Polymerase chain reaction (pcr) steps

We owe the discovery of the polymerase chain reaction to Kary B Mullis in the year 1983. He was the actual proponent of PCR. Few people are aware that in 1971, Kleppe and the Nobel laureate Gobind Khorana published studies including a description of techniques that are now known to be the basis for nucleic acid replication. However, it is unfortunate that Kleppe and Khorana were ahead of their times. Oligonucleotide synthesis wasn't as simple as it is today; genes had not been sequenced and the idea of thermostable DNA polymerases had not been described. Hence, the credit for discovering the PCR remains with Kary Mullis.

The Polymerase Chain Reaction is essentially a cell-free method of DNA and RNA cloning. The DNA or RNA is isolated from the cell and replicated upto a million times. At the end, what you get is a greatly amplified fragment of DNA. The PCR is quick, reliable and sensitive and its variations have made it the basis of genetic testing.

WHAT KARY B MULLIS SAYS ABOUT HOW HE DISCOVERED THE POLYMERASE CHAIN REACTION

> " I was just driving and thinking about ideas and suddenly I saw it. I saw the polymerase chain reaction as clear as if it were up on a blackboard inside my head, so I pulled over and started scribbling." A chemist friend of his was asleep in the car. Mullis says that " Jennifer objected groggily to the delay and the light, but I exclaimed I had discovered something fantastic. Unimpressed, she went back to sleep."

Mullis kept scribbling calculations, right there in the car. He convinced the small California biotech company, Cetus, he was working for at that time, that he was up to something big. They finally listened. They sold the patent of PCR to Hoffman-LaRoche for a staggering $300 million – the maximum amount of money ever paid for a patent. Mullis meanwhile received a $10, 000 bonus.

BASIS OF THE METHOD

The purpose of PCR is to generate a huge number of copies of a segment of DNA, which could be a gene, a portion of a gene, or an intronic region. There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can either heat or cool the tubes containing the reaction mixture, as required, in a very short period of time. There are three major steps in a PCR, which are repeated for 30 or 40 cycles.

Denaturation–During this process, the double stranded DNA melts and opens to form single stranded DNA. All enzymatic reactions, such as those carried over from a previous cycle, stop. This will be explained in the next paragraph. The temperature for denaturation is not fixed but it usually occurs at about 95°C. It is important to realize that the denaturation temperature is largely dependent on G: C (guanine: cytosine) content of the DNA fragment to be analyzed. This is reasonable when one considers that the G: C bond is a triple hydrogen bond and the AT bond is a double bond. Logic dictates that a triple bond should be 1. 5 times harder to break than a double bond. Therefore, when the segment of DNA to be analyzed has a very high G: C content, the denaturation temperature can reach even upto 99°C.

Annealing–This requires temperatures lower than those required for denaturation. In this process, the primers anneal to that very specific segment of DNA that is to be amplified. The primers are jiggling around, caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of what is now double stranded DNA (template and primer); the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.

Extension–This is done at 72°C. This is the ideal temperature for working with polymerase. The primers, which are complementary to the template, already have a strong ionic attraction to the template. This force is stronger than the forces breaking these attractions i. e. the high temperature. Primers that are on positions with no exact match (non complementary) get loose again (because of the higher temperature) and don't give an extension of the fragment. The nucleotide bases are added from the 5′ end to the 3′ end. The phosphate group of the dNTPs is coupled with the hydroxyl group of the extending DNA strand. The extension time depends on two factors; the type of polymerase used and the length of the DNA fragment to be amplified. Usually, Taq polymerase adds dNTPs at the rate of about 1000 bases per minute.

It is important to realize that each component of the PCR including the input DNA, the oligonucleotide primers, the thermostable polymerase, the buffer

and the cycling parameters has a profound impact on the sensitivity, specificity and fidelity of the reaction.

The three steps of the first cycle are shown, that is, denaturation, annealing and extension. At the end of the first cycle, two strands have been synthesized. At the end of the second cycle, four strands have been synthesized (the three steps of the cycle have not been shown). At the end of the third cycle, eight strands have been synthesized. The number of strands increases exponentially with each cycle.

Nuggets

- The Polymerase Chain Reaction is essentially a cell-free method of cloning DNA and RNA.
- There are three steps involved in every cycle; these are denaturation, annealing and extension.
- At the end of each cycle, the DNA doubles. Therefore, theoretically, if there are ‘ n’ cycles in a reaction, the number of DNA fragments at the end of the reaction will be 2n.

COMPONENTS OF THE POLYMERASE CHAIN REACTION

The components that are essential for a successful PCR are elaborated here.

TEMPLATE DNA

This is that portion of the DNA/gene that is to be amplified. Usually the concentration is 100 ng genomic DNA per PCR reaction. However, this can vary depending on the target gene concentration and the source of DNA. The PCR reaction is inherently sensitive. It is not necessary for the template DNA

to be abundant or highly purified. Higher amounts of template DNA can increase the yield of nonspecific PCR products, but if the fidelity of the reaction is crucial, one should limit both template DNA quantities as well as the number of PCR cycles.

DNA in solution may contain a large number of contaminants. These contaminants may inhibit the PCR. Some of these reagents are phenol, EDTA, and proteinase K, which can inhibit Taq DNA polymerase. However, isopropanol precipitation of DNA and washing of DNA pellets with 70% ethanol is usually effective in removing traces of contaminants from the DNA sample.

Effects of Fixation

This is of particular interest to the pathologist since he has to deal with formalin fixed tissue. DNA extracted from fresh tissue or cell suspensions forms an optimal template for PCR. The tissue is best stored at -70°C at which the nucleic acids can be stored indefinitely. A temperature of -20°C is sufficient to preserve the DNA for several months and at 4°C, the DNA can be stored for several weeks. At room temperature, the DNA has been successfully stored for hours to days; however, mitochondrial DNA is very sensitive to temperature and may degrade in thawed tissues.

DNA extracted from fixed tissue has been used successfully for PCR. The type of fixative and the duration of fixation are of critical importance. Non crosslinking fixatives like ethanol provide the best DNA. Formaldehyde is variable in its DNA yield. Carnoy’s, Zenker’s and Bouin’s are poor fixatives as far as DNA preservation is concerned.

Not surprisingly, formaldehyde is the fixative which has been evaluated the most, because it is more commonly used worldwide. The studies have demonstrated that a successful PCR depends on the protocol to extract the DNA and the length of fixation. Formaldehyde reacts with DNA and proteins to form labile hydroxymethyl intermediates which give rise to a mixture of end products which include DNA-DNA and DNA-protein adducts. Purification of DNA from formalin fixed tissue, therefore, includes heating to reverse the hydroxymethyl additions and treatment with a proteinase to hydrolyze the covalently linked proteins. However, there is no way to reverse the DNA-DNA links and these links inhibit the DNA polymerases. This accounts for the low PCR yield which is seen with formalin fixed tissue. Usually, the PCR reaction with formalin fixed DNA as a template yields products which are not more than 600 bp in size.

Nuggets

- Template DNA is required in a concentration of 100ng for each PCR reaction. Contaminants in DNA may inhibit the reaction.
- Fixation of tissues provides DNA which is not as good as DNA obtained from fresh/ frozen tissues.
- Different fixatives give different DNA yields. Alcohol is the best fixative and Carnoy’s, Zenker’s and Bouin’s are poor fixatives as far as DNA preservation is concerned. Formalin is intermediate in DNA yield.
- Purification of DNA from formalin fixed tissue involves heating to reverse the attachment of hydroxymethyl intermediates and treatment with a proteinase to hydrolyze the covalently linked proteins.

- The DNA obtained after fixation can be used for reactions in which the PCR product is not more than 600 bp.

PCR BUFFER

The purpose of using buffers in PCR is to provide optimum pH and potassium ion concentration for the DNA polymerase enzyme (usually obtained from bacteria ‘ Thermus aquaticus’) to function. Most buffers are available in a 10X concentration and require dilution before use. Although most protocols recommend the final buffer concentration of 1X, a concentration of 1. 5X might result in increased PCR product yield.

The PCR buffer contains many components. Some important ones are discussed here:

Divalent and monovalent cations – These are required by all thermostable DNA polymerases. Mg^{2+} is the divalent cation that is usually present in most of the PCR buffers. Some polymerases also work with buffers containing Mn^{2+}. Calcium containing buffers are ineffective and therefore, rarely used. Buffers can be divided into first and second generation buffers on the basis of their ionic component. The second generation buffers, as opposed to first generation buffers, also contain $(NH_4)_2SO_4$ and permit consistent PCR product yield and specificity over a wide range of magnesium concentration (1. 0 to 4. 0 mM $MgCl_2$). The overall specificity and yield of PCR products is better with second generation buffers, as compared with first generation PCR buffers. Buffers also contain KCl. Salts like KCl and NaCl may help to facilitate primer annealing, but concentration of 50 mM will inhibit Taq polymerase activity. Interactions between K^+ and NH_4^+ allow specific primer

hybridization over a broad range of temperatures. Magnesium is one of the most important components of the buffer. Mg^{2+} ions form a soluble complex with dNTPs which is essential for dNTP incorporation; they also stimulate polymerase activity and influence the annealing efficiency of primer to template DNA. The concentration of $MgCl_2$ can have a dramatic effect on the specificity and yield of PCR products. Optimal concentration of $MgCl_2$ is between 1. 0 to 1. 5 mM for most reactions. Low $MgCl_2$ concentration helps to eliminate non-specific priming and formation of background PCR products. This is desirable when fidelity of DNA synthesis is critical. At the same time, however, too few Mg^{2+} ions can result in low yield of PCR products. High $MgCl_2$ concentration helps to stabilize interaction of the primers with their intended template, but can also result in nonspecific binding and formation of non specific PCR products. It is important to be aware that many PCR buffers (often sold in 10X stocks) already contain some amount of $MgCl_2$. Therefore, the addition of further amounts must be carefully monitored. In the best possible scenario, the PCR would work well with the amount of Mg^{2+} already present in the buffer solution. However, if this does not occur, it is necessary to standardize the amount of Mg^{2+} in the reaction mix. This can be difficult because the dNTPs and the oligonucleotide primers bind to Mg^{2+}. Therefore, the molar concentration of Mg^{2+} must exceed the molar concentration of the phosphate groups contributed by dNTPs and the primers. As a rule of thumb, the magnesium concentration in the reaction mixture is generally 0. 5 to 2. 5 mM greater than the concentration of dNTPs. The optimal concentration of Mg^{2+} should, therefore, be standardized for each reaction.

Tris-Cl – The concentration of tris-Cl is adjusted so that the pH of the reaction mixture is maintained between 8. 3 and 8. 8 at room temperature. In standard PCR reactions, it is usually present in a concentration of 10mM. When incubated at 72°C which is the temperature for extension, the pH of the reaction mixture falls by more than a full unit, producing a buffer whose pH is 7. 2.

Other components – Some buffers also contain components like BSA (Bovine serum albumin) and DMSO (dimethyl sulphoxide). BSA reduces the amount of template sticking to the side of the tube, making it available for amplification and reducing the risk of primer dimer. Primer dimers are products obtained when the primers anneal to each other instead to to the template DNA. DMSO has been shown to facilitate DNA strand separation (in GC rich difficult secondary structures) because it disrupts base pairing and has been shown to improve PCR efficiency.

In effect, it is wise not to tamper with the buffer provided with the Taq polymerase. The buffer is usually standardized for the vial of Taq and there is no need to add additional MgCl2 or stabilizers like DMSO and BSA. However, some Taq buffers come with the buffer in one vial and MgCl2 in a separate vial. Under such circumstances, it is advisable to start with 1µL of MgCl2 and increase its concentration in aliquots of 0. 5 µL, if the initial reaction fails.

Nuggets

- The PCR buffer contains divalent and monovalent cations, Tris Cl and other components.

- The PCR buffer is used to give the correct pH and potassium concentration for the DNA polymerase to function.
- The most common divalent ion used is magnesium in the form of MgCl2. MgCl2 concentration is vital for PCR.
- Tris Cl is used to maintain the pH between 8. 3 and 8. 8 at room temperature.
- Salts like NaCl and KCl may facilitate primer annealing
- Other components like BSA and DMSO help to increase the sensitivity and specificity of the reaction.

OLIGONUCLEOTIDE PRIMERS

What are Oligonucleotide Primers?

PCR primers are short fragments of single stranded DNA (17-30 nucleotides in length) that are complementary to DNA sequences that flank the target region of interest. The purpose of PCR primers is to provide a free 3′-OH group to which the DNA polymerase can add dNTPs.

There are two primers used in the reaction. The forward primer anneals to the DNA minus strand and directs synthesis in a 5′ to 3′ direction. The sequence of primers is always represented in a 5′ to a 3′ direction. The reverse primer anneals to the other strand of the DNA.

How to design a primer?

The predominant goal kept in mind while designing a primer is specificity. Each member of the primer must anneal in a stable fashion to its target sequence in the template DNA. The longer the primer, the higher is its

specificity. Unfortunately, the longer the primer, the less likely it is to anneal to a particular sequence in the template DNA. Conversely, if the primer length is small, it is likely to anneal, but its specificity will be poor. A compromise is reached by designing primers between 20 and 25 nucleotides long. Inclusion of less than 17 nucleotides often leads to non specific annealing, while presence of more than 25 nucleotides may not allow annealing to occur at all.

Remember that the DNA sequence in the human genome appears to be a random sequence of nucleotides. When designing primers, it is important to calculate the probability that a sequence exactly complementary to a string of nucleotides in the human genome will occur by chance. Several formulae are designed to calculate such probabilities. However, mathematical expressions are not necessarily correct and in this case, the predictions maybe wildly wrong. The distribution of codons is non random with repetitive DNA sequences and gene families. It is advisable to use primers longer than the statistically indicated minimum. It is also advisable to scan DNA databases to check if the proposed sequence occurs only in the desired gene.

For a practicing pathologist, it is best not to attempt designing of primers. What a pathologist requires is the primer sequence for an established test. If, for example, a pathologist requires primer sequence for the diagnosis of sickle cell anemia, all he has to do is search the web for papers related to molecular testing of sickle cell anemia. The primer sequences will be provided in the paper. Custom made primers can be commercially synthesized. Several biotechnology companies provide this facility. Before

the primers are ordered, it is essential to check that the sequence is correct and that there are no missing nucleotides in the sequence. That is where, BLAST is invaluable.

Before the intricacies of the BLAST search are elaborated upon, it is necessary to mention that designing a primer does not depend only on the sequence of nucleotides. Other factors like the GC content and melting point are also important considerations. They will be dealt with later in the chapter.

BLAST and its uses

BLAST is an acronym for Basic Local Alignment Search Tool. It is an algorithm comparing information about primary biological sequences with a library or database of sequences.

A BLAST can be performed for different organisms, but in this book, we will concern ourselves with nucleotide BLAST in humans only. BLAST searches the database for sequences similar to the sequence of interest (the " query" sequence) by using a 2-step approach. The basic concept is that the higher the number of similar segments between two sequences, and the longer the length of similar segments, the less divergent the sequences are, and therefore, likely to be more genetically related (homologous).

Before perfoming a BLAST search the oligonucleotide sequence is first identified. The sequence is fed into the programme. BLAST first searches for short regions of a given length called " words" (W). It then searches for substrings which are compared to the query sequence. The program then

aligns with sequences in the database (“ target sequences”), using a substitution matrix.

For every pair of sequences (query and target) that have a word or words in common, BLAST extends the search in both directions to find alignments that score greater (are more similar) than a certain score threshold (S). These alignments are called high scoring pairs or HSPs; the maximal scoring HSPs are called maximum segment pairs (MSPs).

The BLAST search as outlined in fig 7. 2 shows the results of the search. If we scroll down further, we can see the sequences producing significant alignments. Note that in this BLAST search, there are 49 BLAST hits in the query sequence.

In the list shown in figure 7. 2, there is a list of hits starting with the best (most similar). To the right of the screen is the E-value. This is the expected number of chance alignments; the lower the E value, the more significant the score. First in the list is the sequence finding itself, which obviously has the best score. To the left is the accession number. This refers to a unique code that identifies a sequence in a database.

It is important to know that there is no set cut-off that determines whether a match is significant or “ similar enough”. This must be determined according to the goals of the project.

The sequences provided in the figure 7. 2 show a significant alignment with Pseudomonas japonica. It shows a high score (bits) and a low E-value. Note

that the lower the E value, the greater the likelihood that the sequence is a good match.

BLAST output can be delivered in a variety of formats. These formats include HTML, plain text and XML formatting. For the NCBI's web-page, the default format for output is HTML. When performing a BLAST on NCBI (National Centre for Biotechnology Information), the results are displayed in a graphical format showing the following:

1. The hits found
2. A tabular form showing sequence identifiers for the hits with scoring related data
3. Alignments for the sequence of interest and the hits received with corresponding BLAST scores for these.

The easiest to read and most informative of these is probably the table. The main idea of BLAST is that there are often high-scoring segment pairs (HSP) in a statistically significant alignment. BLAST searches for these high scoring sequence alignments between the query sequence and the sequences in the database. The speed and relatively good accuracy of BLAST are among the key technical innovations of the BLAST programs.

Sequence of events to be followed when performing a BLAST search.:

- Go to PUBMED (http://www. ncbi. nlm. nih. gov/pubmed/)
- Scroll down to reach a heading called ' POPULAR'
- Under ' POPULAR' click on ' BLAST'
- Click on ' nucleotide blast'

- Under the heading, enter accession number(s), gi(s), or FASTA sequence(s), type or paste the sequence that you want matched.
- Click BLAST
- Wait for the results. Analyse the nucleotide sequence as it appears.

Calculation of Melting Temperature

The melting temperature or Tm is a measure of stability of the duplex formed by the primer and the complementary target DNA sequence and is an important consideration in primer design. Tm corresponds to the midpoint in transition of DNA from the double stranded to its single stranded form. A higher Tm permits an increased annealing temperature that makes sure that the annealing between the target DNA and the primer is specific. The Tm is dependent on the length of the oligonucleotides and the G+C content of the primer. The formula for calculation of Tm is given in table 7. 1.

Table 7. 1: Formula for calculation of the melting temperature.

Length of Primer

Tm (°C)

Less than 20 nucleotides long

2(effective length*)

20 to 35 nucleotides long

22 + 1. 46(effective length)

*Effective length = 2(number of G+C) + number of (A + T)

Primers are usually designed to avoid matching repetitive DNA sequences. This includes repeats of a single nucleotide.. The two primers in a PCR reaction are not homologous to each other and their complementarity can lead to formation of spurious amplification artifacts called primer dimers. The 3′ end of a primer is most critical for initiating polymerization.

The rules for selecting primers in addition to those already mentioned are as follows:

The C and G nucleotides should be distributed uniformly throughout the primer and comprise approximately 40% of the bases. More than three G or C nucleotides at the 3′-end of the primer should be avoided, as nonspecific priming may occur.

The primer should be neither self-complementary nor complementary to any other primer in the reaction mixture, in order to avoid formation of primer-dimer or hairpin-like structure.

All possible sites of complementarity between the primer and the template DNA should be noted.

The melting temperature of flanking primers should not differ by more than 5°C. Therefore, the G+C content and length must be chosen accordingly (a higher G+C content means a higher melting temperature).

The PCR annealing temperature (TA) should be approximately 5°C lower than the primer melting temperature.

G+C content in each primer should not be more than 60% to avoid formation of internal secondary structures and long stretches of any one base.

Primer extension will occur during the annealing step. Primers are always present in an excess concentration in conventional (symmetric) PCR amplification and, typically, are within the range of 0. 1M to 1M. It is generally advisable to use purified oligomers of the highest chemical integrity.

Primer Dimers

A Primer Dimer (PD) consists of primer molecules that have attached or hybridized to each other because of strings of complementary bases in the primers. As a result, the DNA polymerase amplifies the PD, leading to competition for PCR reagents, thus potentially inhibiting amplification of the DNA sequence targeted for PCR amplification.

In the first step of primer dimer formation, two primers anneal at their respective 3′ ends. The DNA polymerase will bind and extend the primers. In the third step, a single strand of the product of step II is used as a template to which fresh primers anneal leading to synthesis of more PD product.

Primer dimers may be visible after gel electrophoresis of the PCR product. In ethidium bromide stained gels, they are typically seen as 30-50 base-pair (bp) bands or smears of moderate to high intensity. They can be easily distinguished from the band of the target sequence, which is typically longer than 50 bp.

One approach to prevent PD formation consists of physical-chemical optimization of the PCR system, i. e., changing the concentration of primers, MgCl2, nucleotides, ionic strength and temperature of the reaction. Reducing PD formation may also result in reduced PCR efficiency. To overcome this limitation, other methods aim to reduce the formation of PDs only. These include primer design, and use of different PCR enzyme systems or reagents.

Nuggets

- Oligonucleotide primers are short fragments of single stranded DNA (17-30 nucleotides in length) that are complementary to DNA sequences that flank the target region of interest. They dictate which region of DNA in the PCR will be amplified.
- Primer sequences can be obtained by reviewing previously published literature. A confirmation of the sequence can be done by using BLAST (Basic Local Alignment Search Tool).
- The melting temperature is the midpoint in the observed transition from a double stranded to a single stranded form. A higher annealing temperature ensures that the annealing between the target DNA and the primer is specific.
- A primer dimer consists of primer molecules that have attached or hybridized to each other because of strings of complementary bases in the primers. Taq polymerase amplifies the primer dimer leading to competition for the PCR products.
- Several methods are used to reduce primer dimer formation including changing the concentrations of primers, MgCl2, nucleotides, ionic strength and temperature of the reaction.

TAQ DNA POLYMERASE

The initial PCR reaction used the Klenow fragment of Escherichia coli DNA polymerase. However, this was unstable at high temperatures and it was necessary to add a fresh aliquot of enzyme after every denaturation step. The annealing and extension temperatures had to be kept low and as a result, there was formation of non specific products in abundance. The discovery of the thermostable Taq DNA polymerases ensured that the PCR did not remain a laboratory curiosity. The extension and annealing temperatures could now be kept high and the formation of non specific products was greatly reduced.

Taq became famous for its use in the polymerase chain reaction and was called the ‘ Molecule of the Year’ by the journal ‘ Science’.

Why Taq?

Taq is the enzyme of choice in PCR because of the following reasons:

Taq works best at 75°C–80°C, allowing the elongation step to occur at temperatures which make non-Watson-Crick base pairing a rare event.

It can add upto 1, 000 nucleoside triphosphates to a growing DNA strand.

Taq has a half-life of 40 minutes at 95°C and 9 minutes at 97. 5°C, and can replicate a 1000 base pair strand of DNA in less than 10 seconds at 72°C.

Because of all these properties, Taq is the enzyme of choice in the PCR.

How does Taq polymerase act?

The first requirement is a primer. The primer is annealed to the template strand having free hydroxyl group at its 3′ end. During the extension phase, the Taq synthesizes a new DNA strand complementary to the template by adding dNTPs in a 5′ to 3′ direction condensing the 5′ phosphate group of the dNTPs with the 3′ hydroxyl group of the end of the extending DNA strand. Since Taq works best between 70°C- 80°C, a temperature of 72°C is usually chosen as the optimum annealing temperature.

Where does Taq come from?

In Thermus aquaticus, Taq polymerase is expressed at very low levels and commercial production is not economically viable. However, the enzyme can now be produced from different versions of the engineered Taq gene so as to obtain high levels of expression in E coli.

What other polymerases are available for use in PCR?

Taq is not the only polymerase; other polymerases are available but Taq is the one that is generally used in a PCR. A few other polymerases with their uses are as follows:

PFU DNA polymerase -Found in Pyrococcus furiosus, it functions in vivo to replicate the organism’s DNA. The main difference between Pfu and alternative enzymes is the Pfu’s superior thermostability and ‘ proofreading’ properties compared to other thermostable polymerases. Unlike Taq DNA polymerase, Pfu DNA polymerase possesses 3′ to 5′ exonuclease proofreading activity, meaning that it works its way along the DNA from the 3′ end to the 5′ end and corrects nucleotide-misincorporation errors. This

means that Pfu DNA polymerase-generated PCR fragments will have fewer errors than Taq-generated PCR inserts. As a result, Pfu is more commonly used for molecular cloning of PCR fragments than the historically popular Taq. However, Pfu is slower and typically requires 1-2 minutes to amplify 1kb of DNA at 72° C. Pfu can also be used in conjunction with Taq polymerase to obtain the fidelity of Pfu with the speed of Taq polymerase activity.

TFL DNA polymerase – Obtained from Thermus flavus, it is useful for the amplification of large segments of DNA.

WHAT IS FIDELITY?

All DNA polymerases have an intrinsic error rate that is highly dependant on the buffer composition, pH of the buffer, dNTP concentration and the sequence of the template itself. The types of errors that are introduced are frameshift mutations, single base pair substitutions, and spontaneous rearrangements. Therefore, the PCR reaction generates a product that is very similar, but in many cases, not identical to the original sequence. The quantity of dissimilar product obtained is obviously related to the cycle in which the mismatch took place. Under normal circumstances, this does not make any difference; however, these errors may become significant during sequencing when the role of fidelity comes into play.

Fidelity is the ability of the polymerases to avoid the incorporation of wrong nucleotides during the reaction. Under normal circumstances, it really does not make a difference if a wrong nucleotide is incorporated because the size of the PCR product remains the same and that is what we have to look for. However, there are some polymerases like Pfu which have a high fidelity. In

addition to reading from the 5′ to the 3′ direction, they can also read from the 3′ to the 5′ direction and correct the wrong nucleotides wh