Doctor 022

Biochemistry

Sheet no. 4

POLYMERASE CHAIN REACTION (PCR)

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POLYMERASE CHAIN REACTION (PCR)

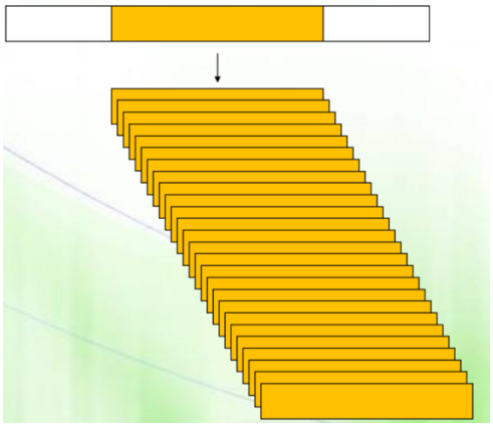
➢ CHALLENGES IN RESEARCH AND MEDICINE

- Genetic variation
  - STR (short tandem repeats), VNTR (variable number of tandem repeats), SNPs (single nucleotide polymorphism), and mutations. the presence of these things was a challenge on its own, how can we identify them?
- Minute amounts of genetic material. how can we handle them?
  - Dinosaurs and early humans (e.g., old bones, a hair in crime scene).
- Identification of organisms (e.g., infectious agents such as SARS-CoV-2 (corona virus))

➢ POLYMERASE CHAIN REACTION

PCR: which stands for polymerase chain reaction, is a repetitive biochemical enzymatic reaction that utilizes DNA polymerase to synthesize DNA. It is a consecutive or chain reaction, meaning that the reaction is repeated multiple times.

- 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.
- It is extremely sensitive; it can detect a single DNA molecule in a sample.

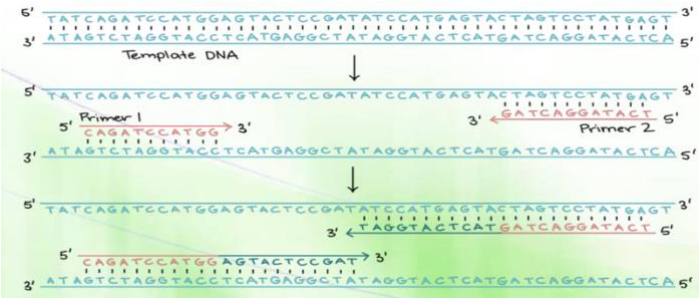


Side note: The piece of DNA that needs to be amplified, can be linear or circular, but the final product is linear DNA.

➢ COMPONENTS OF PCR REACTION

- The DNA templates (circular or linear). The DNA sample that I want to amplify
- A pair of DNA primers

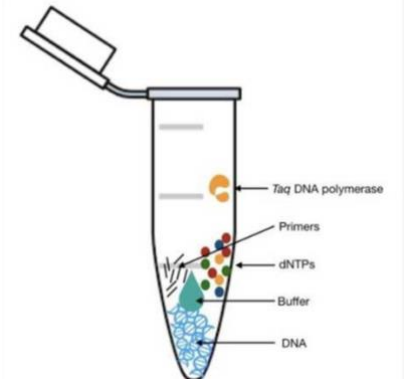
DNA primers are short nucleotide sequences that serve as starting points for DNA synthesis during PCR. They bind to the target DNA sequence and provide a template for DNA polymerase to initiate DNA replication.



We need 2 DNA primers:

- **Forward (1) primer: 5' to 3'.**
- **Reverse (2) primer: 3' to 5'.**

- **The 15 – 25 nucleotides-long primers should surround the target sequence.**
- **The newly synthesized DNA contains the old (parental) strand and the newly synthesized strand.**
- **All four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP) (tri because we need energy).**
- **A heat-stable DNA polymerase**



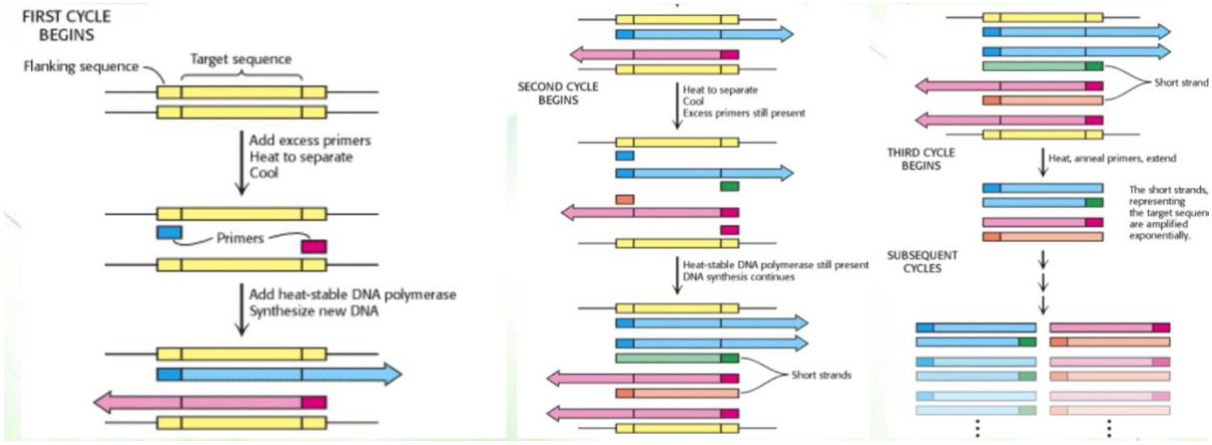
➢ THE DNA POLYMERASE

- **We deal here with high temperatures which can't be handled by normal DNA polymerase, so we use a special 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.**

➢ THE PCR CYCLES

Each PCR reaction has cycles that are repeated, each cycle contains:

- **Denaturation (at 95°C): DNA is denatured into single-stranded molecules.**
- **Annealing (50°C to 70°C): the primers anneal to the DNA.**
- **Polymerization or DNA synthesis (at 72°C): optimal for the polymerase.**



In the first picture (the first cycle), there are:

- **Denaturation.**
- **Annealing: there are primers that anneal to their complementary sites, and they must surround the region that needs to be amplified.**
- **Polymerization.**
- **In the end, we get four strands. Notice that the product is extending to the left and right, so this is not the product we are looking for.**
  - **The second cycle also doesn't result in the needed products.**
  - **The third cycle results in the needed products. Then, these products are amplified.**

➢ PCR CYCLES

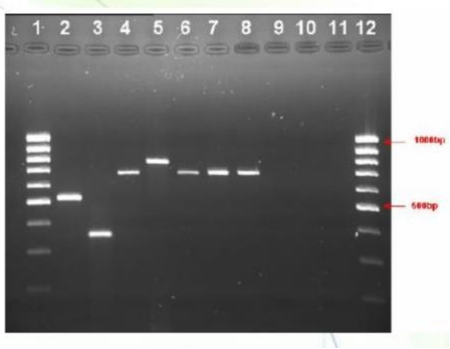
- **20-30 cycles of reaction are required for DNA amplification.**

***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) derived from each starting molecule.

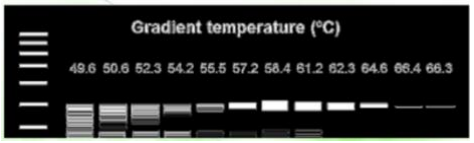
➢ DETECTION OF DNA FRAGMENTS



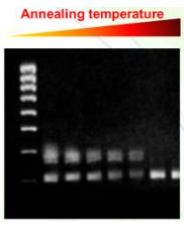
- This DNA fragment can be easily visualized as a discrete band of a specific size by agarose gel electrophoresis.

➢ IMPORTANCE OF PRIMERS

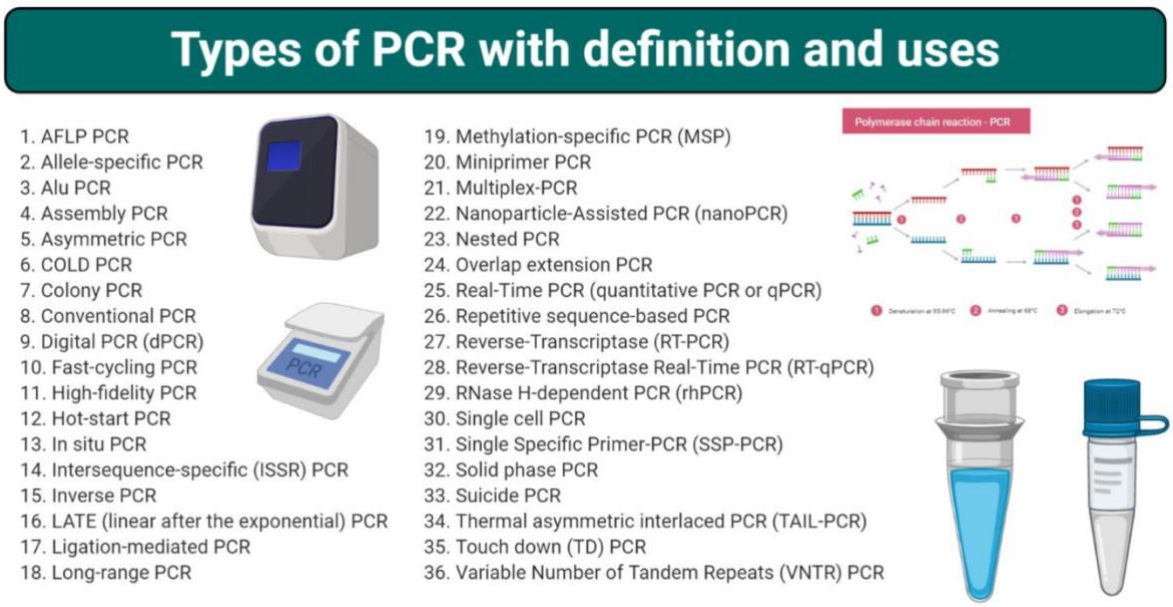
- The higher the temperature the more hydrogen bonds get broken.
- We get 50% of DNA hydrogen bonded (double stranded) and 50% is not (single stranded) at the melting point. (look at the figure bellow)
- As temperature increases, the DNA becomes more single stranded.
- We talked about the factors that affect melting point in last semester, you can go back and check them.
- If the annealing temperature is low, this allows for nonspecific hybridization between the primer and the DNA strand to take place resulting in nonspecific amplification.
- We have regions in our genome that are homologous to each other (having the same sequence). Thus, we can have nonspecific amplification.
- 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.
- The following picture demonstrates that the lower the temperature, the more nonspecific amplification takes place.



- Last two bands (on the right) represent specific amplification because the bands are single (thin).
- If there is so high temperature, there will be no hybridization between the primers and the templates. Therefore, we won't notice any bands.



This slide is not for memories, so cheer up!!



➢ REAL-TIME QUANTITATIVE PCR (QPCR)

Quantitative PCR method utilizes a chemical known as SYBR green, this

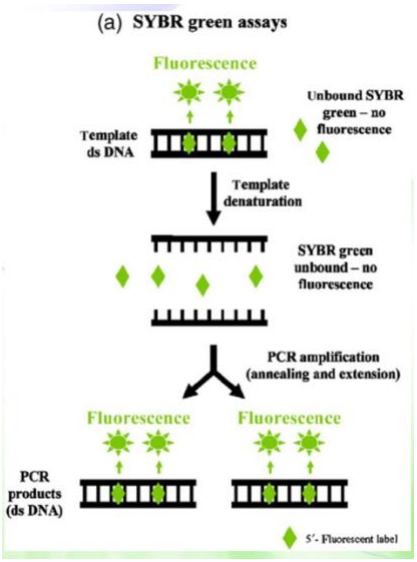
SYBR green binds to double-stranded DNA and fluoresces only when bound.

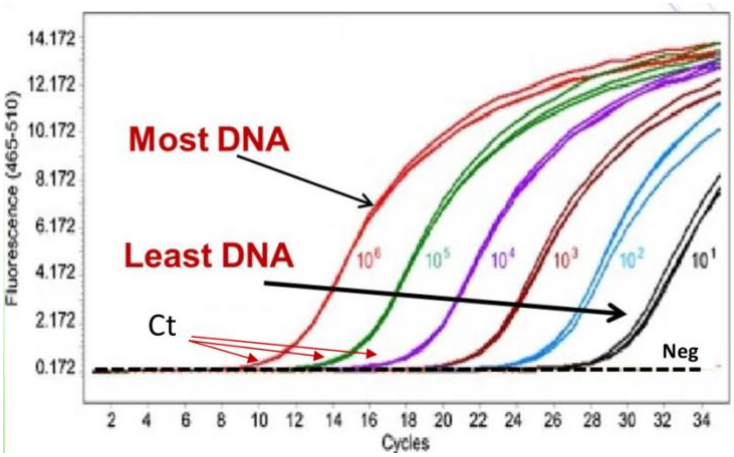
A way of relative quantitation of amount of DNA in a sample is by amplifying it in the presence of SYBR green. (we measure how much DNA gets amplified during amplification using Syper green assays)
So, the more double-stranded DNA you have in a sample, the stronger the fluorescence, again in PCR, DNA is amplified so at the end of the cycle when DNA is renatured you have SYBR green bound to this double- stranded, and since it's amplified, you have at the end of the cycle more and more fluorescence.

So, the idea is if we have more DNA to start with, you will have the fluorescence early on, again the higher the amount of DNA, the sooner it is detected.

Now listen to the story: let's say we have two individuals and one of them has severe symptoms versus the other, and you expect -cuz u r very smart- the one with severe symptoms has a higher bacterial or viral load, then we extract DNA from the two individuals and isolate it from the sample and we amplify it, and we use specific primers for the bacterial DNA or viral DNA,

(so that only the bacterial DNA or viral DNA will be amplified), keep in mind that primers are really important and temperature as well.





Ponder this plot here:

x-axis represents cycles, and the y-axis represents the intensity of these signals, you notice that the sample with the 1 million DNA molecules (the red one in the chart), its signals is detected at cycle 10, why not earlier? Because that's the limitation of the instrument dummy-it cannot detect a lower signal-, now regarding the sample with the hundred thousand DNA molecules, we have the signal detected let's say at cycle 14, regarding the next sample, we will have the signal detected at cycle 17, and so on (booooring).

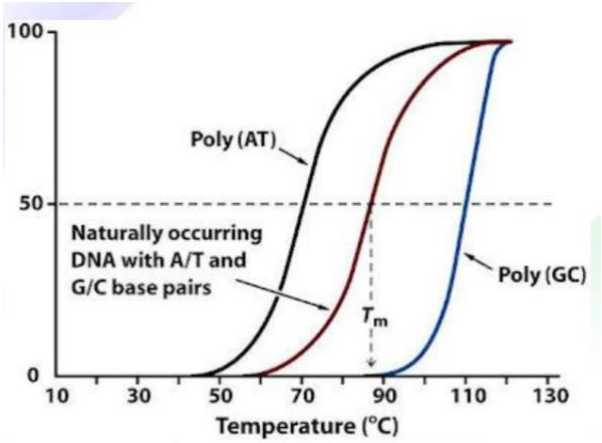
So, the lower the starting DNA concentration is, the later the signal will be detected, and that gives you an indication for how much starting material you have in the sample.

We place a line and it's arbitrary, in other words: it's not a scientific line, a threshold line above the signal of negative sample of course, and that tells us where the signal is detected, this is known as the CT.

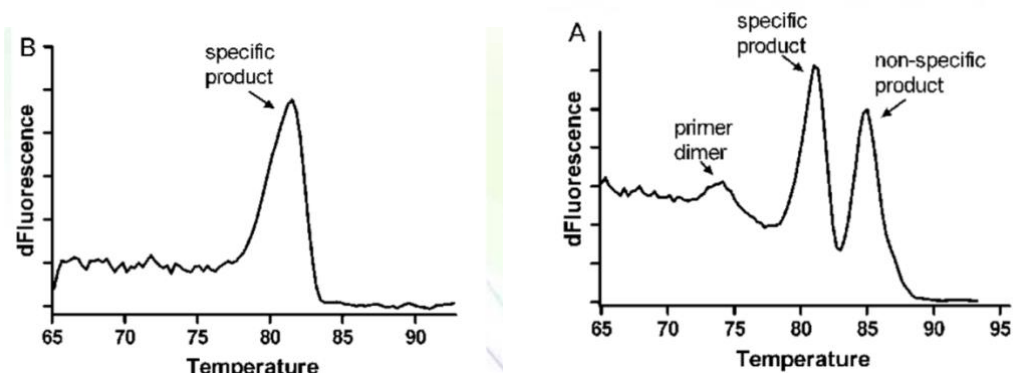
Threshold cycle (Ct) tells us at which cycle the signal is detected and is a measure of starting amount of DNA.

➢ MELTING CURVE ANALYSIS OF QPCR

By qPCR you can get non-specific amplification along with the amplified DNA, so how can we be sure that we're amplifying our DNA, well we can at the end do gel electrophoresis and see where the bands are, but the instrument can do this by using the melting curve analysis.



Again, it depends on the melting point, the point where 50% of the DNA is denatured, and what a melting curve does for us is that it tells us the change in fluorescence observed when double-stranded DNA (dsDNA) with incorporated dye molecules dissociates, or "melts" into single- stranded DNA (ssDNA) as the temperature of the reaction is raised.



For instance, we have the instrument detects change in the fluorescence, and at the end of the reaction we increase temperature, and the DNA becomes single stranded so it gets denatured, if we have a single product then we should have a single peak, (like in the graph B), represents the highest change of fluorescence as the temperature is raised, if we have two or more peaks, that tells that there are two or more products because each one of them would have a different sequence or different composition of A(s) and T(s), (don't dare ask about what the A(s) and T(s), those are the nitrogenous bases dummy, what a beautiful mind in your head you don't use!)

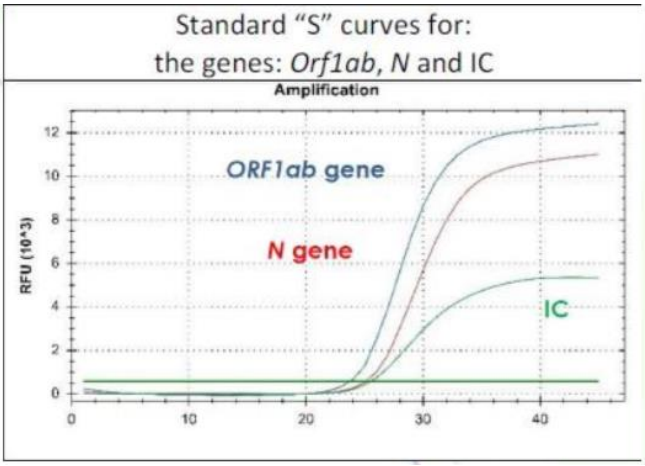
➢ TAQMAN QPCR

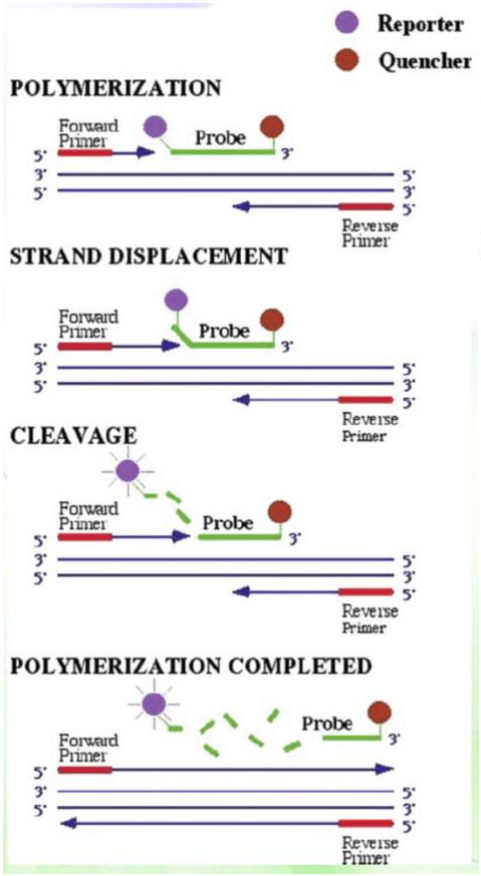
Advantages (versus SYBR chemistry) -
More specific

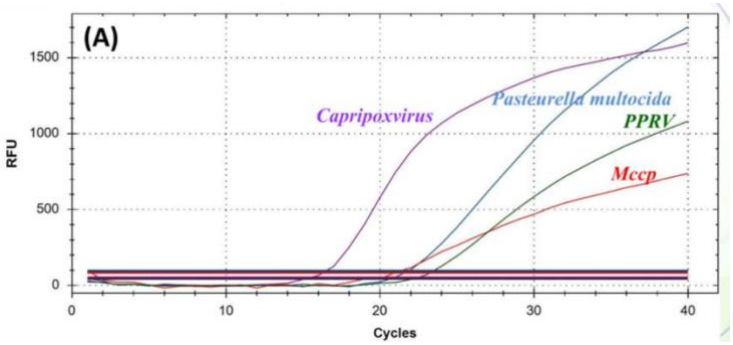
-More sensitive

-More reproducible

-Multiplexing







(STAND BY, IT'S GONNA BE ROUGH, AHRAM talks a lot!), in addition to primers, we also use probes, what's special about probe is that it's specific to the target, it also has a reporter and quencher attached to, basically, the reporter is a chemical that fluoresces, and the quencher is a suppressor of the reporter, it suppresses the fluorescence that can be emitted from the reporter if it is close to it, so, if the quencher is close to the reporter that is as close as the picture in the left above, being attached to the same probe, then the reporter doesn't fluoresce, now if they get separated then the reporter can fluoresce, so, we use a special taq polymerase which has a 5` to 3` exonuclease activity in addition to the polymerase activity, so as it extends and synthesizes the DNA, once it reaches the probe, it activates the exonuclease activity, and starts degrading and cleaving the phosphodiester bonds between the nucleotides of the probe,

releasing the reporter and now it gets away from the quencher, now it fluoresces, so we have a lot of probe and more and more of probe molecules are degraded by the taq polymerase, and you get more and more signal coming out, so it's more specific, the probe is more specific to target DNA that is amplified, so if you have amplification of another target that is not specific, the probe does not bind to it and as a result there would be no fluorescence, it's more sensitive because the signal that is emitted from the reporter is stronger than the signal that is emitted from the SYBR green, also it's more reproducible; because you get the same results over and over because we're not only using primers that are specific to the target we want to amplify, we're also using a probe that is specific to it, it also allows for multiplexing, which means that you can amplify different targets, you can use different sets of primers in the same exact reaction, so you can have a tube that contains the same template, taq polymerase, same deoxyribonucleotides, but you also have different primers that can amplify different regions in the template, and different probe molecules that would bind to this amplified region, and this amplified DNA and that amplified DNA, and each probe would have a different reporter emitting different signals, (look at the biggest picture in the previous page), you can also do a sort of like quantifications as well, because you have signals emitted at different threshold cycles.

Side note: This horrific story was done in the corona virus except that the corona virus genetic material was RNA not DNA.