Result Variation and Efficiency Kinetics in Real-Time PCR

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Abstract- Fluorescent monitoring of DNA amplification is the basis of real-time PCR. Absolute quantification can be achieved using a standard curve method. The standard curve is constructed by amplifying known amounts of standards under identical conditions to that of the samples.The objective of the current study is to propose a mathematical model to assess the acceptability of PCR resulys.Four commercial standards for HCV-RNA (hepatitis C virus RNA) along with 6 patient samples were measured by real-time PCR, using two different RT-PCR reagents. The standard deviation of regression ($S_{y,x}$) was calculated for each group of standard and compared by F-Test. The efficiency kinetics was computed by logistic regression, χ^2 goodness of fit test was preformed to assess the appropriateness of the efficiency curves.Calculated efficiencies were not significantly different from the value predicted by logistic regression model. Reactions with more variation showed less stable efficiency curves, with wider range of amplification efficiencies.Amplification efficiency kinetics can be computed by fitting a logistic regression curve to the gathered fluorescent data of each reaction. This model can be employed to assess the acceptability of PCR results calculated by standard curve method.

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Key words: Polymerase chain reaction; efficiency; regression analysis; logistic model

Introduction

Fluorescent monitoring of DNA amplification is the basis of real-time PCR. Template concentration is determined from the fractional cycle at which a threshold amount of amplicon DNA is reached (threshold cycle or Ct), set at a point where amplicon DNA just becomes detectable, but is still within the exponential phase of the amplification. Absolute quantification can be achieved using a standard curve. The standard curve is constructed by amplifying known amounts of standards under identical conditions to that of the sample (1). It has been shown that reactions with higher amplification efficiency, primed by well-performing primers proceed with lower variability and are therefore better suited for measurement purposes (2).

Amplification efficiency on a cycle to cycle basis can be written as:

$$E_n = \left(\frac{R_n - R_{n-1}}{R_{n-1}}\right) \quad (1).$$

Where E_n is the amplification efficiency at cycle n, R_n and R_{n-1} are the fluorescence dye strength at cycle n and n- 1.

Because the equation has a small denominator, during the exponential phase of PCR reaction, small random fluctuations in efficiency are greatly magnified, and hence the apparent large fluctuations in calculated efficiency are not reflective of true changes (3). Paradoxically, efficiency variation is important in this phase as it determines the amount of product and influences the quantification.

Four-parameter sigmoid function can be used to model PCR amplification. In this method the amplification efficiency for each individual reaction can be calculated from the kinetic curve (4).

Logistic regression is another approach for computing the amplification efficiency curves. In the present study this method is used to verify the assumption of constant amplification efficiencies in standard curve quantification.

The aim of the current study is to suggest a method

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to apply to clinical practice in order to assess the quality of real-time PCR results.

Patients and Methods

Four commercial standards for HCV-RNA (5000, 500, 50 and 5 IU/μL) along with 6 patient samples were measured by real-time PCR, using two different RT-PCR reagents (Two different LOT numbers of Qiagen HCV RNA quantitative kit) in two separate groups. The resulting values were calculated by standard curve method. The standard deviation of regression ($S_{y,x}$) was calculated for each group of standards and compared by F-Test.

Predicted efficiency curves are computed as follows:

1. The normalized Fluorescence data of reactions were exported.
2. Fluorescent values below the threshold were omitted because there is high signal/noise ratio in this phase and the efficiencies if calculated, are not representative of the real efficiencies.
3. Amplification efficiencies were calculated in each reaction using the remaining fluorescent data by equation 1.
4. A logistic regression curve was fit to the fluorescent values of each reaction according to logit equation: logit (En) = Ln[En/(1-En)] where E_n is the amplification efficiency in cycle n and Ln is natural logarithm. The result of this method is shown in figure 1.
5. The entire rate of changes in amplification efficiencies, including the early phase of the reaction (below the threshold), was obtained.

χ^2 goodness of fit test was preformed to assess the appropriateness of the predicted efficiency curves.

Results

Calculated concentrations are shown in Table 1. The standard deviation of regression ($S_{y,x}$) of the first and second group of the standards were 4.79×10^{-2} and 2.77×10^{-1} respectively. The difference between the $S_{y,x}$ of results was statistically significant ($P < 0.02$). The range of the efficiency curves of the samples and standards are shown in Figure 2. χ^2 goodness of fit test showed that the calculated efficiencies were not significantly different from the predicted model ($P > 0.995$). The second group of the results shows less stable curves with wider range of amplification efficiencies.

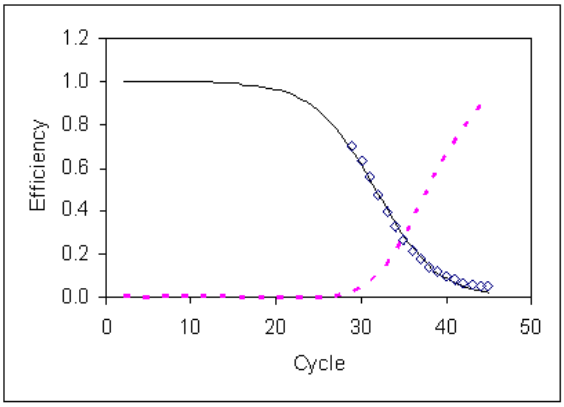

Figure 1. The Real time PCR kinetics and corresponding amplification efficiency curves are shown. Calculated efficiency values in the late phase of reaction (after threshold) are used to predict the whole range of changes (including the exponential phase of the reaction). Boxes represent the calculated efficiencies, solid line represents the efficiencies predicted by logistic regression and dashed line represents the fluorescent values of the real time PCR reaction. The threshold cycle (Ct) is at about cycle 30.

Table 1. The standards and samples analyzed with two different reagents in two separate groups. The concentration of the standards as well as Ct and calculated concentration of each reaction is shown

No	Given concentration		Group 1		Group 2	
			Ct	Calc Conc (IU/μL)	Ct	Calc Conc (IU/μL)
1	standards	5000	28.91	4498.26	28.3	3477.43
2		500	31.77	574.01	31.64	576.91
3		50	35.12	52.10	34.68	111.64
4		5	38.48	4.64	41.53	2.79
5	samples	-	26.46	1306.92	32.59	344.95
6		-	-	0	-	0
7		-	-	0	-	0
8		-	25.81	2061.83	30.99	817.88
9		-	27.70	546.48	32.92	288.31
10		-	39.26	0.16	-	0

Calc Conc: Calculated Concentration, Ct: Threshold Cycle

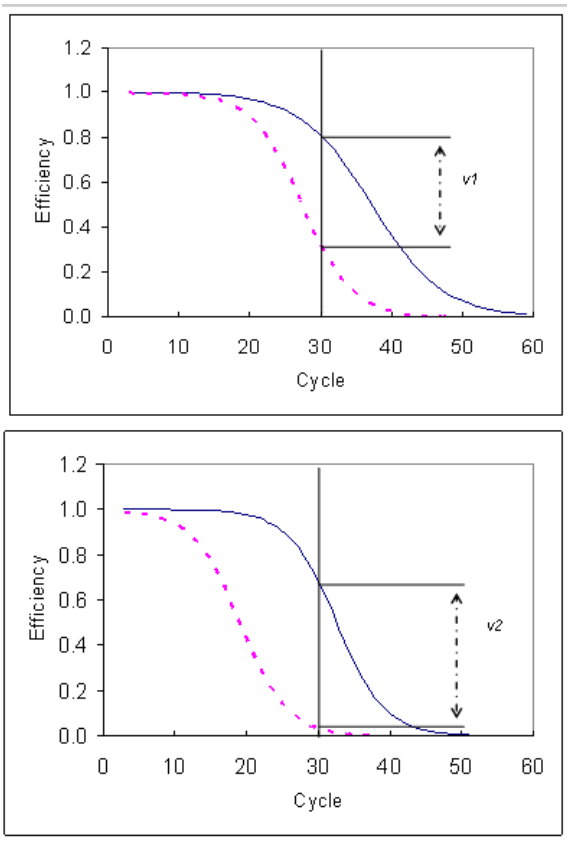

Figure 2. The greatest variation in amplification efficiency curves of the first and second reaction groups are shown in the upper and lower panels respectively. The threshold cycles (Ct) are adjusted to cycle 30. The efficiency curves are less stable in the second reaction (lower panel), therefore efficiency values are distributed in a wider range.
v1, v2: variation in efficiency at Ct between different samples.

Discussion

The amplification of template by PCR is a process involving multiple components including amount of templates, primers, ions, nucleotides, enzyme activity and reaction temperature. Except for the reaction temperature which is well controlled, all of these components are likely to be dynamically changed as the reaction progresses and to subsequently affect amplification efficiency. As well as the assumption of constant amplification efficiency, current quantification methods also rely on the assumption of equal amplification efficiencies among standards and between measured samples (3). Thus, in practical real time PCR, if the above assumptions are not actually satisfied, one could not expect a reliable quantification.

The vast amount of data gathered in the process of amplification was to verify these assumptions by fitting logistic regression curve to the data gathered in the process of amplification. This is reasonable because of the binary outcome of PCR reaction in which a proportion of templates are amplified in each cycle. In other words in each cycle the probability of a template to be amplified represented by E_n and probability of the same template to be not amplified is represented by 1-E_n, where E_n is the amplification efficiency in cycle n. Logistic regression provides a method for modeling such a binary response variable (5).

Generally internal controls are usually used to ensure that there is no PCR inhibitor in the reaction. Designing appropriate internal control is an issue since the internal control itself can compete with the target by consuming the primers and nucleotides. Inhibitors affect the efficiency and their absence in each reaction can be assured by calculating the efficiency curves.

Variation in the reaction kinetics is a source of random error in samples. Random error that occurs in standards can cause systematic error in samples. When there is a large variation, increasing the number of standards might improve the result by lowering the systematic error (6). Meanwhile, there is a limit for bias and variability of each diagnostic test beyond which the results are not acceptable (7,8). The difference observed between the reactions can not be solely related to the variation in amplifications efficiencies since reverse transcription is another source of variation. Despite the limited number of assays preformed,This study proves that a logistic regression model can be applied to the PCR kinetic to assess its efficiency .Moreover, it is well known that pushing the reaction to a better kinetics will result in improved CV (2). Study on a larger series is needed to determine the mathematical relationship between the CV and efficiency curves. In conclusion, amplification efficiency kinetics can be computed by fitting a logistic regression curve to the gathered fluorescent data of each reaction. This model can be employed to asses the acceptability of PCR results calculated by standard curve method.

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