POLYMERASE CHAIN REACTION

Introduction

PCR (Polymerase Chain Reaction) is a revolutionary method developed by Kary Mullis in the 1980s.In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. PCR amplifies a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of between 0.1 and 10 kilo base pairs (kbp), although some techniques allow for amplification of fragments up to 40 kbp in size. Principle PCR is based on thermal cycling process, which consists of repeated heating and cooling cycles of the reaction for DNA melting and enzymatic replication of the DNA. Key components for these selective and repeated amplifications are primers and Taq DNA polymerase. As the process continues, the DNA generated is itself used as a template for replication and amplified exponentially in a chain reaction. PCR can be extensively modified to perform a wide array of genetic manipulations.

Components of PCR DNA template is the sample DNA that contains the target sequence. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strands from each other.

DNA polymerase is a type of heat resistant enzyme that synthesizes new strands of DNA complementary to the target sequence. The first and most commonly used of these enzymes is **Taq DNA polymerase** (from Thermus aquaticus), whereas PfuDNA polymerase (from Pyrococcus furiosus) is used widely because of its higher fidelity when copying DNA.

Primers are short pieces of single-stranded DNA that are complementary to the target sequence. DNA synthesis begins at the end of primer by DNA polymerase enzyme. Nucleotides (dNTPs or deoxynucleotide triphosphates) are single units of the bases A, T, G, and C, which are essentially "building blocks" for new DNA strands. Buffer solution and bivalent cations providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

Reaction of 10–200 µl volume of PCR is carried out in a thermal cycler. The thermo cycler is a laboratory instrument used to perform polymerase chain reaction of DNA with controlled temperature settings of different cycles which heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Procedure Process of PCR includes the sample preparation, components for reaction and number of cycles which consists different temperature steps mostly three. The temperature plays very crucial role in the complete process as DNA denaturation, primer annealing and extension all depends upon the temperature in each cycle. To decide the temperature and the length of time number of parameters has to be considered these includes the enzyme used for DNA synthesis, the concentration of divalent ions , dNTPs in the reaction, and temperature (Tm) of the primers. Sample preparation:- Before initiating PCR, DNA must be isolated from a sample. DNA extraction is a multi-step process that may be done manually or by miniprep kit. Following sample preparation, the three-step PCR process is initiated.

1. Denaturation: - During the first step of PCR, called denaturation, the tube containing the sample DNA is heated to more than 90 degrees Celsius which separates the double-stranded DNA into two separate strands. Because of high temperature weak bonds between the nucleotides broken down and results into single stranded DNAknown as DNAmelting.

2. Annealing: - Next important step is annealing of the primers which are specific to the DNA sequence to be amplified. It is well known that PCR amplifies only specific segment of DNA bordered by the primers on either side of the target DNA region. During step two, the tube is cooled and primer binding occurs between 40 and 60 degrees Celsius. Annealing of primer is the most important step for further proceeding of PCR reaction so the correct temperature is must for the hybridization of the primer to the strand. 3. Extension: - In the third phase of the reaction, the temperature is increased to approximately 72-80 degrees Celsius as it depends upon the DNA polymerase used. Taq polymerase has its optimum activity at 72-80°C. As the process begins DNA polymerase starts adding nucleotide in 5' to 3' direction at the regions marked by the primers to synthesize a new strand of DNA complementary to each of the single template strands. As the process of extension completes, two identical copies of the original DNA have been made. After this step cycle begins again and creates more than one billion copies of the original DNA segment after 30-

40 cycles. Because the PCR is automated, it can be completed in just few hours. The complete process of PCR can be divided into three stages: Exponential amplification: In this stage the amount of product is doubled at every cycle. Leveling off stage: In this stage of PCR reaction slows down as the Taq polymerases loses its activity and also consumption of reagents like dNTPs and primers become limiting. Plateau effect: Due to exhaustion of reagents and polymerase enzymes there is no product accumulation in this stage.

Application of PCR is an indispensable technique used in medical and biological research labs for a variety of applications.

In a healthcare sector PCR have number of application like DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes and diagnosis of hereditary diseases due to its ability to make enough copies of target DNA from the clinical sample to allow analysis.

The identification of genetic fingerprints used in forensic science, DNA paternity testing and the detection and diagnosis of infectious diseases can also be done using PCR.

PCR is an established tool for DNA amplification, quantification and it can be used to measure , analyze extremely small amount of DNA sample which is often critical for forensic analysis.

. Limitation:

DNA polymerase is prone to error, which in turn causes mutations in the PCR fragments that are made. Primer synthesis needs prior information of the DNA sequence. □ Early “plateau effect” may occurs due to reagent limitation, self-annealing of the accumulating product or by inhibitors of the polymerase reaction. □ Due to this false plateau effect no more amplification of target sequence occurs at exponential phase, make product quantification unreliable.

Molecular Markers:

A genetic marker can be defined in one of the following ways: (a) a chromosomal landmark or allele that allows for the tracing of a specific region of DNA; (b) a specific piece of DNA with a known position or (c) a gene whose phenotypic expression is used to identify an

individual or a cell that carries it, or as a probe to mark a nucleus, chromosomes, or locus. Based on the mode of transmission markers fall into one of the three broad classes:

Morphological and agronomic traits which are those based on visually assessable traits. Biochemical markers are those which are based on gene product. Molecular markers are those relying on a DNA assay.

Here we will discuss molecular markers or genetic markers.

Due to advancement in molecular biology techniques, large numbers of highly informative DNA markers have been developed for the identification of genetic polymorphism. Molecular markers can be dominant or co dominant type depends upon the gene action.

Based on the method of analysis they can be categorized in two groups like

1. Molecular markers based on Restriction digestion.

2. Molecular markers based on Polymerase chain reaction (PCR).

Molecular marker based on Restriction digestion

RFLP (Restriction fragment length polymorphism)

RFLP is the most widely used hybridization-based molecular marker which is based on the restriction endonuclease enzymes to determine the difference in homologues DNA sequences of individuals. Although two individuals of the same species have almost identical genomes, they will always differ at a few nucleotides. This difference may be due to one or more of the following causes: point mutation i.e. changes in single nucleotide, insertion/deletion of a single nucleotide in the sequence, translocation, inversion and duplication of the nucleotides. Some of these differences in DNA sequences at the restriction sites can result in the gain, loss, or relocation of a restriction site. Due to this difference digestion of DNA with restriction enzymes results in fragments whose number and size can vary among individuals, populations, and species.

In this technique fragment of different length generated due to restriction digestion of DNA sequence and are detected and analyzed by gel electrophoresis.

Procedure

Sample preparation: - First step for RFLP analysis is extraction of DNA from sample. Digestion or fragmenting of sample: The basic technique for the detecting RFLPs involves fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs, and the process is known as a restriction digest.

Agarose gel electrophoresis: -The digested fragments are separated by agarose gel electrophoresis and a sheet of either nitrocellulose or nylon paper (membrane) is laid over the gel, and the separated DNA fragments are transferred to the sheet from gel in a process known as southern blotting.

Hybridization:- Membrane with transferred DNA fragments is now incubated with radioactive labelled DNA probe which hybridized only to those fragments which are complementary to the probe. Unbounded probes are washed away with buffer.

Autoradiography: -Exposure to X-ray film is given to visualize the DNA that has been hybridized to the labelled probe as a band on the autoradiograph and the process is known as autoradiography.

The RFLP pattern generated after the hybridization is unique to the individual and by comparing these banding patterns of different samples one can identify the difference and similarity between the individuals. Each fragment length is considered an allele, and can be used in genetic analysis. Most RFLP markers are codominant and highly locus specific.

Applications of RFLP analysis was the first DNA profiling technique inexpensive enough to see widespread application.

RFLP analysis was an important tool in marker assisted selection, genome mapping, localization of genes for genetic disorders, determination of risk for diseases.

RFLP analysis was also the basis for early methods of genetic fingerprinting, useful in the identification of samples retrieved from crime scenes, in the determination of paternity, and in the characterization of genetic diversity or breeding patterns in animal populations. Limitations.

The technique requires a large amount of sample DNA, and the combined process of probe labelling, DNA fragmentation, electrophoresis, blotting, hybridization, washing, and

autoradiography could take up to a month to complete. The technique for RFLP analysis is, however, slow and cumbersome and has been replaced by newer, faster techniques.

Molecular marker based on PCR RAPD (Random amplified polymorphic DNA)

Introduction: Random amplified polymorphic DNA (RAPD) is one of the most commonly used molecular technique to develop DNA markers based on PCR for identifying genetic variation. PCR is a molecular biology technique for enzymatically replicating (amplifying) small quantities of DNA.RAPD involves the use of a single, short and arbitrary oligonucleotide primer in a reaction and thus do not require prior knowledge of a DNA sequence, resulting in the amplification of many discrete DNA products. RAPD technique was developed independently by two different laboratories (Williams et. al., 1990; Welsh and McClelland, 1990) and called as RAPD and AP-PCR (Arbitrary primed PCR) respectively. Low expense, efficiency in developing a large number of DNA markers in a short time no requirement of probes, hybridization steps, make it quick simple and efficient. Requirement for less sophisticated equipment has made the RAPD technique valuable although reproducibility of the RAPD profile is still the centre of debate. RAPD markers are dominant type markers and cannot used to identify heterozygotes.

Principle: The RAPD technique is based on the polymerase chain reaction (PCR). A target DNA sequence is exponentially amplified with the help of short synthetic oligonucleotide as primer which binds to many different loci, and amplifies random sequences from a complex DNA template. The PCR generated amplified fragments depends upon the length and size of both the primers and the target genome. With the use of single primer generation of different DNA fragments are considered to originate from different genetic loci. Mutation or rearrangements at or between the primer binding sites may results into absence or presence of particular RAPD band which results into polymorphism. This means that RAPDs are dominant type marker. Apart from primers other important component of reaction are thermostable DNA polymerase, dideoxy nucleotide tri - phosphates, magnesium and reaction buffer. The reaction involves repeated cycles, each consisting of a denaturation, a primer annealing and an elongation step already described in previous topic PCR. After the completion of cycles the amplified products are separated on agarose gel electrophoresis and stained with ethidium bromide for visualization. The most crucial factors that need to

be optimized in a RAPD reaction are the magnesium, enzyme, DNA concentration and annealing temperature of the primer.

Procedure Sample preparation: -The first step is the extraction of DNA sample. DNA isolation is done either manually or by using miniprep kit. For RAPD analysis less amount of DNA is required as it is PCR based technique but DNA must be clean and of high molecular weight. If minimal quality of DNA isnot achieved, the reproducibility of results will be hard to ensure.

PCR reaction and DNA amplification: -Next step for RAPD analysis is to perform PCR with the purified sample. Mix the DNA with reaction mixture and keep the tubes in thermo cycler with set temperatures for each cycle.

Separating DNA fragments by Agarose gel electrophoresis: -After completion of the amplification reaction in thermocycler sample is run on agarose gel electrophoresis for the separation of DNA fragments with reference to DNA ladder of standard molecular weight.

Visualization of DNA bands: -To visualize the amplified regions of DNA ethidium bromide (EtBr) dye an intercalating agent is used during agarose gel electrophoresis and because of which the DNA bands illuminated under the presence of UV-transilluminator. Applications of RAPD Analysis RAPD as a molecular marker has a wide range of applications ranging from study of genetic diversity/polymorphism, germplasm conservation. Due to its requirement of very less amount of sample and also generates high number of fragments makes it an efficient tool to study plant and animal breeding, animal-plant-microbe interactions, pesticide/herbicide resistance, and also detection of soma clonal variations. RAPDs have also been applied in gene mapping studies to fill gaps not covered by other markers.

Limitations: RAPD analysis is depends upon the PCR which is highly sensitive to changes in DNA quality, PCR components and conditions. Mismatch between the primer and template may results into absence of PCR product due to it become difficult to interpret the data. RAPD reproducibility is very less and sometimes it is not possible to differentiate

whether the DNA fragment amplified is from homozygous locus or heterozygous locus.
RAPD technique is highly laboratory dependent and expensive too.

Conclusion:

Due to advancement in molecular biology techniques like PCR, electrophoresis, oligonucleotide synthesis it's become possible to develop molecular markers such as RFLP and RAPD. Advancement in the area of molecular markers opens the way to explore the area of genetic mapping, linkage, genetic diversity. These markers have found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding.