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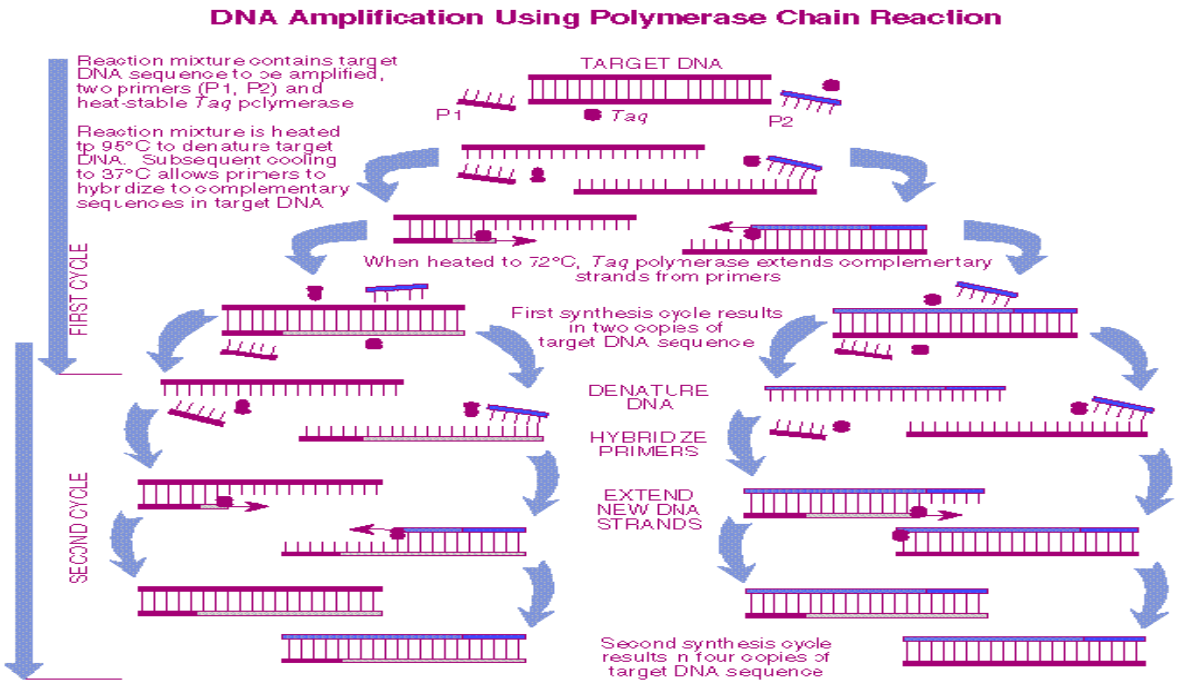
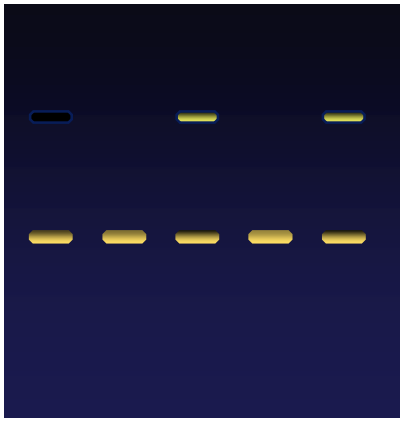
# Real-Time PCR

- **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**

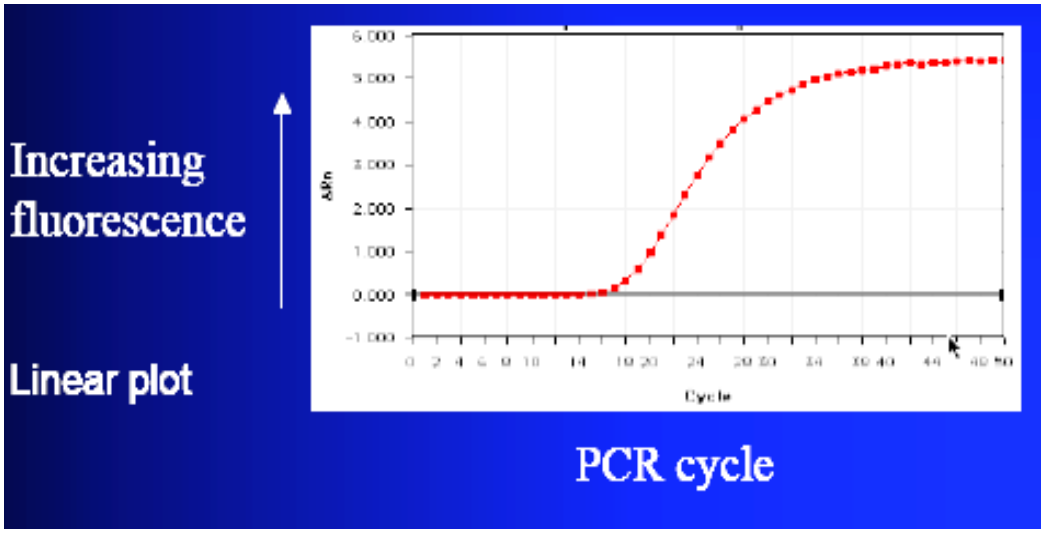
# Real-Time PCR

## 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 1010-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

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# Popular Real-Time PCR Systems

ABI PRISM® 7900HT Sequence Detection System

BioRad iCycler

Roche

LightTyper & LightCycler