Real-Time PCR:
Practical Issues and Troubleshooting

Mehmet Tevfik DORAK, MD PhD

Dept of Environmental & Occupational Health
Robert Stempel College of Public Health and Social Work
Florida International University
Miami, Florida
USA

FIU FLORIDA INTERNATIONAL UNIVERSITY

MOBGAM, Istanbul, Turkey
June 3, 2011

Quantification of mRNA using real-time RT-PCR

Tania Nolan[1], Rebecca E Hands[2] & Stephen A Bustin[2]

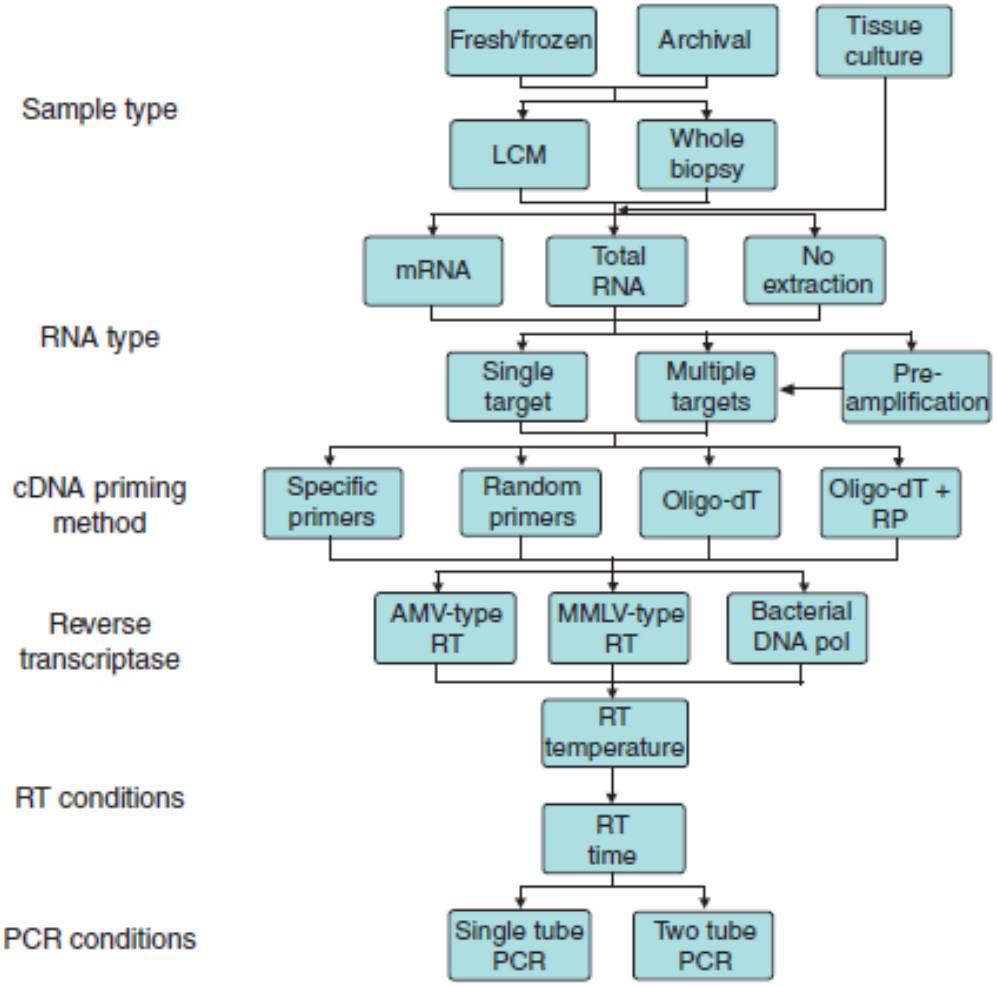


Figure 1 | Steps involved in planning a RT-qPCR assay. The numerous permutations illustrate the alternatives and potential for variability associated with this technique.

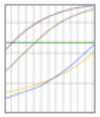
(www)

Real-Time PCR Troubleshooting Tool

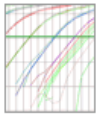
Having problems with your gene expression or SNP genotyping experiments? Do your amplification curves look sigmoidal, or do you have no curves at all? Do your allelic discrimination plots have diffuse or trailing clusters? Our interactive troubleshooting tool will guide you step by step to a solution.

Gene Expression/Quantitation Experiments

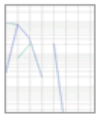
I am having problems with...



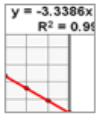
Abnormal Amplification
Your curves are sigmoidal or amplification occurs later than you expected.



NTC Positive Amplification
You have an amplification product in your no template control (NTC).

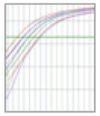


No Amplification
You have no detectable PCR product.



Poor PCR Efficiency
If the slope of your standard curve is below -3.6, you have poor PCR efficiency.

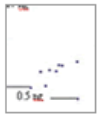
Example of a normal amplification curve



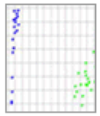
Normal Amplification Curve
Your amplification curve should look something like this.

SNP Genotyping Experiments

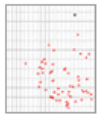
I am having problems with...



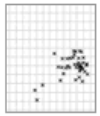
SNP Not Amplifying
You cannot amplify a SNP.



Trailing Clusters
Not using ROX or not designating it as a passive reference dye may cause trailing clusters.



Diffuse Clusters
You have diffuse clustering in your SNP analysis.



No Autocalling
Failure of autocalling of SNP results is usually due to outlier wells or software problems.

Example of a normal allelic discrimination plot

Normal Allelic Discrimination Plot
Your cluster plot should look something like this.

applied biosystems
by life technologies

Ten Most Common Real-Time qRT-PCR Pitfalls

1 **Poor primer and probe design.** For the most efficient design of PCR primer and probe sets for real-time qRT-PCR, we strongly recommend using primer design software. Most primer design programs include adjustable parameters for optimal primer and probe design. These parameters consider primer/probe T_m, complementarity, and secondary structure as well as amplicon size and other important factors. Restricting the number of identical nucleotide runs is also recommended. When designing amplicons in eukaryotic targets, choose PCR primers that span at least one exon-exon junction in the target mRNA to prevent amplification of the target from contaminating genomic DNA.

2 **Using poor quality RNA.** Degraded or impure RNA can limit the efficiency of the RT reaction and reduce yield. RNA should either be prepared from fresh tissue, or from tissue treated with an RNA stabilization solution such as RNA*later*® (see **www.ambion.com/techlib/resources/rnalater** for more information). The importance of using full length RNA for reverse transcription depends on the application. Amplicons for real-time qRT-PCR are typically short (70-250 bp). As a result, some degradation of the RNA can be tolerated. If it is not possible to use completely intact RNA, design primers to anneal to an internal region of the gene of interest. Note that for truly quantitative RT-PCR, partially degraded RNA may not give an accurate representation of gene expression.

TechNotes Archive

Ordering Information

Related Links:

Avoiding DNA Contamination in RT-PCR
[read]

Designing a Successful qRT-PCR Experiment
[read]

Real-Time Relative RT-PCR: How It's Done
[read]

RT-PCR Problem Solver
[read]

RT-PCR: The Basics
[read]

(www)

Quantification of mRNA using real-time RT-PCR

Tania Nolan[1], Rebecca E Hands[2] & Stephen A Bustin[2]

TABLE 3 | Troubleshooting table.

Problem	Possible reason	Solution
General		
Normalization of RNA samples based on A_{260} measurement results in samples of unequal concentration.	RNA may be degraded – absorbance increases in the presence of free nucleotides, leading to overestimation RNA concentration.	Check RNA sample using automated or agarose gel electrophoresis.
Determination of RNA concentration by RiboGreen is unreliable.	False readings can be obtained if RiboGreen absorbs to the sides of the tube.	Use non-stick, nuclease-free polypropylene tubes for RiboGreen assays.
No amplification detected.	No amplification occurring.	Resolve PCR products by agarose gel electrophoresis.
	Probe-based detection failing.	Try fluorescent nucleic acid binding dye detection.
Amplification plot increases very little when using probes for detection.	High background – instrument problem.	Probe inefficiently quenched.
	Low background.	Probe poorly labeled; instrument not detecting dye; Probe has G as 5′ base; Probe is designed to overlap primer site; probe is too far from 3′ of primer and so is being displaced rather than cleaved.
Amplification plot dips under baseline.	Baseline setting is using an incorrect end cycle.	Examine raw data and reset baseline cycles.
Replicates show high variability.	Poor assay efficiency. Poor pipetting.	Redesign assay.
C_t recorded in NTC.	Contamination. Primer dimers.	Use fresh reagents. Redesign assay.
C_t recorded in –RT control.	DNA contamination.	DNase treat samples.
RNA dilution standard curve is not linear.	RT was performed using oligo-dT or random primers.	Use specific priming or dilute cDNA for standard curve.

TABLE 3 | Troubleshooting table (continued).

Problem	Possible reason	Solution
SYBR Green I		
No increase in fluorescence with cycling.	A reagent is missing from the PCR reaction.	Gel analyze PCR product to determine if there was successful amplification and repeat the PCR.
	The $MgCl_2$ concentration is not optimal.	Increase up to 5.0 mM in 0.5 mM increments.
	Hot-start DNA polymerase was not activated.	Ensure that the appropriate initial incubation at 95 °C was performed as part of the cycling parameters.
	Is SYBR Green I concentration too high?	Ensure the correct dilution of SYBR Green I was used.
	Too high a template concentration was used.	Dilute 1 in 10^5 and repeat PCR.
	Insufficient annealing and extension times.	Check the length of the amplicon and increase the extension time if necessary.
	Too few cycles in the PCR reaction.	Increase cycle number; redesign more efficient assay.
	Inappropriate annealing temperature.	Reduce annealing temperature.
Multiple peaks in melt curve.	Abundance of primer-dimer and nonspecific PCR products.	Increase the annealing temperature; lower the Mg^{2+} concentration; always use a hot start polymerase; design more optimal primers.
	Amplification of splice variants/pseudogenes.	Analyze products on gel; sequence different size bands; redesign primers.
Probe		
No increase in fluorescence with cycling.	The probe is not binding to the target efficiently because the annealing temperature is too high.	Verify the calculated T_m, using appropriate software. Note that Primer Express T_ms can be significantly different than T_ms calculated using other software packages.
	The probe is not binding to the target efficiently because the PCR product is too long and is folding in solution.	Design the primers so that the PCR product is no longer than 120.
	The probe is not binding to the target efficiently or being cleaved effectively because the Mg^{2+} concentration is too low.	Perform a Mg^{2+} titration to optimize the concentration.
	The probe has a nonfunctioning fluorophore.	Verify that the fluorophore functions by detecting an increase in fluorescence in the denaturation step of thermal cycling or at high temperatures in a melting curve analysis. If there is no increase in fluorescence, redesign and/or resynthesize probe.
	The reaction is not optimized and no or insufficient product is formed.	Verify formation of enough specific product by gel electrophoresis.

(www)

Probe QPCR Troubleshooting Guide

No Amplification	
Pipetting error / reagents missing	Repeat experiment checking concentration of all reagents.
Enzyme not activated	Check that the full 15 minute activation step is performed before cycling.
Annealing step	Check that you have the optimal annealing temperature by performing a temperature gradient (2°C increments). Annealing time should be carried out as specified in the protocol.
Extension step	Extension time can be increased for longer amplicons. Amplification of products over 300bp is not recommended.
Primers	**Poor primer design** – Check for primer dimers on gel. **Wrong primer concentration** – 0.4µM recommended. **Primers degraded** – Check on polyacrylamide gel. Re-order new primers if necessary.
Detection step	Detection reading taken at wrong step. Reading should be taken in the annealing step for Molecular Beacons or the detection/annealing step for hybridisation probes.
Product too long	The ideal amplicon size is between 80–200bp. Amplification of products over 300bp is not recommended.
Too few cycles	40 cycles is recommended.
Template	**Impure template** – Purify template before use. For templates isolated from difficult sources (such as plant) use a commercial mix containing enhancers/detergents. **Wrong concentration** – A concentration of up to 500ng can usually be used. **Degraded** – Make fresh dilution from stock. Check storage conditions.
Wrong dye layer	Check that machine settings correspond with experiment.
Probes	**Poor probe synthesis** – Check with rDNase I digestion. Once digested the fluorescence should increase greatly as the quencher and fluorophore become separated. **Poor probe design** – See "Designing Dual Labelled Probes" http://www.abgene.com/downloads/Guide_QPCR-probedesign.pdf

Probe QPCR Troubleshooting Guide

Wavy/Erratic Lines

No ROX	Check machine settings. ABI PRISM® requires ROX for normalisation. ROX is available included in ABsolute™ mixes or as a separate vial.
Too many cycles performed	Reduce number of cycles.
Wrong detection step	Check detection step is set in the correct stage of the cycle.
Machine needs calibrating	Wavy lines can be caused by mirror misalignment or lamp problems. Consult machine manufacturer.
Baseline for ΔRn set at wrong cycle	ΔRn should be set between 3-15 cycles and at least 2 cycles before 1st dilution amplifies.
Reaction volume too low.	Some QPCR instruments (e.g ABI® PRISM 7700) are set to read accurately only at volumes of at least 15µl.

High Well-to-Well Variance

Poor plate choice	Do not use frosted or black plates.
Low quality sealing material	Use only high quality optically clear seals that have been specifically designed for fluorescence applications.
Machine needs re-calibrating	Follow manufacturer's guidelines.
Evaporation	Do not use corner wells or use a more robust seal.
Concentration gradient formed in vial	Invert the mixture a couple of times before use.

Probe QPCR Troubleshooting Guide

Amplification in No Template Control	
Primer dimers	Primer dimers can also be identified by using a serial dilution of your template and running products on a gel. As template concentration increases, the primer dimer bands should decrease in intensity. If the presence of primer dimers is observed, then it may help to do one of the following: 1) re-design the primers, 2) try increasing the annealing temperature, or 3) decrease the primer concentration. If using a standard curve, a sub-optimal gradient can indicate inhibition from primer dimers.
Contamination	**Template contaminated** - Purify template before use. If doing QRT-PCR, treat RNA template with recombinant DNase I or design exon-spanning primers. **DNA polymerase contaminated** – All recombinant DNA polymerases will contain small amounts of *E.coli* DNA. Thermo-Start® contains minimal amounts compared to competitor enzymes. However, if contamination remains a problem, a 'BLAST' search can be performed to check for homology to the *E.coli* genome. **Reagents contaminated** – Repeat with fresh reagents and always use filter tips.

Probe QPCR Troubleshooting Guide

Low Sensitivity (High Cycle Threshold (Ct))	
Evaporation	Do not use corner wells or use more robust seal.
Low quality sealing material	Use only high quality optically clear seals that have been specifically designed for fluorescence applications.
Primer dimers	Primer dimers can also be identified by using a serial dilution of your template and running products on a gel. As template concentration increases, the primer dimer bands should decrease in intensity. If the presence of primer dimers is observed, then it may help to do one of the following: 1) re-design the primers, 2) try increasing the annealing temperature, or 3) decrease the primer concentration. If using a standard curve, a sub-optimal gradient can indicate inhibition from primer dimers.
dUTP	dUTP is not as efficient a substrate as dTTP (Longo *et al*, Gene 1993). This reduction in PCR efficiency will reduce QPCR sensitivity, resulting in higher Ct values. ABgene® recommend the use of dTTP for most applications.
Annealing step	Check that you have the optimal annealing temperature by performing a temperature gradient (2°C increments). Annealing time should be carried out as specified in the protocol.
Extension step	Extension time can be increased for longer amplicons. Amplification of products over 300bp is not recommended.
Primers	Wrong primer concentration – 0.4µM recommended.

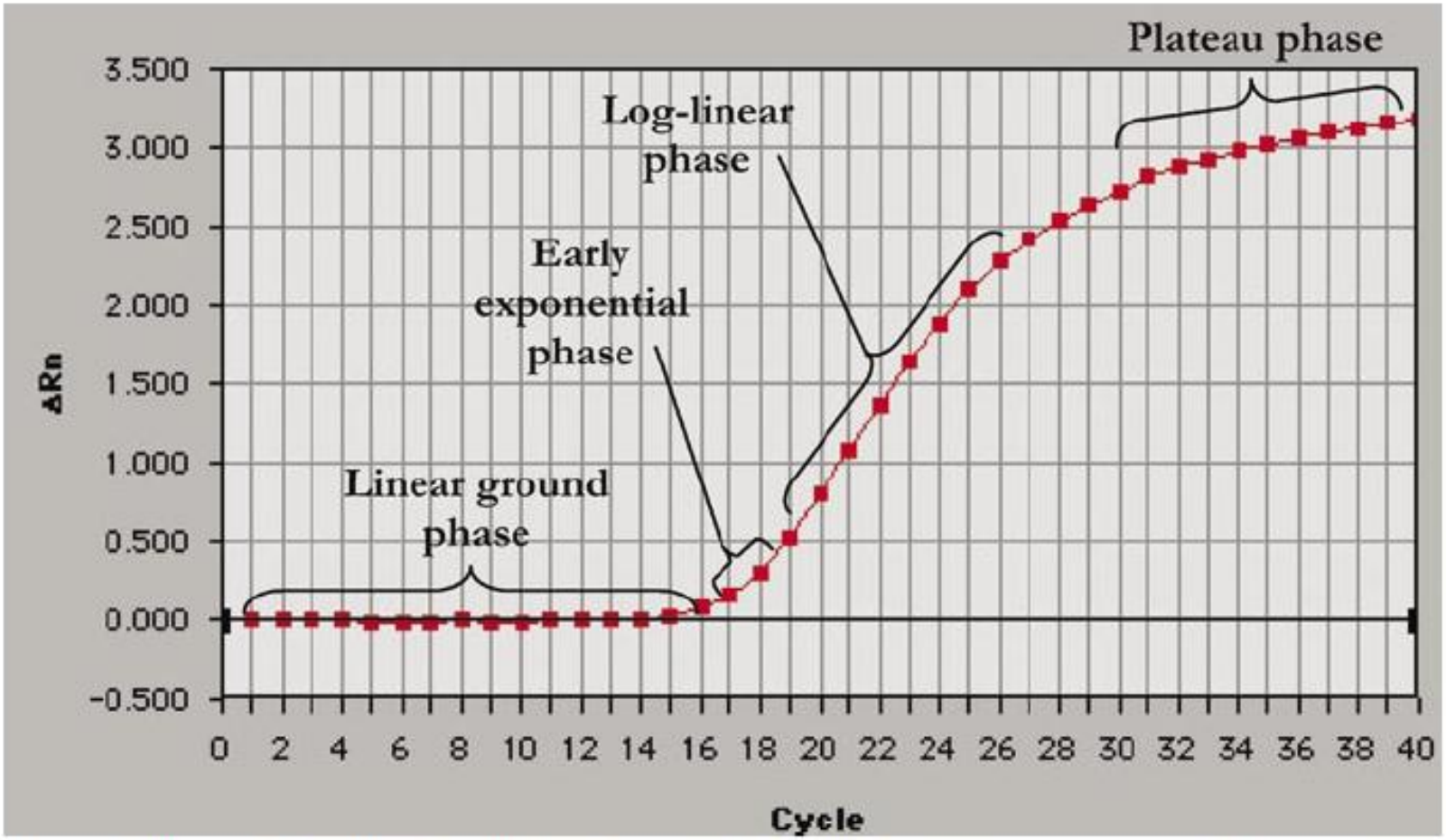


Figure 2. Phases of the PCR amplification curve. The PCR amplification curve charts the accumulation of fluorescent emission at each reaction cycle. The curve can be broken into four different phases: the linear ground, early exponential, log-linear, and plateau phases. Data gathered from these phases 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).

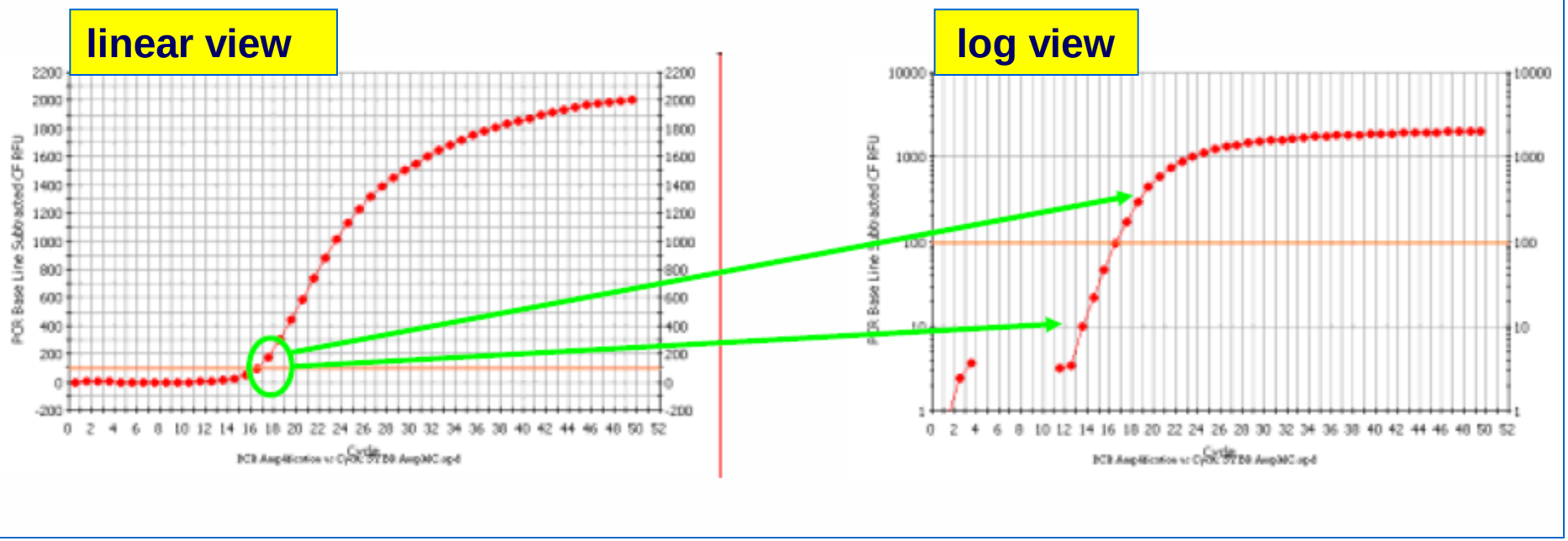
Real-time PCR for mRNA quantitation

Marisa L. Wong and Juan F. Medrano

BioTechniques 39:75-85 (July 2005)

(www)

Linear vs Log View



Linear vs Log View

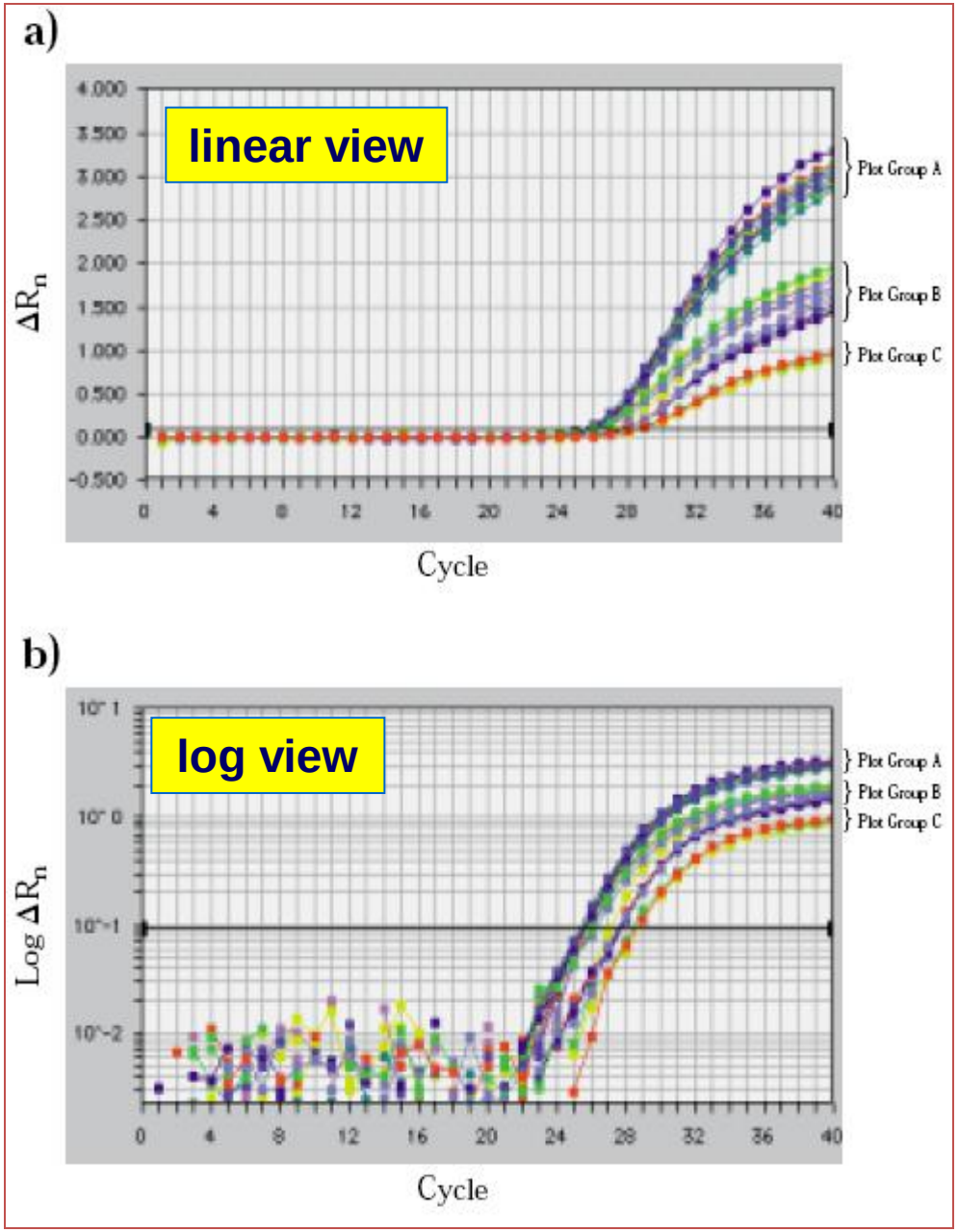


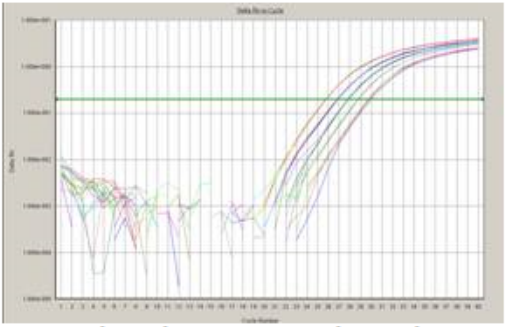
Figure 1. Primer optimization experimental results showing amplification plots of all nine primer combinations in the Primer Optimization Matrix. (a) linear view (b) log view.

Plot Group A: combinations that contain at least a 300 nM concentration of forward and reverse primer.

Plot Group B: combinations that contain a 50 nM concentration of forward or reverse primer.

Plot Group C: 50 nM concentrations of forward and reverse primer.

A Normal Amplification Curve



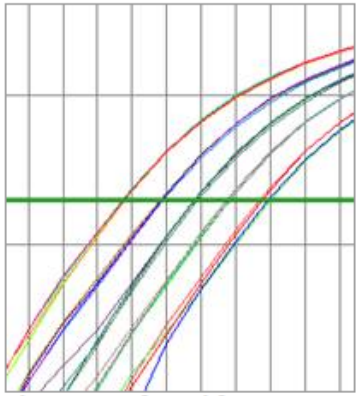
A normal amplication curve obtained using a dilution series of a sample

A normal amplification curve from a dilution series of a sample is shown above. The curve is a semi-log view (Y-axis is a log scale). The initial PCR cycles (1&endash;19, above) produce low fluorescent signals that cannot be detected by the CCD camera. The linear portion of each curve is in the exponential phase of PCR, where the amount of product, and therefore the signal, doubles after each cycle. The top portion of the curves shows minimal signal increase, as PCR slows due to the depletion of reaction components, such as primers and dNTPs.

The curves should be smooth during the exponential phase of the PCR. Any spikes in the curves may be the result of unstable light sources from the instrument, or sample preparation problems, such as the presence of bubbles in the reaction wells.

applied biosystems™
by life technologies™

A Normal Amplification Curve



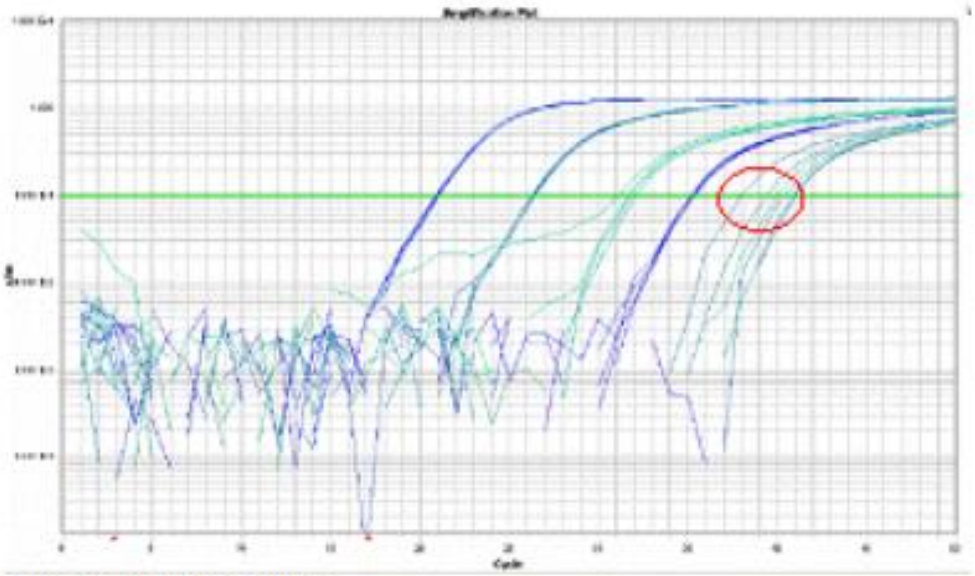
Clustering of amplification curves

The clustering of the amplification curves for each dilution should be tight. You can achieve this by careful pipetting, using a master mix to minimize sample-to-sample variation, and using a passive reference dye to normalize non-PCR-related fluorescence fluctuations. All Applied Biosystems real-time PCR master mixes contain the ROX™ dye that acts as a passive reference; for a good replicate, you should not observe a C_T difference greater than 0.3 cycles.

A good amplification should produce a C_T value based on the template input. The amplification curves shown above are based on a dilution series of a sample using a dilution factor of 1:2. At the exponential phase, the difference in C_T values is 1 cycle from one dilution to the next. Additionally, in the exponential phase, the amplification curves are parallel to each other, indicating that the amplification efficiency is the same for all the dilutions.

applied biosystems
by life technologies

Amplification occurs later than expected



Late amplification

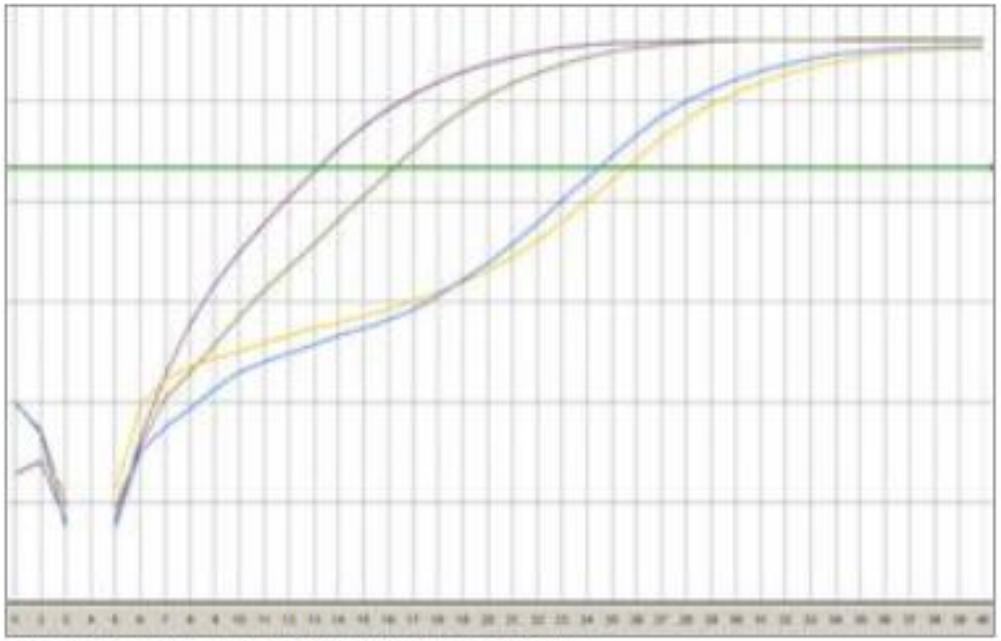
Possible causes (ranked by frequency observed by Applied Biosystems support):

- Your RNA sample may be of poor quality
- You may not have enough template sequence
- Your reverse transcription may not be optimal
- Your assay design may not detect all of the splice variants for the gene of interest

6.6 picogram template & C_T > 40

applied biosystems by life technologies

Your amplification curves have a sigmoidal shape



Sigmoidal amplification curves

If your amplification curves look sigmoidal, it is likely you have one of these problems:

- The baseline setting in your instrument's data analysis software may be too low
- You may have a high level of fluorescent noise during the early cycles of PCR

applied biosystems
by life technologies

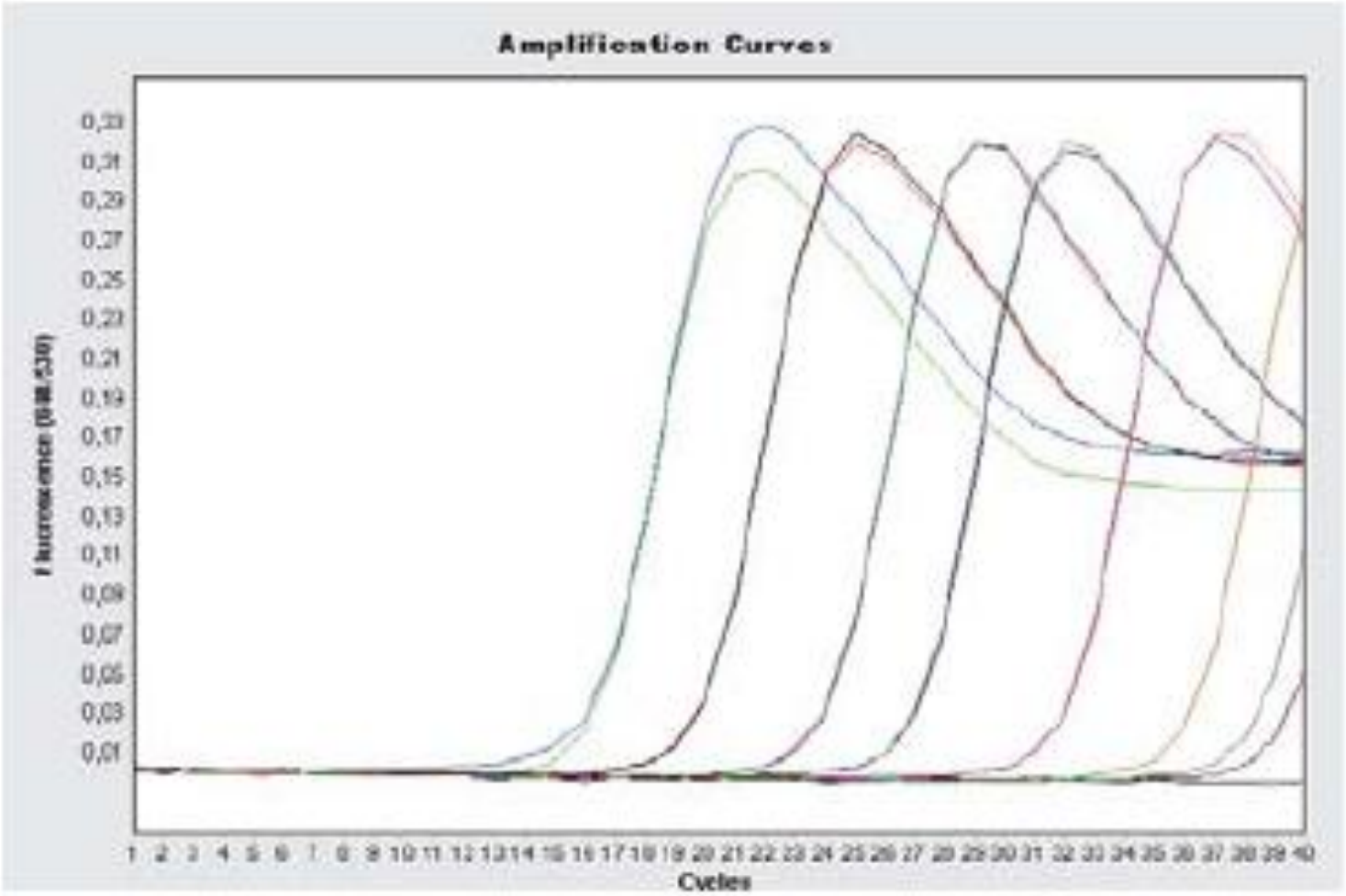
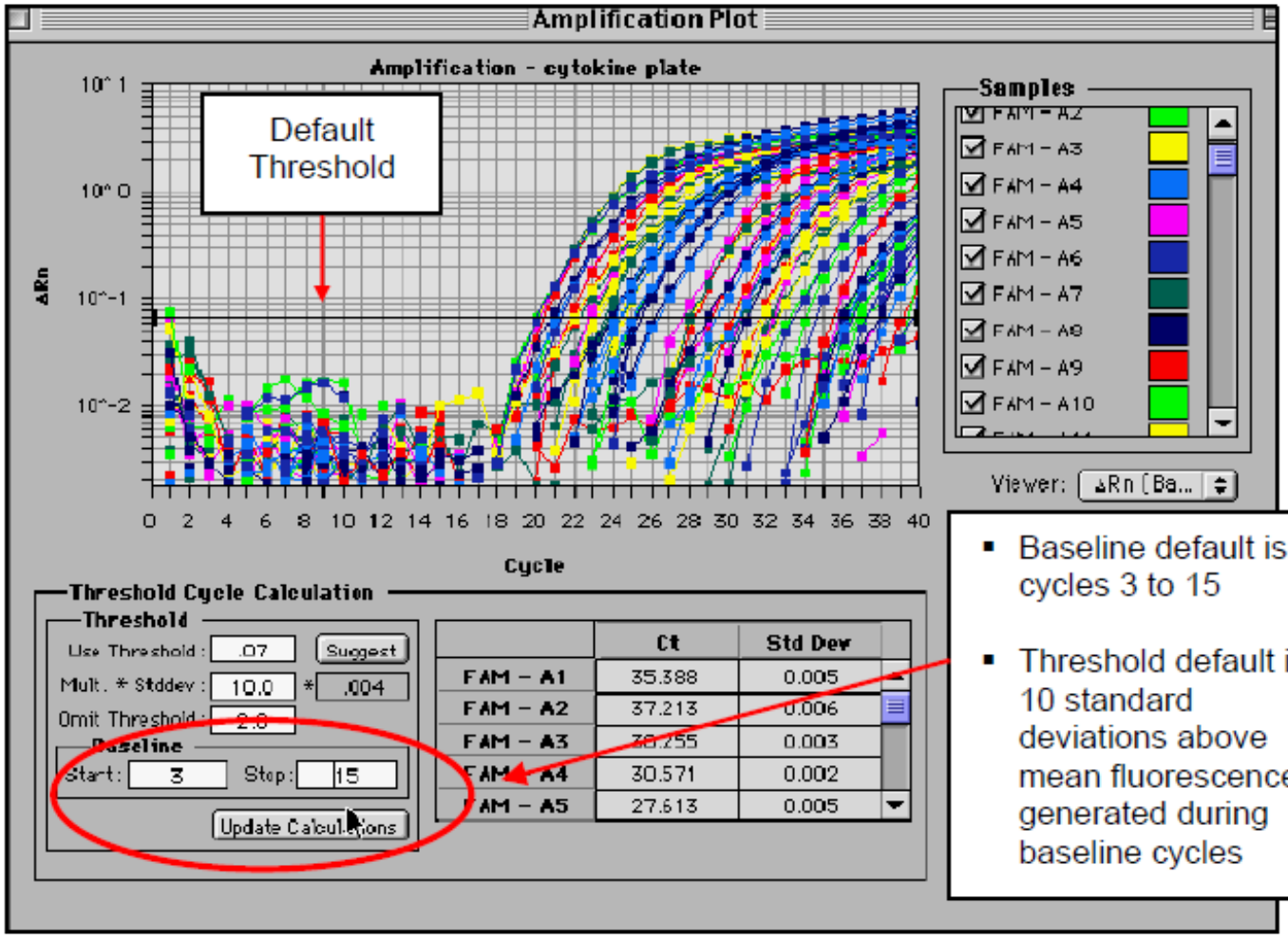


Figure 49. Hook-shaped amplification plot. Amplification plot showing apparent decrease in yield of PCR product toward the end of PCR. This phenomenon is typical for LightCycler instruments.

Baseline Setting

Figure 1: Default Baseline

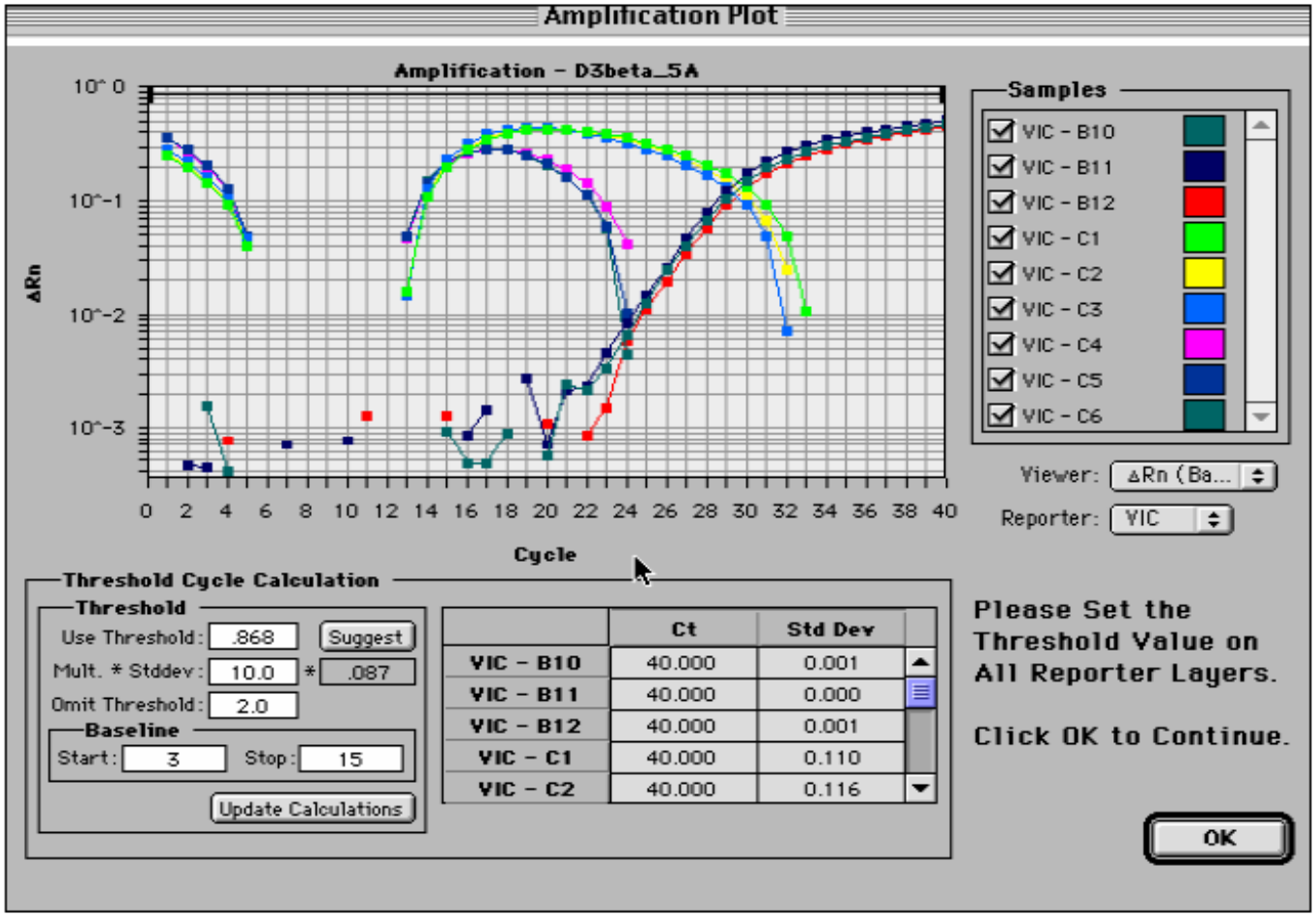


- Baseline default is cycles 3 to 15
- Threshold default is 10 standard deviations above mean fluorescence generated during baseline cycles

Applied Biosystems

Baseline Setting

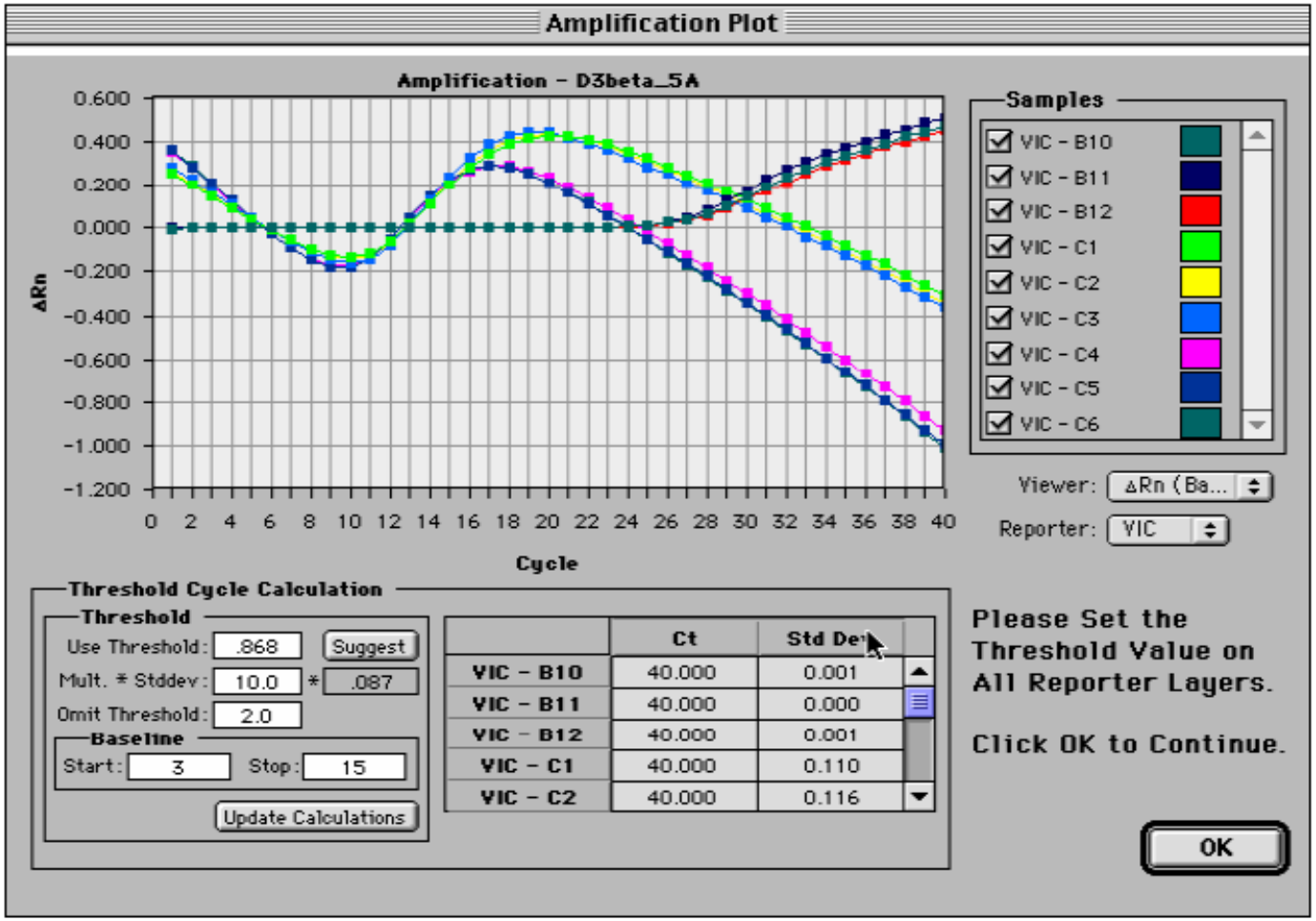
Figure 3: Log View of Early Amplification



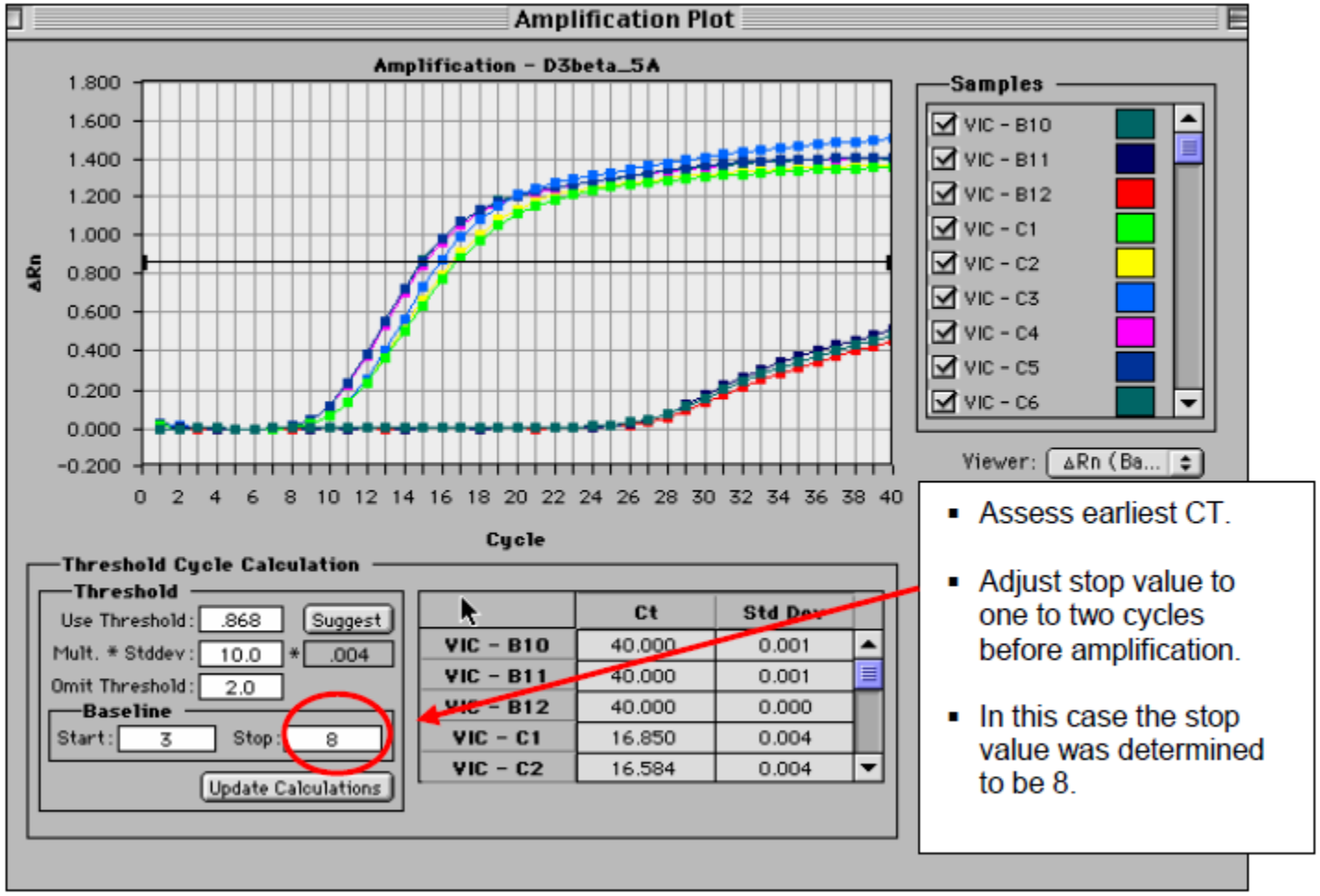
Applied Biosystems

Baseline Setting

Figure 4: Linear View



Baseline Setting

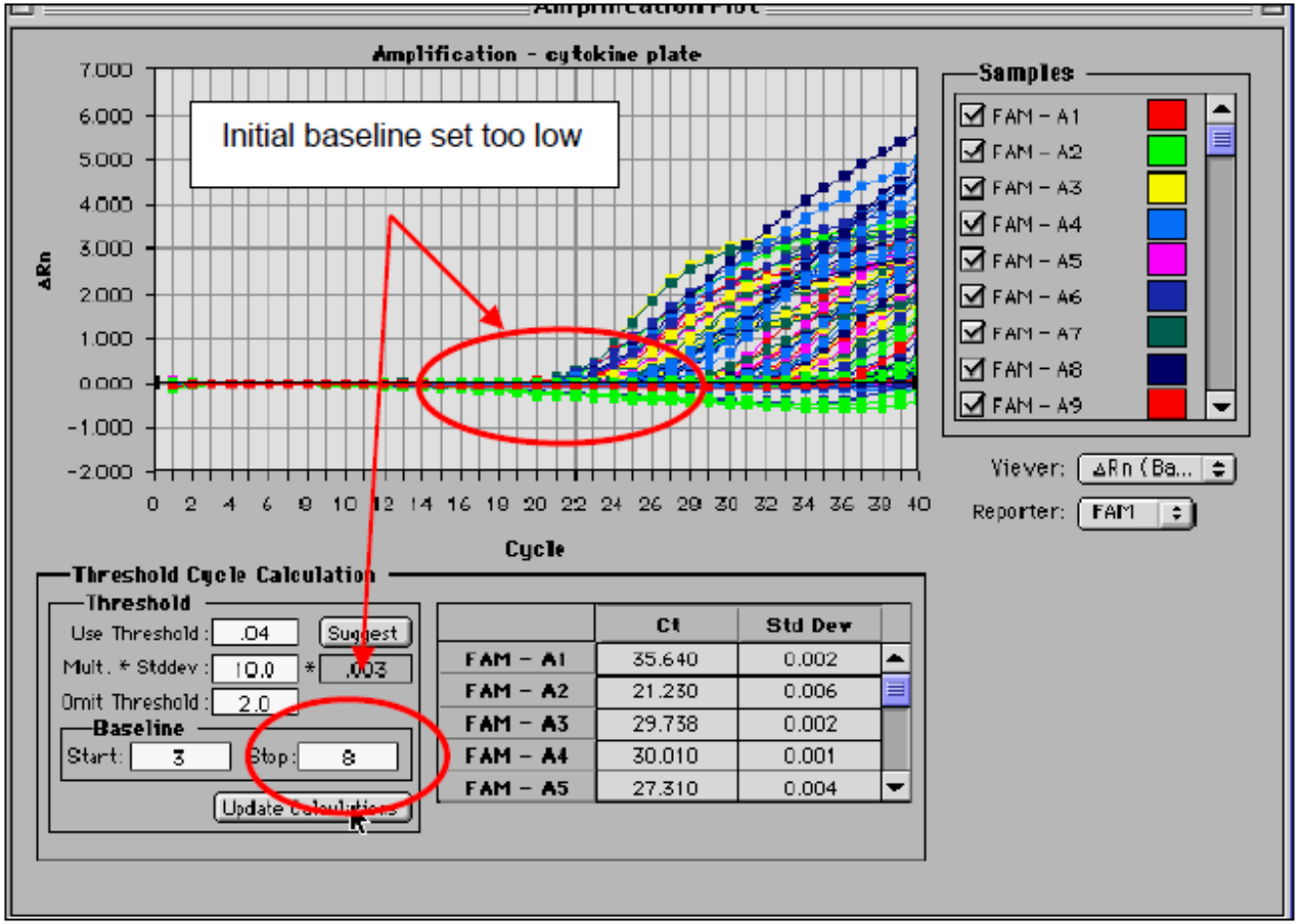


- Assess earliest CT.
- Adjust stop value to one to two cycles before amplification.
- In this case the stop value was determined to be 8.

Applied Biosystems

Baseline Setting

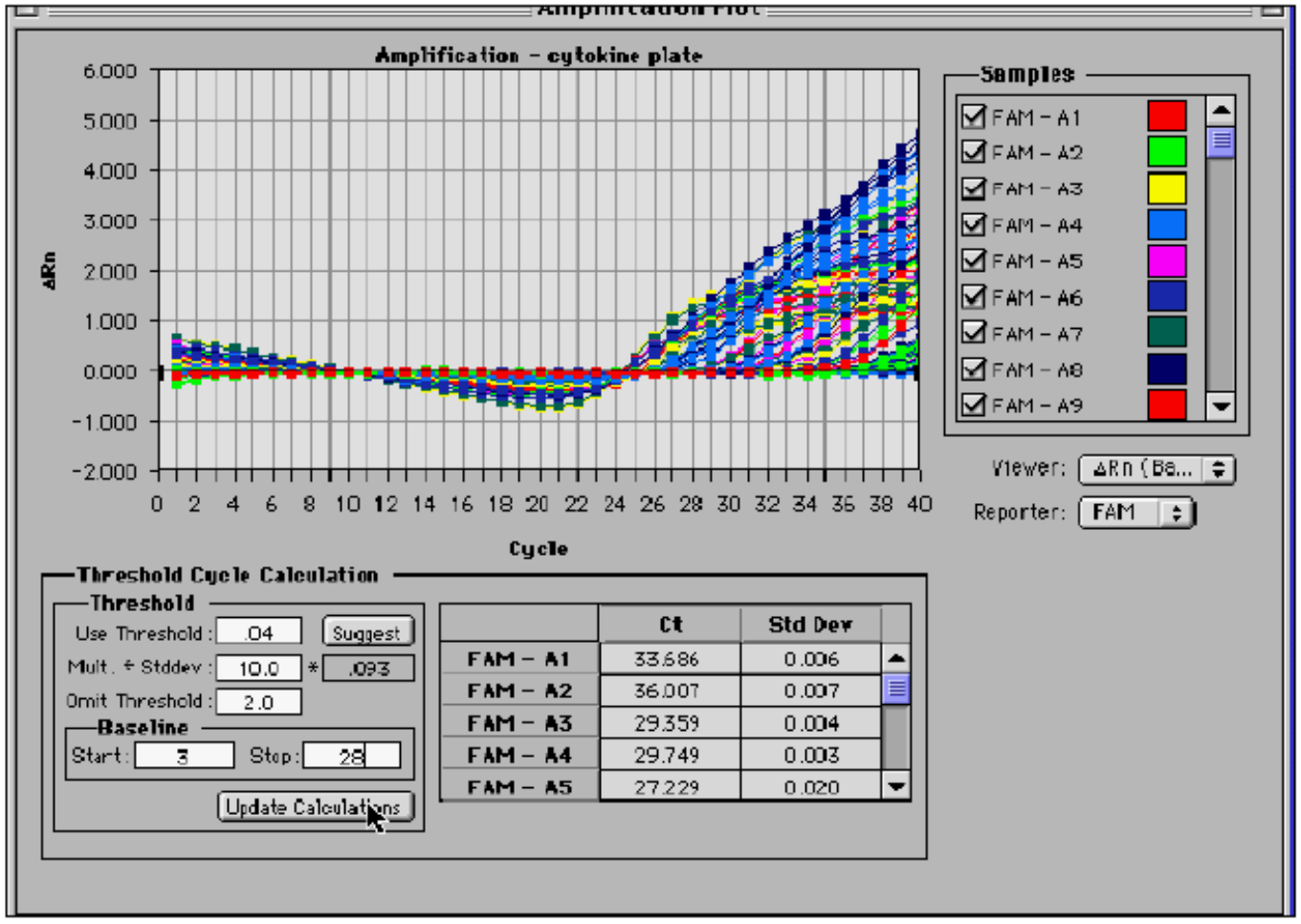
Figure 6: Baseline Setting Too Low



Applied Biosystems

Baseline Setting

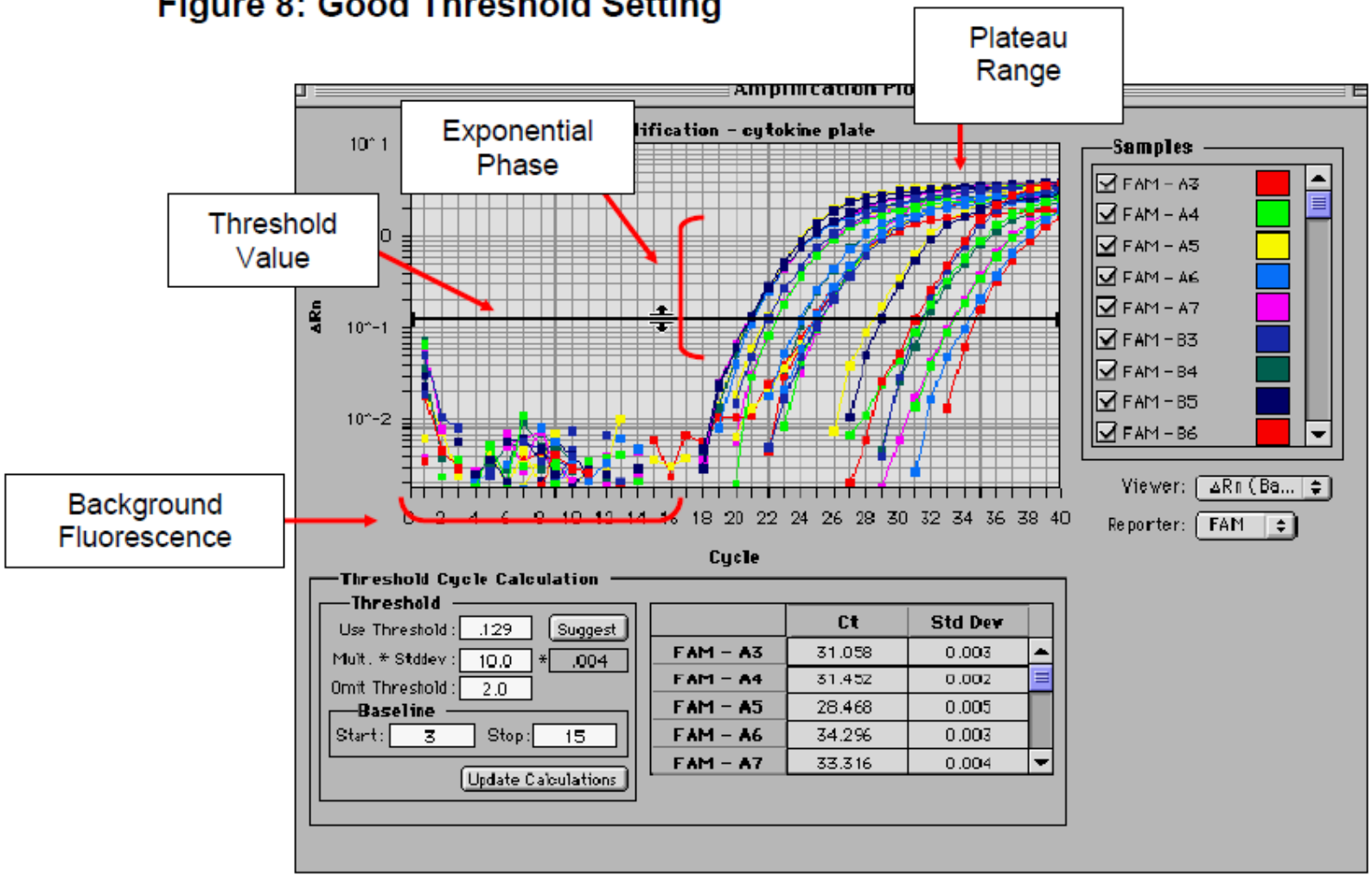
Figure 7: Baseline Setting Too High



Applied Biosystems

Threshold Setting

Figure 8: Good Threshold Setting

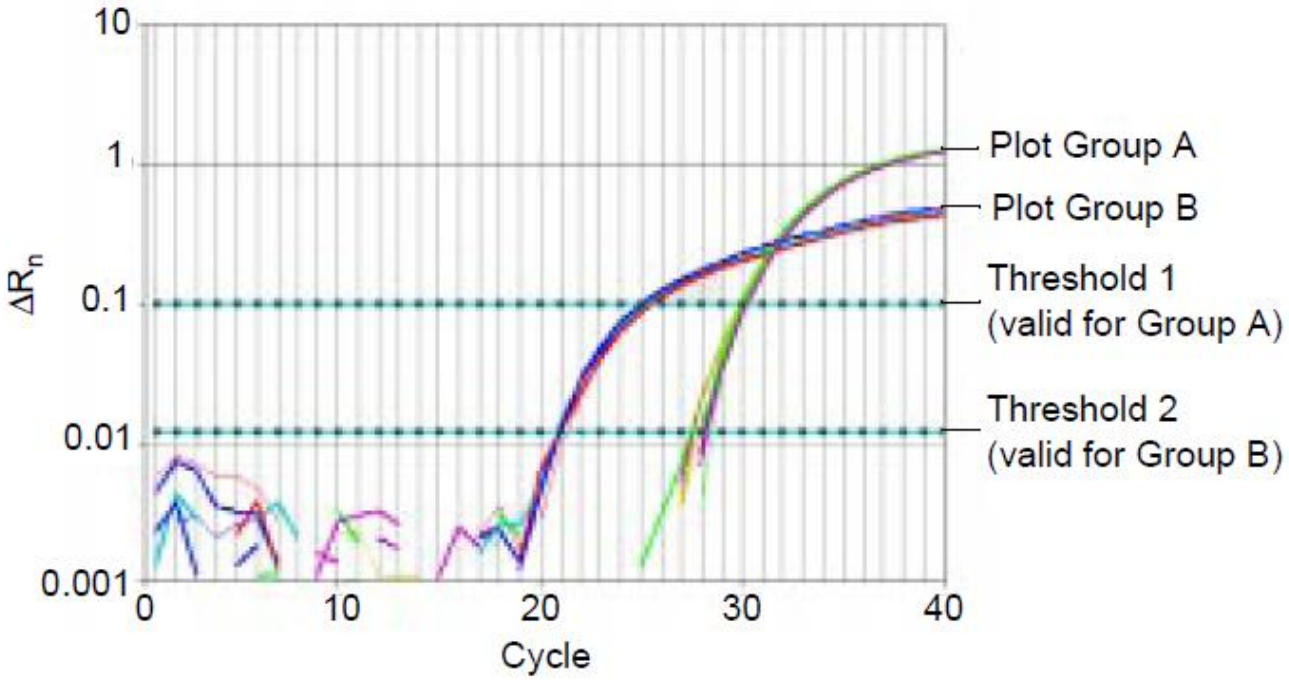


Easier to do in log-view

Applied Biosystems

Threshold Setting

Multiple Thresholds May be Necessary



Easier to do in log-view

TaqMan® Human Endogenous Control Plate

Applied Biosystems

Baseline and Threshold Setting

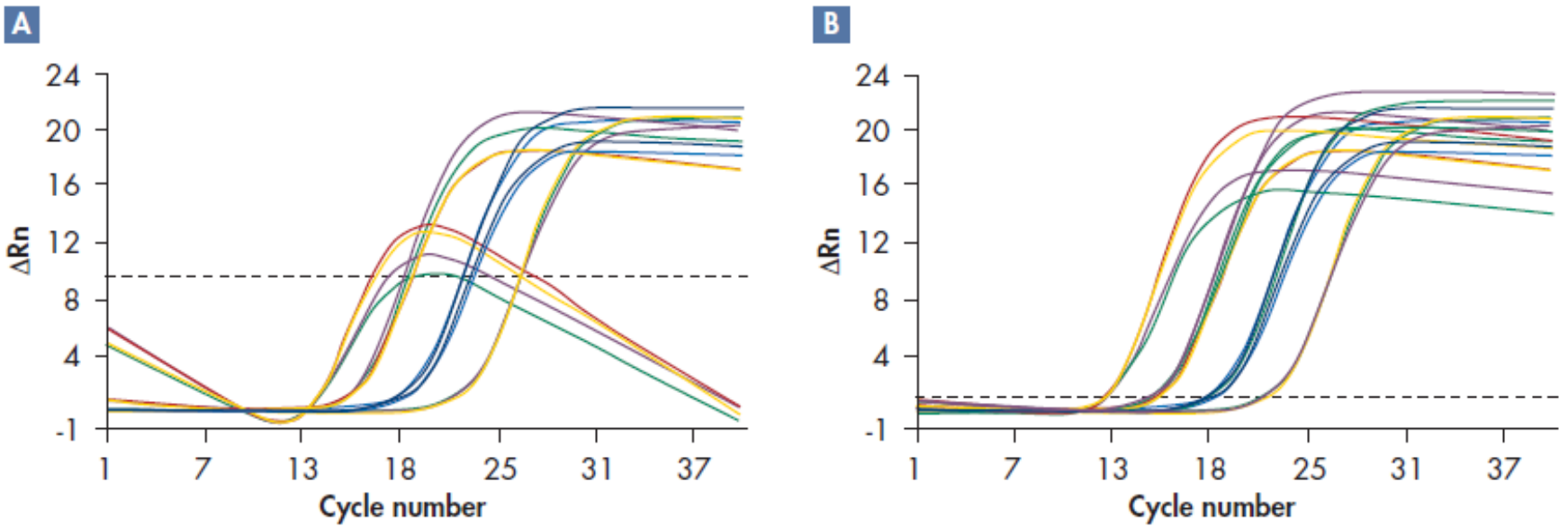


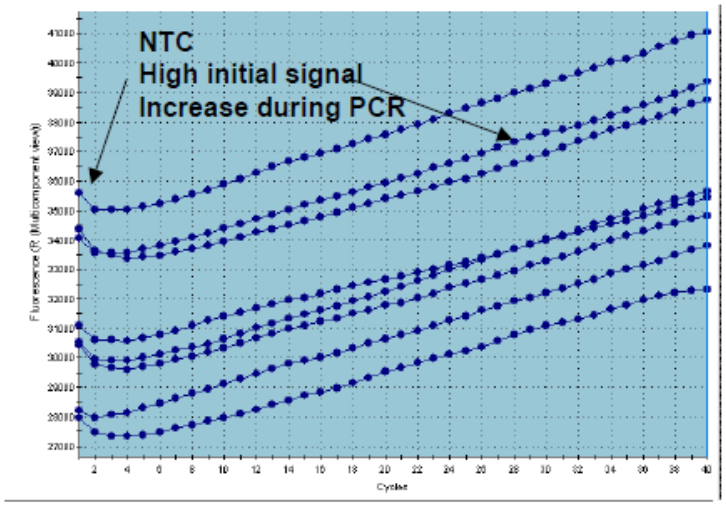
Figure 9. Correct baseline and threshold settings are important for accurate quantification. A Amplification product becomes detectable within the baseline setting of cycles 6 to 15 and generates a wavy curve with the highest template amount. B Setting the baseline within cycles 6 to 13 eliminates the wavy curve. The threshold is set at the beginning of the detectable log-linear phase of amplification.

High Background

High Background

There are several potential reasons for high background:

- SYBR based:
  - Too much SYBR
  - Too much template
- Probe based:
  - Insufficient quenching
    - → quencher doesn't fit to dye
    - → quencher too far from dye
  - probe concentration too high
  - probe degraded
  - free dye in your probe



High background leads to higher variability and lower sensitivity!

STRATAGENE

Standard Curve

Note: A 100% efficient reaction will yield a 10-fold increase in PCR amplicon every 3.32 cycles during the exponential phase of amplification ($\log_2 10 = 3.3219$).

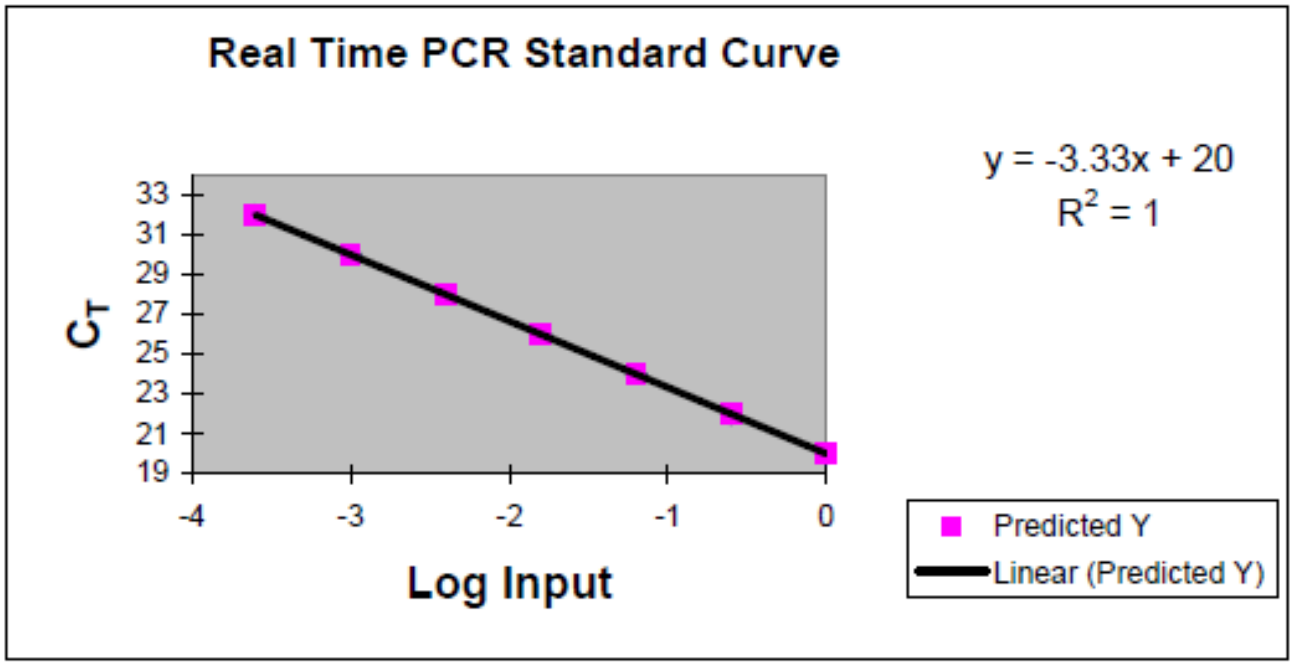


Figure 1: Real-Time PCR Standard Curve representing 100% PCR Efficiency

applied biosystems by life technologies

Low ΔRn/RFU

- **Imperfect assay design**
- **Incorrect quencher**
- **Bad template quality**

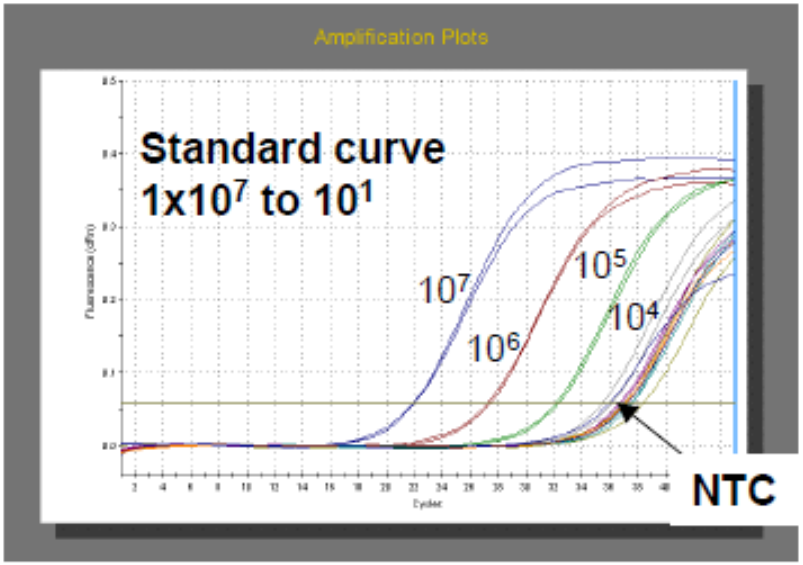
(long probe, G at 5’ end, incorrect choice of reporter and quencher pair, wrong primer-pair concentrations, probe has mismatches)

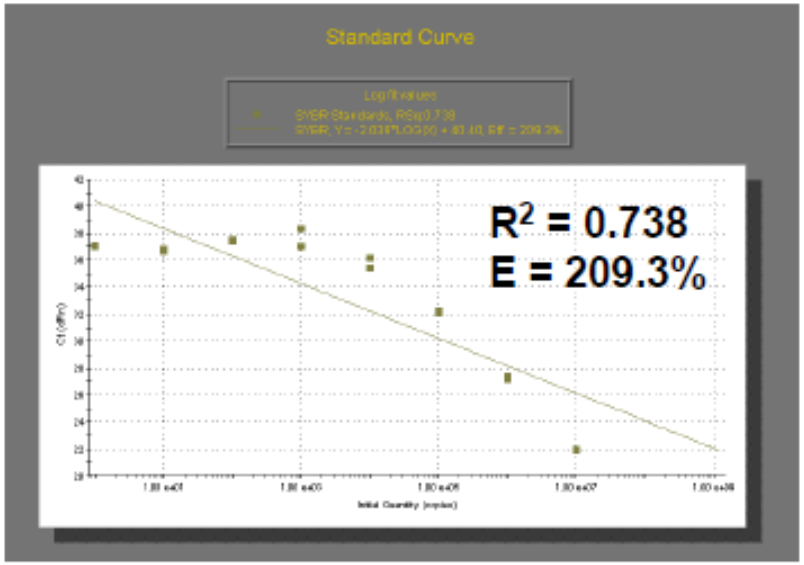
High efficiency?

Efficiency > 110% (slope <-3.1) is due to:

- **Pipetting errors**
- **Wrong threshold setting (not in the log-linear phase)**
- **Primer-dimers in SYBR green assays**
- **Probe degradation in TaqMan assays**
- **High variability at low concentrations**
- **PCR inhibition by reverse transcriptase**

High efficiency?



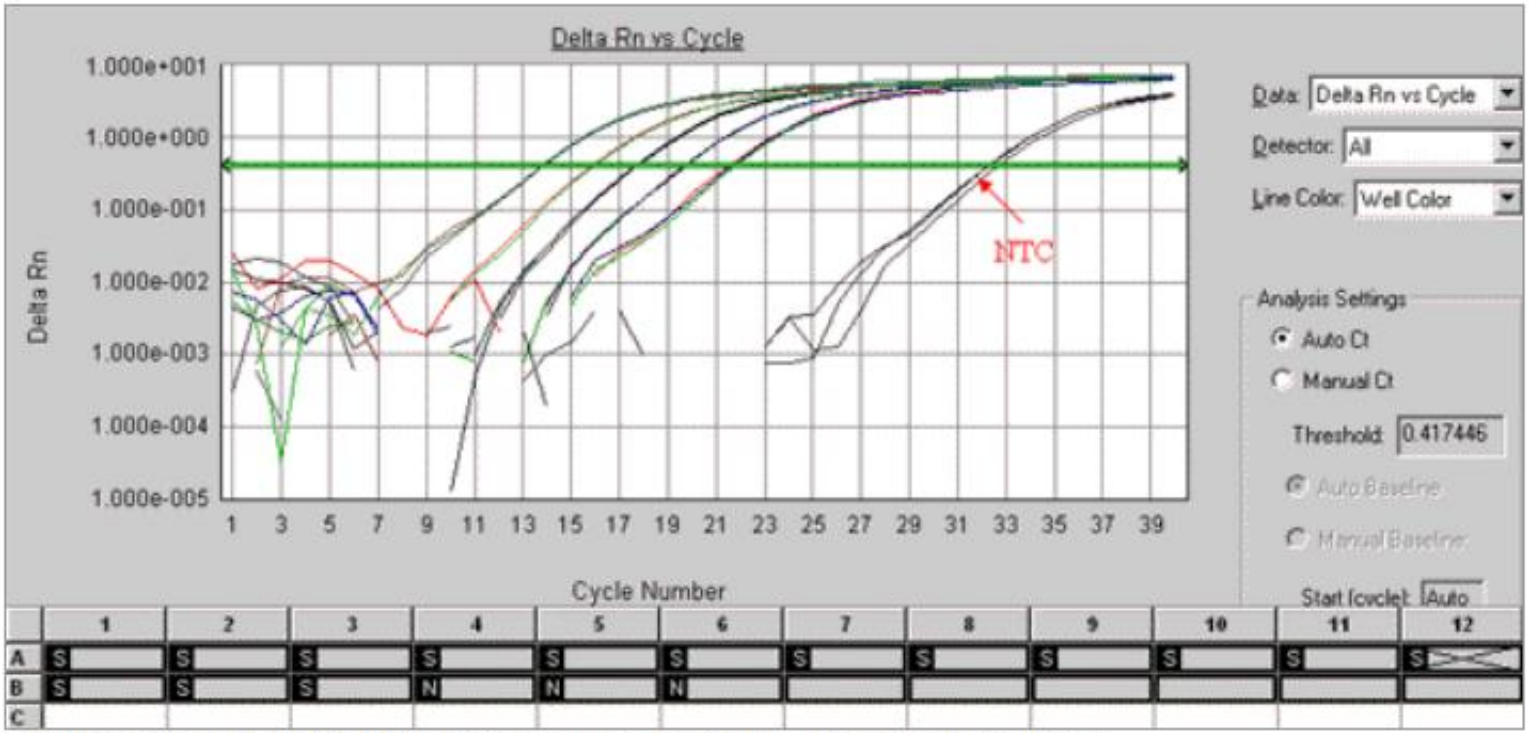


ΔCt: 10^7 → 10^6 5.4; 10^6 → 10^5 5.0; 10^5 → 10^4 3.6

Primer dimers, in combination with inefficient priming/inhibition

STRATAGENE

Amplification of the No Template Control (NTC)



Amplification of the No Template Control Due to Reagent Contamination

If you get an amplification product in your no template control (NTC), you may have one of these problems (select one):

- Contamination of your reactions by DNA
- Primer dimer formation (SYBR® Green chemistry only)

- **Five C_T difference rule**
- **SYBR green vs TaqMan**
- **Melting curve analysis**

applied biosystems by life technologies

RNA quality

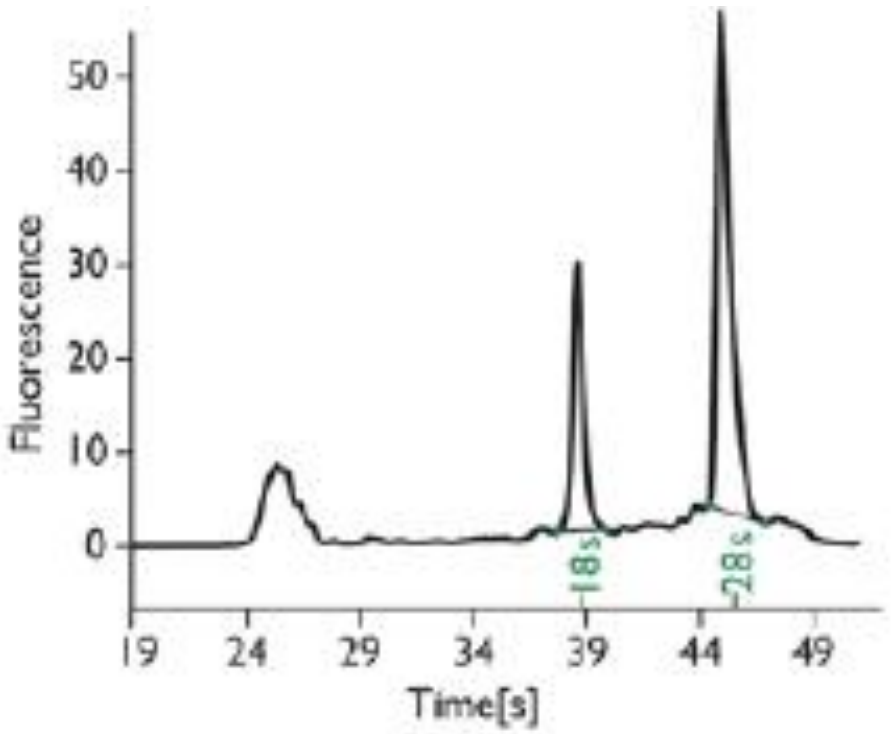


Figure 4. Agilent® 2100 Bioanalyzer Scans of High Integrity Total RNA. The 18S and 28S peaks are clearly visible at 39 and 46 seconds, respectively.

applied biosystems by life technologies

RNA quality: integrity

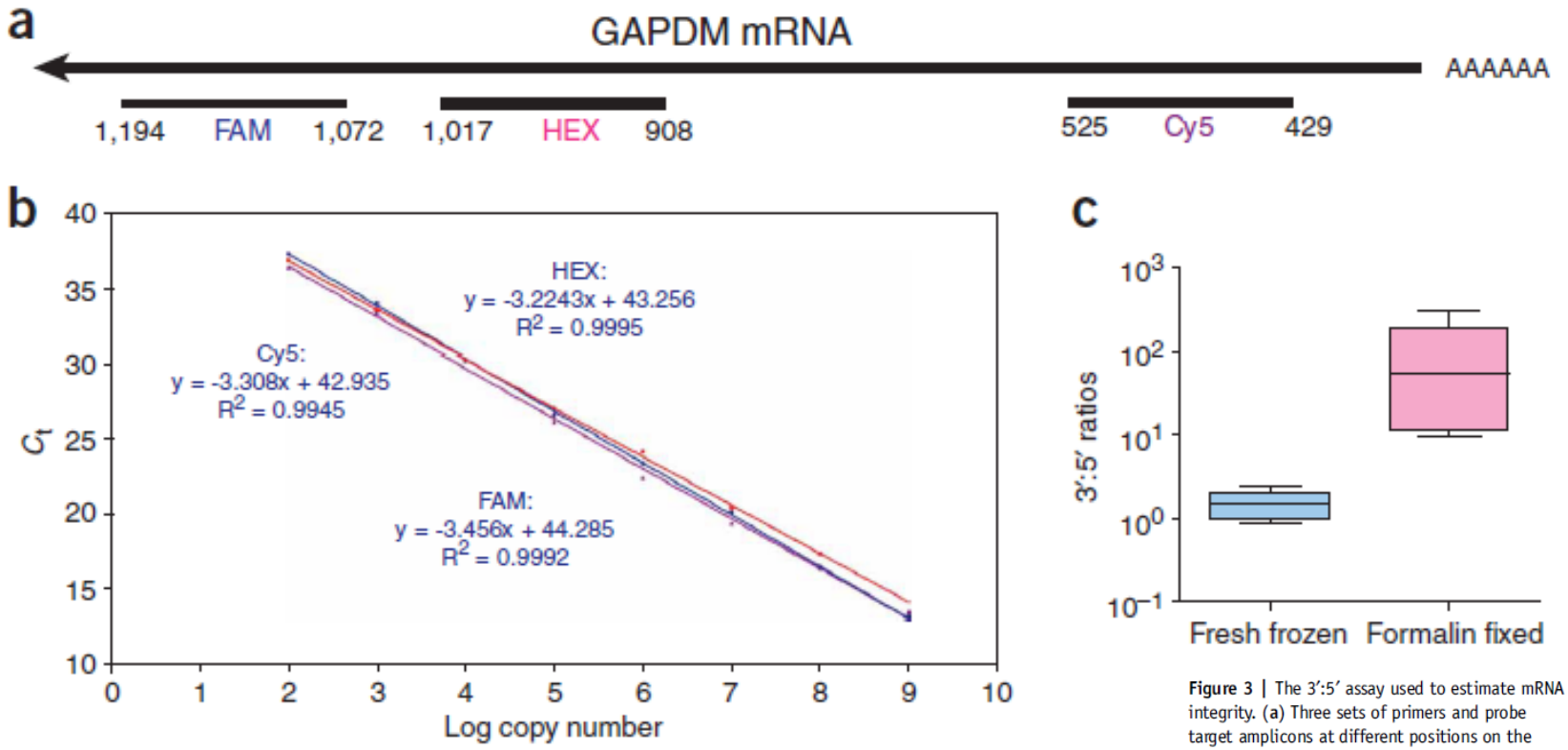


Figure 3 | The 3′:5′ assay used to estimate mRNA integrity. (**a**) Three sets of primers and probe target amplicons at different positions on the GAPDH (NM_002046) mRNA. (**b**) When run in a multiplex reaction, it is apparent that the amplification efficiencies of the three reactions are approximately the same, with similar y-intercepts indicating roughly similar sensitivity. (**c**) Difference in integrity between RNA extracted from fresh colonic biopsies on the left and RNA extracted from formalin-fixed, paraffin-embedded (FFPE) material on the right. Perfectly intact mRNA should result in a 3′:5′ ratio of 1–5, with denatured mRNA resulting in ratios of 10 or above. The median 3′:5′ ratio for GAPDH for the fresh biopsies is 2, indicative of high quality mRNA, whereas the median ratio for the FFPE biopsies is 90.

PROTOCOL

Quantification of mRNA using real-time RT-PCR

Tania Nolan[1], Rebecca E Hands[2] & Stephen A Bustin[2]

(www)

RNA quality: purity

TABLE 1 | Oligonucleotide sequences for RNA integrity and inhibition detection assays.

	Oligonucleotides (5′–3′)	Stock concentration
5′-GAPDH (FAM)	P: CCTCAAGATCATCAGCAATGCCTCCTG	5 μM
	F: GTGAACCATGAGAAGTATGACAAC	10 μM
	R: CATGAGTCCTTCCACGATACC	10 μM
Center GAPDH (HEX)	P: CCTGGTATGACAACGAATTTGGCTACAGC	5 μM
	F: TCAACGACCACTTTGTCAAGC	10 μM
	R: CCAGGGGTCTTACTCCTTGG	10 μM
3′-GAPDH (CY5)	P: CCCACCACACTGAATCTCCCCTCCT	5 μM
	F: AGTCCCTGCCACACTCAG	10 μM
	R: TACTTTATTGATGGTACATGACAAGG	10 μM
SPUD amplicon	AACTTGGCTTTAATGGACCTCCAATTTTGAGTGTGCACAAGCTATGGAACACCACGTAAGACATAAAACGGCCACATATG GTGCCATGTAAGGATGAATGT	5 μM
SPUD (FAM)	P: TGCACAAGCTATGGAACACCACGT	5 μM
	F: AACTTGGCTTTAATGGACCTCCA	10 μM
	R: ACATTCATCCTTACATGGCACCA	10 μM

P, dual labeled or TaqMan Probe (HPLC purified); F, forward primer (desalt purified); R, reverse primer (desalt purified).

PROTOCOL

Quantification of mRNA using real-time RT-PCR

Tania Nolan[1], Rebecca E Hands[2] & Stephen A Bustin[2]

(www)

RNA quantity

As long as you are staying within the dynamic range of the assay established by initial validation, use any amount, but no less than 10 picogram and no more than 1 microgram. Usually 1 to 100 nanogram is sufficient.

High template amount may increase background.

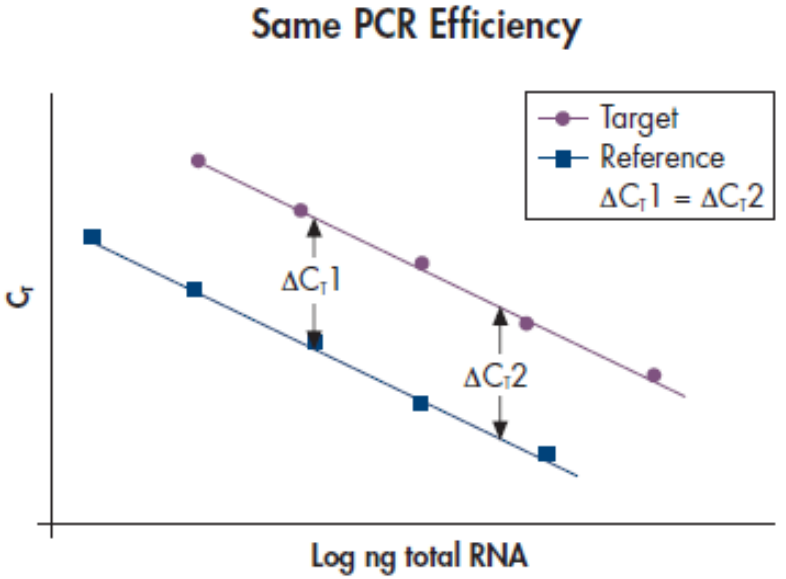
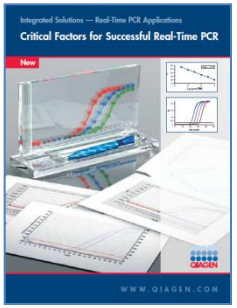


Figure 17 Typical standard curve showing amplification of two targets with similar PCR efficiencies.



Quantification of mRNA using real-time RT-PCR

Tania Nolan[1], Rebecca E Hands[2] & Stephen A Bustin[2]

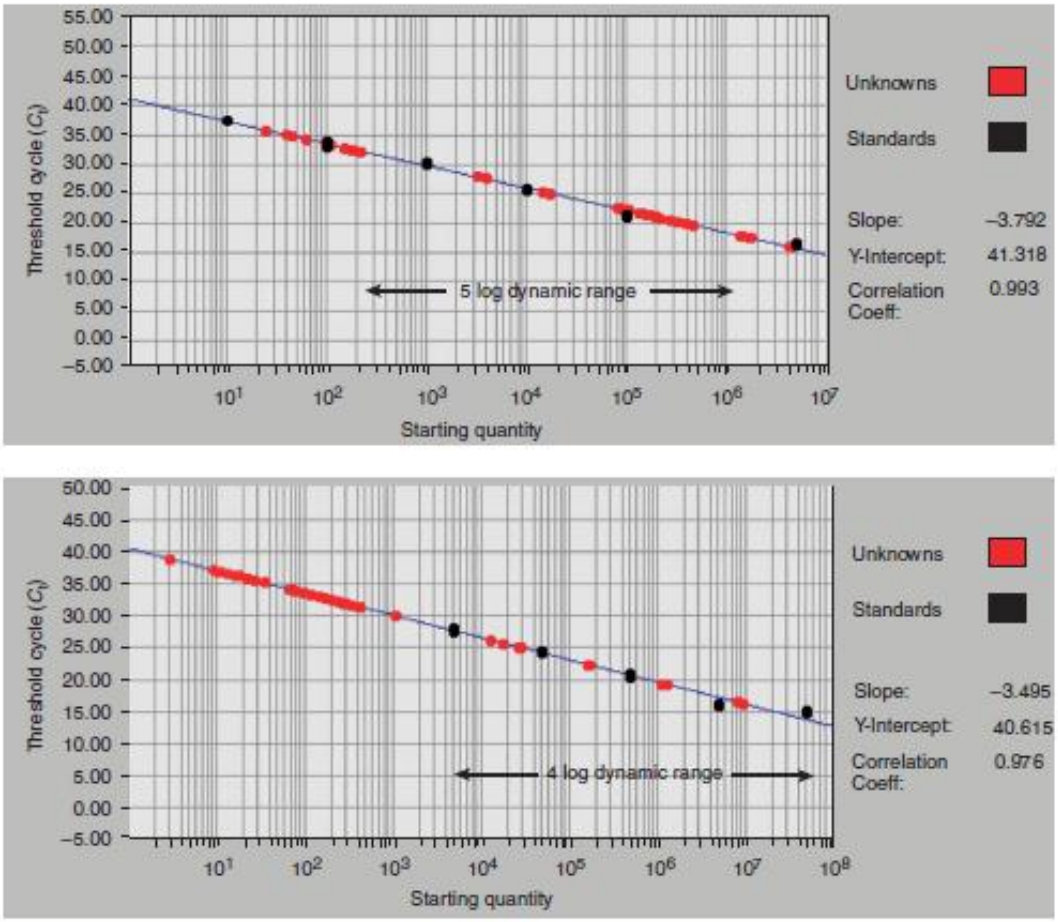


Figure 5 | Acceptable and unacceptable standard curves. All the unknowns (red dots) in the upper picture are contained within the dynamic range of the standard curve, which is demarcated by the two outermost points of the standard represented by black dots. This allows accurate quantification of the corresponding mRNAs. In the lower picture, a majority of the unknowns is below the highest dilution of the standards (4×10^3 copies).

(www)

Optimal template amount

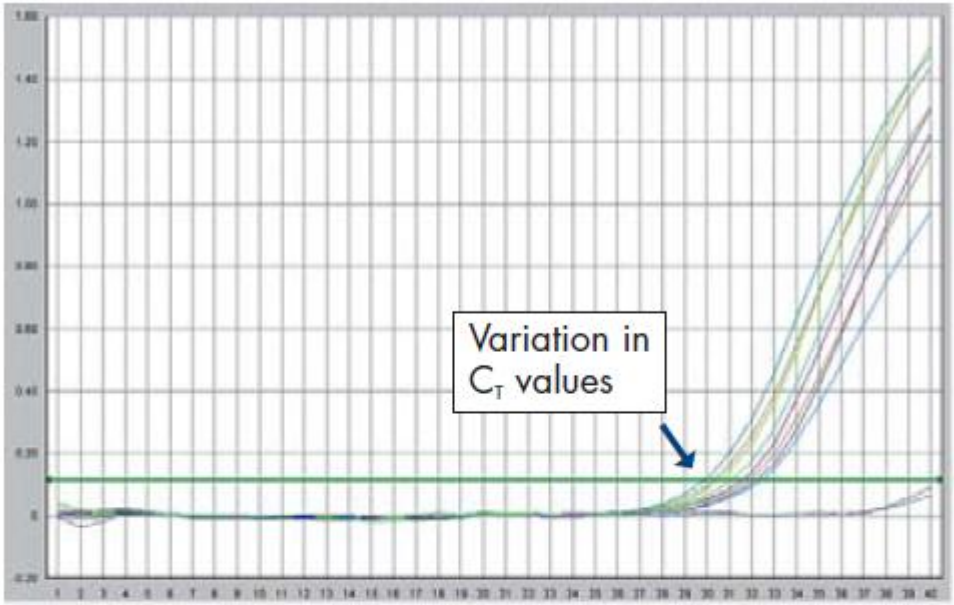


Figure 22. Stochastic problems when analyzing a low-copy transcript. Eight replicate RNA samples (1 ng each) were amplified using the QuantiTect Whole Transcriptome Kit. Real-time PCR analysis of NFκB transcript was then performed using 10 ng amplified cDNA and the QuantiFast Probe PCR Kit. The resulting C_T values were in the range of 30–32.5. This significant variation occurred because the stochastic variation in low-copy NFκB transcript in the replicate RNA samples is amplified, resulting in widely differing amounts of NFκB cDNA.

Table 9. Transcript representation in different cell amounts

	10^3 cells*	10^2 cells†	10 cells‡
Amount of RNA (ng)	20	2	0.2
No. of high-copy transcripts	10^7	10^6	10^5
No. of medium-copy transcripts	10^5	10^4	10^3
No. of low-copy transcripts	10^3	10^2	**10**
No. of mosaics transcripts	10^2	**10**	**1**

* Complete representation of all transcripts.

† Stochastic problems for mosaic transcripts.

‡ Stochastic problems for low-copy and mosaic transcripts.

§ Stochastic problems for low-copy transcripts and loss of mosaic transcripts.

Optimal template amount

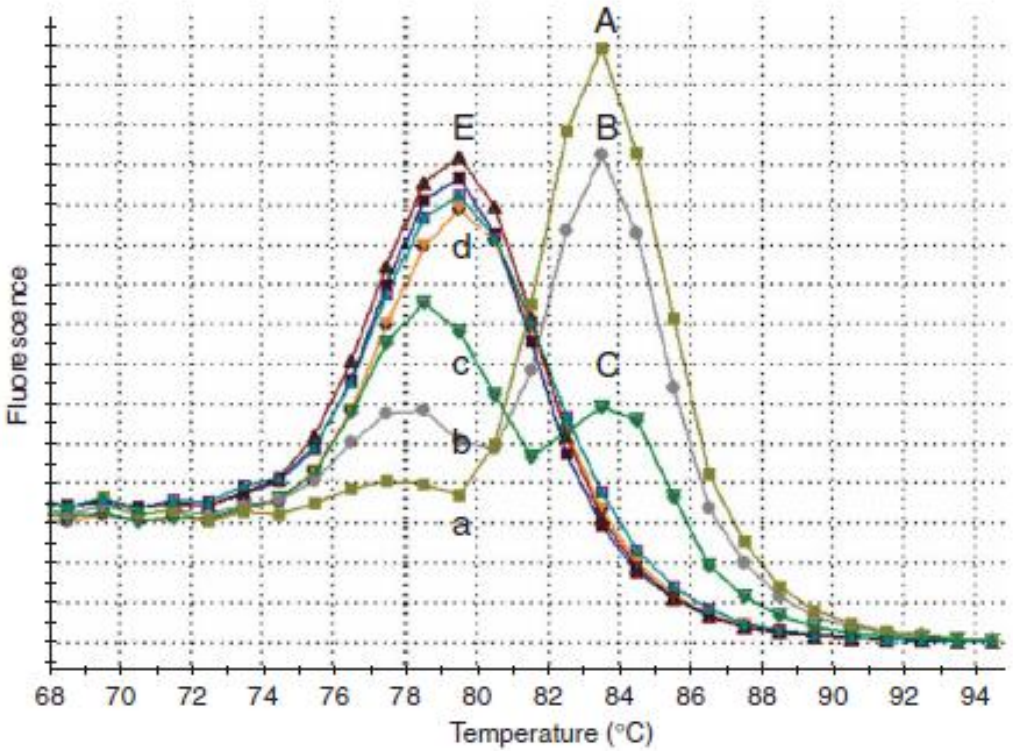


Figure 11 | SYBR Green melt curve demonstrating the appearance of primer dimers in a poorly designed assay. At high RNA concentration (50 ng), a single melt curve appears at around 83 °C (trace A), with no primer dimer (trace a). Two serial 10-fold dilutions result in a reduction in the 83 °C peak (B,C) and the appearance of a second peak at around 79 °C (b,c). Further dilution results in the absence of any target-specific amplification and an increase in the nonspecific peak (d). The position of peaks b–d is identical to the no-template control (NTC) (E).

PROTOCOL

Quantification of mRNA using real-time RT-PCR

Tania Nolan[1], Rebecca E Hands[2] & Stephen A Bustin[2]

(www)

Reaction volume

- Manufacturers recommend 50 microL
- 25 microL is fine
- 10 microL is generally fine
- 5 microL may be fine (for allelic discrimination) but not recommended for qPCR
- Aim for 3-5 microL template volume in the reaction

- Aim for duplicates unless using so little template (around 10 picogram or Ct > 35); then you need to use triplicates

cDNA synthesis

Table 3: Comparison of RT primers for cDNA synthesis

Primers for cDNA synthesis	Considerations
Sequence-specific primer	• Use to reverse transcribe gene specific RNA sequences only • Only primer type recommended for one-step RT-PCR • Can also be used in two-step RT-PCR
Oligo $d(T)_{15}$	• Use to reverse transcribe only eukaryotic mRNAs and retroviruses with poly-A tails • Not all genes have polyA-tails. Cannot reverse transcribe 18S rRNA • May have trouble transcribing long mRNA transcripts (> 1500 bp) or transcripts containing hairpin loops (secondary structure) • May not efficiently reverse transcribe degraded RNA due to loss of intact poly-A tail. • Tends to show bias towards 3' end of transcript
Random Primers (ex. hexamers)	• Can simultaneously reverse transcribe all mRNAs as well as 18S rRNA (i.e. targets + endogenous controls) • Try first for use with long transcripts, transcripts containing hairpin loops, and degraded RNA • Not biased to 3' end of transcript

Applied Biosystems recommends the use of random primers for performing the two-step RT-PCR method. With this approach, you can generate cDNA from a library of transcripts in a single reverse transcription reaction; therefore, you can perform real-time PCR quantitation of many different targets from one pool of cDNA.

mRNA in formalin fixed samples may have lost their poly-A tails.

applied biosystems by life technologies

cDNA synthesis

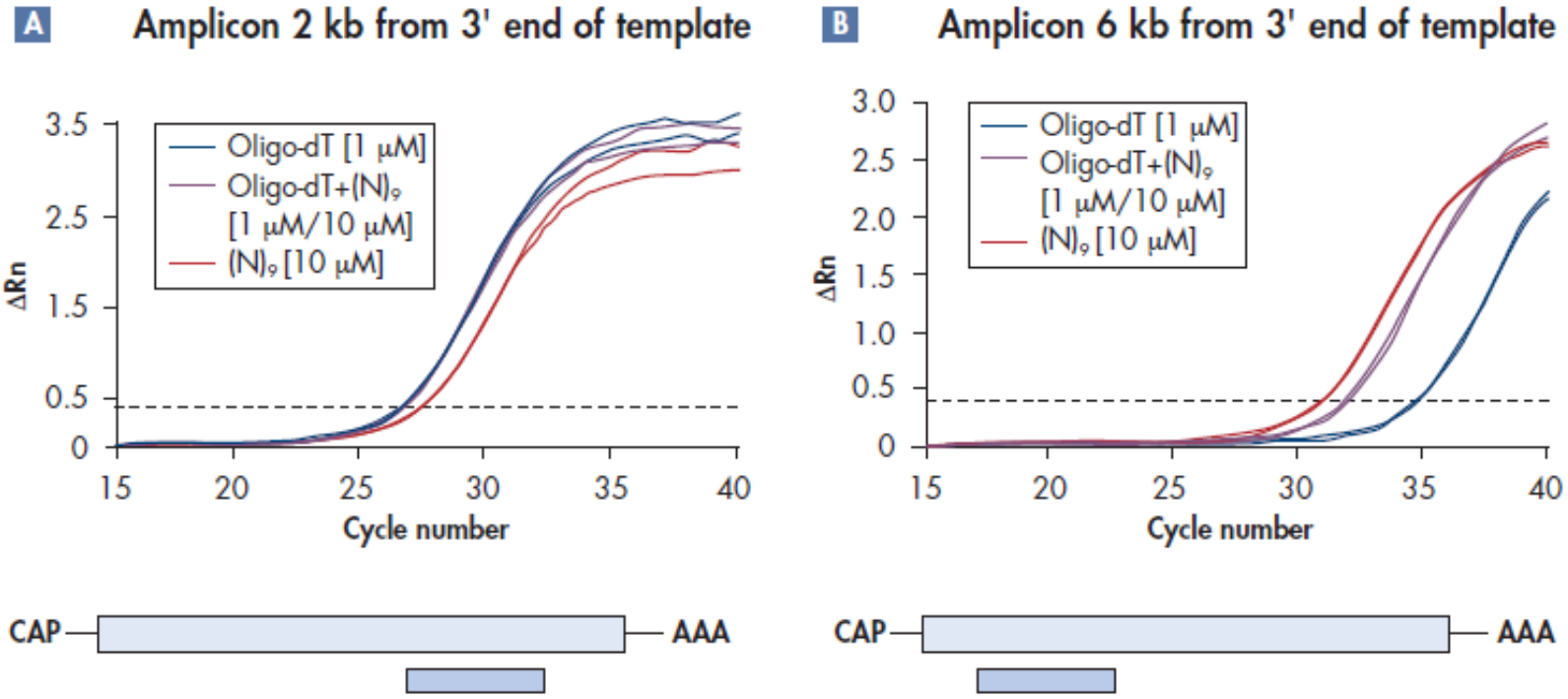


Figure 23. Effect of RT primer choice on RT-PCR. Two-step RT-PCR was carried out using the QuantiTect SYBR Green PCR Kit and the primer combinations shown. The amplicon was **A** 2 kb from the 3' end or **B** 6 kb from the 3' end of the template RNA.

Primer design

Table 13. Primer design for real-time PCR

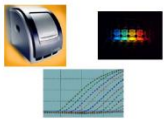
Sequence:	■ Length of PCR product should ideally be less than 150 bp
	■ Avoid complementary sequences within and between primers and probes
	■ Avoid mismatches
	Avoid a 3'-end T as this has a greater tolerance of mismatch
Length:	18–30 nucleotides
GC content:	40–60%
T_m: (simplified)	$T_m = 2°C \times (A+T) + 4°C \times (C+G)$

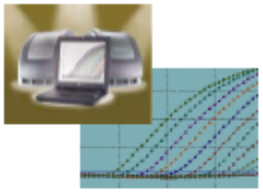
Primer design

Primer Selection

- Try to achieve similar Tm for all primers: Ideal ~60°C. (Future multiplexing or use of Taqman™ assays in mind)
- Forward and reverse primer should have ΔTm <2°C
- 40-60% GC content to prevent G/C region self-hybridization
- ΔG of primer dimer/cross primer dimer formation > -4 kcal/mol to avoid stable primer dimers
- Design via software (Always use the same one):
- Always perform a BLAST search with your amplicon and primers
  (→ Specificity of the PCR)

STRATAGENE QPCR Systems





Step 5: Real-time PCR

Assay Design

Amplicons

Amplicon length affects assay performance:
QPCR uses small amplicons between 70-200 bp

Primers

Avoid long primers:
Primers should be in the range of 17-25 bp
Design cDNA specific primers over exon junctions:
Avoids amplification of genomic DNA
T_m of Primers should be at 60°C
Reduces risk of primer dimers and enables running multiple assays on the same plate or using primers in a probe based assay

Probes

Avoid long probes:
Probes should be in the range of 17-30 bp
Probes should have a T_m that is 10°C higher than primers
Probes should bind before primers do

Quantification of mRNA using real-time RT-PCR

Tania Nolan[1], Rebecca E Hands[2] & Stephen A Bustin[2]

" Primer concentrations may result in severe changes in CT values and should remain constant in all experiments for the same assay "

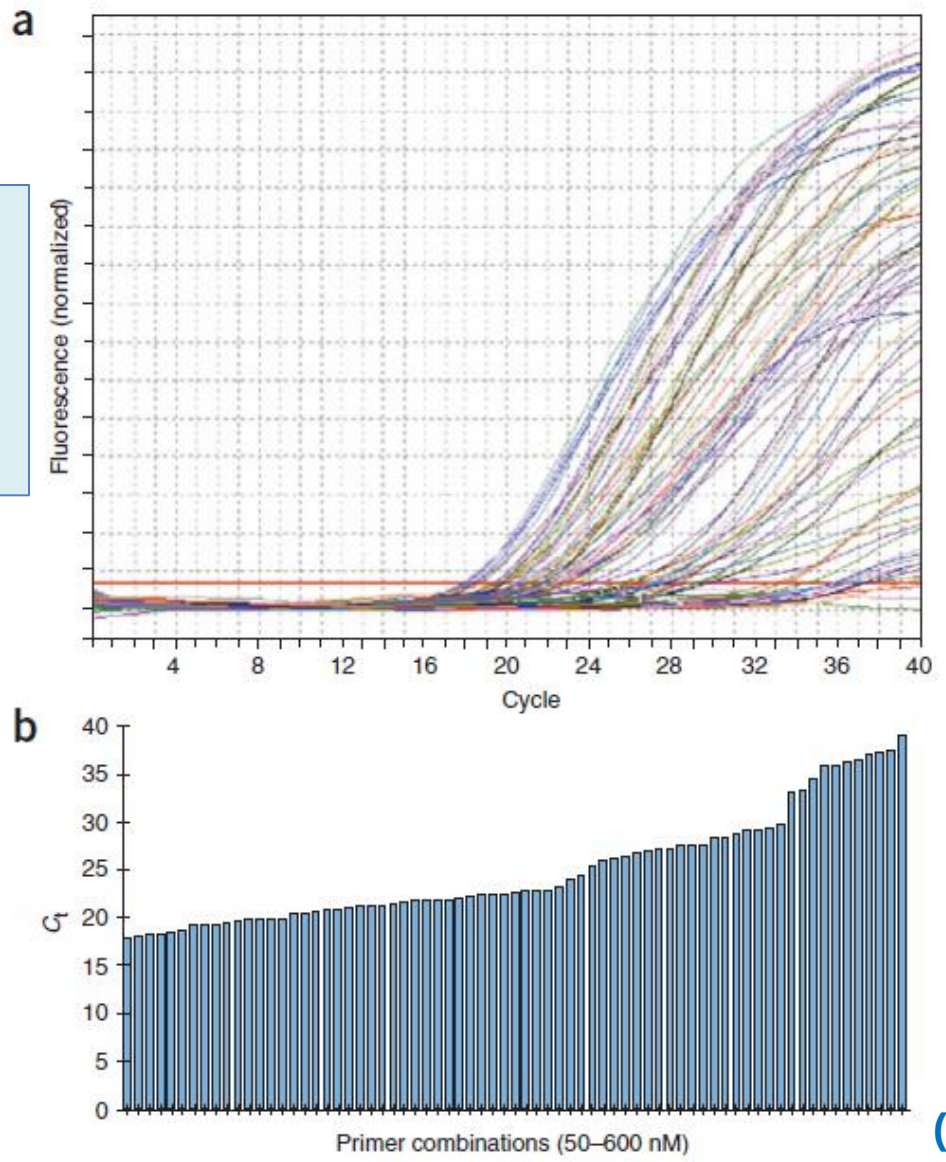


Figure 4 | Optimization of primer concentration. cDNA derived from total RNA (mouse) was amplified using PCR primers specific to Hepcadin 1 (ref. 62). Since primer conditions were not specified, all combinations of forward and reverse primer concentrations ranging between 50 nM and 600 nM were used, similar to the abridged mismatched primer concentration matrix (**Table 2**). (**a**) The amplification plots reveal the huge differences in C_t, slope and plateau obtained using identical templates. Each amplification plot represents a different combination of primer concentration. The horizontal red line represents the threshold, which was set automatically by the instrument. (**b**) The different primer concentrations resulted in a C_t range of more than 20 cycles with low concentrations of the forward primer corresponding to high C_t values. The most appropriate primer combination is the one that gives the lowest C_t and the highest normalized fluorescence.

(www)

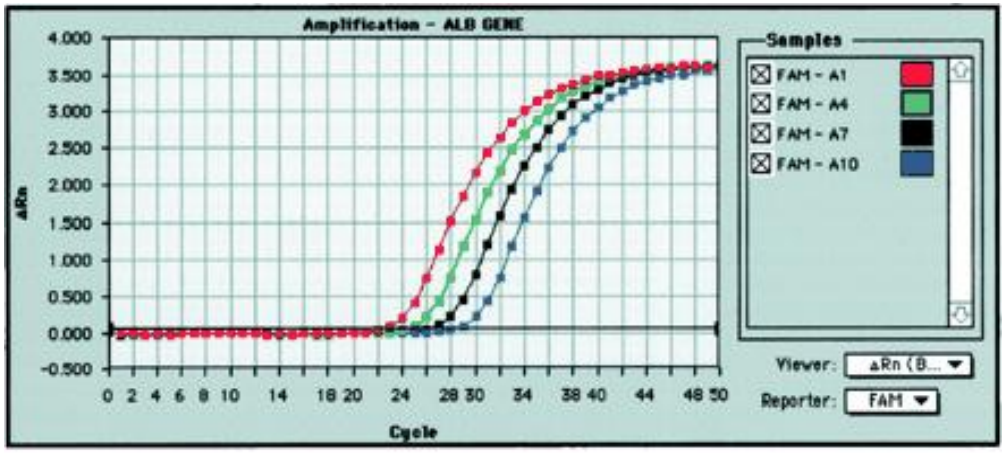
Pipetting

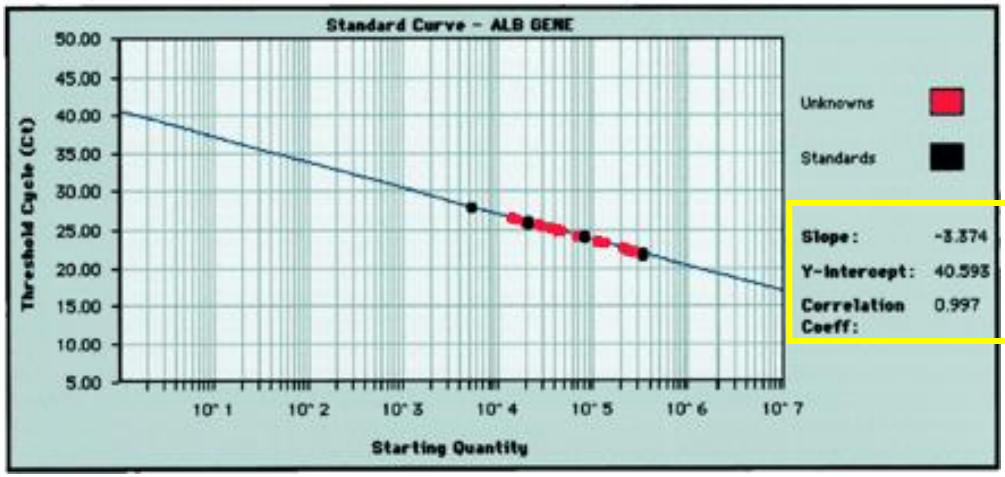
Table 2: Consequences of inaccurate pipetting

Pipetting problem	Consequence
Sample: Poor pipetting of identical replicates	High C_T standard deviations
Standards: Poor pipetting of standards	High C_T standard deviations (identical replicates), R^2 value <0.99
Standards: Consistent pipetting excess of diluent in serial dilution (ex. 100 µL instead of 90 µL)	Potentially good R^2 value ≥0.99, however slope of standard curve will be inaccurate; perceived lower PCR efficiency of assay
Standards: Consistent pipetting deficit of diluent in serial dilution (ex. 80 µL instead of 90 µL)	Potentially good R^2 value ≥0.99, however slope of standard curve will be inaccurate; perceived higher PCR efficiency of assay
Standards: Consistent pipetting excess of standard sample in serial dilution (ex. 12 µL instead of 10 µL)	Potentially good R^2 value ≥0.99, however slope of standard curve will be inaccurate; perceived higher PCR efficiency of assay
Standards: Consistent pipetting deficit of standard sample in serial dilution (ex. 8µL instead of 10 µL)	Potentially good R^2 value ≥0.99, however slope of standard curve will be inaccurate; perceived lower PCR efficiency of assay

applied biosystems by life technologies

Good Assay





Good efficiency, good sensitivity and good predictive power.

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

Laurendeau et al. Clin Chem 1999 (www)

Good Assay

TABLE 1. Performance Evaluation of Real-Time PCR

Factors	Recommendations	Criteria
Efficiency	Serial dilution with 5-log dilutions	Slope~ -3.3
		$R^2 > 0.99$
Precision	Minimum of 3 replicates	Standard deviation < 0.167
Sensitivity	High replicate number of reactions for low copy number sample input due to Poisson distribution	Statistical test analysis

ABI Understanding C_T (www)

Good Assay

- Increase specificity: Get rid of unspecific amplification
  eg. primer dimers

- Increase sensitivity: Get earlier Ct values, detect lower
  concentrations

- Increase reproducibility: Low replicate variability,
  high amplification efficiency

Optimized for:

- **High signal intensity: High RFU / ΔRn**
- **Low background (noise)**
- **Low Ct values**
- **Maximum Ct = 40 for lowest template amount in dilution series**

The Benefit of Controls

Controls enable you to understand unexpected results and are necessary components of assay validation:

No Template Control (NTC):
Template contamination, primer dimers, probe degradation

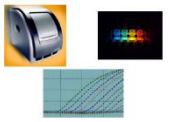
No Reverse Transcriptase Control (noRT):
Amplification from genomic DNA

Negative Sample:
Non-specific amplification (non-specific primer/probe binding)

Positive Controls:
Validating assay performance, control for Inhibition

Only controls will tell you which data is good or bad!

STRATAGENE

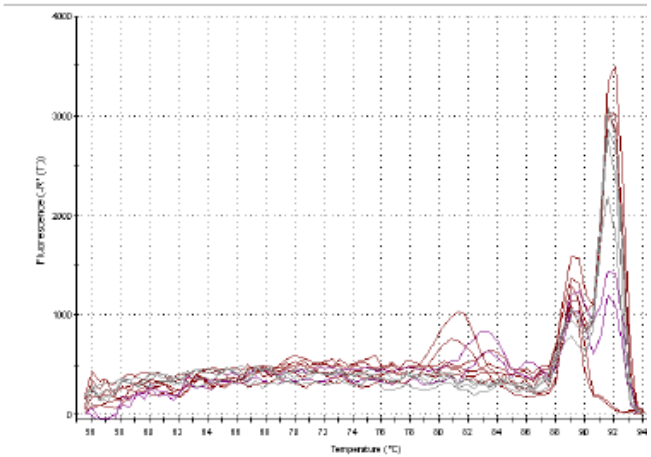


Check your Controls

There are several reasons for positive negative controls:

- Primer dimers seen in SYBR
- Probe degradation during PCR process
- Template contamination
- non-specific amplification due to mispriming or non-specific probe binding

SYBR meltcurves are a good tool to find out about the reasons for positive negative controls



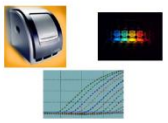
STRATAGENE

STRATAGENE

Controls

- No Template controls (NTC)
  - No cDNA added to QPCR reaction
  - Detects primer dimer, contaminating template, or probe degradation across cycles
- No Reverse Transcription Control (NoRT)
  - RNA sample undergoing reaction w/o RT
  - Detects contaminating gDNA in RNA
- No Amplification Control (NAC)
  - No Taq DNA polymerase added to QPCR reaction
  - May indicate high background

STRATAGENE QPCR Systems



$\Delta\Delta C_T$ Assay

a. A Validation Experiment Is Necessary to Determine If Your $\Delta\Delta C_T$ Calculation Is Valid.

Note: TaqMan® Gene Expression Assays have amplification efficiencies of 100%, in high quality samples. AB has extensively tested the design parameters and is confident that the resulting assays will have 100% efficiency (±10%) when measured over a 6-log dilution range, in samples that are free of are free contaminants (carryover phenol, proteins, inhibitors etc).
Note: The experimental values presented are taken from User Bulletin #2, page 14.

Guidelines for a Validation Experiment

Note: Accurate real-time PCR results depend on a variety of reagent, experimental set-up, sample quality, and analysis factors. For a listing of factors involved in quality real-time PCR results, see Factors Affecting Accurate Real Time PCR Results.

The guidelines for running an effective validation experiment are:

1. The input of cDNA should ideally span 5 to 6 logs (i.e.100 ng to 10 pg) and span the expression levels of your target(s). The initial results will help you in assessing the dynamic range of each assay. Additional details on assessing the dynamic range are in "Determination of input RNA amounts to be used in a relative quantitation study".

Note: It may not be possible to include all datapoints in your validation experiment

3. Run the target and endogenous control reactions in separate wells (singleplex reactions).
4. The primer and probe concentrations should be at the recommended levels of 900 nM for each primer and 250 nM for each probe. If optimization experiments yield alternative optimum primer and/or probe concentrations, adjust the concentrations accordingly.

applied biosystems by life technologies

$\Delta\Delta C_T$ Assay: Validation

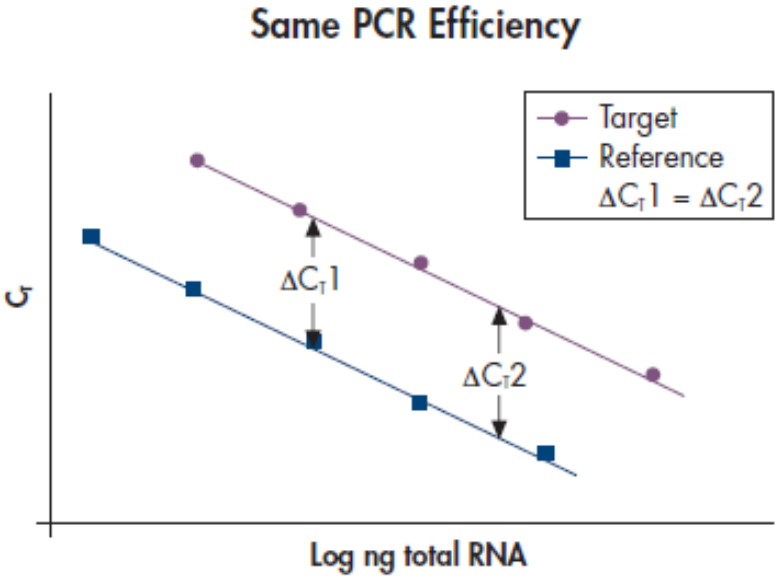


Figure 17 Typical standard curve showing amplification of two targets with similar PCR efficiencies.

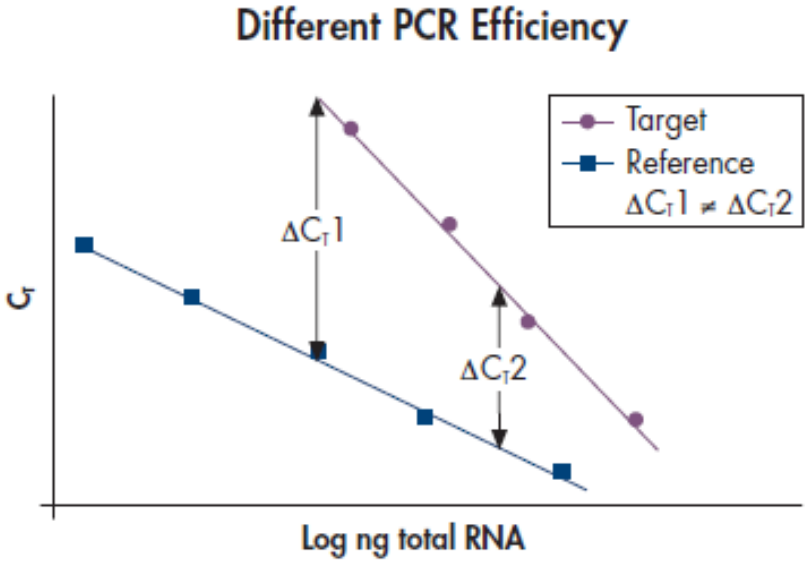
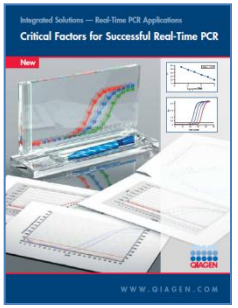


Figure 15 Typical standard curve showing amplification of two targets with different PCR efficiencies.



$\Delta\Delta C_T$ Assay: Validation

Therefore, the slope of the line in this example is 0.0472 (see Figure 6). This would be considered a passing validation experiment because the absolute value of the slope of ΔC_T vs. log input is < 0.1.

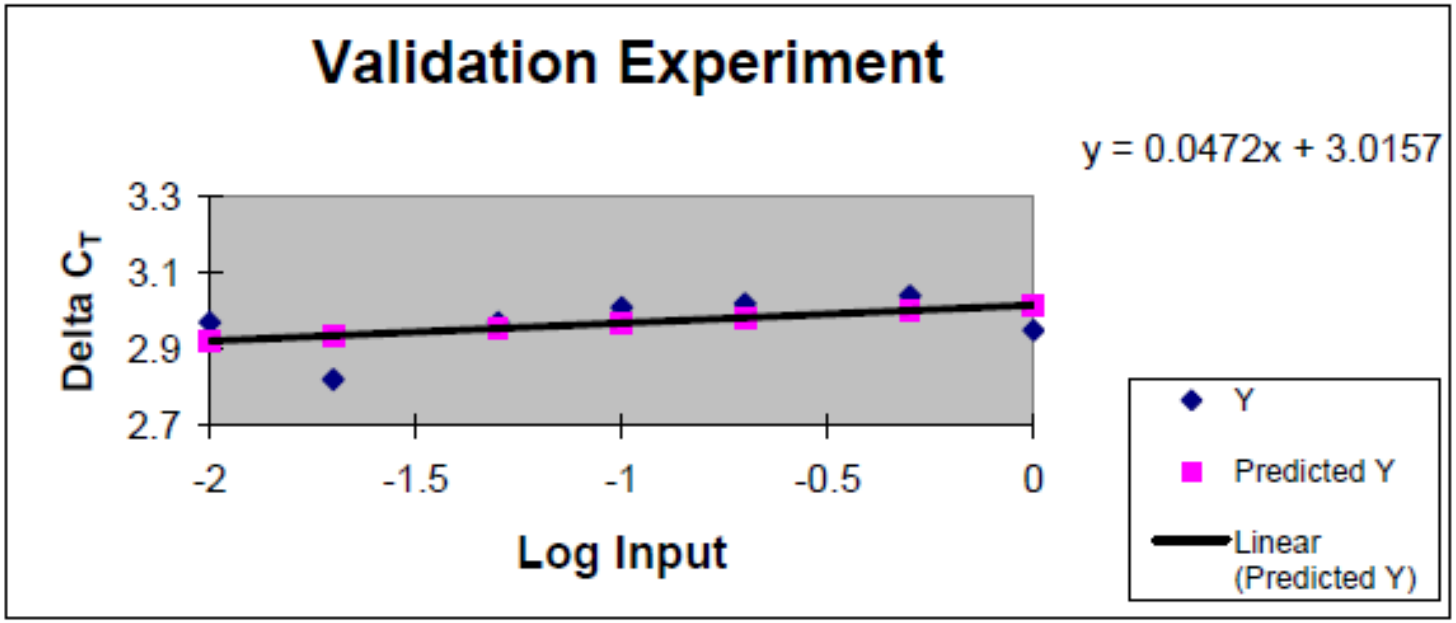


Figure 6: Validation plot of ΔC_T vs. log input amount of RNA

applied biosystems by life technologies

$\Delta\Delta C_T$ Assay

Table 11: Fold change expression of c-myc after treatment, calculated by $\Delta\Delta C_T$ method

Sample	c-*myc* Average C_T	GAPDH Average C_T	ΔC_T c-*myc*-GAPDH	$\Delta\Delta C_T$ ΔC_T treated -ΔC_T untreated	Fold difference in c-*myc*$_N$ relative to untreated
untreated	30.49±0.15	23.63±0.09	6.86±0.19	0.00±0.19	1 (0.9-1.1)
Drug treatment A	27.03±0.06	22.66±0.08	4.37±0.10	–2.4±0.10	5.6 (5.3-6.0)
Drug treatment B	26.25±0.07	24.60±0.07	1.65±0.10	–5.11±0.10	37 (34.5-39.7)
Drug treatment C	25.83±0.07	23.01±0.07	2.81±0.10	–3.95±0.10	16.5 (15.4-17.7)

applied biosystems by life technologies

Dye selection

Table 15. Combinations of reporter dyes for multiplex assays using QuantiFast and QuantiTect Multiplex Kits

Cycler	Reference dye	Dye 1†	Dye 2†	Dye 3†	Dye 4†
ABI PRISM 7700	ROX	FAM	HEX, JOE, VIC	—	—
ABI PRISM 7000 and 7900, Applied Biosystems 7300, StepOnePlus	ROX	FAM	HEX, JOE, VIC	Bodipy TMR, NED	—
Applied Biosystems 7500	ROX	FAM	HEX, JOE, VIC	Bodipy TMR, NED	Alexa Fluor 647, Cy5
iCycler iQ and iQ5	Not required	FAM	HEX, JOE, TET, VIC	Texas Red, ROX	Cy5
LightCycler 2.0	Not required	FAM	HEX, JOE, VIC	Texas Red, ROX	Alexa Fluor 660, Bodipy 630/650, Pulsar® 650
Mx3000P®, Mx3005P®	Not required	FAM	HEX, JOE, VIC	Texas Red, ROX	Cy5
Rotor-Gene 6000	Not required	FAM	HEX, VIC	ROX	Quasar® 705

* Visit www.qiagen.com/multiplex to view dye combinations for other cyclers, including dye combinations for 5-plex PCR.

† Preferably, select Dye 1 for the least abundant target, Dye 2 for the second least abundant target, and Dyes 3–4 for the most abundant targets.

Normalizer selection

Table 7. Housekeeping genes commonly used as endogenous references

	Gene symbol		Relative expression level*	
Gene	**Human**	**Mouse**	**Human**	**Mouse**
18S ribosomal RNA	RRN18S	Rn18s	++++	++++
Actin, beta	ACTB	Actb	+++	+++
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Gapdh	+++	+++
Phosphoglycerate kinase 1	PGK1	Pgk1	+++	++
Peptidylprolyl isomerase A	PPIA	Ppia	+++	+++
Ribosomal protein L13a	RPL13A	Rpl13a	+++	+++
Ribosomal protein, large, P0	RPLP0		+++	
Acidic ribosomal phosphoprotein PO		Arbp		+++
Beta-2-microglobulin	B2M	B2m	++ – +++	++ – +++
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	YWHAZ	Ywhaz	++ – +++	+
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	SDHA	Sdha	++	+
Transferrin receptor	TFRC	Tfrc	++	+
Aminolevulinate, delta-, synthase 1	ALAS1	Alas1	+	+
Glucuronidase, beta	GUSB	Gusb	+	+
Hydroxymethylbilane synthase	HMBS	Hmbs	+	++ – +++
Hypoxanthine phosphoribosyltransferase 1	HPRT1	Hprt1	+	+
TATA box binding protein	TBP	Tbp	+	+
Tubulin, beta	TUBB		+	
Tubulin, beta 4		Tubb4		+

* "+" indicates relative abundance of the transcripts.

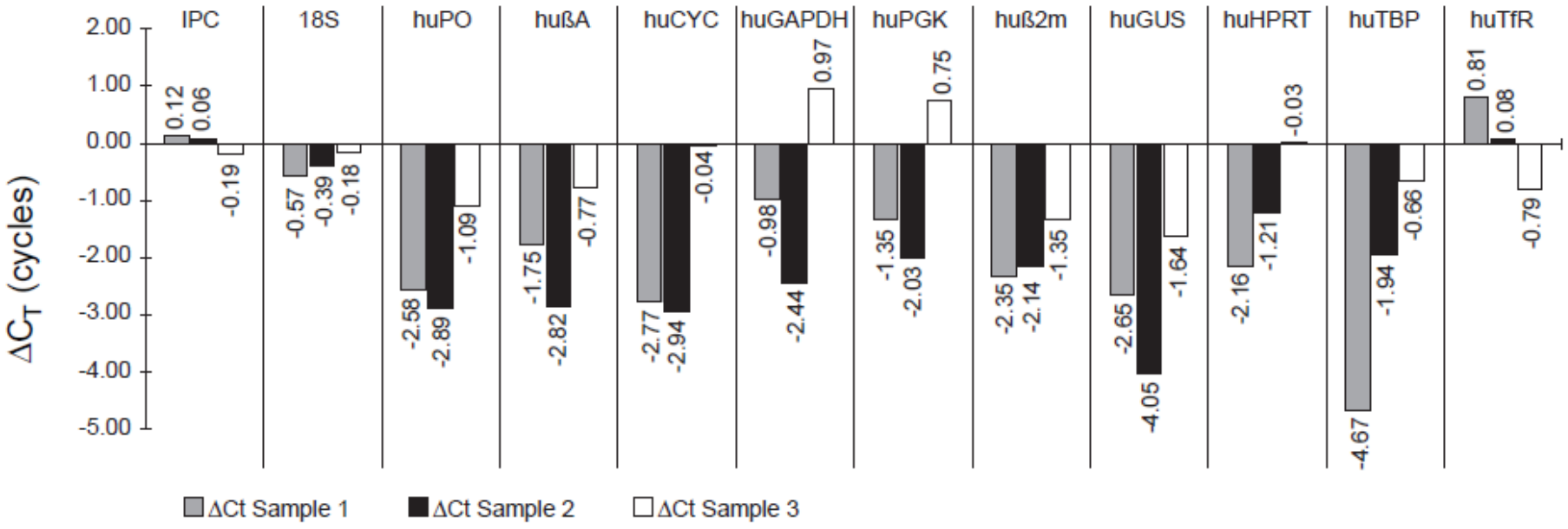
Normalizer selection

Column	Control Assay	Abbreviation
1	Internal Positive Control	IPC
2	18S rRNA	18S
3	Acidic ribosomal protein	huPO
4	Beta-actin	huβA
5	Cyclophilin	huCYC
6	Glyceraldehyde-3-phosphate dehydrogenase	huGAPDH
7	Phosphoglycerokinase	huPGK
8	β_2-Microglobulin	huβ2m
9	β-Glucronidase	huGUS
10	Hypoxanthine ribosyl transferase	huHPRT
11	Transcription factor IID, TATA binding protein	huTBP
12	Transferrin receptor	huTfR

TaqMan® Human Endogenous Control Plate

Applied Biosystems

Normalizer selection



Stable endogenous controls do not yield ΔCt values greater than that of IPC and do not show much variation.

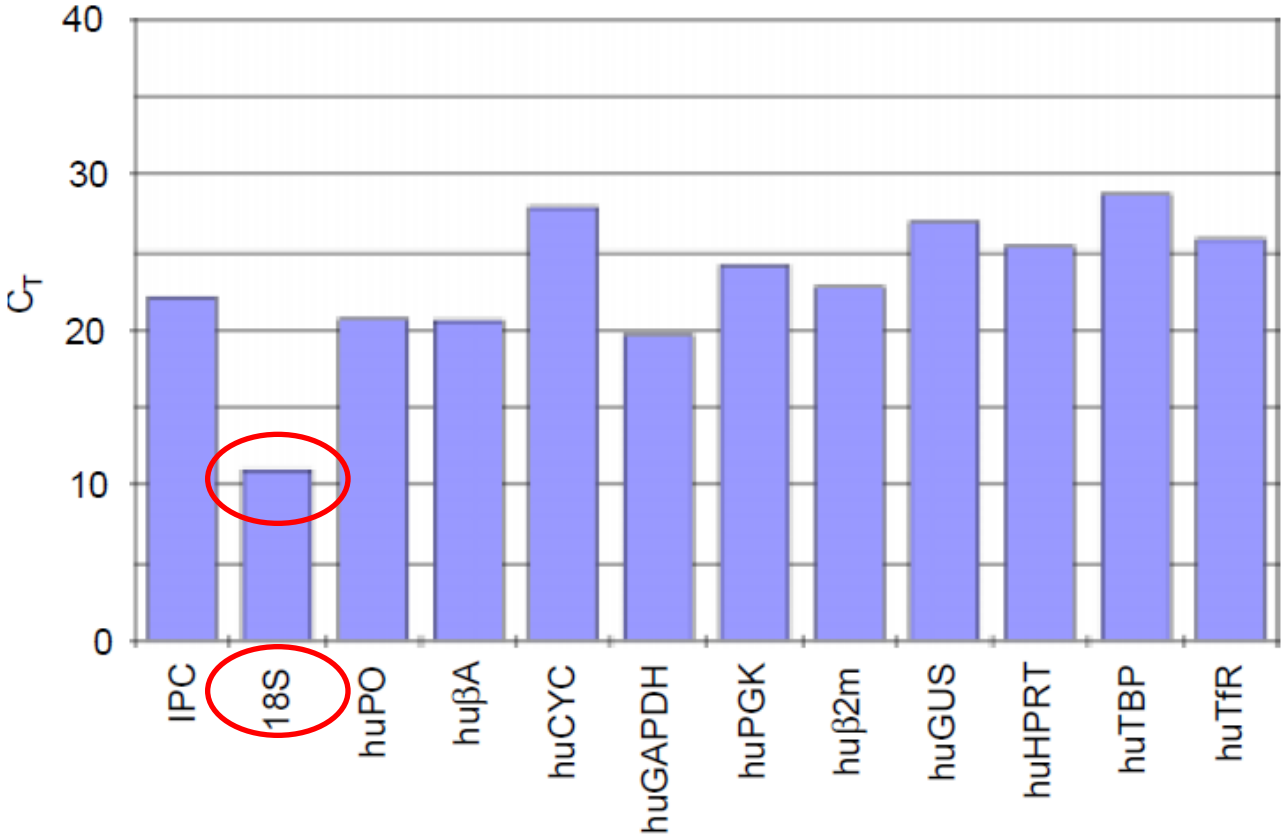
TaqMan® Human Endogenous Control Plate

Applied Biosystems

Normalizer selection

Demonstrating Performance with TaqMan Human Control Total RNA

TaqMan Human Control Total RNA is available to demonstrate the performance of the TaqMan Human Endogenous Control Plate. The figure below shows a typical gene expression profile for the sample.



TaqMan® Human Endogenous Control Plate

Applied Biosystems

Normalizer selection

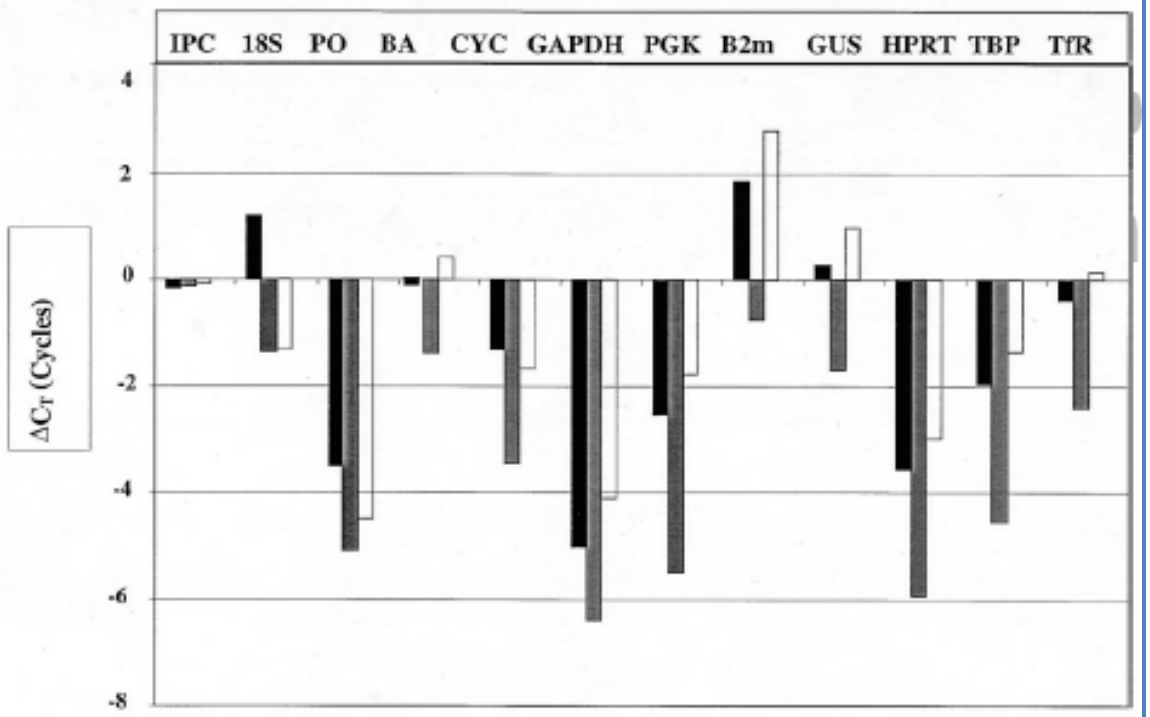
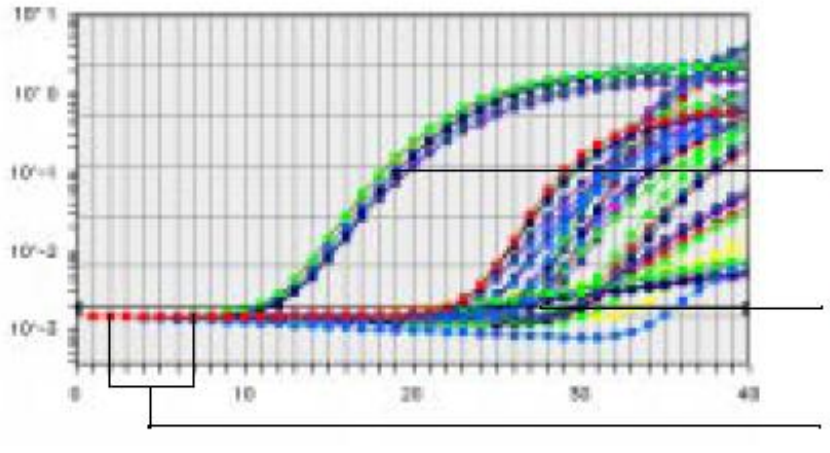


FIGURE 1. Variation in housekeeping gene expression in healthy individuals. The calibrator served as a baseline for the assay and is shown as zero on the graph. Samples with values above zero indicate lower levels of target gene expression, whereas those below zero indicate a higher level of expression 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)

18S as a Normalizer



- **Most abundant RNA: may need singleplex runs using diluted samples or competimers (Ambion); not suitable for rare target transcripts**
- **Forces separate baseline settings in some instruments**
- **Not mRNA**
- **Does not have 3' poly-A tail**
- **Ct value should be smaller than 22 for valid results**

TaqMan® Human Endogenous Control Plate

Applied Biosystems

GAPDH as a Normalizer

- The most unstable and inconsistent normalizer!

- Just don’t use it!

- A case of majority not always being right!

Interpretation

" Any assessment of the biological consequences of variable mRNA levels must include additional information regarding regulatory RNAs, protein levels and protein activity "

PROTOCOL

Quantification of mRNA using real-time RT-PCR

Tania Nolan[1], Rebecca E Hands[2] & Stephen A Bustin[2]

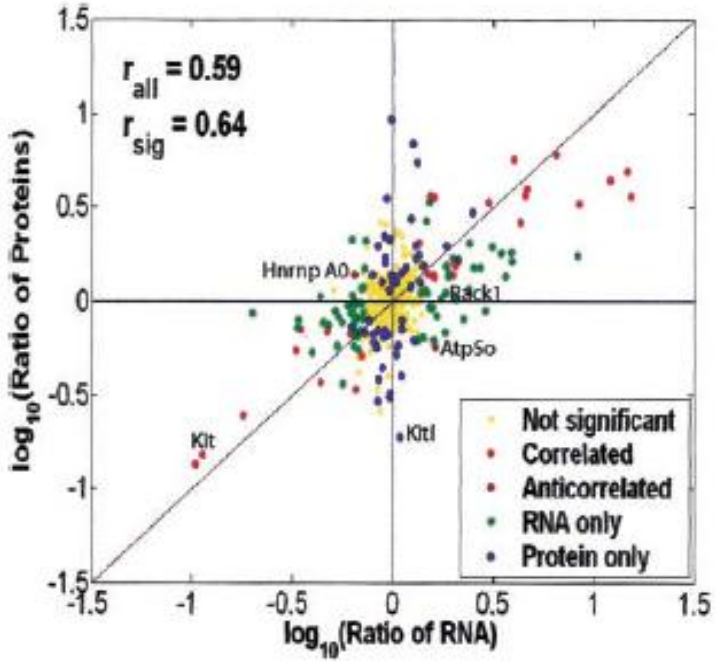


FIG. 1. **Scatter plot of mRNA *versus* cognate protein expression ratios ($\log_{10}$) of MPRO:EML.** The correlation coefficient for the 150 signature genes between mRNA and protein is r_{sig} = 0.64, and the overall correlation for all the genes (425 in total) in the analysis is r_{all} = 0.59.

Weak Correlation Between mRNA and Protein Levels in Eukaryotes

A total of 150 signature genes showed significant changes at either the protein and/or the mRNA level in two bovine bone marrow derived cell lines. 113 signature genes (76%) exhibited changes for mRNAs and their cognate proteins in the same direction (1st and 3rd quadrants), only 29 of them changed significantly at both mRNA and protein levels and were thus dubbed correlated genes (red). In contrast, 67 genes showed significant changes at the mRNA but not the protein level (green), whereas 52 genes showed significant changes at the protein but not the mRNA level (blue). Another two genes showed opposite expression patterns of mRNA and protein (brown). The correlation coefficient between mRNA and protein is 0.64 for the signature genes and 0.59 for all the genes examined. Tian, 2004 (www)

(www)

Quantification of mRNA using real-time RT-PCR

Tania Nolan[1], Rebecca E Hands[2] & Stephen A Bustin[2]

BOX 1 | CONSIDERATIONS FOR RT-qPCR PROCEDURES

General

- For all procedures use DNase/RNase-free consumables.
- Maintain a dedicated set of micropipettes and use filter barrier tips for all qPCR reactions.
- Dilute the template so that between 3 µl and 10 µl are added to each qPCR reaction. This reduces inaccuracies due to attempting to pipette very low volumes.
- Always aliquot all reaction components and use fresh aliquots if product is detected in the no template control (NTC).
- Always include a NTC after all reagents have been dispensed to reveal potential cross contamination.
- Defrost all reagents on ice and mix well prior to making up reaction mixes (we prefer inversion to vortexing, followed by a quick spin).
- Avoid exposing fluorescent probes and fluorescent nucleic acid binding dyes to light.
- When preparing mastermixes take care to ensure that all samples and controls have been accounted for. Make extra mix to allow for pipetting irregularities (usually around 10% or an extra sample is sufficient).
- When using a block-based PCR system briefly spin reaction tubes to ensure the removal of bubbles.

Reverse transcription

- In general, use total RNA as the template for the reverse transcription.
- Add the same amount of total RNA into each reaction.
- When possible use target specific primers; if using random primers use 15-mers[39].
- Use a thermostable RT enzyme such as Superscript III.
- If possible process samples simultaneously to avoid batch-to-batch variation.
- Always include no-RT controls to reveal the presence of contaminating gDNA. This is important if the target transcript is present in low copy numbers, where the assay cannot be designed across intron-exon boundaries or where the intron sequences are less than 1 kb and so might amplify during the PCR step.

PCR

- Positive template control (PCR product, synthetic amplicon, linearized plasmid) to check for consistency of reaction.
- A NTC (i.e., water control for PCR) is essential to check for non-specific signal arising from primer dimers or template contamination.
- Perform reactions in duplicate (triplicate if C_ts are >35). If the data from these differ by >0.5 C_t, the reactions should be repeated. If the reproducibility is consistently low, the assay should be re-optimized. Carrying out reactions in duplicate is generally sufficient. It is more important to run biological duplicates, as these will identify the true variability within the data.

(www)

REAL-TIME PCR

M. Tevfik Dorak, MD, PhD

Glossary of Terms Used in Real-Time PCR
PowerPoint Presentation on Real-Time PCR
Webinars on qPCR (by Mikael Kubista)

GLOSSARY OF REAL-TIME PCR TERMS

M.Tevfik Dorak

Real-Time PCR

M.Tevfik DORAK, MD PhD

updated on April 19, 2011

(www)

www.dorak.info

www.dorak.info/mobgam2011.pdf

FIU FLORIDA INTERNATIONAL UNIVERSITY