PRINCIPAL OF REAL TIME PCR

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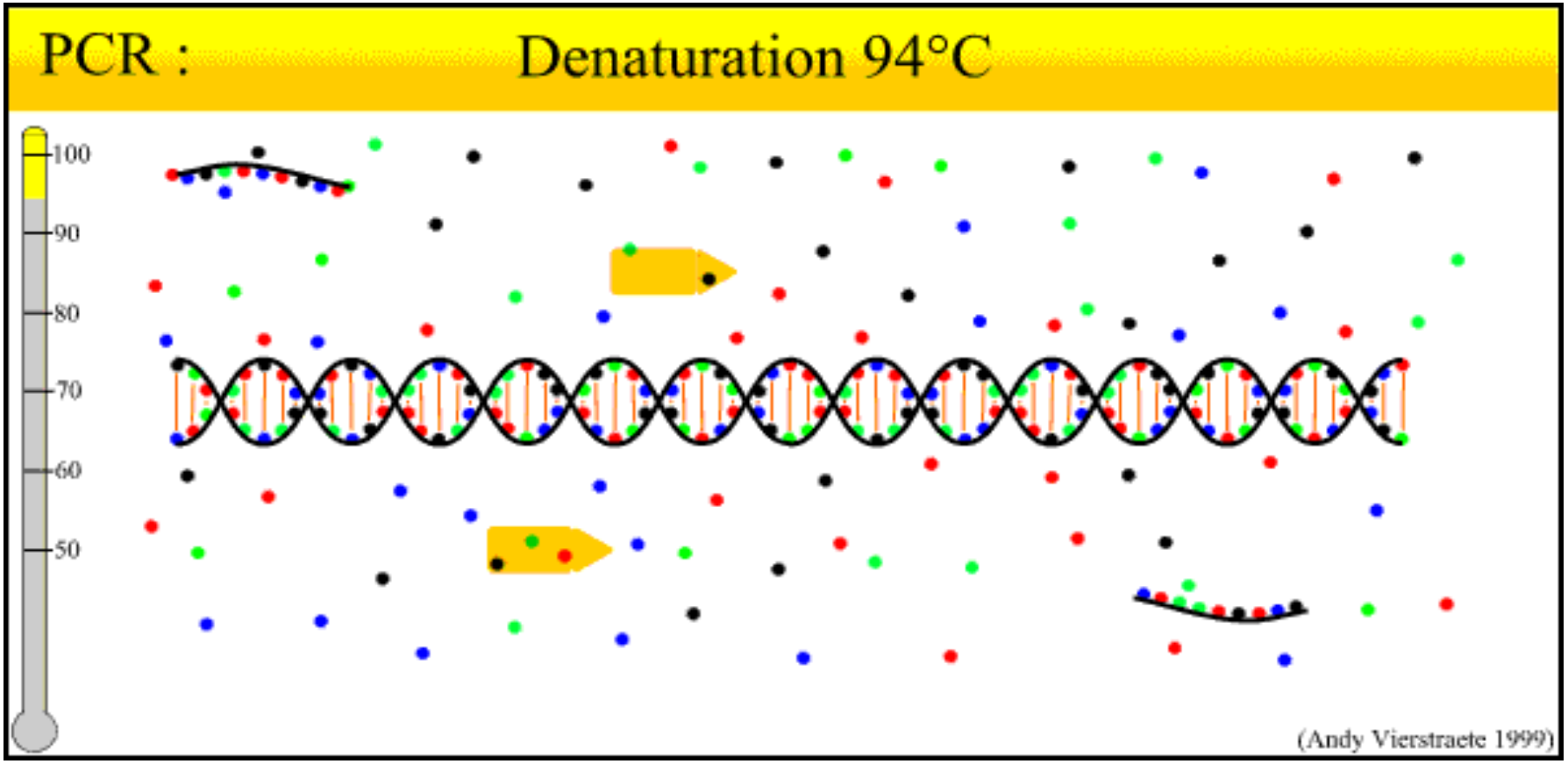
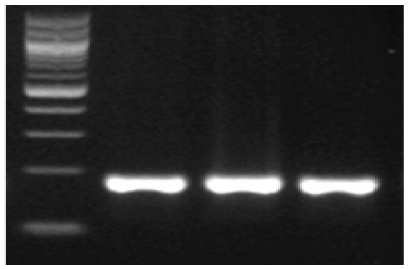
PCR (POLYMERASE CHAIN REACTION)



What the diffrences between conventional PCR and Real-Time PCR

- Conventional PCR amplified DNA and the product detected by visualizing on agarose gel.



- In contrast, Real-time PCR the accumulation of amplification product is measured as the reaction progresses, (product quantification after each cycle).

HOW DOES REAL-TIME PCR WORKS?

- **A fluorescent reporter molecule included in each reaction/ or Syber green dye → Increasing the fluorescence with the increasing of the amount of DNA.**
- **Specialized thermal cyclers equipped with fluorescence detection modules are used.**
- **The measured fluorescence is proportional to the total amount of amplicon; the change in fluorescence over time is used to calculate the amount of amplicon.**

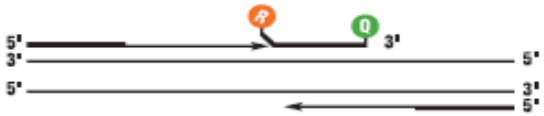
HOW DOES REAL-TIME PCR WORKS?

- **Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable (cycles 1–18/20) even though product accumulates exponentially.**
- **Eventually, enough amplified product accumulates to yield a detectable fluorescence signal.**

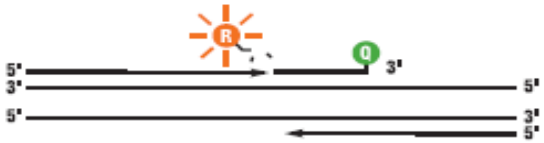
TAQMAN® PROBE-BASED ASSAY CHEMISTRY

1. **Polymerization:** A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan® probe, respectively.

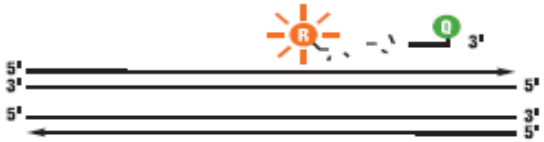
2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



3. **Cleavage:** During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.



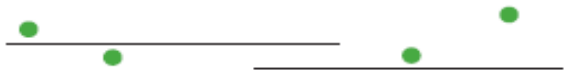
4. **Polymerization completed:** Once separated from the quencher, the reporter dye emits its characteristic fluorescence.



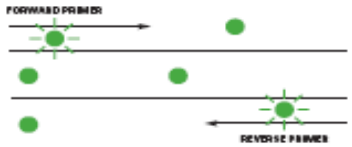
SYBR® GREEN I DYE ASSAY CHEMISTRY

1. **Reaction setup:** The SYBR® Green I Dye fluoresces when bound to double-stranded DNA.

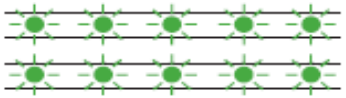
2. **Denaturation:** When the DNA is denatured, the SYBR® Green I Dye is released and the fluorescence is drastically reduced.



3. **Polymerization:** During extension, primers anneal and PCR product is generated.



4. **Polymerization completed:** When polymerization is complete, SYBR® Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the 7900HT system.



QUANTIFICATION CYCLE (CQ)/THRESHOLD CYCLE (CT):

Definition:

The cycle at which fluorescence from amplification exceeds the background fluorescence.

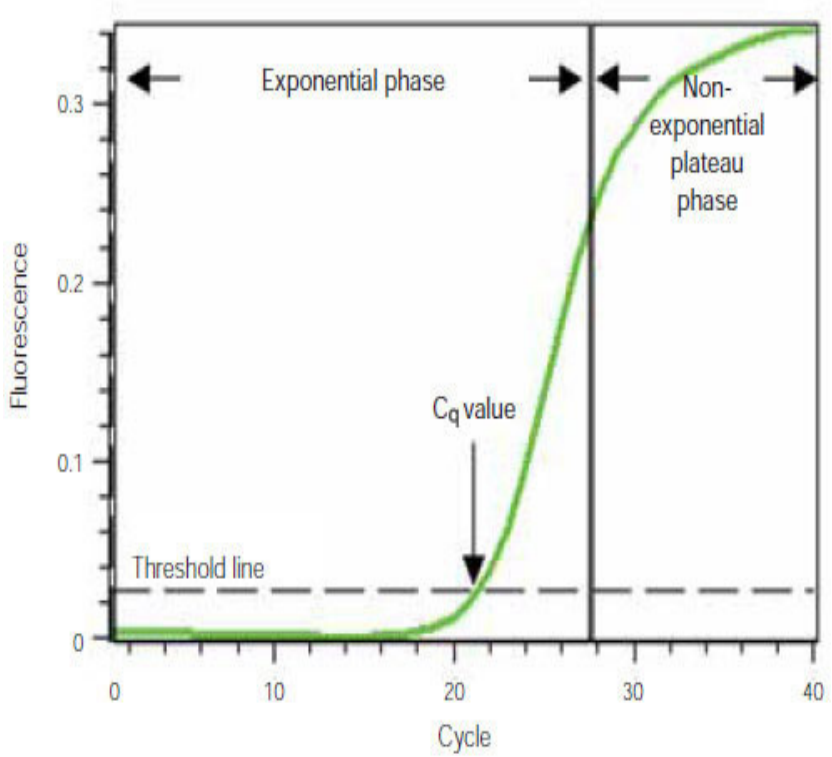
Referred to threshold cycle (Ct), crossing point (Cp) and take-off point (TOF) by different instrument manufacturers.

But is now standardized by the MIQE guidelines as the quantification cycle (Cq).

A lower Cq correlates with higher target expression in a sample.

How Does Real-Time PCR Works?

In this Curve, the number of PCR cycles is shown on the x-axis, and the fluorescence from the amplification reaction, which is proportional to the amount of amplified product in the tube, is shown on the y-axis.



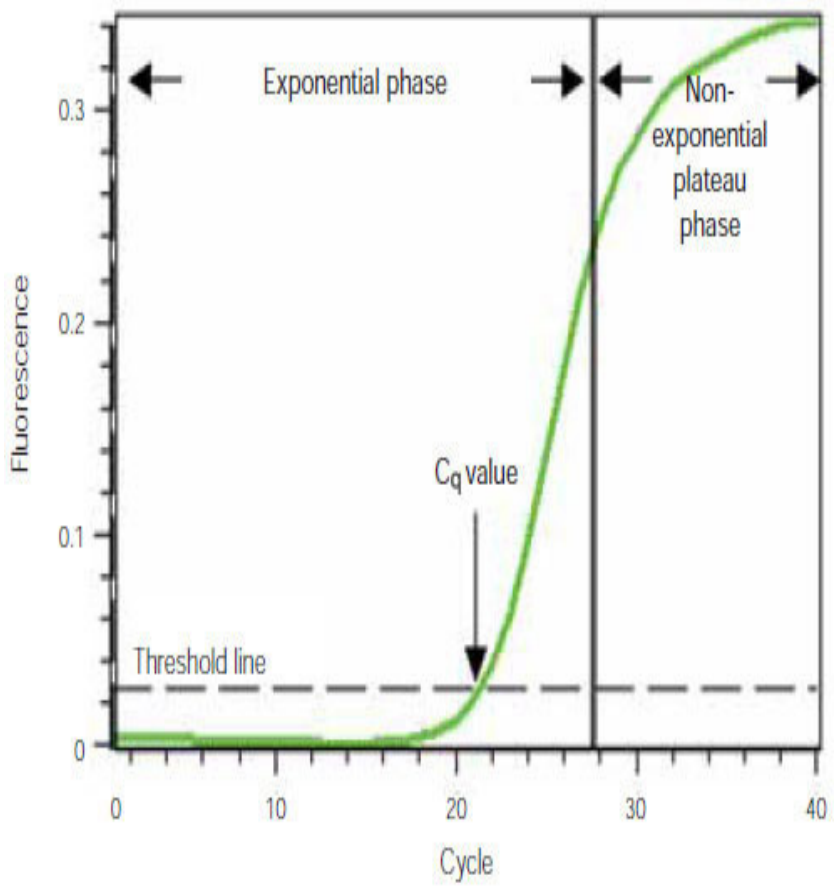
C_Q/C_T AND THE AMOUNT OF TEMPLATE

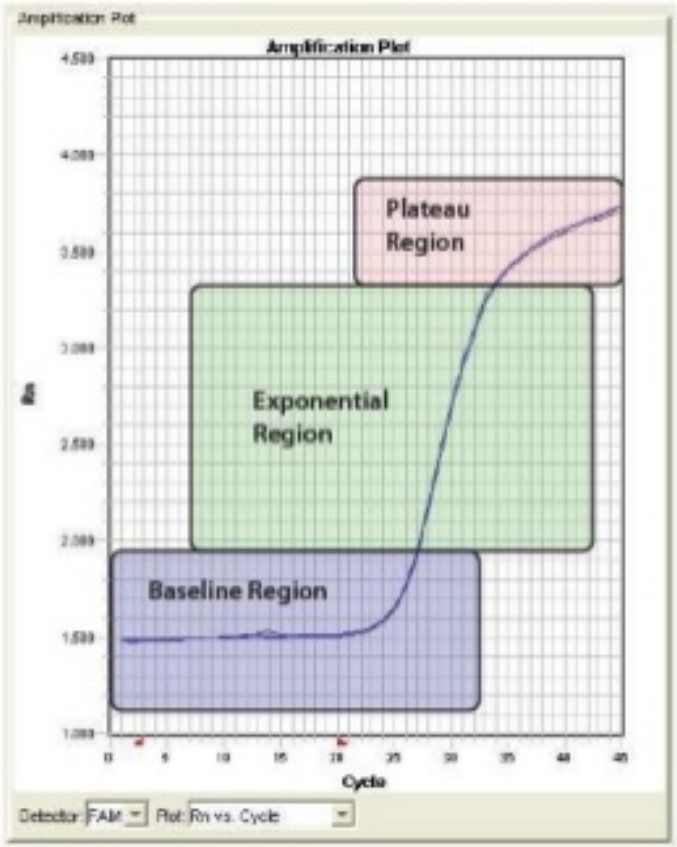
The C_q of a reaction is determined mainly by the amount of template present at the start of the amplification reaction.

- **If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescence signal above background. Thus, the reaction will have a low, or early, C_q.**

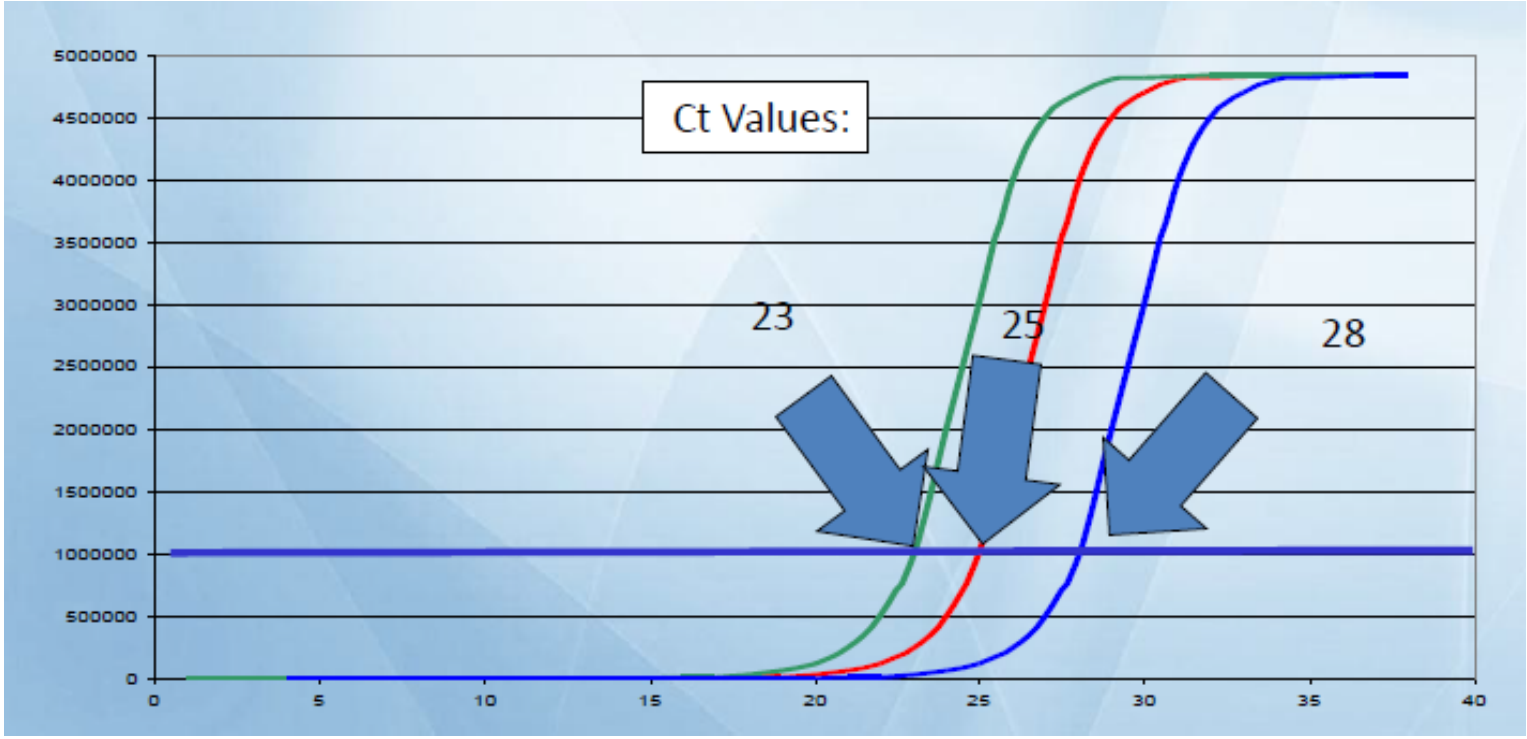
- **If a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescence signal to rise above background. Thus, the reaction will have a high, or late, C_q.**

qPCR analysis typical amplification plot.





The cycle at which fluorescence from amplification exceeds the background fluorescence has been referred to as threshold cycle (Ct).



ACCURACY/RELIABILITY /STANDARDIZATION OF Q-PCR WHEN USE TAQ-MAN PROB OR SYBER GREEN

ACCURACY & RELIABILITY WITH TAQ-MAN PROB QPCR

The traditional method for determining amplification efficiency requires a calibration curve (Standard Curve).

1. Serially dilute an artificial template of known concentration.

2. Plot the Ct values versus the initial amounts of input material on a semi-log10 plot, fit the data to a straight line and calculate the slope.

The closer the slope is to -3.33, the closer the amplification efficiency is to the 100% ideal.

STANDARD CURVE

Note: A 100% efficient reaction will yield a 10-fold increase in PCR amplicon every 3.32 cycles during the exponential phase of amplification ($\log_2 10 = 3.3219$).

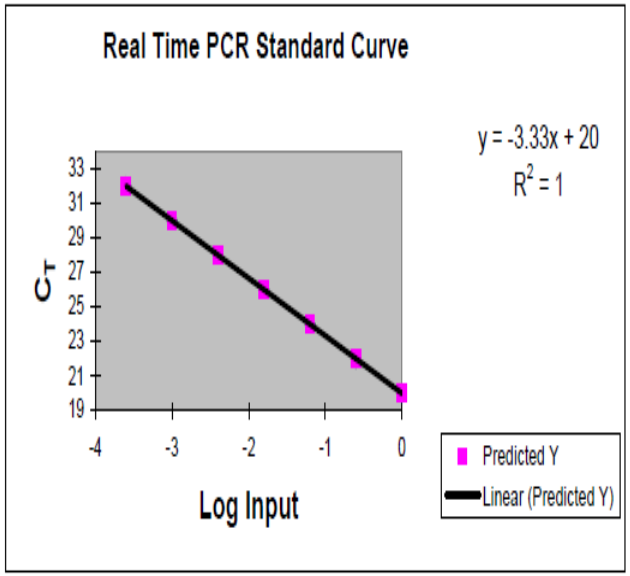
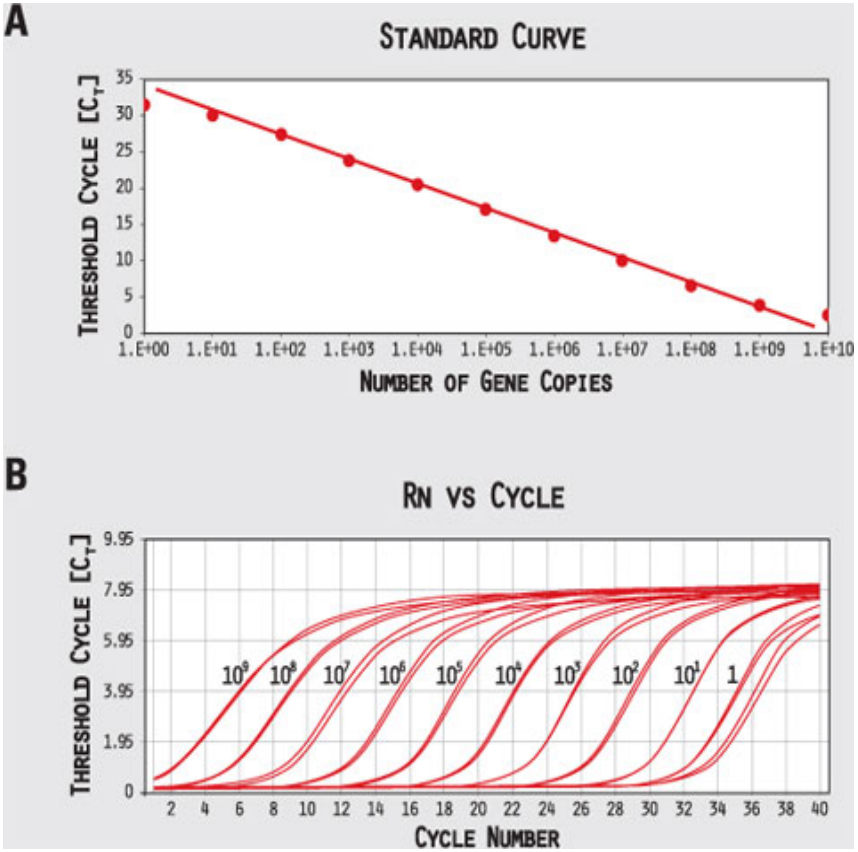


Figure 1: Real-Time PCR Standard Curve representing 100% PCR Efficiency



SPECIFICITY AND MELTING CURVE: SYBER GREEN Q-PCR

The intercalating dyes used in qPCR fluoresce only when they are bound to double-stranded DNA (dsDNA).

They do not fluoresce in the presence of single-stranded DNA (ssDNA), or when the dyes are free in solution.

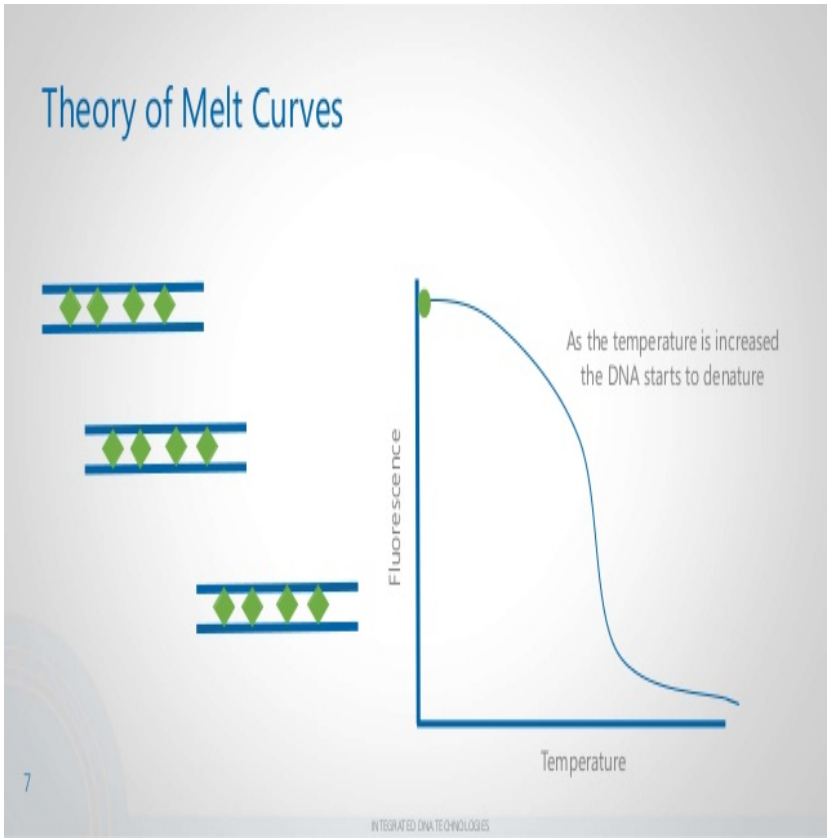
Typically, the thermal cycler being used to run the qPCR is programmed to produce the melt curve after the amplification cycles are completed.

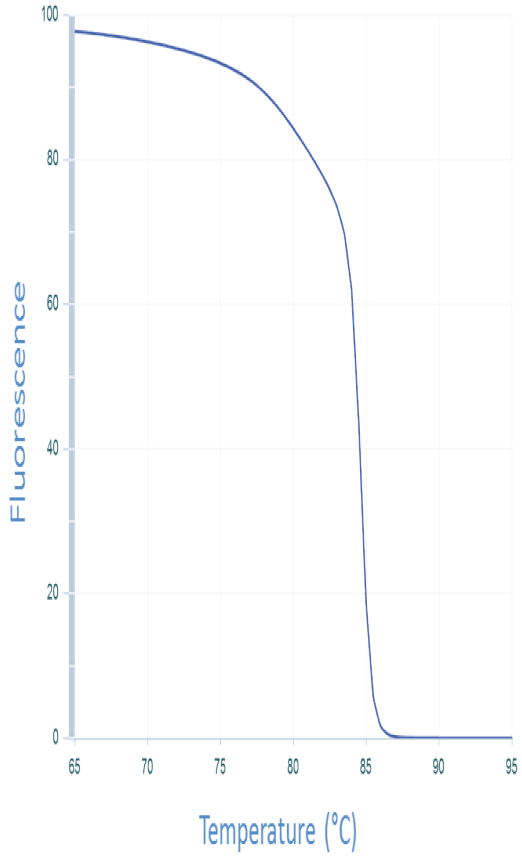
At the end of the qPCR run, the thermal cycler starts at a preset temperature (usually above the primer T_m; e.g., 65°C) and measures the amount of fluorescence.

The temperature of the sample is then increased incrementally as the instrument continues to measure fluorescence. As the temperature increases, dsDNA denatures becoming single-stranded, and the dye dissociates, resulting in decreasing fluorescence.

SPECIFICITY AND MELTING CURVE:

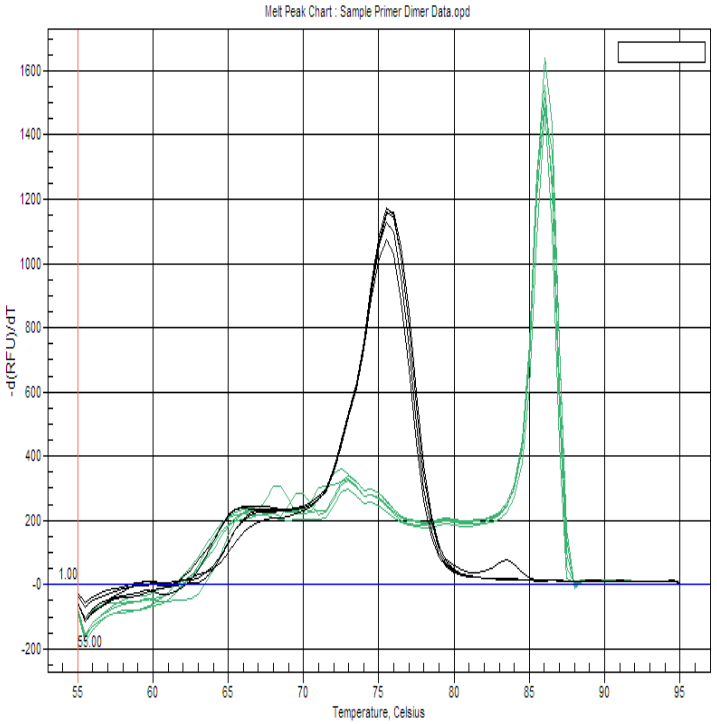
MELT CURVE ANALYSIS IN QPCR EXPERIMENTS.MP4





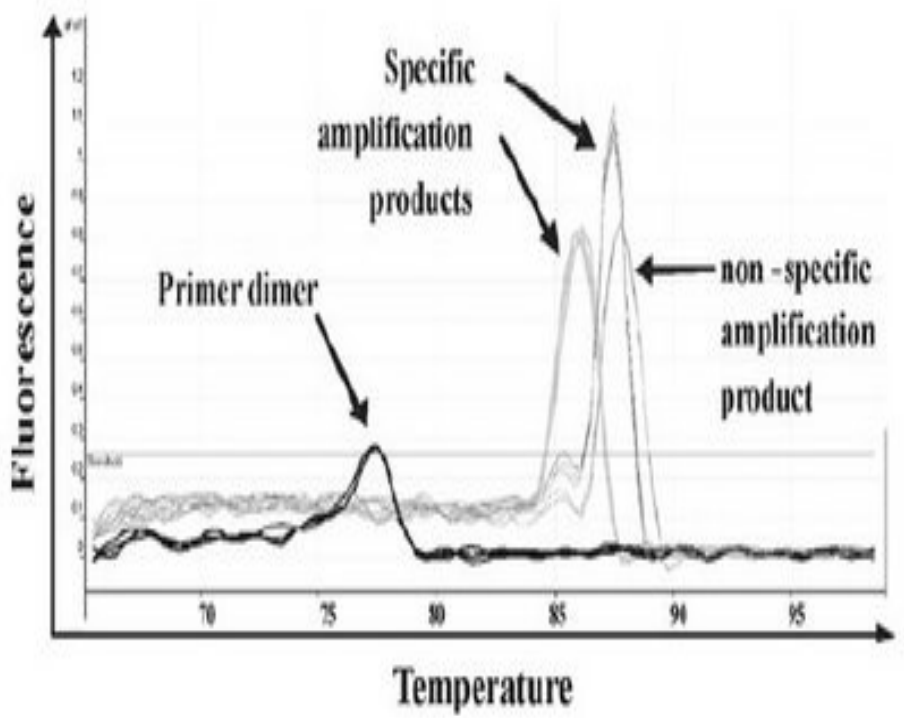
SPECIFICITY AND MELTING CURVE:

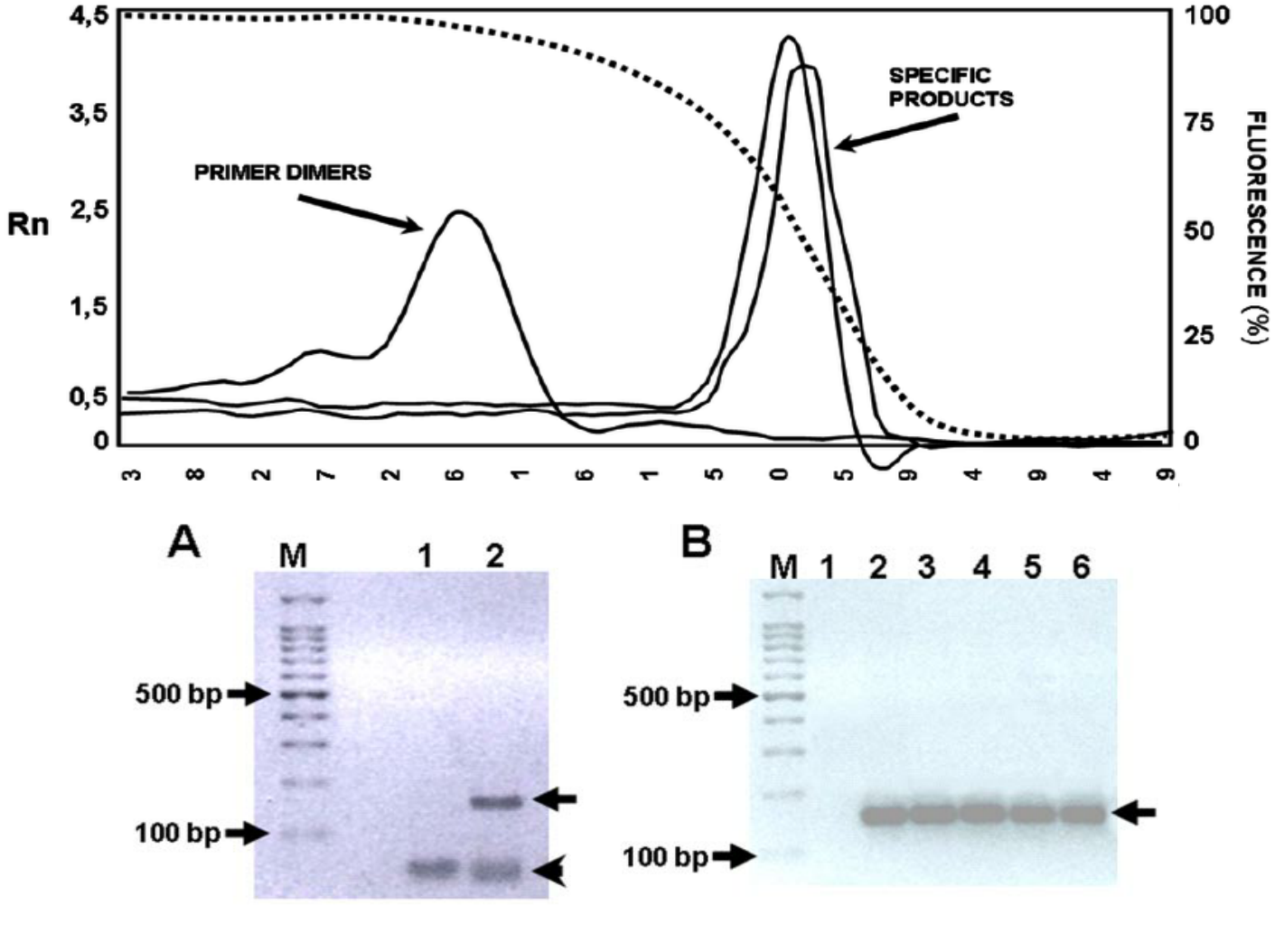
- **Pure, homogenous PCR products produce a single, sharply defined melting curve with a narrow peak.**



SPECIFICITY AND MELTING CURVE:

- **Primer dimers melt at relatively low temperatures and have broader peak**




4,5
3,5
2,5
1,5
0,5
0
Rn
100
75
50
25
0
FLUORESCENCE (%)
PRIMER DIMERS
SPECIFIC PRODUCTS
A
M
1
2
500 bp
100 bp
B
M 1 2 3 4 5 6
500 bp
100 bp

THANKS A LOT

WITH MY BEST REGARDS AND MY BEST WISHES