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The Polymerase Chain Reaction

The polymerase chain reaction (PCR) is the most powerful laboratory technique ever invented. The ease with which it can be done, the relatively low cost, and it's unique combination of specificity and sensitivity coupled with great flexibility has led to a true revolution in genetics.

The polymerase chain reaction was conceptualized and operationalized by Kary Mullis and colleagues at Cetus Corporation in the early 1980's. The method was first formally presented at the American Society of Human Genetics Conference in October of 1985 and the first clinical application for PCR, an analysis of sickle cell anemia, was published the same year. In its initial form, PCR was tedious and labor intensive. However, the advent of a method by which a specific DNA sequence could be isolated from its genomic context and amplified virtually without limit would not long remain a useful tool. The breakthrough came with the isolation and purification of thermo-stable DNA polymerases . This allowed for PCR to be automated and soon the first programmable PCR thermal cyclers appeared on the market. Since that time, PCR has spread to literally every corner of the world and to every conceivable aspect of biology and chemistry. So profound was the impact of PCR that Kary Mullis was awarded the 1993 Nobel Prize in Chemistry, not even ten years after its introduction.

The PCR Reaction Components

Despite the numerous variations on the basic theme of PCR, the reaction itself is composed of only a few components. These are as follows:

1. Water
2. PCR Buffer
3. MgCl2
4. dNTPs
5. Target DNA
6. Taq Polymerase
7. Forward Primer
8. Reverse Primer

Considering each of these components, we can begin with **Water**. While it may seem trivial, water can be a source of concern and frustration. Water is present to provide the liquid environment for the reaction to take place. It is the matrix in which the other components interact. For most people and in most labs sterile, deionized water is the choice.

The next component is the **PCR Reaction Buffer**. The primary purpose of this component is to provide an optimal pH and monovalent salt environment for the final reaction volume. Many commercially supplied PCR buffers already contain **magnesium chloride** (MgCl2). MgCl2 supplies the Mg++ divalent cations required as a cofactor for Type II enzymes, which include DNA polymerases used in PCR. The standard final concentration of this reagent for polymerases used in PCR is 1.5mM. Sometimes it is necessary to change this concentration in order to optimize the PCR reaction. For this reason we choose to obtain PCR buffer without MgCl2 and to add it ourselves. 3.0ul of the standard 25mM MgCl2 provided commercially will yield a 1.5mM final concentration in a 50ul reaction volume.

The purpose of the **deoxynucleotide triphosphates (dNTPs)** is to supply the "bricks." Since the idea behind PCR is to synthesize a virtually unlimited amount of a specific stretch of double-stranded DNA, the individual DNA bases must be supplied to the polymerase enzyme.

The next component is, of course, the **target DNA**. The quality and quantity of the target DNA is important. The DNA used as the PCR target should be as pure as possible and also it should be uncontaminated by any other DNA source. Thus care must be taken to ensure that the target DNA only contains the target of interest. We have settled upon a maximum of 100ng of genomic DNA for PCR amplifications from a genomic background.

DNA polymerases should be presented, during the essential DNA denaturation step, 94ºC or 95ºC for up to a several minute, the DNA target was rendered single stranded, it also destroyed the polymerase. The answer to this problem was, as are all good solutions, blindingly simple. There exists in nature organisms that are perfectly happy at very high temperatures. Such organisms, called **thermophiles**. The first of these thermophilic organisms to be exploited was the bacterium *Thermus aquaticus*. The DNA polymerase from *Thermus aquaticus* is stable at 95ºC and allowed for automation of the PCR process. The nomenclature rule for enzymes derived from microorganisms is to use the first letter of the genus and the first two letters of the species. Thus, the DNA polymerase from *Thermus aquaticus* is called Taq polymerase.

In general, there are three aspects of a DNA polymerase that should be considered. These are;

1. **processivity**
2. **fidelity**
3. **persistence**.

Processivity refers to the rate at which that polymerase enzyme makes the complementary copy of the template. The standard here is Taq polymerase, which has a processivity of 50-60 nucleotides (nt) per second at 72 oC. One of the most important of these other features is **fidelity**. This refers to the accuracy of the complementary copy being made. Taq DNA polymerase has among the highest error rates of the thermophilic polymerases at 285 x 10^{-6} errors per template nucleotide. Finally, the attribute of **persistence,** which refers to the stability of the enzyme at high temperature, Taq polymerase has a half-life of about an hour and a half at a sustained 95ºC.

Taq DNA polymerase have a very unusual property not shared by other DNA polymerases. These enzymes do not possess 3'→5' proof reading ability whereas other polymerases do possess this ability. The consequence of the lack of 3'→5' proof reading ability is that Taq polymerase adds a single 3' nucleotide (Adenosine) on both strands of every amplicon. This 3' extension permits direct cloning of a PCR product using one of the various commercially available PCR cloning vectors.

The success of a PCR reaction will ultimately depend upon the **primers** and the **reaction conditions**. The purpose of a PCR primer is to specify a unique address in the background of the target DNA. In order to do this, two aspects must be considered. First is the **length** of the primer and second is the **actual sequence** of the primer. Regardless of the actual sequence of a PCR primer, its length must be sufficient to guarantee that it will occur in the background target DNA less than once by chance alone. Here, we must now consider the actual sequence of primers attributes of **melting temperature** (**Tm**), **Tm** used to refer to the thermal denaturation of duplex nucleic acid strands. PCR reaction need two deferent primers one for each DNA strand; Forward primer and reverse primer.

The PCR Reaction procedure

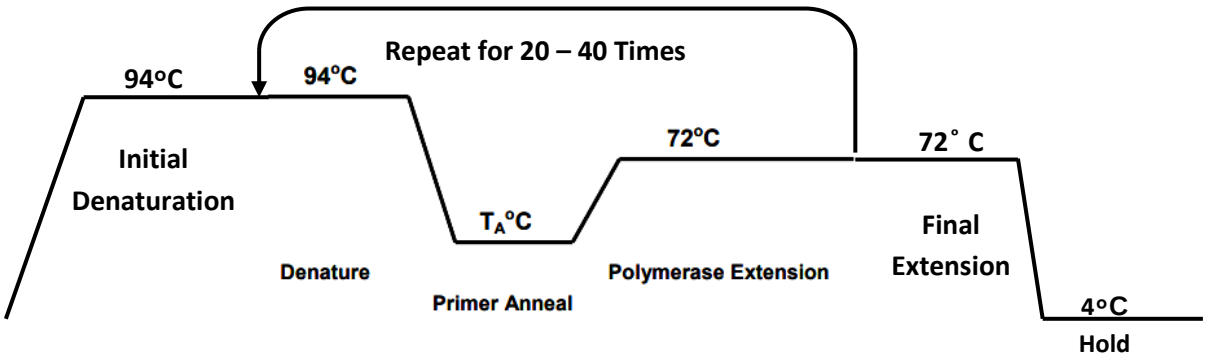
Typically, PCR consists of a series of 20–40 repeated temperature changes, called cycles, with each cycle commonly consisting of three discrete temperature steps. The cycling is often preceded by a single temperature step at a very high temperature (94-95 °C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters, including the enzyme used for DNA synthesis, the concentration of bivalent ions and dNTPs in the reaction, and the melting temperature (*Tm*) of the primers. The individual steps common to most PCR methods are as follows:

- **Initialization** (**Pre-Denaturation**): This step is only required for DNA polymerases that require heat activation by hot-start PCR. It consists of heating the reaction chamber to a temperature of 94–95 °C, at the end of this step the double strand DNA become denaturized .
- **Denaturation**: This step is the first regular cycling event and consists of heating the reaction chamber to 94–95 °C for 0.5-1 min. this causes DNA melting or denaturation of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.
- **Annealing**: In the next step, the reaction temperature is lowered to 50–65 °C for 20–60 seconds, allowing annealing of the primers to each of the single-stranded DNA templates. Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region. The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand.

  It is critical to determine a proper temperature for the annealing step because efficiency and specificity are strongly affected by the annealing temperature. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, the primer should bind *only* to a perfectly complementary part of the template. If the temperature is too low, the primer may bind imperfectly. If it is too high, the primer may not bind at all. A typical annealing temperature is about 3–5°C below the *Tm* of the primers used. Stable hydrogen bonds between complementary bases are formed only when the primer sequence very closely matches the template sequence. During this step, the polymerase binds to the primer-DNA template hybrid and begins DNA formation.
- **Extension**: The temperature at this step depends on the DNA polymerase used; the optimum activity temperature for the thermostable DNA polymerase of Taq (*Thermus aquaticus*) polymerase is approximately of 72 °C. In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxy group at the end of the nascent (elongating) DNA strand. The precise time required for elongation depends both on the DNA polymerase used and on the length of the DNA target region to amplify. As a rule of thumb, at their optimal temperature, most DNA polymerases polymerize a thousand bases per minute. Under optimal

conditions, at each extension step, the number of DNA target sequences is doubled. With each successive cycle, the original template strands plus all newly generated strands become template strands for the next round of elongation, leading to exponential (geometric) amplification of the specific DNA target region.

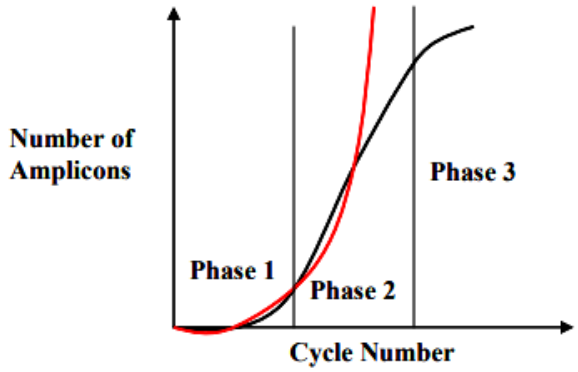
The processes of denaturation, annealing and elongation constitute a single cycle.



Multiple cycles are required to amplify the DNA target to millions of copies. The formula used to calculate the number of DNA copies formed after a given number of cycles is:

Copies No. = 2^n (where *n* is the number of cycles)

Thus, a reaction set for 30 cycles results in 2^{30}, or 1073741824, copies of the original double-stranded DNA target region.



- **Final Extension**: This single step is performed at a temperature of 72°C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.
- **Final hold**: The final step cools the reaction chamber to 4–10°C for an indefinite time, and may be employed for short-term storage of the PCR products.