بسم الله الرحمـٰن الرحيم

(وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ)

Cytology & Molecular Biology | FINAL 7

Polymerase Chain Reaction (PCR)

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NST member

وَلِلَّهِ الْأَسْمَاءُ الْحُسْنَى فَادْعُوهُ بِهَا

المعنى: العظيم ذو الكبرياء، المتعالي عن صفات خلقه، القاهر لعتاتهم، ولا يوصف بهذا الاسم على سبيل المدح سواه سبحانه وتعالى.

الورود: ورد مرة واحدة في القرآن.

الشاهد: ﴿ٱلْعَزِيزُ ٱلْجَبَّارُ ٱلْمُتَكَبِّرُ ۚ سُبْحَٰنَ ٱللَّهِ عَمَّا يُشْرِكُونَ﴾ [الحشر:٢٣].

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اضغط هنا لشرحٍ أكثر تفصيلًا

Challenges in research and medicine

- Genetic variation — RFLP can detect those variations but it's very slow.
  - STR, VNTR, SNPs, and mutations.
- Minute amounts of genetic material
  - Dinosaurs and early humans, — We don't have enough genetic material to perform regular techniques, especially if samples were taken from Dinosaurs, mummies, people dead for long time.
- Identification of organisms (e.g. infectious agents)

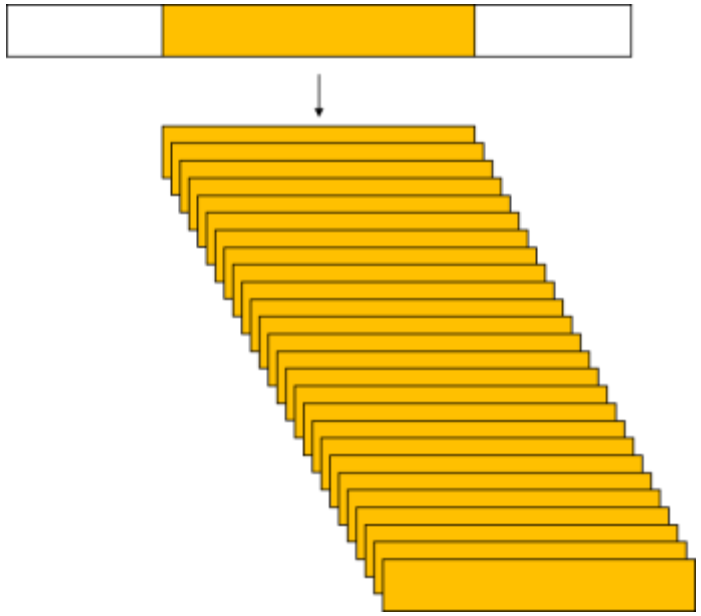
If their amount is little, we cannot detect them easily, so we needed a method to make the amount of the DNA more.

Before PCR we used the southern blotting technique to view genetic variations, but it had some problems:
1.It was slow.
2. It need radioactivity techniques that can be dangerous if the human body, especially the human DNA, got exposed to it.

Polymerase Chain Reaction

- A Chain reaction: same reaction is repeated for many times.
- Catalyzed by DNA Polymerase.

- Polymerase chain reaction (PCR) allows the DNA from a selected region of a genome ((even if it's tiny)) 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.



Kary Mullis invented the PCR, prof Mamoun didn't care much about this info.

DNA pol is used in this technique but not alone, we need an enzyme that's more specific.

PCR is incredibly good when we want to study a specific gene or a specific sequence important for DNA regulation, instead of studying the whole genome.
It's also good for amplification of STR, VNTR, SNP, etc.
We usually use PCR when we want a big amount of a specific DNA sequence, like:

1. Sequencing
2. Cloning

Components of PCR reaction

An enzymatic reaction, so sensitive that it can work on a **single** DNA molecule.

- The DNA template, which needs to be fully amplified, or a part of it.
- A **pair** of DNA primers,
  - The 15-25 nucleotides-long primers should surround the target sequence.

We design complementary DNA primers to perform PCR. Normally in cells, RNA primers are used, because DNA pol can't start working without primers, naturally the primers are made of RNA in physiologic conditions.

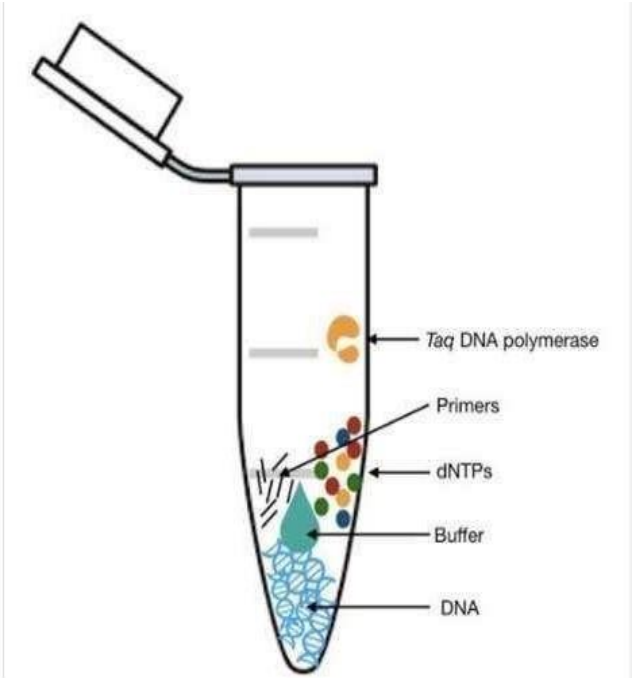
A question may pop up, why do cells use RNA primers?? It's unknown, but scientist thinks that life started with RNA due to its dual rule: genetic material and enzyme.

All four deoxyribonucleoside triphosphates

- as substrates for the enzyme, ATP/CTP/GTP/TTP

A heat-stable DNA polymerase we need it to be

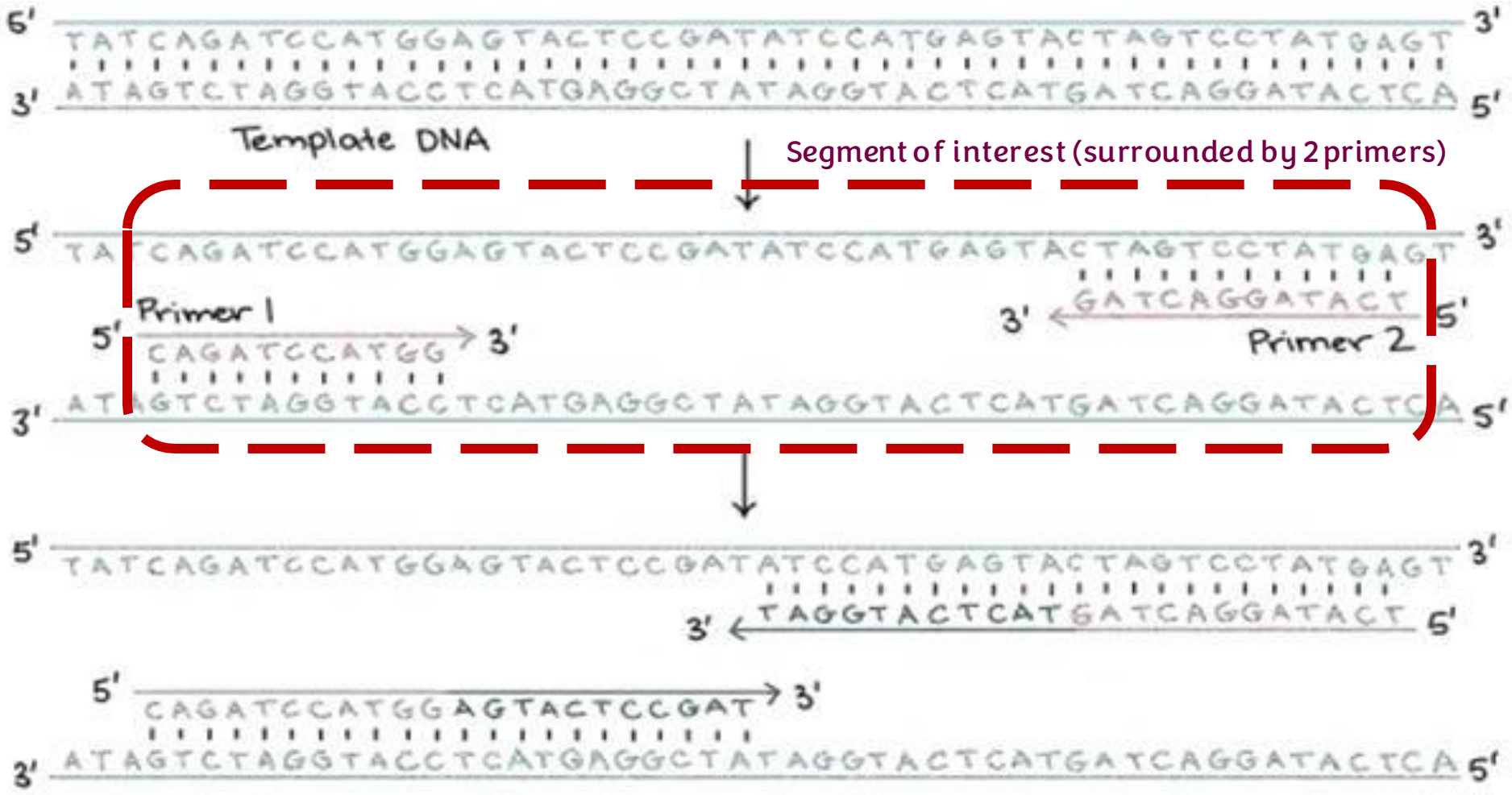
- heat-table because the temperature used in the PCR technique is very high.



Components of PCR reaction

Always from 5' to 3'
On both directions, so the primer should be antiparallel for the strand that it binds.

Highly suggested to rewatch this part for good understanding.



This technique differs from DNA replication that it doesn't have a leading strand and a lagging strand.

1. The 2 strands should be separated.
2. The primer then binds to each one.
3. DNA pol starts synthesizing the DNA wanted.

A keyword for this technique is:
DNA amplification.

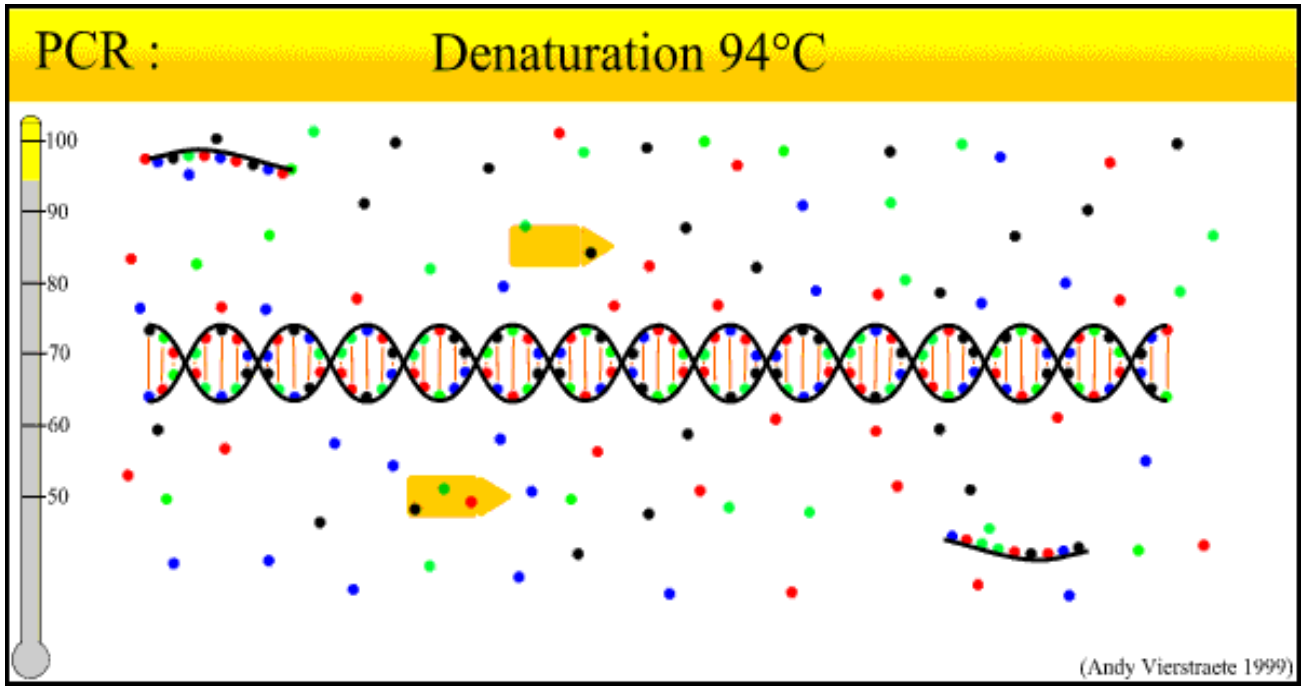
The PCR cycles

The chain reaction consists of 3 steps, but those 3 steps are repeated in a cycle, because again the word here is AMPLIFICATION.

Those PCR cycles are repeated around 25 times

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

Our DNA pol enzymes work at 37°C, but in this technique the temperatures are nowhere near that.



The doctor advises us to watch animations on the process for further understanding.
Here's a good animation on youtube.

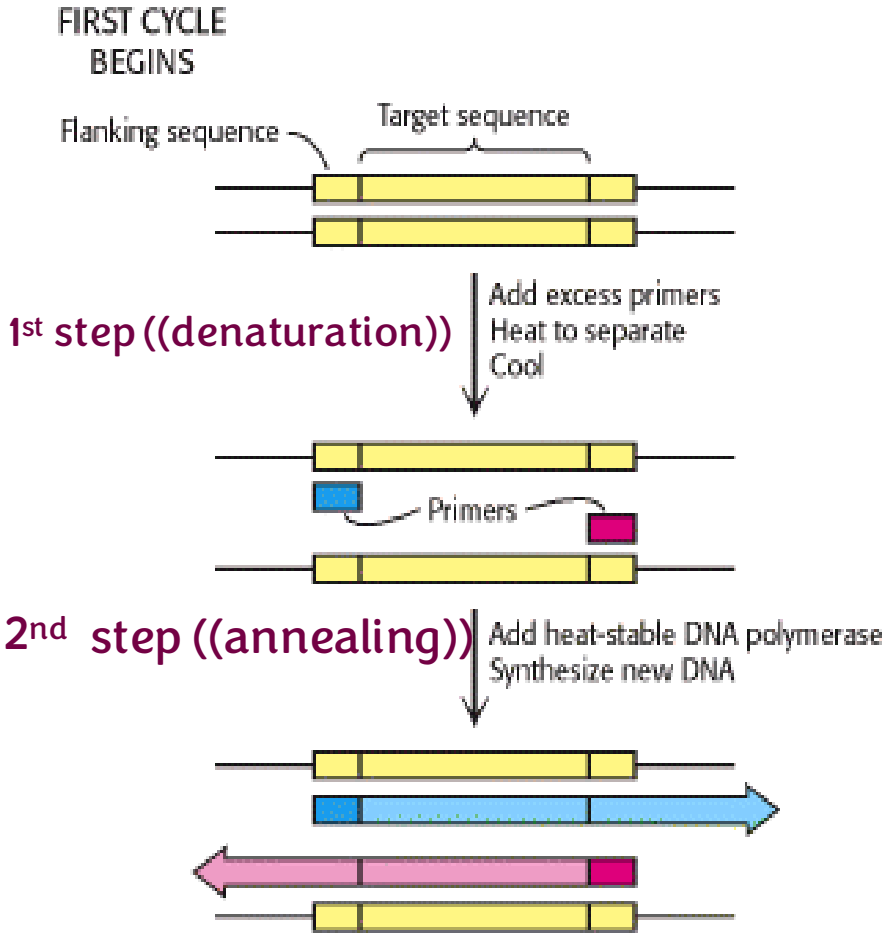
The DNA polymerase

عنا بالأردن ممكن نستثمر البكتيريا الموجودةِ في حمامات ماعين

- Suitably heat-stable DNA polymerases that 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.

Therma = Heat
Aqua = water

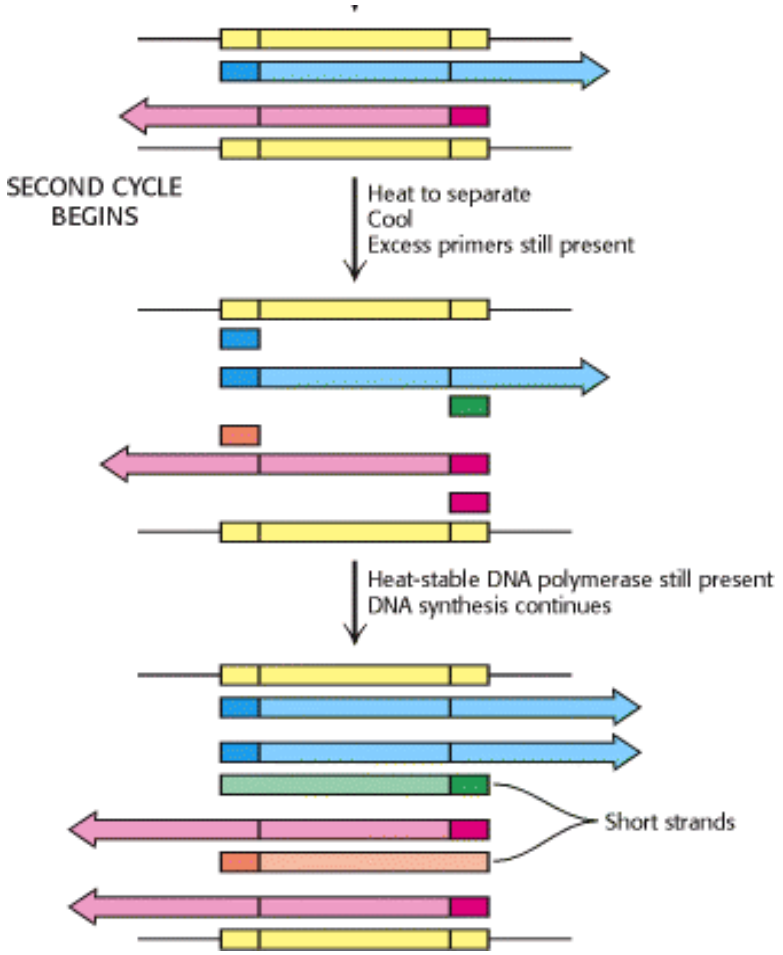
These 3 steps are repeated for multiple cycles, doubling the amount of DNA with every cycle.

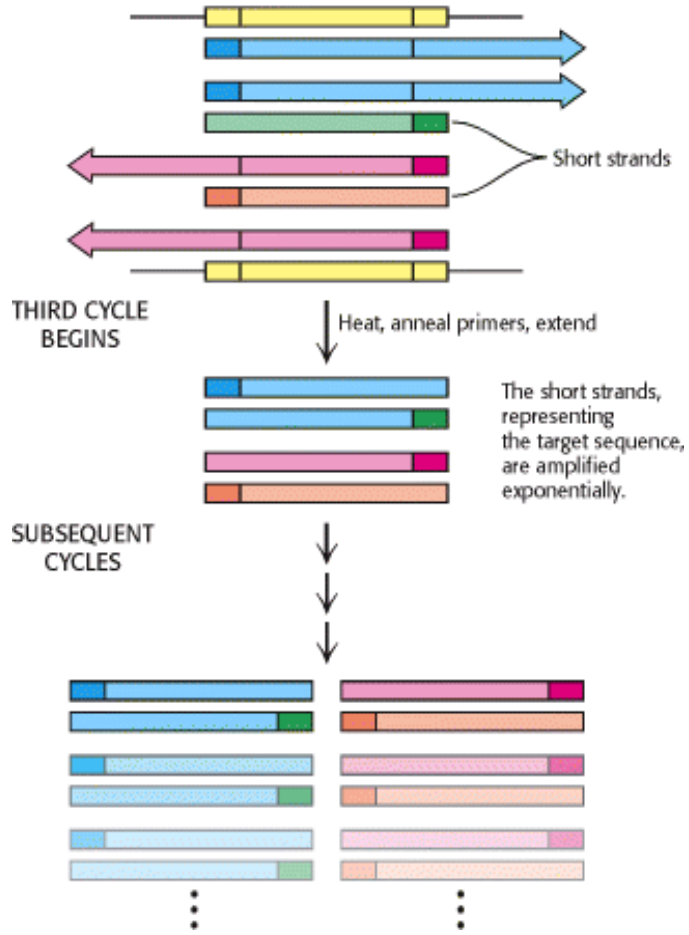


1st step ((denaturation))

2nd step ((annealing))

3rd step ((synthesis))





First step,
Denaturation, we raise the temperature up to 95 C, resulting in separation of the 2 strands
Second step,
Annealing, we reduce the temperature down to (50-70 C) depending on the binding of primers to the DNA,
Third step,
Synthesis, we raise the temperature up to 72 C for the DNA pol to start synthesizing the DNA
In the first cycle the outcome is 2 DNA molecules

These 3 steps are repeated in a 2nd cycle giving 4 molecules, then a 3rd cycle giving 8 molecules, and so on, **and on the 3rd cycle onward, the target molecule starts to appear and amplification happens only to this Target DNA** ((very important)), watch the video on slide 17.

After 25 cycles the reaction gives enough DNA molecules to proceed to the next goal.
Why 25 cycles are sufficient?
Because the Equation used is 2^n, and $2^{25} \approx 33.6$ million. n = number of cycles.

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 products, 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.

It’s relatively small

The process used to take relatively long times because of the ever-going fluctuations of temperature that must be applied on the apparatus used (the temperature changes are the rate limiting steps).

Recently, new metal has been used, which can change its temperature fast (low specific temperature), leading to the completion of the PCR process in about 2 hours.

Detection of DNA fragments

This is done after PCR

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

Recall what we took in electrophoresis

wells

Lane number 1 is the molecular weight standard, and lanes (2-9) are PCR products.
If we applied PCR followed by gel elctrophoresis for the VNTRs of several people, you could see that the VNTR size differs from each other.

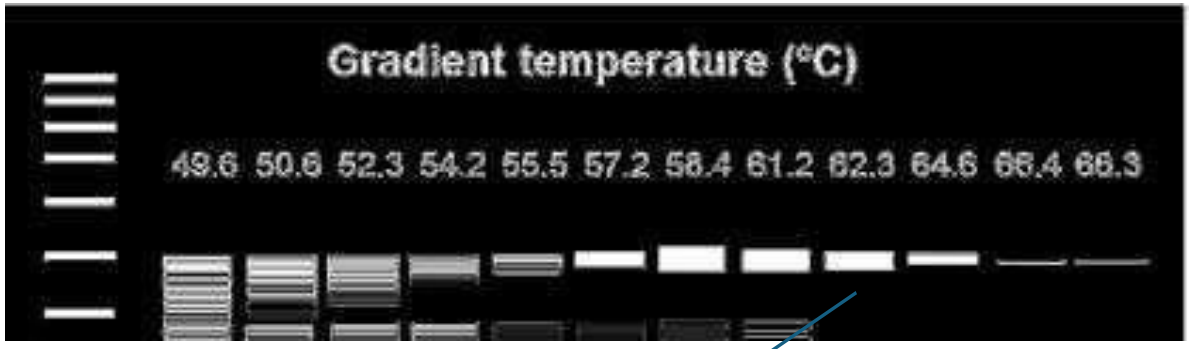
Notice that at the end of any PCR reaction
we have very specifically one product.

The primers used in the PCR are the factor that determines the DNA region that undergoes amplification.
If one primer only worked no amplification can happen, so they should bind to the region from left end and right end.

Extra note: The primers aren't the same sequence when read from 5' to 3', not even a complementary sequence.
Extra note: The primers used in PCR are DNA primers, whereas is our cells it's an RNA primer, DNA pol can work with any type, but DNA primers are more stable, that's why they're used in PCR.

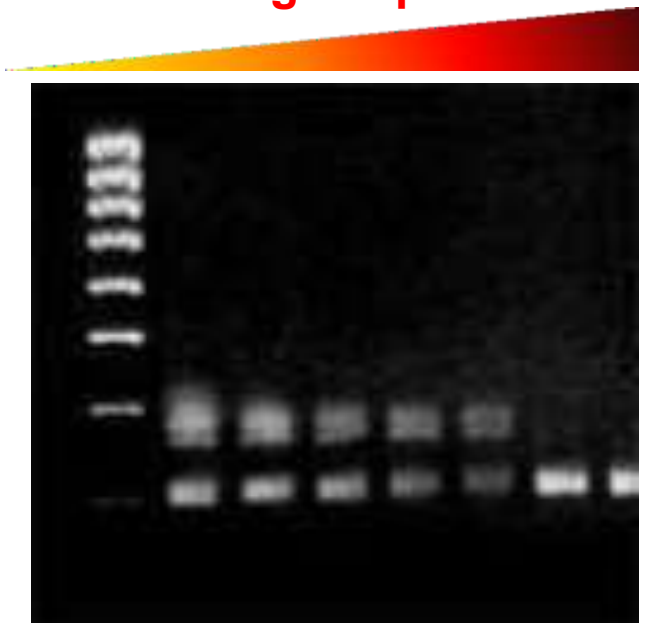
Importance of primers

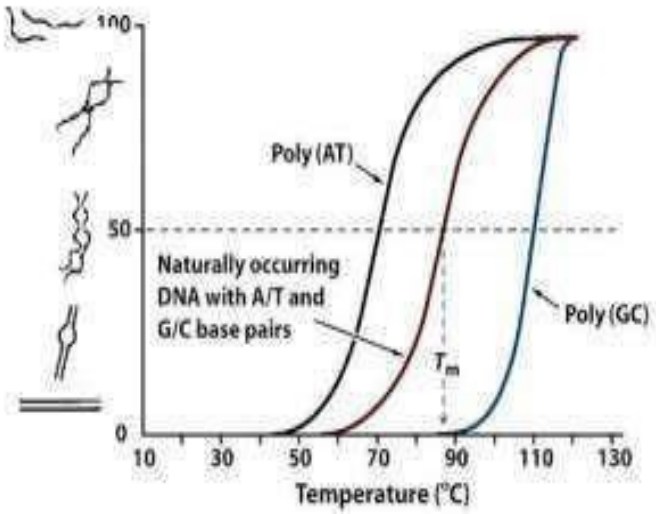
- The specificity of amplification depends on the specificity of the primers to not recognize and bind to sequences other than the intended target DNA sequences.
- How can you prevent it?
- How can you take advantage of it?



The wanted one, e.g.: very specific.

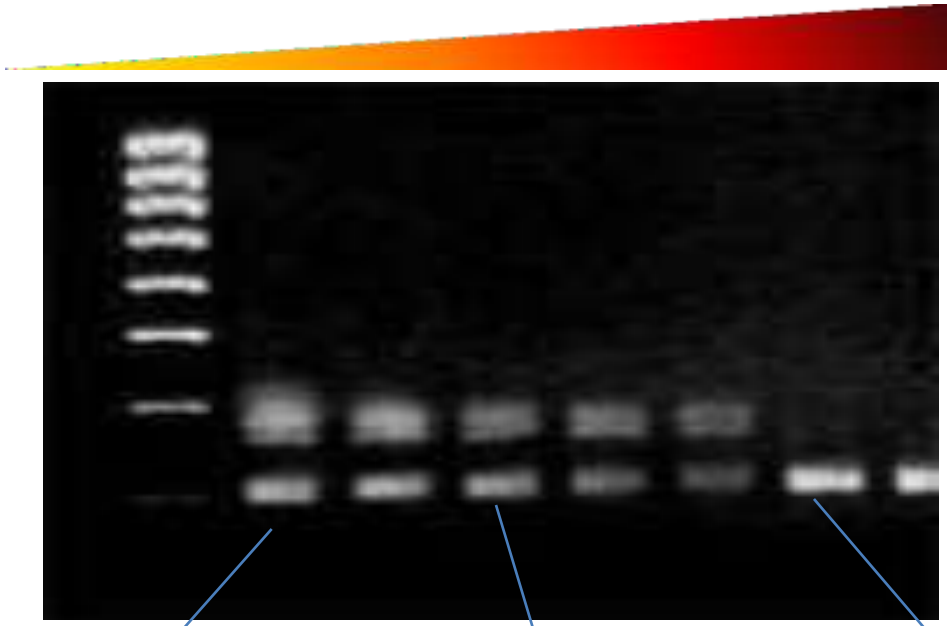
Annealing temperature





Primers are not absolutely specific to a single genomic location because sequences complementary to the primers can exist at multiple regions across the genome. As a result, primers may bind to different sites, leading to the amplification of different PCR products.

The temperature used in step 2 (annealing) typically ranges between 50 °C and 70 °C and depends on the specificity of the primers for the target DNA. As discussed in previous lectures, primers can bind to non-target regions even without perfect complementarity due to imperfect hybridization. Therefore, the annealing temperature is a critical factor, just as primer specificity is essential for targeting a specific region.

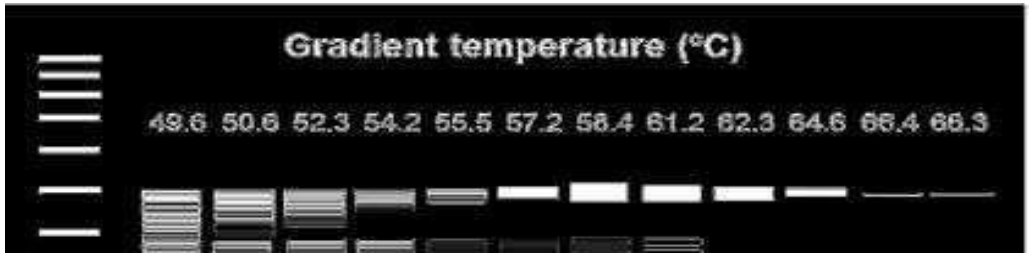


As you can see in this reaction, the annealing temperature used for the same PCR reaction was the only different thing, and because of that the number of products differed at each temperature, and as we raised the temperature one product was obtained, at lower temperatures imperfect hybridization occurred.

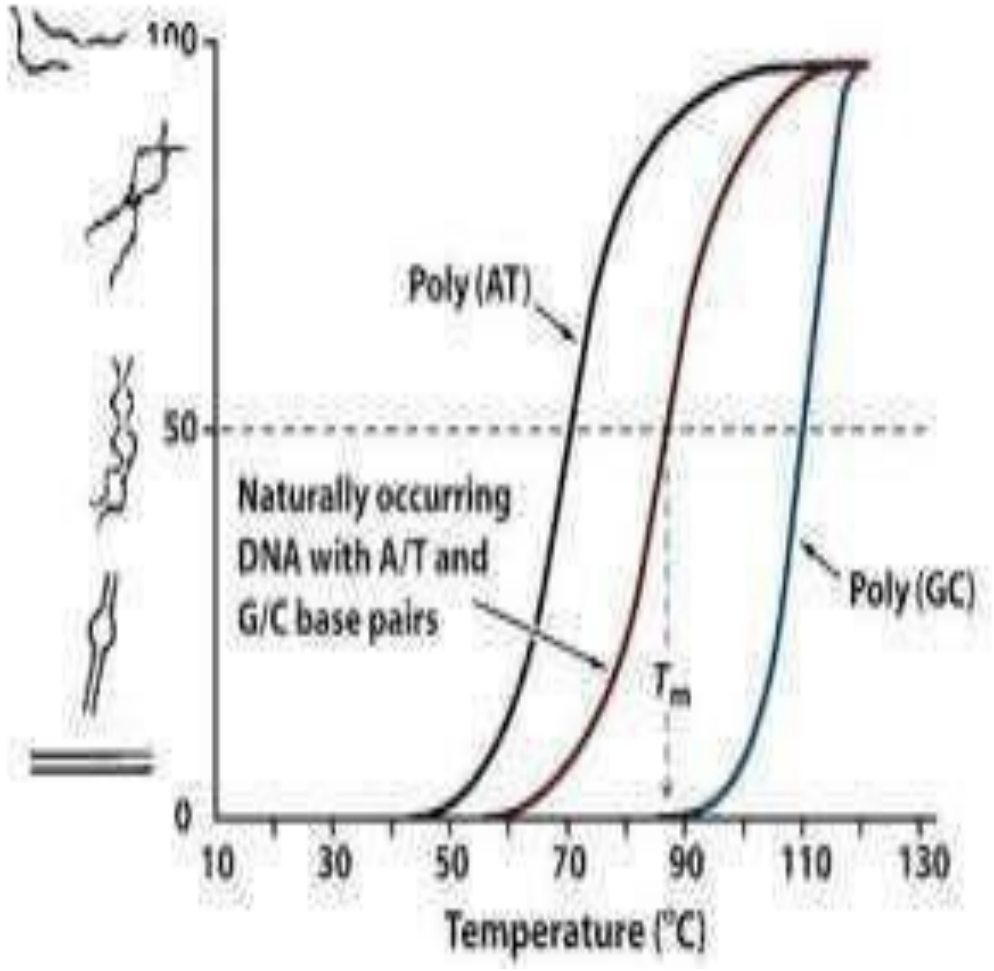
At low temperature, there are 2 PCR products.

As temperature increases on product is being produced more.

At this specific annealing temperature, 1 product was obtained, because only perfect hybridization was allowed.



Here, the perfect hybridization is 62.3, anything below that gives more than one product, and when temperature used exceeds 62.3, let's say 68, there won't be amplified DNA because the primers didn't bind to the DNA.



This image illustrates the concept of melting temperature (Tm), which is the temperature at which **50% of a DNA duplex becomes single-stranded**.

Therefore, it is necessary to use an appropriate annealing temperature that allows the primer to bind to the DNA template **with high specificity**.

The temperature does not magically make primers “very specific.”
Higher annealing temperature **reduces non-specific binding** by destabilizing mismatched hybrids.

Eventually we'll get a DNA molecule with a specific size that we want.

Watch this animation from Prof. Mamoun's lecture.

It's a summary for what we've discussed from this lecture, but it's **crucial** for understanding it.

Uses of PCR

In the Human Genome Project, we determine the correct DNA sequence of an individual, which enables us to study specific genes and design precise primers.

This aids in choosing the right primers in PCR.

- Molecular fingerprinting
- Genotyping — Alleles present for a gene
- Genetic matching
- Detection of Mutations
- Prenatal diagnosis
- Cloning
- Detection of organisms
- Classification of organisms

Molecular fingerprinting is based on STRs and VNTRs, which differ among individuals in the number of repeated DNA units. Primers are designed to bind to conserved sequences flanking the repeats, on the left and right sides of the STR or VNTR region, allowing PCR amplification of the entire locus.

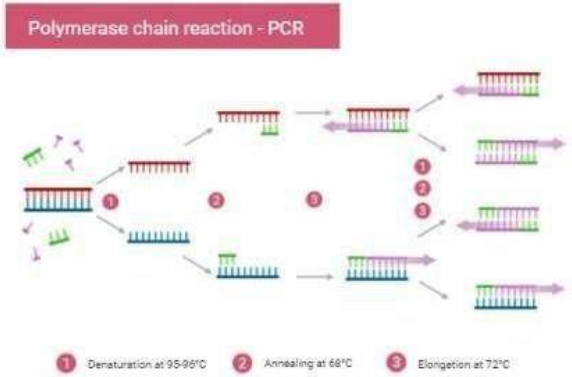
} Like in detecting and classifying infectious agents.

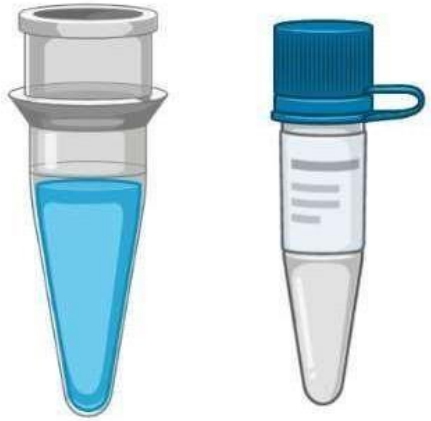
- Mutagenesis — the process of inducing changes or mutations in the DNA of an organism.
- Molecular archaeology — Study ancient biological organisms.

You don’t need to memorize the uses of PCR from this slide or the previous one, but the uses on the following slides are important to learn.

Types of PCR with definition and uses

1. AFLP PCR
2. Allele-specific PCR
3. Alu PCR
4. Assembly PCR
5. Asymmetric PCR
6. COLD PCR
7. Colony PCR
8. Conventional PCR
9. Digital PCR (dPCR)
10. Fast-cycling PCR
11. High-fidelity PCR
12. Hot-start PCR
13. In situ PCR
14. Intersequence-specific (ISSR) PCR
15. Inverse PCR
16. LATE (linear after the exponential) PCR
17. Ligation-mediated PCR
18. Long-range PCR
19. Methylation-specific PCR (MSP)
20. Miniprimer PCR
21. Multiplex-PCR
22. Nanoparticle-Assisted PCR (nanoPCR)
23. Nested PCR
24. Overlap extension PCR
25. Real-Time PCR (quantitative PCR or qPCR)
26. Repetitive sequence-based PCR
27. Reverse-Transcriptase (RT-PCR)
28. Reverse-Transcriptase Real-Time PCR (RT-qPCR)
29. RNase H-dependent PCR (rhPCR)
30. Single cell PCR
31. Single Specific Primer-PCR (SSP-PCR)
32. Solid phase PCR
33. Suicide PCR
34. Thermal asymmetric interlaced PCR (TAIL-PCR)
35. Touch down (TD) PCR
36. Variable Number of Tandem Repeats (VNTR) PCR

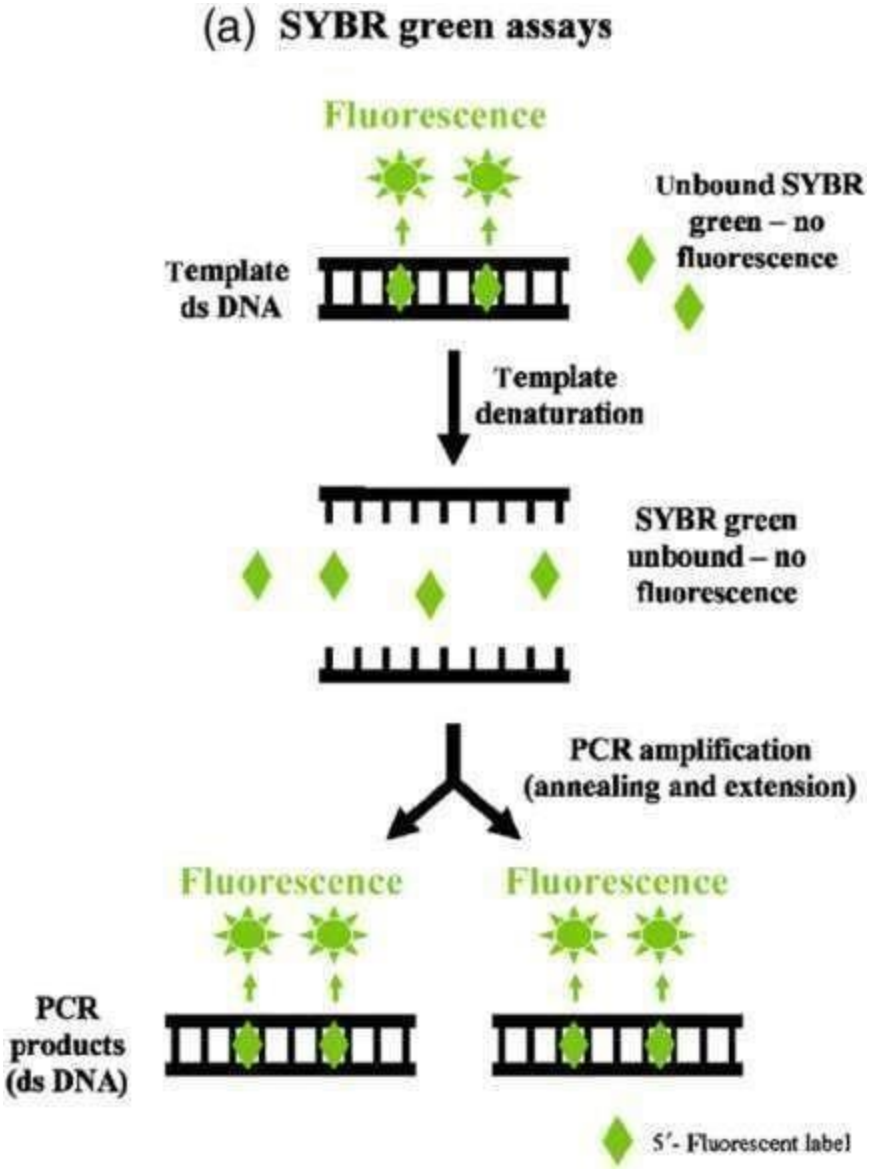




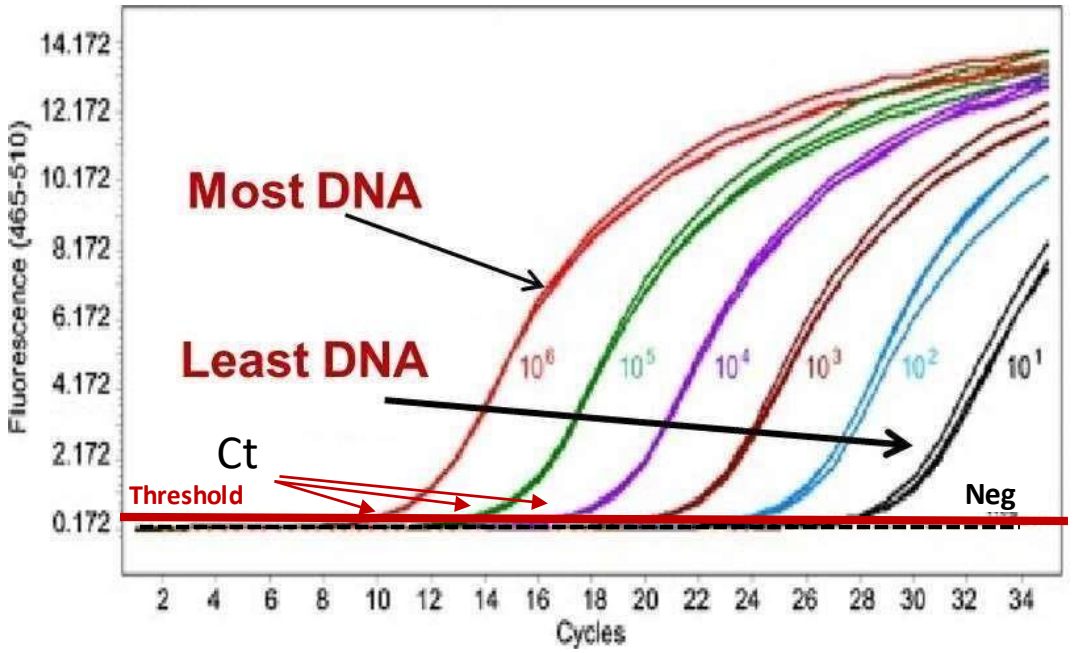
The uses of PCR have become so diverse that it is now possible to amplify DNA from a **single cell**.

Currently, to understand how an individual cell functions within a scientific context, **single-cell DNA sequencing** is performed, which requires PCR beforehand.

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



SYBR green is a chemical substance that binds to double-stranded DNA (dsDNA) making the DNA fluorescent.

PCR by itself cannot be used for DNA quantification, but when combined with other methods, it allows DNA measurement in what is called **quantitative PCR (qPCR)**, **real-time quantitative PCR**, or **qPCR**.

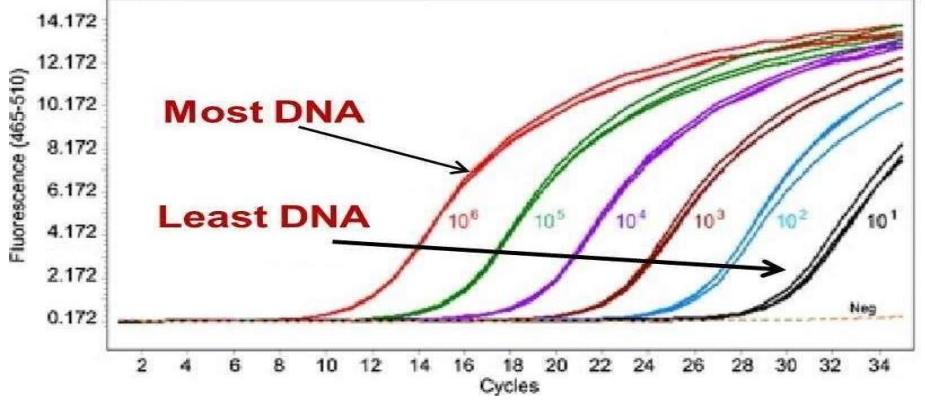
As the amount of double-stranded DNA (dsDNA) increases, the fluorescence from **SYBR Green** also increases. This fluorescence can be converted into the concentration of dsDNA.

In the first cycle, only a very small signal is produced, but the detection device is not sensitive enough to measure it. With each subsequent cycle, more dsDNA is generated, and more SYBR Green binds, but the signal may still remain below the detection threshold. After a certain number of cycles—around cycle 10, though this can vary depending on the reaction—the device begins to detect the fluorescence. By the following cycles, the signal continues to rise.

Eventually, the reaction reaches a **plateau**, where the device can no longer accurately detect changes in fluorescence.

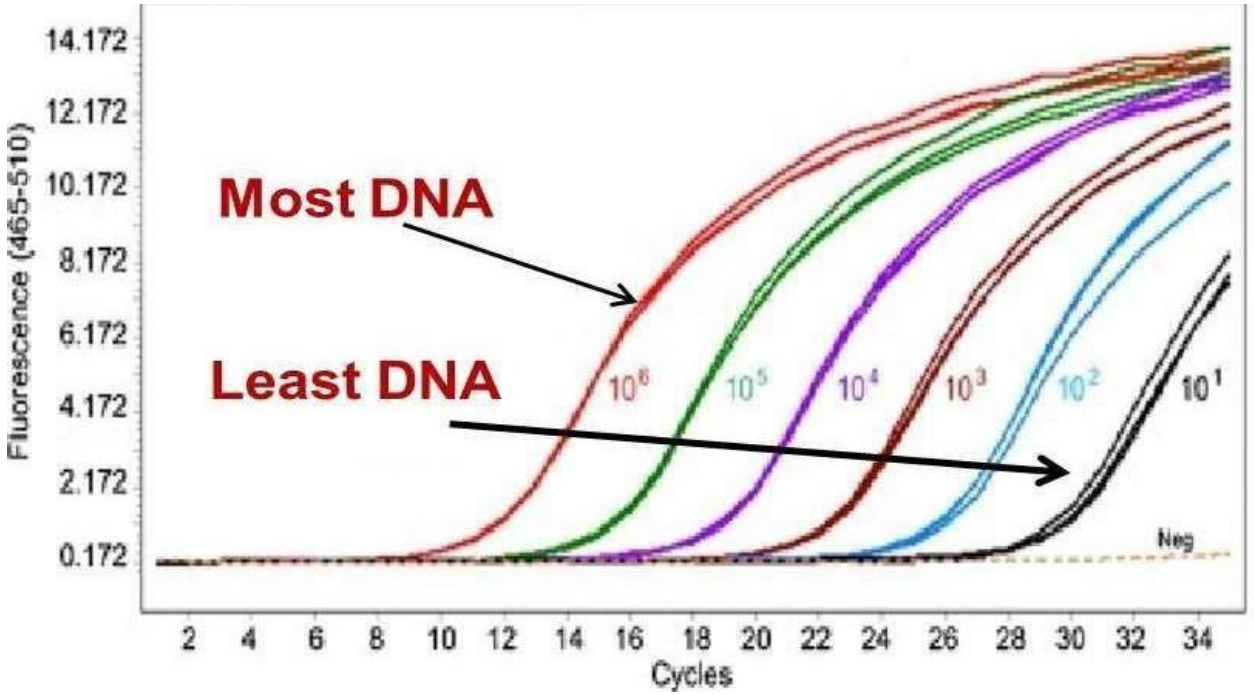
The key idea is that if you start with a small amount of DNA in one reaction and a larger amount in another, the reaction with more DNA will reach detectable levels sooner. For instance, if one sample begins with 10 DNA molecules and another with 100, the sample with 100 molecules will be detected earlier than the one with only 10 molecules, which makes intuitive sense.

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



- The point where the signal is first detected is called the Threshold, or Ct (Cycle threshold). If the fluorescence signal surpasses this threshold, DNA is detected by the machinery.

- A higher Ct or threshold indicates less detection, meaning the starting DNA material is lower. Conversely, a lower Ct or threshold means earlier detection, indicating a higher amount of starting DNA material. So, a lower Ct corresponds to more starting DNA, while a higher Ct means there is less starting DNA.

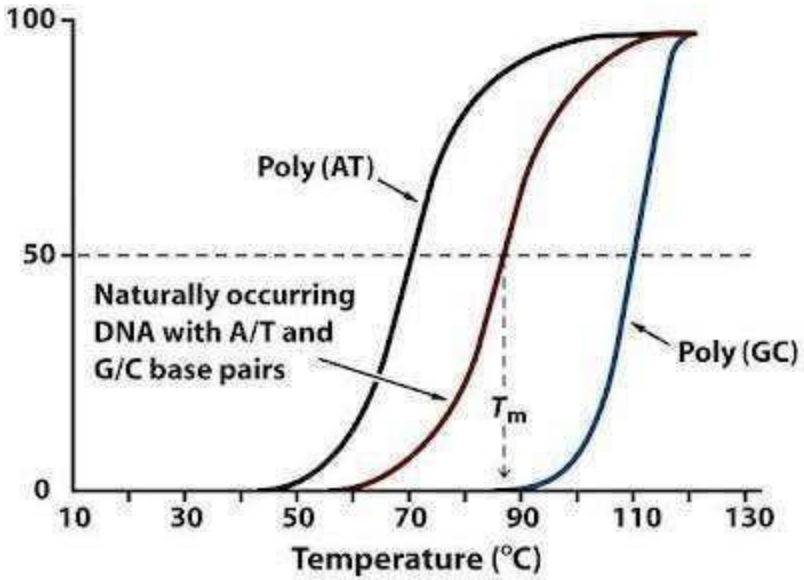


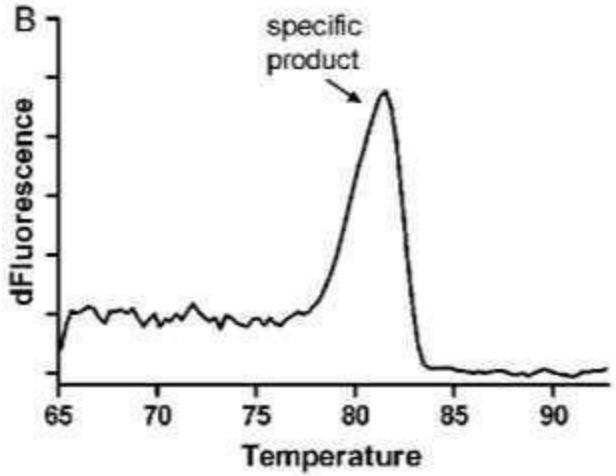
For example, consider several samples with different starting amounts of DNA: one with 1,000,000 templates, another with 100,000, then 10,000, 1,000, 100, and 10. There is also a Negative Control sample, which contains only primers and polymerase but no DNA template. The Negative Control will not undergo any amplification because it lacks a starting template, so no signal will be detected.

Now, the sample that started with 1,000,000 DNA molecules will be detected earliest, for instance at cycle 10. The sample with 100,000 molecules might be detected at cycle 14, 10,000 at cycle 18, 1,000 at cycle 22, and so on, which aligns with expectations. Therefore, if a sample is detected at cycle 22, we can infer that its starting amount of DNA was approximately 1,000 molecules.

By measuring the cycle at which detection occurs, we can estimate the initial quantity of DNA. This cycle is called the threshold cycle (Ct). Importantly, the threshold cycle is not the same as the plateau phase; it represents the point at which the amplification signal becomes detectable. This principle underlies quantitative PCR (qPCR), allowing us to determine the starting amount of DNA based on the Ct value.

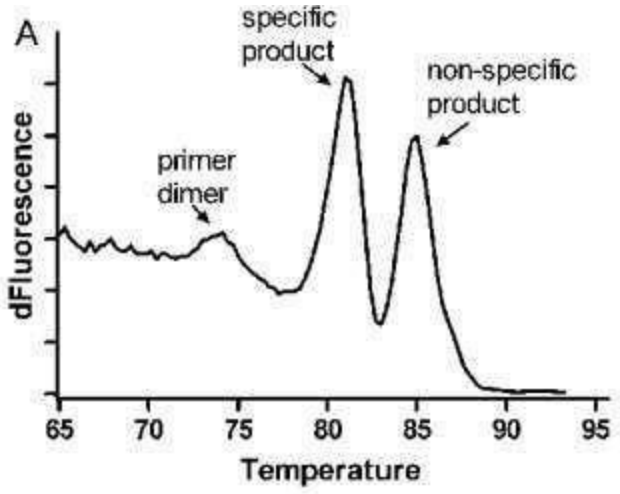
Melting curve analysis of qPCR



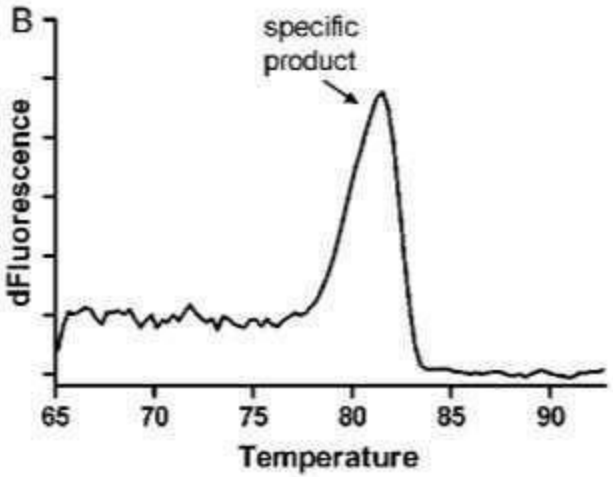


Melting point: is the point at which 50% of DNA is single stranded and 50% of DNA is double stranded. DNA with Poly GC has a higher melting point than DNA with poly AT.

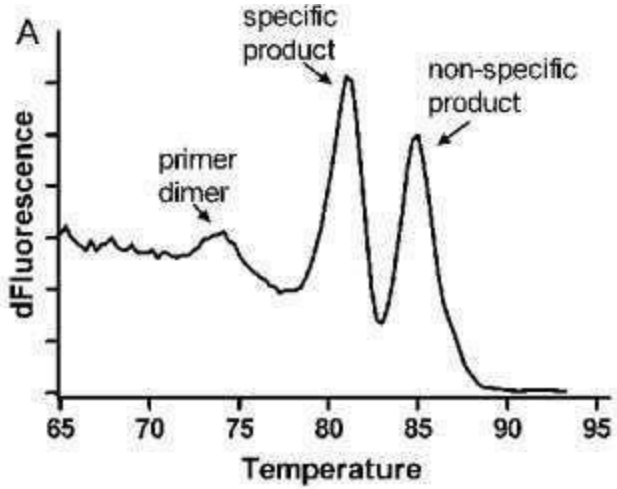
- A melting curve charts the change in fluorescence observed when double-stranded DNA (dsDNA) with incorporated dye molecules dissociates, or “melts” into single-stranded DNA (ssDNA) as the temperature of the reaction is raised.



Melting curve analysis of qPCR

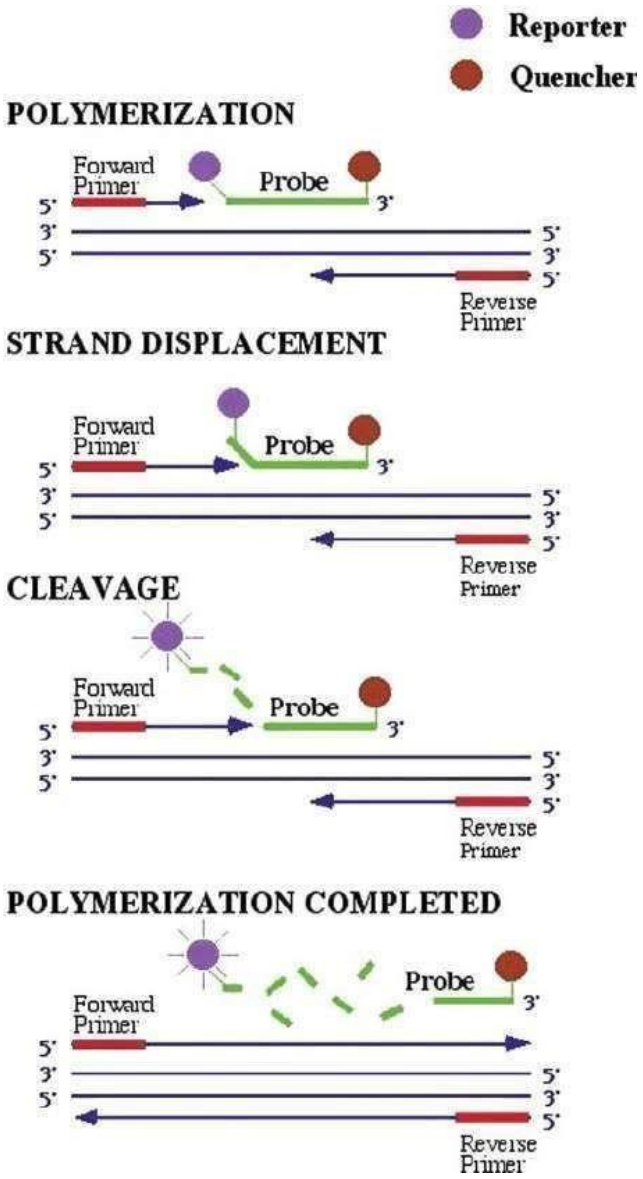


Sometimes, PCR amplification can be non-specific. There are two methods to assess this.
First method: using **gel electrophoresis** to see if a single band is present.
One band on the gel indicates a single PCR product.
Second method: melting curve analysis, performed by gradually increasing the temperature at the end of the reaction. As double-stranded DNA denatures, **SYBR Green** is released.
If there are multiple PCR products, their **melting temperatures (Tm)** may differ due to differences in **sequence composition, GC content, and length**. By measuring when the SYBR Green signal disappears, we can determine the number of products:
A single DNA product produces a **single Tm**.
Two or more products produce **distinct Tms**, which appear as separate peaks on the melting curve. For example, one peak may be at 83 °C and another at 85 °C, reflecting differences in sequence and length.
This analysis is called the **melting curve**, and it allows us to determine whether the PCR yielded a single product or multiple products by observing whether there is **one Tm or multiple Tms**.

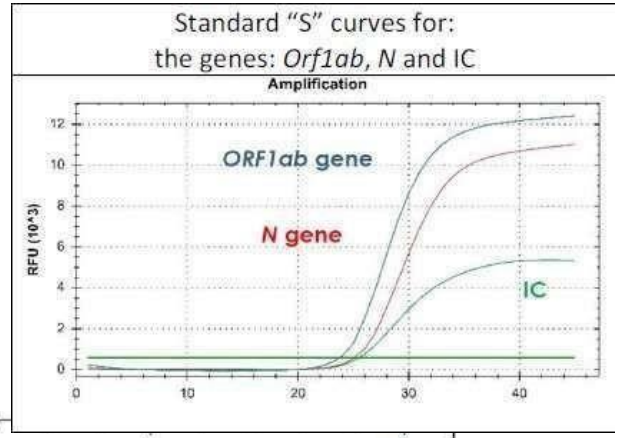


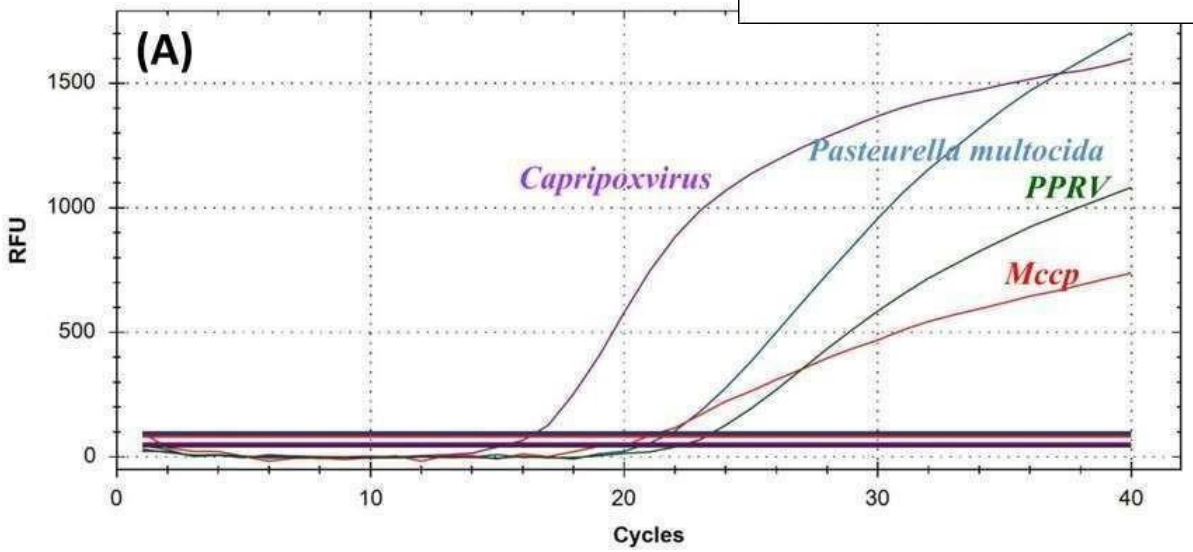
Taqman qPCR

SEE NEXT SLIDE FOR EXPLANATION



- Advantages (versus SYBR chemistry)
  - More specific
  - More sensitive
  - More reproducible
  - Multiplexing





The TaqMan qPCR, named after the video game Pac-Man, also performs DNA amplification, but with a specific purpose: it allows us to monitor whether the amplification is specific.

The components used in this technique are:
1. The DNA template for amplification.
2. The enzyme, Taq polymerase.
3. Substrates (nucleotides).
4. Forward and reverse primers.
5. The component unique to this method: the probe, which binds to a specific region within the DNA, located between the forward and reverse primers.

The probe contains two chemical groups: a reporter at the 5' end and a quencher at the 3' end. The reporter generates a signal, while the quencher suppresses this signal when they are close together. As long as the reporter remains part of the intact probe, no signal is emitted.

During PCR, Taq polymerase not only synthesizes DNA but also possesses 5' to 3' exonuclease activity, similar to the activity seen in DNA replication when RNA primers are removed. As the polymerase encounters the probe, it cleaves the probe nucleotide by nucleotide, separating the reporter from the quencher. Once released, the reporter emits a detectable signal.

Unlike SYBR Green, which binds to any double-stranded DNA regardless of specificity and requires a melting curve analysis to confirm correct amplification, TaqMan probes inherently indicate specificity. The probe only hybridizes to its perfectly complementary sequence, and the signal is only generated if amplification occurs and the reporter is released.

Therefore, the specificity of TaqMan qPCR arises from two factors: the primers, which direct amplification to the correct target, and the probe, which confirms that the amplification is specific.

Multiplexing in TaqMan PCR allows simultaneous amplification and detection of multiple DNA targets within a single reaction. This is accomplished using different sets of primers and probes, each specific to a particular gene or pathogen. Each probe carries a distinct reporter dye, so the signals from different targets can be distinguished.

For example, consider a patient with an infection where the causative bacterial species is unknown. Each bacterial gene, specific to a particular bacterium, is targeted with a corresponding primer pair and probe. When bacteria X is present, its probe is cleaved by Taq polymerase, releasing a signal unique to that bacterium. If multiple bacteria are present—say X, Y, and Z—each probe emits its specific signal, allowing simultaneous identification of all three.

Multiplexing can also be used to confirm the presence of a single pathogen by targeting multiple genes of that organism. For instance, to confirm infection with bacteria X, three different genes can be amplified using three primer pairs and three probes, each with a distinct reporter. Detection of signals from all three probes confirms the presence of bacteria X, although in some cases, a single gene target may be sufficient.

This video is great for taqman qPCR.

Additional Resources:

رسالة من الفريق العلمي:

Extra References for the Reader to Use:

1. https://youtu.be/qPQfhAlR8Rs?si=T6bIdiMGETmJhRbM

Look for something positive each day,
Even if some days you have to look a little harder.
Let the challenges make you stronger.

For any feedback, scan the code or click on it.

Corrections from previous versions:

Versions	Slide # and Place of Error	Before Correction	After Correction
V0 → V1			
V1 → V2			