# Precision in Real-Time PCR: Implementing a New Function for Accurate Quantification and Outlier Detection

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## Introduction

Real-time PCR is an essential technique for absolute and relative quantification of nucleic acids. The standard quantification method (Ct) assumes that the compared samples have similar PCR efficiency. However, in some biological samples, inhibitors can be co-purified with DNA, making them difficult to remove and causing bias in real-time PCR quantification [1]. To avoid quantification errors due to differences in amplification efficiency among samples, outlier detection methods based on amplification estimation have been developed.

## Objectives

The Cy0 method has demonstrated greater accuracy in the presence of inhibitors compared to the Ct method [1], but the goodness of fitting is maximum only when the amplification curve does not exceed a critical asymmetry value (1-2/e). Consequently, the SOD (shape outlier detection) technique can be extended, increasing the reliability, by developing an approach in which the SOD value can be calculated with greater accuracy even in cases where the asymmetry of the curve exceeds the limits manageable by the Richards curve.

## Materials and Methods

The Cy0 method is based on the intersection between the abscissa axis and the tangent at the inflection point of the sigmoidal amplification curve obtained through nonlinear fitting [1,2]. Traditionally, the Richards curve has been used for this purpose, but it has limitations when the curve's asymmetry exceeds a critical value of 1-2/e. To overcome these issues, a new function derived from the solution of a differential equation has been introduced.

The classical logistic growth differential equation is:

$$\frac{dy}{dx} = ay\left(1 - \frac{y}{k}\right),$$

Introducing an additional parameter allows for varying the shape of the sigmoidal curve. The resulting differential equation is:

$$\frac{dy}{dx} = ay\left(1 - \frac{y}{k}\right)^{b}$$

where b is a constant that varies the shape of the curve. The solution of this equation leads to the Richard function. The goodness of fitting of Richards' curve is maximum only when the amplification curve does not exceed a critical asymmetry value.

Then, the new proposed differential equation is:

$$\frac{dy}{dx} = ay\left(1 - \frac{cy}{K - y + cy}\right)$$

This new function allows for modelling amplification curves with a higher degree of asymmetry compared to the Richards curve, thereby improving the accuracy of the Cy0 method even in the presence of strong inhibitors. The Cy0 value is now calculated as the intersection between the abscissa axis and the tangent at the inflection point of the sigmoidal curve obtained through nonlinear fitting using the new proposed function.

Shape-based Outlier Detection (SOD)

The SOD (Shape-based Outlier Detection) method compares the shape of the experimental amplification curve with the standard curve without comparing efficiency. The shape parameters used in the new nonlinear regression function are critical for accurately modelling the amplification curves in real-time PCR. These parameters include the plateau value, which represents the maximum fluorescence signal achieved during the reaction, the slope of the tangent at the inflection point and the y-coordinate of the inflection point [3]. Additionally, the new function introduces parameters that allow for greater flexibility in handling curve asymmetry, particularly when it exceeds the critical value of 1-2/e. This flexibility is achieved by adjusting the constants in the differential equation to accommodate variations in the amplification kinetics. These adjustments ensure that the curve fitting remains accurate even in the presence of significant inhibitors, thereby improving the reliability of the Cy0 quantification method.

The SOD formula is given by:

$D^2=(\boldsymbol{x}-\boldsymbol{\mu})^T\boldsymbol{S}^{-1}(\boldsymbol{x}-\boldsymbol{\mu})$

where:

$\boldsymbol{x}$ is the vector of shape parameters for the experimental sample,

$\boldsymbol{\mu}$ is the mean vector of shape parameters for the standard samples,

$\boldsymbol{S}$ is the covariance matrix of the shape parameters for the standard samples,

$\boldsymbol{D^2}$ is the Mahalanobis distance.

Indeed, the SOD value is based on the Mahalanobis distance and is used to determine the similarity of an unknown multivariate sample set to a known one [3]. If the SOD value exceeds 7.81, the null hypothesis can be rejected, and it can be established that the shape of the amplification curve differs from that of the standard samples, considering all three parameters.

## Results and Conclusion

The results show that the new function provides excellent fitting of the amplification curves, even when the asymmetry exceeds the critical limit for the Richards curve. This approach allows for the calculation of the Cy0 value with greater precision and reliability, minimizing errors due to the limitations of the previous model. The distribution of residuals and run tests confirm the effectiveness of the new function. Consequently, using the new function for fitting the amplification curves, the SOD value can be calculated with greater accuracy even in cases where the asymmetry of the curve exceeds the limits manageable by the Richards curve. This allows for more effective identification of outliers in real-time PCR analyses.

## Bibliography

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