Polymerase Chain Reaction (PCR)

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Polymerase chain reaction (PCR) is a technique that is used for amplification of DNA. The double stranded DNA is denatured by heating at ~94^{o}C. As a result the two strands separate from each other. Each strand acts as template for the synthesis of a new second strand. The synthesis of the new strands is marked by a pair of primers (short sequences of DNA) that are complementary to each of the two DNA strands. The primers are allowed to anneal with the target regions at around 60^{o}C. In the presence of heat stable Taq DNA polymerase, deoxy-nucleotides (dNTPs), $MgCl_2$ and reaction buffer, the synthesis of new strand takes place at 72^{o}C which is the optimum temperature of Taq polymerase. During synthesis or extension step the dNTPs are added to the complementary bases of the target template. At the end of the extension step two molecules of double stranded DNA are formed from a single starting molecule (Fig.3.1). If a similar cycle of heating and cooling is repeated again the newly synthesized double stranded DNA molecules act as templates for further DNA synthesis and from two molecules four molecules of DNA are formed.

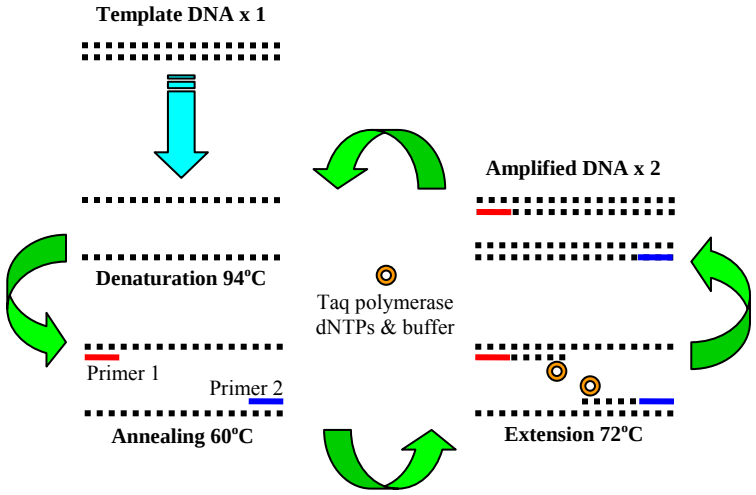


Fig: 3.1. Basic principle of polymerase chain reaction. At the end of one cycle of sequential heating and cooling one molecule of template DNA is amplified to produce two molecules of DNA. In each successive cycle the newly formed (amplified DNA) molecules also act as templates for further amplification.

Sequential repetition of the denaturation, annealing and extension cycles leads to doubling of the DNA molecules with each cycle. In thirty cycles one molecule of DNA can be amplified to produce approximately one billion molecules of DNA. The number of

DNA molecules produced from one molecule of target DNA can be calculated by the formula 2^n, where n is the number of cycles.

Size of the new double stranded DNA molecules is defined by the two primers used for amplification. In the initial few cycles the newly synthesized DNA strands are a little longer than the actual boundaries defined by the two primers. However, in the subsequent cycles when more and more of the newly synthesized strands are used as templates the amplified DNA fragments of exactly the same size, defined by the two primers, are produced.

The initial outburst of amplification in a PCR is gradually reduced to a platue. This is due to the consumption of dNTPs and primers and progressive denaturation of Taq polymerase at very high temperatures. Many other factors like the quality and the quantity of the template DNA, presence of inhibitors of PCR, and the quality of the consumables also play important role in the overall performance of a PCR.

PCR Primers

Primer design

The amplification of DNA essentially requires a short sequence of DNA or "primer" to anneal with the template. Primer or oligo is the main determinant of specificity in a PCR It also has important bearing on the sensitivity of the reaction. Therefore it is essential to understand the basic parameters of designing a PCR primer. The primers may be designed manually or by many computer software packages available for this purpose. The latter also provide simulation data about the performance of the primers.

Primer length

The length of a primer is measured in bases. The usual length ranges from 20-25 bases. The number of bases in a primer is often called "mer" e.g. 20 or 30 mer. A short primer is more likely to anneal non-specifically at sequences that are even partially identical. Increasing the length of the primer increases its specificity but reduces the sensitivity.

Primer ends (5' and 3')

Primers, like any other DNA sequence, have a 5' (5 prime) and a 3' (3 prime) end. For example the primer 5'-CAATGTATCATGCCTCTTTGCA begins at C (5' end) and ends at A (3' end).

The 3' end of the primer is the most critical region as the extension (addition of dNTPs) occurs at this end. The last few bases, especially the last base, at the 3' end are most critical in annealing of a primer. A mismatch at the last base could seriously impair the ability of a primer to anneal. It is also important that the last five bases at the 3' end should not have more than three G or C. The 5' end of the primer is less significant. This end is often used for tagging the primers with compounds like fluorescent dyes etc.

Primer direction (forward and reverse primers)

The two strands of DNA run in opposite directions. Similarly the primer and the template DNA also run in opposite directions. In amplification of double stranded DNA a primer

each is used for the two strands i.e. one for the forward strand and the other for the reverse strand. It is customary to name the two primers by their direction. The primer that anneals with the reverse strand of the DNA is called forward or sense primer while the primer that anneals with the forward strand is called the reverse or ante-sense primer.

DNA is a double stranded molecule but for writing its sequence only the forward strand is written. The primer shown on the forward strand is meant to anneal with the reverse strand. The primer for the other (forward) strand can be understood if the reverse strand is also written. However, to mark the location of the two primers these are often shown on the same strand (Fig 3.3).

Primer sequence

The sequence of nucleotides (G, A, C & T) in a primer that is complementary to the target is called its sequence. In a good primer the numbers of G, A, C and T should ideally be equal. But in practice it is not always possible. However, the total GC content in a primer should remain between 40-60%. Too many G and C can lead to formation of secondary structures and impair the performance. The presence of repeats like ATATAT can lead to non specific annealing.

Primer Tm and annealing temperature

The melting temperature (Tm) is the temperature at which half of the primer is annealed to its target. Tm is dependent on the nucleotide sequence of the primer. Greater the numbers of G and C in a primer higher is its Tm. There are many formulae that can be used for calculation of Tm. For example:

Tm = 2 x AT + 4 x GC

(AT is the sum of A and T nucleotides, and GC is the sum of G and C nucleotides).

The primer designing software and primer vendors also provide data on the Tm of a primer. It is essential that two or more primers being used together in a PCR should have nearly equal Tms. The optimum annealing temperature of a primer is generally 3-5^{o}C below the theoretically calculated Tm.

Primer dimers and secondary structures

Partial similarities in the sequence of primers can make them anneal with each other to form primer dimers. This may include self-dimers (between themselves) or cross-dimers (with other primers) (Fig 3.2). The primer dimers may become difficult to control in a multiplex PCR (PCR with more than two primers). Primers may also form secondary structures like loops and hair pins (Chapter 1). Strong primer dimers and secondary structures may reduce the bioavailability of a primer for the actual target leading to impaired amplification. The problem can become serious when primers are designed manually. The primer designing softwares are useful in tackling this issue.

a. 

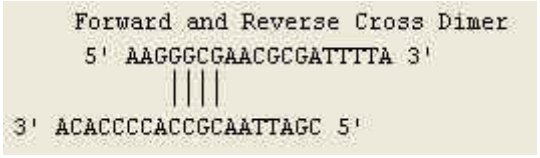
b. 

Fig. 3.2. Primer self-dimers (a) and cross-dimers (b).

Primer cross-homology

A good primer should be specific only for its own target. Occasionally a primer may have partial or rarely a complete sequence homology with another site in the genome. This would cause nonspecific amplification. The problem may be overcome by BLAST searching of the newly designed primer sequence (Chapter 1).

Primer designing by computer software

The primers can be designed on computer software or by manual method. For some applications like allele specific PCR the primers are better designed manually. Many commercially available software packages are available with numerous options. A simple free of cost primer designing software "Primer3" is available at the following web address:

http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

The sequence for which the primers are to be designed can be uploaded as a file or it can be pasted in the software window. Particular regions of the sequence can be marked for inclusion or exclusion or as a target. The software provides numerous options for a large number of primer parameters that can be chosen as per requirement. A simple interface for the Primer3 software called"Primer3Plus" is also available at the following web address:

http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

Primer labeling

Primers are often required to be labeled with dyes like fluorochromes etc. All such labels are attached at the 5' end which is also the non performing end of the primer. A typical fluorescent dye "FAM" labeled primer would be written as:

5' FAM-CAGTAGCATCTGACTTTGAGCCTCAGGGTCT

Primer purification

During the process of primer synthesis the yield is often contaminated with many short length sequences. This is especially common when primers longer than 30 bases are ordered. The presence of short length sequences can cause non specific amplification. This can consume other ingredients like dNTPs and Taq and may lower the specific amplification. The short length primers can also falsely elevate the final concentration of the working primer solution. In applications where it is critically important to get only specific amplification the primers may have to be purified before use. The primer

manufacturers also provide option of gel or HPLC purification of a primer at an extra cost. However, primers shorter than 30 bases usually do not require extra purification.

Primer concentration and dilution

An optimum concentration of primer is critical in a PCR. Too much primer causes it to anneal non-specifically whereas too little primer reduces its availability for annealing and the amplification. Most PCRs are done with at least one pair of primers. It is important that the two primers of a pair are used in equi-molar ratios. The primer concentration is usually determined by the optical density (OD) at 260 nm. In general 1µg of a primer with 25 bases has an OD of 25. The primer manufacturers provide concentration of primers in µg and OD when reconstituted in one ml of water. The primer is usually supplied in a lyophilized form that is reconstituted in water and is diluted as per requirement. In the lyophilized form it is very stable at room temperature. The lyophilized primer is usually diluted in 1ml of water and it may be stored in aliquots at -20^{o}C for several months or -80^{o}C for several years.

It is convenient to use primer concentration in micro-moles (µM) or pico-moles per micro-liter (pM/µl). The optimum concentration of a primer in most PCRs ranges between 5-10 pM/µl. The following example can explain the dilution of a lyophilized stock primer to make a final concentration of 5pM/µl:

1. OD of the lyophilized stock primer: 13.5 (provided with the stock primer vial).
2. Reconstitute the lyophilized stock primer by adding 1ml water (preferably DNase free).
3. Gently mix by inverting several times.
4. Aliquot and store the stock at -20 to -80^{o}C.
5. To make 100µL of 5pM/µl primer use the following volumes:
    - Volume of the stock primer = 1/OD x volume required

      1/13.5 x 100 = 7.4µl
    - Volume of distilled water to be added: 100-7.4 = 92.6µl
6. Using 1µl of the diluted primer will give a final concentration of 5pM per reaction.
7. To make 10pM/µl concentration, double the volume of the stock primer (14.8µl stock primer + 85.2µl water).
8. Two or more primers to be used in a PCR reaction may also be diluted together in one vial. For example four primers with ODs of 10.5, 13.4, 12.5 and 15.1 may be diluted together in one vial, at concentration of 5pM each in 100µL final volume, as follows:
    - Calculate the volume of the individual primers as above.

- Add the volume of the four stock primers (1/10.5 x 100) + (1/13.4 x 100) + (1/12.5 x 100) + (1/15.1 x 100).
- Add distilled water equal to 100 – volume of four stock primers.

Ordering the primer for synthesis

The designed primers are synthesized on a DNA synthesizer. One may synthesize the primers in the lab if a DNA synthesizer is available. But the process is not cost effective unless very large numbers are required on a regular basis. The best option is to order the primer synthesis from a good commercial company. The request for primer synthesis should include the sequence of the primer, the choice of purification and the synthesis scale. The primers are usually synthesized at a scale of 50 or 200nM. The labeling of the primer, if required, is also indicated.

Taq polymerase

Synthesis of DNA requires a polymerase. Since the temperature in a PCR is raised to 94^{o}C an ordinary polymerase would be denatured at this temperature. The problem is overcome by using bacterial polymerase derived from *Thermus aquaticus*, that grow in hot water springs. The optimum temperature of Taq polymerase is 72^{o}C.

The *Taq polymerase* also has 5' to 3' exonuclease activity that can remove any dye attached to the 3' end of a primer. This function is used in removing a dye/quencher attached to the 3' end of a TaqMan® probe used in real time PCR.

***Tth* DNA polymerase**

It is another heat stable polymerase that is derived from *Thermus thermophilus*. The enzyme has dual function of reverse transcriptase and DNA polymerase. This is useful in cDNA synthesis from RNA and its amplification in a single tube reaction.

High fidelity *Taq polymerase*

The *Taq polymerase* lacks 3'to 5' exonuclease activity and therefore is unable to proof read any errors in DNA synthesis. The enzyme *Pfu DNA polymerase* by virtue of its 3' to 5' exonuclease activity can do the proof reading. It is especially useful in doing PCR of very long templates (high fidelity amplification).

Deoxyneucleotides (dNTPs)

Deoxynucleotides (dNTPs) are the building blocks in new DNA synthesis. These are available from many commercial sources as a set of four nucleotides i.e. dATP, dTTP, dGTP and dCTP. These are usually available in 10 or 100mM concentrations. The four nucleotides are also available as a premixed solution. If a set of four separate nucleotides is purchased then these may be mixed in equal proportions and stored as aliquots at -20 or -80^{o}C.

Magnesium chloride

Mg^{2+} ions increase the solubility of dNTPs and their incorporation in the template. These ions also stimulate Taq polymerase. Therefore the concentration of $MgCl_2$ in a PCR is

critical. The amplification can be enhanced by increasing the concentration of Mg^{2+}. The optimum results are achieved at concentration between 1.5-2.0mM $MgCl_2$.

PCR mix (buffer)

PCR buffer without dNTPs is mostly supplied with Taq polymerase. It may also be prepared in the lab. The following buffer formulation gives consistently good results in a wide range of PCR applications:

Stock Solutions:

2M KCl:	14.9 g/dl
1M Tris:	12.1 g/dl
1M $MgCl_2$:	9.52 g/dl
1M Spermidine:	14.52 g/dl

10 X PCR Buffer

2M KCl:	1.25 ml
1M Tris (pH 8.3):	0.5 ml
1M $MgCl_2$:	75 µl
Gelatin (300 bloom):	5 mg
Distilled water:	3.2 ml

PCR mix (1 ml)

10 X PCR buffer:	50 µl
10mM dNTPs (all):	25 µl
1M Spermidine:	1 µl
Distilled water:	925 µl

Inhibitors and enhancers of PCR

Haemoglobin, heparin, porphyrin, SDS, phenol, and proteinase-K are potent inhibitors of PCR. SDS, and phenol must be completely removed during DNA extraction. Proteinase K can be inactivated by heating. PCR may also be inhibited by chocolate that might contaminate the DNA extracted from a mouthwash. Enhancers of PCR include formamide, DMSO, tetramehylammonium chloride (TMAC), polyethylene glycol (PEG), glycerol, tween 20 and 7 deaza-dGTP.

Setting up a PCR

There is a wide variety of PCR applications that differ in the reaction conditions. An example of amplification of β-globin gene containing C-T polymorphism is described. The sequence of the gene was downloaded (GenBank NG_000007.3) and the primers flanking the C-T polymorphism were designed using the Primer3 software (Fig 2.1).

TTTAGACATAATTTATTA**GCATGCATGAGCAAATTAAGA**AAAACAACAA
CAAATGAATGCATATATATGTATATGTATGTGTGTA**[C/T]**ATATACAC
ACATATATATATATATTTTTTCTTTTCTTACCAGAAGGTTTTAATCCAA
ATAAGGAGAAGATATGCTTAGAACCGAGGTA**GAGTTTTCATCCATTCTG**
TCCTGTAA

Fig. 3.3. PCR amplification of β-globin gene (GenBank NG_000007.3) containing C-T polymorphism. The primers flanking the polymorphism were designed using the Primer3 software.

Locus: β-globin gene

GenBank accession: NG_000007.3

Forward primer: 5'-GCATGCATGAGCAAATTAAGA

(Length: 21, Tm: 59^{o}C, GC 38%)

Reverse primer: 5'-CAGGACAGAATGGATGAAAACTC

(Length: 23, Tm: 60^{o}C, GC 43%)

Amplified product size: 179bp

PCR master mix

For 10 reactions (7 test samples + 1 positive control + 1 negative control + 1 reagent blank) make 260µl master mix (25µl/reaction + 10µl extra for pipetting errors). Mix the following in a 0.5ml plastic tube:

- PCR buffer with dNTPs: 240µL (25µL per reaction)
- Forward primer 10µL (5pmol/µL) (1µL per reaction)
- Reverse primer 10µL (5 pmol/µl) (1µL per reaction)
- Taq polymerase (5 units/µL) 1.0µL (0.1µL/reaction)
- Template DNA (~200ng/µl): 1µL/reaction

1. Take 10 x 0.2ml PCR reaction tubes in a rack and label these 1-10.
2. Dispense 25µL master mix to each of the ten labeled tubes.
3. Add 1µL test DNA samples to tubes 1-7.
4. Add 1µL control DNA positive for C-T polymorphism in tube 8.
5. Add 1µL control DNA Negative for C-T polymorphism in tube 9.
6. Add 1µL water instead of DNA in tube 10.

7. Firmly close the lids of the tubes and put these in thermal cycler.
8. Programme the thermal cycler and run PCR as follows:
    a. Initial denaturation 94^{o}C for 5 minutes.
    b. Thirty cycles each of denaturation at 94^{o}C for 1 minute, annealing at 60^{o}C for 1 minute and extension at 72^{o}C for 1 minute.
    c. Final extension at 72^{o}C for 3 minutes.
9. Electrophoresis:
    - 10 x 10 cm 6% polyacrylamide, 30 minutes at 150 volts.
10. Staining:
    - 0.1% silver nitrate

DNA Thermal Cycler

Thermal cycler is a machine that can be programmed to heat and cool rapidly for PCR amplification. A wide variety of machines are available from different sources. The choice of machine depends on the requirement of a lab. An average thermal cycler can heat or cool at about 1^{o}C per second. More expensive machines with very fast heating or cooling (ramping) are also available. Very fast ramping can shorten the time required in thermal cycling. The fast machines also require special thin walled PCR tubes that can allow heat transfer at an equally fast rate. Most PCR machines have 96 well capacities. Smaller machines with capacity of 32 or 48 well are also available.

Hot start PCR

At the start of PCR when the temperature in the reaction vial is rising the primers may anneal non specifically with a partially denatured template resulting in nonspecific amplification. This may be prevented if the Taq polymerase is added after the template is completely denatured. In a hot start PCR Taq polymerse is added when the contents of the PCR reaction mix have reached above 80^{o}C. The process may be helped by using Ampliwax^{TM}. A small wax bead is placed above the reaction mix in a PCR tube. Taq polymerase mixed in buffer is added on top of the wax bead. When the temperature in the vial reaches above 80^{o}C the wax melts and the Taq polymerase is mixed with the reaction mixture.

Nested PCR

When the target DNA is low in concentration the amplification may be done in two steps. In the first step a pair of flanking primers is used to amplify the target. In the second step the amplified product of the first step is re-amplified with another set of primers located within the outer/flanking primers used in the first step. The nested PCR is extremely sensitive. The first step amplified product may easily contaminate the other PCR reagents and may be very difficult to handle.

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