Primers
PCR primers are important as they are complementary to the beginning and end of the DNA fragment of interest which one needs to amplify. Primers therefore select the boundaries of the region to be amplified by PCR. During the PCR annealing cycle, PCR primers anneal to the complementary region of the DNA. DNA polymerase binding and the 3' OH of the oligo allows the synthesis of DNA to occur.
PCR Primers are short single-stranded, synthetically synthesized oligonucleotides usually shorter than 50 nucleotides (often 18-25 nucleotides)

Important Factors To Consider When Choosing Primers:

- Optimal Tm (annealing T) ~ 50-60°C
- Oligonucleotide GC-content should be between 40-60%.
- Calculated Tm (melting temperature) for both primers used in reaction should not differ >5°C.
- Primer annealing temperature is usually 5°C below the calculated lower Tm. However it should be chosen empirically for individual conditions.
- Inner self-complementary hairpins of >4 and of dimers >8 should be avoided.
- 3' terminus is extremely case sensitive - it must not be complementary to any region of the other primer used in the reaction and must provide correct base matching to template.
- If possible, primers should start and end with 1-2 G or C residues
- Primers should NOT have any extensive secondary structure or self-complementarity (can result in Primer-dimers).

PCR types

- *Allele-specific PCR*: a diagnostic or cloning technique which is based on single-nucleotide polymorphisms (SNPs) (single-base differences in DNA). It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3' ends encompass the SNP. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.

- *Assembly PCR* or *Polymerase Cycling Assembly (PCA)*: artificial synthesis of long DNA sequences by performing PCR on a pool of long oligonucleotides with short overlapping segments. The oligonucleotides alternate between sense and antisense directions, and the overlapping segments determine the order of the PCR fragments, thereby selectively producing the final long DNA product.

- *Asymmetric PCR*: preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in sequencing and hybridization probing where amplification of only one of the two complementary strands is required. PCR is carried out as usual, but with a great excess of the primer for the strand targeted for amplification. Because of the slow (arithmetic) amplification later in the

reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification on this process, known as *L*inear-*A*fter-*T*he-*E*xponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

- Colony PCR the screening of bacterial (E.Coli) or yeast clones for correct ligation or plasmid products. Selected colonies of bacteria or yeast are picked with a sterile toothpick or pipette tip from a growth (agarose) plate. This is then inserted into the PCR master mix or pre-inserted into autoclaved water. PCR is then conducted to determine if the colony contains the DNA fragment or plasmid of interest.
- The *Digital polymerase chain reaction* simultaneously amplifies thousands of samples, each in a separate droplet within an emulsion.

- *Helicase-dependent amplification*: similar to traditional PCR, but uses a constant temperature rather than cycling through denaturation and annealing/extension cycles. DNA helicase, an enzyme that unwinds DNA, is used in place of thermal denaturation.

- *Hot-start PCR*: a technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the melting temperature (e.g., 95°C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that only dissociate after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.
- *In Situ* PCR (ISH) is a polymerase chain reaction that actually takes place inside the cell on a slide. *In situ* PCR amplification can be performed on fixed tissue or cells.

- *Intersequence-specific PCR* (ISSR): a PCR method for DNA fingerprinting that amplifies regions between simple sequence repeats to produce a unique fingerprint of amplified fragment lengths.

- *Inverse PCR*: is commonly used to identify the flanking sequences around genomic inserts. It involves a series of DNA digestions and self ligation, resulting in known sequences at either end of the unknown sequence. Inverse PCR has numerous applications in molecular biology including the amplification and identification of sequences flanking transposable elements, and the identification of genomic inserts.

The inverse PCR method includes a series of digestions and self-ligations with the DNA being cut by a restriction endonuclease. This cut results in a known sequence at either end of unknown sequences.

Inverse PCR Steps

1) Target DNA is lightly cut into smaller fragments of several kilobases by restriction endonuclease digestion.
2) Self-ligation is induced under low concentrations causing the phosphate backbone to reform. This gives a circular DNA ligation product.
3) Target DNA is then restriction digested with a known endonuclease. This generates a cut within the known internal sequence generating a linear product with known terminal sequences. This can now be used for PCR (polymerase chain reaction).
4) Standard PCR is conducted with primers complementary to the now known internal sequences.

- *Ligation-mediated PCR* uses small DNA oligonucleotide 'linkers' (or adaptors) that are first ligated to fragments of the target DNA. PCR primers that anneal to the linker sequences are then used to amplify the target fragments. This method is deployed for DNA sequencing, genome walking, and DNA footprinting A related technique is *Amplified fragment length polymorphism*, which generates diagnostic fragments of a genome.

- *Methylation-specific PCR* (*MSP*) is used to identify patterns of DNA methylation at cytosine-guanine (CpG) islands in genomic DNA.Target DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is complementary to adenosine in PCR primers. Two amplifications are then carried out on the bisulfite-treated DNA: One primer set anneals to DNA with cytosines (corresponding to methylated cytosine), and the other set anneals to DNA with uracil (corresponding to unmethylated cytosine). MSP used in Q-PCR provides quantitative information about the methylation state of a given CpG island.

- **Long PCR** is a PCR is which extended or longer than standard PCR, meaning over 5 kilobases (frequently over 10 kb). Long PCR is usually only useful if it is accurate. Thus, special mixtures of proficient polymerases along with accurate polymerases such as Pfu are often mixed together. **Applications of Long PCR** Long PCR is often used to clone larger genes or large segments of DNA which standard PCR cannot.

- *Miniprimer PCR*: uses a thermostable polymerase (S-Tbr) that can extend from short primers ("smalligos") as short as 9 or 10 nucleotides. This method permits PCR targeting to smaller primer binding regions, and is used to amplify conserved DNA sequences, such as the 16S (or eukaryotic 18S) rRNA gene.

- *Multiplex Ligation-dependent Probe Amplification* (*MLPA*): permits multiple targets to be amplified with only a single primer pair, thus avoiding the resolution limitations of multiplex PCR (see below).

- *Multiplex-PCR*: consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, i.e., their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis.

- ***Nested PCR*:** increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets (instead of one pair) of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

- *Overlap-extension PCR*: a genetic engineering technique allowing the construction of a DNA sequence with an alteration inserted beyond the limit of the longest practical primer length.

- *Quantitative PCR (Q-PCR)*: used to measure the quantity of a PCR product (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA or RNA. 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. *Quantitative real-time PCR* has a very high degree of precision. QRT-PCR methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. It is also sometimes abbreviated to RT-PCR (*R*eal *T*ime PCR) or RQ-PCR. QRT-PCR or RTQ-PCR are more appropriate contractions, since RT-PCR commonly refers to reverse transcription PCR (see below), often used in conjunction with Q-PCR.

- *R*everse *T*ranscription PCR (RT-PCR): for amplifying DNA from RNA. Reverse transcriptase reverse transcribes RNA into cDNA, which is then amplified by PCR. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be used to map the location of exons and introns in the gene. The 5' end of a gene (corresponding to the transcription start site) is typically identified by RACE-PCR (*Rapid Amplification of cDNA Ends*).

- *Solid Phase PCR*: encompasses multiple meanings, including Colony Amplification (where PCR colonies are derived in a gel matrix, for example), 'Bridge PCR' (primers are covalently linked to a solid-support surface), conventional Solid Phase PCR (where Asymmetric PCR is applied in the presence of solid support bearing primer with sequence matching one of the aqueous primers) and Enhanced Solid Phase PCR (where conventional Solid Phase PCR can be improved by employing high Tm and nested solid support primer with optional application of a thermal 'step' to favour solid support priming).

- *Thermal asymmetric interlaced PCR (TAIL-PCR)*: for isolation of an unknown sequence flanking a known sequence. Within the known sequence, TAIL-PCR uses a nested pair of primers with differing annealing temperatures; a degenerate primer is used to amplify in the other direction from the unknown sequence.

- *Touchdown PCR* (*Step-down PCR*): a variant of PCR that aims to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (3-5°C) above the T_m of the primers used, while at the later cycles, it is a few degrees (3-5°C) below the primer T_m. The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles.

- *Universal Fast Walking*: for genome walking and genetic fingerprinting using a more specific 'two-sided' PCR than conventional 'one-sided' approaches (using only one gene-specific primer and one general primer - which can lead to artefactual 'noise') by virtue of a mechanism involving lariat structure formation. Streamlined derivatives of UFW are LaNe RAGE (lariat-dependent nested PCR for rapid amplification of genomic DNA ends), 5'RACE LaNe and 3'RACE LaNe.

- *Variable Number of Tandem Repeats (VNTR) PCR* targets areas of the genome that exhibit length variation. The analysis of the genotypes of the sample usually involves sizing of the amplification products by gel electrophoresis. Analysis of smaller VNTR segments known as Short Tandem Repeats (or STRs) is the basis for DNA Fingerprinting databases such as CODIS.

Pretreatments and extensions

Adjustments to the synthetic oligonucleotides used as primers in PCR are a rich source of modification:

- Normally PCR primers are chosen from an invariant part of the genome, and might be used to amplify a polymorphic area between them. In **Allele-specific PCR** the opposite is done. At least one of the primers is chosen from a polymorphic area, with the mutations located at (or near) its 3'-end. Under stringent conditions, a mismatched primer will not initiate replication, whereas a

matched primer will. The appearance of an amplification product therefore indicates the genotype.

- **InterSequence-Specific PCR** (or **ISSR-PCR**) is method for DNA fingerprinting that uses primers selected from segments repeated throughout a genome to produce a unique fingerprint of amplified product lengths. The use of primers from a commonly repeated segment is called **Alu-PCR**, and can help amplify sequences adjacent (or between) these repeats.

- Primers can also be designed to be 'degenerate' - able to initiate replication from a large number of target locations. **Whole genome amplification** (or **WGA**) is a group of procedures that allow amplification to occur at many locations in an unknown genome, and which may only be available in small quantities. Other techniques use **degenerate primers** that are synthesized using multiple nucleotides at particular positions (the polymerase 'chooses' the correctly matched primers). Also, the primers can be synthesized with the nucleoside analog inosine, which hybridizes to three of the four normal bases. A similar technique can force PCR to perform Site-directed mutagenesis.

- Normally the primers used in PCR are designed to be fully complementary to the target. However, the polymerase is tolerant to mis-matches away from the 3' end. **Tailed-primers** include non-complementary sequences at their 5' ends. A common procedure is the use of **linker-primers**, which ultimately place restriction sites at the ends of the PCR products, facilitating their later insertion into cloning vectors.

- An extension of the 'colony-PCR' method (above), is the use of **vector primers**. Target DNA fragments (or cDNA) are first inserted into a cloning vector, and a single set of primers are designed for the areas of the vector flanking the insertion site. Amplification occurs for whatever DNA has been inserted.

- PCR can easily be modified to produce a **labeled product** for subsequent use as a hybridization probe. One or both primers might be used in PCR with a radioactive or fluorescent label already attached, or labels might be added after amplification. These labeling methods can be combined with 'asymmetric-PCR' (above) to produce effective hybridization probes.