MOLECULAR BIOLOGY

بسم الله الرحمن الرحيم

DOPAMINE SCIENTIFIC TEAM

FINAL – Lecture 6

Polymerase Chain Reaction (PCR)

﴿ وَإِن تَتَوَلَّوْاْ يَسْتَبْدِلْ قَوْمًا غَيْرَكُمْ ثُمَّ لَا يَكُونُوٓاْ أَمْثَٰلَكُم ﴾

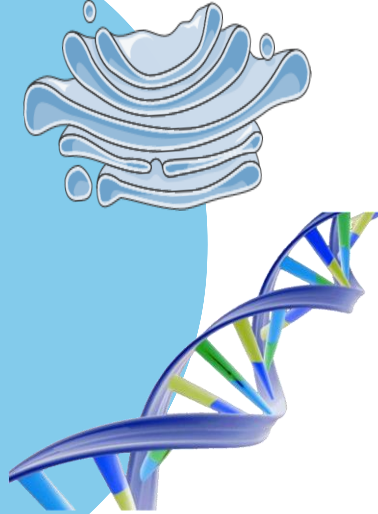
اللهمّ استعملنا ولا تستبدلنا

Written by :

- Zaid Abu Hassan
- Zaid Bassam

Reviewed by :

- Muthanna Khalil



Challenges in research and medicine

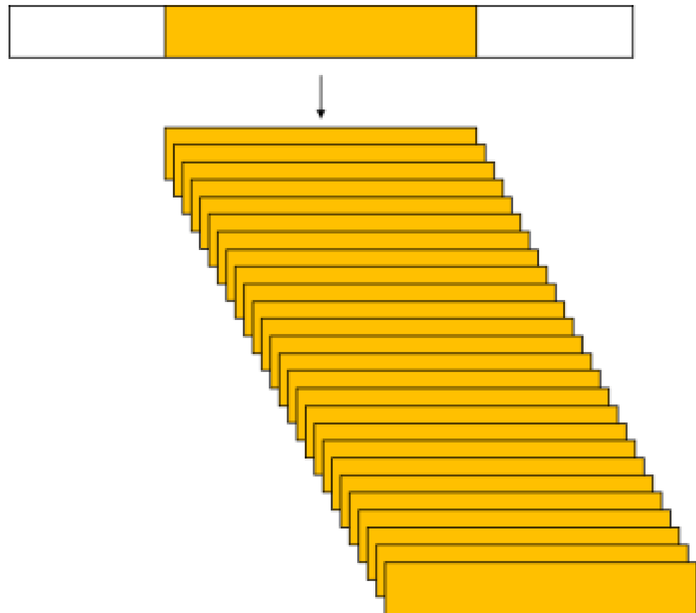
- Genetic variation → RFLP can detect those variations but it's very slow.
  - STR, VNTR, SNPs, and mutations.
- Minute amounts of genetic material
  - Dinosaurs and early humans, → We don't have enough genetic material to perform regular techniques.
- Identification of organisms (e.g. infectious agents)

If their amount is little, we cannot detect them easily.

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.



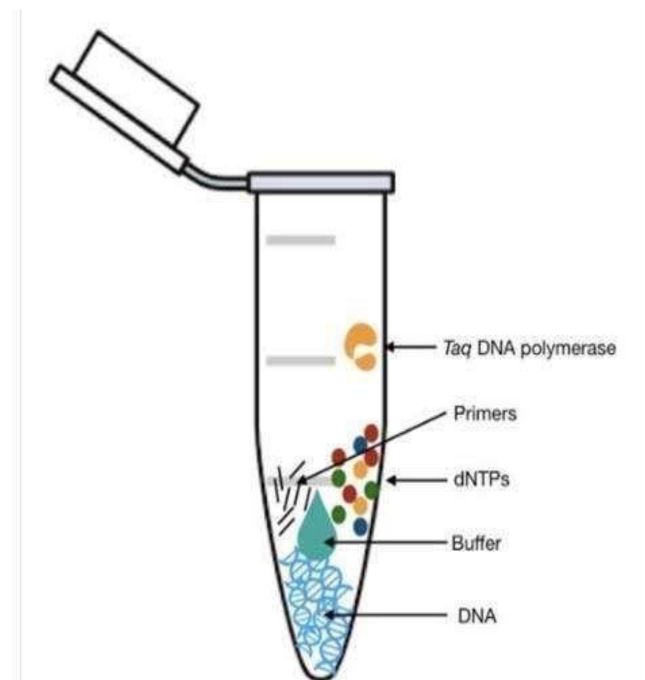
Components of PCR reaction

- The DNA template, which needs to be amplified.
- 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 (recall primase in L5).

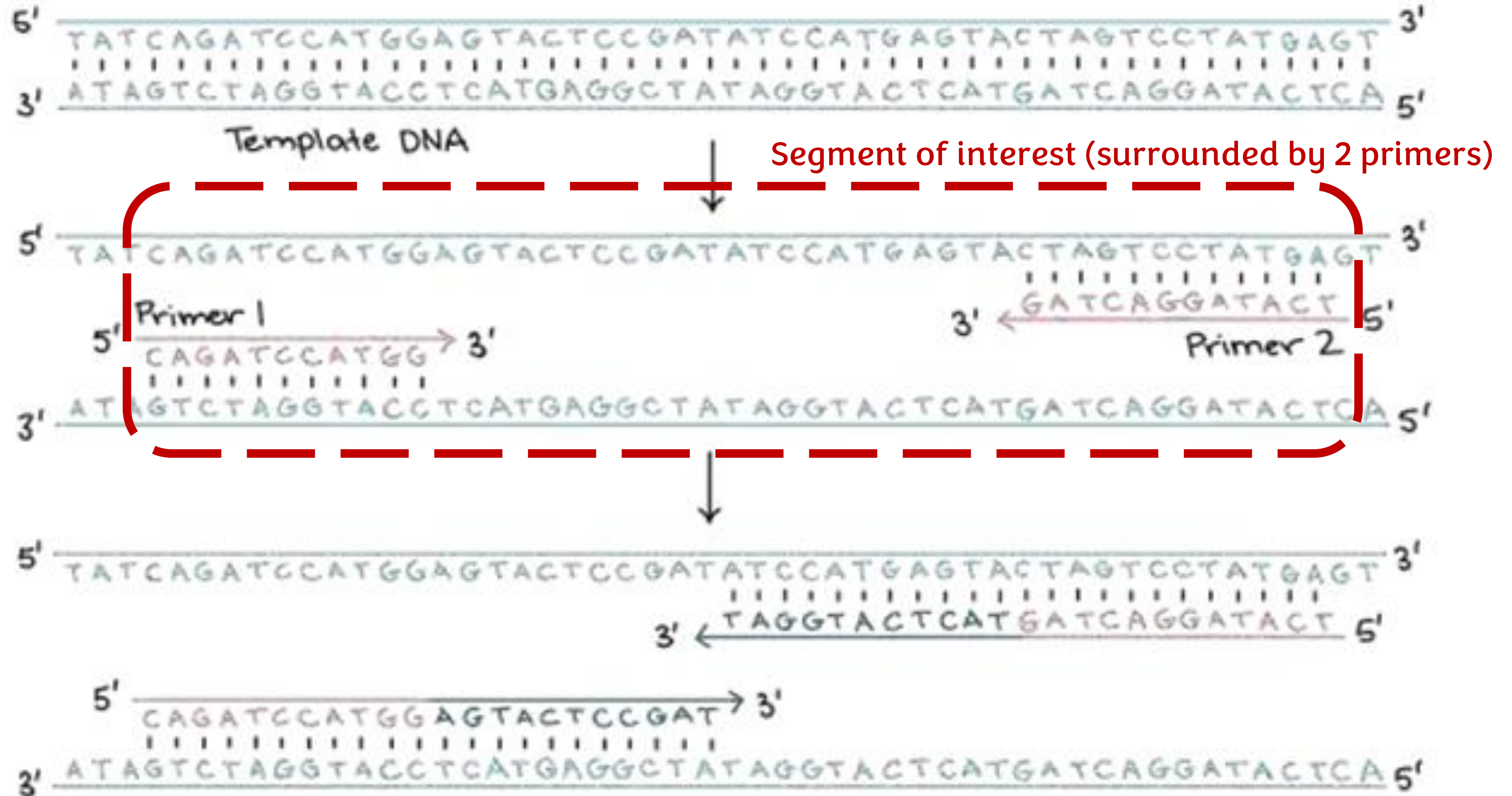
A question may pop up, why do cells use RNA primers??
It's unknown, but scientist thinks that life started with RNA due to its dual rule: genetic material and enzyme.

- All four deoxyribonucleoside triphosphates,
- A heat-stable DNA polymerase



Components of PCR reaction

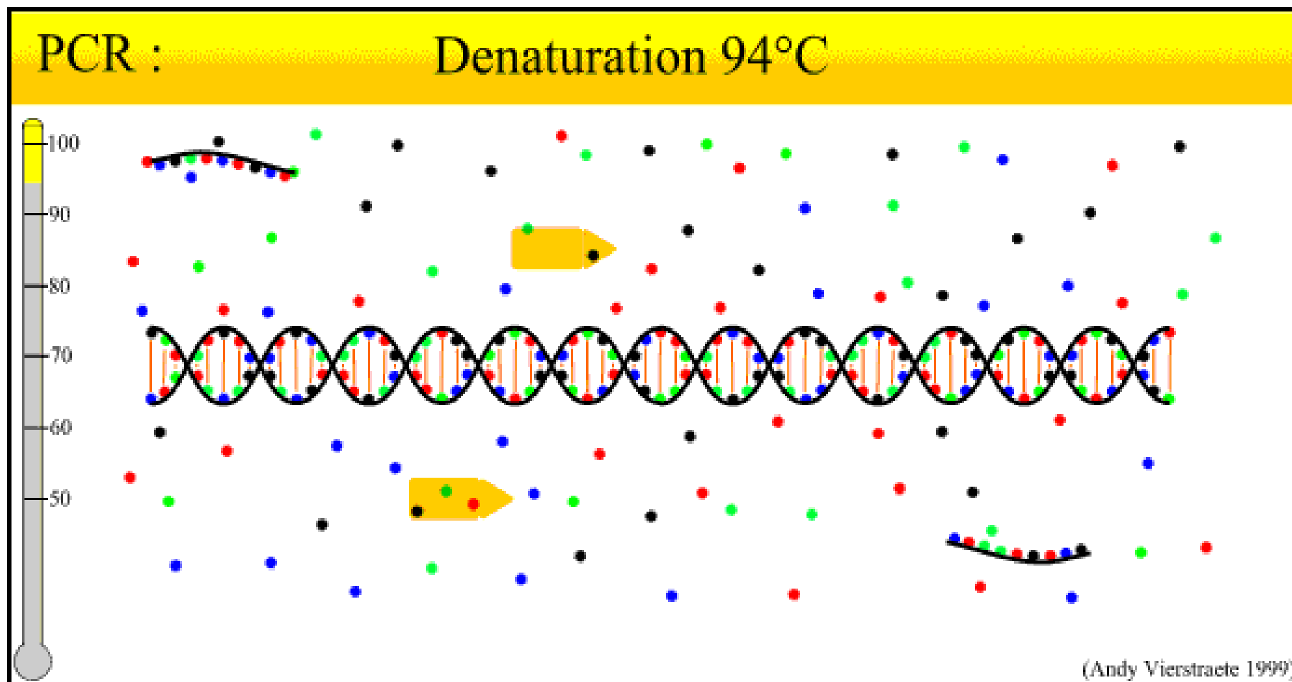
Always from 5’ to 3’
On both directions



The PCR cycles

Those PCR cycles are repeated around 30 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.



The doctor advises us to watch animations on the process for further understanding.

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

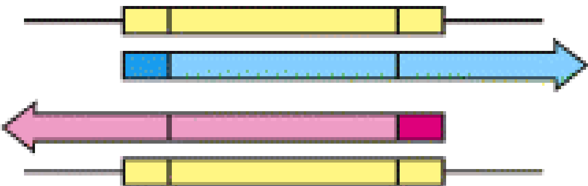
FIRST CYCLE BEGINS

1st step ((denaturation))

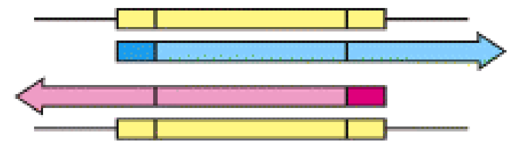
Add excess primers
Heat to separate
Cool

2nd step ((annealing))

Add heat-stable DNA polymerase
Synthesize new DNA

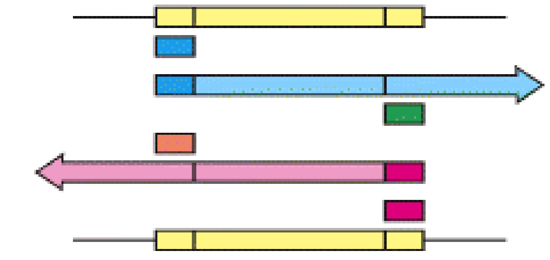


3rd step ((synthesis))

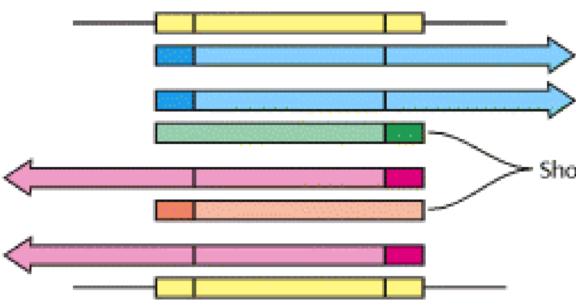


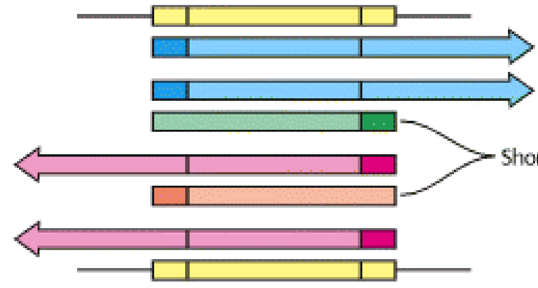
SECOND CYCLE BEGINS

Heat to separate
Cool
Excess primers still present



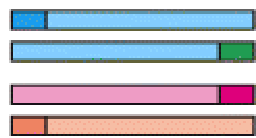
Heat-stable DNA polymerase still present
DNA synthesis continues





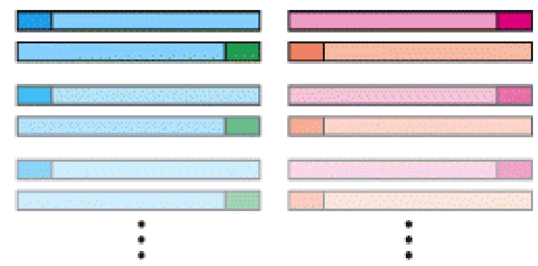
THIRD CYCLE BEGINS

Heat, anneal primers, extend



The short strands, representing the target sequence, are amplified exponentially.

SUBSEQUENT CYCLES



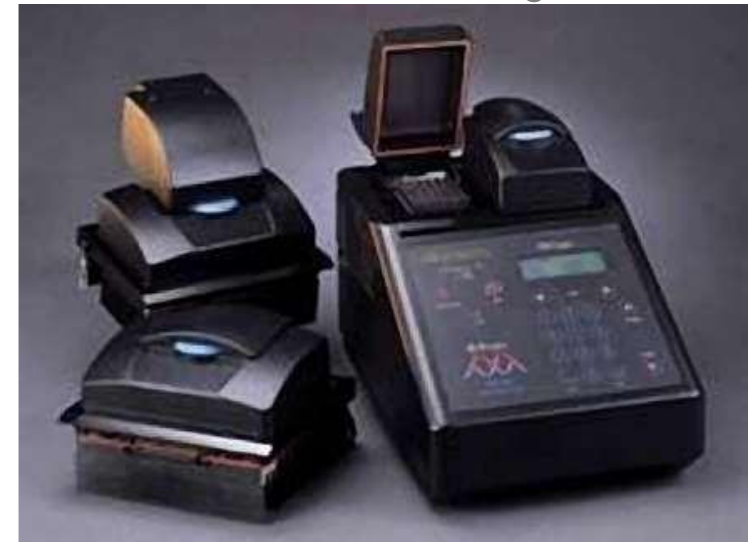
The DNA polymerase

- 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.

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 one hour.

Detection of DNA fragments

This is done after PCR

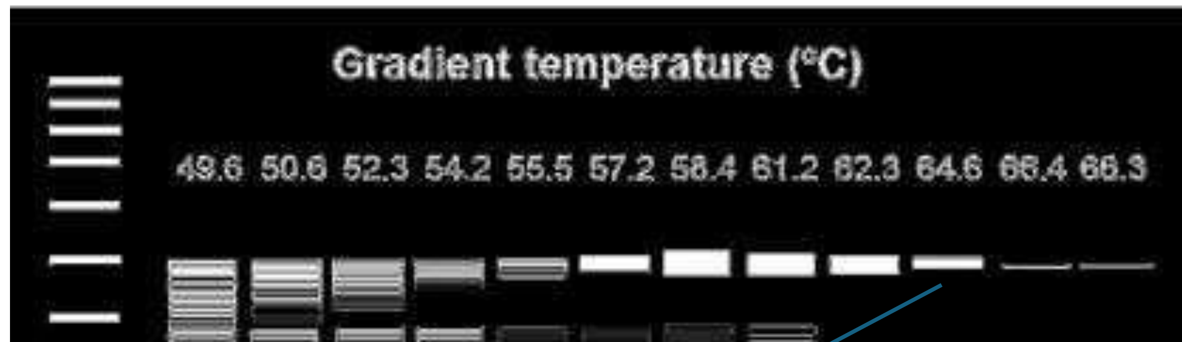
- This DNA fragment can be easily visualized as a discrete band of a specific size by agarose gel electrophoresis.

Recall what we took in electrophoresis

Notice that at the end of any PCR reaction we have very specifically <u>one product</u>

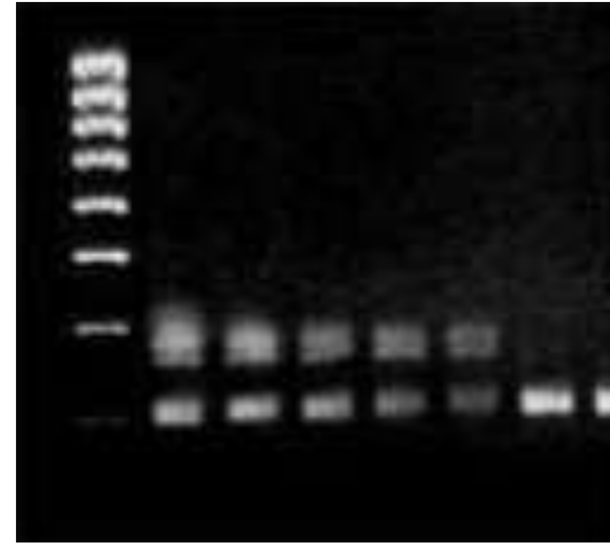
Importance of primers

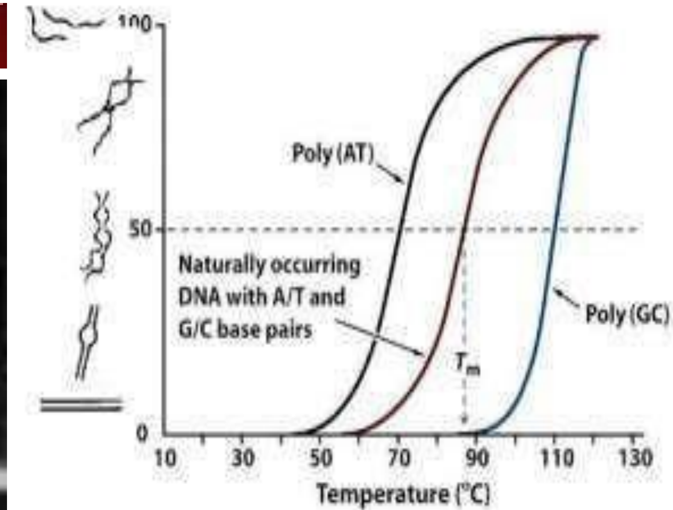
- The specificity of amplification depends on the specificity of the primers to not recognize and bind to sequences other than the intended target DNA sequences.
- How can you prevent it?
- How can you take advantage of it?



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

Annealing temperature





The annealing temperature is the only variable one (50 – 70) depending on the primer's binding to DNA template, which in it's turn depends on Tm (melting temperature).

Imperfect hybridization can take place in primer annealing when we decrease the temperature; as a result, non-specific amplification occurs. By increasing the temperature, you'll have more specific ((accurate)) results. On the other hand, if we increase the temperature beyond the limit, primers will start losing their ability to bind (we have passed the Tm).

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

→ Like in detecting and classifying infectious agents.

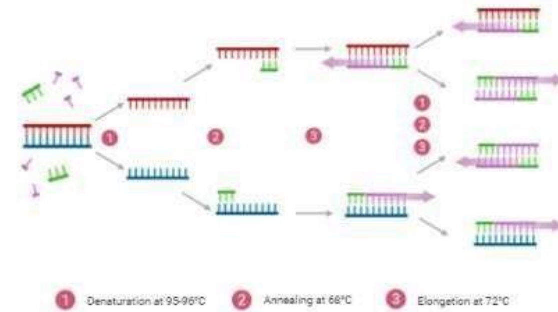
- Mutagenesis → the process of inducing changes or mutations in the DNA of an organism.
- Molecular archaeology → Study ancient biological organisms.

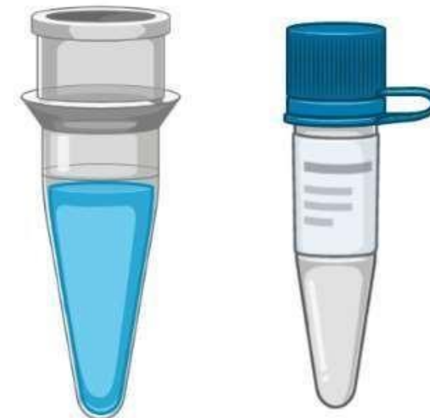
You do <u>not</u> need to know all of these, but only know the ones in the slides next.

Types of PCR with definition and uses

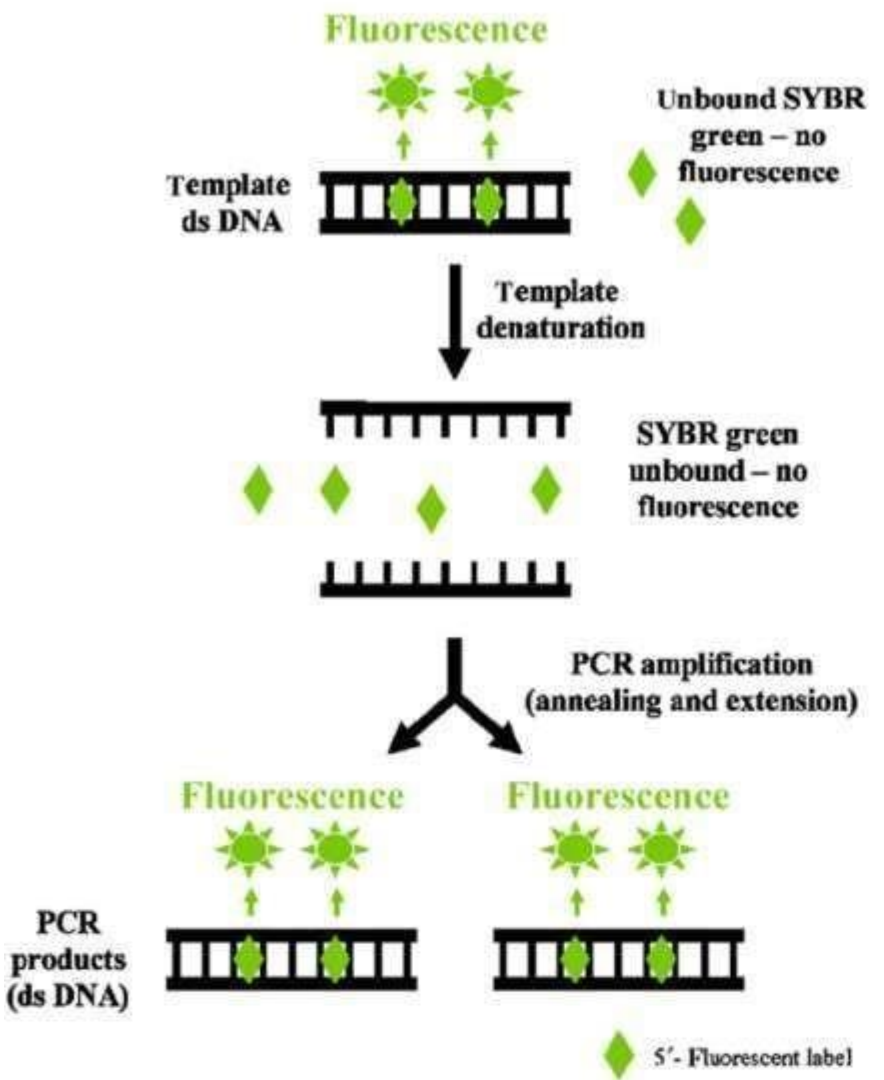
1. AFLP PCR
2. Allele-specific PCR
3. Alu PCR
4. Assembly PCR
5. Asymmetric PCR
6. COLD PCR
7. Colony PCR
8. Conventional PCR
9. Digital PCR (dPCR)
10. Fast-cycling PCR
11. High-fidelity PCR
12. Hot-start PCR
13. In situ PCR
14. Intersequence-specific (ISSR) PCR
15. Inverse PCR
16. LATE (linear after 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

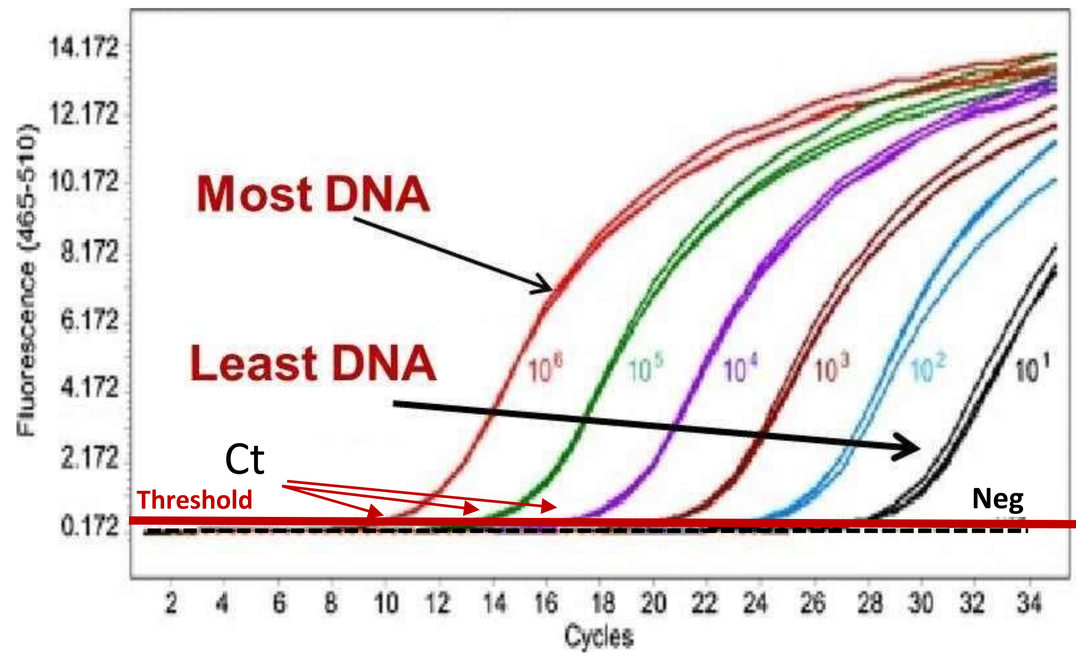




Real-time quantitative PCR (qPCR)



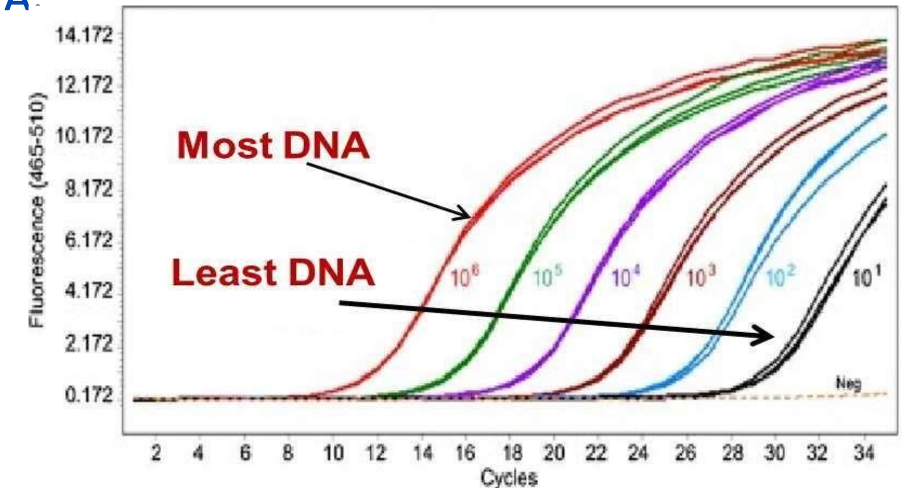
- SYBR green binds to double-stranded DNA and fluoresces only when bound.
- A way of relative quantitation of amount of DNA in a sample is by amplifying it in the presence of SYBR green.
- The higher the amount of DNA, the sooner it is detected.
- Threshold cycle (Ct) tells us at which cycle the signal is detected and is a measure of starting amount of DNA.



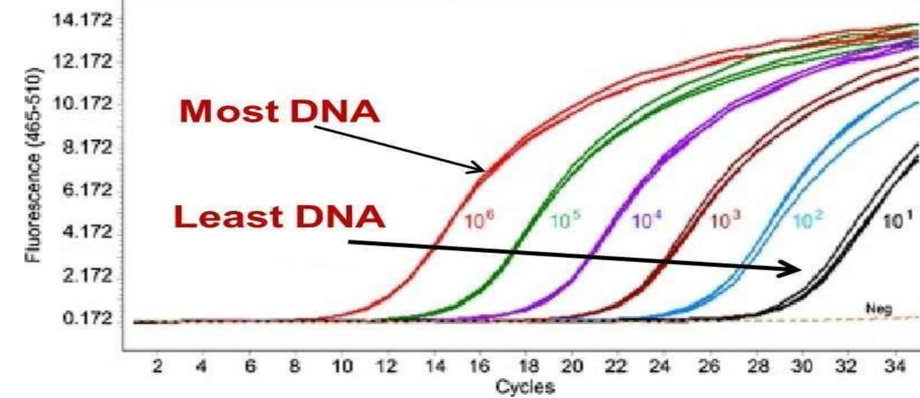
SYBR green is a chemical substance that binds to double-stranded DNA (dsDNA) making the DNA fluorescent.

Real-time quantitative PCR (qPCR). Explanation

- The x-axis represents the PCR cycles, while the y-axis shows the fluorescence intensity. The PCR machine detects fluorescence but has a limitation—it cannot detect very low signals. However, it can detect fluorescence once it reaches a certain level, such as 1,000,000 copies of DNA.
- Now, let's consider two samples:
- 1. A sample with 10 copies of DNA as the starting material.
- 2. Another sample with 100 copies of DNA as the starting material.
- At the end of every PCR cycle, the amount of DNA doubles:
- · The sample with 10 copies doubles to 10, 20, 40, 80, and so on.
- · The sample with 100 copies doubles to 100, 200, 400, 800, and so on.
- The sample with 100 copies will reach 1,000,000 copies first because it started with more DNA. This means the PCR machine will detect the fluorescence from the sample with initially 100 copies earlier than the sample with initially 10 copies.
- When the fluorescence is detected, the PCR machine can determine the starting amount of DNA. For instance:
- · If the signal is detected earlier, it means the sample started with more DNA (e.g., 100 copies).
- · If the signal is detected later, it means the sample started with less DNA (e.g., 10 copies).
- This process allows us to quantify the starting material of DNA in each sample based on when the fluorescence is detected.



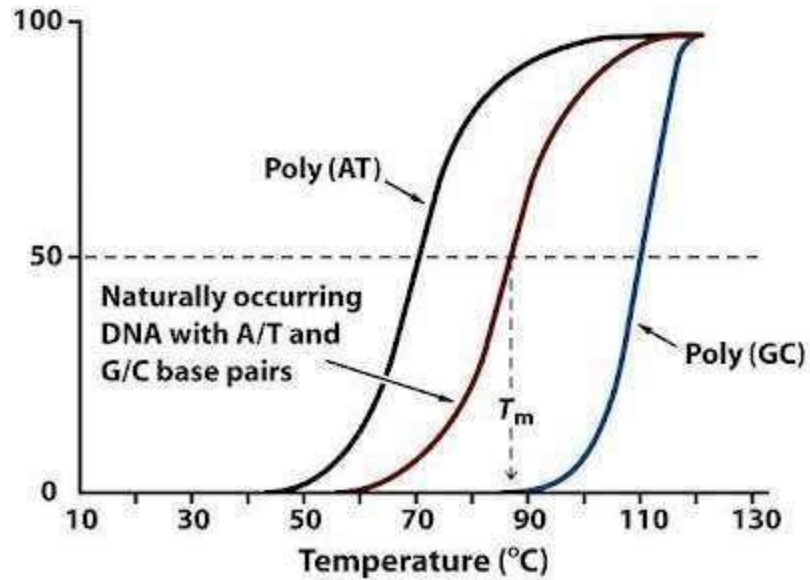
Threshold cycle (Ct) tells us at which cycle the signal is detected and is a measure of starting amount of DNA.

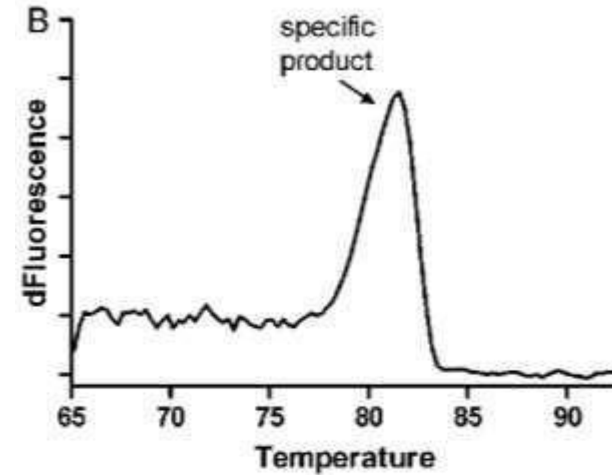


- The point where the signal is first detected is called the Threshold, or Ct (Cycle threshold). If the fluorescence signal surpasses this threshold, DNA is detected by the machinery.

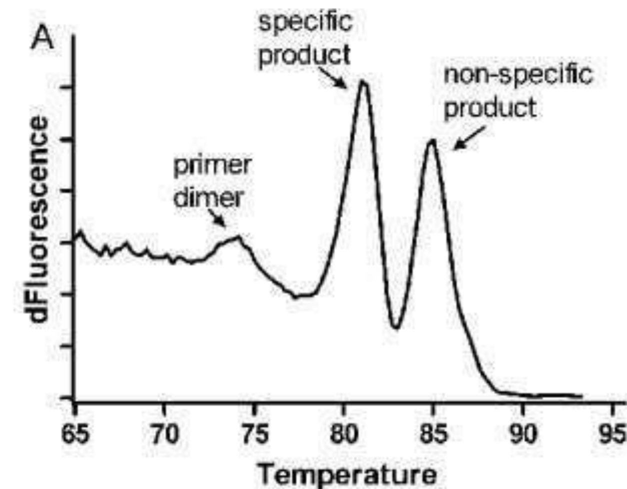
- A higher Ct or threshold indicates less detection, meaning the starting DNA material is lower. Conversely, a lower Ct or threshold means earlier detection, indicating a higher amount of starting DNA material. So, a lower Ct corresponds to more starting DNA, while a higher Ct means there is less starting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

Melting curve analysis of qPCR



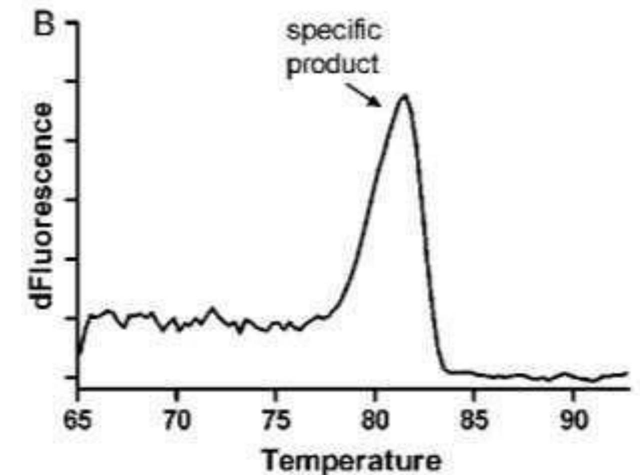


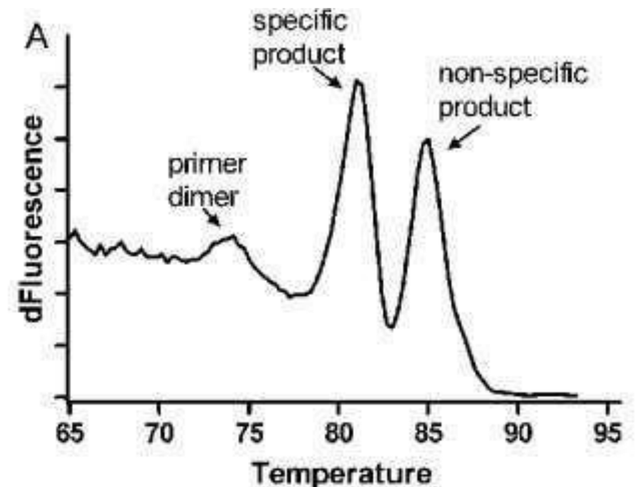
- A melting curve charts the change in fluorescence observed when double-stranded DNA (dsDNA) with incorporated dye molecules dissociates, or “melts” into single-stranded DNA (ssDNA) as the temperature of the reaction is raised.



Melting curve analysis of qPCR

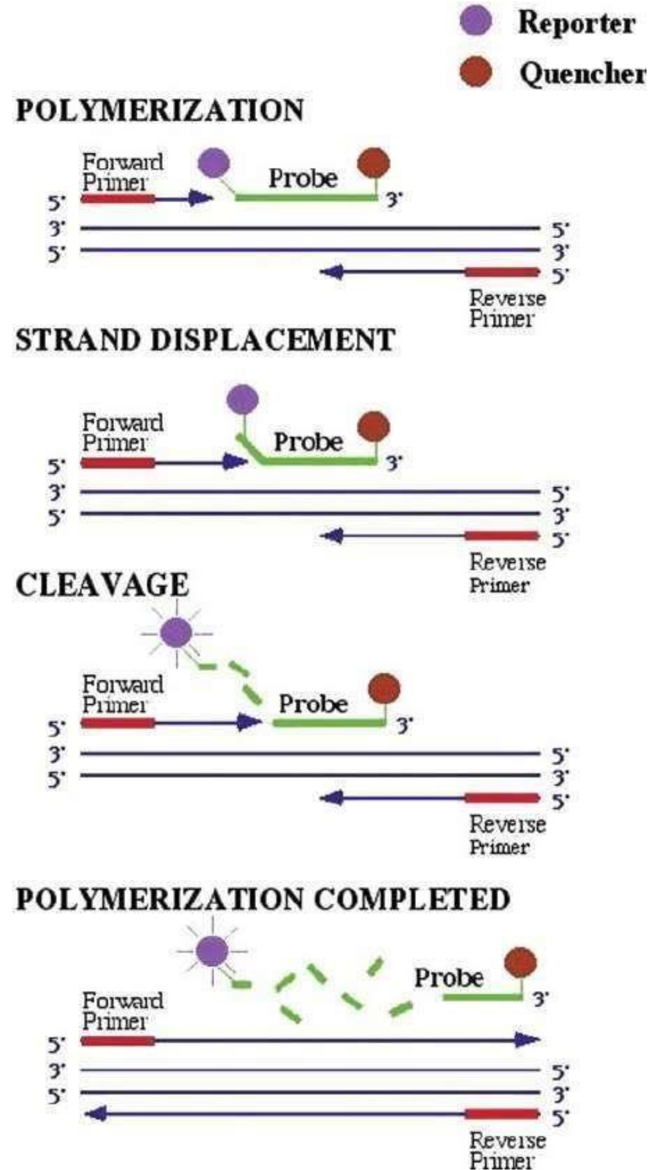
- Sometimes, amplification in PCR can be non-specific. To assess this, we have two methods. The first method involves using SYBR Green. When the sample is placed in the machine, SYBR Green can be used to check how many bands are present, which gives an initial indication of specificity.
- The second method is melting curve analysis, which is done by increasing the temperature at the end of the reaction. When double-stranded DNA is denatured, SYBR Green will be released, and the melting temperature of the DNA products will be observed.
- · If there is only one DNA product, there will be a single melting temperature.
- · If there are two products, they will likely have different sequences and thus different melting temperatures. The instrument detects these differences and displays them as peaks on the melting curve:
- · One peak indicates a single DNA product.
- · Two peaks indicate two DNA products with different melting temperatures. For example, one peak may be at 83°C and another at 85°C.



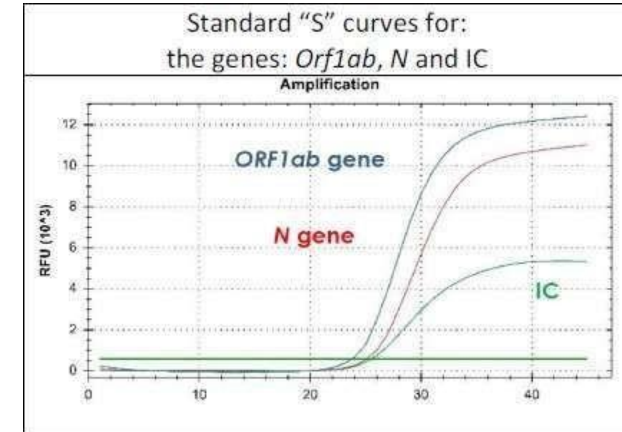


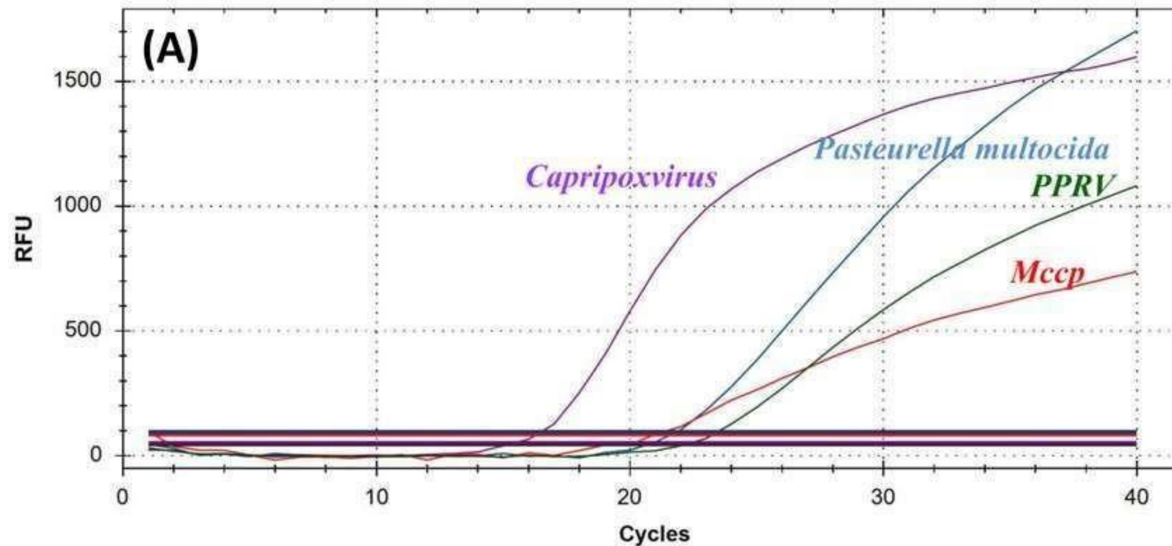
Taqman qPCR

SEE NEXT SLIDE FOR EXPLANATION



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





Taqman qPCR

- A probe in the TaqMan PCR technique is a short DNA sequence (oligonucleotide) specifically designed to bind to a particular region of the target DNA. It contains two chemical groups: a fluorescent reporter which gives a signal at one end and a quencher at the other. When the reporter and quencher are in proximity, the quencher suppresses the reporter's fluorescence. During the PCR reaction, the Taq DNA polymerase encounters the probe while synthesizing the DNA strand and degrades it using its (5' to 3') exonuclease activity. This process separates the reporter from the quencher, allowing the reporter to emit a fluorescence signal. This signal is directly proportional to the amount of target DNA, making the technique highly specific and more effective than SYBR Green, as it eliminates the risk of detecting non-specific products or primer dimers.

TaqMan allows for **Multiplexing** (Complex signaling):
Detecting more than 1 sequence at once using different probes
Each probe (and its signal intensity) tells us information about a specific sequence.

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			

Additional Resources:

رسالة من الفريق العلمي:

اللهم الطف بعبادك المستضعفين

لا تنسوا إخوانكم من الدعاء