PCR

Q.What is the purpose of PCR?

i)This polymerase chain reaction (PCR) results in the selective application of a chosen region of DNA molecule.

ii)Any region of any DNA molecule can be chosen, so long as the sequences at the borders of the region are known.

iii)Two short oligonulceotides must hybridize to the DNA molecule, one to each strand of the double helix (**Figure1**). These oligonucleotides, which act as primers for the DNA synthesis reactions, delimit the region that will be amplified.

Q.What is the significance of using Taq polymerase in PCR?

This is a DNA polymerase I enzyme from *Thermus aquaticus* which lives in hot springs and **Taq polymerase** is thermostable, that means they are resistant to denaturation by heat treatment. The thermostability of Taq polymerase is an essential requirement in PCR methodology.

Q.Describe the PCR with proper illustrations.

1. The target DNA is mixed with Taq polymerase, the two oligonucleotide, primers and a supply of nucleotides.

2.The reaction is started by heating the mixture to 94^{o}C. AT this temperature the hydrogen bonds that hold together the two polynucleotides of the double helix are broken, so the target DNA becomes denatured into single-stranded molecules. (**Figure 2**)

3.The temperature is then reduced to 50-60^{o}C, which results in some rejoining of the single strands of the target DNA, but also allows the primers to attach to their annealing positions.

4.DNA synthesis can now begin, so the temperature is raised to 74^{o}C, just below the optimum for Taq polymerase.

5.In the first stage of the PCR, a set of long products is synthesized from each strand of the target DNA. These polynucleotides have identical 5' ends but random 3' ends, the latter representing positions where DNA synthesis terminates by chance.

6.The cycle of denaturation-annealing-synthesis is now repeated. (**Figure3**) The long products denature and the four resulting strands are copied during the DNA synthesis stage.

7.This give 4 double stranded molecules, two of which are identical to the long products from the first cycle and two of which are made entirely of new DNA.

8.During 3rd cycle, the latter gives rise to 'short products', the 5' and 3' ends of which are both set by the primer annealing positions.

9.IN subsequent cycles, the number of short products accumulates in an exponential fashion (doubling during each cycle).

10.At the end of a PCR a sample of the reaction mixture is usually analyzed by agarose gel electrophoresis, sufficient DNA having been produced for the amplified fragment to be visible as a discrete band after staining with **ethidium bromide**.

The sequence of the primers are critical to the success of the experiment, as are the precise temperatures used in the heating and cooling stages of the reaction cycle.

Q.How to design a primer prior to a PCR?

i)Primers must correspond with the sequences flanking the target region on the template molecule . Each primer must be complementary (not identical) to its template strand in order for hybridization to occur and the 3' ends of the hybridized primers should point towards one another (**Figure4**).

ii)If the primers are too short they might hybridize to non-target sites and give undesired amplification products.

Q.Why not simply make the primers as long as possible?

The length of the primer influences the rate at which it hybridizes to the template DNA, long primers hybridizing at a slower rate. The efficiency of the PCR, measured by the number of amplified molecules produced during the

experiment, is therefore reduced if the primers are too long, as complete hybridization to the template molecule cannot occur in the time allowed during the reaction cycle. In practice, primers longer than 30-mer are rarely used.

Q.Is there any restriction on size of DNA fragment to be amplified by PCR?

The DNA fragment to be amplified should not be greater than about 3kb in length and ideally less than 1 kb. Fragments up to 10 kb can be amplified by standard PCR techniques, but the longer he fragment the less efficient the amplification and more difficult it to obtain consistent results.

Q.Is there any restriction for fixed temperature in PCR?

During each cycleof a PCR, the reaction mixture is transferred between 3 temperatures (**Figure5**):

1.The denaturation temperature, usually 94^{o}C, which breaks the base pairs and releases single-stranded DNA to act as template in the next round of DNA synthesis.

2.The hybridization or annealing temperature, at which the primers attach to the templates.

3.The extension temperature, at which DNA synthesis occurs. This is usually set at 74^{o}C, just below the optimum for taq polymerase.

Conditions:

(a)The annealing temperature is the important one because again, this can affect the specificity or the reaction. DNA-DNA hybridization is a temperature-dependent phenomenon. If the temperature is too high no hybridization takes place instead the primers and templates remain dissociated (**Figure6**)

(b)If the temperature is too low, mismatched hybrids—ones in which not all the correct base pairs have formed—are stable (**Figure7**)

(c)The ideal annealing temperature must be low enough to enable hybridization between primer and template, but high enough to prevent mismatched hybrids from forming (**Figure8**). This temperature can be estimated by determining the **melting temperature or Tm** of the primer-template hybrid.

Q.How to determine the Tm value prior to PCR?

The Tm value is calculated from the simple formula—

$$\mathbf{Tm = (4\times [G + C]) + (2 \times [A+T])^{o}C}$$

Q.Determine the Tm of a primer with the following sequence –
5'AGACTCAGAGAGAACCC3'.

Hints:

4Gs, 5Cs, 7As and 1 T.

Tm= (4×9) +(2×8) = 52^{o}C.

The annealing temperature for a PCR experiment is therefore determined by calculating the Tm for each primer and using a temperature of 1-2^{o}C below this figure. Two primers should be designed so that they have identical Tms.

Q.What are the possible ways of studying PCR products?

Key points:

1.Gel electrophoresis of PCR products,

2.Cloning of PCR products

3.Sequencing of PCR products.