Principles of Real Time PCR

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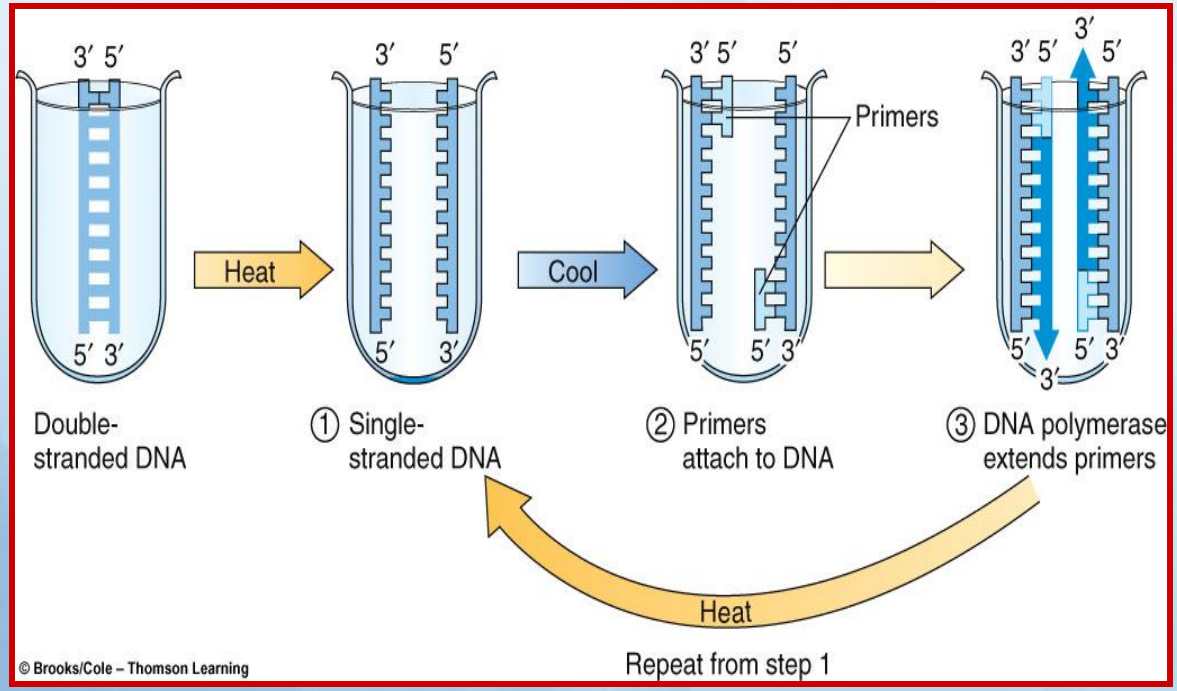
Types of PCR

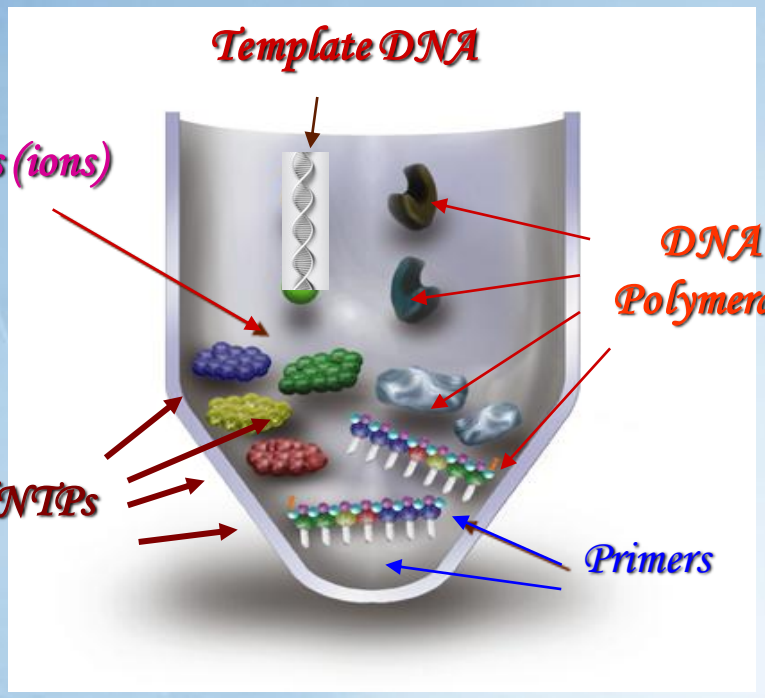
- Standard PCR (conventional)
- RT-PCR (Reverse Transcriptase PCR)
- Real Time PCR (qRT-PCR)

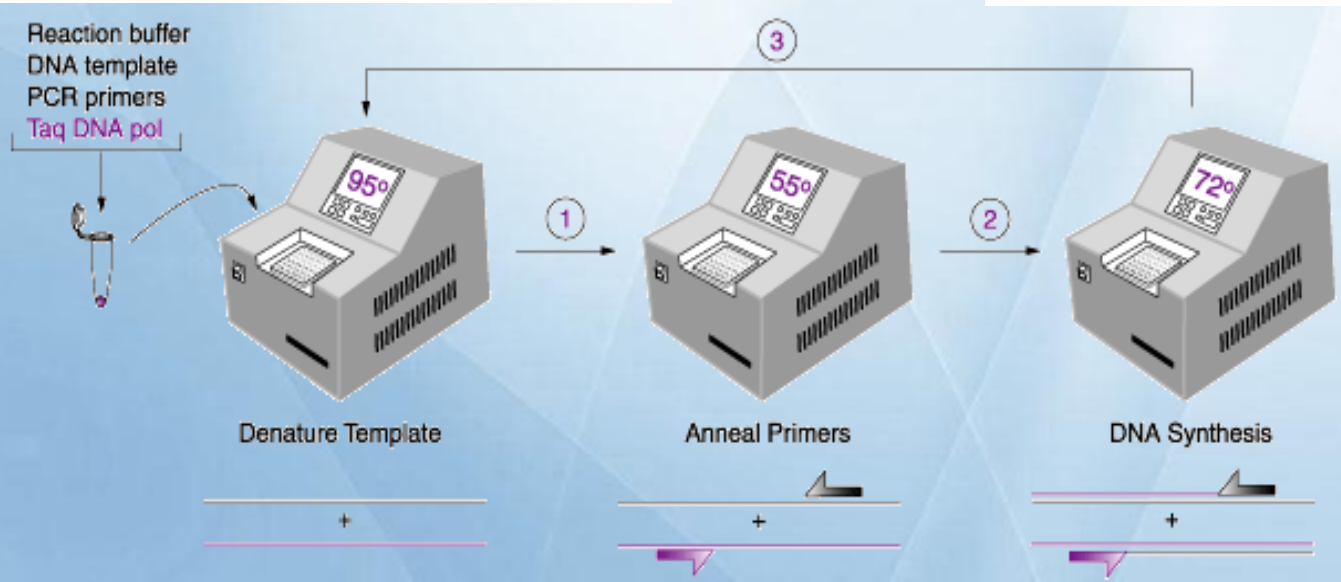
Conventional PCR

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

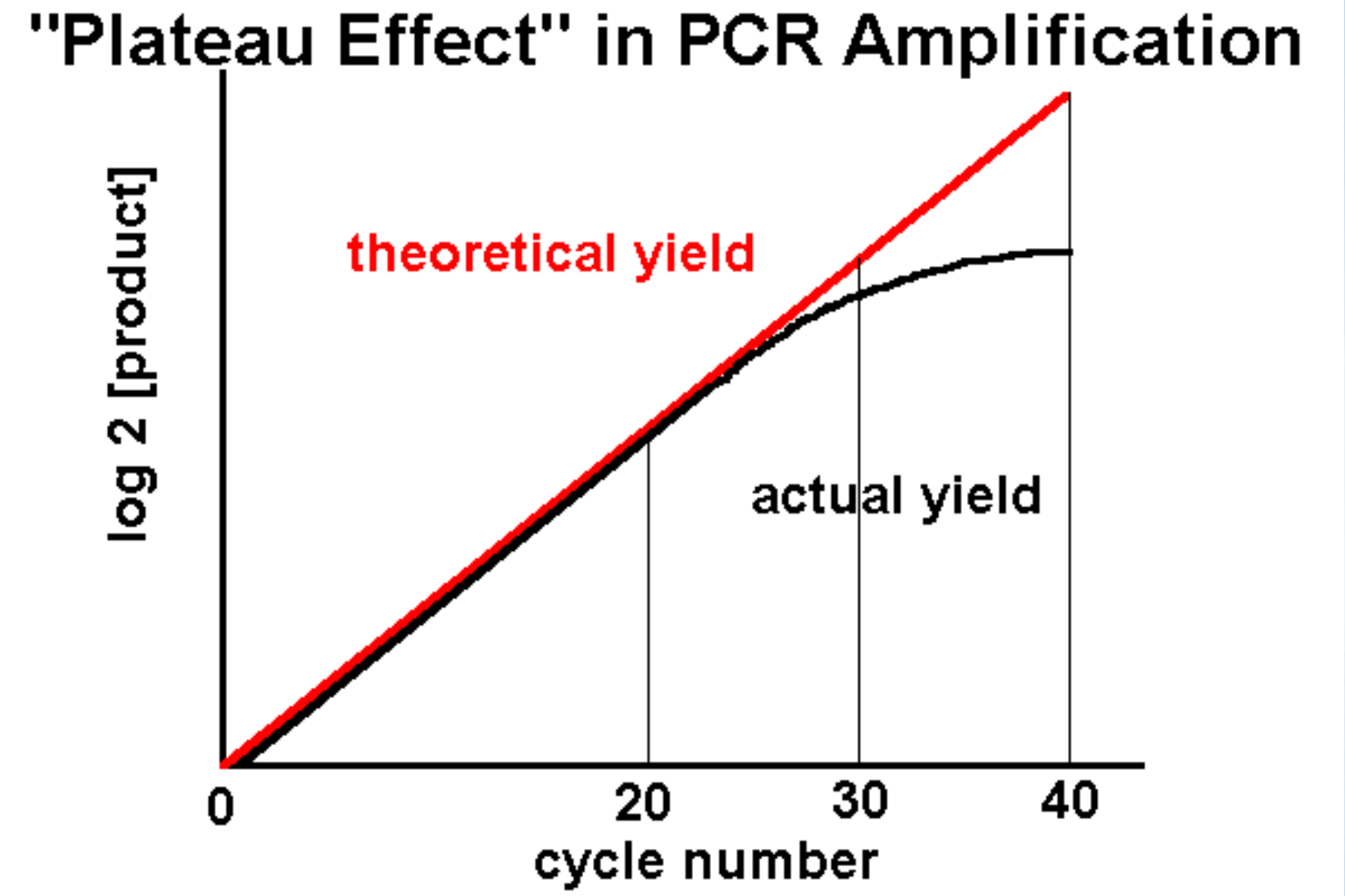


PCR Procedure

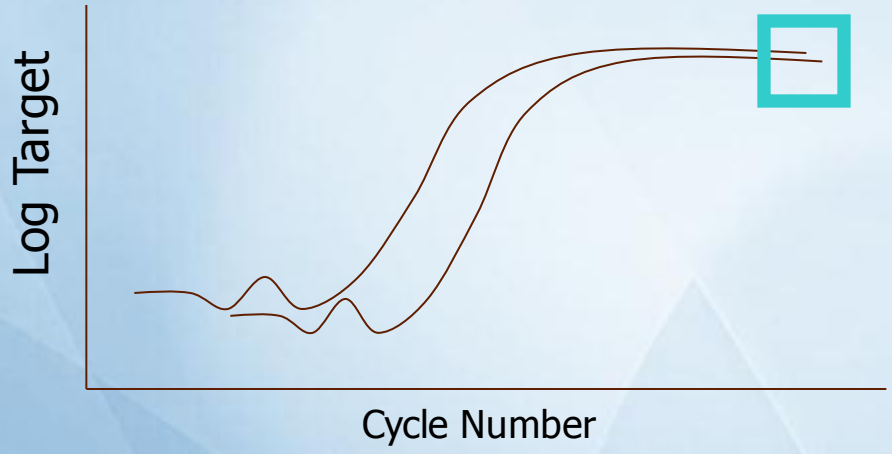




Cycle Number

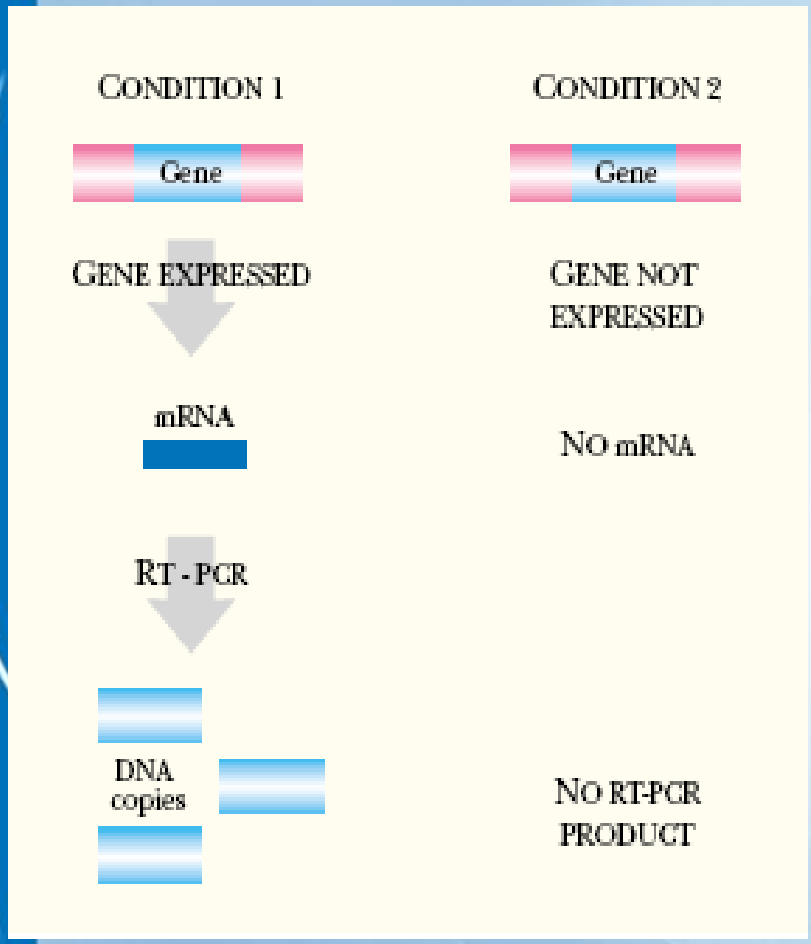


Conventional PCR problem



Agarose gel electrophoresis following PCR

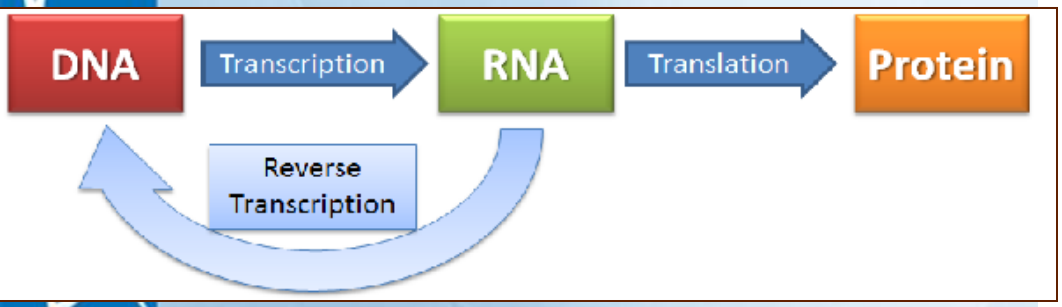
Why qRT-PCR?

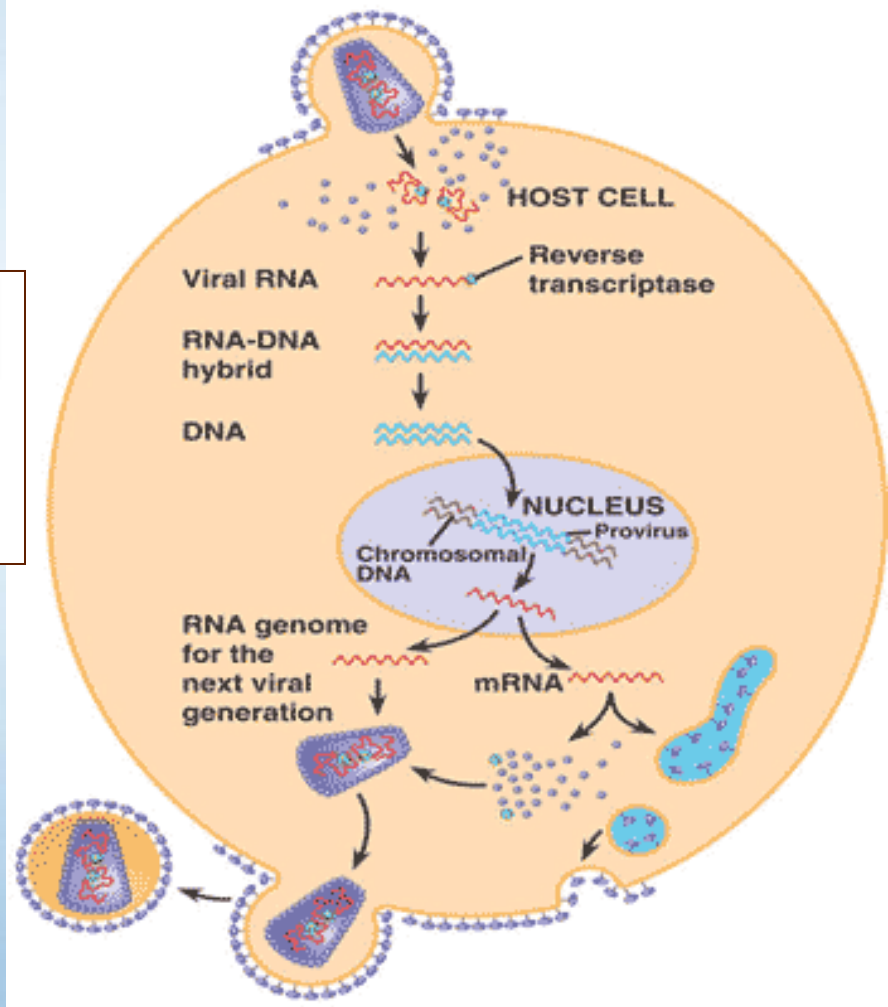


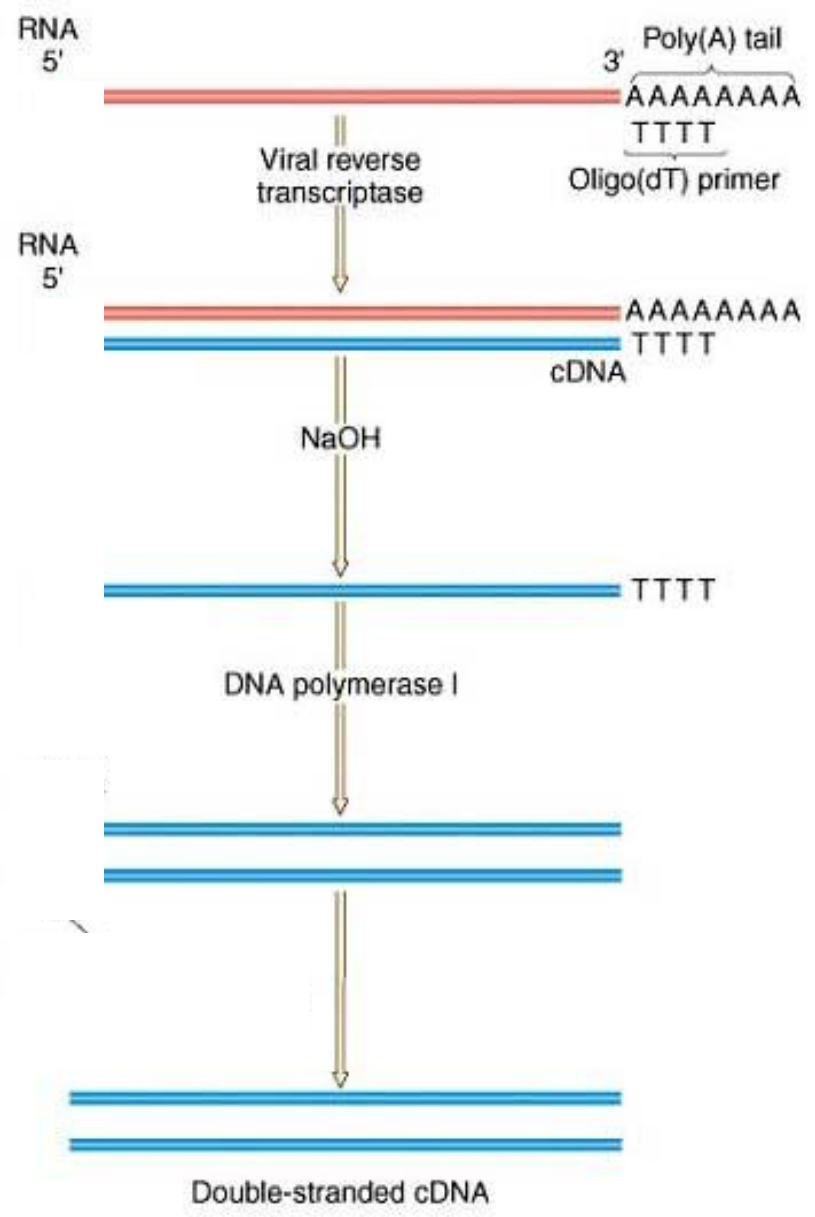
- **Gene expression analysis**
  - Cancer research
  - Drug research
- **Disease diagnosis and management**
  - Viral quantification
- **Food testing**
  - Percent GMO food
- **Animal and plant breeding**
  - Gene copy number

qRT-PCR is used to qualitatively detect gene expression

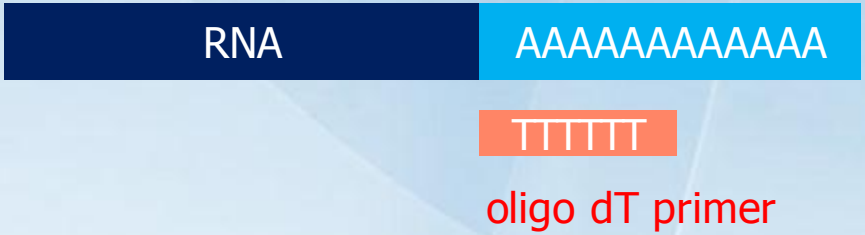
Reverse transcription







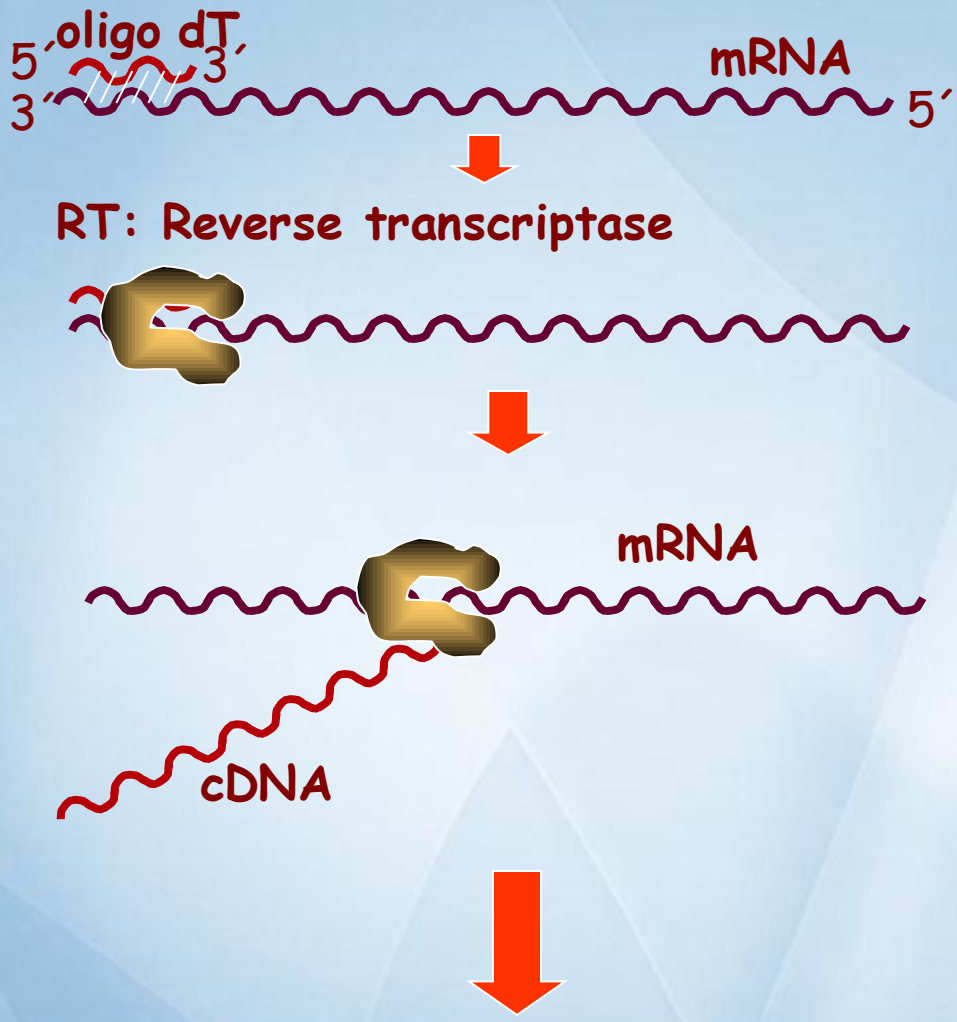
All messenger RNAs (mRNA) have a poly A tail



The RT reaction can be primed by a:

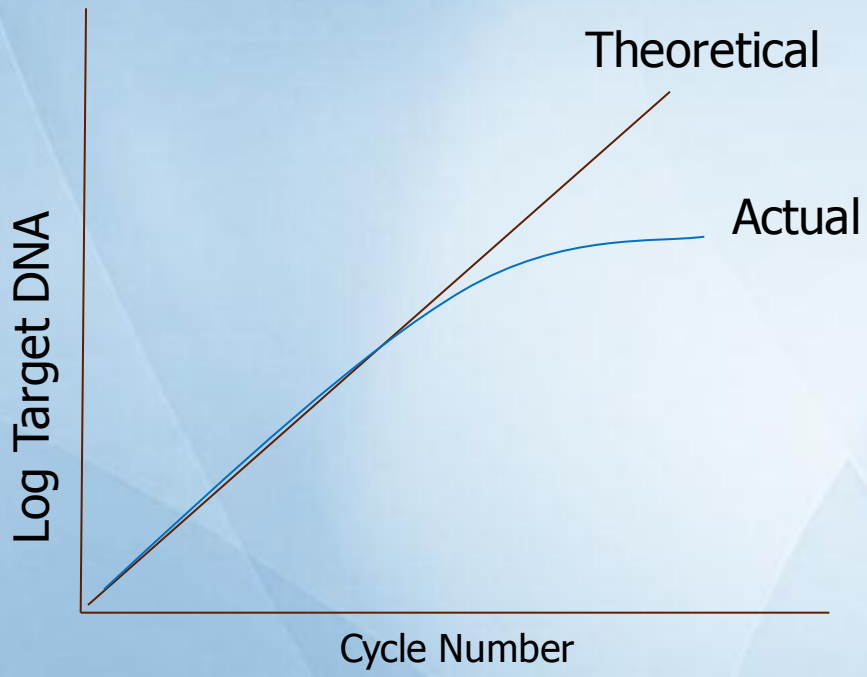
- ✓target specific primer (i.e. primer targeting VHSV nucleocapsid (N) gene)
- ✓oligo dT primer (a primer consisting of a run of T's that targets the mRNA poly A tail)
- ✓random primers (a mix of 6 base primers consisting of random nucleotides)

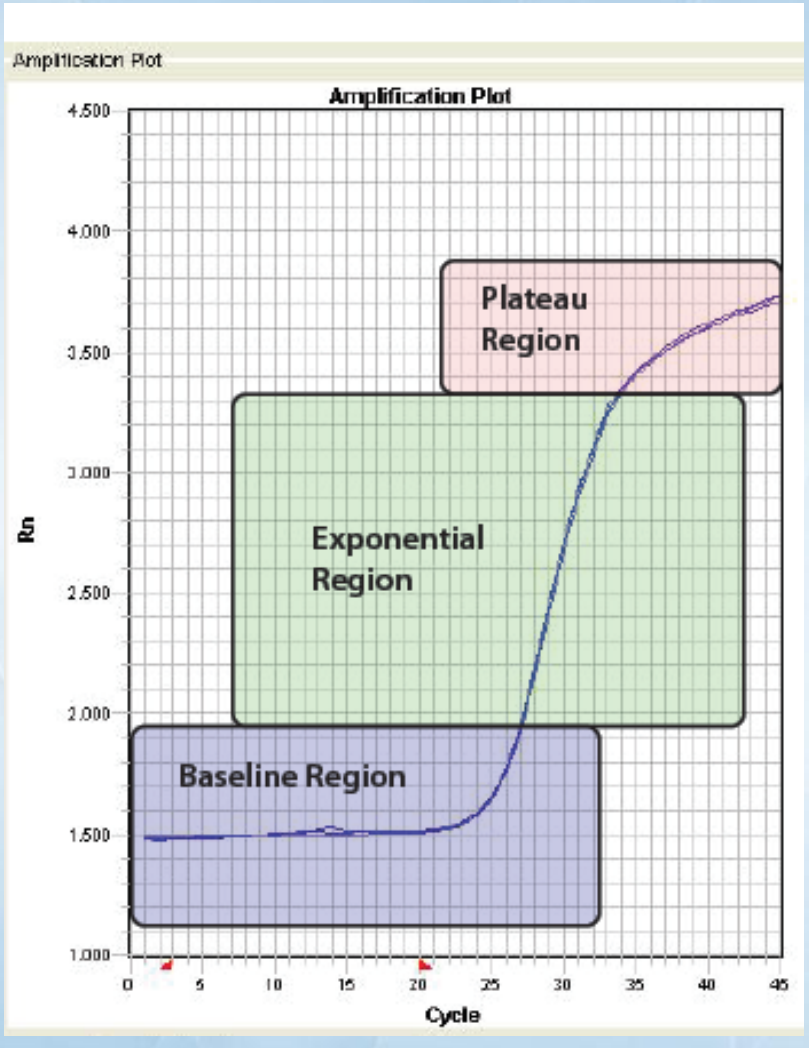
RT-PCR



Templat for RT- PCR

How does PCR work?

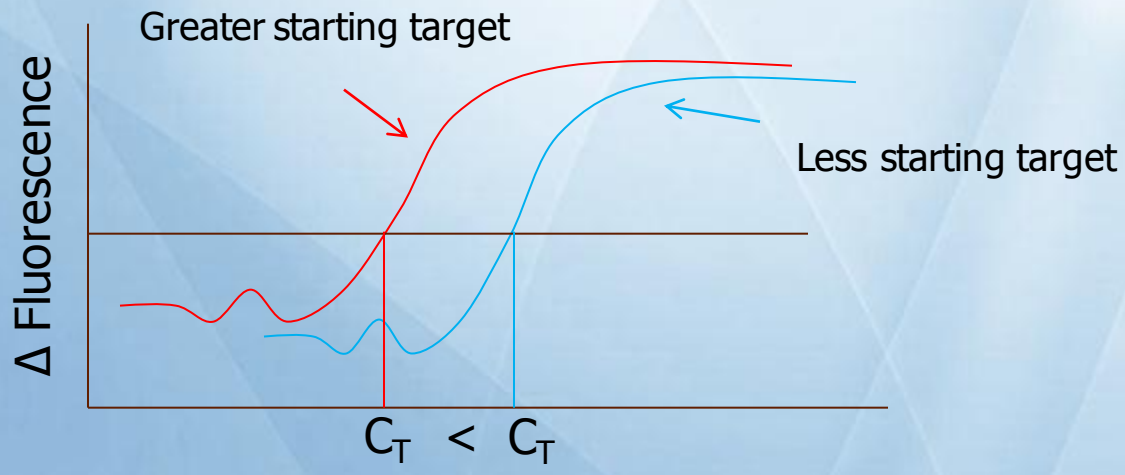




Real-Time PCR

- 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?

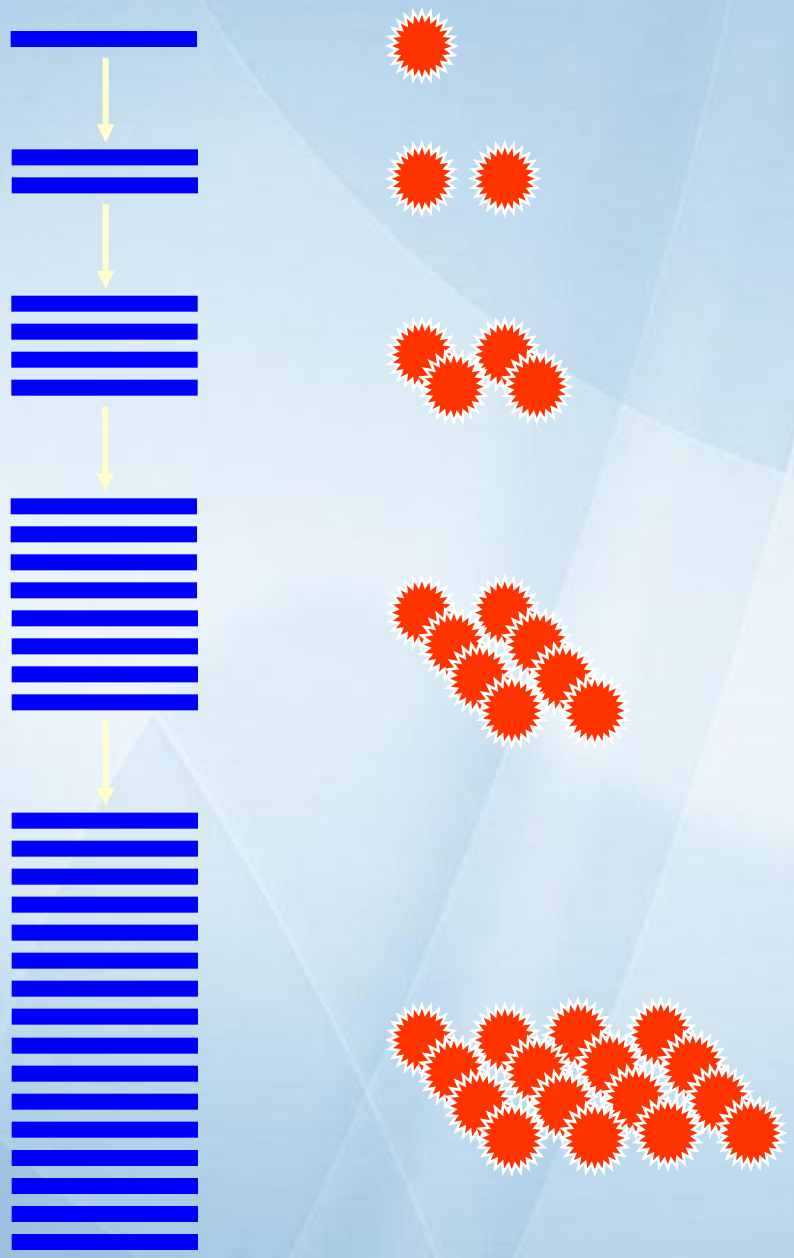
Initial DNA strand

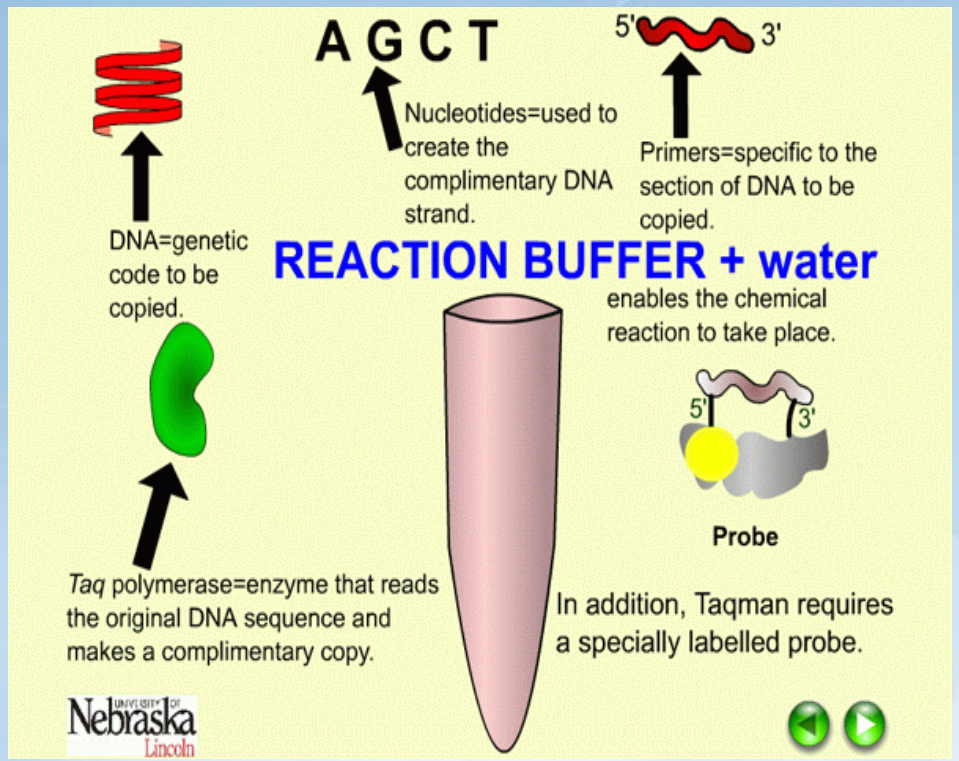
First PCR cycle

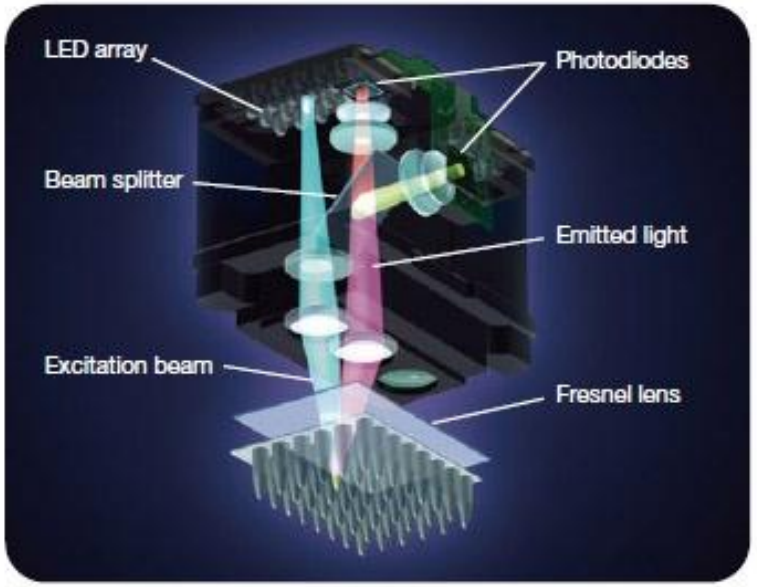
Second PCR cycle

Third PCR cycle

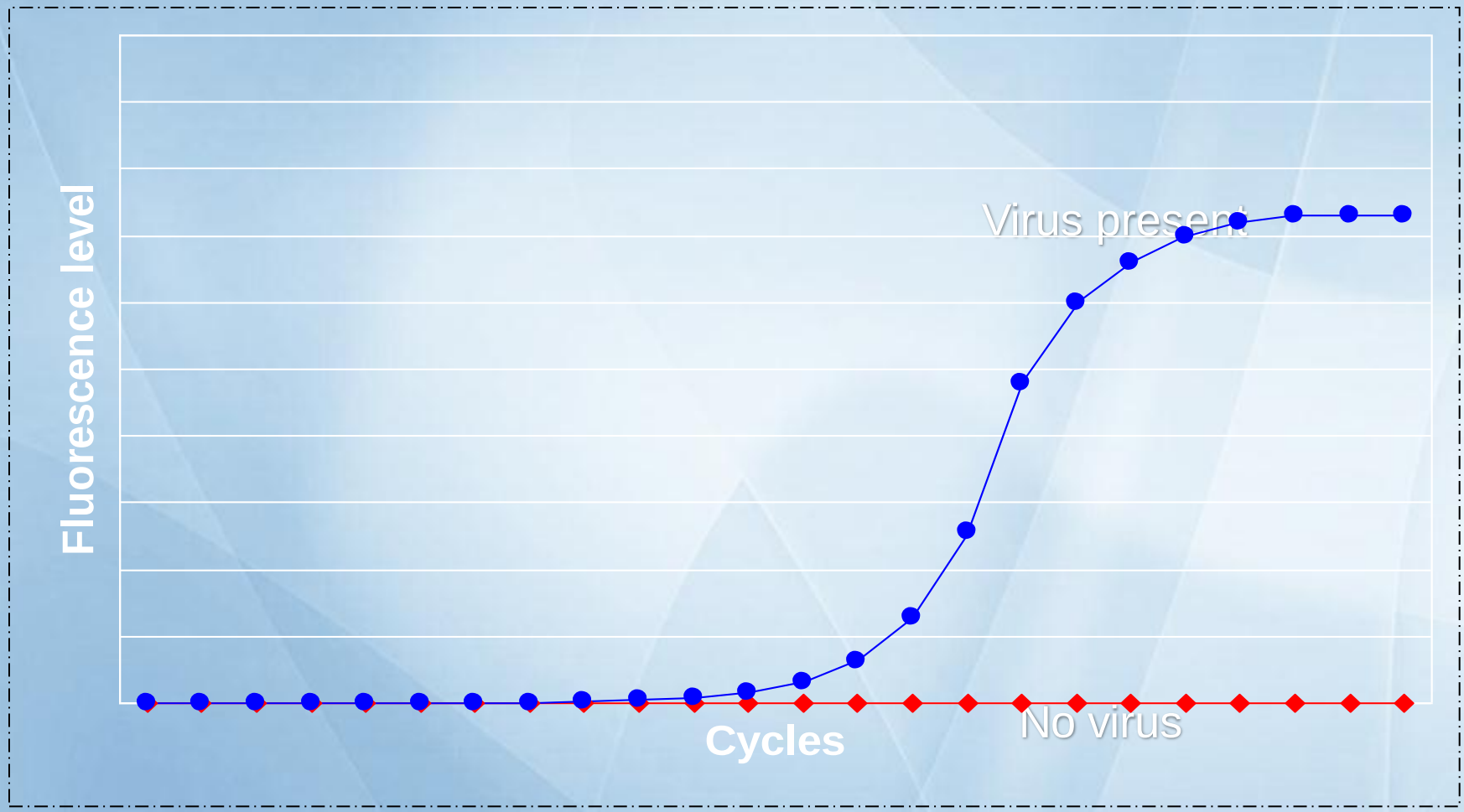
Fourth PCR cycle

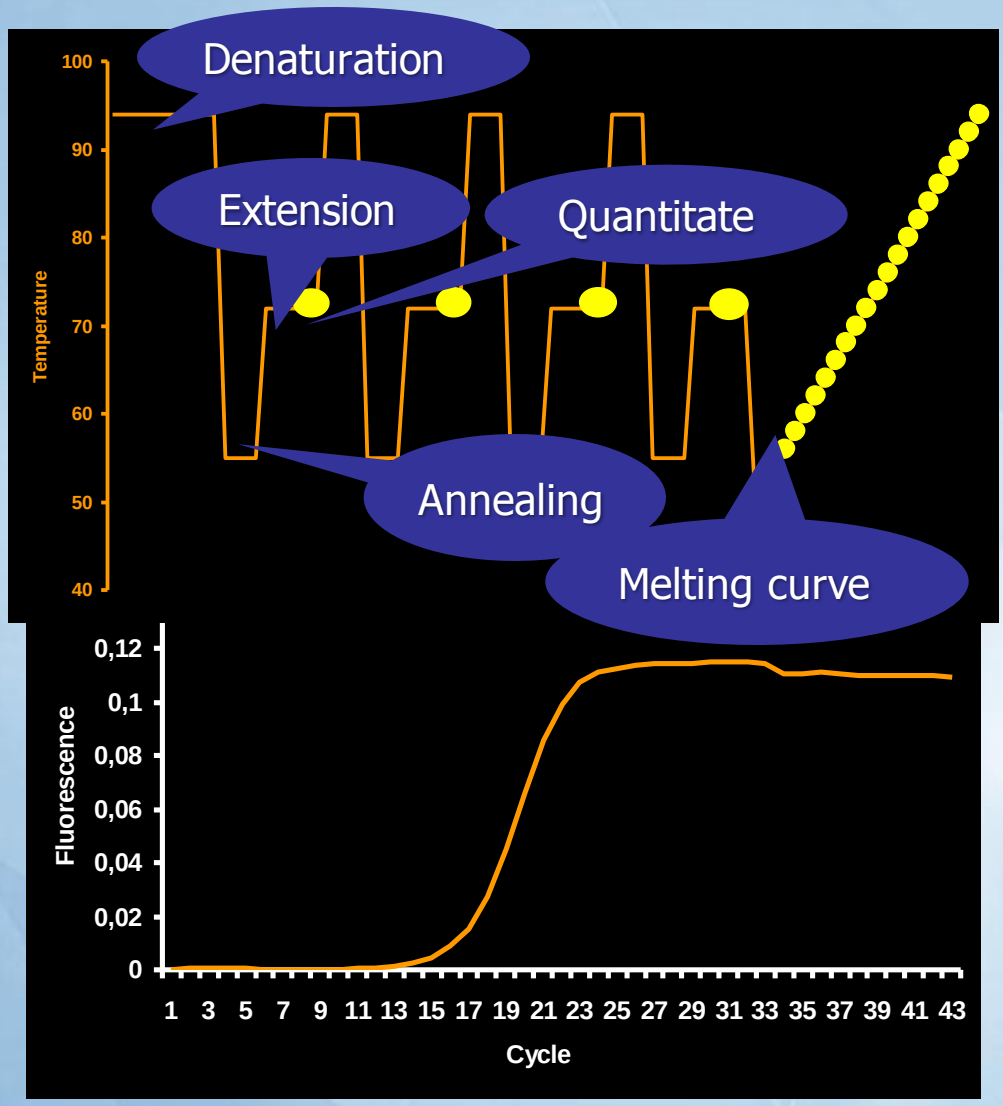



A G C T
5'
3'
Nucleotides=used to create the complimentary DNA strand.
Primers=specific to the section of DNA to be copied.
DNA=genetic code to be copied.
REACTION BUFFER + water
enables the chemical reaction to take place.
5'
3'
Probe
Taq polymerase=enzyme that reads the original DNA sequence and makes 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

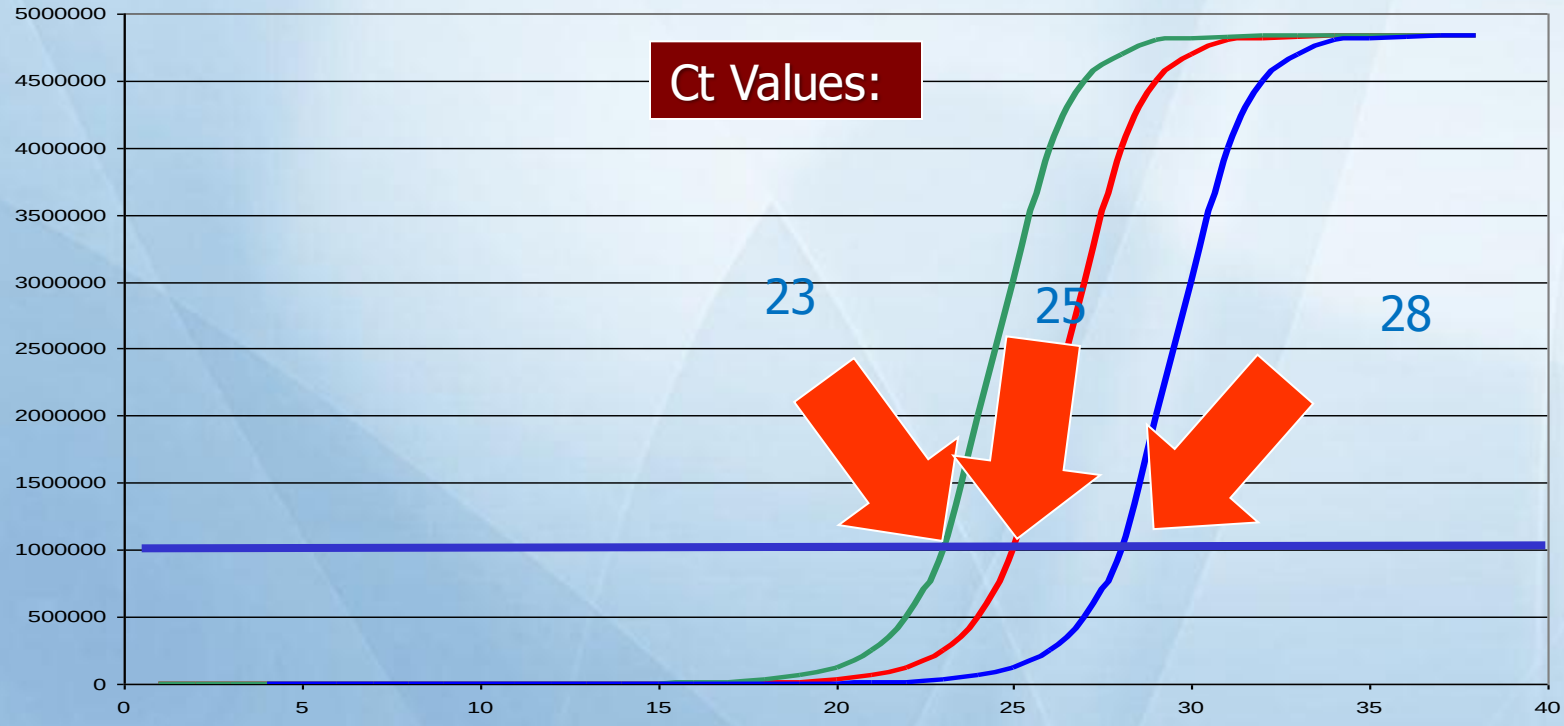



Denaturation
Extension
Quantitate
Annealing
Melting curve
Temperature
100
90
80
70
60
50
40
Fluorescence
0,12
0,1
0,08
0,06
0,04
0,02
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43
Cycle

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

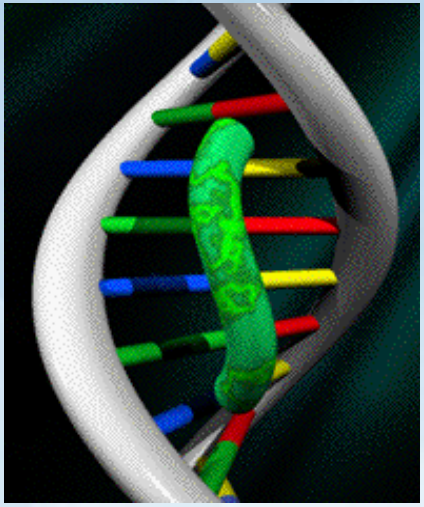
This is called the cycle threshold “Ct Value”.

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

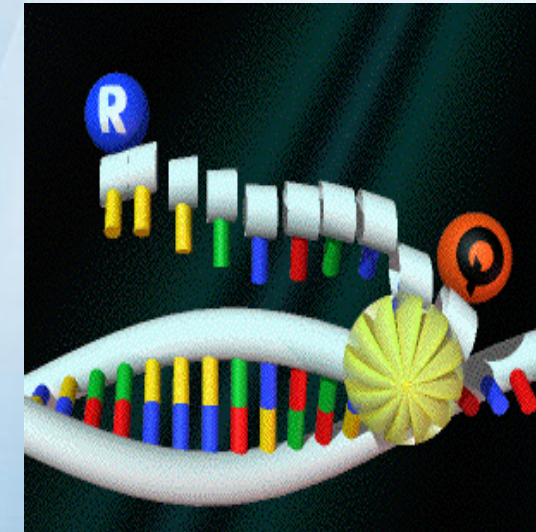


Quantitative PCR – in depth

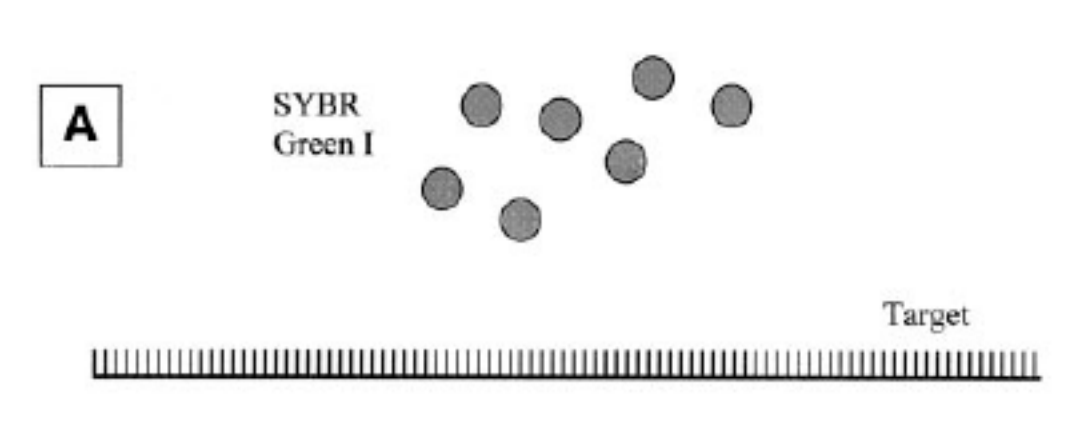
- Major assay types
  - **Hydrolysis probes**
    - Basis of TaqMan® chemistry
    - Uses two primers and an internal hydrolysis probe
    - Most commonly used for fish health diagnostics

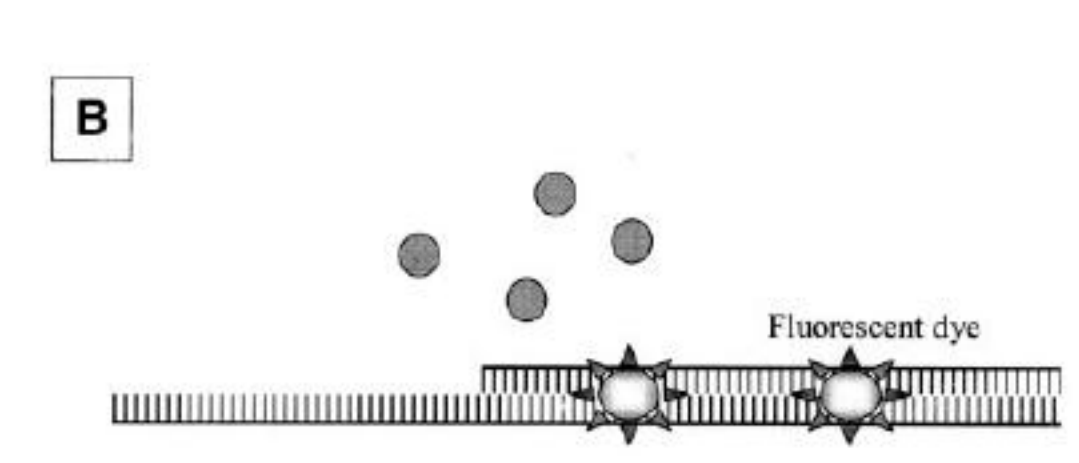


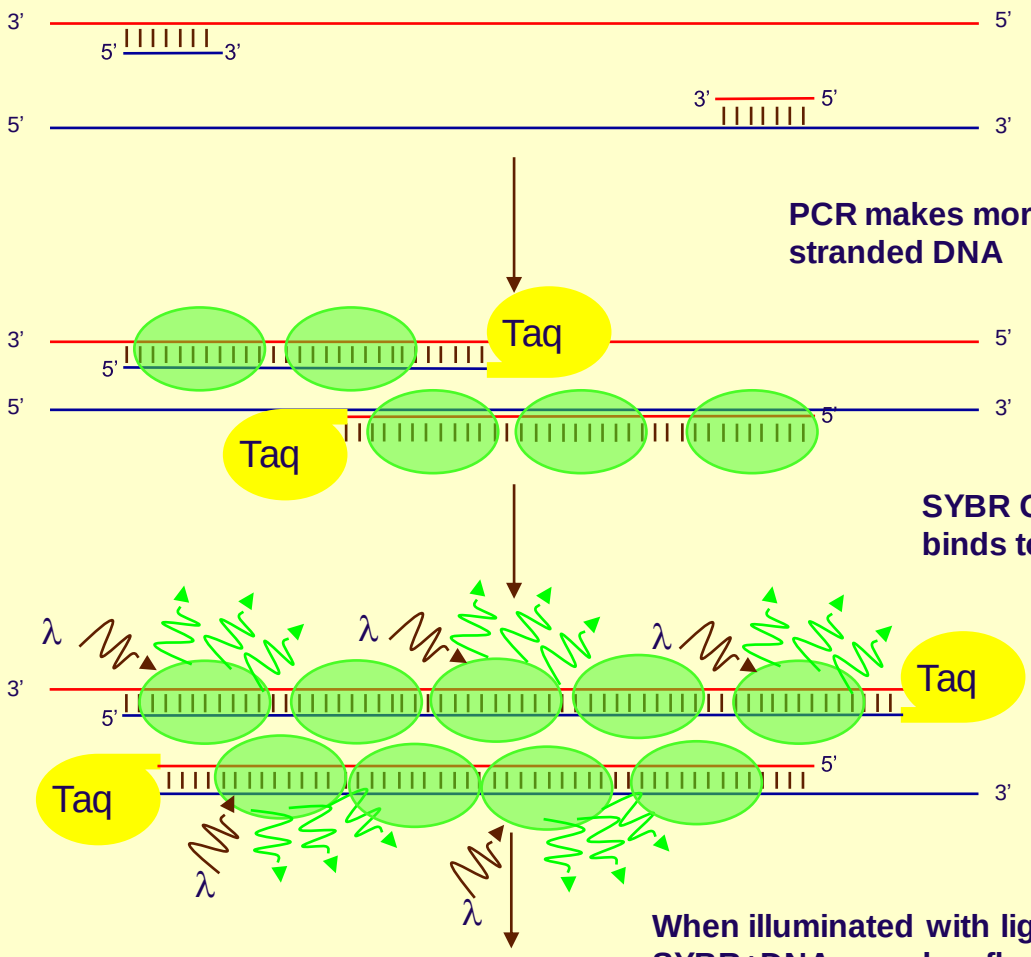
  - **SYBR ® green dye**
    - Increased fluorescence when bound to dsDNA
    - Slightly lower specificity
    - Costs less
    - May not be as sensitive as the 5’ nuclease assays



SYBRGreen

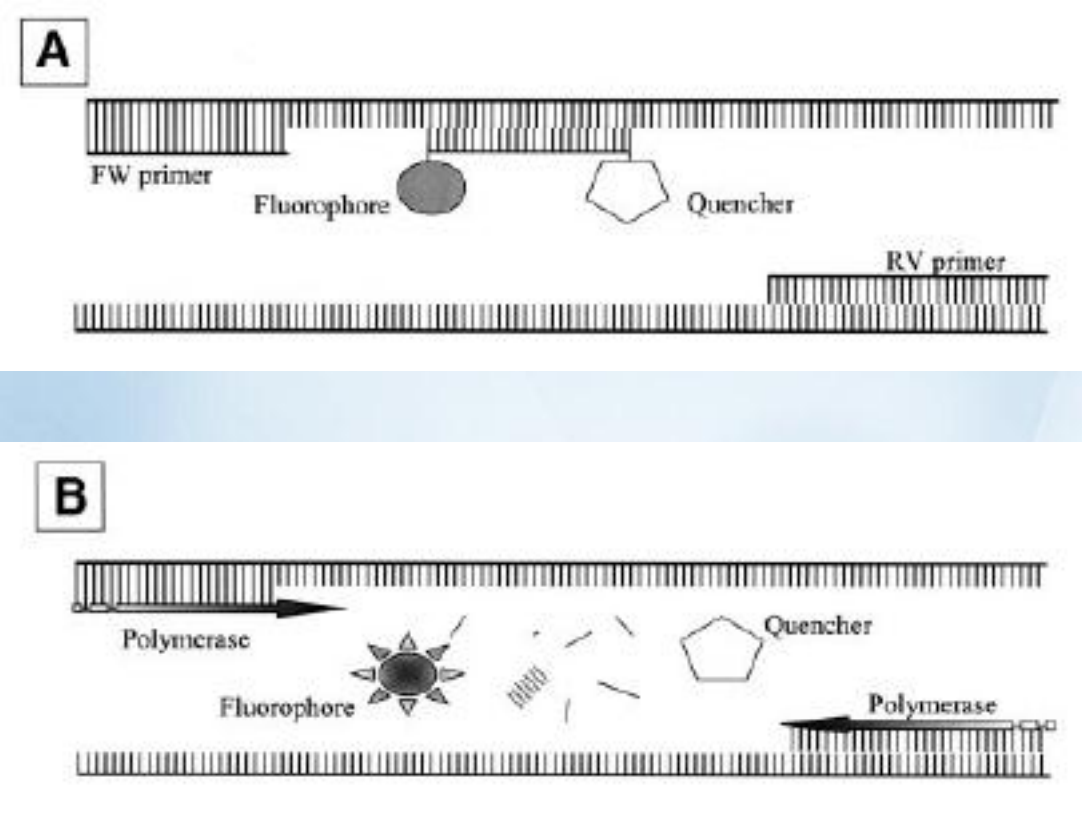




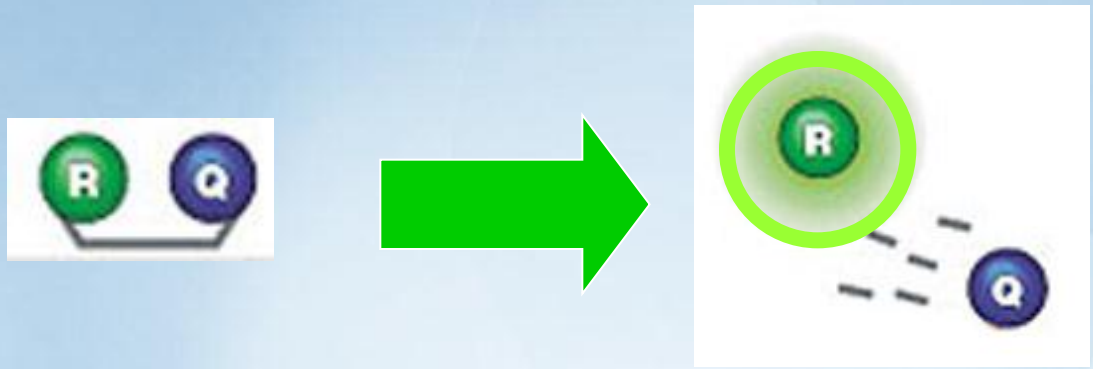

3'
5'
5'
3'
3'
5'
5'
3'
PCR makes more double-stranded DNA
Taq
3'
5'
5'
3'
Taq
5'
SYBR Green dye binds to dsDNA
λ
λ
λ
Taq
3'
5'
5'
Taq
3'
λ
λ
When illuminated with light at 490nm, the SYBR+DNA complex fluoresces at 520nm.

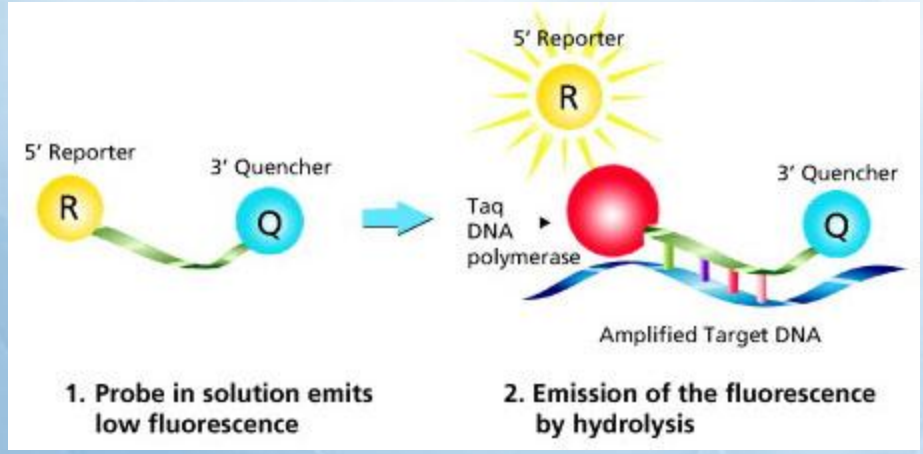
BackMan vs TaqMan

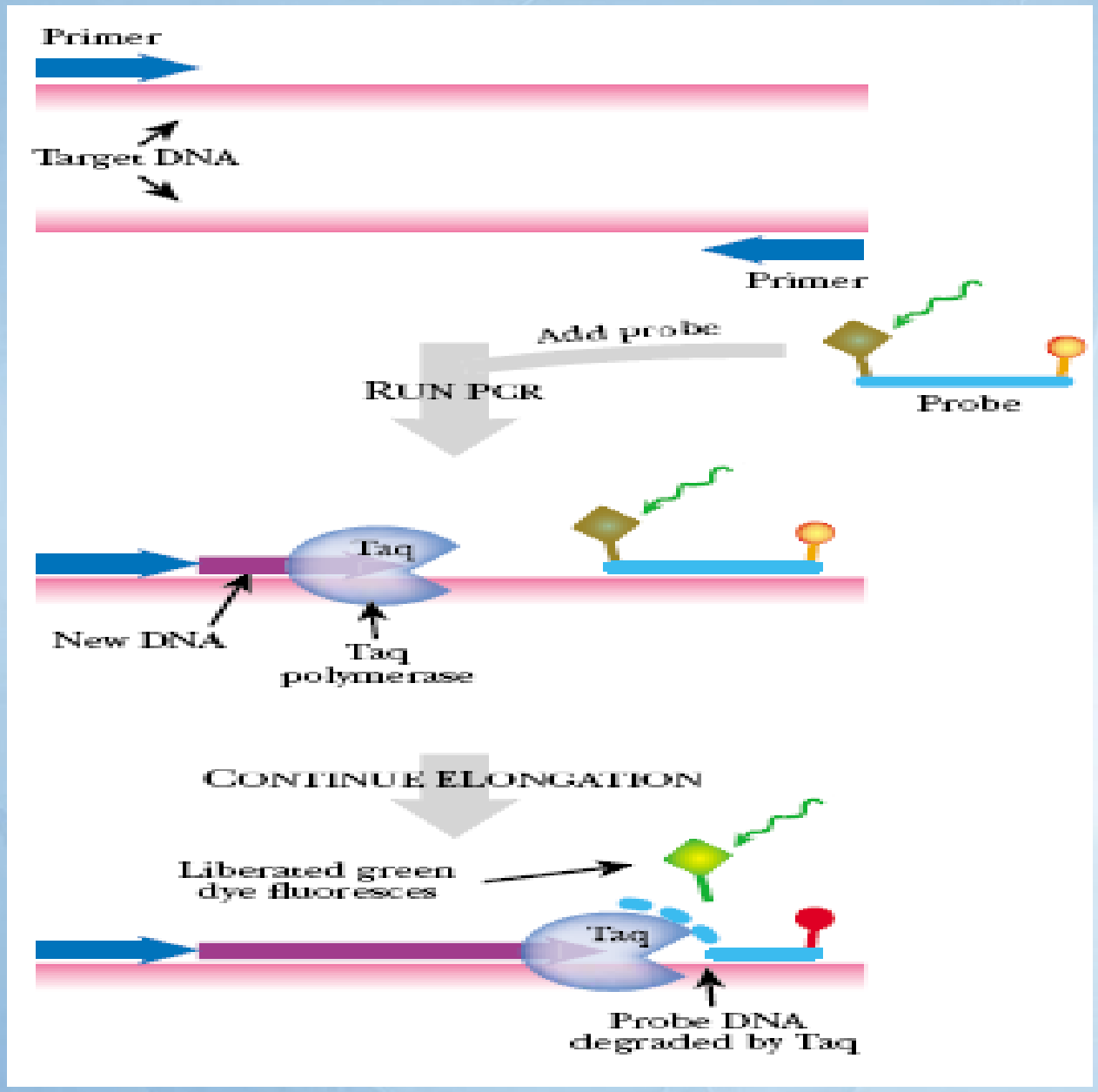
TaqMan


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

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:

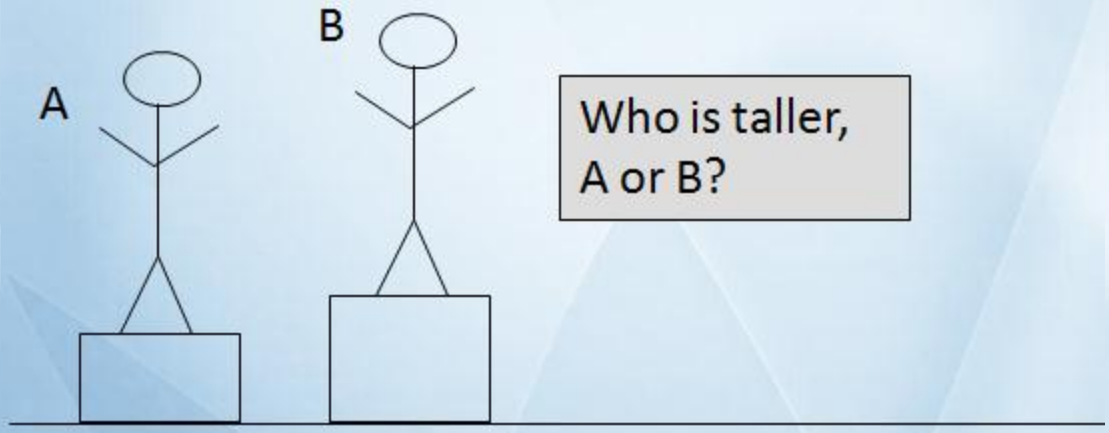
- Relative high cost of labeled probe.

- **SYBR Green**

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

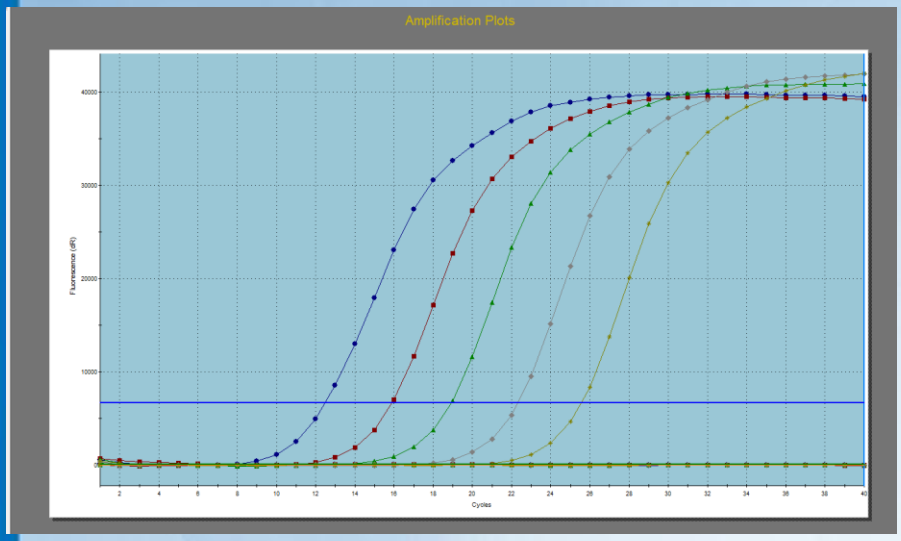
- **Disadvantages:**
- Less specific – only primers determine specificity.
- Specific and non-specific double-stranded PCR products generate the same fluorescence signal upon binding SYBR Green I dye.
- Not possible to multiplex multiple gene targets.

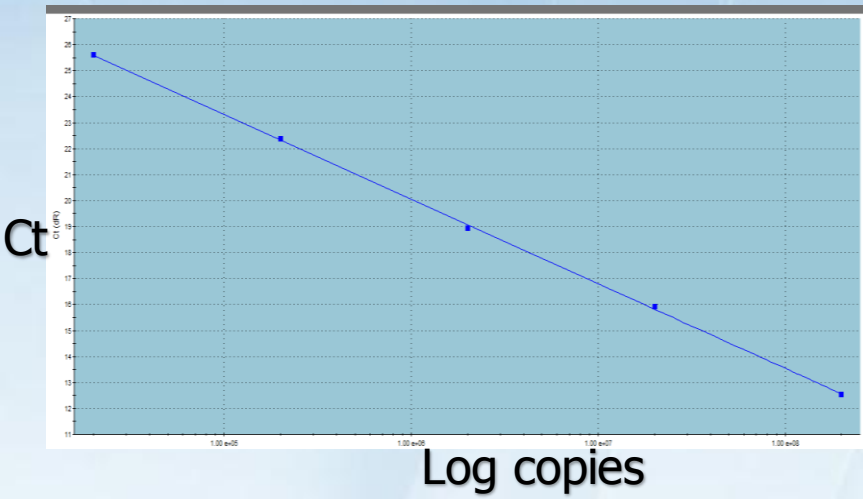
Normalization



Normalization: corrects for variation in template quantity and/or template quality

Standard Curves

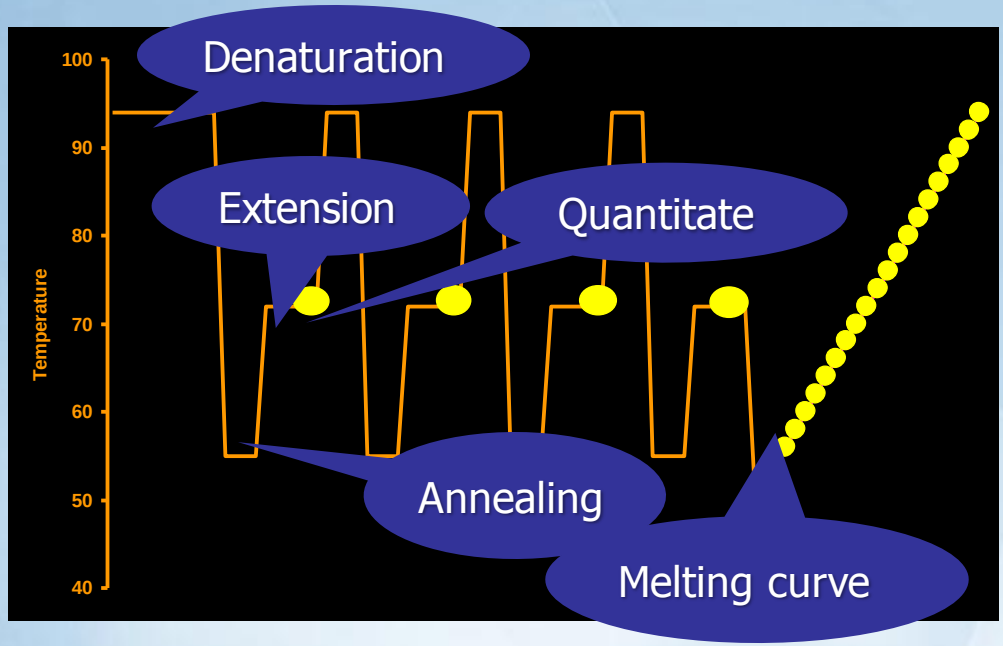


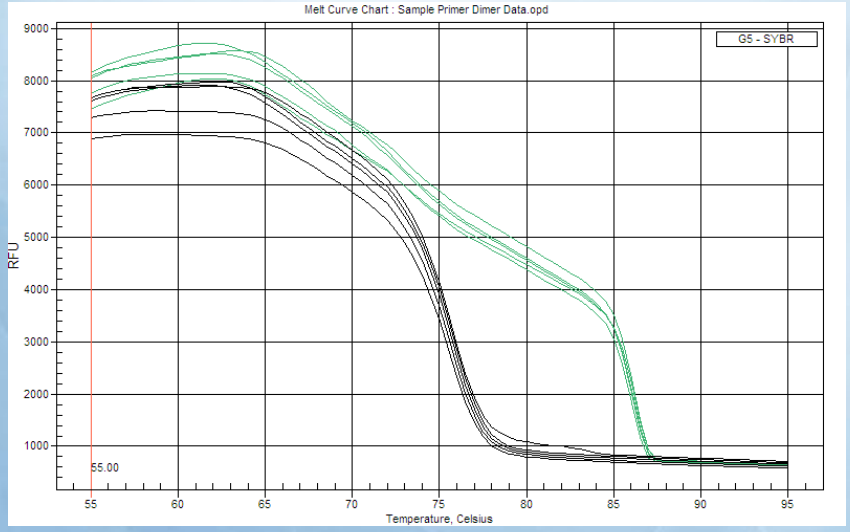


- Use at least 5 concentrations for a standard curve
- Use serial dilutions that are one order of magnitude apart
  _ 1:10, 1:100, 1:1000,…

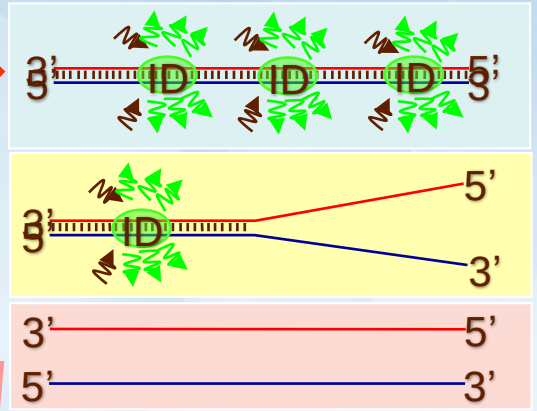
Housekeeping Genes

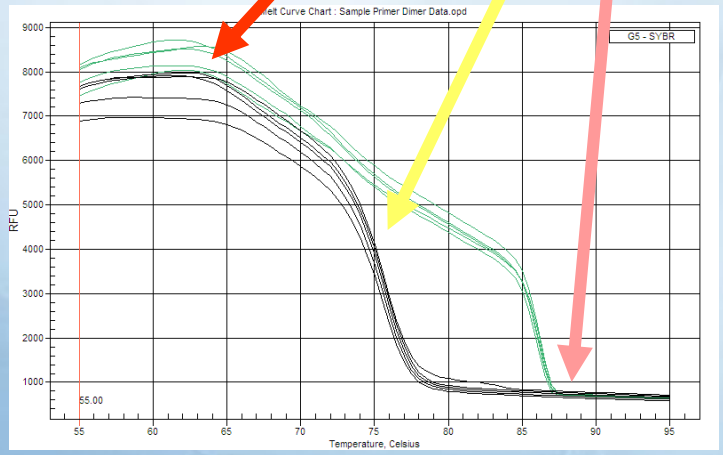
Gene	Genomic structure / pseudogenes	Regulation e.g.
ß-actin	multigene family; > 20 genes; 1 active locus 20 pseudogenes	↑: hormones of tyroid gland ↑: stomach tumor
γ-actin	multigene family; pseudogenes	
GAPDH	multigene family; 10-30 genes; > 200 in mouse mostly pseudogenes	↑: lung, pancreatic, colon cancer ↑: insulin, EGF
5.8S,18S, 28S RNA	pseudogenes	
ß2-microglobulin	no pseudogenes	↑: Non-Hodgkin lymhoma abnormal expression in tumors
G6PDH	no pseudogenes	↑: kidney, stomach tumor ↑: hormones, oxidant stress, growth factors
PBGD	no pseudogenes	
aldolase	pseudogenes	
HPRT	pseudogenes	
U3, U8, ...	Pseudogenes	
ornithin decarboxylase		↑: tumors
...		


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

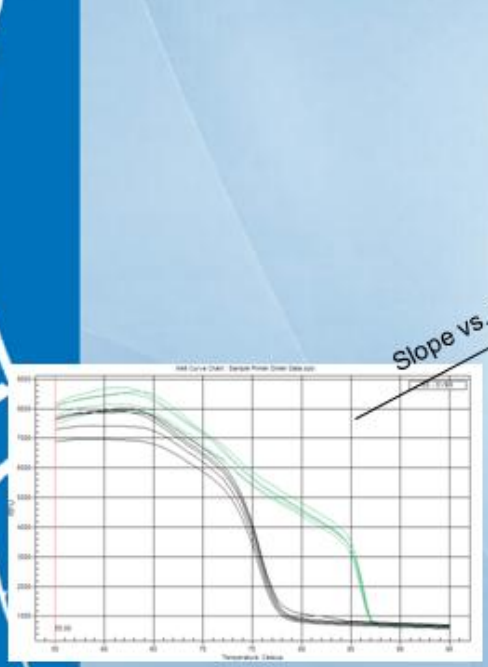

Melt Curve Chart : Sample Primer Dimer Data.opd
G5 - SYBR
RFU
9000
8000
7000
6000
5000
4000
3000
2000
1000
55.00
55
60
65
70
75
80
85
90
95
Temperature, Celsius

- **Melt curves can tell us what products are in a reaction.**
- PCR products that are shorter will melt at lower temperatures.
- Different PCR products will therefore have different shaped curves.

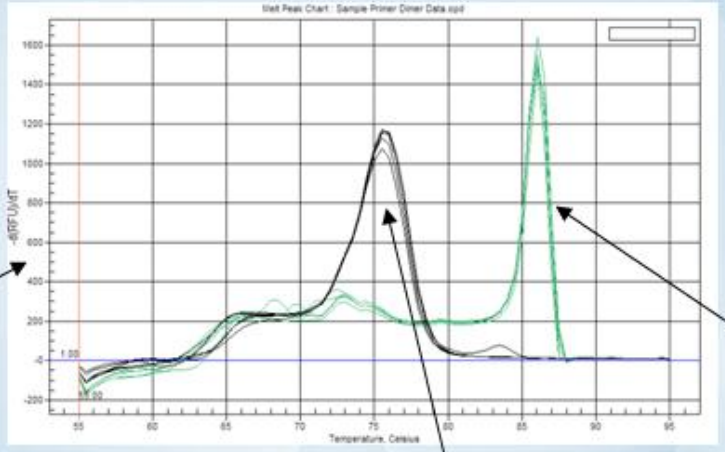




- For convenience, we typically view the derivative *(slope)* of the actual melt curve data.
- The resulting graph looks like a chromatogram, with peaks that represent different PCR products.

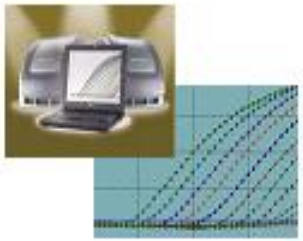


Slope vs. Temp

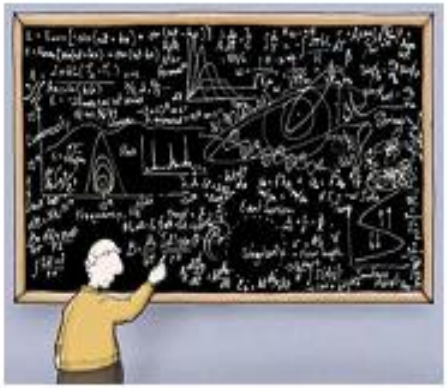


These PCR products melt at a relatively low temperature, are likely primer-dimers.

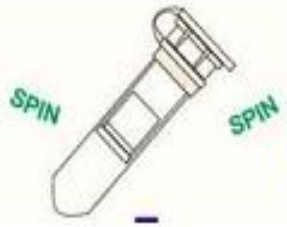
These PCR products melt at a higher temperature, and are likely to be the main PCR product.



Steps towards successful Real-time QPCR experiments



1.Experimental Design



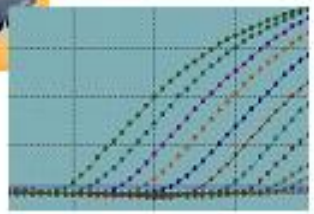
2.Sample preparation and purification

3. RNA/DNA quantification and quality

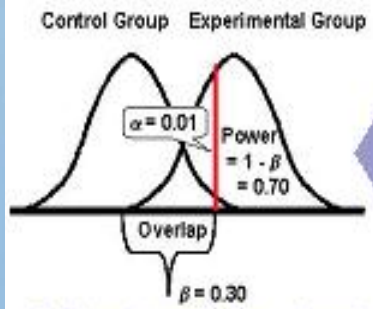
Total RNA

cDNA

4.Reverse Transcription



5.Real time QPCR



6.Post-run Analysis