Relative quantification

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3.1   Introduction

Reverse transcription (RT) followed by a polymerase chain reaction (PCR) represents the most powerful technology to amplify and detect trace amounts of mRNA (Heid *et al*., 1996; Lockey, 1998). To quantify these low abundant expressed genes in any biological matrix the real-time quantitative RT-PCR (qRT-PCR) is the method of choice. Real-time qRT-PCR has advantages compared with conventionally performed 'semi-quantitative end point' RT-PCR, because of its high sensitivity, high specificity, good reproducibility, and wide dynamic quantification range (Higuchi *et al*., 1993; Gibson *et al*., 1996; Orland *et al*., 1998; Freeman *et al*., 1999; Schmittgen *et al*., 2000; Bustin, 2000). qRT-PCR is the most sensitive and most reliable method, in particular for low abundant transcripts in tissues with low RNA concentrations, partly degraded RNA, and from limited tissue sample (Freeman *et al*., 1999; Steuerwald *et al*., 1999; Mackay *et al*., 2002). While real-time RT-PCR has a tremendous potential for analytical and quantitative applications in transcriptome analysis, a comprehensive understanding of its underlying quantification principles is important. High reaction fidelity and reliable results of the performed mRNA quantification process is associated with standardized pre-analytical steps (tissue sampling and storage, RNA extraction and storage, RNA quantity and quality control), optimized RT and PCR performance (in terms of specificity, sensitivity, reproducibility, and robustness) and exact post-PCT data proccesion (data acquisition, evaluation, calculation and statistics) (Bustin, 2004; Pfaffl, 2004; Burkardt, 2000).

   The question which might be the 'best RT-PCR quantification strategy' to express the exact mRNA content in a sample has still not been answered to universal satisfaction. Numerous papers have been published, proposing various terms, like 'absolute', 'relative', or 'comparative' quantification. Two general types of quantification strategies can be performed in qRT-PCR. The levels of expressed genes may be measured by an 'absolute' quantification or by a relative or comparative real-time qRT-PCR (Pfaffl, 2004). The 'absolute' quantification approach relates the PCR signal to input copy number using a calibration curve (Bustin, 2000; Pfaffl and Hageleit, 2001; Fronhoffs *et al*., 2002). Calibration curves can be derived from diluted PCR products, recombinant DNA or RNA, linearized plasmids, or spiked tissue samples. The reliability of such a an absolute real-time RT-PCR assay depends on the condition of 'identical' amplification efficiencies

for both the native mRNA target and the target RNA or DNA used in the calibration curve (Souaze et al., 1996; Pfaffl, 2001). The so-called 'absolute' quantification is misleading, because the quantification is shown relative to the used calibration curve. The mRNA copy numbers must be correlated to some biological parameters, like mass of tissue, amount of total RNA or DNA, a defined amount of cells, or compared with a reference gene copy number (e.g. ribosomal RNA, or commonly used house keeping genes (HKG)). The 'absolute' quantification strategy using various calibration curves and applications are summarized elsewhere in detail (Pfaffl and Hageleit, 2001; Donald *et al.*, 2005; Lai *et al.*, 2005; Pfaffl *et al.*, 2002).

This chapter describes the relative quantification strategies in quantitative real-time RT-PCR with a special focus of relative quantification models and newly developed relative quantification software tools.

3.2 Relative quantification: The quantification is relative to what?

Relative quantification or comparative quantification measures the relative change in mRNA expression levels. It determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of another RNA. Relative quantification does not require a calibration curve or standards with known concentrations and the reference can be any transcript, as long as its sequence is known (Bustin, 2002). The units used to express relative quantities are irrelevant, and the relative quantities can be compared across multiple real-time RT-PCR experiments (Orlando *et al.*, 1998; Vandesompele *et al.*, 2002; Hellemans *et al.*, 2006). It is the adequate tool to investigate small physiological changes in gene expression levels. Often constant expressed reference genes are chosen as reference genes, which can be co-amplified in the same tube in a multiplex assay (as endogenous controls) or can be amplified in a separate tube (as exogenous controls) (Wittwer *et al.*, 2001; Livak, 1997, 2001; Morse *et al.*, 2005). Multiple possibilities are obvious to compare a gene of interest (GOI) mRNA expression to one of the following parameters. A gene expression can be relative to:

- an endogenous control, e.g. a constant expressed reference gene or another GOI
- an exogenous control, e.g. an universal and/or artificial control RNA or DNA
- an reference gene index, e.g. consisting of multiple averaged endogenous controls
- a target gene index, e.g. consisting of averaged GOIs analyzed in the study

To determine the level of expression, the differences (Δ) between the threshold cycle (C_t) or crossing points (C_p) are measured. Thus the mentioned methods can be summarized as the ΔC_P methods (Morse *et al.*, 2005; Livak and Schmittgen, 2001). But the complexity of the relative quantification procedure can be increased. In a further step a second relative parameter can be added, e.g. comparing the GOI expression level relative to:

- a nontreated control
- a time point zero
- healthy individuals

These more complex relative quantification methods can be summarized as $\Delta\Delta C_P$ methods (Livak and Schmittgen, 2001).

3.3 Normalization

To achieve optimal relative expression results, appropriate normalization strategies are required to control for experimental error (Vandesompele *et al.*, 2002; Pfaffl *et al.*, 2004), and to ensure identical cycling performance during real-time PCR. These variations are introduced by various processes required to extract and process the RNA, during PCR set-up and by the cycling process. All the relative comparisons should be made on a constant basis of extracted RNA, on analyzed mass of tissue, or an identical amount of selected cells (e.g. microdissection, biopsy, cell culture or blood cells) (Skern *et al.*, 2005). To ensure identical starting conditions, the relative expression data have to be equilibrated or normalized according to at least one of the following variables:

- sample size/mass or tissue volume
- total amount of extracted RNA
- total amount of genomic DNA
- reference ribosomal RNAs (e.g. 18S or 28S rRNA)
- reference messenger RNAs (mRNA)
- total amount of genomic DNA
- artificial RNA or DNA molecules (= standard material)

But the quality of normalized quantitative expression data cannot be better than the quality of the normalizer itself. Any variation in the normalizer will obscure real changes and produce artefactual changes (Bustin, 2002; Bustin *et al.*, 2005).

It cannot be emphasized enough that the choice of housekeeping or lineage specific genes is critical. For a number of commonly used reference genes, processed pseudogenes have been shown to exist, e.g. for β-actin or GAPDH (Dirnhofer *et al.*, 1995; Ercodani *et al.*, 1988). Pseudogenes may be responsible for specific amplification products in a fully mRNA independent fashion and result in specific amplification even in the absence of intact mRNA. It is vital to develop universal, artificial, stable, internal standard materials, that can be added prior to the RNA preparation, to monitor the efficiency of RT as well as the kinetic PCR respectively (Bustin, 2002). Usually more than one reference gene should be tested in a multiple pair-wise correlation analysis and a summary reference gene index be obtained (Pfaffl *et al.*, 2004). This represents a weighted expression of at least three reference genes and a more reliable basis of normalization in relative quantification can be postulated.

There is increasing appreciation of these aspects of qRT-PCR software tools were established for the evaluation of reference gene expression levels. *geNorm* (Vandesompele *et al.*, 2002) and *BestKeeper* (Pfaffl *et al.*, 2004) allows

for an accurate normalization of real-time qRT-PCR data by geometric averaging of multiple internal control genes (http://medgen.ugent.be/ ~jvdesomp/genorm). The *geNorm* Visual Basic applet for Microsoft Excel® determines the most stable reference genes from a set of 10 tested genes in a given cDNA sample panel, and calculates a gene expression normalization factor for each tissue sample based on the geometric mean of a user defined number of reference genes. The normalization strategy used in *geNorm* is a prerequisite for accurate kinetic RT-PCR expression profiling, which opens up the possibility of studying the biological relevance of small expression differences (Vandesompele *et al.*, 2002). These normalizing strategies are summarized and described in detail elsewhere (Huggett *et al.*, 2005; LightCycler® Relative Quantification Software, 2001).

3.4 Mathematical models

The relative expression of a GOI in relation to another gene, mostly to an adequate reference gene, can be calculated on the basis of 'delta C_p' (ΔC_p, 24) or 'delta delta C_t' ($\Delta\Delta C_t$) values (Livak and Schmittgen, 2001). Today various mathematical models are established to calculate the relative expression ratio (R), based on the comparison of the distinct cycle differences. The C_p value can be determined by various algorithms, e.g. C_p at a constant level of fluorescence or C_p acquisition according to the established mathematic algorithm (see Section 3.6).

Three general procedures of calculation of the relative quantification ratio are established:

1. The so-called 'delta C_t' (eqs. 1–2 using ΔC_p) or 'delta-delta C_t' method (eqs. 3–4 using $\Delta\Delta C_p$) without efficiency correction. Here an optimal doubling of the target DNA during each performed real-time PCR cycle is assumed (Livak, 1997, 2001; Livak and Schmittgen, 2001). Such expression differences on basis of ΔC_p values are shown in *Figure 3.1*.

$$R = 2^{[C_P \text{ sample} - C_P \text{ control}]} \qquad \text{(eq. 1)}$$

$$R = 2^{\Delta C_P} \qquad \text{(eq. 2)}$$

$$R = 2^{-[\Delta C_P \text{ sample} - \Delta C_P \text{ control}]} \qquad \text{(eq. 3)}$$

$$R = 2^{-\Delta\Delta C_P} \qquad \text{(eq. 4)}$$

2. The efficiency corrected calculation models, based on ONE sample (eqs. 5–6) (Souaze *et al.*, 1996; LightCycler® Relative Quantification Software, 2001) and the efficiency corrected calculation models, based on MULTIPLE samples (eqs. 7) (Pfaffl, 2004).

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta C_P \text{ target (control} - \text{sample)}}}{(E_{\text{Ref}})^{\Delta C_P \text{ Ref (control} - \text{sample)}}} \qquad \text{(eq. 5)}$$

$$\text{ratio} = \frac{(E_{\text{Ref}})^{C_P \text{ sample}}}{(E_{\text{target}})^{C_P \text{ sample}}} \div \frac{(E_{\text{Ref}})^{C_P \text{ calibrator}}}{(E_{\text{target}})^{C_P \text{ calibrator}}} \qquad \text{(eq. 6)}$$
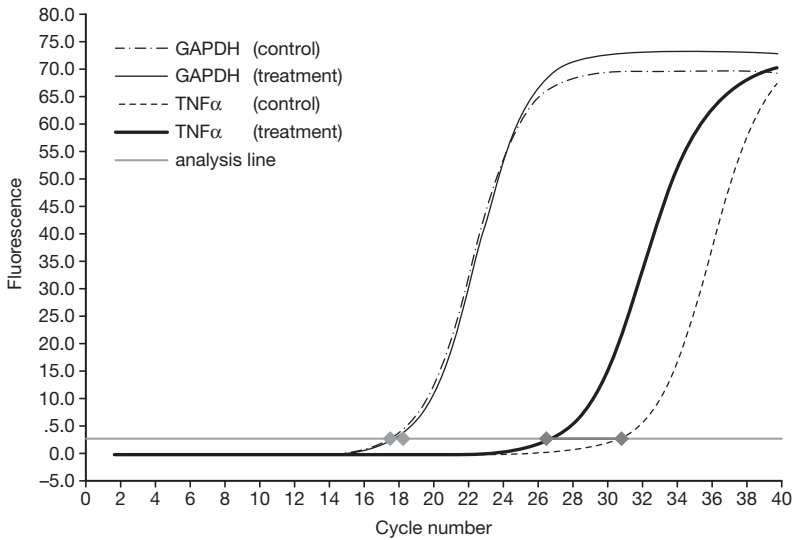
Figure 3.1

Effect of LPS treatment of TNFα target gene expression and on GAPDH reference gene expression in bovine white blood cells. Expression differences are shown by ΔC_P values.

$$\text{ratio} = \frac{(E_{target})^{\Delta C_P\ target\ (MEAN\ control\ -\ MEAN\ sample)}}{(E_{Ref})^{\Delta C_P\ Ref\ (MEAN\ control\ -\ MEAN\ sample)}} \qquad \text{(eq. 7)}$$

3. An efficiency corrected calculation models, based on MULTIPLE sample and on MULTIPLE reference genes, so-called REF index, consisting at least of three reference genes (eq. 8) (Pfaffl, 2004).

$$R = \frac{(E_{target})^{\Delta C_P\ target\ (MEAN\ control\ -\ MEAN\ sample)}}{(E_{Ref\ index})^{\Delta C_P\ Ref\ index\ (MEAN\ control\ -\ MEAN\ sample)}} \qquad \text{(eq. 8)}$$

In these models, the target-gene expression is normalized by one or more non-regulated reference gene (REF) expression, e.g., derived from classical and frequently described reference genes (Bustin, 2000; Vandesompele *et al.*, 2002; Pfaffl *et al.*, 2005). The crucial problem in this approach is that the most common reference-gene transcripts from so-called stable expressed housekeeping gene are influenced by the applied treatment. The detected mRNA expressions can be regulated and these levels vary significantly during treatment, between tissues and/or individuals (Pfaffl, 2004; Schmittgen and Zakrajsek, 2000).

Thus always one question appears: which is the right reference to normalize with and which one(s) is (are) the best housekeeping- or reference gene(s) for my mRNA quantification assay? Up to now no general answer can be given. Each researcher has to search and validate each tissue and treatment analyzed for its own stable expressed reference genes. Further,

each primer and probe combination, detection chemistry, tubes and the real-time cycler platform interfere with the test performance. However, qRT-PCR is influenced by numerous variables and appears as a multi-factorial reaction. Thus, relative quantification must be highly validated to generate useful and biologically relevant information.

The main disadvantage of using reference genes as external standards is the lack of internal control for RT and PCR inhibitors. All quantitative PCR methods assume that the target and the sample amplify with similar efficiency (Wittwer *et al*., 2001; Livak and Schmittgen, 2001). The risk with external references is that some analyzed samples may contain substances that significantly influence the real-time PCR amplification efficiency of the PCR reaction. As discussed earlier (Pfaffl, 2004), sporadic RT and PCR inhibitors or enhancers can occur.

3.5   Real-time qPCR amplification efficiency

Each analyzed sample generates an individual amplification history during real-time fluorescence analysis. As we know from laboratory practice, biological replicates, even technical replicates, result in significantly different fluorescence curves as a result of sample-to-sample variations (*Figure 3.2*). Changing PCR efficiencies are caused by RT and PCR inhibitors or enhancers, and by variations in the RNA pattern extracted. Thus the shapes of fluorescence amplification curves differ in the background level (noisy, constant or increasing), the take-off point (early or late), the steepness (good
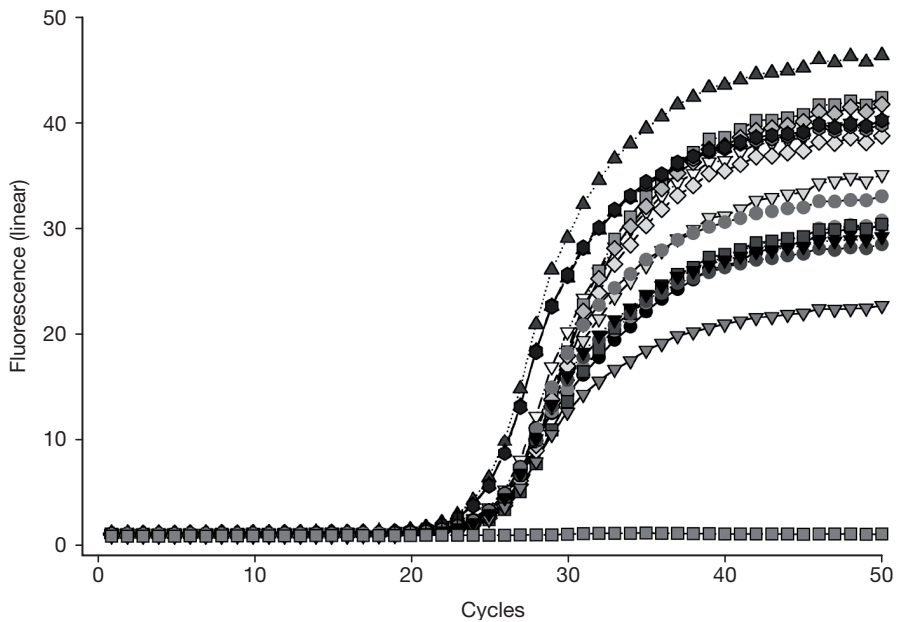
Figure 3.2

Variation of fluorescence amplification plot of three different genes run in quadruplicates.

or bad efficiency), the change-over to the plateau phase (quick or steady), and in the appearance of the PCR plateau (constant, in or decreasing trend) (Tichopad *et al.*, 2003; Tichopad *et al.*, 2004). The PCR amplification efficiency bears the biggest impact on amplification kinetics and is critically influenced by PCR reaction components. Therefore C_p determination of the threshold level and in consequence the accuracy of the quantification results are influenced by the amplification efficiency. The efficiency evaluation is an essential marker and the correction is necessary in real-time gene quantification (Rasmussen, 2001; Liu and Saint, 2002a; Liu and Saint, 2002b; Tichopad *et al.*, 2003; Meijerink *et al.*, 2001).

A constant amplification efficiency in all compared samples is one important criterion for reliable comparison between samples. This becomes crucially important when analyzing the relationship between an unknown and a reference sequence, which is performed in all relative quantification models. In experimental designs employing standardization with reference genes, the demand for invariable amplification efficiency between target and standard is often ignored, despite the fact that corrections have been suggested in the recent literature (Pfaffl, 2001; Pfaffl *et al.*, 2002; Liu and Saint, 2002a; Liu and Saint, 2002b; Soong *et al.*, 2000; Wilhelm *et al.*, 2003). A correction for efficiency, as performed in efficiency corrected mathematical models (eqs. 5–8), is strongly recommended and results in a more reliable estimation of the 'real' expression changes compared with NO efficiency correction. Even small efficiency differences between target and reference generate false expression ratio, and the researcher over- or underestimates the initial mRNA amount. A theoretic difference in qPCR efficiency (ΔE) of 3% ($\Delta E = 0.03$) between a low copy target gene and medium copy reference gene generate falsely calculated differences in expression ratio of 242% in case of $E_{target} > E_{ref}$ after 30 performed cycles. This gap will increase dramatically by higher efficiency differences $\Delta E = 0.05$ (432%) and $\Delta E = 0.10$ (1,744%). The assessment of the sample specific efficiencies must be carried out before any relative calculation is done. Some tools are available to correct for efficiency differences. The LightCycler® Relative Expression Software (2001), Q-Gene (Muller *et al.*, 2002), qBase (Hellmans *et al.*, 2006), SoFar (Wilhelm *et al.*, 2003), and various REST software applications (LightCycler® Relative Quantification Software, 2001; Pfaffl *et al.*, 2002; Pfaffl and Horgan, 2002; Pfaffl and Horgan, 2005) allow the evaluation of amplification efficiency plots. In most of the applications a triplicate determination of real-time PCR efficiency for every sample is recommended. Therefore efficiency corrections should be included in the relative quantification procedure and the future software applications should calculate automatically the qPCR efficiency (Pfaffl, 2004).

3.6 Determination of the amplification rate

Up to now only one software package can automatically determine the real-time PCR efficiency sample-by-sample. In the Rotor-Gene™ 3000 software package (Corbett Research), it is called the comparative quantification. Amplification rate is calculated on the basis of fluorescence increase in the PCR exponential phase. Further algorithms and methods are described in recent publications to estimate the real-time PCR efficiency. These can be

grouped in direct and indirect methods. Direct methods are based on either a dilution method or a measurement of the relative fluorescence increase in the exponential phase. On the other hand, indirect methods are published, doing the efficiency calculation on basis of a fit to a mathematical model, like sigmoidal, logistic models or an exponential curve fitting (for details see http://efficiency.gene-quantification.info).

3.6.1 Dilution method

The amplification rate is calculated on the basis of a linear regression slope of a dilution row (*Figure 3.3*). Efficiency (*E*) can be determined based on eq. 9 (Higuchi *et al.*, 1993; Rasmussen, 2001). But the real-time PCR efficiency should be evaluated sample-by-sample, which is quite laborious and costly, wastes template, and takes time if the dilution method is used. Alternatively, the pool of all sample RNAs can be used to accumulate all possible 'positive and negative impacts' on kinetic PCR efficiency. Applying the dilution method, usually the efficiency varies in a range of *E* = 1.60 to values over 2 (*Figure 3.3*) (Souaze *et al.*, 1996).

$$E = 10^{[-1/\text{slope}]} \qquad \text{(eq. 9)}$$

Typically, the relationship between C_P and the logarithm of the starting copy number of the target sequence should remain linear for up to five orders of magnitude in the calibration curve as well as in the native sample
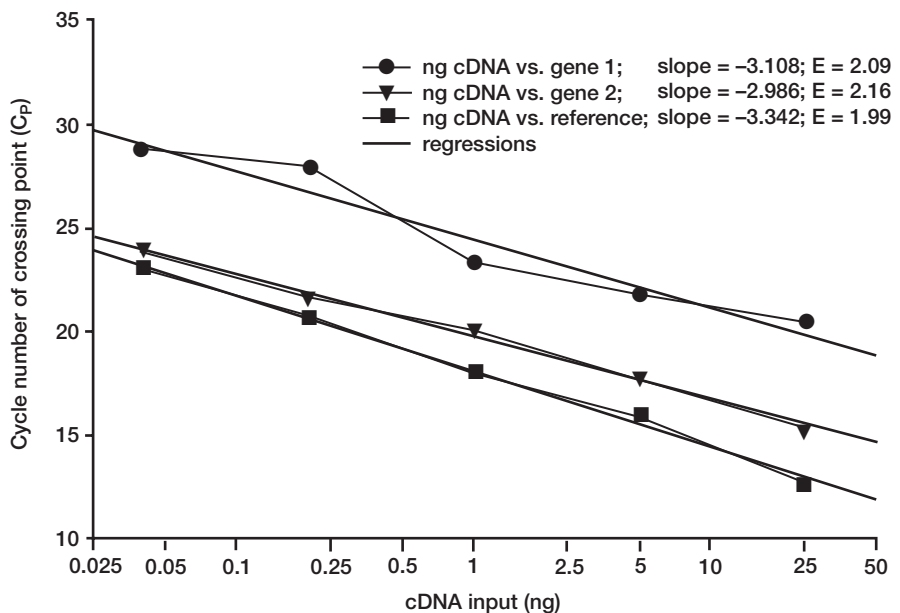
Figure 3.3

On the basis of a dilution row the real-time efficiency is calculated according to eq. 9 (Higuchi *et al.*, 1993; Rasmussen, 2001).

RNA (Muller *et al.*, 2002). The advantage of the dilution method is that it is highly reproducible and constant within one transcript and tissue. The disadvantage of this approach is the high efficiencies, often higher than two ($E > 2.0$), which is practically impossible on the basis of the PCR amplification theory. This indicates that this efficiency estimation is more or less not the best one and it will overestimate the 'real' amplification efficiency.

3.6.2 Fluorescence increase in exponential phase

Efficiency calculation from the fluorescence increases in the exponential phase of fluorescence history plot (in log. scale) (*Figure 3.4*). Fitting can be done by eye, or more reliably by software applications like LinRegPCR (Ramakers *et al.*, 2003) or DART-PCR (Peirson *et al.*, 2003). The investigator has to decide which fluorescence data to include in the analysis and which to omit. A linear regression plot is drawn from at least four data points, where the slope of the regression line represents the PCR efficiency. Therefore this method is more or less arbitrary and dependent on the chosen data points. Resulting efficiencies range between $E = 1.45$, and $E = 1.90$, and seem more realistic than the results mentioned above. This efficiency calculation method might be good estimator for the 'real efficiency,' because data evaluation is made exclusively in exponential phase.

The advantage of both direct methods is the independency of the background fluorescence. We know from several applications that a rising
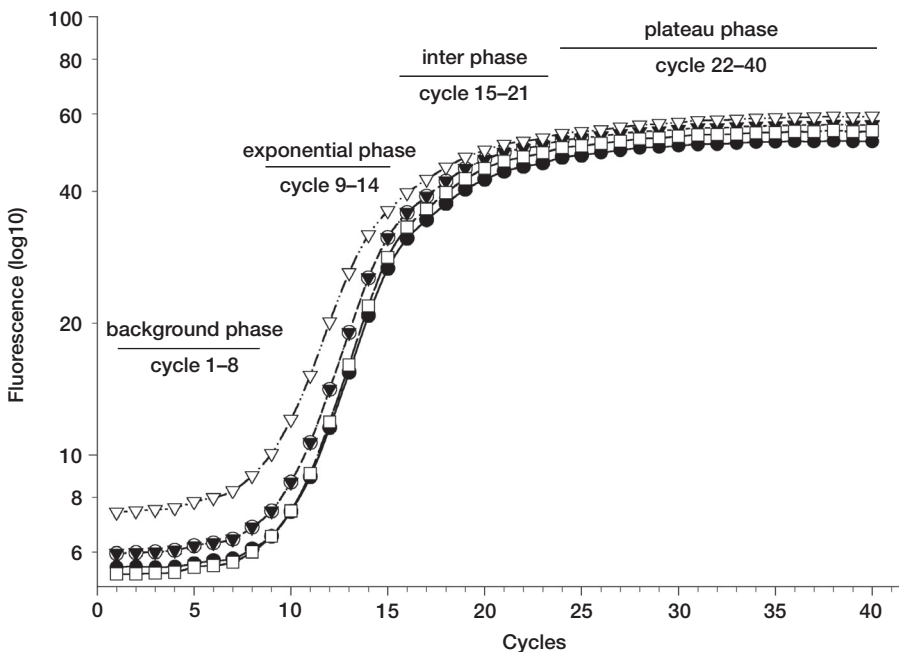
Figure 3.4

Efficiency calculation in the exponential phase.

trend in the background fluorescence will interfere with the indirect curve fit, like sigmoidal, logistic and exponential models. Probe based detection in particular exhibits high and noisy background levels, whereas SYBR® Green I applications show low and constant background fluorescence (*Figure 3.5*).

3.6.3 Sigmoidal or logistic curve fit

A number of publications have suggested an efficiency calculation on the basis of all fluorescence data points (starting at cycle 1 up to the last cycle), according to a sigmoidal or logistic curve fit model (Tichopad *et al.*, 2003; Tichopad *et al.*, 2004; Liu and Saint, 2002a; Liu and Saint, 2002b; Rutledge, 2004). The advantage of such models is that all data points will be included in the calculation process and no background subtraction is necessary. The efficiency will be calculated at the point of inflexion (cycle 27.06 shown in *Figure 3.5*) at absolute maximum fluorescence increase.

$$f(x) = y_0 + \frac{a}{1 + e^{-(\frac{x-x_0}{b})}} \qquad \text{(eq. 10)}$$

In the four-parametric sigmoid model (eq. 10), x is the cycle number, $f(x)$ is the computed function of the fluorescence in cycle number x, y_0 is the background fluorescence, a is the difference between maximal fluorescence reached at plateau phase and background fluorescence (i.e. the plateau height), e is the natural logarithm base, x_0 is the co-ordinate of the first derivative maximum of the model or inflexion point of the curve, and b describes the slope at x_0 in the log–linear phase (Tichopad *et al.*, 2004). But
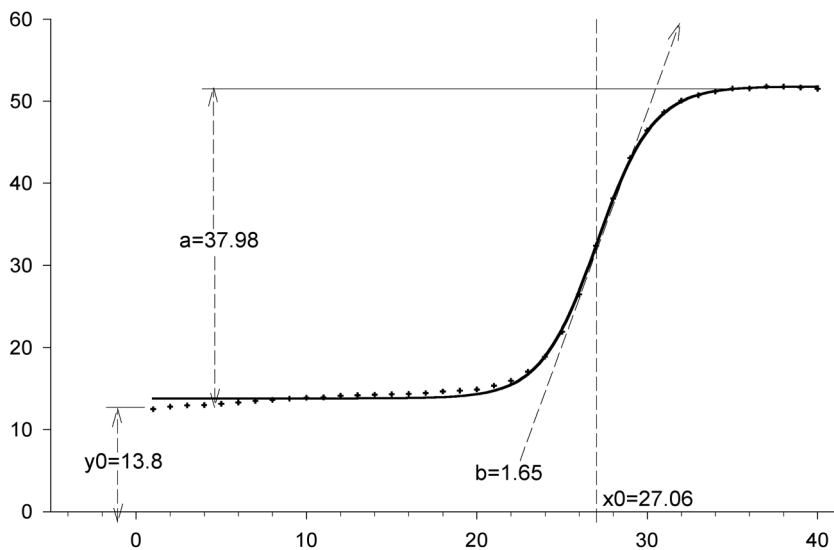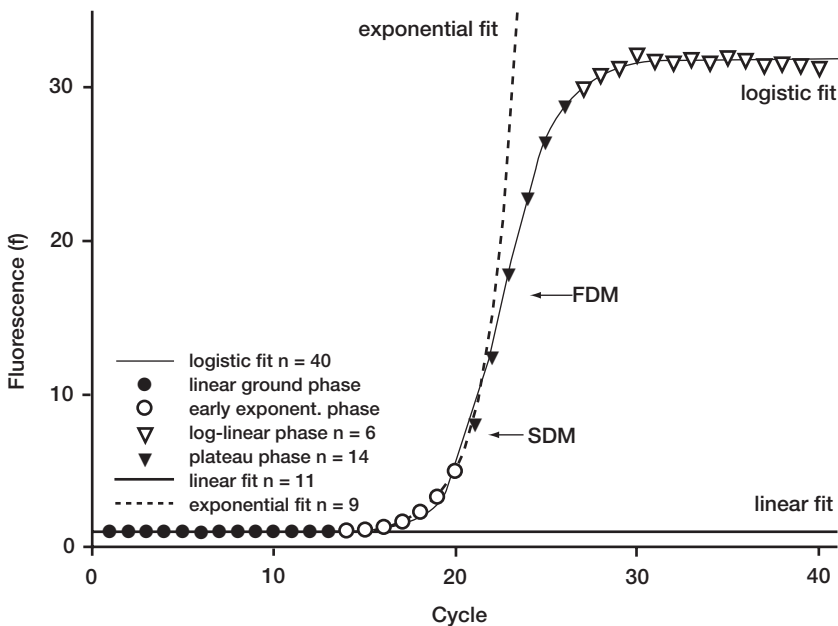

Figure 3.5

Efficiency calculation on the basis of a four-parametric sigmoid model (eq. 10).

the derived slope parameters generated by the sigmoidal or logistic models, e.g. *b*, can not directly compared with the 'real PCR efficiency.' The advantages of the four-parametric sigmoid model is that it is easy to perform, is a good estimator for the maximum curve slope with high correlation between replicates (r > 0.99) and the algorithm can easily implemented in analysis software. The resulting efficiencies are comparable to the latter method and range from 1.35 to 1.65.

3.6.4 Efficiency calculation in the exponential phase using multiple models

Here we describe the efficiency calculation in the exponential phase using multiple models, first, a linear, second, a logistic and third, an exponential model (Tichopad *et al.*, 2003). The background phase is determined with the linear model using studentized residual statistics. The phase until the second derivative maximum (SDM) of the logistic fit exhibits a real exponential amplification behavior (*Figure 3.6*). The phase behind including the first derivative maximum (FDM) shows suboptimal and decreasing amplification efficiencies and therefore has to be excluded from the analysis. Efficiency calculation is only performed between the background and before SDM. Here an exponential model according to a polynomial curve fit is performed, according to eq. 11.

$$Y_n = Y_0 \, (E)^n \qquad\qquad (\text{eq. 11})$$

Figure 3.6

Efficiency calculation in the exponential phase using multiple model fitting: linear, logistic and exponential model (Tichopad *et al.*, 2003).

In the polynomial model, Y_n is fluorescence acquired at cycle n, and Y_0 initial fluorescence, and E represents the efficiency. Here in the exponential part of the PCR reaction, kinetic is still under 'full amplification power' with no restrictions. The calculation is performed on each reaction kinetic plot and the amplification efficiency can be determined exactly. They range from $E = 1.75$ to $E = 1.90$, in agreement with the other methods.

A comparable multi-factorial model is used in the SoFAR software application (Wilhelm *et al.*, 2003). Here the background is corrected by a least square fit of the signal curve. Efficiency is determined by an exponential growth function (eq. 11) or a logistic or sigmoidal fit (eq. 10). The sigmoidal exponential function was the most precise one and could increase the amplification efficiency, before and after correction, from around 62% up to 82% (Wilhelm *et al.*, 2003).

All models lead to efficiency estimates, but which model results in the 'right', most accurate and realistic real-time amplification efficiency estimate has to be evaluated in further experiments. From our experiment we know that the detection chemistry, the type of tubes (plastic tubes or glass capillaries), the cycling platform as well the optical system has considerable influence on the estimates of real-time efficiency. Better dyes and much more sensitive optical and detection systems are needed to guarantee a reliable efficiency calculation. In *Table 3.1* an overview of the existing efficiency calculation methods is shown.

Table 3.1 Overview of existing efficiency calculation methods.

Summary	Sample individual determination	Overestimation + Intermediate Ø Underestimation −	Combination of efficiency and C_P determination
Dilution series (fit point or SDM) Rasmussen (2001)	no	+ n = 3–5	
Fluorescence increase Various authors	+	− n = 3–6	
Fluorescence increase Peccoud and Jacob (1996)	+	− n = 3	
Sigmoidal model Lui and Saint (2002a, 2002b) Tichopad *et al.* (2004) Wilhelm *et al.* (2003) Rutledge (2004)	+	− n = 1	+
LinRegPCR Ramakers *et al.* (2003)	+	Ø n = 4–6	
KOD Bar *et al.* (2003)	+	Ø n = 3–5	
Logistic model Tichopad *et al.* (2003) Wilhelm *et al.* (2003)	+	Ø n > 7	+
Rotor-Gene™ 3000 Comparative quantitation analysis	+	Ø n = 4	+

3.7   What is the right crossing point to determine?

The C_P value is the central value in real-time PCR applications. Everything is related to this single point. But not much effort has been put into standardizing and optimizing the determination of this parameter that is so central to quantification. Most software use the so-called 'threshold cycle method' or 'fit point method' and measure the C_P at a constant fluorescence level. But there are other possibilities and options to consider. Let us first think about the background:

- What kind of background fluorescence is evident, a noisy, a constant, a rising or a decreasing background?
- Does the software show me my real raw-fluorescence-data or are the data already manipulated, e.g., additional ROX adjustment?
- What about the curve smoothing of the fluorescence data?
- Which kind of fluorescence background correction and/or subtraction is applied?

Most real-time platforms show pre-adjusted fluorescence data and pre-adjusted C_P. After doing an automatic background correction the C_P value are determined by various methods, e.g., at a constant level of fluorescence. These constant threshold methods assume that all samples have the same DNA concentration at the threshold fluorescence. But measuring the level of background fluorescence can be a challenge. Often real-time PCR reactions with significant background fluorescence variations occur, caused by drift-ups and drift-downs over the course of the reaction. Averaging over a drifting background will give an overestimation of variance and thus increase the threshold level (Livak, 1997, 2001; Rasmussen, 2001; Wilhelm *et al.*, 2003). The threshold level can be calculated by fitting the intersecting line at 10 standard deviations above baseline fluorescence level. This acquisition mode can be easily automated and is very robust (Livak, 1997, 2001). In the fit point method the user has to discard uninformative background points, exclude the plateau values by entering the number of log-linear points, and then fit a log line to the linear portion of the amplification curves. These log lines are extrapolated back to a common threshold line and the intersection of the two lines provides the C_P value. The strength of this method is that it is extremely robust. The weakness is that it is not easily automated and so requires a lot of user interaction, which are more or less arbitrary (Rasmussen, 2001, LightCycler® Software, 2001).

The real problem lies in comparing numerous biological samples. The researcher will have problems in defining a constant background for all samples within one run or between runs. These sample-to-sample differences in variance and absolute fluorescence values lead to the development of a new and user-friendly C_P acquisition model. As discussed in the previous section there are several mathematical models to determine the amplification rate, using a logistic or sigmoidal model. These mathematically fit models can also be used to determine the optimal C_P (*Table 3.1*). They are more or less independent of the background level or calculated on the basis of the background fluorescence and implement the data in the C_P determination model (Tichopad *et al.*, 2004; Wilhelm *et al.*, 2003).

In LightCycler® (Roche Applied Science) and Rotor-Gene™ (Corbett

Research) software packages these approaches are already implemented. In second derivative maximum method the C_P is automatically identified and measured at the maximum acceleration of fluorescence (Ramussen, 2001; LightCycler® Software, 2000). The exact mathematical algorithm applied is still unpublished, but is very comparable to a logistic fit. In the Rotor-Gene family using comparative quantification the 'take of point' is also calculated on basis of a sigmoidal model. Both the sigmoidal and polynomial curve models' work well with high agreement ($P<0.001$; $r>0.99$) (Tichopad *et al*., 2004; Liu and Saint, 2002a; Liu and Saint, 2002b; Rutledge, 2004). The sigmoidal exponential function was the more precise and could increase the exactness and precision of the C_P measurement as well as the amplification efficiency rate (Wilhelm *et al*., 2003). Peirson further discusses the importance of threshold setting in relative quantification in Chapter 6.

3.8 Relative quantification data analysis and software applications

A major challenge is the development of exact and reliable gene expression analysis and quantification software. A 'one-fits-all' detection and application software is the target for future developments and seems the optimal solution. But can we implement various detection chemistries with varying background and fluorescence acquisition modes in one software package? Should we not think about optimized models on each real-time platform and for each applied chemistry? In biological research and in clinical diagnostics, real-time qRT-PCR is the method of choice for expression profiling. On the one hand cycler and chemistry developed much faster than detection and analysis software. However, accurate and straightforward mathematical and statistical analysis of the raw data (cycle threshold/crossing point values or molecules quantified) as well as the management of growing data sets have become the major hurdles in gene expression analyses. Now the 96-well applications are the standard in the research laboratories, but in the near future high throughput 384-well applications will generate huge amounts of data. The data need to be grouped (Hellemans *et al*., 2006) and standardized by intelligent algorithms. Real-time qPCR data should be analyzed according to automated statistical method, e.g. Kinetic Outlier Detection (KOD), to detect samples with dissimilar efficiencies (Bar *et al*., 2003). Mostly the statistical data analysis or C_P values is performed on the basis of classical standard parametric tests, such as analysis of variance or t-tests. Parametric tests depend on assumptions, such as normality of distributions, whose validity is unclear (Sheskin, 2000). In absolute or relative quantification analysis, where the quantities of interest are derived from ratios and variances can be high, normal distributions might not be expected, and it is unclear how a parametric test could best be constructed (Pfaffl *et al*., 2002; Sheskin, 2000). At present, the following relative quantification data analysis and software applications are available.

3.8.1 LightCycler® Relative Quantification Software

The first commercially available software was the LightCycler® Relative Quantification Software (2001). It can be used to calculate and compare

relative quantification results of triplicates of a target versus a calibrator gene. Target genes are corrected via a reference-gene expression and calculates on the basis of the median of the performed triplets. Real-time PCR efficiency correction is possible within the software and is calculated from the calibration curve slope, according to the established eq. 9, ranging from $E = 1.0$ (minimum value) to $E = 2.0$ (theoretical maximum and efficiency optimum). A given correction factor and a multiplication factor, which are provided in the product specific applications by Roche Molecular Biochemicals (LightCycler® Relative Quantification Software, 2001), have to be incorporated in eq. 6. Importantly, no statistical comparison of the results by a statistical test is possible.

3.8.2 REST

In 2002, the relative expression software tool (REST, http://rest.gene-quantification.info) was established as a new tool (Pfaffl *et al.*, 2002). The first REST version is Excel®-based and programmed in Visual Basic to compare several gene expressions on C_P level. It compares two treatment groups, with multiple data points in the sample versus control groups, and calculates the relative expression ratio between them. The mathematical model used is published and is based on the mean C_P deviation between sample and control group of target genes, normalized by the mean C_P deviation of one reference gene as shown in eq. 7 (Pfaffl *et al.*, 2002). Further an efficiency correction can be performed, either based on the dilution method (eq. 9) or an optimal efficiency of $E = 2.0$ is assumed. The big advantage of REST is the provision of a subsequent statistical test of the analyzed C_P values by a *Pair-Wise Fixed Reallocation Randomization Test* (Pfaffl *et al.*, 2002). Permutation or randomization tests are a useful alternative to more standard parametric tests for analyzing experimental data (Manly, 1997; Horgan and Rouault, 2000). They have the advantage of making no distributional assumptions about the data, while remaining as powerful as conventional tests. Randomization tests are based on one we know to be true: that treatments were randomly allocated. The randomization test repeatedly and randomly reallocates at least 2000 times the observed C_P values to the two groups and notes the apparent effect each time, here in the expression ratio between sample and control treatment. The REST software package makes full use of the advantages of a randomization test. In the applied two-sided *Pair-Wise Fixed Reallocation Randomization Test* for each sample, the C_P values for reference and target genes are jointly reallocated to control and sample groups (= pair-wise fixed reallocation), and the expression ratios are calculated on the basis of the mean values. In practice, it is impractical to examine all possible allocations of data to treatment groups, and a random sample is drawn. If 2000 or more randomizations are taken, a good estimate of *P*-value (standard error <0.005 at $P = 0.05$) is obtained. Randomization tests with a pair-wise reallocation are seen as the most appropriate approach for this type of application. In 2005 various new REST versions were developed, calculating with a geometric mean averaged REF index (Vandescompele *et al.*, 2002; Pfaffl *et al.*, 2004), according to the mathematical model described in eq. 8, which can analyze 15 target and reference genes (REST-384) (LightCycler® Relative Quantification Software,

2001). Specialized REST versions can compare six treatment group with one non-treated control (REST-MCS, REST – Multiple Condition Solver) (LightCycler® Relative Quantification Software, 2001), or take individual amplification efficiency into account, exported from the Rotor-Gene (REST-RG). A stand alone application REST-2005 was developed, running independent of Excel® or Visual Basic, comparing 'unlimited' target and reference genes, using newly developed bootstrapping statistical tool, and graphical output showing 95% confidence interval (TUM and Corbett Research, 2005) (Pfaffl and Horgan, 2005).

3.8.3 Q-Gene

Recently a second software tool, Q-Gene, was developed, which is able to perform a statistical test of the real-time data (Muller *et al.*, 2002). Q-Gene manages and expedites the planning, performance and evaluation of quantitative real-time PCR experiments. The expression results were presented by graphical presentation. An efficiency correction according to the dilution method is possible (eq. 9). Q-Gene can cope with complex quantitative real-time PCR experiments at a high-throughput scale (96-well and 384-well format) and considerably expedites and rationalizes the experimental set-up, data analysis, and data management while ensuring highest reproducibility. The *Q-Gene Statistics Add-In* is a collection of several VBA programs for the rapid and menu-guided performance of frequently used parametric and non-parametric statistical tests. To assess the level of significance between any two groups' expression values, it is possible to perform a paired or an unpaired Student's test, a Mann-Whitney U-test, or Wilcoxon signed-rank test. In addition, the Pearson's correlation analysis can be applied between two matched groups of expression values. Furthermore, all statistical programs calculate the mean values of both groups analyzed and their difference in percent (Muller *et al.*, 2002).

3.8.4 qBASE

Comparable software application qBASE was recently developed by colleagues to offer solutions to compare more real-time set-ups (Hellemans *et al.*, 2006). QBASE is an Excel®-based tool for the management and automatic analysis of real-time quantitative PCR data (http://medgen.ugent.be/qbase). The qBASE browser allows data storage and annotation while keeping track of all real-time PCR runs by hierarchically organizing data into projects, experiments, and runs. It is compatible with the export files from many currently available PCR instruments and provides easy access to all the data, both raw and processed. The qBASE analyzer contains an easy plate editor, performs quality control, converts C_P values into normalized and rescaled quantities with proper error propagation, and displays results both tabulated and in graphs. One big advantage of the program is that it does not limit the number of samples, genes and replicates, and allows data from multiple runs to be combined and processed together (Hellemans *et al.*, 2006). The possibility of using up to five reference genes allows reliable and robust normalization of gene expression levels, on the basis of the *geNorm* normalization procedure (Vandescompele *et al.*, 2002). qBASE

allows the easy exchange of data between users, and exports tabulated data for further statistical analyses using dedicated software.

3.8.5 SoFAR

The algorithms implemented in SoFAR (distributed by Metralabs) allow fully automatic analysis of real-time PCR data obtained with a Roche LightCycler® (Roche Diagnostics) instrument. The software yields results with considerably increased precision and accuracy of real-time quantification. This is achieved mainly by the correction of amplification independent fluorescence signal trends and a robust fit of the exponential phase of the signal curves. The melting curve data are corrected for signal changes not due to the melting process and are smoothed by fitting cubic splines. Therefore, sensitivity, resolution, and accuracy of melting curve analyses are improved (Wilhelm *et al.*, 2003).

3.8.6 DART-PCR

DART-PCR (Data Analysis for Real-Time PCR) provides a simple means of analyzing real-time PCR data from raw fluorescence data (Peirson *et al.*, 2003) (http://nar.oxfordjournals.org/cgi/content/full/31/14/e73/DC1). This allows an automatic calculation of amplification kinetics, as well as performing the subsequent calculations for the relative quantification and calculation of assay variability. Amplification efficiencies are also tested to detect anomalous samples within groups (outliers) and differences between experimental groups (amplification equivalence). Data handling was simplified by automating all calculations in an Excel® worksheet, and enables the rapid calculation of threshold cycles, amplification rate and resulting starting values, along with the associated error, from raw data. Differences in amplification efficiency are assessed using one-way analysis of variance (ANOVA), based upon the null hypotheses, that amplification rate is comparable within sample groups (outlier detection) and that amplification efficiency is comparable between sample groups (amplification equivalence) (Peirson *et al.*, 2003).

3.9 Conclusion

Facilitating data management and providing tools for automatic data analysis, these software applications address one of the major problems in doing real-time quantitative PCR-based nucleic acid quantification. Nevertheless, successful application of real-time RT-PCR and relative quantification depends on a clear understanding of the practical problems. Therefore a coherent experimental design, application, and validation of the individual real-time RT-PCR assay remains essential for accurate, precise and fully quantitative measurement of mRNA transcripts. An advantage of most described software applications (except SoFAR) is that they are freely available and scientists can use them for their academic research. qBASE intends to be an open source project and interested parties can write their own analysis or visualization plug-ins. All calculation- and statistical-software applications are summarized and described in detail at http://bioinformatics.gene-quantification.info.

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