Chapter 12

Polymerase chain reaction and its types

Minimol V A, Sivaraman G K and Toms C Joseph

Polymerase chain reaction (PCR) is one of the fundamental techniques in various molecular microbiology experiments and refers to a set of procedures for the *in vitro* enzymatic amplification of a desired DNA fragment or gene from the whole genome of an organism. PCR enables the synthesis of more than 10 million copies of a target DNA sequence from a few initial copies of the sequence. PCR technique is used widely in various diagnostics and forensic investigations, and becomes essential for many common procedures such as cloning, sequencing, microaarays etc.

History

PCR was discovered by Dr. Kerry Mullis in 1983 at the Cetus Corporation in Emeryville, CA and he was awarded the Nobel Prize in Chemistry for his work on PCR in 1993. Moreover, the *in vitro* DNA synthesis using two primers was initiated by Gobind Khorana in 1971, but the main constraints were primer synthesis and polymerase purification issues. Though the previous techniques were used for isolating a specific gene by gene cloning method, it is laborious and time-consuming processes. Prior to 1988, a PCR reaction was carried out by a series of water baths by adding a fresh aliquot of Klenow fragment of *E. Coli* DNA polymerase I after each denaturation step. The Klenow fragment was not highly specific, can amplify only about 400bp and could produce an incompletely pure target product and the target sequence should be confirmed by specific hybridization probe. Obviously, it is a tedious process and was eliminated by the introduction of a thermostable DNA polymerase, the Taq DNA polymerase and is highly specific to the target sequence as well as can withstand in the repeated heating PCR cycling conditions. This increased specificity also could increase the yield of the target sequence and can amplify the PCR products up to 10 kb. The introduction of DNA polymerase enzymatically assembles a new DNA strand

from target DNA and nucleotides, for initiation of DNA synthesis. Recently, *Pyrococcus furiosus* (Pfu) DNA polymerases and *Thermococcus Litoralis* (VENT) are becoming more widely used because of the proof reading 3' to 5' exonuclease activity which is lacking in *Taq* polymerase.

Components and Reagents used in PCR Mixture

Typical PCR mixtures consists of a 10X reaction buffer for the Taq polymerase, forward and reverse primers, deoxyribonucleotide triphosphates (dNTPs), Taq polymerase and the template DNA. Each of these components is described below.

Template DNA

DNA template is nothing but the target DNA to be amplified. The quantity, quality and integrity of the template DNA is an important factor to determine the success of PCR. About 1 ng of plasmid or phage DNA and 10-100 ng of pure genomic DNA generally yield expected amplifications. Higher amounts of template DNA usually result in nonspecific PCR amplifications. Still higher quantities might result in the inhibition of amplification. The contaminants such as heparin, heme, formalin, Mg^{2+}-chelating agents, as well as detergents etc. if any will also result in the inhibition of amplification process and need to be eliminated.

10X Buffer for *Taq* polymerase

10X buffer provide suitable condition for optimum activity and stability of the DNA polymerase. The reaction buffer consists of Tris-HCl, EDTA, KCl, Nonidet P40, tween 20 and glycerol. However, this can vary depending on the type of polymerase and the source. Buffers are supplied at 10× concentration along with the polymerase. It is not advisable to interchange buffers and polymerases from different suppliers, as this may not yield expected results.

$MgCl_2$ concentration

The range of $MgCl_2$ concentration in a reaction mixture varies between 1 to 4 mM, with a standard concentration of 1.5 mM. The 10X buffers are available either with or without $MgCl_2$. If the buffer does not contain

concentration of $MgCl_2$, it has to be added separately in to the reaction mixture. The Mg^{2+} ions form a soluble complex with dNTPs which is essential for dNTP incorporation. It stimulates polymerase activity, and increase the T_m of primer/template interaction (and therefore they stabilise the duplex interaction). The optimal concentration of $MgCl_2$ prevent Mg^{2+} ions to form complexes with dNTPs, primers and DNA templates. Too low Mg^{2+} ions result in low yield of PCR product, and too high increase the yield of non-specific products.

Primers

Primers are complementary to the 3' ends of each of the sense and anti-sense strand of the DNA target. PCR primers are usually 18-25 nucleotides in length with an average GC content of 40-60%. To avoid non-specific priming, primers should not have more than three G or C nucleotides at the 3'-end. The primer should not be self-complementary or complementary to any other primer in the reaction mixture, in order to avoid primer-dimer and hairpin loop formation. The standard concentration of primer/oligonucleotide is usually 1μM which will be sufficient for at least 30 cycles of amplification. The presence of higher concentration of pimer can can cause amplification of undesirable non-target sequences. Conversely, the PCR is inefficient with limiting primer concentration.

Deoxynucleoside triphosphates (dNTPs)

Deoxynucleoside triphosphates (dNTPs) are the building blocks from which the DNA polymerases synthesize a new DNA strand. The equimolar concentration of each dNTP (dATP, dCTP, dGTP & dTTP) is required in a PCR mixture and usually at 200 μM each. Unequal concentrations lead to misincorporations of nucleotides resulting in altered sequences which might affect post-PCR experiments such as sequencing, cloning and expression of protein.

***Taq* DNA polymerase**

Taq polymerase attaches to the target sequence and can withstand repeated heating PCR cycling conditions. Typically, DNA polymerases can only incorporate nucleotides from the 3' end of a polynucleotide. The first thermostable DNA polymerase used was the *Taq* DNA polymerase isolated from the bacterium *Thermus aquaticus*. Even though this enzyme is probably the most widely used in PCR applications, several other DNA polymerases are commercially available. The concentration of *Taq* polymerase is very crucial in a PCR. The recommended concentration is 1-1.5 units in a 50 µl reaction mixture involving amplifications of up to 1 kb. When larger than 1 kb is to be synthesized, the concentration of *Taq* may be increased. It is important to follow manufacturer's recommendations regarding the concentrations to be used since this can vary considerably depending on the source of the enzyme.

PCR Cycle and Principles

The PCR process consists of repetitive series of three fundamental steps called PCR cycle. The cycling is often preceded by a single temperature step called Initialization at a high temperature (>90°C) and one final elongation for any remaining single-stranded DNA is fully extended. The Final hold step is at 4°C for short term storage of the amplified PCR products.

In PCR, the double stranded DNA is denatured by heat and then the temperature is lowered to allow annealing of two specific primers by complementary base pairing on the opposite strands of the DNA. *Taq* polymerase directs the synthesis of the new strand from the primed sites in both directions that results in double stranded DNA and the procedure is repeated for 25-40 times in a thermocycler. In each cycle, the target DNA is replicated by a factor of 2 so that, after the completion of PCR, millions of copies of DNA are available for subsequent manipulations.

Initial Denaturation

Initial Denaturation ensures complete denaturation of the double stranded DNA at the start of the PCR cycling. A 5 min denaturation at 95°C should be sufficient for templates with a GC content of 50% or less. The denaturation time should be extended up to 10 min for GC-rich templates.

After initial denaturation, the following 3 steps are repeated 30-40 times.

1. **Denaturation**/ The double-stranded DNA template denaturation: The double-stranded DNA template denatured into two complementary single strands of DNA by disrupting the hydrogen bonds between complementary bases. Usually DNA undergoes rapid denaturation at 94–98 °C with 30 seconds to 2 minutes.

2. **Annealing**/ Annealing of two oligonucleotide primers to the single-stranded template: After denaturation, temperature is lowered to 50–65°C for 20–60 seconds allowing annealing of the primers to the single-stranded DNA template and preventing immediate reannealing of long DNA strands. The primers rapidly anneal to the single strands of DNA because of their small size and Taq polymerase will binds to them. Generally the annealing temperature is 136 about 3-5 degrees Celsius below the Tm of the primers used. Once the stable DNA-DNA hydrogen bonds are formed, the polymerase binds to the primer-template hybrid and begins DNA synthesis.

Annealing temperature of the primers is calculated using the following formula

Tm = 4 (G + C) + 2 (A + T)

Annealing temperature (°C) = Tm – 5°C

Where, Tm = Melting temperature; G, C, A, T =number of respective nucleotides in the primer.

3. Extension

Enzymatic extension of the primers to produce copies that can serve as templates in subsequent cycles: The DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the extending DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. The DNA polymerase will polymerize a thousand bases per minute. At each

extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

Final extension

After the completion of cycling, a final extension for 5-15 min at 72°C is done to complete incompletely synthesized strands.

Preparation of PCR Reaction Mixture

A master mix can be prepared by adding the required concentration reagents of PCR reaction in a given volume and then be aliquoted appropriately to individual tubes depending upon the testing requirement. This procedure can minimize the possibility of pipetting errors in case of handling low volume and also saves time. Transfer the required water, buffer, $MgCl_2$ dNTPs, primers and *Taq* DNA polymerase in a single tube be aliquoted into individual tubes. Various template DNA to be amplified are then added to the individual tubes and to be labelled for further recording of the results. A positive control (known DNA sample) & a negative PCR control (water) should also be included to ensure the reliability of the procedure. PCR reaction mix preparation is follows

Thaw all the reagents such as water, buffer, $MgCl_2$ dNTPs, primers and template DNA except *Taq* DNA polymerase (it should keep always under ice) vortex gently and spin all solutions. Prepare master mix by adding the following reagents except template and transfer aliquot to individual tubes (PCR tubes 0.2ml) placed on ice. Then add DNA templates to the respective tubes. The final concentration for 30 ml reaction mixture is given in the table

Component	**Final concentration**	**Volume required (µl)**
Sterile deionized water	-	15.4
10X *Taq* buffer	1X	3
10 mM dNTP mix	0.2 mM of each	0.5
Primer Forward	1 µM	3

Primer Reverse	1 µM	3
Taq DNA Polymerase	1.5 u	0.3
25 mM $MgCl_2$	1.5 mM	1.8
Template DNA	100 ng	3
Total volume		**30**

Vortex each PCR tubes and spin to collect all drops from the walls of tube. Place the PCR tubes in thermocycler and start PCR. Once the PCR cycle is over, the PCR product is visualized by electrophoresis in agarose gel stained with 0.5 µg/ml of ethidium bromide.

Agarose gel electrophoresis

Agarose gels are cast by melting the appropriate percentage of agarose (1-2%) in convenient buffer (1X TAE) until a clear, transparent solution is achieved. The melted gel is then cooled (50°C), poured on a tray containing combs and allowed to solidify. Upon solidification, the comb is removed and gel is placed in the electrophoresis chamber and then pour the buffer to cover the gel. DNA samples are loaded into the sample wells by mixing with loading dye (2 µl for 10 µl of the PCR product) and gel is run at a voltage for a time period that will perform optimal separation. When electric field is applied across the gel, DNA migrates toward the anode. When running of the gel is completed, the separated bands on agarose gel are labeled or stained for interpreting. One method of staining DNA is to expose it to the dye ethidium bromide (EtBr) (0.5 µg/ml). EtBr intercalates between the stacked bases of nucleic acids and fluoresces red–orange when illuminated with ultraviolet (UV) light. EtBr is a carcinogen and should be handled with care.

Specialised PCR

In addition to the amplification of a target DNA sequence by the typical PCR procedures already described, several specialized types of PCR have been developed for specific applications.

RT-PCR (or Reverse Transcription PCR)

RT-PCR (or Reverse Transcription PCR) is used when the target nucleic acid is RNA. The central dogma in molecular biology explains about the direction or flow of information in which the DNA of the organism encodes the genetic information, intern transfer to RNA by the process of transcription and then to protein via translation process. As RNA is highly unstable and enzymatic amplification is difficult and need to reverse transcribed to cDNA for amplification. The reverse transcriptase, an enzyme that converts RNA into cDNA. This cDNA can be used for PCR and reverse transcription process may be combined in a tube, as the initial heating step of PCR being used will inactivate the transcriptase enzyme. The Tth polymerase is used for the enzymatic amplification due to its inherent RT activity, and can carry out the entire reaction. As the phenotype of an organism is explained by the RNA or protein fractions. So, RT-PCR is used in expression profiling of specific gene or gene products. It can also used in RNA transcript analysis where in transcription start and termination sites are determined. Also it enables the mapping of exons and introns of the gene sequence.

RT-PCR can also be very useful in the insertion of eukaryotic genes into prokaryotes. Because most eukaryotic genes contain introns, which are present in the genome but not in the mature mRNA, the cDNA generated from a RT-PCR reaction is the exact (without regard to the error-prone nature of reverse transcriptase) DNA sequence that would be directly translated into protein after transcription. When these genes are expressed in prokaryotic cells for the sake of protein production or purification, the RNA produced directly from transcription need not undergo splicing as the transcript contains only exons. (Prokaryotes, such as *E. coli*, lack the mRNA splicing mechanism of eukaryotes). RT-PCR is commonly used in studying the genomes of viruses whose genomes are composed of RNA, such as Influenza virus A and retroviruses like HIV.

Nested PCR

Nested sets of primers can be used to improve PCR yield of the target DNA sequence. In nested PCR, two primer sets are used in which the first round of PCR is performed with one primer set for 15-30 cycles, then second set of primer is used for second round PCR, for an internal region of the first amplified DNA for an additional 15 to 30 cycles. The PCR product of the first round of PCR is used as DNA template for the second PCR. Thus, the nested PCR method increases the sensitivity and specificity of DNA amplification. The specificity is particularly enhanced because this technique almost always eliminates any spurious non-specific amplification products. This is because after the first round of PCR any non-specific products are unlikely to be sufficiently complementary to the nested primers to be able to serve as a template for further amplification, thus the desired target sequence is preferentially amplified. However, the increased risk of contamination is a drawback of this extreme sensitivity, and great care must be taken when performing such PCRs, particularly in a diagnostic laboratory.

Multiplex PCR

Multiplex PCR enables simultaneous amplification of many sequences or gene using two or more set of primers in one PCR. The presence of many PCR primers in a single tube could cause many problems, such as the increased formation of misprimed PCR products, "primer dimers", and the amplification discrimination of longer DNA fragments. For this type of PCR amplification, primers are chosen with similar annealing temperatures. The lengths of amplified products should be similar; large differences in the lengths of the target DNAs will favour the amplification of the shorter target over the longer one, resulting in differential yields of amplified products. In addition, Multiplex PCR buffers contain *Taq* polymerase additive, which decreases the competition among amplicon and the discrimination of longer DNA fragments during Multiplex PCR. Multiplex PCR products can be further hybridised with a gene-specific probe for verification.

Quantitative PCR

Quantitative PCR is used to measure the amount or quantity of target nucleic acid (DNA or RNA) in a sample. The amount of fluorescence generated during the the phase of true exponential stage is directly measures the amount of nucleic acid or target. Special thermal cyclers fitted with light source and suitable filters for wavelength selection are used for the real time detection or monitoring of PCR product. Fluorescent dyes used are Sybr Green, or flurorophore-anchored DNA probes, such as TaqMan, to measure the amount of amplified product as the amplification progresses. Quantitative PCR is also used by microbiologists working in the fields of food safety, food spoilage and fermentation and for the microbial risk assessment of water quality (drinking and recreational waters) and in public health protection.

The antibacterial assay Virtual Colony Count utilizes a data quantification technique called Quantitative Growth Kinetics (QGK) that is mathematically identical to QPCR, except bacterial cells, rather than copies of a PCR product, increase exponentially. The QGK equivalent of the threshold cycle is referred to as the "threshold time".

Colony PCR

In Colony PCR, bacterial colonies are screened directly by PCR, for example, the screen for correct DNA vector constructs. Colonies are sampled with a sterile pipette tip and a small quantity of cells transferred into a PCR mix. To release the DNA from the cells, the PCR is either started with an extended time at 95 °C (when standard polymerase is used), or with a shortened denaturation step at 100 °C and special chimeric DNA polymerase.

Applications of PCR

1. Selective DNA amplification

- PCR allows selective amplification of a specific region of DNA/ gene from genomic DNA which can be utilized for direct sequencing, genomic cloning, DNA typing, detection of infectious microorganisms, sitedirected mutagenesis, prenatal genetic disease diagnosis and analysis of sequence variations.

• PCR 'fingerprints' methods can be used to identify genetic relationships between individuals (parentchild, between siblings and paternity testing) and microbial identification, evolutionary relationships among individuals/ organisms and forensic analysis. • PCR may also be used in the analysis of ancient DNA.

• Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample and quantitative determine the levels of gene expression. 2. PCR in diagnosis of diseases

• A diagnostic application in microbiology for the detection of infectious agents and the differentiation of non-pathogenic from pathogenic strains. • Identification of non-cultivatable or slow-growing microorganism 's like viruses mycobacteria, anaerobic bacteria.

• Viruses can be detected before the onset of disease and/ or immediately after the infection.

• Utilize for early diagnosis of cancer research

. • Amount of virus in an infected patient can be quantified.

****