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mRNA and microRNA quality control for RT-qPCR analysis

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ABSTRACT

The importance of high quality sample material, i.e. non-degraded or fragmented RNA, for classical gene expression profiling is well documented. Hence, the analysis of RNA quality is a valuable tool in the preparation of methods like RT-qPCR and microarray analysis. For verification of RNA integrity, today the use of automated capillary electrophoresis is state of the art. Following the recently published MIQE guidelines, these pre-PCR evaluations have to be clearly documented in scientific publication to increase experimental transparency.

RNA quality control may also be integrated in the routine analysis of new applications like the investigation of microRNA (miRNA) expression, as there is little known yet about factors compromising the miRNA analysis. Agilent Technologies is offering a new lab-on-chip application for the 2100 Bioanalyzer making it possible to quantify miRNA in absolute amounts [pg] and as a percentage of small RNA [%]. Recent results showed that this analysis method is strongly influenced by total RNA integrity. Ongoing RNA degradation is accompanied by the formation of small RNA fragments leading to an overestimation of miRNA amount on the chip. Total RNA integrity is known to affect the performance of RT-qPCR as well as the quantitative results in mRNA expression profiling. The actual study identified a comparable effect for miRNA gene expression profiling. Using a suitable normalization method could partly reduce the impairing effect of total RNA integrity.

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1. Introduction

The expression level of RNAs serves as a good indicator of the physiological status of a cell or tissue. Various studies showed a distinct influence of total RNA integrity on the performance of gene expression profiling using RT-qPCR or microarrays [1–3]. RNAs are very sensitive molecules and the ubiquitous occurrence of nucleases poses a constant risk of RNA degradation. For this reason cautious handling in every single pre-PCR step of the gene expression analysis (e.g. sampling, storage and extraction) is important as only experiments conducted with high quality starting material provide reliable results. The recently published guidelines for "minimum information for publication of quantitative real-time PCR experiments" (MIQE guidelines) demand a higher transparency of the pre-PCR steps like the documentation of sample quality [4]. These guidelines are supposed to give recommendations for authors, which details are necessary to be declared in a publication. This should guarantee to get a standardized paperwork for gene expression experiments to help the reader to evaluate and reproduce published results, to promote consistency between laboratories, and to increase experimental transparency.

1.1. Total RNA quality control

RNA quality control arose the interest in gene expression analysis as it was shown to strongly influence the performance and quantitative data of RT-qPCR, which is the method of choice to study gene regulation. The term RNA quality is defined as the composition of RNA purity and RNA integrity.

1.1.1. RNA purity

RNA purity can be measured photometrically using the NanoDrop (peqLab Biotechnologie GmbH, Erlangen, Germany), the NanoVue (GE Healthcare, Munich, Germany) or other sensitive spectrophotometers e.g. the NanoPhotometer (Implen, Munich, Germany), which is an optimal solution for application of very small volumes. The optical density (OD) is measured at different wave lengths: 230 nm (absorption of contaminants & background absorption), 260 nm (absorption maxima of nucleic acids), 280 nm (absorption maxima of proteins), and 320 nm (absorption of contaminants & background absorption). The $OD_{260/280}$ ratio is used as indicator for RNA purity. A ratio higher than 1.8 is assumed as suitable for gene expression measurements [5,6]. The $OD_{260/230}$ and the $OD_{260/320}$ should be maximized as these represent the degree of background absorption and contaminants.

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Classical quality control of nucleic acids uses high resolution 4% agarose gel electrophoresis to separate the different fractions (5S, 18S, 28S) of ribosomal RNA (rRNA) subunits. For RNA of good quality a 28S/18S ratio of 2.0 is assumed. The subjective interpretation of these agarose gel images strongly depends on the experience and examination of the individual researcher and can hardly be compared between different users and laboratories.

1.1.2. Total RNA integrity control

Today, lab-on-chip technology for automated capillary electrophoresis is state of the art and is recommended for standardized RNA integrity control. Different lab-on-chip instruments are commercially available like the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and the Experion (Bio-Rad Laboratories, Munich, Germany). Both devices are sensitive, highly reproducible and suitable for a reliable quality control of RNAs [19]. For visualization and better interpretation, an electropherogram and a virtual gel image are generated. The 28S/18S ratio is calculated by assessing the peaks recorded in the electropherogram and the bands occurring on the gel-like image. Additionally, to simplify the assessment of RNA integrity the instrument software calculates a numerical value: RNA integrity number (RIN) on the 2100 Bioanalyzer and RNA quality index (RQI) on the Experion. A RQI/RIN of 1 represents almost fragmented and degraded RNA and a RQI/RIN of 10 represents intact and non-fragmented RNA [7].

1.2. Quality control in miRNA analysis

MicroRNAs (miRNAs) are small RNAs with a length of approximately 22 nucleotides, those are thought to be involved in the regulation of many physiological processes like growth and development. These molecules were already described in 1993 [8], the name "miRNAs" was primary alluded in 2001, and the analytical interest in valid miRNAs quantification arose over the past years. Concerning functional studies, especially the investigation of miRNA expression profiles is of great interest, because miRNAs are implicated in the genesis of different cancer types and therefore could be used as clinical markers in diagnosis [9–11]. As miRNAs belong to the group of nucleic acids, they are examined with the same technologies as long RNAs like mRNAs. Problems start with the quantification and quality control of miRNAs, as classical photometrical methods for measuring the concentration of nucleic acids do not allow discriminating between different fractions of RNAs. For quantitative expression profiling of mRNAs, RT-qPCR has become the gold standard. Concerning mRNA, factors influencing RT-qPCR like inhibitors or RNA quality are well investigated and the immane influence of RNA integrity on the performance of RT-qPCR and quantitative results is stated [1,2,12]. The evaluation of RNA integrity should also be integrated as a routine step in pre-PCR for expression profiling of miRNAs, as little is known about the accessibility of miRNA to degradation and the influence of total RNA integrity as a factor possibly compromising the expression profiling of miRNAs [13]. Agilent Technologies offers a new small RNA tool on the 2100 Bioanalyzer making it possible to analyze small RNA (<200 nt) with the lab-on-chip technology. Within this small RNA fraction, fragments with a size of 15–40 nt are defined as miRNA (Fig. 1A). The concentration of miRNA is calculated as absolute amount [pg] and as a percentage of small RNA [%]. By now, this chip offers one of the few possibilities to quantify miRNA.

1.3. Aim of the current study

A study was conducted to investigate the influence of total RNA quality on mRNA and miRNA quantification with the small RNA Assay on the Bioanalyzer and the miRNA expression measured using RT-qPCR. Also, an adequate normalization method for miRNA expression data should be validated, as normalization is an essential step in RT-qPCR analysis to avoid technical variations and to prove that the evaluated miRNA expression differences are of biological kind.

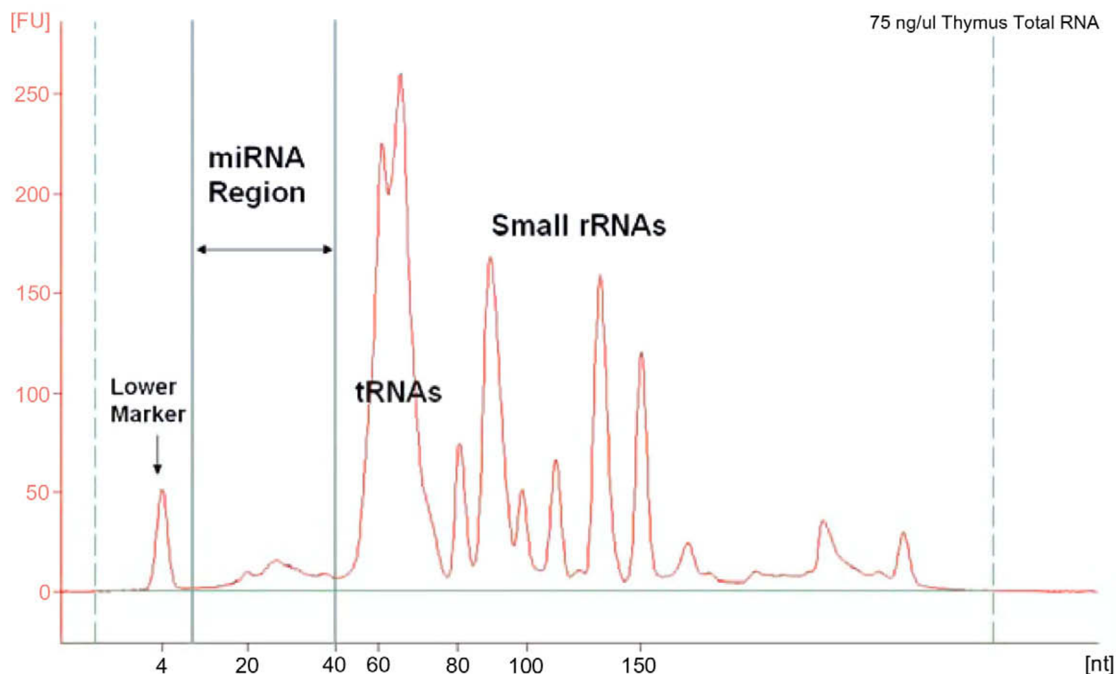

Fig. 1A. Image of a typical electropherogram for small RNA analysis performed with the Small RNA Assay on the 2100 Bioanalyzer (Agilent Technologies) (http://www.chem.agilent.com/Library/technicaloverviews/Public/5989-7002EN.pdf).

2. Description of methods

2.1. RNA extraction

Total RNA has been extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. Extractions were done from different bovine tissues [liver, muscle, white blood cells (WBC)] in six replicates per tissue ($n = 6$).

2.2. RNA degradation

For artificial RNA degradation, the six replicates of each tissue were pooled and the pool divided into two equal portions. One portion was degraded by exposure to UV light for 90 min to create a fragmented and degraded RNA fraction. The second portion remained untreated and served as intact and non-degraded RNA fraction. To create a linear gradient between intact RNA and degraded RNA from the identical transcriptome, the two fractions were mixed in changing ratios. A serial dilution with 11 degradation steps was created, whereby step 1 being intact RNA (consisting of 100% intact RNA, 0% fragmented RNA) going down in 10% steps with the intact RNA to step 11 being the most degraded RNA (consisting of 0% intact RNA; 100% fragmented RNA). This was done for all three tissues separately.

2.3. RNA quantification and RNA integrity control

Total RNA concentration has been quantified with the NanoDrop ND-1000 (peqLab Biotechnologie GmbH) by measuring the extinction at 260 nm. Additionally, the $OD_{260/230}$ and the $OD_{260/280}$ ratio showing RNA purity were examined. Quality control has been done with the 2100 Bioanalyzer using "Eukaryote total RNA Nano Assay" (Agilent Technologies). The RNA integrity number (RIN) served as RNA integrity parameter. Quantification and quality assessment of small RNA including the miRNA fraction were undertaken with the "Small RNA Assay" (Agilent Technologies). All chips were done as duplicates.

2.4. Primer design

Primer pairs for mRNA expression (Table 1) analysis were either newly designed using published bovine nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) or previously established primer sequences were used. Newly designed primers were ordered and synthesized at MWG (Ebersberg, Germany).

"miScript Primer Assays" for specific miRNA targets were synthesized and ordered at Qiagen. As no specific bovine primer assays are available, human assays were used after checking sequence homology using http://www.mirbase.org/. The investigated miRNAs showed 100% homology between human and bovine sequences, except miR-195, which showed a single nucleotide aberration at the 3′-end.

2.5. Reverse transcription

RNA samples were converted to cDNA using MMLV H^{minus} reverse transcriptase (Promega, Regensburg, Germany). Therefore, 500 ng total RNA were diluted to a final volume of 13 µL. The master mix for the reverse transcription was prepared as follows: 4 µL 5× reaction buffer (Promega), 1 µL random primers (Invitrogen, Karlsruhe, Germany), 1 µL dNTP (Fermentas, St. Leon-Rot, Germany), 1 µL MMLV H^{minus} reverse transcriptase (Promega). After adding 7 µL of the mastermix to the diluted sample the plate was inserted in the Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany) and the here stated temperature protocol was started: 21 °C, 10 min; 48 °C, 50 min; 90 °C, 2 min; 4 °C hold. After reverse transcription, all samples were diluted to a final volume of 60 µL.

miRNA samples were poly-adenlyated, elongated and reverse transcribed in a separated step using the "miScript" system (Qiagen). The following mastermix: 2 µL 5× miScript buffer, 1 µL miScript reverse transcriptase was mixed with 500 ng total RNA diluted to a volume of 7 µL to a final volume of 10 µL. The plate was inserted in the Eppendorf Gradient Cycler (Eppendorf) and the here stated temperature protocol was started: 37 °C, 60 min; 95 °C, 5 min; 4 °C hold. After reverse transcription, all samples were diluted 6-fold to a final volume of 60 µL.

2.6. Quantitative PCR

Quantitative PCR (qPCR) was performed in the Realplex ep gradient S Mastercycler (Eppendorf). For automation, pipetting was done with the epMotion 5075 LH pipetting robot (Eppendorf). For mRNA, RealMasterMix SYBR ROX (5Prime, Hamburg, Germany) was used by a standard protocol recommended by the manufacturer. The mastermix was prepared as follows: 7.5 µL 2× RealMasterMix SYBR ROX, 0.75 µL forward primer (10 pmol/µL), 0.75 µL reverse primer (10 pmol/µL), 4.0 µL RNAse free water (5Prime). 13 µL of the mastermix were filled in a well and a 2 µL volume of 12.5 ng cDNA was added for a total volume of 15 µL. The qPCR protocol was started: denaturation step (94 °C, 2 min), cycling program (95 °C, 5 s; annealing temperature according to Table 1 and 10 s; 68 °C, 20 s) and melting curve analysis.

qPCR for miRNA was done using "miScript" system (Qiagen) according to the manufacturer's recommendation. The following

Table 1
Primer sequences used for mRNA expression analysis with gene name, sequence (3′ → 5′), annealing temperature (T_M), product length and accession number.

Gene	Sequence		T_M [°C]	Product length [bp]	Accession number
Ubiquitin	for	AGA TCC AGG ATA AGG GAA GGC AT	60	198	Z18245
	rev	GCT CCA CCT CCA GGG TGA T			
p53	for	ATT TAC GCG CGG AGT ATT TG GAC	60	174	NM_174201
	rev	CCAGTGTGATGATGGTGAGGA			
LDH	for	GTG GCT TGG AAG ATA AGT GG	60	155	NM174099
	rev	ACT AGA GTC ACC ATG CTC C			
Caspase 3	for	GAC AGT GGT GCT GAG GAT GA	60	164	NM_001077840
	rev	CTG TGA GCG TGC TTT TTC AG			
ACTB	for	AAC TCC ATC ATG AAG TGT GAC	60	202	AY141970
	rev	GAT CCA CAT CTG CTG GAA GG			
IL-1β	for	TTC TCT CCA GCC AAC CTT CAT T	60	198	M37211
	rev	ATC TGC AGC TGG ATG TTT CCA T			
Histon H3	for	ACTGCTACAAAAGCCGCTC	60	233	NM_001034034
	rev	ACTTGCCTCCTGCAAAGCAC			

Table 2
RIN values and miRNA quantification data from Bioanalyzer exemplary for WBC.

Good quality RNA [%]	Mean RIN	SD RIN	Mean miRNA/small RNA ratio [%]	SD ratio [%]	Mean miRNA concentration [pg/μL]	SD miRNA concentration [pg/μL]
100	9.15	0.07	3.80	0.45	592.54	17.76
90	8.75	0.07	4.54	0.66	765.47	69.21
80	8.65	0.35	5.69	0.54	1031.72	222.49
70	8.40	0.14	5.74	1.65	1141.24	129.56
60	7.90	0.42	7.59	0.37	1455.04	380.61
50	7.75	0.07	8.59	0.31	1643.46	563.81
40	6.95	0.07	10.49	0.44	1586.82	475.17
30	6.65	0.21	11.52	0.20	1897.65	773.89
20	5.85	0.21	11.69	0.44	1805.73	339.21
10	4.25	0.35	10.57	3.78	1623.96	808.92
0	2.65	0.07	13.11	0.74	2053.08	47.11

mastermix was prepared with all necessary components for PCR: 10 μL 2× QuantiTect SYBR Green PCR mastermix, 2 μL 10× universal primer, 10× miScript primer assay, 4 μL RNAse free water. Eighteen microliters of the prepared mastermix were filled in a well and 2 μL template from miRNA reverse transcription were added for a total volume of 20 μL and the following PCR protocol was started: denaturation step (95 °C, 15 min), cycling program (95 °C, 15 s; 55 °C, 30 s; 70 °C, 30 s) and melting curve analysis.

2.7. Data analysis

Quantification and expression data were statistically processed with SigmaStat 3.0 (SPSS, Chicago, IL). The determined p-values of the statistical significance were examined using linear regression and coefficient determination (r^2). Significance of linear regression was analyzed by Students's t-test by testing the slope to be different from zero. Level of significance was set for $p < 0.05$. All data were graphically plotted using SigmaPlot 11.0 (SSPS).

Suitable reference genes (RG) for normalization of gene expression data for mRNA and miRNA were evaluated by Cq stability and variability testing using the GenNorm and Normfinder algorithm in GenEx v. 4.3.6 (MultiD Analyses AB, Gothenburg, Sweden). The geometric mean of two RG was used as reference index.

Data were normalized according to the ΔCq model [14] with the following formula:

$$\Delta Cq = Cq_{(\text{target gene})} - Cq_{(\text{reference index})}$$

3. Results

3.1. RNA degradation

RNA degradation via UV light was successful in all tissues and a quality gradient could be created (exemplary the results for WBC are shown in Table 2 and Fig. 1B). As RNA quality consists of RNA integrity and RNA purity, also the $OD_{260/280}$ ratio and the $OD_{260/230}$ ratio have been checked photometrically to ensure good RNA purity. This examination showed constant RNA purity for all degradation steps with a mean $OD_{260/280}$ of 2.033 ± 0.027 and a mean $OD_{260/230}$ of 1.925 ± 0.14 ($n = 28$) indicating that the different degradation levels are solely caused by a shift in RNA integrity.

3.2. miRNA quantification

miRNA quantification using the 2100 Bioanalyzer "Small RNA Assay" showed a clear relation between the miRNA amount and

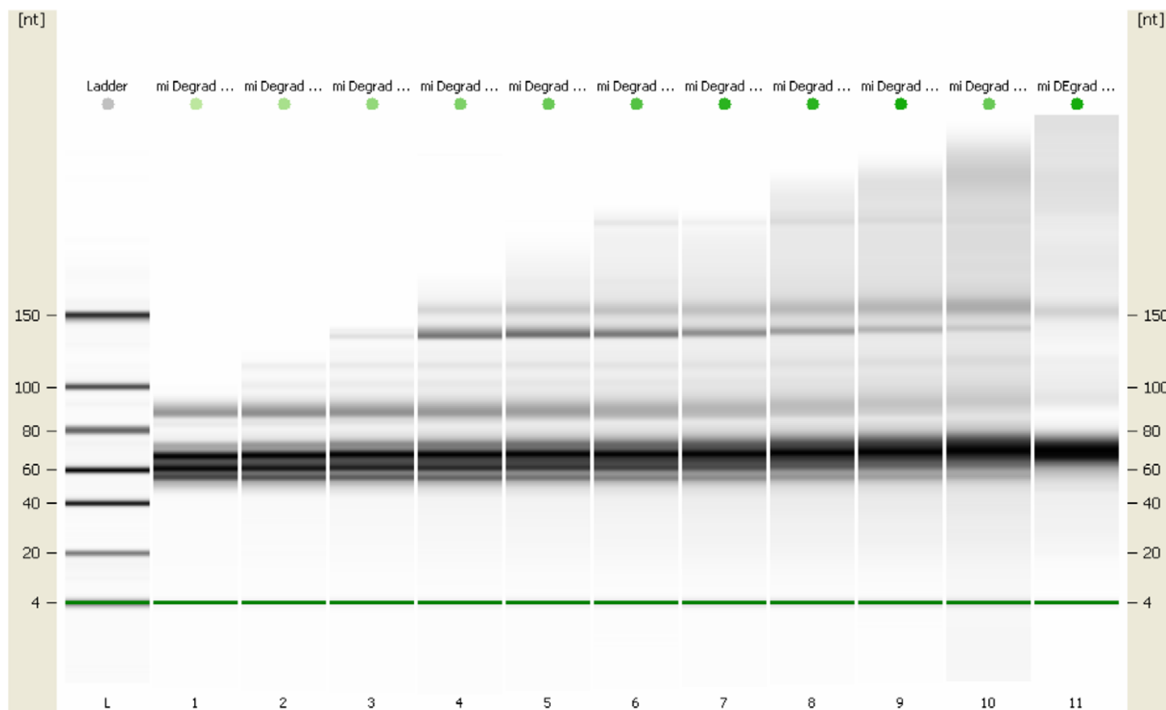

Fig. 1B. Electronic gel image on the small RNA assay from the results of UV-based degradation of WBC.

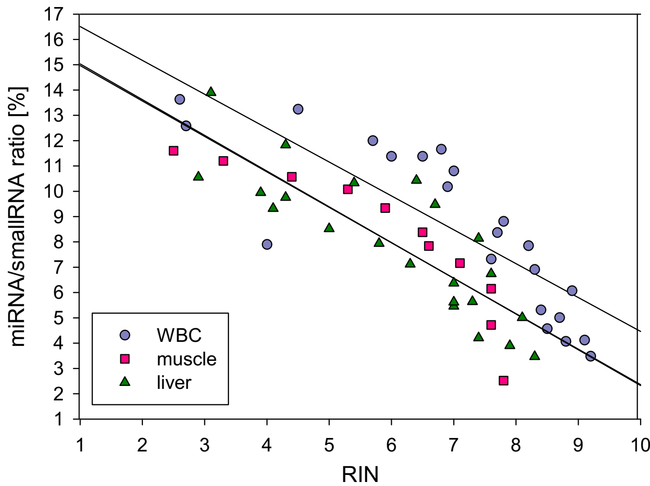

Fig. 2. Highly significant correlation between RIN and miRNA/small RNA ratio for liver, muscle and WBC with $p < 0.001$ for all subsets (regression lines for liver and muscle are overlapping).

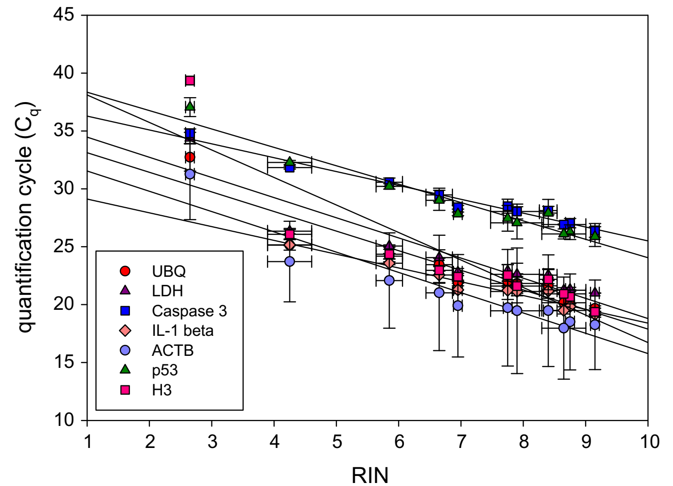

Fig. 3. mRNA expression data obtained from WBC showing a highly significant correlation between RIN and Cq value with $p < 0.001$ for all subsets.

the state of degradation for all tissues. With ongoing RNA degradation a significant rise ($p < 0.001$) in miRNA/small RNA ratio appeared for all tissues (Fig. 2). In liver and WBC, also a significant increase ($p < 0.001$) in the miRNA concentration occurred. An increase could also be shown for muscle tissue, but the rise was not statistically significant.

In WBC, the miRNA/small RNA ratio ascended from 3.80 ± 0.45% to 13.11 ± 0.74% showing a clear increase due to the formation of short RNA fragments during RNA degradation. Similar results were acquired for WBC and muscle. For muscle, an increase from 2.19 ± 0.47% to 11.08 ± 0.74% and for liver, an increase from 3.69 ± 0.30% to 12.23 ± 2.36% was shown. A comparable relationship was obtained for the correlation between RIN and miRNA concentration ($p < 0.001$). The miRNA concentration in liver rose from 999.92 ± 43.63 pg/μL to 2697.35 ± 616.21 pg/μL, in muscle from 814.49 ± 163.73 pg/μL to 2902.54 ± 306.89 pg/μL and in WBC from 592.54 ± 17.76 pg/μL to 2053.08 ± 47.11 pg/μL. An exemplary summary of the miRNA quantification results for WBC on the 2100 Bioanalyzer is stated in Table 2.

3.3. Results of gene expression analysis

Gene expression of mRNA and miRNA was measured using RT-qPCR. As expected, a distinct and highly significant, negative correlation between RIN and quantification cycle (Cq) could be shown for mRNA in all tissues and for all quantified genes ($r^2_{mean} = 0.837$; $p < 0.001$) with a mean slope of the regression line of 1.578. Exemplary, the gene expression results for WBC are demonstrated in Fig. 3. Analog results were obtained for muscle and liver tissue.

Comparable to mRNA expression, a highly significant correlation between the RIN and the Cq value ($r^2_{mean} = 0.835$; $p < 0.001$) could also be observed for miRNA expression with a mean slope of the regression line of 0.784. Expression results for WBC are shown in Fig. 4.

3.4. Normalization of expression data

mRNA expression data have been normalized using a reference index consisting of the geometric mean expression of two suitable RG, which were determined for every tissue by GenEx software. Optimal number of RG was selected using pairwise variation analysis integrated in geNorm algorithm implemented in GenEx [15]. For WBC, βActin (ACTB) and Lactatdehydrogenase (LDH); for muscle tissue, Ubiquitin (UBQ) and ACTB and for liver tissue, ACTB and Caspase 3 were used for calculation of an RG index. Normalized expression data were linearly regressed with the RIN. For almost all data no statistically significant correlation between the RIN and the ΔCq value could be shown. Significant correlations were consistent after normalization in muscle for Caspase 3 and IL-1β, in liver for UBQ and IL-1β and in WBC for Caspase 3 and Histon H3. For these genes, the correlation was no more significant, when elimination the results from degradation step 10 and 11 (10% and 0% good quality RNA), clearly showing that samples with a very low RIN are not suitable for qPCR analysis.

Two strategies were applied for normalization of miRNA expression data. Similar to the determination of RG for mRNA, suitable RG for miRNA were detected using GenEx software. For WBC, miR-122 and miR-191, for muscle and liver tissue, miR-122 and let-7a were used for calculation of an RG index [15].

As second strategy, normalization using the geometric mean expression value of all measured miRNAs was applied [18]. Examples for both normalization methods obtained in WBC are shown in Figs. 5 and 6.

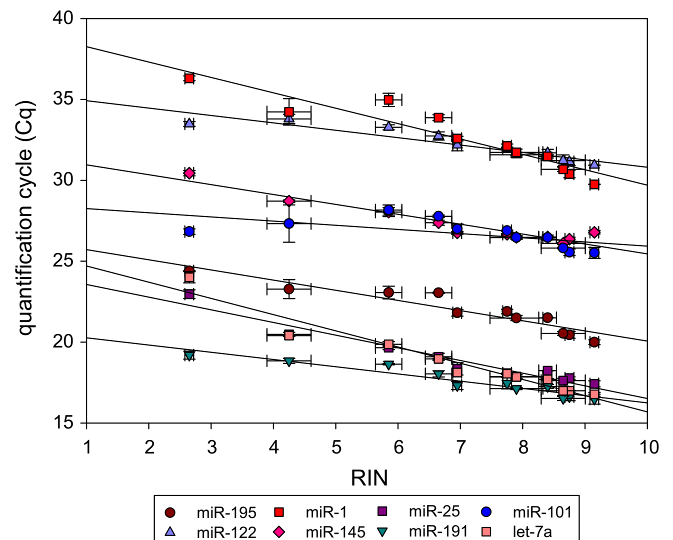

Fig. 4. miRNA expression data obtained from WBC showing a highly significant correlation between RIN and Cq value with $p < 0.001$ for all subsets.

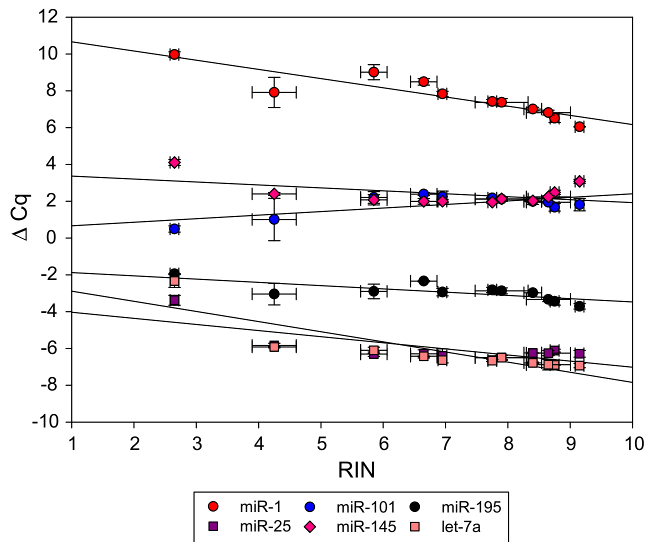

Fig. 5. miRNA expression data for WBC normalized with an RG index according to the ΔCq model.

The positive impact of normalization on biased expression data was not as effective in miRNA as in mRNA. Despite normalization, for almost all results a statistically significant correlation between the RIN and the ΔCq value occurred.

4. Discussion

It is generally accepted that sustaining of high RNA quality is one of the keys to get reliable and reproducible results from mRNA expression analysis [2,12]. This finding should be kept in mind for new applications also dealing with nucleic acids, e.g. expression profiling of miRNAs. Interestingly, samples with low total RNA quality showed the highest concentrations of miRNA. These data suggest an impairing influence of total RNA also for miRNA quantification and raised the question, if these results show a biological phenomenon or are due to a technical bias. Thus, in the actual study the influence of total RNA integrity on miRNA quantification and expression analysis was to be investigated.

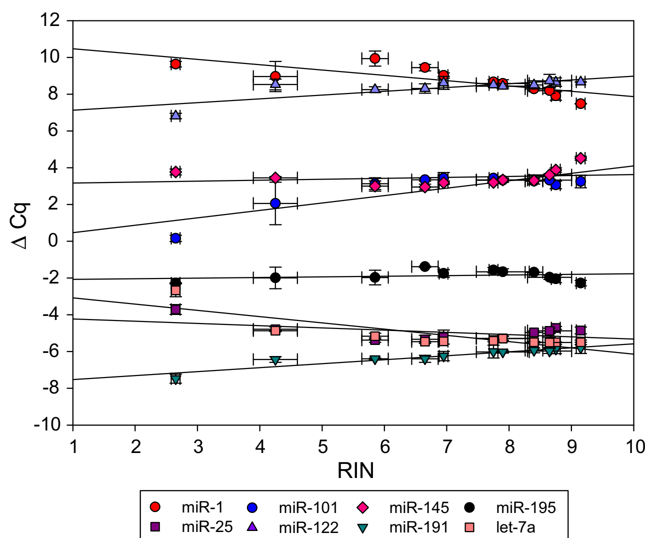

Fig. 6. miRNA expression data for WBC normalized with the mean expression value according to the ΔCq model.

As expected, a significant rise in miRNA amount occured with ongoing RNA degradation. This is caused by the formation of small RNA fragments by degradation of longer RNAs. Due to their length, these small fragments could reach the analytical range analyzed by the small RNA lab-on-chip assay and therefore lead to an overestimation of the miRNA amount. Hence, the concentration measurement using the 2100 Bioanalyzer is just reliable for RNA samples with good RNA quality. In consequence, it is recommended to consider the miRNA not as an isolated solitaire fraction, but always in combination with the mRNA and total RNA. Also, the definition of the miRNA fraction on the applied small RNA assay is questionable. Herein, all fragments with a length of 15–40 nt are defined as miRNAs, although in literature miRNAs are considered to have a length of 18–25 nt [16]. Thereby, it may be expected that even in samples with a low degree of fragmentation undefined RNA fragments are accounted as miRNAs and the miRNA amount may be overestimated even in samples with good RNA integrity. These results show clearly that it is hardly possible to quantify exactly the real amount of miRNA in a biological sample with existing methods. Coming technical innovations may give the possibility to solve these complex problems.

For gene expression analysis, mRNA as well as miRNA profiles have been investigated in this study to get a whole view over gene expression and to verify former results. A highly significant, negative correlation between the RIN and the Cq value for mRNA could be observed. Therefore, the results of earlier studies of Fleige and coworkers could be clearly confirmed [1,2]. Similar to mRNA, there is also a highly significant, negative correlation between the RIN and the Cq value for miRNA. From the lower slope of the regression line (1.578 vs. 0.784) it could be inferred that the compromising effect is less pronounced in comparison to mRNA. Due to their length miRNAs seem to be more stable and exhibit less recognition sites for nucleases. We conclude that miRNAs might be less affected by the overall degradation of total RNA compared to longer mRNAs.

To deal with factors adversely affecting the performance of RT-qPCR, it is important to apply a suitable normalization strategy in data analysis. The normalization of expression data can partly reduce the impairing influence of RNA quality on the performance of RT-qPCR [15]. For mRNA, the use of an RG index calculated as the geometric mean of multiple RG is generally accepted [15]. By now, there is no universally valid guideline for normalization of miRNA expression data. In literature, different strategies are described. Other endogenous small RNAs (nuclear and nucleolar RNAs) are used as internal control and also universal “reference miRNAs” (miR-17-5p, miR-103, miR-191) have been described [17]. miR-191 is also defined as a proper normalizer by GenEx in the current study, but just for one tissue. This finding suggests that normalizers for miRNAs are tissue and species specific just like normalizers for mRNA studies. They should not be determined generally, but tested for each experiment separately. Recently published data proved that normalization using the mean expression value or stable endogenous miRNAs used similarly to the RG index showed the best reduction of technical variances in RT-qPCR data [18]. The mean expression value is mostly used for high-throughput experiments like microarrays or qPCR array setups, whereas an RG index serves as normalizer in experiments focusing on limited number of genes like RT-qPCR. Both methods have been tested for normalization of the expression data from the current study. Looking at the genes investigated in the actual experiment, the normalization with the mean expression value gave comparable results to the normalization using RG. For the actual study, we could show that the normalization using the mean expression value may also be practicable for experiments with a limited number of genes and its application seems not just to be valid for profiling of large numbers of genes.

Obviously, the normalization of miRNA is much more sensitive to RNA quality than the normalization of mRNA. In spite of normalization, for almost all measured miRNAs and all tissues a significant correlation of the RIN and the ΔCq remained. This result was comparable for normalization with an RG index or either the mean expression value. It is evident that the linearity is just interrupted by outliers in the range of very low RNA quality (degradation step 10/11). Thus, the threshold of a RIN = 5 for gaining reliable PCR results, which was stated by Fleige et al. [1] could be confirmed for mRNA and also stated for miRNA by the actual study.

In conclusion, it could be shown that mRNA and miRNA quantification using the lab-on-chip technology is influenced by the overall total RNA integrity. Ongoing RNA degradation is accompanied by the formation of small RNA fragments leading to an overestimation of the miRNA amount. Thus, miRNA should not be considered as a solitaire fraction, but always as a part of the entire total RNA in respect to mRNA and total RNA quality. Using a small RNA assay is not an optimal method for exact quantification of the real miRNA amount. This assay is exceedingly influenced by the total RNA integrity and only reliable for samples with good RNA quality. Furthermore, the definition of the miRNA fraction in the entire small RNA fraction is questionable. The performance of RT-qPCR is impaired by decreasing RNA quality for miRNA similar to mRNA, but to a lower magnitude. The application of an appropriate normalization method can partly reduce the comprising degradation problem in RT-qPCR.

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