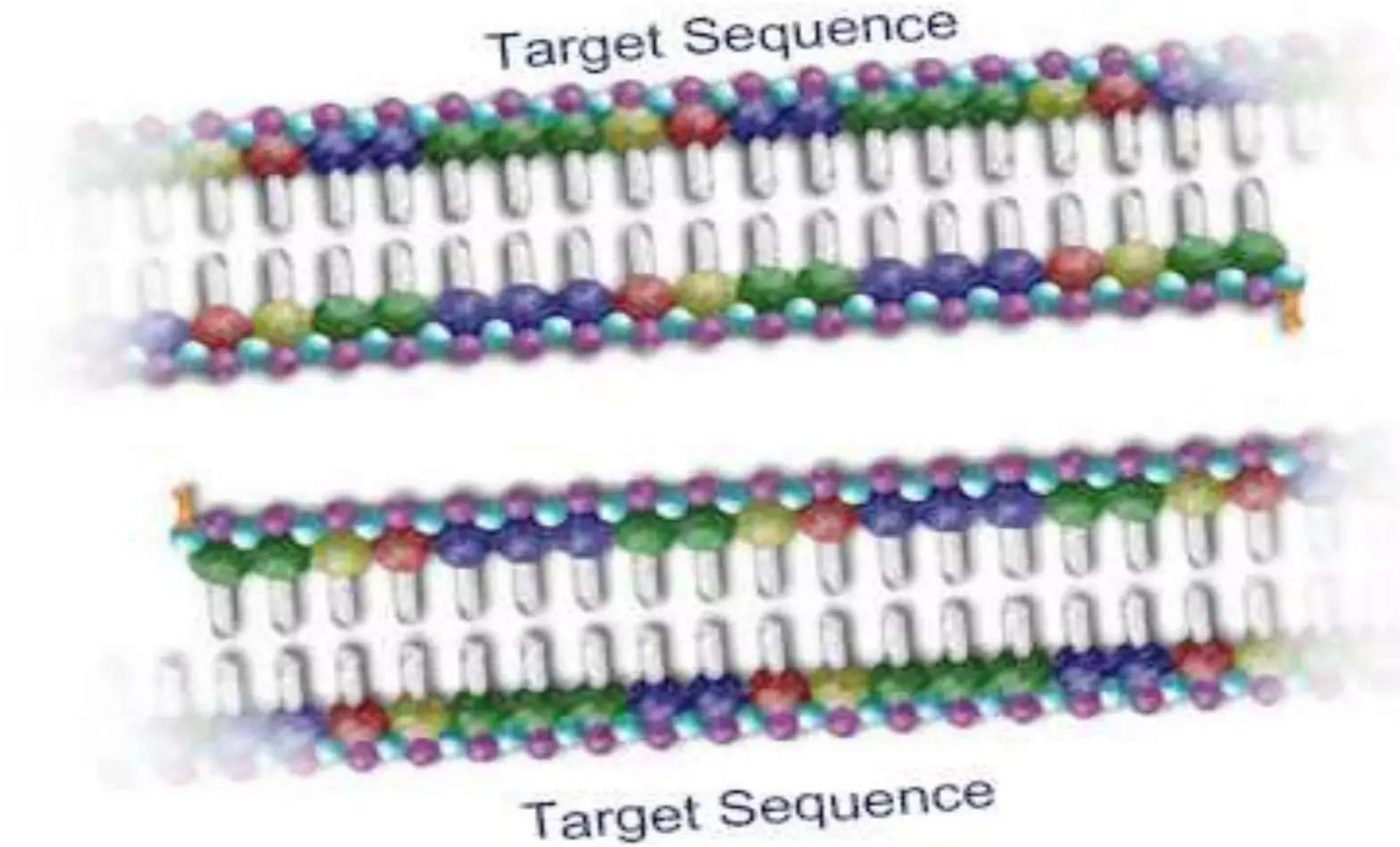


SYNTHESIS OF NEW DNA

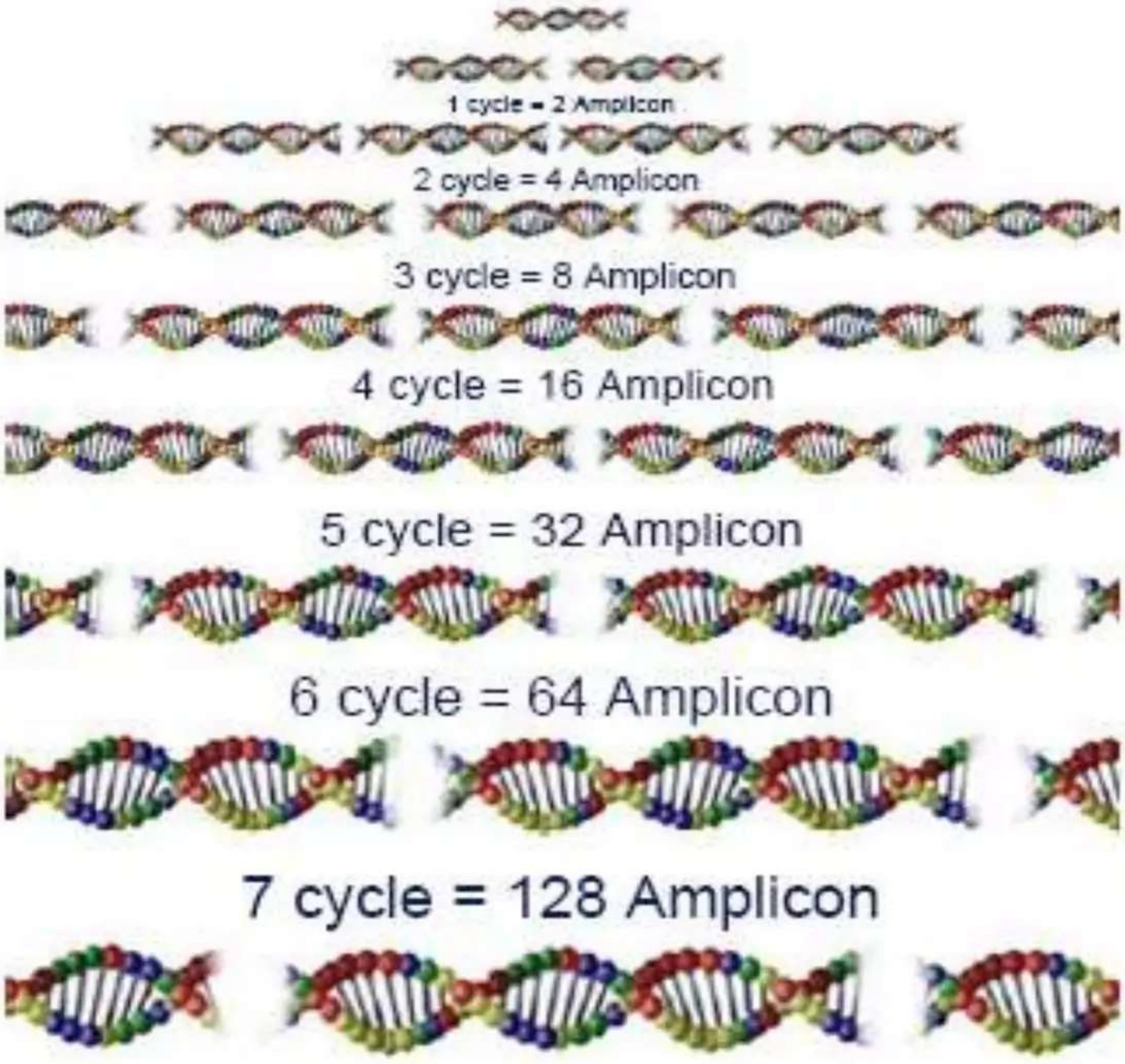
- The temperature is raised to 72-75 °C .
- At this elevated temperature , Taq polymerase shows optimum activity and initiates DNA synthesis at 3 hydroxyl end of each primer.

End of the 1st PCR Cycle –

Results in two copies of target sequence

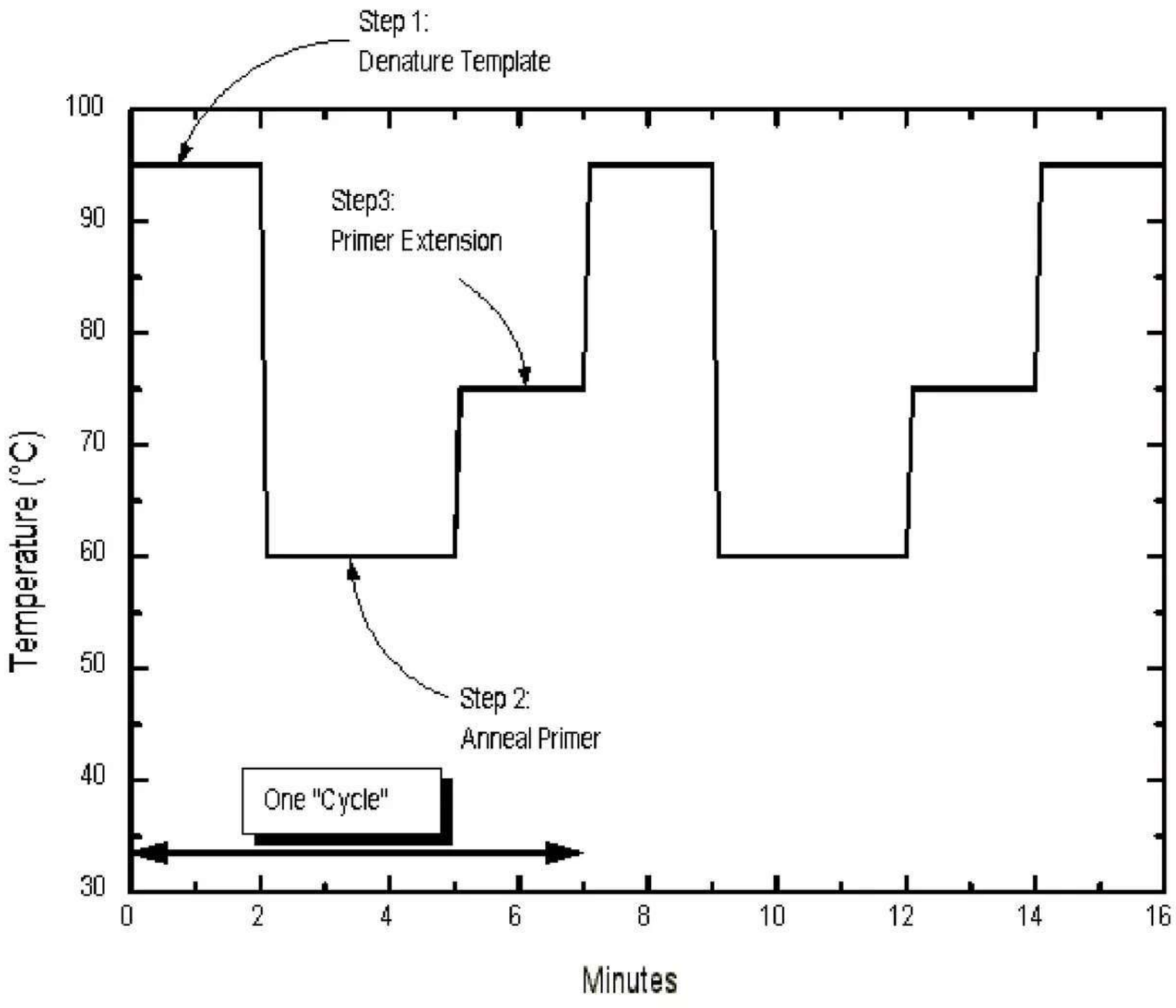


Target Amplification



No. of Cycles	No. Amplicon Copies of Target
1	2
2	4
3	8
4	16
5	32
6	64
20	1,048,576
30	1,073,741,824

STANDARD THERMOCYCLE



TYPES OF PCR

- Several modifications of PCR have been developed for specific applications

- Another reason for the modification is to overcome problems or trouble shooters during amplification.

- Following are some of the improved version of PCR.

- Nested PCR
- Inverse PCR
- Hot start PCR

1. NESTED PCR

➢ Nested PCR is a variation of the polymerase chain reaction , in that 2 pairs (instead of 1 pair) of PCR primers are used to amplify a fragment.

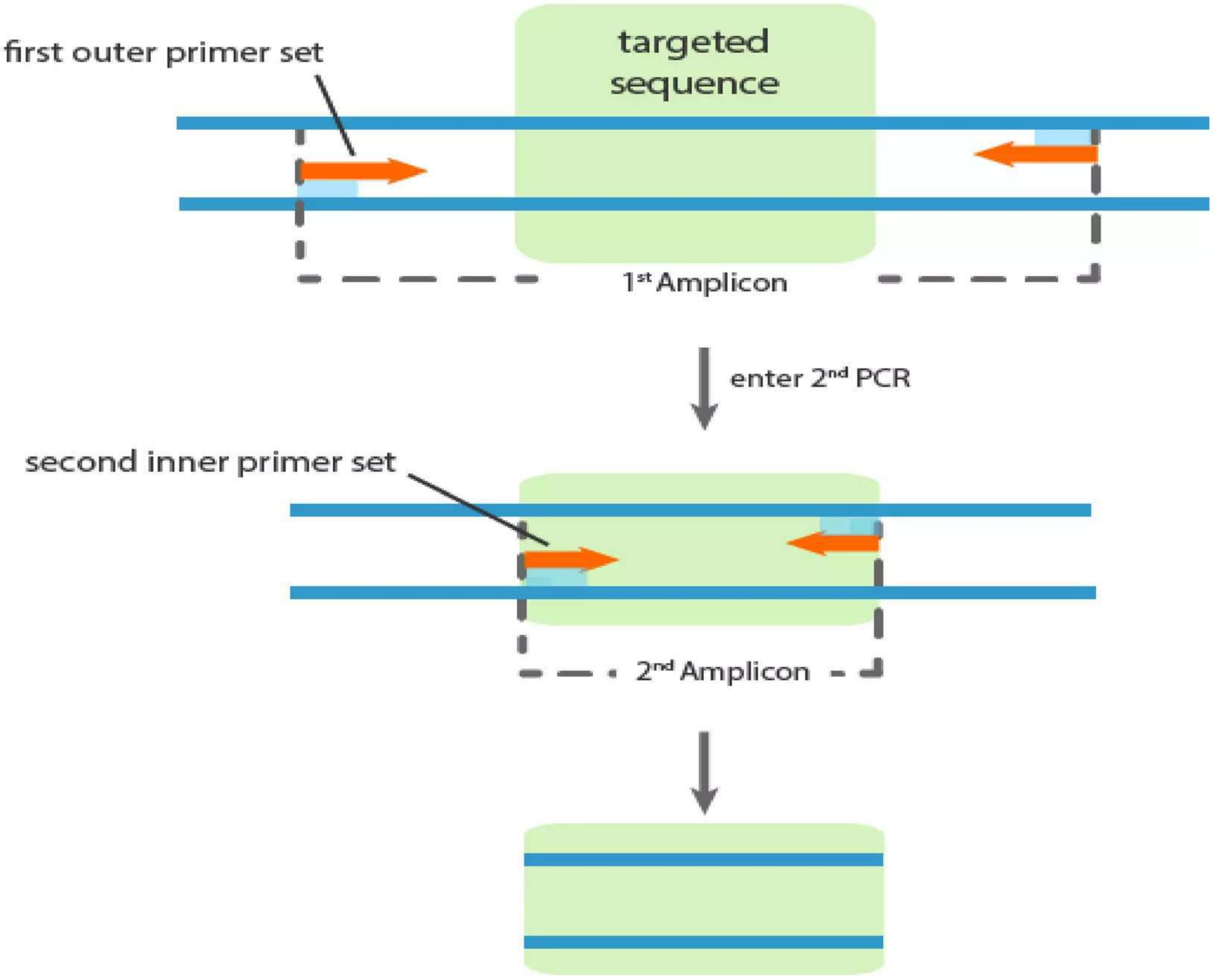
❑TECHNIQUE

➢Step 1 : primers binds to template DNA and PCR start.

➢Step 2 : PCR products from the first PCR reaction are subjected to a second PCR run.

➢Step 3 : we can get multiple copies

Nested PCR


first outer primer set
targeted sequence
1st Amplicon
enter 2nd PCR
second inner primer set
2nd Amplicon

2. HOT-START PCR

➢A technique that reduces non-specific amplification during the initial set up stages of the PCR.

➢It may be performed manually by heating the reaction components to the melting temperature (eg.95°C) before adding the polymerase.

➢ 2 types

a. Mechanical hot start b. non-mechanical hot start

❑ MECHANICAL HOT START PCR

➢All components of PCR are added to the PCR vial except for the DNA polymerase enzyme which will be added just at the first denaturation step.

❑ NON-MECHANICAL HOT START PCR

➢The use of a form of Taq DNA polymerase , for example , Amplitaq Gold which is activated only if the reaction mixture is heated at about 94°C (the first denaturation step)

➢ Other methods depend on covalent linking of the polymerase enzyme to certain inhibitors.

➢The enzyme becomes dissociated from these inhibitors at the first denaturation step.

PPase

The pyrophosphatase (Ppase) is activated (>70 °C) and hydrolyzes the PPi to 2 phosphate groups and facilitates the release of Mg2+.

PCR reaction start

Mg^{2+} Free

Mg++

Pfu

Pi Pi

Mg^{2+} Mg^{2+}

Mg2+

Pyrophosphate (PPi) has high affinity for Mg^{2+}. This Ppi-Mg^{2+} Complex suppresses non-specific before starting the PCR reaction

pfu DNA Polymerase is fully active at the temperatures above 70°C via pyrophosphate hydrolysis with our thermostable pyrophosphatase.

Pfu DNA Polymerase

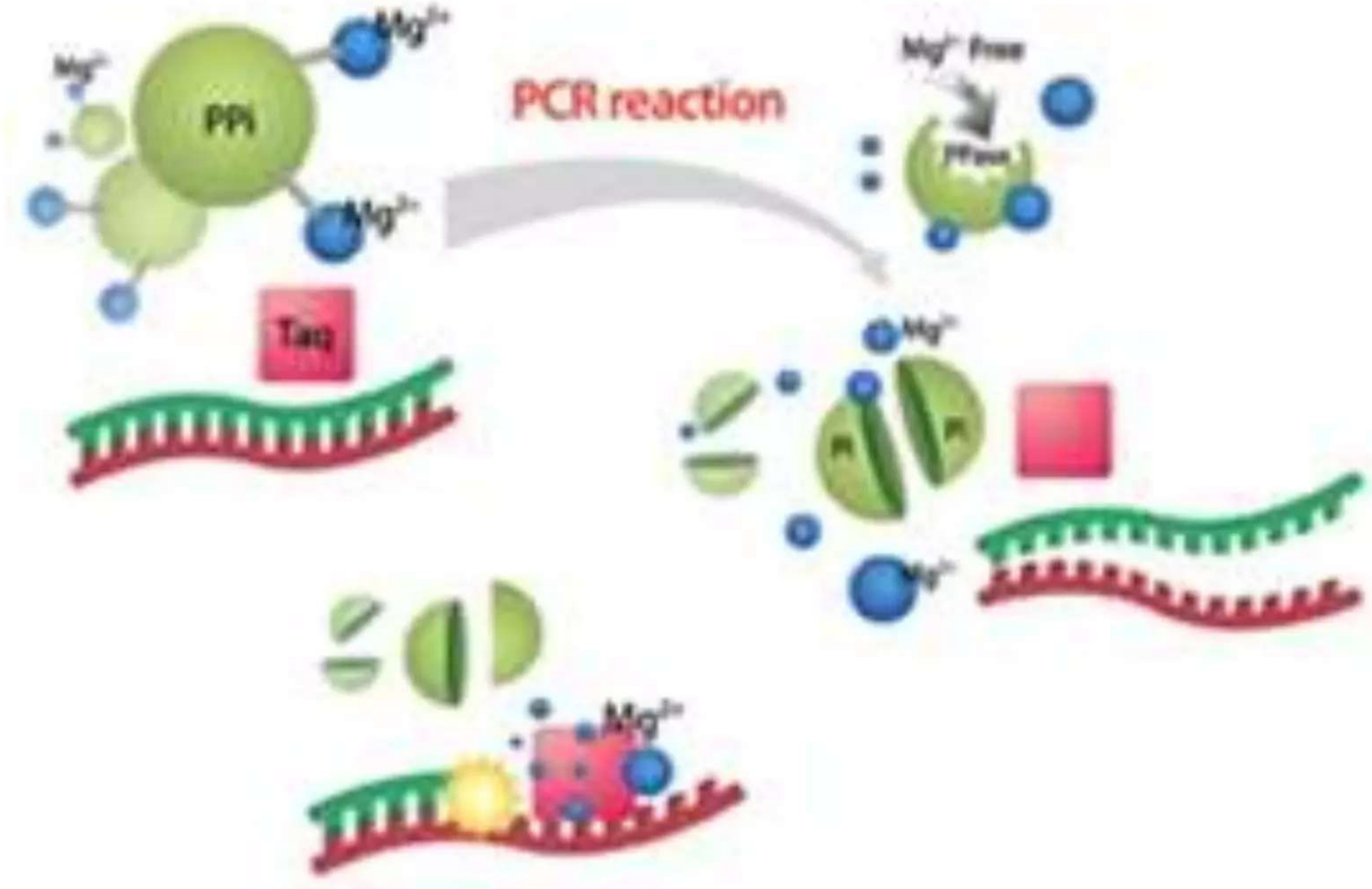
Pyrophosphate (PPi)

2 phosphate groups (Pi+Pi)

Mg^{2+}

DNA template

HOT START PCR



3. INVERSE PCR

- Target DNA is lightly cut into smaller fragments of several kilobases by restriction endonuclease digestion.

- Self-ligation is induced under low concentrations causing the phosphate backbone to reform.

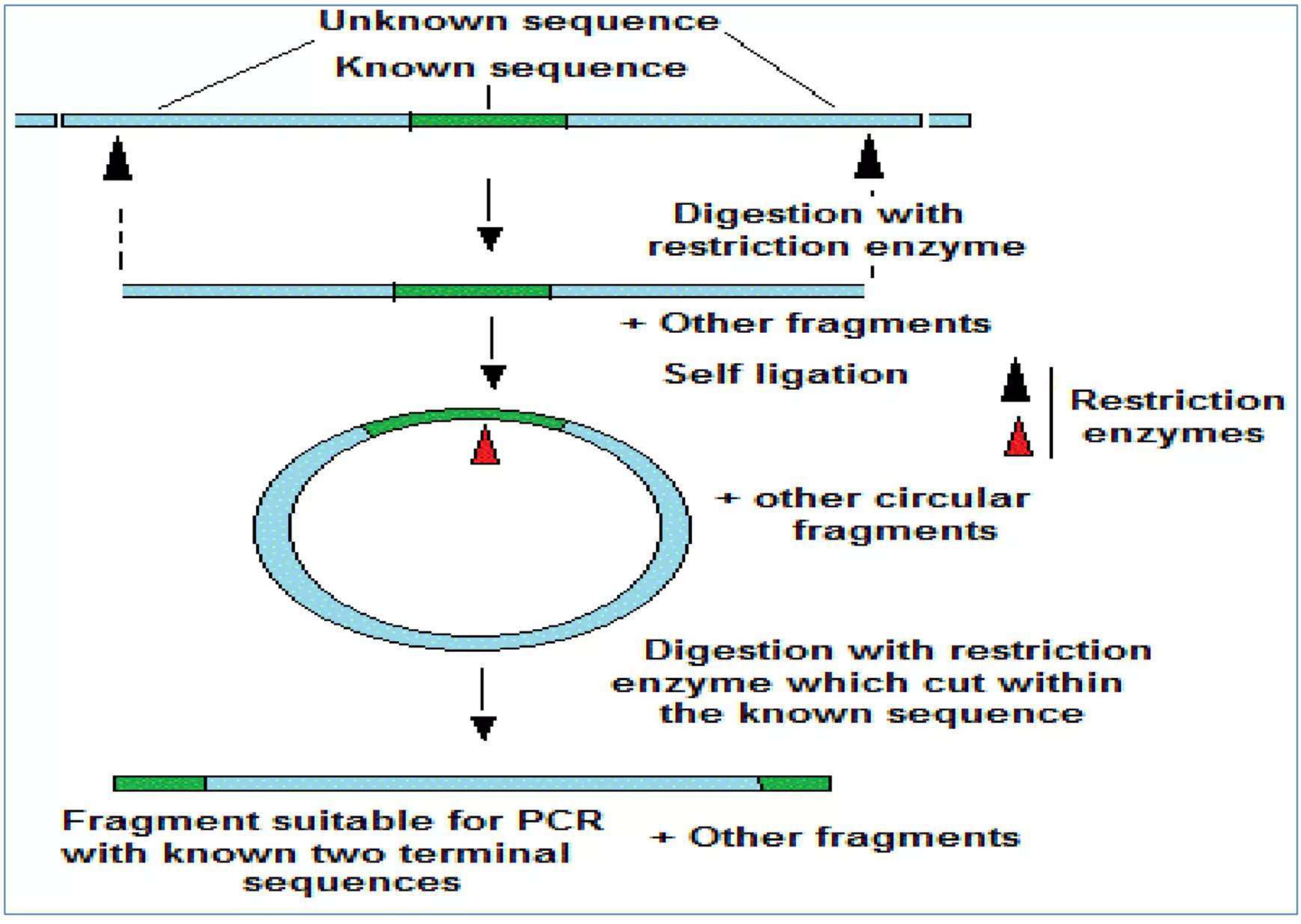
- This gives a circular DNA ligation product.

- Target DNA is then restriction digested with a known endonuclease.

- This generates a cut within the known internal sequence generating a linear product with known terminal sequences.

- This can now be used for PCR (polymerase chain reaction)

INVERSE PCR


Unknown sequence
Known sequence
Digestion with restriction enzyme
+ Other fragments
Self ligation
Restriction enzymes
+ other circular fragments
Digestion with restriction enzyme which cut within the known sequence
Fragment suitable for PCR with known two terminal sequences
+ Other fragments

OTHER VARIATIONS OF THE PCR

- Colony PCR
- Multiplex PCR
- Asymmetric PCR
- Long PCR
- Allele specific PCR
- Real time PCR
- Reverse transcriptase PCR (RT-PCR)
- In situ PCR
- Long accurate PCR
- Touch down PCR
- AFLP PCR

ADVANTAGES

- Automated, fast, reliable (reproducible) results
- High output
- Sensitive
- Broad uses
- Defined, easy to follow protocols
- Commercial kits are now available for easy PCR reaction setup and amplification

DISADVANTAGES

- Extreamly liable to contamination
- High degree of operator skills required
- False positive results due to amplifications
- Expensive to the developing world

APPLICATIONS

- It is extensively used for the amplification of a particular target DNA sequence
- Genome mapping and gene function determination
- Biodiversity studies (e.g. evolution studies)
- Detection of drug resistance genes
- In plant biotechnology, PCR is used in plant disease identification , presence of any target gene in crop plants

- The PCR is widely employed in disease diagnosis (early detection of cancer, viral infections...) .

- The speed and sensitivity of PCR helps in prenatal diagnosis of inherited disease.

- Forensic (DNA fingerprinting)

- PCR has an important role in gene manipulation and expression studies.

THANK YOU