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Polymerase Chain Reaction- The DNA Copying Machine

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Introduction

It is possible to obtain large quantities of a particular DNA sequence merely by selective replication. The method for selective replication is called the polymerase chain reaction (PCR). PCR is an in vitro procedure that amplifies enzymatically a particular DNA sequence which is flanked by two oligonucleotide primers that share identity to the opposite DNA strands. The launching of PCR as a revolutionary new technology was enabled by the development of the programmable thermocycler. These instruments are based on metal heating blocks with holes for the PCR tubes. The blocks are designed to switch between the programmed series of temperature steps with great speed and precision by a combination of heating and cooling systems.The use of small (0.2–0.5 ml), thin-walled, tubes help to ensure a rapid change of temperature. Alternatively, for larger numbers of samples, microtiter plates

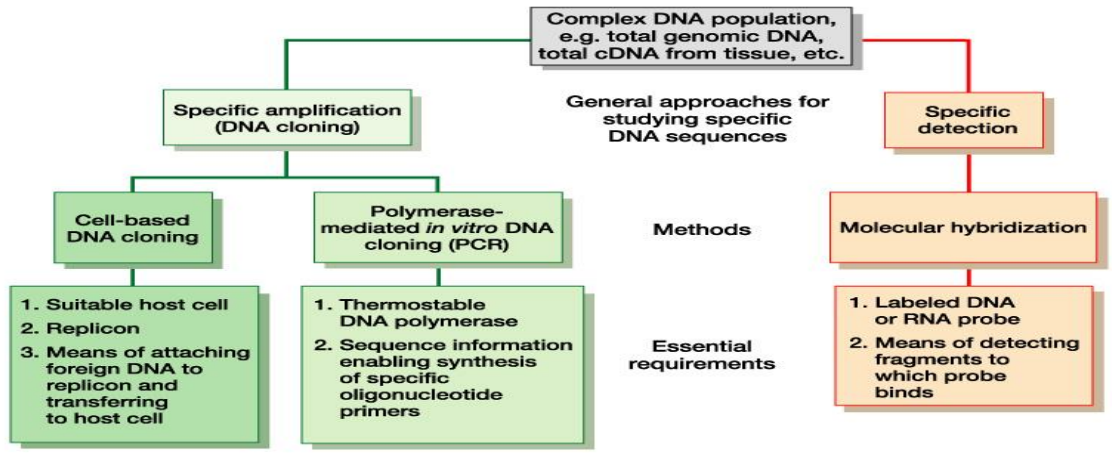

Figure 5-1 Human Molecular Genetics, 3/e. (© Garland Science 2004)

are used.

On 10 December 1993, Kary B. Mullis received the Nobel Prize in Chemistry from King Carl XVI Gustaf of Sweden for the invention of the polymerase chain reaction (PCR) method. Mullis had published and patented this invention only eight years earlier, in 1985.

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The same year, the Physiology and Medicine prize was awarded to Richard J. Roberts and Phillip A. Sharp for their independent discovery that eukaryotic genes are split into introns and exons. These laureates had waited twice as long, 16 years since their fundamental discovery made in 1977. This will give you some idea of the immense impact PCR has had. The key factor in transforming the initial method into one that could have such an impact was the introduction of a thermostable DNA polymerase called Taq polymerase. It was originally isolated from *Thermophilus aquaticus*, a thermophilic archaebacterium that thrives in hot springs at temperatures close to the boiling point of water. As a result, all enzymes in this organism have evolved to withstand high temperatures where most proteins from most other organisms would denature immediately and irreversibly. Apart from this distinct feature, Taq polymerase (not to be confused with TaqI, a restriction enzyme from the same species) is a normal DNA polymerase. It will synthesize a new DNA strand complementary to a single stranded DNA template. Like all other DNA polymerases, it requires a primer, a more or less short strand of complementary DNA, to start its synthesis from. In fact, as we will see, it is not a particularly outstanding DNA polymerase. Although it has high processivity (which means it has a propensity for remaining bound to the DNA and continuing to add successive nucleotides without dissociating from the DNA), it lacks proof-reading activity, so it is unable to correct erroneously incorporated nucleotide bases. This is significant for some applications, as the product may not be a completely accurate copy of the original sequence, and other thermostable polymerases are available which have proof-reading ability. PCR uses Taq polymerase (or other thermostable DNA polymerases) for the exponential amplification of a DNA fragment from a longer initial template, which could be as long as a whole chromosome. The amplified fragmentis defined by two short synthetic oligonucleotides, primers, that arecomplementary to the opposing DNA strands of the template that is being amplified. This introduces another limitation to the method. You must knowthe sequence for at least part of the DNA molecule you wish to amplify – or you must at least be able to make an educated guess.

So how do we go from a small amount of, say, total human genomic DNAto a large amount of one short region that has been exponentially amplifiedto the extent that it entirely dominates the reaction mixture?This will be clear if you go through the first few cycles in a PCR amplification. Most PCR methods typically amplify DNA fragments of up to ~10 kilo

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base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size.

Steps involved in PCR reaction

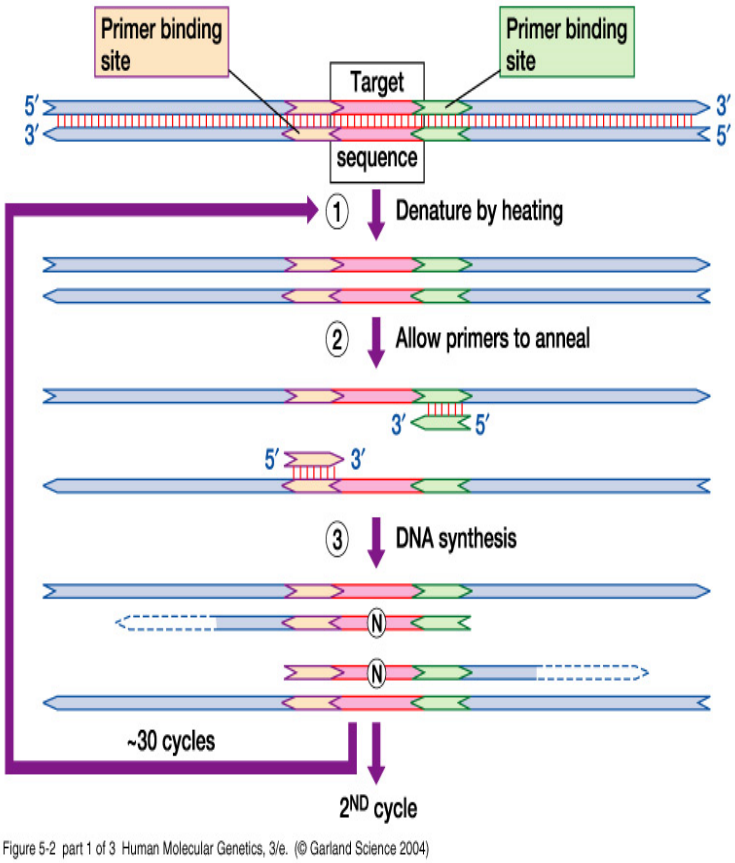

Figure 5-2 part 1 of 3 Human Molecular Genetics, 3/e. (© Garland Science 2004)

It uses DNA polymerase and a pair of short, synthetic oligonucleotide primers, usually about 20 nucleotides in length, that are complementary in sequence to the ends of any DNA sequence of interest. Starting with a mixture containing as little as one molecule of the fragment of interest, repeated rounds of DNA replication increase the number of molecules exponentially. For example, starting with a single molecule, 25 rounds of DNA replication will result in 225 = 3.4 ´ 107 molecules. This number of molecules of the amplified fragment is so much greater than that of the other unamplified molecules in the original mixture that the amplified DNA can often be used without further purification. The binding, annealing, of the primer to the template is a typical DNA: DNA hybridization reaction, and follows similar principles to the hybridization of probes. Firstly, the initialization process which consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes and is only required for DNA polymerases that require heat activation by hot start PCR . Further, the double-stranded template needs to be denatured. The temperature used for this in PCR is 94-98°C for 20-30 sec., that hardly does any damage to the Taq polymerase molecule during the minute or so that the PCR reaction is heated to this temperature causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules. The temperature is

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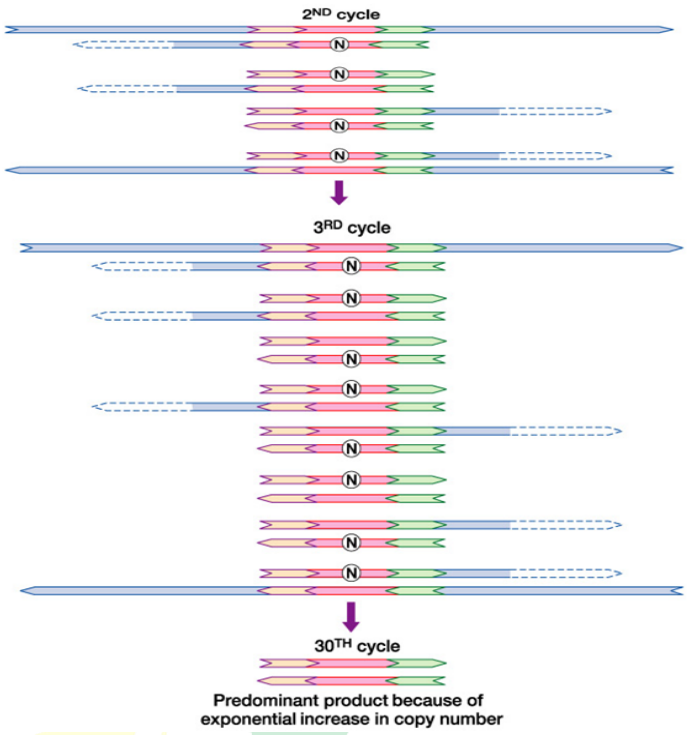
then lowered to the optimal annealing temperature of 50–65 °C for 20–40 seconds where the two primers can bind to the opposing DNA strands. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. This is the only temperature in a PCR cycle that can be varied widely. It is chosen for maximum binding of the primer to the correct template, and minimum binding to other sequences. If the annealing temperature is too low, the primers will bind at other positions on the template, resulting in false products or no detectable product at all. If the annealing temperature is too high, the primers may fail to bind at the correct site, resulting in no amplification. The temperature needed will depend on the sequence and length of the primers, as discussed later. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis. Because the primers are short, and at relatively high molar concentrations, annealing is rapid, taking less than a minute. The temperature is then raised to approximately 72°C, which is normally the optimum extension temperature for a PCR reaction. The Taq polymerase will now synthesise a new complementary DNA strands by starting from the primers and adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand.



The extension proceeds at approximately 1000 bases per minute. Because the template in this case is many times larger than that, the polymerization will proceed until it is interrupted. This happens when the temperature is yet again raised to 94°C in order to start the next cycle in the PCR reaction, which is normally identical in temperature and duration to

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the previous ones. As we finish the first PCR cycle, we have two double-stranded DNA molecules for each one that we started with.

Three Stages of P.C.R Cycle:-

Exponential amplification:

• At every cycle, the amount of product is doubled (assuming 100% reaction efficiency).

• The reaction is very sensitive: only minute quantities of DNA need to be present.

Leveling off stage:

• The reaction slows as the DNA polymerase loses activity and as consumption of reagents such as dNTPs and primers causes them to become limiting.

Plateau:

• No more product accumulates due to exhaustion of reagents and enzyme

Basic Requirements for P.C.R

• DNA template that contains the DNA region (target) to be amplified.

• Two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the target DNA .

• Taq polymerase or another DNA polymerase.

• dNTPs; nucleotides containing triphosphate groups.

• Buffer solution.

Primer design

The most important PCR parameter by far is the design of the primers. Assuming that we know the sequence of the target gene, we want to pick a region of a suitable length for amplification. For analytical purposes, 200–500 base pairs is adequate. If it is too small, it will be difficult to detect on an agarose gel; if it is too big, the amplification will be inefficient.We then select two sequences (usually 20–25 bp) either side of this region for binding our complementary primers. It is essential to get the orientation of the primers right. The ‘left-hand’ (forward) primer will be the same as the sequence of the ‘top’ strand – remember that double stranded DNA is conventionally represented with the top strand in the 5′ to3′ direction, reading from left to right; this is the strand that is shown if yourDNA is shown in single-stranded format. The ‘right-hand’ (reverse) primer must be the sequence of the complementary strand, read from right to left (which is the 5′ to 3′ direction for the lower strand).

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DNA labeling by PCR

The standard PCR reaction can be modified to permit incorporation of labeled nucleotides. Two methods are commonly used:

Standard PCR-based DNA labeling: PCR reaction is modified to include one or more labeled nucleotide precursors which become incorporated into PCR product throughout its length.

• **Primer-mediated 5′ end labeling**:- PCR is conducted using a primer in which a labeled group is attached to the 5′ end. As PCR proceeds the primer with its 5′ end-label is incorporated into the PCR product. This method is often used with fluorophore labels during DNA sequencing and is an example of a general PCR mutagenesis method known as 5′ add-on mutagenesis which has many applications.

Analysis of PCR products

The normal way of analysing the products of a PCR reaction is to separate the samples in an agarose electrophoresis gel. This allows you to ascertain that only one fragment is obtained in each reaction,which is usually the objective. By comparing the size of the amplified fragment to a molecular weight standard, it is also possible to make sure that the molecular weight is the same as the predicted one (which is usually known). The assumption is that, if the fragment is the predicted size, then it probably corresponds to the predicted fragment

Optimization of PCR

PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions

Nested primers

The products of an initial amplification reaction are diluted and used as the target DNA source for a second reaction in which a different set of primers is used, corresponding to sequences located close, but internal, to those used in the first reaction

Hot-start PCR

Mixing of all PCR reagents prior to an initial heat denaturation step allows more opportunity for non-specific binding of primer sequences. To reduce this possibility, one or more components of the PCR are physically separated until the first denaturation step. A

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popular approach is to use a specially formulated wax bead designed to fit snugly within a PCR reaction tube. The reaction components minus the enzyme and reaction buffer are added to the tube followed by the molten wax bead which floats on top and then solidifies on cooling. The thermostable polymerase is then added with buffer. At the initial denaturation step the wax melts again and rises to the surface causing all the reaction components to come into contact with each other.

Touch-down PCR

Most thermal cyclers can be programed to perform runs in which the annealing temperature is lowered incrementally during the PCR cycling from an initial value above the expected Tm to a value below the Tm. By keeping the stringency of hybridization initially very high, the formation of spurious products is discouraged, allowing the expected sequence to predominate.

Reverse-transcription PCR

Use of reverse transcriptase is to obtain a cDNA copy of mRNA, and the construction of cDNA libraries. Combining reversetranscriptase (RT) with PCR, a procedure known as RT-PCR, extends the application of PCR into the analysis of gene expression, either qualitatively or quantitatively, as well as greatly facilitating the construction of cDNAlibraries or the cloning of specific cDNAs. One problem with RT-PCR is that the initial mRNA preparation may be contaminated with genomic DNA. The PCR step can then result in amplification of the contaminating DNA. When working with eukaryotic material, this can often be overcome by designing the primers so that the amplicon spans at least one intron. In this way, amplification of any genomic DNA will either be prevented altogether (because the presence of the intervening intron makes the sequence too large to be amplified), or at least it will be readily distinguished from amplified cDNA (because of the different size of the product). Generally, it is preferable to remove all traces of DNA from the mRNA, usually by treatment with RNase-free DNase.

Major Advantages

Speed and ease of use: DNA cloning by PCR can be performed in a few hours, using relatively unsophisticated equipment.

Sensitivity: PCR is capable of amplifying sequences from minute amounts of target DNA, even the DNA from a single cell.

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Robustness: PCR can permit amplification of specific sequences from material in which the DNA is badly degraded or embedded in a medium from which conventional DNA isolation is problematic.

Gene tagging: PCR is utilized for developing molecular markers closely linked to specific genes of economic importance

Confirmation of presence of transferred gene: Gene of interest transferred to vector-primer can be designed to cloned PCR for amplification of gene of interest.

Human Genetics: Prenatal diagnosis of sickle cell anemia and Sex determination of embryos – since DNA sequence in single cells can be studied using PCR.

DNA fingerprinting: Identification of criminals & disputed parentage PCR is used to generate single strand used for DNA sequencing and thermal cycle sequencing, which is extensively used in automated DNA sequencing**.**PCR is being used to analyze the preserved tissue of extinct species.It is particularly useful in monitoring retroviral infections.It can be used for detection of infection by such pathogen which are very difficult to culture *in vitro*.

Major Disadvantages

- In order to construct specific oligonucleotide primers that permit selective amplification of a particular DNA sequence, some prior sequence information is necessary.
- A clear disadvantage of PCR as a DNA cloning method has been the size range of the DNA sequences that can be cloned.
- PCR is very sensitive technique and is prone to generating false signals, which in many cases are the result of contaminants from previous PCR amplifications being present in the amplification reaction contaminants

Conclusion

The use of PCR has an advantage in that it gives you the option to re-amplify the target DNA each time your DNA supplies dwindle without ligation into a vector or transformation into *E. coli.* Alternatively, PCR products can be ligated into a suitable vector, which can then be transformed into and replicated by *E. coli*. The PCR process is a useful tool to quickly and easily amplify the desired sequences. With the successful sequencing of whole and partial genomes of organisms across all biological kingdoms, DNA cloning by PCR is an easily attainable option.

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