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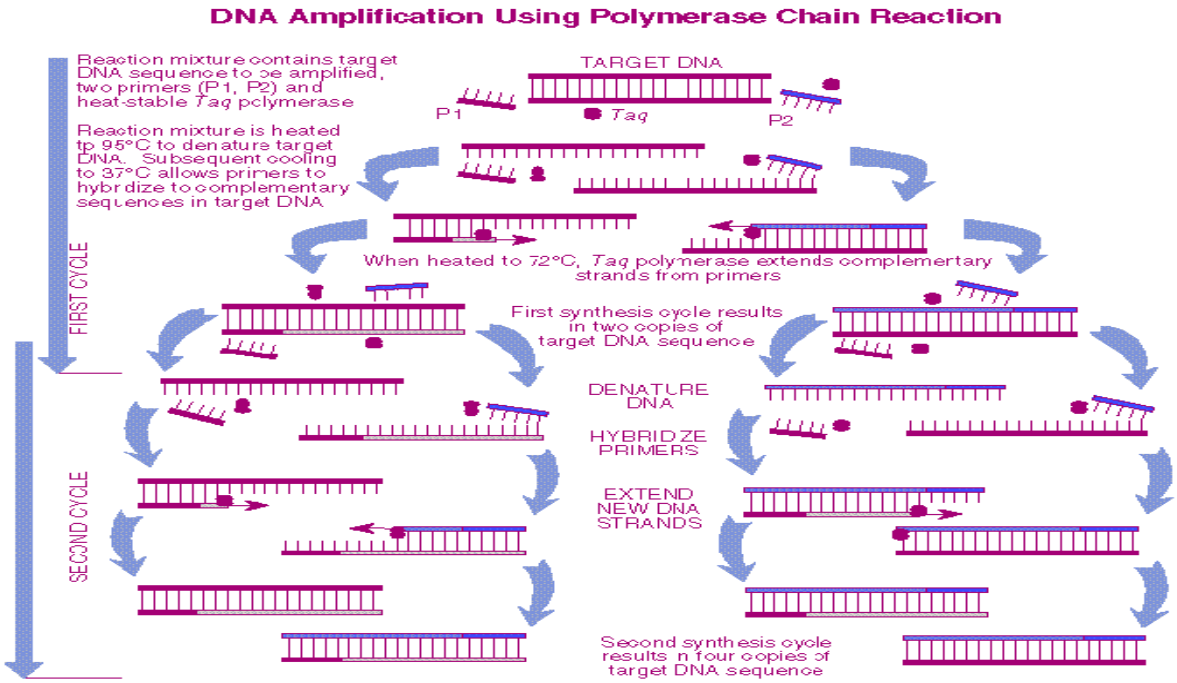
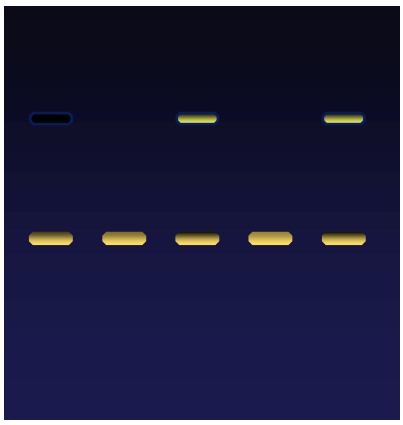
Real-Time PCR

- **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**

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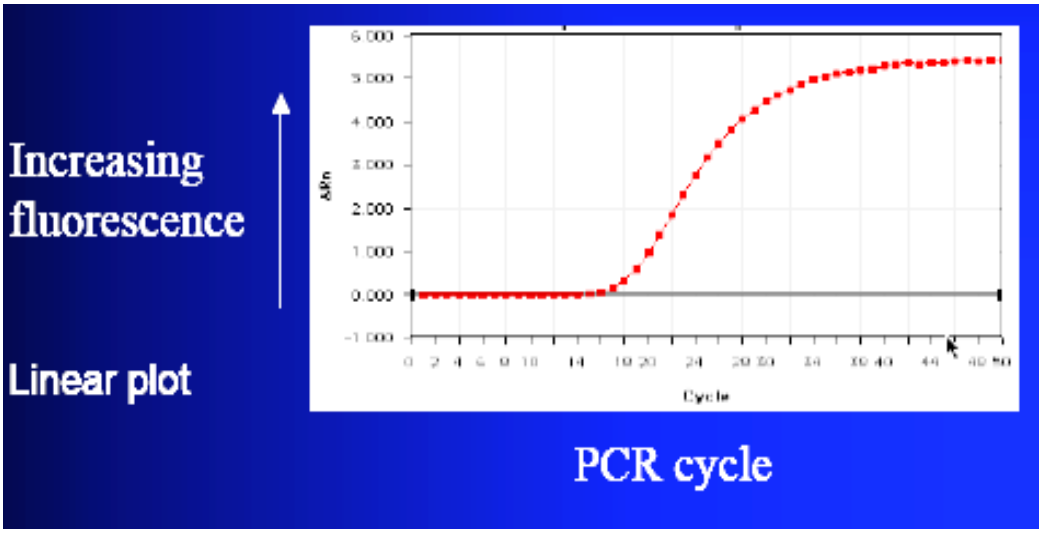
Real-Time PCR

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)

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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