Real-Time PCR

Professor
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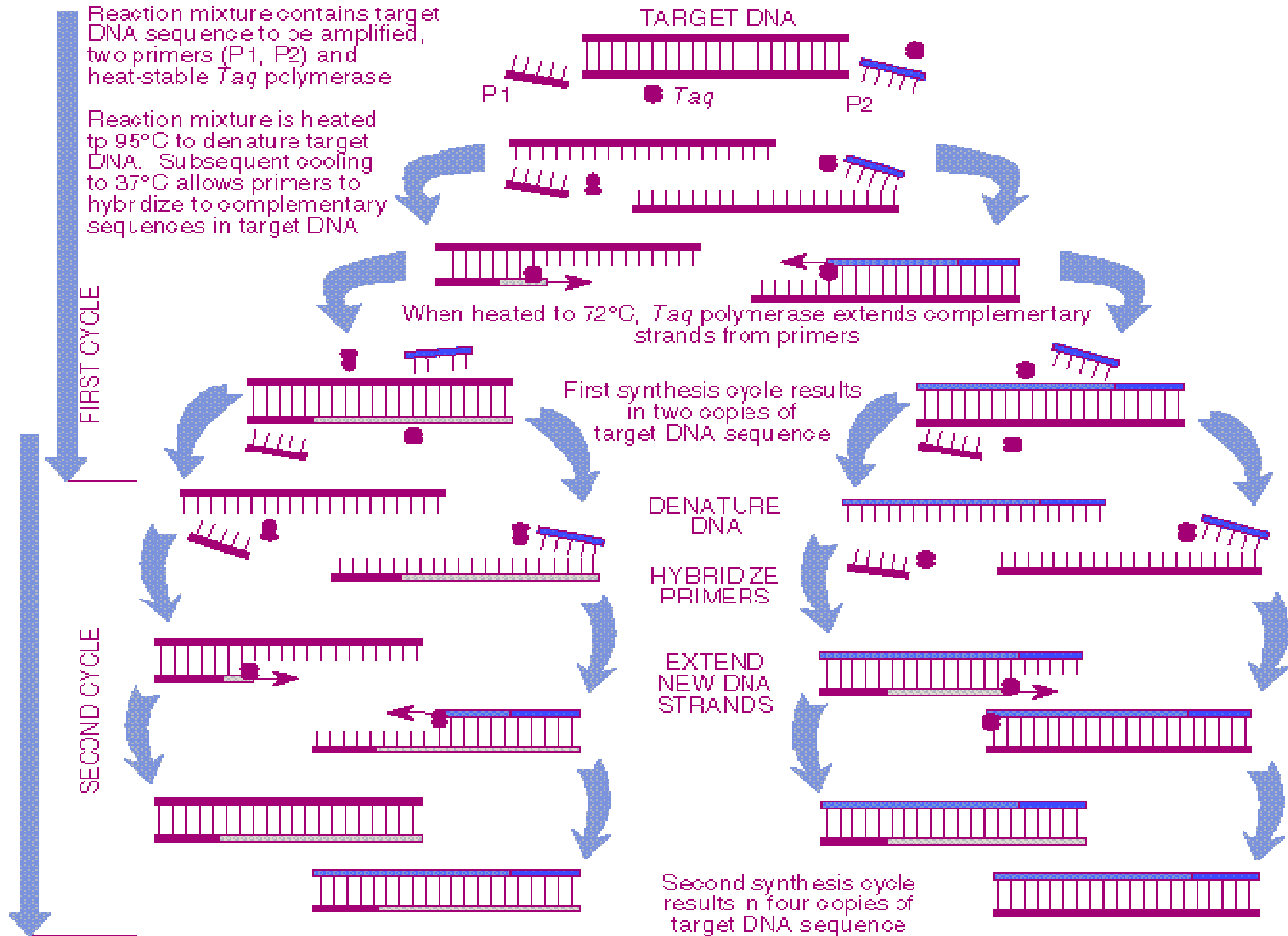
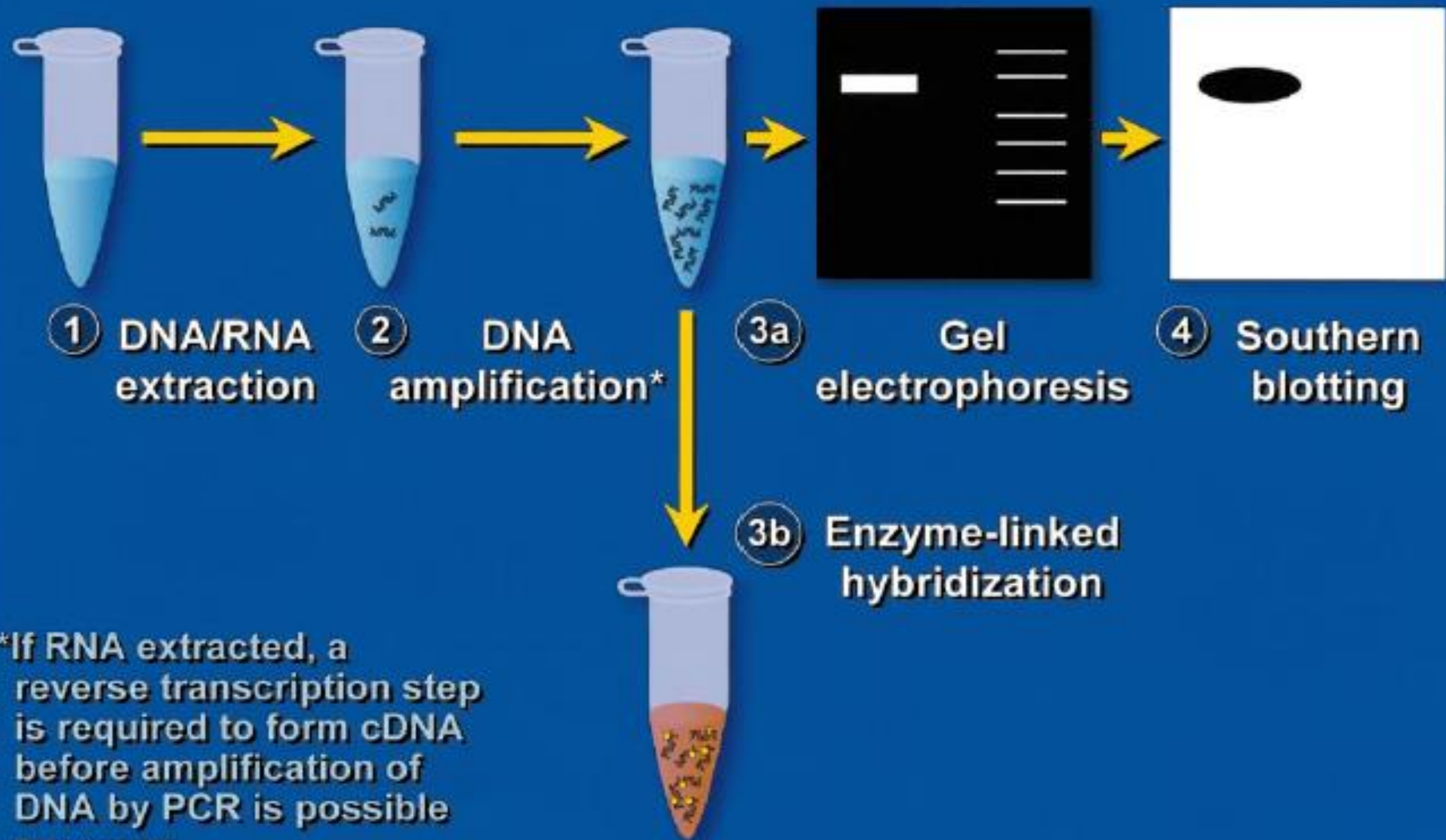
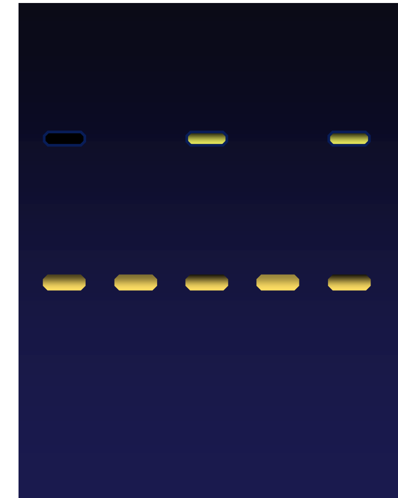

DNA Amplification Using Polymerase Chain Reaction
Reaction mixture contains target DNA sequence to be amplified, two primers (P1, P2) and heat-stable Taq polymerase
TARGET DNA
P1
Taq
P2
Reaction mixture is heated to 95°C to denature target DNA. Subsequent cooling to 37°C allows primers to hybridize to complementary sequences in target DNA
When heated to 72°C, Taq polymerase extends complementary strands from primers
FIRST CYCLE
First synthesis cycle results in two copies of target DNA sequence
DENATURE DNA
HYBRIDIZE PRIMERS
EXTEND NEW DNA STRANDS
SECOND CYCLE
Second synthesis cycle results in four copies of target DNA sequence



Cockerill FR III. Arch Pathol Lab Med. 2003;127:1112 (www)

What is 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

ABI: Real-Time PCR vs Traditional PCR (www)

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

TheScientist tuesday
MAGAZINE OF THE LIFE SCIENCES | EVERY DAY, ONLINE

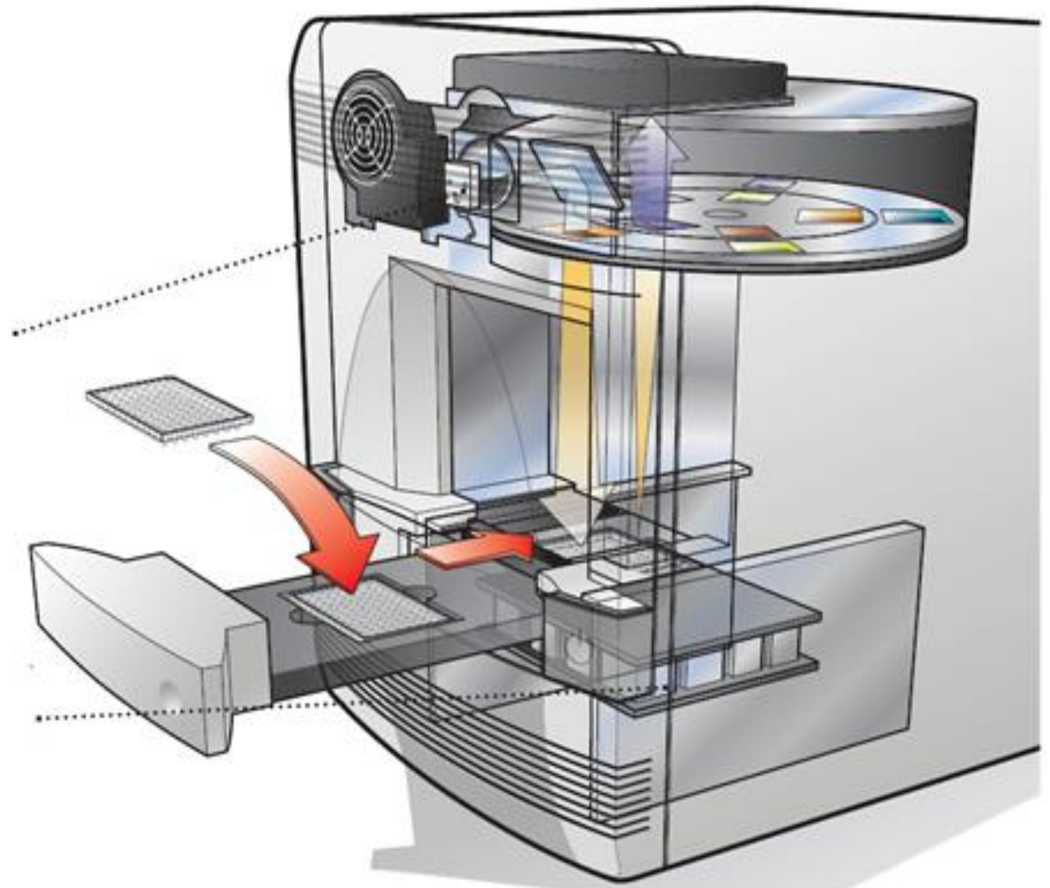
How it Works: Real Time PCR

Brendan Maher

The instrumentation is basic: a thermal cycler for amplification, a light source for excitation of fluorescent probes (see chemistries below), a camera for recording, and a computer to control the instrument and record data. Increasingly sophisticated instruments, such as those capable of multiplex experiments, are becoming affordable in academic labs.

The light source in the Applied Biosystems 7500 (represented here) is a simple halogen lamp shone through one of five different excitation filters over the entire sample. A CCD camera positioned above the sample records fluorescence from behind one of five emission fi lters. Some makes and models use a scanning head that moves over the plate, exciting and reading fluorescence in the wells individually.

Many qPCR instruments including the ABI 7500 use a Peltier element for heating and cooling. Peltier coolers use electron flow between semiconductor couples to heat or cool one side of a plate depending on the direction of current. Other systems use liquid or air fl ow or mechanical transition between blocks of different temperatures to cycle the samples.



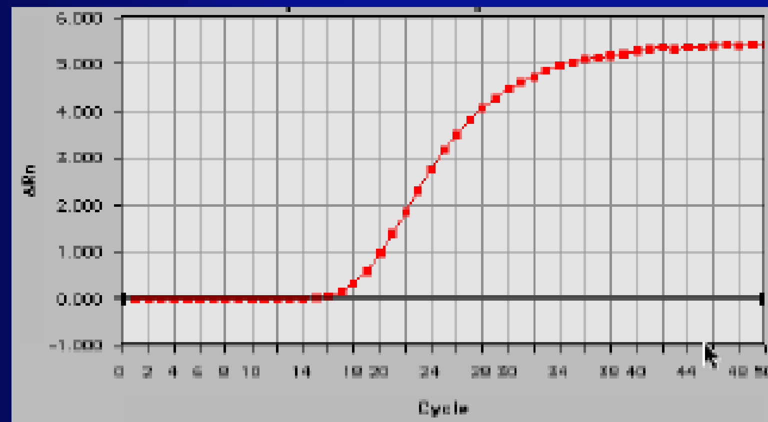
(www)

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 time course

Increasing fluorescence

Linear plot



PCR cycle

} Log-view augments this part

Nigel Walker, NIEHS (www)

Real-time PCR advantages

* not influenced by non-specific amplification

* 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^{10}-fold

* requirement of 1000-fold less RNA than conventional assays
(6 picogram = one diploid genome equivalent)

* detection is capable down to a two-fold change

* confirmation of specific amplification by melting curve analysis

* most specific, sensitive and reproducible

* not much more expensive than conventional PCR
(except equipment cost)

Wider Dynamic Range

Example 1: Linear Dynamic Range Analysis

Figure 4 illustrates an example of the linear dynamic range for a one-step RT-PCR run using a 1–10^5 pg range of initial template concentrations.

- Five log dilutions
- Triplicates are used
- Six dilutions

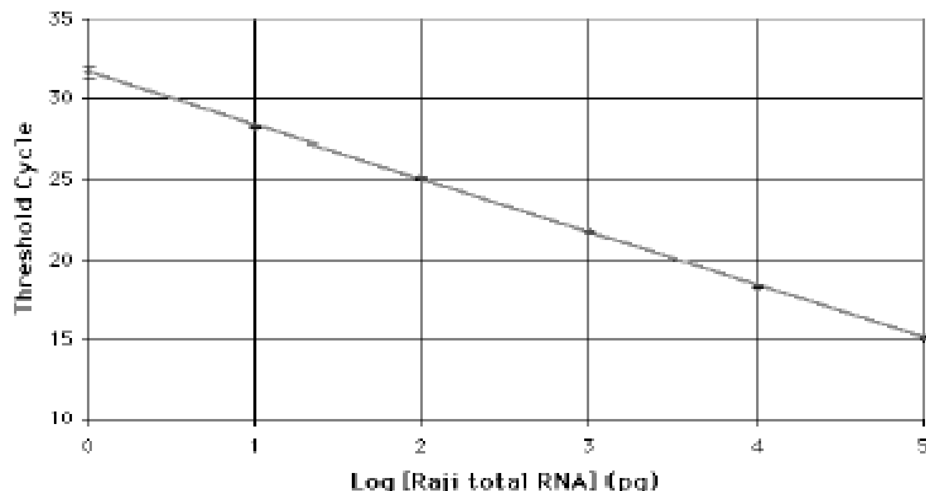


Figure 4. Standard Curve (Human GAPDH mRNA System). Each data point represents the average of triplicate reactions.

All initial template concentrations plotted on the graph appear to be in the linear dynamic range for the system. Therefore, any total RNA concentration within the 1–10^5 pg range can be used. However, the greatest sensitivity will be achieved if the limiting primer experiment is run at 1 pg initial total RNA.

ABI-7700 User Bulletin #5

Applied Biosystems

Real-time PCR disadvantages

* not ideal for multiplexing

* setting up requires high technical skill and support

* high equipment cost

* * *

* intra- and inter-assay variation

* RNA lability

* DNA contamination (in mRNA analysis)

Real-time PCR Principles

* based on the detection and quantitation of a fluorescent reporter

* the first significant increase in the amount of PCR product (C_T - threshold cycle) correlates to the initial amount of target template

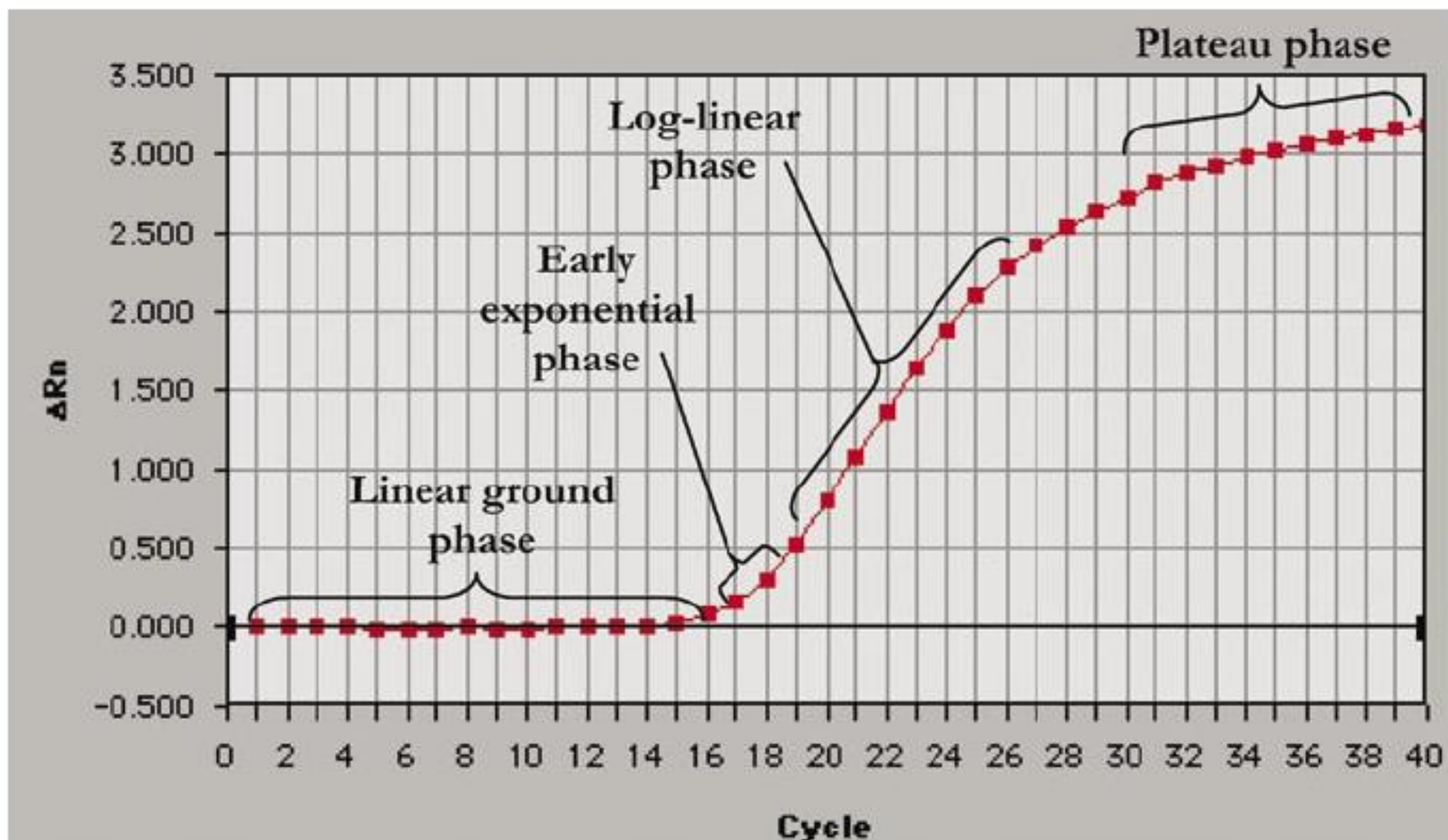


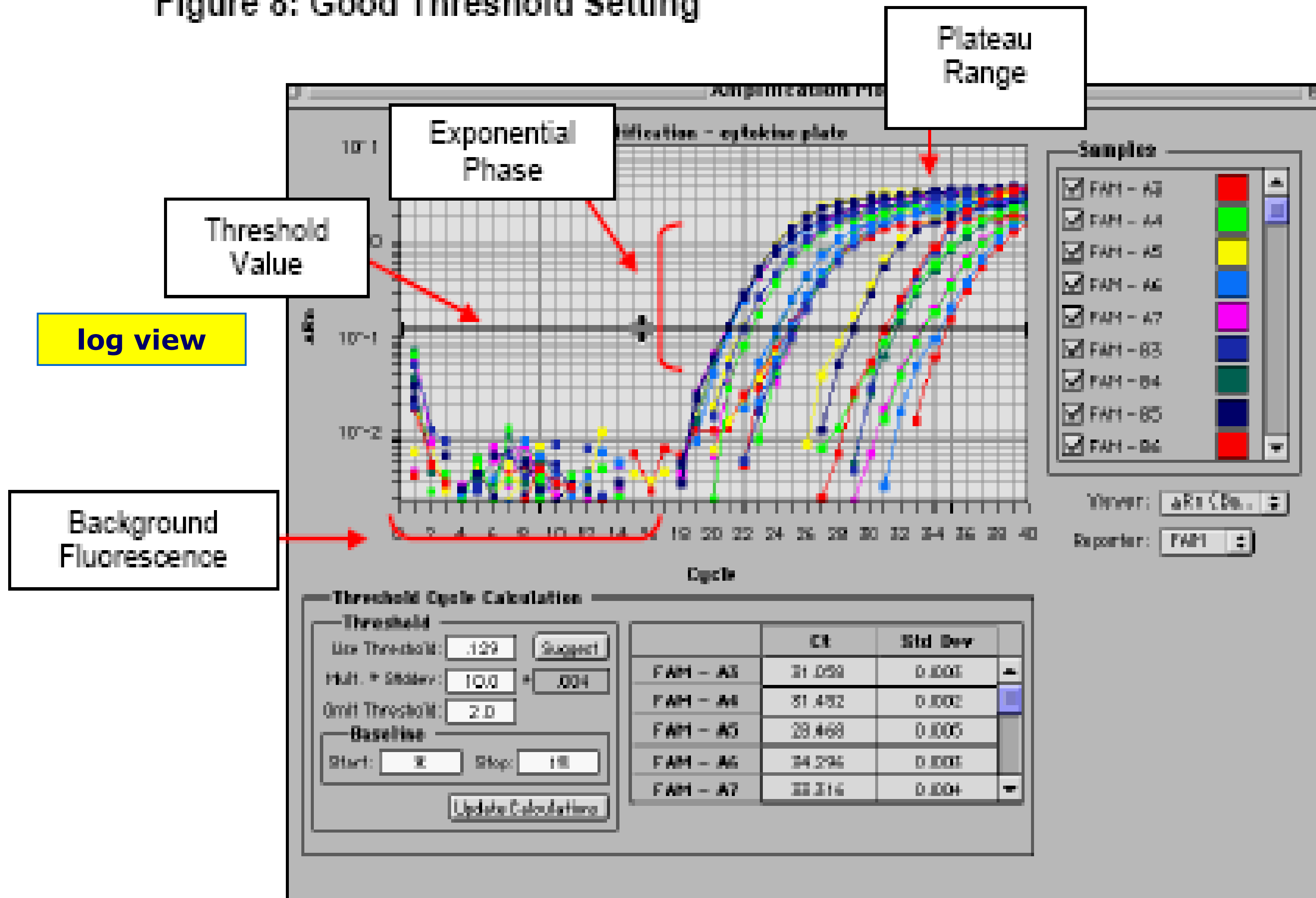
Figure 2. Phases of the PCR amplification curve. The PCR amplification curve charts the accumulation of fluorescent emission at each reaction cycle. The curve can be broken into four different phases: the linear ground, early exponential, log-linear, and plateau phases. Data gathered from these phases are important for calculating background signal, cycle threshold (C_t), and amplification efficiency. Rn is the intensity of fluorescent emission of the reporter dye divided by the intensity of fluorescent emission of the passive dye (a reference dye incorporated into the PCR master mix to control for differences in master mix volume). ΔRn is calculated as the difference in Rn values of a sample and either no template control or background, and thus represents the magnitude of signal generated during PCR. This graph was generated with ABI Prism SDS version 1.9 software (Applied Biosystems).

Real-time PCR for mRNA quantitation

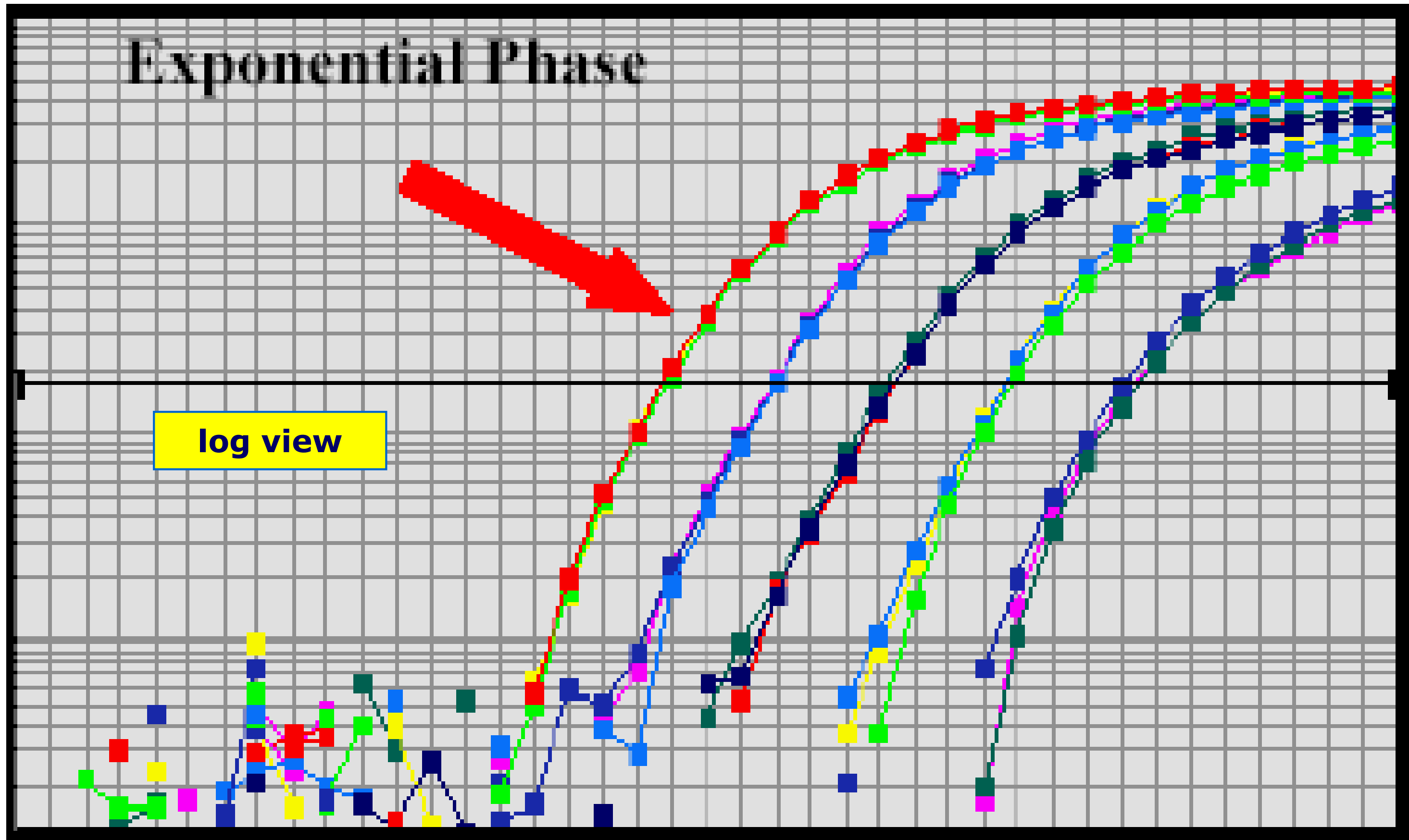
Marisa L. Wong and Juan F. Medrano

BioTechniques 39:75-85 (July 2005)

(www)



Applied Biosystems



The five-fold dilution series 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.

Applied Biosystems

Real-Time PCR Principles

Three general methods for the quantitative assays:

1. Hydrolysis probes

(TaqMan, Beacons)

2. Hybridization probes

(Light Cycler)

3. DNA-binding agents

(SYBR Green)

Principles of Real-Time Quantitative PCR Techniques

(a) **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.

(b) **Hydrolysis probe technique:** The hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the Taq polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.

(c) **Hybridization probes technique:** In this technique one probe is labelled with a donor fluorochrome at the 3′ end and a second –adjacent- probe is labelled with an acceptor fluorochrome. When the two fluorochromes are in close vicinity (1–5 nucleotides apart), the emitted light of the donor fluorochrome will excite the acceptor fluorochrome (FRET). This results in the emission of fluorescence, which subsequently can be detected during the annealing phase and first part of the extension phase of the PCR reaction. After each subsequent PCR cycle more hybridization probes can anneal, resulting in higher fluorescence signals.

Van der Velden, Leukemia 2003 (www)

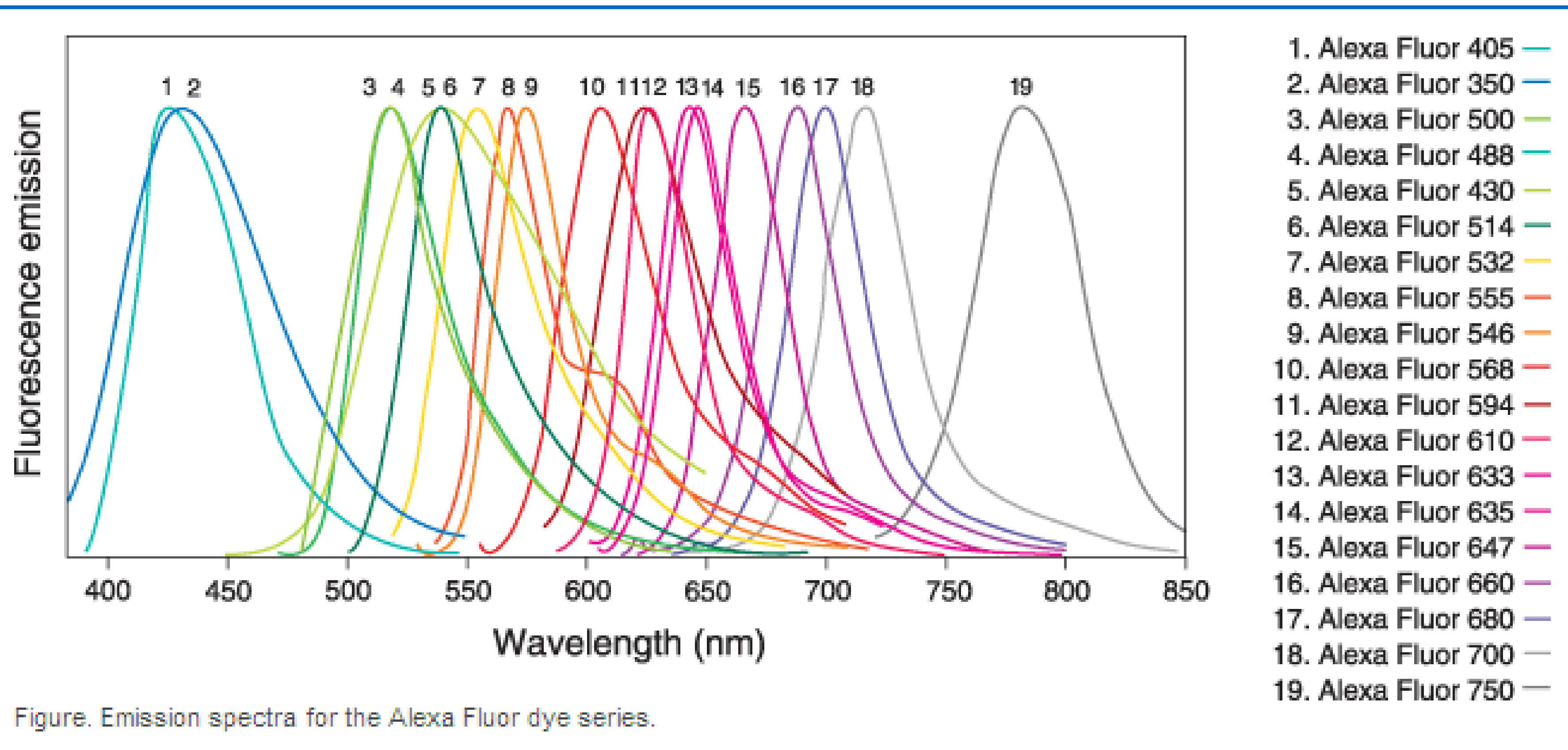


Figure. Emission spectra for the Alexa Fluor dye series.

invitrogen™

Threshold Cycle

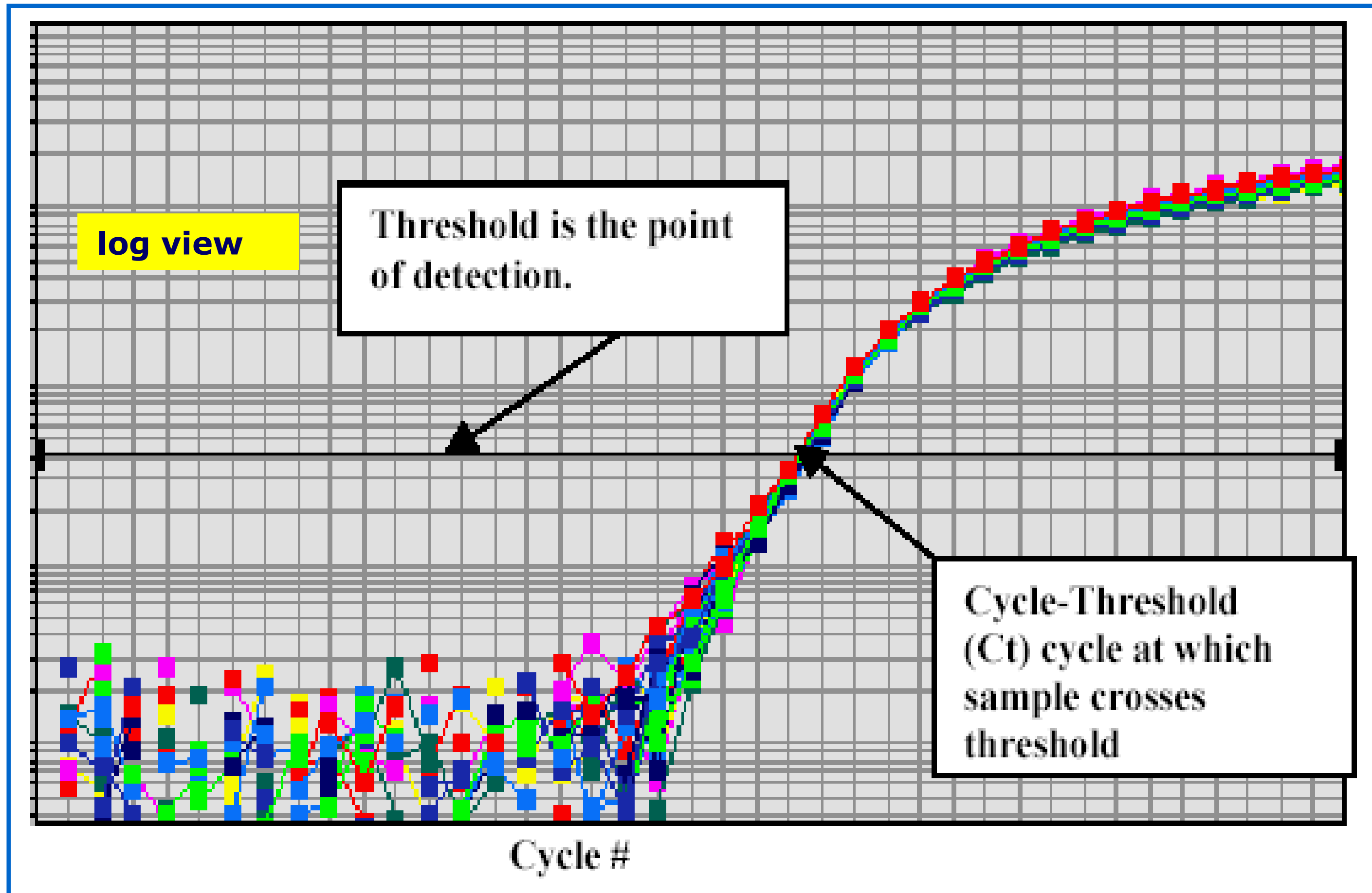
* threshold cycle or the C_T value is the cycle at which a significant increase in ΔRn is first detected

* it is the parameter used for quantitation

* C_T value of 40 or more means no amplification and cannot be included in the calculations

* theoretically a single copy of the target should create a C_T value of 40 (if efficiency is 100%), which is the y-intercept in a standard curve experiment

What is C_T?



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, C_T. These two values are very important for data analysis using the 5' nuclease assay.

Applied Biosystems

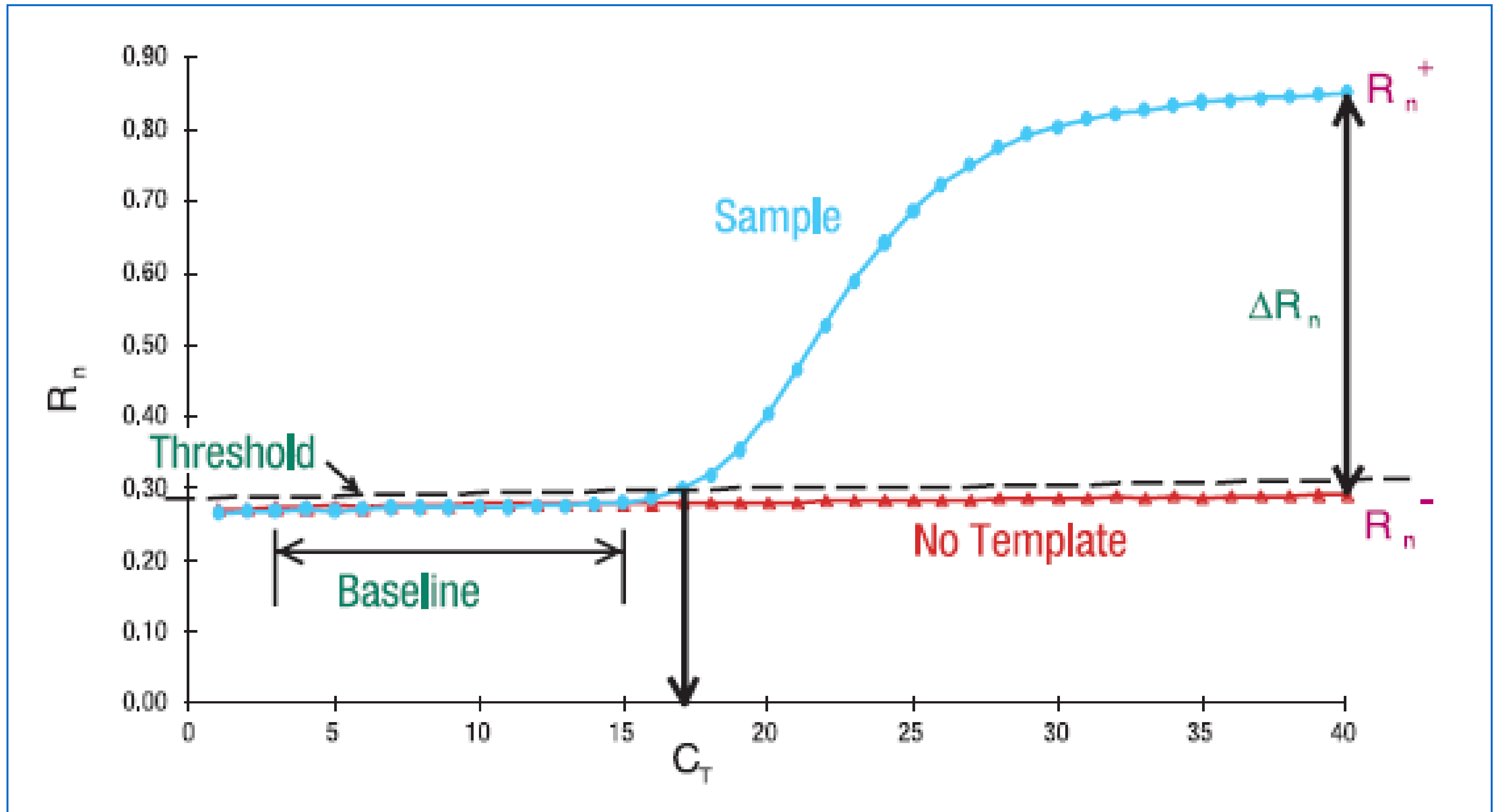
∆Rn

* Rn^+ is the Rn value of a reaction containing all components (the sample of interest); Rn^- is the Rn value detected in NTC (baseline value)

* ∆Rn is the difference between Rn^+ and Rn^-. It is an indicator of the magnitude of the signal generated by the PCR

* ∆Rn is plotted against cycle numbers to produce the amplification curves and to estimate the C_T values

What is ΔRn?



(www)

Efficiency

The slope of the log-linear phase is a reflection of the amplification efficiency

The efficiency of the reaction can be calculated by the following equation: $Eff = 10^{(-1/slope)} - 1$. The efficiency of the PCR should be 90-110% (ideal slope = 3.32)

A number of variables can affect the efficiency of the PCR. These factors can include length of the amplicon, secondary structure and primer design, to name a few

Approximation vs Pfaffl method

(Efficiency Determination)

Issues of assay design

- RNA specific sets -ie Primers spanning intron location
  - If you know the gene and have the time go for it.
  - Not all genes in database and annotated esp. rat
- Do you need RNA specific sets?
  - RNA expression 10^3-10^8 copies/100ng total RNA
  - 100 ng RNA approx = 100 single gene copies (assuming 1% DNA contam)
- Reverse transcription
  - Gene specific primer is best especially if using a synthetic RNA standard
  - Oligo d(T)-may not be good for 5' end targets
  - Random hexamers - poor for synthetic RNA standard

Nigel Walker, NIEHS (www)

Assay Validation

* Test primer pairs in all combinations with the probe with a known template (plasmid clone, cDNA, RNA)

* Use standard assay conditions: 300-400 nM primers, 100 nM probe, 4 mM $MgCl_2$ (higher for multiplex reactions)

* Choose the primer pair that gives the highest ΔRn and the lowest C_T

* Make at least three (1:10) dilutions of a template, either cDNA, RNA or total RNA (in triplicates) for a standard curve

* If the slope of the standard curve of the best primer pair is around -3.5 increase the $MgCl_2$ concentration to 5 mM

* If the slope is higher than -3.6, change primers

* An ideal assay will have a slope of -3.32, R^2 (coefficient of determination) >0.99, SD<0.250 and y-intercept ~ 40

* Target and normalizer standard curves should be parallel (same slope = efficiency)

* In a well-optimized multiplex reaction, the target CT values should be the same as obtained in singleplex reactions for each target

Real-Time PCR Applications - I

* quantitation of gene expression

* array verification

* quality control and assay validation

* biosafety and genetic stability testing

* drug therapy efficacy / drug monitoring

* viral quantitation

* pathogen detection

Real-Time PCR Applications - II

* DNA damage (microsatellite instability) measurement

* radiation exposure assessment

* in vivo imaging of cellular processes

* mitochondrial DNA studies

* methylation detection

* detection of inactivation at X-chromosome

* linear-after-the-exponential (LATE)-PCR: a new method for real-time quantitative analysis of target numbers in small samples, which is adaptable to high throughput applications in clinical diagnostics, biodefense, forensics, and DNA sequencing

Real-Time PCR Applications - II

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Real-Time PCR Applications - III

* Determination of identity at highly polymorphic HLA loci
* Monitoring post transplant solid organ graft outcome
* Monitoring chimerism after HSCT
* Monitoring minimal residual disease after HSCT
* Genotyping (allelic discrimination)
  - Trisomies and copy number variations
  - Microdeletion genotypes
  - Haplotyping
  - Quantitative microsatellite analysis
  - Prenatal diagnosis from fetal cells in maternal blood
  - Intraoperative cancer diagnostics