Polymerase Chain Reaction - A versatile diagnostic tool

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The Polymerase chain reaction (PCR) is an *in vitro* technique for the amplification of specific DNA sequences by simultaneous primer extension of complementary strands of DNA. The method was invented by Karry Mullis in 1985 who received the Nobel Prize for chemistry for his work in 1993. PCR is a major development in the analysis of DNA and RNA because it has both simplified the existing technology and enabled the rapid development of new techniques which would not other wise have been possible.

PRINCIPLE

The PCR amplification of DNA is achieved by using oligonucleotide primers, also known as amplimers. These are short, single stranded DNA molecules which are complementary to a defined sequence of DNA template. The primers are extended of a single stranded denatured DNA by a DNA polymerase, in the presence of deoxynucleotide triphosphates (dNTPs) under suitable reaction conditions.

PCR involves a series of steps. DNA denaturation, primer hybridization and extension represent one PCR cycle. Each of the three steps is carried out at an appropriate temperature. This results in *de novo* synthesis of the target DNA

The dNTPs and primers are present in large excess, so the synthesis step can be repeated by heating the newly synthesized DNA to separate the strands and cooling to allow the primers to anneal to their complementary sequences.

REACTION COMPONENTS FOR PCR

i) Target sequence

There are different targets for nucleic acid amplification. It may be RNA or single /double stranded DNA. If a circular DNA (e.g. plasmid) is to be used it should be linearized prior to amplification. It is not necessary to know the nucleotide sequence of the target template, but that of the nucleotide flanking the target sequence must be known to design the primers. Use of purified primers is not mandatory. Even crude cell lysates provide excellent templates, enhancing its applications in clinical and forensic investigations. The DNA chosen must be intact over the length which is to be amplified. This may mean that only shorter fragments can be amplified from sources where the DNA is degraded.

II) DNA polymerase

In the original PCR protocol, Klenow fragment of *E.coli* DNA polymerase I was used which was subsequently replaced by Taq DNA polymerase (purified from the thermophilic bacterium *Thermus aquaticus*). A variety of DNA polymerases are now available (Table 1).The most commonly used ones are: native enzyme purified from *Thermus aquaticus* and a genetically engineered form of the enzyme synthesized in *E. coli* (AmplitaqR) .Their advantage includes heat stability and high temperature optimum. It also pocesses a broad pH range from 8.2 – 9.0 in 10 mM Tris. Both forms of polymerase carry a 5 ' to 3 ' polymerization – dependant exonuclease activity. But they lack 3 ' to 5 ' exonuclease activity. Approximately 2 units of either of these enzymes are required to catalase a typical PCR. Addition of excess of enzymes may lead to amplification of non-target sequences.

For RNA amplification, the initial step is to create a complementary DNA (cDNA) from the RNA in the sample. This is achieved through the use of a reverse transcriptase enzyme.

Table 1. Thermostable DNA polymerase and their sources

DNA polymerase	Source
Taq	*Thermus aquaticus*
Amplitaq^{R}	*Thermus aquaticus*
HotTub^{TM}	*Thermus flavus*
Vent^{TM}	*Thermococcus litoralis*
Tth	*Thermus thermophilus*
Pfu	*Pyrococcus furiosus*
UlTma^{TM}	*Thermotoga maritima*

III) Primers

Oligonucleotides ranging from 18 – 30 bases are commonly used as primers. These oligonucleotides are too short to form stable hybrids at the temperature used for polymerization. Primer sequence should have similar G + C content; minimal secondary structure and low complimentarity to each other, particularly in the 3 ' region. Ideally, both primers should anneal at the same temperature.

Usually in case of paired primers equal quantity of upstream and down stream primers are used. This is usually sufficient for at least 30 cycles of amplification. The presence of higher concentration of oligonucleotides can cause priming at ectopic sitesth consequent amplification of non- target sequence.

IV) Buffers

The standard buffer of PCR contains

50 mM KCl

10 mM Tris.Cl. (pH 8.3 at RT)

1.5 mM $MgCl_2$.

The presence of divalent cations is critical. Mg^{++} ions are superior to Mn^{++} and Ca^{++} is ineffective. The optimal concentration of Mg^{++} is quite low (1.5 mM). The template DNA preparation should not contain high concentration of chelating agent such as EDTA or of negatively charged ionic groups such as phosphates.

The concentration of Mg^{++} should be optimized whenever a new combination of target and primer is first used or when the concentration of dNTPs or primers is altered. Too little free Mg^{++} may produce a variety of unwanted products.

v) dNTPs

Deoxynucleotide triphosphates are dATP, dCTP, dGTP, dTTP and dUTP. These are the major source of phosphate groups in the reaction. Any change in their concenteration effects the concentration of available Mg^{++} . These are used at saturating concentrations (200µM of each dNTPs). A stock solution of dNTPs (50mM) should be adjusted to pH 7.0 with 1N NaOH to ensure that the pH of the final reaction does not fall below7.1.

BASIC PROTOCOL

The components of PCR in appropriate volume are mixed in reaction tubes and kept in the thermal cycler. The PCR cycle is preprogrammed as per the user requirement.

A typical PCR cycle begins with initial denaturation at 94°C for 5 minutes for complete strand separation of template DNA and inactivation of endogenous polymerases. This is followed by the thermal cycles consisting of:

Denaturation at 94°C for 20-30 seconds.

Primer annealing at about 50°C for 20-60 seconds.

Extension at 72°C for 30-60 seconds.

In case of double stranded DNA template, cycling concludes with a final extension step at 72°C for 5 minutes.

The quantum of amplification by PCR thermal cycling is 2^n , where 'n' is the number of cycles employed for DNA amplification. Thus DNA will continue to accumulate exponentially until one of the reaction product is exhausted or the enzyme is unable to synthesis new DNA quickly enough. At high concentrations the DNA may at times begin to prime itself and result in synthesis of nonspecific product. The number of cycles required for optimum amplification varies depending on the amount of starting material and efficiency of each of the amplification steps. Generally 20-35 cycles will generate optimum quantity of DNA for further analysis.

PCR for diagnosis

PCR can be considered as revolutionary tool to the molecular biologists. This ingenious method has profound application in several fields including veterinary disease diagnosis. PCR as veterinary diagnostic practice appeared in late 1980's (Belak *et.al.*, 1993). It was applied for the detection and differentiation of several pathogens present in environment or biological or histological materials (Fratamico *et.al.*, 2000; Gillespie *et.al.*, 1994).

The main advantage of using PCR in the detection of pathogens is that a single cell/ pathogen can be detected. Conventional method of characterization of pathogens involving their *in vitro* culture may take weeks. A PCR assay can be performed within hours. PCR is a viable option for the detection of fastidious organisms and those that cannot be grown *in vitro*, which are particularly difficult to be detected by conventional methods.

The specificity and high sensitivity of the test produce a valuable tool for early diagnosis of the disease. This is useful to detect sub-clinical and carrier status of the host (Fahrimal *et.al.*, 1992).

Table 2. Detection of specific pathogens using PCR

Pathogen	*Reported by*
viral	
FMD	Meyer *et.al.*,1991
Swine fever	Roche and Woodward,1991
ILT	Williams *et.al.*,1992
Marek's Disease	Silva,1992
Newcastle disease	Jestin and Jestin,1991
Blue tongue	Gould *et.al.*,1989
Canine parvo	Mochizuki *et.al.*,1993
Rabies	Shanker *et.al.*,1991
Duck hepatitis	Qiao *et.al.*,1990
Bacterial	
Leptospira	Bal *et.al.*,1994
Campylobacter jejuni	Itoh *et.al.*,1995
Mycoplasma pneumonia	Kai *et.al.*,1993
Mycobacterium tuberculosis	De Wit *et.al.*,1990
Fungus	
Micosporum sp	Faggi *et.al.*,2001
Trychophyton sp	
Epidermophyton sp	
Parasites	
Theileria annulata	D'Oliveira *et.al.*,1995
Ehrlichia canis	Iqbal and Rikibrisa,1994
Babesia bigemina	Figeroa *et.al.*,1992

(Source: Ramadass & Meerarani in Text book of Animal Biotechnology)

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