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POLYMERASE CHAIN REACTION: AN EMERGING TOOL FOR RESEARCH IN PHARMACOLOGY

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ABSTRACT Polymerase chain reaction (PCR) is a technique that can amplify a specific DNA segment *in vitro* using two site specific primers that hybridise to opposite DNA strands. The method produces large amounts of specific DNA from a complex DNA template in a single enzymatic reaction within a matter of hours. Reaction mixture contains a double helix DNA, two target specific primers, dNTPs, a DNA polymerase and a Mg^{2+} containing buffer. PCR is carried out in three distinct steps *i.e.* denaturation step, annealing step and extension step, which are repeated many times. The efficiency of PCR is measured in terms of its specificity, yield and fidelity. Most of the disease pathologies are associated with an altered gene expression leading to a resultant increase or decrease in the activity of cellular proteins. PCR can be employed to quantify alterations in cellular mRNA encoding for such proteins. These proteins may serve as novel target sites for therapeutic intervention. This modified form of PCR is termed as reverse transcriptase PCR (RT-PCR). *In situ* PCR, another modified form of PCR, not only permits the quantification of mRNA but also the exact localisation of mRNA changes in a particular cell type in a given tissue. PCR has also been employed to amplify a family of related genes using degenerate oligo nucleotide primers (DOP). This strategy is known as homology screening or DOP-PCR and has successfully expanded several gene families such as cyclins and cyclin-dependent kinases. Besides these application of PCR in pharmacology, it also serves as a useful diagnostic tool.

KEYWORDS DNA amplification gene expression PCR thermocycler

Introduction

Recombinant DNA technology has emerged as a powerful tool for *in vivo* DNA amplification in dividing cells of micro-organisms. However, the technique is labour intensive and time consuming. In 1984, Karry Mullis while working with human-β-globin at Cetus Corporation developed an *in vitro* method for DNA amplification[1]. This technique was termed as polymerase chain reaction (PCR). PCR is an enzymatic technique to amplify a specific DNA segment *in vitro* using two site specific primers that hybridise to complimentary DNA strands. The method produces large amount of specific DNA from a complex DNA template in a single enzymatic reaction within a matter of hours. Therefore, PCR is much faster and simpler procedure as compared to recombinant DNA technology. Moreover, PCR is a highly sensitive technique which can amplify even picograms of DNA and starting DNA need not be pure or free from contamination of cell components. On the other hand, PCR amplifies DNA in a cell free system and this has greatly simplified subsequent cloning, analysis and modification of DNA. These advantages of PCR are reflected in an exponential increase in its use in the diverse scientific fields. The present review intends to describe the basic methodology of PCR with its possible application to pharmacological sciences.

Principle and methodology

A typical PCR protocol is carried out in a reaction mixture of 20-100 µl, either in individual tubes or multiwell plates. Reaction mixture contains a double helix DNA, two target specific primers, deoxynucleotide triphosphates (dNTPs), a DNA polymerase and

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a Mg^{2+} containing buffer. Mg^{2+} ions are required as a cofactor for DNA polymerase. Primers are 17-30 base containing oligonucleotides that hybridise to 5' end of regions flanking the target DNA sequence. PCR is carried out in three distinct cycles which are repeated many times. Each cycle begins with a denaturation step in which the reaction mixture is heated to 92-96°C to denature the double stranded DNA. Time required to denature DNA depends on its complexity, geometry of tubes and volume of reaction mixture. For G≡C rich DNA sequence, addition of glycerol, longer denaturation time and use of nucleotide analogues has been reported to improve yield of PCR[3]. Insufficient heating during denaturation step is the most common cause of PCR failure. The denaturation step is followed by annealing step in which the primers are allowed to hybridise to their complementary DNA sequences. The temperature of this step varies from 37°C to 65°C depending on the homology of the primers for the target sequence and the composition of the primers. Since the primers are present in high concentration and are short in length, they hybridize to their target sequence at a much higher rate than the complementary strands of the target DNA. In the final step, the annealed primer is extended at its 3' hydroxy position by a DNA polymerase. This three step cycle is repeated many times until a sufficient amount of target DNA is produced. Each extension product generated in one PCR cycle can serve as a template for extension in the next cycle (Figure 1). Therefore, PCR product increases exponentially as a function of the cycle number. Earliest PCR experiments employed klenow's fragment of *E.coli* DNA polymerase for DNA extension step[2]. This enzyme has optimal activity at 37°C and often amplifies non-specific DNA target products. Moreover, the enzyme gets inactivated at higher temperature, therefore fresh enzyme has to be added after each denaturation cycle. Later on, a heat resistant DNA polymerase named as Taq polymerase, was isolated from a thermophilic bacterium *Thermus aquaticus*[3]. Several thermostable DNA polymerases have now been isolated from various other thermophilic bacteria (Table 1). Unlike klenow fragment, these enzymes escape inactivation during the denaturation cycle. Therefore, they need to be added just once in the reaction mixture at the beginning. This has allowed automation of PCR using programmable machines named as thermocyclers. If the annealing step is carried out at low temperature, primers may hybridize to non-specific targets due to looping out of primers at low temperature. Thermostable polymerases allow annealing and extension at an elevated temperature, therefore amplify target DNA by minimising primer mismatch. Moreover, these polymerases allow amplification of much bigger DNA fragments as compared to 400bp with klenow's fragments[4].

The efficiency of PCR is measured in terms of its specificity, yield and fidelity.

Optimization of PCR specificity

Specificity is achieved by designing primers of sufficient length so that their sequence is virtually unique in the genome. Various additives such as DMSO[1,5,6] (1-10%), glycerol[5-7] (5-20%), non-ionic detergents, formamide[5,6,8] (1.25-10%), bovine serum albumin[5-7] (10-100 μg/ml) and polyethyleneglycol 6000 (5-15%)[9,10] can be incorporated into the reaction to increase PCR specificity. It has been proposed that these reagents enhance specificity by lowering strand separation temperature of DNA. At lower temperature, looping out of primers promotes formation of primer template complexes that are not perfectly matched and allow extension of mismatched target. This can be avoided by hot start PCR[11-13]. Use of klenow's fragment does not permit hot start and accounts for non-specific DNA amplification as was mentioned above.

PCR specificity can also be enhanced by the use of nested PCR. In this technique, products from first PCR amplification are subsequently amplified using a second set of primers which flank the same target site but internal to original primers. Any spurious product amplified in the first reaction by mispriming are unlikely to possess the identical nucleotide sequence for the internal primer. Therefore, only genuine product will be amplified in the second reaction[1,13-16].

Optimization of PCR yield

To maximize PCR yield, primers and dNTPs should always be present in molar excess. However, if the quantity of primers and dNTPs is excessively large, PCR is prone to low specificity and fidelity respectively. The average yield of a PCR protocol can be described by the following equation[17]:

Table 1. Various thermostable DNA polymerases.

Enzyme	Source	Temperature (ºC)	General applications
Taq™	Thermus aquaticus	70-80	Broad range
Stoffel Fragment™	Thermus aquaticus	70-80	
Tth™	Thermus thermophilus	110-120	RT-PCR
Tli/Vent™	Thermococcus litoralis	72-80	High fidelity PCR, LM PCR
pfu™	Pyrococcus furiosus	72-80	High fidelity PCR, DNA sequencing
Bst™	Bacillus stearothermophilus	60-65	High sensitivity
Sac	Sulfolobus acidocaldarius	70-80	DNA sequencing
Tac	Thermoplasma acidophilum	65	AT rich DNA PCR
Tfl/Tub™	Thermus flavus	70	Large template DNA PCR
Tru	Thermus ruber	50	DNA sequencing
Tsp	Thermotoga sp	80	DNA sequencing
Mth	Methanobacterium thermoautotrophicum	65	DNA sequencing

TM: Trademark

$N=n(1+E)^c$ where N= final amount of target DNA

n=initial amount of DNA

E=efficiency of amplification

c=number of PCR cycles

However, the yield of PCR varies significantly depending upon the nature of the target sequence, primer sequence and reaction conditions[18,19].

Optimization of PCR fidelity

PCR infidelity, defined as rate of nucleotide misincorporation, has been the subject of exhaustive investigation. Taq polymerase lacks 3'-5' exonuclease proofreading activity and has a high error rate in PCR. Nucleotide incorporation in PCRs employing Taq polymerase is further dependent upon dNTP concentration. Certain other thermostable DNA polymerases such as Pfu or T4 polymerase have 3'-5' proofreading activity and are reported to improve fidelity[18,19]. In general, rate of misincorporation can be reduced by minimizing the annealing or extention time, reducing dNTP and Mg^{2+} ion concentration and maximizing annealing temperature[20]. Unfortunately, the reaction conditions that minimize misincorporation also reduce PCR yield.

Analysis of PCR Product

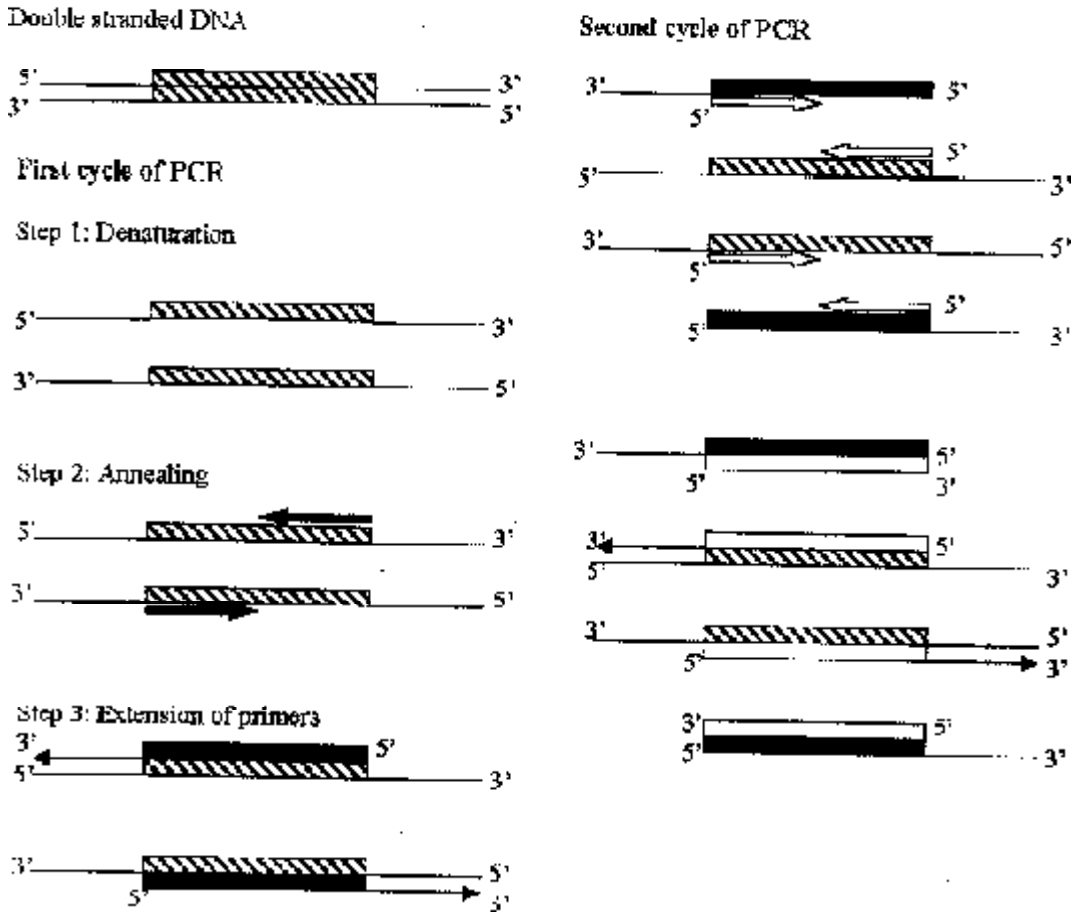
(a) Electrophoresis

Agarose gel (typically 1-1.5% w/v) electrophoresis followed by staining with ethidium bromide represents the simplest and most common method to analyse PCR product. PCR product is of a defined length. The size of PCR product may be calculated by simultaneous running of DNA molecular weight marker on the agarose gel. Excess primers may appear as diffuse bands close to the leading edge of the gel[21].

(b) Southern blotting and hybridisation

After electrophoresis, PCR amplified DNA template is transferred (blotted) onto a nitrocellulose or nylon membrane. Blotted DNA is denatured to single strand and fixed onto the membrane by heating at 80ºC or by U.V. crosslinking. The membrane is then placed in a solution contaning labelled (Radioactive or flouresent) complementary single stranded

Figure 1. Schematic representation of steps involved in PCR.



oligonucleotide known as probe. Hybridisation occurs between the lablled probe and complementary single stranded sequence present on membrane. The position of probe can be visualised by autoradiography or chemilumnisense[22].

Modified versions of PCR

The basic technique of PCR has been modified in several ways which has broadened its applications. The detailed discussion of the modifications of PCR techniques is beyond the scope of this review and therefore, they are being mentioned only in brief. Allele specific PCR is employed to detect point mutations responsible for genetic disorders. Primers are designed to anneal at the site which is probable to undergo mutation. Therefore, primers will not anneal to this site if it has suffered from mutation and DNA amplification will not take place in case of mutated genes[23]. Inverse PCR can be used to amplify the unsequenced DNA template that lie adjacent to a core DNA sequence for which the primer sequence is already known[24-26]. Pan handle PCR allows the amplification of DNA when the sequence for primer design is known only on one end of target DNA sequence[27,28]. Race PCR is a technique to obtain full length cDNA while starting from limited nucleotide

sequence. PCR has also been employed to introduce point mutations in the target DNA. This technique termed as PCR mutagenesis, employs primers which are not exactly complementary to their target sequence but still undergo annealing under appropriate reaction conditions[29-31]. Ligation-mediated PCR (LM PCR) is an extremely sensitive technique to map single-stranded DNA breaks, DNA methylation and nucleosome position. Therefore, the technique is quite useful for toxicological studies[32]. Arbitrarily primed PCR (AP PCR) or DNA amplification finger printing is a technique which can be employed to identify genotypes or different strains of a single species. The technique is of great importance in diagnosis of microbial strains. In AP PCR, stringency of primer annealing is sufficiently lowered. Therefore, primer binds to more than one target sites in the DNA and amplifies more than one PCR products. This set of PCR products is specific and reproducible. After electrophoresis, it may be viewed as finger print to identify source DNA[33].

Applications of PCR in pharmacological research

1. Quantification of gene expression

Discovery of a new target site for therapeutic intervention is every pharmacologist`s dream. Most of the disease pathologies are associated with an altered gene expression leading to an increase or decrease in the activity of cellular proteins. PCR can be employed to detect very small alterations in cellular mRNA encoding for such proteins. This modified form of PCR is termed as reverse transcriptase PCR (RT-PCR). In this technique, cellular RNA is initially reverse transcribed to cDNA in a reaction mixture containing oligo dT primers, a viral reverse transcriptase, all four dNTPs and $MgCl_2$ containing buffer. The cDNA thus generated, can be amplified by PCR using primers specific to a particular mRNA. The final amount of PCR product generated from mRNA, depends on the starting amounts of mRNA and efficiency of the reverse transcription and PCR reactions. In order to rule out the variability due to later two factors, two basic methods have been employed to quantitative mRNA using RT-PCR.

Noncompetitive RT-PCR

In this method, a known quantity of standard DNA template is added exogenously. Standard DNA and target cDNA are co-amplified simultaneously using RT-PCR. From the ratio of amplified DNA, absolute value of target cDNA can be calculated. Alternatively, an endogenous gene transcript with high tissue expression can act as an internal standard. Such gene transcripts are termed as "House keeping genes". Genes for β-actin, HPRT and GAPDH are some of the examples of house keeping genes routinely employed as internal standards[34-37]. However, the quantification relies on the empirical assumption that efficiency of PCR is same for target cDNA and standard DNA or endogenous gene. Therefore, this method of mRNA quantification is less accurate.

Competitive RT-PCR

In contrast to non-competitive RT-PCR, this method employs an external DNA standard that closely mimics target cDNA with respect to primer binding and other PCR variables[38-40]. Therefore, standard and target cDNA get amplified with the same efficiency. However, the method doesn't take into account the efficiency of reverse transcription reaction. To overcome this limitation, samples have been spiked with a known quantity of mRNA transcripts which are reverse transcribed and amplified in a manner analogous to target mRNA. This method is reported to yield better results.

RT-PCR has been employed to quantitate the mRNA for many proteins. PGI_2 synthase[41], Cox 2[41], iNOS[42,43], AT_1 receptors[44], peroxisome proliferation activated receptors[45], matrix metalloprotinases (MMP-1, MMP-3 and MMP-9)[46], cGMP dependent protein kinase[47], cardiac background K^+ channel[48], human renin[49], NPY and NPY receptors[50], endothelial leptin receptors[51], human $P2X_7$ receptor subtype[52] mRNA are just a few to name. Intrinsic expression of multidrug resistance gene is a major impediment in the treatment of cancer by chemotherapy. The MDR phenotype may be caused by expression of transporters of P glycoprotein family or multidrug resistance protein family[53]. Expression of MDR transporters serves as an important predictor for clinical prognosis of cancer[54]. RT-PCR has been extensively employed to quantify the expression of MDR transporters in several types of carcinomas[53-57].

2. Cellular localisation of gene expression

Although RT-PCR is a highly sensitive technique to detect small alteration in gene expression, it does not permit the cellular localization of such gene

alteration. Homogenized tissue samples employed for RT-PCR mostly consist of heterogeneous group of cells. Therefore, in a given pathological condition it is difficult to ascribe the mRNA expression alteration to a particular cell type. *In situ* PCR overcomes this short coming of RT-PCR. In this technique, a tissue section is fixed on a glass slide in formalin[58]. Cellular proteins are digested on the slide with the help of pepsin, trypsin or proteinase K. DNA is digested with help of DNAase. The total messenger RNA is reversed transcribed to cDNA as described in RT-PCR by putting all the reagents on slide. The cDNA thus generated is amplified using two specific set of primers, Taq polymerase and streptavidin containing dNTPs. Finally, this slide is stained with nuclear fast red stain and viewed under microscope.

3. Identification of gene families

PCR has been employed to amplify a family of related genes using degenerate oligo nucleotide primers (DOP). DOP are mixture of primers designed around a conserved domain of a gene family but with alternative nucleotides at certain positions[58]. Therefore, these primers will bind to closely related DNA sequences in the genome. This strategy is known as homology screening or DOP-PCR and has successfully expanded several gene families such as cyclins and cyclin-dependent kinases[58].

PCR has also revolutionised the detection of genetic disorders such as haemoglobinopathies, X-linked disorders, cystic fibrosis, cancers such as acute myeloid leukemia, chronic myeloid leukemia and infectious disorders such as tuberculosis, HIV and hepatitis B. However, this use of PCR is more of a diagnostic rather than pharmacological interest.

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