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THE POLYMERASE CHAIN REACTION

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Introduction

The Polymerase Chain Reaction (PCR) is a technique for the *in vitro* synthesis of billions of copies of a specific nucleic acid sequence by performing successive rounds of *in vitro* nucleic acid replication. This is achieved by using two oligonucleotide primers that hybridize (annealing) to the opposite strand of the target DNA at positions that flank the region to be amplified through simultaneous extension of both primers. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by 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, yields about million-fold amplification.

The PCR with tremendous applications was possible because of the availability of nucleic aid sequence information and the thermostable DNA polymerase enzyme. The components required for the PCR viz., the template (the DNA to be amplified), the primers, *Taq*. polymerase, the four types of de-oxynucleotide triphosphates and buffer containing magnesium ions are assembled in a tube and the amplification reaction carried out by cycling the temperature within the reaction tube. For any given pair of oligonucleotide 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.

The standard PCR mixture in addition to the sample (template) DNA contains 50mM KCl, 10 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$, 100 µg/ml gelatin, 0.25-100 p moles of each primer, 200 µm of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP and dTTP) and 2.5 units of *Taq* polymerase. The sample DNA generally contains 10^2 to 10^5 copies of template. The volume is made up to 25 or 50µl. The amplification is performed in a DNA thermal cycler, each cycle consisting of denaturation at 94°C for 30- 60 sec, annealing at 55°C for 30- 90 sec and extension at 72°C for 60- 120 sec for total of 30 cycles. Cycling could include an initial denaturation at 94°C and a final extension at 72°C for 5 min. At the end reactions are stopped by chilling at 4°C or by addition of EDTA at 10mM.

Materials and Reagents for PCR

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. 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. Concentration of template DNA also affects the degree of amplification. 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.

The Primers

Oligonucleotide primers in the range of 18 to 30 bases are generally used for the PCR. The sequence of the primers should be complementary to the 3' and of the target (template) DNA strands to be amplified. Primers are the most important components that determine the success of an amplification reaction. 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 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. 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 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 25 to 100 p moles of each primer should be used.

The Reaction Buffer

The components of PCR buffer, particularly the concentration of $MgCl_2$ have a profound effect on the specificity and yield of an amplification product. Concentration of about 1.5 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. Though several buffer formulations have been published, a consensus is beginning to emerge. The recommended PCR buffer should contain 10mM

Tris-HCI (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 μ mg) 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.

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 −20°C for months. The doxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) is generally used at concentrations of 200 μM 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, 20μM of each dNTP in a 100ul reaction is sufficient to synthesise 10 pMol 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 polymerase

The most commonly used thermostable polymerase isolated from the organism *Thermus aquaticus* that is available commercially. Many other thermostable DNA polymerases are also available. The required concentration of Taq DNA polymerase concentration is between 1 and 2.5 units per 100 μl reaction when other parameter are optimum. When optimizing a PCR, enzyme concentration ranging from 0.5 to 5 units/ 100μl are tried and results assayed 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.

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 25 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°C for 30 to 60 seconds. Higher temperatures e.g. 97°C may be necessary for G + C rich targets. Denaturation steps that are too 1ong or too high lead to unnecessary loss of enzyme activity.

Primer annealing

At temperatures ranging from 55°C to 72°C, 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.

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 of 5°C below the true Tm of the amplification primers can be attempted. Annealing temperature in the range of 55 to 72°C 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 72°C. 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°C. The rate of nucleotide incorporation at 72°C vary from 35 to 100 nucleotides per second depending upon the buffer, pH, salt concentration and the nature of the DNA template. An extension time of one minute at 72°C is considered sufficient for products up to 2 Kb in length.

Cycle number

The optimum number of cycles will depend mainly upon the starting concentration of 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. Suppose there is only one copy of the wanted gene before the cycling starts, 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.

Detection and analysis of PCR product

The PCR product will be a 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.

Nested PCR

Carrying out nested PCR can further enhance the reliability of the PCR. The process utilizes two consecutive PCRs. 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. 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.

RAPD-PCR

Arbitrary primed PCR (AP-PCR) / Random amplified polymorphic DNA (RAPD) has been increasingly reported as a method for the genetic characterization. It can be useful for species/strain identification. The underlying theory in AP-PCR/RAPD is that single primer of arbitrary sequence is used that target complimentary sequence on the two DNA strands used as template and amplify the intervening regions in order to generate a genetic profile. The rapid technique, which was developed by Williams *et al.*, (1990) can be used to produce simple and reproducible DNA fingerprints. This is made possible using randomly designed short primers. 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.

PCR- application:

- Site-specific mutagenesis.
- *In situ* PCR: amplification and detection of DNA *in situ* from cells.
- Genomic subtraction.
- Analysis of protein functions and intermolecular assembly.
- DNA fingerprinting using AP-PCR for evaluation of genetic heterogeneity & relationship.
- Generation of single chain antibody fragments by PCR
- Rapid disease diagnosis.
- CDNA synthesis from RNA.
- Production of clones for sequencing.
- Paternity verification
- Molecular taxonomy etc.,

Suggested Reading

- PCR Strategies. Ed.Innis,M.A.,Gelfand,D.H and Sninsky.J.J.,Academy Press, New York.
- PCR - A practical approach. Ed. Mc. Pherson *et al*; Oxford Univ. Press, New York.
- Molecular Methods for virus detection. Ed. Wiedbrauk and Farkas.D.H., Academy Press, New York.