

The tilted threshold line. An alternative to the horizontal counterpart which is commonly used for dilution curve quantification?

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Introduction

The estimation of real-time efficiencies has been shown to be conducted most accurately by utilizing calibration curve analysis. For this purpose, serial dilutions of the gene of interest are subjected to real-time PCR and the threshold cycles obtained from the dilutions are plotted against the logarithmized dilution factors (relative approach) or copy numbers (absolute approach). The threshold cycles (or otherwise denoted as crossing points) for the above procedure are usually acquired from the software accompanying the real-time PCR instrument. This value is instrument specific and may often be not very intuitive for the user.

A major drawback is to our opinion, that the threshold cycles are always calculated from a fluorescence value which is constant over all dilutions and samples (threshold line or border). This results in suboptimal regression curves when the difference in the threshold cycles (delta-ct's) between two dilutions gets larger with increasing dilutions, which is almost always seen for higher cycles. Being able to accurately quantify samples with very low abundance of transcripts is crucial, as these often belong to the most interesting biological groups (i.e. transcription factors or receptors).

Algorithm

Our method substitutes the horizontal threshold line by a line which is tilted downwards from the sample of lowest dilution to the sample of highest dilution. This line with slope X and intercept Y is calculated iteratively through all intercepts and slopes (calculation time about 2-10 minutes, depending on the number of steps chosen within each iteration).

Ultimately a threshold line with defined X and Y is selected that optimizes the performance of a regression curve, either by maximizing the coefficient of determination R^2 or by minimizing the Akaike Information Criterion (AIC). Constraints are applied to this curve such that the iteration starts at the second derivative maximum of the lowest dilution sample and stops at the first outlier cycle of the highest dilution curve, so that values in the baseline region of the curves are not accidentally included (**Box 1**).

Results and Discussion

The paradigm of our new method is to maximize the goodness-of-fit of the regression curve from the dilution analysis by means of an iterative method (**Figure 2**). The resulting threshold line from which the efficiency and also the threshold cycles are derived is in most cases not horizontal (**Figure 1A**) but tilted downwards after the optimization process (**Figure 1B**). This results in optimized regression curves with higher R^2 and lower AIC values (**Figure 3**). The latter can be used to estimate the gain in goodness-of-fit by using Akaike weights of the common method and the new method. For example, a decrease of the AIC from 3.2 to -1.8 (**Figure 3**) means 12 times more weight of evidence in the fit.

A desired property of the new method must be to exhibit a gain of accuracy and precision in the quantitation of unknown samples. A Leave-One-Out approach (omitting a dilution and quantitating its dilution factor from the remaining samples) shows that the new method displays similar accuracy but increased precision/reproducibility (smaller interquartil-ranges in the boxplots) than the common procedure (**Figure 4**).

Box 1: The algorithm in detail, starting from a series of background subtracted real-time PCR dilution curves:

Step 1: Set the initial y-axis intersection to the second derivative maximum of the lowest dilution.

Step 2: Iterate the slope in steps of $-n^\circ$ until the line intersects the outlier cycle of the highest dilution curve.

Step 3: For each iteration collect a figure-of-merit, such as R^2 or the Akaike Information Criterion (AIC) of the resulting linear regression curve.

Step 4: Decrease the y-axis intersection by $n\%$ and continue at **Step 2**. If y-axis intersection is near the outlier cycle of the lowest dilution curve, go to **Step 5**.

Step 5: Show a heatmap representation with the figure-of-merit from all iterations and select the combination with highest R^2 (or lowest AIC).

Figure 3: Comparison of performance between existing (red) and new (blue) method using replicate dilution data (6 dilutions, 20 replicates)

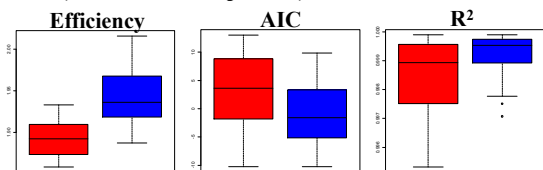


Figure 4: Accuracy and precision in estimating unknown quantities from the same data as above

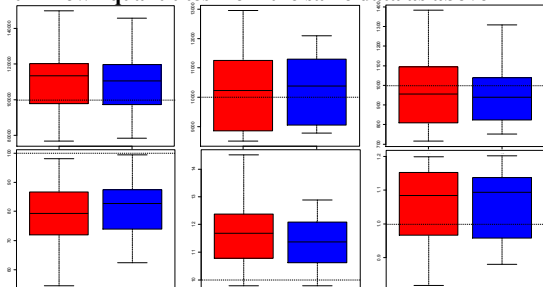


Figure 2: Heatmap matrix with figure-of-merit (AIC) for each iteration. Best iteration highlighted with black box.

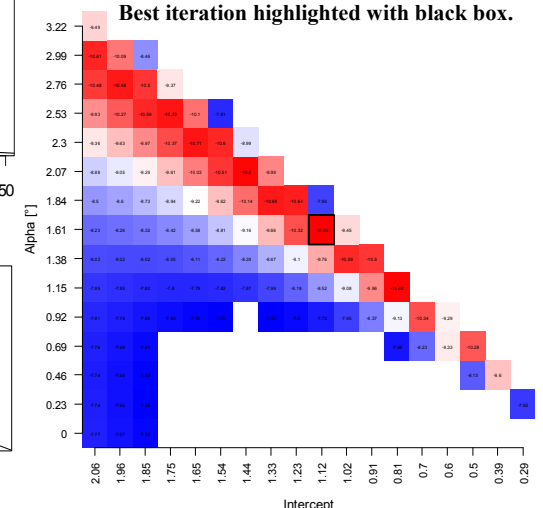


Figure 1A: Common procedure with horizontal threshold line

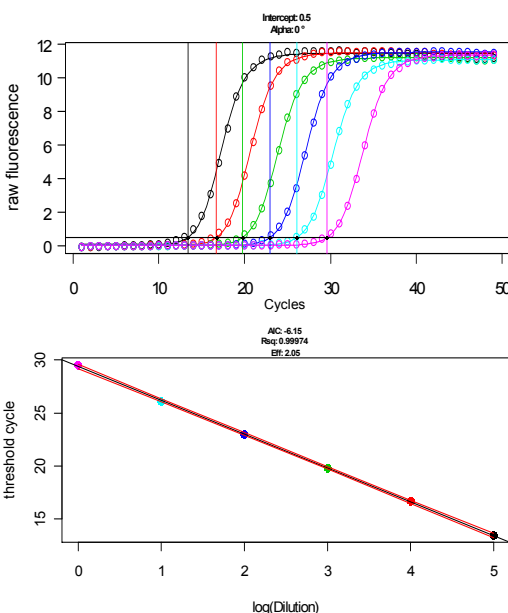


Figure 1B: New procedure with optimized tilted threshold line

