Polymerase Chain Reaction

Polymerase chain reaction (PCR) was invented by Karry Mullis in 1983 and was patented in 1985. Its principle is based on the **use of DNA polymerase which is an in vitro replication of specific DNA sequences. This method can generate tens of billions of copies of a particular DNA fragment** (the sequence of interest, DNA of interest, or target DNA) from a DNA extract (DNA template).

If the sequence of interest is present in the DNA extract, it is possible to selectively replicate it (we call it amplification) in very large numbers i.e., billions of copies within no time.

Principle of the PCR

PCR makes it possible to obtain, by in vitro replication, multiple copies of a DNA fragment from an extract. Matrix DNA can be genomic DNA as well as complementary DNA obtained by RT-PCR from a messenger RNA extract (poly-A RNA), or even mitochondrial DNA. It is a technique for obtaining large amounts of a specific DNA sequence from a DNA sample. This amplification is based on the replication of a double-stranded DNA template. It is broken down into three phases: a denaturation phase, a hybridization phase with primers, and an elongation phase. The products of each synthesis step serve as a template for the following steps, thus exponential amplification is achieved.

The polymerase chain reaction is carried out in a reaction mixture which comprises the DNA extract (template DNA), Taq polymerase, the primers, and the four deoxyribonucleoside triphosphates (dNTPs) in excess in a buffer solution. The tubes containing the mixture reaction are subjected to repetitive temperature cycles several tens of times in the heating block of a thermal cycler (apparatus which has an enclosure where the sample tubes are deposited and in which the temperature can vary, very quickly and precisely, from 0 to 100°C by Peltier effect). The apparatus allows the programming of the duration and the succession of the cycles of temperature steps. Each cycle includes three periods of a few tens of seconds. The process of the PCR is subdivided into three stages as follows:

1. The denaturation

It is the separation of the two strands of DNA, obtained by raising the temperature. The first period is carried out at a temperature of 94°C, called the denaturation temperature. At this temperature, the matrix DNA, which serves as matrix during the replication, is denatured: the hydrogen bonds cannot be maintained at a temperature higher than 80°C and the double-stranded DNA is denatured into single-stranded DNA (single-stranded DNA).

2. Hybridization

The second step is hybridization. It is carried out at a temperature generally between 40 and 70°C, called primer hybridization temperature. Decreasing the temperature allows the hydrogen bonds to reform and thus the complementary strands to hybridize. The primers, short single-strand sequences complementary to regions that flank the DNA to be amplified, hybridize more easily than long strand matrix DNA. The higher the hybridization temperature, the more selective the hybridization, the more specific it is.

3. Elongation

The third period is carried out at a temperature of 72°C, called elongation temperature. It is the synthesis of the complementary strand. At 72°C, Taq polymerase binds to primed single-stranded DNAs and catalyzes replication using the deoxyribonucleoside triphosphates present in the reaction mixture. The regions of the template DNA downstream of the primers are thus selectively synthesized. In the next cycle, the fragments synthesized in the previous cycle are in turn matrix and after a few cycles, the predominant species corresponds to the DNA sequence between the regions where the primers hybridize. It takes 20–40 cycles to synthesize an analyzable amount of DNA (about 0.1 µg).

Each cycle theoretically doubles the amount of DNA present in the previous cycle. It is recommended to add a final cycle of elongation at 72°C, especially when the sequence of interest is large (greater than 1 kilobase), at a rate of 2 minutes per kilobase. PCR makes it possible to amplify sequences whose size is less than 6 kilobases. The PCR reaction is extremely rapid, it lasts only a few hours (2–3 hours for a PCR of 30 cycles).

Primers

To achieve selective amplification of nucleotide sequences from a DNA extract by PCR, it is essential to have least one pair of oligonucleotides. These oligonucleotides, which will serve as primers for replication, are synthesized chemically and must be the best possible complementarity with both ends of the sequence of interest that one wishes to amplify. One of the primers is designed to recognize complementarily a sequence located upstream of the fragment 5'–3' strand DNA of interest; the other to recognize, always by complementarity, a sequence located upstream complementary strand (3'–5') of the same fragment DNA.

Primers are single-stranded DNAs whose hybridization on sequences flanking the sequence of interest will allow its replication so selective. The size of the primers is usually between 10 and 30 nucleotides in order to guarantee a sufficiently specific hybridization on the sequences of interest of the matrix DNA.

Taq polymerase

DNA polymerase allows replication. We use a DNA polymerase purified or cloned from of an extremophilic bacterium, *Thermus aquaticus*, which lives in hot springs and resists temperatures above 100°C. This polymerase (Taq polymerase) has the characteristic remarkable to withstand temperatures of around 100°C, which are usually sufficient to denature most proteins. *Thermus aquaticus* finds its temperature of comfort at 72°C, optimum temperature for the activity of its polymerase.

The reaction conditions

The volumes of reaction medium vary between 10 and 100 µl. There are a multitude of reaction medium formulas. However, it is possible to define a standard formula that is suitable for most polymerization reactions. This formula has been chosen by most manufacturers and suppliers, who, moreover, deliver a ready-to-use buffer solution with Taq polymerase. Concentrated 10 times, its formula is approximately the following: 100 mM Tris-HCl, pH 9.0; 15 mM $MgCl_2$, 500 mM KCl.

It is possible to add detergents (Tween 20, Triton X-100) or glycerol in order to increase the conditions of stringency that make it harder and therefore more selective hybridization of the primers. This approach is generally used to reduce the level of nonspecific amplifications due to the hybridization of the primers on sequences without a relationship with the sequence of

interest. We can also reduce the concentration of KCl until eliminated or increase the concentration of $MgCl_2$. Indeed, some pairs of primers work better with solutions enriched with magnesium. On the other hand, with high concentrations of dNTP, the concentration of magnesium should be increased because of stoichiometric interactions between magnesium and dNTPs that reduce the amount of free magnesium in the reaction medium. dNTPs (deoxyribonucleoside triphosphates) provide both the energy and the nucleotides needed for DNA synthesis during the chain polymerization. They are incorporated in the reaction medium in excess, that is, about 200 µM final. Depending on the reaction volume chosen, the primer concentration may vary between 10 and 50 pmol per sample.

Matrix DNA can come from any organism and even complex biological materials that include DNAs from different organisms. But to ensure the success of a PCR, it is still necessary that the DNA matrix is not too degraded. This criterion is obviously all the more crucial as the size of the sequence of interest is large. It is also important that the DNA extract is not contaminated with inhibitors of the polymerase chain reaction (detergents, EDTA, phenol, proteins, etc.). The amount of template DNA in the reaction medium initiate that the amplification reaction can be reduced to a single copy. The maximum quantity may in no case exceed 2 µg. In general, the amounts used are in the range of 10–500 ng of template DNA. The amount of Taq polymerase per sample is generally between 1 and 3 units. The choice of the duration of the temperature cycles and the number of cycles depends on the size of the sequence of interest as well as the size and the complementarity of the primers. The durations should be reduced to a minimum not only to save time but also to prevent risk of nonspecific amplification. For denaturation and hybridization of primers, 30 seconds are usually sufficient. For elongation, it takes 1 minute per kilobase of DNA of interest and 2 minutes per kilobase for the final cycle of elongation. The number of cycles, generally between 20 and 40, is inversely proportional to the abundance of DNA matrix.

4. PCR product detection and analysis

The product of a PCR consists of one or more DNA fragments (the sequence or sequences of interest). The detection and analysis of the products can be very quickly carried out by agarose gel electrophoresis (or acrylamide). The DNA is revealed by ethidium bromide staining. Thus, the products are instantly visible by ultraviolet transillumination (280–320 nm). Very small products are often visible very close to the migration front in the form of more or less diffuse bands. They correspond to primer dimers and sometimes to the primers themselves. Depending on the reaction conditions, nonspecific DNA fragments may be amplified to a greater or lesser extent, forming net bands or “smear”. On automated systems, a fragment analyser is now used. This apparatus uses the principle of capillary electrophoresis. Fragment detection is performed by a laser diode. This is only possible if the PCR is performed with primers coupled to fluorochrome].

Applications:

1. PCR is widely used for diagnostic purposes to detect the presence of a specific DNA sequence of this or that organism in a biological fluid.

2. It is also used to make genetic fingerprints, whether it is the genetic identification of a person in the context of a judicial inquiry, or the identification of animal varieties, plants, or microbial for food quality testing, diagnostics, or varietal selection.

3. PCR is essential for whole genome sequencing or detection of site-directed mutagenesis.

Finally, there are variants of PCR such as real-time PCR, competitive PCR, PCR in situ, RT-PCR, etc.

At present, the revolutionary evolutions of molecular biological research are based on the PCR technique which provides suitable and specific products, especially in the field of the characterization and conservation of genetic diversity.

Several applications are possible in downstream of the PCR technique:

(1) the establishment of a complete sequence of the genome of the most important livestock breeds;

(2) development of a technology measuring scattered polymorphisms at loci throughout the genome (e.g., SNP detection methods); and

(3) the development of a microarray technology to measure gene transcription on a large scale.

The study of biological complexity is a new frontier that requires high throughput molecular technology, high speed and computer memory, new approaches to data analysis, and the integration of interdisciplinary skills.

Reference

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