Principals of Real-Time PCR

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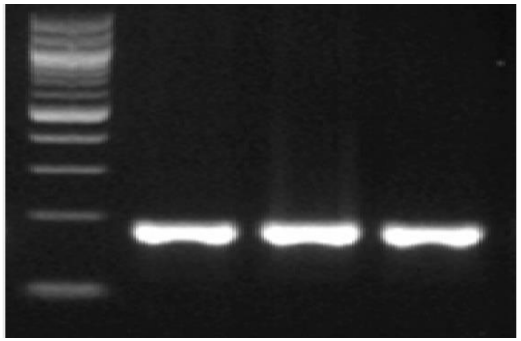
What Is Real-Time PCR?

- **Nucleic acid (DNA) amplification and detection technique considered as one of the most valuable tools in biological research today.**
- **Real-time PCR can be used for both qualitative and quantitative analysis**

What Is Real-Time PCR?

- **In conventional PCR, the amplified DNA product is detected by visualizing the DNA product on an agarose gel.**



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

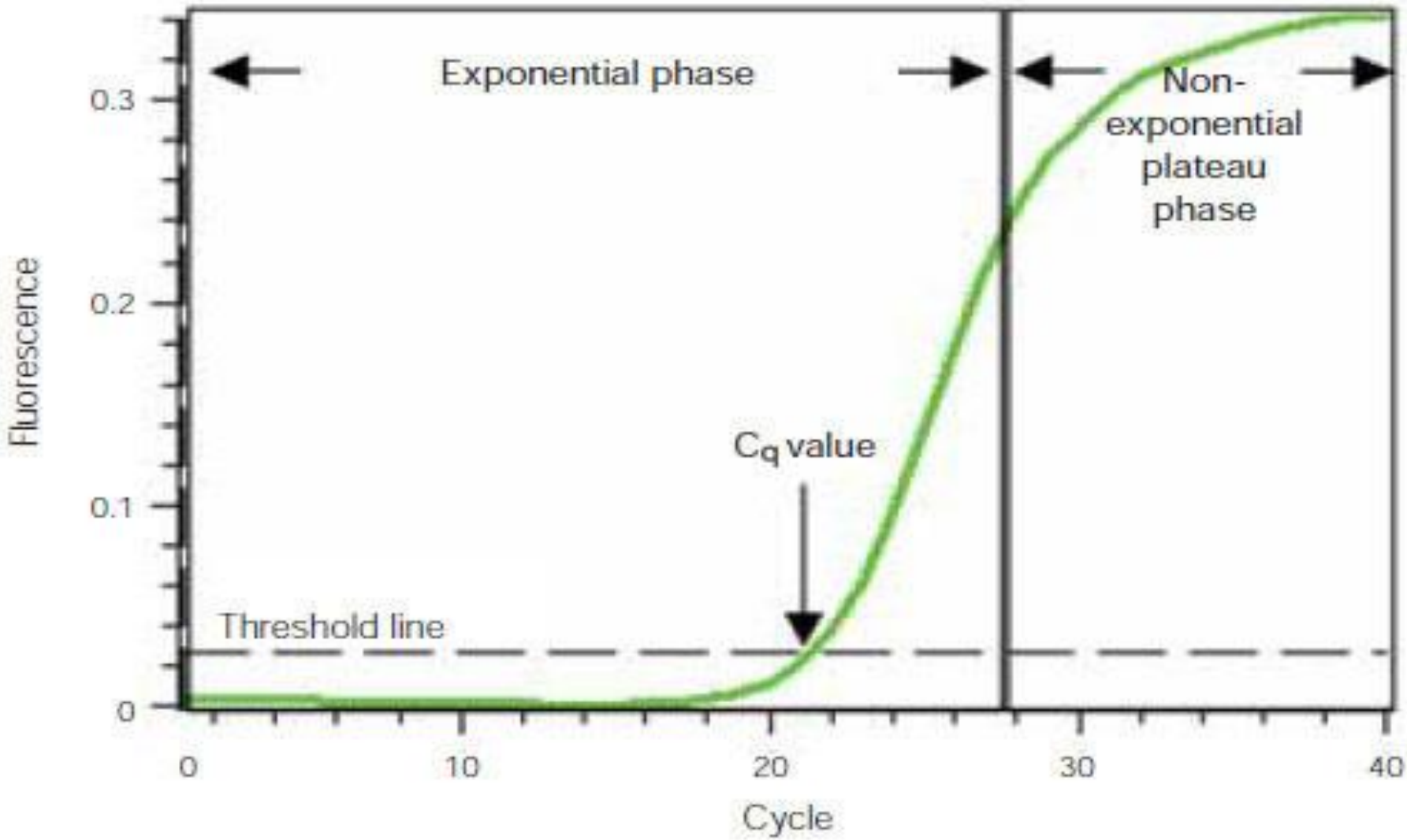
What Is qRT-PCR?

- **Quantitative real-time PCR is thus also known as qRT-PCR analysis.**
- **qRT-PCR reactions are run and data are evaluated in a unified, closed system, opportunities for contamination are reduced.**

How Does Real-Time PCR Work?

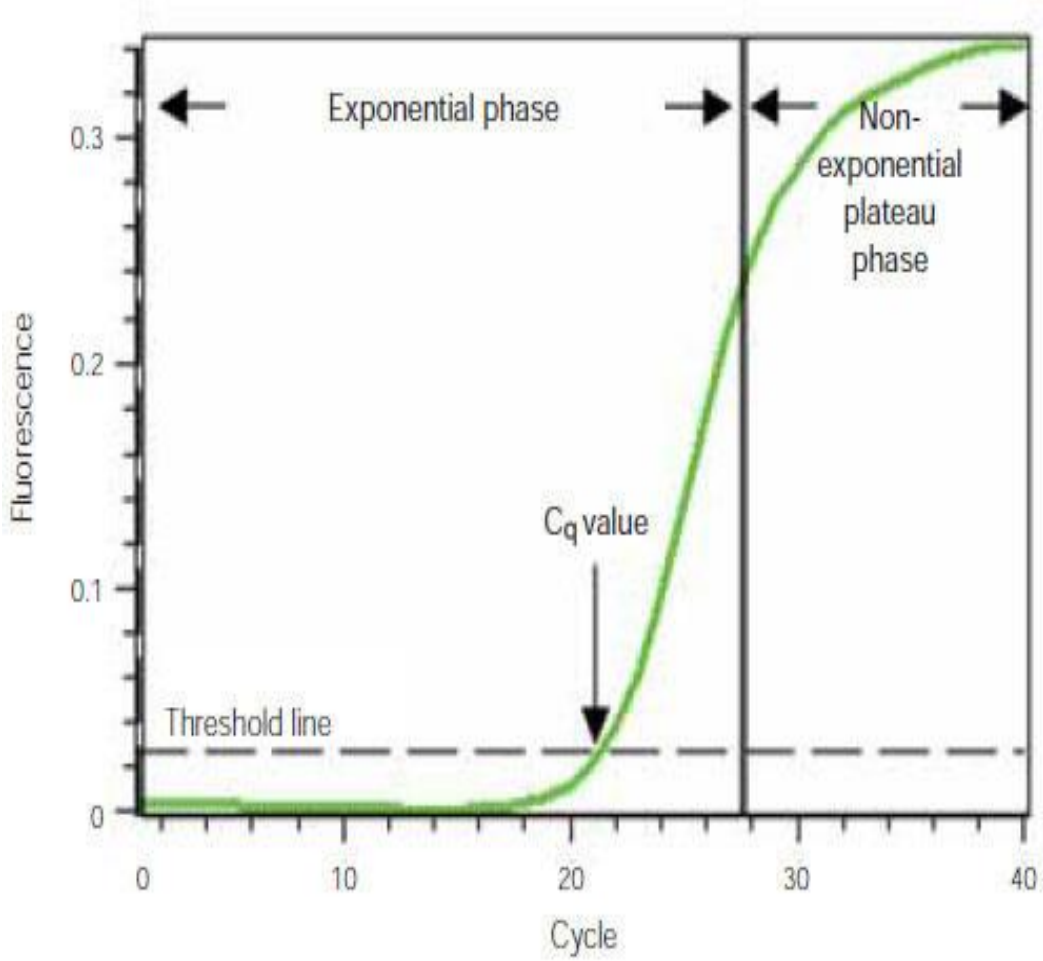
- **A fluorescent reporter molecule included in each reaction → Increasing the fluorescence with the increasing of the amount of DNA.** Real Time PCR - Basic simple animation - part 1 intro HD.mp4
- **Specialized thermal cyclers equipped with fluorescence detection modules are used.**
- **The measured fluorescence is proportional to the total amount of amplicon; the change in fluorescence over time is used to calculate the amount of amplicon.**

- **qPCR analysis typical amplification plot.**



How Does Real-Time PCR Work?

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.



How Does Real-Time PCR Work?

The amplification plot shows two phases:

1. **An exponential phase.**
2. **A non-exponential plateau phase.**

During the exponential phase, the amount of PCR product approximately doubles in each cycle.

As the reaction proceeds, the reaction's components are consumed and ultimately one or more of the components becomes limiting.

At this point, the reaction slows and enters the plateau phase.

PCR and Real-time PCR Animation - Animation PCR et PCR en temps réel.mp4

How Does Real-Time PCR Work?

- **Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable (cycles 1–18) even though product accumulates exponentially.**
- **Eventually, enough amplified product accumulates to yield a detectable fluorescence signal.**
- **The cycle number at which this occurs is called the quantification cycle, or C_q.**

- **Because the C_q value is measured in the exponential phase when reagents are not limited, real-time qPCR can be used to reliably and accurately calculate the initial amount of template present in the reaction based on the known exponential function describing the reaction progress**

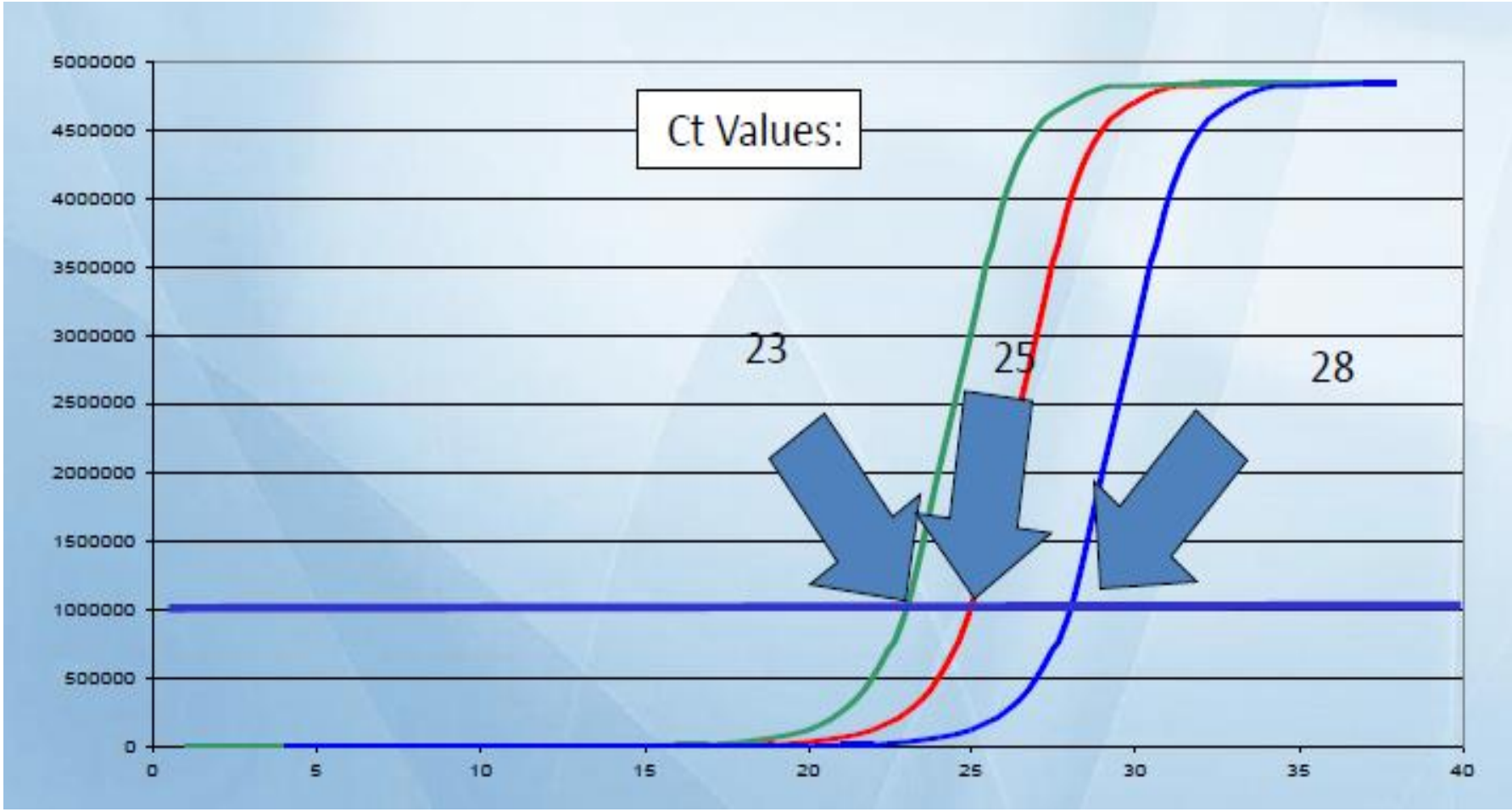
C_q and the amount of template

- **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.**

C_q and the amount of template

- **In contrast, 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.**

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



Quantification Cycle (Cq)/Threshold Cycle (Ct):

Definition:

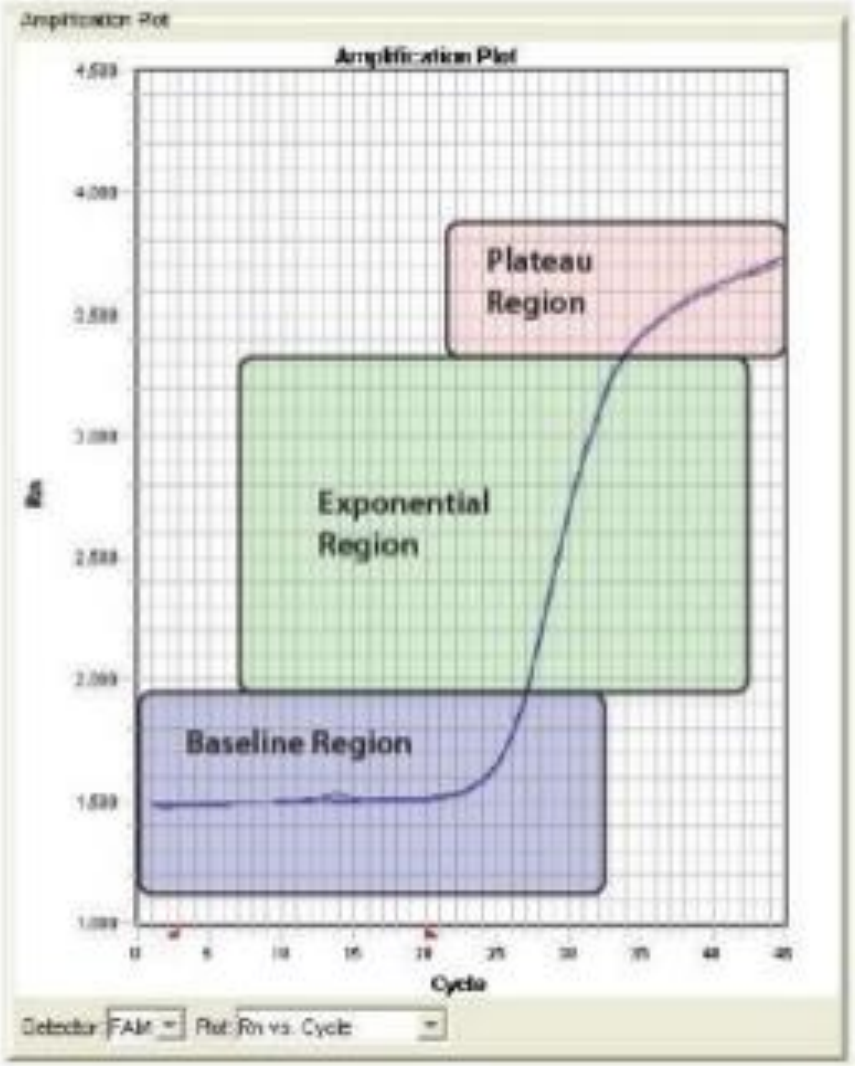
The cycle at which fluorescence from amplification exceeds the background fluorescence.

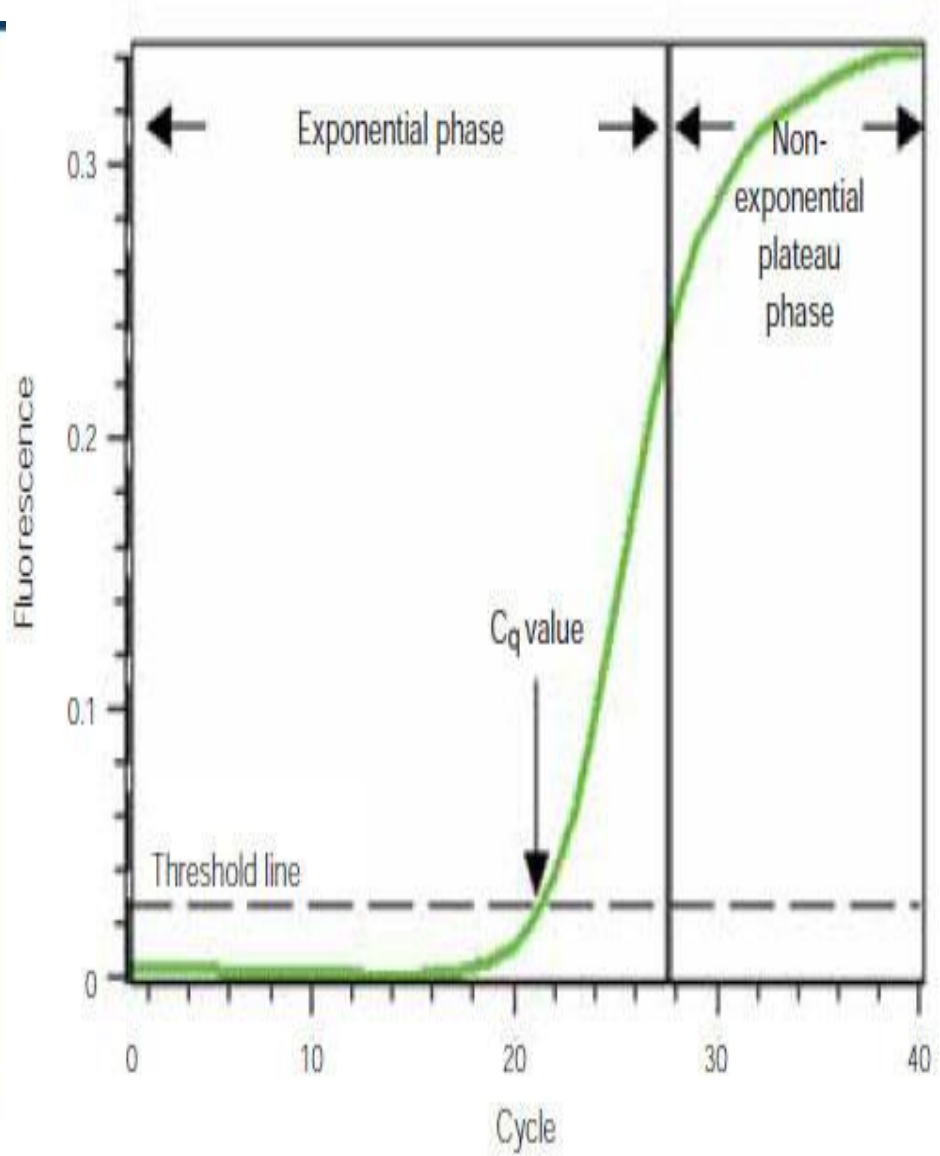
Referred to threshold cycle (Ct), crossing point (Cp) and take-off point (TOF) by different instrument manufacturers.

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 Work?



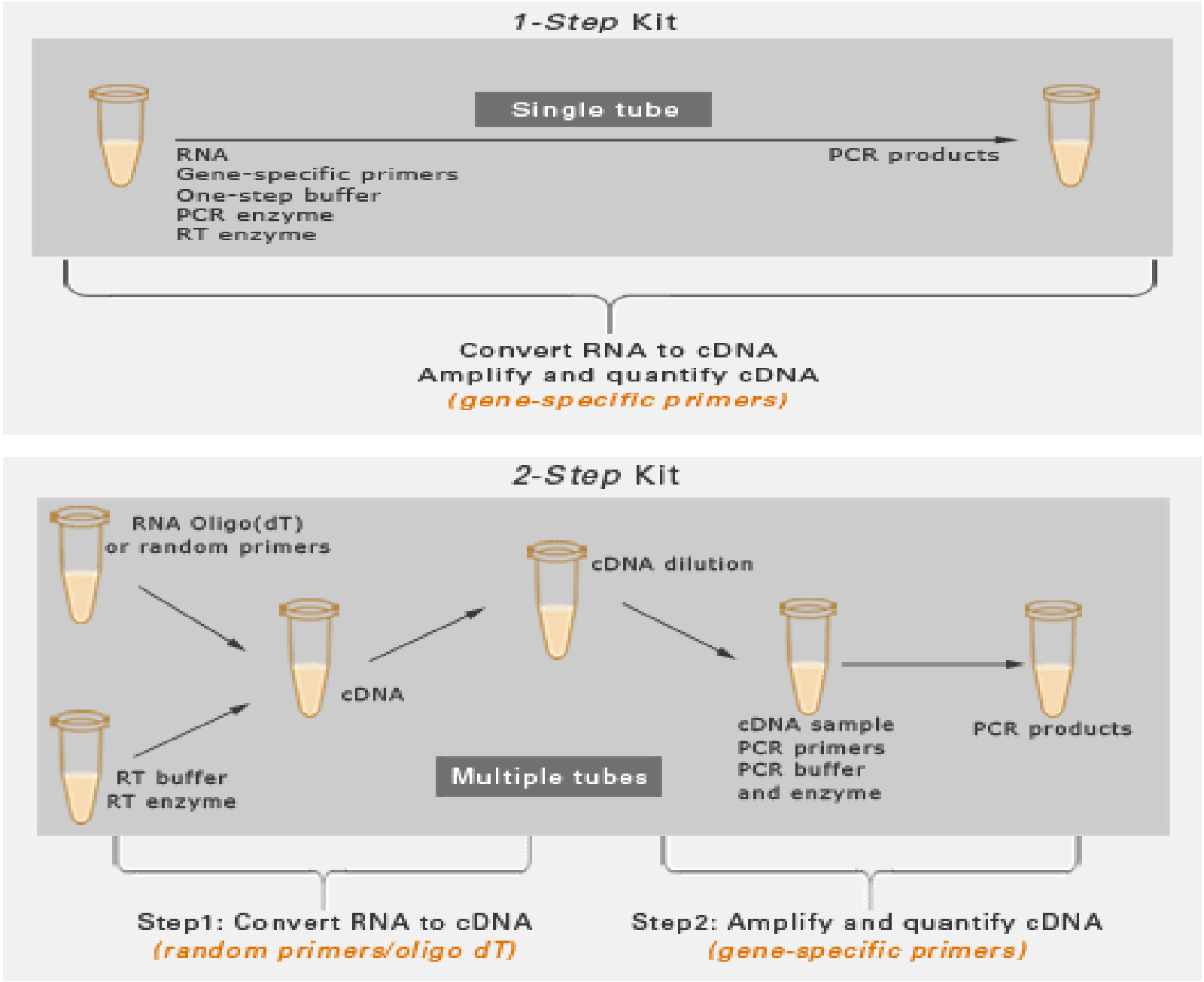


Two-step qRT-PCR and one-step qRT-PCR.

In both cases, RNA is reverse transcribed into cDNA, and the cDNA is then used as the template for qPCR amplification.

One-step: refer to whether the RT and real-time PCR amplification are performed in the same or separate tubes.

Two-step RNA is first transcribed into cDNA in a reaction using reverse transcriptase. An aliquot of the resulting cDNA is then used as a template for multiple qPCR reactions.

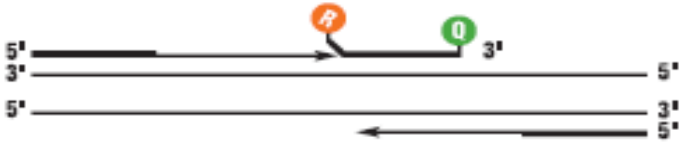

1-Step Kit
Single tube
RNA
Gene-specific primers
One-step buffer
PCR enzyme
RT enzyme
PCR products
Convert RNA to cDNA
Amplify and quantify cDNA
(gene-specific primers)
2-Step Kit
RNA Oligo(dT)
or random primers
cDNA dilution
cDNA
RT buffer
RT enzyme
Multiple tubes
cDNA sample
PCR primers
PCR buffer
and enzyme
PCR products
Step1: Convert RNA to cDNA
(random primers/oligo dT)
Step2: Amplify and quantify cDNA
(gene-specific primers)

Types

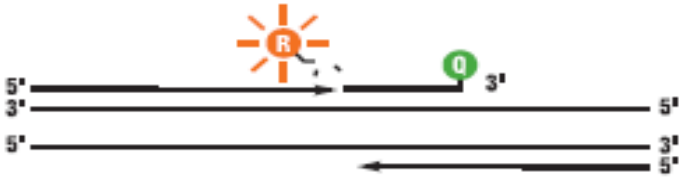
TAQMAN® PROBE-BASED ASSAY CHEMISTRY

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

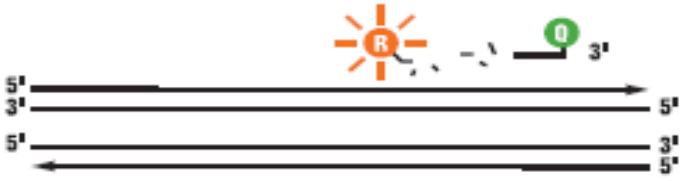
2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



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



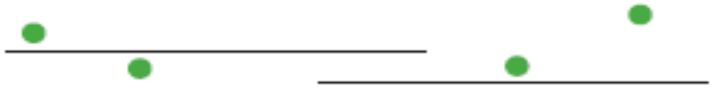
4. **Polymerization completed:** Once separated from the quencher, the reporter dye emits its characteristic fluorescence.



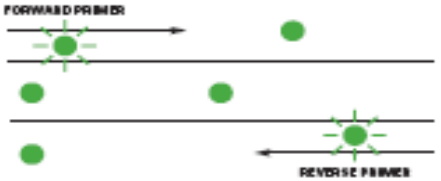
SYBR® GREEN I DYE ASSAY CHEMISTRY

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

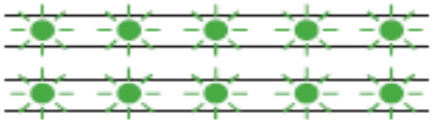
2. **Denaturation:** When the DNA is denatured, the SYBR® Green I Dye is released and the fluorescence is drastically reduced.



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.



Accuracy, Reliability and standardization of TR-PCR & qRT-PCR

Accuracy & Reliability

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

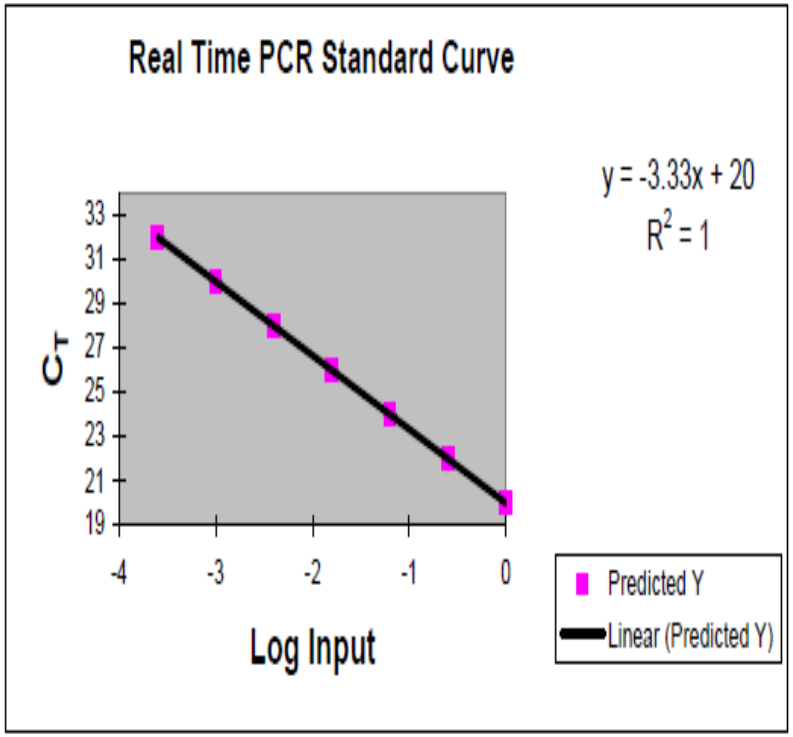
1. Serially dilute an artificial template of known concentration.

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 ($\log_2 10 = 3.3219$).



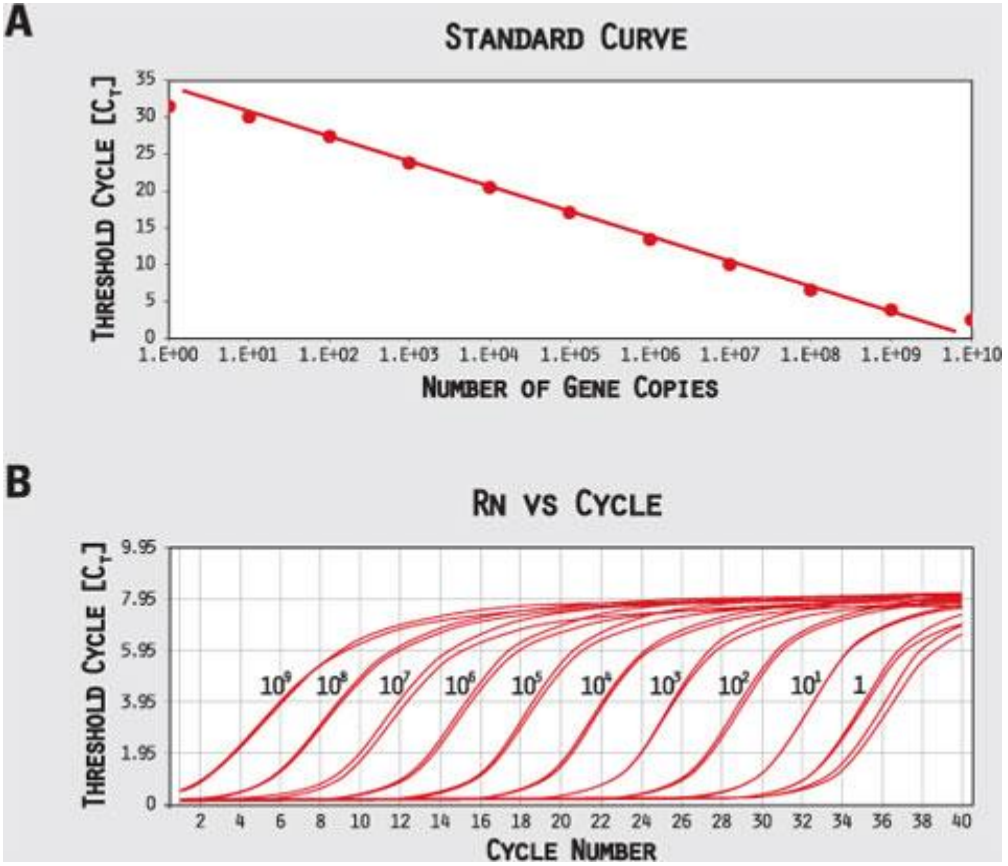


Figure 1: Real-Time PCR Standard Curve representing 100% PCR Efficiency

Specificity and Melting Curve:

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.

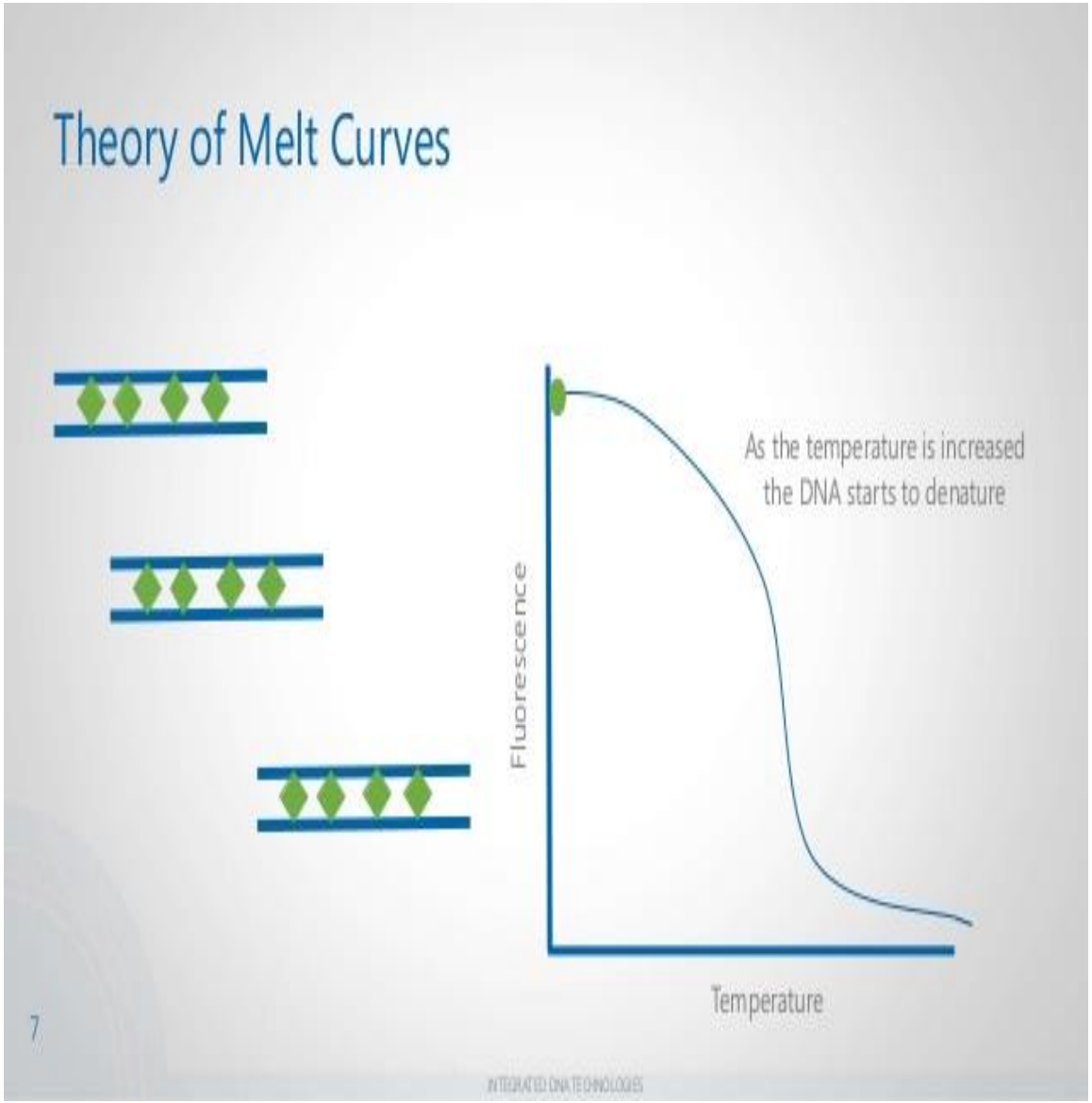
Typically, the thermal cycler being used to run the qPCR is programmed to produce the melt curve after the amplification cycles are completed.

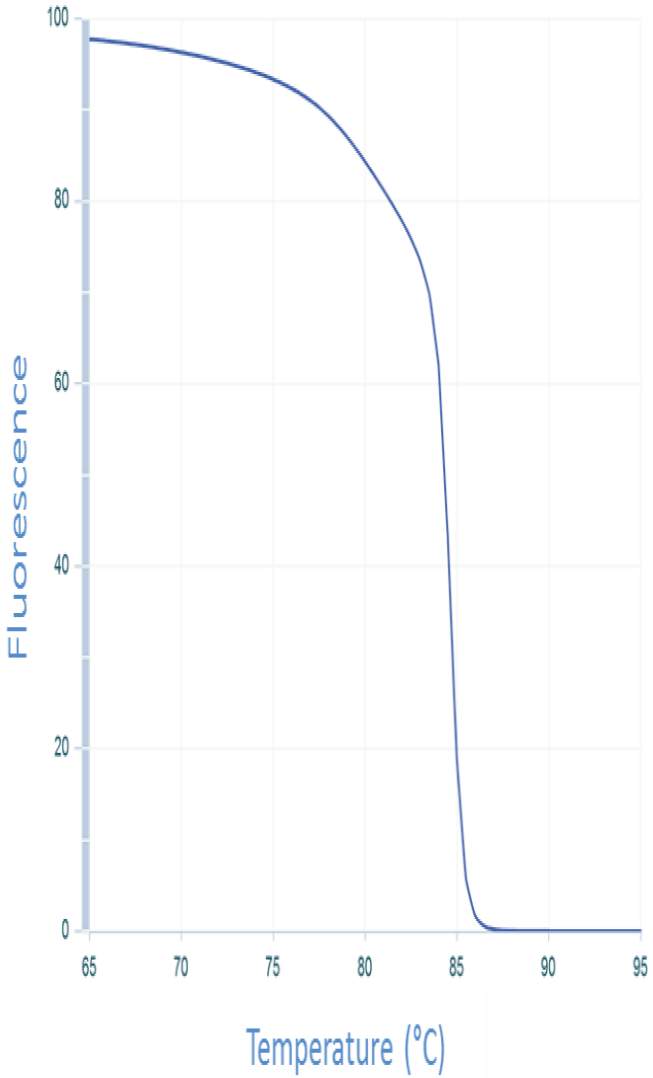
At the end of the qPCR run, the thermal cycler starts at a preset temperature (usually above the primer T_m; e.g., 65°C) and measures the amount of fluorescence.

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:

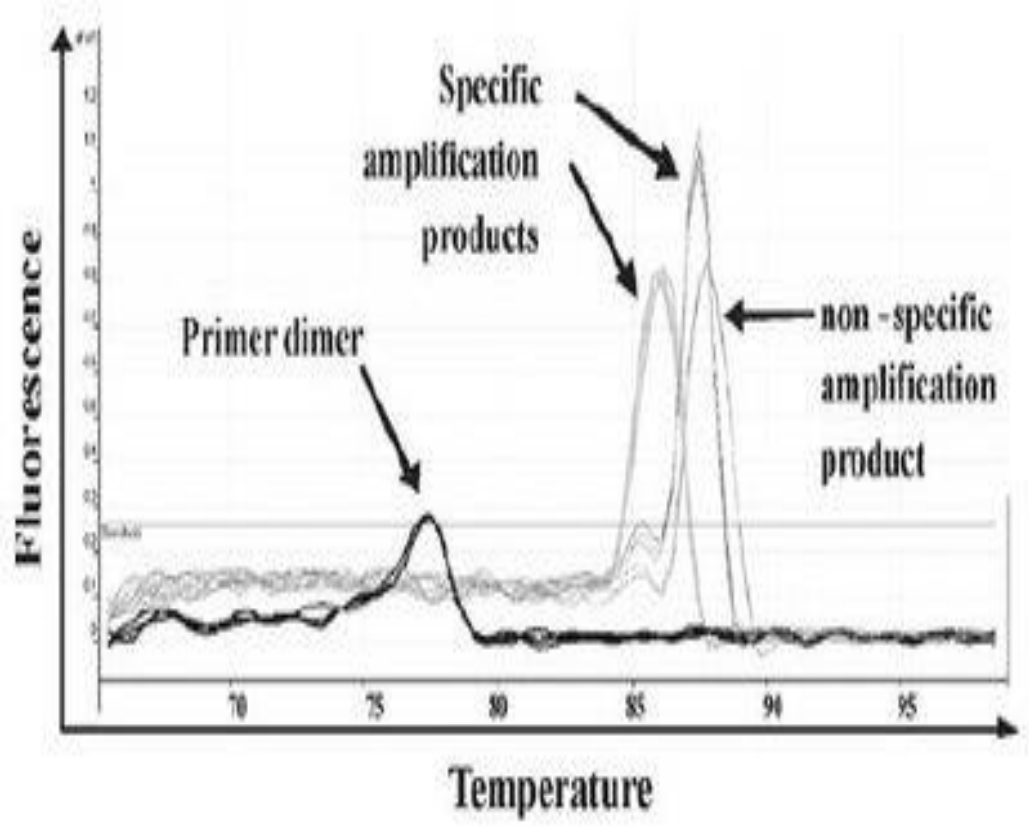
[Melt curve analysis in qPCR experiments.mp4]

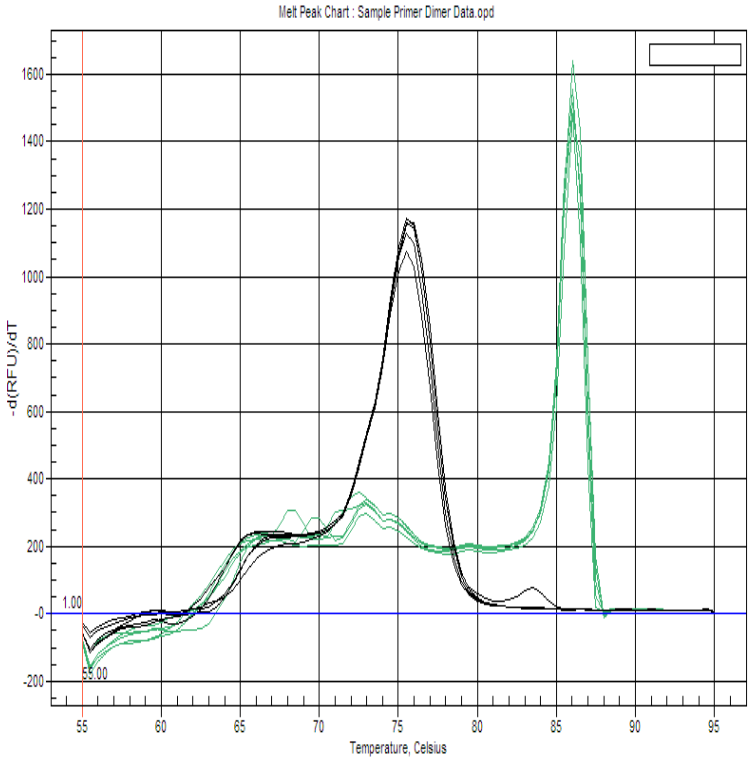


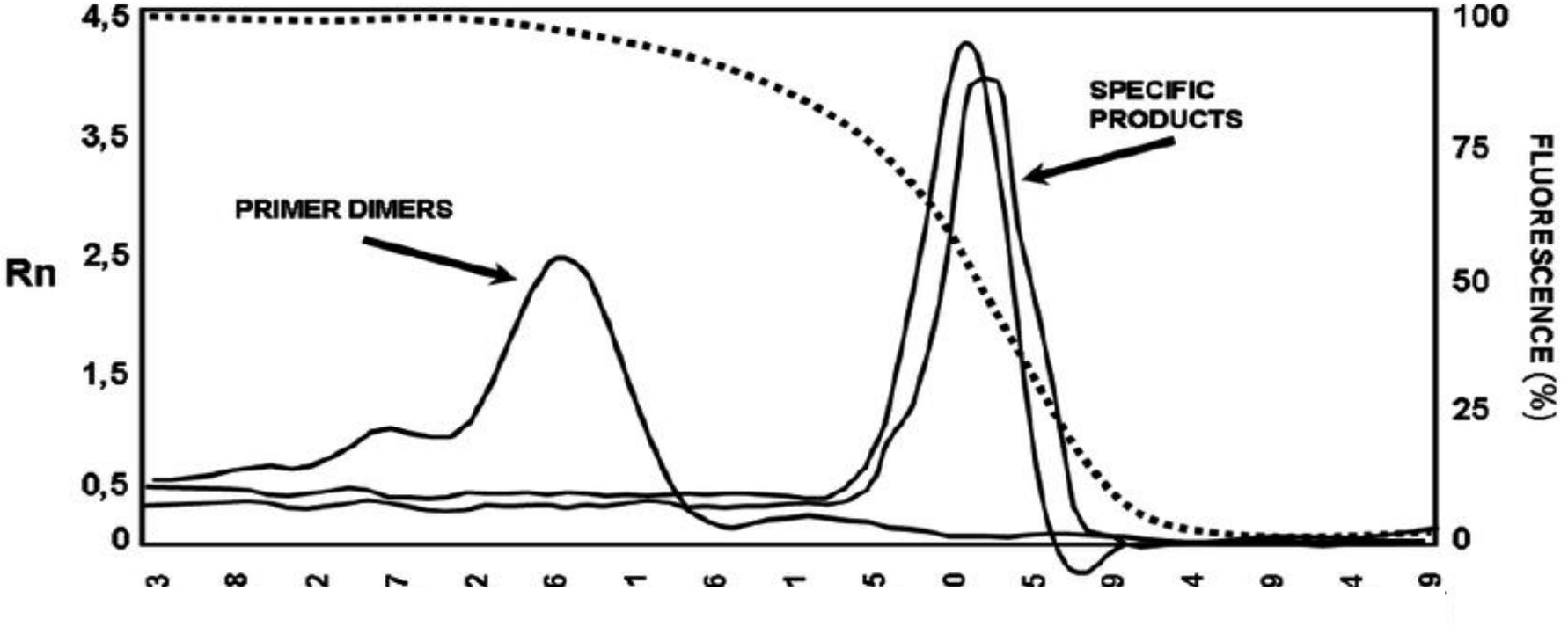


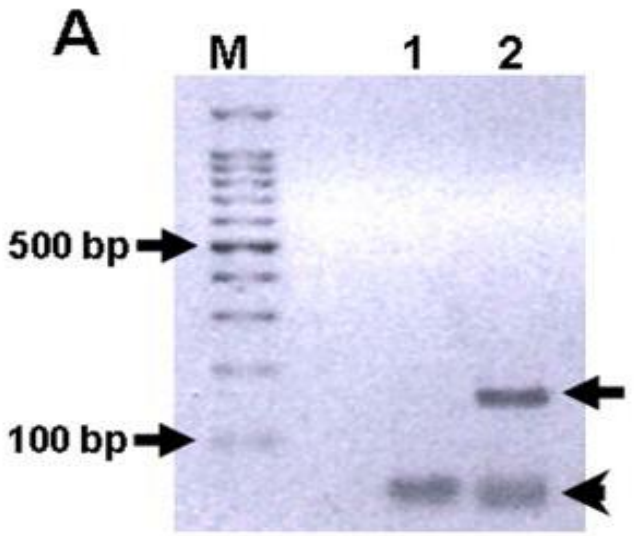
Specificity and Melting Curve:

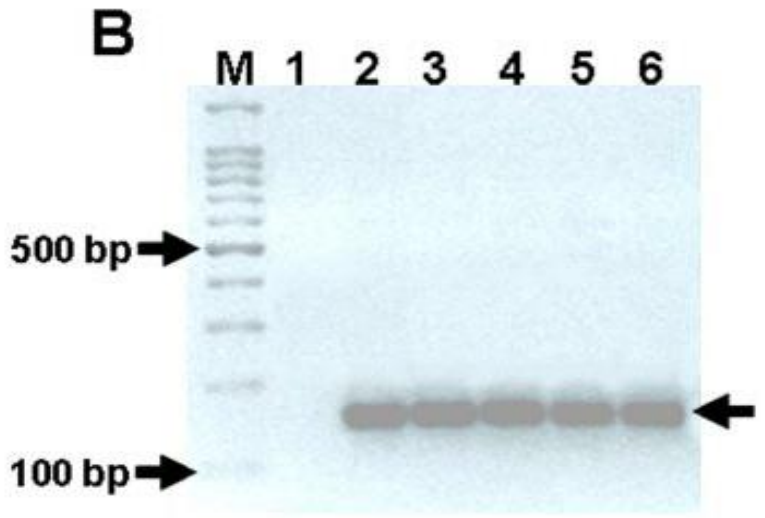
- **Pure, homogenous PCR products produce a single, sharply defined melting curve with a narrow peak.**
- **Primer dimers melt at relatively low temperatures and have broader peak**






4,5
3,5
2,5
1,5
0,5
0
Rn
100
75
50
25
0
FLUORESCENCE (%)
PRIMER DIMERS
SPECIFIC PRODUCTS
3 8 2 7 2 6 1 6 1 5 0 5 9 4 9 4 9


A
M 1 2
500 bp
100 bp


B
M 1 2 3 4 5 6
500 bp
100 bp

Step-by-Step Design Strategy for qPCR Assays

When designing a qPCR assay, follow these steps:

1. **Check the literature and databases for existing primers.**
2. **Choose a target sequence**
3. **Design primers and probes**
4. **Check primer specificity**
5. **Assess primer and probe properties: melting temperature (T_m).**
6. **Determine PCR product properties**
7. **Validate the primers and/or probes and optimize the protocol**

Thanks a lot

with my Best Regards and My Best wishes

Amira A. T. AL-Hosary