Quantification Strategies in Real-time Polymerase Chain Reaction

3

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Abstract

The present chapter describes the quantification strategies used in real-time RT-PCR (RT-qPCR), focusing on the main elements that are essential to fulfil the MIQE guidelines. The necessity of initial proper data adjustment and background correction is discussed to allow reliable quantification. The advantages and disadvantages of the absolute and relative quantification approaches are also described. In conjunction with relative quantification, the importance of an amplification efficiency correction is shown, and software tools that are available to calculate relative expression changes are presented.

Introduction

In general, two quantification strategies can be applied to analyse real-time RT-PCR (RT-qPCR) expression data. The levels of expressed genes may be measured by absolute quantification, using a standard curve, or relative quantification, using validated reference genes in RT-qPCR.

Absolute quantification relates the PCR signal to input copy number using a calibration curve with known concentrations of the amplified gene of interest (Fig. 3.1). Relative quantification instead measures the expression changes between two or more analysed experimental groups. The reliability of an absolute RT-qPCR assay depends mainly on the validity of the 'calibration curve' used and the 'identical' amplification efficiencies for both the native target in the biological sample and the artificial standard material used in the calibration curve, in the RT and qPCR reactions (Souaze *et al.*, 1996; Bustin *et al.*, 2005).

From the handling perspective, the relative quantification approach seems easier to perform than the absolute one, because a calibration curve is not needed. But it bears other essential problems, such as the changing amplification efficiencies of the analysed target and reference genes, and lacks of amplification variability at low transcript concentrations. It is based on the expression levels of a gene of interest versus one (or more) reference gene(s), whereby the reference gene selection is not trivial (Vandesompele *et al.*, 2002, 2009). To guarantee optimal quantification results, the recently published MIQE guidelines – Minimum Information for Publication of Quantitative Real-Time PCR Experiments – are highly recommended and a prerequisite for any quantitative PCR study (Bustin *et al.*, 2009).

Data adjustment

Prior to any quantification, a proper data adjustment is necessary so that the biological samples and the standards are comparable. Between reaction vessel differences are obvious, which are introduced by the operator, by the handling errors or are derived from the complex enzymatic reaction steps. Furthermore, some instruments excite all reaction vials individually and detected the fluorescence independently, which also may introduce some variability. To overcome this potential variation between reaction set-ups and replicates, it is possible to incorporate a passive reference dye, such as ROX or Fluorescein (FL), in each reaction mix. The passive reference signal is monitored during the course of the amplification, and each

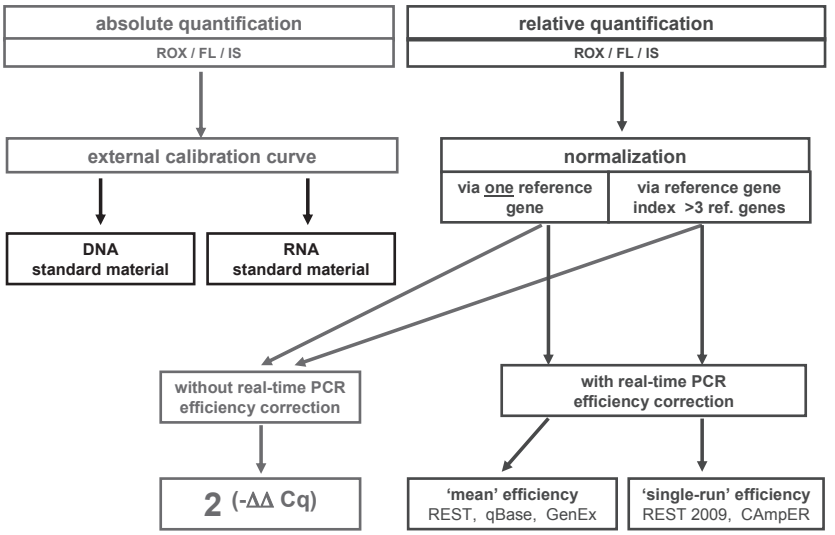

Figure 3.1 Quantification strategies in quantitative real-time RT-PCR.

raw data fluorescence reading is then corrected and equalized using the unchanging reference signal (ROX or FL). An advantage of the data normalization strategy is that the impact of any optical variability across the instrument block will be minimized, but as an additional fluorescence channel is introduced, this adds additional variability in the quantification workflow. The use of a passive reference dye may however help to remove background fluorescence and reduce the noise in the background phase from the measurements. This can be done by the software using the 'automatic background correction' option. An optimal fluorescence history shows low initial background signal, a significant and exponential fluorescence increase resulting from a stable PCR amplification, and a stable plateau (Keer, 2008; Pfaffl *et al.*, 2009).

After proper data adjustment, all samples under investigation are comparable and can be analysed dependent on the applied quantification strategy selected according to the scientific or analytical question. Either an absolute quantification approach, which results in defined units, e.g. copies per defined nucleic acid concentration, or a relative quantification, leading to gene expression ratios between different treatment groups, is used. In this later case, absolute units remain unknown.

Absolute quantification

The so-called 'absolute quantification' is misleading because the method is never 'absolute', as such, it is relative to the created calibration or standard curve. Hence everything depends on the reliability and validity of the standard curve and the used standard material. In particular, the external calibration curve model has to be thoroughly validated as the fidelity of 'absolute quantification' in real-time RT-qPCR depends entirely on the accuracy and quality of the standard used. It should be pure, not degraded, stable, and for this reason yield highly reproducible results over time and the entire quantification range. The standard curve model should allow the generation of highly specific, sensitive, and reproducible quantification data (Reischl and Kochanowsky, 1995; Bustin, 2000; Pfaffl and Hageleit, 2001; Reiter *et al.*, 2011). The standard composition, sequence design, purity, determination of the exact concentration, and stability over long storage time is not straightforward and can be problematic. In addition, the matrix of the biological samples is important. Some biological samples under investigation bear inhibitors for the enzymatic RT and/or qPCR reaction. Therefore, the standard curve itself should reflect as well the biological sample which is the focus for quantification. What is the real benefit of a suboptimal quantification model

if the standard curve and the biological do not show the same amplification behaviour? Often the standard curve shows perfect properties with pure standard material and a perfect amplification history whereas the biological samples demonstrate high background, low amplification efficiency and seem to be inhibited by any unknown substance. Very importantly, both the standard curve dilutions and the unknown biological samples have to amplify in the same manner and with the same amplification characteristics. Elsewise the quantification is NOT reliable and will result in wrong quantification findings (Pfaffl, 2004).

What are the characteristics of a good standard curve? The dynamic range of the performed calibration curve should be as broad as possible. It can be up to nine orders of magnitude, starting at one theoretical molecule in the lowest, up to billions of molecules in the highest concentration. If plotted, all standard points and their replicates should be equally spread over the quantification range. All standard points will be summarized in a linear regression which should be linear with low variation, expressed in the confidence interval with a high correlation coefficient (r^2), and a slope, ideally –3.33, which corresponds to an amplification efficiency of 100% or a perfect doubling in each cycle.

The quantification range depends much on the applied standard material stability whereas the biggest difference appears between RNA and DNA (Pfaffl and Hageleit, 2001; Reiter *et al.*, 2011). The analytical question determines the type of the standard material, either DNA or RNA. If the focus is on the quantification of DNA, e.g. quantification of microorganisms in a sample, the calibration curve should be made out of DNA. If the interest is on the quantification of expressed transcripts or to measure RNA viruses concentrations, the standard substance must be RNA based.

For a DNA standard curve, the type of standard material is always based on known concentrations of double- or single-stranded DNA molecules. In most cases, serial dilutions with 10-fold dilution steps are sufficient (Fig. 3.2). Furthermore, the stability and reproducibility of the absolute quantification curve depends on the type of standard material applied, e.g. genomic DNA, RT-PCR product, recombinant plasmid DNA, commercially synthesized oligo-nucleotide or artificial genes (Morrison *et al.*, 1998, Pfaffl, 2004). Cloned

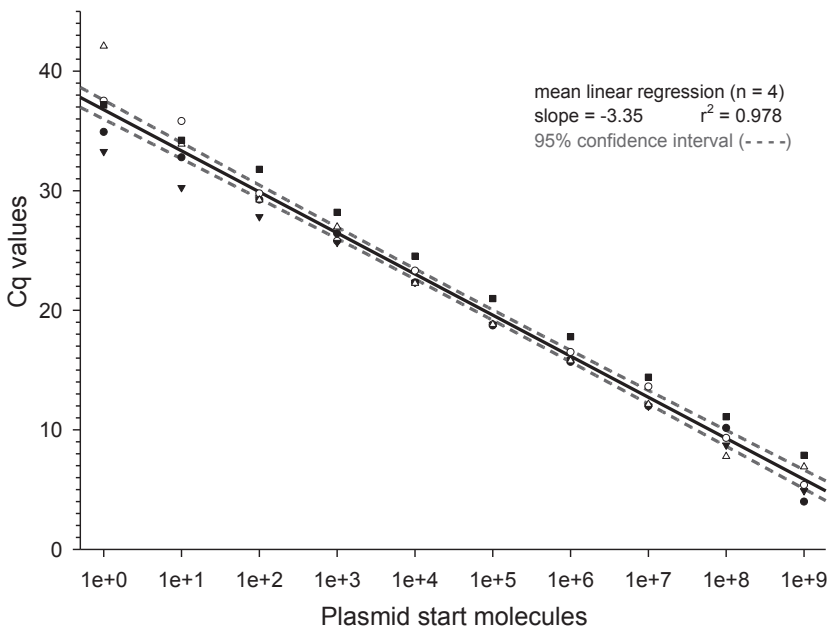

Figure 3.2 DNA standard curve.

recombinant DNA, artificial genes, and genomic DNA are very stable and generate highly reproducible standard curves even after a long storage time up to several years in a –20°C freezer (own findings).

RNA standards are much more sensitive to RNAse activity and degradation, especially at low concentration. Owing to the stability problem, it will introduce variability in the quantification of low abundant transcripts, and low concentration cannot be measured with high confidence. Furthermore, the longer templates derived from artificial genes mimic the average native length of messenger RNA of roughly 2 kb better than shorter templates or oligo-nucleotides. These longer standards have longer overhanging ends and are hence more resistant against unspecific enzymatic cleavage during RT or PCR reaction (Rasmussen, 2004).

A major problem with DNA-based calibration curves is that they can only be applied to quantify at the DNA level, and they are not suitable to quantify any RNA. DNA is subject to the PCR step only, unlike the unknown mRNA samples that must first be reverse transcribed to convert them to complementary DNA (cDNA). The RT step efficiency is usually around 30–40% and bears high variability between technical RT replicates (additional 30%) (Stahlberg *et al.*, 2004a,b). This decreases the amount of synthesized cDNA dramatically; pushes the variability in the quantitative RT-qPCR study and the derived quantification results may not reflect the reality of RNA initially present in the biological sample. If RNA is in the focus of the quantification, a recombinant RNA standard material *in vitro* transcribed from a plasmid, or artificially synthesized is mandatory (Pfaffl *et al.*, 2001; Fronhoffs *et al.*, 2002; Reiter *et al.*, 2011).

Independent of the standard material used, an absolute quantification model requires careful optimization of its precision. The reverse transcription efficiencies for both, standard curve material and native biological sample, must be tested thoroughly and should be confirmed by multiple technical RT replicates (Tichopad *et al.*, 2003b). We recommend performing replicates in the same RT reaction run, to get information about the RT assay variability, and replicates in separate runs, to get the inter-assay variation or reproducibility. This will help to find the detection and quantification limitations within the given absolute quantification procedure, and will show exactly the technical variance (Pfaffl and Hageleit, 2001; Tichopad *et al.*, 2009).

A special focus should be on the existence of inhibitors in the biological sample, which hampers either RT and/or PCR reactions. Inhibitors may be present in the biological sample but not in the standard dilutions used in the calibration curve. Known and unknown biological matrix compounds interact with the enzymatic reaction and therefore impact the validity and trueness. If present, a strong bias is introduced, leading to an overestimation of the standard curve and an underestimation of the unknown sample concentration. This will lead to completely wrong quantification results. The presence of inhibitors in the biological matrix should always be tested first. Many factors present in samples as well as exogenous contaminants have been reported to inhibit enzymatic reactions, either RT or PCR. For example, the presence of humic acids, haemoglobin, fatty acids, glycogen, cell components, calcium ions, and nucleic acid binding proteins are known inhibiting compounds (Rossen *et al.*, 1992; Wilson, 1997; Tichopad *et al.*, 2004). Additionally, exogenous contaminants such as glove powder, DNA or RNA degrading decontamination chemicals, and ethanol or phenolic compounds from the extraction process will also have an inhibitory effect (Rossen *et al.*, 1992; Wilson, 1997). Furthermore, low-quality and impure plasticware with high nucleic acid-binding capacity interact with the quantification procedure and should be avoided. To test for such inhibitors, a SPUD assay can be performed, which allows determining the degree of inhibition in the biological sample (Nolan *et al.*, 2006).

To get the most correct and valid results in an absolute quantification approach, either the user must take actions to get rid of any interfering compounds in the unknown biological samples during nucleic acid purification, or the calibration curve must be adapted. This means that the calibration curve amplification behaviour and the overall RT and qPCR efficiency must be brought to the same level as the biological sample material. In the

end, both the standard curve and the biological samples should show most comparable amplification behaviour. The so called 'biological matrix background effect' in the standard curve model should be obtained from the sample under investigation. Eventually, identical background levels in the calibration curve and the biological samples should result in comparable levels of inhibition and therefore leads to a more realistic and valid quantification (Pfaffl, 2004).

Regardless on how accurate the concentration of the standard material is determined, the final result is always reported compared with a defined unit of interest, e.g. copies per defined RNA or DNA concentration, copies per genome, copies per solitaire-cell, copies per tissue unit, copies per millilitre blood, copies per gram of soil, etc. If absolute changes in copy number are important then the denominator must still be shown to be fully stable across the entire study. The applicability depends strongly on the adherence to the MIQE guidelines, the general 'good laboratory practice' and the personal skills of the researcher (Bustin *et al.*, 2009; Health Protection Agency, 2010). Failure to comply with the mentioned rules may introduce enormous additional variability to the 'absolute' quantification system and will result in artificial quantification results.

Relative quantification

Relative quantification determines the relative changes in steady-state messenger RNA (mRNA) levels of an expressed gene across multiple biological samples. Today the use of unregulated and stable expressed reference genes for normalization of transcript levels is certainly the gold standard (Vandesompele *et al.*, 2002). It is adequate for most purposes to investigate even low expression changes of minute amounts of mRNA or microRNA. The units used to express relative quantities are irrelevant, and the relative quantities can be compared across multiple real-time RT-qPCR experiments (Vandesompele *et al.*, 2009). These controls are internally grown, named reference genes (formerly known as housekeeping genes), and can be co-amplified in a multiplex assay or can be amplified in separate tubes (Wittwer *et al.*, 2001). Therefore, relative quantification does not require standards with known concentrations. The controls can be one or more reference gene(s), which can be any transcript as long as it is expressed in all biological samples and properly validated according to the MIQE guidelines (Bustin *et al.*, 2009).

To calculate the expression difference of a target gene in relation to an adequate reference gene, two general mathematical calculation models are established in the scientific qPCR community. The so called 'delta-delta Cq model', developed by Livak and Schmittgen (2001) or the efficiency corrected model, developed by Pfaffl (2001). Both calculations models are based on the comparison of the distinct cycle of quantification (Cq). The most applied and common calculation model is the 'delta-delta Cq method' which is assuming optimal amplification efficiency of 100% in any cycle, over the entire PCR cycles, with all target transcripts. It does not take any suboptimal PCR efficiency or its variation into account, derived from PCR inhibition or handling errors, either for the target or reference genes. As described in the previous 'absolute quantification' section, inhibitors or suboptimal reaction conditions may interfere with the reaction performance and hence the reaction efficiency is never exactly 100%. Therefore, a model was modified by Pfaffl and co-workers in 2001 by adding a real-time PCR efficiency correction. This correction is applied separately for each gene studied, e.g. the target gene (E_{target}) and the reference gene (E_{ref}), as shown in the equation:

$$\text{ratio} = \frac{(E_{target})^{\Delta CP_{target}(control - sample)}}{(E_{ref})^{\Delta CP_{ref}(control - sample)}}$$

This efficiency corrected relative quantification model was further developed, driven by innovations from other groups. It was expanded by adding multiple reference genes for normalization (summarized by Vandesompele *et al.*, 2009), by applying the model to group measurements (Pfaffl *et al.*, 2002), or by adding new statistical analysis tools to get more significant valid relative expression results (Qiagen, 2009).

We now know that reference genes have to be carefully selected and validated, which can be

done using free available software applets, e.g. Genorm (Vandesompele, 2002), BestKeeper (Pfaffl *et al.*, 2004) or Normfinder (Andersen *et al.*, 2004). The main goal of the normalization procedure is to remove experimental induced variation, each with their own advantages and considerations (Huggett *et al.*, 2005). While most of these methods cannot completely reduce all sources of variation, they have been shown to be very important to control most sources of variation along the entire workflow of PCR-based gene expression analysis. If one does not meticulously try to standardize each step, variation can and will be introduced in the results which cannot be eliminated by applying a final normalization (Stahlberg *et al.*, 2004b). It is thus recommended to ensure that similar biological sample sizes and extraction volumes are used, and also to standardize the workflow. The amount of time invested in DNase treatment and reverse transcription optimization is important and can impact the cDNA conversion efficiency. Furthermore, artificial RNA molecules can be spiked into the sample prior to extraction or to the RNA extract prior to reverse transcription (Smith *et al.*, 2003; Gilsbach *et al.*, 2006). This will give an indication of the RT and qPCR efficiencies, and will reveal any inhibition. We however need to keep in mind that the spiked artificial RNA may not be extracted with the same yield as natural mRNAs and will not fully control for the final amount of input material in the reaction (Stahlberg *et al.*, 2004a).

Summarizing the qPCR literature from the last decade, it has been agreed that the reference gene concept is the currently preferred way of normalizing real-time PCR data for relative expression profiling (Huggett *et al.*, 2005; Vandesompele *et al.*, 2009). The reference gene concept is particularly attractive because the reference genes are grown internal controls that are affected by all sources of variation during the experimental workflow, from sampling, storage, extraction, nucleic acid stabilization, and enzymatic reaction (RT, DNAse digestion, and PCR) in the same way as the genes of interest. Furthermore, PCR based quantification findings for a gene of interest are best normalized using a factor that is measured using the same quantitative methodology set-up and chemicals.

After selecting the right references, the relative expression difference of the target genes can be calculated by multiple software tools. Either a software application is already implemented in the real-time cycler software package or the data can be exported and further analysed using proper quantification tools. The oldest available software is the REST (Relative Expression Software Tool), established by Pfaffl and colleagues (Pfaffl *et al.*, 2002; http://REST.gene-quantification.info), qBase and qBASEPlus, developed around the group of Vandesompele and co-workers (Hellemans *et al.*, 2007; http://www.biogazelle.com), or GeneEx set up by Kubista and colleagues (Bergkvist *et al.*, 2010; http://www.multid.se). In these software, the target gene expression is normalized by multiple validated non-regulated reference gene expressions, and even more advanced applications are implemented to evaluate if the target gene is expressed as part of a gene cluster, via cluster analysis, principal component analysis or neural networks (summarized in Pfaffl *et al.*, 2009; Bergkvist *et al.*, 2010). This way, relative quantification can generate useful and biologically relevant information when used appropriately according to the MIQE guidelines (Bustin *et al.*, 2009).

PCR amplification efficiency

Constant PCR amplification efficiency in all investigated biological samples is one important criterion for reliable comparison between samples and genes. Individual reaction set-ups can however generate different fluorescence histories in quantitative real-time PCR. The shapes of amplification curves differ in the background fluorescence level, the steepness of fluorescence increase, and in the absolute fluorescence levels at plateau. PCR efficiency has a major impact at each cycle and finally in the overall cycle fidelity or accuracy of the assay. Therefore, efficiency evaluation is an essential and requested criterion in gene quantification procedure (Tichopad *et al.*, 2003a; Bustin *et al.*, 2009). Some quantification models assume that the analysed genes amplify optimally with 100% efficiency (Livak and Schmittgen, 2001) or at least with similar efficiency. But this is

often not the case. As discussed, sporadic RT and PCR inhibitors inhibitor, sample impurity in the reaction set-up, or nucleic acid degradation may reduce the efficiency of the RT or the PCR reaction (Stahlberg *et al.*, 2004b; Becker *et al.*, 2010). To determine the level of inhibition, a dilution series can be performed and the inhibitory factors can often be diluted out, causing a non-linear standard curve (Morrison *et al.*, 1998; Rasmussen, 2001).

A correction for PCR efficiency and its variation, as performed in efficiency corrected mathematically models, is strongly recommended and is one of the key element of the MIQE guidelines (Bustin *et al.*, 2009). It results in a more reliable estimation of the 'real expression ratio' compared with no efficiency correction in the relative quantification model (Pfaffl, 2001). Small efficiency differences between target and reference genes, or between biological samples, generate false expression ratio and results in wrong scientific findings. Overall, the researcher over- or under-estimates the 'real and initial' mRNA amount present in the biological sample (LightCycler Relative Quantification Software; Roche Applied Science, 2001). This is why efficiency quantification corrections should be included in the calculation procedure, and is a major goal to implement it in any real-time qPCR cycler relative quantification software.

The most applied method to evaluate the PCR efficiency is from the slopes of the calibration curve or dilution row from a pool of biological samples. From the regressed expression data versus the concentration, the PCR efficiency (E) can be calculated according to the equation $E = 10^{[-1/slope]}$, as described earlier (Higuchi *et al.*, 1993; Rasmussen, 2001). Efficiency determination by the dilution method can be performed sample by sample, but this is very time consuming and needs lots of RNA. If not available, the efficiency can be evaluated using a pool of all starting total RNAs to accumulate all possible positive and negative 'impacts' on kinetic PCR efficiency estimation. Usually, this so called 'mean PCR efficiency' (Fig. 3.2) vary around the ideal regression line, behave linearly ($0.980 > r^2 > 0.999$), and range from $E = 1.75$ to maximal values up to $E = 2.10$ for cDNA input ranges over at least three orders of magnitude (Wittwer *et al.*, 1991; Pfaffl *et al.*, 2002). Typically, the relationship between Cq and the logarithm of the starting copy number of the target sequence should remain linear in the calibration curve as well as in the native sample RNA (Muller *et al.*, 2002).

Over the years further methods were established, for a more realistic estimation of the real-time PCR efficiency. These models are based on the analysis of all available data points in the fluorescence history. Raw fluorescence data reading points were fitted according to a given mathematical model. Often, sigmoidal, logistic or exponential curve model were used in different approaches and even multiple fitting models were established (Liu and Saint, 2002a,b; Tichopad *et al.*, 2003a; Zhao and Fernald, 2005). The advantage of such models is that all data points will be included in the calculation process and the 'single run' efficiency can be estimated from each biological sample individually. No work consuming and cost intensive dilution row has to be performed, and no background subtraction is necessary (Raeymaekers, 2000; Tichopad *et al.*, 2004). It can be measured at different points, either at the point of inflexion or at the absolute maximum fluorescence increase which equals the 'first derivate maximum' point. Alternatively, it can be measured earlier than or at the so called 'second derivate maximum' point, where the optimal reaction point is defined. However, the derived slope parameters generated by such more advanced models are in the eyes of a small group of researchers more reliable and 'directly' comparable with the real PCR efficiency (Liu and Saint, 2002a,b; Tichopad *et al.*, 2003a). These 'single run' efficiency methods can be fully automated, are easier to perform for the operator, but bear the problem of higher variability in a set of biological samples. Thus, PCR efficiency is different according to biological samples and has a variability which should be taken into account in the quantification model. To cope with the knowledge of individual 'single-run' PCR efficiencies in each biological sample, new software tools were established, e.g. REST 2009 (Pfaffl *et al.*, 2002; Qiagen, 2009) and Champer (Blom, 2006).

Conclusion

Any calculation model, any applied algorithm, and any quantification strategy are making assumptions which try to describe the nature of the qPCR reaction at its best. Which standard curve material is the most stable? Which biological background best mimics the biological reality? Which normalization approach is the most reliable? Which PCR efficiency calculation method is 'the most correct one'? In the end, the quantification model that shows the most valid expression results is still unknown. With the latest progress, we are however in a good position to find it in the near future.

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