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How to evaluate and compare real-time PCR reactions using key performance parameters

Real-time PCR

Real-time PCR, also called quantitative PCR or qPCR, can provide a simple and elegant method for determining the amount of a target sequence that is present in a sample. Because qPCR provides a relative measure of the concentration of a target in a reaction, it is important to note that results from reactions run under different conditions or with different reagents cannot be compared directly. In these instances, evaluating key performance parameters is necessary to ensure valid comparison of the reactions. In this application note, we highlight template-independent factors that can influence real-time PCR data and provide guidelines for comparing reaction performance when run conditions or reagents change.

Real-time PCR: understanding C_t

The threshold cycle, commonly referred to as the C_t value (or sometimes C_q for quantification cycle), is a critical parameter in qPCR analysis. It is defined as the cycle number at which the fluorescence generated within a reaction crosses a predetermined threshold, signaling the detection of the target nucleic acid.

Figure 1 shows several parameters of the real-time reaction amplification plot and how the C_t value is determined using qPCR data. In Figure 1A, the normalized reporter signal (Rn) is plotted against the PCR cycle number to generate the amplification curve. ΔRn is then calculated by subtracting the baseline fluorescence signal from Rn. In Figure 1B, ΔRn is plotted against PCR cycle number and the threshold is set in the exponential phase of the amplification plot. The point where the threshold line intersects with the amplification plot is the C_t value of the reaction. Note, the threshold must be set in the exponential phase of the amplification plot in Figure 1B, which also must be in the linear phase of the amplification plot in Figure 1C. The exponential phase in Figure 1B corresponds to the linear phase in Figure 1C.

C_t is a relative measure of the concentration of target in the PCR reaction, and its value increases with a decreasing amount of template. The observation that the C_t value produced from one sample is higher than the C_t value of another sample 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 when different instruments, reagents, primers and probes, or reaction volumes are involved in producing the two C_t values. Therefore, the absolute C_t value comparison is only meaningful when comparing experiments using the same reaction conditions.

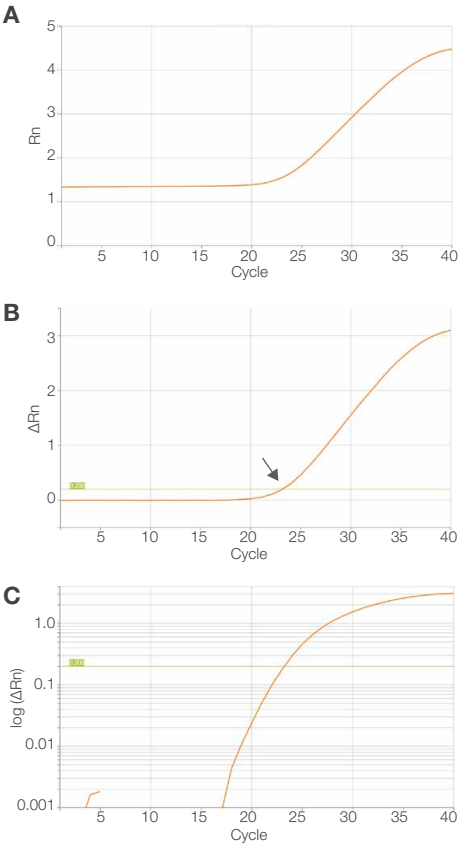

Figure 1. Graphical representation of real-time PCR data and the C_t. Rn is the fluorescence of the reporter dye divided by the fluorescence of the passive reference dye. **(A)** Rn is plotted against PCR cycle number. **(B)** ΔRn is Rn minus the baseline; ΔRn is plotted against PCR cycle number and the threshold (green line) is set in the exponential phase of the amplification plot. **(C)** An amplification plot shows the variation of log (ΔRn) with PCR cycle number and the threshold (green line) is set in the linear phase of the amplification plot.

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Factors that can influence C_t

Many factors impact the absolute value of C_t besides the concentration of the target. For example, artifacts from the reaction mix or an instrument that changes 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 with different components or run conditions cannot be compared directly.

The effect of master mix components

The fluorescence emission of any molecule depends on environmental factors such as pH and salt concentration. Figure 2 shows the raw fluorescence data of an Applied Biosystems™ TaqMan™ probe in the background of 2 different master mixes. Note that the fluorescence intensity is higher in master mix A even though the target, probe, and Applied Biosystems™ ROX™ dye concentrations are the same in both cases.

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

ROX passive reference dye

The Rn value is calculated as the ratio of the fluorescence of Applied Biosystems™ FAM™ dye divided by the fluorescence of ROX dye. Therefore, a lower amount of ROX dye would produce a higher Rn value assuming the fluorescence signal from the 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 that follows).

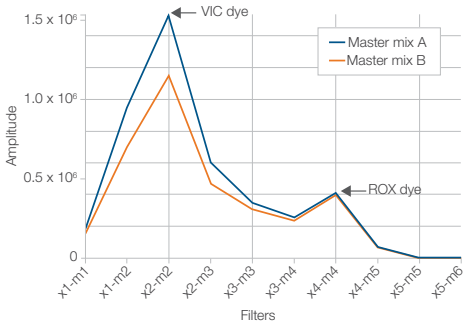

Figure 2. Raw fluorescence data obtained from 1 assay using 2 master mixes with the same ROX dye concentration. The difference in signal is due to the master mix composition. The reaction was performed on an Applied Biosystems™ real-time PCR system using an Applied Biosystems™ VIC™ MGB probe. The x-axis shows the emission wavelength of the fluorophore, and the y-axis shows the intensity of the emission.

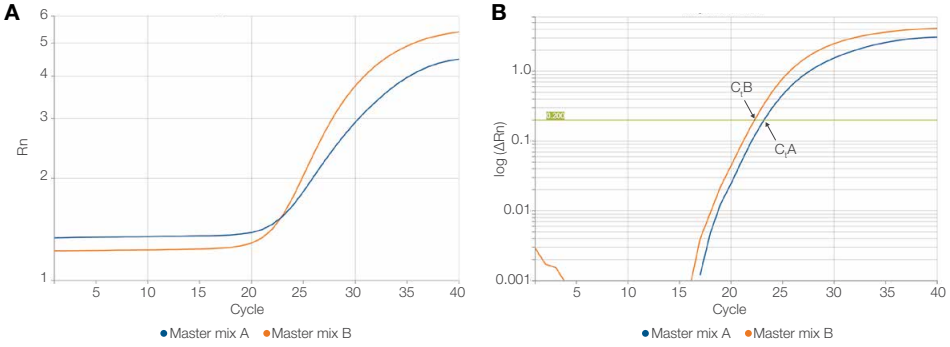

Figure 3. Amplification of an RNase P target gene in equal amounts of human gDNA using master mixes A and B. (A) Rn is plotted against cycle number; master mix A (blue) has a higher baseline than master mix B (orange). **(B)** log (ΔRn) is plotted against cycle number. The threshold (green line) is set at the same level for both master mixes. The C_t of master mix B (C_tB) is earlier than that of master mix A (C_tA) for identical concentrations of target, reflecting the lower baseline of master mix B. All amplifications were performed using an Applied Biosystems real-time PCR system.

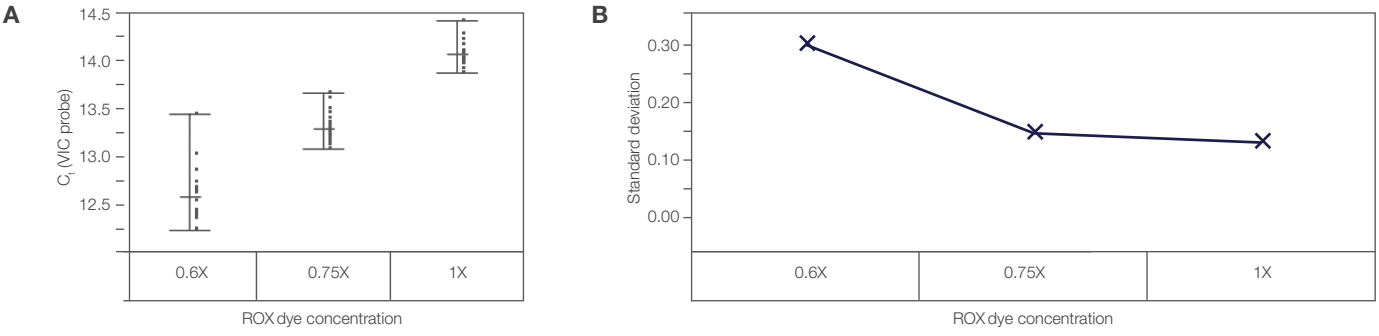

Figure 4. Amplification of TGF-beta using master mixes containing 3 different concentrations of ROX dye. The variation of **(A)** C_t and **(B)** standard deviation with ROX dye concentration is shown. Decreasing the ROX dye concentration gives an earlier C_t value, but increases the standard deviation. All amplifications were performed using an Applied Biosystems real-time PCR system.

Efficiency of a PCR reaction

The efficiency of a PCR reaction can also affect C_t values. 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) gives a later C_t at high concentrations, it results in better sensitivity at low target concentrations. The PCR efficiency depends on the assay, the master mix performance, and sample quality. Generally, an efficiency between 90% and 110% is considered acceptable.

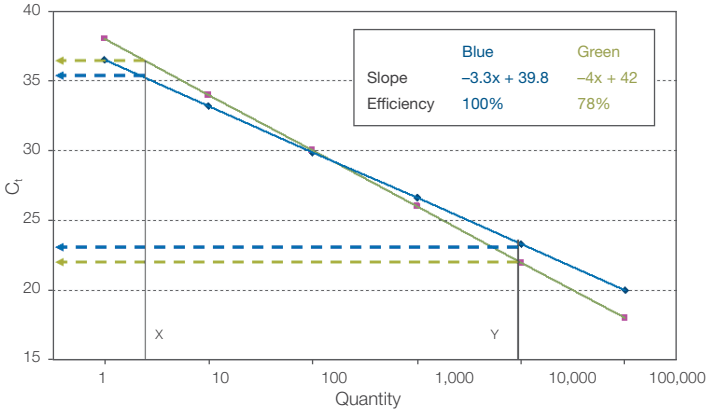

Figure 5. Variation of C_t with PCR efficiency. The blue standard curve has an efficiency of 100% (the slope is –3.3). The green standard curve has an efficiency of 78% (the slope is –4.0). Amplification of quantity Y gives an earlier C_t under low-efficiency conditions (green) compared to the high-efficiency 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).

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

To compare 2 reactions in which a condition is changed (for example 2 different master mixes or 2 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. Even if the assay is 100% efficient, a range from 70% to 168% can be obtained when testing a dilution series of a single log, due to the standard deviation in 1 dilution. On a 5-log range, the potential artifact for 6 dilutions or replicates is only ±8%. That means for 94% efficiency on a 5-log range, the assay would have a range of 92% to 108% 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.

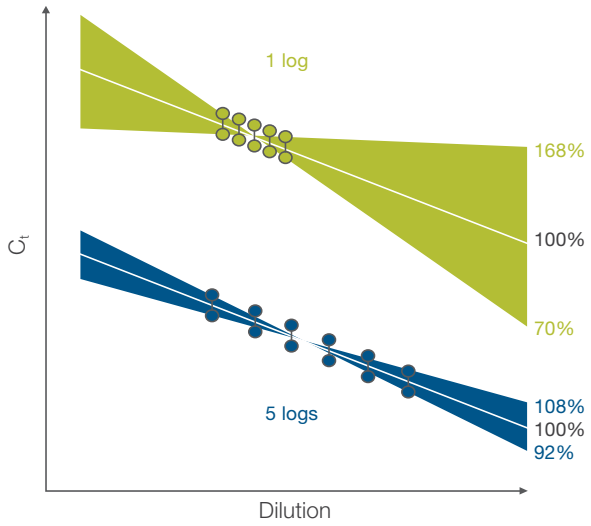

Figure 6. Accurate calculation of PCR efficiency depends on the range of template amount used for the dilution series. For a 2-fold dilution with 5 points (green), the potential artifact is higher than for the 10-fold dilution with 6 points (blue).

R^2 value

Another critical parameter in 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 that 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 2 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).

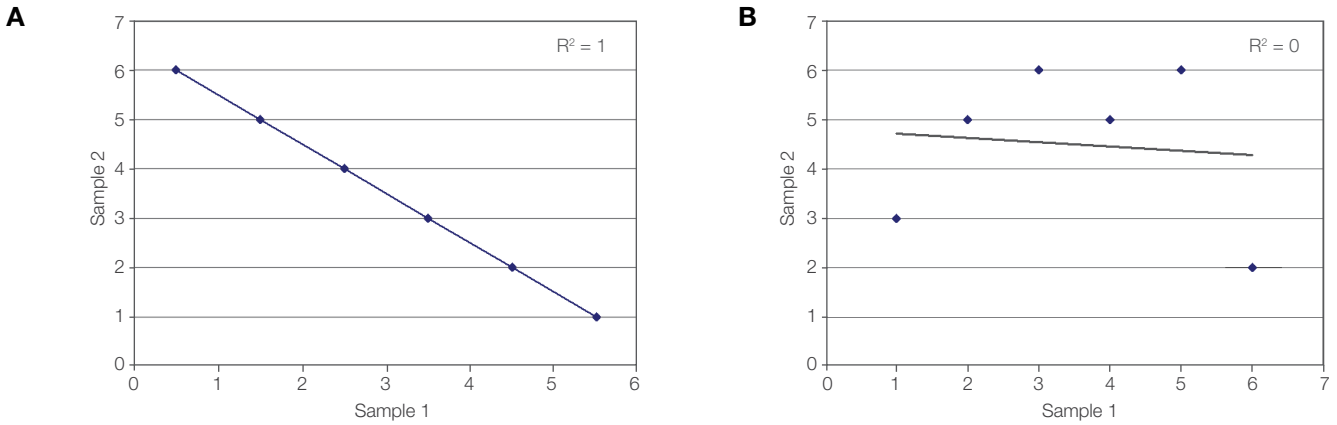

Figure 7. Examples of R^2 values calculated for 2 straight lines. (A) There is a direct relation between x and y values. **(B)** There is no linear relation between x and y values.

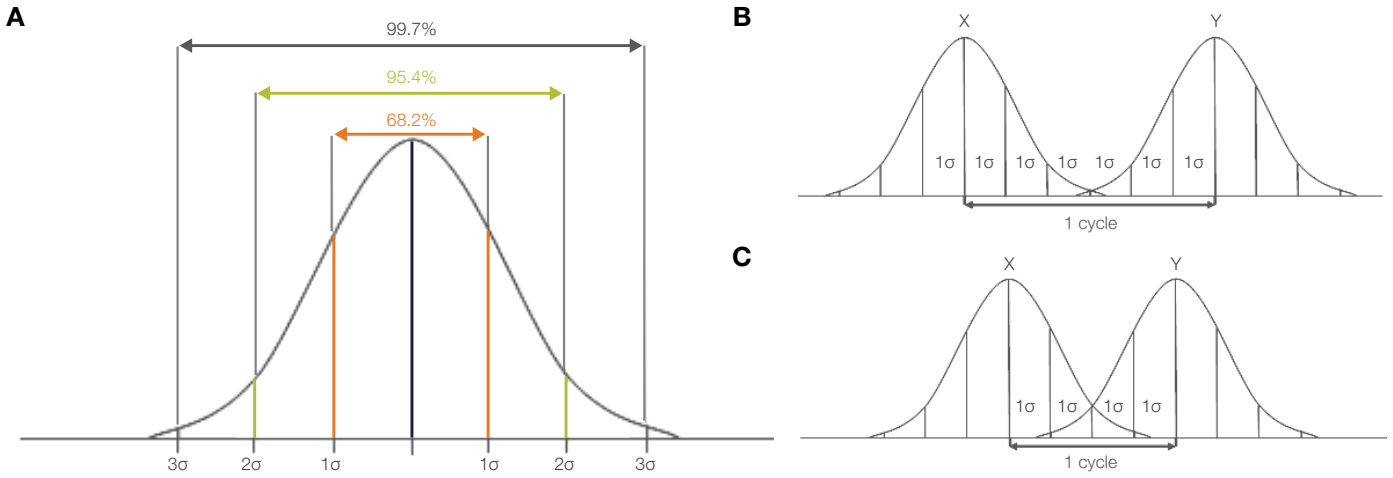

Figure 8. Normal distribution and standard deviation. (A) Normal distribution of data is shown. **(B)** For a PCR efficiency of 100%, the difference in C_t between the means of 2 successive samples in a 2-fold dilution series is 1 (sample X and sample Y). To be able to quantify both samples in 99.7%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

Sensitivity

Any system capable of effectively amplifying and detecting 1 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 a 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 1 copy of starting template, approximately 37% should actually have no copies, only 37% should contain 1 copy, and 18% should contain 2 copies (see Figure 9). Thus, for reliable low-copy detection, a large number of replicates is necessary to provide statistical significance and to overcome the Poisson distribution limitation.

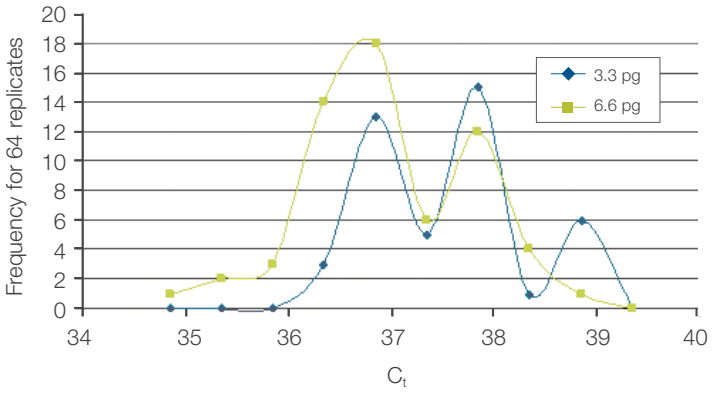

Figure 9. Poisson distribution for low copy number. The blue curve represents the Poisson distribution for 3.3 pg of DNA (1 copy of DNA). The green curve represents the Poisson distribution for 6.6 pg of DNA (1 cell, 2 copies of DNA).

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 a no-template control and a no-RT control) and template quality must be evaluated and validated.

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

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.

Delta Rn (ΔRn)

ΔRn is the normalization of Rn obtained by subtracting the baseline:
(ΔRn = Rn – baseline).

Passive reference

A dye, such as ROX dye or Applied Biosystems™ Mustang Purple™ 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

A reporter dye is used to measure target amplification during qPCR. TaqMan probes have a fluorescent reporter dye on the 5′ end and a quencher on the 3′ end, which suppresses reporter signal until cleaved during qPCR. Several reporter dyes are offered with TaqMan probes, including Applied Biosystems™ ABY™, JUN™, FAM, and VIC dyes. For dye-based qPCR, Applied Biosystems™ SYBR™ Green reporter dye enables quantification of qPCR product by producing a fluorescent signal when bound to dsDNA.

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 ΔRn 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.

Threshold cycle (C_t)

The fractional cycle number at which the fluorescence generated within a reaction crosses a predetermined threshold, signaling the detection of the target nucleic acid.

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