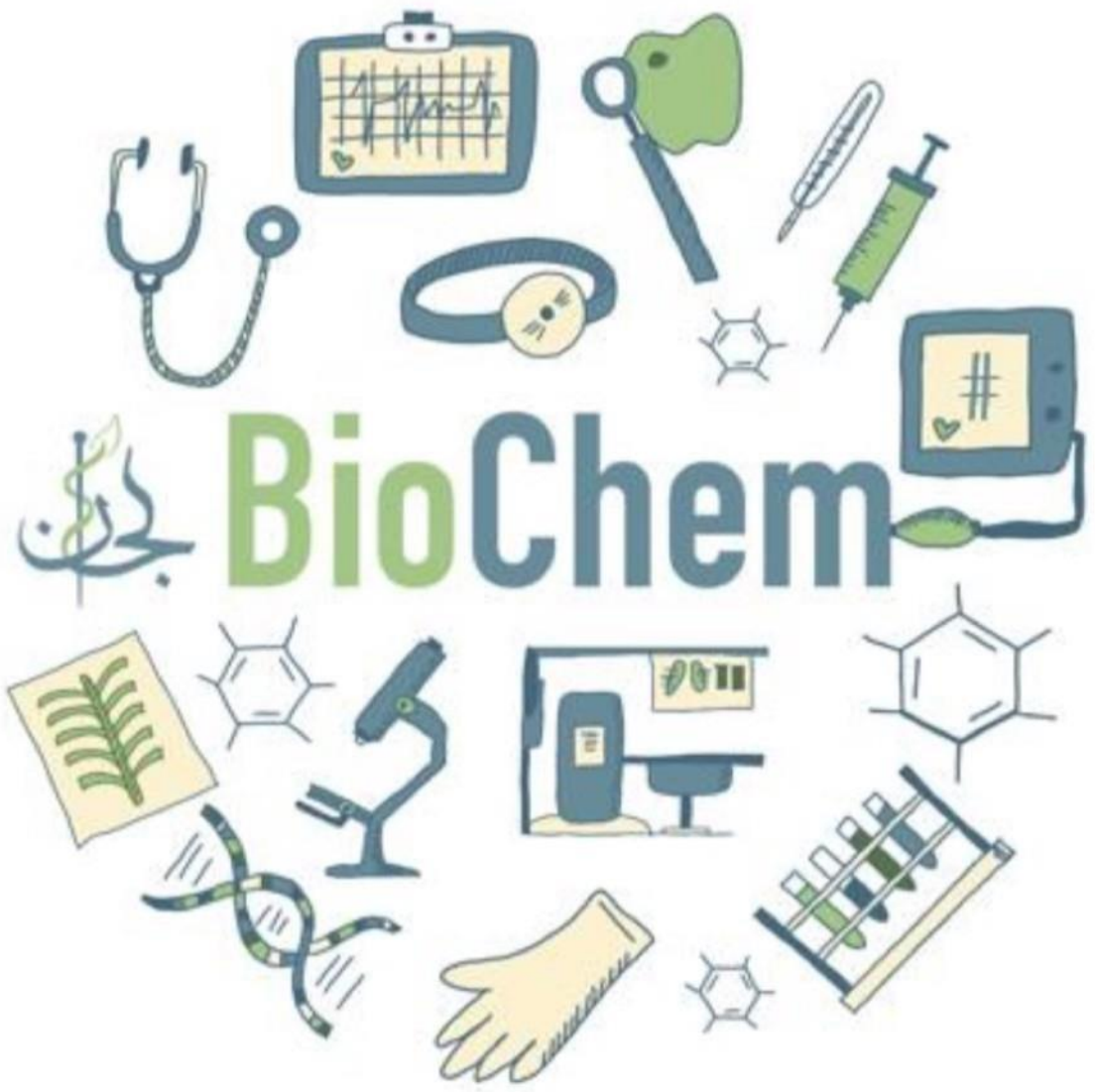

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Enzyme-based molecular techniques

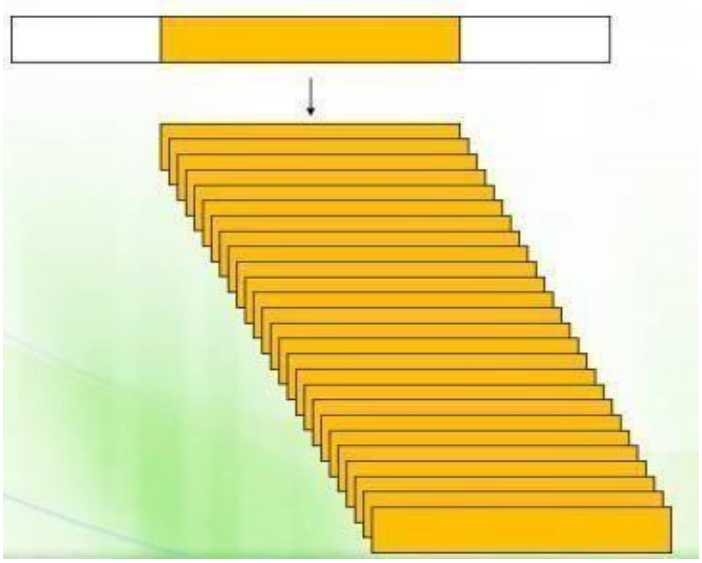
Polymerase chain reaction (PCR):

what is polymerase chain reaction?

it is a chemical reaction that occurs in cycles over & over (chain), and it is catalyzed by DNA polymerase enzyme .

Polymerase chain reaction (PCR) allows the DNA from a selected regionof a genome to be amplified a billionfold, effectively "purifying" this DNA away from the remainder of the genome.

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



PCR is like cloning but faster(as no need to prepare plasmid) and more specific & sensitive (as specific regions are detected and amplified)

What are the components of PCR reaction?

1-**DNA template** to start with.

2-**A pair of DNA primers**, to initiate the DNA synthesis(as DNA polymerase needs a primer) it is DNA primer not RNA primer, the primer is[15-25] nucleotide-long primers should surround the target sequence.

3-**substrate**: All four deoxyribonucleoside triphosphates.

4-**enzyme**: A heat-stable DNA polymerase.

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

The PCR cycles

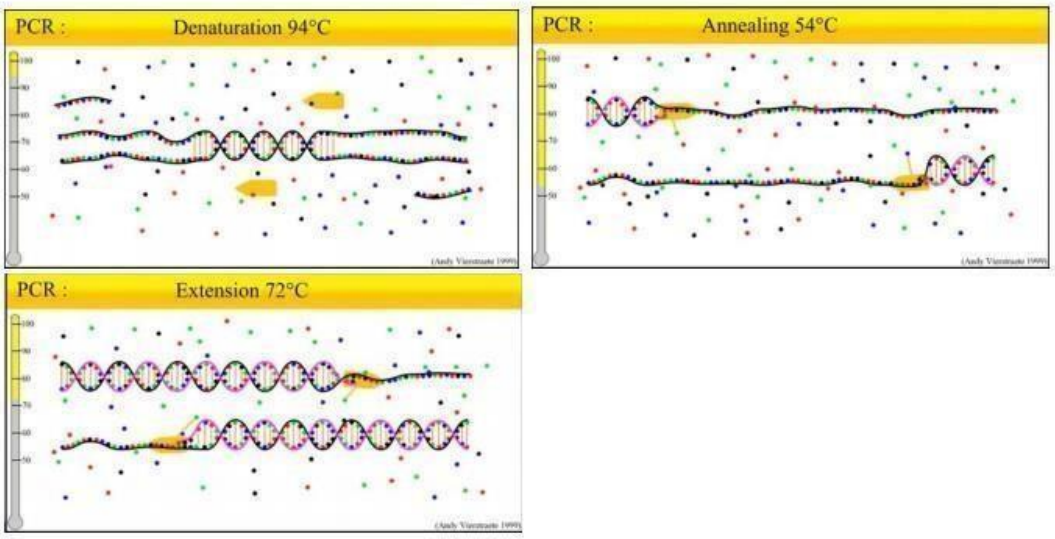
The idea here that the PCR reaction is made of a number of cycles that arebetween 25 to 30 cycles in which each cycle is composed of three steps :

Denaturation (at 95C): DNA is denatured into single-stranded molecules (separate2 strand from each other).

Reannealing (50C to 70C): the primers anneal (bind) to the DNA, temperature goesdown in this step allowing primer to bind DNA template .

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

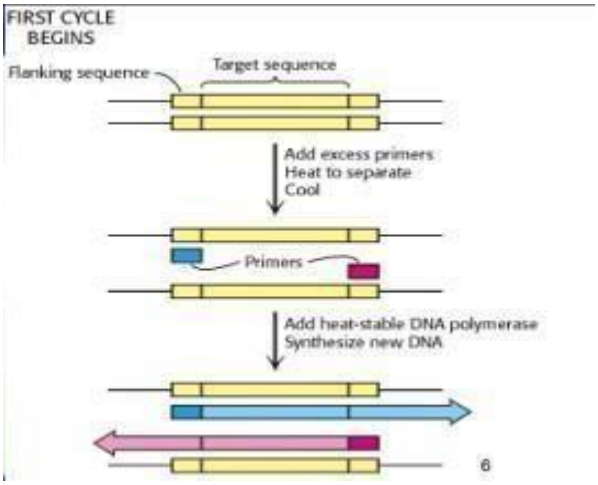
The reannealing temperature varies due to many factors ,that affects hydrogen bonds between primer and DNA fragments (primer binding), such as length of primer, CG pairs, salt conc..... (factors that affect melting temperature)



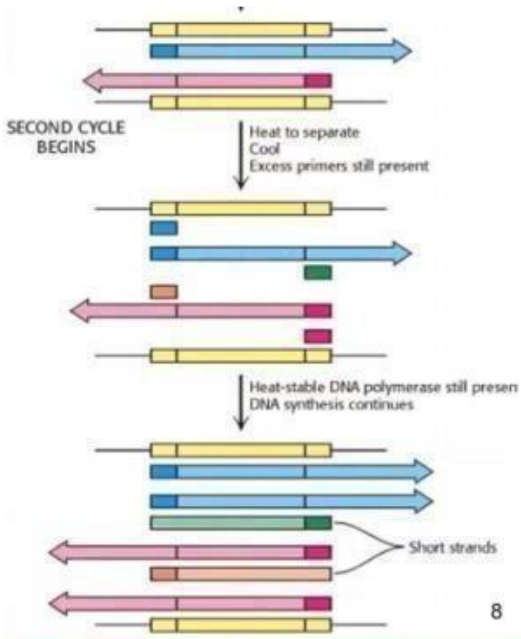
The DNA polymerase Suitably heat-stable DNA polymerases have been obtainedfrom microorganisms whose natural habitat is hot springs. The DNA polymerase that is to be used, which is called Taq DNA polymerase, is a special one obtained from a thermophilic bacteria known as Thermus aquaticus, and is thermostable up to 95C. Note: The optimal temperature for this enzyme is 72 C.

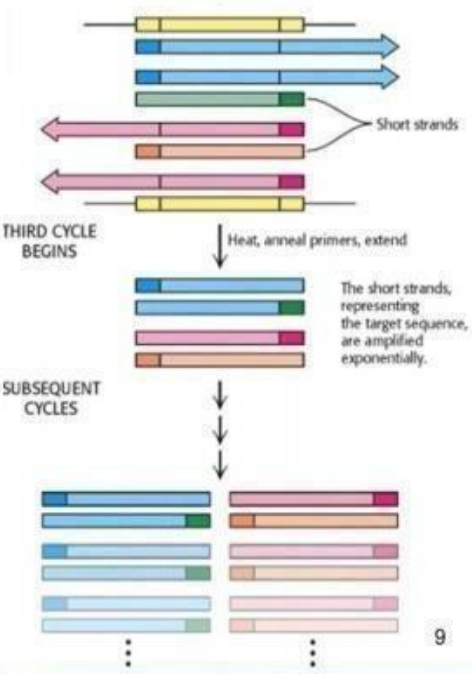
Human enzymes wouldn't survive at that temperature (up to 95°), it would be denatured and non-functioning, but this enzyme can stay stable at a temperature up to 95° c.

Note: Aqua means water, Thermus refers to temperature.



So again, in each cycle, DNA is denatured (at a temperature of 95°) and 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 just one molecule of DNA, we have many molecules here with a lot of primers). These primers would anneal (or hybridize) with the DNA template at 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.

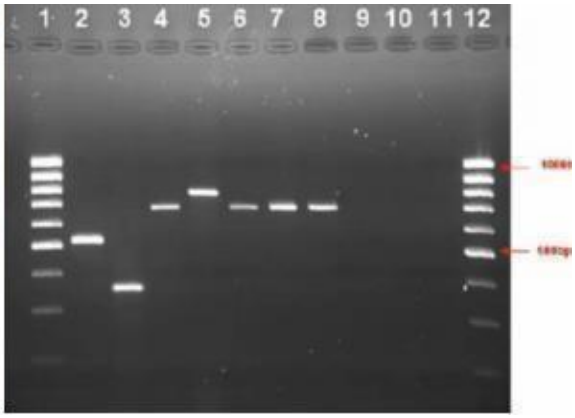
Notice that in the first three cycles all DNA is amplified (replicated by DNA polymerase). At the end of the third cycle, the wanted region is separated from the whole DNA. Thus, after the third cycle the wanted region is amplified significantly. (Make sure to watch the extra videos given by the doctor to understand this point)

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

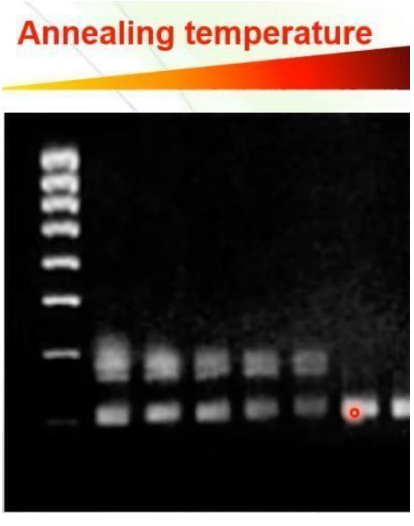
This is the instrument that used for the PCR reaction. Modern instruments take an hour to finish all 25 cycle, old instruments would take 18 hours. The efficiency of PCR instruments depends on how fast temperature are changing (not how fast reactions take place because reactions would happen in seconds.

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 foreach numbered sample only one DNA band is in its lane. So, DNA amplification is very specific. (The template DNA will not appear, just the required DNA, because template is not amplified a lot (only in the first 3 cycles). However, primers may appear.



Importance of primers :The specificity of amplification depends on the specificity of the primers to 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 and having only one primer that anneal will not be enough (both of them are needed to amplify the specific region). However, conditions like low temperature or using a common primer will cause primer to anneal to more than different DNA, and more than one DNA fragment is amplified (**imperfect hybridization**)



At lower temperature , more than one DNA fragment appear, this is because primer anneal to more than one DNA. As temperature increases, primer anneal to a specific DNA . However, if temperature kept on increasing , both fragments will not appear because primer will not anneal at higher temperatures .

How can we prevent this? By choosing the right temperature, salt concentration…, which will make the primer specific to a certain DNA fragment.

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). These Similarities may mean that both DNAs are in the same gene family, for example histone genes (H2A and H2B) 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 geneH2B 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 helps us in the identification of genes within a family.

Uses of PCR

1. Discovery of gene families

2. Disease diagnosis , for example: let say we are try to amplify gene in normal individual we will get a signal and we will get an amplicon (amplified DNA region) but if we want to amplify the same gene in a patient where this gene has mutation (deletion) we will not be able amplify this gene or amplicon will be shorter more than normal because part of it is deleted .

Paternity and criminal cases. How? Certain genes are able to distinguish humans (Recall human genome lecture from last semester, that there are individual differences in number of repeats of VNTRs and STRs,) from others, we try to identify these genes in individuals by trying to amplify them, if they are present a signal will be produced.

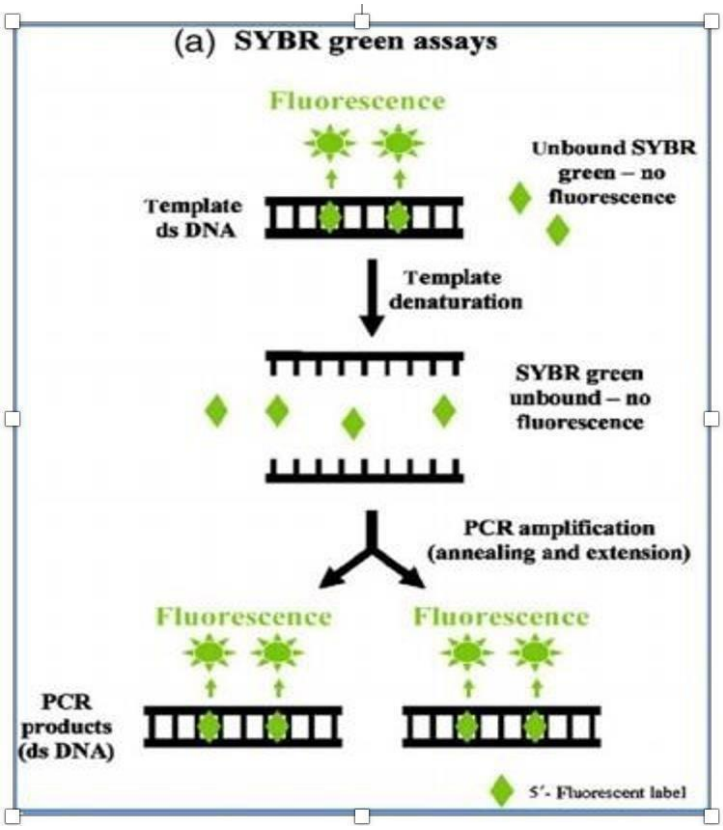
** we use PCR instead of RFLP to reduce time and effort.

3- determine Viral and bacterial load: the quantity of virus in a given volume. This is done using 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.

Before discussing Quantitative PCR, we can use PCR to identify if a person is infected with a certain virus or bacteria.

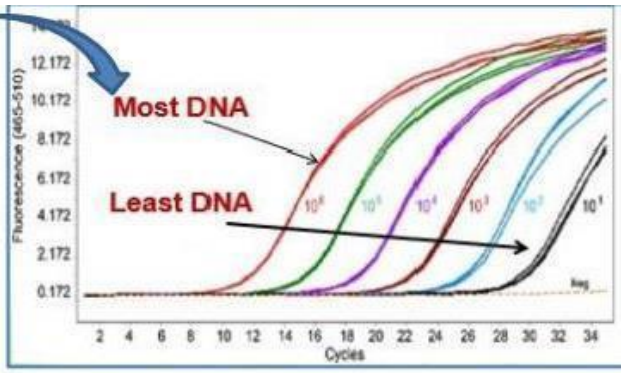
First: we extract a DNA from a blood sample from this person. Then, we use specific primers for bacterial/viral DNA so that only the bacterial/viral DNA will be amplified and we can confirm whether the person has an infection or not, and recognizing the type of the bacteria (due to primer because it is specialized for certain type of bacteria)

Quantitative PCR (qPCR)



- Quantitative PCR uses SYPR green
- 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 and measuring fluorescence
- However, the limitation in detecting instrument will prevent it from detecting any fluorescence until fluorescence levels reaches a specific limit. THUS:
- The higher the amount of DNA, the sooner it is detected (less cycles) .

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.

THE END