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Improving quantitative real-time RT-PCR reproducibility by boosting primer-linked amplification efficiency

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Abstract

The effect of primer selection on real-time polymerase chain reaction (RT-PCR) performance was tested. Primer sets of varying length of product were used to amplify the sequence of β-actin. Variability in length caused variability in RT-PCR performance. Kinetic parameters of PCR were studied by mathematical approximation of real-time data by means of a four-parametric sigmoid model. This model describes the full kinetics of the amplification trajectory. Statistical exploration of parameters yielded by this model revealed that reactions with higher amplification efficiency – primed by well-performing primers – proceed with lower variability and are therefore better suited for measurement purposes.

Introduction

Reverse transcription (RT) polymerase chain reaction (PCR) is, because of its sensitivity, the method of choice for quantifying low abundance mRNAs in cells and tissues (Schmittgen 2001, Gibson *et al.* 1996, Rasmussen 2001).

There are two major disadvantages of using PCR for measurements: first, since the initial information is amplified exponentially, any error is also amplified in the same way. Second, the inherent stochastic character of the chain reaction is responsible for some initial information loss during amplification and thus the reproducibility can never be 100% (Peccoud & Jacob 1996). Nevertheless, optimising PCR conditions and data processing can increase its reproducibility. A myriad of optimisation protocols have been published but not many of them mention the problem of reaction priming. Routinely, reaction conditions are considered good when the trajectory of reaction (fluorescence curve) is steep, although this does not imply that a worse performing but well standardized reaction (Pfaffl 2002, Meijerink *et al.* 2002) cannot yield good quantitative results. We have now tested if a higher amplification efficiency achieved by primer selection can improve the reproducibility during amplification and had therefore has a direct impact on the reliability of the assay.

Materials and methods

RNA extraction and RT-reaction

Tissue samples of liver, jejunum, heart and spleen from six sheep were stored in liquid N_2 after animals had been slaughtered. Total RNA was extracted with commercially available preparation TriPure (Roche Diagnostics). Constant amounts of 1000 ng RNA were reverse-transcribed to cDNA using 200 units Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) according to the manufacturer's instructions.

PCR amplification

Sequences of β-actin gene coding region varying in length, summarized in Table 1, were amplified in

Table 1. Primer characteristics used to amplify five various sequences of β-actin gene coding region.

Primer[a]	Sequence	GC content (%)	Melting temperature (°C)	Product length (bp)
	for CAC GGA ACG TGG TTA CAG CTT TAC C	52	64	
P1				56
	rev TGT CAC GCA CAA TTT CCC GCT C	54	62	
	for GAA CGT GGT TAC AGC TTT AC	45	55	
P2				99
	rev ATC TCC TGC TCG AAG TCC A	53	57	
	for ATC CTC ACG GAA CGT GGT TAC AGC	54	64	
P3				159
	rev ATC GGG CAG CTC ATA GCT CTT CTC	54	64	
	for GTG CGT TGA CAT CAA GGA GAA GCT C	54	64	
P4				217
	rev TTG AAG GTG GTC TCG TGA ATG CCG	54	64	
	for AAG GCC AAC CGT GAG AAG ATG ACC	54	64	
P5				298
	rev TGT CAC GCA CAA TTT CCC GCT C	55	62	

[a]for = forward; rev = reverse.

25 ng cDNA in LightCycler instrument (Roche Diagnostics) (Wittwer *et al.* 1997). Five different beta-actin primer sets were used in all 24 samples (4 tissues $\times$ 6 animals) to generate variability in amplification kinetics. All primer sets were designed to generate PCR products from 50 to 300 bp in nearly equal steps of 50 bp difference. RT-PCR products should differ only in their length. The primer characteristics, like GC content and annealing temperature of all sets were adjusted to nearly constant values (Table 1).

The master-mix for each PCR run was prepared as follows: 6.4 μl water, 1.2 μl $MgCl_2$ (25 mM), 0.2 μl each primer (20 pmol), 1 μl Fast Start DNA Master SYBR Green I (Roche Diagnostics) mix, 9 μl of master-mix and 25 ng reverse transcribed total RNA. The following amplification protocol was used for all runs with all five primer sets: denaturation program (95 °C for 10 min), a three-segment amplification and quantification program repeated 40 times (95 °C for 15 s, 60 °C for 10 s and 72 °C for 20 s), melting curve program (95 °C for 10 s, 60 °C for 10 s and then slow heating with a rate of 0.1 °C per s to 99 °C with continuous fluorescence measurements) and finally cooling program down to 40 °C. All fluorescence measurements during the above mentioned program were performed in fluorescence acquisition channel 1 set at value 3. In order to prevent inter-assay variation, samples with the same primer set were always amplified within one run.

Data processing

Fluorescence observations of all samples were taken directly from LightCycler software 3 (Roche Diagnostics). Using SigmaPlot 2000 (SPSS Inc, Chicago, USA) they then were fitted with four-parametric sigmoid model

$$f = y_0 + \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}}. \quad (1)$$

In Equation (1) f is the value of function computed (fluorescence at cycles x), y_0 is the ground fluorescence, a is the difference between maximal fluorescence acquired in the run and the ground fluorescence, e is the natural logarithm base, x is the actual cycle number, x_0 is the first derivative maximum of the function or the inflexion point of the curve and b describes the slope of curve (Figure 1). In this model, the smaller is the value of b, the higher is the amplification efficiency. All PCR kinetics data produced by this model fit were finally statistically processed in SigmaStat 2.0 (Jandel Corporation, San Rafael, USA).

Table 2. Influence of different primer sets and different tissues on amplification efficiency computed using two-way ANOVA.

Source of variance	DF[a]	SS[b]	MS[c]	F[d]	*P*[e]
Primer	4	0.354	0.0886	34.056	<0.001
Tissue	3	0.0338	0.0113	4.332	0.006
Interaction	12	0.0551	0.0046	1.764	0.065
Residual	1000	0.26	0.0026		
Total	119	0.703	0.0059		

[a]Degrees of freedom.
[b]Sum of squares.
[c]Mean square.
[d]F-test ratio.
[e]Probability.

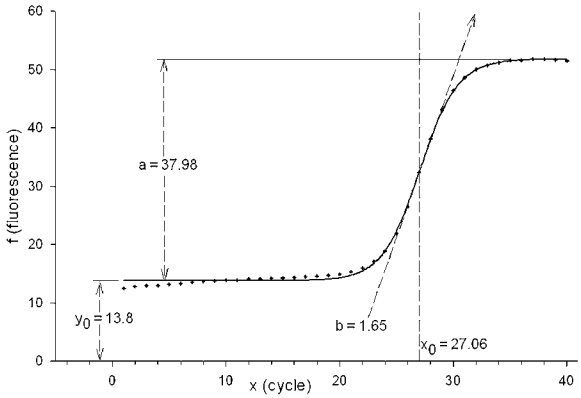

Fig. 1. Four-parametric sigmoid model. Model is described by Equation (1). One fluorescence data set from this study was used as an example. In this model, y_0 is the ground fluorescence, a is the difference between maximal fluorescence acquired in the run and the ground fluorescence, x_0 is the first derivative maximum of the function or the inflexion point of the curve and b describes the slope of curve.

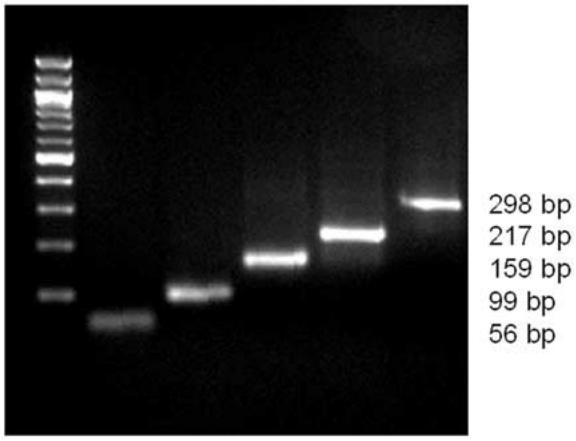

Fig. 2. Product purity inspection. cDNA sample from liver was amplified with five β-actin primer sets P1–P5 (lanes 1–5). Product lengths on 4% agarose gel match the theoretical designed length.

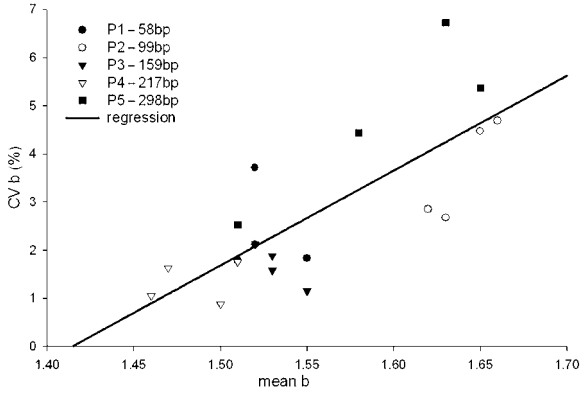

Fig. 3. Linear trend between mean value of parameter b and its variability. Each point represents six PCR runs with the same primer set and the same tissue-derived samples, but from different animals. Different primer sets are distinguished by various symbols. After fitting fluorescence data with a four-parametric sigmoid model, *mean b* (ordinate) and coefficient of *variance CV b* (abscissa) were computed and plotted.

Results and discussion

All five primer pairs generated highly specific products at the indicated lengths. Melting curve analysis (Ririe *et al.*1997) and gel inspection did not detect any primer dimers or other side-product (Figure 2). The applied mathematical model (Figure 1) is very suitable for so-called '*optimal PCR runs*' with high amplification efficiencies, low ground level fluorescence, high and constant plateau fluorescence, where the b value indicates very small variations (A. Tichopad, unpublished work). Its sensitivity is very much greater than other often used methods of serial dilutions or other methods of amplification efficiency detection (Liu & Saint 2002) As to our personal findings, the model gives justified fit and good b resolution if coefficient of determination $r^2 > 0.999$ (A. Tichopad, unpub-

lished work). Such a sigmoid estimator, however, must be considered relative as the slope of the curve does not directly indicate the amplification efficiency, e.g., the proportion between current and previous product amounts. Nevertheless, this point is not important if the various efficiencies are simply being compared and exact calculation of the differences is not required.

The significant trend ($P < 0.001$) shows that there is a lower variance of amplification efficiency in groups where b value is lower and therefore the PCR efficiency itself is higher. No such a trend is observed if variance of parameter a (e.g., high of plateau reached) was plotted against mean b. A two-way ANOVA test computed on b parameters of all runs with factors of *primer* and *tissue* showed that most of the variance between b parameters was caused by the primers (Table 2). There was also no trend between primer length and its amplification efficiency (Figure 3).

In LightCycler software, the second derivative maximum CP_{sdm} is an often used computing procedure to obtain the initial number of copies in sample. We can simulate this in the four-parametric sigmoid model, where second derivative maximum CP (SM_{sdm}) is computed from Equation (1) as follows: the first, the second, and the third derivations of the model are calculated (not shown). To calculate a second derivative maximum the third derivation has to be null: $f'''(x) = 0$. There are two second derivative maximums for $x \approx x_0 \pm 1,317 \cdot b$, whereas only the first '*positive maximum*' is relevant for an intelligent approximation of the CP. From the calculation, therefore:

$$CP(SM_{sdm}) \Rightarrow x = x_0 - 1,317 \cdot b. \qquad (2)$$

Equation (2) indicates a linear relationship between b and the value of the second derivative maximum CP (SM_{sdm}). Therefore, variability in CP (SM_{sdm}) is also linearly related to amplification efficiency in this simulation. The higher amplification efficiency, the lower variability of CP (SM_{sdm}).

To summarize and apply the above mentioned findings. Sample-specific factors such as fat, blood etc. as well as contaminants from extraction alter the PCR amplification parameters and hence introduce variability even when the same tissue samples are analysed but from different animals. We believe an error induced in this way can be minimized by boosting amplification efficiency. In this way, if compared samples undergoing PCR are forced towards their potential chemical-kinetic limits they can only vary over a smaller range. Therefore, when several primer sets are available, the set with highest amplification efficiencies should be chosen. It is likely that this concerns all other optimisation parameters (e.g., annealing temperature, Mg^{2+} concentration etc.). Several estimators of amplification efficiencies have been suggested. Here the suggested model is the most sensitive one for efficient reactions with steep trajectories. Also other sigmoid models can be used as relative estimators of amplification efficiency but their use must be considered and optimized according to the data analysed.

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