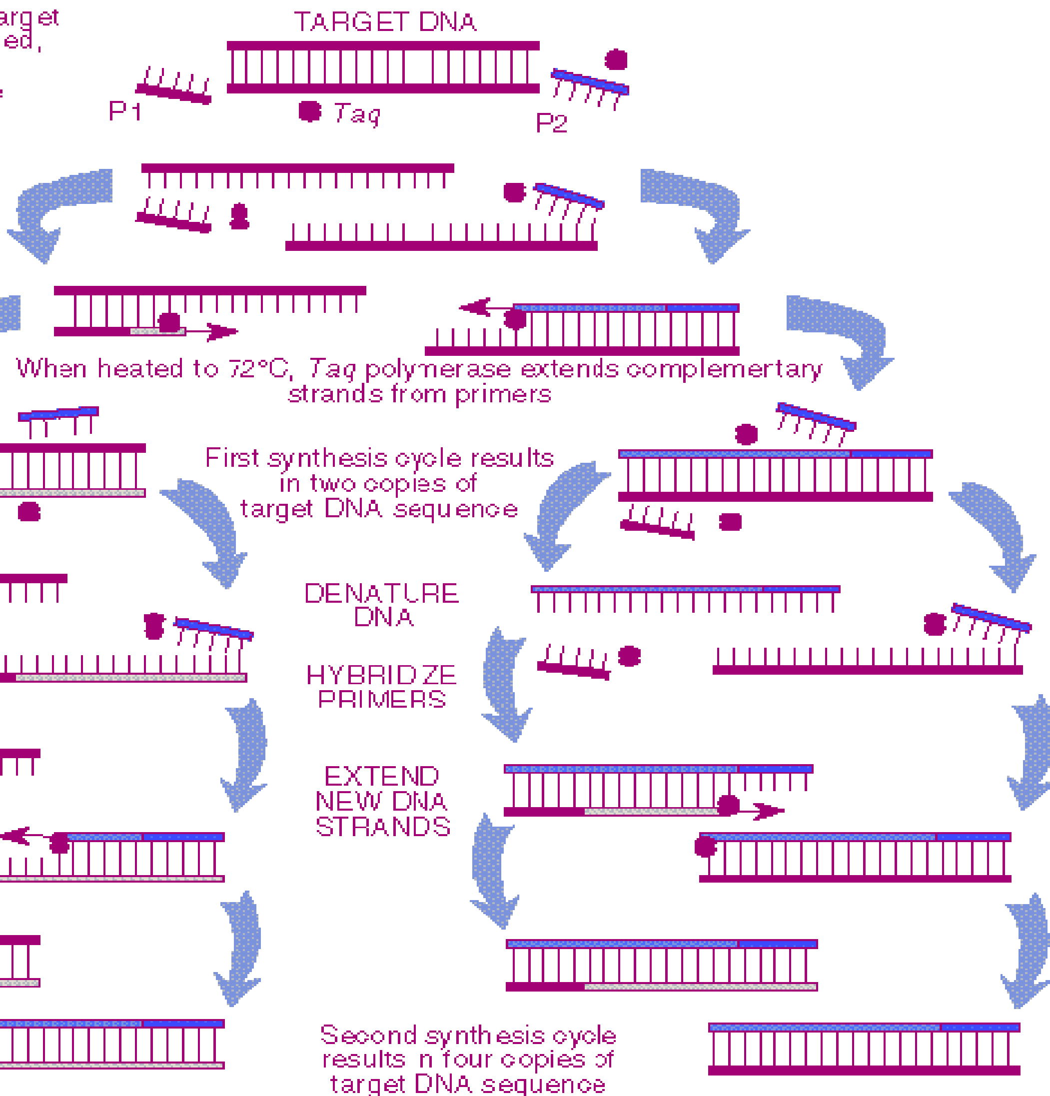
Real-Time PCR

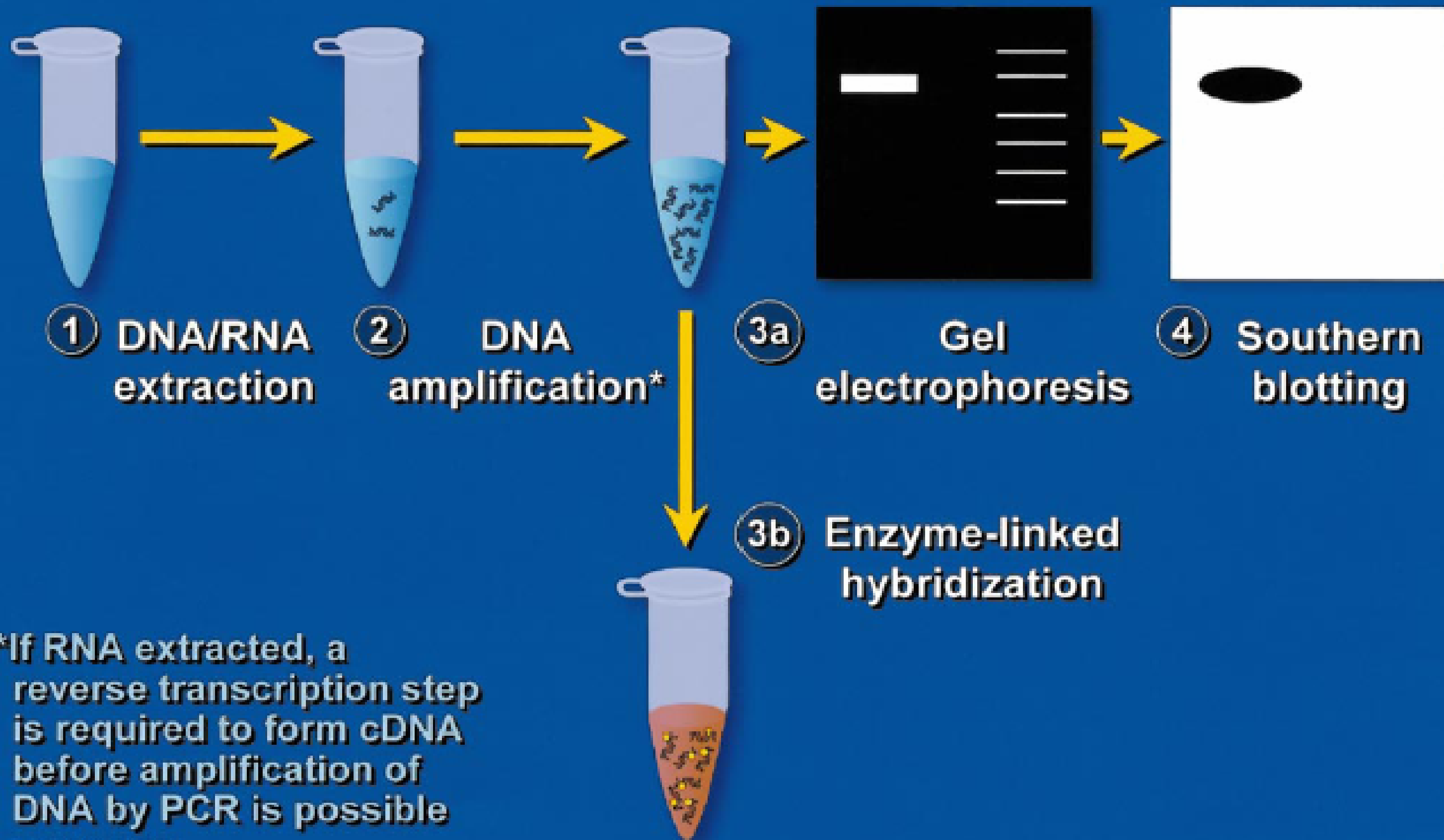
M.Tevfik DORAK, MD PhD

DNA Amplification Using Polymerase Chain Reaction

Reaction mixture contains target DNA sequence to be amplified, two primers (P1, P2) and heat-stable *Taq* polymerase

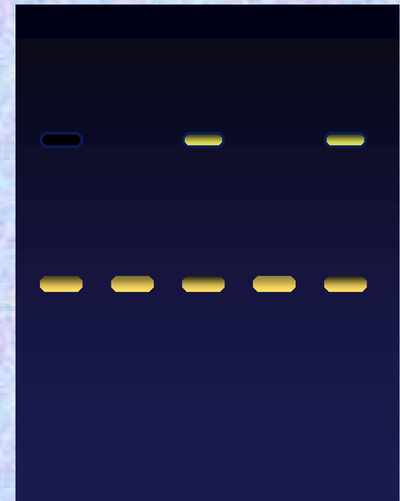
Reaction mixture is heated tp 95°C to denature target DNA. Subsequent cooling to 37°C allows primers to hybridize to complementary sequences in target DNA





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

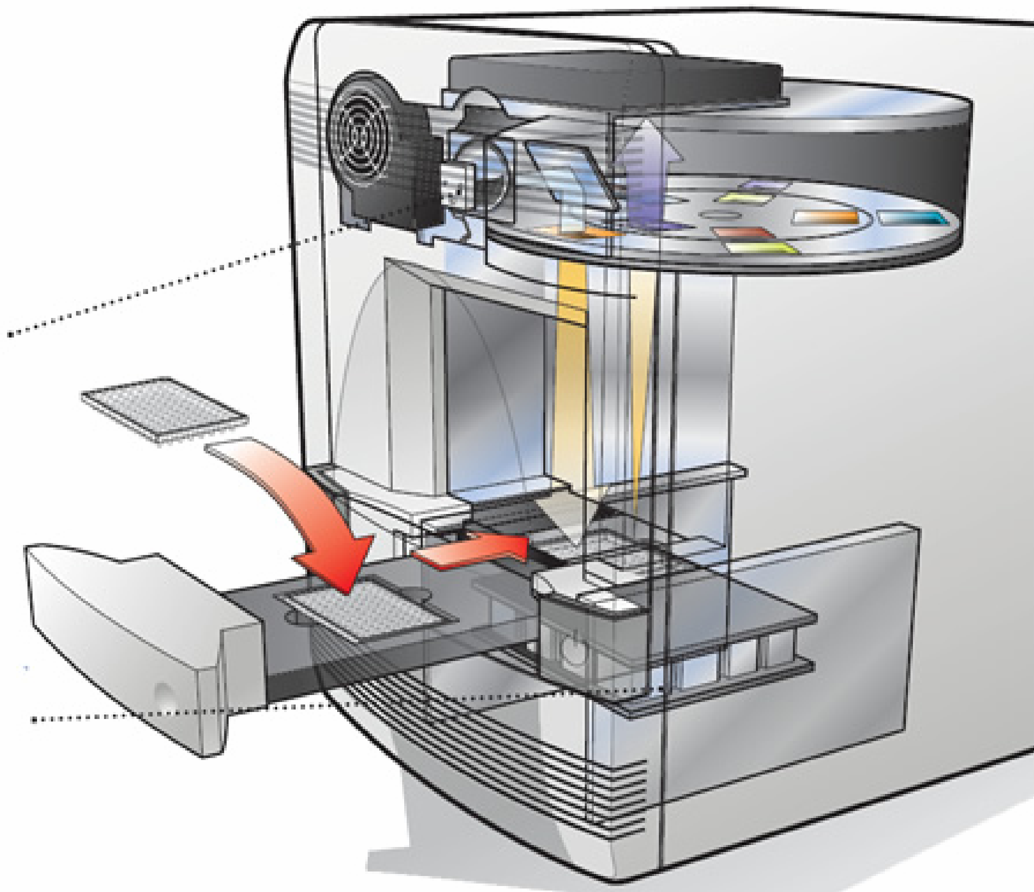
Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection

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.



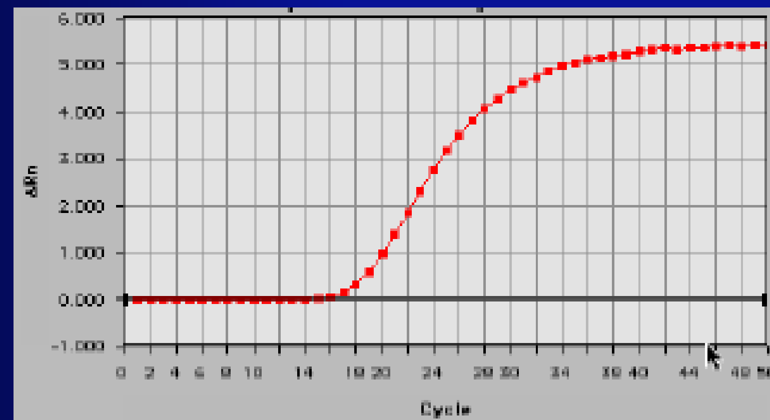
The light source in the Applied Biosystems 7500 (represented here) is a simple halogen lamp shone through one of five different excitation filters over the entire sample. A CCD camera positioned above the sample records fluorescence from behind one of five emission fi lters. Some makes and models use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

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

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
(3 picogram = one genome equivalent)

* detection is capable down to a 2-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.

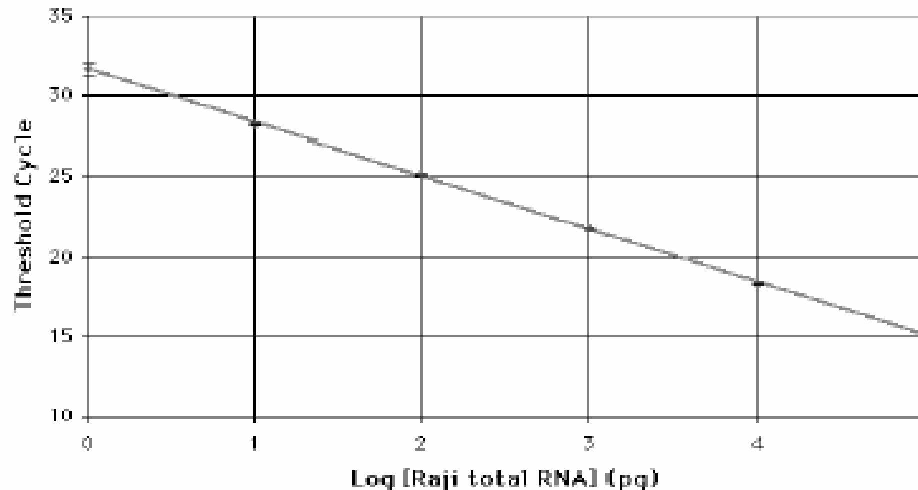


Figure 4. Standard Curve (Human GAPDH mRNA System). Each data point represents the average of triplicate reactions.

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

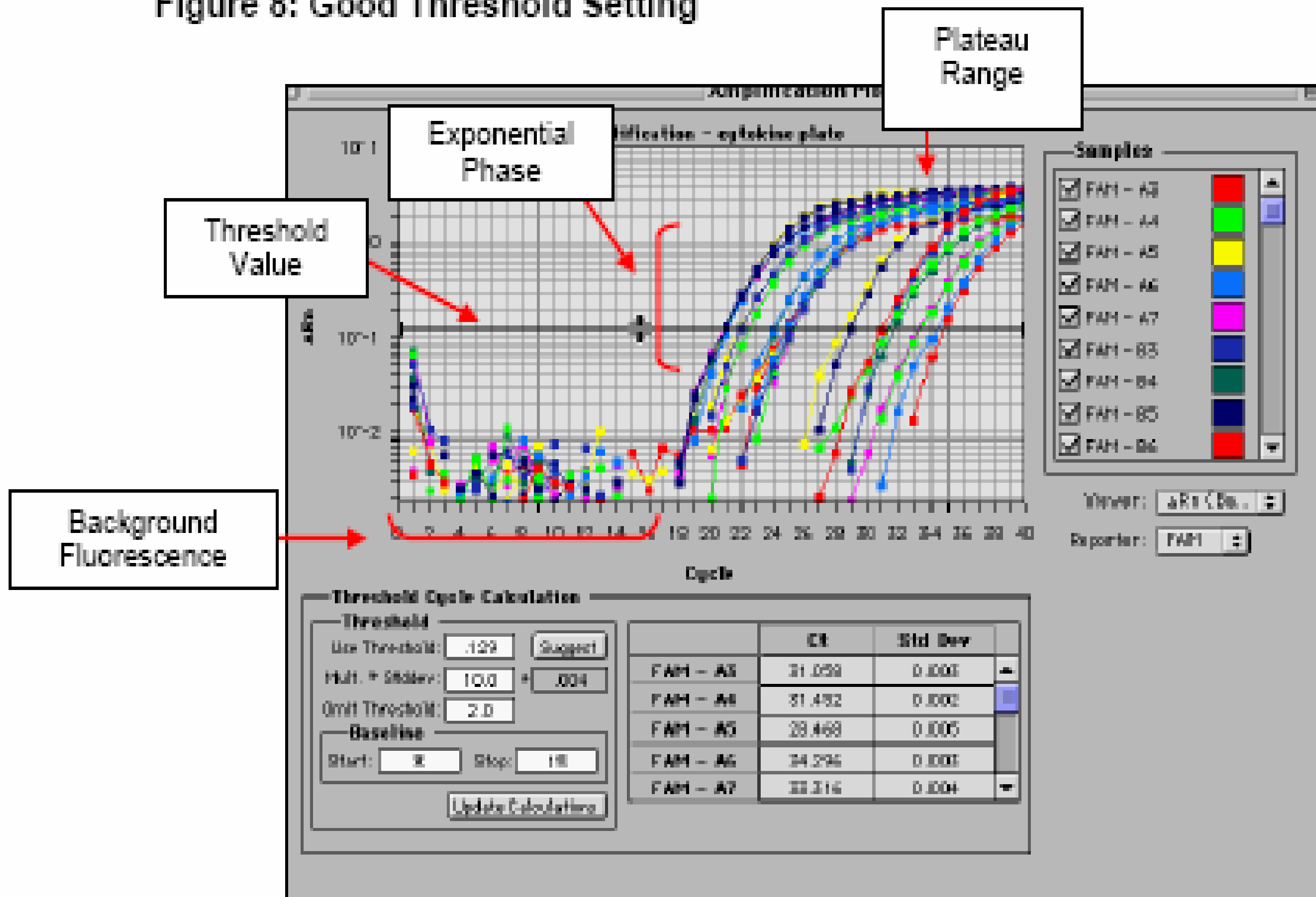
* RNA lability

* DNA contamination (in mRNA analysis)

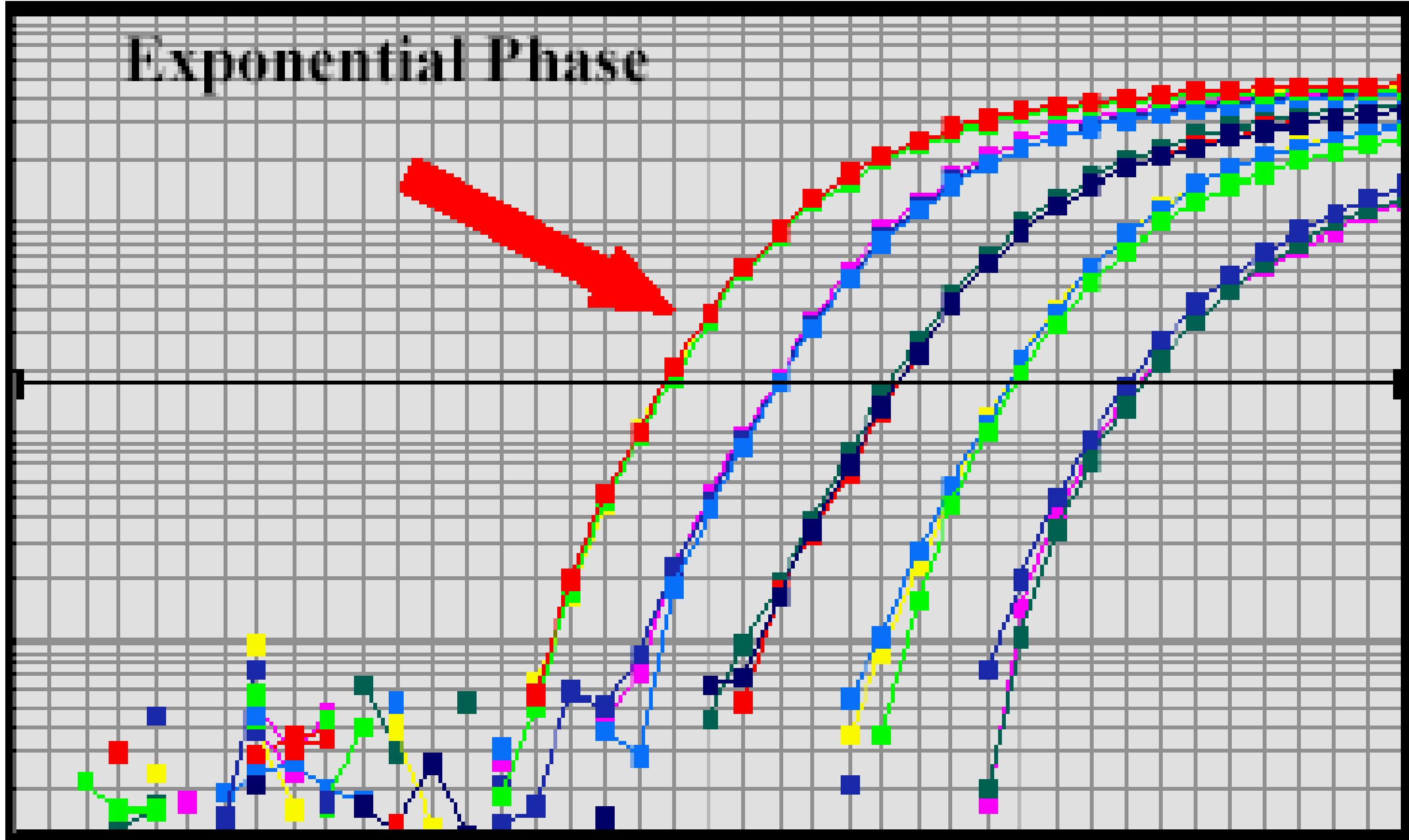
Real-time Principles

* 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



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 Principles

Three general methods for the quantitative assays:

1. Hydrolysis probes

(TaqMan, Beacons, Scorpions)

2. Hybridization probes

(Light Cycler)

3. DNA-binding agents

(SYBR Green)

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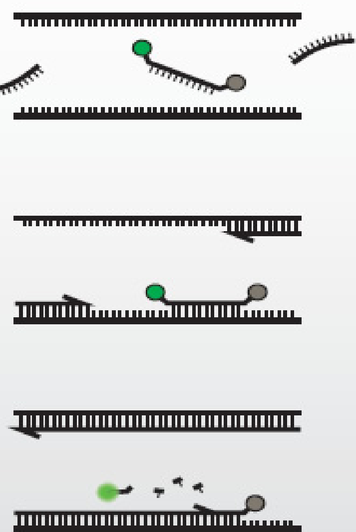
How it Works: Real Time PCR

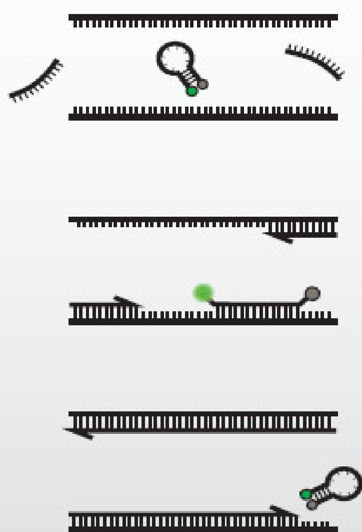
Brendan Maher

TaqMan requires a sequence-specific probe that connects fluorophore and quencher.



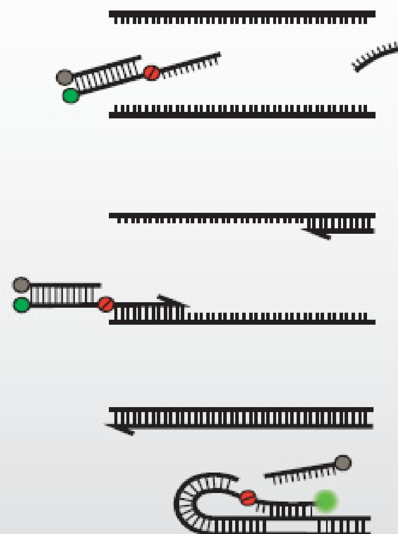
* Pros: specificity, different colors can be used in multiplex assays
* Cons: some background noise due to irreversibility of the reaction

Molecular Beacons uses sequence specific probes that take on a hairpin structure.



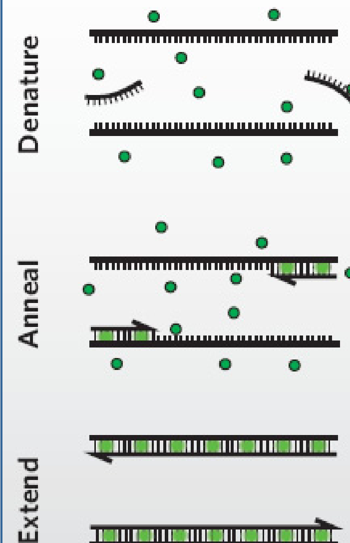
* Pros: greater specificity, reversible fluorescence means lower background
* Cons: some non-specific interactions of the hairpins can lead to false positives

Scorpions chemistry combines probe and primer. A polyermase blocker prevents unwanted replication.



* Pros: high specificity, faster cycling
* Cons: probe/primer design is involved and pricey

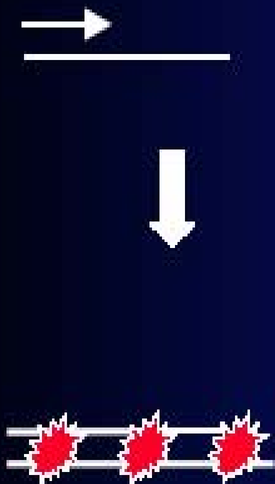
SYBR Green I fluoresces only when boun to dsDNA.

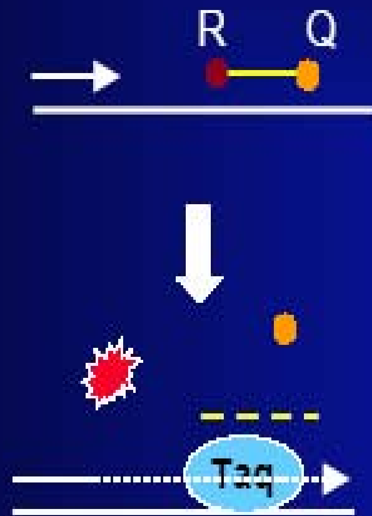


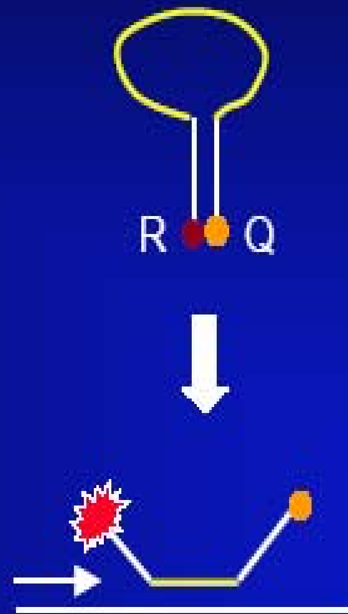
* Pros: relatively cheap, doesn't require probe design
* Cons: nonspecificity can lead to false p tives, not attuned for complex protocol

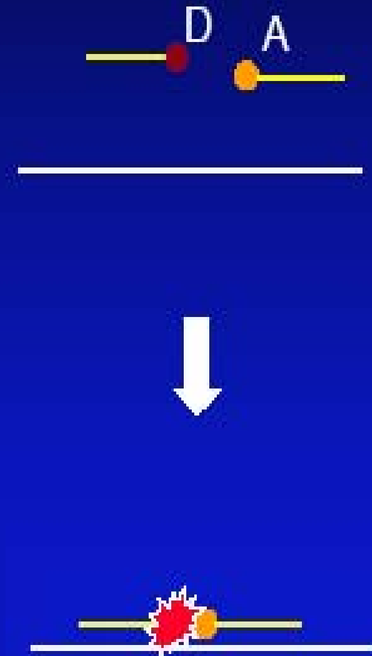
(www

Methods of fluorescence detection





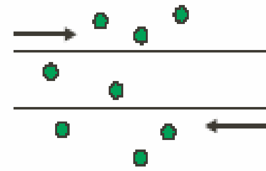
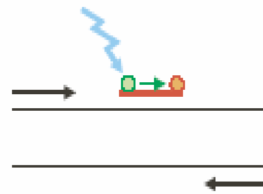
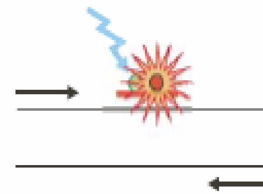




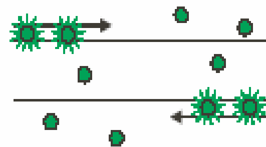
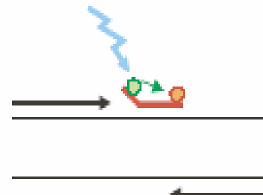
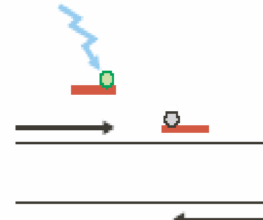
Nigel Walker, NIEHS (www)

a SYBR Green I | b Hydrolysis probe | c Hybridization probes

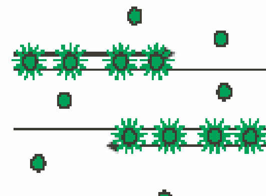
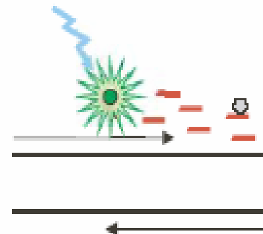
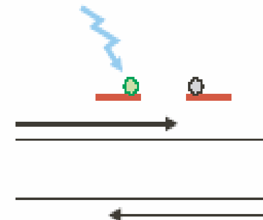
Annealing phase

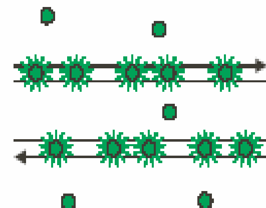
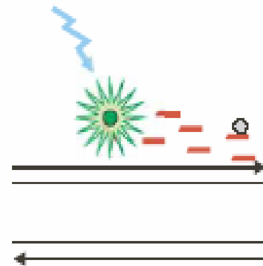
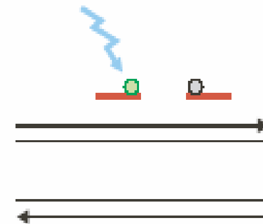
Extension phase (I)

Extension phase (II)

End of PCR cycle

Van der Velden, Leukemia 2003 [(www)]

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.

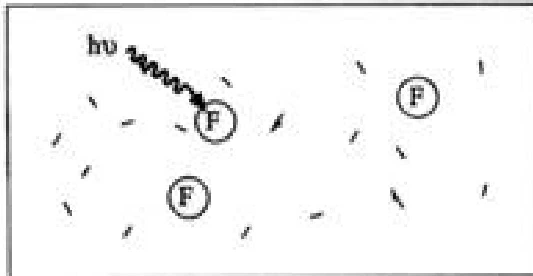
(b) **Hydrolysis probe technique:** The hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed 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.

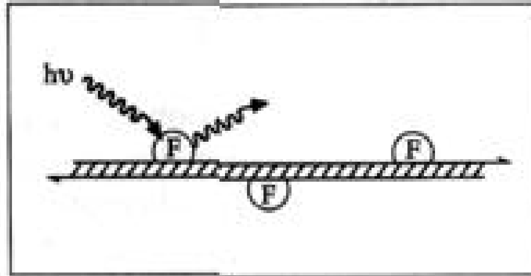
Van der Velden, Leukemia 2003 (www)

A. Increased fluorescence by binding double stranded DNA.

• Minimal fluorescence before amplification

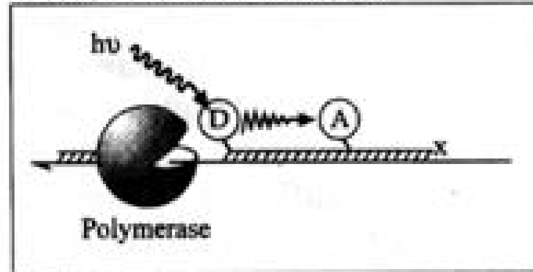


• Increased fluorescence after amplification

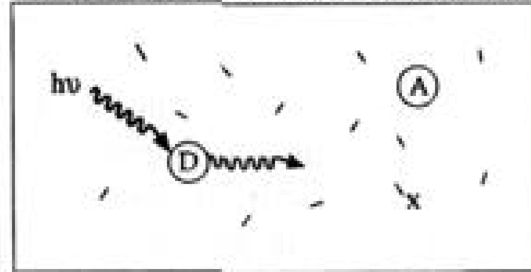


B. Release from quenching by hydrolysis.

• Quenching of donor by acceptor

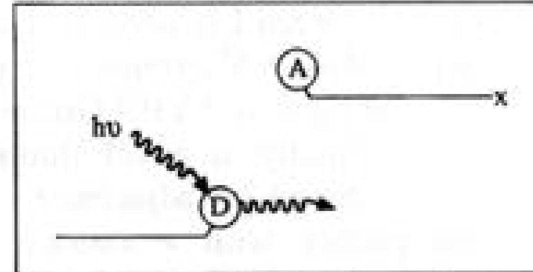


• Increased donor fluorescence after hydrolysis

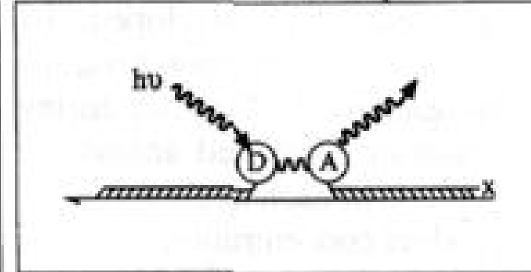


C. Increased resonance energy transfer by hybridization.

•Base line donor fluorescence



•Increased donor transfer to acceptor



Wittwer, 1997 (www)

Schematic diagram comparing three different fluorescence-monitoring systems for DNA amplification. System A uses dsDNA-specific dyes (F) such as SYBR"Green I, which increase in fluorescence when bound to accumulating amplification product. System B uses dual-labelled probes and depends on the 5'-exonuclease activity of the polymerase to separate donor (D) and acceptor (A) by hydrolysis. Donor fluorescence is increased by removing acceptor quenching. System C depends on the independent hybridization of adjacent donor (D) and acceptor (A) probes. Their approximation increases resonance energy transfer from the donor to the acceptor. Other symbols are "hv" for excitation light and "x" for a 3'-phosphate.

Wittwer, 1997 (www)

TaqMan Probes

FRET = Förster/fluorescence resonance energy transfer & DNA Polymerase 5' exonuclease activity

*** Tm value 10^0 C higher than primers**

*** runs of identical nucleotides (no consecutive Gs)**

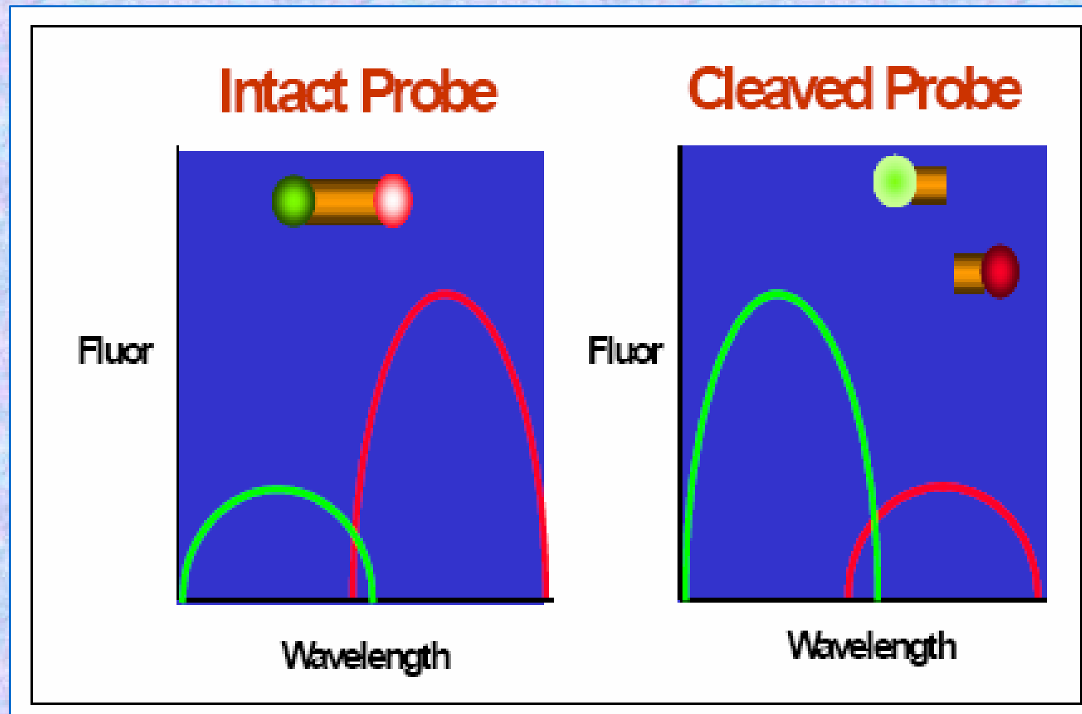
*** G+C content 30-80%**

*** more Cs than Gs**

*** no G at the 5' end**

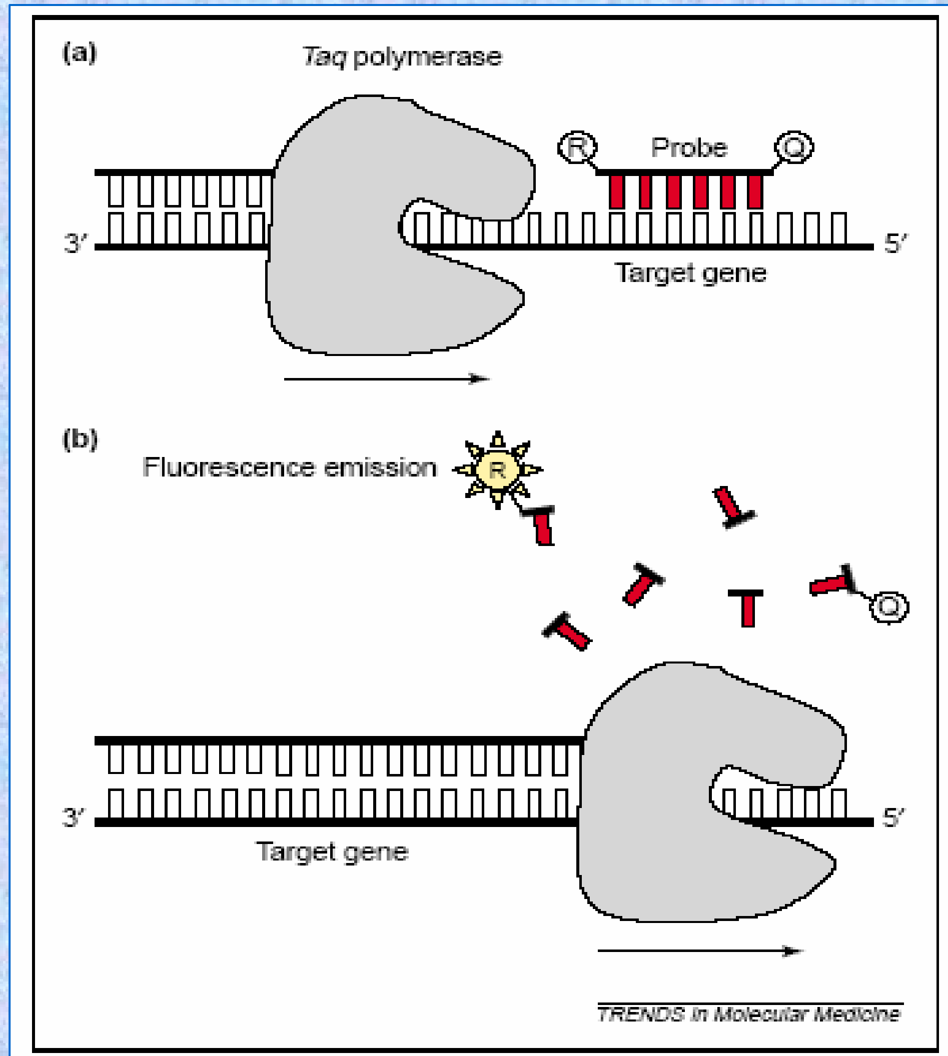
ABI Primer Express Software Tutorial (www)

FRET = Förster/fluorescence resonance energy transfer

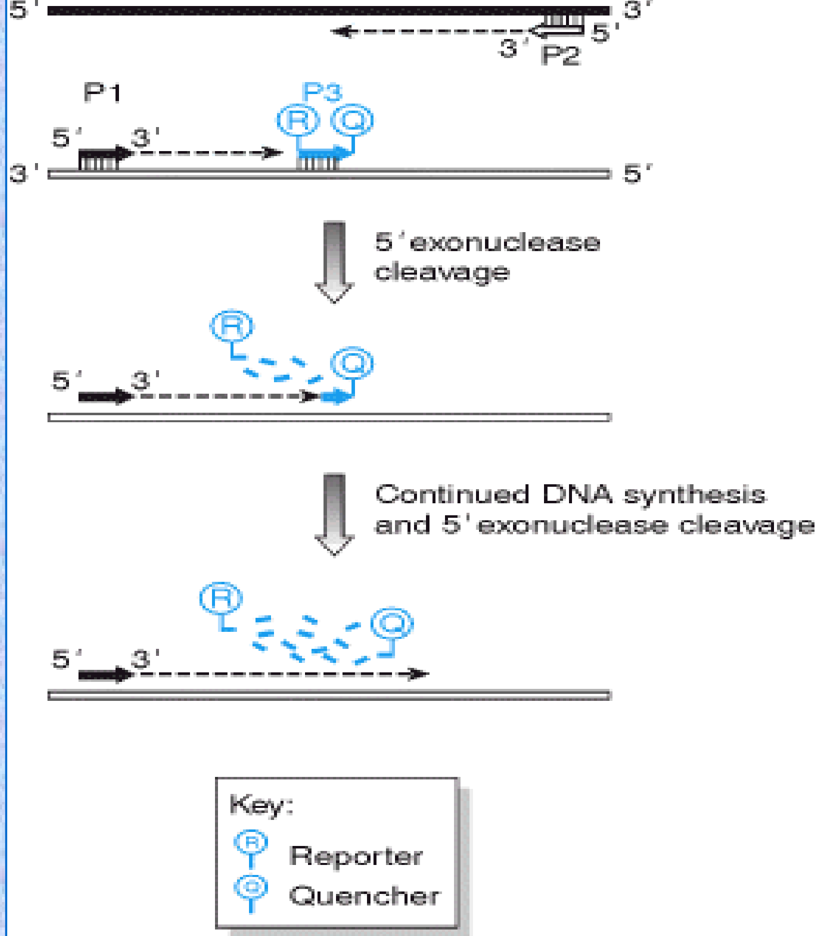


ABI: Real-Time PCR vs Traditional PCR (www)

DNA Polymerase 5' Exonuclease Activity



Mocellin et al. Trends Mol Med 2003 (www)



The TaqMan 5′ Exonuclease Assay

In addition to two conventional PCR primers, P1 and P2, which are specific for the target sequence, a third primer, P3, is designed to bind specifically to a site on the target sequence downstream of the P1 binding site. P3 is labelled with two fluorophores, a reporter dye (R) is attached at the 5 end, and a quencher dye (D), which has a different emission wavelength to the reporter dye, is attached at its 3 end. Because its 3 end is blocked, primer P3 cannot by itself prime any new DNA synthesis. During the PCR reaction, *Taq* DNA polymerase synthesizes a new DNA strand primed by P1 and as the enzyme approaches P3, its 5 3 exonuclease activity processively degrades the P3 primer from its 5 end. The end result is that the nascent DNA strand extends beyond the P3 binding site and the reporter and quencher dyes are no longer bound to the same molecule. As the reporter dye is no longer in close proximity to the quencher, the resulting increase in reporter emission intensity is easily detected.

Human Molecular Genetics 2, NCBI Books (www)

Dye and Quencher Choice

When designing a fluorescent probe, it is necessary to ensure that the fluorophore and quencher pair is compatible, given the type of detection chemistry. In addition, when designing multiplexed reactions the fluorophores and quenchers chosen for the different targets should minimize the spectral overlap between them, to avoid possible crosstalk issues (Table 1).

Filter Set	Ex Wavelength	Em Wavelength
Alexa 350	350	440
FAM/SYBR Green	492	516
TET	517	538
HEX/JOE/VIC	535	555
CY3	545	568
TAMRA	556	580
ROX/Texas Red	585	610
CY5	635	665
FR 640	492	635
FR ROX	492	610
FR CY5	492	665

Table 1

Parameters of the Mx3000P system filter sets. FR 640, FR ROX and FR Cy5 are available only as a custom set.

(www)

TaqMan Primers

* equal Tm (58-60^{0} C)

* 15-30 bases in length

* G+C content 30-80%

* no runs of four or more Gs (any nucleotide)

* no more than two G+C at the 3′ end

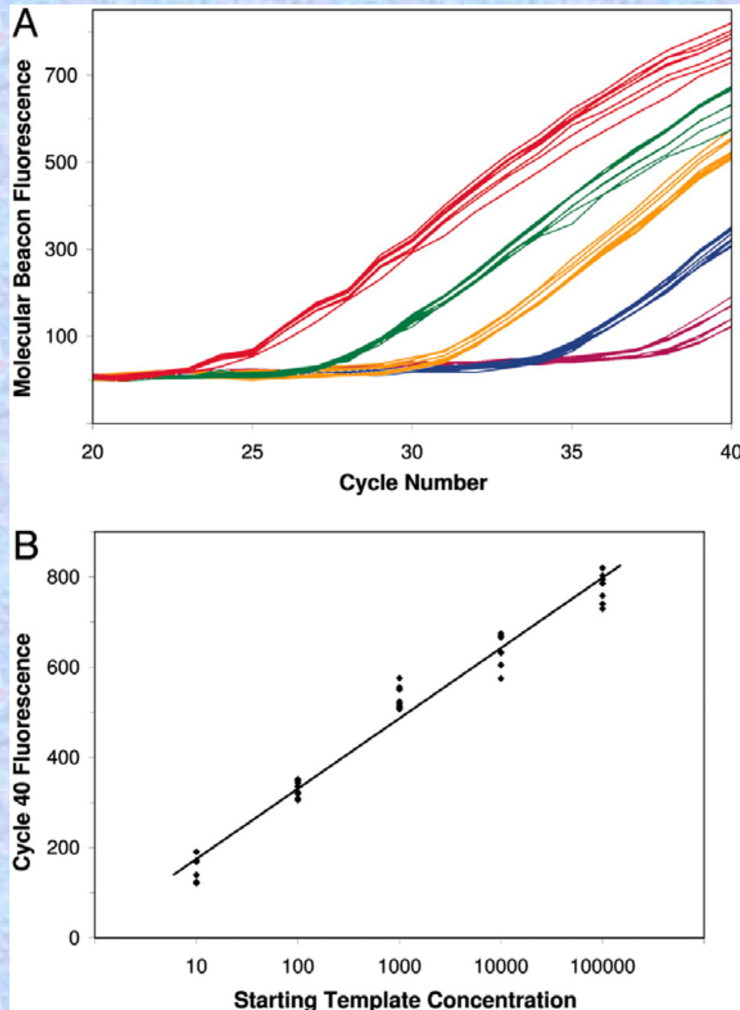
* no G at the 5' end

* amplicon size 50-150 bp (max 400)

* span exon-exon junctions in cDNA

ABI Primer Express Software Tutorial (www)

Linear After The Exponential (LATE) PCR



Detection of CFTR-specific product in samples containing different initial concentrations of DNA. (*A*) Optimized LATE-PCR was carried out by using 100,000 (red), 10,000 (green), 1,000 (orange), 100 (blue), and 10 (purple) copies of human genomic DNA. Curves show molecular beacon fluorescence increase in eight replicate samples at each starting template concentration. (*B*) Plots of initial DNA concentration vs. cycle 40 fluorescence demonstrates the quantitative nature of these endpoint values (R^2 = 0.974) (www)

Pierce, 2005 (www)

Locked Nucleic Acid (LNA) Primers & Probes

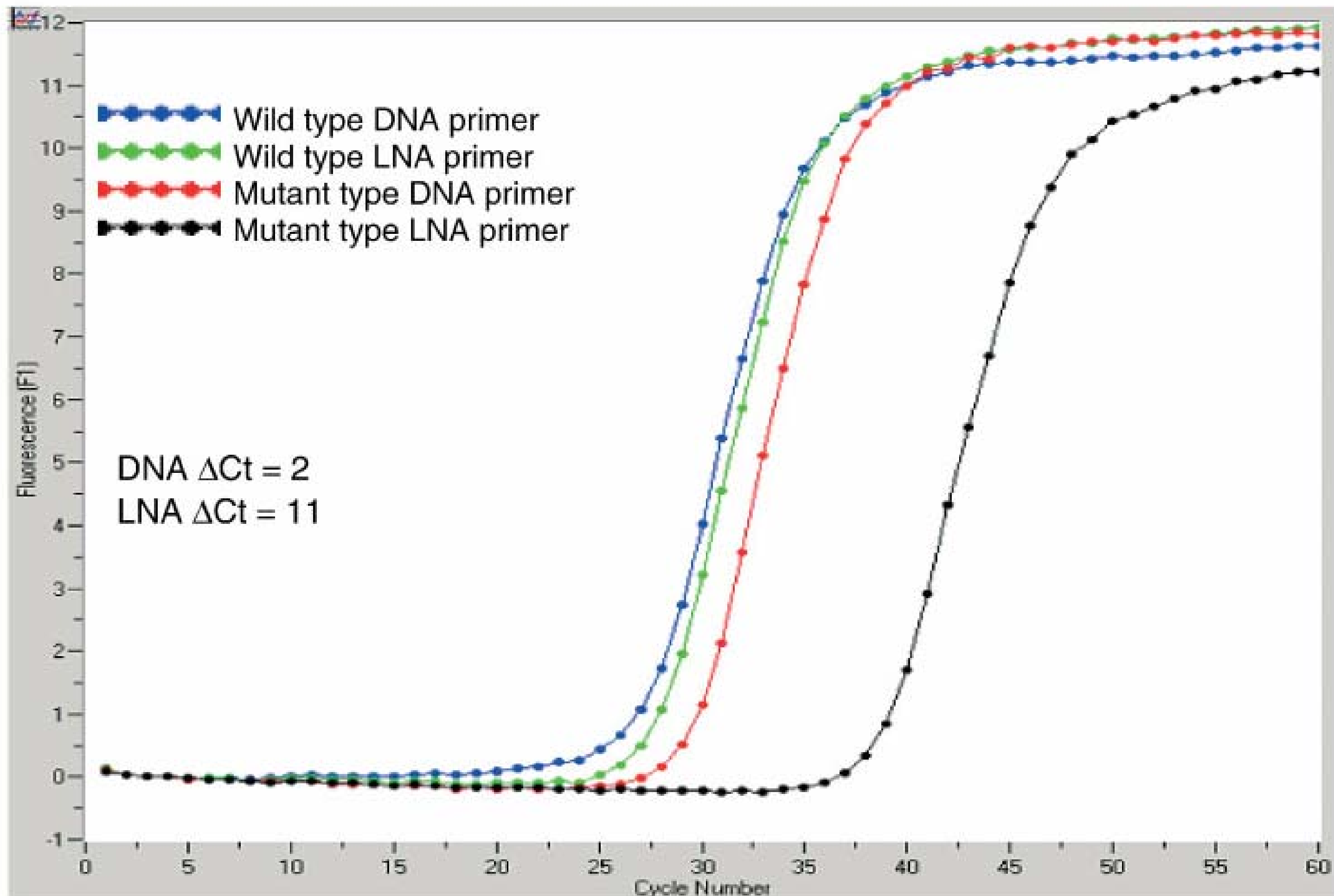


FIGURE 5. Real-time PCR results with CFTR polymorphism 1717−1G>A. SYBR Green fluorescence curves from LightCycler software analysis of cystic fibrosis SNP 1717−1G>A are displayed for wild-type template with DNA and 3′ LNA wild- and mutant-type primers as indicated. PCR and thermal cycling conditions are given in the Materials and Methods section. The difference in cycle threshold value (ΔCt) between matched and mismatched primer is two for the DNA primer and 11 for the LNA primer.

Latorra, 2003 (www)

SYBR Green

(double-stranded DNA binding dye)

* emits a strong fluorescent signal upon binding to double-stranded DNA

* nonspecific binding is a disadvantage

* requires extensive optimization

* requires melting point curve determination

* longer amplicons create a stronger signal

* may be multiplexed when coupled with melting curve analysis

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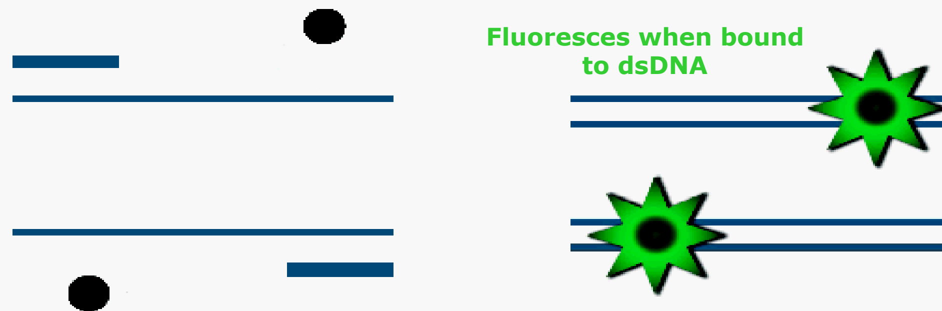
98-075b.pdf

SYBR® Green Assay



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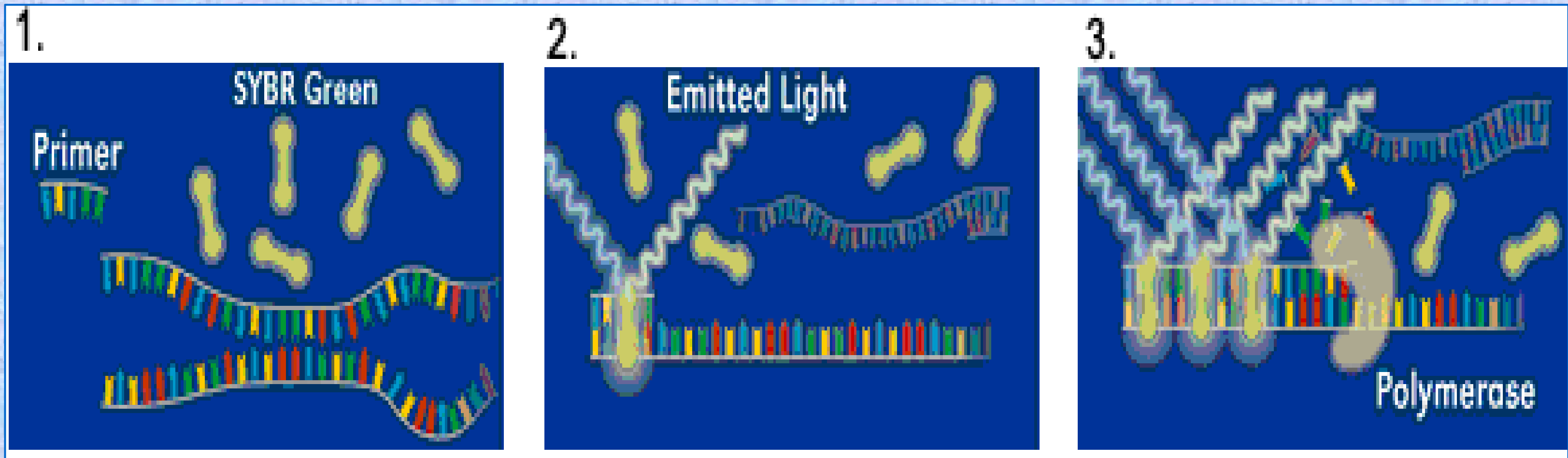

SYBR Green

(1) At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis. (2) After annealing of the primers, a few dye molecules can bind to the double strand. DNA binding results in a dramatic increase of the SYBR Green I molecules to emit light upon excitation. (3) During elongation, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.

Mapping Protein/DNA Interactions by Cross-Linking (NCBI Books) (www)

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

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2. Hybridization probes

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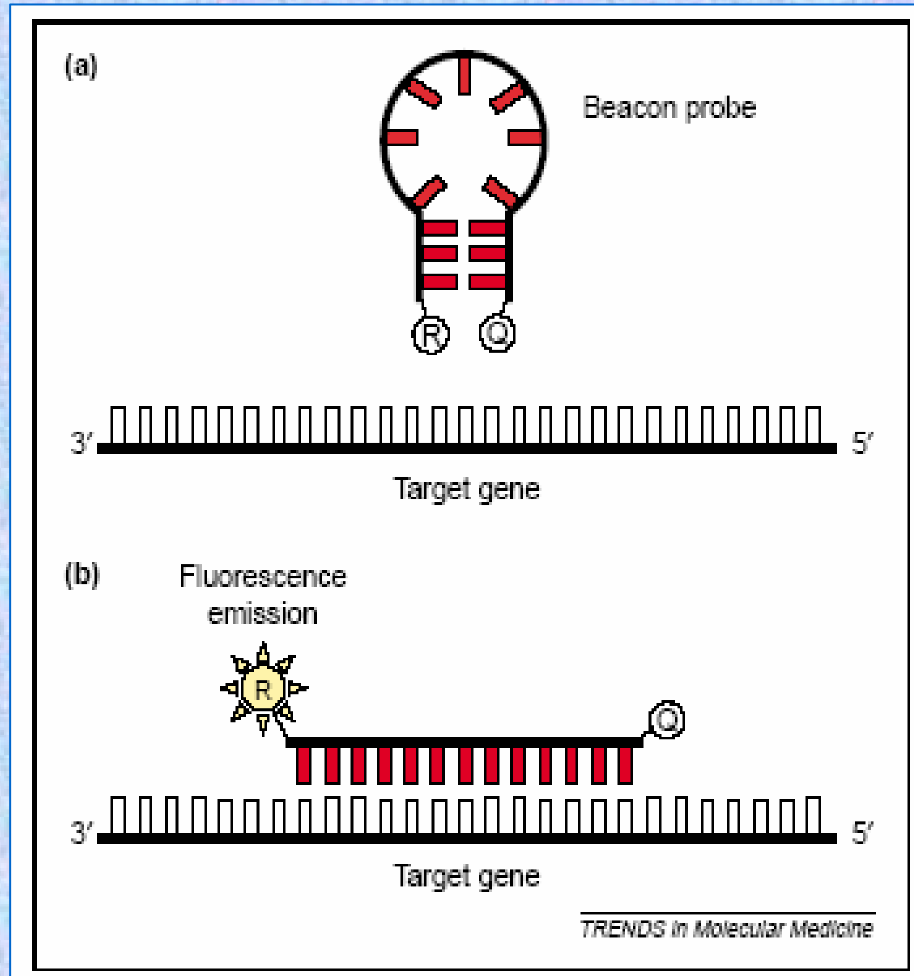
(SYBR Green)

Molecular Beacons



Mocellin et al. Trends Mol Med 2003 (www)

Real-Time Principles

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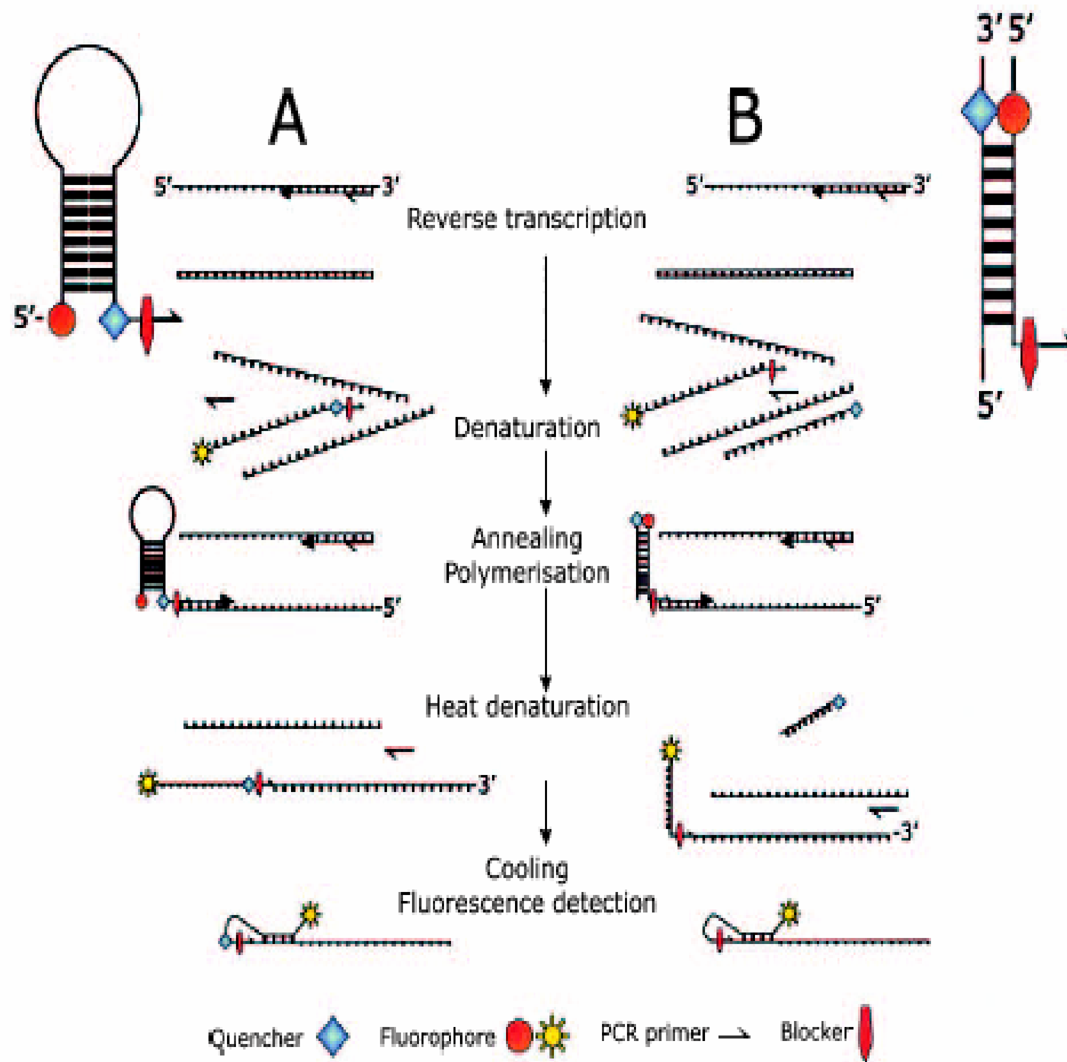
(SYBR Green)

Scorpions



Bustin SA. J Mol Endocrinol 2002 (www)

Scorpions

Figure 2. Real-time RT-PCR with Scorpions. A standard RT step is followed by denaturation of the double-stranded template. (A) In the hairpin loop structure, the fluorophore attached at the 5′ end forms a non-fluorescent complex with the quencher at the 3′ end. The hairpin loop is linked to the 5′ end of a specific primer through a PCR stopper that prevents read-through of the hairpin loop. During PCR, the Scorpion primers are extended to become part of the amplicon. During the annealing/extension phase of the PCR reaction, the probe sequence in the Scorpion hybridises to the newly formed complementary target sequence in the PCR product separating the fluorophore from the quencher and resulting in a fluorescent signal. As the tail of the Scorpion and the PCR product are now part of the same strand of DNA, the interaction is intramolecular. The target sequence is typically chosen to be within three bases of the 3′ end of the Scorpion primer. (B) Separation of fluorophore and quencher onto different oligonucleotides improves signal intensity. The quencher oligonucleotide has the quencher at its 3′ end and is complementary to the probe sequence. Following denaturation and polymerisation, intramolecular interaction of probe and newly synthesised product is more favourable than intermolecular binding between quencher oligonucleotide and the probe.

Bustin SA. J Mol Endocrinol 2002 (www)

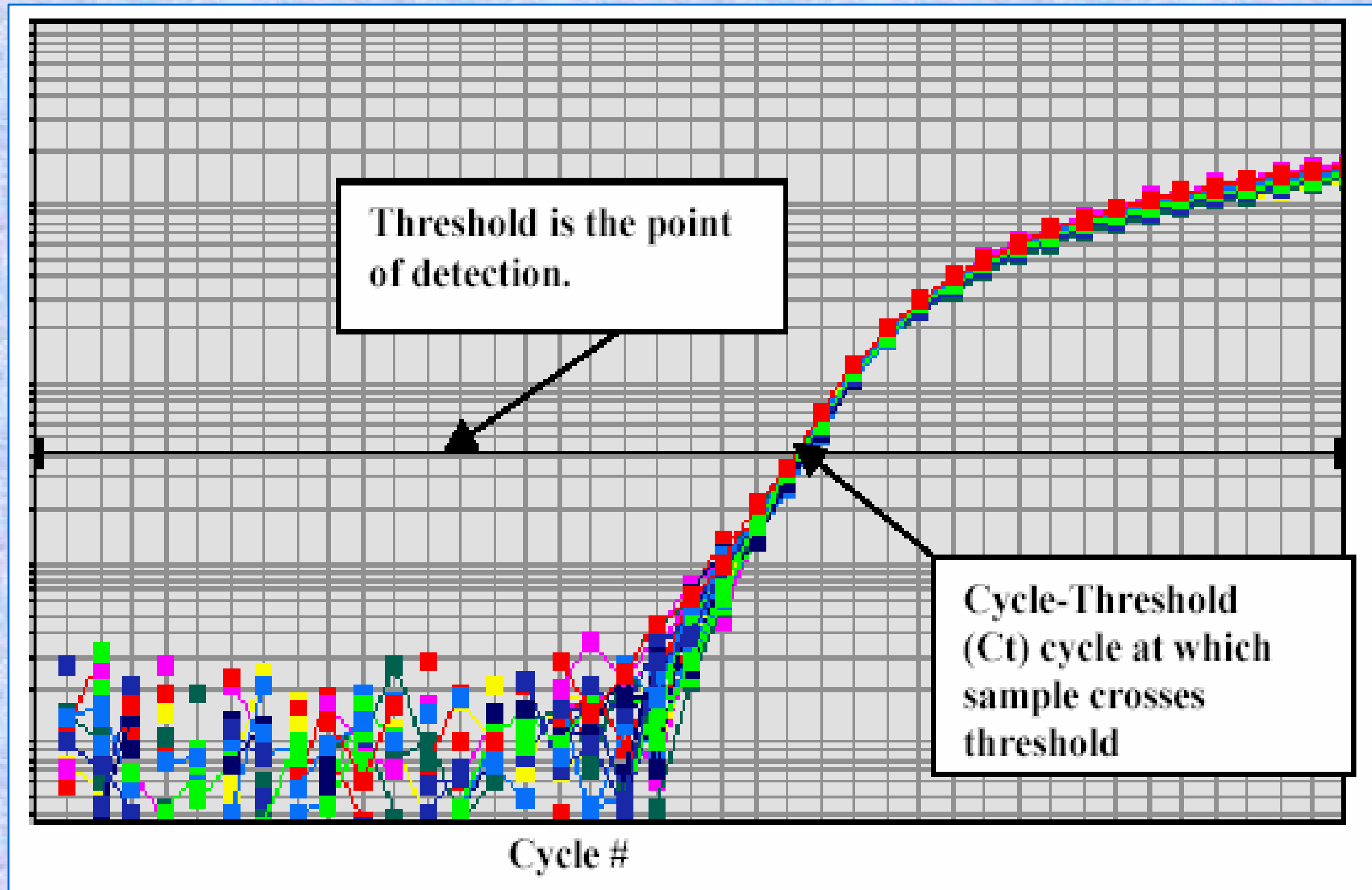
Threshold Cycle

* 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

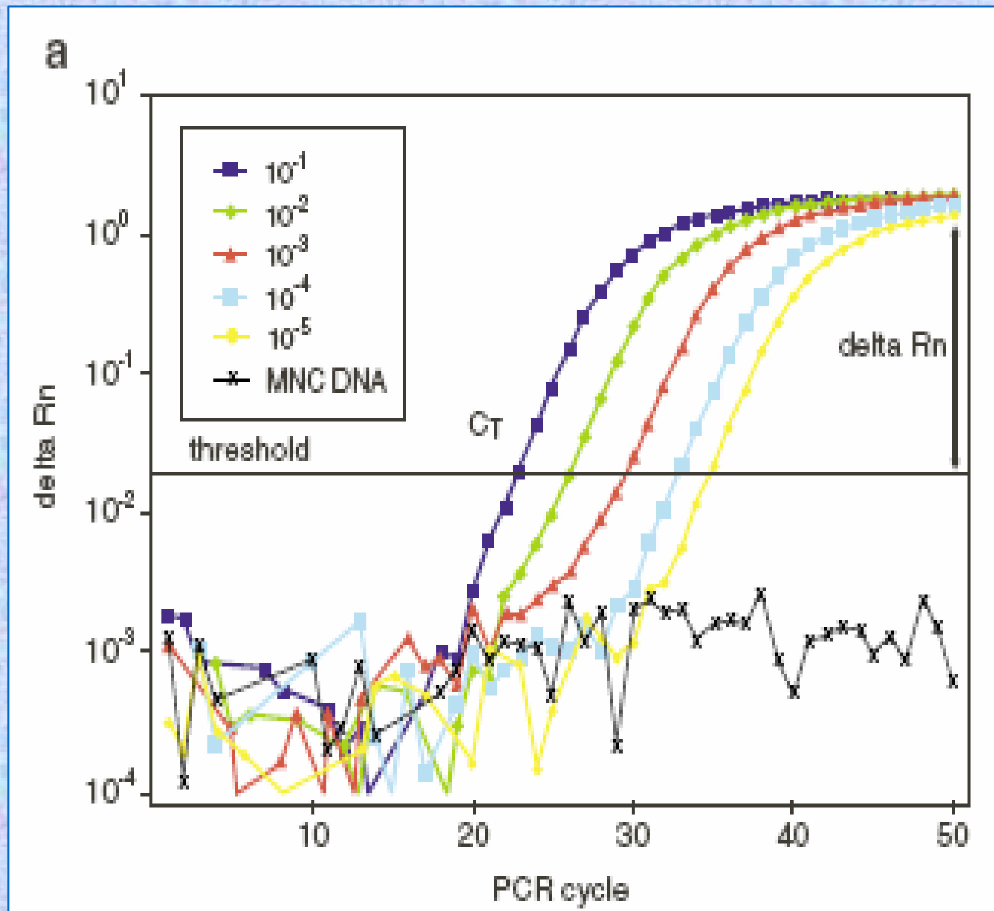
* C_T value of 40 or more means no amplification and cannot be included in the calculations

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

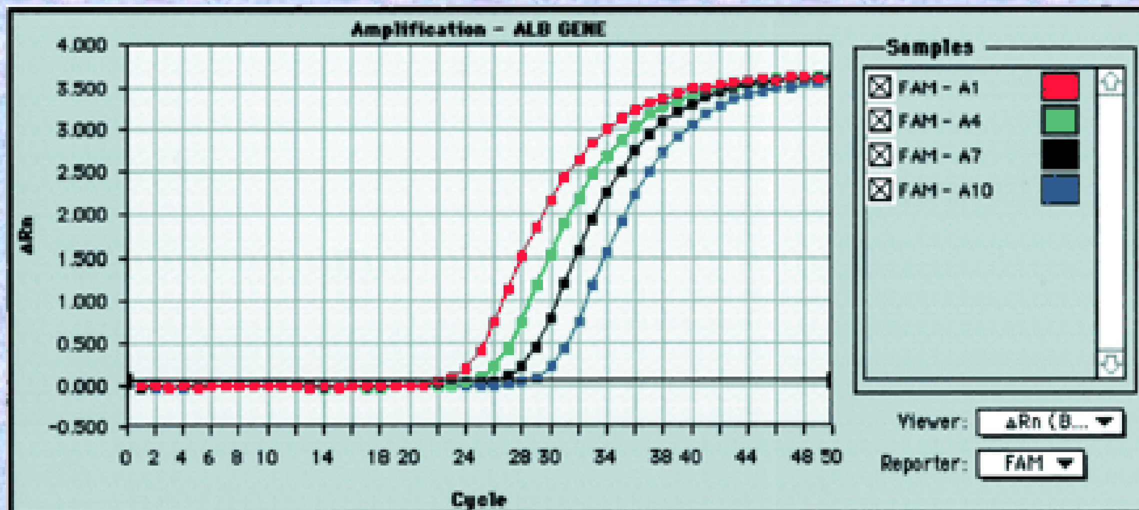


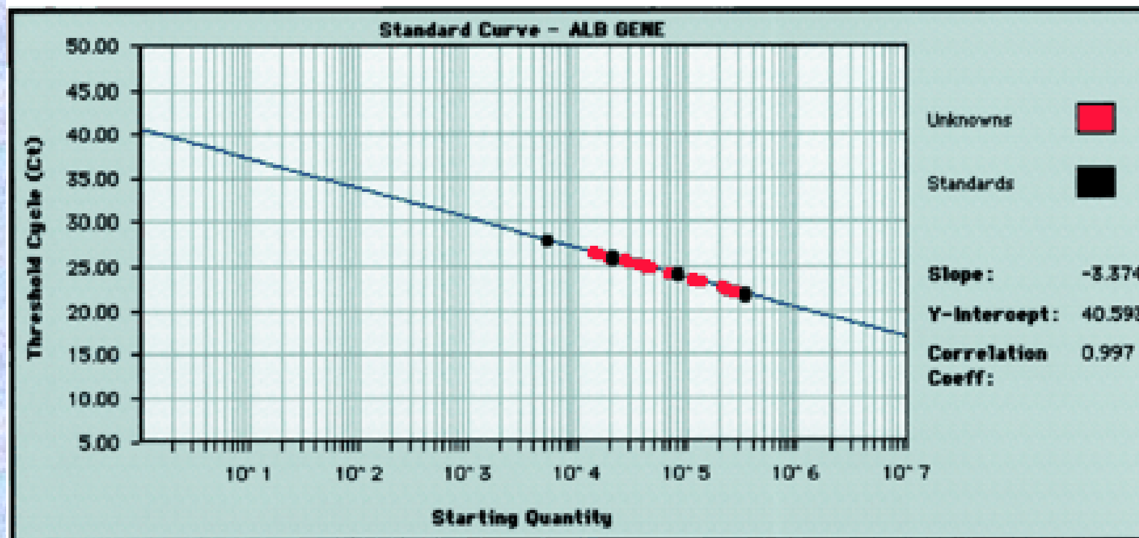
Van der Velden. Leukemia 2003 (www)

- 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 a 1x standard
  - Best used when particular ratios are expected or to verify trends

Ambion
THE RNA COMPANY®

(www)





Albumin (*ALB*) gene dosage by real-time PCR

Laurendeau et al. Clin Chem 1999 (www)

Albumin (*ALB*) Gene Dosage by Real-Time PCR

Top, amplification plots for reactions with starting ***ALB*** gene copy number of 33 000 (***A1***, 100 ng), 8250 (***A4***, 25 ng), 2062 (***A7***, 6.25 ng), or 515 (***A10***, 1.56 ng). The cycle number is plotted vs the change in normalized reporter signal (***Rn***). For each reaction tube, the fluorescence signal of the reporter dye (FAM) is divided by the fluorescence signal of the passive reference dye (ROX) to obtain a ratio defined as the normalized reporter signal (Rn). ***Rn*** represents the normalized reporter signal (Rn) minus the baseline signal established in the first 15 PCR cycles. ***Rn*** increases during PCR as ***ALB*** PCR product copy number increases until the reaction reaches a plateau. C_t represents the fractional cycle number at which a significant increase in Rn above a baseline signal (***horizontal black line***) can first be detected. Three replicates were performed for each reference DNA sample, but the data for only one are shown here. ***Bottom***, calibration curve plotting log starting copy number vs C_t. The ***black symbols*** represent the triplicate PCR amplification of the reference DNA samples and ***red symbols*** the triplicate PCR amplification of unknown genomic DNA, all included inside the calibration curve. The copy number of ***ALB*** (x) can be calculated as follows: $y = -3.374x + 40.593$, where the C_t value is substituted as y.

Laurendeau et al. Clin Chem 1999 (www)

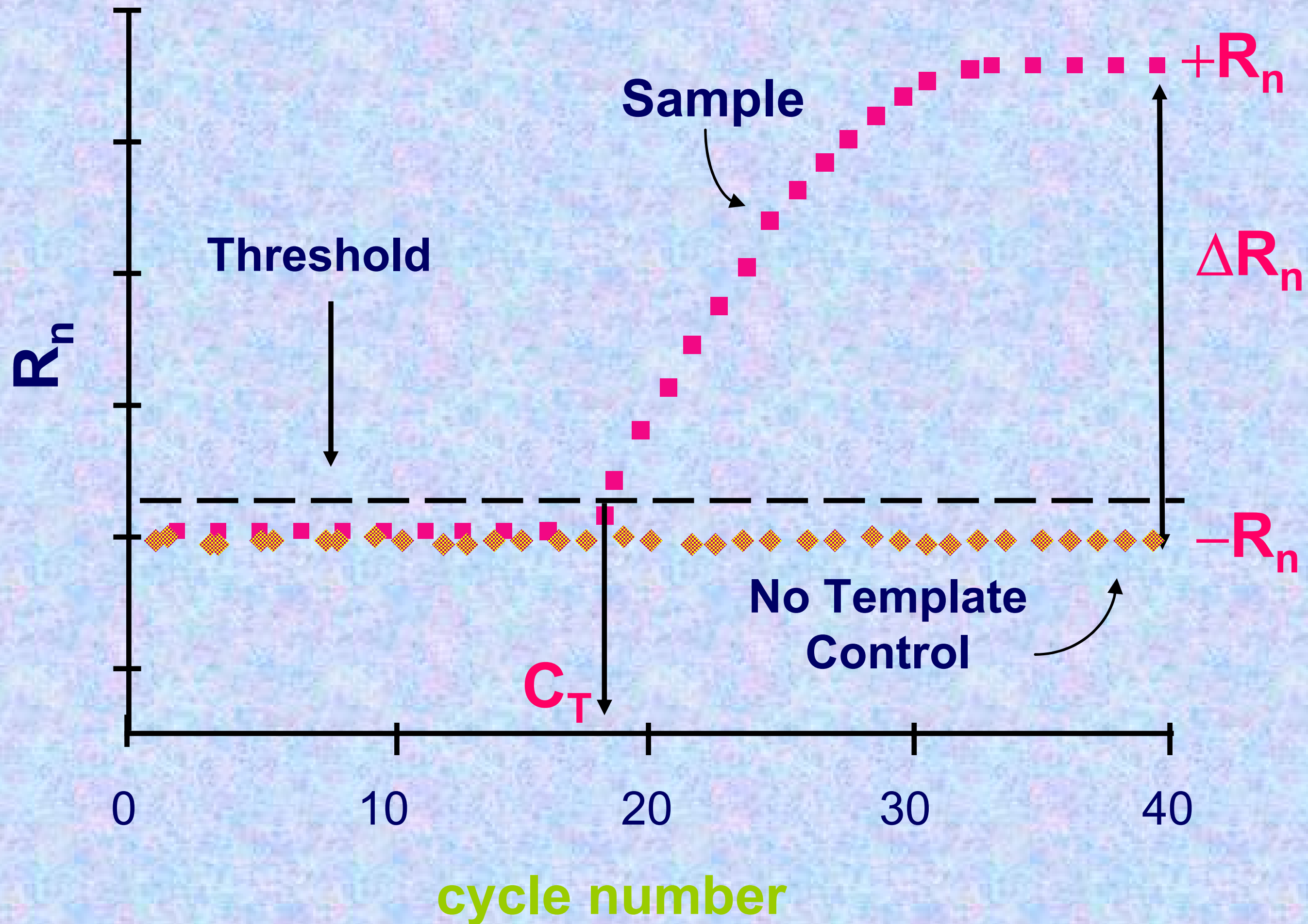
ΔRn

* 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)

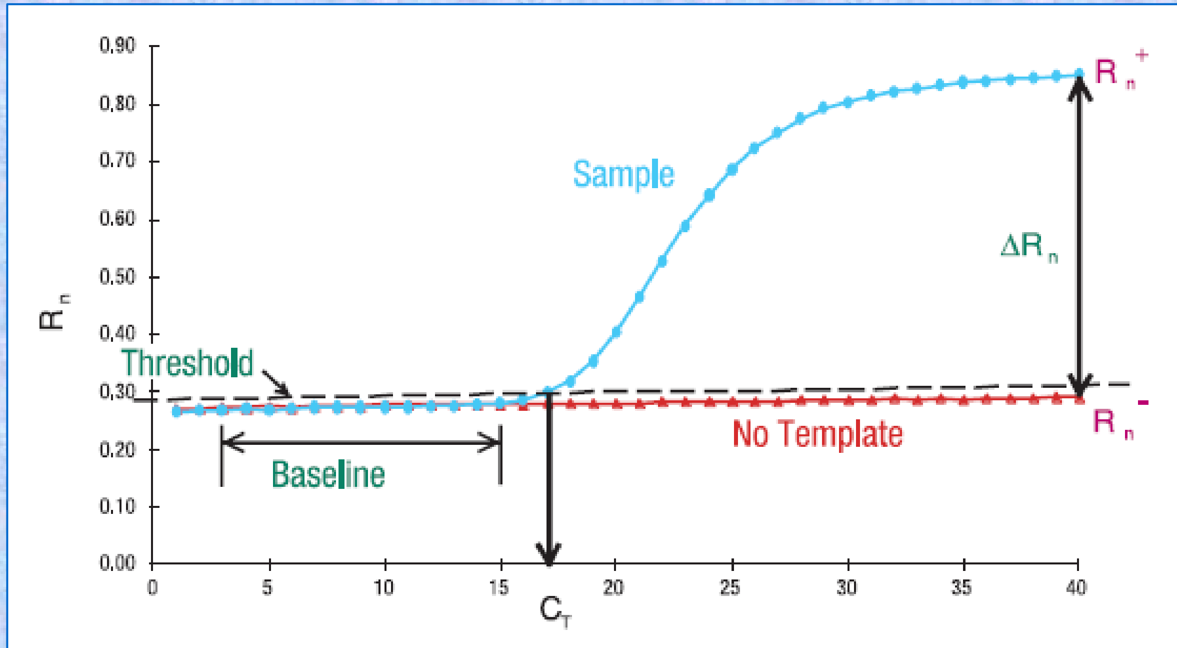
* ΔRn is the difference between Rn^+ and Rn^-. It is an indicator of the magnitude of the signal generated by the PCR

* ΔRn is plotted against cycle numbers to produce the amplification curves and gives the C_T value

What is ∆Rn?

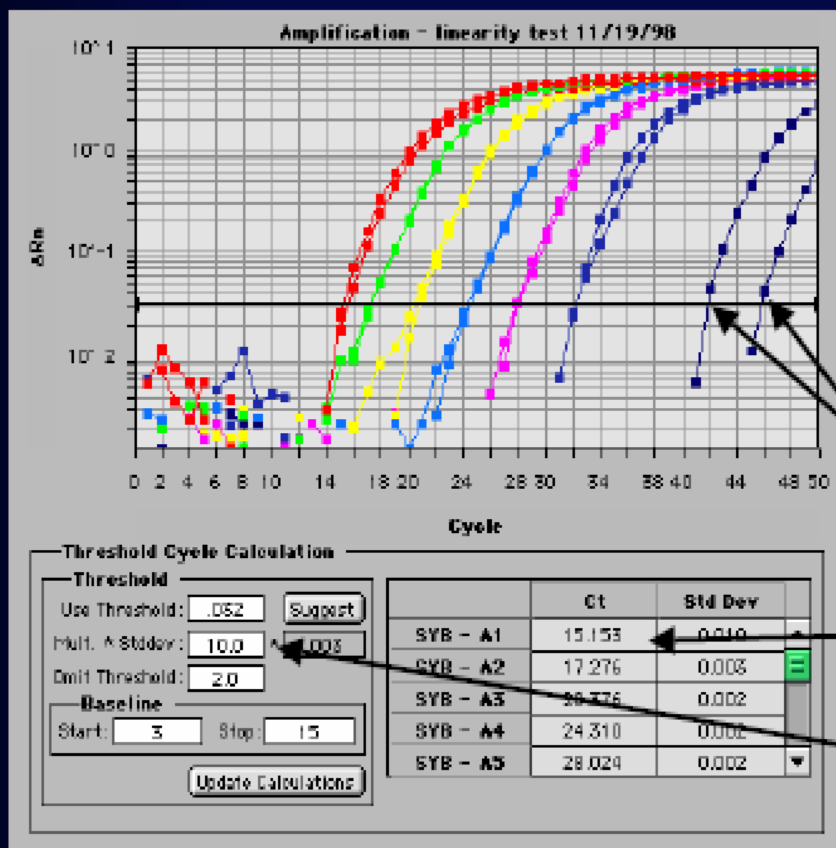


What is ΔRn?



(www)

Example analysis of CYP1A1



- SYBR Green detection
- 10-fold dilution series

No RNA controls

C_T values

Threshold

Nigel Walker, NIEHS (www)

Endogenous/Internal Control (Normalization)

* usually an abundantly and constantly expressed housekeeping gene

* most commonly used ones are the least reliable ones

* best to run a validity test for the selected endogenous control

* combination may/should be used

Endogenous Control Selection

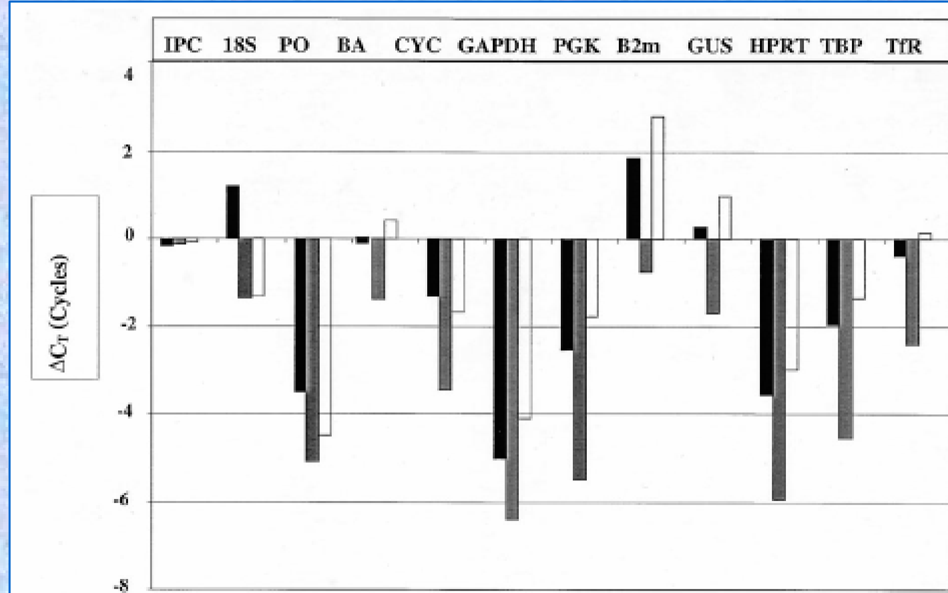


FIGURE 1. Variation in housekeeping gene expression in healthy individuals. The calibrator served as a baseline for the assay and is shown as zero on the graph. Samples with values above zero indicate lower levels of target gene expression, whereas those below zero indicate a higher level of expression of the specific gene compared with the calibrator. Genes that show little variation from the calibrator (zero line) in multiple sample analysis were chosen for use as reliable internal housekeeping control genes.

Sabek et al. Transplantation 2002 (www)

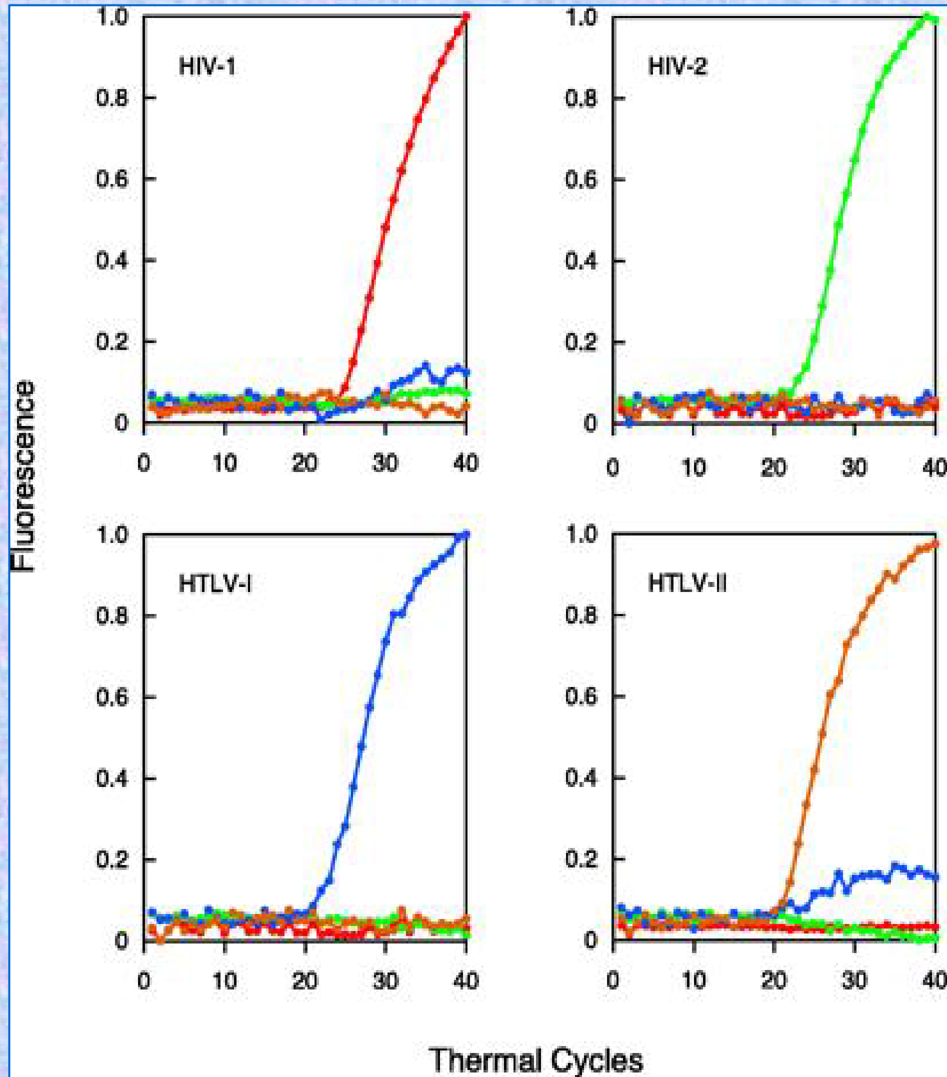
Multiplexing

* TaqMan: different dyes for each target (FAM, TET, VIC and JOE)

* SYBR green: different melting points for each target

* extensive optimization is required

Multiplex Real-Time PCR
(fluorescein-labeled molecular beacon)



Real-time detection of four different retroviral DNAs in a multiplex format. Four assays were carried out in sealed tubes, each initiated with 100,000 molecules of a different retroviral DNA. Each reaction contained four sets of PCR primers specific for unique HIV-1, HIV-2, HTLV-I, and HTLV-II nucleotide sequences and four molecular beacons, each specific for one of the four amplicons and labelled with a differently coloured fluorophore. Fluorescence from the fluorescein-labeled molecular beacon (HIV-1-specific) is plotted in red, fluorescence from the tetrachlorofluorescein-labelled molecular beacon (HIV-2-specific) is plotted in green, fluorescence from the tetramethylrhodamine-labelled molecular beacon (HTLV-I-specific) is plotted in blue, and fluorescence from the rhodamine-labelled molecular beacon (HTLV-II-specific) is plotted in brown. The slight HTLV-I signal seen in the assay initiated with HTLV-II DNA is an artefact that resulted from a portion of the rhodamine fluorescence being interpreted by the spectrofluorometric thermal cycler as tetramethylrhodamine fluorescence. Vet JA et al. PNAS 1999 (www)

Multiplex Real-Time PCR
(fluorescein-labeled molecular beacon)

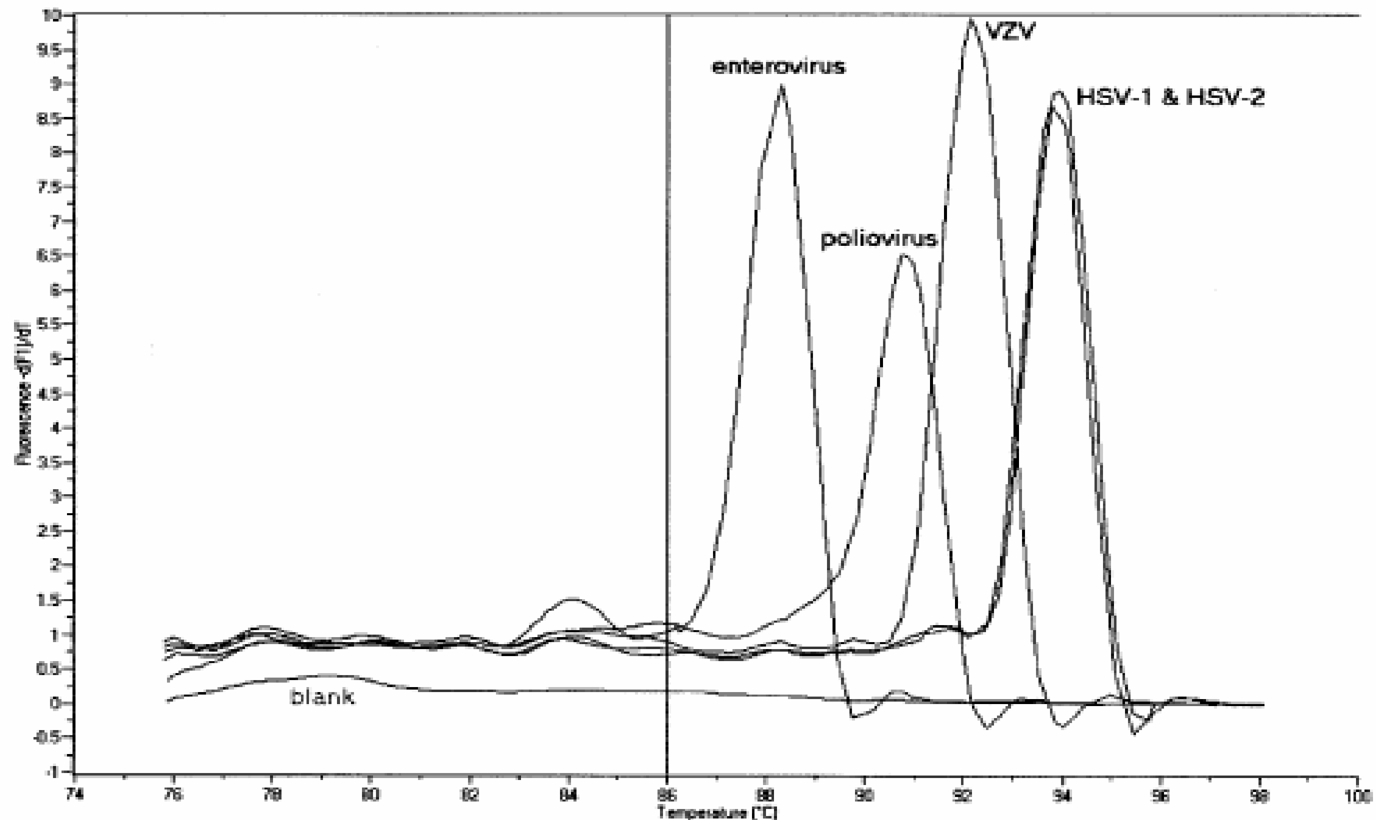


FIG. 1. LightCycler system melting curve analysis of ATCC isolates amplified by multiplex PCR. Enterovirus is echovirus type 1, ATCC VR-31, T_m of 88.20°C; poliovirus is poliovirus type 2 (attenuated), ATCC VR-301, T_m of 90.79°C; VZV is ATCC VR-586, T_m of 92.17°C; HSV-1 is ATCC VR-260, T_m of 93.83°C; and HSV-2 is ATCC VR-734, T_m of 93.91°C. The line designated "blank" represents the analysis of a PCR mixture without the addition of a target. The vertical line at 86°C represents the incubation temperature at which the levels of fluorescent signals were measured during each cycle of PCR. The y axis is the negative differential of fluorescence over temperature ($-dF/dT$).

Read SJ et al. J Clin Microbiol 2001 (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 90-100% (ideal slope = -3.3)

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)

Calculation

The slope of the standard curve can be used to determine the exponential amplification and efficiency of the PCR reaction by the following equations:

$$\text{Exponential Amplification} = 10^{(-1/\text{slope})}$$
$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

The following table shows the amplification and efficiency for various values of the slope:

Slope	Amplification	Efficiency
-3.60	1.8957	0.8957
-3.55	1.9129	0.9129
-3.50	1.9307	0.9307
-3.45	1.9492	0.9492
-3.40	1.9684	0.9684
-3.35	1.9884	0.9884
-3.30	**2.0092**	**1.0092**
-3.25	2.0309	1.0309
-3.20	2.0535	1.0535
-3.15	2.0771	1.0771
-3.10	2.1017	1.1017

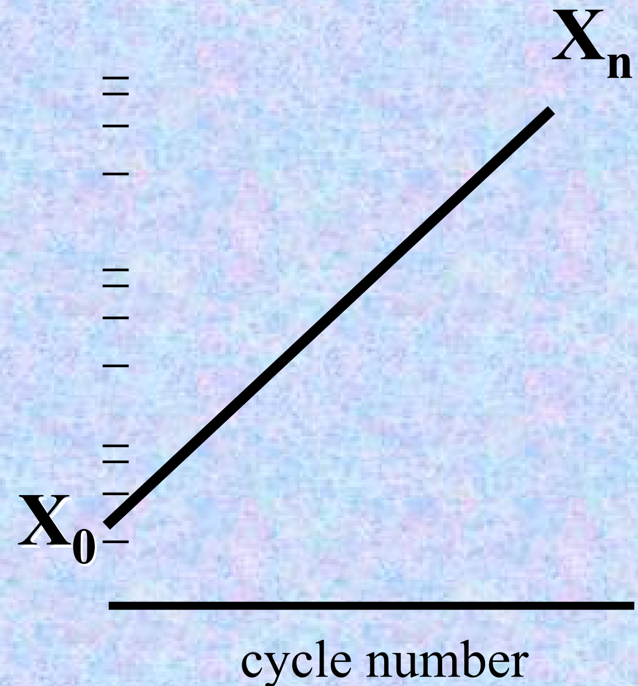
As the table illustrates, optimal PCR efficiency is indicated by a slope of –3.3.

Stratagene Application Notes #10 (www)

Using the PCR Equation

$$X_n = X_0(1 + E)^n$$

X_n = PCR product after cycle n
X_0 = initial copy number
E = amplification efficiency
n = cycle number



Effect of Amplification Efficiency

$X_n = X_0(1+E)^n$

Case 1: E = 0.9

$X_n = 100\ (1+0.9)^{30}$

$X_n = 2.3 \times 10^{10}$

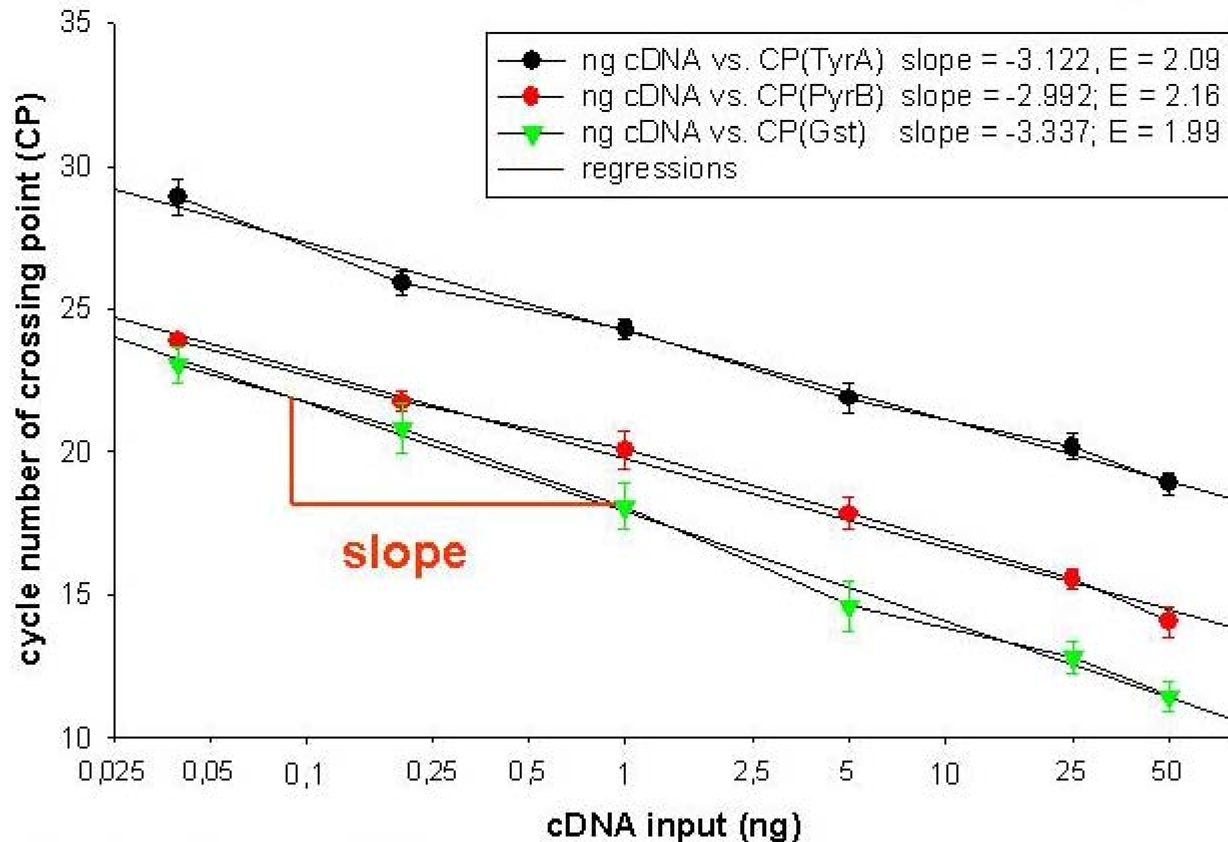
Case 2: E = 0.8

$X_n = 100\ (1+0.8)^{30}$

$X_n = 4.6 \times 10^{9}$

Result

A difference of 0.1 in amplification efficiencies created a five-fold difference in the final ratio of PCR products after 30 cycles



Determination of real-time PCR efficiencies of reference gene (Gst), target gene 1 (TyrA) and target gene 2 (PyrB). CP cycles versus cDNA (reverse transcribed total RNA) concentration input were plotted to calculate the slope (mean ± SD; *n* = 3). The corresponding real-time PCR efficiencies were calculated according to the equation: $E = 10^{[-1/slope]}$

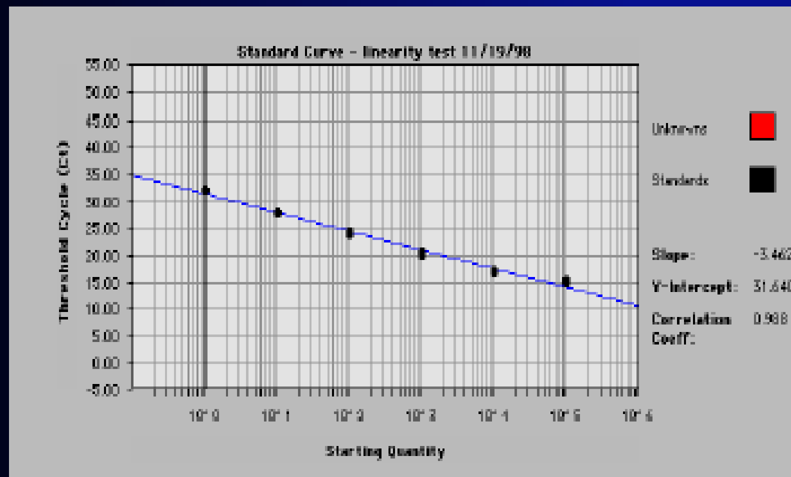
From: Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 2001 (www)

If the C_T values for each of the dilutions are plotted against concentrations, the result should be a linear graph with a high correlation coefficient (> 0.99). The slope of this graph is also a measure of efficiency, and can be readily used to calculate efficiency - this is done by most software (iCycler, for example).

Real-Time PCR Tutorial (University of South Carolina) (www)

Calculation of Efficiency

- Based on a linear plot of C_T vs. log copies:
- Efficiency(e%) = $10^{(-1/slope)}$
- 100% efficiency (2 copies each cycle) slope of –3.3219.



Slope = -3.462

e = $10^{(-1/3.462)}$ = 1.95

1.95 copies per cycle

ΔCt = 3.3

Fold= $(1.95)^{3.31}$ = 9.1 fold

Nigel Walker, NIEHS (www)

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

Assay Validation

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

* Use standard assay conditions: 300-400 nM primers; 100 nM probe, 3 mM $MgCl_2$

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

* Make a dilution of a template, either sDNA, sRNA or total RNA for a standard curve

* Correlation coefficient of the standard curve > 0.99?

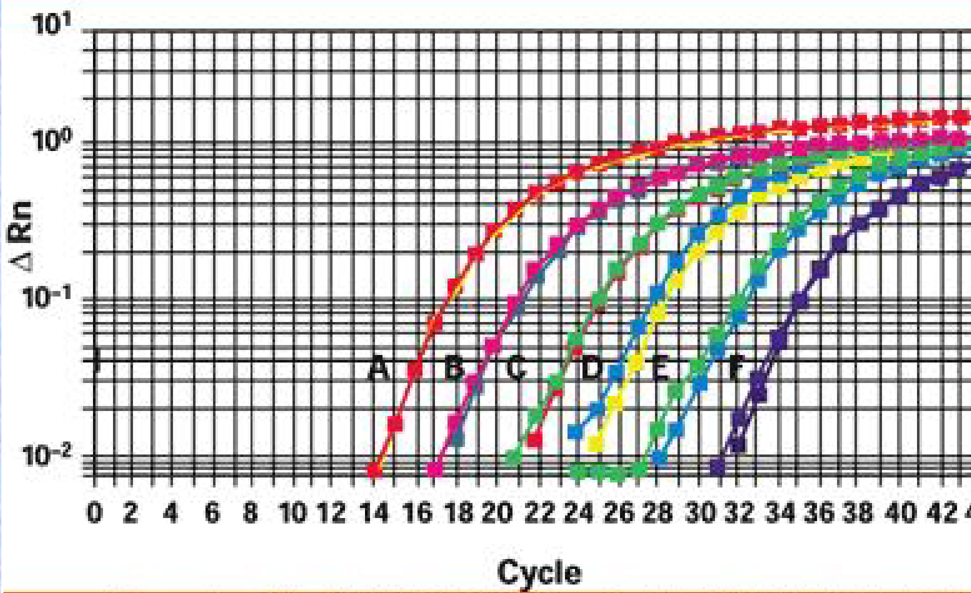
* If the slope of the standard curve of the best primer pair is around -3.5 increase the $MgCl_2$ to 5 mM

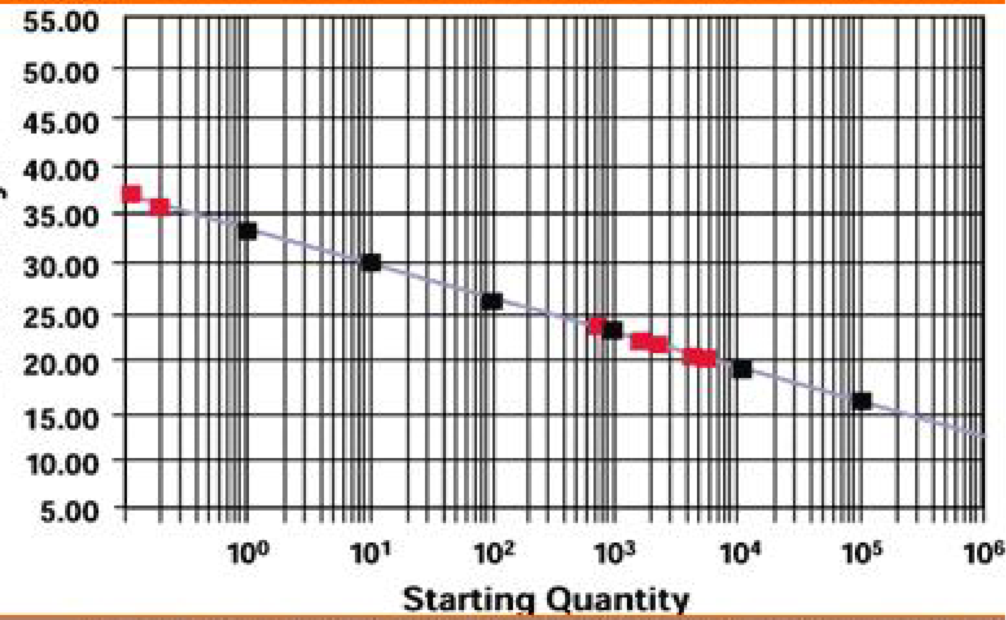
* If the slope is higher than -3.6, change primers

* An ideal assay will have a slope of -3.3

Validation of bcr-abl p210 real-time PCR

A, Amplification, bcr032801. Standards were as follows: A, 10^5; B, 10^4; C, 10^3; D, 10^2; E, 10^1; and F, 10^0. ΔRn, change in fluorescence. B, Standard curve, bcr032801. Slope, -3.499; Y-intercept, 33.670; correlation coefficient, 0.998. Red, unknown; black, standards.





Jones et al, Am J Clin Pathol 2003 (www)

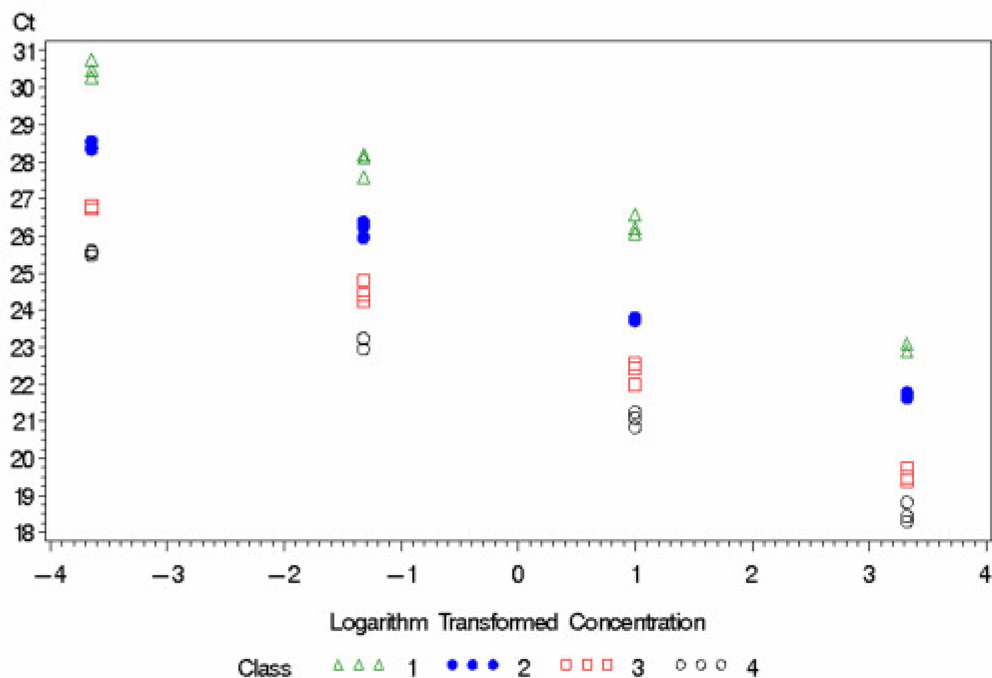


Figure 2

Data quality control. The four classes represent four different combinations of sample and gene, which are reference gene in control sample, target gene in control sample, reference gene in treatment sample, and target gene in treatment sample. Each class should derive a linear correlation between Ct and logarithm transformed concentration pf PCR product with a slope of -1.

Yuan, 2006 (www)

I. Assay Development

- A. Sequence selection
- B. Primer & probe selection
- C. Quencher dye and internal reference
- D. Assay validation

II. Assay Setup

- A. One- or two-Step PCR
- B. Thermocycler settings

III. Data Analysis

- A. Baseline and threshold settings
- B. Standard Curves
- C. Inter- vs intra-assay variability
- D. Sample normalization

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One-Step or Two-Step PCR

* one-step real-time RT-PCR performs reverse transcription and PCR in a single buffer system and in one tube

* in two-step RT-PCR, these two steps are performed separately in different tubes

I. Assay Development

- **A. Sequence selection**
- **B. Primer & probe selection**
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II. Assay Setup

- **A. One- or two-Step PCR**
- **B. Thermocycler settings**

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Reporter, Quencher and Internal Reference Dyes

* The classical reporter dye is 6-FAM (fluorescein)

* Other reporters used for multiplexing are Joe and Vic.

* Some other real-time machines, such as the Stratagene Mx4000, can use red dyes as reporters

* The classic quencher dye has been TAMRA (rhodomine)

* Newer quenchers are the dark dyes, DABYCL and the black hole quenchers (Biosearch Technologies)

* TAMRA-quenched probes do not require a reference dye; they can use the TAMRA itself

* Single probe reactions quenched by dark dyes should use an internal reference dye, classically ROX (dark red)

* Multiplex reactions usually use dark quenchers and ROX

Sample Layout

20 unknowns in triplicate, standard curve, NACs and NTC

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND Std #1 2.0e+07	STND Std #1 2.0e+07	STND Std #2 2.0e+06	STND Std #2 2.0e+06	STND Std #2 2.0e+06	STND Std #3 2.0e+05	STND Std #3 2.0e+05	STND Std #3 2.0e+05	STND Std #4 2.0e+04	STND Std #4 2.0e+04	STND Std #4 2.0e+04	NTC A12
B	STND Std #5 2.0e+03	STND Std #5 2.0e+03	STND Std #5 2.0e+03	NTC B4	UNKN Smpl #7 2.6e+05	UNKN Smpl #7 2.1e+05	UNKN Smpl #7 1.8e+05	NAC Smpl #7	UNKN Smpl #14 2.2e+05	UNKN Smpl #14 2.3e+05	UNKN Smpl #14 1.8e+05	NAC Smpl #14
C	UNKN Smpl #1 2.7e+05	UNKN Smpl #1 2.1e+05	UNKN Smpl #1 1.9e+05	NAC Smpl #1	UNKN Smpl #8 2.7e+05	UNKN Smpl #8 2.0e+05	UNKN Smpl #8 2.3e+05	NAC Smpl #8	UNKN Smpl #15 2.1e+05	UNKN Smpl #15 2.2e+05	UNKN Smpl #15 1.7e+05	NAC Smpl #15
D	UNKN Smpl #2 2.3e+05	UNKN Smpl #2 2.1e+05	UNKN Smpl #2 1.9e+05	NAC Smpl #2	UNKN Smpl #9 2.2e+05	UNKN Smpl #9 2.3e+05	UNKN Smpl #9 2.4e+05	NAC Smpl #9	UNKN Smpl #16 2.3e+05	UNKN Smpl #16 2.2e+05	UNKN Smpl #16 2.1e+05	NAC Smpl #16
E	UNKN Smpl #3 2.9e+05	UNKN Smpl #3 2.2e+05	UNKN Smpl #3 2.4e+05	NAC Smpl #3	UNKN Smpl #10 2.6e+05	UNKN Smpl #10 2.8e+05	UNKN Smpl #10 1.8e+05	NAC Smpl #10	UNKN Smpl #17 2.5e+05	UNKN Smpl #17 2.1e+05	UNKN Smpl #17 2.1e+05	NAC Smpl #17
F	UNKN Smpl #4 2.2e+05	UNKN Smpl #4 2.3e+05	UNKN Smpl #4 2.4e+05	NAC Smpl #4	UNKN Smpl #11 2.2e+05	UNKN Smpl #11 1.5e+05	UNKN Smpl #11 2.8e+05	NAC Smpl #11	UNKN Smpl #18 2.2e+05	UNKN Smpl #18 2.3e+05	UNKN Smpl #18 2.1e+05	NAC Smpl #18
G	UNKN Smpl #5 3.0e+05	UNKN Smpl #5 2.5e+05	UNKN Smpl #5 2.7e+05	NAC Smpl #5	UNKN Smpl #12 2.5e+05	UNKN Smpl #12 2.1e+05	UNKN Smpl #12 2.9e+05	NAC Smpl #12	UNKN Smpl #19 2.8e+05	UNKN Smpl #19 2.0e+05	UNKN Smpl #19 2.5e+05	NAC Smpl #19
H	UNKN Smpl #6 3.2e+05	UNKN Smpl #6 2.7e+05	UNKN Smpl #6 2.0e+05	NAC Smpl #6	UNKN Smpl #13 2.9e+05	UNKN Smpl #13 2.3e+05	UNKN Smpl #13 2.2e+05	NAC Smpl #13	UNKN Smpl #20 2.3e+05	UNKN Smpl #20 2.3e+05	UNKN Smpl #20 2.7e+05	NAC Smpl #20

Interpretation

* Melting curve analysis

* Absolute quantification

* Relative quantification

    i. Relative standard method (relative fold change)

    ii. Comparative threshold method

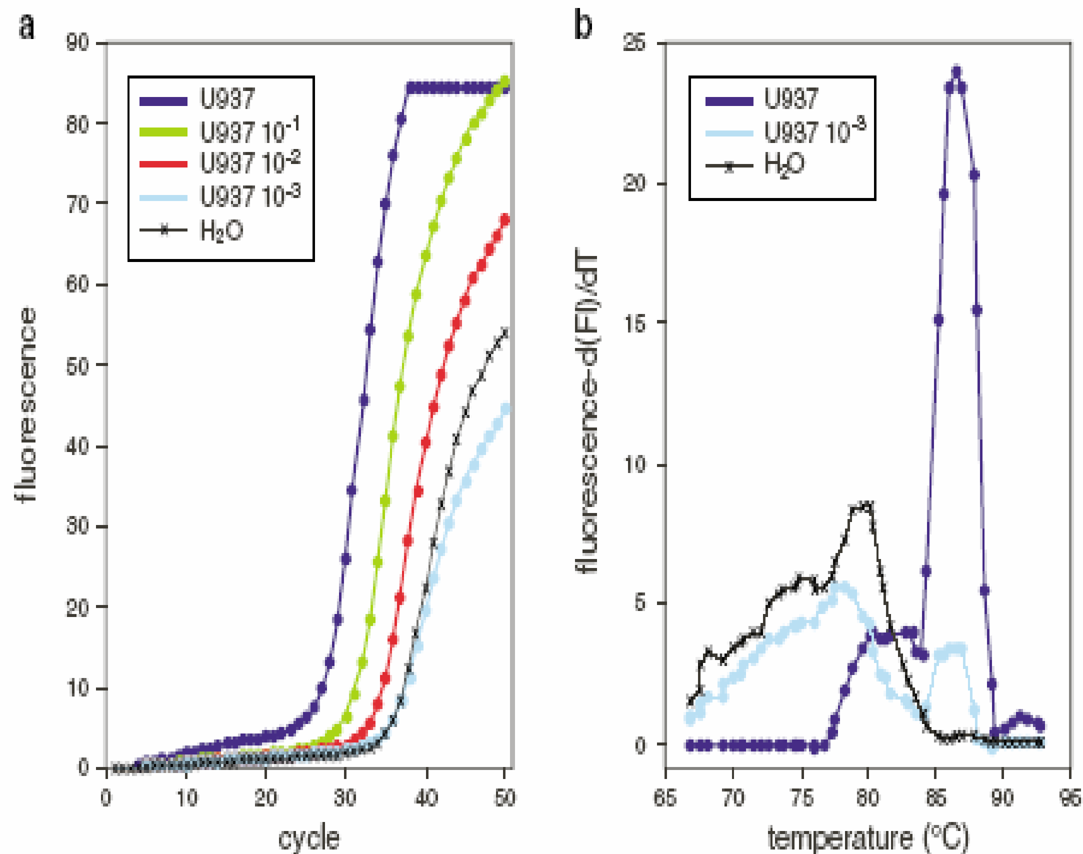


Figure 2 Melting curve analysis. (a) Amplification curves of several dilutions of the U937 cell line using SYBR Green I-based RQ-PCR analysis of the *ABL* gene. An increase in fluorescence is observed for all U937 dilutions, but also for the water control, suggesting nonspecific amplification. (b) Melting curve analysis of the same samples shows the presence of the specific PCR product (melting temperature approximately 86°C) in the U937 samples, but not in the water control, indicating that no specific PCR product has been formed. The increase in fluorescence apparently was because of nonspecific amplification or the formation of primer dimers.

Van der Velden. Leukemia 2003 (www)

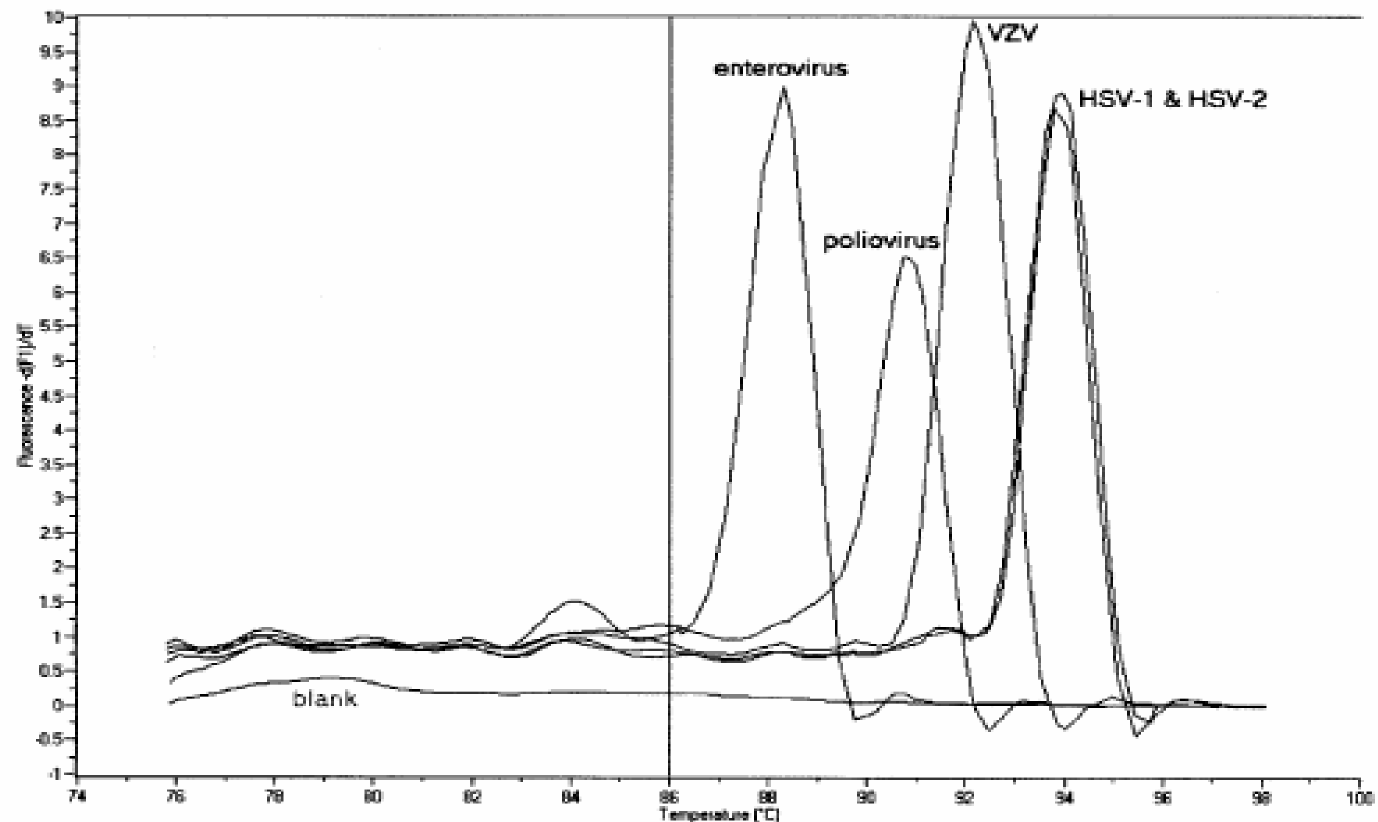
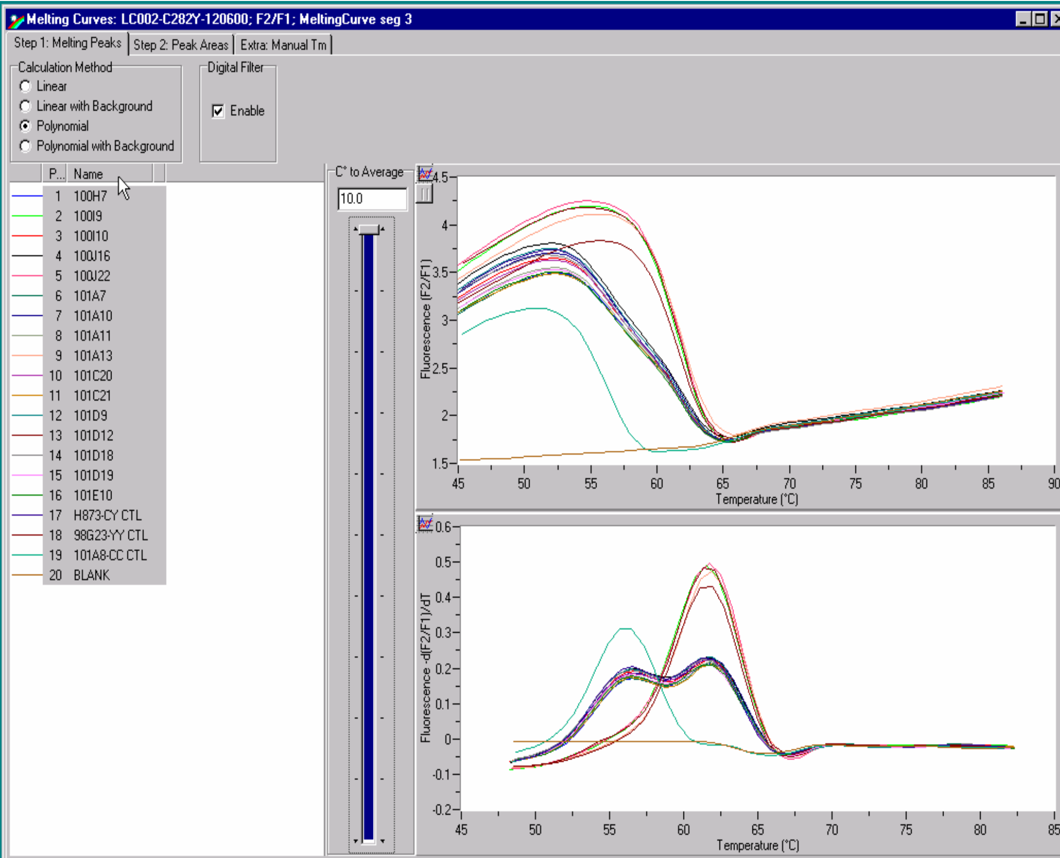


FIG. 1. LightCycler system melting curve analysis of ATCC isolates amplified by multiplex PCR. Enterovirus is echovirus type 1, ATCC VR-31, T_m of 88.20°C; poliovirus is poliovirus type 2 (attenuated), ATCC VR-301, T_m of 90.79°C; VZV is ATCC VR-586, T_m of 92.17°C; HSV-1 is ATCC VR-260, T_m of 93.83°C; and HSV-2 is ATCC VR-734, T_m of 93.91°C. The line designated "blank" represents the analysis of a PCR mixture without the addition of a target. The vertical line at 86°C represents the incubation temperature at which the levels of fluorescent signals were measured during each cycle of PCR. The y axis is the negative differential of fluorescence over temperature ($-dF/dT$).

Read SJ et al. J Clin Microbiol 2001 (www)



Genotyping for the haemochromatosis G845A mutation using melting curve analysis of FRET hybridization probes

AA, G845A homozygotes; GA, G845A heterozygotes; GG, or “wild-type” homozygotes. Right upper panel: Plot of red fluorescence relative to reference (F2/F1) versus temperature (T) for the three genotypes. Three different melting curves are shown for the three possible genotypes. These represent changes in fluorescence of the FRET complexes as they are heated through their melting temperature at the end of PCR amplification. Right Lower panel: -d(F2/F1)/dT versus temperature (T). The apex of the curves represents the melting point for the fluorescent complexes. The FRET probes bind to both alleles to form a fluorescent complex; however they are complementary to the A allele but mismatched to the G allele by one base. Consequently the melting temperature of the fluorescent complex is higher for the A allele than the G allele. Heterozygotes have two peaks representing both alleles.

Interpretation

* Melting curve analysis
* Absolute quantification
* Relative quantification
    i. Relative standard method (relative fold change)
    ii. Comparative threshold method

Cloned GOI/PCR Product

Genomic cDNA

GOI cDNA

Universal Reference RNA T7 Transcript

Synthetic Oligo

Perform serial dilutions (5-7 orders of magnitude) for every plate run

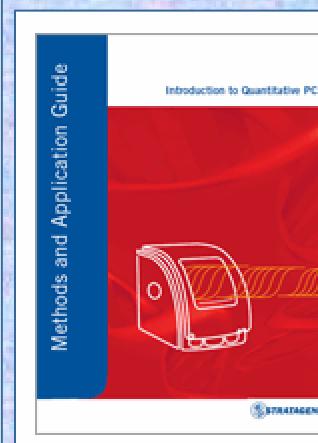
Stock 10^1 10^2 10^3 10^4...

Run reaction

Quantitate → Use to control for sample isolation

Figure 8

Experimental setup for standard curve quantification. Using a known starting concentration of template from one of a variety of sources, a dilution series is performed. These samples are run under the standard well type on the same plate as your unknowns. By comparing the Ct values of the unknowns to the Ct values of the standards, the starting template quantities for the unknown samples can be calculated.



(www)

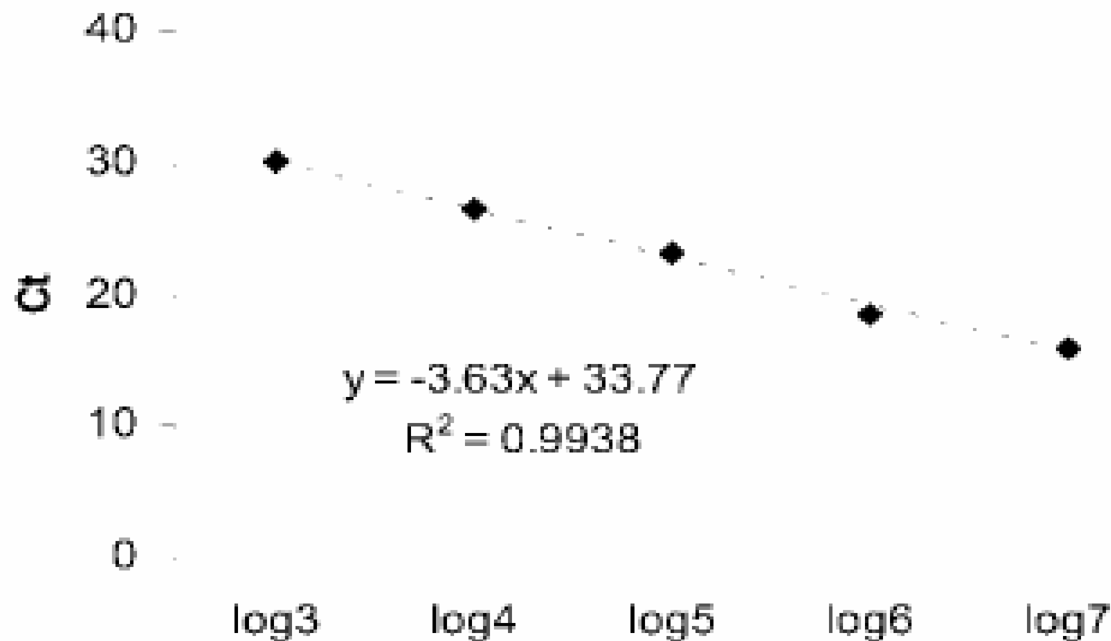


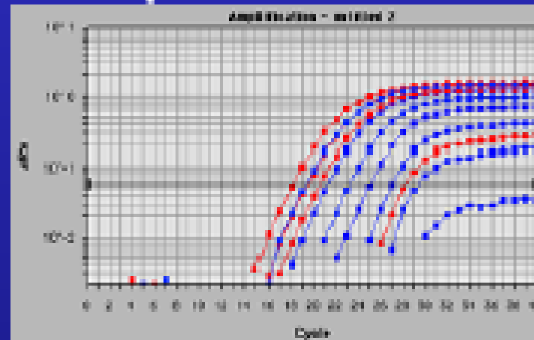
Figure 3. When the known concentrations (expressed in logarithmic form, X axis) of target gene are plotted against the corresponding cycle threshold (Ct, Y axis) obtained by qr-PCR, the result is a line representing the linear correlation between the two parameters. The equation describing this relationship is used to extrapolate the gene copy number in experimental samples.

Example of a Real-Time RT-PCR Experiment

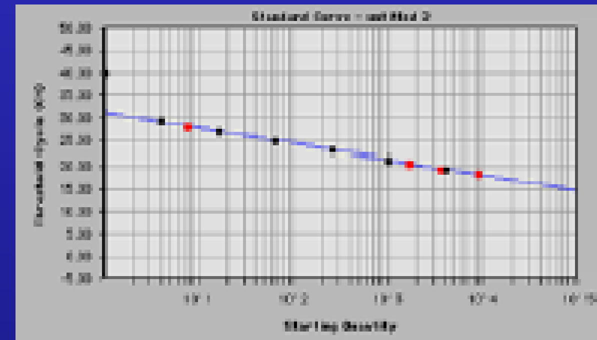
18S rRNA

standard curve, unknowns

Amplification Profile



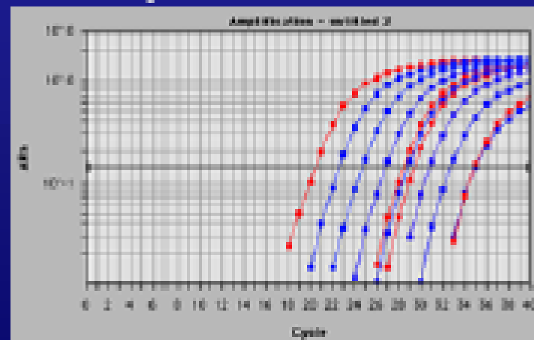
Standard Curve



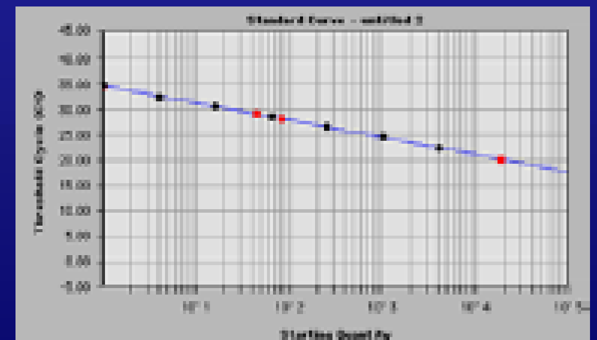
α2-macroglobulin

standard curve, unknowns

Amplification Profile



Standard Curve



Ambion

Interpretation

* Melting curve analysis
* Absolute quantification
* Relative quantification
    i. Relative standard method (relative fold change)
    ii. Comparative threshold method

Table 1. Characteristics of Relative Quantitation Methods

Methods (Reference)	Amplification Efficiency Correction	Amplification Efficiency Calculation	Amplification Efficiency Assumptions	Automated Excel-Based Program
Standard Curve (31)	no	standard curve	no experimental sample variation	no
Comparative C_t ($2^{-\Delta\Delta Ct}$) (21)	yes	standard curve	reference = target	no
Pfaffl et al. (26)	yes	standard curve	sample = control	REST[a]
Q-Gene (23)	yes	standard curve	sample = control	Q-Gene[b]
Gentle et al. (7)	yes	raw data	researcher defines log-linear phase	no
Liu and Saint (22)	yes	raw data	reference and target genes can have different efficiencies	no
DART-PCR (30)	yes	raw data	statistically defined log-linear phase	DART-PCR[c]

C_t, cycle threshold, DART-PCR, data analysis for real-time PCR; REST, relative expression software tool.

[a]www.gene-quantification.info
[b]www.BioTechniques.com
[c]nar.oupjournals.org/cgi/content/full/31/14/e73/DC1

Wong & Medrano, 2006 (www)

Relative quantitation

- ΔC_T between "control" and "treated" RNAs on a single plate
  - Fold-difference
  - Cannot compare Ct between samples on different plates
- ΔC_T between "calibrator" RNA sample and unknown RNA
  - Same calibrator RNA can be on multiple plates
- $\Delta\Delta C_T$ between "control" and "treated"
  - Fold change-normalized to a separate reference gene/sample

Nigel Walker, NIEHS (www)

Relative fold change

- C_T inversely correlated with starting copies
- Each cycle there is a "doubling" of amplicons (assuming 100% efficiency)
- Difference in 1 cycle therefore a 2=fold difference in copies

Fold change = $2^{\Delta CT}$

$\Delta Ct = 3.31$

Fold difference in starting copy number = $2^{3.31} = 9.9$

Nigel Walker, NIEHS (www)

Interpretation

* Melting curve analysis
* Absolute quantification
* Relative quantification
    i. Relative standard method (relative fold change)
    ii. Comparative threshold method

No Normalizer

CONTROL

EXPERIMENTAL

Assumes RNA extraction is perfect

RT-PCR

Calculate Fold Change

Endogenous Normalizer

CONTROL

EXPERIMENTAL

Controls for RNA extraction, but assumes reference expression constant

RT-PCR

Calculate Fold Change

Exogeneous Normalizer

CONTROL

EXPERIMENTAL

No RNA extraction control, but removes expression assumptions

Spike

RT-PCR

Calculate Fold Change

= GOI from control sample

= Reference gene (housekeeping) from control/experimental

= GOI from experimental sample

Figure 10

The use of exogenous and endogenous normalizers in QRT–PCR.

(www)

Validation Experiment for Comparative C_T Method - I

Relative Efficiency of Target and Reference

For the $\Delta\Delta C_T$ calculation to be valid, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal. A sensitive method for assessing if two amplicons have the same efficiency is to look at how ΔC_T varies with template dilution. The standard curves for c-myc and GAPDH used in the previous section provide the necessary data. Table 2 shows the average C_T value for c-myc and GAPDH at different input amounts.

Table 2. Average C_T Value for c-myc and GAPDH at Different Input Amounts

Input Amount ng Total RNA	c-myc Average C_T	GAPDH Average C_T	ΔC_T c-myc – GAPDH
1.0	25.59±0.04	22.64±0.03	2.95±0.05
0.5	26.77±0.09	23.73±0.05	3.04±0.10
0.2	28.14±0.05	25.12±0.10	3.02±0.11
0.1	29.18±0.13	26.16±0.02	3.01±0.13
0.05	30.14±0.03	27.17±0.06	2.97±0.07
0.02	31.44±0.16	28.62±0.10	2.82±0.19
0.01	32.42±0.12	29.45±0.08	2.97±0.14

Figure 6 on page 14 shows a plot of log input amount versus ΔC_T. If the efficiencies of the two amplicons are approximately equal, the plot of log input amount versus ΔC_T has a slope of approximately zero.

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Validation Experiment for Comparative C_T Method - II

Validation Experiment

Before using the $\Delta\Delta C_T$ method for quantitation, perform a validation experiment like that in Figure 6 to demonstrate that efficiencies of target and reference are approximately equal. The absolute value of the slope of log input amount vs. ΔC_T should be < 0.1. The slope in Figure 6 is 0.0492, which passes this test. Once this is proven, you can use the $\Delta\Delta C_T$ calculation for the relative quantitation of target without running standard curves on the same plate.

If the efficiencies of the two systems are not equal, perform quantitation using the standard curve method. Alternatively, new primers can be designed and synthesized for the less efficient system to try to boost efficiency.

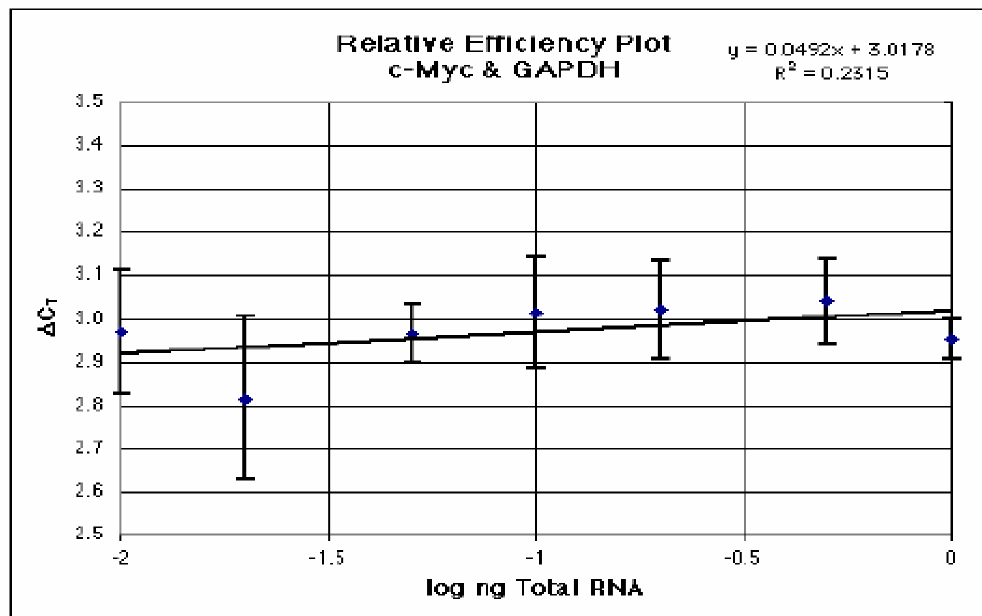

Figure 6. Plot of log input amount versus ΔC_T

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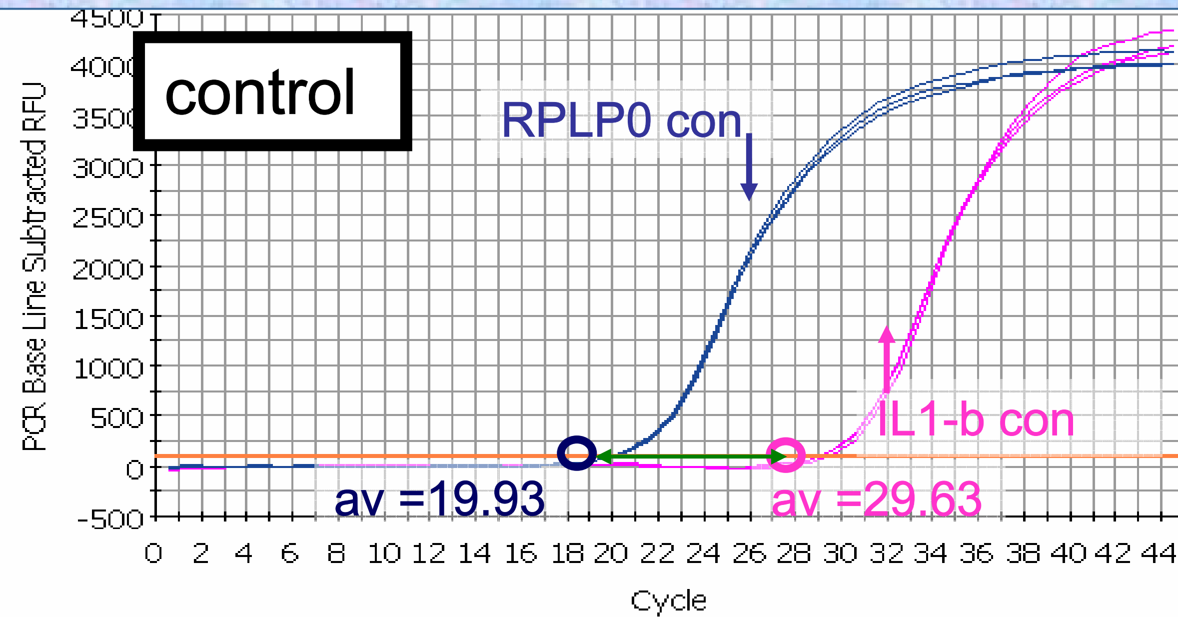
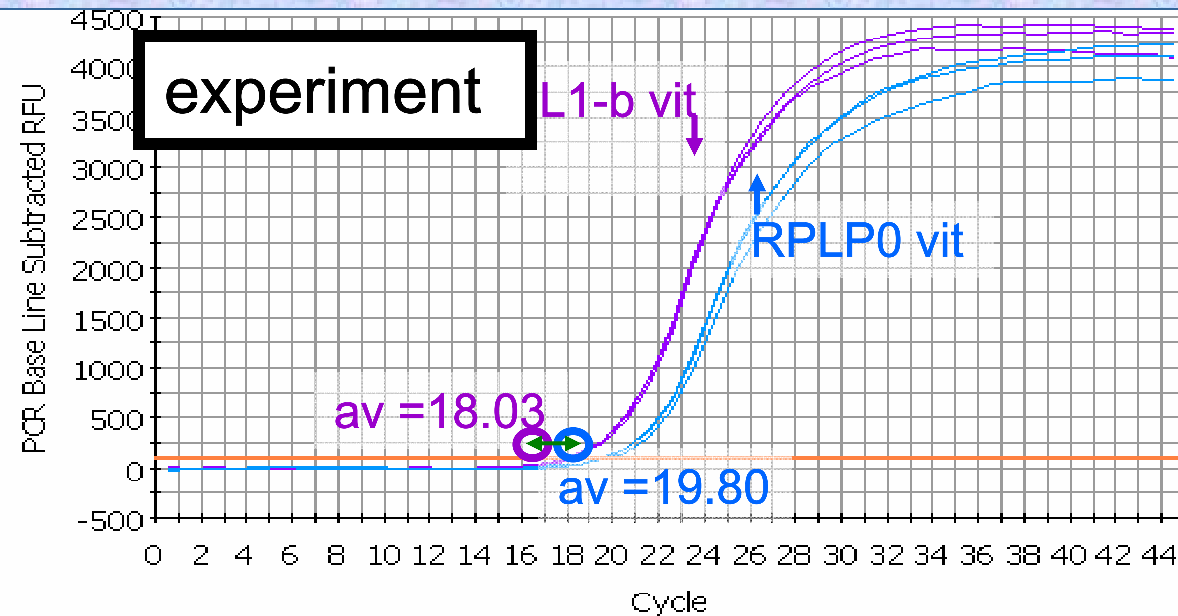


Δ Ct = target - ref

Δ Ct = 9.70



Δ Ct = target - ref

Δ Ct = -1.7

Difference = ΔCt-ΔCt
= $\Delta\Delta$Ct
= 9.70-(-1.7)
= 11.40

$\Delta\Delta Ct = 11.40$ for IL1-beta

$2^{\Delta\Delta Ct}$ variant: assumes efficiency is 100%

Fold change = $2^{11.40} = 2702$

But our efficiency for IL1-beta is 93%

Fold change = $1.93^{11.40} = 1800$

Pfaffl equation corrected for RPLP0 efficiency

Fold change = 1901

EFFICIENCY $^{\Delta\Delta Ct}$ METHOD

- **assumes**
  - **minimal correction for the standard gene, or**
  - **that standard and target have similar efficiencies**
    - **2 $^{\Delta\Delta Ct}$ variant assumes efficiencies are both 100%**

- **approximation method, but need to validate that assumptions are reasonably correct - do dilution curves to check ΔC_Ts do not change**

- **The only extra information needed for the Pfaffl method is the reference gene efficiency, this is probably no more work than validating the approximation method**

Efficiency adjusted Normalization

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(control - sample)}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(control - sample)}}$$

- Fold-change can be “normalized” relative to a “reference gene”
- Reference can be a separate sample on the plate
- Beware of the interpretation of a normalized fold change
  - ↖ Assumption that the reference gene is “unaffected” by treatment

Nigel Walker, NIEHS (www)

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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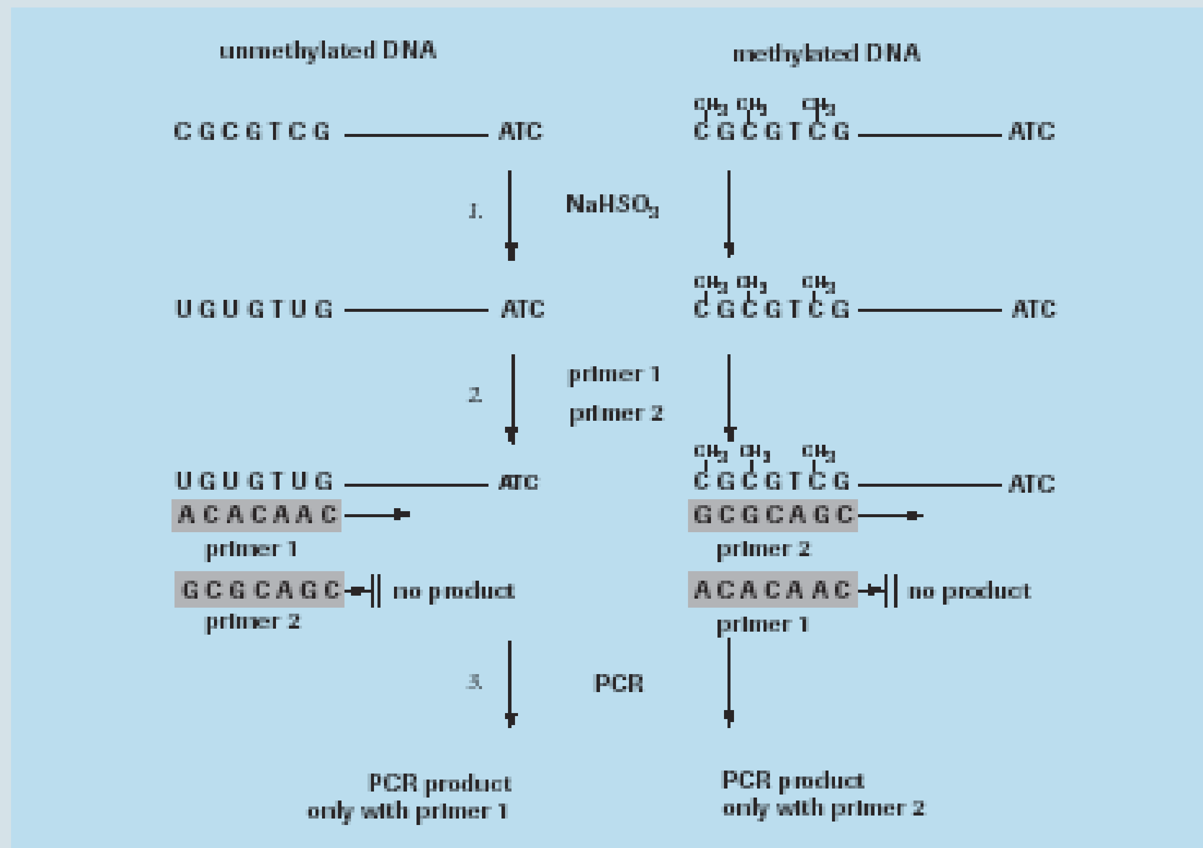
Identifying inactivated genes: methylation-specific PCR (MSP)



In many tumours important genes that control cell growth are switched off by methylation of the promoter region. These changes can be detected by means of methylation-specific PCR (MSP).
In MSP all the normal cytosines (C) of the original DNA are converted to the RNA building block uracil. The methylated cytosines, by contrast, remain unchanged (1). The subsequent PCR procedure then uses specific primers for the various products formed (2). Hence, either the original methylated or the unmethylated DNA is copied. The original DNA was therefore methylated or not (3) depending on the primer used to obtain a product.

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 single-gene copy numbers

  - Microdeletion genotypes

  - Haplotyping

  - Quantitative microsatellite analysis

  - Prenatal diagnosis from fetal cells in maternal blood

  - Intraoperative cancer diagnostics

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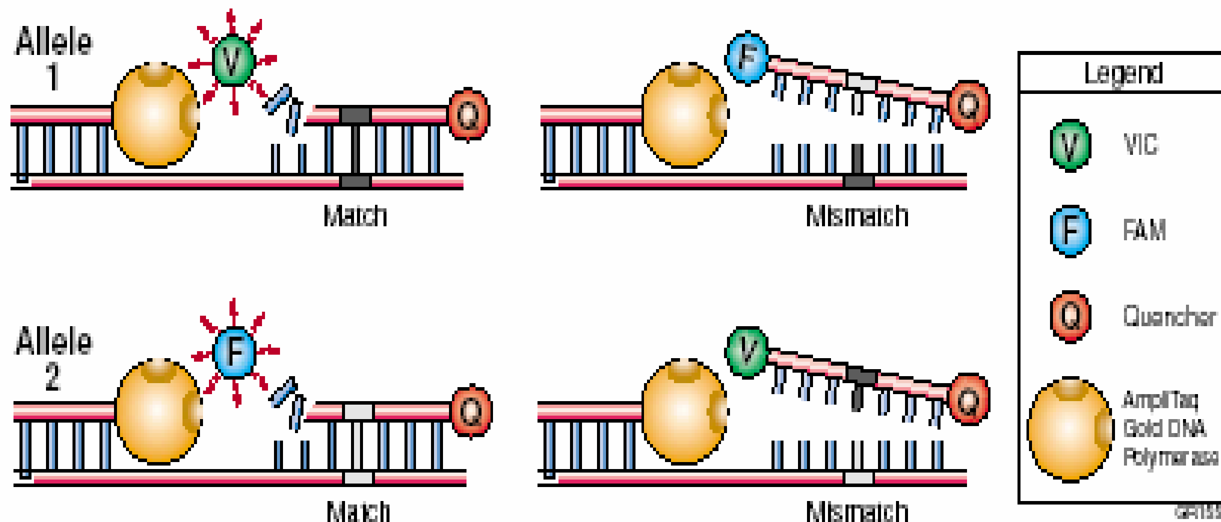
Allelic Discrimination Using TaqMan Probes



The table below summarizes the possible results of the example allelic discrimination assay shown above.

A substantial increase in...	Indicates...
VIC fluorescence only	homozygosity for Allele 1.
FAM fluorescence only	homozygosity for Allele 2.
both fluorescent signals	heterozygosity.

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Allelic Discrimination Using TaqMan Probes

Two Types of TaqMan® Probes

Applied Biosystems offers two types of TaqMan probes:

- TaqMan® probes (with TAMRA™ dye as the quencher dye)
- TaqMan® MGB probes

TaqMan® MGB Probes Recommended for Allelic Discrimination Assays

Applied Biosystems recommends the general use of TaqMan MGB probes for allelic discrimination assays, especially when conventional TaqMan probes exceed 30 nucleotides. The TaqMan MGB probes contain:

- A nonfluorescent quencher at the 3′ end - The SDS instruments can measure the reporter dye contributions more precisely because the quencher does not fluoresce.
- A minor groove binder at the 3′ end - The minor groove binder increases the melting temperature (T_m) of probes, allowing the use of shorter probes.

Consequently, the TaqMan MGB probes exhibit greater differences in T_m values between matched and mismatched probes, which provides more accurate allelic discrimination.

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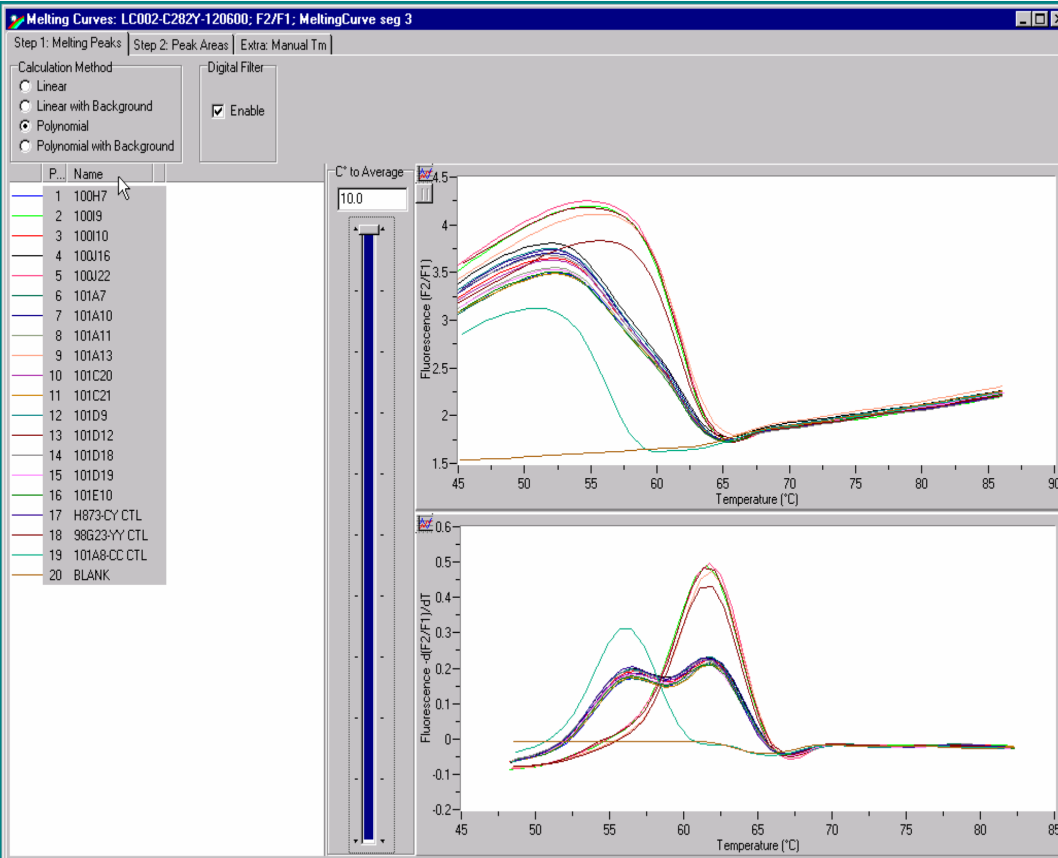
Allelic Discrimination Using SYBR Green



Genotyping for the haemochromatosis G845A mutation using melting curve analysis of FRET hybridization probes

AA, G845A homozygotes; GA, G845A heterozygotes; GG, or "wild-type" homozygotes. Right upper panel: Plot of red fluorescence relative to reference (F2/F1) versus temperature (T) for the three genotypes. Three different melting curves are shown for the three possible genotypes. These represent changes in fluorescence of the FRET complexes as they are heated through their melting temperature at the end of PCR amplification. Right Lower panel: -d(F2/F1)/dT versus temperature (T). The apex of the curves represents the melting point for the fluorescent complexes. The FRET probes bind to both alleles to form a fluorescent complex; however they are complementary to the A allele but mismatched to the G allele by one base. Consequently the melting temperature of the fluorescent complex is higher for the A allele than the G allele. Heterozygotes have two peaks representing both alleles.

Mamotte, Langan & Pocathikorn. DCIBG, Royal Perth Hospital, June 2004 (www)

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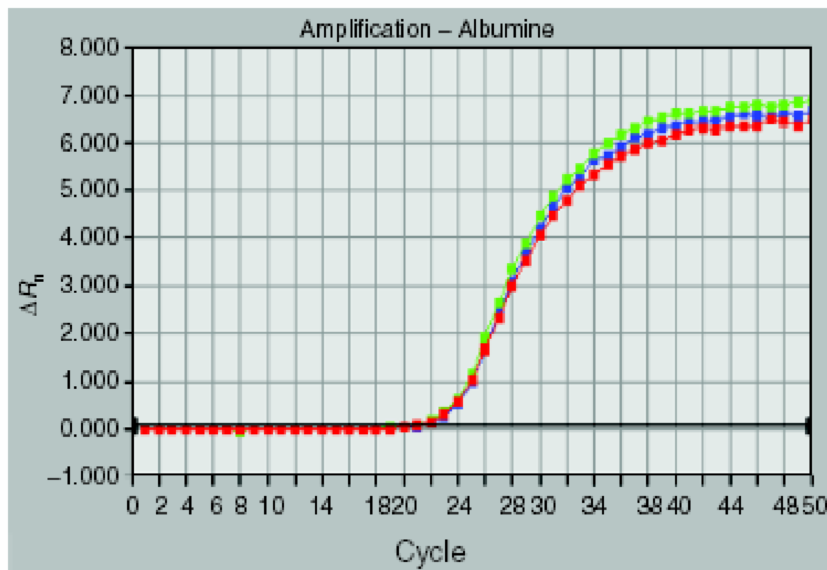

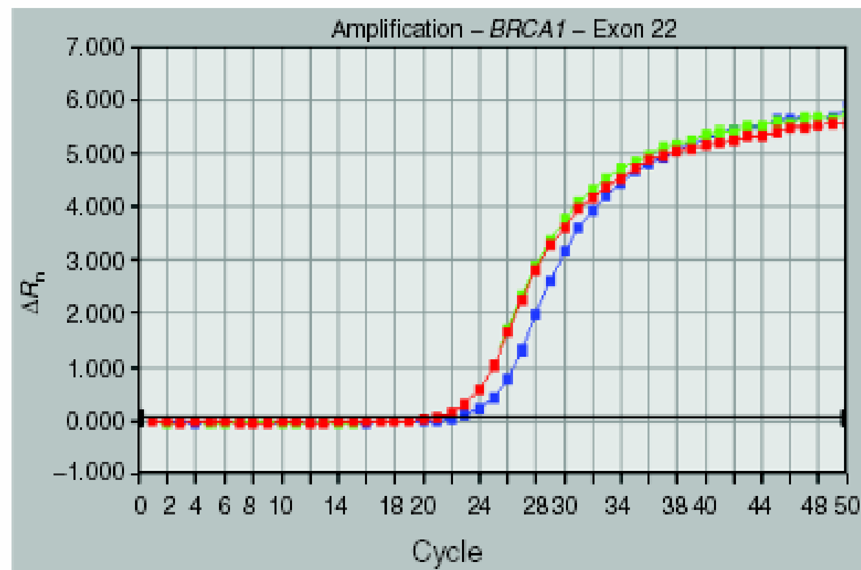

Sample	C_t*ALB*	C_t*Ex22*	N*Ex22*
Calibrator	21.72	21.77	1.00
IC1712	21.81	21.75	1.03
IC557	21.93	23.01	0.47

Fig. 2. *BRCA1* exon 22 and *ALB* gene dosage assay by real-time polymerase chain reaction on two DNA samples from patients and one from a control subject (calibrator). From the C_t of each sample, the N*Ex22* value was calculated as described in *Patients and methods*. Each DNA sample was analyzed in triplicate, and the results for one analysis are shown here. Sample IC557 bears a heterozygous deletion of exon 22 (N*Ex22* = 0.47), while exon 22 is not deleted in sample IC2171 (N*Ex22* = 1.03).

Barrois M et al. Clin Genet 2004 (www)

Essentials of Genetics [M.Tevfik DORAK] - Windows Internet Explorer

http://www.dorak.info/genetics/genetics.html

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Viral and Bacterial Genetics Plant Genetics

Biotechnology Genetic Engineering & Cloning

Real-Time PCR

Basic Population Genetics Genetic Epidemiology Evolution

Bioethics

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Genetics Education: ROCHE GLAXO WELLCOME TRUST Centre for Genetics Education (Australia) (Fact Sheets)

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