Quantification strategies in real-time PCR

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Abstract

This chapter analyzes the quantification strategies in real-time RT-PCR and all corresponding *markers of a successful real-time RT-PCR*. The following aspects are describes in detail: RNA extraction, reverse transcription (RT), and general quantification strategies—absolute vs. relative quantification, real-time PCR efficiency calculation, data evaluation, automation of quantification, data normalization, and statistical comparison. The discussion turns into practical considerations with focus on specificity and sensitivity.

3.1. Introduction

Reverse transcription (RT) followed by polymerase chain reaction (PCR) represents a powerful tool for the detection and quantification of mRNA. Real-time RT-PCR (or kinetic RT-PCR) is widely and increasingly used because of its high sensitivity, good reproducibility, and wide dynamic quantification range.[1-4] The first practical kinetic PCR technology, the 5'-nuclease assay, was established 1993 and combines the exponential PCR amplification of a specific transcript with the monitoring of newly synthesized DNA in each performed PCR cycle.[5-7] It is the most sensitive method for the detection and quantification of gene expression levels, in particular for low abundant transcripts in tissues with low RNA concentrations, from limited tissue sample and for the elucidation of small changes in mRNA expression levels.[1-4,8-12] While kinetic RT-PCR has a tremendous potential for analytical and quantitative applications, a comprehensive understanding of its underlying principles is important. Fidelity of real-time RT-PCR is associated with its "true" specificity, sensitivity, reproducibility, and robustness and, as a fully reliable quantitative method, it suffers from the problems inherent in RT and PCR, e.g., amplification of unspecific products, primer-dimers, amplification efficiencies, hetero-duplex formation, etc.[13]

This chapter analyzes the quantification strategies in real-time RT-PCR and all corresponding markers of a successful real-time RT-PCR.

3.2. Markers of a Successful Real-Time RT-PCR Assay

3.2.1. RNA Extraction

The integrity of purified RNA is critical to all gene expression analysis techniques. The preparation of intact cellular total RNA or pure mRNA is the *first marker in gene quantification*. For successful and reliable diagnostic use, real-time RT-PCR needs high-quality, DNA-free, and undegraded RNA.[14,15] Accurate quantification and quality

assessment[30] of the starting RNA sample is particularly important for absolute quantification methods that normalize specific mRNA expression levels against total RNA ("molecules/g total RNA" or "concentrations/g total RNA").[28,29] RNA, especially long mRNA up to 10 kb,[14] is easily degraded by cleavage of RNases during tissue sampling, RNA purification, and RNA storage. The source of RNA, sampling techniques (biopsy material, single cell sampling, and laser microdissection),[2,16,17] as well as RNA isolation techniques (either total RNA or poly-adenylated RNA) often vary significantly between processing laboratories.[15] RNA extracted from adipose or collagen-rich tissues often has a lower yield and is of lesser quality, and contains partly degraded RNA sub-fractions (own unpublished results). Particular RNA extraction techniques can work more effectively in one specific tissue type compared with another one, and result in up to 10-fold variations in total RNA yield.[15] RNA may contain tissue enzyme inhibitors that result in reduced RT and PCR reaction efficiencies and generate unreliable and "wrong" quantification results.[14,15]

Most RNA preparations are contaminated with DNA and protein at very low levels. Even high-quality commercially obtained RNAs contain detectable amounts of DNA.[15] While this is not a problem for some applications, the tremendous amplification power of kinetic PCR may result in even the smallest amount of DNA contamination to interfering with the desired "specific amplification." To confirm the absence of residual DNA either a "minus-RT" or "water control" should always be included in the experimental design. It may be necessary to treat the RNA sample with commercially available RNase-free DNase, to get rid of residual DNA. However, unspecific side reactions of the DNase often result in RNA degradation (own unpublished results). It is always necessary to remove the DNase prior to any RT or PCR step.

Furthermore, the design of the PCR product should incorporate at least one exon-exon splice junction to allow a product obtained from the cDNA to be distinguished on electrophoresis from genomic DNA contamination. However, processed pseudogenes (e.g., -actin, GAPDH or 18S rRNA) can be present and lead to confusion in data interpretation. In addition, intron-lacking pseudogenes (e.g. -actin) with equal sequence length to endogenous mRNA have been described.[18-24] They prevent a distinction between products originating from genomic DNA versus mRNA, which poses a significant problem in qualitative and quantitative gene quantification. Therefore, various housekeeping genes must be tested or multiplex assays of reference genes as internal controls for the assessment of RNA and cDNA quality must be performed.[25-27]

3.2.2. Reverse Transcription

The *second marker in quantitative RT-PCR* is the production of a single-stranded (ss) complementary DNA copy (cDNA) of the RNA through the reverse transcriptase (RT) and its dynamic range, sensitivity, and specificity are prime consideration for a successful kinetic RT-PCR assay.[31-34] For many quantitative applications, MMLV H^{-} RT is the enzyme of choice,[31,35,36] as its cDNA synthesis rate is up to 40-fold greater than that of AMV (own unpublished results). Newly available thermostable RNAse H^{-} RT maintains its activity up to 70°C, thus permitting increased specificity and efficiency of first primer annealing. However, this enzyme may be less robust than more conventional ones as it appears to be more sensitive to inhibitors present in RNA preparation.[28,36,37]

The RT step is the source of most of the variability in a kinetic RT-PCR experiment and for each enzyme the specific reaction conditions has to be optimized. Salt contamination, alcohol, phenol, and other inhibitors carried over from the RNA isolation process can affect the apparent RT efficiency.[13,31,34] Another source of variability is the choice of priming method used to initiate cDNA synthesis, which can be either target gene-specific or non-specific. Target gene-specific primers work well in conjunction with elevated RT-reaction temperatures to eliminate spurious transcripts.[36,37] The same reverse primer is used for the subsequent PCR assay in conjunction with the corresponding gene-specific sense primer (forward primer). However, the use of gene-specific primers necessitates a separate RT reaction for each gene of interest. It cannot be assumed that different reactions have the same cDNA synthesis efficiency; the result can be high variability during multiple RT reactions.

To circumvent these high inter-assay variations in RT, target gene unspecific primers, e.g., random hexamer, octamer or decamer primers, can be used and a cDNA pool can be synthesized. Similarly, poly-T oligonucleotides (consisting solely of 16-25 deoxythymidine residues) can anneal to the polyadenylated 3' (poly-A) tail found on most mRNAs.[13,30] cDNA pools synthesized with unspecific primers can be split into a number of different target-specific kinetic PCR assays. This maximizes the number of genes that can be assayed from a single cDNA pool, derived from one small RNA sample. Therefore the gene expression results are directly comparable between the applied assays, at least within one and the same RT pool. In conclusion, a rank order of RT efficiency can be shown for the applied different primers for ONE specific gene: random hexamer primers > poly-dT primer > gene-specific primer (own unpublished results).

Importantly, not only RNA quantity and quality, but also yield and quality of cDNA can be highly variable. Certainly, there is evidence that cDNA yield from sequences near the 5' end of partially degraded mRNAs is significantly less than from sequences near the poly-A tail and assays aimed at identifying RNA degradation are being developed.[3,14,34,38] Thus, reliable

internal quality control of cDNA synthesis is essential. Controls are generally performed by PCR amplification of reference genes, mostly common housekeeping genes (GAPDH, albumin, actins, tubulins, cyclophilin, microglobulins, 18S ribosomal RNA (rRNA) or 28S rRNA).[11,27,39-43] The chosen reference genes used as well as the expression levels vary between different laboratories, and only few of them have been critically evaluated (see Section 3.4. *Normalization*).

3.2.3. Comparison of Real-Time RT-PCR with Classical End-Point Detection Method

The efficacy of kinetic RT-PCR is measured by its specificity, low background fluorescence, steep fluorescence increase, high amplification efficiency, and high level plateau.[44] Typically, the PCR reaction can be divided in four characteristic phases:[45] 1st phase is hidden under the background fluorescence where an exponential amplification is expected; 2nd phase with exponential amplification that can be detected and above the background; 3rd phase with linear amplification efficiency and a steep increase of fluorescence; and finally 4th phase or plateau phase, defined as the attenuation in the rate of exponential product accumulation, which is seen concomitantly in later cycles.[46,47] The amount of amplified target is directly proportional to the input amount of target only during the exponential phase of PCR amplification. Hence the key factor in the quantitative ability of kinetic RT-PCR is that it measures the product of the target gene within that phase.[10,45,48-51] Since data acquisition and analysis are performed in one and the same tube, this increases sample throughput, reduces the chances of carryover contamination, and removes post-PCR processing as a potential source of error.[52]

In contrast, during the plateau phase of the PCR there is no direct relation of "DNA input" to "amplified target"; hence classical RT-PCR assays have to be stopped at least in linear phase.[44, 53] The exponential range of amplification has to be determined for each transcript empirically by amplifying equivalent amounts of cDNA over various cycles of the PCR or by amplifying dilutions of cDNA over the same number of PCR cycles.[10, 53] Amplified RT-PCR end product is later detected by ethidium bromide gel staining, radioactivity labelling, fluorescence labelling, high-performance liquid chromatography, southern blotting, densitometric analysis, or other post-amplification detection methods.[53-55] This step-wise accumulation of post-PCR variability[10,49, 53] leads to semi-quantitative results with high intra-assay (around 30-40%) and inter-assay variability (around 50-70%; own unpublished results) in endpoint detection assays. Finally, whereas real-time methods have a dynamic range of

greater than eight orders of magnitude, the dynamic range of the endpoint assays is at best two.[10, 49, 56]

3.2.4. Chemistry Developments for Real-Time RT-PCR

The *third marker in kinetic RT-PCR* is the right detection chemistry. Two general methods for the quantitative detection of the amplicon have become established: gene-specific fluorescent probes or specific double strand (ds) DNA binding agents[8,49,52,57,58] based on fluorescence resonance energy transfer (FRET).[11,48,59] The best-know probe-based system is ABI's TaqMan,[6,60,61] which makes use of the 5'-3' exonuclease activity of *Taq* polymerase to quantitate target sequences in the samples. Probe hydrolysis separates fluorophore and quencher and results in an increased fluorescence signal called "Förster type energy transfer."[62,63]. The alternative is a non-sequence specific fluorescent intercalating dsDNA binding dye, e.g., SYBR Green I (Molecular Probes) or ethidium bromide.[58] For single PCR product reactions with well-designed primers, SYBR Green I can work extremely well, with spurious non-specific background only showing up in very late cycles.[4,47,56] Among the real-time detection chemistry, SYBR Green I and TaqMan assays produced comparable dynamic range and sensitivity, while SYBR Green I detection was more precise and produced a more linear decay plot than the TaqMan probe detection.[10]

3.2.5. Real-Time RT-PCR Platforms

A detailed description of all real-time PCR platforms is available under http://cyclers.gene-quantification.info/ These PCR machines differ in sample capacity, up to 96-well and 384-well standard format, others process 72 (RotorGene) or only 32 samples and require specialized glass capillaries (LightCycler), excitation method (lasers and others broad-spectrum light sources with various filters), and fluorescence acquisition channels. There are also platform-specific differences in how the software processes data with focus on absolute or relative quantification strategies.[61,64,65] For at least two systems and chemistries, the ABI PRISM 7700 using "TayMan Probes" and Roche's LightCycler using "Hybridization Probes," there is little difference in accuracy and performance.[66]

3.2.6. Quantification Strategies in Kinetic RT-PCR

The quantification strategy is the principal *marker in gene quantification*. Generally, two strategies can be performed in real-time RT-PCR. The levels of expressed genes may be

measured by absolute or relative quantitative real-time RT-PCR. Absolute quantification relates the PCR signal to input copy number using a calibration curve, while relative quantification measures the relative change in mRNA expression levels. The reliability of an absolute real-time RT-PCR assay depends on the condition of "identical" amplification efficiencies for both the native target and the calibration curve in RT reaction and in following kinetic PCR.[67-69] Relative quantification is easier to perform than absolute quantification because a calibration curve is not necessary. It is based on the expression levels of a target gene versus a housekeeping gene (reference or control gene) and in theory is adequate for most purposes to investigate physiological changes in gene expression levels.[61,64] The units used to express relative quantities are irrelevant, and the relative quantities can be compared across multiple real-time RT-PCR experiments.[1]

3.2.6.1. Absolute Quantification

Calibration curves are highly reproducible and allow the generation of highly specific, sensitive and reproducible data.[3,4,47,54,56] However, the external calibration curve model has to be thoroughly validated as the accuracy of absolute quantification in real-time RT-PCR depends entirely on the accuracy of the standards. Standard design, production, determination of the exact standard concentration, and stability over long storage time is not straightforward and can be problematic. The dynamic range of the performed calibration curve can be up to nine orders of magnitude from $< 10^1$ to $> 10^{10}$ start molecules, depending on the applied standard material.[4,56,71] The calibration curves used in absolute quantification can be based on known concentrations of DNA standard molecules, e.g., recombinant plasmid DNA (recDNA), genomic DNA, RT-PCR product, and commercially synthesized big oligonucleotide.[3,4,49,54,58,71] Stability and reproducibility in kinetic RT-PCR depends on the type of standard used and depends strongly on "good laboratory practice." Cloned recDNA and genomic DNA are very stable and generate highly reproducible standard curves even after a long storage time, in comparison to freshly synthesized DNA. Furthermore, the longer templates derived from recDNA and genomic DNA mimic the average native mRNA length of about 2 kb better than shorter templates derived from RT-PCR product or oligonucleotides. They are more resistant against unspecific cleavage and proofreading activity of polymerase during reaction setup and in kinetic PCR (own unpublished results). One advantage of the shorter templates and commercially available templates is an accurate knowledge of its concentration and length. A second advantage is that their use avoids the very time consuming process of having to produce standard material: standard synthesis, purification, cloning, transformation, plasmid preparation, linearization, verification, and exact determination of standard concentration.[4,47,49,56]

A problem with DNA-based calibration curves is that they are subject to the PCR step only, unlike the unknown mRNA samples that must first be reverse transcribed. This increases the potential for variability of the RT-PCR results and the amplification results may not be strictly comparable with the results from the unknown samples. However, the problem of the sensitivity of the RT-PCR to small variations in the reaction setup is always lurking in the background as a potential drawback to this simple procedure. Therefore, quantification with external standards requires careful optimization of its precision (replicates in the same kinetic PCR run – intra-assay variation) and reproducibility (replicates in separate kinetic PCR runs – inter-assay variation) in order to understand the limitations within the given application.[4,54,56]

A recombinant RNA (recRNA) standard that was synthesized *in vitro* from a cloned RT-PCR fragment in plasmid DNA is one option.[4,7,47,56,72] However, identical RT efficiency, as well as real-time PCR amplification efficiencies for calibration curve and target cDNA must be tested and confirmed if the recRNA is to provide a valid standard for mRNA quantification.[4] This is because only the specific recRNA molecules are present during RT and the kinetics of cDNA synthesis are not like those in native RNA (the unknown sample) that also contain a high percentage of natural occurring subfractions, e.g., ribosomal RNA (rRNA, ~ 80%) and transfer RNA (tRNA, 10-15%). These missing RNA subfractions can influence the cDNA synthesis rate and in consequence RT efficiency rises and calibration curves are then overestimated in gene quantification.[36,73] To compensate for background effects and mimic a natural RNA distribution like in native total RNA, total RNA isolated from bacterial or insect cell lines can be used. Alternatively commercially available RNA sources can be used as RNA background, e.g., poly-A RNA or tRNA, but they do not represent a native RNA distribution over all RNA subspecies.[4] Earlier results suggest, that a minimum of RNA background is generally needed and that it enhances RT synthesis efficiency rate. Low concentrations of recRNA used in calibration curves should always be buffered with background or carrier RNA; otherwise the low amounts can be degraded easily by RNAses. Very high background concentrations had a more significant suppression effect in RT synthesis rate and in later real-time PCR efficiency.[4]

No matter how accurately the concentration of the standard material is known, the final result is always reported relatively compared to a defined unit of interest, e.g., copies per defined ng of total RNA, copies per genome (6.4 pg DNA), copies per cell, copies per gram of tissue, copies per ml blood, etc. If absolute changes in copy number are important, then the denominator still must be shown to be absolute stable across the comparison. This accuracy may only be needed in screening experiments (amount of microorganism in food), to measure the percentage of GMO (genetic modified organism) in food, to measure the viral load or bacterial load in immunology and microbiology. The quality of your gene quantification data cannot be better than the quality of the denominator. Any variation in the

denominator will obscure real changes, produce artificial changes, and wrong quantification results. Careful use of controls is critical to demonstrate that your choice of denominator was a wise one.[49] Under certain circumstances, absolute quantification models can also be normalized using suitable and unregulated references or housekeeping genes (see Section 3.4 *Normalization*).

3.2.6.2. Relative Quantification

Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. This reference gene is often a housekeeping gene and can be co-amplified in the same tube in a multiplex assay or can be amplified in a separate tube.[56,59] Therefore, relative quantification does not require standards with known concentrations and the reference can be any transcript, as long as its sequence is known.[29] Relative quantification is based on the expression levels of a target gene versus a reference gene and in many experiments is adequate for investigating physiological changes in gene expression levels. To calculate the expression of a target gene in relation to an adequate reference gene various mathematical models are established. Calculations are based on the comparison of the distinct cycle determined by various methods, e.g., crossing points (CP) and threshold values (C_t) at a constant level of fluorescence; or CP acquisition according to established mathematic algorithm.[50,51,69] To date, several mathematical models that determine the relative expression ratio have been developed. Two types of relative quantification models are available and published: (1) without efficiency correction (see Eqs. 3.1-3.2).[11,61,70]

$$R = 2^{-[\Delta CP\ \text{sample} - \Delta CP\ \text{control}]} \qquad (3.1)$$

$$R = 2^{-\Delta\Delta CP} \qquad (3.2)$$

and (2) with kinetic PCR efficiency correction (Eqs.3.3-3.6).[50,51,68,69,74-78] Further, the available models allow for the determination of single transcription difference between one control and

one sample, assayed in triplicates (n = 1/3), e.g., LightCycler Relative Quantification Software[65] or Q-Gene[79] or for a group-wise comparison for more samples (up to 100), e.g., REST and REST-XL.[69] The relative expression ratio of a target gene is computed, based on its real-time PCR efficiencies (E) or a static efficiency of 2, and the crossing point (CP) difference (Δ) of one unknown sample (treatment) versus one control ($\Delta CP_{\text{control - treatment}}$). Using REST and REST-XL, the relative calculation procedure is based on the MEAN CP of the experimental groups (Eq. 3.4) http://REST.gene-quantification.info/

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}\,(\text{control} - \text{sample})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}\,(\text{control} - \text{sample})}} \tag{3.3}$$

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}\,(\text{MEAN control} - \text{MEAN sample})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}\,(\text{MEAN control} - \text{MEAN sample})}} \tag{3.4}$$

In these models the target-gene expression is normalized by a non-regulated reference gene expression, e.g., derived from classical and frequently described housekeeping genes.[11,39-41,43] The crucial problem in this relative approach is that the most common reference-gene transcripts from so-called housekeeping genes, whose mRNA expression can be regulated and whose levels vary significantly with treatment or between individuals.[43,80-83] However, relative quantification can generate useful and biologically relevant information when used appropriately.

$$\text{ratio} = \frac{(E_{\text{ref}})^{CP_{\text{sample}}}}{(E_{\text{target}})^{CP_{\text{sample}}}} \div \frac{(E_{\text{ref}})^{CP_{\text{calibrator}}}}{(E_{\text{target}})^{CP_{\text{calibrator}}}} \tag{3.5}$$

$$\text{ratio} = \frac{\text{conc}_{(\text{target sample})} / \text{conc}_{(\text{ref sample})} * \text{MF}}{\text{conc}_{(\text{target cal.})} / \text{conc}_{(\text{ref cal.})} * \text{CF}} \quad (3.6)$$

3.2.7. Advantages and Disadvantages of External Standards

External standard quantification is the method of choice for the nucleic acid quantification, independent of any hardware platform used. The specificity, sensitivity, linearity, and reproducibility allow for the absolute and accurate quantification of molecules even in tissues with low mRNA abundance (< 100 molecules/reaction setup) and detection down to a few molecules (< 10 molecules/reaction setup).[4,12,56,71] The dynamic range of an optimal validated and optimized external standardized real-time RT-PCR assay can accurately detect target mRNA up to nine orders of magnitude or a billion-fold range with high assay linearity (correlation coefficient; r > 0.99).[4,12,49,56,84] In general, a mean intra-assay variation of 10-20% and a mean inter-assay variation of 15-30% on molecule basis (maximal 2-4% variability on CP basis, respectively) is realistic over the wide dynamic range.[6,12,47,56,71,85] At high (> 10^7) and low (< 10^3) template copy input levels the assay variability is higher than in the range between the two.[4,47,49] At very low copy numbers, under 20 copies per tube, the random variation due to sampling error (Poisson's error law) becomes significant.[49,76]

A recDNA calibration curve model can quantify precisely only cDNA molecules derived from the RT step; it says nothing about the conversion to cDNA of the mRNA molecules present in the native total RNA sample. Variability in cDNA synthesis efficiency during reverse transcription must be always kept in mind. Therefore, a recRNA calibration curve model has the advantage that both RNA templates undergo parallel RT and real-time PCR steps. However, a direct comparison suggests that the recDNA quantification model shows higher sensitivity, exhibits a larger quantification range, has a higher reproducibility, and is more stable than the recRNA model.[4] Furthermore, recDNA external calibration curves exhibit lower variation (intra-assay variation < 0.7%; inter-assay variation < 2.6% on CP basis) than the recRNA model (< 2.7% and < 4.5%, respectively). Clearly, the RT step has a profound affect on the overall result obtained from an RT-PCR assay and more thorough consideration of RT efficiency is needed.

The main disadvantage of 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.[61,70] The risk with external standards is that some of the unknown samples

may contain substances that significantly reduce the efficiency of the PCR reaction in the unknown samples. As discussed, sporadic RT and PCR inhibitors or different RNA/cDNA distributions can occur. A dilution series can be run on the unknown samples and the inhibitory factors can often be diluted out, causing a non-linear standard curve.[49,58,68,69]

Real-time assays using SYBR Green I can easily reveal the presence of primer dimers, which are the product of nonspecific annealing and primer elongation events.[58] These events take place as soon as PCR reagents are combined. During PCR, formation of primer dimers competes with formation of specific PCR product, leading to reduced amplification efficiency and a less successful specific RT-PCR product.[86] To distinguish primer dimers from the specific amplicon a melting curve analysis can be performed in all available quantification software.[61,64,65] The pure and homogeneous RT-PCR products produce a single, sharply defined melting curve with a narrow peak. In contrast, the primer dimers melt at relatively low temperatures and have broader peaks.[87] To avoid primer dimer formation, an intensive primer optimization is needed, by testing multiple primer pair using cross-wise combinations.[51] Multiple optimization strategies have been developed and are published.[88-90] The easiest and most effective way to get rid of any dimer structures, at least during the quantification procedure, is to add an additional 4th segment to the classical three-segmented PCR procedure: 1st segment with denaturation at 95°C; 2nd segment with primer annealing at 55-65°C; 3rd segment with elongation at 72°C; and 4th segment with fluorescence acquisition at elevated temperatures.[47,56,91] The fluorescence acquisition in 4th segment is performed mainly in the range of 80-87°C, eliminates the non-specific fluorescence signals derived by primer dimers or unspecific minor products, and ensures accurate quantification of the desired product. High temperature quantification keeps the background fluorescence and the "no-template control" fluorescence under 2-3% of maximal fluorescence at plateau.[47,56]

"Do we need to run a calibration curve in each run?"[49] and *"Do we need a calibration curve at all?"*[64,65,85] are frequently posed questions, together with *"What about the reproducibility between the runs?"* (http://www.idahotec.com/lightcycler_u/lectures/quantification_on_lc.htm). Repeated runs of the same standard curve give minor variations of a 2-3% in the slope (real-time PCR efficiency) and about 10% in the intercept of calibration curve. Since the variation in the standard curve correlates with variation in the unknowns, a detection of a 2-fold difference over a wide range of target concentrations is possible.[49] The slope of the calibration curve is more reproducible than the intercept, hence only a single standard point will be required to "re-register" a previously performed calibration curve level for the new unknowns. The curve can be imported into any run, as done in the LightCycler software.[64] Never changing variations and 100% reproducibility are the big advantages of such a calibration curve import, but there are also disadvantages as variations of reagents, primers, and probe (sequence alterations and fluorescence intensity), day-to-day or sample-to-sample variations will not be

covered in this "copy-and-paste" approach. Since these affect PCR efficiency, such an approach can introduce significant errors into the quantification.

3.2.8. Real-Time PCR Amplification Efficiency

Individual samples generate different and individual fluorescence histories in kinetic RT-PCR. The shapes of amplification curves differ in the steepness of any fluorescence increase and in the absolute fluorescence levels at plateau depending on background fluorescence levels. The PCR efficiency has a major impact on the fluorescence history and the accuracy of the calculated expression result and is critically influenced by PCR reaction components. Efficiency evaluation is an *essential marker in real-time gene quantification procedure.*[45,49-51,77,78] 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 sequence and a standard sequence, which is performed in all relative quantification models. In experimental designs employing standardization with housekeeping genes, the demand for invariable amplification efficiency between target and standard is often ignored, despite the fact that corrections have been suggested.[68,69,77,78] A correction for efficiency, as performed in efficiency corrected mathematical models (Eq. 3.3 – 3.6), is strongly recommended and results in a more reliable estimation of the "real expression ratio" compared to NO-efficiency correction. Small efficiency differences between target and reference gene generate false expression ratio, and the researcher over- or under-estimates the "real" initial mRNA amount. Difference in PCR efficiency (ΔE) of 3% ($\Delta E = 0.03$) between target gene and reference gene generate a falsely calculated differences in expression ratio of 47% in case of $E_{target} < E_{ref}$ and 209% in case of $E_{target} > E_{ref}$ after 25 performed cycles. This gap will increase dramatically by higher efficiency differences $\Delta E = 0.05$ (28% and 338%, respectively) and $\Delta E = 0.10$ (7.2% and 1083%, respectively) and higher cycle number performed.[49,69] Therefore efficiency corrected quantification corrections should be included in the automation and calculation procedure in relative quantification models.

The assessment of the exact amplification efficiencies of target and reference genes must be carried out before any calculation of the normalized gene expression is done. LightCycler Relative Expression Software,[65] Q-Gene,[79] REST, and REST-XL software applications[69] allow the evaluation of amplification efficiency plots. A separate determination of real-time PCR efficiency in triplets for every tissue and each performed transcript is necessary.[64,68,69,79] Different tissues exhibit different PCR efficiencies, caused by RT inhibitors, PCR inhibitors, and by variations in the total RNA fraction pattern extracted. Several methods are described in the literature to calculate real-time PCR efficiency http://efficiency.gene-quantification.info/

A) Efficiency calculation from the slopes of the calibration curve according to the equation: $\mathbf{E = 10^{[-1/slope]}}$, as described earlier.[5,49,61] Determination of efficiency should be evaluated in a pool of all starting RNAs to accumulate all possible "negative impacts" on kinetic PCR efficiency. Usually, real-time PCR efficiency vary with high linearity ($r > 0.989$) from E = 1.60 to maximal values up to E = 2.10 for cDNA input ranges from a few pg to 75 ng cDNA input.[12,56,69,71] Typically, the relationship between CP 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 RNA.[79] This calculation method results, in some cases, in efficiencies higher than ($E > 2.0$), which is practically impossible in the PCR amplification theory. But as shown in given results they are highly reproducible and constant within one transcript and tissue.[68,71] This probably indicates that this efficiency calculation method is not optimal and *overestimates* the "real efficiency."

B) Efficiency calculation from the fluorescence increase in 3rd linear phase of each logarithmic fluorescence history plot. The investigator has to decide which cycle number to include in the analysis and plot a linear regression (similarly to the *Fit Point Method* regression) where the slope of the regression line represents the PCR efficiency. Here efficiencies between E = 1.35 and E = 1.60 are realistic and differ dramatically from the results above.[47,92] This efficiency calculation method might *underestimate* the "real efficiency," because data evaluation is made in linear phase near the plateau where reaction trend to get restrictive.[46]

C) Efficiency calculation on the basis of all fluorescence data points (starting at cycle 1st up to the last cycle), according to a sigmoidal or logistic curve fit model. The advantage of such model is that all data points will be included in the calculation process. No background subtraction is necessary.[45,50,51,77,78] Slope value is "nearly" identical to method B and only measured at the point of inflexion at absolute maximum fluorescence increase ($1.35 < E < 1.60$). But the derived slope parameters generated by the sigmoidal or logistic models are not directly comparable with the "real PCR efficiency." This method is easy to perform and a good estimator for the maximum curve slope with high correlation coefficient ($r > 0.99$) and level of significance ($p < 0.001$).[45,50,51,77,78]

D) Efficiency calculation from the fluorescence increase only in the 2nd real exponential phase, according to a polynomial curve fit, as described earlier $\mathbf{Y_n = Y_0\ (E)^n}$, where $\mathbf{Y_n}$ is fluorescence acquired at cycle n, and Y_0 initial fluorescence, so-called ground fluorescence.[45,74,92-94] This phase around the *"Second Derivate Maximum"* exhibit a real exponential amplification behavior.[45] Here in the exponential part of the PCR reaction, kinetic is still under "full power" with no restrictions.[46] In this method 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, hence are between the other methods.

Which efficiency calculation method is "the right one" and which one shows the realistic real-time PCR kinetic and thereby is highly reproducible, has to be evaluated in further experiments.

3.2.9. Data Evaluation

The next marker in gene quantification using real-time RT-PCR is the data evaluation. The calculation unit in real-time PCR is a sample specific and characteristic crossing points (CP). For CP determination various fluorescence acquisition methodologies are possible. The *"Fit Point Method"* and *"Threshold Cycle Method"* measure the CP at a constant fluorescence level.[5,7,61,64,74] These constant threshold methods assume that all samples have the same cDNA concentration at the threshold fluorescence. Measuring the level of background fluorescence can be a challenge in real-time PCR reactions with significant background fluorescence variations 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.[61,49] The threshold level can be calculated by fitting the intersecting line upon the ten-times value of ground fluorescence standard deviation. This acquisition mode can be easily automated and is very robust.[61] In the *"Fit Point Method"* the user has discard the uninformative background points, exclude the plateau values by entering the number of log-linear points, and then fits 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 CP 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.[49,64] *"Fit Point Method"* or *"Threshold Cycle Method"* can be used on all available platforms with different evaluation of background variability.

The problems of defining a constant background for all samples within one run, sample-to-sample differences in variance and absolute fluorescence values lead to develop a new acquisition modus according to mathematical algorithms. In the LightCycler software the *"Second Derivative Maximum Method"* is performed where CP is automatically identified and measured at the maximum acceleration of fluorescence.[49,64] The kinetic fluorescence histories of individual curves are different. They show individual background variability (1st phase), exponential and linear growth of fluorescence (2nd and 3rd phase), and finally reaction-specific plateau values (4th phase). The amplification reaction and the kinetic

fluorescence history over various cycles is obviously not a smooth and easy function. The mathematical algorithm on which the *"Second Derivative Maximum Method"* in Roche Molecular Biochemicals software is based is unpublished. But it is possible to fit sigmoidal and polynomial curve models[45,50,51,77,78] with high significance ($p < 0.001$) and coefficient of correlation ($r > 0.99$), which can be differentiated, and the second-derivate maximum can be estimated.[45,50,51] This increase in the rate of fluorescence increase, or better called the acceleration of the fluorescence signal, slows down at the beginning of the 3rd linear phase. Therefore the cycle where the second derivative is at its maximum is always between 2nd exponential and 3rd linear phase.[45]

3.3. Automation of the Quantification Procedure

Automation of quantification with any kind of calibration curve using *"Fit Point Method,""Threshold Cycle Method"* or *"Second Derivative Maximum Method"* is fully supported by the software supplied with the real-time instruments. The investigator has to input his individual settings, e.g., threshold level and noise band, import an existing standard curve, and then click for calculation of the CPs and the corresponding concentrations. However, although relative expression is performed by researchers according to several established mathematical models (Eqs. 3.1-3.6),[61,68-70,75,79] up to now relative quantification software has been commercially available only from Roche Molecular Biochemicals *"LightCycler Relative Quantification Software"* (http://www.lightcycler-online.com/lc_sys/soft_ind.htm#quant). The software allows for a comparison of maximal triplets (n = 3) of a target versus a calibrator gene, both 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 calculated from the calibration curve slope, according to the established equation $\mathbf{E = 10^{[-1/slope]}}$, ranging from E = 1.0 (minimum value) to E = 2.0 (theoretical maximum and efficiency optimum). A given correction factor (CF) and a multiplication factor (MF), which are provided in the product specific applications (Eq. 3.6) by Roche Molecular Biochemicals, have to be attended in the equation calculation process.[65]

Importantly, until recently it was not possible to perform a reliable group-wise calculation of the relative expression ratios and a subsequent statistical comparison of the results by a statistical test with more than three repeats or more than three samples. This has changed when new software tools were established (e.g. REST and REST-XL), both Excel®-based and programmed in Visual Basic for Applications.[68,69] Both compare two treatment groups, with multiple data points in sample group versus control group, and calculate the relative expression ratio between them. Four target genes with up to 100 data points can be

calculated in REST-XL. The mathematical model used is published;[69] it is based on the MEAN crossing point deviation between sample and control group of up to four target genes, normalized by the MEAN crossing point deviation of a reference gene (Eq. 3.4). Normalization via endogenous control can be performed according to the users demand, but it is recommended to compensate intra- and inter-RT-PCR variations.[68,69,95] Therefore the requirement for high reproducibility of RT and RT efficiency is not "that important" anymore. cDNA input concentration variation of ±3-fold was evaluated to mimic these huge RT variations and resulted in no significant changes of relative expression ratio.[69] Specific amplification efficiencies of four target-gene genes can be estimated and included in the correction of the quantification ratio. If no real-time PCR efficiency assessment is performed, REST assumes an optimal efficiency of E = 2.0. The big advantage of the software tool is the subsequent statistical test. REST tests the group differences for significance with a newly developed randomization test - *Pair-Wise Fixed Reallocation Randomization Test©*. Variation depends only on CP variation of the investigated transcripts and remains stable between 3% and 12%.[69]

Nevertheless, successful application of real-time RT-PCR and REST 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 and fully quantitative measurement of mRNA transcripts http://REST.gene-quantification.info/

Recently a second software tool, named Q-Gene, was developed.[79] Q-Gene manages and expedites the planning, performance, and evaluation of quantitative real-time PCR experiments, as well as the mathematical and statistical analysis, storage, and graphical presentation of the data. An efficiency correction is possible. The Q-Gene software application is a tool to 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 setup, data analysis, and data management while ensuring highest reproducibility (http://www.biotechniques.com/softlib/qgene.html).

3.4. Normalization

Data normalization in real-time RT-PCR is a further *major marker in gene quantification analysis*. The reliability of any relative RT-PCR experiment can be improved by including an invariant endogenous control in the assay to correct for sample-to-sample variations in RT-PCR efficiency and errors in sample quantification. A biologically meaningful reporting of target mRNA copy numbers requires accurate and relevant normalization to some standard and is strongly recommended in kinetic RT-PCR.[28,29,68-70] 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 artifactual changes.[28,29] Real-time RT-PCR-specific errors in the quantification of mRNA transcripts are easily compounded with any variation in the amount of starting material between the samples, e.g., caused by sample-to-sample variation, variation in RNA integrity, RT efficiency differences, and cDNA sample loading variation.[15,31,95] This is especially relevant when the samples have been obtained from different individuals, different tissues, and different time courses and will result in the misinterpretation of the derived expression profile of the target genes. Therefore, normalization of target-gene expression levels must be performed to compensate intra- and inter-kinetic RT-PCR variations (sample-to-sample and run-to-run variations).

Data normalization can be carried out against an endogenous unregulated reference gene transcript or against total cellular DNA or RNA content (molecules/g total DNA/RNA and concentrations/g total DNA/RNA). Normalization according the total cellular RNA content is increasingly used, but little is known about the total RNA content of cells or even about the mRNA concentrations. The content per cell or per gram tissue may vary in different tissues *in vivo*, in cell culture (*in vitro*), between individuals and under different experimental conditions. Nevertheless, it has been shown that normalization to total cellular RNA is the least unreliable method.[3,28,29] It requires an accurate quantification of the isolated total RNA or mRNA fraction by optical density at 260 nm (OD_{260}), Agilent Bioanalyser 2100, or RiboGreen RNA Quantification Kit. Alternatively the rRNA content has been proposed as an optimal and stable basis for normalization, despite reservations concerning its expression levels, transcription by a different RNA polymerase, and possible imbalances in rRNA and mRNA fractions between different samples.[29,42,80,96,97]

To normalize the absolute quantification according to a single reference gene, a second set of kinetic PCR reactions has to be performed for the invariant endogenous control on all experimental samples and the relative abundance values are calculated for internal control as well as for the target gene. For each target gene sample, the relative abundance value obtained is divided by the value derived from the control sequence in the corresponding target gene. The normalized values for different samples can then be directly compared. The sets of CPs for the reference gene can be easily imported into the REST software application, according to the given equations (Eqs. 3.3-3.4). The calculation process considers them and allows for a normalization of the target genes with the reference gene.[64,68,69,79] Additionally, it will show whether normalization via the chosen reference is useful by showing the factor of regulation and level of significance as result of the randomization test. The investigators can decide if the reference is suitable in this experimental trial or not.

Here a central question arises: "*What is the appropriate reference gene for an experimental treatment and investigated tissue?*"[3,41,42,98] Commonly used housekeeping genes, e.g.,

GAPDH, albumin, actins, tubulins, cyclophilin, microglobulins, 18S rRNA or 28S rRNA[27,39-42,50]may be suitable for reference genes, since they are present in all nucleated cell types and are necessary for basic cell survival. The mRNA synthesis of housekeeping genes is considered to be stable in various tissues, even under experimental treatments.[39-41,50] However, numerous treatments and studies have already shown that the mentioned housekeeping genes are regulated and vary under experimental conditions.[42,43,80-83,99] It remains up to the individual investigator to choose a reference gene that is best for reliable normalization in their particular experimental setting. In addition, the endogenous control should be expressed at roughly the same CP level as the target gene.[3,28] At the same CP level, reference and target experience the same condition and real-time RT-PCR kinetics with respect to polymerase activation (heat activation of polymerase), reaction inactivation, stochastic relation between target and primer concentration, and reaction end product inhibition by the generated RT-PCR product.

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. -actin or GAPDH.[22-24] These pseudogenes may be responsible for specific amplification products in an mRNA-independent fashion and result in specific amplification even in the absence of intact mRNA.[18-21,84] 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.[29] Usually more than one housekeeping genes should be tested in a multiple pair wise correlation analysis and its behavior summarized to a housekeeping gene index called *BestKeeper©* [105]. According to this *BestKeeper©* index, which is based on the weighted expression of at least three housekeeping genes, a more reliable basis of normalization in relative quantification can be postulated.[69,100,101] http://www.gene-quantification.de/bestkeeper.html

There is increasing appreciation of these aspects of quantitative RT-PCR and recently a software tool was established for the evaluation of housekeeping genes expression levels. *GeNorm*[27] allows for an accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes (http://allserv.rug.ac.be/~jvdesomp/genorm/). The *geNorm* VBA applet for Microsoft Excel determines the most stable housekeeping genes from a set of ten 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 housekeeping 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.[27]

3.5. Statistical Comparison

Bio-informatics and bio-statistics on real-time RT-PCR experiment data is a *new subject and a new challenge in gene quantification analysis*. This is because the coordination of the experiments and the efficient management of the collected data has become an additional major hurdle for kinetic RT-PCR experiments. The main challenge remains the evaluation and the mathematical and statistical analysis of the enormous amount of data gained by this technology, as these functions are not included in the software provided by the manufacturers of the detection systems.[79] Normally the statistical data analysis in gene quantification, independent of block, competitive or real-time RT-PCR experiments, are all performed on the basis of classical standard parametric tests, such as analysis of variance or t-tests.[102] Parametric tests depend on assumptions, such as normality of distributions, whose validity is unclear. 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.[69]

Only two free available software packages support statistical analysis of expression results: Q-Gene[79] and REST.[69] 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 nonparametric 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.[102] 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.[79]

Permutation or randomization tests are a useful alternative to more standard parametric tests for analyzing experimental data.[103,104] 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.[103,104] The randomization test is conducted as follows: A statistical test is based on the probability of an effect as large as that observed occurring under the null hypothesis of no treatment effect. If this hypothesis is true, the values in one treatment group were just as likely to have occurred in the other group. The randomization test repeatedly and randomly reallocates the observed values to the two groups and notes the apparent effect (expression ratio in REST) each time. The proportion of these effects, which are as great as that actually observed in the experiment gives us the p-value of the test (http://www.bioss.ac.uk/smart/unix/mrandt/slides/frames.htm).

The REST software package makes full use of the advantages of a randomization test.[69] In the applied two-sided *Pair-Wise Fixed Reallocation Randomization Test* for each sample, the

CP 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 2,000 or more samples are taken, a good estimate of p-value (standard error < 0.005 at p = 0.05) is obtained.[103,104] Randomization tests with a pair-wise reallocation are seen as the most appropriate approach for this type of application. They are more flexible than non-parametric tests based on ranks (Mann-Whitney, Kruskal-Wallis, etc.) and do not suffer a reduction in power relative to parametric tests (t-tests, ANOVA, etc.). They can be slightly conservative (i.e., type I error rates lower than the stated significance level) due to acceptance of randomizations with group differences identical to that observed, but this mainly occurs when used with discrete data.[103,104]

3.6. Conclusion

The recent advances in gene quantification strategies, fluorescence chemistries, and instrumentations have led to the development of various assays whereby mRNA transcripts can be quantified precisely in very short time. The benefits in terms of increased sensitivity, reduced variability, reduced risk of contamination, increased throughput by automation, and meaningful data interpretation are obvious. If done properly, kinetic RT-PCR is the most powerful method for quantifying cellular mRNA levels. The quantification strategy used should be designed according to the researchers demand, but must be highly optimized and precisely validated. In the future, there is a need for greater standardization of the applied assays to make the expression results comparable between runs, between real-time RT-PCR platforms, and between different laboratories worldwide.

Sharing any technical and practical information for sample preparation, assay design, standard materials, and data management will help to improve gene quantification analysis. Therefore various information platforms and discussion forums are available on the internet (summarized under http://infoportal.gene-quantification.info/).

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