Polymerase Chain Reaction (PCR)

PCR is a patented procedure developed originally by Kary Mullis in 1983, while working for Cetus Corporation in USA. For his contribution, he was awarded the Nobel Prize in chemistry in 1993. This process can enzymatically amplify minute quantities of DNA or RNA to large number of copies. Even a single copy of well preserved DNA can act as a template but for efficient duplication 20-30 copies are optimal.

Target: The portion of DNA that needs to be amplified is the target DNA. Its length can vary from 50bp to 5000bp. Efficiency of PCR is less with too short or too long target sequences. It is not absolutely necessary to know the entire nucleotide sequence of the target DNA; however a small stretch of region on either ends of the target will be useful in designing primers. Stretches of chromosomal or plasmid DNA are often used as target in microbial molecular technology.

Primers: Primers are short oligonucleotides that can range from 15-50 nucleotides in length. They are designed to be complementary to regions flanking the target on both of the DNA strands. One primer binds to the 3' end of one strand and the other primer binds to 5' end of the other DNA strand flanking the target. Primers that are too short run the risk of binding non-specifically to non-target regions. Primers, which are always designed in pairs, are often synthetically prepared. Primers are accessible to the complementary region only when the two strands of the target DNA are separated.

Denaturation: The process of separating the two strands of DNA is called melting or denaturation and can the temperature can vary between 90-100^{o}C. Heating serves to disrupt the hydrogen bonds between the bases present on opposite strands. The Mol % G+C content of the target DNA is helpful in calculating the optimum melting temperature (T_m). The melting temperature is calculated by the following formula:
$T_m = 2^{o}C$ (No. of A + No. of T) + $4^{o}C$ (No. of G + No. of C).
The initial heating of the target DNA can take up to 10 minutes and is used in hot-start PCR. For regular cycling the denaturation takes 15 seconds to a minute.

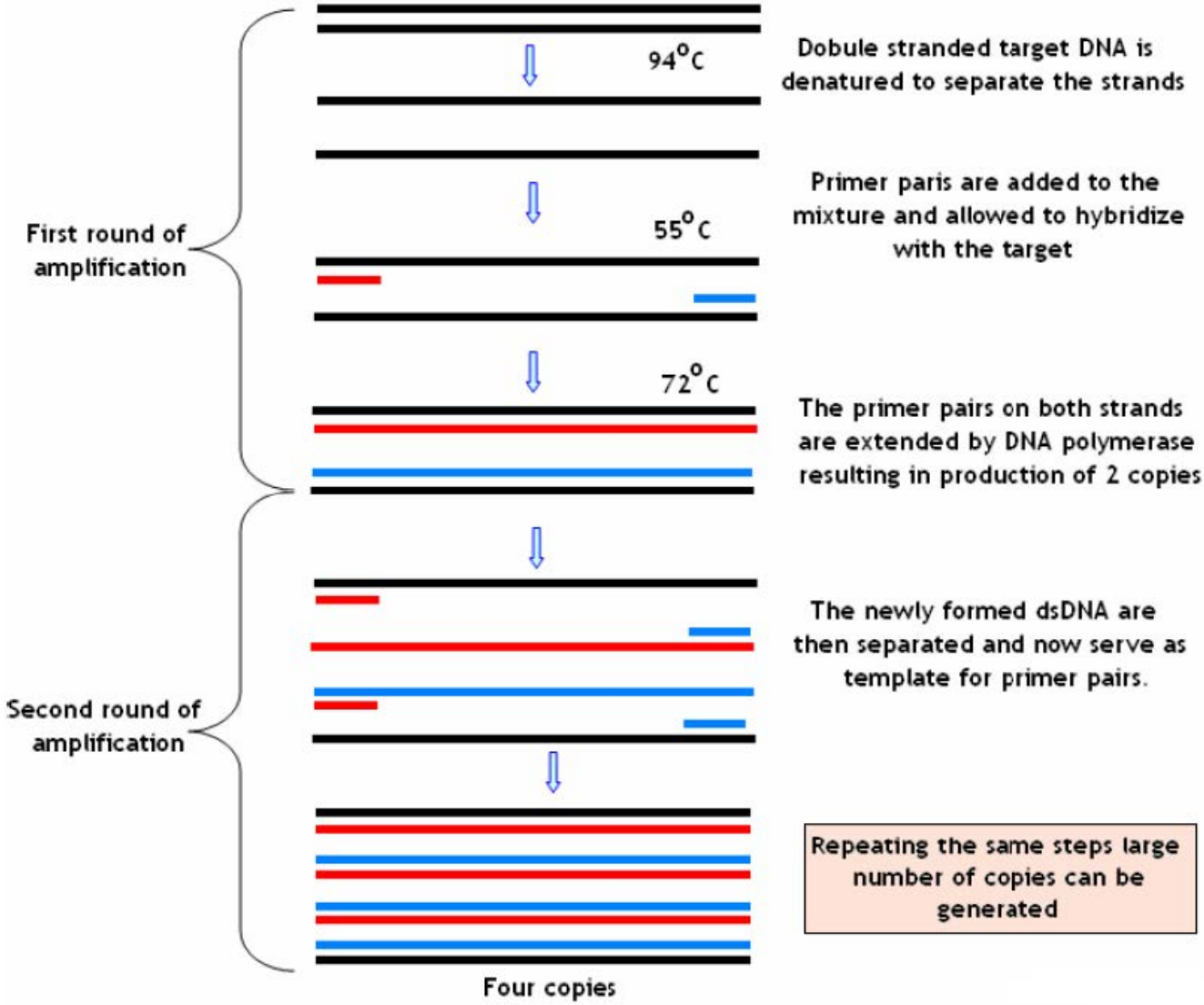
Annealing: Once the two strands are separated, the primers bind to their respective complementary regions flanking the target. This is called annealing or renaturation. But, for this to happen, the temperature has to be reduced to 40-65^{o}C, depending on the primers. Typically, the annealing temperature is about 3-5 degrees Celsius below the T_m of the primers used. The primers have to be designed such a way that the annealing temperatures of either of them are not far apart. This process can take 15 seconds to a minute.

Extension: Once the primers have bound, polymerase enzyme extends the complementary strand using the exogenously supplied dNTPs (dATP, dTTP, dGTP, dCTP). The polymerase enzyme adds new nucleotide to the existing primer in 5'-3' direction. This way, both the strands get complementary strands. Initially, polymerase enzyme derived from *E. coli* was used for extension but the enzyme got destroyed by the heat in the subsequent cycles and had to be replenished after every cycle. Taq polymerase is a thermostable polymerase derived from the bacterium *Thermus aquaticus* that dwells in the hot water springs (~80^{o}C). This enzyme has optimum activity at 70-80^{o}C, hence a temperature of 72^{o}C is often chosen. Depending on the length of target, extension can take 15 seconds to 2 minutes. At its optimum

temperature, the DNA polymerase can polymerize a thousand bases per minute. Taq polymerase has poor fidelity and lacks 3'-5' exonuclease proof reading ability. The 3'-5' exonuclease activity of the enzyme allows the incorrect base pair to be excised (this activity is known as proofreading). The other alternatives are Pfu, Tth and Tli. Pfu is derived from the bacterium *Pyrococcus furiosus* while Tli is derived from *Thermococcus litoralis*. Both of these enzymes have proof reading ability and hence less error rates.

PCR cycle: Some PCR protocols (especially hot-start PCR) have a long melting step. Thereafter a typical cycle of 30-40 rounds consists of following steps:

- ✓ Denaturation at 90-100^{o}C for 15 secs to one minute
- ✓ Annealing at 40-60^{o}C for 15 secs to two minutes
- ✓ Extension at 72^{o}C for 15 secs to 3 minutes



Final elongation: This step is usually 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. The amplified DNA copies are called amplicons.

The process of transferring the tubes to different temperatures has been circumvented by the use of automated machines called thermocyclers.

PCR is an exponential amplification system such that after n number of cycles, there is $(1+x)^n$ times as much target as was present initially; where x is the mean efficiency of the reaction for each cycle. After a standard run of 30 cycles over a period of 2-3 hours, theoretically the original target molecule is amplified 230 times (10^9 fold). However, PCR is usually less efficient than theoretical and the actual practical amplifications are only 10^6-10^7 folds, which is due to various factors such as inactivation of Taq DNA polymerase, shortage of nucleotide substrates, shortage of primer, inhibition by pyrophosphate and re-annealing of amplified DNAs. After certain number of PCR cycles, PCR attains plateau phase. The plateau phase of PCR indicates that almost same amount of amplified products will be obtained, regardless of the initial amount of the templates, by sufficient cycles of PCR. For targets up to 1000 bp, 20-30 cycles of PCR are performed, but if organisms are present in low numbers, 45 cycles may be required. Even though the system has the ability to detect one copy of DNA in the sample, detection is dependent on the ability of the primers to locate and anneal to the target copy and optimum PCR conditions.

Factors affecting PCR:

- Annealing temperature: At lower temperatures, non-specific binding of primers can occur. Taq polymerase can start extension of non-target leading to production of non-specific products. This can be avoided by hot-start PCR.
- Mg2+ concentration: Since thermostable polymerases are Mg dependent enzymes, each combination of target DNA and primer require unique concentration of Mg2+.
- Concentration and source of thermostable polymerase
- Concentration and purity of both target DNA and primer
- Denaturing temperature, annealing temperature and time
- Number of cycles

Common problems in PCR are false negatives due to presence of PCR inhibitors, poor nucleic acid isolation and poor amplification efficiency and false positives due to contaminations.
Common sources of error are:

- False positive reactions are caused by contamination with a new or previously amplified DNA
- Non-specific primer hybridization. Binding of primers to area other than desired region resulting in false positive results.
- Primer-dimer formation. This condition can arise if the two primers used are complimentary to each other and end up hybridizing with each other instead of hybridizing with the target. This may lead to little or no amplification of target sequence.
- Background hybridization occurs as a result of non-specific binding of only one of the primers. This results in the amplification of the target DNA thirty times after 30 cycles and not exponentially.
- Primer artifact formation can occur in conditions of low stringency, small target amounts, too much enzyme in early cycles, high primer concentration and excessive thermal cycling. This often occurs when the polymerase enzyme generates new primer binding sites for the same or other primer. This decreases the efficiency of PCR by consuming primer and competing with target for other reaction components.

Methods to improve sensitivity of PCR:

- Addition of low concentration of dimethyl sulphoxide (DMSO) allows amplification of previously unamplifiable targets and allows larger target DNA to be amplified more efficiently.
- Addition of 20% glycerol allows amplification of up to 2500 bases.
- PCR specificity can be improved by “Hot start” method. If all the reaction mixtures are added at the same time and slowly heated at the start of PCR, the thermostable polymerase may extend any

non-specific primer–template complex before denaturation can begin. This problem can be overcome by adding the polymerase after all the components have reached 70^{o}C.

Setting up PCR laboratory:

Activities such as preparation of PCR mix, extraction of DNA, running of PCR cycles and post-PCR handling must be performed on separate rooms. Some authors have even recommended having separate rooms on different floors. Amplicons produced during previous PCR run can contaminate the subsequent samples thereby giving rise to false positives. In order to avoid this different aspects of PCR are performed in widely separated areas. In any case, the order must be maintained and samples/tubes must never travel to the preceding room. A minimum of three separate rooms must be made for PCR. Preparation of PCR mix (dNTPs, buffer, polymerase, Mg^{2+} etc) must be performed in the first room. Nucleic acid extraction must be made in second room. Transfer of extracted target nucleic acid to PCR mix tube and PCR run may be performed in third room. Electrophoresis and gel documentation may be performed in the fourth room.

Procedure of PCR

The glasswares and the disposable items must be kept ready. Bacterial cultures must be kept ready. Reagents required for the procedure should be prepared and appropriate dilutions should be made. Calculations of the required volumes must be made in advance keeping in mind the number of test samples. Positive and negative controls should also be kept ready. It must be ensured that the electrical appliances are in order.

Materials required

Test tubes, 1.5 ml eppendorf tubes, PCR tubes, ice pack, micropipette (1-500 µl), micropipette tips, tube holders, microcentrifuge, deep freeze, thermocycler, electrophoresis apparatus, microwave oven, UV transilluminator, nitrile gloves

Double distilled RNAse free water, TAE buffer, primers, dNTPs, 2.5 units Taq polymerase, $MgCl_2$ (25 mM), agarose, phosphate buffer, ethidiuim bromide, gel loading dye, 100bp ladder

Extraction of whole nucleic acid

1 ml of PBS is transferred to a 1.5-ml Eppendorf tube. A loopfull of bacteria is picked from a plate and transferred to the Eppendorf tube. The suspension is centrifuged at 14.000 rpm for 5 min. The supernatant is discarded and the pellet is re-suspended in 100 µl TE 10:1. The suspension is boiled for 5-10 minutes and transferred directly to ice. The lysed DNA is diluted 10 fold in TE 10:1.

Plasmid isolation by alkali lysis method

Bacteria is inoculated into 2ml of trypticase soy broth and grown overnight at 37^{o}C. Approximately 1.5 ml of overnight culture is transferred to a microcentrifuge tube and centrifuged at 10,000 rpm for 5 minutes. The supernatant is discarded and the tube is kept on ice. The cell pellet is resuspended in 100 µl of ice cold solution I (50mM Glucose, 25mM Tris; pH: 8.0, 10mM EDTA) and mixed by vortexing gently. It is further kept at room temperature for 5 minutes. Next, 200 µl of solution II (0.2M NaOH, 1% SDS) is added and the contents are gently mixed by inverting the tube 5 times. The tube is kept at room temperature for 5 minutes. Next, 150 µl of solution III (3M potassium acetate; pH: 5.5) is added and the contents are gently mixed by inverting the tube. The tube is placed on ice for 5 minutes. The tube is centrifuged at 10,000 rpm for 10 minutes. The supernatant is transferred into a fresh tube and 450 µl of isopropanol is added to it. The contents are mixed by inverting the tube. The tube is kept at room temperature for 5-10 minutes and then centrifuged at 10,000 rpm for 10 minutes. The supernatant is carefully removed and discarded. DNA would be seen as white precipitate sticking to the wall of the tube. 1 ml of 70% ethanol is added to wash the pellet. The contents are mixed by inverting and centrifuged at 10,000 rpm for 10 minutes. The supernatant is removed completely and pellet is dried by keeping the cap

of the tube open. 30 µl of autoclaved double distilled water is added and gently mixed by tapping the sides of the tube to dissolve the DNA. It should stand at room temperature with intermittent mixing for 15-20 minutes.

Testing the quality of DNA
Impurities (such as proteins) in the sample DNA may affect the performance of PCR. Hence, it is vital to assess the purity of extracted plasmid DNA. This is achieved by measuring the optical densities (OD) at 260nm and 280 nm. The purest sample of DNA has 260/280 ratio of 1.7-2. The OD ratio of DNA preparation should not be below 1.6.

Estimating the quantity of DNA
The DNA solution is diluted 1 in 100 in Tris EDTA buffer. The optical density of the suspension is read at 260nm. The concentration of DNA is calculated by the following formula:
DNA µg/ml = OD x 50 x dilution factor

PCR mix: The PCR mix must be calculated for the number of specimens plus controls. The tube containing PCR mix must be placed in ice. This is then aliquoted in to the required number of PCR tubes. One drop of mineral or silicone oil may be placed in every tube as a lid to avoid the mixture to vaporize and condensate in the lid of the tubes. Mineral oil need not be added if the thermocycler is provided with a heated lid. The PCR Buffer is available as a 10x concentrate and should be diluted 1:10 in the final reaction (e.g., use 5 µl in a 50-µl PCR reaction). It is composed of (10x): 200 mM Tris-HCl (pH 8.4), 500 mM KCl and 50 mM Magnesium Chloride.

An example of reaction mixture:

Components	**Volume**	**Final Concentration**
10X PCR buffer with $MgCl_2$	10 µl	1X
10 mM dNTP mixture	2 µl	0.2 mM each
Primer mix (10 µM each)	5 µl	0.5 µM each
Template DNA	2 µl	-----
Taq DNA Polymerase (5 U/µl)	0.5 µl	2.5 units
Autoclaved distilled water	30 µl	

Generally nanograms of plasmid DNA or 0.1-1.0 µg of chromosomal DNA is taken. Higher amounts of target DNA may significantly hamper the proper amplification. Negative control receives 1 µl of sterile water whereas positive control 1 µl of positive DNA. Similarly, 1 µl of sample DNA is placed in respective tubes. Positive controls must always be added last to prevent any possible contamination. The total volume in each PCR tube is approximately 50 µl. The PCR tubes are then placed in thermocycler and the program is run. An example of PCR run is as follows

Initial denaturation 94^{o}C for 10 minutes
Denaturation at 94^{o}C for 30 seconds
Annealing at 48^{o}C for 30 seconds } 30 cycles
Extension at 72^{o}C for one minute
Final extension at 72^{o}C for 5 minutes

Confirmation of PCR authenticity

There are various methods to validate the PCR generated amplicons, as they serve to differentiate the target amplification from non-specific amplification. These methods include:

- Electrophoresis of the amplicon should be sufficient in most cases to demonstrate similarity between the expected and observed size of amplicons.

- Confirmation of a single restriction site within the amplified DNA; the amplified DNA should contain a restriction site, which can be demonstrated by splicing with restriction enzyme.
- Hybridization with probes for the known sequence within the amplified target sequence
- Nucleotide sequencing of amplicon

Electrophoresis of amplicons

1.5% agarose gel containing 1% ethidium bromide is prepared and set up for electrophoresis with TBE buffer. Following PCR, 5 µl of gel loading (containing 40%sucrose, 0.25% bromophenol blue, xylene cyanol and Tris-EDTA) is transferred into all PCR tubes. Avoiding the mineral oil, 15 µl of amplicons are transferred to the wells in the gel. Similarly positive and negative controls are loaded on the gel.10 µl of 100bp DNA ladder is loaded on the last well of the gel. The order of the samples has to be noted down. The electrophoresis is run at 100 volts/70mA for 1-2 hours or till the tracking dye reaches $3/4^{th}$ of the gel length. The electrophoresis apparatus is switched off. The gel is placed in a straining-bath for about 30 minutes, rinsed briefly in water before visualizing the bands in a UV transilluminator. The amplicons must be in the expected size for tentative confirmation of PCR. Ethidium bromide is deactivated by adding 10g activated charcoal per 2.5 litres of waste.

Amplicon containment

In order to limit the spread of amplicon from one PCR to another, multiple room approach is adopted. The amplified products should never be carried to preceding rooms to avoid contamination. Reusable glasswares and plasticwares should be decontaminated with 0.5M HCl for 1 hour at RT, followed by washing and autoclaving. Tips with aerosol filters must be used.

Another way of ensuring destruction of amplicons of previous cycle is by enzymatic inactivation of contaminating amplicons. dUTP is used instead of dTTP in the reaction mixture and the resulting PCR amplicon contains incorporated dUMP instead of dTMP. If this amplicon contaminates another PCR process, it can be removed before commencement of PCR by treating with uracil N glycosylase. The enzyme removes uracil base from the contaminating amplicon, which gets degraded on commencement of PCR cycle. The enzyme also gets destroyed in the heating process and does not interfere with the next PCR cycle.