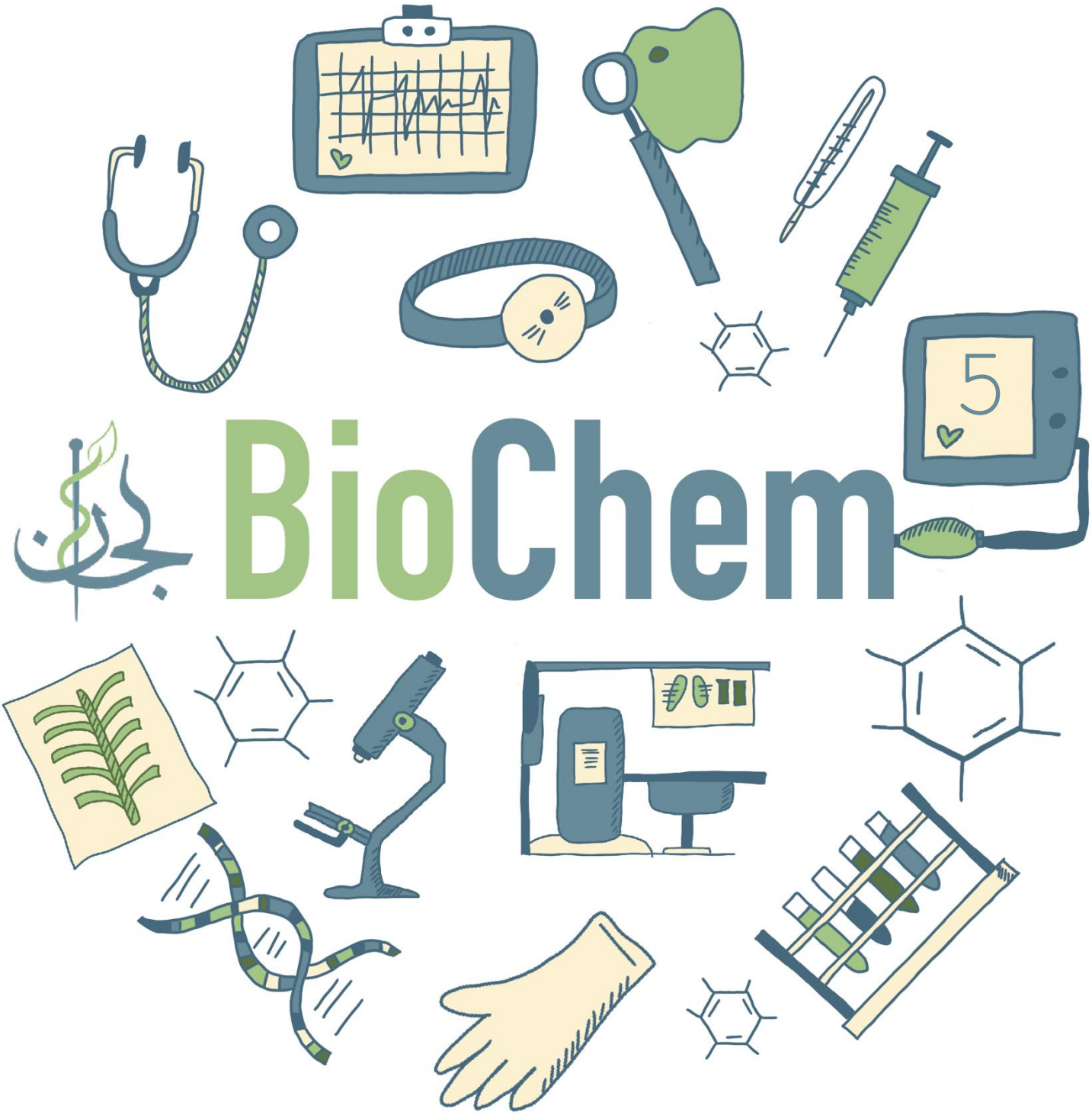

BioChem

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Enzyme-based molecular techniques

Polymerase chain reaction (PCR).

So, what is polymerase chain reaction?

it is a chemical reaction that is a chain reaction (متسلسل), so it proceeds sequentially from the first round to the second to third round and so on… فالتفاعل يحدث بشكل متسلسل ومتتالي

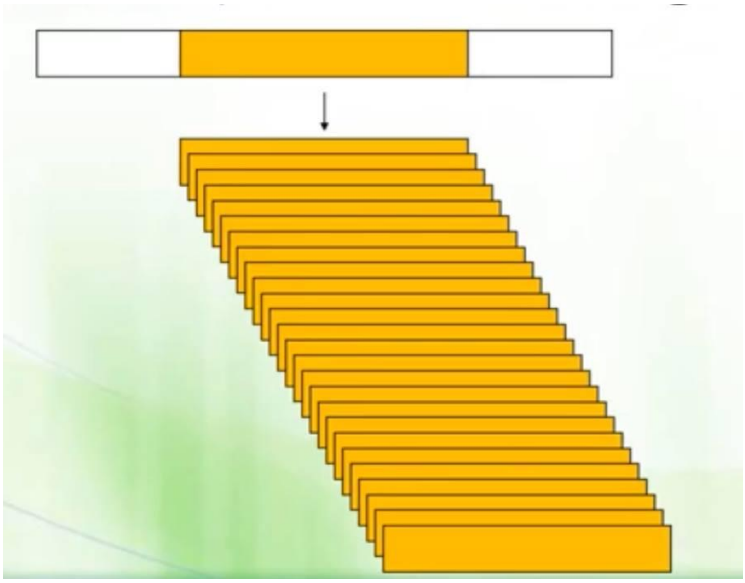
and this reaction allows the DNA from a SELECTED region of a genome to be amplified a billionfold (on a scale of billion), effectively "purifying" this DNA away from the remainder of the genome.

This reaction is catalyzed by the enzyme **DNA polymerase**.

What is the purpose of this reaction?

the purpose is to make lots of copies of specific region in a DNA molecule, so it is almost like cloning, except that it's much faster and very sensitive (meaning that it can detect a single DNA molecule in a sample) with a lot of uses.

The name of the scientist who invented this method is **Kary Mullis**.



What are the components of PCR reaction?

1-DNA template to start with.

2-A pair of DNA primers (not like in DNA synthesis we don't need RNA primers here.)

To initiate the DNA synthesis with it.

-the 15-25 nucleotide-long primers should surround the target sequence.

3-All four deoxyribonucleoside triphosphates.

4-A heat-stable DNA polymerase.

Compared to the sequencing reaction, what is missing here is dideoxynucleotide.

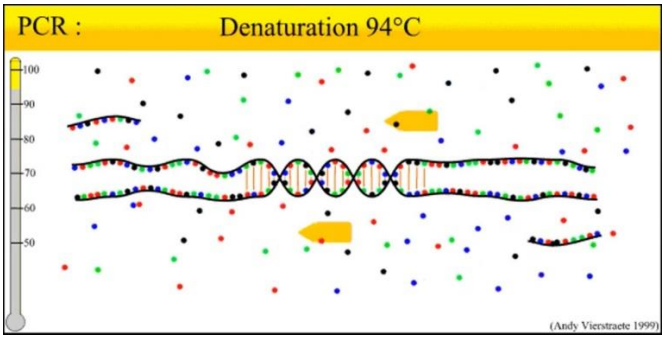
The PCR *cycles*

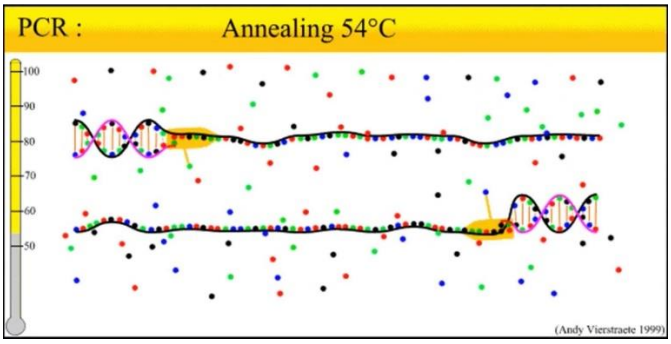
The idea here that the PCR reaction is made of a number of cycles that are between 25 to 30 cycles in which each cycle is composed of three steps which are:

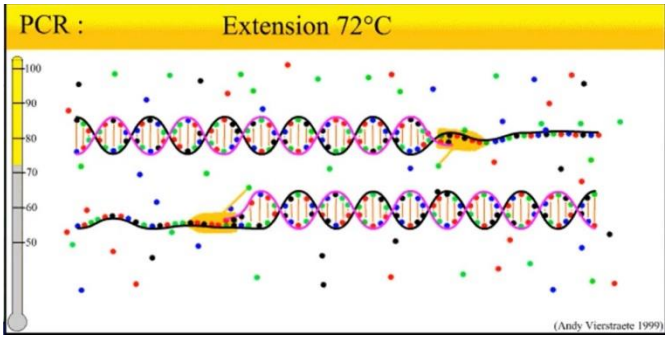
Denaturation (at 95C): DNA is denatured into single-stranded molecules. (we break up these strands).

Reannealing (50C to 70C): the primers anneal to the DNA.

DNA synthesis (at 72C): optimal for polymerase.







The DNA polymerase

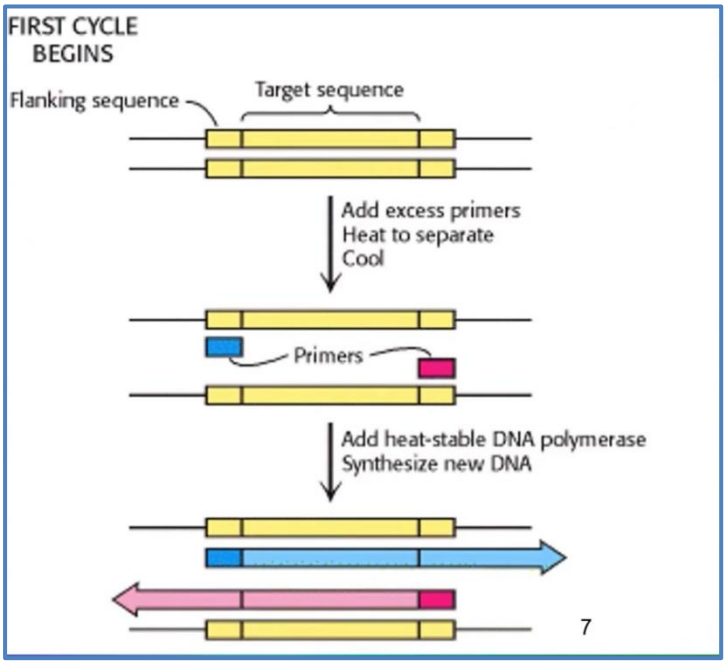
Suitably heat-stable DNA polymerases have been obtained from microorganisms whose natural habitat is hot springs.

The DNA polymerase that is to be used, which is called ***Taq DNA polymerase,*** is a special one obtained from a thermophilic bacteria known as ***Thermus aquaticus***, and is thermostable up to 95C.

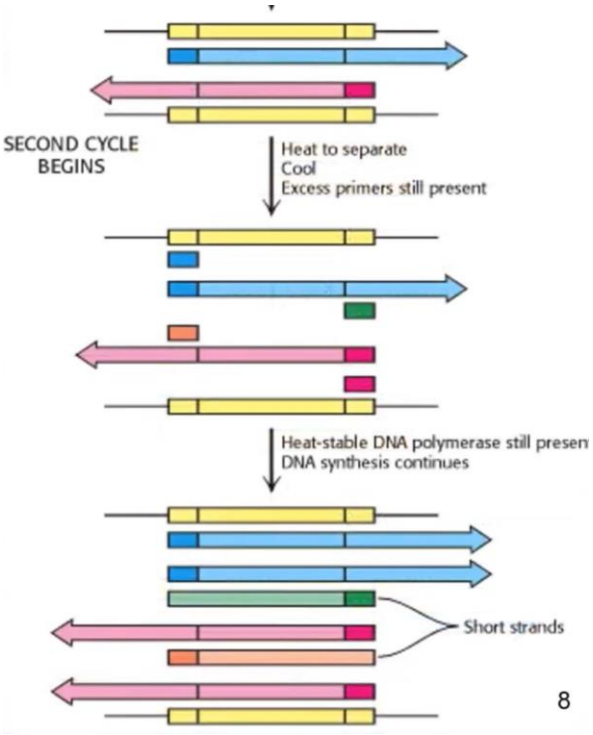
Aqua means water while thermus means temperature so this type lives in hot springs.

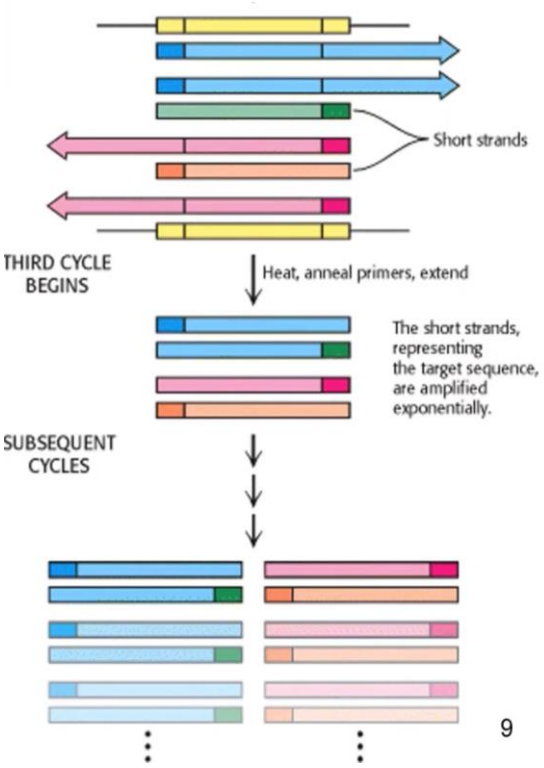
Note: The optimal temperature for this enzyme is 72 C.

These are the steps of the reaction,
First we have the denaturation step whereby the template is denatured so the temperate goes up to 95 degrees and the two strands are separated from each other.
Then temperature goes down to about 50-70C allowing the primers to bind to the DNA template.
Remember that we don't have one DNA molecule here (even though we could) but actually we have many molecules here with a lot of primers.
These primers would anneal (or hybridize) with the DNA template and some of them would bind with regions



Which are flanking regions (meaning that they appear to have wings) (on the sides) surrounding the region of DNA we want to amplify.
The temperature goes up again to 72C allowing the DNA polymerase to start synthesis.



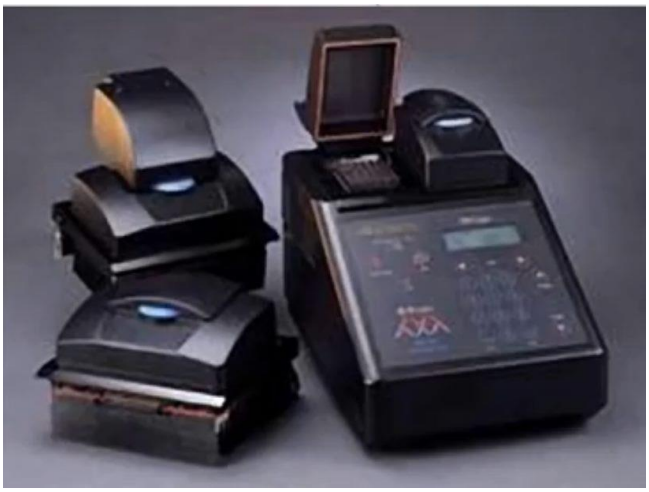


Here we have the second cycle,
we have denaturation again then annealing where primers bind to the DNA and then we have DNA synthesis.

And so on, so right at the third cycle we have the DNA size that we want (the DNA fragment we want). And then subsequently, we have further amplification of this region.

PCR cycles

20-30 cycles of reaction are required for DNA amplification.
the products of each cycle serve as the DNA templates for the next-hence the term polymerase "chain reaction".
Every cycle DOUBLES the amount of DNA.
After 30 cycles, there will be over 250 MILLION short products derived from each starting molecule.



Here's the instrument we use for the PCR reaction,
it is a modern one. VERY efficient.
The reaction used to take 18 hours to finish, but nowadays it would take half an hour to an hour to finish all 25 cycles!

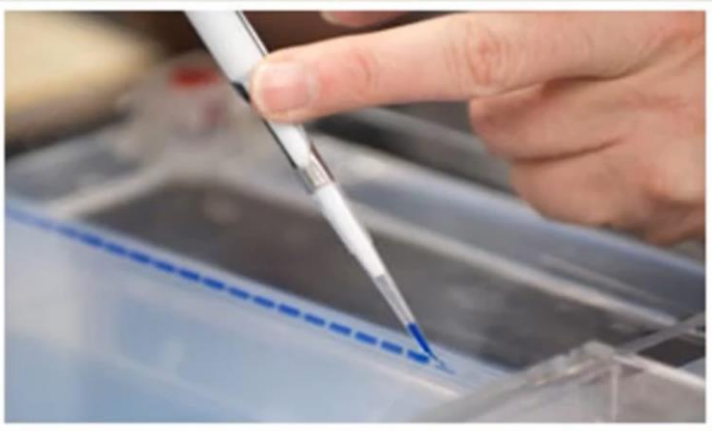
Detection of DNA fragments

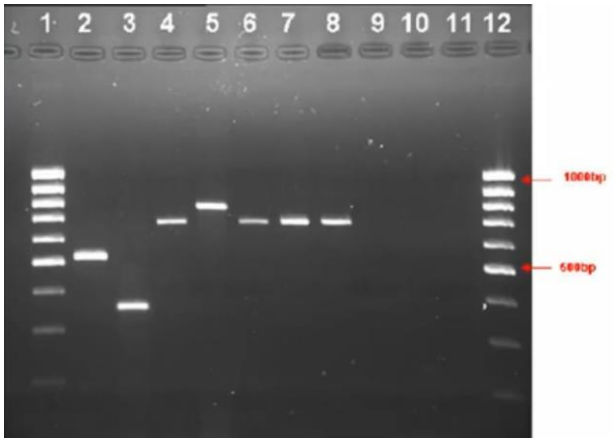
A DNA fragment can be easily visualized as a discrete band of a specific size by agarose gel electrophoresis.

After the DNA reaction is completed, the sample is taken and is ran on a gel. The DNA is then loaded into wells. After DNA is ran, it is to be stained with Ethidium bromide (which is an intercalating agent used as a fluorescent tag) or with whatever dye we use.

Notice in the picture below how for each numbered sample only one DNA band is in its lane.

So DNA amplification is very specific!





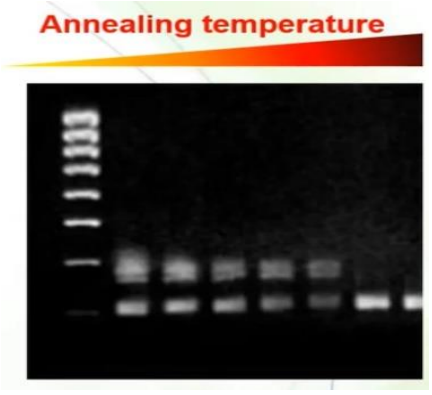
Importance of primers

The specificity of amplification depends on the specificity of the primers in which they do not recognize and bind to sequences other than the intended targeted DNA sequences.

So basically, primers are the main determinants of where DNA is amplified exactly.

Notice how both primers are needed to surround the region of the DNA region we want to be amplified (remember when we talked about flanking regions ☺), and having only one primer doesn't work, BOTH of them are needed to amplify the specific region.

Now, let's say that it is very common that the primers would go somewhere else on the DNA and bind or anneal to DNA of target, but how do we prevent this from happening? By choosing the right temperature and that's why the temperature of the annealing step of any PCR cycle is variable, it depends on the optimal temperature that specifically allows primers to bind to DNA.



How can we take advantage of that (unspecific binding at low temperatures)?

Simply, why do primers go somewhere else on the DNA? It is because there is what we call *homology* (similar sequences within the genome which are scattered and found in different places in the genome) but what causes this homology and creates those homologous sequences? Because sometimes we have gene families like for example we have histone genes (H2A and H2B) for example, as the DNA sequences of those histones are very similar to each other (high homology).

And that is why primers for H2A may in fact bind or anneal to a region within the gene H2B amplifying it as well.

So what we can do is that we can really **identify gene families** by using primers at low temperature of the annealing step allowing those primers to go somewhere else amplifying it and that helps us in the identification of genes within a family.

Recommended: go back and watch the animation video in the lecture.

Uses of PCR

1. **Discovery of gene families**
2. **Disease diagnosis**

➢ for example: deletion of a gene.
What we do is that we amplify the same gene in 2 people a person with a mutation (deletion of the gene) and a NORMAL person, the gene of the normal person will be amplified but the person with the deletion mutation will not be amplified or amplified but in a shorter length than the normal one (partial deletion).

3- **Paternity and criminal cases. Why?**

- An individual DNA profile is highly distinctive because many genetic loci are highly variable within a population.

➡ We amplify certain regions of DNA. Those regions could distinguish individual. Examples are VNTRs and STRs.

** we use PCR instead of RFLB to reduce time and effort.

Notice here that at low temperature for example we have number of products, if we increase the temperature slowly eventually, we would get this perfect temperature that allows primers to bind to DNA specifically.
Thus allowing a single product to be amplified.

4- **Viral and bacterial load**: the quantity of virus in a given volume. How?

- Quantitative PCR

****Remember** the specificity of the PCR is determined by its primers so in this case we design primer specific for viral or bacterial DNA.

➢ So if there's someone that we suspect he has bacterial infection :

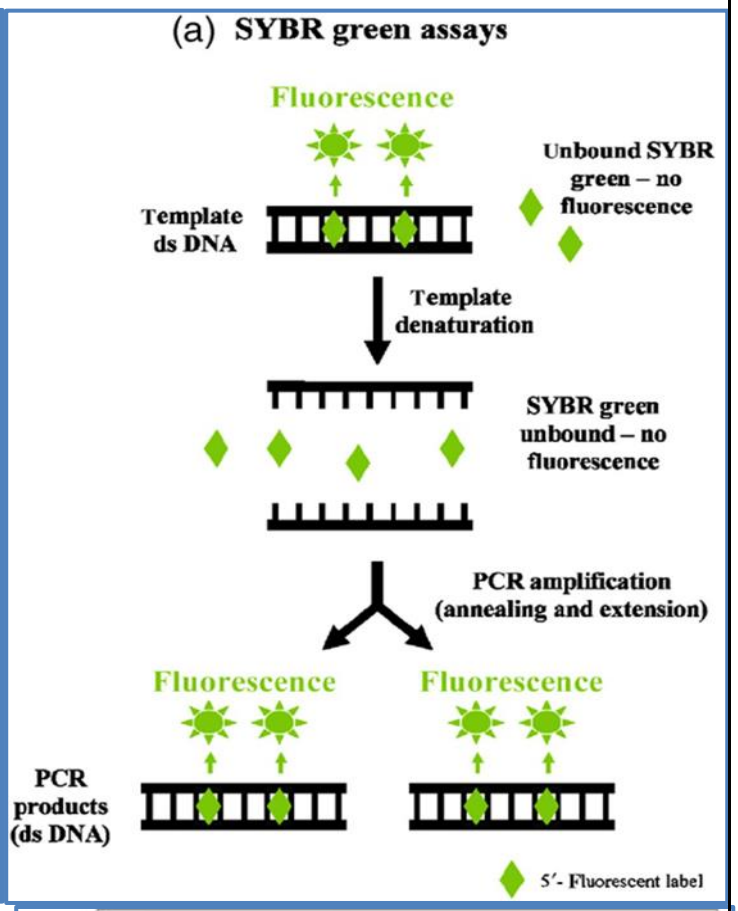
First: we extract a DNA from a blood sample from this person for example.
Then, we use specific primers for bacterial DNA so that only the bacterial DNA will be amplified and we can confirm whether the person has an infection or not, and recognizing the type of the bacteria (due to primer). This method is called Quantitative PCR.

Quantitative PCR (qPCR)

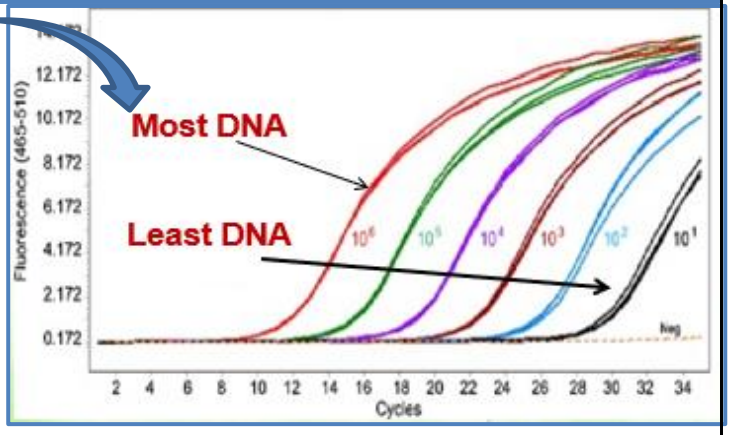
- **SYBR green binds to double-stranded DNA and fluoresces only when bound.**
- **A way of relative quantitation of amount of DNA in a sample is by amplifying it in the presence of SYBR green.**
- **The higher the amount of DNA, the sooner it is detected.**
- **** SYBR green: is a small molecule that can bind specifically to double stranded DNA and fluoresces.**

Remember the steps of PCR

1- **Denaturation: DNA is denatured into single-stranded molecules → NO double stranded DNA → NO fluorescence.**
2- **Reannealing: the primers anneal to the DNA → NO double stranded DNA → NO fluorescence.**
3- **DNA synthesis: after that double stranded DNA is synthesized → it fluoresce.**
**** DNA amplified (more cycles) the more SYBR green bound to double stranded DNA the more fluorescence detected.**



Some notes on the figure:
The Y axis shows the amount of fluorenes (signal) detected.
The X axis shows the no. of cycles of amplification.
Each color or each curve represent different **DNA sample**.



In the figure shown:

- ✓ **In cycle 2-10:** there's no signal detected that's because the amount pf DNA is really low the instrument to detect a signal so it eliminates it.
- ✓ **In cycle 12:** the instrument can detect a signal from SYBR green. As more cycles are proceeded as more fluorescence is detected.

- ✓ **In cycle 30-32**: no more signal detected due to 2 possible reasons 1- the enzyme got tired 2- consumption of nucleotides
    - Different sample get detected at different no. of cycle due to initial DNA template (The concentration of DNA template needed to initiate the PCR).

The lower The concentration of DNA template needed to initiate the PCR the slowest the signal gets detected (at higher no. of cycles).

Let's assume that we have 2 samples: a red sample and a green sample, and that the PCR instrument requires at least 10^4 DNA molecules for them to be detected.

	Initial amount	1st cycle	2nd cycle	3th cycle	4th cycle	5th cycle
Red sample	1000	2000	4000	8000	16000	32000
Green sample	100	200	400	800	1600	3200

And after the 5 cycles, a signal is detected from the red sample but no signal is detected from the green sample; because the red sample has higher concentration of initial DNA template, so the red sample requires less cycles to be detected than the green cycle. This is what we mean by viral load the amount of initial (viral) DNA template in a sample.

Good luck

SHORT QUIZ

TEST BANK

1. Polymerase used for PCR is extracted from _____

a) Escherichia coli
b) Homo sapiens
c) Thermus aquaticus
d) Saccharomyces cerevisiae

2. Primers used for the process of polymerase chain reaction are _____.

a) Single-stranded RNA oligonucleotide
b) Single-stranded DNA oligonucleotide
c) Double-stranded RNA oligonucleotide
d) Single-stranded DNA oligonucleotide

3. What is the process of binding of primer to the denatured strand called?

(a) Annealing
(b) Renaturation
(c) Denaturation
(d) None of the above

4. Which of the following is a mismatch?

a) Polymerase – Taq polymerase
b) Template – double stranded DNA
c) Primer – oligonucleotide
d) Synthesis – 5’ to 3’ direction

5. Which of the statement hold true for Quantitative PCR?

a) A fluorescent dye is used which binds on single stranded DNA molecules
b) SYBR green is an example such type of dye
c) The quantity of DNA is simply measured by measuring the amount of fluorescence
d) This approach is useful if the products are non-specific in nature

6. How many DNA duplex is obtained from one DNA after 4 cycles of PCR ?

a) 64
b) 32
c) 256
d) 128
e) 16

ANSWERS

Q1	Q2	Q3	Q4	Q5	Q6
C	B	A	B	C	E