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ORIGINAL PAPER

Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR

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Abstract Relative quantification in quantitative real-time RT-PCR is increasingly used to quantify gene expression changes. In general, two different relative mRNA quantification models exist: the delta-delta Ct and the efficiency-corrected Ct model. Both models have their advantages and disadvantages in terms of simplification on the one hand and efficiency correction on the other. The particular problem of RNA integrity and its effect on relative quantification in qRT-PCR performance was tested in different bovine tissues and cell lines ($n = 11$). Therefore different artificial and standardized RNA degradation levels were used. Currently fully automated capillary electrophoresis systems have become the new standard in RNA quality assessment. RNA quality was rated according the RNA integrity number (RIN). Furthermore, the effect of different length of amplified products and RNA integrity on expression analyses was investigated. We found significant impact of RNA integrity on relative expression results, mainly on cycle threshold (Ct) values and a minor effect on PCR efficiency. To minimize the interference of RNA integrity on relative quantification models, we can recommend to normalize gene expression by an internal reference gene and to perform an efficiency correction. Results demonstrate that innovative new quantification methods and normalization models can improve future mRNA quantification.

Keywords Gene expression study · Ct · mRNA · qRT-PCR · real-time RT-PCR · RNA integrity · RNA integrity number (RIN)

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Introduction

PCR for gene expression profiling has become the standard technology for the quantification of nucleic acids. For an exact quantitative measurement of low abundant mRNA gene expression real-time quantitative reverse-transcription PCR (qRT-PCR) is the method of choice. 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 control RNA is often a classical reference gene, like GAPDH, ribosomal RNA subunits (18S and 28S rRNA), or β-actin, which are co-amplified in the same tube in a multiplex-assay or amplified in a

separate tube as an external standard (Bustin and Nolan 2004). The relative quantification strategy is adequate for most purposes to investigate physiological changes in gene expression levels.

An essential requirement for a successful quantitative mRNA analysis using qRT-PCR is the usage of intact RNA. Low-quality RNA may compromise the derived expression results. Investigations with low quality RNA as starting material supply results of qRT-PCR (Imbeaud et al. 2005; Raeymarkers 1993). The need to isolate high quality total RNA from wide variety of clinical and/or experimental tissue samples becomes more important for quantitative gene expression studies. After extraction the RNA is unstable. Long mRNA, up to several kilo bases, is very sensitive to degradation (Bustin 2002). This can occur through cleavage by RNAses during handling of RNA samples, otherwise it may also be impaired in samples stored for a long time or under sub-optimal conditions (Schoor et al. 2003). Consequently, the determination and confirmation of RNA quantity and quality is the first critical step in obtaining meaningful gene expression data.

Verification of RNA integrity prior to usage in downstream qRT-PCR application permits experiments to be compared and provide more accurate and reliable results. While methods for the physical isolation of total RNA have evolved significantly over the last two decades, there has been limited advancement in methods used for assessing RNA quality. Today it is well acknowledged that the accuracy of gene expression is influenced by starting RNA quality. RNA purity is normally assessed by its A260/A280 ratio (Baelde et al. 2001). The spectrometric methods often fail in sensitivity, are highly variable and give no results in terms of RNA integrity. In the past, RNA quality could often not be assessed exactly. The further development of the capillary gel electrophoresis methods and spectrophotometer technologies have addressed this issue (Auer et al. 2003).

The micro-fluidic capillary electrophoresis has developed into commonly tool, particularly in the gene expression profiling platforms (Mueller et al. 2000; Livak and Schmittgen 2001). Instruments, such as the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and the Experion (Bio-Rad Laboratories, Hercules, CA), are becoming more and more standard since their use dramatically decreased the amount of RNA needed to evaluate integrity down to the submicrogram scale. On condition that the 18S and 28S ribosomal RNA (rRNA) fragments produce well-defined peaks without any smearing towards a smaller size, the sample can be considered for further analysis. The 28S/18S ratio is automatically generated and is the first criterion for a total RNA quality check. A new and more advanced tool for RNA quality assessment is the RNA Integrity Number (RIN, Agilent Technologies). The algorithm assigns a RIN number score from 1 to 10, where level 10 represents a completely intact RNA, and 1 presents a highly degraded RNA (Mueller et al. 2000). An interpretation of an RNA integrity is facilitated, comparison of samples is possible and repeatability of experiments is ensured.

While it is obvious that intact RNA constitutes the best representation of the natural state of the transcriptome, there are situations in which gene expression analysis even on partially degraded RNA may be desirable, e.g. in ancient, necrotic, clinical or biopsy samples. Yet, little is known about the possibility of obtaining reasonable qRT-PCR data from RNA samples with impaired RNA quality. Until today there are no statistically confirmed studies at which threshold RNA integrity is useless for quantitative downstream applications. With that in mind, and with the aim of anticipating future standards, we identified and analyzed the effect of various artificial and standardized degraded RNA samples on the two most abundant relative quantification models: the delta-delta Ct (Livak and Schnittgen 2001) and the efficiency corrected Ct model (Pfaffl 2001). The procedure of normalizations with an internal reference mRNA standard can reveal the importance of a relative expression approach to exclude compounded errors by variation in RNA quality and quantity. A further aim of the study was to investigate whether a correlation exists between PCR performance, PCR efficiency, length of amplified product and quality of RNA.

Materials and methods

Sample preparation

Two experimental set-ups were processed. In the first experiment total RNA was prepared from eight different bovine tissue types [lymph node, colon, corpus luteum, caecum, spleen, abomasums, reticulum and white blood cells (WBC)] and two primary cell cultures (granulosa and kidney cells). In the second experiment total RNA extracted from bovine spleen, corpus luteum, liver and WBC was investigated.

RNA purification in both experiments was performed by a slightly modified phenol-based extraction method, using peqGOLD TriFast (PeqLab, Erlangen, Germany). 500 µl peqGOLD TriFast and 50 mg tissue (stored at –80°C) were added to impact-resistant 2 ml tubes (MP Biomedicals, Solon, OH) pre-filled with 200 mg specialized lysing matrix particles (Qbiogene, Morgan Irvine, CA). The samples were homogenized two times by mechanical disruption using the FastPrep 120 instrument at speed 6.0 for 30 s. (Qbiogene). RNA extraction was carried out in RNAse-free environment and the purified total RNA was eluted in RNAse-free water (Eppendorf, Hamburg, Germany). Nucleic acid concentrations were measured in triplicate at 260 nm by using the BioPhotometer (Eppendorf). Purity of the total RNA extracted was determined as the A_{260}/A_{280} ratio with expected values between 1.8 and 2.

Artificial total RNA degradation

In order to get RNA samples with different and standardized degradation levels, but with the identical transcriptome and tissue typical mRNA distribution, intact cellular RNA was artificially degraded either enzymatically via ubiquitary skin RNAses for 10 s, or by irradiation with ultraviolet-C radiation (UVC) at 200–280 nm (Kendro UV-C 30, Langenselbold, Germany). In the first experiment reticulum and lymph node$_{(e)}$ were treated enzymatically ($n = 2$). Lymph node$_{(p)}$, colon, corpus luteum, caecum, spleen, abomasums, WBC, kidney cells and granulose cells were treated physically ($n = 9$) by UV-C radiation (Kundu et al. 2004). In the second experiment only physical degradation via UVC was performed ($n = 4$). Depending on the type of tissue each sample was placed under a UVC lamp for a tissue specific period of time up to 120 min. For both experimentals, intact RNA samples from the identical tissue extraction were mixed in various ratios with degraded RNA samples to generate a RIN gradient. Each sample consists of 10–12 denaturation grades (in total 135 samples) and ranging from intact RNA (RIN >7.5) to highly degrade RNA (RIN <3).

RNA integrity number (RIN) algorithm analysis

For the rapid quantification of nucleic acids the Agilent 2100 Bioanalyzer (Agilent Technologies), a chip-based nucleic acid separation system was used. The Bioanalyzer utilizes a combination of micro-fluidics, capillary electrophoresis, and fluorimetry to determine RNA length, distribution and concentration. The RNA Nano 6000 LabChip kit (Agilent Technologies) was used together with a standardized RNA ladder (Ambion, Austin, TX, USA) for RNA analysis and quantification. Altogether 135 RNA samples with different total RNA degradation levels were investigated in triplicates ($n = 405$).

One-step qRT-PCR

In the first experiment the expression levels of four representative genes (18S, 28S and β-actin and IL-1β) were measured. High abundant ribosomal 18S and 28S rRNA subunits, medium abundant β-actin and low abundant IL-1β genes were used to cover all possible abundance levels of a normal distributed transcriptome. Quantification was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 50 ng total RNA of various RNA degradation levels were used as template in the Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia). For ribosomal subunits, 18S and 28S rRNA, 1:10.000 dilutions were used in qRT-PCR. The real-time qRT-PCR master-mix was prepared as follows: 5 µl 2X

SYBR Green Reaction Mix, 0.5 µl forward primer (10 pmol), 0.5 µl reverse primer (10 pmol) and 0.2 µl SYBR Green One-Step Enzyme Mix (Invitrogen).About 6.2 µl of master mix was filled into a tube and a 3.8 µl volume of total RNA was added as PCR template. Bovine sequence-specific primers were synthesized commercially (MWG Biotech, Ebersberg, Germany) as shown in Table 1. A four-step experimental run protocol was used: (1) reverse transcription (10 min at 50°C); (2) denaturation program (5 min at 95°C); amplification and quantification program repeated 40 times (15 s at 95°C; 30 s at 60°C with a single fluorescence measurement; 20 s at 68°C); (3) melting curve program (60–99°C with a heating rate of 0.5°C/s and a continuous measurement); (4) cooling program down to 40°C.

Cycle threshold (Ct) and amplification efficiency

Ct values and qPCR efficiency were computed with the *''comparative quantitation''* method in the real-time qPCR Analysis Software, version 6.0 (Corbett Life Science). The Ct value is defined as the point at which the fluorescence rises above the background fluorescence (Pfaffl et al. 2002).

Relative quantification

The Ct of three target genes (18S, β-actin and IL-1β) was normalized to the chosen reference gene Ct of 28S rRNA. In the first relative quantification approach no efficiency correction was performed according to the delta-delta Ct model (Livak and Schnittgen 2001) shown in Eqs. 1 and 2.

$$R = 2^{-[\Delta Ct\,\text{sample} - \Delta Ct\,\text{control}]}, \quad (1)$$

$$R = 2^{-\Delta\Delta Ct}. \quad (2)$$

In the second approach the efficiency correction was implemented additionally (Eqs. 3, 4) and the advantages of an efficiency corrected quantification model were applied (Pfaffl 2001). The derived ratio values describe the relative expression change of the target gene relative to the 28S reference gene expression:

Table 1 Description of used primers

Gene	Primers	Length [bp]	Annealing temperature [°C]
Study 1			
18S rRNA	for: GAG AAA CGG CtA CCA CAT CCA rev: GAC ACt CAG CtA AGA GCA TCG A	338	60
28S rRNA	for: TAA CAA GCC GGT AGC CCA CG rev: GCA AGG GCt CtT GAT GGC AGA	238	60
β-actin	for: AAC TCC ATC ATG AAG TGT GAC G rev: GAT CCA CAT CtG CtG GAA GG	202	60
IL-1β	for: TTC TCt CCA GCC AAC CtT CAT T rev: ATC TGC AGC TGG ATG TTT CCA T	198	60
Study 2			
β-actin 50	for: ATC CtG CGT CtG GAC CtG rev: ACG CtC CGT GAG GAT CtT C	66	60
β-actin 100	for: GAG CGA GGC TAC AGC TTC A rev: CAT CtC CtG CtC GAA GTC CA	99	60
β-actin 200	for: GGC ATC GTG ATG GAC TCC rev: GAG CtT CtC CtT GAT GTC ACG	201	60
β-actin 400	for: TCt ACA ACG AGC TCC GTG TG rev: GAG CtT CtC CtT GAT GTC ACG	380	60
β-actin 600	for: GGC ATC GTG ATG GAC TCC rev: TCt GCt GGA AGG TGG ACA G	616	60
β-actin 800	for: TCt ACA ACG AGC TCC GTG TG rev: TCt GCt GGA AGG TGG ACA G	795	60
β-actin 950	for: GTC TTC CCG TCC ATC GTG rev: TCt GCt GGA AGG TGG ACA G	976	60

$$R = \frac{(E_{\text{target}})^{\Delta \text{Ct target (control}-\text{sample)}}}{(E_{\text{ref}})^{\Delta \text{Ct ref (control}-\text{sample)}}}, \quad (3)$$

$$R = \frac{(E_{\text{ref}})^{\text{Ct sample}}}{(E_{\text{target}})^{\text{Ct sample}}} \div \frac{(E_{\text{ref}})^{\text{Ct control}}}{(E_{\text{target}})^{\text{Ct control}}}. \quad (4)$$

PCR product length

In the second experiment sample RNA was diluted to a final concentration of 20 ng/µl. Seven PCR primer sets were designed using HUSAR software and synthesized by MWG Biotech (Ebersberg) to amplify different sequence fragments of β-actin (Table 1). Primer characteristics like primer dimer, self binding or false binding ability were minimized. GC content and PCR annealing temperature of all primer sets were adjusted and optimized to constant values. The one-step qRT-PCR was performed and expression levels of β-actin were measured with the standardized protocol as described above. Some minor changes were introduced in the time of denaturation depending on product length. For the product length of 800 and 950 bp attuned to 20 s. Elongation time at 68°C was slightly changed and adapted to different product lengths: 10 s for 50 bp, 20 s for 100 and 200 bp, 30 s for 400 bp, 60 s for 800 and 950 bp. In order to prevent inter-assay variation, samples with the same primer set were always amplified within one run. To ensure that PCR products from qRT-PCR had the expected size, an agarose gel electrophoresis was performed (gel not shown).

Statistical interference

All statistical analyses were performed by using Sigma Stat 3.0 (SPSS Inc., Chicago, IL). The determined *P*-values of the statistical significance were analyzed using linear regression and coefficient of determination (R^2). Significance of linear regression was tested by Student's *t*-test, by testing the slope to be different from zero. Coefficients were recorded when significant at $P < 0.05$. Higher significance levels were considered when available. All data were plotted in Sigma-Plot 8.0 (SPSS) and Microsoft Excel (Microsoft, Redmond, WA).

Results

RNA quality assessment and RNA degradation levels

Isolated total RNA quality was verified by an average A_{260}/A_{280} ratio of 1.88 (range 1.75–2.01). An A_{260}/A_{280} ratio greater than 1.8 is usually considered an acceptable indicator of good RNA (Sambrook et al. 1989). No phenolic contamination or background absorption was reported via the A_{260}/A_{230} ratio. All 135 artificial total RNA degradation gradient samples, were measured in triplicates in the Bioanalyzer 2100, and ranged from integer to degraded quality levels: RIN 7.3–9.5 for integer down to RIN 1.1–3.0 for degraded RNA (total measurements $n = 405$). Furthermore, the dependency of the RNA quality on tissue type, WBC and cell-lines was determined. The average RIN for solid tissues ranged between 5.4 and 9.6, whereas tissues or organs with high content of connecting tissue, for example in the gastrointestinal tract, showed higher variations in RIN values. In cell culture and WBC the RIN ranged between 8.4 and 9.6 with low experimental variance (Fleige and Pfaffl 2006).

Confirmation of primer specificity

The expression levels of four genes (18S, 28S, β-actin and IL-1β) were measured in all RNA quality aliquots. For each analyzed gene a melting curve analysis was performed. All investigated qRT-PCR products showed only single peaks and no primer-dimer peaks or artifacts. The specificity of qRT-PCR products was documented with high resolution gel electrophoresis and resulted in a single product with the desired length (not shown).

Gene expression profiling versus RNA integrity

To determine how qRT-PCR is affected by the integrity of the starting RNA, we compared Ct

Table 2 Correlation between RNA integrity and gene expression

	18S rRNA		28S rRNA		β-actin		IL-1β	
	R^2	P	R^2	P	R^2	P	R^2	P
UV degradation								
Lymph node$_{(p)}$	0.911	< 0.001	0.946	< 0.001	0.812	< 0.001	0.903	< 0.001
	$y = -0.633x + 17.38$		$y = -0.492x + 15.38$		$y = -0.794x + 15.06$		$y = -0.674x + 24.68$	
Colon	0.541	< 0.001	0.911	< 0.001	0.72	< 0.001	0.725	< 0.001
	$y = -0.873x + 26.16$		$y = -0.337x + 14.21$		$y = -0.604x + 15.57$		$y = -0.847x + 24.65$	
Corpus luteum	0.832	< 0.001	0.948	< 0.001	0.912	< 0.001	0.885	< 0.001
	$y = -1.568x + 26.83$		$y = -0.489x + 16.45$		$y = -0.801x + 15.47$		$y = -0.907x + 27.95$	
Caecum	0.743	< 0.001	0.842	< 0.001	0.965	< 0.001	0.947	< 0.001
	$y = -0.614x + 18.91$		$y = -0.589x + 16.45$		$y = -0.901x + 16.99$		$y = -0.608x + 23.07$	
Spleen	0.606	< 0.001	0.686	< 0.001	0.673	< 0.001	0.894	< 0.001
	$y = -1.544x + 25.39$		$y = -0.413x + 14.74$		$y = -0.704x + 15.79$		$y = -0.591x + 21.48$	
Abomasum	0.752	< 0.001	0.876	< 0.001	0.776	< 0.001	0.868	< 0.001
	$y = -1.251x + 22.18$		$y = -0.745x + 16.35$		$y = -1.199x + 20.69$		$y = -0.740x + 29.78$	
WBC	n.a.	n.a.	0.534	< 0.05	0.746	< 0.001	0.799	< 0.001
	n.a.		$y = -0.243x + 27.49$		$y = -0.923x + 21.61$		$y = -0.751x + 27.25$	
Kidney cells	0.907	< 0.001	0.581	0.002	0.833	< 0.001	0.901	< 0.001
	$y = -0.346x + 26.88$		$y = -0.191x + 13.624$		$y = -1.617x + 22.04$		$y = -0.959x + 29.42$	
Granulosa cells	0.182	< 0.001	0.248	< 0.001	0.776	< 0.001	0.868	< 0.001
	$y = -0.114x + 13.22$		$y = -0.104x + 25.67$		$y = -1.199x + 20.69$		$y = -0.740x + 29.78$	
Enzymatic degradation								
Reticulum	0.28	< 0.001	0.352	< 0.001	0.803	< 0.001	0.717	< 0.001
	$y = -0.518x + 22.53$		$y = -0.284x + 13.44$		$y = -0.582x + 13.26$		$y = -0.133x + 21.97$	
Lymph node$_{(e)}$	0.579	< 0.001	0.265	< 0.01	0.594	< 0.001	0.842	< 0.001
	$y = -0.231x + 14.37$		$y = -0.038x + 12.05$		$y = -0.092x + 8.96$		$y = -0.088x + 19.59$	

Impact of RNA integrity (shown as RIN $= x$) on *cycle threshold* (Ct shown as Ct $= y$). Data are representing as linear regression, coefficient of determination (R^2) and the P-values of regression. Four different genes (18S, 28S, β-actin and IL–1β), were analyzed in 11 tissues, sorted by various type of degradation, either UV degradation ($n = 9$) or enzymatic degradation ($n = 2$)

levels or single-run PCR efficiency with the RNA integrity (Tables 2 and 3). Therefore, RIN numbers were linearly regressed with Ct or efficiency values. Each amplified gene was tissue specifically influenced by the RNA integrity, reflected by the level of significance (P-value) and the Pearson correlation coefficient (R^2) in the applied linear regressions. This demonstrates an incomprehensible tissue-matrix-effect between RNA integrity and type of tissue and the analyzed transcript (cited the lymph-node as an example in Fig. 1). The expression data demonstrates that a high-quality, intact RNA will result in a high expression level (low Ct) and a less-quality RNA results in low expression level (high Ct). The mean coefficient of determination in all regressed genes and tissues ($R^2 > 0.812$) shows that there is a causally determined high correlation between RIN and the Ct ($n = 53$). The determined P-values provide a statistical significance, in almost all datasets of $P < 0.001$.

28S/18S rRNA ratio

In a further sub-study all 28S/18S ratios were compared with the RIN. No clear trend of RNA quality compared to 28S/18S ratios could be found, therefore the 28S/18S ratio data are not presented.

Real-time PCR amplification efficiencies

Our research into RNA integrity effect on the single-run qRT-PCR efficiency has been carried out similarly to the above mentioned tissues and artificial RNA qualities. The efficiency variations for the lymph node were diagrammed in Fig. 2. Each analyzed tissue turned out to have significant tissue specific qPCR amplification efficiencies.

Table 3 Correlation between RNA integrity and real-time PCR efficiency

Tissue	18S rRNA		28S rRNA		β-actin		IL-1β	
	R^2	P	R^2	P	R^2	P	R^2	P
UV degradation								
Lymph node$_{(p)}$	0.009	0.58	0.074	0.11	0.004	0.74	0.348	<0.001
	$y = -0.002x + 1.73$		$y = 0.001x + 1.75$		$y = -0.0009x + 1.77$		$y = 0.017x + 1.68$	
Colon	0.036	0.33	0.069	0.13	0.0002	0.94	0.292	<0.001
	$y = -0.005x + 1.53$		$y = 0.004x + 1.74$		$y = -0.0003x + 1.79$		$y = 0.013x + 1.69$	
Corpus luteum	0.212	<0.01	0.024	0.37	0.004	0.06	0.008	0.59
	$y = -0.008x + 1.72$		$y = 0.001 + 1.75$		$y = 0.004x + 1.75$		$y = 0.002x + 1.78$	
Caecum	0.174	<0.05	0.003	0.76	0.231	<0.01	0.074	0.11
	$y = -0.012x + 1.67$		$y = -0.001 + 1.76$		$y = -0.011x + 1.83$		$y = -0.005x + 1.78$	
Spleen	0.048	0.21	0.049	0.19	0.536	<0.001	0.137	<0.05
	$y = 0.011x + 1.52$		$y = 0.004 + 1.74$		$y = 0.017x + 1.72$		$y = -0.006x + 1.81$	
Abomasum	0.020	0.41	0.009	0.57	0.477	<0.001	0.094	0.07
	$y = -0.001x + 1.63$		$y = -0.001 + 1.75$		$y = -0.012x + 1.81$		$y = -0.006x + 1.79$	
WBC	n.a.	n.a.	0.744	<0.001	0.011	0.46	0.088	<0.01
	n.a.		$y = -0.065x + 1.99$		$y = -0.002x + 1.81$		$y = -0.004x + 1.82$	
Kidney cells	0.625	<0.05	0.315	0.051	0.218	0.108	0.161	0.174
	$y = -0.016x + 1.85$		$y = 0.006x + 1.69$		$y = 0.002x + 1.76$		$= -0.006x + 1.86$	
Granulosa cells	0.447	<0.01	0.228	<0.01	0.523	<0.001	0.012	0.66
	$y = 0.002x + 1.69$		$y = 0.020x + 1.39$		$y = 0.015x + 1.63$		$y = 0.003x + 1.76$	
Enzymatic degradationr								
Reticulum	0.424	<0.001	0.039	0.28	0.024	0.39	0.0003	0.93
	$y = -0.024x + 1.62$		$y = -0.002x + 1.76$		$y = -0.002x + 1.81$		$y = 0.0003x + 1.85$	
Lymph node$_{(e)}$	0.41	0.02	0.182	<0.01	0.113	<0.05	0.05	0.19
	$y = 0.001x + 1.70$		$y = 0.005x + 1.75$		$y = 0.005x + 1.72$		$y = 0.004x + 1.77$	

Impact of RNA integrity (shown as RIN $= x$) on *qPCR efficiency* (shown as $E = y$). Data are shown as linear regression, coefficient of determination (R^2) and the P-values of regression. Four different genes (18S, 28S, β-actin and IL–1β), were analyzed in 11 tissues, sorted by various type of degradation, either UV degradation ($n = 9$) or enzymatic degradation ($n = 2$)

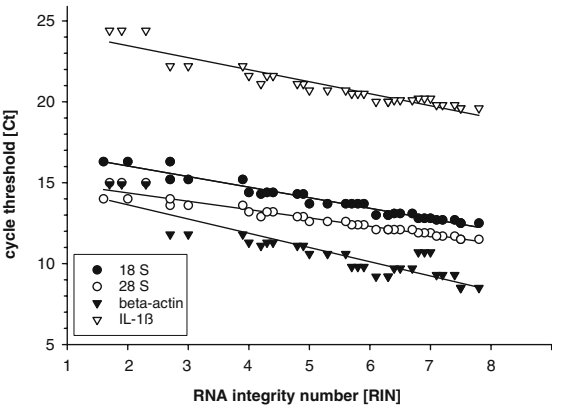

Fig. 1 Correlation between crossing point and RIN Distribution of the RT-PCR absolute expression measured on 12 RNA aliquots in triplicates ($n = 36$) from lymph node (cited as an example for all tested tissues and cell cultures) using 18S, 28S, β-actin and IL-1β. The linear regression lines are indicated and shown in detail in Table 2

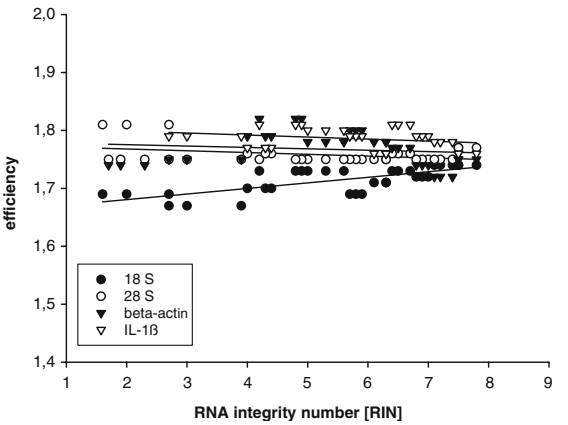

Fig. 2 Single-run PCR efficiency versus RIN Distribution of the RT-PCR efficiency measured on 12 RNA aliquots in triplicates ($n = 36$) from lymph node (cited as an example for all tested tissues and cell cultures) using 18S, 28S, β-actin and IL-1β. The linear regression lines are indicated and shown in detail in Table 3

Some tissues had higher amplification efficiencies with increasing RNA integrity, shown as positive coefficient of regression. Looking over all analyzed genes and tissues (Table 3) the RIN effect on qPCR efficiency was minor, compared to the influence on Ct (Table 2). The data demonstrates that amplification efficiency is highly dependent on the incomprehensible tissue-matrix-effect (represented by the intercept of the regression equation) and only minor affected by the RNA integrity itself (represented by the slope of linear regression). However, the coefficient of regression of the amplification efficiency data showed significance for some tissues and genes.

Effect of normalization

To test the effect of normalization by an internal reference gene, expression results of 18S, β-actin and IL-1β were normalized to the reference gene 28S rRNA. 28S rRNA was chosen as an optimal reference gene, because it showed the lowest variations during the performed RNA degradation study. 28S rRNA expression too, showed the lowest slope in the Ct and efficiency analysis (Tables 2 and 3). The relative quantification alteration in expression of the target gene relative to the reference gene was performed in two ways: first according to the delta-delta Ct method (Livak and Schmittgen 2001), and second by the single-run-specific efficiency-corrected relative expression model (Pfaffl 2001, LightCycler Relative Quantification Software, Version 1.0). For both models the RIN values were regressed versus the relative expression ratios. In the first model an optimal amplification efficiency of two ($E = 2$) was assumed. For nearly all genes and tissues we could show a significant effect on relative expression level depending on the RNA quality (Table 4). In the advanced calculation

Table 4 Correlation between RNA integrity and delta-delta Ct model

Tissue	18S rRNA		β-actin		IL–1ß	
	R^2	P	R^2	P	R^2	P
UV degradation						
Lymph node$_{(p)}$	0.764 $y = 0.071x + 0.03$	< 0.001	0.465 $y = 0.803x + 0.20$	< 0.001	0.666 $y = 0.001x + 0.01$	< 0.001
Colon	0.226 $y = 0.002x - 0.01$	< 0.001	0.412 $y = 0.161x + 0.28$	< 0.001	0.841 $y = 0.001x - 0.01$	< 0.001
Corpus luteum	0.766 $y = 0.064x - 0.16$	< 0.001	0.770 $y = 1.128x + 2.38$	< 0.001	0.768 $y = 0.001x + 0.01$	< 0.001
Caecum	0.263 $y = 0.024x + 0.05$	< 0.001	0.698 $y = 0.541x - 1.130$	< 0.001	0.214 $y = 0.003x + 0.01$	< 0.001
Spleen	0.307 $y = 0.068x - 0.20$	< 0.001	0.564 $y = 0.241x + 0.34$	< 0.001	0.706 $y = 0.003x + 0.01$	< 0.001
Abomasum	0.388 $y = 0.029x - 0.01$	< 0.001	0.624 $y = 0.048x + 0.057$	< 0.001	0.0005 $y = -0.0002x + 0.000$	< 0.001
WBC	n.a. n.a.	n.a.	0.759 $y = 560.16x - 1580$	< 0.001	0.777 $y = 3.795x - 9.47$	< 0.001
Kidney cells	0.741 $y = 0.0002x + 0.000$	< 0.001	0.640 $y = 1.205x - 0.84$	< 0.001	0.718 $y = 0.0002x - 0.000$	< 0.001
Granulosa cells	0.707 $y = 0.035x - 0.13$	< 0.001	0.383 $y = 1.212x - 0.09$	< 0.001	0.399 $y = 0.001x - 0.000$	< 0.001
Enzymatic degradation						
Reticulum	0.026 $y = -0.005x + 0.09$	0.25	0.703 $y = 0.566x + 0.71$	< 0.001	0.198 $y = -0.001x + 0.01$	< 0.001
Lymph node$_{(e)}$	0.398 $y = 0.056x + 0.13$	< 0.001	0.327 $y = 0.394x + 0.35$	< 0.001	0.399 $y = 0.001x + 0.01$	< 0.001

Impact of RNA integrity (shown as RIN $= x$) on relative expression ratio, calculated according the equation delta-delta Ct model 2^Ct(RG)-Ct(TG) (shown as y value). Data are shown as linear regression ($n = 36$), coefficient of determination (R^2) and the P-values of regression. Three efficiency-corrected relative expression ratios are shown (18S, β-actin and IL-1β) and 28S rRNA was used as reference gene. Expression ratios were analyzed in 11 tissues, sorted by various type of degradation, either UV degradation ($n = 9$) or enzymatic degradation ($n = 2$)

model, according to the efficiency corrected model regressed data are shown in Table 5. Significant, positive correlations (mostly $P < 0.001$) and regression coefficients between the RNA integrity and the relative expression of the quantified target genes were determined in the sample-specific efficiency-corrected relative quantification (shown as $E_{(RG)}$^$Ct_{(RG)}$/$E_{(TG)}$^$Ct_{(TG)}$ values). To proof the feasibility of this model a intact RNA at RIN value 8 ($E_{(RG)}$^$Ct_{(RG)}$/$E_{(TG)}$^$Ct_{(TG)}$ = 4.65, defined as sample) and a degraded RNA at RIN value 2 ($E_{(RG)}$^$Ct_{(RG)}$/$E_{(TG)}$^$Ct_{(TG)}$ = 1.16, defined as control) were compared for the β-actin mRNA expression (Fig. 3). The defined sample contained fourfold more β-actin molecules than the control, meaning around 75% β-actin mRNA was degraded.

Effect of length of the amplified product

To test the influence of PCR product length, a gradient with several steps of intact RNA down to degraded RNA was examined with seven primer sets, amplifying qRT-PCR products of various lengths up to 976 bp. To amplify the sequence of β-actin in different tissues and varying RNA integrity levels, seven primer sets were used. In Fig. 4 it is clearly visible for all product length that the crossing point is shifted towards lower cycle numbers using intact total RNA. The best repeatability could be attained in quadrant IV with a RIN value higher than five (even better over eight), and a qRT-PCR product length lower than 200 bp. These results could be confirmed for all analyzed tissues and genes. Quadrant I and III showed low RNA quality having RIN lower than

Table 5 Correlation between RNA integrity and efficiency-corrected relative expression ratio

Tissue	18S rRNA		β-actin		IL-1β	
	R^2	P	R^2	P	R^2	P
UV degradation						
Lymph node$_{(p)}$	0.129 $y = 0.042x + 0.45$	< 0.05	0.543 $y = 0.581x + 0.001$	< 0.001	0.311 $y = 0.0008x + 0.01$	< 0.001
Colon	0.209 $y = 0.053x + 0.000$	< 0.01	0.193 $y = 0.153x + 0.18$	< 0.01	0.157 $y = 0.002x + 0.004$	< 0.05
Corpus luteum	0.659 $y = 0.105x + 0.04$	< 0.001	0.476 $y = 0.397x + 2.30$	< 0.001	0.027 $y = 0.0003x + 0.004$	0.34
Caecum	0.289 $y = 0.164x + 0.17$	< 0.001	0.606 $y = 0.345x + 0.000$	< 0.001	0.215 $y = 0.003x + 0.008$	< 0.05
Spleen	0.603 $y = 0.155x + 0.000$	< 0.001	0.110 $y = 0.104x + 0.59$	< 0.05	0.538 $y = 0.009x + 0.01$	< 0.001
Abomasum	0.333 $y = 0.082x + 0.19$	< 0.001	0.677 $y = 0.073x + 0.02$	< 0.001	0.137 $y = 0.0001x + 0.00$	< 0.05
WBC	n.a. n.a.	n.a.	0.006 $y = 0.946x + 45.71$	0.76	0.031 $y = 0.445x + 1.06$	0.47
Kidney cells	0.642 $y = 0.0003x + 0.01$	< 0.001	0.565 $y = 0.664x + 0.001$	< 0.001	0.567 $y = 0.0001x + 0.001$	< 0.001
Granulosa cells	0.078 $y = 0.082x + 0.38$	0.22	0.432 $y = 0.566x + 0.35$	= 0.001	0.323 $y = 0.002x + 0.003$	< 0.01
Enzymatic degradation						
Reticulum	0.208 $y = 0.047x + 0.15$	< 0.01	0.613 $y = 0.288x + 0.62$	< 0.001	0.153 $y = -0.0004x + 0.01$	< 0.05
Lymph node$_{(e)}$	0.299 $y = 0.089x + 0.33$	< 0.001	0.195 $y = 0.324x + 6.25$	< 0.01	0.032 $y = 0.0004x + 0.01$	0.29

Impact of RNA integrity (shown as RIN = x) on efficiency-corrected relative expression ratio, calculated according the equation $E_{(RG)}$^$Ct_{(RG)}$/$E_{(TG)}$^$Ct_{(TG)}$ (shown as y value). Data are shown as linear regression ($n = 36$), coefficient of determination (R^2) and the P-values of regression. Three efficiency-corrected relative expression ratios are shown (18S, β-actin and IL-1β) and 28S rRNA was used as reference gene. Expression ratios were analyzed in 11 tissues, sorted by various type of degradation, either UV degradation ($n = 9$) or enzymatic degradation ($n = 2$)

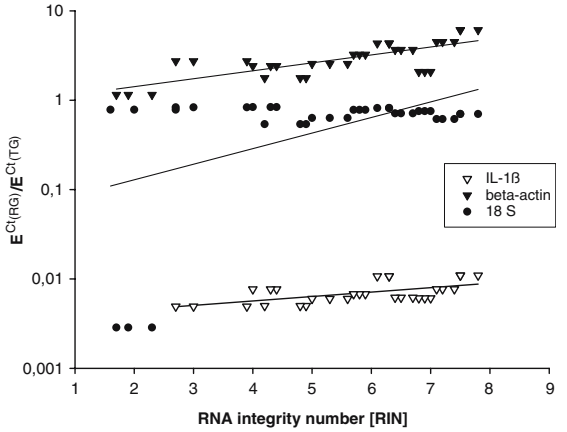

Fig. 3 Single-run efficiency-corrected relative expression ratio $E_{(RG)}{}^{\wedge Ct(RG)}/E_{(TG)}{}^{\wedge Ct(TG)}$ ratio versus RIN Distribution measured on 12 RNA aliquots in triplicates ($n = 36$) from lymph node (cited as an example for all tested tissues and cell cultures) using 28S as reference gene. The linear regression lines are indicated and shown in detail in Table 4

five and high variability in qRT-PCR results. In quadrant II high quality RNA was used, but high amplicon size resulted in late and highly variable Ct, and consequently in inefficient reaction with low PCR efficiency (no figure shown). High Ct values for 800 and 950 bp may result from inefficient amplification as from too long qRT-PCR products and the applied stringent cycle conditions.

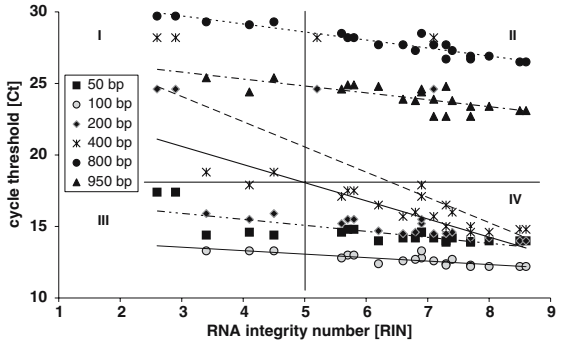

Fig. 4 Relationship between RNA integrity and length of the amplified product Integrity of 23 bovine corpus luteum RNA sample (cited as an example for all tested tissues) profiles was scored using the RIN software. Cycle threshold (Ct) values in dependence on amplicon length and RNA integrity (RIN). Comparative analysis was done using β-actin with different length of the amplified product. Graph is divided in four quadrants (I–IV)

Discussion

Intact RNA is essential for many molecular biotechnology techniques used in gene expression studies. It is universally accepted that RNA purity and integrity are of foremost importance to ensure reliability and reproducibility of qRT-PCR. Despite this valid assumption, there has been minor experimental and statistical proven data to verify this assertion. Spectrophotometer analysis of RNA in particular has been widely accepted as an important quality assurance measures for RT-PCR and microarray experiments (Baelde et al. 2001). The spectrophotometer abnortancy measurement has long been used as a criterion for assessing contamination of RNA samples throughout the development of molecular biology. The A_{260}/A_{280} and A_{260}/A_{230} ratios reflect RNA purity but are not informative regarding the integrity of the RNA (Mueller et al. 2000). A_{260}/A_{280} ratios higher than 1.8 are indicative of limited protein contaminations, whereas low A_{260}/A_{230} ratios are indicative of residual contamination by organic compounds such as phenol, sugars or alcohol, which could be highly detrimental to downstream applications (Sambrook et al. 1989). Today micro-fluidic capillary electrophoresis are more and more used for RNA quality and quantity assessments, particularly in the gene expression profiling platforms (Mueller et al. 2000; Lightfood 2002). From the shape of the electropherogram and the dominant ribosomal RNA subunits peaks, the software automatically generates the 28S/18S rRNA ratio and in newer software versions as well the RIN value (Mueller 2004; Schroeder et al. 2006). The RNA degradation is a gradual process and this is shown in a decrease of the ribosomal 28S/18S rRNA ratio and an increasing base-line signal between the two ribosomal peaks and the 5S rRNA peak. The 28S/18S ratio is calculated automatically, but it may not be used as a gold standard for assessing RNA integrity because of its high variability (CV 19–24%) (Imbeaud et al. 2005; Fleige and Pfaffl 2006; Schroeder et al. 2006). Our dataset could confirm this finding in 11 analyzed tissues showing a high 28S/18S ratio variations (CV 32 ± 18%; $n = 405$). No significant correlation

between the 28S/18S rRNA ratio and real-time RT-PCR performance could be shown. Therefore the 28S/18S rRNA ratio could not be recommended as useful indicator of RNA integrity.

Effect of RNA integrity on PCR performance

Bustin and Nolan (2004) proposed performing a RNA quality control prior to downstream quantification assays, especially if one aims to accurately quantify small expression differences (Perez-Novo et al. 2005). The examination of the RNA integrity before use in different applications enabled to compare experiments and classify the significance of final gene expression results (Imbeaud et al. 2005). Therefore, we focused the influence of degraded RNA on the performance of qRT-PCR. A biologically *''normal and integer''* transcriptome of distinct bovine tissues or cell-lines were isolated and artificially degraded factiously by enzymatic digest or with ultraviolet light UVC (Kundu et al. 2004). A gradient with several steps of intact down to degraded RNA was researched by real-time qRT-PCR expression analysis. A significant negative relationship between the RIN and Ct for all tested samples is proven ($P < 0.001$). A conclusion from Auer et al. (2003) aforesaid, that degradation does not prelude micro array analysis if comparison is done using samples of comparable RNA integrity. Imbeaud et al. (2005) and Schroeder et al. (2006) showed the direct influence of RNA integrity on the absolute gene expression results. We could confirm mentioned studies using an RNA-integrity gradient in two prominent relative quantification models.

Impact of RIN on relative quantification

Normalization by an internal reference gene reduces or even diminishes tissue derived effects on qRT-PCR (Wittwer et al. 1997). Specific errors in the mRNA quantification procedure are easily compounded by any variation in the amount of starting material between samples (Gottwald et al. 2001) and on variation in the RNA integrity (own statement). A normalization of target genes with an endogenous expressed reference standard is strictly recommended. The applied standard should not be regulated or at least be minor regulated, like in the applied study the 28S rRNA expression. Furthermore the sensitivity to RNA degradation must be pointed out, because reference genes varying in theirs sensitivity (Perez-Novo et al. 2005). An accurate and relevant normalization to some internal standard is obligatory for biologically meaningful mRNA quantification (Bustin et al. 2005). With that prospect in mind, we correlate the RIN to normalized expression level values, normalized by an internally expressed reference gene, according to the two most abundant models: the delta-delta Ct method (Livak and Schmittgen 2001) and the efficiency-corrected model (Pfaffl 2001). It is well established that small efficiency differences between target and reference gene generate false expression ratios, resulting in over- or under-estimation of the real initial mRNA amount (Pfaffl 2001).

Ct and qPCR amplification efficiencies were determined sample-specific automatically in each single qRT-PCR reaction. Therefore, a direct RNA integrity influence on qPCR efficiency could be measured directly in each qRT-PCR sample. This method of efficiency determination is very comparable to earlier described methods and based on the single sample analysis, using multiple algorithms in the *''real''* exponential phase of PCR (Tichopad et al. 2003). Minor efficiency differences were found within one analyzed tissues (represented by the slopes of the linear regression), and most stable for 28S rRNA. The analyzed tissue itself is mainly influencing the PCR amplification efficiency (represented by the intercept of regression equation). It is well known from previous publications that many unknown factors in sample and exogenous contaminants inhibit PCR (Wilson 1997). Those tissue-matrix-effects relevant in qRT-PCR can be compounds like hemoglobin, fat, glycogen, cell constituents, or DNA binding proteins. Additionally, exogenous contaminants such as glow powder, phenolic compounds from extraction or plastic ware can have an inhibiting effect on reverse transcription and/or on PCR performance. The existence of an incomprehensible tissue-matrix-effect makes is important

to determine the qPCR efficiency tissue-by-tissue and run-by-run, and correct for it according to established models (Pfaffl et al. 2002, LightCycler Relative Quantification Software, Version 1.0).

Normalization of expression data by an internal reference gene on the basis of varying RNA integrities, showed to be strong RIN dependent. Herein the single-run specific efficiency was added to the model, as shown in Eqs. 3 and 4, to result in an efficiency-corrected relative quantification model. To visualize the results, as shown after normalization, fourfold more β-actin mRNA could be found comparing highly intact to degraded RNA. Statements about importance of normalization and efficiency correction as specified above could certify herewith. Results demonstrate that innovative new quantification methods and normalization models can improve mRNA quantification.

Interrelation between RIN and length of amplified product

Furthermore, the length of the amplified product influences PCR efficiency such as primer length, annealing temperature, and secondary structure (Bustin and Nolan 2004; Perez-Novo et al. 2005). Quantitative RT-PCR involves analysis of smaller mRNA regions and is therefore more tolerant of partially degraded RNA. However, RNA integrity control is often not systematically performed prior to qRT-PCR analyses (Perez-Novo et al. 2005). An interesting question is, if there is an increasing influence on the PCR performance with both variables (RNA quality and length of amplified product). Fragmentation of long mRNA will result in a loss of the molecule for qPCR detection only if the RNA break occurs within the product sequence. This might be a rare event in only moderately degraded RNA (Schoor et al. 2003). Therefore, the sequence of β-actin was assessed in different tissues and by varying RNA integrity. The results of correlation between RIN and Ct fulfilled the expectation for all tested tissues, where Ct value is shifted to lower cycle number with increasing RIN for all product lengths. Similarly low quality RNA pointed a high variability in qRT-PCR expression results. We subdivided the graph in four quadrants, which show the best repeatability in quadrant IV with high RNA integrity (RIN > 5) and product length up to 200 bp. The length of the amplified product is a very important part for primer design. Late and highly variable Ct (quadrant II) is also be due to amplified product length over 400 bp and good RNA quality. Maximum amplicon size should not exceed 400 bp (ideally 80–150 bases). Smaller amplicon give more consistent results because PCR is more efficient and more tolerant of reaction conditions. The research into the relationship between RNA integrity and length of the amplified product onto PCR efficiency show no correlation. With regard to the efficiency of the PCR, those can affect by a number of variables like length of the amplicon, RNA secondary structure and primer quality (Bustin and Nolan 2004; Wong and Medrano 2005).

Conclusion

Our data suggest that RNA quality control prior to qRT-PCR assays is indispensable. Tissue sampling, RNA extraction and storage are very sensitive to RNA integrity and should be designed to keep RNA pure and intact. Total RNA samples of high quality (RIN > 8) can serve as an optimal template whereas for partly degraded RNA ($8 >$ RIN > 5) result in suboptimal qRT-PCR expression results. Degraded RNA interferes with PCR performance as such, expressed as Ct value, whereas PCR efficiency is minor effected by RNA integrity. PCR efficiency seems to be major affected by the tissue type and extraction procedure.

The delta-delta Ct and the efficiency corrected model are both sensitive to RNA integrity. Statements about importance of normalization could be confirmed by our investigations, consequently we commended an efficiency-corrected relative quantification strategy and normalization with an internally reference gene for every quantitative mRNA expression analyses. In view of the observed difference in gene expression

stability between intact and degraded RNA sample, we and other authors (Bustin and Nolan 2004; Auer et al. 2003) propose performing RNA quality control prior to downstream quantification assays. We can recommend a RIN value higher than five and a PCR product length up to 200 bp as a minimal requirement for a successful and reliable real-time RT-PCR quantification.

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