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Savitzky-Golay smoothing and differentiation for PCR quantification

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Abstract

In quantitative PCR (qPCR), replicates can minimize the impact of intra-assay variation; however inter-assay variations must be minimized in order to obtain a robust quantification method. The method proposed in this study uses Savitzky-Golay Smoothing and Differentiation (SGSD) to identify a derivative maximum-based cycle of quantification. It does not rely on curve modeling, as is the case with many existing techniques. PCR fluorescence data sets challenged for inter-assay variations (different thermocycler units, different reagents batches, different operators, different standard curves and different labs) were used for the evaluation. The algorithm was compared to a four-parameters logistic model method (4PLM), the \( C_{\text{y0}} \) method and the threshold method. The SGSD method compared favorably to all methods in terms of inter-assay variation. SGSD was statistically different from 4PLM (p-value = 0.03), \( C_{\text{y0}} \) (p-value = 0.05) and threshold method (p-value = 0.004) on relative error comparison basis. For intra-assay variations, SGSD outperformed the threshold method (p-value = 0.005) and equalled the 4PLM and \( C_{\text{y0}} \) methods (p-value > 0.05) on relative error basis. Our results demonstrate that the SGSD method could potentially be an alternative to sigmoid modeling based methods (4PLM and \( C_{\text{y0}} \)) when PCR data are challenged for inter-assay variations.

Keywords: gene quantification, PCR quantification, quantification cycle, qPCR, Savitzky-Golay
Introduction


Quantitative PCR, which appeared in the 1990s (Higuchi et al. 1992; Higuchi et al. 1993), is based on real-time fluorescence measurement of DNA template amplification. Quantification is performed using mathematical treatment of the raw fluorescence data. High sensitivity, theoretically as low as 3 gene copies (Birch et al. 2001), and high throughput, have made qPCR the gold standard of DNA quantification methods (Dolan et al. 2009; Kephart and Bushon 2009; Jimenez et al. 2000; Espy et al. 2006; Postollec et al. 2011; Jimenez 2011).

Although very sensitive, qPCR is also prone to error from two major sources. The first source of error results from efficiency determination which is used to calculate the output quantity. Fluctuation in the amplification curve shape affects efficiency calculations as well as the quantification process (Sisti et al. 2010). This efficiency can be calculated from a standard curve plot (Wilhelm and Pingoud 2003) as usually performed for absolute quantification. Another way is to calculate it individually by numerical analysis of the reaction exponential phase as performed for relative quantification (Tichopad et al. 2003) and sometime for absolute quantification (Rutledge 2004). Relationship between the efficiency (E), the DNA template concentration (T) and the number of amplification cycle (n) is given by the following equation:

\[ T_n = T_0 (1 + E)^n \]

It is understood that any difference between the calculated efficiency and the real efficiency would have an important effect on the calculated DNA template concentration. This effect becomes more important with higher number of amplification cycles. Standard curves are used under the assumption that efficiency is constant between replicates, while numerical efficiency calculation takes these fluctuations into account.

The second major source of error comes from the shape of the amplification curve subject to experimental variation affecting gene quantification. This is generally true whether quantification is calculated from a standard curve (absolute quantification) or based on a housekeeping gene comparison (relative quantification).
The amplification curve is usually shaped like a logistic curve and is subject to experimental variations in asymmetry (Spiess et al. 2008), slope steepness at inflexion, location of inflexion point and total increase in fluorescence from baseline to plateau (Sisti et al. 2010; Guescini 2013). To eliminate the curve comparison bias, it has been shown that a quantification methods relying on optical calibration rather than on curve comparison can be developed (Rutledge 2004).

In the first decade of the 21st century, efforts were made to increase qPCR precision. Breakthrough in relative quantification paved the way for quantification without standard curves (Pfaffl 2001). This innovation prompted the need to correct the baseline (Wilhelm et al. 2003a) and to model the logistic curve so as to determine the PCR efficiency directly from the amplification curve (Tichopad et al. 2003; Liu and Saint 2002). Meanwhile, absolute quantification, based on standard curves using the threshold method (Wilhelm and Pingoud 2003; Rutledge and Côté 2003) was adapted for logistic curve fitting (Goll et al. 2006) and made more robust against PCR inhibitors (Guescini et al. 2008). Numerous authors have worked to increase precision of both quantification methods (Karlen et al. 2007; Spiess et al. 2008; Sisti et al. 2010; Carr and Moore 2012; Guescini et al. 2013; Ruijter et al. 2013; Ruijter et al. 2015; Tellinghuisen and Spiess 2015a, b). Over the past years, different quantification approaches were compared (Ruijter et al. 2013; Tellinghuisen and Spiess 2014a; Tellinghuisen and Spiess 2014b) and new methods are still being developed and tested (Carr and Moore 2012; Franke and al. 2012; Guescini et al. 2013; Jones et al. 2014; Rao and Huang 2013; Chen and Huang 2015; Tellinghuisen and Spiess 2015b) since qPCR data variance is not yet fully explained. Along new methods, various tools and software have been developed recently to improved qPCR data analysis (Bultmann and Weiskirchen 2014; Rödiger et al. 2015; Baebler et al. 2017; Mallona et al. 2017).

The approach described in this article investigates a non-modeling avenue for quantification. This contrasts with most quantification methods, which are based on PCR curve modeling. In this study, the Savitzky-Golay smoothing and differentiation (SGSD) filter commonly used for spectral band noise removal (Savitzky and Golay 1964; Schafer 2011) was evaluated as a mean to determine a derivative maximum-based cycle of quantification. To the best of our knowledge, this avenue has been scarcely researched and never thoroughly evaluated (Franke et al. 2012; Trampuz et al. 2006). The parameters of the SGSD filter were evaluated and
optimized, and the method was then compared to three other PCR quantification methods: the threshold method, the four-parameters logistic curve model method, and the C\textsubscript{0} method. The investigated methods are compared from an absolute quantification perspective using several criteria (relative bias, error and standard deviation), the effect on outlier and the effect of competimers.

**Methods**

*Fluorescence data*

Data were obtained in two steps. As a first step, series of data were obtained from three different labs using the same equipment and method. Each PCR run consists of duplicated serial dilutions of standard DNA at 10, 50, 100, 1 000 and 10 000 gene copies (G\textsubscript{c}). A total of 20 runs, called data set in this article, were used, for a total of 200 PCR amplification curves as shown by Figure 1. In a second step, the quantification methods were challenged with competimer and different PCR assays using data sets from the literature. Raw fluorescence data are provided in Data File S1.

In order to validate results, three data sets were obtained from literature. The data set from Ruitjer et al. (2013) consists of MYCN gene of 10-fold dilutions from 15 000 to 15 G\textsubscript{c} for 94 replicates. Data sets from Rutledge (2004) consist of 10-fold dilutions of 4.17x10\textsuperscript{7} to 4.17x10\textsuperscript{2} copies in quadruplicate for 5 runs of the K1/K2 and K2/K3 amplicons.

In order to simulate decreased efficiency caused by inhibitors, competitive primers (competimers) have been used by previous authors (Ruijter et al. 2013) to change PCR efficiency in a predictable way. A competimer data set was obtained from the literature [http://qPCRDataMethods.hfrc.nl]. The preparation of this data set has been described in Ruitjer et al. (2013). The data set contained results with 0.0625 µg DNA by 4-fold dilution in triplicate for competimer concentration covering 0 to 50 %.

In order to evaluate the four quantification methods based on published methodology, fluorescence data set from 16 assays covering 5 dilutions replicated thrice were obtained from Ruijter biomarker data set named Data_Vermeulen_A [http://qPCRDataMethods.hfrc.nl].
**Data pre-treatment**

Raw fluorescence data were screened using MATLAB to identify lack of PCR amplification. To do so, the mean fluorescence value of baseline ($n_3$ to $n_7$) was compared to the mean value of plateau ($n_{40}$ to $n_{45}$). The plateau had to be 10 standard deviations (SD) higher than the baseline for the curves to be included into the datasets.

The baseline of the curves obtained using the 4PLM and the $C_0$ methods were adjusted to zero before data processing. The baseline was adjusted with a saturation function as described by Wilhelm et al. (2003b):

$$F_{(n)0} = a(1 - e^{b-n}) + c$$

where $F_{(n)0}$ is the baseline at cycle $n$, $a$ the saturation value, $b$ the number of cycle for baseline saturation, $c$ the fluorescence at saturation. The equation was fitted to the curve using non-linear least-square fitting by varying $a$, $b$ and $c$. Data File S2 describes the baseline adjustment using a MATLAB script.

**Standard curves for calculations of number of gene copies**

Standard curves are made with the fluorescence data in order to correlate calculated $C_q$ with the number of gene copies ($G_c$) originally present in the sample. Usually, a standard curve is prepared once using one data set of ten standard DNA sample and it is used to estimate unknown DNA sample.

Using a single standard curve for our study would potentially favor one quantification method over another; thus in order to minimize the probability of introducing bias in the study, data set were analysed 10 times using 10 standard curves in a similar fashion as done in cross-validation. In this study, 10 iterations were executed. For each iteration, 10 amplification curves were taken out of the data set in order to construct a standard curve that would be used to analyse the remaining 190 amplification curves. In each iteration, different amplification curves were taken out to generate a new standard curve. At the end of the process, each of the 200 amplification curves was calculated 9 or 10 times. The 9 or 10 estimates were then averaged. This data processing method minimizes the possible bias introduced by a single standard curve in order to better compare each quantification method.
Each calibration curve was done with amplification curves obtained with 10, 50, 100, 1 000 and 10 000 Gc from
the same sample preparation. Calibration curves were generated by linear regression of the logarithm of the
standard DNA Gc against the output Cq. The linear regression equation is given by equation 3 which can be
rearranged into the more practical equation 4.

$$G_c = 10^{\frac{(C_q - b)}{m}}$$

where \(m\) and \(b\) are respectively the slope and the intercept of the standard curve. Gene copies calculated from
the standards curves are given in Data File S1.

**Description of 4PLM method**

The algorithm consists of a sigmoid curve fitting to the amplification curve as described by Tichopad et al.
(2003). PCR amplification curves were fitted using the following equation:

$$F(n) = F_0 + \frac{\Delta F}{1 + \left( \frac{n}{n_0} \right)^2}$$

where \(F\) is the fluorescence, \(n\) the cycle number, \(F_0\) the baseline fluorescence, \(\Delta F\) the difference between the
plateau fluorescence and the baseline, \(n_0\) the inflexion point and \(b\) describes the slope at \(n_0\). The equation was
fitted using MATLAB non-linear least-square fitting and the default algorithm called *Thrust Region Effective*
(Coleman and Li 1996). The initial estimates for iteration are: \(F_0 = \min(F(n))\), \(\Delta F = \max(F(n)) - \min(F(n))\), \(b = 1\) and
the estimate for \(n_0\) was given by \(n\) at \(F(n)\) given by equation 6.

$$F_{(n_0)} = \min \left[ \left| F(n) - \frac{[\max(F(n)) - \min(F(n))] }{2} \right| \right]$$

The algorithm’s lower and upper bound were set to:

\[F_0 - \Delta F < F_0 < F_0 + \Delta F\]
\[\Delta F - 0.01 \times \Delta F < \Delta F < \Delta F + 0.01 \times \Delta F\]
\[0 < b < \infty\]
\[0 < \chi_0 < \infty\]

The 4PLM model described here is the same model used by Tichopad to fit the experimental data point;
however, the 4PLM methods used in this article is not the Tichopad’s method. Tichopad’s method models the
amplification curve in order to find the end of the exponential phase, the second derivative maximum, needed later to determine the efficiency of individual amplification. Since this article focus on methods that recover PCR efficiency from standard curves rather than on each individual curve, the Tichopads model was used but not its method. Thus, the C_q obtained using the 4PLM method is the maximum of the 2nd order derivative of the Tichopad’s model. This was realized by first generating new data points using the fitted equation. Then the data points were differentiated twice using the MATLAB differentiate function. The second derivative maximum was found using the MATLAB max function on these differentiated data points. Data File S2 describes the 4PLM method MATLAB script.

**Description of C_y0 method**

The C_y0 method has been described in detail elsewhere (Guescini et al. 2008). The PCR curve is first fitted to a five-parameter logistic model given by the Richard's equation:

\[ F(n) = F_0 + \frac{\Delta F}{1 + e^{-\left(\frac{n-n_0}{d}\right)}} \]

where \( F \) is the fluorescence, \( n \) the cycle number, \( F_0 \) the baseline fluorescence, \( \Delta F \) the difference between the plateau fluorescence and the baseline fluorescence, \( n_0 \) the inflexion point, \( b \) describes the slope at \( n_0 \) and \( d \) is the Richard’s coefficient. As for the 4PLM, the equation was fitted using MATLAB non-linear least-square fitting with initiation estimates and bound parameters. The initial estimates and the bounds were the same as for the 4PLM while initial estimate for \( d \) was 1 bounded as \( 0 < d < 10 \). The cycle of quantification, \( C_y0 \), is the intercept where the tangent of the inflexion point crosses the x-axis with a baseline set to zero. The \( C_y0 \) is given by equation 8. Data File S2 describes the C_y0 method MATLAB script.

\[ C_y0 = n_0 - b \left( 1 + \frac{1}{d} - \ln d \right) \]  

**Description of the threshold method (C_t)**

In this method, the cycle of quantification is defined to be the cycle at which the fluorescence reaches an established threshold. The baseline’s (\( n_{13} \) to \( n_{35} \)) standard deviation (SD) of every amplification curve was calculated and used to establish a threshold set at 10SD. Then, the cycle at which fluorescence minus the
baseline reach the established threshold is identified as the threshold cycle ($C_t$). Nine data points were added between each experimental point using cubic interpolation in order to add decimals to the $C_t$. It is required to add data points by interpolation between experimental points in order to increase precision since gene copies increase roughly by two-fold between two experimental data points. This operation was performed using the `resize` MATLAB function. Data File S2 presents the threshold method MATLAB script.

**Description of the SGSD method**

The Savitzky-Golay smoothing and differentiation method (SGSD) is based on mathematical treatment of spectral bands using convolution coefficients (Savitzky and Golay 1964; Schafer 2011). In summary, the SGSD method uses a moving window of points to re-align each data point according to its neighbors, which reduces noise and allows further treatment such as differentiation. First, a polynomial of order $N$ is fitted to the data in the window of width $W$ centered on the data point being calculated. The center point is then repositioned according to the fitted polynomial, the polynomial is differentiated and the differentiated value of the center point is retained to construct the differentiated curve. The window is then moved over by 1 point to calculate the next point with the same approach, until the whole curve has been re-fitted and differentiated. The new differentiated curve is truncated at the beginning and at the end by $(W-1)x0.5$ points due to the impossibility to fit the polynomial for those points. Figure 2 shows how SGSD affects the differentiated PCR curve.

This operation is realized by calculating convolution coefficients specific to the smoothing polynomial order, derivative order and the number of data point used to smooth each point. Briefly, these coefficients are obtained in two steps. First a Jacobian matrix $J$ of $2M+1$ row with $N+1$ column with values $m^n$ where $M$ is the half-width, $N$ the polynomial order, were $-MsmsM$ and $0sNsN$ is created (Schafer 2011). Then convolution coefficient matrix ($C$) is obtained by computing the equation 9.

$$ C = (J^T)^{-1} * J^T $$  \hspace{1cm} 9

Each row of $C$ have $2M+1$ number of coefficient used to smooth the spectral band and each different row correspond to different derivative power. Each individual smoothed data points are obtained by summing up
the weighted adjacent data points inside the smoothing window. Data File S3 present an example of calculations required for smoothing and Data File S2 describes the SGSD method MATLAB script.

After applying SGSD to the fluorescence curves, the cycle of quantification was found at the derivative maximum. Similarly to the threshold method previously described, data points were added prior using interpolation in order to add decimals to the $C_q$. The derivative maximum was found using the MATLAB `max` function on these differentiated data points.

The parameters that need to be adjusted for the SGSD method are the polynomial and derivative order, and the window width for the smoothing. These parameters were evaluated using factorial designs of experiment (DoE). The results of the experiments demonstrated that the derivative order has to be set to 1 and the same would apply for the polynomial order. Higher level of derivative and polynomial order increased the level of noise in the differentiated curve, which lead to higher errors. The smoothing width of the algorithm was adjusted in a way that the truncation would cover no more than the baseline or the plateau of the amplification curve. Thus, an half-width of 4 to 5 would be sufficient for most assays. Detailed information about the DoE are found in a supplementary data files comprising the raw results, the analysis and the discussion (Data File S4 and S5).

Note that prior applying SGSD, eight data points were added between each experimental point by cubic interpolation using the MATLAB resize function. This is required to give decimals to the determinate $C_q$.

**Method comparison criteria**

The algorithms were compared based on three primary criteria: relative standard deviation (RSD), relative bias (RB) and relative error (RE). These criteria were calculated using the gene copy estimation rather than the cycle of quantification as recommended (Bustin et al. 2009). RSD, equation 10, evaluates the methods’ precision. RB, equation 11, detects systematic error. RE, equation 12, reflects methods’ accuracy. In equation 10, 11, 12, $y$ is the predicted value, $\mu$ is the mean of replicates, $x$ is the actual value.
Moreover, the methods were compared with two secondary criteria: residual normality and effect of
competers. All results were evaluated for statistical significance using Boneferroni-Holm correction for group
comparison.

Normality of the residuals from the amplification reactions results was used to demonstrate their statistical
predictability and to detect outliers. The error between the statistical predicted rate and the actual rate will
depend on the residual normality. For each compared method, a straight line was constructed using residuals
within ±1 z-score. Distance from the straight line was calculated for each residual within ±3 z-score and the SD
(σ) was computed. Residuals at a distance of 1.96σ and more from the straight line were considered outlier.
Residuals were also evaluated for their ability to match a 95 % limit prediction. Residuals located at z-score =
1.96 were compared to statistical prediction of the lower and upper 95 % residual limit (mean ± 1.96σ).

As an example of quantification cycles and genes copies calculation by MATLAB using any of the four methods
cited above, a script is available (Data File S2). Quantification cycles and gene copies calculated from the
standards curves for the four evaluated methods are given in Data File S1.

As a last step, the four methods were compared based on Ruijter et al. (2013) methodology using the
fluorescence data set and the analysis template associated with the author’s article. Since the author supplies
Cq and target quantities from the 11 methods discussed in the original article, our comparison will also include
these methods. Note that Ruijter’s methodology emphasis on bias, linearity, precision and resolution; his
definition of bias is different than in the present article and his evaluation of precision focus on intra essay
variation.

\[
RSD = \sqrt{\frac{1}{N} \sum \left( \frac{y_i - \mu}{\mu} \right)^2}
\]

\[
RB = \frac{1}{N} \sum \left( \frac{y - \mu}{\mu} \right)
\]

\[
RE = \frac{1}{N} \sum \left( \frac{y - x}{y} \right)
\]
Results and discussion

Effect of processing on the amplification curve

As an example, a duplicate of a PCR amplification subjected to high level of PCR inhibition was chosen to demonstrate how the different algorithm performs in harsh conditions. Figure 3 – A shows the amplification curve without processing. For one of these two curves, it is difficult to determine where the amplification fluorescence reaches over its baseline noise; threshold determination is thus not achievable. On figure 3 – B, the SGSD differentiated curve retains a portion of the noise seen on the raw amplification curve. However, the inflexion point of the original curve shown by the maximum of the differentiate fluorescence is easily located and it matches for the duplicates. \( C_q \) of 31.5 and 32.0 are founds. On figure 3 – C, the intensity difference does separate the replicate \( C_q \) from one another when the \( C_y0 \) is determined. \( C_q \) of 20.4 and 24.8 are founds. On figure 3 – D, we see that the four parameters logistic modeling induced modeling error which separated the fluorescence maximum of the derived model. \( C_q \) of 22.5 and 26.6 are founds. This example shows that SGSD processing is more conservative of the amplification curve behavior while curve modeling can lead to biased amplification model when experimental condition are not well controlled. It shows that a SGSD processing can effectively lead to a stable \( C_q \) determination in harsh condition. However, it should be noted that this is an example based on a selected duplicate; the purpose was to show the effect of the SGSD processing on the curve compared to other methods. Examples that show bad performance for SGSD are possible; thus the performance of the SGSD processing will be evaluated in the next sections.

Effect on the standard curves

Slopes, intercepts and \( R^2 \) are shown in Error! Reference source not found.. Determination coefficient demonstrates similar linearity amongst investigated methods (0.98 <\( R^2 \) < 0.99).

From the slope, the PCR reaction efficiency can be obtained by the following equation:

\[
E = \left( 10^{\frac{1}{	ext{slope}}} \right) - 1
\]

The efficiencies \( (E) \) are reported in Figure 4 and were compared together. Results show that the four methods belong to two efficiency groups. SGSD and \( C_t \) would belong to the first group \( (E = 0.93) \) while 4PLM and \( C_y0 \)
would belong to the second group \((E = 0.90)\). Within groups efficiency variations are not statistically different (p-value = 0.36 and 0.18 respectively). However the two groups are different (p-value = 0.008). Amplification curve modeling could explain the efficiency difference. In the first group, quantification is performed using real or smoothed data points, while the second group quantification is performed using sigmoid curve modeling. P-values were calculated using the Bonferroni-Holm post hoc method.

Overall, the analysis of standard curves shows that the SGSD method performs similarly to other methods in term of efficiency determination using standard curve as a mean of absolute quantification.

**Comparison of quantification methods: effect on the \(C_q\)**

The generated \(C_q\) for the four methods were compared for precision. The overall RSD for the SGSD, 4PLM, \(C_{y0}\) and the Threshold method are 1.8, 2.0, 2.2 and 2.5% respectively; these variations are large considering the relationship between the \(G_c\) and the \(C_q\), see equation 4. However, the SGSD gains in precision are not statistically significant when compared to the 4PLM and the \(C_{y0}\) method (p-value of 0.13 and 0.075 respectively. The four methods can be compared for accuracy when plotting the input DNA quantity against the obtained \(C_q\), figure 5. Similar determination coefficients are found for the four methods \((R^2 > 0.99)\). Since errors are amplified from the \(C_q\) to the target quantities, further analysis of bias, error and standard deviation will made on the estimated target quantities or gene copies in the next section, as recommended by the MIQE guideline (Bustin et al. 2009)

**Comparison of quantification methods: global assay**

Results for relative standard deviation (RSD), bias (RB) and error (RE) for the 200 PCR curves are found in Figure . These results express the performance of the four algorithms against multiple uncontrolled factors coming from intra- and inter-assay variations. The RSD of SGSD is the lowest amongst compared method but is only statistically different to the \(C_p\) method (p-value = 0.004). Bias and error are significantly lower for the SGSD method (p-values respectively <0.005 and <0.01). The global performance of SGSD therefore appears to be best of the four investigated methods.
Comparison of quantifications methods: intra-assay

PCR reactions are influenced by various factors regrouped in two categories: intra- and inter-assay. Intra-assay variations result from mostly physical factors that vary between replicates of a PCR batch run, such as temperature, well cleanliness, physical obstruction and alteration of light path for fluorescence measurements. For perfect replicates, chemistry should be the same, but experimentally, slight variations are expected. Intra-assay variations reveal how the algorithm is able to perform with those varying factors. They are calculated by first dividing each $G_c$ prediction by its input $G_c$. Then, all of the ten samples are averaged over the 20 experiments and means are compared to each other.

Figure shows RB, RE and RSD for the four methods. SGSD, 4PLM and $C_0$ have similar RSD and RE (p-value > 0.05). SGSD has different RSD and RE value than the $C_t$ method (p-value = 0.02 and 0.005 respectively). Therefore, it can be concluded that SGSD, 4PLM and $C_0$ performances are similar in a homogenous PCR experiment.

Comparison of quantification methods: inter-assay

Inter-assay variations result from experimental variations and external factors such as reagent variations between batches, PCR inhibitors or activators, PCR thermocycler, different operators and variation in manipulation. Figure give the RB, RE and RSD for the four methods. They are calculated by first dividing each $G_c$ prediction by its input $G_c$. Then, mean of experiments or runs are compared to each other.

The SGSD method statistically outperforms the other methods in terms of error (p-value = 0.03, 0.05 and 0.004 respectively). It is not the case for RSD, for which the SGSD method is not statistically different than 4PLM and $C_0$ (p-value = 0.11 and 0.12 respectively) but still outperforms $C_t$ (p-value = 0.05). To demonstrate that RSD for SGSD is statistically better, a larger data set would be required since PCR variations are large and existing sampling is small (n = 20 experiments). Still, data suggest that the SGSD method performs better than the three other methods for multiple heterogeneous experiments.
Comparison of quantifications methods: summary

In summary, SGSD outperformed other methods on the global comparison because it was superior in inter-assay performance. The SGSD method is expected to perform similarly to other quantification method in homogenous experiments and better in experiments affected by multiple uncontrolled factors that vary from run to run.

Comparison of quantification methods: data normality

Using a normal probability plot (Data File S6) outliers were identified statistically and counted for each method (Error! Reference source not found.). Results indicate that the SGSD and the \( C_y \) method have the fewest statistical outliers.

Deviation from normality can be represented as in Figure , where statistical prediction and actual observation of the residual 95% confidence limit are compared. Positive limits (z-score = 1.96) are identified with the (+) sign and negative limits (z-score = -1.96) are identified with the (-) sign. According to these results, the SGSD and the \( C_y \) methods are the most statistically predictable, with lower and upper statistical limits differing slightly.

Overall, the analyses of residuals and outliers demonstrate that the SGSD and \( C_y \) methods would be less affected by statistical outliers than the other tested methods. It would also be easier to establish statistical specification limits using the SGSD or \( C_y \) methods since their residuals are more normal than the other methods’. False positive rate would thus be lower for these two methods.

Comparison of quantification methods: Effect of competimers

When testing with increasing amounts of competimer, the standard deviation of the calculated \( C_q \) increases. The four method’s precision (SD) was affected in a similar pattern, as shown by Figure . Additionally, the \( C_q \) number increased proportionally with competimers and the SGSD method was distinctly less affected by the
increase, as reported in Figure 5. The C_q increased by 1.95 %, 2.50 %, 2.52 % and 2.25 % for each 1 % of
competimer for, respectively, SGSD, C_y, 4PLM and C_t methods.

**Performance for different PCR assay**

In order to validate the SGSD method on different PCR assay, data sets from the literature were used as
described previously. Results for relative standard deviation (RSD), bias (RB) and error (RE) are shown in Figure
6 (rounded and excluding C_t results). The C_t method showed an RSD of 30 to 33 %, RB of 4 to 17 % and RE of 21
to 24 %. All methods except the C_t method show statistically equivalent performances for all three criteria. This
may results from the data sets coming from similar runs and which minimize the impact of inter assay
variation. These variation were shown to be best controlled by the SGSD method. Thus the SGSD method was
not challenged by these data. These results demonstrate that the SGSD method is valid and can be used with
different assays.

**Method comparison based on Ruijter’s methodology**

Bias, linearity, precision and resolution were evaluated using Ruijter fluorescence data and template for
analysis. As a first step, it was confirmed that our analysis of the 11 quantification methods published by
Ruijter using his template, matched his published results (data not shown), then the four methods evaluated in
this paper were added to the analysis.

Based on this methodology, it is first noted that standard curve based quantification methods (Ruijter C_y,0,
Standard-Cq, SGSD, 4PLM, current works C_y, Threshold) outperform the other methods. It is observed that C_y
from Ruijter and from the current work, yield the similar results; difference might arise from baseline correction
and from the non-linear curve fitting parameters used. It is also noted that the FPLM and 4PLM method yield
much different performances results; as previously said, the FPLM method from Ales Tichopad, was modified in
our work in order to do standard curve based analysis, the latter came to be called the 4PLM.

As Ruijter noted in, his evaluation of the bias is biased toward standard curve based method. For such methods,
bias would be better evaluated using the definition used in the current work.
Based on precision, the SGSD method ranks 9/15 overall and 6/6 compared to its standard curve based method siblings. Since Ruijter precision analysis is based on intra assay variance, it is not surprising and it similar to what is observed from intra assay variance on figure 7. On the other hand, SGSD performance should be better when focusing on inter assay such as shown in figure 8.

Based on resolution, the SGSD method ranks 7/15 overall and 5/6 compared to its standard curve based method siblings. A possible explanation could be that the smoothing of the amplification curve and the truncation of the first and last cycle of quantification somehow limit the resolution.

The overall rank of the SGSD method is 6/15, but ranked 5/6 compared to its standard curve siblings. The results are shown in table 3. Ruijter analysis template filled with SGSD’s \( C_q \) and target quantities can be found as a supplementary data file (Data File S7).

It should be noted that the SGSD method windows width was not optimized for each individual assay for the analysis of Ruijter data set as it was for the evaluation of this paper. Performance may improve with windows width optimization.

**Conclusion**

The Savitzky-Golay smoothing and differentiation (SGSD) method using a derivative and polynomial order of 1, and a smoothing width of 4 to 5 was evaluated for its potential to cope with inter-assays variations. The SGSD method was compared to two methods based on sigmoid curve fitting and to the traditional threshold method. The SGSD method did perform significantly better than other methods when challenged with inter-assay variations. The SGSD method performed similarly to sigmoid curve modeling methods on an intra-assay basis. Residual normality and residual predictability were superior when using the SGSD method. Competimer was found to affect the SGSD method’s precision similarly as the other methods. However, the SGSD method seems to be less affected by the competimer \( C_q \) shift.
Results obtained from in-house data were validated using fluorescence data from the literature for intra-assay variation. On the other hand, inter-assay variation results could not be validated with other PCR chemistry since fluorescence data challenged for inter-assay variation could not be found in the literature. Overall results are sufficient to suggest SGSD uses for assays submitted to inter-assay variations and noise.

**Authors' contributions**

C. Gaudreault planned, executed and analyzed the qPCR experiments. C. Gaudreault also programmed MATLAB scripts for quantification from fluorescence data. C. Gaudreault analyzed results and wrote the manuscript. J. Salvas and J. Sirois supervised the work, the planning, contributed to idea generation and revised critically the manuscript.

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We thank Ryan Gosselin from Université de Sherbrooke for MATLAB and Savitzky-Golay smoothing support and for useful suggestions. We thank Pfizer Montreal’s microbiology lab team for giving us access to lab and equipments and for their wises advices. Part of this work was supported by the BMP-Innovation scholarship (NSERC/FRQNT) and by Pfizer.

**Competing interests’ statement**

The authors declare no competing interests.

**Supporting information**

**Data File S1**

EXCEL data file containing raw fluorescence data, transformed fluorescence, calculated quantification cycles and gene quantities from the four evaluated method.

**Data File S2**

Notepad file containing the following: MATLAB script for baseline adjustments (QuantificationBaseline); MATLAB script for the four parameter logistic model (4PLM) quantification method (Quantification4PLM);
MATLAB script for the $C_{y0}$ quantification method (QuantificationCy0); MATLAB script for the threshold ($C_t$) quantification method (QuantificationThreshold); MATLAB script for the Savitzky-Golay smoothing and differentiation (SGSD) quantification method (QuantificationSGSD) and a MATLAB script example for $C_q$ and $G_q$ generation using any of the four methods cited above (Fluorescence2Cq). The scripts are shown altogether in Notepad and must be copy pasted in MATLAB.

**Data File S3**

Microsoft Word file containing additional explanations about Savitzky-Golay convolution coefficient determination

**Data File S4**

Microsoft excel file containing the DoE raw results from the optimization and evaluation of the windows width, polynomial order and differentiation order for the SGSD method

**Data File S5**

Microsoft Word file presenting the results and discussion of the DoE. 

**Data File S6**

Microsoft Word file containing a supplementary figure

**Data File S7**

A Microsoft Excel file originally created by Ruijter et al. 2013 for the analysis of PCR bias, precision, linearity and resolution. Results from the SGSD method were added to this file for analysis according to Ruijter methodology.

**References**


Figure 1. The 200 raw fluorescence PCR curves, with baseline adjusted to zero, used to evaluate quantification algorithms.

Figure 2. Effect of Savitzky-Golay smoothing on the 1st order differentiated PCR curve using width from 3 to 11 and a first order polynomial fit. The input DNA is 1000 gene copies.

Figure 3. Effect of processing on the amplification curve; A – no processing such as used in threshold method; B – SGSD differentiated curve; C – five parameter logistic model used for the Cy0 method ; D –four parameters logistic model used for the 4PLM method.

Figure 4. PCR mean efficiency (E) of standards curves
Figure 5. Quantification cycles obtained from 200 amplifications curves for the four evaluated methods.

Figure 6. Global algorithm comparison for standard deviation, bias and error on a relative scale.

Figure 7. Intra assay comparison for bias (RB), error (RE) and standard deviation (RSD) on a relative scale.

Figure 8. Inter-essay comparison: relative bias (RB), error (RE) and standard deviation (RSD).

Figure 9. Observed residuals limit and the statistical residuals limit

Figure 10. Effect of increasing concentration of competimer on Cq SD for the evaluated methods

Figure 5. Effect of increasing concentration of competimer on the Cq for the tested methods.

Figure 6. Evaluation of the SGSD, 4PLM and Cy0 method on three different assays. The target gene of the different assays are the k1k2 amplicon, the k2k3 amplicon and MYCN gene. The methods are evaluated based on relative standard deviation (RSD), bias (RB) and error (RE).
The bar graph shows the efficiency of different treatments: SGSD, 4PLM, Cy0, and Ct. The percentages are 0.93, 0.90, 0.90, and 0.93 respectively. Significant differences are indicated by asterisks: * for SGSD and Ct, ** for 4PLM and Cy0.
190x139mm (300 x 300 DPI)
Table 1. Standards curves parameters (n = 10)

<table>
<thead>
<tr>
<th></th>
<th>SGSD</th>
<th>4PLM</th>
<th>$C_0$</th>
<th>$C_t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>-3.5 ± 0.1</td>
<td>-3.6 ± 0.2</td>
<td>-3.6 ± 0.2</td>
<td>-3.5 ± 0.1</td>
</tr>
<tr>
<td>Intercept</td>
<td>40.1 ± 0.5</td>
<td>37.9 ± 0.5</td>
<td>36.5 ± 0.5</td>
<td>35.6 ± 0.5</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.98 ± 0.01</td>
<td>0.98 ± 0.01</td>
<td>0.98 ± 0.01</td>
<td>0.98 ± 0.02</td>
</tr>
</tbody>
</table>

Table 2. Data points that deviate from the normality

<table>
<thead>
<tr>
<th></th>
<th>SGSD</th>
<th>4PLM</th>
<th>$C_0$</th>
<th>$C_t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line SD (-1 to 1)</td>
<td>0.10</td>
<td>0.12</td>
<td>0.08</td>
<td>0.15</td>
</tr>
<tr>
<td>Number of outliers</td>
<td>(95% confidence)</td>
<td>5</td>
<td>12</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3. Analysis of performances for bias, precision, linearity, precision and resolution for the four methods presented in this paper along the 11 methods presented in Ruijter’s 2013 paper. The methods ranks for each performance are averaged over 15 assays or genes; then the global rank for the performance indicator is shown in parentheses.

<table>
<thead>
<tr>
<th>Method</th>
<th>Bias</th>
<th>Linearity</th>
<th>Precision</th>
<th>Resolution</th>
<th>Mean Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_0^*$</td>
<td>1.00 (1)</td>
<td>4.40 (1)</td>
<td>4.87 (4)</td>
<td>4.40 (2)</td>
<td>3.67 (2)</td>
</tr>
<tr>
<td>LinRegPCR*</td>
<td>9.60 (8)</td>
<td>4.40 (1)</td>
<td>4.73 (3)</td>
<td>4.47 (3)</td>
<td>5.80 (7)</td>
</tr>
<tr>
<td>Standard-Cq*</td>
<td>1.00 (1)</td>
<td>7.33 (9)</td>
<td>4.60 (2)</td>
<td>5.27 (5)</td>
<td>4.55 (4)</td>
</tr>
<tr>
<td>PCR-Miner*</td>
<td>10.13 (10)</td>
<td>7.73 (10)</td>
<td>6.80 (6)</td>
<td>6.73 (6)</td>
<td>7.85 (8)</td>
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<tr>
<td>MAK2*</td>
<td>9.53 (7)</td>
<td>6.93 (8)</td>
<td>8.27 (10)</td>
<td>8.33 (11)</td>
<td>8.27 (10)</td>
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<tr>
<td>LRE-E100*</td>
<td>9.60 (8)</td>
<td>6.27 (5)</td>
<td>8.73 (11)</td>
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<tr>
<td>5PSM*</td>
<td>13.87 (15)</td>
<td>8.27 (11)</td>
<td>7.00 (7)</td>
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<td>9.15 (11)</td>
</tr>
<tr>
<td>DART*</td>
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<td>12.33 (12)</td>
<td>10.67 (12)</td>
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<td>11.67 (12)</td>
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<tr>
<td>FPLM*</td>
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<td>12.80 (13)</td>
<td>12.33 (13)</td>
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<td>12.25 (13)</td>
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<tr>
<td>SGSD**</td>
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<td>6.27 (5)</td>
<td>7.80 (9)</td>
<td>7.33 (7)</td>
<td>5.60 (5)</td>
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<tr>
<td>4PLM**</td>
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<td>4.87 (4)</td>
<td>5.40 (5)</td>
<td>4.53 (4)</td>
<td>3.95 (3)</td>
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<tr>
<td>$C_0^*$</td>
<td>1.00 (1)</td>
<td>4.67 (3)</td>
<td>4.47 (1)</td>
<td>4.13 (1)</td>
<td>3.57 (1)</td>
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<tr>
<td>Threshold**</td>
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<td>7.33 (8)</td>
<td>7.93 (9)</td>
<td>5.65 (6)</td>
</tr>
</tbody>
</table>

* Methods from Ruijter et al. 2013  
** Methods from the current work