



Real-Time PCR

Principles and Applications

Dr Esam Ibraheem Azhar

(BSc, MSc, Ph.D Molecular Medical Virology)

Asst. Prof. Medical Laboratory Technology Department

Real-Time PCR

Principles and Applications

Objectives

- This Lecture will provide an understanding of the following:
 - Limitations of traditional PCR
 - Introduction to Real-Time PCR
 - Methods
 - Advantages of Real-Time chemistries over traditional PCR

PCR analysis

From classical to real time

Some definitions

- **Molecular biology** : the study of gene structure and function at the molecular level.
- **Gene** : hereditary unit that is composed of a sequence of DNA and occupies a specific position or locus. The genes carry the instructions for making all the thousands of proteins that are found in a cell.
- **DNA** : is a long fiber made from two strands that stick together with a slight twist.

PCR analysis : refreshment



- The 4 bases are linked together to form a chain or single strand of DNA:

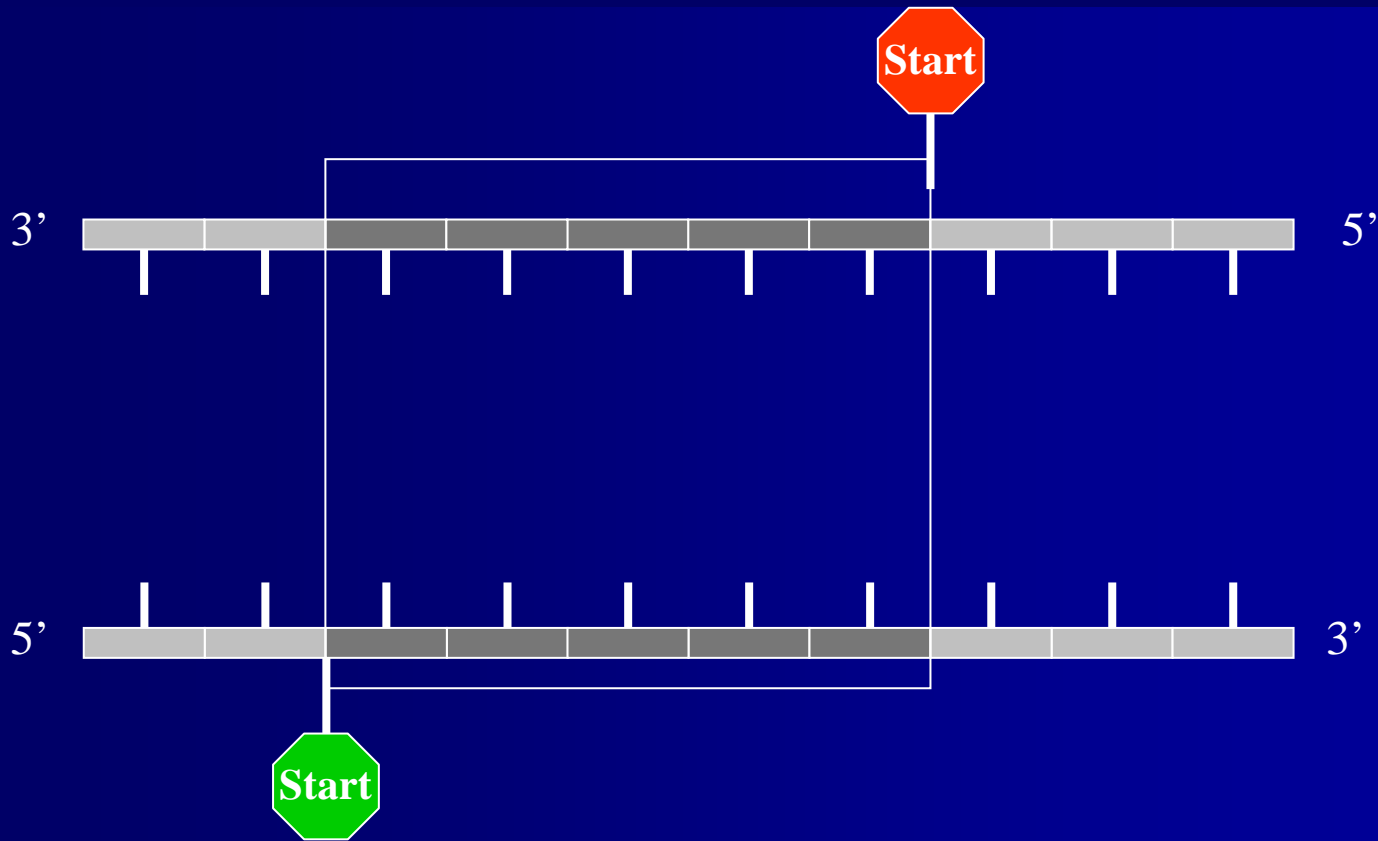


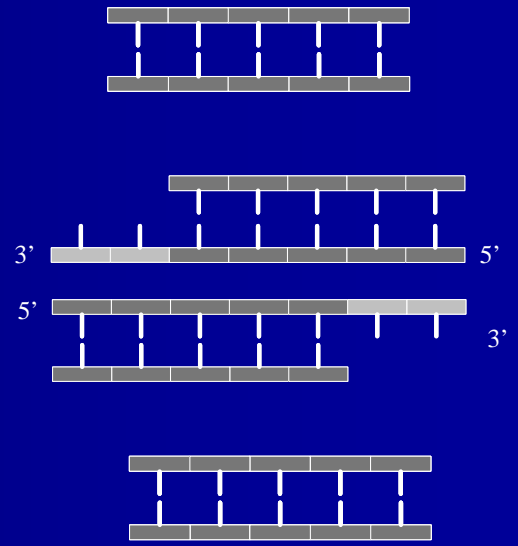
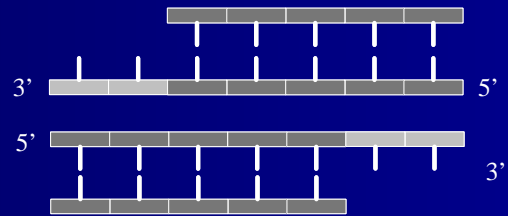
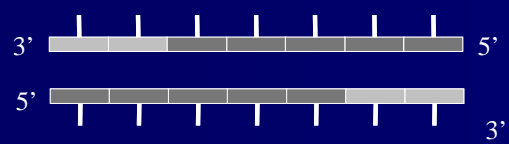
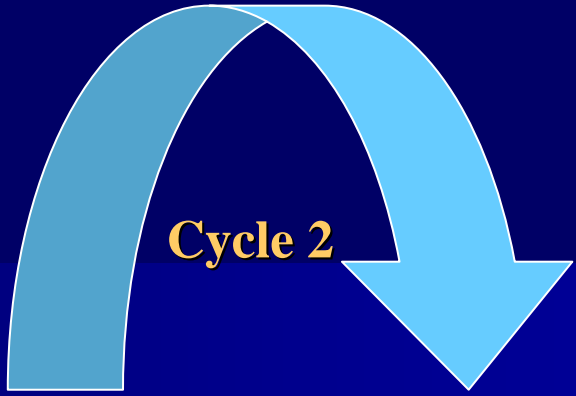
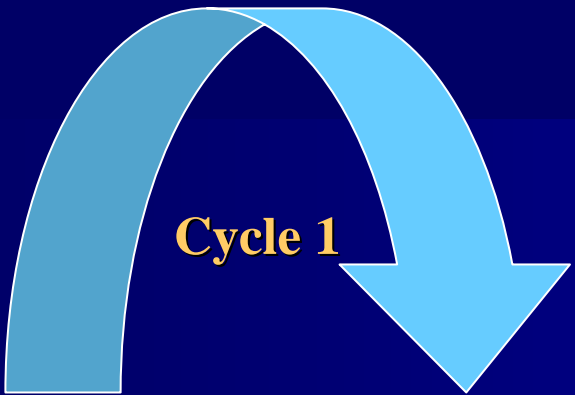
PCR analysis : refreshment

- Most DNA is double stranded and pair in a unique way:

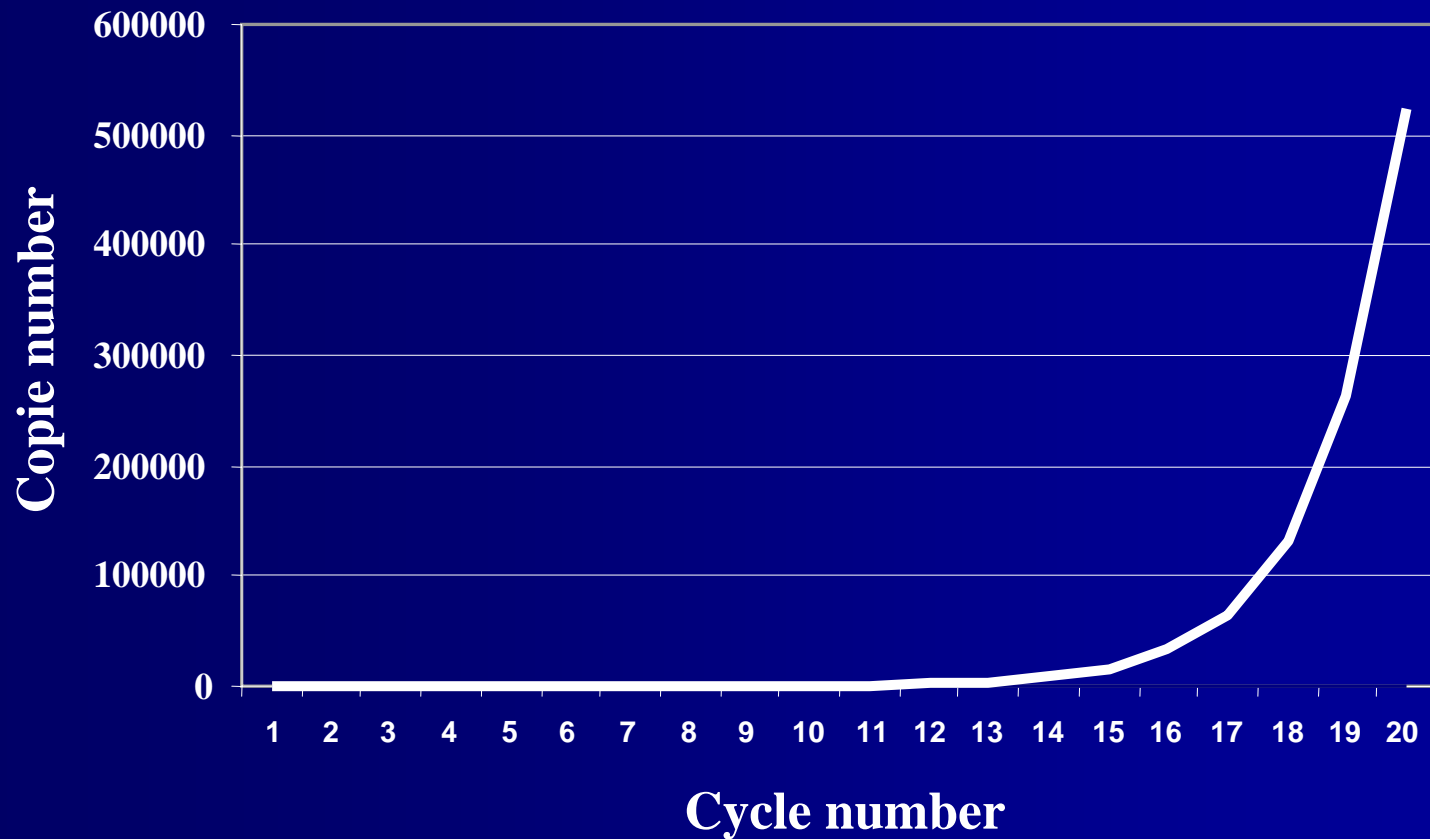


PCR analysis



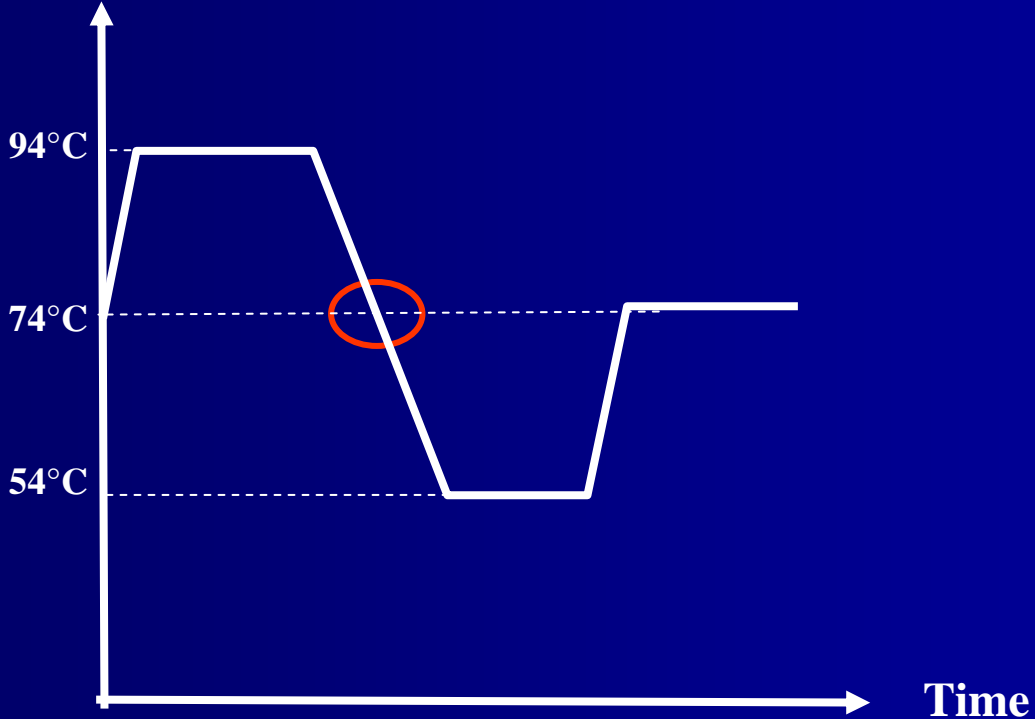


Amplicons production *vs* Cycles



Temperature Curve

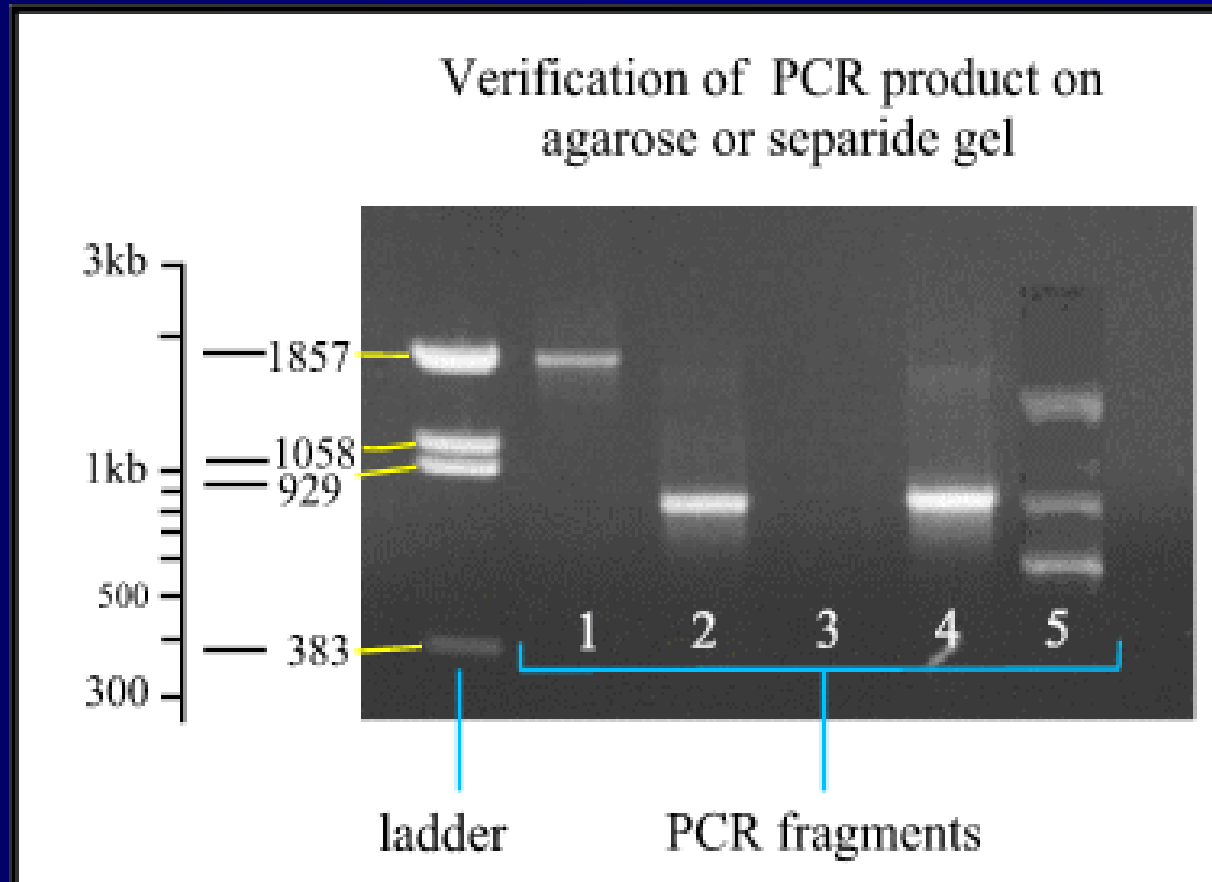
Temperature



The Polymerase Chain Reaction “PCR”

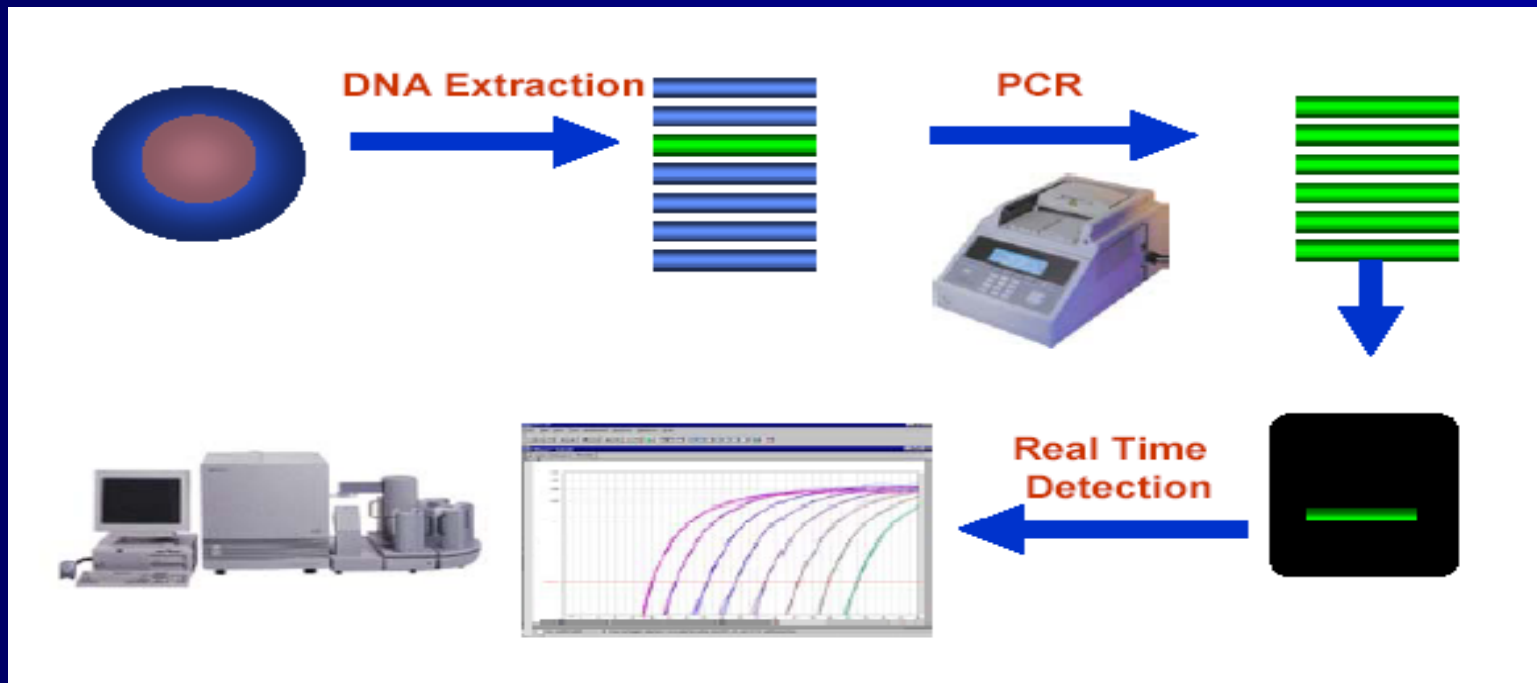
- Traditional PCR; gel-based detection
- Real-Time PCR; fluorescence-based detection

Post-PCR Analysis



The Evolution of PCR to Real-Time

- PCR has completely revolutionized the detection of RNA and DNA. Traditional PCR has advanced from detection at the end-point of the reaction to detection while the reaction is occurring.



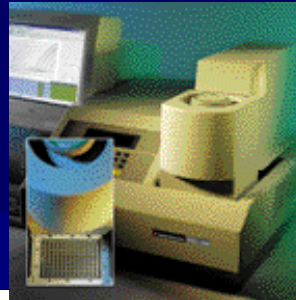
real-time real-time
real-time PCR
real-time
hardware

- Intuitive programming
- Fast and accurate performance
- Flexibility for multiple users
- Small footprint

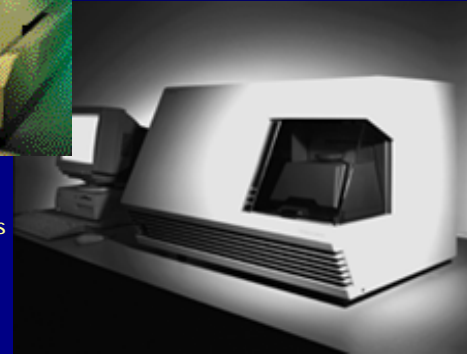


The optical module fits on the iCycler base unit, offering you Real Time Quantitative PCR* capability.

iCycler
BioRad



5700
Applied Biosystems



7700
Applied Biosystems



LightCycler
Roche



FluorTracker
Stratagene



FluorImager
Molecular Dynamics

real-time real-time
LightCycler
real-time
Set Up

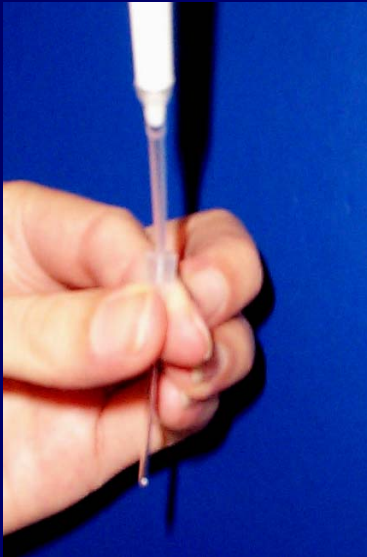
1.



3.



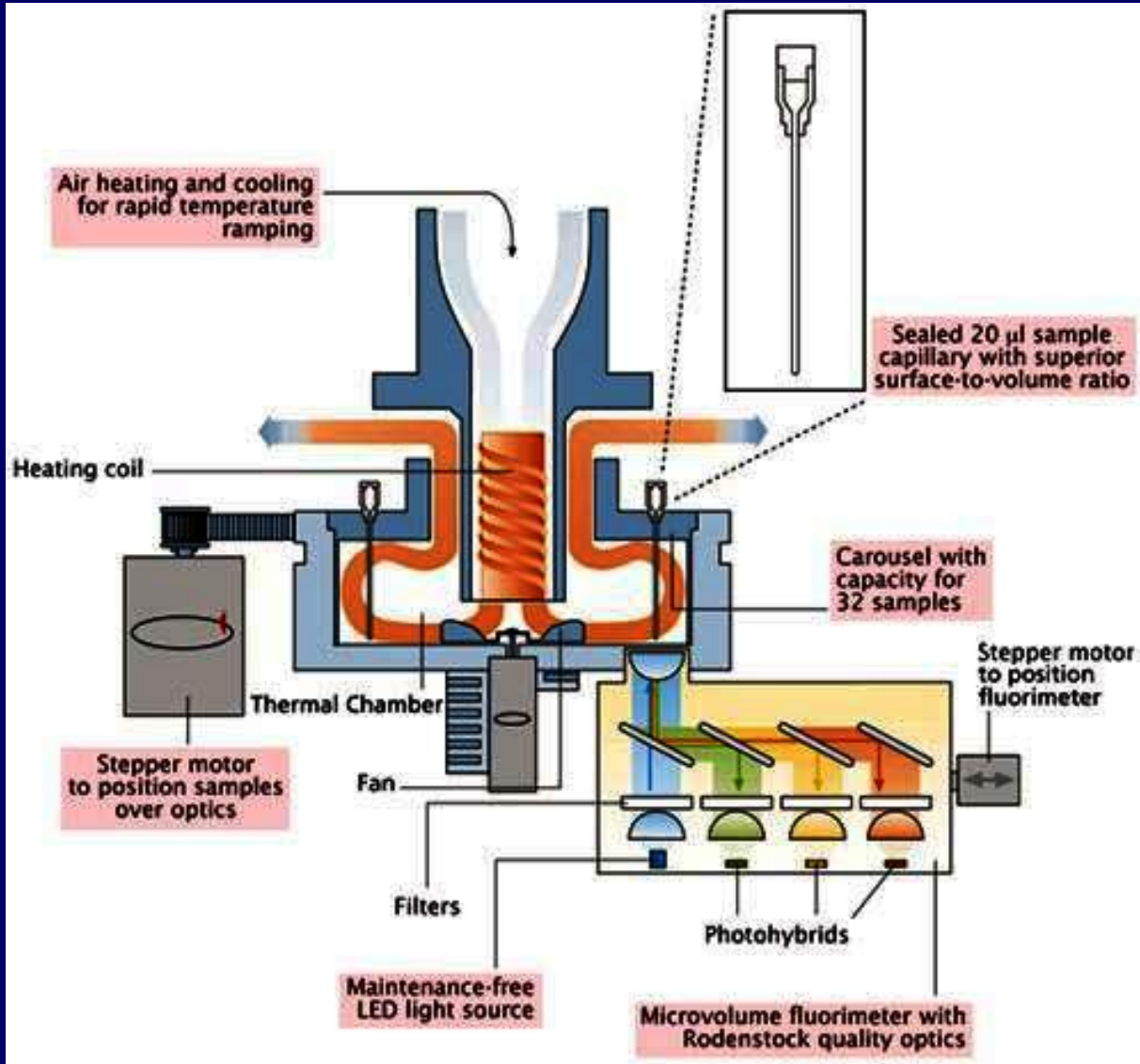
2.



4.



real-time real-time
LightCycler
real-time
Hardware



Real-Time Vs Traditional PCR

- **Real-Time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction.**

Limitations of End-Point PCR

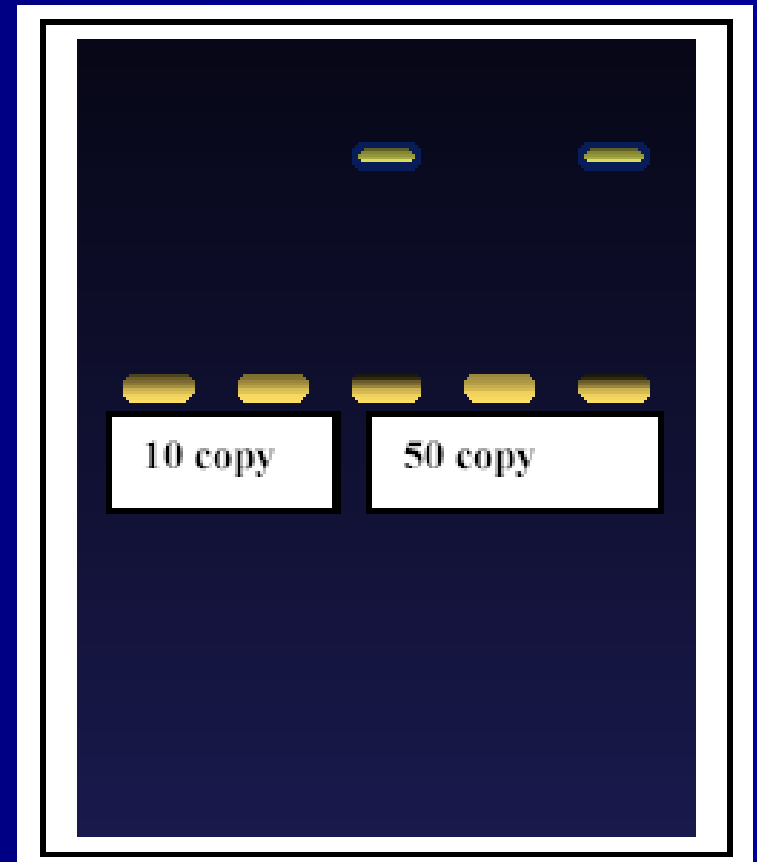
- Agarose gel results are obtained from the end point of the reaction. Endpoint detection is very time consuming.
- Results may not be obtained for days.
- Results are based on size discrimination, which may not be very precise.
- As seen later in the section, the end point is variable from sample to sample.
- While gels may not be able to resolve these variabilities in yield, real-time PCR is sensitive enough to detect these changes.
- Agarose Gel resolution is very poor, about 10 fold.
- Real-Time PCR can detect as little as a two-fold change!

Problems with End-Point Detection

- Poor Precision
- Low sensitivity
- Low resolution
- Non - Automated
- Size-based discrimination only
- Results are not expressed as numbers
- Ethidium bromide for staining is not very quantitative
- Post PCR processing

Agarose Gel

- Samples in the gel contain 10 copies and 50 copies, respectively.
- It is hard to differentiate between the 5-fold change on the Agarose gel.
- Real-Time PCR is able to detect a two-fold change (i.e. 10 Vs. 20 copies).



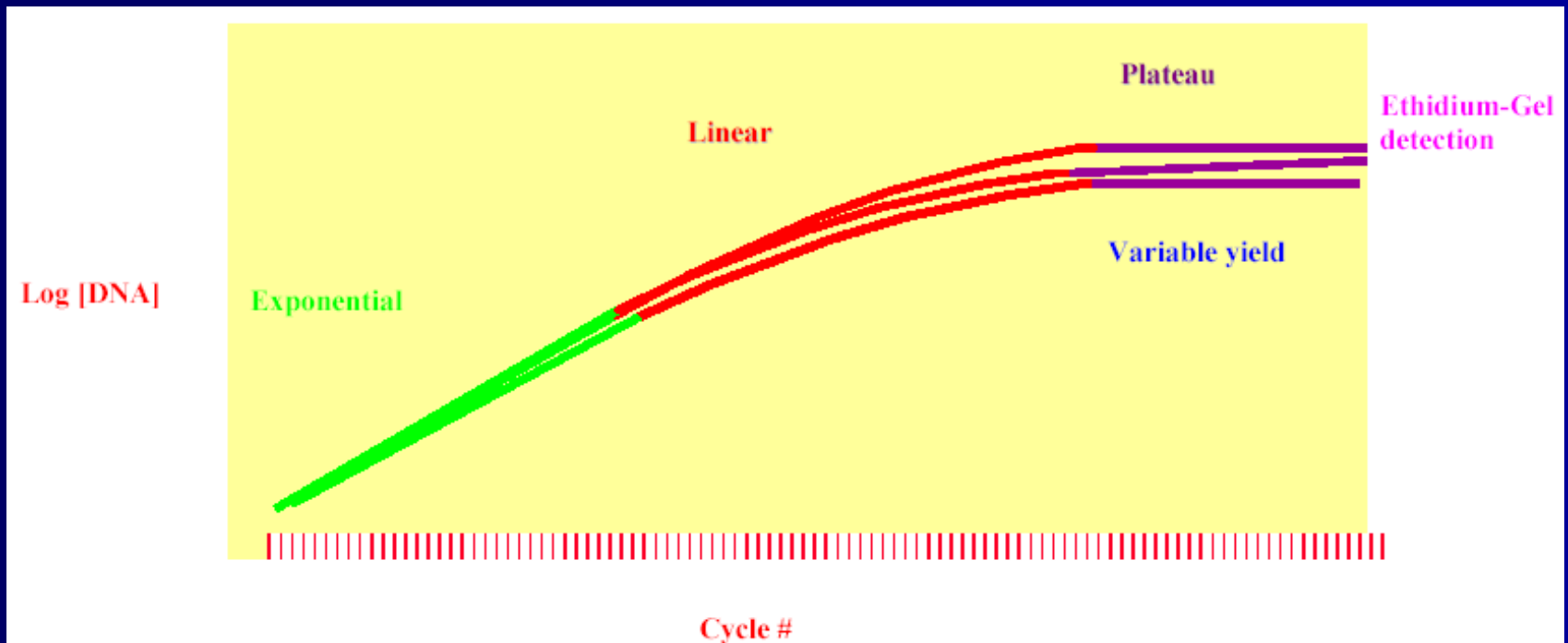
PCR Phases

To understand why end-point PCR is limiting, it is important to understand what happens during a PCR reaction.

- A basic PCR run can be broken up into three phases:
 - **Exponential:** Exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). The reaction is very specific and precise.
 - **Linear (High Variability):** The reaction components are being consumed, the reaction is slowing, and products are starting to degrade.
 - **Plateau (End-Point: Gel detection for traditional methods):** The reaction has stopped, no more products are being made and if left long enough, the PCR products will begin to degrade.

PCR Phases

- Shows three replicates of a sample. The replicates have the same starting quantity. As the PCR reaction progresses, the samples begin to amplify in a very precise manner. Amplification occurs exponentially, that is a doubling of product (amplicon) occurs every cycle. This type of amplification occurs in the exponential phase. Exponential amplification occurs because all of the reagents are fresh and available, the kinetics of the reaction push the reaction to favor doubling of amplicon.



- However, as the reaction progresses, some of the reagents are being consumed as a result of amplification. This depletion will occur at different rates for each replicate. The reactions start to slow down and the PCR product is no longer being doubled at each cycle. This linear amplification can be seen in the linear phase of the reaction. The three samples begin to diverge in their quantities during the linear phase.
- Eventually the reactions begin to slow down and stop all together or plateau. Each tube or reaction will plateau at a different point, due to the different reaction kinetics for each sample. These differences can be seen in the plateau phase. The plateau phase is where traditional PCR takes its measurement, also known as end-point detection.
- Previous Figure shows that the three replicate samples, which started out at the same quantity in the beginning of the reaction, reflect different quantities at the plateau phase. Since the samples are replicates they should have identical quantities. Therefore, it will be more precise to take measurements during the exponential phase, where the replicate samples are amplifying exponentially.
- The amplification phases can be viewed differently to assess the PCR phases. The figures that follow show the phases of PCR in a Logarithmic scale view and a Linear scale view (Figures 4 and 5).

Figure 4: PCR Phases in Log view

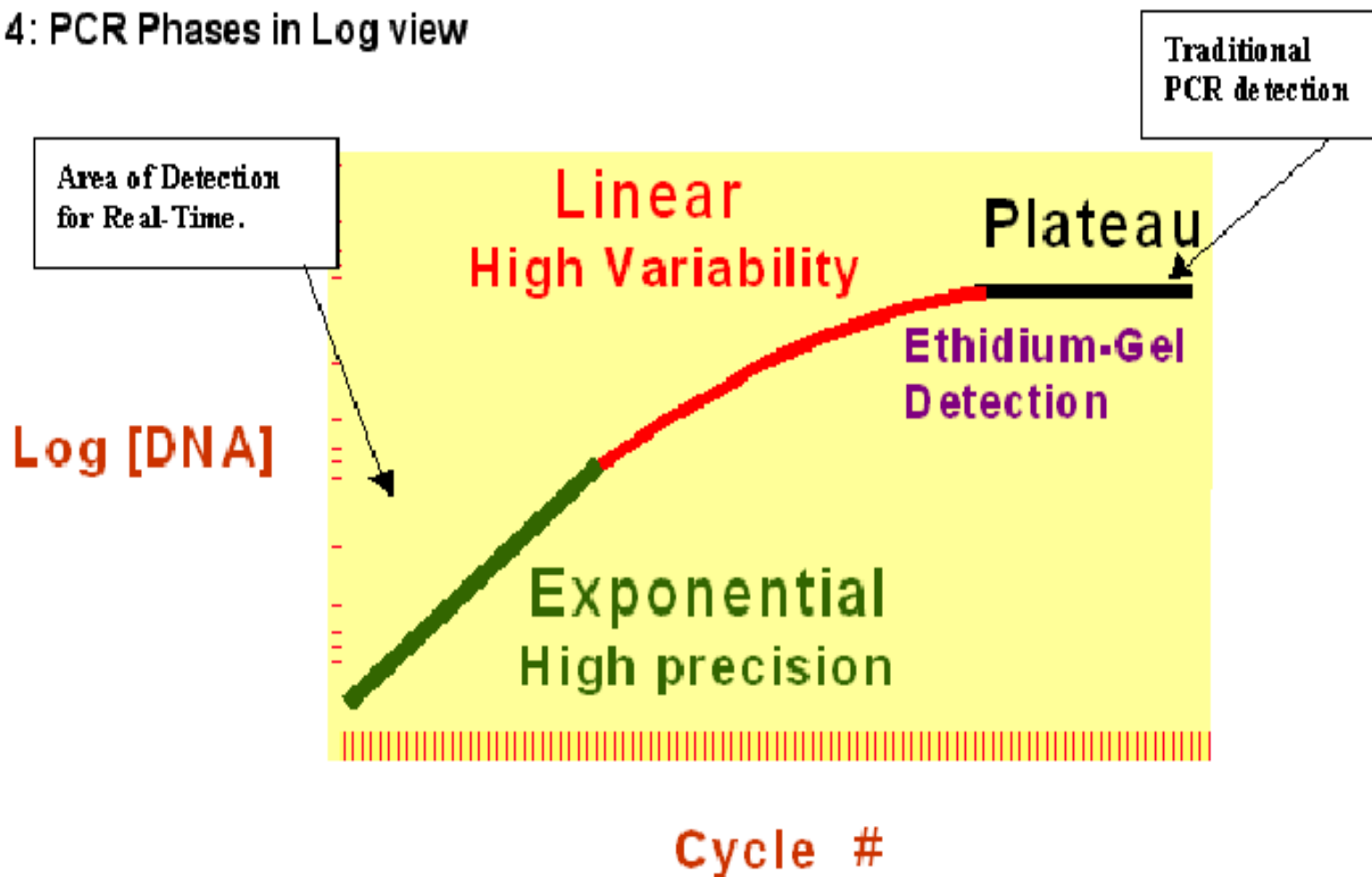
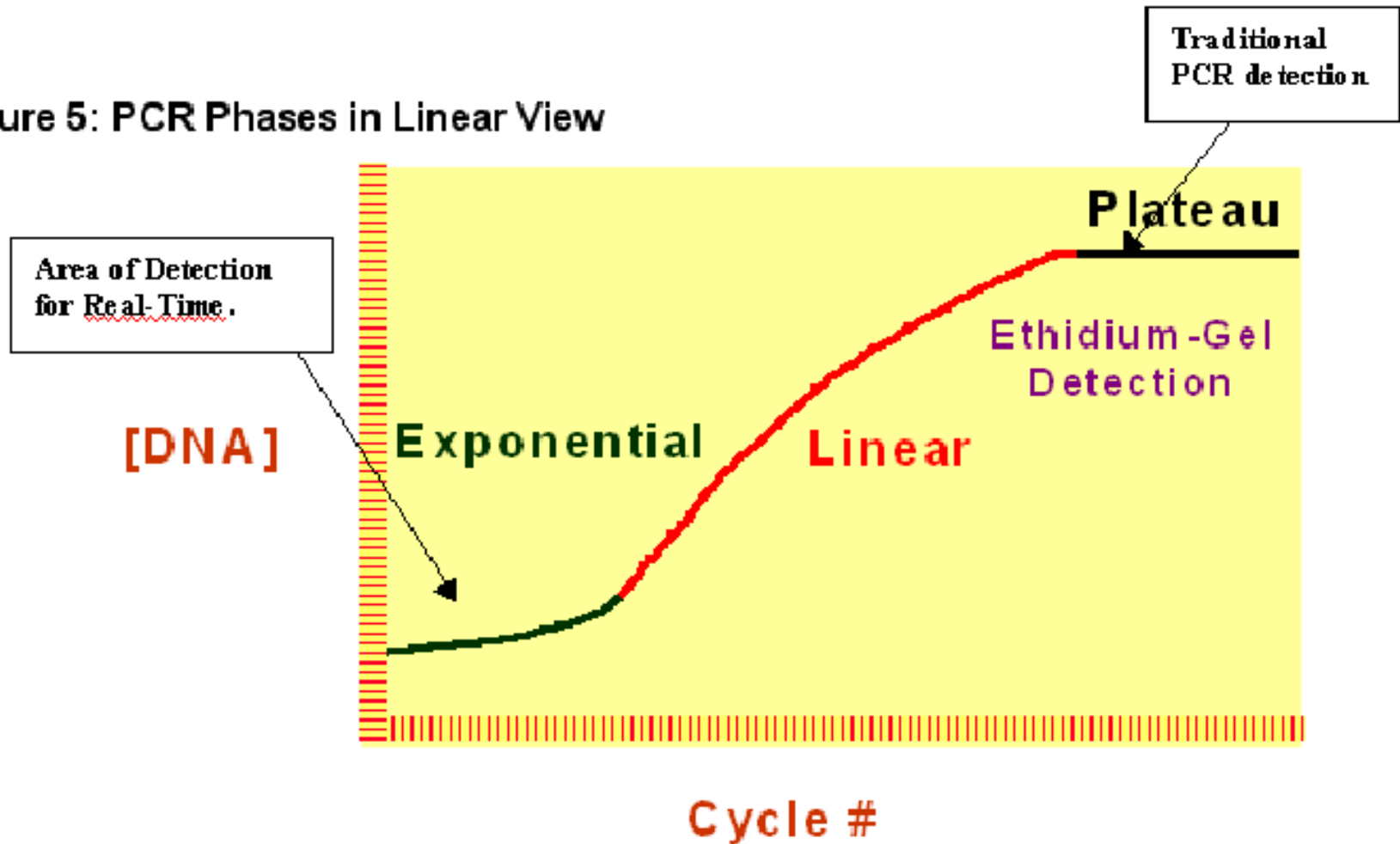


Figure 5: PCR Phases in Linear View



Problems with detection in the Plateau phase of PCR

- As stated earlier, the plateau region is the end-point of the reaction and is representative of the amount of product that you would see on agarose Gels. The 96 replicates in the exponential phase are very tight in both the linear and logarithmic views.

Figure 6: Linear view 96 replicates

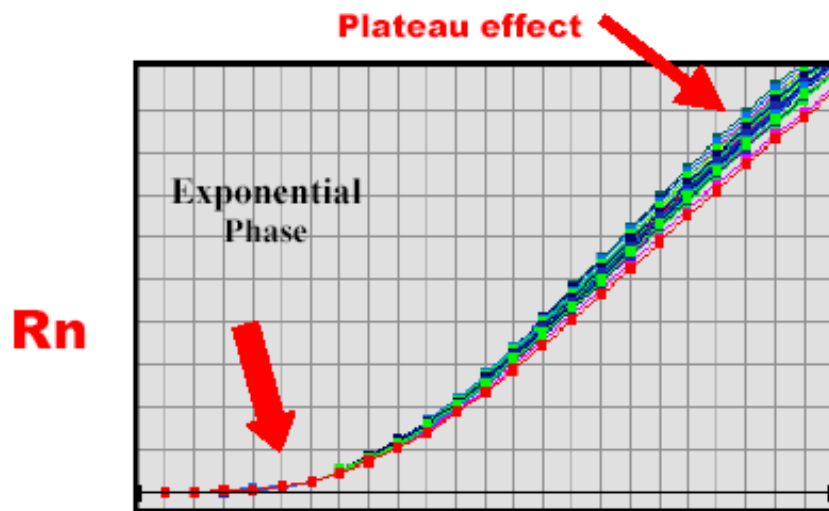
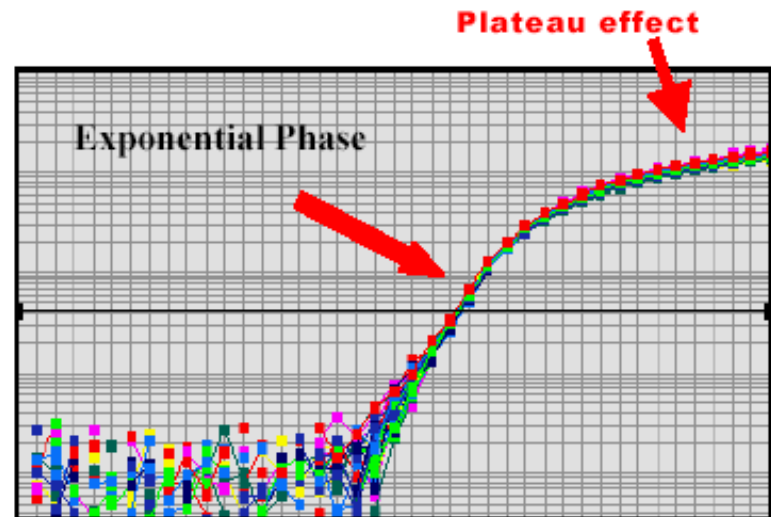


Figure 7: Log view 96 replicates



cycles

Real-time PCR is...

- **The ability to monitor or visualize accumulation of PCR products using fluorescence**
- **Real-Time chemistry provides fast, precise and accurate results. Real-Time PCR is designed to collect data as the reaction is proceeding, which is more accurate for DNA and RNA **quantitation** and does not require laborious post PCR methods.**

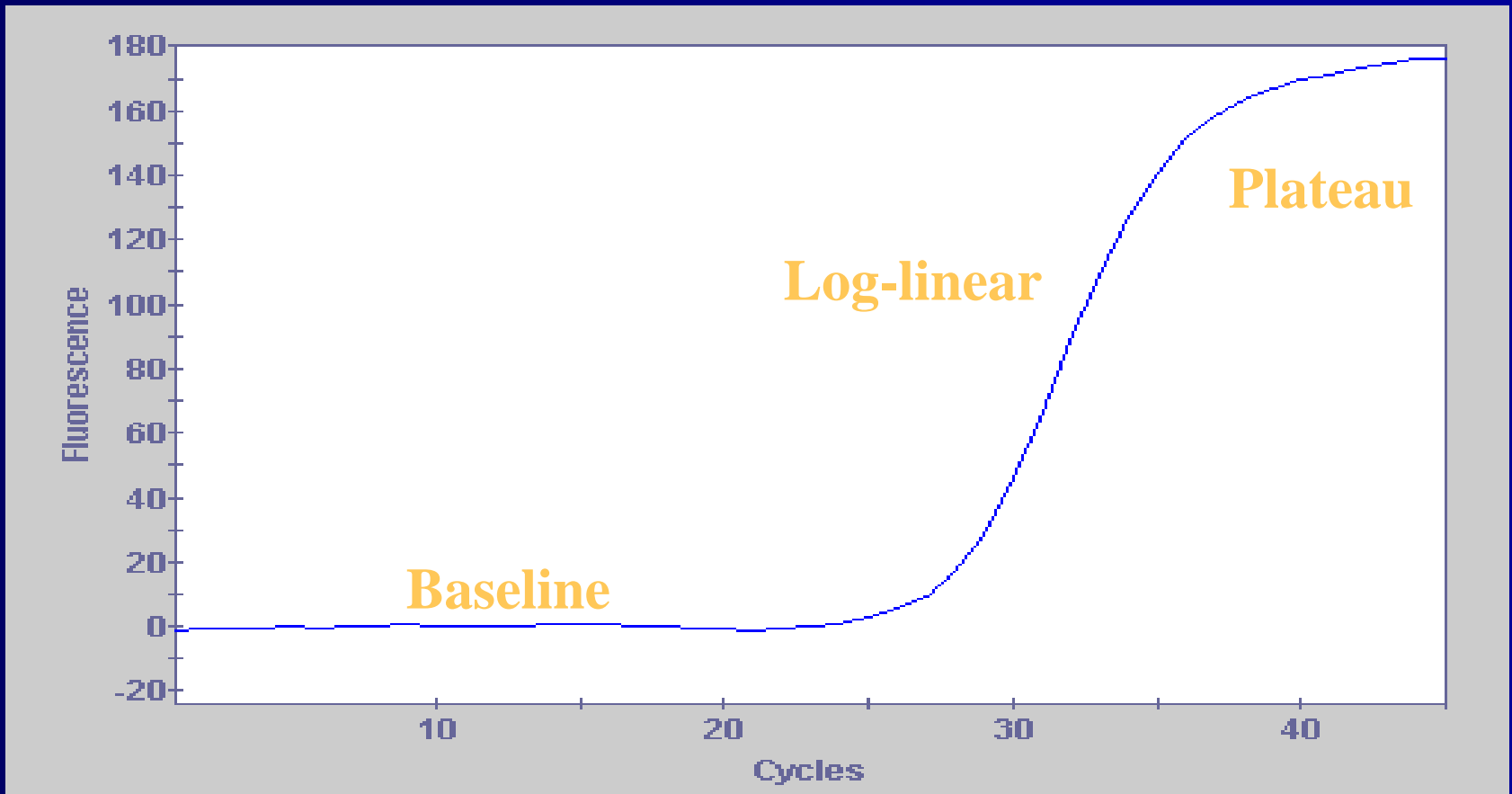
Why Real-Time PCR?

- **To :**
 - **Permit rapid target Id; <30 min**
 - **Eliminate post-PCR processing**
 - **Be highly specific**
 - **Allow multiplexing**
 - **Permit quantification**

Real-Time PCR Definitions

- **Growth Curve**
Plot of Fluorescence vs Cycle #

Real-time Growth Curve: fluorescence vs cycle number



Real-time Requirements...

- **Real Time PCR is : the ability to monitor or visualize accumulation of PCR products using fluorescence**
 - **Target sequence must be amplified in the presence of a fluorescent reporter**
 - **Increase in fluorescence must be proportional to the increased amount of DNA (amplicon)**

Reporter methods

- Intercalating Dyes : SYBR® Green I
- Hybridization Probes
 - FRET probes : LC640, LC705

SYBR[®] Green

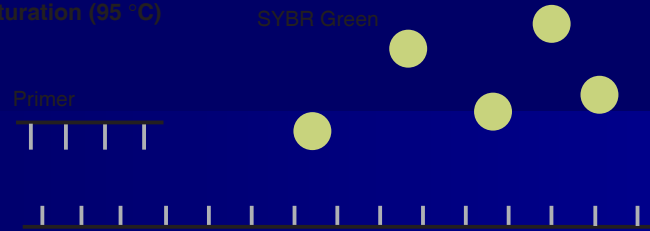
- The simplest and cheapest principle is based on intercalation of double-stranded DNA-binding dyes. This technology can be easily applied to already established PCR assays and does not need any additional fluorescence-labelled oligonucleotide. However, specific and nonspecific PCR products are both detected. Therefore, these assays require careful optimization of the PCR conditions and a clear differentiation between specific and nonspecific PCR products using melting-curve analysis.

Intercalating Dyes

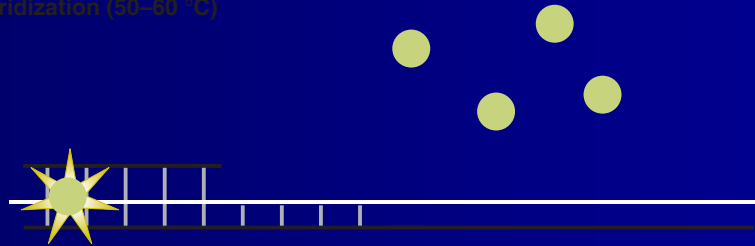
- **Binds to all double stranded DNA, non-sequence specific**
- **Identify amplicon purity and melting temperature (T_m) by melt curve analysis**
- **Example : SYBR Green I**

SYBR[®] Green Dye

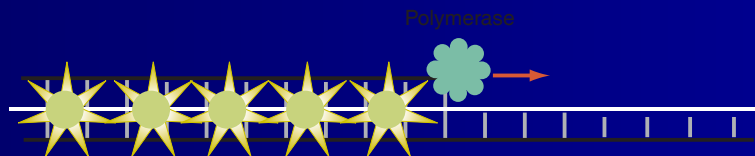
1) Denaturation (95 °C)



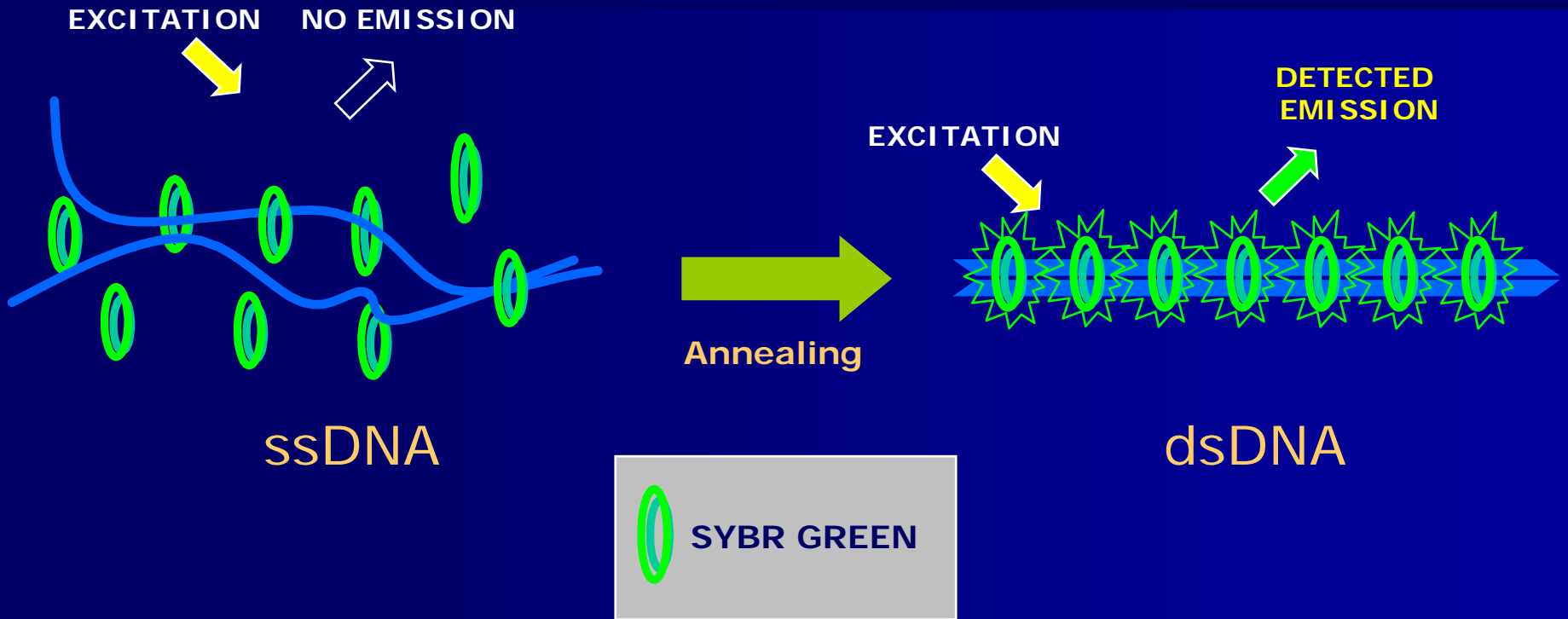
2) Hybridization (50–60 °C)



3) Extension (72 °C)



SYBR Green I



Melt Curve Analysis

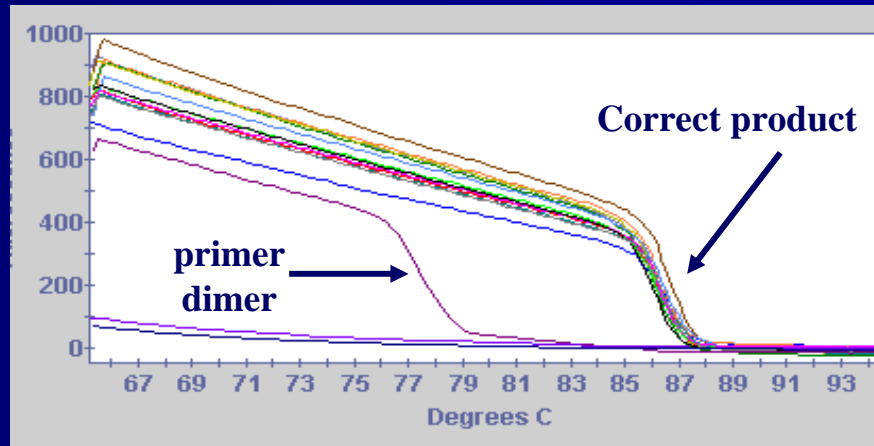
- Following amplification, temperature is slowly increased to separate(melt) paired DNA strands, the intercalating dye is released and fluorescence decreases.
- Melt Curve – plot of temperature vs fluorescence signal

Melting Temperature (T_m)

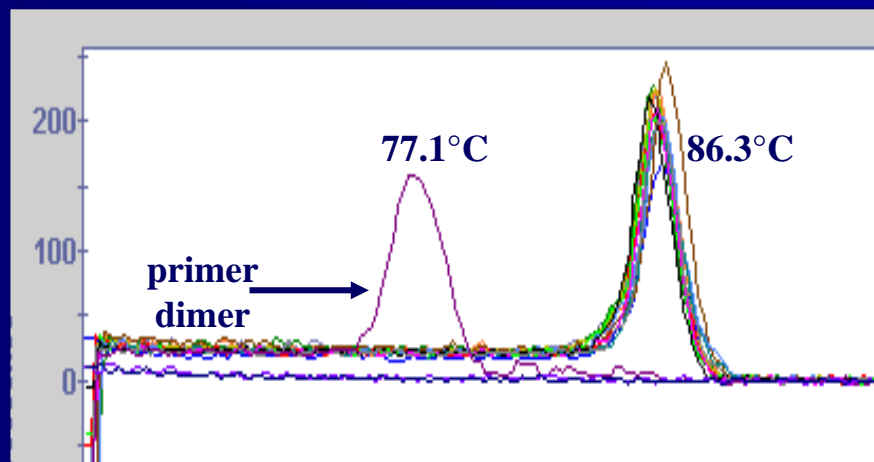
- **Temperature at which 50% of the DNA is double stranded and 50% is single stranded**
- **What is it used for?**
 - **To determine purity of amplification product**
 - **To identify amplification product(s)**
 - **To optimize assay annealing temperature**

Post-PCR Melt Analysis of Amplification with SYBR[®] Green

Fluorescence



Negative
first
derivative

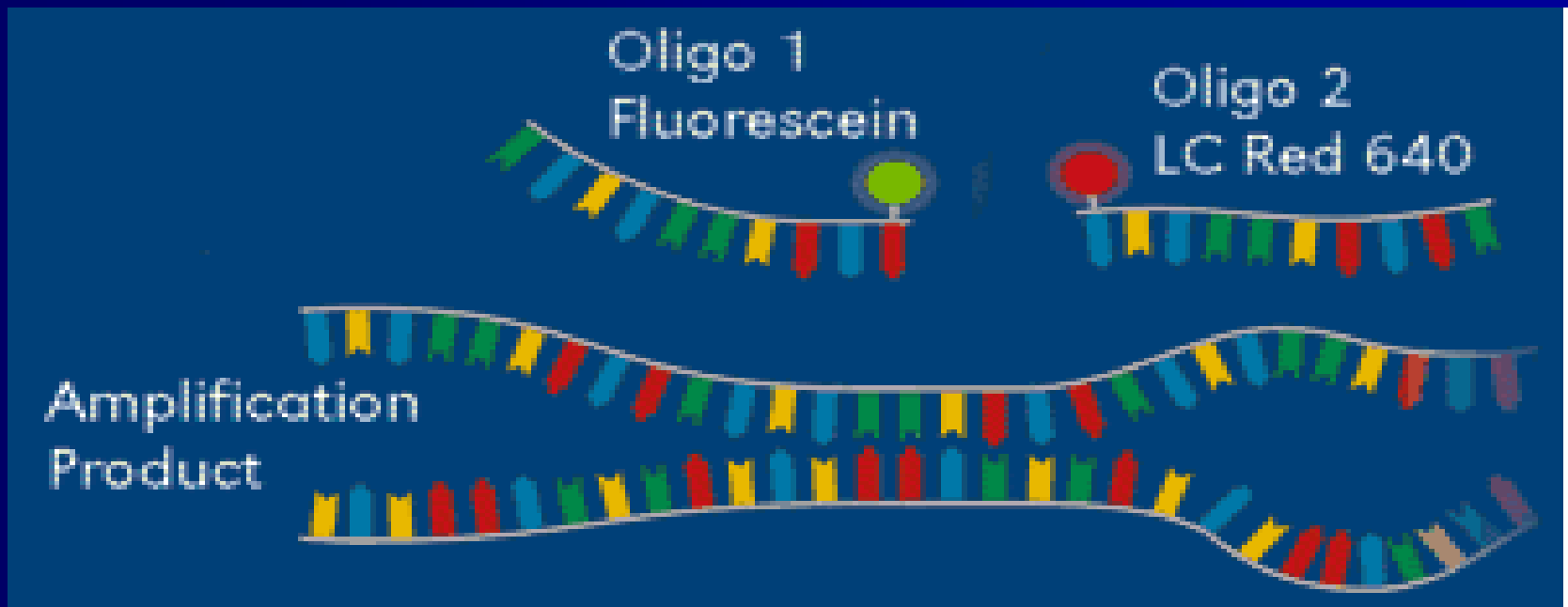


Quantitation

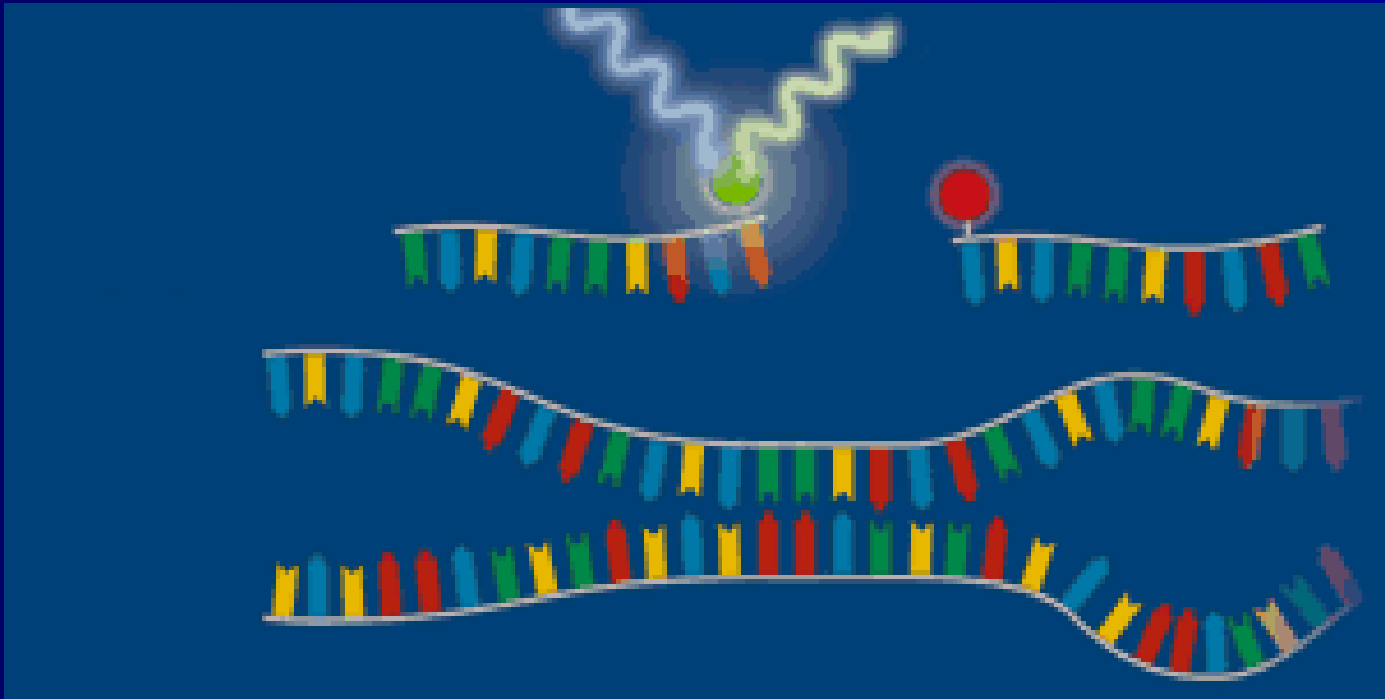
- Theoretically, there is a quantitative relationship between amount of starting target sample and amount of PCR product at any given cycle number.
- Real-Time PCR detects the accumulation of amplicon during the reaction. The data is then measured at the exponential phase of the PCR reaction. Traditional PCR methods use Agarose gels or other post PCR detection methods, which are not as precise. As mentioned earlier, the exponential phase is the optimal point for analyzing data. Real-Time PCR makes quantitation of DNA and RNA easier and more precise than past methods.

PCR Monitoring with Hybridization Probes

- The Hybridization Probe format is used for DNA detection and quantification and provides a maximal specificity for product identification. In addition to the reaction components used for conventional PCR, two specially designed, sequence specific oligonucleotides labeled with fluorescent dyes are applied for this detection method. This allows highly specific detection of the amplification product.



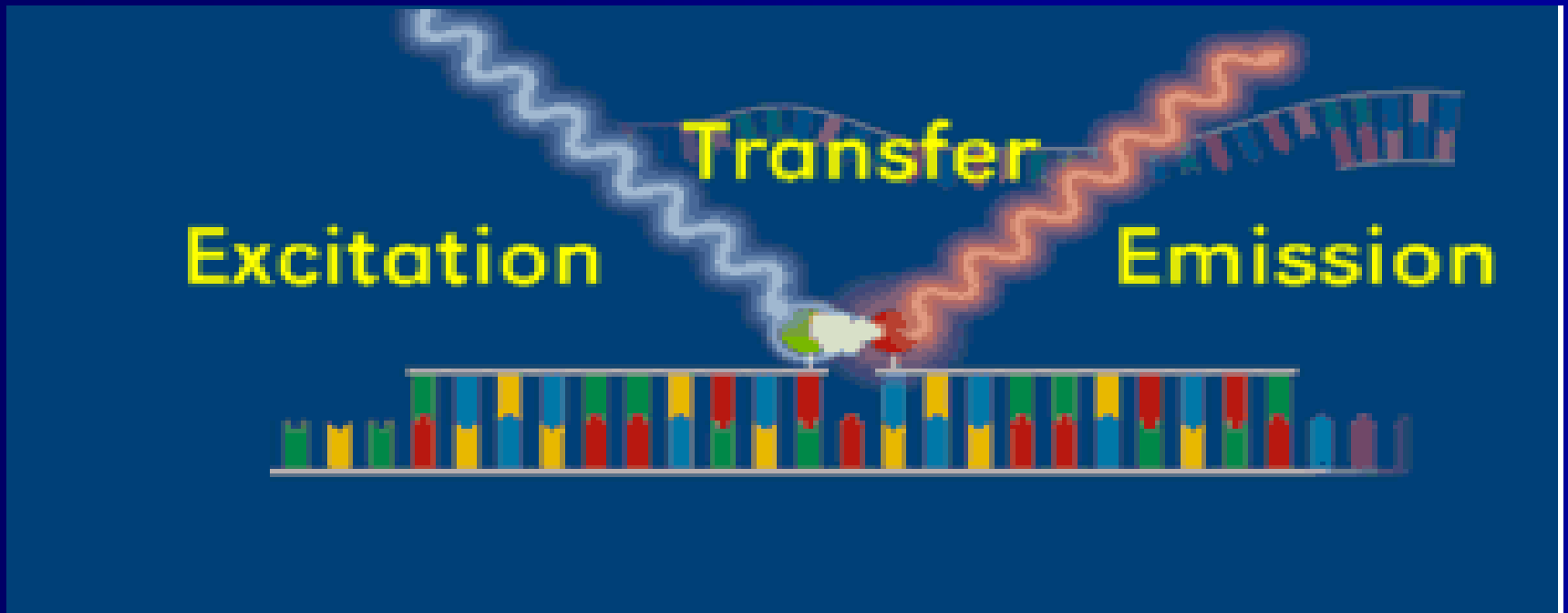
The sequences of the two oligonucleotides are selected such that they hybridize to the amplified DNA fragment in a head to tail arrangement. Why is this design important? When the oligonucleotides hybridize in this orientation, the two fluorescence dyes are positioned in close proximity to each other.



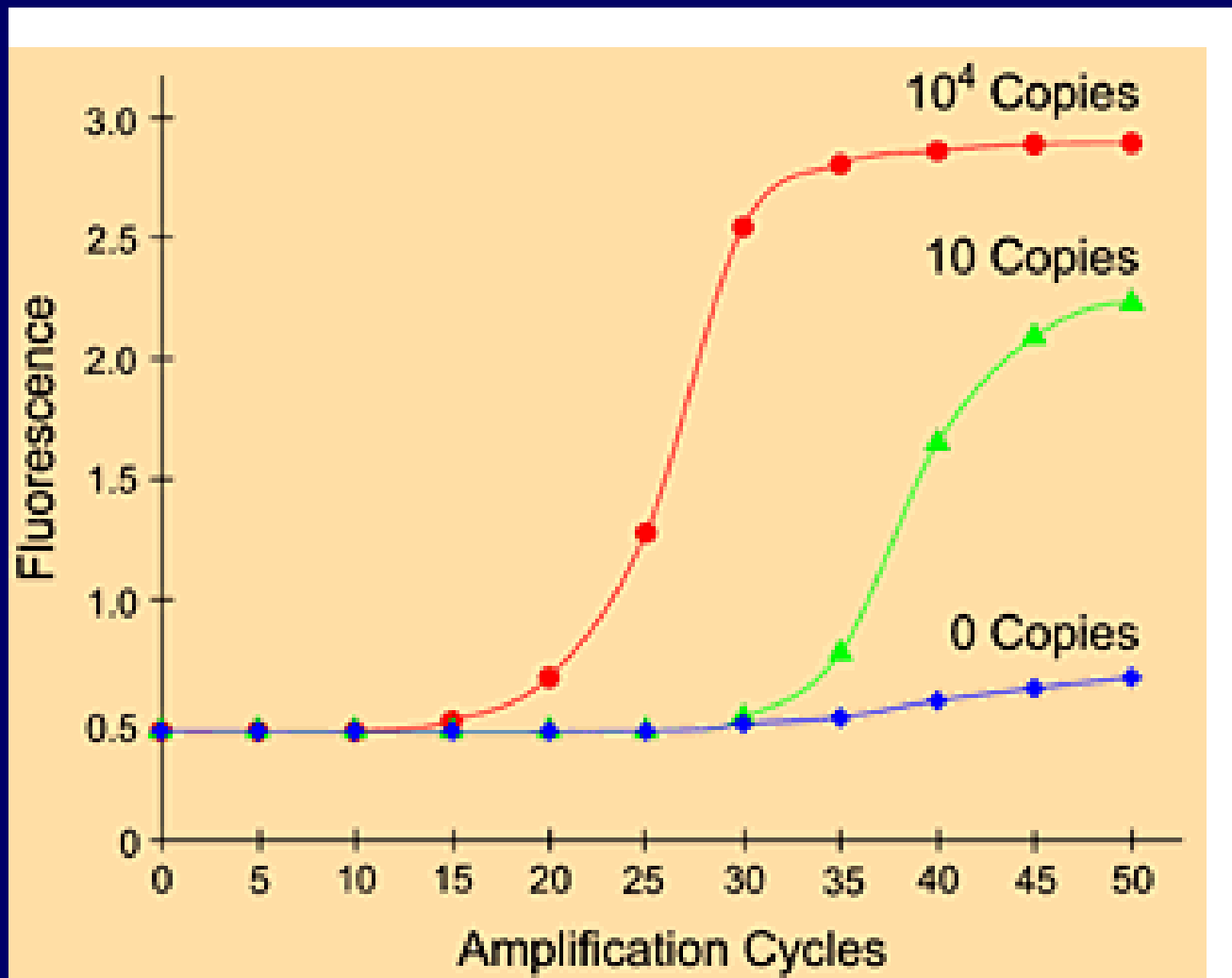
When the two dyes are in close proximity, the emitted energy excites the LC Red 640 attached to the second hybridization probe that subsequently emits red fluorescent light at an even longer wavelength.

This energy transfer, referred to as FRET (Fluorescence Resonance Energy Transfer) is highly dependent on the spacing between the two dye molecules. Only if the molecules are in close proximity (a distance between 1–5 nucleotides) is the energy transferred at high efficiency.

The increasing amount of measured fluorescence is proportional to the increasing amount of DNA generated during the ongoing PCR process. Since LC Red 640 only emits a signal when both oligonucleotides are hybridized, the fluorescence measurement is performed after the annealing step.



PCR Monitoring with Hybridization Probes

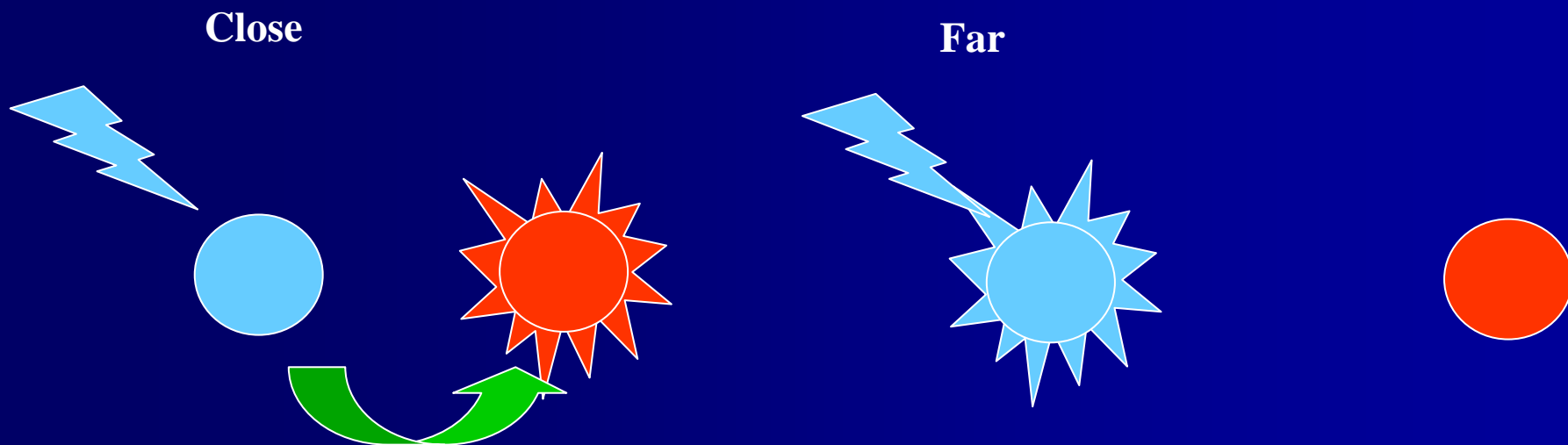


Hybridization Probes

- Based on complementary sequence
- Sequence specific
- Use Fluorescence Resonance Energy Transfer (FRET) technology.
- FRET is affected by two factors
 - Distance between the donor and acceptor
 - Spectral overlap between donor fluorescence emission and acceptor excitation

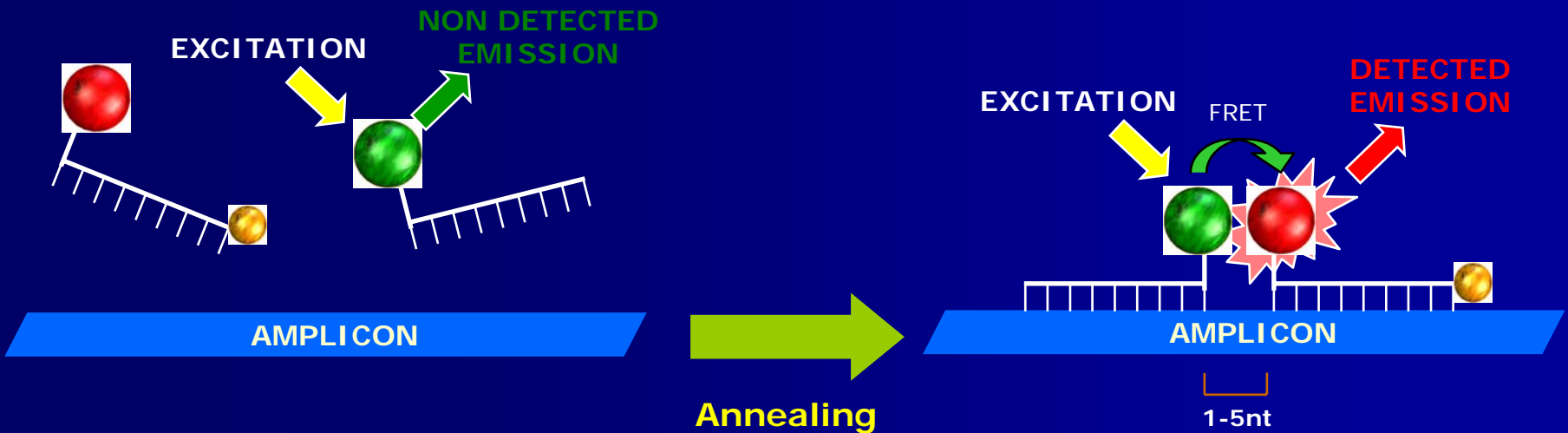
Fluorescence Resonance Energy Transfer

- Fluorescence Resonance Energy Transfer occurs when electronic excitation of a molecule is transferred to another molecule without emission of photon



Hybridization probes

Principle: adjacent hybridisation and FRET



Fluorescein (donor)



LC RED640/705 (acceptor)



Phosphate

Hybridization system

Option 2 : FRET probes

- **Second fluorochrome is the Reporter**
 - **Advantage : lower background**
 - **Disadvantage : Use large part of the spectrum**
- =>limited multiplex capability (two targets mean four emissions wavelengths to manage)**

Hybridization system

FRET emission probes



- **Requirements:**

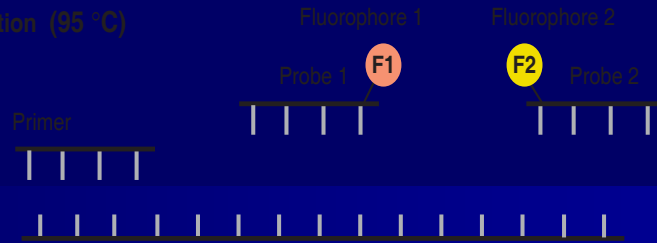
- 2 probes one labeled with an acceptor dye and one labeled with a donor dye
- Both probes must hybridize to target head to tail within 1-5 bases

- **Mechanism:**

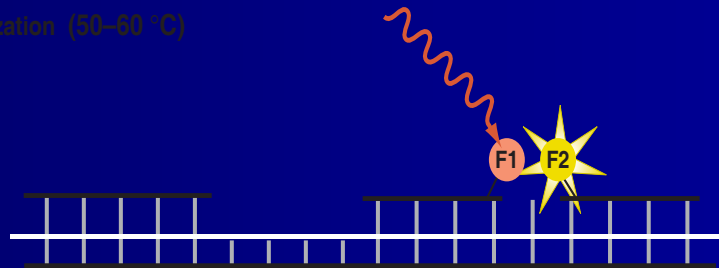
- Donor dye excited
- Energy transferred to acceptor dye which emits at longer wavelength

FRET Technology

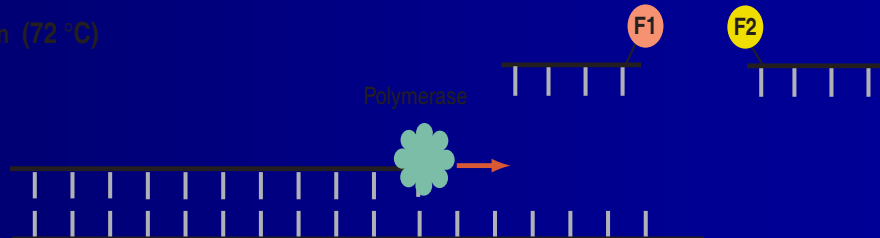
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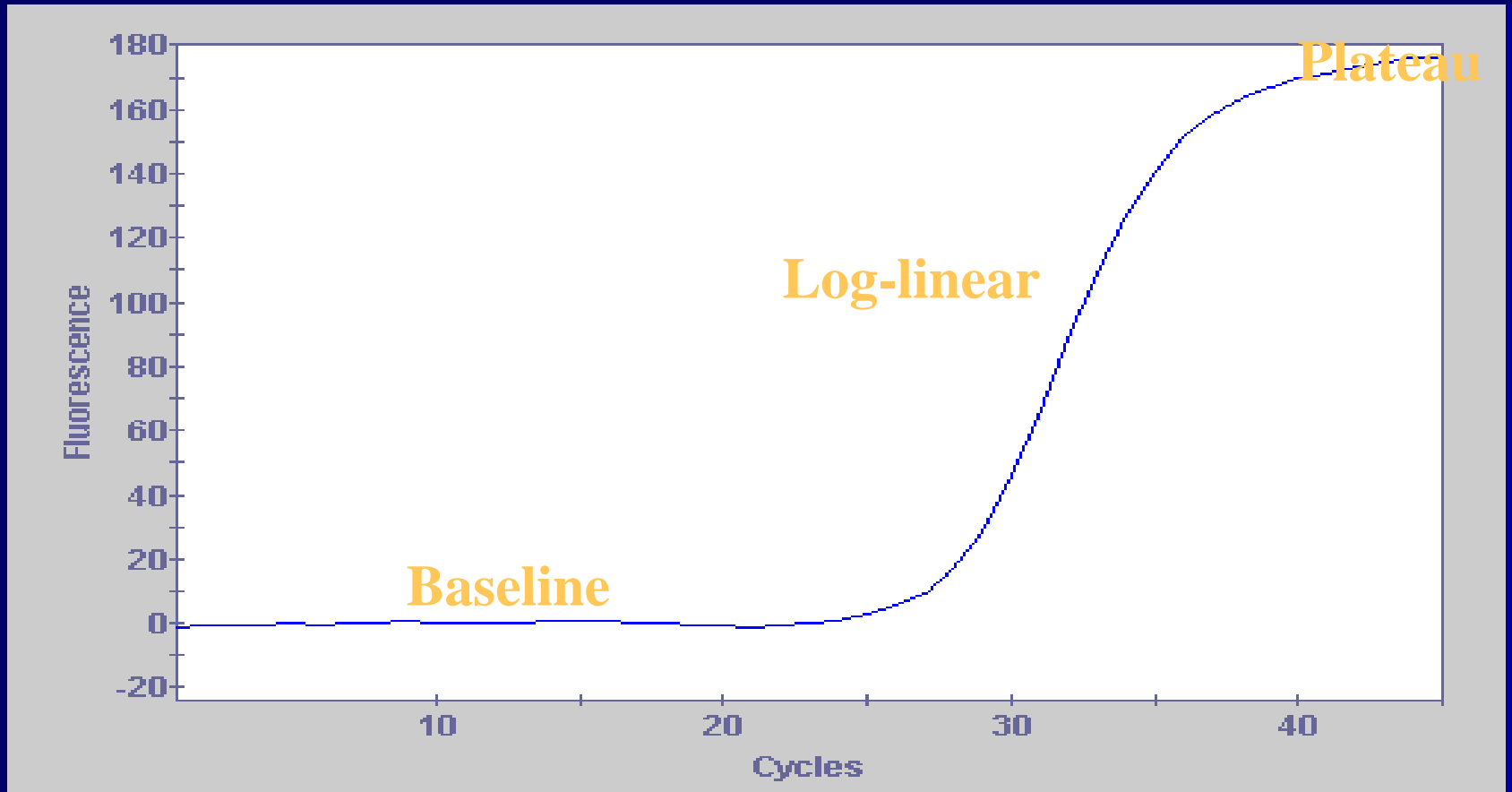
2) Hybridization (50–60 °C)



3) Extension (72 °C)



Real-time Growth Curve: Plot of fluorescence vs cycle number

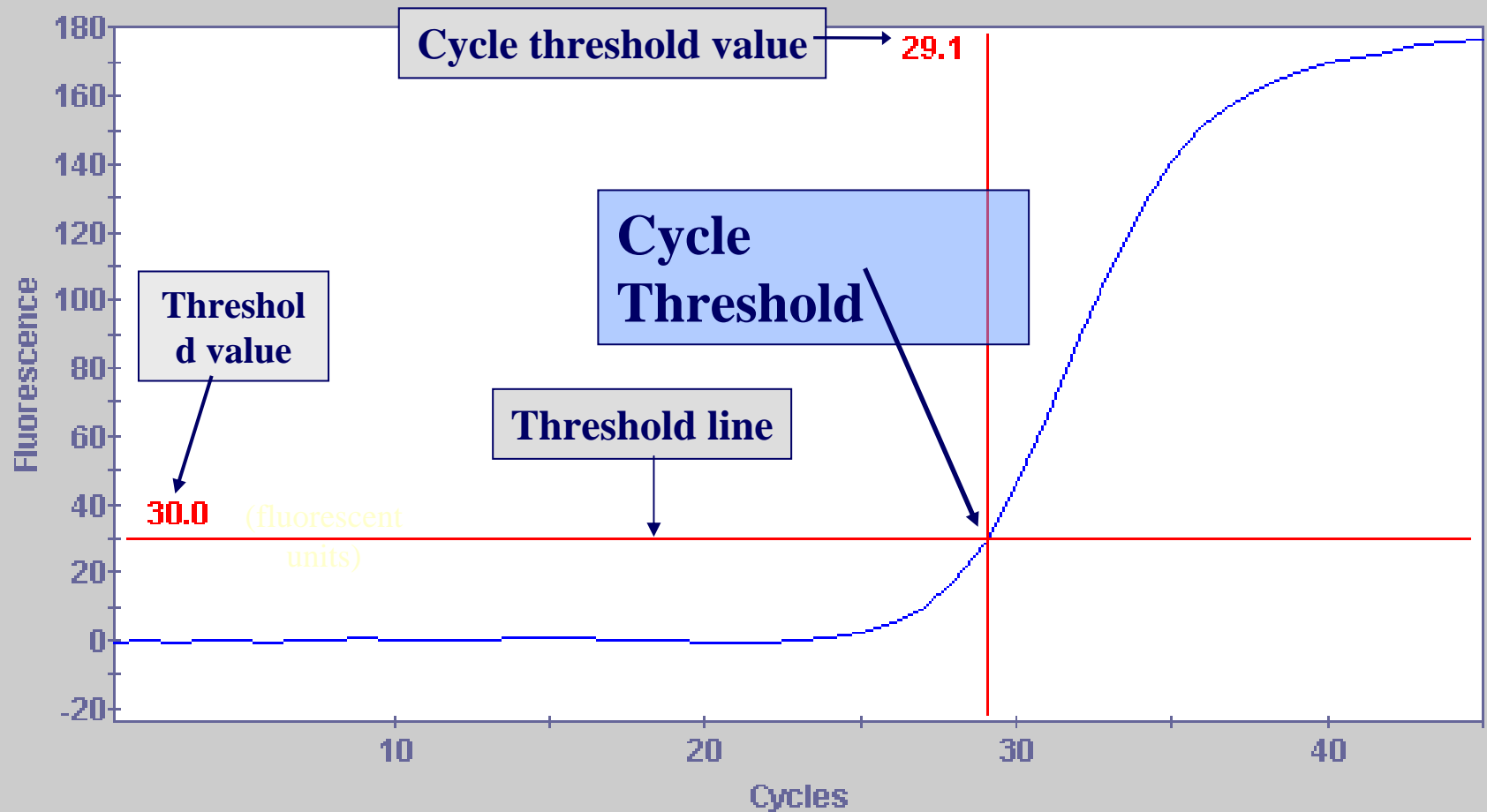


Real-Time PCR Definitions

- **Cycle Threshold (C_T)**

The first cycle in which there is a significant increase in fluorescence above a specified threshold.

Primary Signal with Threshold



Advantages and limitations of real-time PCR

Advantages

- Wide dynamic range of quantification (7-8 log decades)
- High technical sensitivity (< 5 copies)
- High precision (< 2% CV of C_T values)
- No post-PCR steps
- Minimized risk of cross contamination
- High throughput
- Multiplex approach possible

Limitations

- PCR product increases exponentially
- Variation increases with cycle number
- Increased variation after transformation to linear values
- Overlap of emission spectra^a
- Maximal four simultaneous reactions^a
- Increased risk of false negative results^b

^aIt is expected that new technology will improve this.

^bIn particular for pathogen detection (e.g. new emerging pathogens, highly variable pathogens).

Applications of real-time PCR in molecular diagnostics

Clinical microbiology

Viral load (e.g. HIV, HCV, HBV)

Bacterial load (e.g. EHEC, *Salmonella*,
Mycobacterium)

Fungal load (e.g. *Candida*, *Cryptococcus*,
Aspergillus)

Food microbiology

Bacterial load (e.g. *Listeria*, *Salmonella*,
Campylobacter)

Veterinary microbiology

Viral load (e.g. FIV, CSFV, FCV)

Zoonotic agents

Clinical oncology

Minimal residual disease

Chromosomal translocations

Single nucleotide polymorphisms (SNPs)

Gene therapy

Gene transfer estimation

Biodistribution of vectors

Gene expression

Cytokines, receptors, etc.