

Supplement to the RQmin/RQmax Algorithms in Applied Biosystems Real-Time PCR Systems Software

Introduction

The $\Delta\Delta C_T$ method (also known as the Comparative C_T method) is a quantitative PCR method used to determine the relative quantity (RQ) of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample. The $\Delta\Delta C_T$ method was invented by Applied Biosystems scientists ^{1,2}.

Applied Biosystems real-time PCR systems software programs have two different algorithms to calculate RQ and the statistical variation of RQ (RQmin/RQmax). The default algorithm is the **Advanced** or **Confidence Level** algorithm and the alternative algorithm is the **Basic** or **Standard Deviation** algorithm (the algorithm name may be different in different systems or different software versions). This document is used as a supplement to the RQ algorithm User Bulletin ² by focusing on the differences between these two algorithms.

	Advanced or Confidence Level Algorithm	Basic or Standard Deviation Algorithm
ΔC_T ^a	$\Delta C_T = C_{T \text{ target}} - C_{T \text{ endogenous control}}$	$\Delta C_T = C_{T \text{ target}} - C_{T \text{ endogenous control}}$
Statistical variability (SV) of ΔC_T ^b	Standard error of the averaged ΔC_T values (ΔC_T SE)	Standard deviation of the averaged ΔC_T values (ΔC_T SD)
ΔC_T degrees of freedom ^c	Calculated	Not calculated
$\Delta\Delta C_T$ ^d	Calculated the same way in both algorithms	Calculated the same way in both algorithms
Statistical variability of $\Delta\Delta C_T$ ^e	It is the same as ΔC_T SE	It is the same as ΔC_T SD
Relative Quantity (RQ)	$RQ = 2^{-\Delta\Delta C_T}$	$RQ = 2^{-\Delta\Delta C_T}$
RQmin/RQmax ^f	Multiplied by t-factor in Student's t-distribution ^g	Based on user selected number of standard deviation away from the RQ mean ^h

a. The ΔC_T value is calculated differently depending on the study method (singleplex or multiplex).

In multiplex studies, because the target and endogenous control detectors are contained in the same well, normalization takes place at the well level, i.e., the ΔC_T value is calculated for each sample/target combination in each well (well-level ΔC_T). All ΔC_T descriptive statistics (arithmetic mean, statistical variability, and degrees of freedom) can be calculated directly from the well-level ΔC_T values.

In singleplex studies, because the target and endogenous control detectors are not contained in the same well, the normalization between these two is more complicated than in multiplex studies. Instead of normalizing the target and endogenous control values within a well, the average of the C_T values for each target and endogenous control are made first within plates, and then the plate-local values are used to find an average of the values over all plates contained in a study. The details of the calculation are described in the RQ algorithm User Bulletin ².

b. Standard error (SE) is a measure of variability representing the deviation of sample means around the actual mean of a population. Standard error (SE) is calculated as the standard deviation (SD) divided by the square root of the number of data points: $SE = SD/\sqrt{N}$. In comparison, standard deviation (SD) is a measure of variability representing the deviation of the measured values from the arithmetic mean of the measured values. It is calculated as the square root of the mean of squared deviations from the sample mean. For the **Advanced** or **Confidence Level** algorithm, the software calculates the variability of ΔC_T values using the standard error of the mean ². For the **Basic** or **Standard Deviation** algorithm, the software calculates the variability of ΔC_T using standard deviation of the mean ¹.

c. The degrees of freedom are an index of the amount of random variability that can be present in a particular situation. The degrees of freedom values are used to statistically account for small sample sizes. The software calculates the degrees of freedom values only when **Advanced** or **Confidence Level** algorithm is used.

d. The software calculates $\Delta\Delta C_T$ values for each target detector in each sample by subtracting the study-level ΔC_T arithmetic mean, $\mu(\Delta C_T)$, of the calibrator sample from the corresponding ΔC_T arithmetic mean of each test sample (including the calibrator sample) for each target detector.

Equation #1:

$$(\Delta\Delta C_T)_{\text{sample, target}} = \mu(\Delta C_T)_{\text{sample, target}} - \mu(\Delta C_T)_{\text{calibrator, target}}$$

Where: $\mu(\Delta C_T)$ = the study-level ΔC_T arithmetic mean.

e. The calculation of $\Delta\Delta C_T$ involves subtraction of the ΔC_T calibrator value. Since this is the subtraction of an arbitrary constant, the standard deviation of the $\Delta\Delta C_T$ value is the same as the standard deviation of the ΔC_T value.

f. RQmin/RQmax represents the variability of RQ value for each sample/target detector combination in the study. It gives a confidence interval based on the desired confidence set by the user. The confidence interval is an estimated range of values that should contain the actual relative quantity of the target in the sample with the specified level of confidence. The RQmin/RQmax values are graphically represented in the Gene Expression Plot as error bars.

g. For the **Advanced** or **Confidence Level** algorithm, the software calculates the confidence interval where T is the Student's t-factor corresponding to the number of degrees of freedom in the study-level ΔC_T arithmetic mean for the confidence level $\alpha = (1 - CI\%)$ selected in the Analysis Settings dialog box. The t-factor is used in creating the confidence interval to indicate uncertainty due to a small number of sample data. Generally, the normal distribution and a corresponding z-value are used to generate confidence intervals in statistics, but the Student's t-distribution is a distribution related to the normal distribution that reflects a greater variation in data that is found using very small samples. The t-factor, in contrast to the z-value for a normal distribution, varies in relation to the degrees of freedom in a calculation.

Equation #2 (RQmin):

$$2^{-((\Delta\Delta C_T)_{\text{sample, target}} + T(\alpha, \nu(\Delta C_T)_{\text{sample, target}}) \times \sigma_{SV}(\Delta C_T)_{\text{sample, target}})} < RQ_{(\text{sample, target})}$$

Equation #3 (RQmax):

$$RQ_{(\text{sample, target})} < 2^{-((\Delta\Delta C_T)_{\text{sample, target}} - T(\alpha, \nu(\Delta C_T)_{\text{sample, target}}) \times \sigma_{SV}(\Delta C_T)_{\text{sample, target}})}$$

Where: T = Student's t-factor; α = confidence level; ν = degree of freedom; σ_{SV} = statistical variability

h. For the **Basic** or **Standard Deviation** algorithm, the software does not use the t-factor in Student's t-distribution. Instead, the user selects the desired number of standard deviations away from the mean to be contained in the confidence interval, and the software uses this number directly to calculate the interval.

Equation #4 (RQmin):

$$2^{-((\Delta\Delta C_T)_{\text{sample, target}} + S \times \sigma_{SV}(\Delta C_T)_{\text{sample, target}})} < RQ_{(\text{sample, target})}$$

Equation #5 (RQmax):

$$RQ_{(\text{sample, target})} < 2^{-((\Delta\Delta C_T)_{\text{sample, target}} - S \times \sigma_{SV}(\Delta C_T)_{\text{sample, target}})}$$

Where: S = number of standard deviations to include; σ_{SV} = statistical variability

Since the **Basic** or **Standard Deviation** algorithm does not involve confidence-level considerations typically used for small experiments (for example, 2 to 4 replicates). Therefore, RQmin/RQmax error bar for the calculation does not necessarily correlate to any implied statistical confidence.

Glossary

Δ	Delta. A symbol indicating a normalized value.
α	Alpha. The confidence level selected in the Analysis Settings dialog box
σ	Sigma. Summation.
σ_{SD}	Standard deviation, calculated as the square root of the mean of squared deviations from the sample mean.
σ_{SV}	Statistical variability, a calculated value used to estimate the variability associated with measured or calculated values.
μ	Mu. Arithmetic mean.
$\mu(\Delta Ct)$	The study-level ΔCt arithmetic mean
RQ	Relative quantity
SD	Standard deviation
SE	Standard error

Notes

To find these algorithms in the software go to Analysis > Analysis Settings > Relative Quantification Settings. The 7900 system software (RQ Manager) has only the Confidence Level algorithm.

References

1. User Bulletin #2: ABI Prism 7700 Sequence Detection System. December 1997 (updated 10/2001), Part Number 4303859B.
2. User Bulletin: Applied Biosystems Real-Time PCR Systems - Relative Quantification (RQ) Algorithms in Applied Biosystems Real-Time PCR Systems Software. July 2007, Part Number 4378622.