POLYMERASE CHAIN REACTION (PCR)

PCR stands for the Polymerase Chain Reaction and was developed in 1987 by Kary Mullis (which won him a Nobel Prize) and associates. With this technique it is possible to make virtually unlimited copies of a single DNA molecule even though it is initially present in a mixture containing many different DNA molecules. It is used to amplify a specific DNA (target) sequence lying between known positions (flanks) on a double-stranded (ds) DNA molecule. The polymerase chain reaction can be used to amplify both double and single stranded DNA.

In order to perform PCR, one must know at least a portion of the sequence of the target DNA molecule that has to be copied. Generally, PCR amplifies small DNA targets 100-1000 base pairs (bp) long. It is technically difficult to amplify targets >5000 bp long. A pair of single stranded oligonucleotide primers, which have DNA sequences complementary to the flanking regions of the target sequence, must be synthesized. The primers are complementary to either end of the target sequence but lie on opposite strands. The primers are usually 20-30 nucleotides long and bind to complementary flanking region at 3' end.

Flanking region 5'		flanking region
	ATATTGGGCCTA'	TTATGGTTTAAT
TGGGCAAACCC	TATAACCCGGAT.	AATACCAAATTA
		5 [*]

Requirements:

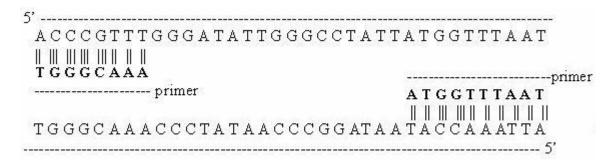
- Thermal cycler (thermocycler)
- PCR amplification mix typically containing:
- Sample dsDNA with a target sequence
- Thermostable DNA polymerase
- Two oligonucleotide primers
- Deoxynucleotide triphosphates (dNTPs)
- Reaction buffer containing magnesium ions and other components

Procedure:

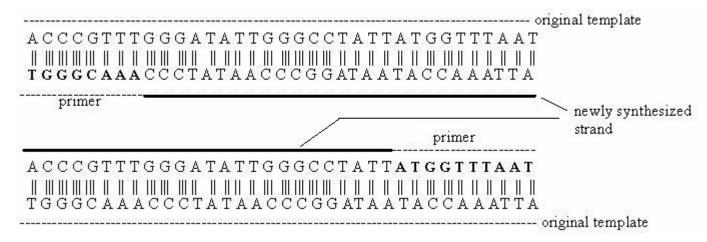
1. The DNA molecule carrying a target sequence is denatured by heat at 90-95°C for 20 seconds. The two strands separate due to breakage of the hydrogen bonds holding them together. Oligonucleotide primers are added.

5	5'	
	ACCCGTTTGGGATATTGGGCCTATTATGG	
	TGGGCAAACCCTATAACCCGGATAATACC	
_		5'

- 2. A reaction mixture containing all four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) and a thermostable DNA polymerase is added. A DNA polymerase (Taq) that is not denatured by the high temperature needed to separate the DNA strands is used. It is usually sourced from *Thermus aquaticus*, a bacterium isolated from hot springs.
- 3. The mixture is allowed to cool to a lower temperature (50-65°C). Each strand of DNA molecule becomes annealed with an oligonucleotide primer complementary to either end of the target sequence. Primer annealing takes 20 seconds.



4. The temperature is raised to 60-75°C and primers are extended by the action of DNA polymerase for 30 seconds. The polymerase synthesizes complementary sequence the 5' to 3' direction away from each of the primers. If the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain. Polymerization continues until each newly synthesized strand has proceeded far enough to contain the site recognized by the other primer. At this point there would be exactly two copies of the target DNA sequence.



5. The mixture is heated again at 90-95°C to denature the molecules and separate the strands and the cycle repeated. Each new strand then acts as a template for the next cycle of synthesis. Thus amplification proceeds at an exponential (logarithmic) rate, i.e. amount of DNA produced doubles at each cycle. The amplified product at the end of PCR is called amplicon.

A typical thermal cycle might be as follows:

- Heat denaturation at 94°C for 20 seconds
- Primer annealing at 55°C for 20 seconds
- Primer extension at 72°C for 30 seconds

Average time for each cycle is approximately 4-5 minutes, considering the fact that heating and cooling between each stage also have to be considered.

Initially these steps at three different temperatures were carried out in separate water baths but nowadays a thermal cycler is used (a machine that automatically changes the temperature at the correct time for each of the stages and can be programmed to carry out a set number of cycles). After the introduction of thermocycler, each cycle of replication can be completed in less than 5 minutes. After 30 cycles, a single molecule of DNA is amplified into more than a billion copies ($2^{30} = 1.02 \times 10^{9}$).

Post amplification detection: Following PCR, the amplification product can be detected using gel electrophoresis followed by ethidium bromide staining and visualization with uv transillumination. Visualization of a band containing DNA fragments of a particular size can indicate the presence of the target sequence in the original DNA sample. Absence of a band may indicate that the target sequence was not present in the original DNA sample. Confirmation of the amplicons can be made by southern blotting using specific probes.

Modifications and different types of PCR are:

Nested PCR, Multiplex PCR, RT-PCR, Touchdown PCR, Arbitrarily Primed PCR, Inverse PCR, Allele Specific PCR, Asymmetric PCR, "Hot Start" PCR, Core Sample PCR, Degenerate PCR and PCR-Elisa.

Applications of PCR:

- Amplification of small amounts of DNA for further analysis by DNA fingerprinting.
- The analysis of ancient DNA from fossils.
- Mapping the human (and other species) genome.
- The isolation of a particular gene of interest from a tissue sample.
- Generation of probes: large amount of probes can be synthesized by this technique.
- Production of DNA for sequencing: Target DNA in clone is amplified using appropriate primers and then its sequence determined. Helpful in conditions where amount of DNA is small.
- Analysis of mutations: Deletions and insertions in a gene can be detected by differences in size of amplified product.
- Diagnosis of monogenic diseases (single gene disorders): For pre-natal diagnosis, PCR is used to amplify DNA from foetal cells obtained from amniotic fluid. PCR has also proved very important in carrier testing.
- Detection of microorganisms: Especially of organisms and viruses that are difficult to culture or take long time to culture or dangerous to culture.
- The PCR has even made it possible to analyze DNA from microscope slides of tissue preserved years before.
- Detection of microbial genes responsible for some aspect of pathogenesis or antibiotic resistance.
- Crucial forensic evidence may often be present in very small quantities, e.g. one human hair, body fluid stain (blood, saliva, semen). PCR can generate sufficient DNA from a single cell.

Limitations of PCR: PCR is an extremely sensitive technique but is prone to contamination from extraneous DNA, leading to false positive results. Another potential problem is due to cross-contamination between samples. It is for this reason that sample preparation, running PCR and post-amplification detection must be carried out in separate rooms. Concentration of Mg is very crucial as low Mg²⁺ leads to low yields (or no yield) and high Mg²⁺ leads to accumulation of nonspecific products. Non-specific binding of primers and primer-primer dimmer formation are other possible reasons for unexpected results. Reagents and equipments are costly, hence can't be afforded by small laboratories.

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