**Exp 6.**

**Amplification of specific parts of a gene by PCR**

**Aim: Amplification of different segments of a yeast gene by polymerase chain reaction (PCR) on plasmid DNA**

**Theory**

**PCR** (**P**olymerase **C**hain **R**eaction) is a revolutionary method developed by Kary Mullis in the 1980s. It is a technique used to make multiple copies of a segment of DNA. PCR is very precise and can be used to amplify, or copy, a specific DNA target from a mixture of DNA molecules. As the DNA amplification requires cycles of amplification that includes denaturation at 95°C, a thermo-stable polymerase is very critical to the process. The method thus uses a specific DNA polymerase called Taq DNA polymerase. Taq DNA polymerase is isolated from bacteria *Thermus aquaticus* found in hot springs and is stable at high temperature.

PCR has the following steps:

1. Initial template DNA denaturation: It’s a process of separation of two strands of DNA in template (genomic DNA) by heating usually at 95°C for 5 minutes.This step follows a program with 25-35 cycles of the following steps 2-4.
2. Primer annealing: In this step forward and reverse primers anneal (attach) to their complementary sequences in the separated template DNA strands. This step is usually performed at 55°C for 30 sec.
3. Elongation of copied DNA: During this step Taq DNA polymerase synthesizes (polymerization) new DNA strands by adding dNTPs (Deoxy-ribonucleotide triphosphates: dATP,dCTP,dGTP,dTTP) complementary to the template nucleotides at 3’ end of the primers. This step is usually performed at 72°C for 1-2 minutes depending of expected length of the product (usually 1 kilo-base length of PCR product requires 1min elongation time; this however may vary for different templates or enzymes).
4. Denaturation: This step is performed usually at 95°C for 30 seconds to separate the double stranded products from earlier cycles.
5. Final elongation is done usually for 5-10min at 72°C to complete any unfinished polymerization reaction.



MATERIALS Required:

For PCR: PCR machine/Themocycler, Ice, Vortex, PCR tubes, Micropipettes, milli-Q water, DNA template (yeast genomic DNA), 10x concentrated Deoxy-ribonucleotide triphosphates (dNTPs, mixture of dATP, dCTP, dGTP and dTTP), 10x concentrated PCR Buffer, 10x concentrated forward primer, 10x concentrated reverse primer, Taq DNA polymerase enzyme.

PROCEDURE:

**PCR for amplification of various fragments of gene**

1. In this experiment we will amplify parts of a gene from yeast (see the schematic below). A group of two students should setup four PCR reactions as given in the following table. Keep the reagents and PCR tubes on ice at all times until start of the PCR. We will be doing the reactions for first 2 fragment amplifications



PCR setup : Keep tubes on Ice. Add all reagents with tube on ice.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Reaction 1** | **Reaction 2** | **Reaction 3** | **Reaction 4** |
|  | **Control PCR -1** | **PCR for 1st part of yeast gene (Expected length 725bp )** | **PCR for 2nd part of yeast gene (Expected length 441 bp)** | **Control** **PCR-2** |
| Template(plasmid with gene) | - | 2µl | 2µl | - |
| 10x PCR buffer with MgCl2 | 2.5 µl | 2.5 µl | 2.5µl | 2.5 µl |
| Forward Primer | 2µl( F1) | 2µl(F1) | 2µl (F1) | 2µl ( F1) |
| Reverse Primer | 2µl(R1) | 2µl(R1) | 2µl (R2) | 2µl (R2) |
| dNTPs | 2µl | 2µl | 2µl | 2µl |
| MgCl2(25mM) | 2 µl | 2 µl | 2 µl | 2 µl |
| H2O (sterile water) | 12.5 µl | 10.5 µl | 10.5 µl | 12.5 µl |
| Taq DNA polymerase | 2µl | 2µl | 2µl | 2µl |
| Total volume | 25µl | 25 µl | 25 µl | 25 µl |

1. Set the following program in a PCR machine (Thermocycler) as follows. With the help of teaching assistants learn how to make a new program in the PCR machine.

|  |  |  |
| --- | --- | --- |
| Initial Denaturation | 95˚C | 5 min. |
| Denaturation | 95˚C | 30sec.30 cycles |
| Annealing | 56˚C | 30sec. |
| Elongation | 72˚C | 1 min. |
| Final Elongation | 72˚C | 10min |

1. After PCR is completed (which will take around two hours), store the samples at -20˚C.
2. You would perform agarose gel electrophoresis to confirm if the PCR has worked and estimate size of the amplicon in the next class.
3. In the next class: Add 5µl of DNA loading dye to the 25µl PCR samples, mix by tapping the tubes. Load in 1.5% agarose gel, perform electrophoresis and visualize DNA bands under UV light. Estimate size of each amplifications and compare with the schematic given in point #1 of PCR. In one well load 10µl chromosomal DNA (2 ul of Gel loading dye has to be added to the chromosomal DNA).