Introduction:

1) The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.
2) PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications.
3) There are three major steps involved in the PCR technique: denaturation, annealing, and extension.
4) It is called "polymerase" because the only enzyme used in this reaction is DNA polymerase.
5) It is called "chain" because the products of the first reaction become substrates of the following one, and so on.
6) Polymerase Chain Reaction was developed in 1984 by the American biochemist, Kary Mullis. Mullis received the Nobel Prize and the Japan Prize for developing PCR in 1993.

Basic concept of PCR

1) The basic PCR principle is simple. As the name implies, it is a chain reaction: One DNA molecule is used to produce two copies, then four, then eight and so forth.
2) This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands.
3) To do their job polymerases require a supply of:
   a) DNA building blocks, i.e. the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G).
   b) They also need a small fragment of DNA, known as the primer, to which they attach the building blocks

c) DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates.

4) PCR is based on the mechanism of DNA replication in vivo: dsDNA is unwound to ssDNA, duplicated, and rewound. This technique consists of repetitive cycles of:
   a) Denaturation of the DNA through melting at elevated temperature to convert double-stranded DNA to single-stranded DNA
   b) Annealing (hybridization) of two oligonucleotides used as primers to the target DNA
   c) Extension of the DNA chain by nucleotide addition from the primers using DNA polymerase as catalyst in the presence of Mg^{2+} ions.

- **Steps in PCR**:

- **Initialization step:**

It consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes.

- **Denaturation step:**

This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

- **Annealing step:**

The reaction temperature is lowered to 50–65 °C and held for 20–60 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 ^{o}C below the temperature of the primers used. Stable DNA-DNA hydrogen bonds are

only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

- **Extension/elongation step:**

The temperature depends on the DNA polymerase used; Taq polymerase has its optimum activity at 75–80 °C, and commonly a 72 °C is used with this enzyme. The DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs in 5' to 3' direction. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. At its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

- **Final elongation:**

This single step is occasionally 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.

- **Final hold:**

This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

▪ The components of a PCR reaction:

1) DNA template that contains the DNA region (target) to be amplified.
2) Two primers that are complementary to the 3' end of each of the sense and anti-sense strand of the DNA target.
3) Taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C. This enzyme used to catalyze the reaction.

4) Deoxynucleoside triphosphates (the four nucleotides containing triphosphate groups; dNTPs), the building blocks from which the DNA polymerase synthesizes a new DNA strand.
5) Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
6) Monovalent cation potassium ions and Divalent cations, magnesium or manganese ions, which are used as cofactor of the enzyme.
7) The reaction is set up in a thin walled PCR tube permit suitable thermal conductivity to allow for rapid thermal equilibration in a thermal cycler.

- **Checking fragment identity:**

To control whether the PCR generated the expected DNA fragment, 1-2% agarose or 6% acrylamide gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a length marker, several DNA fragments of known size) run on the same gel alongside the PCR products.

- **Applications of PCR:**

- **Basic Researches:**
  1) Mutation screening
  2) Drug discovery
  3) Classification of organisms
  4) Genotyping
  5) Molecular Archaeology
  6) Molecular Epidemiology
  7) Molecular Ecology
  8) Bioinformatics
  9) Genomic cloning

10) Site-directed mutagenesis
11) Gene expression studies

- **Applied Researches:**
  1) Genetic matching
  2) Detection of pathogens
  3) Pre-natal diagnosis
  4) DNA fingerprinting
  5) Gene therapy

▪ Types of PCR technique

Type	Features	Purpose
Allele-specific PCR	1) It based on single-nucleotide polymorphisms (SNPs). 2) It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3' ends encompass the SNP.	Study of mutations and polymorphisms if site of them is known.
Assembly PCR or Polymerase Cycling Assembly (PCA)	1) Artificial synthesis of long DNA sequences from long oligonucleotides with short overlapping segments. 2) The oligonucleotides alternate between sense and anti-sense directions. 3) The overlapping segments determine the order of the PCR fragments.	Study the gene expression
Helicase-dependent amplification PCR	1) Similar to traditional PCR, but uses a constant temperature rather than three different temperatures denaturation, annealing and extension. 2) DNA helicase an enzyme used in place of thermal denaturation.	Detection and diagnosis studies.
Hot start PCR	1) It is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature. 2) It may be performed manually by heating the reaction components to the denaturation temperature (e.g., 95°C) before adding the polymerase.	It used to reduce non-specific amplification during the initial set up stages of the PCR
Inter-sequence-specific PCR (ISSR)	It amplifies regions between simple sequence repeats to produce a unique fingerprint of amplified fragment lengths.	DNA fingerprinting
Inverse PCR	It involves a series of DNA digestions and self-ligation, resulting in known sequences at either end of the unknown sequence.	Identify the flanking sequences around genomic inserts

Ligation-mediated PCR	1) It uses small DNA linkers ligated to the DNA of interest. 2) It uses multiple primers annealing to the DNA linkers	DNA sequencing, genome walking and DNA foot-printing.
Methylation-specific PCR (MSP)	1) DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil that recognized as thymine by PCR primers. 2) Using primer sets identical except at any CpG islands within the primer sequences. 3) One primer set recognizes DNA with cytosines to amplify methylated DNA, and other set recognizes DNA with uracil or thymine to amplify unmethylated DNA.	Detect methylation of CpG islands in genomic DNA
Mini-primer PCR	It uses a thermostable polymerase that can extend from short primers as short as 9 or 10 nucleotides.	Amplify conserved DNA sequences, such as the 16S (or eukaryotic 18S) *rRNA* gene.
Multiplex-PCR	1) It consists of multiple primer sets within a single PCR mixture. 2) Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes.	Produce amplicons of varying sizes that are specific to different DNA sequences.
Nested PCR	1) Two sets of primers are used in two successive PCRs. 2) In the first reaction, one pair of primers is used to generate DNA products, which consist of target and non-specifically amplified DNA fragments. 3) In the second reaction, set of primers bind completely or partially different from and located 3' end of each of the primers used in the first reaction.	Increases the specificity of DNA amplification
Overlap-extension PCR or Splicing by overlap extension (SOE)	A genetic engineering technique	a) Used to splice together two or more DNA fragments that contain complementary sequences. b) Used to join DNA pieces containing genes, regulatory sequences, or mutations. c) Enables creation of specific and long DNA constructs.

Quantitative PCR (Q-PCR)	1) It has a very high degree of precision. 2) It uses fluorescent dyes, such as Sybr Green, Eva Green or fluorophore-containing DNA probes, such as TaqMan. 3) It is sometimes abbreviated to RT-PCR (Real Time PCR)	a) Measure the quantity of a PCR product. b) It quantitatively measures starting amounts of DNA, cDNA, or RNA. c) Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample.
Reverse Transcription PCR (RT-PCR)	Reverse transcriptase reverse transcribes RNA into cDNA, which is then amplified by PCR	a) Used for amplifying DNA from RNA. b) Used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript. c) Used to map the location of exons and introns in the gene.
Thermal asymmetric interlaced PCR (TAIL-PCR)	1) It uses a nested pair of primers with differing annealing temperatures. 2) A degenerate primer is used to amplify in the other direction from the unknown sequence.	Isolation of an unknown sequence flanking a known sequence.
Universal Fast Walking PCR	It uses specific 'two sided' PCR than conventional 'one-sided' approaches (using only one gene-specific primer and one general primer	It used for genome walking and genetic fingerprinting.