

An Introduction to Real Time PCR

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● Techniques for quantifying mRNA level

- Northern blot analysis
- RNase protection assay
- RT-PCR (used to quantify mRNA levels from much smaller samples)
- most sensitive technique for mRNA detection and quantitation
- method of choice for quantitating changes in gene expression

Real-Time Vs Traditional PCR

- Real-Time chemistries - detection of PCR amplification during the early phases of the reaction.
- Measuring the kinetics of the reaction in the early phases of PCR - 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.

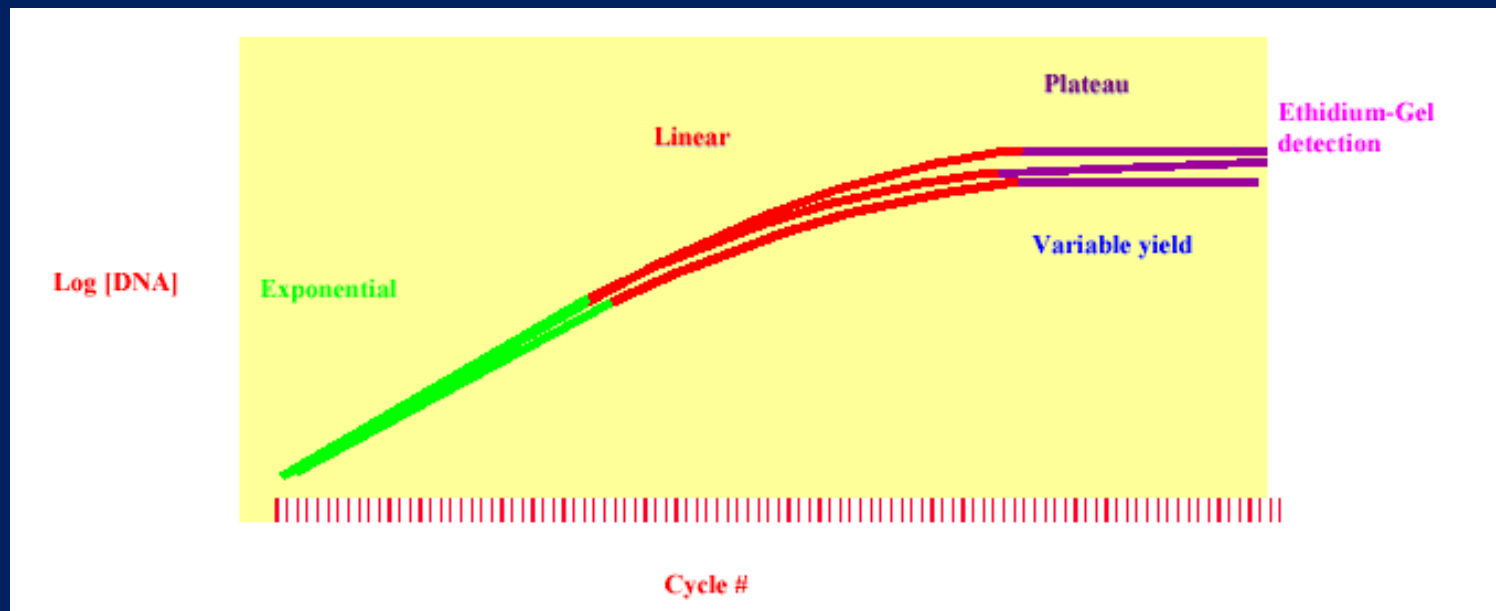
Limitations of End-Point PCR

- 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.
- 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!

Some of the problems with End-Point Detection:

- Poor Precision
- Low sensitivity
- Short dynamic range < 2 logs
- 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

PCR phases



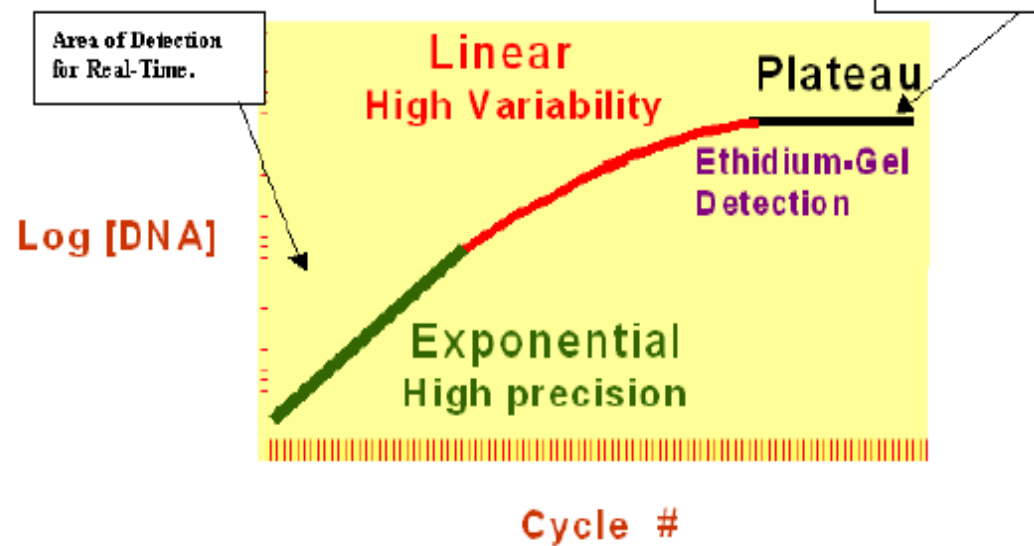
Exponential amplification occurs because all of the reagents are fresh and available, the kinetics of the reaction push the reaction to favor doubling of amplicon.

- 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.

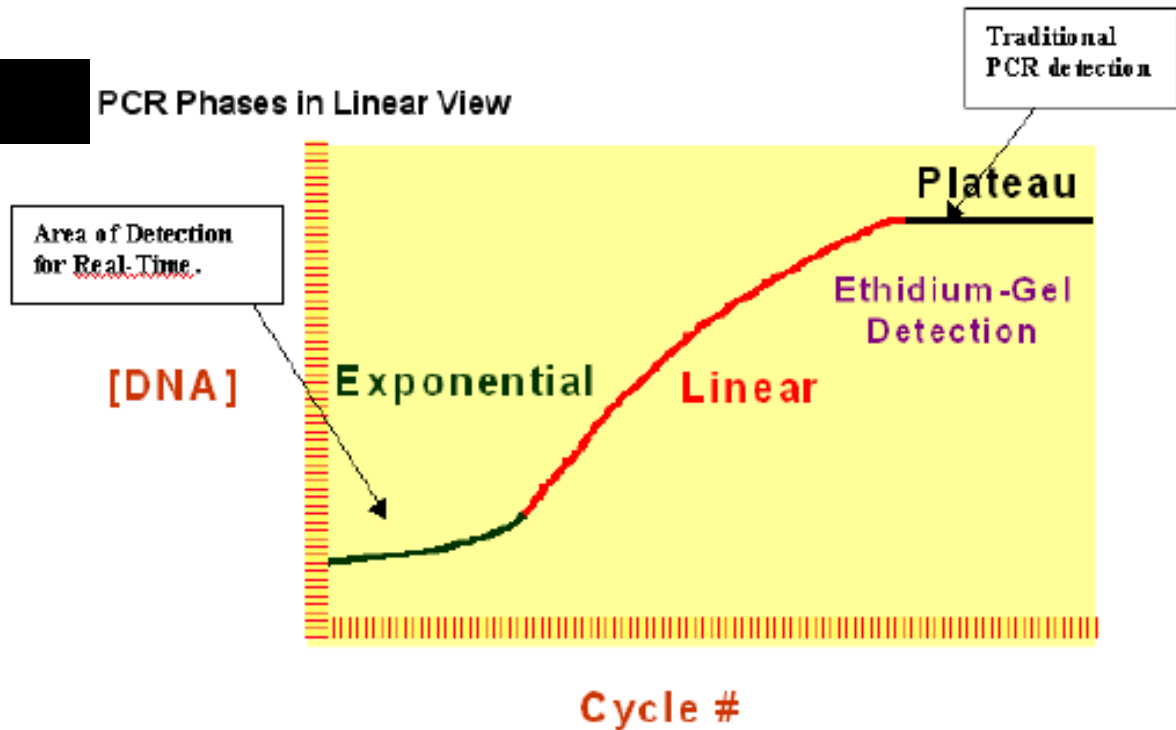
- Eventually the reactions begin to slow down and stop all together or plateau.
- Each tube or reaction will plateau at a different point, due to the different reaction kinetics for each sample.
- These differences can be seen in the plateau phase.
- The plateau phase is where traditional PCR takes its measurement, also known as end-point detection.

- three replicate samples
 - started out at the same quantity in the beginning of the reaction, reflect different quantities at the plateau phase.
 - samples are replicates they should have identical quantities.
- more precise to take measurements during the exponential phase, where the replicate samples are amplifying exponentially.

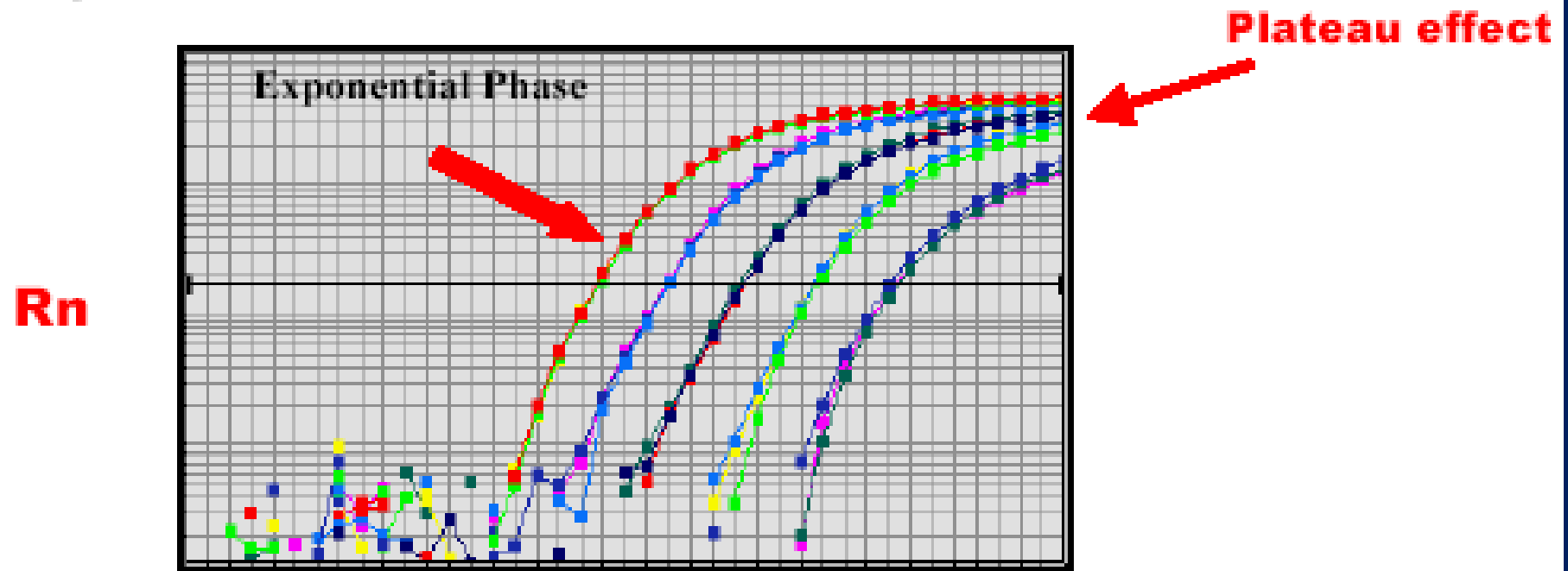
PCR Phases in Log view



PCR Phases in Linear View



Log view 5-fold dilution series



- The 5-fold dilution series seems to plateau at the same place
- the exponential phase clearly shows a difference between the points along the dilution series.
- This reinforces the fact that if measurements were taken at the plateau phase, the data would not truly represent the initial amounts of starting target material.

Real-Time PCR

- Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection
- the first significant increase in the amount of PCR product (CT - threshold cycle) correlates to the initial amount of target template

How Real-Time PCR works?

- Instrumentation is basic

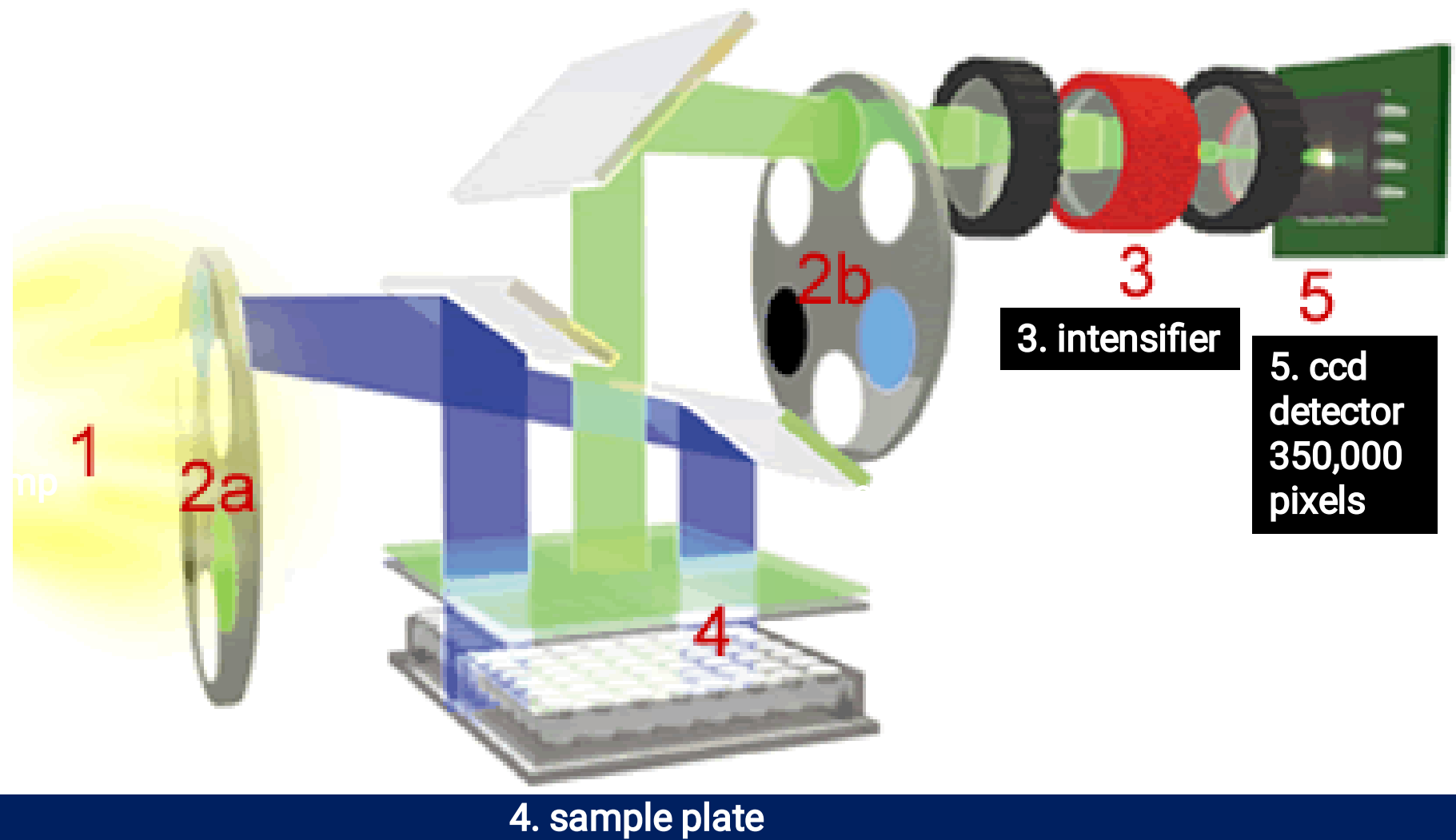


Fig. 1.2. Representation of Optical Detection System layout.

Principles of Real-Time Quantitative PCR Techniques

- Three general methods for the quantitative assays:

1. Hydrolysis probes

(TaqMan, Beacons, Scorpions)

2. Hybridization probes

(Light Cycler)

3. DNA-binding agents

(SYBR Green)

- 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.

Advantages of using Real-Time PCR:

- Traditional PCR is measured at End-Point (plateau), while Real-Time PCR collects data in the exponential growth phase
- An increase in Reporter fluorescent signal is directly proportional to the number of amplicons generated
- The cleaved probe provides a permanent record amplification of an amplicon
- Increase dynamic range of detection
- No-post PCR processing
- Detection is capable down to a 2-fold change

● Real-Time PCR Applications

- Real-Time PCR can be applied to traditional PCR applications as well as new applications that would have been less effective with traditional PCR.
- With the ability to collect data in the exponential growth phase, the power of PCR has been expanded into applications such as:
 - Viral Quantitation
 - Quantitation of Gene Expression
 - Array Verification
 - Drug Therapy Efficacy
 - DNA Damage measurement
 - Quality Control and Assay Validation
 - Pathogen detection
 - Genotyping