PCR AND REAL TIME PCR

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a method widely used to rapidly make millions to billions of copies of a specific DNA sample, allowing scientists to take a very small sample of DNA and amplify it to a large enough amount to study in detail. Shortly, using PCR, copies of very small amounts of DNA sequences are exponentially amplified in a series of cycles of temperature changes. PCR is now a common and often indispensable technique used in medical laboratory and clinical laboratory research for a broad variety of applications including biomedical research and criminal forensics.

PCR is one of the most powerful tools in Molecular Biology. It is defined as a technique used for *in vitro* amplification of specific target DNA sequence using a set of specific and unique primers for primer extension of complementary strands by Taq Polymerase. PCR acts like a 'copying machine' creating a large number of duplicated copies of DNA molecules from a minute amount of starting material. It is a rapid, inexpensive, extraordinarily powerful and most commonly used versatile technique in all day to day lab experiments. Kary Mullis from Cetus Corporation was awarded Nobel Prize in chemistry in 1993 for his invention of PCR in 1983.

PCR reaction consists of the following components as depicted in Fig. 1.

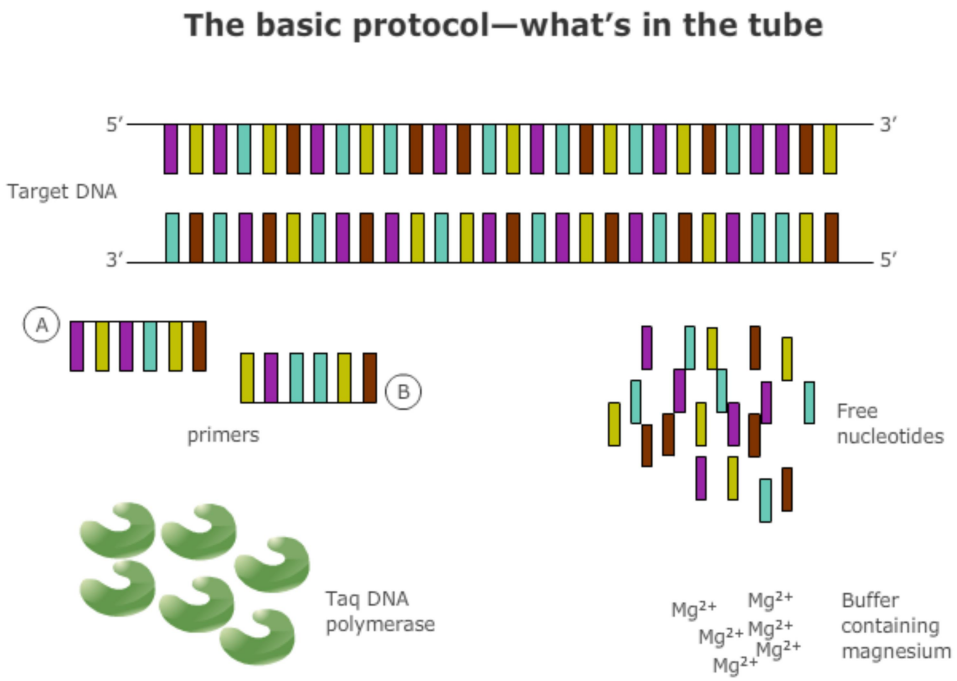


Fig. 1 The basic protocol - what is required for a PCR reaction

Components of PCR

1-Template (Target) DNA

Template DNA is the sample DNA that contains the target sequence. It can also be said as the resource material for the amplification of the target gene. It is extracted by following different DNA extraction protocols. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strands from each other. It could be either genomic DNA, plasmid DNA or cDNA prepared from RNA from any source like microbes, viruses, animals and plants. The beauty of this technique is such that because of its high specificity, it can pick up the target amongst the large background of the non-specific DNA also and hence template DNA need not be absolutely pure for PCR amplification.

At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies, those are called amplicons.

2-Nucleotides (Deoxynucleotide triphosphates= dNTPs)

The deoxynucleotide triphosphates (dNTPs) are the nucleotide building blocks (adenine, guanine, cytosine and thymine) which are essentially "building blocks" for new DNA strands.

3-Primers

A pair of primers (Forward and Reverse) is required for amplification of the target DNA from both the strands i.e. 5'---3' and 3'----5'. In brief, primer is a short single-stranded oligonucleotide sequence of DNA that is required to initiate the synthesis of new strand of DNA in a polymerase chain reaction. Generally, these are about 20 to 22 bp in length for general PCR reaction but may vary according to the designed experiment (upto even 50 bp). The primers are identical to the 5' ends of sense and antisense strands of DNA. These are designed to flank the terminal regions of gene to be amplified as shown below also:

Forward primer : 5' GGC TAG CGT CAT ----- 3'

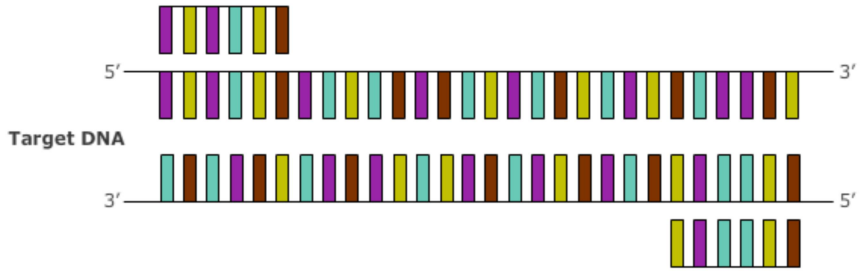
Reverse primer : 5' AGC GGC CGA AGC ------3'

Melting temperature (T_m) is defined as the minimal temperature at which two strands separate as a result of denaturation

Annealing temperature (T_{anneal}) is the temperature at which primers anneal optimally to the template DNA.

Primers

- Usually about 20 nucleotides in length
- Designed to flank the region to be amplified
- Melting point determined by G-C and A-T content
  - Tm = 4⁰C (G+C) + 2⁰C (A+T)
  - Ex: a primer with 10 G/C and 10 A/T would have a Tm of 60⁰C--------->4(10) + 2(10)=60⁰C



4-Taq DNA polymerase

Taq DNA polymerase is a type of enzyme that synthesizes new strands of DNA complementary to the target sequence. The first and most commonly used of these enzymes is*Taq*DNA polymerase (because of its higher fidelity when copying DNA. It can generate new strands of DNA using a DNA template and primers, and are heat resistant. It catalyses DNA polymerization i.e incorporates / adds nucleotides during the synthesis of new strands of DNA during extension at 72°C. However, Taq DNA polymerase requires the use of a buffer containing $MgCl_2$ for its optimal functionality.

Most of the enzymes/proteins get inactivated at higher temperature but Taq DNA polymerase withstands high temperature of denaturation. Taq DNA polymerase was isolated from a thermophilic organism *Thermus aquaticus*, which normally lives in hot springs at temperatures close to 100°C. Hence, this enzyme can remain stable at high temperatures as close as 100°C i.e. it is stable under the extreme temperature conditions of PCR and hence does not need to be supplied afresh in the PCR reaction mix.

5- Magnesium Chloride (MgCl2)

It is used as a buffer mixture to provide appropriate pH and ion conditions. The Mg + 2 ion is also important as a cofactor in the process. Its concentration should be in certain proportions and different for each primer. If its concentration is excessive, mismatches will occur. A small amount of buffer decrease the specifity of taq polymerase.

THERMAL CYCLER

The Thermal cycler also known as PCR machine or DNA amplifier is an equipment which is used for amplification of a gene using polymerase chain reaction.

Procedure/ Cycling parameters

Typically, PCR consists of a series of 20–40 repeated temperature changes, called thermal cycles, with each cycle commonly consisting of two or three discrete temperature steps. The

cycling is often preceded by a single temperature step at a very high temperature (>90 °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 (T_m) of the primers. The individual steps common to most PCR methods are as follows:

1-Denaturation

Template DNA is heated at high temperatures (94-98°C) at which the denaturation of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules. These strands then become accessible to primers annealing for synthesis of new strands during amplification.

2-Annealing

In the next step, the reaction temperature is lowered to 50–65 °C for 20–40 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.

3-Extension/Elongation

Taq DNA polymerase then starts adding nucleotides to the respective primer using information from the DNA template strand at 72°C (approximately 75–80 °C). These steps are followed again and again to generate multiple copies of the DNA template i.e amplified PCR product or amplicons (Fig.).

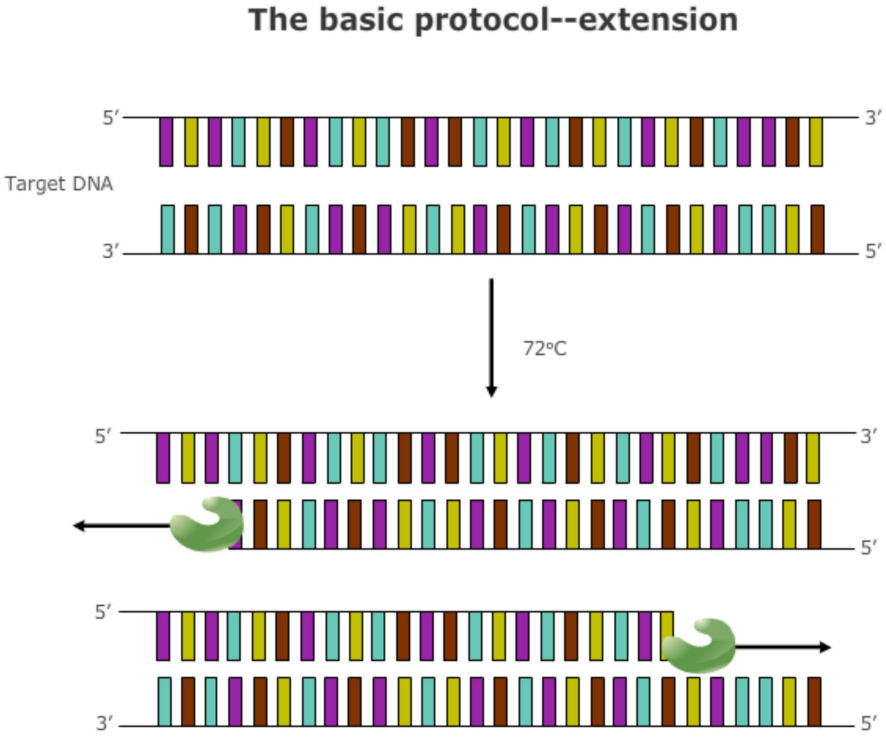


Fig. The basic protocol of PCR reaction

The amount of amplified product at the end of PCR cycles is $2_n^{\#\ of\ cycles}$ e.g. if we start with a single molecule of DNA, after 25 cycles, the amount will be 2^{25} i.e. 3.4x107 molecules of DNA.

-Quantitative or Rreal-time PCR (qPCR or real-time PCR) (quantitative RT-PCR or qRT-PCR.)

It is widely used to quantify (to measure the amount of a specific RNA) RNA transcript levels in cells and tissues. This is achieved by monitoring the amplification reaction using fluorescence, a technique called real-time PCR or quantitative PCR (qPCR). The combination of real-time PCR (qPCR) and reverse transcription PCR is known as quantitative RT-PCR or qRT-PCR. Combined RT-PCR and qPCR are routinely used for analysis of gene expression and quantification of viral RNA in research and clinical settings.

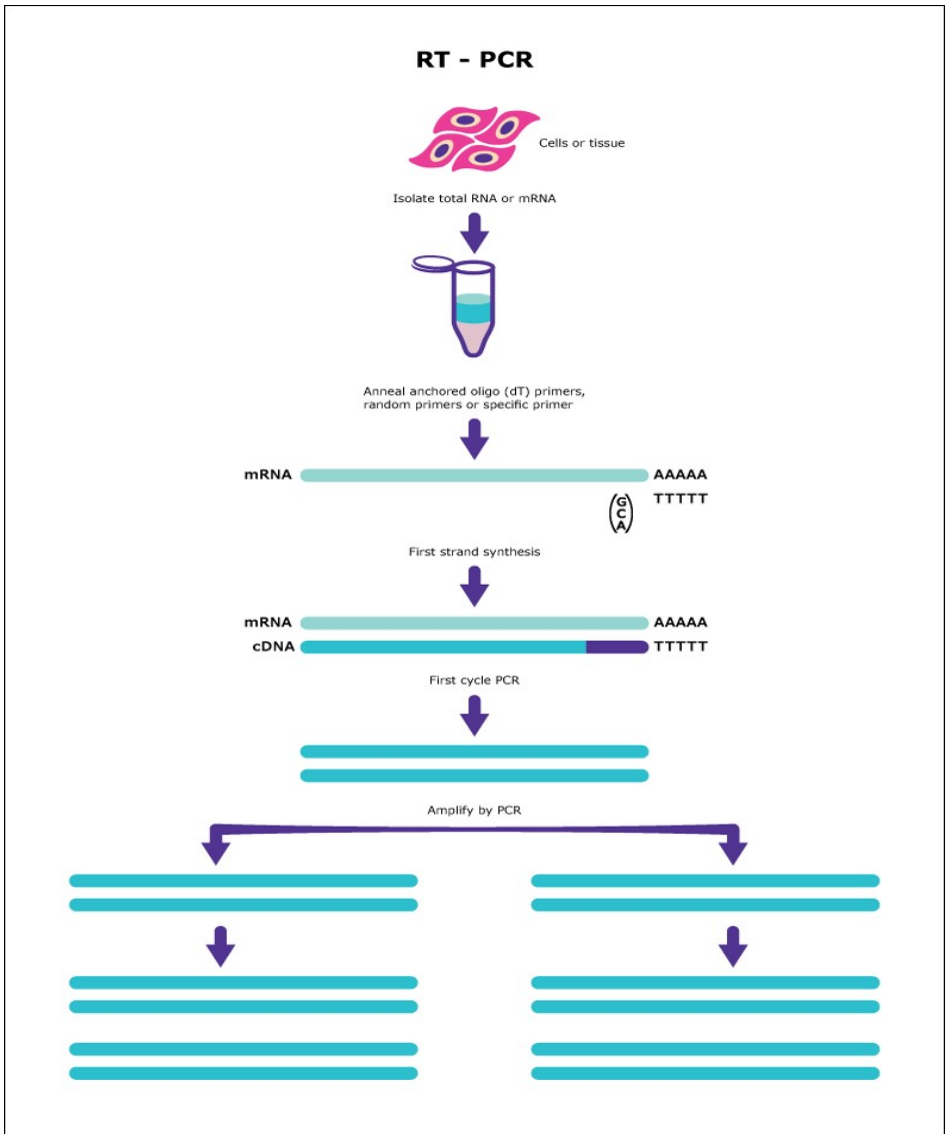
Limitations of PCR and RT-PCR
The PCR reaction starts to generate copies of the target sequence exponentially. Only during the exponential phase of the PCR reaction is it possible to extrapolate back to determine the starting quantity of the target sequence contained in the sample. Because of inhibitors of the polymerase reaction found in the sample, reagent limitation, accumulation of pyrophosphate molecules, and self-annealing of the accumulating product, the PCR reaction eventually ceases to amplify target sequence at an exponential rate and a "plateau effect" occurs, making the end point quantification of PCR products unreliable. This is the attribute of PCR that makesReal-Time Quantitative RT-PCR so necessary.

-Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a laboratory technique combining reverse transcription of RNA into DNA (in this context called complementary DNA or cDNA) and amplification of specific DNA targets using polymerase chain reaction (PCR).

In other words, RT-PCR (Reverse Transcription PCR) is PCR preceded with conversion of sample RNA into cDNA with enzyme **reverse transcriptase or it** allows the use of RNA as a template. The RNA is reverse transcribed into complementary DNA (cDNA), using reverse transcriptase.

Reverse transcription polymerase chain reaction (RT-PCR) is a variation of standard PCR that involves the amplification of specific mRNA obtained from small samples. It eliminates the need for the tedious mRNA purification process required for conventional cloning techniques. In RT-PCR, reverse transcriptase and a RNA sample are used in addition to the standard PCR reagents. The reaction mixture is heated to 37 ˚C, which enables the production of cDNA from the RNA sample by reverse transcription. This cDNA anneals to one of the primers leading to first-strand synthesis. Standard PCR proceeds and dsDNA is produced.


RT - PCR
Cells or tissue
Isolate total RNA or mRNA
Anneal anchored oligo (dT) primers,
random primers or specific primer
mRNA
AAAAA
TTTTT
G
C
A
First strand synthesis
mRNA
cDNA
AAAAA
TTTTT
First cycle PCR
Amplify by PCR