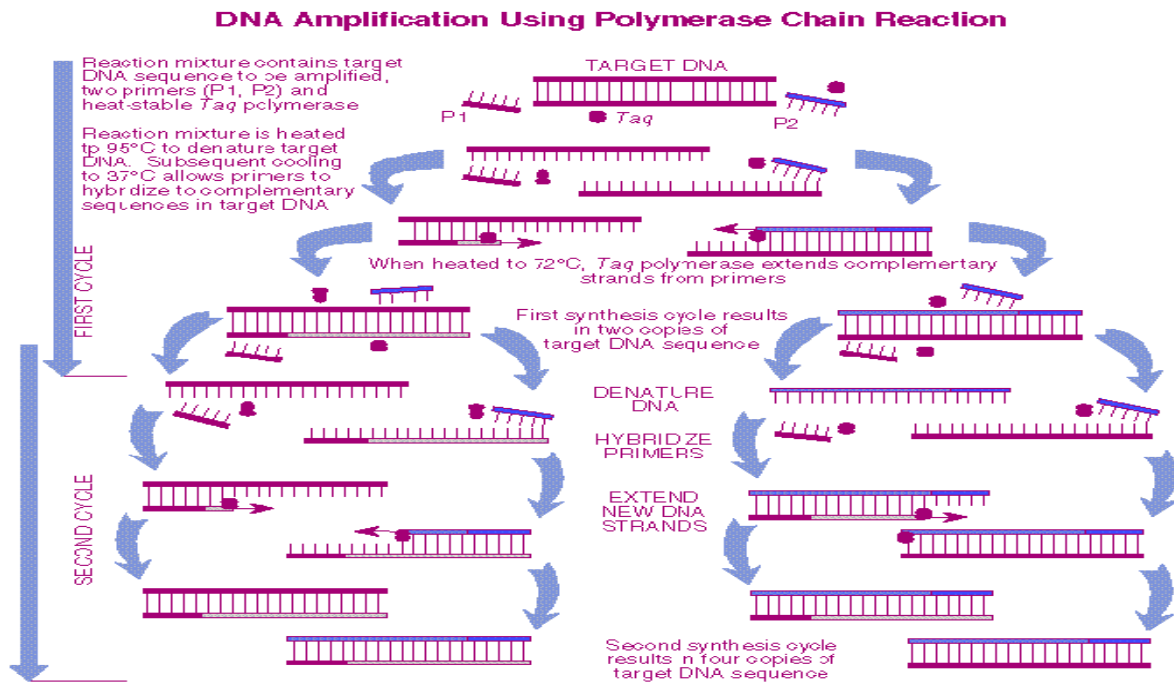


## Real-Time PCR

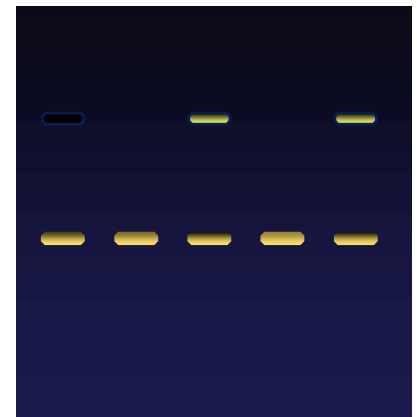
- What is it?
- How does it work?

### Principle of PCR



### What's Wrong with Agarose Gels?

- \* 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 staining is not very quantitative



## Real-Time PCR

### Definition:

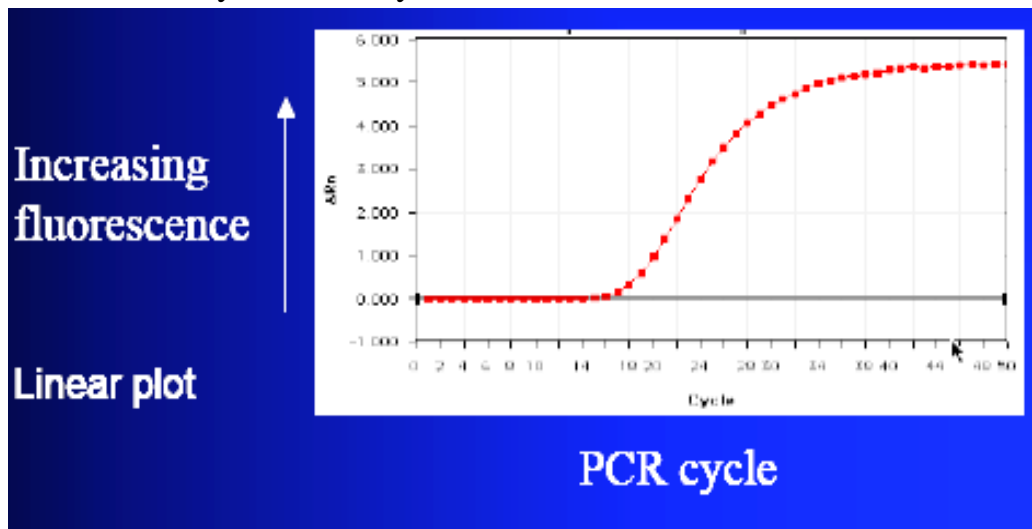
Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection.

### Real Time PCR is kinetic.

Detection of “amplification associated fluorescence” at each cycle during PCR.

No gel-based analysis at the end of the PCR reaction

Computer based analysis of the cycle- fluorescence tie course.



### Real-time PCR advantages

- \* Amplification can be monitored real-time.
- \* No post-PCR processing of products.  
(high throughput, low contamination risk)
- \* ultra-rapid cycling (30 minutes to 2 hours).
- \* Wider dynamic range of up to 10<sup>10</sup>-fold.
- \* requirement of 1000-fold less RNA than conventional assays  
(3 picogram = one genome equivalent)
- \* Confirmation of specific amplification by melting point analysis.
- \* Most specific, sensitive, and reproducible.
- \* Not much more expensive than conventional PCR.  
(except equipment cost)



## Real-time PCR disadvantages

- \* Not ideal for multiplexing.
- \* Setting up requires high technical skill and support.
- \* High equipment cost.

## Real-time Principles

- \* Based on the detection and quantitation of a fluorescent reporter.
- \* The first significant increase in the amount of PCR product (CT - threshold cycle) correlates to the initial amount of target template

### Three general methods for the quantitative detection:

1. Hydrolysis probes  
(**TaqMan, Beacons, Scorpions**)
2. Hybridisation probes  
(**Light Cycler**)
3. DNA-binding agents  
(**SYBR Green**)

### Principles of Real-Time Quantitative PCR techniques

\*SYBR Green I technique: SYBR Green I fluorescence is enormously increased upon binding to double-stranded DNA. During the extension phase, more and more SYBR Green I will bind to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal will be detected.

### When to choose SYBR Green

- \* Assays that do not require specificity of probe-based assays. Detection of 1000s of molecules.
- \* General screening of transcripts prior to moving to probe based assays.
- \* When the PCR system is fully optimized -no primer dimers or non-specific amplicons, e.g. from genomic DNA.
  
- \*When not to choose SYBR Green.
- \* Allelic discrimination assays (not an absolute one).
- \* Multiplex reactions (not an absolute one).



- \* Amplification of rare transcripts.
- \* Low level pathogen detection.

### **Absolute quantitation:**

- Standard curve.
- Standards must be accurately quantitated.
- Best used for viral load determination.

### **Relative quantitation:**

- Standard curve.
- Standards are serial dilutions of a calibrator template.
- Best used for gene expression studies.

### **Comparative quantitation:**

- Mathematical determination
- Calibrator sample used as 1x standard.
- Best used when particular ratios are expected or to verify trends.

### **Applications:**

- Viral quantitation
- Quantitation of gene expression
- Microarray verification
- Drug therapy efficacy.
- Pathogen detection
- Genotyping



# Popular Real-Time PCR Systems



ABI PRISM®  
**7900HT**  
Sequence Detection System



**BioRad iCycler**



**Roche**



**LightTyper & LightCycler**