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Review

RNA integrity and the effect on the real-time qRT-PCR performance

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Abstract

The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data. Working with low-quality RNA may strongly compromise the experimental results of downstream applications which are often labour-intensive, time-consuming, and highly expensive. Using intact RNA is a key element for the successful application of modern molecular biological methods, like qRT-PCR or micro-array analysis. To verify RNA quality nowadays commercially available automated capillary-electrophoresis systems are available which are on the way to become the standard in RNA quality assessment. Profiles generated yield information on RNA concentration, allow a visual inspection of RNA integrity, and generate approximated ratios between the mass of ribosomal sub-units. In this review, the importance of RNA quality for the qRT-PCR was analyzed by determining the RNA quality of different bovine tissues and cell culture. Independent analysis systems are described and compared (OD measurement, NanoDrop, Bioanalyzer 2100 and Experion). Advantage and disadvantages of RNA quantity and quality assessment are shown in performed applications of various tissues and cell cultures. Further the comparison and correlation between the total RNA integrity on PCR performance as well as on PCR efficiency is described. On the basis of the derived results we can argue that qRT-PCR performance is affected by the RNA integrity and PCR efficiency in general is not affected by the RNA integrity. We can recommend a RIN higher than five as

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good total RNA quality and higher than eight as perfect total RNA for downstream application.

Keywords: RNA quality; RNA quantity; qRT-PCR; 2100 Bioanalyzer; Experion; Lab-on-chip

Contents

1. The particular importance of RNA quality

1.1. Introduction

The accuracy of gene expression evaluation is recognised to be influenced by the quantity and quality of starting RNA. Purity and integrity of RNA are critical elements for the overall success of RNA-based analyses. Starting with low quality RNA may strongly compromise the results of downstream applications which are often labour-intensive, time-consuming and highly expensive (Raeymarkers, 1993; Imbeaud et al., 2005). It is preferable to use high-quality intact RNA as a starting point in molecular biological as well as in diagnostic applications. Especially in quantitative RT-PCR, micro-arrays, ribonuclease-protection-assay, in situ hybridization, northern blot analysis, RNA mapping, in vitro translation, cDNA library construction and any kind of array applications the integrity of the used total RNA should be checked. Especially in clinical application with unique or limited tissue material, e.g. obtained after surgery, a reliable RNA quality analysis is necessary (Bustin and Nolan, 2004b). As a consequence, several steps during tissue handling have to be carefully controlled in order to preserve the quality and integrity of the RNA material. It is well known that RNA is sensitive to degradation by postmortem processes and inadequate sample handling or storage (Perez-Novo et al., 2005). Thus the competency to quickly assess RNA quality using minor amounts has become increasingly important as the following measures of mRNA transcripts have become more expensive and more comprehensive.

1.2. RNA extraction

The quality and quality of purified RNA is variable and after the extraction during long storage rather unstable (Bustin et al., 2005). Especially long mRNA fragments up to 10 kb are very sensitive to degradation. This can happen through cleavage of RNAses introduced by handling with RNA samples. The most obvious problem concerns the degradation of the RNA and this is best addressed by insisting that every RNA preparation is rigorously assessed for quality and quantity. The extraction and purification procedure of total RNA must fulfill the following criteria (Bustin and Nolan, 2004b; Pfaffl, 2005a):

- free of protein (absorbance 260 nm/280 nm);
- free of genomic DNA;
- should be undegraded (28S:18S ratio should be roughly between 1.8 and 2.0, with low amount of short fragments);
- free of enzymatic inhibitors for RT and PCR reaction, which is strongly dependent on the purification and clean-up methods;
- free of any substances which complex essential reaction co-factors, like Mg^{2+} or Mn^{2+};
- free of nucleases for extended storage;

There are a substantial quantity of problems that affect reproducibility, and hence the relevance of results. The source of RNA, sampling techniques (biopsy material, single cell sampling, laser micro-dissection) as well as RNA isolation techniques (either total RNA or poly-adenylated RNA isolation techniques) often vary significantly between processing laboratories (Bustin and Nolan, 2004b; Pfaffl, 2004). The RNA quality can be different between two extraction methods, e.g. performed by hand or by an automatic extraction system. The isolated total cellular RNA with the liquid extraction, e.g. Trizol (Roche Diagnostics, Germany) or TriFast (peqlab, Germany), has different RNA quality, whereas only the type of homogenization is changed (Fleige and Pfaffl, 2006). Due to its inherent susceptibility to ubiquitous RNases and its chemical instability, RNA is readily endangered by base- or enzyme-catalyzed hydrolysis. Researchers must take into account a variety of factors, which influence their ability to obtain high-quality RNA that is free of contamination such as RNases, proteins and genomic DNA. These factors include yield variations, processing requirements, and sample availability of different cells or tissues. The best RNA yield is obtained from tissue that has been diced into small fragments with a scalpel prior to being frozen by submerging in liquid nitrogen. The samples must be homogenized using a bead mill or a mechanical homogenizer (Bustin and Nolan, 2004b).

Further problem may arise in the case of research on human or animal tissue sampling techniques and the time dependency until the tissue is stored safely in RNase inhibitors or RNA-later (Ambion, USA). It is often very challenging to decrease this sampling time to a minimum within the framework of clinical routine procedures, or in animal experiments during a slaughtering process. The RNA quality may also be

impaired in samples stored for a long time or under sub-optimal conditions (Schoor et al., 2003).

1.3. RNA quantity and quality assessment

Conventional methods are often not sensitive enough, not specific for single-stranded RNA, and disposed to interferences from contaminants present in the sample (Imbeaud et al., 2005). The assessment of RNA integrity can do by various methods: the classical gel OD measurement, modern OD measurement via NanoDrop, old fashioned denaturating agarose gel-electrophoresis or with high innovative lab-on-chip technologies like Bioanalyzer 2100 (Agilent Technologies, USA) and Experion (Bio-Rad Laboratories, USA). Quantity and quality assessment using a UV/VIS spectrophotometer should be performed at multiple wave lengths at 240 nm (background absorption and possible contaminations), 260 nm (specific for nucleic acids), 280 nm (specific for proteins), and 320 nm (background absorption and possible contaminations). On basis of the OD 260 the quantity, and by the ratio of the optical density (OD) of OD 260/280 the quality, OD 260/240 or OD 260/320 the purity and the extraction performance can be verified. An OD 260/280 ratio greater than 1.8 is usually considered an acceptable indicator of good RNA quality (Sambrook et al., 1989; Manchester, 1996). By the presence of genomic DNA the OD 260 measurement can compromised and leading to over-estimation of the actual and real RNA concentration. Further the used buffer and high salt concentrations will interfere with the result of the optical measurement and therefore the calculated RNA concentrations might be over- or under-estimated (own unpublished results). The accuracy of the OD 260/A280 method has been questioned, with a value of 1.8 corresponding to only 40% RNA, with the remainder accounted for by protein (Bustin and Nolan, 2004b).

More modern spectrometric methods, like the NanoDrop (ND-3300, NanoDrop Technologies, USA) in combination with RNA RiboGreen dye (Molecular Probes, Invitrogen, USA) can be used as an UV/VIS spectrophotometer for ultra sensitive quantification of RNA. A major advantage of the system is the very low sample consumption of 1–2 μl, which is especially important when using precious materials like human biopsy or laser dissected samples. Since the sample is not contained in a secondary vessel, the sample directly wets the system optics, reducing the variations and contaminations resulting from changing or repositioning the cuvettes. Further the ND-3300 measure a spectra of your sample covering 400–750 nm, giving you more information about the RNA integrity and other chemical contamination or the extracted RNA (ND-3300 user manual V2.5, NanoDrop Technologies, USA).

An additional check involves gel electrophoresis with RNA either stained with SYBR Green dye (Molecular Probes) or the less sensitive ethidium bromide (Bustin and Nolan, 2004b). But the assessment of RNA integrity by inspection of the 18S and 28S ribosomal RNA bands using denaturating gel electrophoresis is a cumbersome, low-throughput method and requires significant amounts of precious RNA (Bustin and Nolan, 2004a). Using the RiboGreen (Molecular Probes) reagent, the detection as little as 1 ng RNA/ml is possible, and can be measured reproducible.

In contrast to UV absorbance measurements at 260 nm, where proteins and free ribonucleotides in the mixture interfere with accurate quantitation, the RiboGreen reagent only measures polymeric nucleic acids (Jones et al., 1998; LePecq and Paoletti, 1966; Karsten and Wollenberger, 1977).

Today high innovative lab-on-chip technologies like micro-fluidic capillary electrophoresis were used to do RNA quality and quantity assessments. Certainly, in terms of routinely analyzing large numbers of RNA preparations, it is by far the most convenient and objective way of assessing the quality of RNA. This method has become widely used, particularly in the gene expression profiling platforms (Bustin, 2002; Lightfood, 2002; Mueller et al., 2000).

The Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and the Experion (Bio-Rad Laboratories, USA) provide a framework for the standardization of RNA quality control. Therefore RNA samples are electrophoretical separated on a micro-fabricated chip and subsequently detected via laser induced fluorescence detection. It requires only a very small amount of RNA sample down to 200 pg total RNA. The use of a RNA ladder as a mass and size standard during electrophoresis allows the estimation of the RNA band sizes. Integrity of the RNA may be assessed by visualization of the 18S and 28S ribosomal RNA bands. An elevated threshold baseline and a decreased 28S:18S ratio, both are indicative of degradation (Mueller et al., 2004). The intact RNA preparation (Fig. 1) shows high 18S and 28S rRNA peaks as well as a small amount of 5S RNA. Degradation of the RNA sample

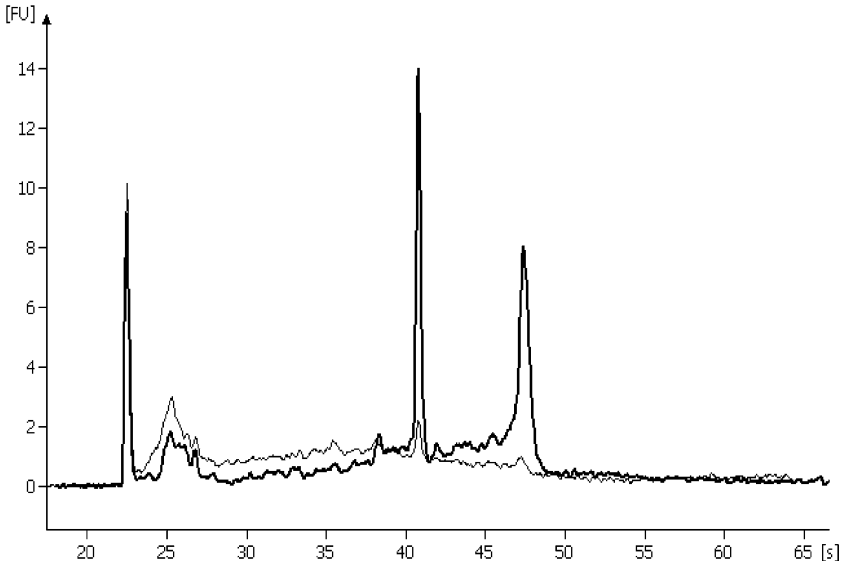

Fig. 1. Chromatograms of micro-capillary electrophoresis from RNA samples showing different degrees of degradation. A typical electropherogram of high-quality RNA (*solid black line*, RIN = 7.5) include a clearly visible 28/18S rRNA peak ratio and a small 5S RNA. Partially degraded sample (*thin grey line*; RIN = 4.5) was indicated by a shift in the electropherogram to shorter fragment sizes and produce a decrease in fluorescence signal as dye intercalation sites are destroyed.

produces a shift in the RNA size distribution toward smaller fragments and a decrease in fluorescence signal as dye intercalation sites are destroyed. The 28S/18S ratios are automatically generated by the both software applications in Experion and Bioanalyzer 2100. The RNA measurement using the lab-on-chip technology appears stable and relatively uninfluenced by contamination. RNA from tissue samples are typically classified according to the observation that the 28S rRNA peak area should be approximately twice the quantity of that of the 18S in total RNA samples for the mRNA quality to be acceptable (Sambrook and Russel, 2001). In general a 2.0 ribosomal ratio is regarded as perfect (Sambrook and Russel, 2001; Mueller et al., 2004). But in practice this value hardly is obtained. The 28S/18S ratio may reflect unspecific damage to the RNA, including sample mishandling, postmortem degradation, massive apoptosis or necrosis, but it can reflect specific regulatory processes or external factors within the living cells. As it is apparent from a review of the literature, the standard 28S/18S rRNA ratio of a 2.0 is difficult to meet, especially for RNA derived from clinical samples, and it now appears that the relationship between the rRNA electropherogram profile and mRNA integrity is up to now unclear (Monstein et al., 1995).

Furthermore, the generated ribosomal ratios are dependent on the used capillary-electrophoresis. In an intern study comparing Bioanalyzer 2100 (Agilent Technologies) with Experion (Bio-Rad) both capillary-electrophoreses systems showed differences in the generated ratio value, sensitivity, variation, and reproducibility (data not shown). Nevertheless, both platform showed more or less the same results.

However, it is unable to locate the original data for this commonly accepted premise. Based on structural differences alone, it might be expected that the in situ stability of mRNA differs from rRNA. Certainly, RNases will eventually result in the loss of both components, although there are other factors under which in situ rRNA will be completely degraded but mRNA remains intact (Mayne et al., 1999). Santiago et al. (1986) described that the mRNA integrity correspond more closely to the 28S than to the 18S integrity. This would mean that with increased length, there is a greater statistical chance of cleavage. Contrary to this assumption, Miller et al. (2004) expected that the 18S integrity correlated better than 28S with the mRNA, as the length of 18S is more closely aligned with that of the average mRNA. From our findings we can confirm the mRNA quality is more related to the 28S rRNA, which is often much faster degraded than the 18S. In a time dependent total RNA degradation via UV light the 28S rRNA disappeared very quickly (data not shown). Therefore the 28S/18S ratio has to be assessed for every single experiment and this is regarded as inadequate for the assessment of the quality (Marx, 2004). Altogether, it appears that the total RNA with lower rRNA ratios is not necessarily of poor quality especially if no degradation products can observe in the electrophoretic trace (Imbeaud et al., 2005).

A new tool for RNA quality assessment is the RNA Integrity Number (RIN, developed by Agilent Technologies) for the lab-on-chip capillary gel-electrophoresis used in the Bioanalyzer 2100 (Mueller et al., 2004). This tool is based on a neuronal network which determines the RIN number from the shape of the curve in the

electropherogram (Fig. 1). The software and the algorithm allows the classification of total RNA on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. In this way, interpretation of an electropherogram is facilitated, comparison of samples is enabled and repeatability of experiments. The verification of the RNA integrity before use in different applications permits to compare experiments and classify the significance of results (Mueller et al., 2004; Imbeaud et al., 2005).

The dependence of the RNA integrity on various calf tissue samples, white blood cells and four cell lines was determined (Fleige and Pfaffl, 2006). As shown (Table 1) for solid tissues the average RIN is between 6 and 8. Tissues or organs with high content of connecting tissue, e.g. in the gastrointestinal tract like rumen, omasum and jejunum, underlie high RNA degradation through the sampling and extraction procedure and show great RIN variations. The reason for this variability might be the solid and tough structure of the tissues, e.g. connecting or fatty tissue, the RNase enzymatic activity and problems during tissue sampling and storage. Furthermore tissues from the gastrointestinal tract have been washed in physical saline solution to get rid of any disturbing gut substances. Thus the physiological constitution of the tissue, the time and management of tissue sampling has a bearing on the degradation level of RNA. In contrary single cells like white blood cells (WBC) or cell derived from cell lines have higher RIN. Cell sampling and RNA extraction is much faster and easier, because cells are better accessible and were not kept in any suboptimal conditions. Therefore RNA integrity based on the RIN classification is

Table 1
Average RNA integrity numbers (RIN) of various bovine tissues and cell lines analyzes with the bioanalyzer 2100 (Agilent Technologies)

Tissue	Quality metrics		
	Mean	Std. dev.	*n*
Liver	6.49	±0.86	28
Heart	6.03	±1.19	23
Spleen	7.28	±0.60	17
Lung	6.55	±0.67	22
Rumen	**4.70**	**±2.81**	23
Reticulum	5.47	±1.29	21
Omasum	6.64	±1.87	18
Abomasum	7.30	±0.86	17
Ileum	7.35	±1.53	17
Jejunum	**4.56**	**±2.13**	20
Colon	7.52	±0.62	19
Caecum	7.28	±0.86	16
Lymph node	6.93	±0.65	26
Kidney cell	8.87	±0.32	3
Corpus luteum	9.62	±0.32	5
Granulosa cell	8.38	±0.41	5
Oviduct	9.40	±0.29	5
WBC	9.36	±0.13	5

much better and lay around RIN 9. The importance of isolation technique for a good RNA quality is shown in detail in Fleige and Pfaffl (2006).

2. Integrity of RNA and its effect on real-time qRT-PCR

2.1. General aspects

For a sensitive and reliable quantitative measurement of low abundant mRNA gene expression real-time quantitative reverse-transcription polymerase-chain-reaction (qRT-PCR) reaction is the method of choice. qRT-PCR shows high sensitivity, good reproducibility and a wide quantification range (Bar et al., 2003; Wang and Brown, 1999). For successful qRT-PCR and micro-array experiments it is important to use intact RNA. It is not known how this image is influenced by sample preparation factors which such as RNA quality, cDNA synthesis and labeling efficiency. Therefore the determination of RNA quality is a critical first step in obtaining meaningful data of gene expression.

Many factors present in samples as well as exogenous contaminants have been shown to inhibit the RT as well as the PCR. Some of them derive from the extracted tissue, others stem from inefficient or messy lab management. For example, the presence of haemoglobin, fat, glycogen, cell constituents, Ca^{2+}, high genomic DNA concentration, and DNA binding proteins are important factors (Wilson, 1997; Rossen et al., 1992). Exogenous contaminants such as glove powder and phenolic compounds from the extraction process or the plastic ware can have an inhibitory effect. Also unknown tissue-specific factors can influence amplification kinetics but this affect can be ameliorated, in part, by appropriate primer selection (Wilson, 1997; Rossen et al., 1992; Tichopad et al., 2004). There nevertheless, little is known about the possibility of obtaining reasonable qRT-PCR data from RNA samples with impaired quality. Expression differences for some genes can independently confirmed by real-time qRT-PCR. Gene Expression profiles obtained from partially degraded RNA samples with still visible ribosomal bands exhibit a high degree of similarity compared to intact samples and that RNA samples of sub-optimal quality. This might therefore still lead to meaningful results if used carefully (Schoor et al., 2003).

2.2. Effect on the mRNA quantification

In view of the observed difference in gene expression stability between intact and degraded RNA samples from the same tissue and the higher gene-specific variation in degraded samples, we propose performing RNA quality control prior to downstream quantification assays (Bustin and Nolan, 2004a). Especially if one aims to accurately quantify small expression differences (Perez-Novo et al., 2005). With that prospect in mind, and with the aim of anticipating future standards by pre-normative research, it is connotatively too identified and analyzed the influence of degraded RNA on the performance on qRT-PCR. In a study from Fleige and Pfaffl (2006) the purity and integrity of RNA samples was assessed, derived from different bovine

tissues and cell lines, using the Bioanalyzer 2100 (Agilent Technologies). To test the influence of the RNA integrity (numbered according to the RIN classification), the intact transcriptome of one distinct bovine tissue was degraded factitiously by enzymatic digest or with ultraviolet light. This leads to enzymatic cutoffs or breaks in the native RNA strand resulting in fragments of different lengths. A gradient with several steps of intact RNA (RIN 8–10) down to degraded RNA (RIN = 1–3) was investigated. The effect of RIN on qRT-PCR performance was investigated by correlating the RIN values with the crossing points (CP) of the PCR runs. The expression levels of four genes were assessed, all of different abundance levels ranging from high abundant 18S and 28S rRNA, intermediate abundant β-actin, down to very low expressed IL-1β mRNA samples. The importance of using high-quality RNA is demonstrated by the results shown in Fig. 2. A high-quality RNA (high RIN) determined a lower CP than by a less-quality (lower RIN). High significant relation between RIN and CP ($p < 0.01$ for the trend) could be shown for all examined genes ($n = 4$) and tissues ($n = 14$). With increasing RNA quality the variability of the qRT-PCR result was decreased (Huch et al., 2005).

It is well known, that normalization by an internal reference gene reduce or even diminish tissue derived effects on qRT-PCR (Wittwer et al., 1997). 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. So called relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and gives a result relative to the levels of an internal control RNA (Pfaffl, 2001). For many

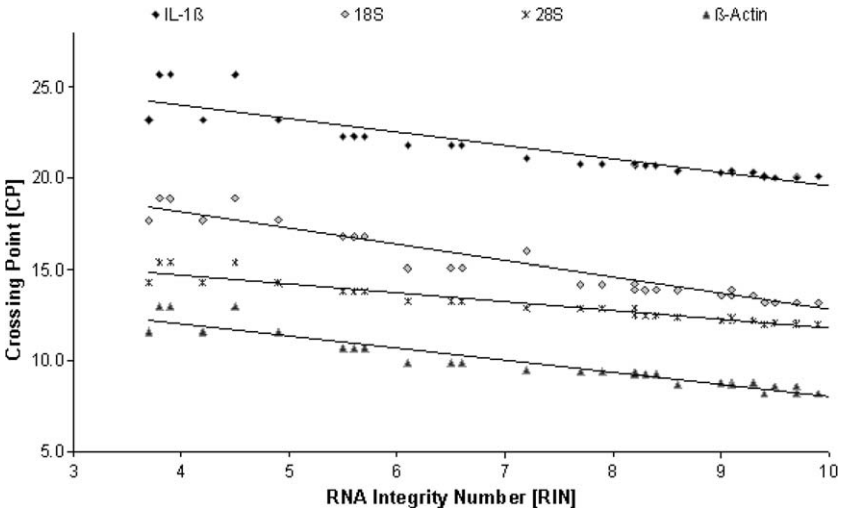

Fig. 2. Influence of RNA integrity (RIN) on the Crossing Point (CP): Amplification curves from three HKG (18S, 28S, β-Actin) and IL-1β with different quality of employed RNA from corpus luteum. An increase of RNA degradation correlates significantly to the amplified product, such that the CP is decrease with increasing RNA integrity number (RIN). Quantitative analyses use the threshold cycle number (Ct), at which the signal is detected above the background and is in the exponential phase.

experiments this method is most adequate for investigating physiological changes in gene expression levels. It is based on the expression levels of a target gene versus an internal reference gene, often non-regulated housekeeping gene are prominent candidates. To get rid of the RIN dependency the CP data were normalized by an internally expressed reference gene (Fleige and Pfaffl, 2006), according to the ΔCP method described earlier (Livak and Schmittgen, 2001). The normalized results (Fig. 3), expressed as RIN compared to ΔCP values showed minor influence of RNA quality on the expression results, and the significant effects could be reduced to a minimum.

Sometimes, even intact RNA does not guarantee good results because RNA sample may contain inhibitors that can reduce reaction efficiency (Bustin and Nolan, 2004a; Wong and Medrano, 2005). These factors include length of the amplicon, secondary structure and primer quality. 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. Therefore PCR efficiency has a major impact on the fluorescence history and the accuracy of the calculated expression result and in critically influenced by PCR reaction components. Efficiency evaluation is an essential marker in real-time gene quantification procedure (Tichopad et al., 2003, 2004). The effect of RIN on PCR efficiency was investigated similarly to the above mentioned tissues and various RNA qualities. The efficiency of all investigated genes was not affected by the RNA quality, independent of gene or tissue. A causally determined correlation between the RIN and the CP is shown in Fig. 4 (Fleige and Pfaffl, 2006).

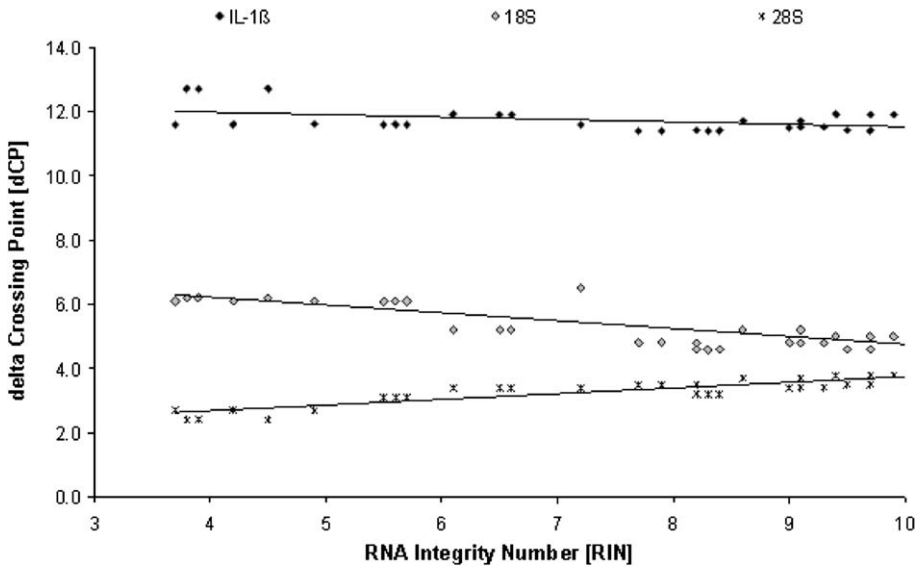

Fig. 3. Influence of RNA integrity (RIN) on the delta CP. The results (CP) from Fig. 2 are normalized with β-Actin. The significant effect of RNA integrity could reduce to a minimum.

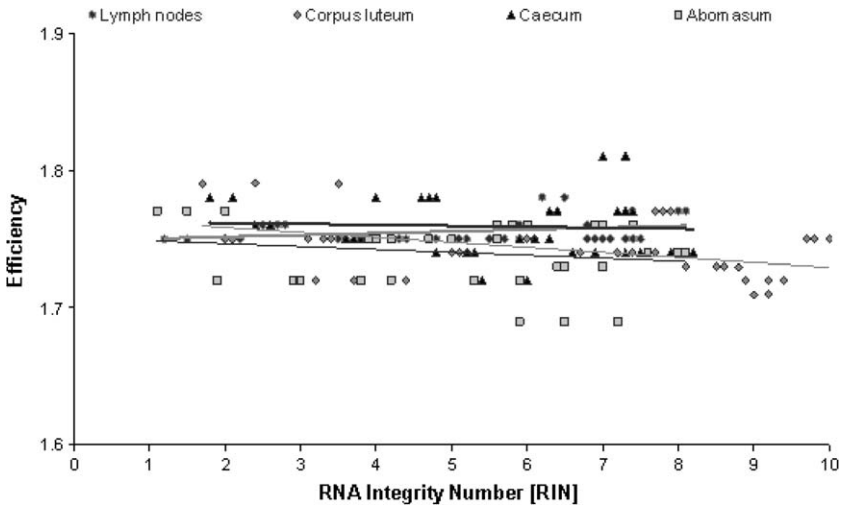

Fig. 4. Influence of RNA integrity (RIN) on PCR Efficiency: The Efficiency was generated by Rotor-Gene 3000 software (Corbett-Research). Only four tissues (lymphnode, corpus luteum, caecum, abomasums) were graph, additional results show the same trend.

2.3. Association between RNA quality and length of the amplified product

The PCR efficiency is also influenced by various factors, among other things by the annealing temperature, the primer length or by the length of the amplified product. And because of exponential amplification of the initial information, any extant error is amplified, too (Tichopad et al., 2002). The new question is, if the PCR efficiency during real-time qRT-PCR is influenced by the RNA quality or not? Therefore again, a gradient with several steps of intact RNA down to degraded RNA were examined with different primer sets, amplifying qRT-PCR products of various lengths. Primer sets for varying lengths of product (50–950 bases) were used to amplify the sequence of β-actin in different tissues and RNA integrity levels. The correlation between RNA integrity and CP were examined. The results of the correlation between the RIN and CP fulfilled the expectations. It is clearly visible that the crossing point is shifted towards lower cycle numbers using intact total RNA or higher RIN. With increasing length of the amplified product, the importance of RNA quality rises. Regarding the results concerning the correlation between the RIN and the CP values, there were some differences in the tested tissues. In some tissues a correlation between the RIN and the crossing point was visible for shorter products and in WBC and corpus luteum this correlation was visible as well for longer products.

In general we can point out, that amplification of long product over 400 bp is strongly dependent on a good RNA quality, which should show at least a RIN of 5. Shorter qRT-PCR products, mostly used with the length of 70–250 bp, are more or less "*independent*" of the RNA quality. Viewing the correlation between the RIN

and the efficiency of PCR, it is noticeable that the efficiency does not vary within one amplicon length, despite some exceptions. No correlation between the RIN and PCR efficiency (ranging between 1.6 and 1.7) was given (Pfaffl, 2005b).

Other studies showed as well an inhibitory effect of poor RNA quality on real-time PCR results. Degraded or impure RNA can limit the efficiency of the RT reaction and reduce yield. RNA should either be prepared from fresh tissue, or from tissue treated with an RNA stabilization solution such as RNA later (Labourier, 2003, 2004). The importance of using full length RNA for reverse transcription depends on the application. As a result, some degradation of the RNA can be tolerated. If it is not possible to use completely intact RNA, a design of primers to anneal an internal region of the gene of interest is useful. Note that for truly quantitative RT-PCR, partially degraded RNA may not give an accurate representation of gene expression (Wang, 2005).

3. Conclusion

In conclusion, while all efforts should be made to obtain high-quality RNA samples that reflect the natural state most reliably, moderately degraded samples with a degradation signature may still lead to a reasonable qRT-PCR expression profile. The normalized expression differences measured with the real-time RT-qPCR are similar to those obtained from high-quality samples. Only the non-normalized values show a correlation between RNA integrity and CP. This findings show the importance of the normalization. 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. Furthermore, RNA samples of optimal quality can serve as a template for all product lengths whereas for degraded RNA primer pairs for shorter amplicon are more suitable. To be on the safe side with primer pairs it would be helpful to prove the RNA quality before starting the run.

Up to now it is still questionable if we can use the 28S/18S ratio or the RIN, both based on the quantity and quality check of the ribosomal sub-units, to make a definite statement on the mRNA quality which is our target in qRT-PCR. We are looking forward for sensitive methods, comparable to an intelligent algorithm, which prove the real mRNA integrity to have a reliable answer on mRNA quantity and quality.

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