Principles of Real Time PCR

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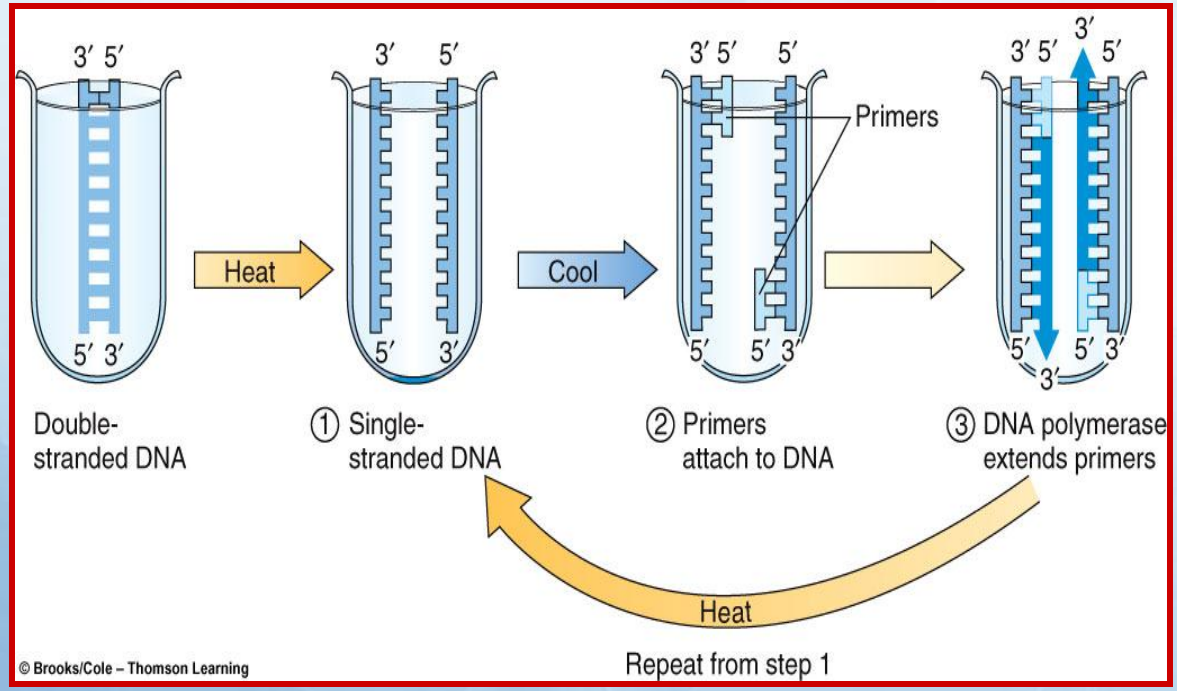
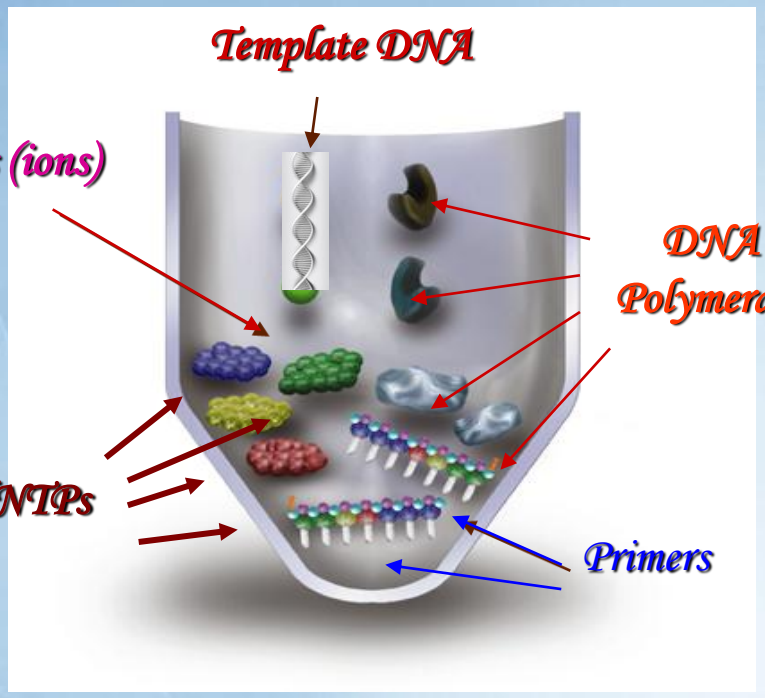
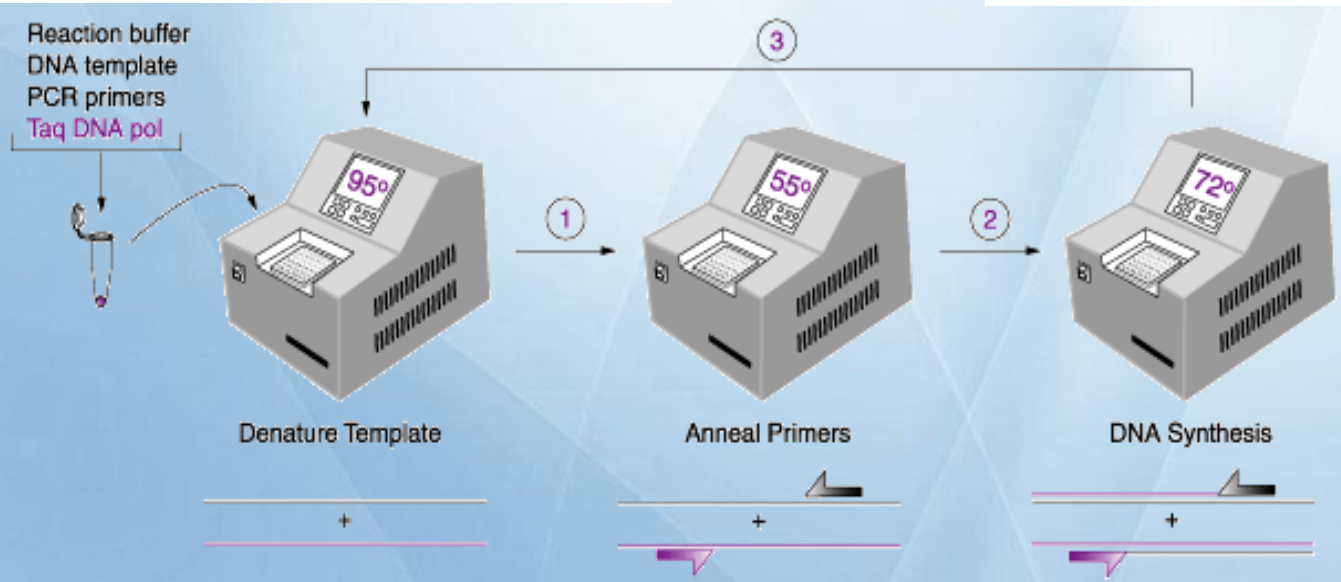
Conventional PCR

- **Each cycle (Round) of PCR contains 3 steps:**
  - **1- Denaturation**
  - **2- Primer annealing**
  - **3- Primer extension**
- **The cycle usually repeated for 25 – 40 times.**



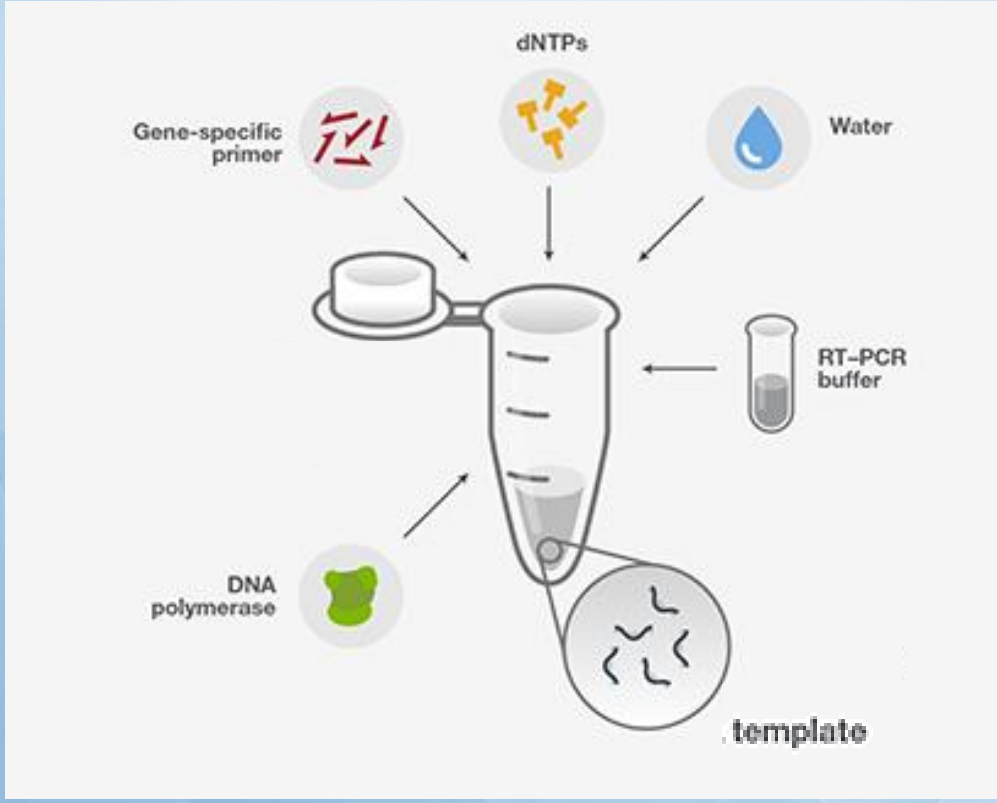
PCR Procedure



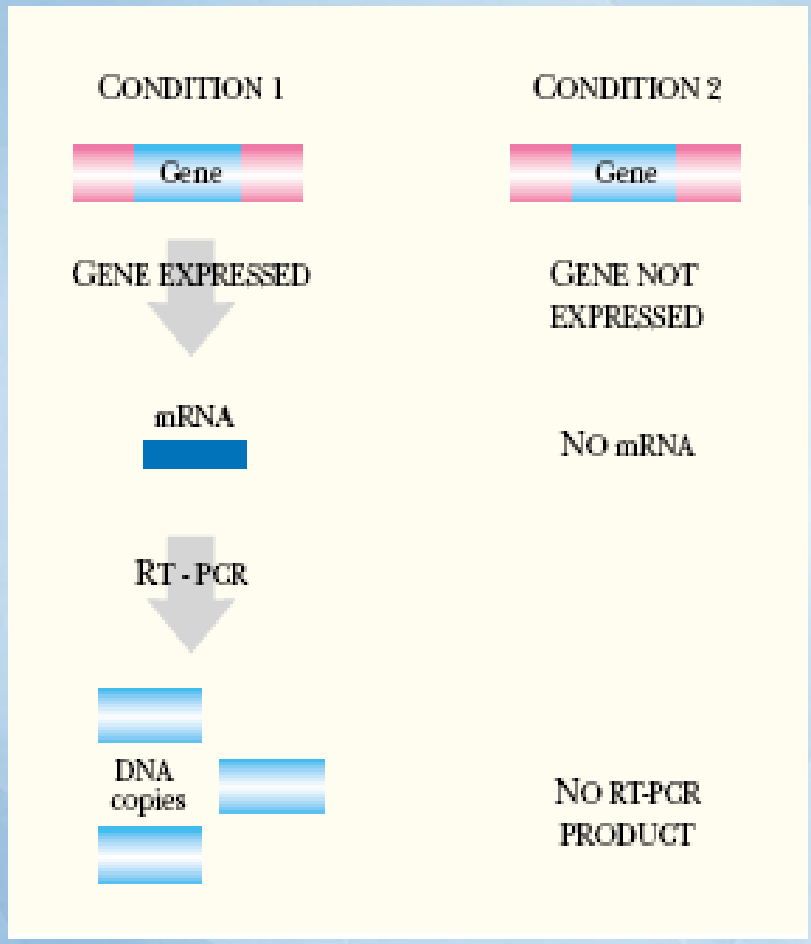


Types of PCR

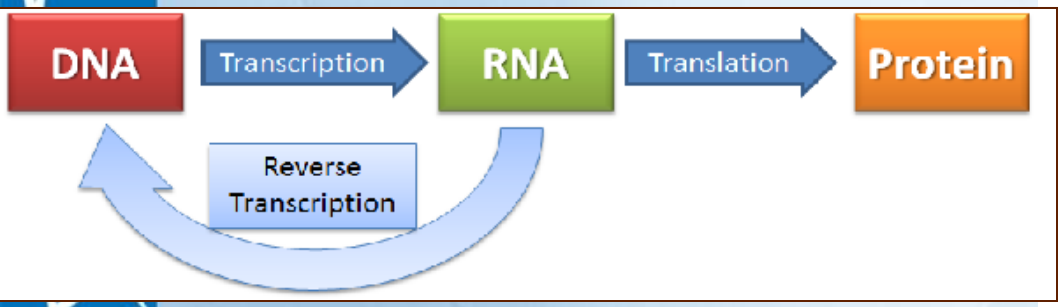
Technique	Abbreviation	Quantitative	Template
Polymerase chain reaction	PCR	No	DNA
Reverse transcriptase polymerase chain reaction	RT-PCR	No	RNA
Real-time polymerase chain reaction	qPCR	Yes	DNA
RT-PCR / qPCR combined technique	qRT-PCR	Yes	RNA

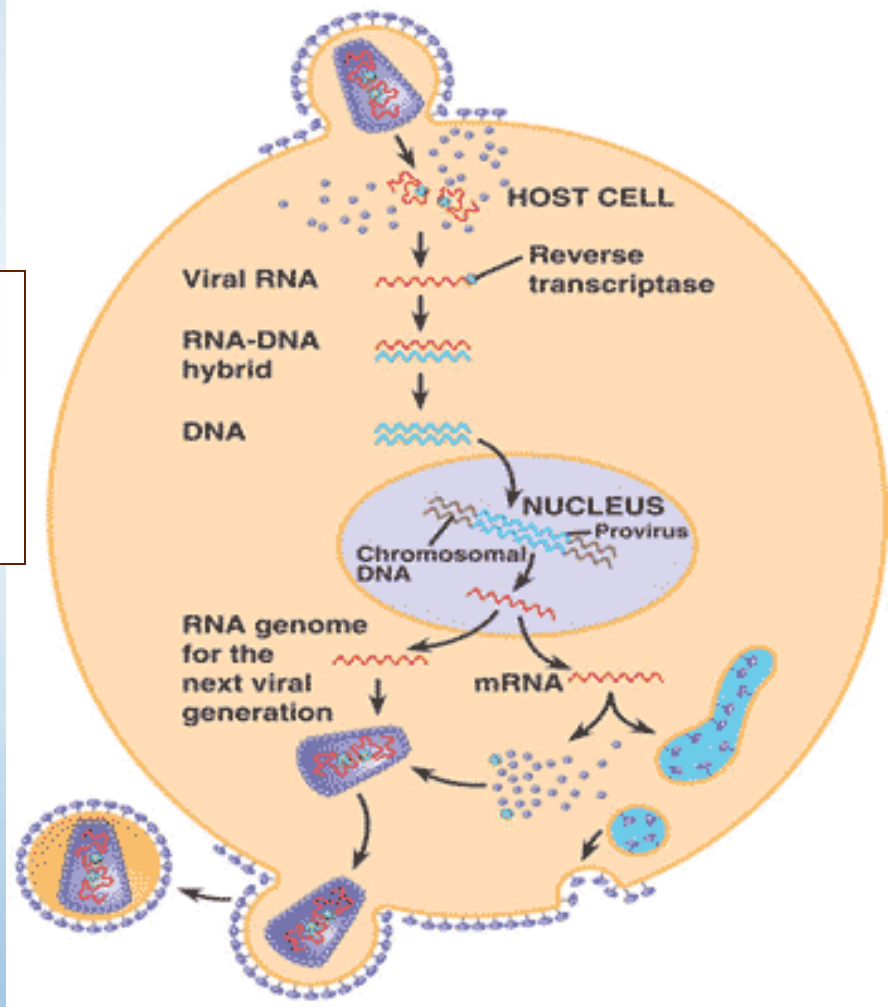


Why RT-PCR?


CONDITION 1
CONDITION 2
Gene
Gene
GENE EXPRESSED
GENE NOT EXPRESSED
mRNA
NO mRNA
RT - PCR
DNA copies
NO RT-PCR PRODUCT

Reverse transcription





Why qPCR?

Technique	Abbreviation	Quantitative	Template
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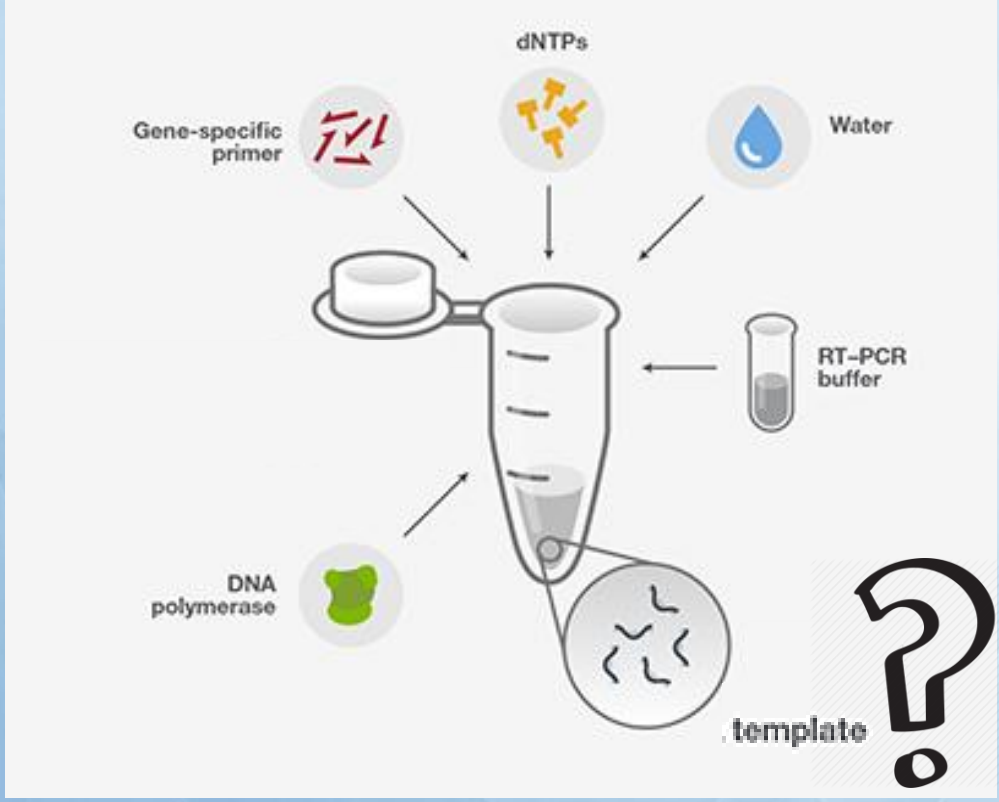
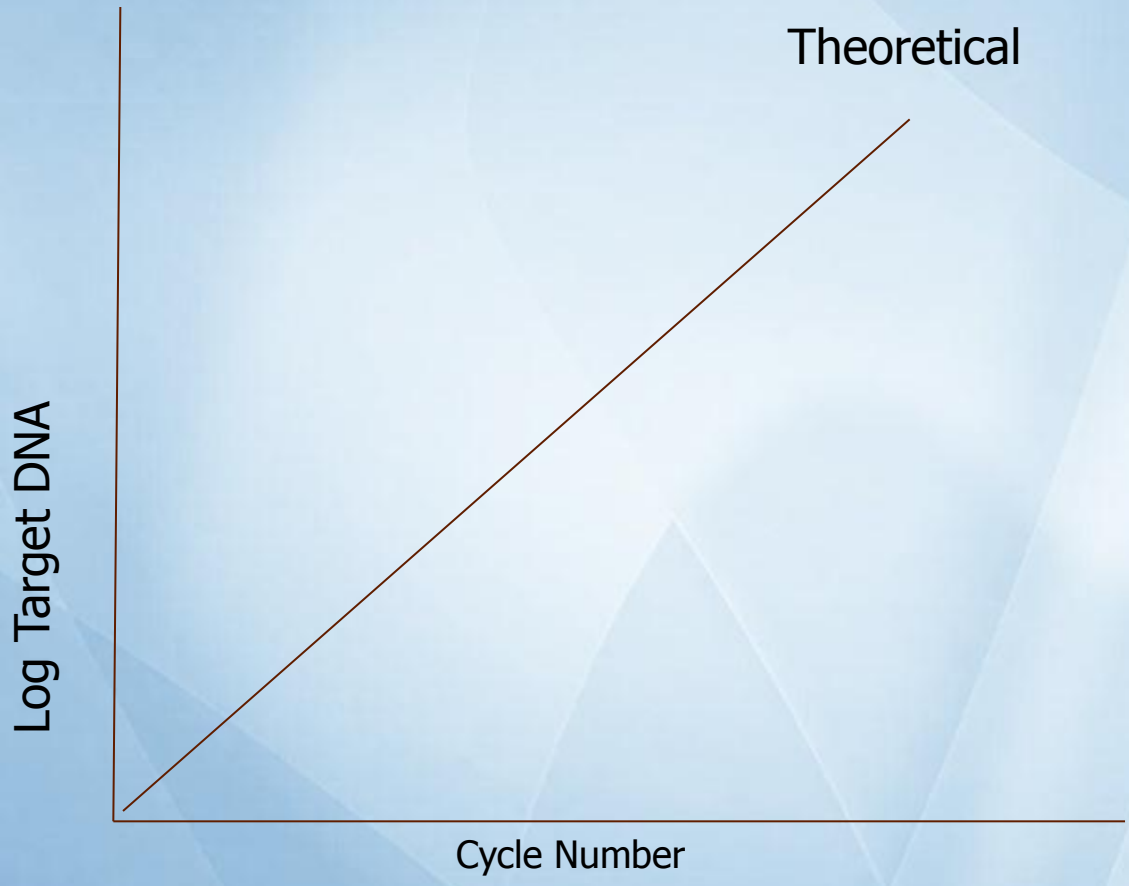
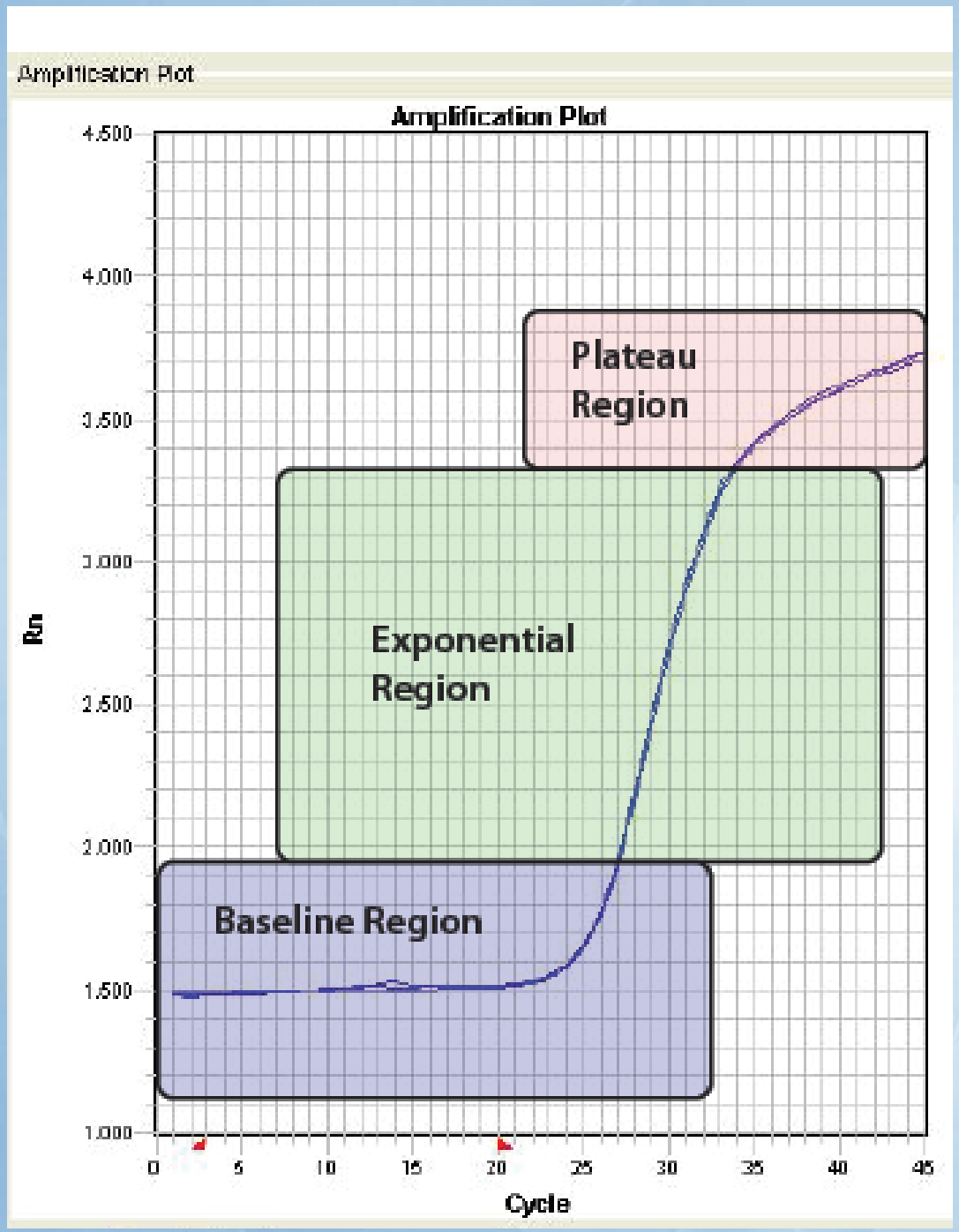


Why qPCR?

- **Gene expression analysis**
  - Cancer research
  - Drug research
- **Disease diagnosis and management**
  - Viral quantification
- **Food testing**
  - Percent GMO food
- **Animal and plant breeding**
  - Gene copy number


Theoretical
Log Target DNA
Cycle Number

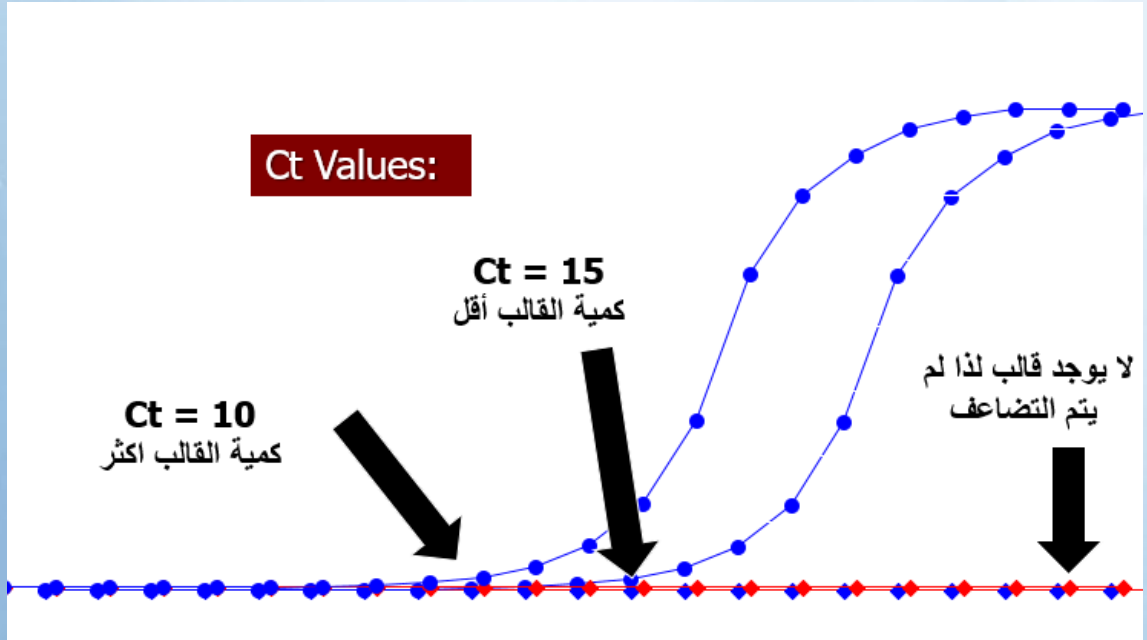

Amplification Plot
Amplification Plot
Rn
Cycle
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
0
5
10
15
20
25
30
35
40
45
Plateau Region
Exponential Region
Baseline Region

How does PCR work?

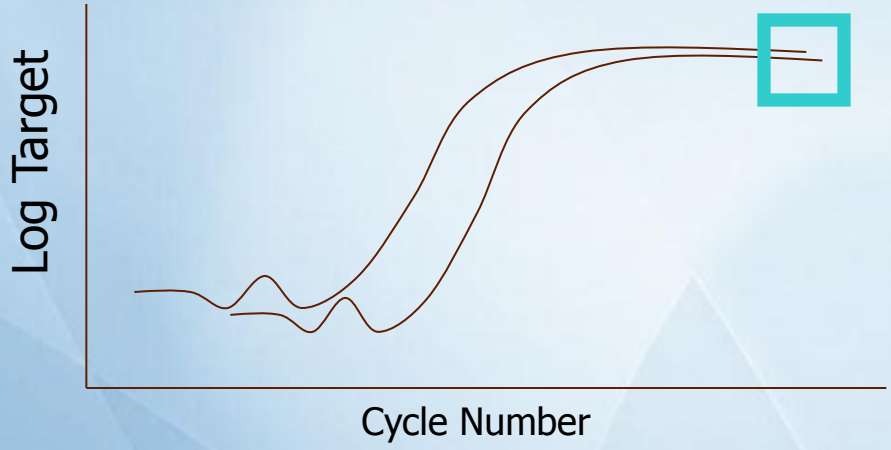
We describe the position of the lines with a value that represents the cycle number where the trace crosses a threshold.

This is called the cycle threshold “Ct Value”.

Ct values are directly related to the starting quantity of DNA, by way of the formula:



Conventional PCR problem

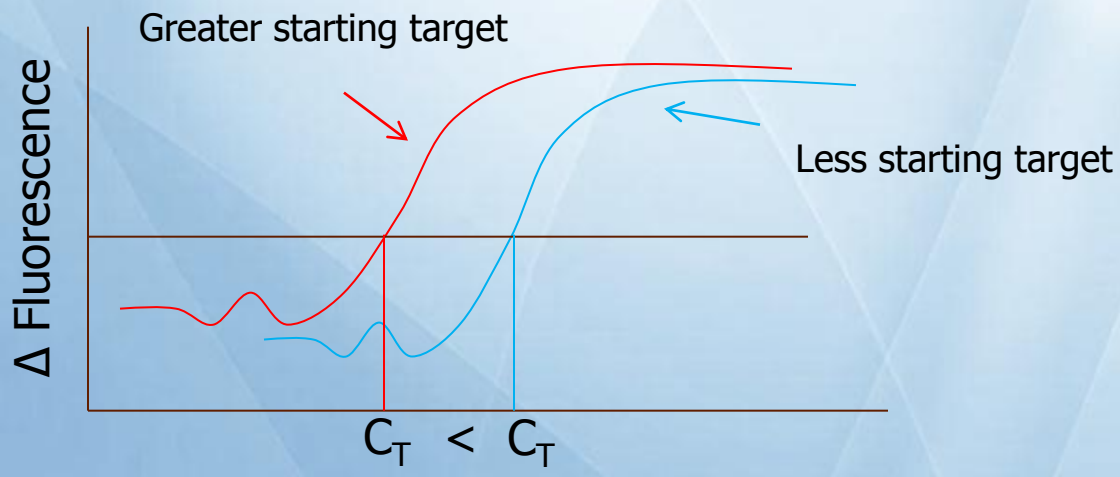


Agarose gel electrophoresis following PCR

Real-Time PCR

- Real-Time PCR a specialized technique that allows a PCR reaction to be visualized "in real time" as the reaction progresses.

- Quantitative PCR relies on the principal that the quantity of target at the start of the reaction is proportional to amount of product produced during the exponential phase



How to measure the PCR product?

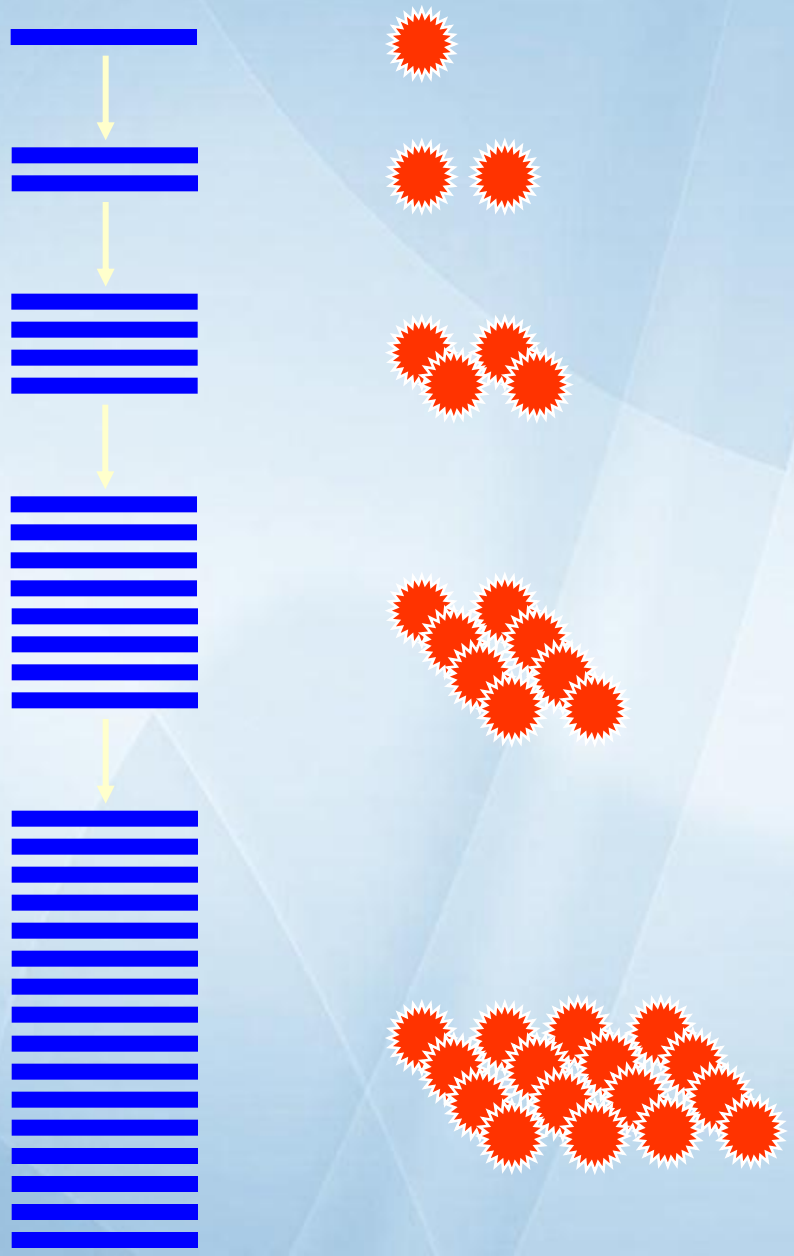
Initial DNA strand

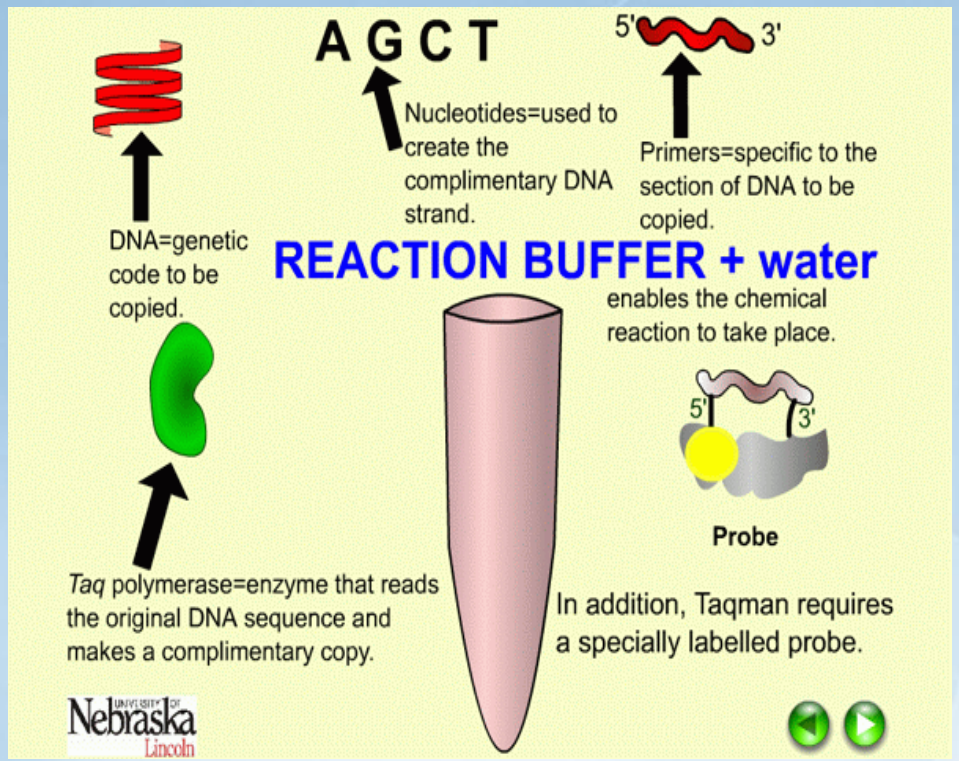
First PCR cycle

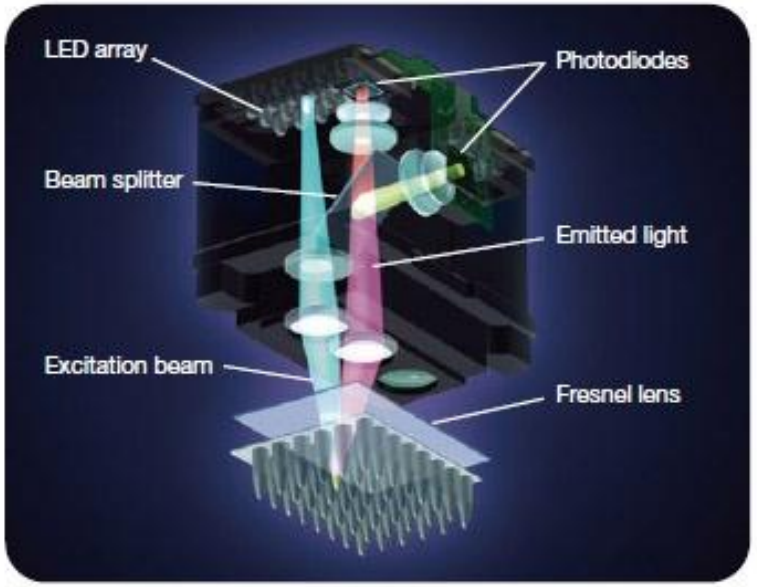
Second PCR cycle

Third PCR cycle

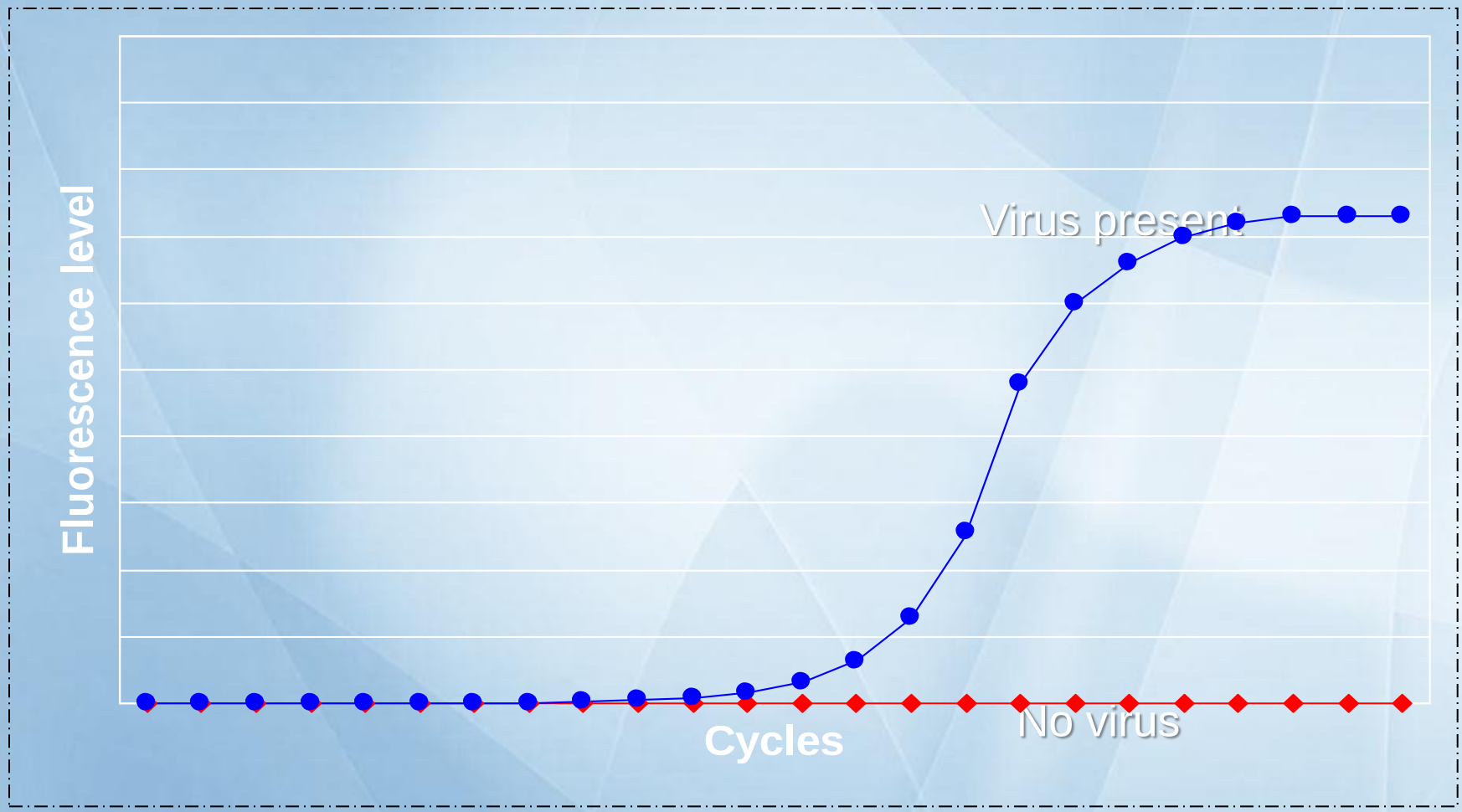
Fourth PCR cycle




A G C T
5'
3'
Nucleotides=used to create the complimentary DNA strand.
Primers=specific to the section of DNA to be copied.
DNA=genetic code to be copied.
REACTION BUFFER + water
enables the chemical reaction to take place.
5'
3'
Probe
Taq polymerase=enzyme that reads the original DNA sequence and makes a complimentary copy.
In addition, Taqman requires a specially labelled probe.
Nebraska
Lincoln


LED array
Photodiodes
Beam splitter
Emitted light
Excitation beam
Fresnel lens

Any increase in fluorescence level can be plotted onto a graph and easily interpreted



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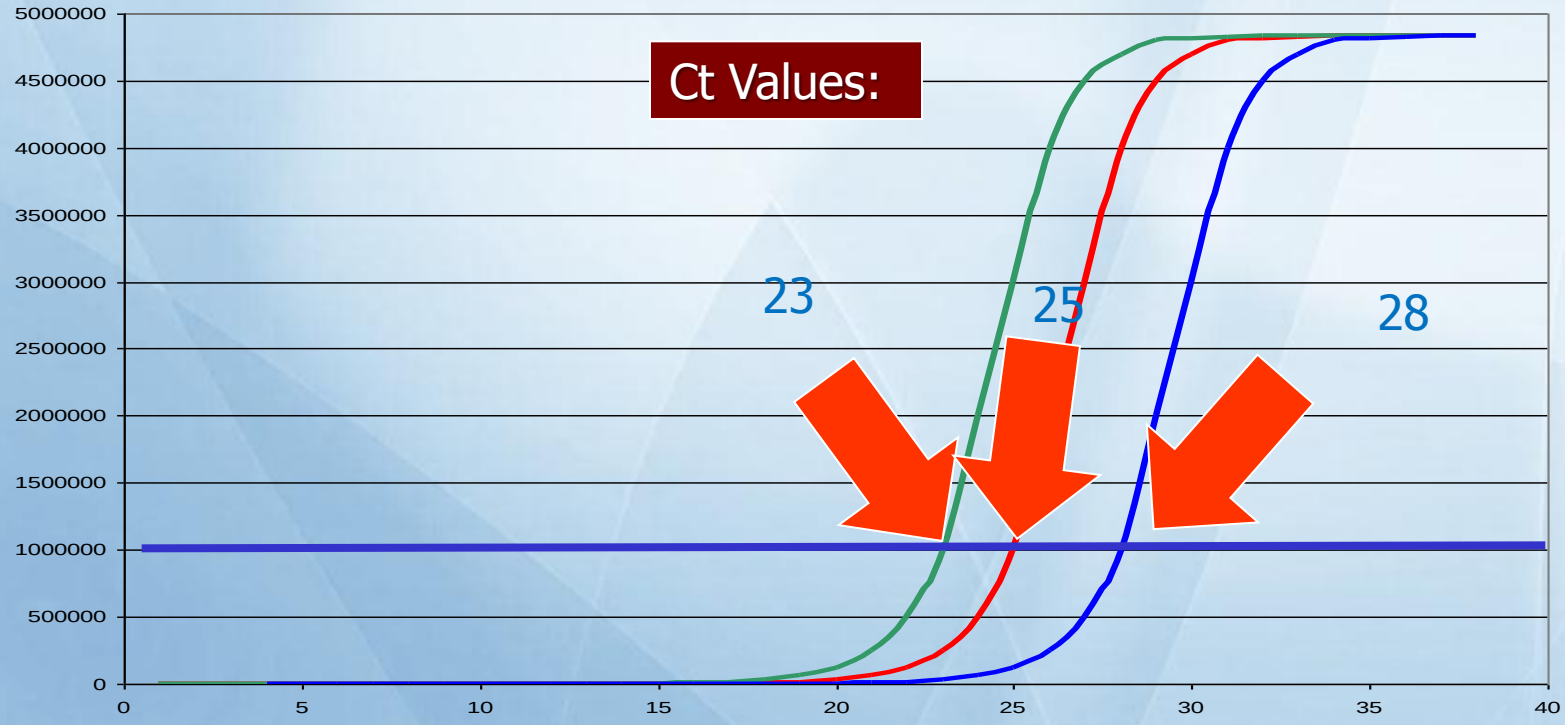
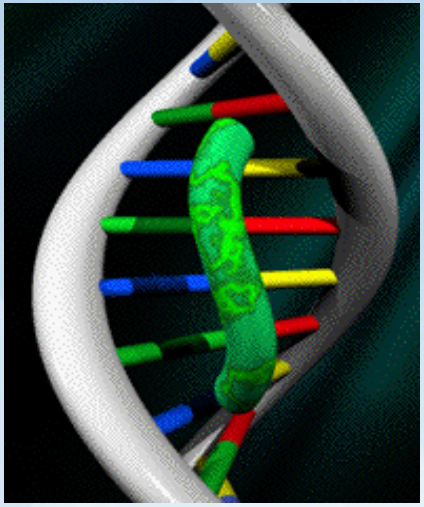
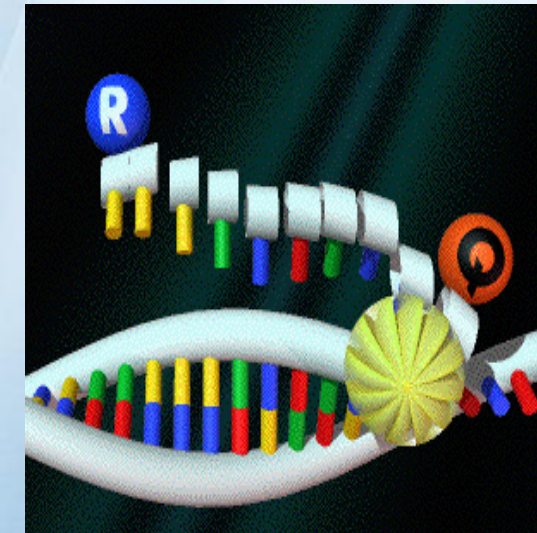


Quantitative PCR – in depth

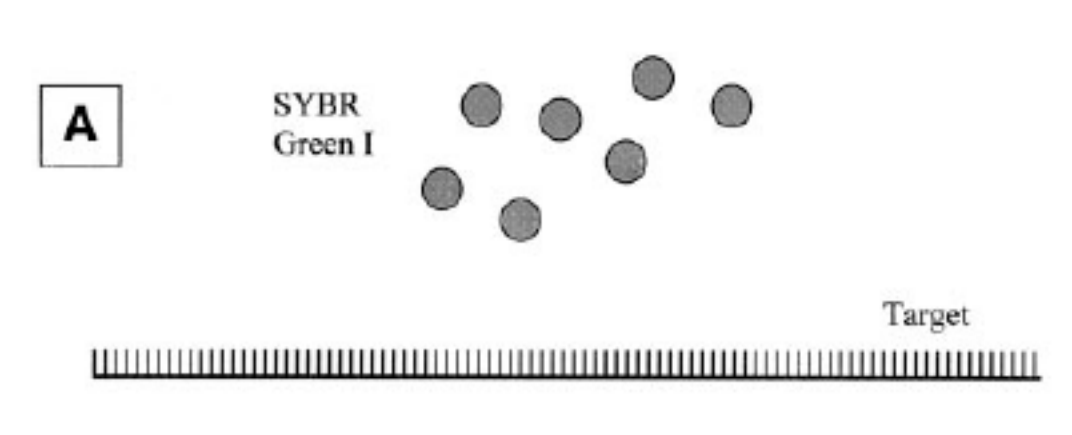
- Major assay types
  - **Hydrolysis probes**
    - Basis of TaqMan® chemistry
    - Uses two primers and an internal hydrolysis probe
    - Most commonly used for fish health diagnostics

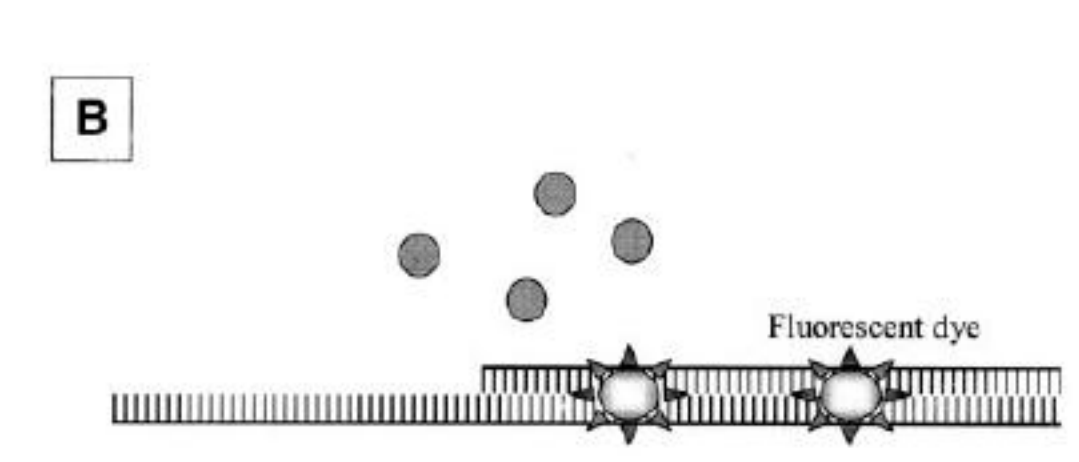


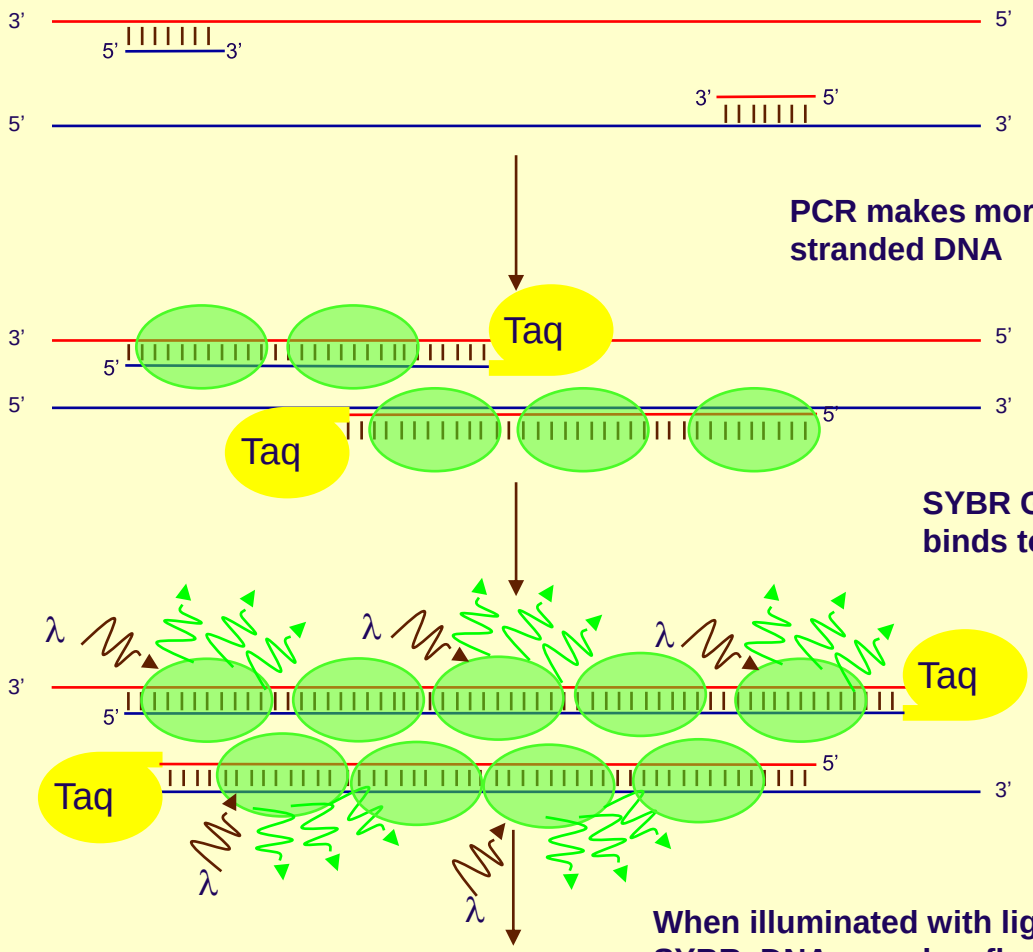
  - **SYBR ® green dye**
    - Increased fluorescence when bound to dsDNA
    - Slightly lower specificity
    - Costs less
    - May not be as sensitive as the 5’ nuclease assays



SYBRGreen

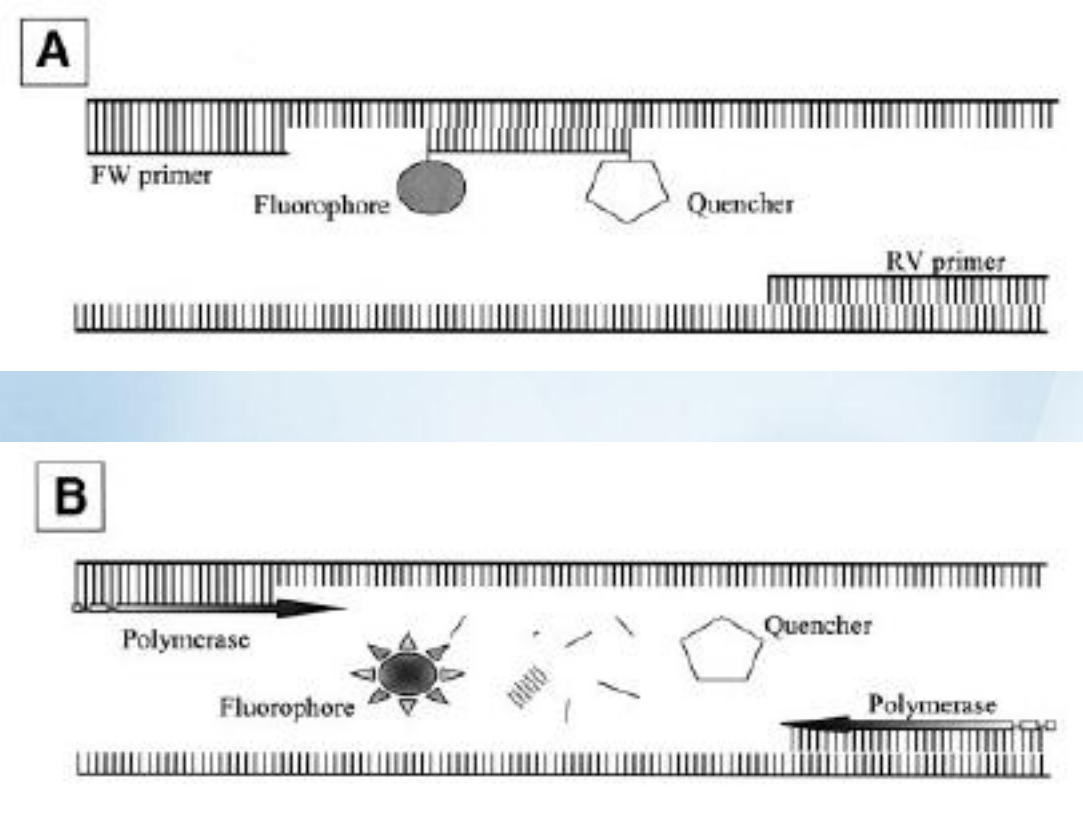




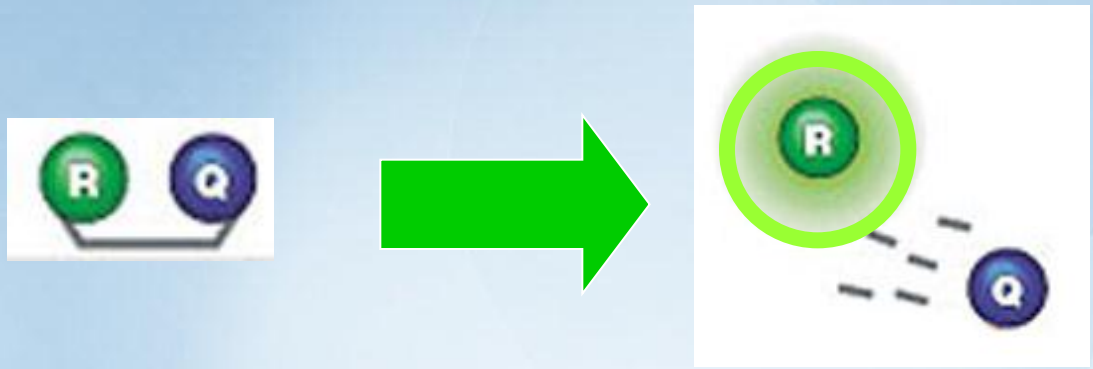

3'
5'
5'
3'
5'
3'
3'
5'
PCR makes more double-stranded DNA
Taq
Taq
SYBR Green dye binds to dsDNA
λ
λ
λ
λ
λ
Taq
Taq
When illuminated with light at 490nm, the SYBR+DNA complex fluoresces at 520nm.

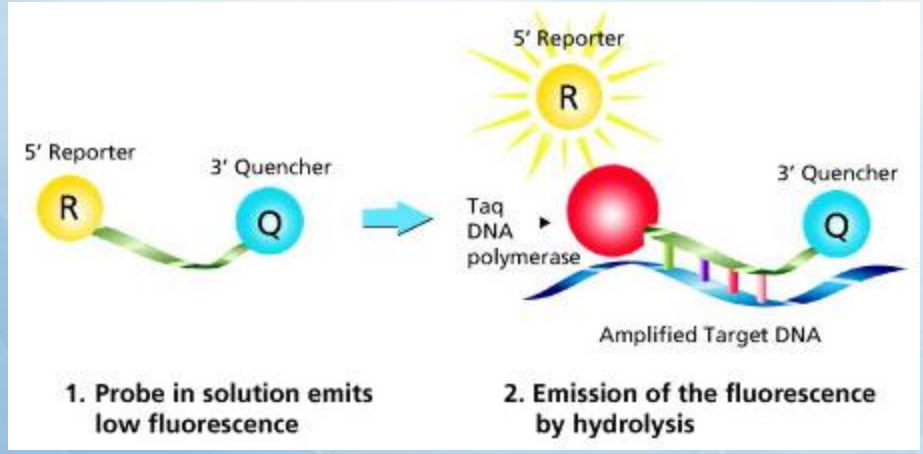
BackMan vs TaqMan

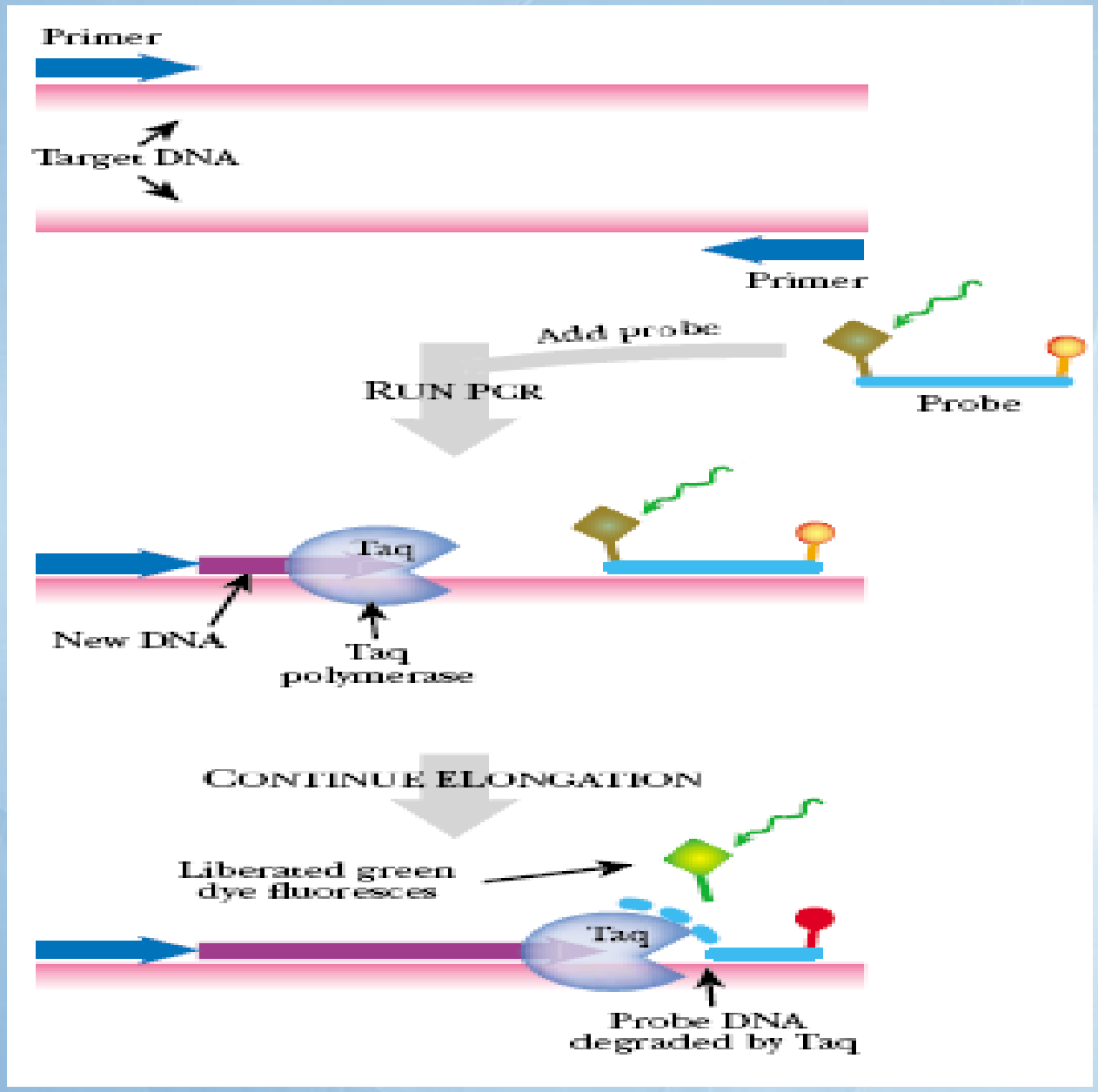
TaqMan

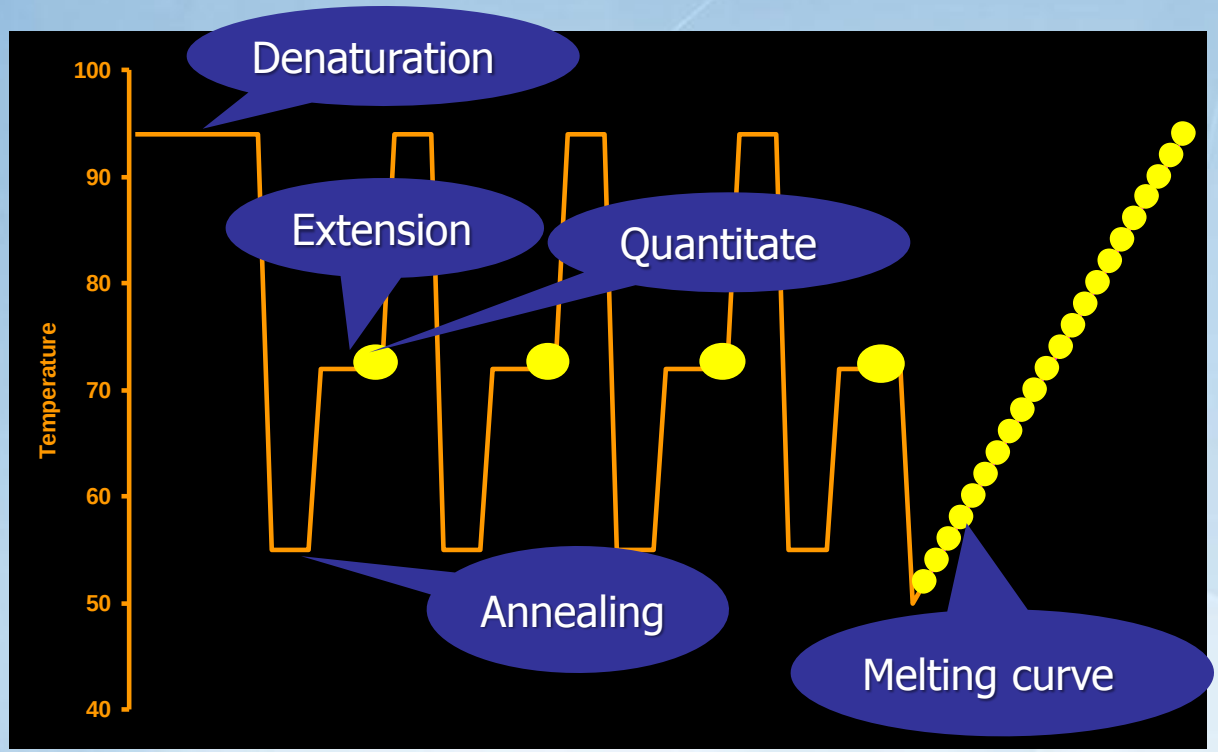

A
FW primer
Fluorophore
Quencher
RV primer
B
Polymerase
Quencher
Fluorophore
Polymerase

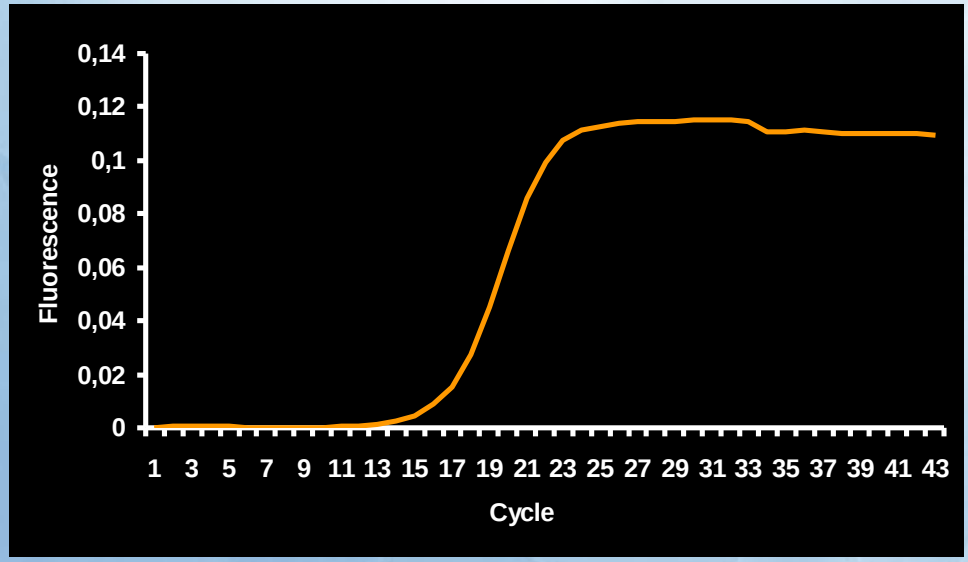
Probe hydrolysis






Primer
Target DNA
Primer
Add probe
Probe
RUN PCR
Taq
New DNA
Taq
polymerase
CONTINUE ELONGATION
Liberated green
dye fluoresces
Taq
Probe DNA
degraded by Taq


Denaturation
Extension
Quantitate
Annealing
Melting curve
Temperature
100
90
80
70
60
50
40


Fluorescence
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43
Cycle

Taqman vs. SYBR Green

TaqMan Probe

Advantages:

- Increased specificity
- Use when the most accurate quantitation of PCR product accumulation is desired.
- Option of detecting multiple genes in the same well (multiplexing).

Disadvantages:

- Relative high cost of labeled probe.

- **SYBR Green**

- **Advantages:**
- Relative low cost of primers.
- No fluorescent-labeled probes required.

- **Disadvantages:**
- Less specific – only primers determine specificity.
- Specific and non-specific double-stranded PCR products generate the same fluorescence signal upon binding SYBR Green I dye.
- Not possible to multiplex multiple gene targets.