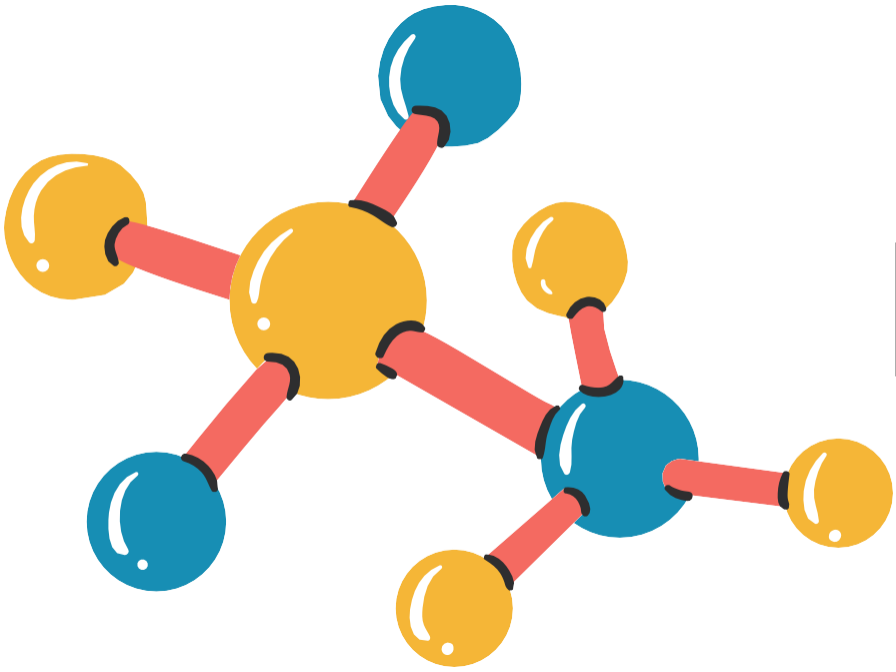
Sheet no.22

Biochemistry



Slides
Doctor`s notes
Writer's notes

Summer 2022

Writer: Noor Abu Hantash

Corrector: Correct team

Doctor : Mamoun Ahram

Enzyme-based molecular techniques (part II)

Polymerase chain reaction (PCR)

We have listened about PCR a lot during past three years according to covid 19 polymeric.

PCR has revolutionized molecular biology, molecular medicine and also molecular archaeology

It has done tremendous improvement in analysing DNA.

Challenges in research and medicine

There are may challenges were faced in studying DNA:

1)Genetic variations:

Existence of short tandem repeats(STR), variable number of tandem repeats(VNTR) also

existence of variations: SNP(single nucleotide polymorphism) and mutations.

-how could we identify these things? How could we take advantages of genetic variations?

2)minute (small) amounts of genetic material

Like the small amount of DNA found in Dinosaurs ,ancient humans, hair in crime

scene& blood spot…

3)identification of organisms :

Identifying existing known or novel infectious agents

such as(SARS COV 2: infectious novel coronavirus)

Polymerase Chain Reaction

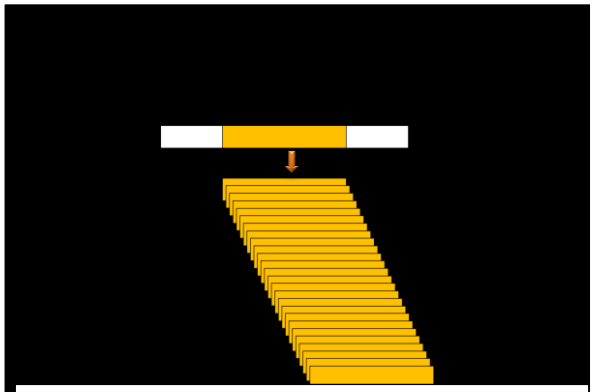
-The invention of PCR has revolutionized everything related to nucleic acid in general containing DNA.

*this technology was invented by Kary Mullis in 1980s, and he got Noble prize for this.

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

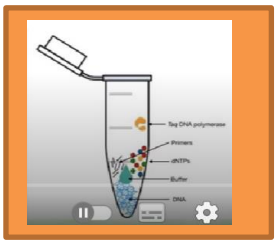
*the point of this technique is to allow the amplification of The DNA either the whole DNA or a piece of the region. It can amplify billion folds.

*it is extremely sensitive ; it can detect a single DNA molecule in a sample

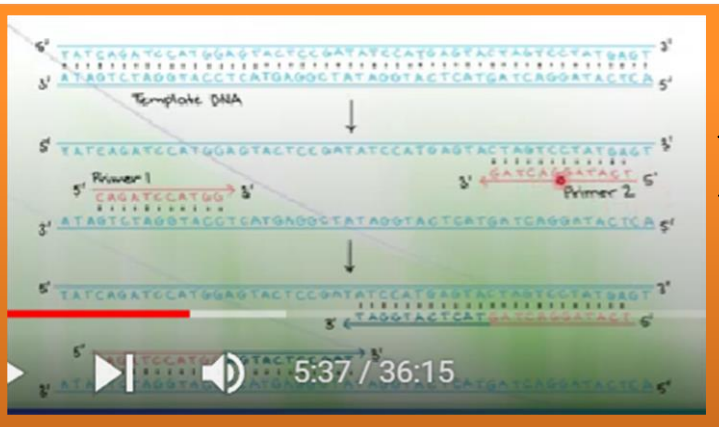


A linear or circular DNA can be amplified
But the final products are linear pieces!

Component of PCR reaction



*PCR is a biochemical enzymatic reaction.
*it is a chain (consecutive) rxn :متسلسل
Means it goes the same rxn repeatedly for several times.
*the components:
1) A heat stable DNA pol:
As it is enzymatic rxn, it needs a special enzyme
2)All four deoxyribonucleoside triphosphate:
(d A, d G, d C, d T): they are the substrates for the enzyme (DNA pol)
3) DNA template:circular or linear)
4) A pair of DNA primers:
The 15-25 nucleotides-long primers should surround the target sequence.
*the DNA pol needs a point to start the synthesis so it needs a pair of primers, we call them (primer 1 & primer 2) or (forward & reverse primers).



The synthesis has directionality 5` to 3`

We want to amplify this region of DNA, so the pair of primers surround the region.
*the DNA is antiparallel

Primer1: the forward primer
Primer2: the reverse primer

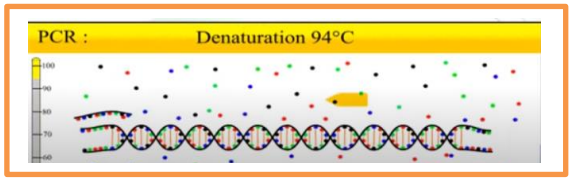
The newly synthesized DNA has 2 strands (the parent, old strand) and the newly synthesized strand.

The PCR cycles

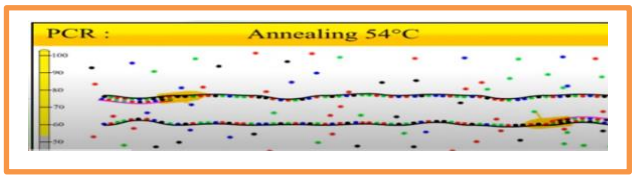
**each PCR reaction goes into cycles, these cycles are repeated (cycle1,2,3,4…)

*each cycle consists of 3 steps, phases:

1) Denaturation (at 95°C):DNA is denatured into single- stranded molecules, the 2 strands separate from each other.

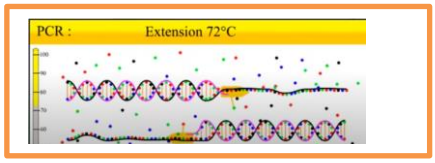


2)annealing (50°C to 70°C): the primers anneal to the DNA.The forward and reverse primers surround the amplified region, and they bind specifically to their strands.



**the annealing occurs at 50 to 60 C and it may reach to 70C depending on the primers and their annealing.

3) Polymerization or extension or DNA synthesis (at 72°C): optimal for the DNA polymerase to start synthesis.



Denaturation(95 C)→ annealing (50-70 C)→ DNA synthesis (72 C)

The DNA polymerase

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

-For example, the widely used Taq DNA polymerase is obtained from a thermophilic bacterium, Thermus aquaticus, and is thermostable up to 95°C.

*according to the temp. needed in the cycle, we notice that they are really high temp. (95, 70 C,…), and they cant be handled by human DNA pol, so we have to use special DNA pol that is isolated from bacteria known as 'Thermus Aquaticus'(Thermus stands for thermometer and aqua stands for water), so it is isolated from hot spring in the USA in Williston park.

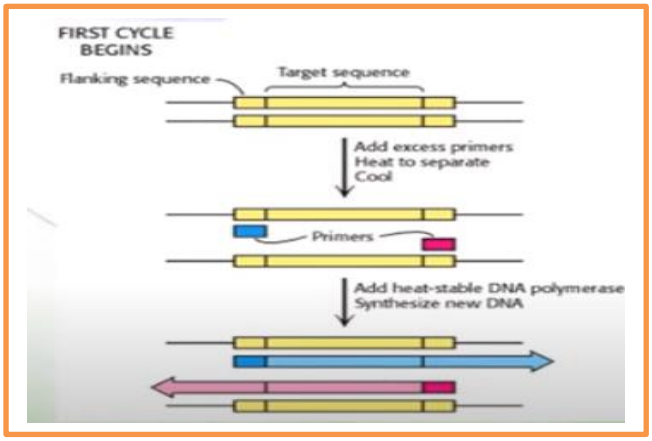
*Also, we can isolate bacteria from Main spring in Jordan

*the doctor then starts promoting tourism in Jordan! :)

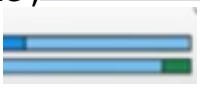
So we use DNA pol from this bacteria and it is called (Taq DNA pol) Taq :thermal aquaticus

PCR mechanism

1st cycle:

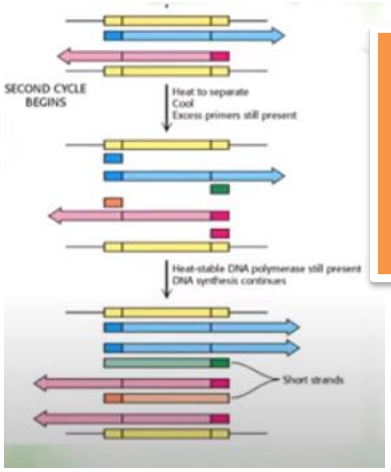


Denaturation starts by separating the 2 strands, then reverse and forward primers anneal(sit, hybridize) to the complementary sites and Taq pol start synthesis

*notice: the product that we gain doesn't the needed one ,  they are 

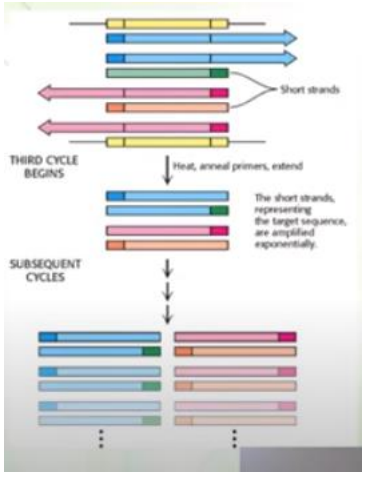
extending to right and left, but we need the short ones.

2nd cycle



The same steps of the 1st cycle, starting with 4 templates, so gaining 8 strands.
And again!They are not our products.

3rd cycle



Here we get our products, then they are amplified in many cycles*-*

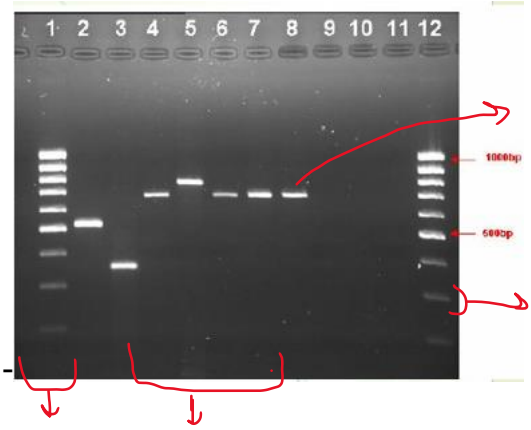
PCR cycles

* 20-30 cycles of reaction are required for DNA amplification.
the products of each cycle serving 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(amplicons or amplified DNA) derived from each starting molecule
2^n : (n: the number of cycle)
2^30=250 million


this is the instrument for PCR, where temperature can change Dramatically
-changing the temperature is what really takes time
* while the steps of cycle happen in few minutes.

Detection of DNA fragments

This DNA fragment can be easily visualized as a discrete band of a specific size by agarose gel electrophoresis.
-at the end of the rxn, the sample is analyzed by gel electrophoresis



One band with multiple fragments.

These shade bands may be the primers alone

-

The Standard Marker (Indicates The sizes)

the amplified sample from PCR, it shows that we can specifically amplify Certain region

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 target DNA sequences.
How can you prevent it?
How can you take advantage of it?

The annealing temp. has a great importance, it determines the amount of primers anneal to the complementary region.

Let`s remember some memories from Molecular course

*Melting temp: Is the temp where 50% of DNA are double stranded and 50% are single stranded.

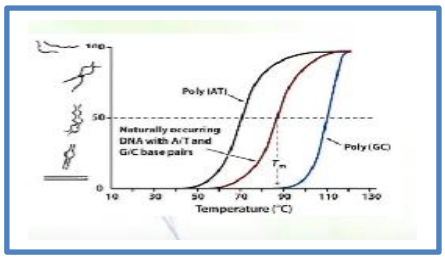
*the highest the temp the more denaturized DNA (more single strands)(more hydrogen bonds are broken)

* the high temp makes hybridization between the primer and the strand more specific, in other word if the temp is lower then the non specific annealing can occur.

*Some factors that affect melting temp:

1)ions and salts: they mask the -ve charge, making the DNA more stable thus the highest melting temp.

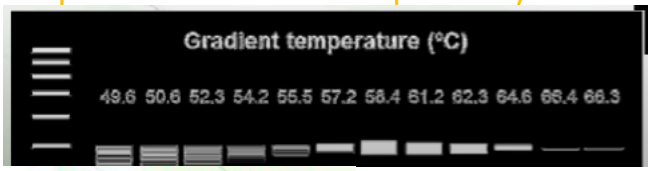
2)the composition of DNA: if it is rich with GC then the temp is higher cause there are 3 hydrogen bonds instead of 2.

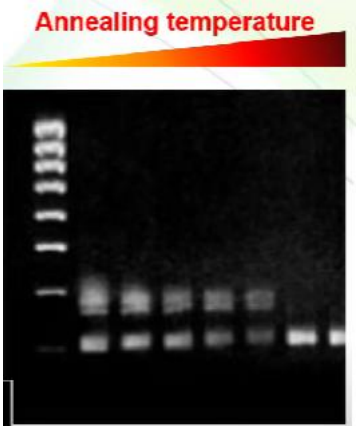


Of course you are wondering: What is the relation between what was mentioned and the annealing temp:

There are regions in genome that are homologues(have the same sequence) so the non specific hybridization could take place.So,the highest temp allows less non specific hybridization or annealing, if we increase the temp we could identify more specific type, for clarification:

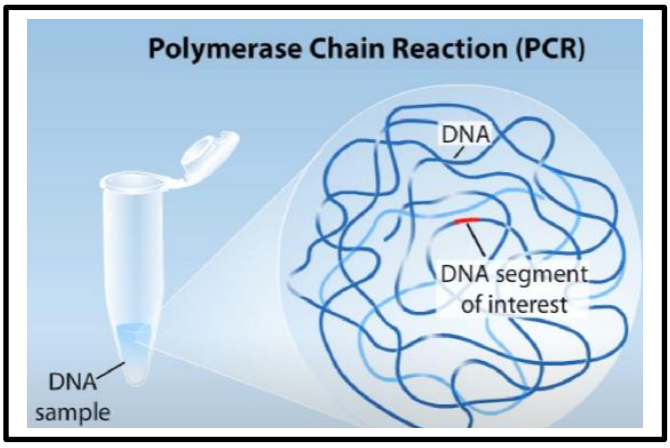
There are many types of histones like H2A and H2B and they are similar to each other so the primer could bind non specifically to them at low temp, see the figure bellow:



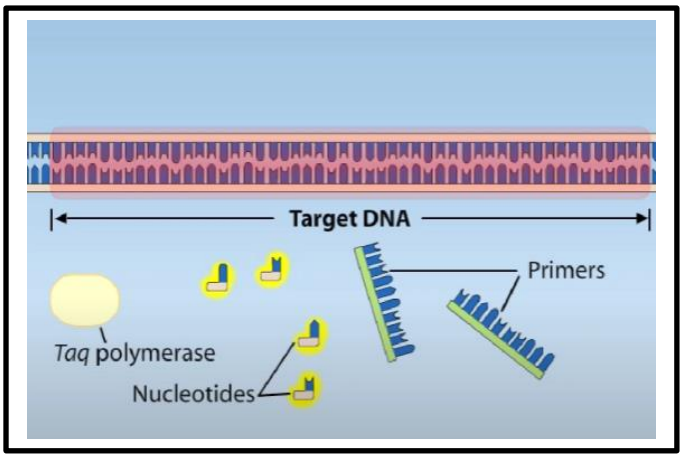


Lower temp→more non specific annealing
Higher temp→ more specific amplification would take place
For eg at 49.6 the thickness of the bind was bigger cause imperfect hybridization has high opportunity to happen
At 66.3 C:higher temp so less imperfect hybridization (more specific binding)

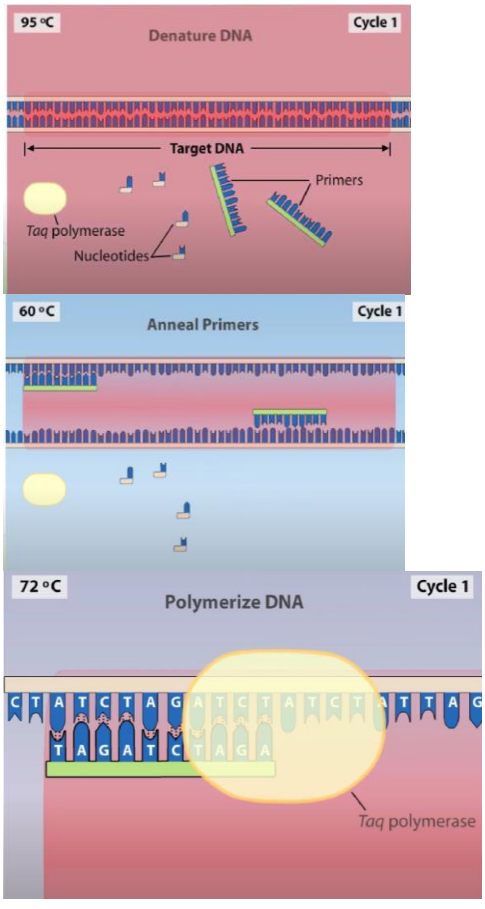
From min 16 to 21 in the video, there is an animation video, try to watch it!
I will write the main ideas in it (بس عشان المعزّة وإلا المفروض اتشوفوه لحالكم)



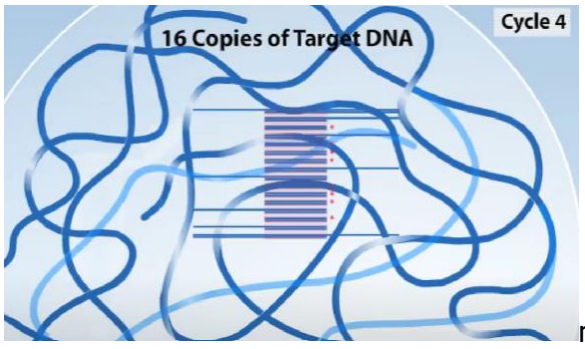
Here is the sample



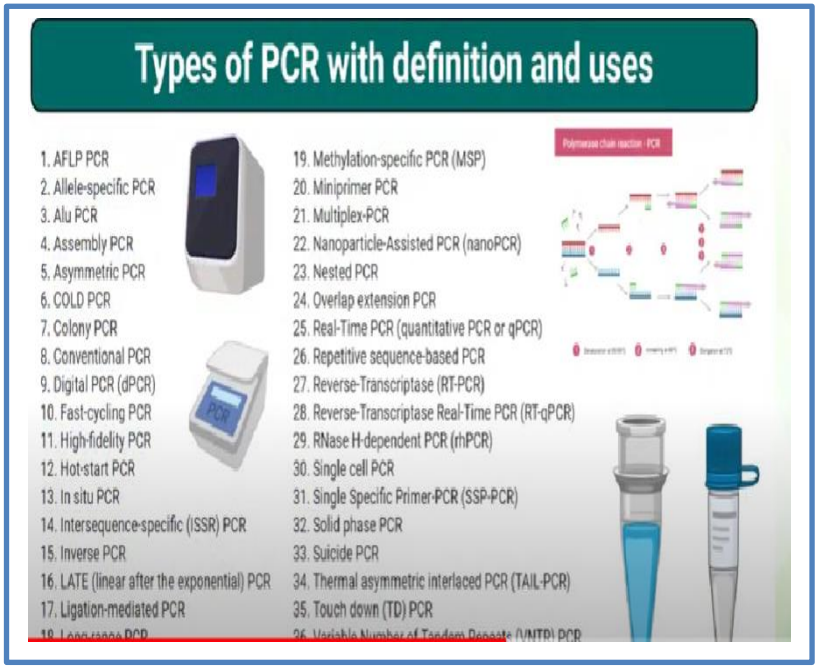
The components

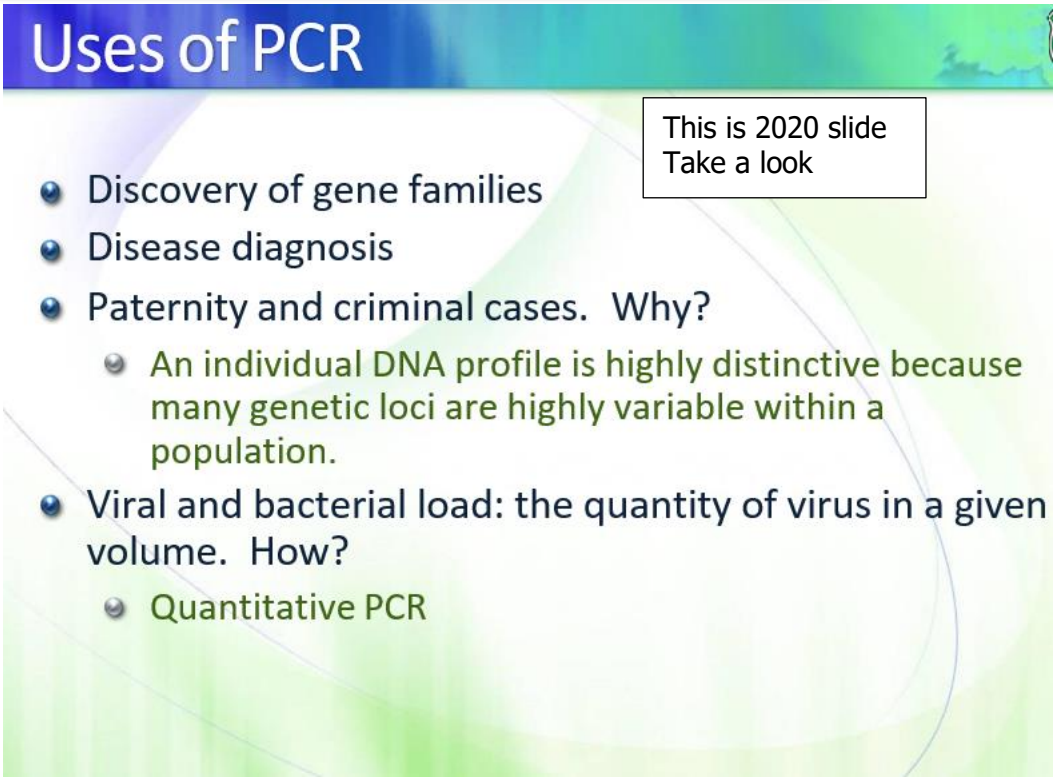


The 3 steps take place
1)denaturation at 95
2)annealing according to base pair rules at 50-70
3)extension at 72



many cycles happen, amplifying DNA regions to many copies





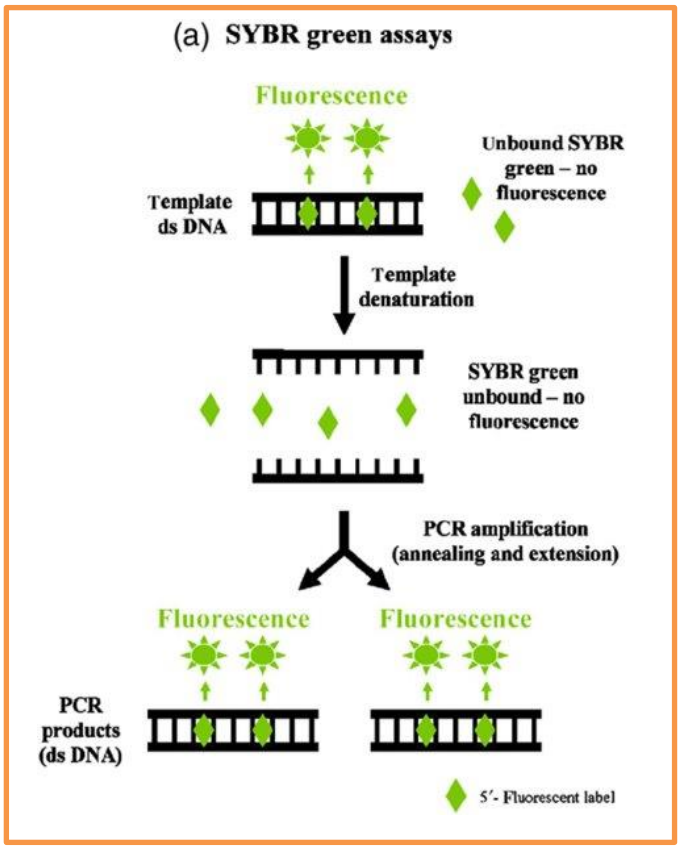
Real-time quantitative PCR (qPCR)

One of the usage in PCR is the qPCR:
It stands on the same mechanisms of PCR with a small addition which is using SYBR green: it is a florescent chemical that is added to the denaturized strands in PCR to fluoresce,(it fluoresces only when binding to DNA, but if it doesn't anneal it isn't fluorescing).

-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 advantage of SYBR is to detect the amount(quantity) of the amplified DNA
*the more double stranded DNA, the stronger the florescence (it is detected by a special instrument).
* For clarification: if we take 2 samples of 2 individuals, the first has a viral infection, while the second is normal, then we isolate the DNA from the sample and amplify it using

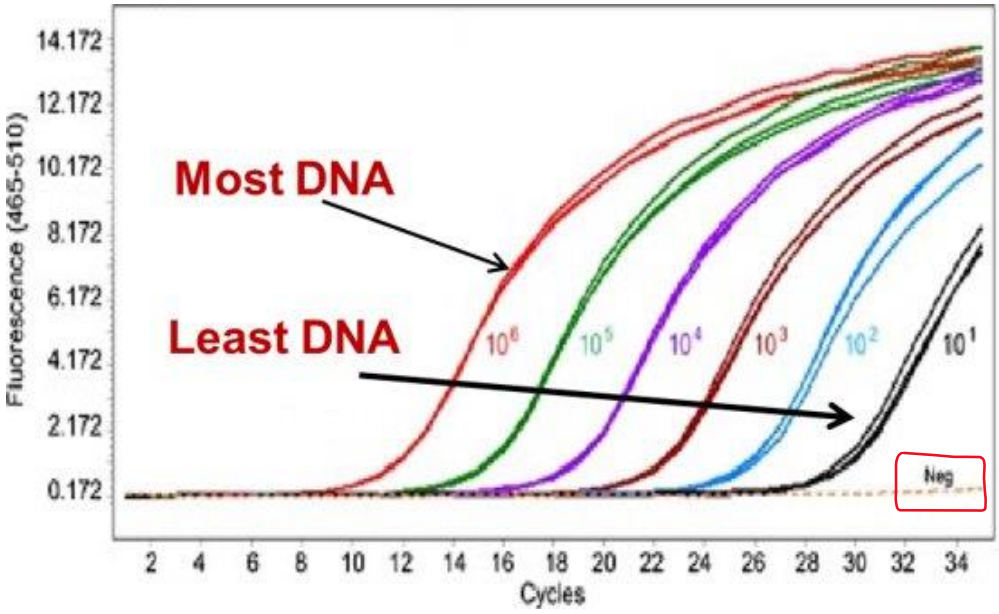
specific primers for the viral DNA and adding SYBR, the sample of the infectious person will give higher signal than the normal one.



The DNA denatures
The SYBR green anneals to the strands
It detects the quantity of the amplified DNA

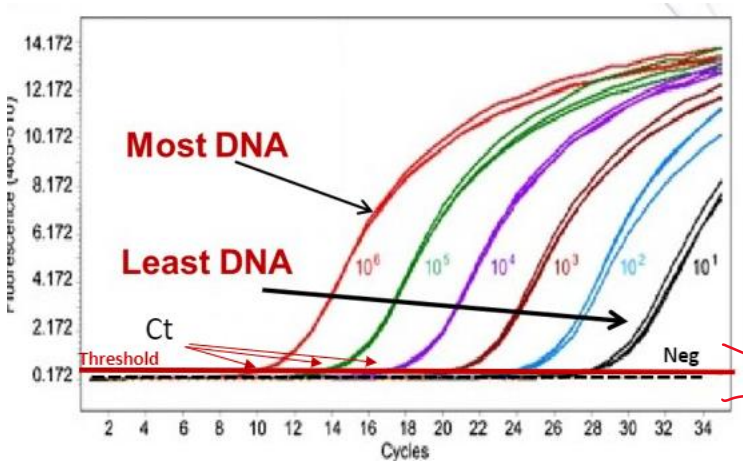
*the more cycles of PCR happens, the more annealed PCR, so the bigger detected signal

-The higher the amount of DNA, the sooner it is detected.
*see the curve bellow:



The x-axis indicates the number of cycles
The Y-axis indicates the quantity of florescence
-lets deal that the more DNA you add in the PCR, the earlier detection you get
So the 10^6 sample was able to be detected at 10th cycle(why not earlier? Cause it is the limit, to get an adequate amount)
While the 10^5 at the 14th cycle
The 10^4 at the 17th cycle
**the conclusion: the lower the starting material(DNA), the later the signaling appears.

- Notice there is a horizontal line (Neg), it means that no signalling is detected, so if it is a viral DNA, the person is normal.



Threshold cycle (Ct) tells us at which cycle the signal is detected and is a measure of starting amount of DNA.

We add threshold line above the Neg line
And it tells us where the detection appears!
It is also called (CT)

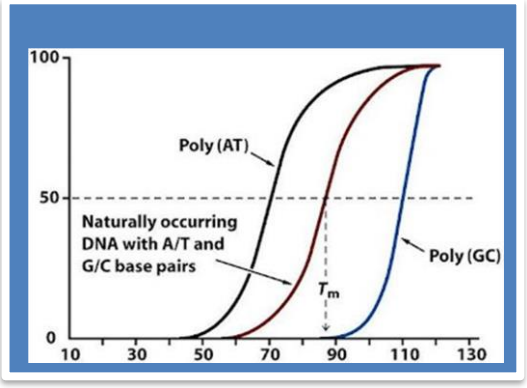
Melting curve analysis of qPCR

SYBR green isn't this specific and it is limited! You wont know if the target DNA that was amplified. You hope that it is your DNA, but you are not sure! The reason is that SYBR green anneal to dsDNA, it cant differentiate if the specific or nonspecific amplification happens.

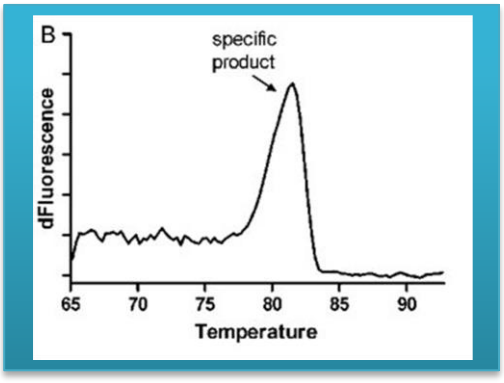
The question is: How can we make sure that our DNA that is amplified?

Either by gel electrophoresis, then we can see where the single band is expected to be

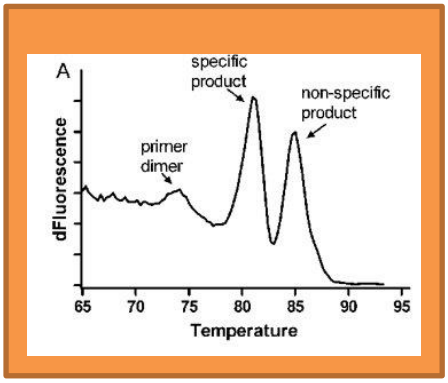
OR by our topic: the PCR instrument does the 'melting curve analysis', depending on melting point(the point where 50% of DNA is denatured).



*Melting curve tells us: the change in fluorescence observed when double-stranded DNA (dsDNA) with incorporated dye molecules (SYBR green) dissociates, or "melts" into single-stranded DNA (ssDNA) as the temperature of the reaction is raised.



After detecting the change in florescence
At the end the temp is increased,
obtaining single stranded DNA(ssDNA),
So if the curve has a single peak it means
That it is a single product.
(specific amplification)



If the curve has 2 or more peaks it indicates that there are 2 or more products (specific and non specific), we can differentiate between them using melting temp, cause they have different content of GC, so they would have different melting temp.

Taqman qPCR

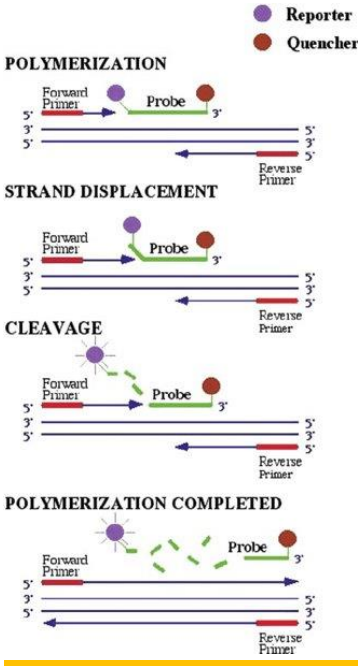
Another method that is more specific, sensitive and reproducible than SYBR green

In this technique: in addition of using primers, we also use probes characterized by:

1) Specific: it targets only what we want to amplify.
2) Each probe has:
   a)reporter: it gives florescence
   b) quencher: a suppressor of the reporter(supresses the florescence of the reporter provided that it is close to it)
   (if the quencher is separated from the reporter, then the reporter will emit florescence.

Close= no florescence

Separated= florescence



We use a special taq polymerase that has 5`→3`
Exonuclease activity in addition to polymerase activity, as the pol extends and synthesises the DNA, once it reaches the prob, it activates the exonuclease activity starting cleaving the phosphodiester bond between the nucleotide of the prob, as a result the reporter is released getting away from the quencher, so the reporter fluoresces
*Having a lot of probes as a result of the repeating cycles means more degraded probe
By Taq pol and more emitted signals.

- Advantages (versus SYBR chemistry)

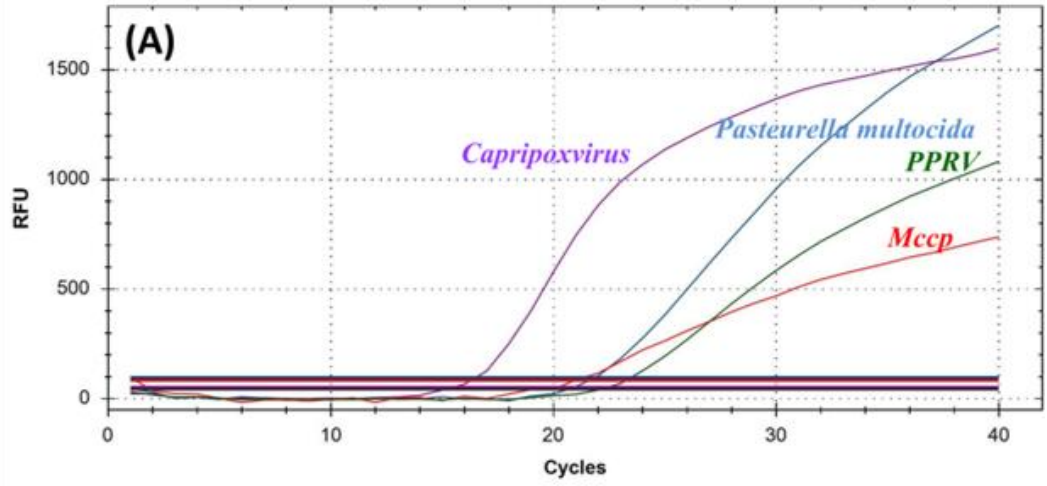
1)More specific:probe is specific to the target DNA that has amplified, the probe wont bind to another amplified DNA and wont emit a signal.

2)More sensitive : the signals that are emitted by the reporter are stronger than those mitted by SYBR.

3)More reproducible:getting the same result over and over again, Not only the primers are specific to the target DNA we want to amplify, but also the probe is specific!

4)Multiplexing: you can amplify different targets, using different sits of primers in the same exact rxn,

For eg: you have a tube contains : same template, Taq pol, same deoxyribonucleotides, **different** sits of primers that can amplify different regions in the template and **different** probes that can bind to different amplified DNA, each probe has different reporters emitting different signals(red, blue, yellow, green…)so you can amplify different regions in same tube in one rxn!



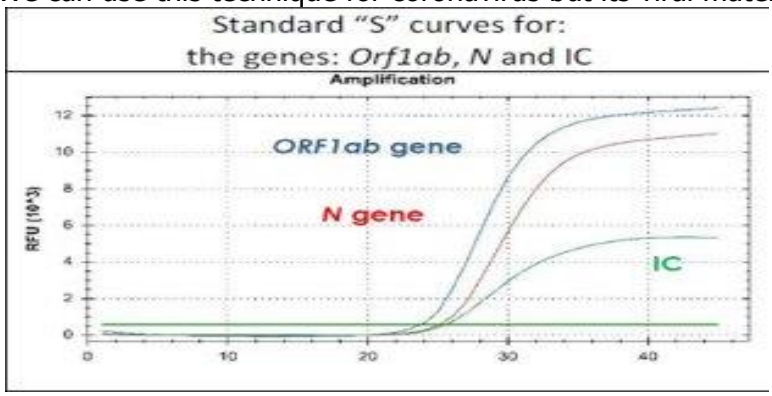
In the digram above,

We have different signals emitted from different bacterial species, it confirms that taqman qPCR is multiplexing.

If you were a geological microbiologist and you found a sample of sand with different bacterial species, you want to detect these different types, so you would have to use different primers and probes each one is spesific for a type of bacteria, at the end you would have amplification for 4 different regions, 4 species, 4 genomic DNA molecules

Also you can do quantification (signals are emitted at different CT(threshold cycles), knowing how much bacteria is in this sample. 😊

We can use this technique for coronavirus but its viral material is RNA not DNA.



We can amplify different regions of SARS COV2 (The doctor says it is a coracoid, coracod DNA or sth else I really didn't get the word, I am sorry) in a single sample to confirm that this sample contains the SARS.

Such a multiplexing, beautiful technology:)

PAST PAPERS:

Answer: A

4) the variation of the annealing temprature of PCR allows for :

A. better selectivity of amplified regions of DNA
B. amplifying GC- rich or AT- rich DNA sequences
C. synthesis of amplicons of certain lengths
D. activation of the taq polymerase
E. controlling speed of PCR reaction

Answer: A

13) Why cannot we detect any signal in the first few cycles of quantitative PCR ?

A. SYBR green is not yet activated
B. The taq polymerase is not active
C. Limitation in the sensitivity of the instrument
D. There is no amplification taking place
E. The proper size of the amplicon has not been reached

Answer: C

19) What is SYBR green ?

A. It is a molecule that terminate DNA synthesis in sequencing reaction
B. It is a molecule that activates and stabilize DNA polymerase
C. it is a molecule that binds to double-stranded DNA and fluoreces
D. it is a molecule that activates and stabilize DNA
E. it is a molecule that tags proteins

Answer : C

24) Taq polymerase is specifically used in PCR due to its

A. accuracy
B. High efficiency
C. Low price
D. Availability
E. Stability at high temperature

Answer : E