applied biosystems™
by *life* technologies™

# Real-time PCR: Understanding $C_t$

Real-time PCR, also called quantitative PCR or qPCR, can provide a simple and elegant method for determining the amount of a target sequence or gene that is present in a sample. Its very simplicity can sometimes lead to problems of overlooking some of the critical factors that make it work. This review will highlight these factors that must be considered when setting up and evaluating a real-time PCR reaction.

### Factors that can influence $C_t$

$C_t$ (threshold cycle) is the intersection between an amplification curve and a threshold line (Figure 1B). It is a relative measure of the concentration of target in the PCR reaction. Many factors impact the absolute value of $C_t$ besides the concentration of the target. We will discuss the most common template-independent factors that can influence $C_t$ and describe how to evaluate the performance of a real-time PCR reaction.

Figure 1 shows several parameters of the real-time reaction amplification plot. The exponential phase in Figure 1B corresponds to the linear phase in Figure 1C. The threshold must be set in the linear phase of the amplification plot in Figure 1C. The $C_t$ value increases with a decreasing amount of template. However, artifacts from the reaction mix or instrument that change the fluorescence measurements associated with the $C_t$ calculation will result in template-independent changes to the $C_t$ value. Therefore, the $C_t$ values from PCR reactions run under different conditions or with different reagents cannot be compared directly.
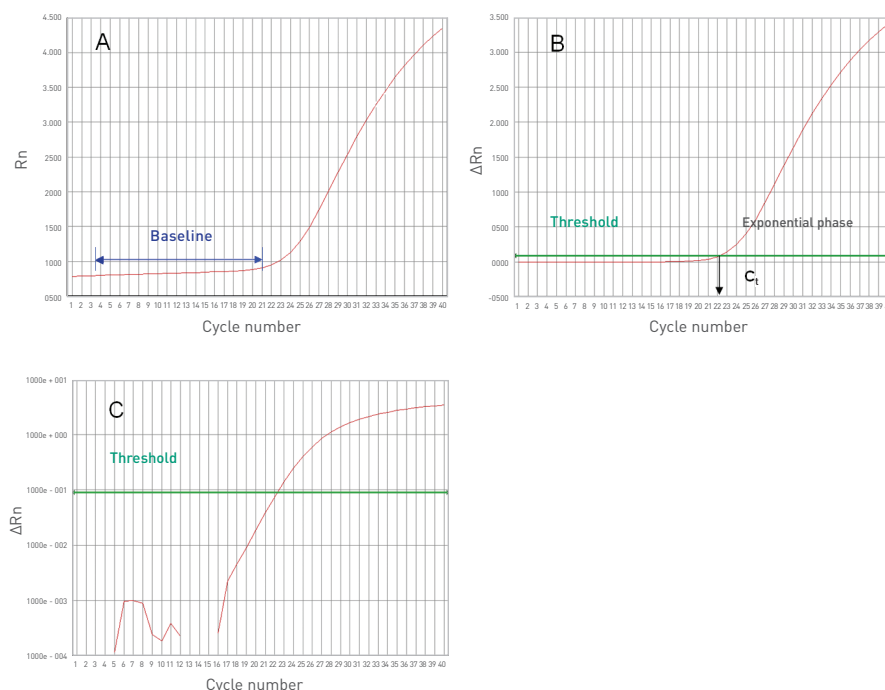


Figure 1. Graphical representation of real-time PCR data. Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye; i.e.,Rn is the reporter signal normalized to the fluorescence signal of ROX™. (A) In this view, Rn is plotted against PCR cycle number. (B) ΔRn is Rn minus the baseline; ΔRn is plotted against PCR cycle number. (C) An amplification plot shows the variation of log (ΔRn) with PCR cycle number.
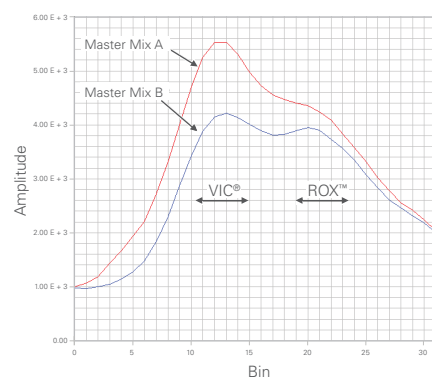


Figure 2. Raw fluorescence data obtained from one assay using two master mixes with the same ROX™ level. The difference in signal is due to the master mix composition. The reaction was performed on an Applied Biosystems® 7900HT Fast Real-Time PCR System using a VIC® MGB probe. The x-axis shows the emission wavelength of the fluorophore and the y-axis shows the intensity of the emission.

### The effect of master mix components

The fluorescence emission of any molecule is dependent on environmental factors such as the pH of a solution and salt concentration. Figure 2 shows the raw fluorescence data of a TaqMan® probe in the background of two different master mixes. Note that the fluorescence intensity is higher in Master Mix A even though the target, probe, and ROX™ dye concentrations are the same in both cases.

The resulting ΔRn value will, therefore, vary as shown in Figure 3. Note that the baseline fluorescence signals, in a template-independent factor, are different for the two master mixes (Figure 3A). Variations in the $C_t$ value do not reflect the overall performance of the reaction system (Figure 3B). Master mixes with equivalent sensitivity may have different absolute $C_t$ values.

### ROX™ passive reference dye

The Rn value is calculated as the ratio of the fluorescence of FAM™ dye divided by the fluorescence of ROX™ dye. Therefore, a lower amount of ROX™ dye would produce a higher Rn value assuming fluorescence signal from FAM™ dye is unchanged. This would lead to an increase in baseline Rn and subsequently a smaller ΔRn as well as a different $C_t$ value. The new $C_t$ value obtained by lowering the level of ROX™ dye has no bearing on the true sensitivity of the reaction, but can have other unintended consequences. Low concentrations of ROX™ dye can result in increased standard deviation of the $C_t$ value, as shown in Figure 4. The greater the standard deviation, the lower the confidence in distinguishing between small differences in target concentration (see the precision section on the next page).

### Efficiency of a PCR reaction

The efficiency of a PCR reaction can also affect $C_t$. A dilution series amplified under low efficiency conditions could yield a standard curve with a different slope from one amplified under high efficiency conditions. In Figure 5, two samples (X and Y) amplified under low and high efficiency conditions show different $C_t$ values for the same target concentration. In this example, although the high-efficiency condition (the blue curve in Figure 5) gives a later $C_t$ at high concentrations, it results in better sensitivity at low target concentrations. The PCR efficiency is dependent on the assay, the master mix performance, and sample quality. Generally, an efficiency between 90 and 110% is considered acceptable.

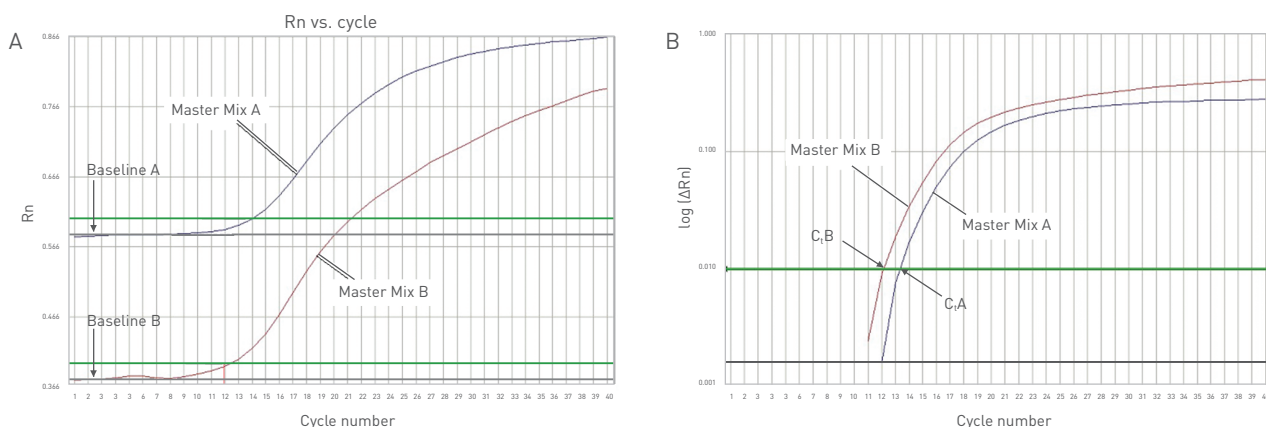The observation that the $C_t$ value produced from one sample is higher than that of



**Figure 3. Amplification of RNase P in equal amounts of human gDNA using Master Mix A and Master Mix B.** (**A**) Rn is plotted against cycle number and the baselines for both reactions are shown. (**B**) log (ΔRn) is plotted against cycle number. The threshold (green line) is set at the same level for both master mixes. The $C_t$ value of Master Mix B ($C_t$B) is earlier than that of Master Mix A ($C_t$A) for identical concentrations of target, reflecting the lower baseline of Master Mix B. All amplifications were performed using the Applied Biosystems® 7500 Real-Time PCR System.
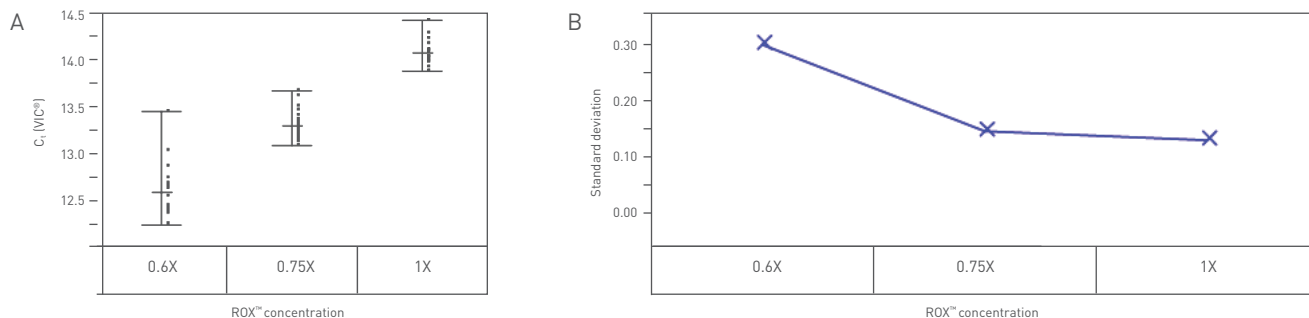


**Figure 4. Amplification of TGF-beta using master mixes containing 3 different concentrations of ROX™ dye.** The variation of (**A**) $C_t$ value and (**B**) standard deviation with ROX™ dye concentration is shown. Decreasing the ROX™ dye concentration gives an earlier $C_t$, but increases the standard deviation. All amplifications were performed using the Applied Biosystems® 7500 Real-Time PCR System.
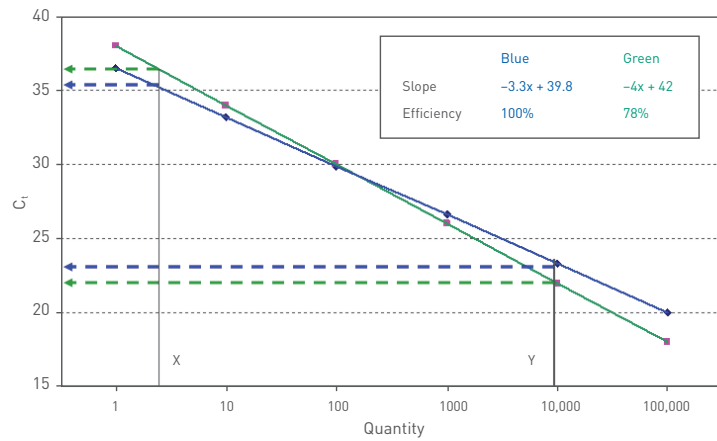
Figure 5. Variation of $C_t$ with PCR efficiency. The blue standard curve has an efficiency of 100% (the slope is −3.3). The green standard curve has an efficiency of 78% (the slope is −4). Amplification of quantity Y gives an earlier $C_t$ under low efficiency conditions (green) compared to the high efficiency condition (blue). With a lower quantity (X) there is an inversion and the low efficiency condition (green) gives a later $C_t$ than the high efficiency condition (blue).
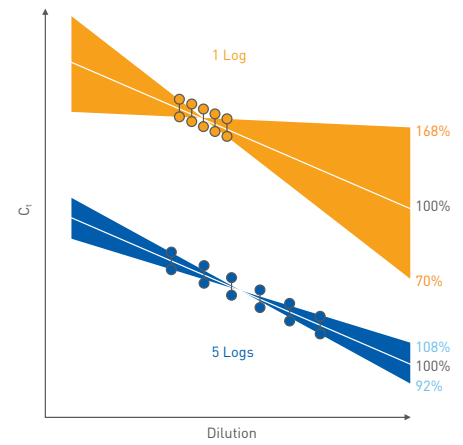


Figure 6. Accurate calculation of PCR efficiency depends on the range of template amount used for the dilution series. For a 2-fold dilution with 5 points (orange), the potential artifact is higher than for the 10-fold dilution with 5 points (blue).

the other, could be valuable in concluding that there is less template in the first sample, assuming all other factors such as instruments, reagents, and assays are equal. However, this is not true if different instruments, reagents, primers and probes, or reaction volumes are involved in producing the two $C_t$s. Therefore, the absolute $C_t$ value comparison is only meaningful when comparing experiments using the same reaction conditions as defined above.

## How to evaluate the performance of a real-time PCR reaction

In order to compare two reactions where a condition is changed (for example two different master mixes or two different instruments), the following parameters must be evaluated.

### Dynamic range

To properly evaluate PCR efficiency, a minimum of 3 replicates and a minimum of 5 logs of template concentration are necessary. The reason for this suggested level of rigor is illustrated in Figure 6, which demonstrates the possible mathematical variation of slope or efficiency obtained when testing dilutions over 1 log vs. 5 logs. Thus, even if the assay is 100% efficient, a range from 70 to 170% can be obtained when testing a dilution series of a single log, due to the standard deviation in one dilution. For the same number of dilutions or replicates on a 5-log range, the potential artifact is only ±8 %. That means for 94% efficiency on a 5-log range, the assay would have a range of 88% to 100% efficiency.

To accurately determine the efficiency of a PCR reaction, a 5-log dilution series must be performed. A slope of −3.3 ±10% reflects an efficiency of 100% ±10%. A PCR reaction with lower efficiency will have lower sensitivity.

### $R^2$ value

Another critical parameter to evaluating PCR efficiency is $R^2$, which is a statistical term that indicates how good one value is at predicting another. When $R^2$ is 1, the value of Y ($C_t$) can be used to accurately predict the value of X (Figure 7A). If $R^2$ is 0, the value of X cannot be predicted from the value of Y (Figure 7B). An $R^2$ value >0.99 provides good confidence in correlating two values.

### Precision

The standard deviation (square root of the variance) is the most common measure of precision. If many data points are close to the mean, the standard deviation is small; if many data points are far from the mean, the standard deviation is large.

In practice, a data set with a sufficient number of replicates forms an approximately normal distribution. This is frequently justified by the classic central limit theorem which states that sums of many independent, identically distributed random variables tend towards the normal distribution as a limit. As shown in Figure 8A, approximately 68% of the values are within 1 standard deviation of the mean, 95% are within two standard deviations, and 99.7% lie within 3 standard deviations.

If a PCR is 100% efficient, the $C_t$ difference between two successive concentrations in a 2-fold dilution is 1 (Figure 8B). To be able to quantify a 2-fold dilution in more than 99.7% of cases, the standard deviation has to be ≤0.167. The greater the standard deviation, the lower the ability to distinguish between 2-fold dilutions. To be able to discriminate between a 2-fold dilution in more than 95% of cases, the standard deviation has to be ≤0.250 (Figure 8C).

### Sensitivity

Any system capable of effectively amplifying and detecting one copy of starting template has achieved the ultimate level of sensitivity, regardless of the absolute value of the $C_t$.

As described earlier, efficiency is a key factor in determining the sensitivity of a reaction (Figure 5). Another important consideration when detecting very low copy numbers is that normal distribution of template is not expected. Instead, a Poisson distribution is followed, which predicts that in a large number of replicates containing an average of one copy of starting template, approximately 37% should actually have no copies, only 37% should contain one copy, and 18% should contain two copies (see Figure 9). Thus, for a reliable low copy detection, a large number of replicates are necessary to provide statistical significance and to overcome the Poisson distribution limitation.
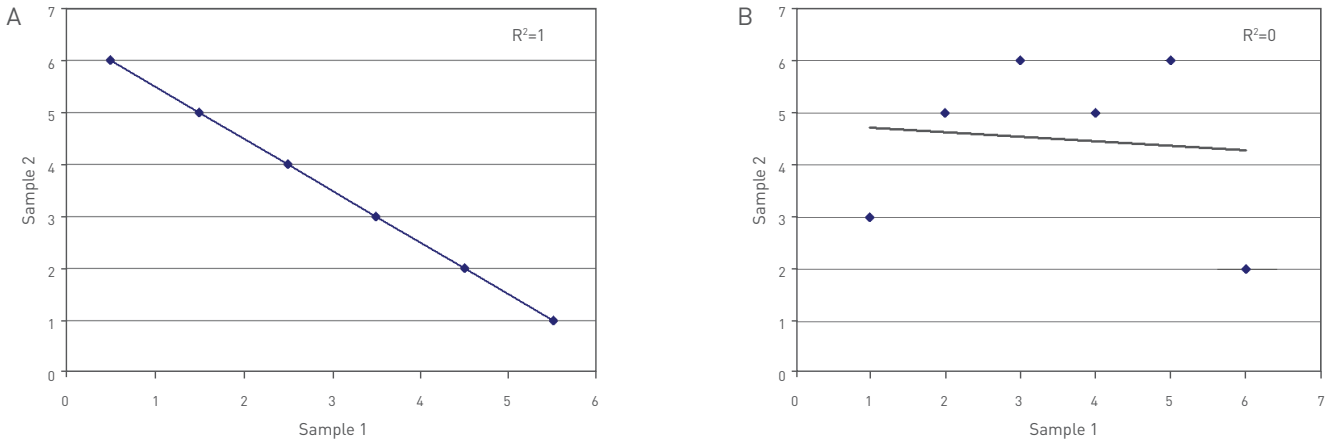
**Figure 7. Examples of R² values calculated for 2 straight lines.** (**A**) There is a direct relation between x and y values. (**B**) There is no relation between x and y values.
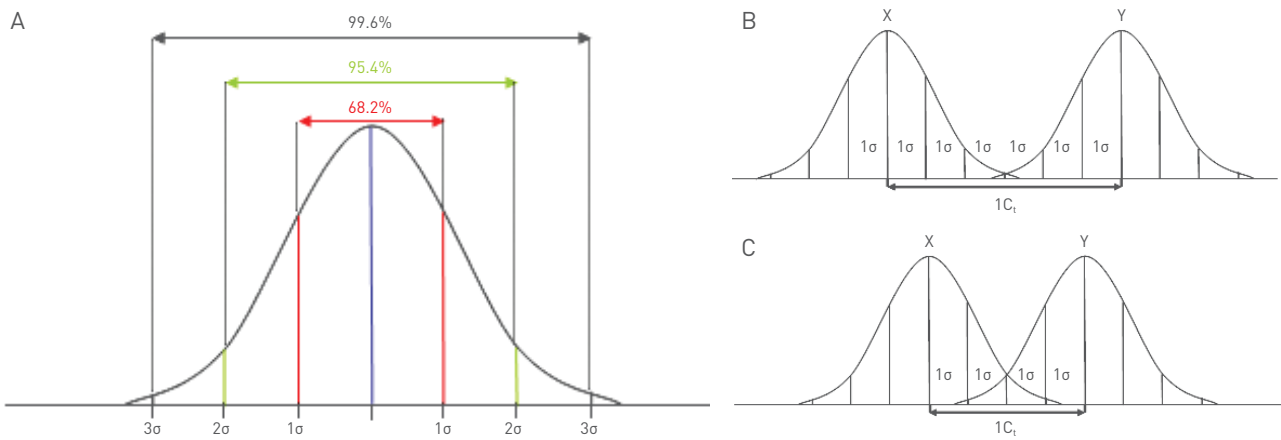


**Figure 8. Normal distribution and standard deviation.** (**A**) Normal distribution of data is shown. For a PCR efficiency of 100%, the difference in $C_t$ between the means of two successive samples in a 2-fold dilution series is 1 (sample X and sample Y). (**B**) To be able to quantify both samples in 99.7% of cases, the standard deviation has to be less than 1 $C_t$ divided by 6 standard deviations (1/6 = 0.167). (**C**) To be able to quantify both samples in 95% of cases, the standard deviation has to be less than 1 $C_t$ divided by 4 standard deviations (1/4 = 0.25).
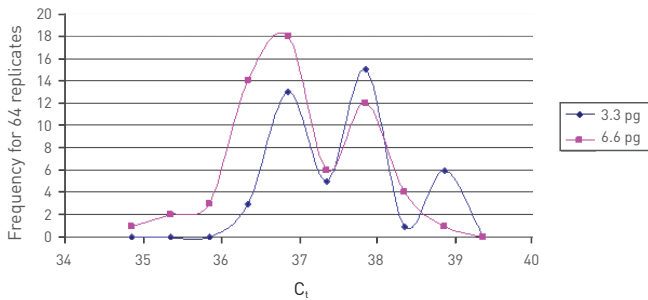


**Figure 9. Poisson distribution for low copy number.** The blue curve represents Poisson distribution for 3.3 pg of DNA (1 copy of DNA). The pink curve represents Poisson distribution for 6.6 pg of DNA (1 cell, 2 copies of DNA).

**Table 1. Performance evaluation of real-time PCR.**

| Factors | Recommendations | Criteria |
|---------|-----------------|----------|
| Efficiency | Serial dilution with 5-log dilutions | Slope: ~ –3.3 <br> $R^2$ >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 |

## Conclusion

Efficiency, $R^2$, precision, and sensitivity are used to determine performance of a PCR reaction when comparing different reaction conditions. For a rigorous evaluation, all factors listed in Table 1 must be evaluated together.

In addition to these factors, proper experimental controls (such as no template control, no RT control) and template quality must be evaluated and validated.

## APPENDIX

### Amplification plot

An amplification plot is the plot of fluorescence signal versus cycle number. Reactions are characterized by the point in time during cycling when amplification of a PCR product is first detected. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

### Baseline

In the initial cycles of PCR there is little change in fluorescence signal. This defines the baseline for the amplification plot. In these cycles the fluorescence background of the reaction is observed. This is subtracted from the results when setting the baseline. For information on how to set up the baseline, download the document *Applied Biosystems 7300/7500 Real-Time PCR System* (Part no. 4347825) at www.appliedbiosystems.com.

### Delta Rn ($\Delta$Rn)

$\Delta$Rn is the normalization of Rn obtained by subtracting the baseline:
($\Delta$Rn = Rn – baseline).

### Passive reference

A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations caused by change in concentration, volume, or sample effects.

### PCR efficiency

The equations below describe the exponential amplification of PCR:
$C_n = C_i \times (1 + E)^n$
$C_i$ = initial copy number
$C_n$ = copy number at cycle n
n = number of cycles
E = efficiency of target amplification

When efficiency is maximum (=1) the equation is: $C_n = C_i \times 2^n$ and the fold increase will be 2 at each cycle. The quantity of PCR product generated at each cycle decreases with decreasing efficiency, and the amplification plot is delayed. The recommended efficiency is from 90 to 110%.

### Reporter dye

The reporter dye is the dye attached to the 5' end of the TaqMan® probe. The dye provides a fluorescence signal that indicates specific amplification. For SYBR® Green I, the reporter dye binds double-stranded DNA, and the increase in fluorescence is also a measure of amplification. Specificity should be checked with a melt curve (Power SYBR® Green PCR Master Mix and RT-PCR Protocol, Part no. 4367218) or gel analysis of the PCR product.

### Rn

Normalized reporter is the ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye.

### Threshold

A level of $\Delta$Rn used for the determination of the threshold cycle ($C_t$) in real-time assays. The level is set above the baseline, but sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the amplification plot defines the $C_t$. For information on how to set up the threshold, download the document *Applied Biosystems 7300/7500 Real-Time PCR System* (Part no. 4347825) at www.appliedbiosystems.com.

### Threshold cycle ($C_t$)

The fractional cycle number at which the fluorescence passes the threshold.

Life Technologies offers a breadth of products   DNA  |  RNA  |  PROTEIN  |  CELL CULTURE  |  INSTRUMENTS

**For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.**

**Headquarters**
5791 Van Allen Way  |  Carlsbad, CA 92008 USA  |  Phone +1.760.603.7200  |  Toll Free in the USA 800.955.6288
www.lifetechnologies.com

*life* technologies™