Amplification of specific DNA fragment by Polymerase Chain Reaction

Aim:

1. To amplify a specific DNA fragment by Polymerase Chain Reaction.

2. To determine the molecular weight of amplified fragment.

Introduction:

Polymerase Chain Reaction (PCR) is an in vitro method of enzymatic synthesis of specific DNA fragment developed by Kary Mullis in 1983. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons).

Components of PCR:

1. DNA template that contains the region to be amplified.
2. Two primers complementary to the 3' ends of each of the sense and anti-sense strand of the DNA
3. Thermostable DNA polymerase like Taq (from *Thermis aquaticus*), Pfu (from *Pyrococcus furiosus*) etc.
4. Deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), the building blocks from which the DNA polymerase synthesizes a new DNA strand.
5. Buffer solution which provides a suitable chemical environment for optimal activity and stability of DNA polymerase.
6. Bivalent magnesium/manganese ions, which are necessary for maximum Taq polymerase activity and influences the efficiency of primer to template annealing.

Principle:

PCR comprises the following steps:

Initialization step (Initial denaturation): This step consists of heating the reaction mixture to 95 ^{o}C for 2-5 minutes (up to 10 min depending on enzyme characteristics and template complexity) to ensure that all complex, double-stranded DNA (dsDNA) molecules are separated into single strands for amplification.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction mixture to 95 ^{o}C for 10 seconds – 1 minute. As a result the template DNA denatures due to disruption of the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

Annealing step: The annealing step (30 sec to 1 min, at temperatures 45–60 °C), is required so that the primers bind to the complementary sequence on each of the DNA single strands. The primers are designed such that they bracket the target of interest and the region of sequence that lies between them is referred to as the amplicon. In general, the annealing temperature may be estimated to be 5 °C lower than the melting temperature of the primer-template DNA duplex.

Extension/Elongation step: The final stage is the extension step (20 sec to 1 min at 72 °C), which is performed so that the DNA polymerase extends the primer sequences from the 3' of each primer to the end of the amplicon. A 1 min extension is typically sufficient to synthesize PCR fragments up to 2 kilobases (kb). To amplify larger fragments, the elongation step is extended at a rate of 1 min per kb. During the first extension, the template will not be length limiting and so templates will be synthesized that exceed the amplicon length. In subsequent extension steps, the amplicon length will be defined by the primer sequence at each end.

Final elongation: This single step is performed at a temperature of 72 ^{o}C for 5- 10 min minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Denaturation, annealing and extension steps are repeated 20-35 times in an automated thermocycler that can heat and cool the reaction mixture in tubes within a very short time.

The amplification, as a final number of copies of the target sequence, is expressed by the following equation: **$(2^n - 2n)\ x$**

Where n is the number of cycles, 2n is the first product obtained after the first cycle and second products obtained after the second cycle with undefined length, x is the number of copies of the original template.

Final hold: This step may be employed for short-term storage of the reaction mixture at 4°C for an indefinite time.

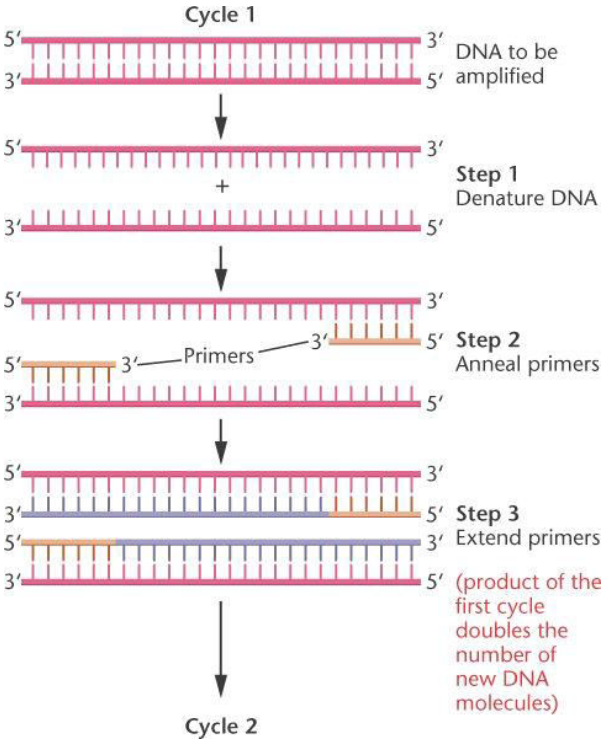

Fig 1: Basic steps of PCR Reaction

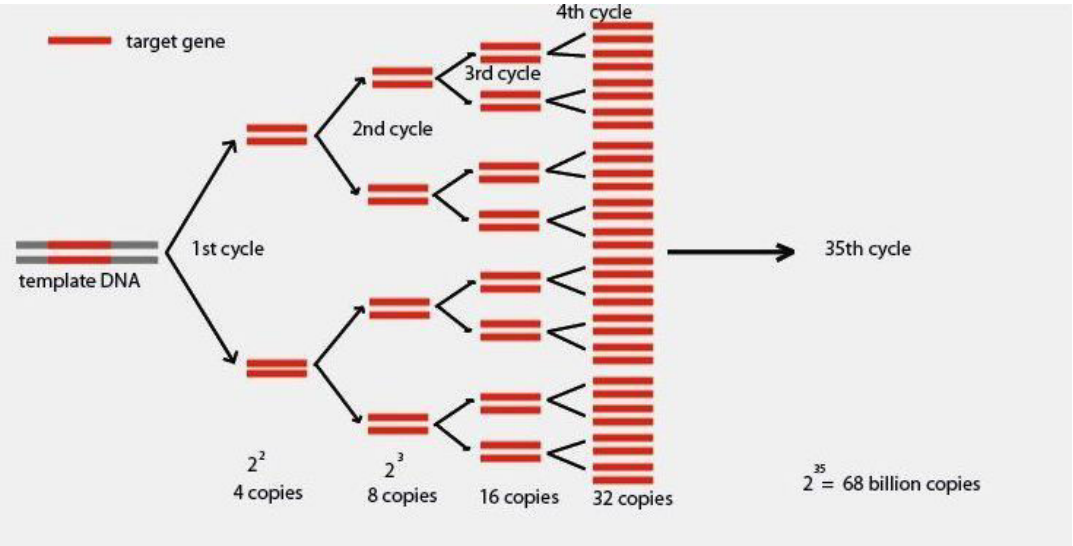

Fig 2: Amplification process of a particular template DNA

Procedure:

1. Preparation of master mix for PCR

To a PCR tube add all the following ingredients in order

S. No.	Components of PCR master mix	Volume (µL)
1.	Molecular Biology Grade Water	10.8
2.	10X Assay Buffer	2
3.	Template DNA (1ng–1µg)	1
4.	Forward Primer (10 nM)	1
5.	Reverse Primer (10 nM)	1
6.	25 mM MgCl2	2
7.	2.5 mM dNTP Mix	2
8.	Taq DNA Polymerase (~ 1 unit per 20 µL reaction)	0.2
	Total Volume	20

2) Tap the tube for 6-8 times to mix the contents thoroughly and do short spin.

3) Add 25 µl of mineral oil in the tube to avoid evaporation of the contents **(optional)**.

4) Place the tube in the thermocycler block and set the program to get DNA amplification.

NOTE: It is not required to add mineral oil if the thermocycler is equipped with a heating lid.

Carry out the amplification in a thermocycler for 30-35 cycles using the following reaction conditions.

Set the PCR programme as follows:

Initial denaturation at 94 ^{o}C for 5 minutes

Denaturation at 94^{o}C for 30 seconds

Annealing at 58^{o}C for 30 seconds

Extension at 72^{o}C for 45 seconds

} **30 – 35 cycle**

Final Extension at 72^{o}C for 10 minutes

Cooling at 4^{o}C

Observation and Result:

After completion of the PCR, perform agarose gel electrophoresis. Compare the amplified product with the ladder and determine the size.