5

Polymerase chain reaction and its various modifications

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Introduction

Polymerase Chain Reaction or PCR is a molecular technique which allows *in vitro* synthesis of billions of copies of a target DNA fragment within hours using a simple enzymatic reaction. This is achieved by using a pair oligonucleotide primers that hybridize (anneal) to the opposite strands of the target DNA at positions flanking the region to be amplified. New strands are made through the simultaneous extension of both the primers by addition of nucleotides to the primers. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by the enzyme DNA polymerase results in the exponential accumulation of the DNA whose termini are defined by the 5' ends of the primers. Since the primer extension products synthesized in one cycle can serve a template for the next, the number of target DNA copies approximately doubles at every cycle. Thus 20 cycles of PCR can yield about a million-fold amplification. The method is simple, as the PCR can be performed in a single tube. It can be performed on relatively crude DNA containing samples. These factors have made the PCR an attractive method for amplification of specific sequences. This method is extremely rapid; it takes only 3 hours to amplify a known sequence of interest. PCR generates sufficient copy numbers of target DNA sequences for their routine visualization through standard procedures such as electrophoresis followed by staining with ethidium bromide. The PCR products may be sequenced to determine the exact sequence of the nucleotides within the amplified product. As a result, PCR permits routine analysis of DNA from single egg and larvae, and from non-invasively secured tissues such as fin clips and scales. Even partially degraded DNA from poorly preserved sources can be analyzed if sufficiently small PCR products are identified.

Discovery of PCR

The concept of PCR was first conceived by Dr. Kerry Mullis in 1983, first reported in1985, while working at the Cetus Corporation in Emeryville, CA, along with other researchers at Cetus Corporation (Molecular Station, 2006). Kerry Mullis discovered that by harnessing one component of molecular reproduction technology, ie a basic principle of replicating a piece of DNA using two primers, a target DNA of interest could be amplified exponentially. This DNA amplification procedure was an *in vitro* process (meaning in a test-tube). The first ever PCR product was the 110 base pair DNA fragment of a cloned segment of the human beta-globulin gene at the company labs, being the beginning of PCR as a basic technique in molecular biology (Mullis et al.,1986, Mullis and Faloona 1987). Dr. Mullis was awarded the Nobel Prize in Chemistry in 1993 for his development of the Polymerase

Chain Reaction (PCR), a central technique in biochemistry and molecular biology. Dr. Mullis subsequently was awarded the Japan Prize that same year.

Materials and reagents for PCR

The components required for the PCR are the template (the DNA to be amplified), a pair of primers, thermostable polymerase, the four types of de-oxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) and appropriate reaction buffer containing magnesium ions (KCl, Tris-HCl (pH 8.4), $MgCl_2$ and gelatin). They are assembled in a tube and the amplification reaction is carried out by manipulating the temperature within the reaction tube, in cyclic manner, using a thermal cycler. For any given pair of primers, the optimal concentrations of all the above ingredients and parameters have to be standardized. Even though there is no single set of conditions and concentrations that will be optimal for all reactions, the parameters outlined below defines a common starting point from where modifications can be attempted.

Target DNA (Template): An advantage of PCR is that it can amplify relatively impure DNA or DNA from blood spots, archival material and ancient DNA. Concentration of template DNA also affects the degree of amplification. The sample DNA generally contains 10^2 to 10^5 copies of template. Too high or too low concentration will result in poor amplification. Therefore, it is useful to optimize the template concentration in a PCR reaction to obtain maximum product. While typically DNA quantity is measured in ng, the relevant unit is actually moles, i.e., how many copies of the sequence that will anneal with the primers are present. Thus, the amount of DNA in ng that is needed to add is a function of its complexity. In theory, a single molecule of DNA can be used in PCR but normally between 1000 and 100,000 molecules for eukaryotic nuclear DNA are used. The nucleotide composition of target DNA also affects the PCR amplification. Extremely GC rich DNA strands are difficult to separate. Addition of denaturing agents like formamide or DMSO can help to overcome the problem.

Primers: Primers are the most important components of PCR, and the success of a PCR largely depends on the primers. Primers are short, single stranded DNA molecules which will bind (anneal) to either ends of region to be amplified (one on each strand) and serve as the starting point for building a new complementary nucleic acid strand. Primers are generally made in pairs, called “forward” and “reverse”. These primers are complimentary to the regions flanking the DNA segment to be amplified such that they can be extended toward one another with DNA polymerase, forming new DNA molecules. The most important property of a primer is its sequence specificity, which determines what nucleic acid sequence it can bind to, how well it will bind, and how well it will serve as a site for extension of new nucleic acid molecules. Generally, a “specific” primer is designed to target a DNA sequence in a closely related group of organisms, while not matching organisms outside that group. “Universal” primers are designed to target DNA sequences shared by any species that contains the sequence of interest. Thus, care should be taken while designing the primers for a particular experiment. Oligonucleotide primers in the range of 18 to 30 bases are generally used for the PCR. Though there are no set rules that will ensure the synthesis of an effective primer pair, the following guidelines are useful.

(a) Wherever possible, select primers with a random base distribution and with a GC content similar to that of the fragment being amplified. Avoid primers with stretches of polypurines, polypyrimidines or other unusual sequences.

(b) Check the primers against each other for sequence complementarity. Use primers with low complementarity to each other. Avoid primers with 3' end overlaps in particular. This will reduce incidence of "primer dimers". Most primers are generally 18 to 30 bases in length and the optimal length to be used in an amplification will vary. Longer primer may be synthesized but are seldom necessary. If shorter primers or degenerate primers are used, the thermal profile should be modified considering the lower stability of the primed target. However, the 3' end of the primer should match the template exactly. Generally, concentrations ranging from 10 to 50 p moles of each primer should be used.

The optimum length of a primer depends upon its (A+T) content, and the Tm of its partner. A prime consideration is that the primers should be complex enough so that the likelihood of annealing to sequences other than the chosen target is very low. For example, there is a ¼ chance (4^{-1}) of finding an A, G, C or T in any given DNA sequence; there is a 1/16 chance (4^{-2}) of finding any dinucleotide sequence (eg. AG); a 1/256 chance of finding a given 4-base sequence. Thus, a given sixteen base sequence will statistically be present only once in every 4^{16} bases (= 4 294 967 296, or 4 billion): this is about the size of the human or maize genome, and 1000x greater than the genome size of *E. coli*. Thus, the association of a greater than 17-base oligonucleotide with its target sequence is an extremely sequence-specific process. Generally, 17-mer or longer primers are routinely used for amplification from genomic DNA of animals and plants. Long primers will result in mismatch pairing and non-specific priming even at high annealing temperatures

Melting temperature (Tm) of primers: The annealing temperature is dependent on the **Tm** of primer. Annealing temperature can be the Tm value calculated using the following formulae:

(1) **Tm** = [(number of A+T residues) x 2 °C] + [(number of G+C residues) x 4 °C]

This formula was determined originally from oligonucleotide hybridization assays, which were performed in 1 M NaCl, and appears to be accurate in lower salt conditions only for primers less than or about 20 nucleotides in length.

(2) **Tm** p = 22 + 1.46 ([2 x (G+C)] + (A+T))

This formula is reportedly useful for primers of 20-35 bases in length. The calculated annealing temperature is only a reference temperature from which to initiate experiments. The actual annealing temperature may be 3-12 °C higher than the calculated Tm. The actual annealing temperature condition should be determined empirically. The optimum annealing temperature which gives the best PCR product should be used.

Deoxynucleotide triphosphate: The dNTPs are the building blocks of DNA. Once the primer binds to its target site, synthesis of the complementary strand of DNA takes place through primer extension by linking of nucleotide to its 3'end with the help of Taq DNA polymerase. Precursor dNTPs can be obtained as a neutralized solution, which are stable at –20ºC for months. The doxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) is generally used at concentrations of 200 mM (0.2mM) each. Higher concentrations may lead to mis-incorporations. Low dNTP concentration reduces mispriming at non-target sites. The lowest dNTP concentration appropriate for the length and composition of the target must be standardized. As a thumb rule, 20mM of each dNTP in a 100 ml reaction is sufficient to synthesize 10 p Mol of a 400 bp sequence. In the standard

reaction, all four triphosphates are added to a final concentration of 0.8mM; this leaves 0.7 mM of the original 1.5mM $MgCl_2$ not complexed with dNTP. Therefore, if dNTP concentration is changed significantly, a compensatory change in $MgCl_2$ may be necessary.

Taq* DNA polymerase:** The discovery of thermostable DNA polymerase has revolutionized the PCR technology. They are obtained from organisms that thrive in extreme temperatures and have an optimum activity at 72° C. It is able to withstand the denaturing conditions (over 90 °C) required during PCR cycling. Thus, unlike thermo-labile polymerase, with *Taq* polymerase there is no need for extra addition of enzymes during cycling process where strands separation required heating to over 90°C .There are now a plethora of commercially available enzymes to choose from that differ in their thermal stability, processivity, and fidelity. The choice of the DNA polymerase employed by PCR is determined by the goals of the experiment. The most commonly used thermostable polymerase is *Taq* DNA polymerase isolated from the bacterium *Thermus aquaticus* which inhabit the hot springs with extremely high temperatures. Isolation of the DNA polymerase from this bacterium yielded a PCR polymerase that was not rapidly inactivated at high temperatures. In 1986, Dr. David Gelfand and Ms. Susanne Stoffel of Cetus Corporation purified such a thermostable DNA polymerase, referred to as native *Taq* (T****hermophilus* ***aq****uaticus* in short), from the organism *Thermus aquaticus*. Taq polymerase was shown to work successfully in PCR, enabling the process to be performed much more easily. Today, almost all PCR is done using recombinant *Taq*, a cloned version of the enzyme, as it is less expensive to manufacture than the native form of the enzyme (Roche Diagnostics, 2007). *Taq* was the first polymerase that was able to withstand the denaturing conditions (over 90 °C) required during PCR cycling. *Taq* has an enzymatic half life at 95°C of about 40 min. *Taq* DNA polymerase is unique in that it produces PCR products with A (Adenine) overhangs. This was found to be quite useful, and was exploited to produce TA Cloning and TOPO cloning. One of *Taq* polymerases' major disadvantages is its low replication fidelity. As Taq does not have 3' to 5' exonuclease proofreading mechanism to replace an accidental mismatch in the newly synthesized DNA strand, Taq produces more errors than proofreading polymerases, such as Pfu (Roche Diagnostics, 2007) *Pyrococcus furiosus*, where it functions in vivo to replicate the organism's DNA. Pfu's have superior thermostability and 'proofreading' properties compared to other thermostable polymerases. Unlike *Taq* DNA polymerase, Pfu DNA polymerase possesses 3' to 5' exonuclease proofreading activity and corrects nucleotide-misincorporation errors. Thus Pfu DNA polymerase-generated PCR fragments will have fewer errors than Taq-generated PCR inserts. It also results in blunt-ended PCR products.

The required concentration of *Taq* DNA polymerase is between 1 and 2.5 units per 100 ml reaction when other parameters are optimum. When optimizing a PCR, enzyme concentration ranging from 0.5 to 5 units/ 100ml are tried and resultant products are visualized by agarose gel electrophoresis. If the enzyme concentration is too high, non-specific background products may accumulate and if too low, an insufficient amount of desired product is made.

Examples of thermostable DNA polymerases:

DNA Polymerase	**Source**
Taq	*Thermus aquaticus*
Amplitaq®	*T. aquaticus*

Amplitaq (Stoffel fragment)®	*T. aquaticus*
Hot *Tub*™	*Thermus flavis*
Pyrostase™	*T. flavis*
Vent™	*Thermococcus litoralis*
Deep Vent™	*Pyrococcus GB-D*
Tth	*Thermus thermophilus*
Pfu	*Pyrococcus furiosus*
ULTma™	*Thermotoga maritima*

The Reaction Buffer: The PCR buffer contains KCl, Tris HCl (pH 8.4), $MgCl_2$ and gelatin. The components of PCR buffer, particularly the concentration of $MgCl_2$ have a profound effect on the specificity and yield of an amplification product. Success of PCR is dependent on $MgCl_2$ concentration in the reaction to a great extent. Mg_2+ ions form a soluble complex with dNTPs which is essential for dNTP incorporation, stimulate polymerase activity and increase the Tm (melting temperature) of primer / template interaction (i.e. it serves to stabilize the duplex interaction). Concentration of about 1.0 to1.5 mM is usually optimal (when 200uM each of dNTPs are used). Excess of Mg^{2+} will result in the accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce the yield. Optimization by titration of $MgCl_2$ concentration is recommended to establish an optimum concentration for a particular reaction.

Several buffer formulations have been published & a consensus is emerging. The recommended PCR buffer should contain 10mM Tris-HCl (pH 8.4) also. KCl up to 50mM can be included in the reaction mixture to facilitate primer annealing. Excess KCl inhibits Taq polymerase activity. Gelatin or bovine serum albumin (100 g/ml) and nonionic detergents such as Tween- 20 and NP40 (0.05 - 0.1%) are included to help stabilize the enzyme. The nonionic detergents can be replaced by 0.1% Triton X-100, but some detergent is essential.

Thermal Cycles for PCR:

Amplification of a target DNA is achieved by repeated cycles of denaturation, primer annealing and extension. These events are controlled by manipulation of temperature. The above three major steps in a PCR are repeated for 35 to 40 cycles. This is done using an automated thermal cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation: Double stranded DNA used for the PCR is separated into single strands in the initial denaturation step. Typical denaturation temperature is $94^{O}C$ for 15 to 60 seconds. Higher temperatures e.g. $97^{0}C$ may be necessary for G + C rich targets. Denaturation steps that are too long or too high lead to unnecessary loss of enzyme activity. Denaturation of nucleic acid (NA) is carried out to make it single-stranded for the purpose of annealing with primers. It is done by heating it to a point above the “melting temperature” of the double or partially double stranded form, and then flash-cooling it: this ensures the “denatured” or separated strands do not re-anneal. Additionally, if the NA is heated in buffers of ionic strength lower than 150 mM NaCl, the melting temperature is generally less than $100^{o}C$ - which is why PCR works with denaturing temperatures of 91-$97^{o}C$. The main reason of importance of denaturing temperature and time in relation to number of cycles is because Taq polymerase has a half-life of 30 min at $95^{o}C$. This half life supports not more than about 30 amplification

cycles. However, it is possible to reduce the denaturation temperature after about 10 rounds of amplification, as the mean length of target DNA is decreased. "Time at temperature" is the main reason for denaturation / loss of activity of *Taq*. Thus, with reduction of time, increases the number of cycles are possible, whether the temperature is reduced or not. It is possible, for short template sequences, to reduce this to 30 sec or less.

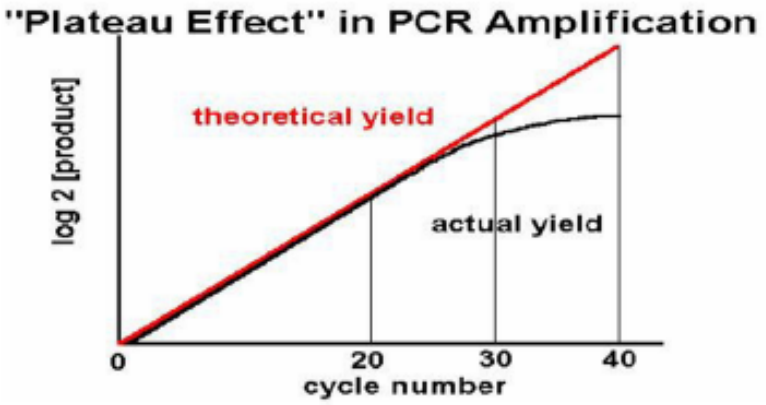
Primer annealing: At temperatures ranging from 47ºC to 62ºC, the primers anneal to its complimentary region on the template. The complimentary sequences will form hydrogen bonds between their complimentary bases (G to C, and A to T or U) and form a stable double stranded, anti-parallel molecule. During PCR, the primers are moving around, caused by the Brownian motion in the reaction mix. Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bond lasts a little bit longer (primer that fit exactly) and on that little piece of doubling stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the hydrogen bond is so strong between the template and the primer that it does not break any more. This is usually performed at temperatures between 47°C and 65°C for 30 to 60 seconds.

The temperature and length of time required for primer annealing depends upon the base composition, length and concentration of the primers. As a rule of the thumb, annealing temperature (Ta) of 5^0C below the lowest melting temperature (Tm) of the amplification pair of primers can be attempted. The annealing temperature chosen for a PCR depends directly on length and composition of the primer(s). Annealing temperature in the range of 55 to 65^0C generally yield the best results. At the optimal primer concentration annealing will require only a few seconds. Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces mis-extension of incorrect nucleotides at the 3' end of the primers. Therefore, stringent annealing temperature, especially during initial few cycles will help to increase specificity.

Primer Extension: The DNA polymerase works ideally at temperature 72ºC. The annealed primers, to which a few bases have been added, have a stronger attraction to the template, created by hydrogen bonds, than the forces breaking these attractions. Primers that are on positions with no exact match get loose again (because of the higher temperature) and do not give an extension of the fragment. The nucleotides (complementary to the template) are linked to the primer on the 3'side by the polymerase, from 5' to 3', reading the template from 3' to 5' side and bases are added complimentary to the template. Extension time depends on the length and concentration of the target sequence and upon the temperature. Primer extensions are usually performed at 72^0C. The rate of nucleotide incorporation at 72^0C varies from 35 to 100 nucleotides per second depending upon the buffer, pH, salt concentration and the nature of the DNA template. The length of the elongation step (30 seconds to three minutes) is determined by the speed of the enzyme, its ability to continue moving down the template DNA and the length of the DNA segment to be amplified. At around 70ºC, the polymerase activity is optimal, and primer extension occurs at upto 100 bases/sec. A general guideline is 1 minute / kb of product length. An extension time of one minute at 72^0C is considered sufficient for products up to 2 Kb size. Longer products require longer times: approximately 3 min for 3kb and longer products.

Cycling could include an initial denaturation at 94^OC and a final extension at 72^OC for 5 min. At the end reactions are stopped by chilling at 4^OC or by addition of EDTA at 10mM.

Cycle number: The optimum number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA when other parameters are optimal. Because both strands are copied during PCR, there is exponential increase of the number of copies of the gene. For example if the PCR is initiated with one copy of the gene, after one cycle there will be 2 copies, after two cycles there will be 4 copies, three cycles will result in 8 copies and so on. Too many cycles may increase the amount and complexity of non-specific background products. Too few cycles give low product yield. Innis and Gelfand (1990) recommend from 40 - 45 cycles to amplify 50 target molecules, and 25 - 30 to amplify $3x10^5$ molecules to the same concentration. This non-proportionality is due to a so-called *plateau effect* (Rybicki, 2001), which is the attenuation in the exponential rate of product accumulation in late stages of a PCR, when product reaches 0.3 - 1.0 nM. This may be caused by degradation of reactants (dNTPs, enzyme); reactant depletion (primers, dNTPs - former a problem with short products, latter for long products); end-product inhibition (pyrophosphate formation); competition for reactants by non-specific products; competition for primer binding by re-annealing of concentrated (10nM) product (Innis and Gelfand, 1990).



(Source: Rybicki, 2001)

Detection and analysis of PCR product: The PCR product will be DNA fragments (amplicons) of defined length. The simplest way to check the PCR product is to load a portion of it into an agarose gel containing ethidium bromide along with molecular weight markers and carry out an electrophoresis. The DNA fragments generated by the PCR should be readily visible over an ultraviolet transilluminator. Hybridizing the PCR product with suitable DNA probe is also in practice for conformation.

Common Types of PCR

PCR has been adapted to fit many different applications and hence there are many different types and each one is unique to the application for which it was designed. There are six common types of PCR: conventional PCR, multiplex PCR, reverse transcription (RT)-PCR, nested PCR, real-time PCR and random primed PCR. And there are many other types for specific purposes.

Conventional PCR: Conventional PCR uses a thermostable DNA polymerase to amplify a region of the DNA defined at each end by a specific primer. The exponential replication of the same target sequence produces enough DNA product or amplicons for use in subsequent analyses. PCR typically consists of three basic steps, as mentioned earlier.

Multiplex PCR: Multiplex PCR is a modification of conventional PCR in which two or more different PCR products are amplified simultaneously within the same reaction. This type of PCR consists of the same steps as conventional PCR, except that multiple sets of primers are used, each one priming a PCR product. 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. The advantage is that it requires less time and effort in amplifying multiple target templates or regions than individual reactions and may be a useful screening assay. However, significant optimization is required to obtain all of the products with equal efficiency and sensitivity. By simultaneously amplifying more than one locus in the same reaction, multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory. Since its first description in 1988 (Chamberlain *et al*., 1988), this method has been successfully applied in many areas of DNA testing, including analyses of deletions, mutations and polymorphisms, or quantitative assays and reverse transcription PCR.

Nested PCR: Nested PCR is a very specific PCR amplification and is a variation of the conventional PCR, in that two pairs (instead of one pair) of PCR primers are used to amplify a fragment. The first PCR utilizes a pair of primes flanking the gene in question while the second PCR uses another pair of primers having complementarity to an internal segment of the gene, which was amplified in the first PCR. The fragment produced by the first reaction is used as the template for the second PCR. The second set of primers called nested primers (as they lie / are nested within the first fragment) is specific to the DNA sequence found within the initial PCR product. The use of a second amplification step with the "nested" primer set results in a reduced background due to the nested primers' additional specificity to the region. The amount of product produced, which is shorter than the first one, is increased as a result of the second round of amplification. Carrying out nested PCR can further enhance the reliability of the PCR. Therefore, when information on the sequence of specific genes is available, amplification and visualization of that gene using a nested PCR could be carried out for confirmation.

Reverse Transcription Polymerase Chain Reaction (RT-PCR): This is based on the processes of reverse transcription and polymerase chain reaction. RT-PCR is a two step process. The first step consists of the formation of complementary or copy DNA (cDNA) from RNA (generally mRNA). This is followed by the second step which is a conventional PCR using the cDNA as the template. The first step referred to as the "first strand reaction" uses enzyme reverse transcriptase for the production of the cDNA from the RNA. In the second step, the cDNA sequence is amplified by using primers specific to it. The RT-PCR forms a high sensitivity detection technique, where low copy number or less abundant RNA molecules can be detected. It is also used to clone mRNA sequences in the form of complementary DNA, allowing cDNA libraries to be created which contain all sequences of all the genes expressed in a cell. It allows the creation of cDNA constructs for the gene expression studies.

Real Time PCR: Real-time PCR is different from other PCR as it quantifies the initial amount of the template instead of detecting the amount of final amplified product (Freeman *et al*., 1999; Raeymaekers, 2000). Real Time PCR is characterized by the point in time during cycling when amplification of the PCR product of interest is first detected *rather* than the amount of the PCR product which has accumulated at the end point. Real Time PCR does this by using fluorescent

dyes such as Sybr Green, or fluorophore-containing DNA probes such as Taq Man which get incorporated into each of the new strand, and monitoring the amount of fluorescence emitted during the PCR. This acts as an indicator of the amount of PCR amplification that occurs during each PCR cycle. Thus, in Real Time PCR machines, one can visually see the progress of the reaction in "real time". Quantification using real-time PCR can be 'relative or absolute.

Random /Arbitrary primed PCR: Random primed PCR conceived by Williams *et al* (1990) is unique in that only single short primer (usually 10 bases long) is used instead of the primer pair in the conventional PCR. Prior knowledge of the sequence of the target DNA is not required and primer with any sequence can be employed. The underlying theory in AP-PCR is that the primer may find complimentary sequence at different locations on the two DNA strands used as template, and amplify the intervening regions at low PCR stringency conditions (36 – 40°C).

This is used to generate Random amplified polymorphic DNA (RAPD) profile which is increasingly being used as a method for the DNA finger printing and genetic characterization where prior knowledge of the sequence of the target DNA is not required. RAPD is used as a marker system, where sequence of the target DNA is not known. This is a rapid technique and can be useful for species/strain identification. Genomic variations between and within species could be identified as the difference in the molecular size and number of DNA fragments amplified. The PCR products variations shall be resolved by agarose gel electrophoresis.

Modifications of PCR for specific purposes:

Degenerate PCR: Degenerate PCR is in most respects identical to ordinary PCR, but with one major difference. It is in a situation where the sequence of the gene to be amplified is not known, insert "wobbles" in the PCR primers are inserted. So, instead of using specific PCR primers with a given sequence, mixed PCR primers are used. For example, when a protein motif is back-translated to the corresponding nucleotide motif (Protein —> Sequence), there will be more than one codon coding for particular amino acid, due to degeneracy of genetic code. Thus there will be more than one nucleotide sequence deciphered for a particular protein sequence.

Asymmetric PCR: Asymmetric PCR is used to preferentially amplify one strand of the original DNA more than the other. It finds use in some types of sequencing and hybridization probing where having only one of the two complementary stands is required. PCR is carried out as usual, but with a great excess of the primers for the chosen strand. Due to 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 known as Linear-After-The-Exponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR): Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) is a fast and efficient method initially developed to amplify unknown sequences adjacent to known insertion sites in Arabidopsis. Nested, insertion-specific primers are used together with arbitrary degenerate primers (AD primers), which are designed to differ in their annealing temperatures. Alternating cycles of high and low annealing temperature yield specific products bordered by an insertion-specific primer on one side and an AD primer on the other. Further specificity is obtained through subsequent rounds of TAIL-PCR, using nested insertion-

specific primers. The increasing availability of whole genome sequences renders TAIL-PCR an attractive tool to easily identify insertion sites in large genome tagging populations through the direct sequencing of TAIL-PCR products. For large-scale functional genomics approaches, it is desirable to obtain flanking sequences for each individual in the population in a fast and cost-effective manner.

Hot Start PCR: The technique may be performed manually by heating the reaction components to the melting temperature (e.g., 95ÚC) 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 Hot Start PCR, polymerase activity during PCR reaction preparation is inhibited by using included chemical modifications, wax-barrier methods, and inhibition by a *Taq*-directed antibody. By limiting polymerase activity prior to PCR cycling, non-specific amplification during the initial set up stages are reduced and the yield of desired PCR product is increased.

Touchdown PCR: Non-specific primer binding obscures polymerase chain reaction results, as the non-specific sequences to which primers anneal in early steps of amplification will "swamp out" any specific sequences because of the exponential nature of polymerase amplification. Touchdown PCR or touchdown style polymerase chain reaction is a variant of PCR that aims to reduce non-specific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (3-5ÚC) above the Tm of the primers used, while at the later cycles, it is decreased in increments for every subsequent set of cycles (the number of individual cycles and increments of temperature decrease is chosen by the experimenter) a few degrees (3-5ÚC) below the primer Tm. 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.

Inverse PCR: Inverse PCR (IPCR), variant of PCR, was first described by Ochman *et al.* (1988). It is used when only one internal sequence of the target DNA is known. It is therefore very useful in identifying flanking DNA sequences of genomic inserts. Inverse PCR uses standard polymerase chain reaction, however it has the primers oriented in the reverse direction of the usual orientation. The template for the reverse primers is a restriction fragment that has been ligated upon itself to form a circle. Applications of Inverse PCR in molecular biology include the amplification and identification of sequences flanking transposable elements, and the identification of genomic inserts.

Long PCR: Long PCR is used when large segments of DNA (frequently over 10 kb) is to be amplified. For the accuracy of PCR, special mixtures of proficient polymerases such as *Pfu* are often used, which possesses 3' to 5' exonuclease activity or proofreading activity. The efficiency drastically declines when incorrect bases are incorporated. The 3' to 5' exonuclease activity removes these mis-incorporated bases and makes the further reaction proceed smoothly. Therefore, the amplification of long DNA fragments can be achieved. Long PCR is often used to clone larger genes or large segments of DNA which standard PCR cannot.

Gradient PCR: When a set of PCRs are run with different annealing temperatures all in the same block of the thermal cycler, simultaneously it is referred to as Gradient PCR. This is generally carried out for

standardizing PCR conditions for heterologous primers. In many models of thermal cyclers, blocks are available with gradient annealing temperatures. This saves time and multiple blocks are not needed.

AFLP PCR: Amplified Fragment Length Polymorphism PCR, also called AFLP PCR was originally described by Zabeau and Vos, 1993. AFLP is a highly sensitive PCR-based method for detecting polymorphisms in DNA. AFLP can be also used for genotyping individuals for a large number of loci using a minimal number of PCR reactions.

Alu PCR: PCR using a primer that anneals to *Alu* repeats to amplify DNA located between two oppositely oriented *Alu* sequences. Used as a method of obtaining a fingerprint of bands from an uncharacterized human DNA.

Colony PCR: Colony PCR is mostly used after a transformation, to screen colonies for the desired plasmid. Primers which generate a PCR product of known size are used. Thus, colonies which give rise to an amplification product of the expected size are likely to contain the correct DNA sequence. Colony PCR is used for the screening of bacterial (*E. coli*) or yeast clones for correct ligation or plasmid products. Selected colonies of bacteria or yeast are picked 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.

***In Situ* PCR:** *In Situ* PCR (ISH) is a polymerase chain reaction that actually takes place inside the cell fixed on slide. *In situ* PCR amplification can be performed on fixed tissue or cells. ISH applies the methodology of the nucleic acid hybridization technique to the cellular level. Combining cytochemistry and immunocytochemistry, it allows the identification of cellular markers to be identified and further permits the localization of to cell specific sequences within cell populations, such as tissues and blood samples.

Single Cell PCR: The advent of the polymerase chain reaction (PCR) has revolutionized the way in which molecular biologists view their task at hand, for it is now possible to amplify and examine minute quantities of rare genetic material: the limit of this exploration being the single cell. It is especially in the field of prenatal diagnostics that this ability has been readily seized upon, as it has opened up the prospect of preimplantation genetic analysis and the use of fetal cells enriched from the blood of pregnant women for the assessment of single-gene Mendelian disorders. However, apart from diagnostic applications, single-cell PCR has proven to be of enormous use to basic scientists, addressing diverse immunological, neurological and developmental questions, where both the genome but also messenger RNA expression patterns were examined. Furthermore, recent advances, such as optimized whole genome amplification (WGA) procedures, single-cell complementary DNA arrays and perhaps even single-cell comparative genomic hybridization will ensure that the genetic analysis of single cells will become common practice, thereby opening up new possibilities for diagnosis and research.

Single nucleotide polymorphism PCR (SNP PCR): SNP PCR involves real-time PCR using single nucleotide polymorphisms (SNPs) as markers. It is a very sensitive and accurate method to quantify the percentage of recipient and donor cells to monitor the effect of stem cell transplantation (SCT) and sequential adoptive immunotherapy by donor lymphocyte infusions (DLI).

Digital PCR: Digital PCR represents an example of the power of PCR and provides unprecedented opportunities for molecular genetic analysis in cancer. The technique is to amplify a single DNA template from minimally diluted samples, therefore generating amplicons that are exclusively derived from one template and can be detected with different fluorophores or sequencing to discriminate different alleles (e.g., wild type vs. mutant or paternal vs. maternal alleles). Thus, digital PCR transforms the exponential, analog signals obtained from conventional PCR to linear, digital signals, allowing statistical analysis of the PCR product. Digital PCR has been applied in quantification of mutant alleles and detection of allelic imbalance in clinical specimens, providing a promising molecular diagnostic tool for cancer detection.

Assembly PCR: Assembly PCR is the 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.

Helicase-dependent amplification: This technique is 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.

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

Ligation-mediated PCR: This method uses small DNA linkers ligated to the DNA of interest and multiple primers annealing to the DNA linkers; it has been used for DNA sequencing, genome walking, and DNA fingerprinting.

Methylation-specific PCR (MSP): The MSP method was developed by Stephen Baylin and Jim Herman at the Johns Hopkins School of Medicine, and is used to detect methylation of CpG islands in genomic DNA. DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine. Two PCR reactions are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences. At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA, and one set recognizes DNA with uracil or thymine to amplify unmethylated DNA. MSP using qPCR can also be performed to obtain quantitative rather than qualitative information about methylation.

PCR applications

PCR has transformed the way that most studies requiring the manipulation of DNA fragments and DNA cloning may be performed as a result of the simplicity and usefulness of PCR. Cell-free DNA amplification by PCR is able to simplify many of the standard procedures for DNA cloning, DNA analysis, and the modification of DNA. Previous molecular biology techniques for isolating a specific piece of DNA had relied on gene cloning, which is a tedious and slower procedure. An alternative to cloning, PCR, can be used to directly amplify rare specific DNA sequences in a complex mixture when the ends of the sequence are known. This method of amplifying rare sequences from a mixture has numerous applications in basic research, human genetics testing and forensics. Some of the PCR applications include site-specific mutagenesis studies, amplification and detection of

DNA *in situ* from cells for rapid diagnosis, genomic subtraction, analysis of protein functions and intermolecular assembly, DNA fingerprinting (RAPD/AFLP/VNTR/) for evaluation of genetic heterogeneity & relationship, paternity verification, forensic application, generation of single chain antibody fragments for immunology, sensitive disease diagnosis, cDNA synthesis from RNA for cDNA library construction, production of clones for sequencing, molecular epidemiology, molecular taxonomy and many more.