Experiment (5): Polymerase Chain Reaction (PCR)

Aim:

- Amplification of a specific region on DNA.
- Primer design.
- Determine the parameters that may affect he specificity, fidelity and efficiency of PCR.

Introduction:

Nucleic acid **amplification** is an important process in biotechnology and molecular biology and has been widely used in research, medicine, agriculture and forensics. [1] In order to study individual genes or specific DNA regions of interest, it is often necessary to obtain a large quantity of nucleic acid for study, rather than isolate a single copy of the target DNA from a large number of cells. It is often more useful to **generate multiple copies of a target from a single molecule of DNA** or mRNA, **via an in vitro amplification method.** [2]

What is PCR (DNA photocopier)?

Polymerase chain reaction (PCR), a process conceived by Kary Mullis in 1983.[3] It is a laboratory version of DNA replication in cell where **particular piece of DNA** can be **amplified** in billions of copies in a short time. The PCR amplify a precise fragment of DNA from a complex mixture of starting material **termed the template DNA** which controlled by heating and cooling. It does require the knowledge of some DNA sequence information which flanks the fragment of DNA to be amplified (**target DNA**). [4]

PAUSE AND THINK ➔ How you will determine your target sequence?

To amplify a specific piece of DNA (target DNA), **two synthetic oligonucleotides are synthesised** called **primers** each complementary to a stretch of DNA to the 3’ side of the target DNA, one oligonucleotide for each of the two DNA strands (DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group). [5]

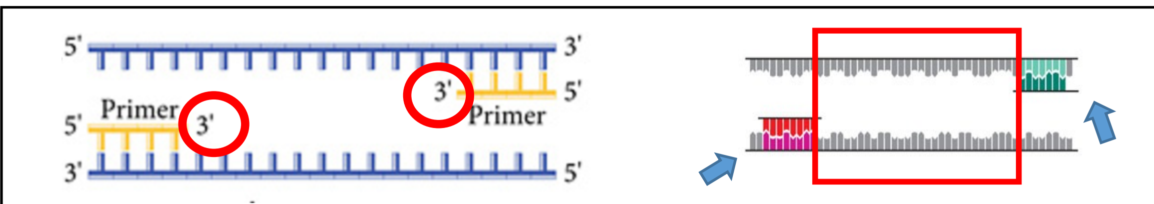


Figure.1. Two primers are prepared, complementary to sequences on opposite strands of the target DNA.

So, PCR **does not copy all of the DNA** in the sample. It copies only a very specific sequence, targeted by the PCR **primers**.

PCR components:

Basic PCR reaction requires four components:

(1) DNA template.

(2) Primers.

(3) Deoxynucleotide triphosphates (dNTPs).

(4) Thermostable DNA polymerase.

The below table illustrate the function of each components and other needed PCR components.

Table.1. Basic PCR components and the function of each one.

Components	Function
Template DNA	The template carries the DNA segment or (target) you wish to amplify.
Forward and Reverse Primers	A primer is a short, single-stranded piece of DNA that anneals (attaches) to its complementary sequence on the template. A pair of primers will bind to either side of the target DNA segment providing initiation sites for DNA synthesis. ➔ e.i. Providing initiation site + specify the amplification to the target DNA segment.
Thermostable DNA polymerase	This is the enzyme used to synthesize new strands of DNA. The DNA polymerase adds nucleotides onto the end of an annealed primer. ➔ PCR uses a heat-stable DNA polymerase, such as the Taq polymerase, which remains active after every heating step and does not have to be replenished. It is named after the thermophilic bacterium *Thermus aquaticus* from which it was originally isolated.[7]
dNTPs	These are the four nucleotides used by DNA polymerase to extend an annealed primer (building blocks).
Magnesium	DNA polymerase requires magnesium for activity (co-factor). Magnesium is usually supplied to a PCR amplification in the form of magnesium chloride.
PCR buffer	PCR buffer is necessary to create optimal conditions for activity of Taq DNA polymerase.

* Additional reagents may include like, DMSO, BSA, potassium salt K^+ or glycerol.

In addition PCR require a special PCR tubes and a device called **thermal cycler** to perform the heating and cooling cycles (see below).

PCR cycle:

PCR proceeds in THREE distinct steps Governed by **Temperature**:

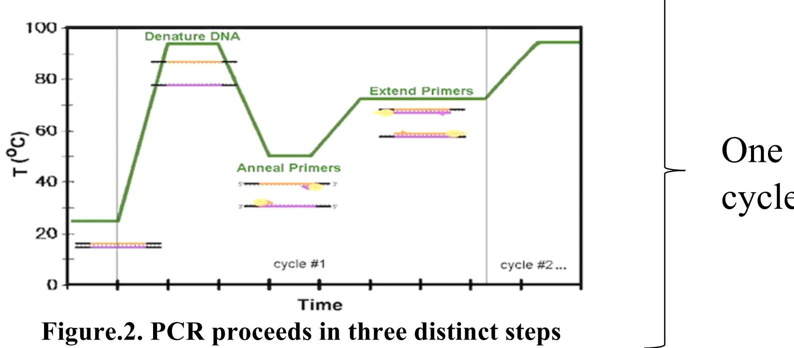


Figure.2. PCR proceeds in three distinct steps

1. **Denaturation:**

This is the first step in cycling event and consists of heating the reaction to 94–97 °C for 20–30 seconds. 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.

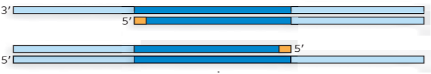
2. **Annealing:**

The reaction temperature is cooled to (50-65°C)* for 15-60 seconds allowing annealing of the primers to the single-stranded DNA template. The single strands of the template are too long and complex to be able to reanneal during this rapid cooling phase. Stable hydrogen bonds are only formed when the primers anneal to sequences that are complementary to them. During this annealing step the thermostable DNA polymerase will be active to some extent and will begin to extend the primers as soon as they anneal to the template.

PAUSE AND THINK ➔ What determine the annealing temperature? See PCR optimization

3. **Extension/elongation:**

The reaction is heated to a temperature depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 72-80°C and commonly a temperature of 72°C is used with this enzyme (optimum temperature). At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTP's that are complementary to the template in 5' to 3' direction. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified.



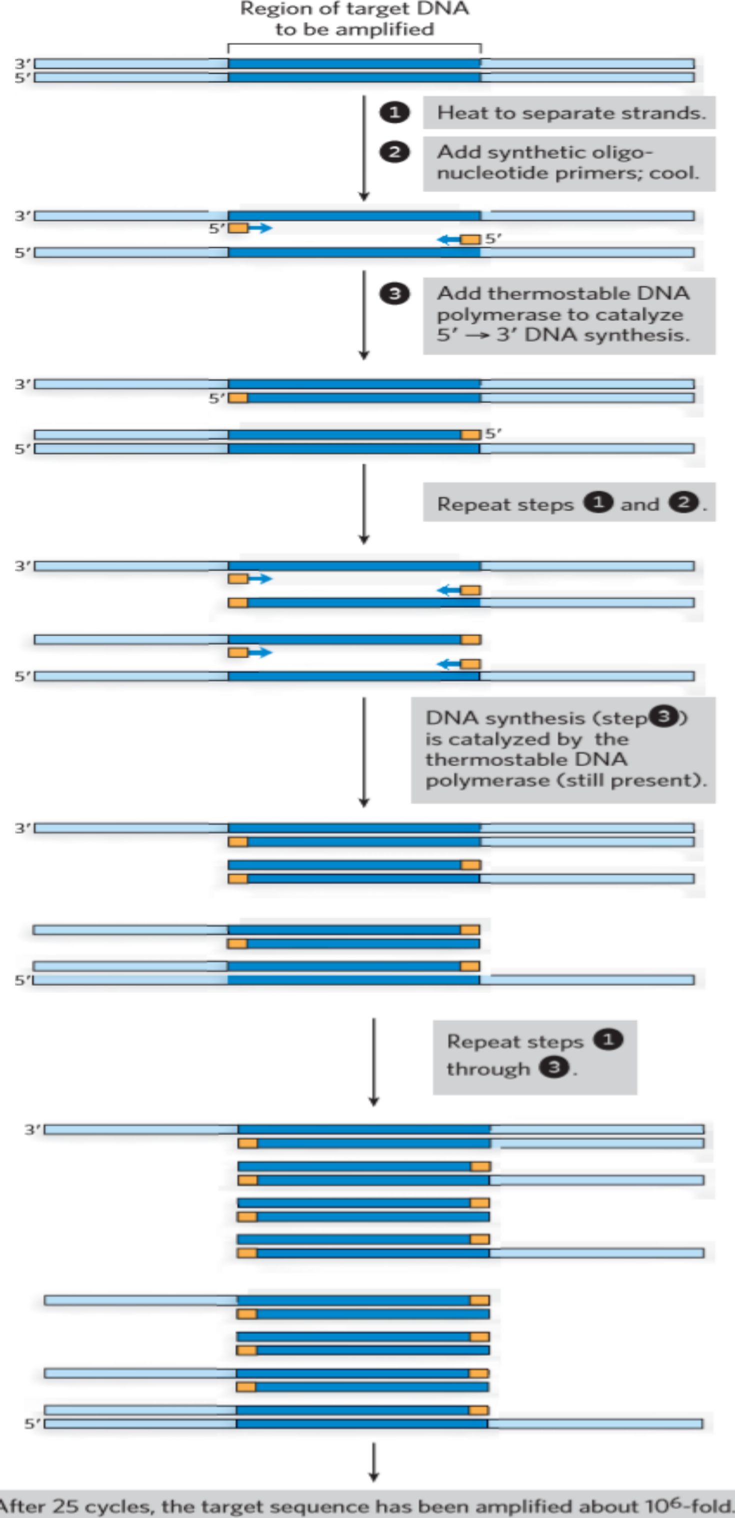


Figure.3. Amplification of DNA segment by PCR.[5]

At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies called **amplicons.** In only 20 cycles, PCR can product about a million (2^{20}) copies of the target. [Number of copies -amplicons- = $2^{\text{number of cycles}}$]

Performing PCR steps:

1. Identification the location of the target sequence in the DNA template.
2. Primer design and primer specificity.
3. PCR optimization.
4. Post-PCR analysis results using agarose gel electrophoresis (AGE).
5. PCR troubleshooting.
6. Start your PCR and visualize the results by AGE.

Primer design:

To design a primer, many parameters should be considered including: primer sequence, length, GC content, melting temperature, annealing temperature and GC clamp.[8]

1. Primer sequence:

Primers must be complementary to flanking sequences of target region. Avoid repeat (ex: ATATATAT) and run (ex: AGCGGGGGAT) sequence which leads to misprime, maximum number is 4 di/nucleotides respectively. It is important to be sure that primer is match to the target sequence at the 3’ end. In addition, complementary sequences between primers should be avoided to prevent primer dimer (self and cross dimers). Avoid cross homology sequences that lead to nonspecific amplification.

2. Primer length:

It is generally accepted that the optimal length of primers is 18-25 bp. Length should be long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature. It prefers that the two primers have a similar length.

3. GC content:

GC%= the number of G's and C's in the primer as a percentage of the total bases. Optimal GC content should be ranged 40-60%.

4. Melting temperature (Tm):

The melting temperature (Tm) is defined as the temperature at which half of the DNA strands are in the single-stranded (ssDNA) state. Melting temperatures in the range of 50-60 °C generally produce the best results. The Tm for both primers should differ by no more than 5 °C (difference between forward and reverse primer).The Tm of the primer can be calculated by the following formula:

$$T_m = [(G + C) \times 4] + [(A + T) \times 2]$$

As shown, the GC content of the sequence gives a fair indication of the primer Tm. In addition there are some online tools that calculate the Tm.

5. Annealing temperature (Ta):

The primer melting temperature is the estimate of the DNA-DNA hybrid stability and critical in determining the annealing temperature. Too high Ta will produce insufficient primer-template hybridization resulting in low PCR product yield. Too low Ta may possibly lead to non-specific products caused by a high number of base pair mismatches.

6. GC clamp:

Presence of G or C bases within the last five bases from the 3' end of primers. It promote a specific binding at the 3' end. GC clamp should be not more than 2 G's or C's .

5'-CAACATAATAGCGACAACA**CTAGA**-3'

There are different tools for primer design, the following are two of the most used tools:

1. NCBI Primer design tool: https://www.ncbi.nlm.nih.gov/tools/primer-blast/
2. Primer3 or primer3Plus: http://bioinfo.ut.ee/primer3-0.4.0/primer3/

After designing the primer, the **specificity of the primer** should be checked to avoid amplification of related pseudogenes or homologs. It could be useful to run a BLAST on NCBI to check for the target specificity of the primers.

PCR Optimization :

There is no single set of conditions that is optimal for all PCR reactions. PCR optimization means to find the most effective condition. This part will be discussed in the next lab.

Post-PCR analysis:

Once the PCR has finished, you need to analyze the products (amplicons). The usual way of doing this is to size fractionate the DNA through an agarose gel. Examining the gel provides evidence for success or failure. The concentration of agarose depends on the product size. [9]

- PAUSE AND THINK → How you will make sure that you target sequence is amplified?

By knowing the target sequence size (product size).

Advantages of PCR:

Simplicity, easier methodology, sensitive, extensively validated standard operating procedure and availability of reagents and equipment. [1]

PCR application:

The polymerase chain reaction has been elaborated in many ways since its introduction and is now commonly used for a wide variety of applications including: genotyping, cloning, mutation detection, sequencing, microarrays, RT-PCR, forensics, and paternity testing. (6)

Supporting materials:

1. Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies: http://www.bio-rad.com/en-sa/applications-technologies/pcr-troubleshooting
2. PCR animation: https://www.youtube.com/watch?v=DkT6XHWne6E
3. PCR troubleshooting: http://www.bio-rad.com/en-sa/applications-technologies/pcr-troubleshooting
4. History of PCR: http://siarchives.si.edu/research/videohistory_catalog9577.html

References:

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