HiPer®PCR Teaching Kit

Product Code: HTBM016

Number of experiments that can be performed: 10

Duration of Experiment:
Protocol: 2 hours
Agarose Gel Electrophoresis:45 minutes

Storage Instructions:

- The kit is stable for 12 months from the date of receipt
- Store Control PCR Product, DNA Ladder and all the PCR reagents at -20°C
- Store 6X Gel Loading Buffer at 2-8°C
- Other kit contents can be stored at room temperature (15-25°C)

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Aim:

To amplify a specific DNA fragment by Polymerase Chain Reaction.

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:

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

Principle:

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

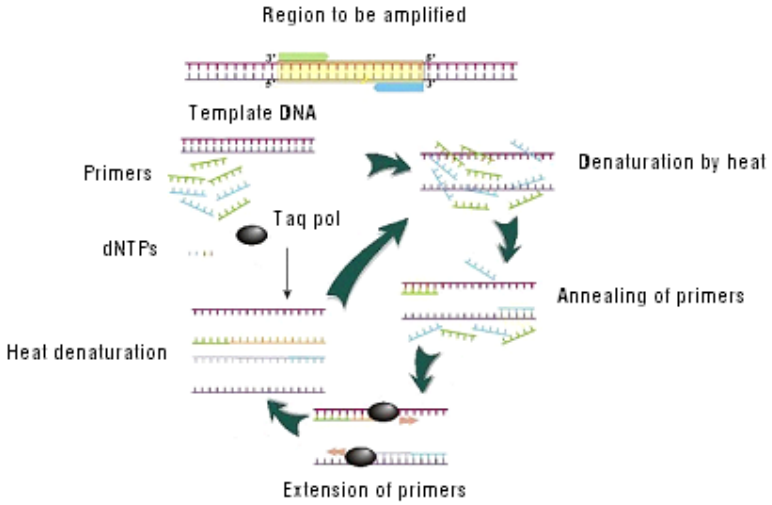


Fig 1: A PCR amplifies a specific DNA fragment by the incorporation of primers, dNTPs and Taq polymerase with periodic denaturation and renaturation of the template DNA.

- **Initialization step:** This step consists of heating the reaction mixture to 94–96°C for 1–9 minutes to break the hydrogen bonds in DNA strands.
- **Denaturation step:** This step is the first regular cycling event and consists of heating the reaction mixture to 94–98°C 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.

- **Annealing step**: In this step the reaction temperature is lowered to 50–65°C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is 3-5°C below the T_m (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.

- **Extension/Elongation step:** In this step, the temperature depends on the DNA polymerase used. Taq polymerase has its optimum activity at 75–80°C. Commonly a temperature of 68-72°C 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.

- **Final elongation:** This single step is occasionally performed at a temperature of 70–74°C 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 = 2^n, where n = No. of cycles.

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

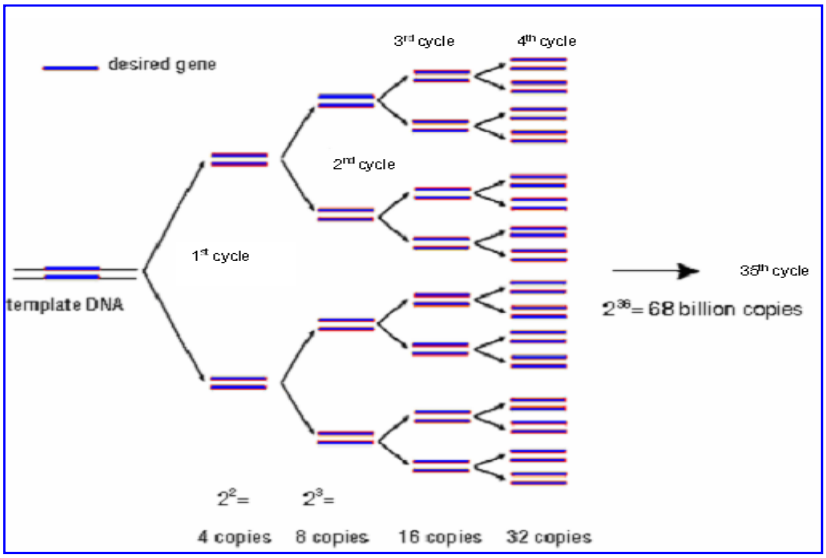


Fig 2: Amplification process of a particular template DNA

Kit Contents:

The kit can be used to perform amplify a particular template DNA using PCR.

Table 1: Enlists the materials provided in this kit with their quantity and recommended storage

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	*TKC116	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	RM1310	Mineral oil (optional)	0.3 ml	R T
15	CG282	Polypropylene Tubes, 0.2 ml (PCR Tubes)	11 Nos.	R T

*** Always give a quick spin before opening the vial as the liquid material may stick to the wall or to the cap of the vial.**

Materials Required But Not Provided:

Glasswares: Measuring cylinder, Beaker
Reagents: Ethidium bromide (10 mg/ml)

Other requirements: Thermocycler, Electrophoresis apparatus, UV Transilluminator, Vortex Mixer, Micropipettes, Tips, Adhesive tape, Microwave/ Hotplate/ Burner, Crushed ice

Storage:

HiPer® PCR Teaching Kit is stable for 12 months from the date of receipt without showing any reduction in performance. On receipt, store Control PCR Product, 1 Kb DNA Ladder and all PCR reagents at -20°C and 6X Gel Loading Buffer should be stored at 2-8°C. Other reagents can be stored at room temperature (15-25°C).

Important Instructions:

- Read the entire procedure carefully before starting the experiment.
- Keep all the solutions in the icebox during use.
- The 1 Kb DNA ladder supplied in the kit is ready to use and can be directly loaded onto the agarose gel.

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	**50µl**

2) Tap the tube for 1 – 2 seconds to mix the contents thoroughly.

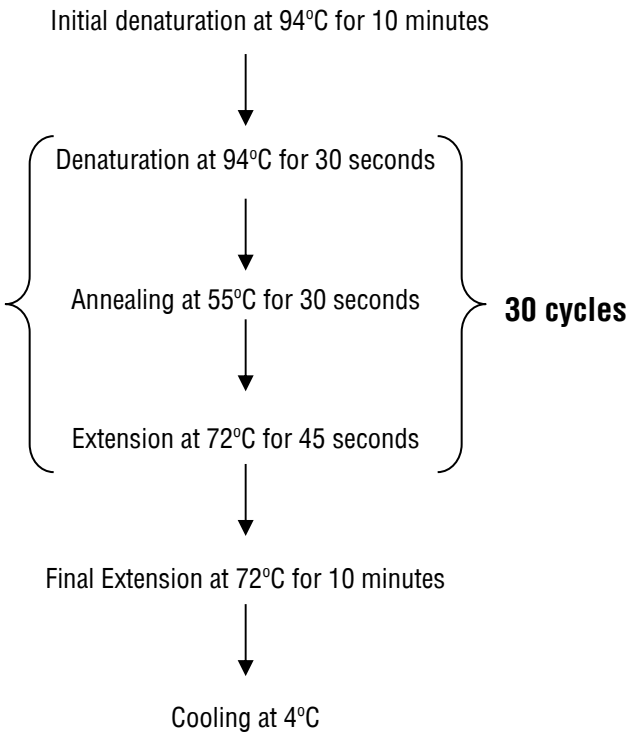
3) Add 25 µl of mineral oil in the tube to avoid evaporation of the contents.

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

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

PCR Amplification Cycle:

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



Agarose Gel Electrophoresis:

Preparation of 1X TAE: To prepare 500 ml of 1X TAE buffer, add 10 ml of 50X TAE Buffer to 490 ml of sterile distilled water*. Mix well before use.

Preparation of agarose gel: To prepare 50 ml of 0.8% agarose gel, add 0.4 g agarose to 50ml of 1X TAE buffer in a glass beaker or flask. Heat the mixture on a microwave or hot plate, swirling the glass beaker/flask occasionally, until agarose dissolves completely (Ensure that the lid of the flask is loose to avoid buildup of pressure). Allow the solution to cool down to about 55-60°C. Add 0.5µl Ethidium bromide, mix well and pour the gel solution into the gel tray. Allow the gel to solidify for about 30 minutes at room temperature.

Loading of the DNA samples: Load 3 µl of ready to use DNA ladder into the first well. Add 2 µl of 6X Gel loading buffer to 10 µl of PCR product. Load the PCR samples into the following wells.

Note: Care should be taken while pipetting out the PCR product from the tube so as to avoid the mineral oil layer.

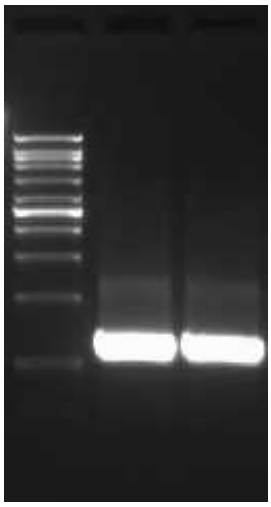
Electrophoresis: Connect the power cord to the electrophoretic power supply according to the conventions: Red-Anode and Black-Cathode. Electrophorese at 100-120 volts and 90 mA until dye markers have migrated an appropriate distance, depending onthe size of DNA to be visualized.

* Molecular biology grade water is recommended (Product code: ML024).

Observation and Result:

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

Lanes: **1** **2** **3**



Lane 1: 1Kb DNA Ladder

Lane 2: Control PCR Product

Lane 3: PCR Product

Interpretation:

After performing agarose gel electrophoresis, one can check the amplification of a specific PCR product. The optimized conditions result in the amplified PCR product of desired size.

Troubleshooting Guide:

Sr. No.	Problem	Possible Cause	Solution
1	Non –specific/ spurious bands observed	Template DNA or dNTPs concentration inappropriate	Take the same amount of template DNA and dNTPs as specified in the procedure
		Template DNA damaged	Minimize damage to template DNA by avoiding vortexing or vigourous mixing
2	No or poor amplification yield	Template or dNTPs may be degraded, enzymes may have been inactive	Store the kit at -20°C and avoid repeated freeze thaw. Also keep all the materials in ice while performing the experiment
		Thermoycler operation or program improper	Ensure proper functioning of Thermocycler. Run positive control with every reaction
		Inadequate mixing of the reaction tube	Mix the reaction mixture using a micropipette, avoid air bubble
3	Smearing of the product	DNA degraded	Work in sterile conditions to avoid contamination. Avoid vigorous mixing of the DNA samples
4	Primer-Dimer observed	Concentration of primers and dNTPs may be inappropriate	Use recommended concentration of primers and dNTPs

Technical Assistance:

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail at mb@himedialabs.com