Real-Time PCR Vs. Traditional PCR

Description

This tutorial will discuss the evolution of traditional PCR methods towards the use of Real-Time chemistry and instrumentation for accurate quantitation.

Objectives

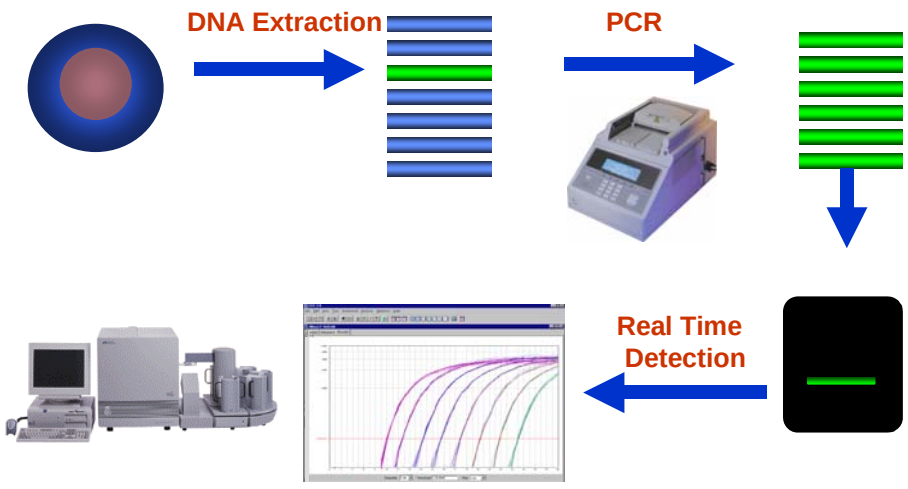
This tutorial will provide an understanding of the following:

- Limitations of traditional PCR
- Introduction to Real-Time PCR
- Advantages of Real-Time chemistries over traditional PCR methods

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.

Figure 1: Real-Time PCR Evolution



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.

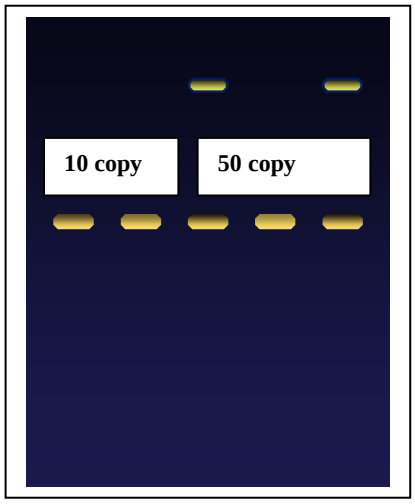
Limitations of End-Point PCR

Agarose gel results are obtained from the end point of the reaction. End-point 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!

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

Figure 2: Agarose Gel



As you can see from the figure, the 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 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.

Figure 3: PCR Phases

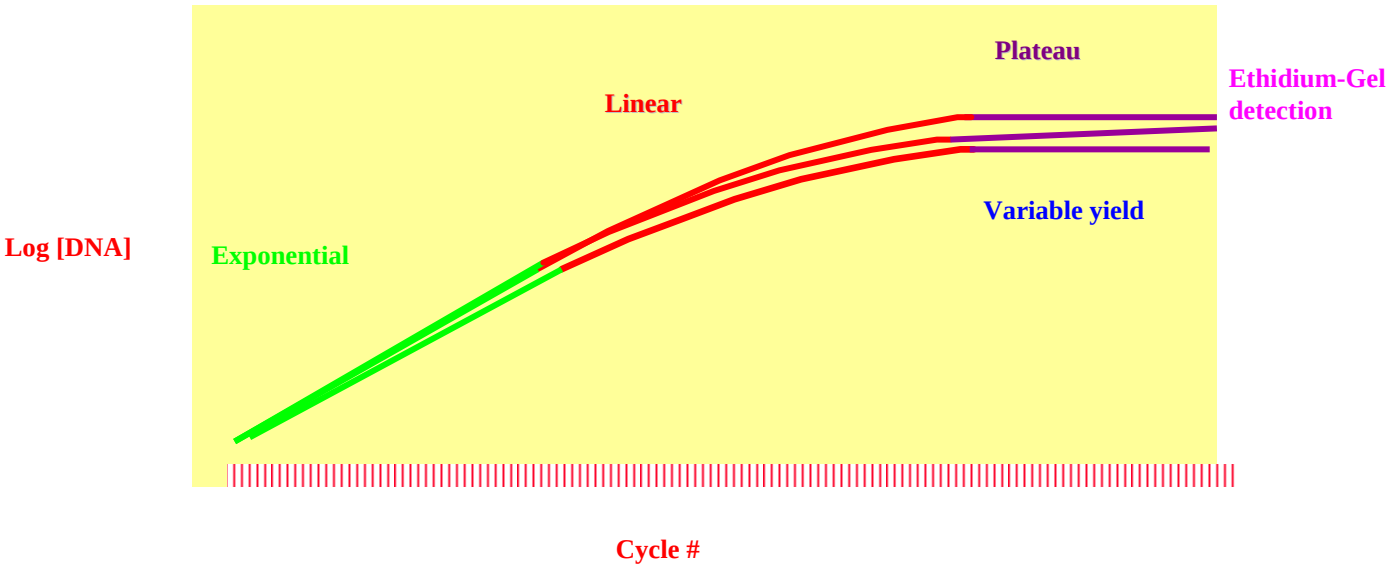


Figure 3 shows three replicates of a sample. The replicates have the same starting quantity. As the PCR reaction progresses, the samples begin to amplify in a very precise manner. Amplification occurs exponentially, that is a doubling of product (amplicon) occurs every cycle. This type of amplification 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.

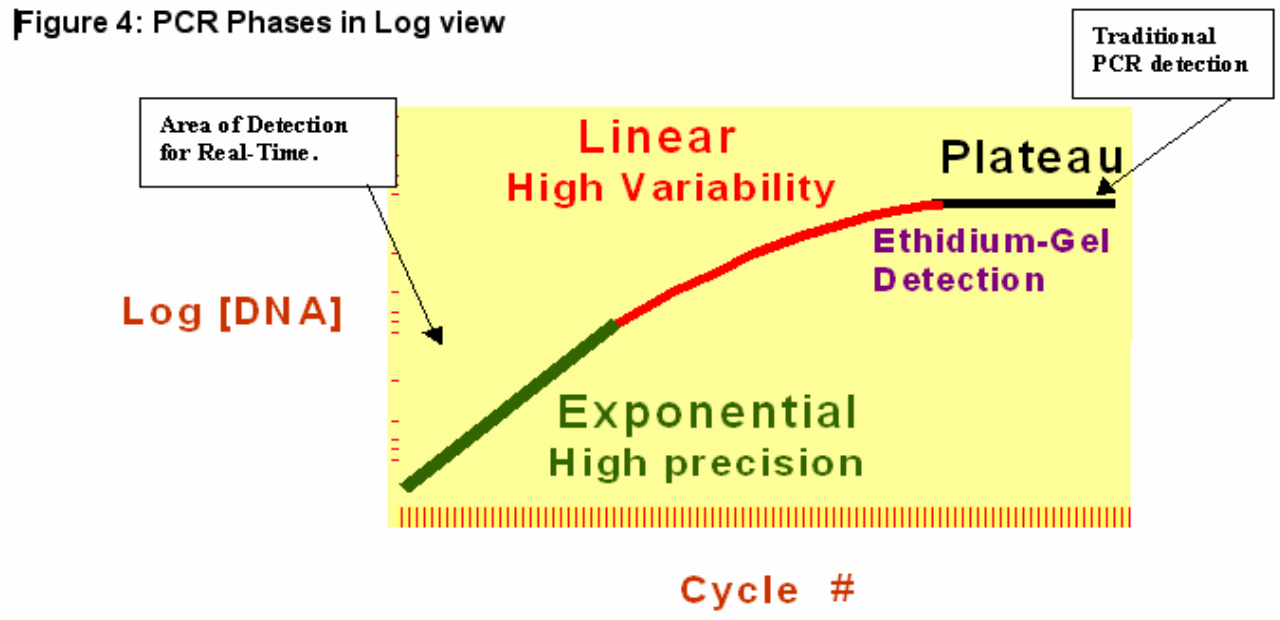
However, as the reaction progresses, some of the reagents are being consumed as a result of amplification. This depletion will occur at different rates for each replicate. The reactions start to slow down and the PCR product is no longer being doubled at each cycle. This linear amplification can be seen 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.

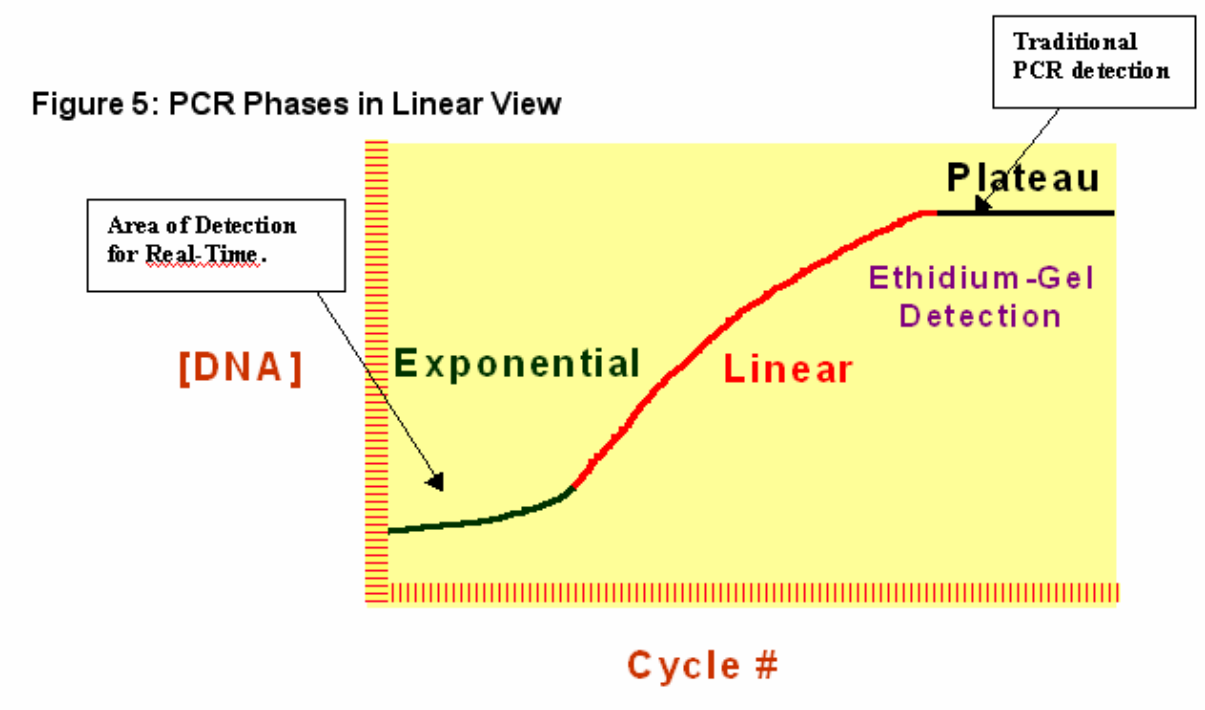
Figure 3 also shows that the three replicate samples, which started out at the same quantity in the beginning of the reaction, reflect different quantities at the plateau phase. Since the samples are replicates they should have identical quantities. Therefore, it will be more precise to take measurements 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). Applied Biosystems Sequence Detection System Instruments can view the data in both forms.

Figure 4: PCR Phases in Log view



Applied Biosystems



Problems with detection in the Plateau phase of PCR

The following three figures show the plateau affect on 96 replicates and a five- fold dilution series. 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.

In the logarithmic view, Figure 7, the plateau for each reaction seems to occur in the same place, but this is solely due to the log scaling of the plot. Figure 6 shows the same 96 replicates in linear view. The reactions show a clear separation in the plateau phase; therefore, if the measurements were taken in the plateau phase, quantitation would be affected.

Figure 6: Linear view 96 replicates

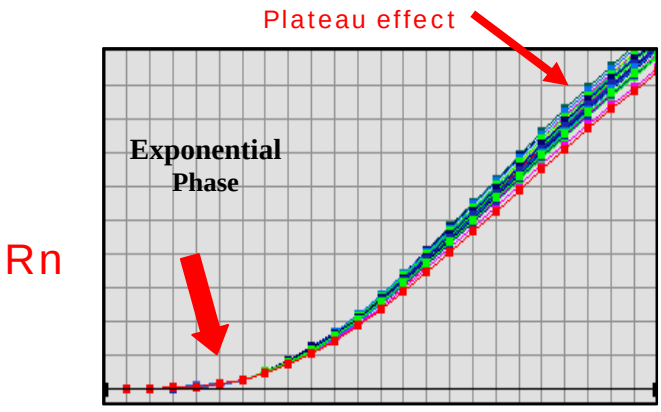


Figure 7:Log view 96 replicates

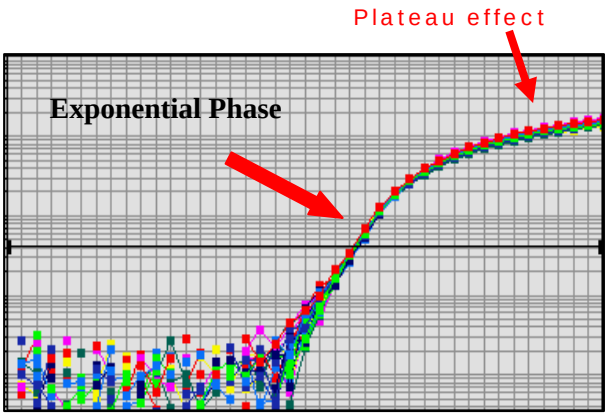
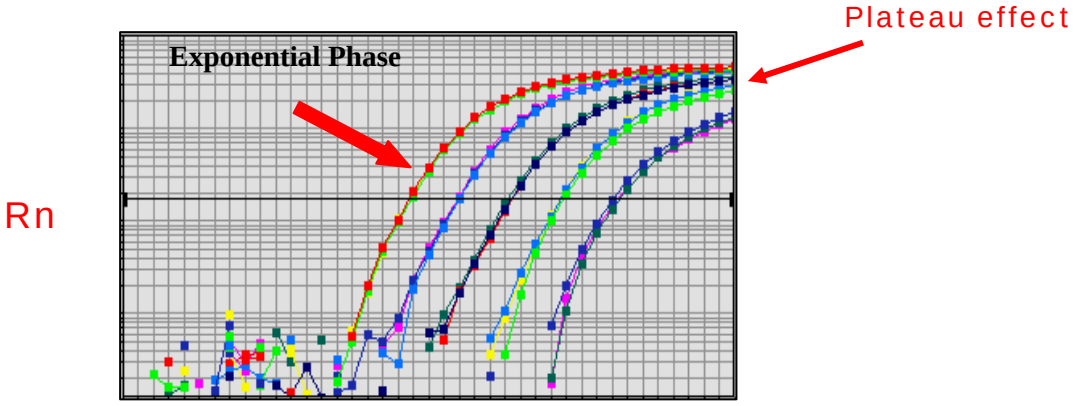


Figure 8: Log view 5-fold dilution series



The 5-fold dilution series, seen in Figure 8, seems to plateau at the same place even though 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 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.

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.

The 5′ Nuclease Assay

5′ Nuclease Activity

AmpliTaq Gold® DNA Polymerase has 5′ exo-nuclease activity. The **5′ exo-nuclease activity** of AmpliTaq® Polymerase and **FRET** (*Fluorescent Resonant Energy Transfer*) makes it possible to detect PCR amplification in Real-Time. The 5′ exo-nuclease activity of the enzyme acts upon the surface of the template to remove obstacles downstream of the growing amplicon that may interfere with its' generation. The 5′ nuclease assay uses this activity in real time detection.

Figure 9: Taq polymerase activity

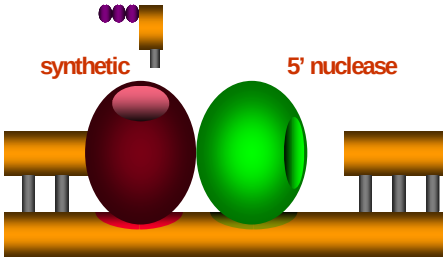
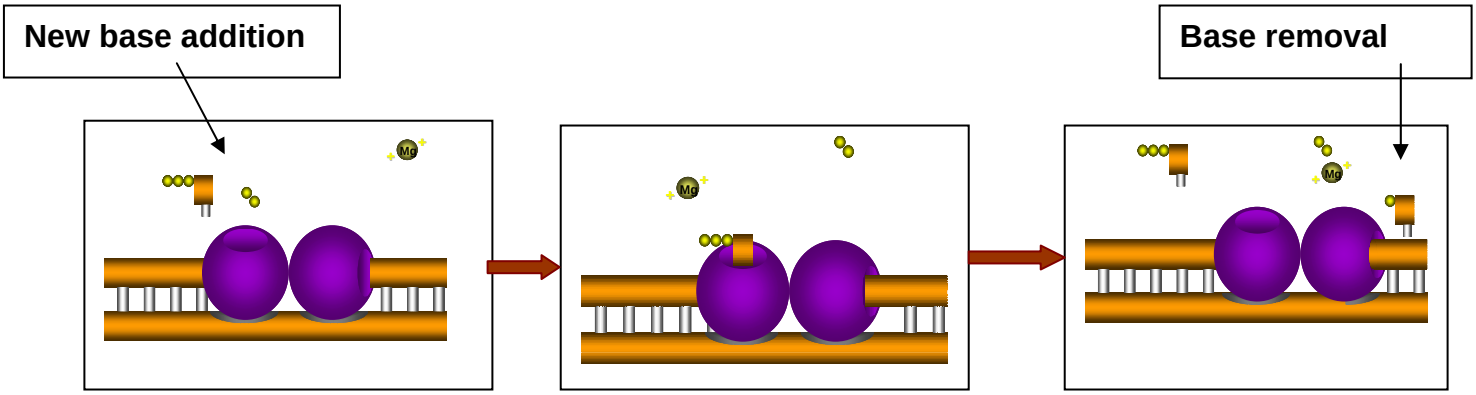


Figure 10: 5′ Exo-Nuclease Activity of Taq Polymerase:

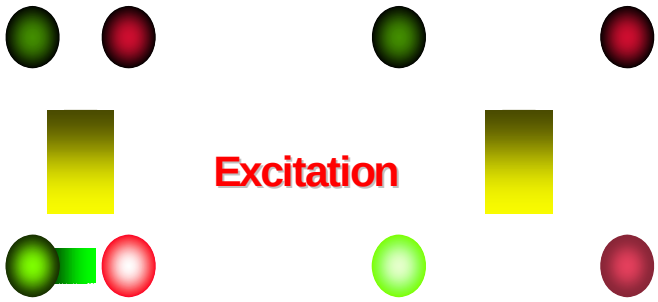
Here the polymerase is adding bases to a growing chain of DNA. Subsequently, the polymerase is removing DNA that is downstream, impeding its' capability to synthesize the new strand.



FRET (Fluorescent Resonance Energy Transfer)

FRET or Florescent Resonance Energy Transfer technology is utilized in the 5′ nuclease assay. The principle is that when a high-energy dye is in close proximity to a low-energy dye, there will be a transfer of energy from high to low, Figure 11.

Figure 11: FRET



The 5′ Nuclease Assay

In the 5′ nuclease assay, an oligonucleotide called a **TaqMan® Probe** is added to the PCR reagent master mix. The probe is designed to anneal to a specific sequence of template between the forward and reverse primers. The probe sits in the path of the enzyme as it starts to copy DNA or cDNA. When the enzyme reaches the annealed probe the 5′ exo-nuclease activity of the enzyme cleaves the probe, Figure 12 through 14.

Figure 12: The 5′ Nuclease Assay

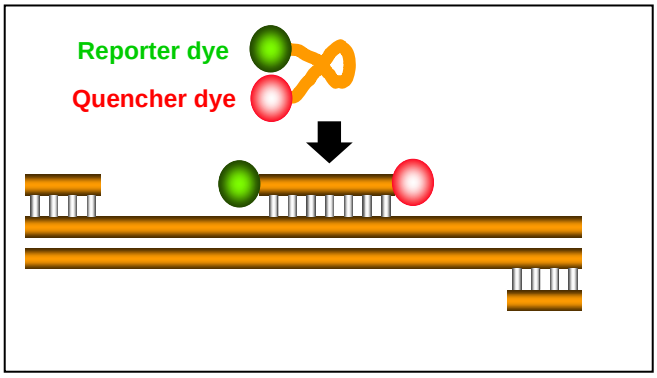
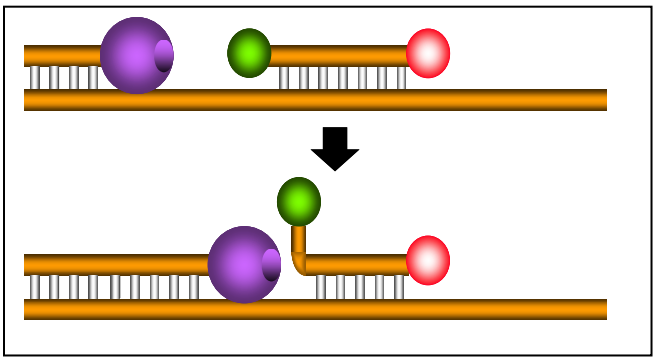


Figure 13: Polymerase collides with TaqMan® Probe



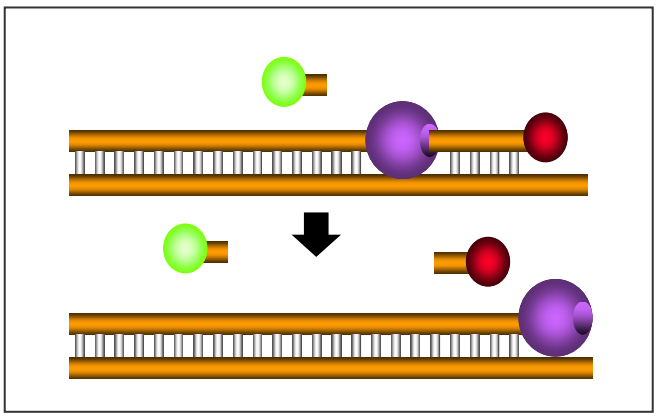
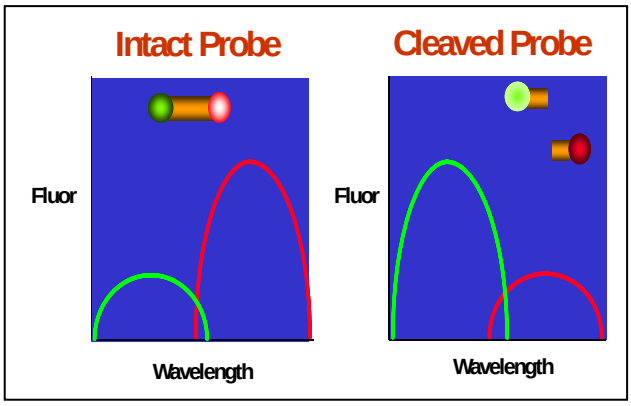


Figure 14: Cleavage of the TaqMan® Probe

The **TaqMan® Probe** is designed with a high-energy dye termed a **Reporter** at the 5′ end, and a low-energy molecule termed a **Quencher** at the 3′ end. When this probe is intact and excited by a light source, the Reporter dye's emission is suppressed by the Quencher dye as a result of the close proximity of the dyes, Figure 15.

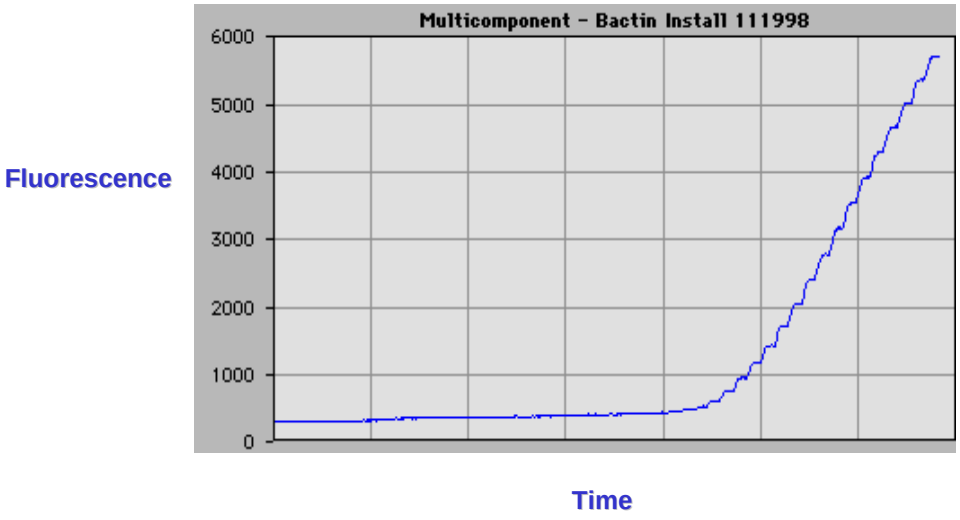
When the probe is cleaved by the 5′ nuclease activity of the enzyme, the distance between the Reporter and the Quencher increases causing the transfer of energy to stop. The fluorescent emissions of the reporter increase and the quencher decrease.

Figure 15: Increased florescence activity due to the cleaved probe



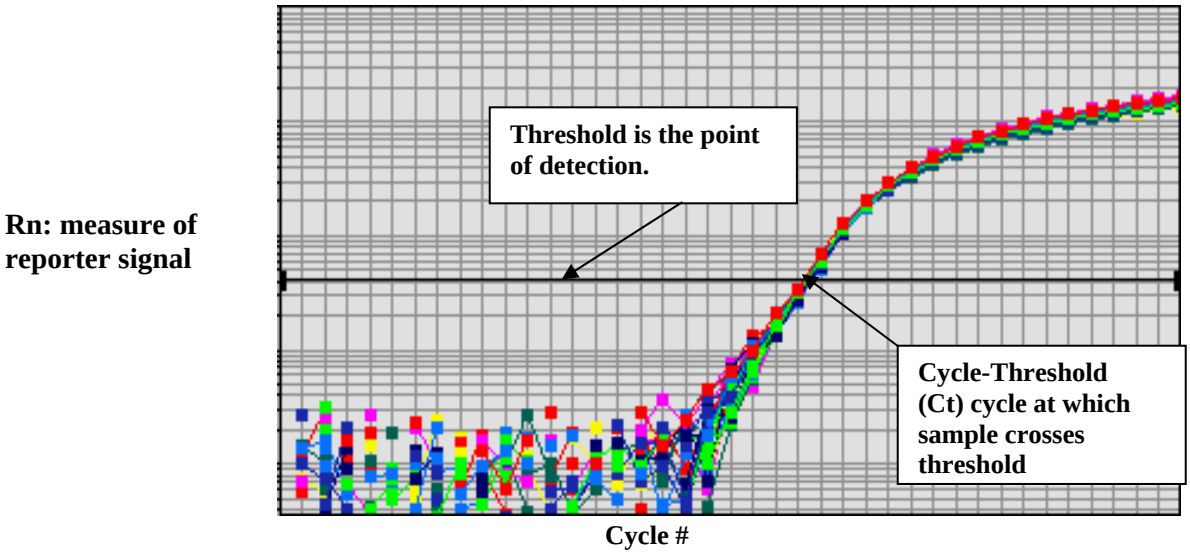
The increase in reporter signal is captured by the Sequence Detection instrument and displayed by the software. Figure 16 shows an increase in the reporter signal over time. The amount of reporter signal increase is proportional to the amount of product being produced for a given sample.

Figure 16: Increase in Reporter Signal



The combination of FRET and the 5′nuclease activity of AmpliTaq Gold® DNA Polymerase enables the 5′ nuclease assay and the SDS instrumentation to collect data in real time. When the fluorescent signal Reporter increases to a detectable level it can be captured and displayed as an **Amplification Plot**, Figure 17.

Figure 17: Amplification Curve



The Amplification Plot contains valuable information for the quantitative measurement of DNA or RNA. The **Threshold** line is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the **Cycle Threshold, Ct.** These two values are very important for data analysis using the 5′ nuclease assay.

SYBR Green Dye

SYBR Green chemistry is an alternate method used to perform real-time PCR analysis. SYBR Green is a dye that binds the Minor Groove of double stranded DNA. When SYBR Green dye binds to double stranded DNA, the intensity of the fluorescent emissions increases. As more double stranded amplicons are produced, SYBR Green dye signal will increase. Figures 18 & 19 show the entire process of each type of real-time chemistry. SYBR Green dye will bind to any double stranded DNA molecule, while the 5′ Nuclease assay is specific to a pre-determined target.

Figure 18: SYBR Green Dye

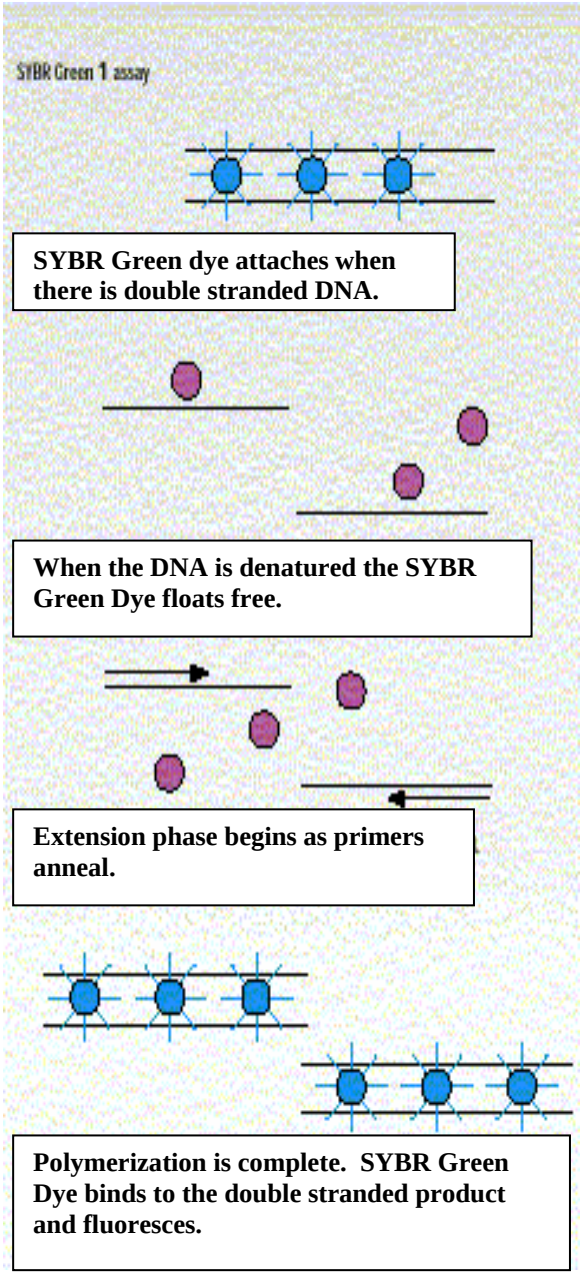
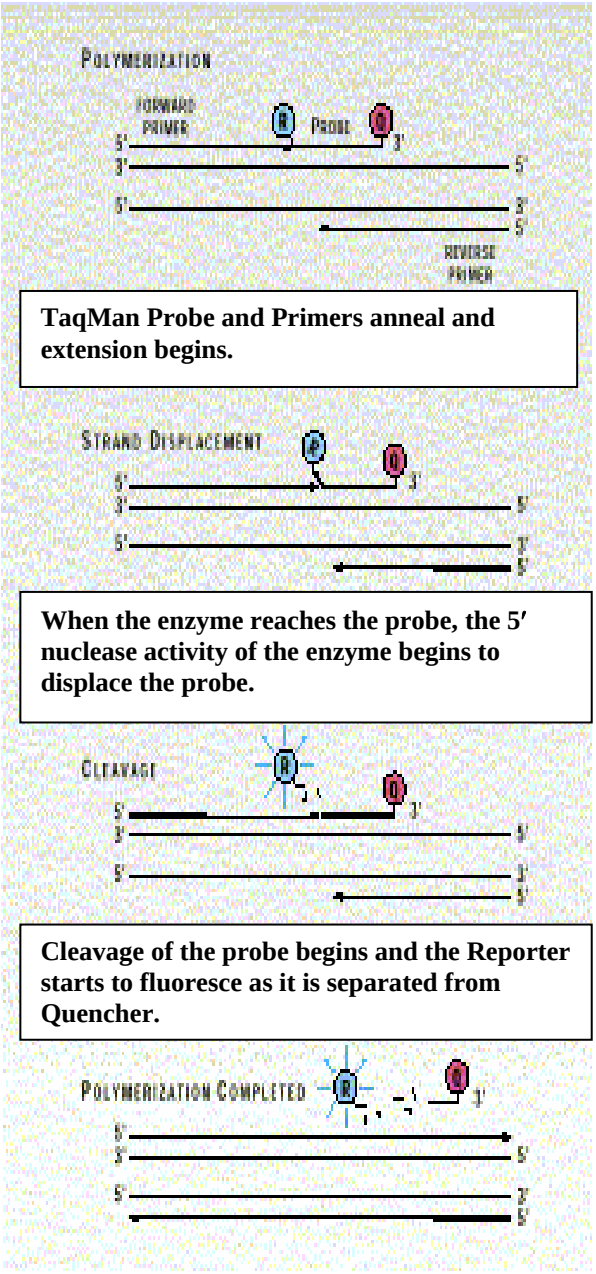


Figure 19: 5' Nuclease Assay



Applied Biosystems

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

Summary

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

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The PCR process and 5’ nuclease process are covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd.

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