PCR Amplification from genomic DNA and analysis by agarose gel electrophoresis.

Introduction

- Polymerase Chain Reaction (PCR) is an in vitro method of enzymatic synthesis of specific DNA fragment developed by Kary Mullis in 1983. It is a very simple technique for characterizing, analyzing and synthesizing DNA from virtually any living organism (plant, animal, virus, bacteria). PCR is used to amplify a precise fragment of DNA from a complex mixture of starting material called as template DNA.
- A basic PCR requires the following components:

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, Vent, Pfu 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.

- Thermal cycler to maintain constant reaction temperature throughout the cycles.

Principle

- The purpose of a PCR is to amplify a specific DNA or RNA fragment.
- PCR comprises of three basic steps:

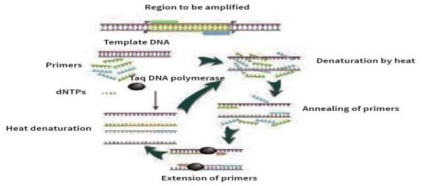


Fig 1: A PCR amplifies a specific DNA fragment by the incorporation of primers, dNTPs and Taq polymerase with periodic denaturation and renaturation of the template DNA.

1. **Initialization step:** This step consists of heating the reaction mixture to 94–96℃ for 1–9 minutes to initiate breaking of the hydrogen bonds in DNA strands.
2. **Denaturation step:** This step is the first regular cycling event and consists of heating the reaction mixture to 94–98℃ for 20–30 seconds. 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.
3. **Annealing step:** In this step the reaction temperature is lowered to 50–65℃ for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is 3-5℃ below the Tm (melting temperature) of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.
4. **Extension/Elongation step:** In this step, the temperature depends on the DNA polymerase used. Taq polymerase has its optimum activity at 75–80℃. Commonly a temperature of 68-72℃ is used with this enzyme. The DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by incorporating dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both upon the DNA polymerase used and on the length of the DNA fragment to be amplified. The DNA polymerase will polymerize a thousand bases per minute at its optimum temperature. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential amplification of the specific DNA fragment.

5. **Final elongation:** This single step is occasionally performed at a temperature of 70–74℃ for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended. Denaturation, annealing and extension steps are repeated 20-30 times in an automated thermocycler that can heat and cool the reaction mixture in tubes within a very short time. This results in exponential accumulation of specific DNA fragments, ends of which are defined by 5' ends of the primers. The doubling of the number of DNA strands corresponding to the target sequences allows us to estimate the amplification associated with each cycle using the formula;

   Amplification = 2n, where n = No. of cycles.
6. **Final hold:** This step may be employed for short-term storage of the reaction mixture at 4°C for an indefinite time.

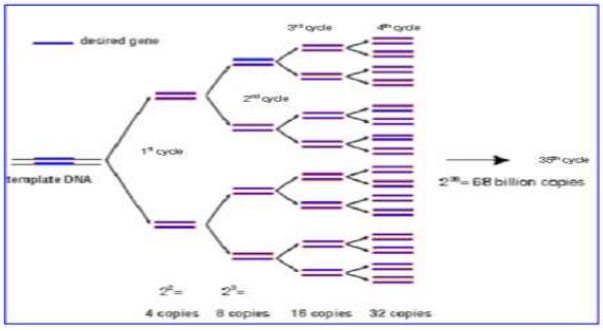


Fig 2: Amplification process of a particular template DNA

Requirements

Sr. No.	Product Code	Materials Provided	Quantity	Storage
			10 expts	
1	TKC117	10X Assay Buffer	0.07 ml	-20°C
2	TKC014	Control PCR Product	0.13 ml	-20°C
3	TKC118	2.5 mM dNTP mix	0.07 ml	-20°C
4	*MBT051	I Kb DNA Ladder	0.035 ml	-20°C
5	*TKC017	Forward Primer (10 nM)	0.015 ml	-20°C
6	*TKC018	Reverse Primer (10 nM)	0.015 ml	-20°C
7	*TKC120	Taq DNA Polymerase	0.01 ml	-20°C
8	*TKC022	Template DNA	0.025 ml	-20°C
9	ML024	Molecular Biology Grade Water	0.5 ml	R T
10	*TKC119	25 mM $MgCl_2$	0.07 ml	-20°C
11	MB002	Agarose	4.8 g	R T
12	ML016	50X TAE	120 ml	R T
13	ML015	6X Gel Loading Buffer	0.05 ml	2-8 °C
14	MB161	Mineral oil (optional)	0.3 ml	R T
15	CG282	Polypropylene Tubes, 0.2 ml (PCR Tubes)	10 Nos.	R T

Other Requirements

- **Glassware's:** Measuring cylinder, Beaker
- **Reagents:** Ethidium bromide (10 mg/ml), Distilled water
- **Other requirements:** Thermocycler, Electrophoresis apparatus, UV Transilluminator, Vortex Mixer, Micropipettes, Tips, Adhesive tape, Microwave/ Hotplate/ Burner, Crushed ice

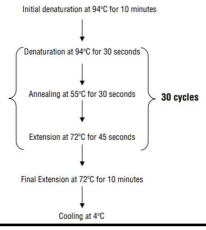
Procedure

1. Preparation of master mix for PCR: To a PCR tube add all the following ingredients in order.

Sr. No.	Ingredients for PCR	Volume in µl
1	Molecular Biology Grade Water	30.5 µl
2	10X Assay Buffer	5 µl
3	Template DNA	2µl
4	Forward Primer (10 nM)	1 µl
5	Reverse Primer (10 nM)	1 µl
6	25 mM $MgCl_2$	5 µl
7	2.5 mM dNTP Mix	5 µl
8	Taq DNA Polymerase	0.5 µl
	Total volume	**50 µl**

2. Tap the tube for 1–2 seconds to mix the contents thoroughly.
3. Add 25 µl of mineral oil in the tube to avoid evaporation of the contents.
4. Place the tube in the thermocycler block and set the program to get DNA amplification.

- ***PCR Amplification Cycle:*** Carry out the amplification in a thermocycler for 30 cycles using the following reaction conditions.



Agarose Gel Electrophoresis

1. Preparation of 1 X TAE buffer.
2. Preparation of Agarose gel.
3. Loading of DNA sample.
4. Electrophoresis.

• Observation and Results: