RNA quality control in miRNA expression analysis

Application Note

Genomics

Authors

Christiane Becker, Michael W. Pfaffl
TU München, ZIEL
Physiology Weihenstephan
Weihenstephaner Berg 3
85384 Freising, Germany

Martina Reiter, Michael W. Pfaffl
BioEPS GmbH, Lise-Meitner-Str. 30
85354 Freising, Germany

Abstract

It is generally known that total RNA quality has a distinct influence on the validity and reliability of quantitative PCR results. In addition, the recently published MIQE guidelines focus on the pre-PCR steps and state the importance of RNA quality assessment.

Various studies showed the impairing effect of ongoing RNA degradation on mRNA expression results. Therefore, the verification of RNA integrity prior to downstream applications like RT-qPCR and mircroarrays is indispensable. A fast and reliable assessment of RNA integrity can be done with the Eukaryote Total RNA Nano Assay of the Agilent 2100 Bioanalyzer.

The importance of RNA quality should also be considered in new applications such as the investigation of miRNA expression profiles. With the Agilent Small RNA Assay, Agilent is offering one of the few possibilities for selectively estimating miRNA before expression analysis. However, by now little is known about factors affecting miRNA analysis. Herein, the important impact of total RNA quality on quantification of mRNA and miRNA should be considered.

Agilent Technologies

Introduction

After extraction, RNAs are very unstable and sensitive to degradation due to the ubiquitous occurrence of nucleases. It is well known that expression profiling using microarrays or qPCR is influenced by RNA quality[1, 2]. Therefore, this hazard has to be considered during all preprocessing steps and should be minimized with cautious handling. The recently published MIQE Guidelines[3] recommend which data are essential for scientific publication. These guidelines focused on the pre-PCR steps and stated the importance of RNA quality. The term RNA quality is defined by the composition of RNA purity and RNA integrity. Therefore, the RNA purity should be tested by the estimation of the $OD_{260/280}$ and the $OD_{260/230}$ using photometrical methods. A fast and reliable analysis of total RNA integrity can be performed with the lab-on-a-chip technology of the Agilent 2100 Bioanalyzer using automated capillary electrophoresis. This is the current gold standard for standardized RNA integrity assessment.

The measurement of RNA quality should also be integrated in the routine analysis of new applications like the investigation of microRNAs (miRNAs)[4]. miRNAs are non-coding, regulatory RNA molecules with a length of approximately 22 bp. There is interest in miRNA expression profiles especially in clinical diagnostics because these could be used as biomarkers in pre-diagnosis of cancer and other diseases. miRNAs belong to the nucleic acids group, so they are quantified with the same analytical methods as long RNAs, such as mRNAs. Currently, little is known about factors compromising miRNA analysis[4, 5]. Therefore, this study determined the influence of RNA quality on quantitative expression analysis.

RNA quality and miRNA concentration measurement

Challenges start with concentration measurement of miRNAs because pho-
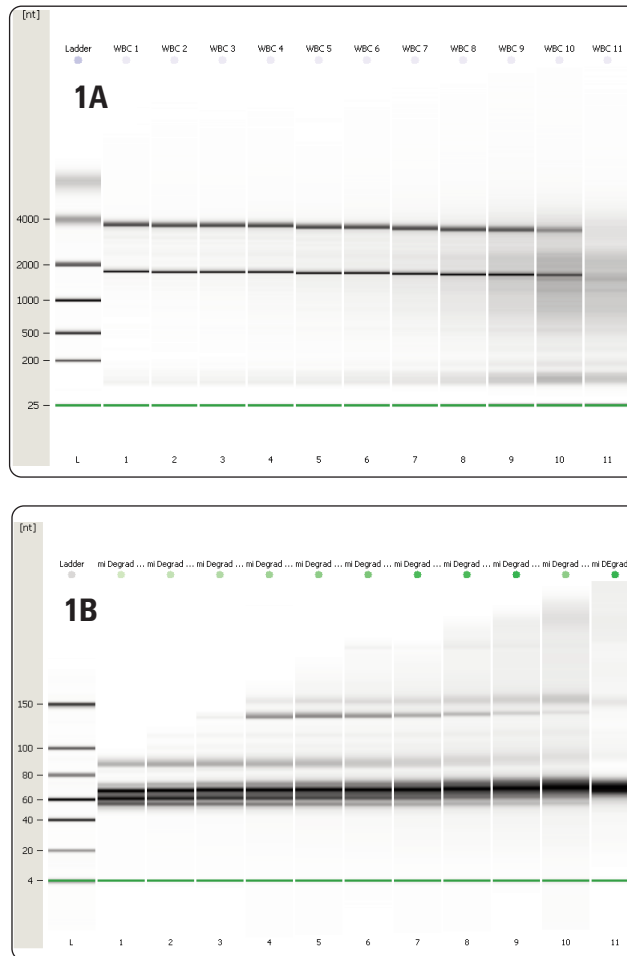
Figure 1
Results from WBC in 11 degradation steps. Gel-like image generated by the 2100 Expert Software for the (a) Eukaryote Total RNA Nano Assay and the (b) Small RNA Assay.

tometrical methods do not allow the discrimination between different RNA fractions. The Agilent Small RNA Assay is one of the few analyzers available that can measure miRNA concentration. This chip can selectively quantify miRNAs in absolute amounts [pg/µL] and as a relative percentage of small RNA [%]. In addition to these numerical results, the Small RNA Assay also provides an electropherogram and an electronic virtual gel-like image (Figures 1A and 1B) of the measured samples.

Recent results showed that RNA degradation has a significant effect on mRNA and miRNA fragmentation patterns. A negative correlation between the miRNA percentage and total RNA integrity was shown for all investigated tissues. This relationship was also demonstrated for the absolute miRNA concentration in liver and white blood cells (WBC)[4].

With ongoing total RNA degradation, the amount of smaller RNA fragments increases, whereas longer RNA molecules decrease (Figures 1A and 1B). These unspecific fragments reach the analytical range of the Small RNA Assay and thereby lead to an overestimation of the absolute miRNA amount. Consequently, the miRNA should not be considered as a single fraction, but as a part of the entire total RNA. It is recommended therefore, to combine the Small RNA Assay and the Eukaryote Total RNA Nano Assay for a complete evaluation of every total RNA sample.

RNA quality and mRNA expression analysis

Previous studies indicated a distinct effect of RNA quality on the performance of later reverse transcription quantitative PCR (RT-qPCR). Different samples showing a degradation gradient with several steps of intact RNA down to degraded RNA were analyzed using RT-qPCR. With ongoing RNA degradation a highly significant rise in the quantification cycle (Cq) value occurs for all analyzed mRNAs (Figure 2) independent of tissue type[2]. The expression data then demonstrates an apparent high expression level for intact starting material and in contrast a low expression level for degraded RNA samples. Each amplified gene was tissue specifically influenced by RNA integrity demonstrating an incomprehensible tissue-matrix effect between RNA integrity and the type of tissue and the analyzed transcript[1, 4]. Also, the influence of RNA integrity on single-run PCR efficiency was determined as minor compared to its influence on Cq[1,2]. Additionally, the effect of the length of amplified product was tested. In this analysis, samples with different levels of RNA integrity were amplified using primers producing PCR product of different lengths. Best results could be obtained for samples with RIN values higher than 5 and PCR amplicon lengths of lower than 200 bp[2].

The impairing effect of low RNA integrity on the results of gene expression analysis can partly be reduced by a valid normalization method. For mRNA, a reference gene (RG) index calculated as the geometric mean of multiple reference genes selected by Genorm[6], Normfinder[7] or GenEx software (MultiD, Gothenburg) is generally accepted[8]. This strategy has been used for mRNA expression results. For nearly all genes and all tissue, a significant reduction of the technical variance due to different RNA integrity levels could be shown after normalization[1, 4].
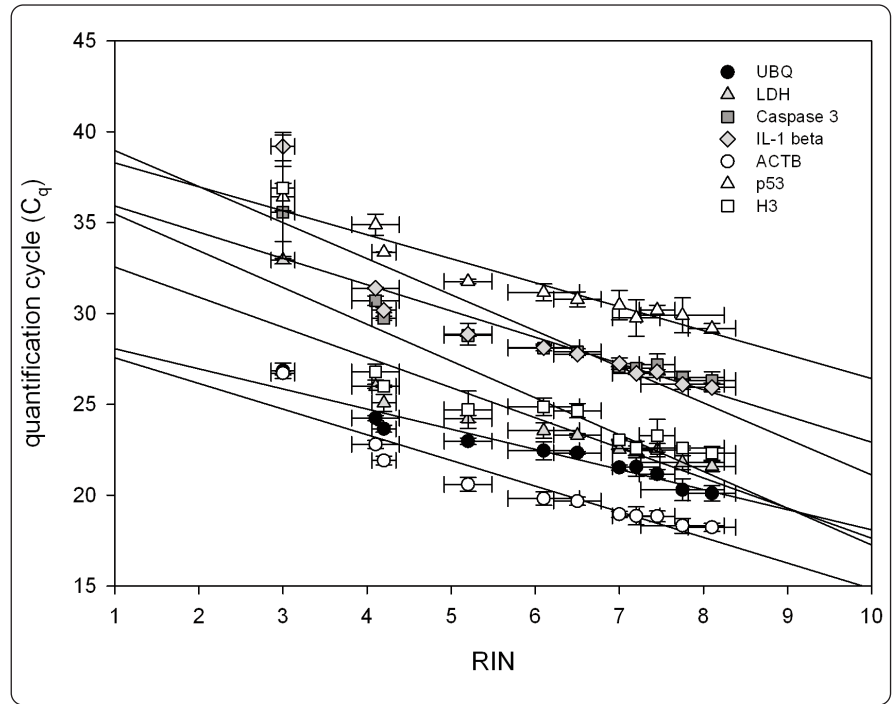
Figure 2
Highly significant, negative correlation between the RIN and the Cq in liver tissue with p<0.001 for all analyzed mRNAs[4].
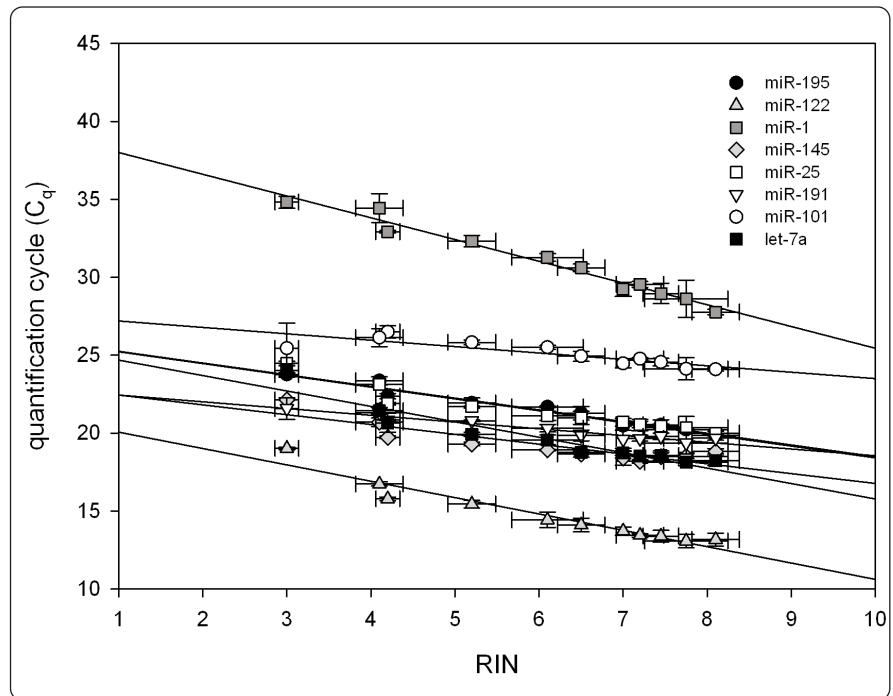
Figure 3
Highly significant, negative correlation between the RIN and the Cq in liver tissue with p<0.001 for all analyzed miRNAs[4].

Therefore, the application of a valid normalization method is indispensable in the processing of RT-qPCR expression data.

RNA quality and miRNA expression analysis

This test also illustrated a distinct influence of RNA degradation on miRNA expression analysis. Eight different miRNAs were quantified using RT-qPCR. For all tissues and in all eight genes, a highly significant, negative correlation (p<0.001) between the RIN and Cq occured. The results gained for liver tissue are shown in Figure 3. With ongoing RNA degradation the Cq increased, showing an impairing influence of the RNA integrity on the performance of the qPCR. As represented by the lower slope of the regression line, the expression analysis of miRNAs is influenced by RNA integrity to a lesser degree than mRNA expression (1.521 vs. 0.709, respectively). Due to their small length, miRNAs[4] seem to be less susceptible to degradation because they show less binding sites for nucleases. Consequently, it can be assumed that the amplification of miRNA in qPCR might be less affected by a low RNA integrity compared to the longer mRNAs. Currently, there are no precise recommendations for the normalization of miRNA expression data. Two strategies of normalization have been applied for testing. RG comparable to that of mRNA were determined for miRNA using GenEx software. Normalization using the geometric mean expression, as it is used for microarray data analysis, has been used as a second strategy. Both methods could minimize the bias of low RNA quality, but a highly significant correlation between the RIN and the Δ Cq remains despite normalization for all subsets. It is obvious that the linearity is interrupted only by samples with very low RNA quality (degradation step 10/11). There is no shared trend between the RIN and the Cq value after eliminating the results of

these degradation steps from the calculation. Therefore, the previously determined threshold level for gaining reliable PCR results[2] (RIN=5) was confirmed for mRNA and stated for miRNA[4]. Samples with a lower RIN are not recommended for expression analysis because the results may not be reliable.

Conclusion

In conclusion, it is shown that sustaining high quality RNA is critical for valid and reliable quantification results in miRNA and mRNA expression analysis. The specific concentration measurement of miRNAs is challenging and reliable for nondegraded samples with high overall RNA quality. It is recommended to consider miRNA in combination with the total RNA, as opposed to a single fraction.

The performance of RT-qPCR is impaired by decreasing RNA quality for miRNA similar to mRNA, but to a lower magnitude. The use of biological samples showing a RIN higher than 5, and primers producing short PCR product are recommended. The application of an appropriate normalization method can partly reduce the degradation problem in RT-qPCR.

References

1.
"Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR," *Biotechnol.Lett. 19, 1601-1613*, **2006**

2.
"RNA integrity and the effect on the real-time qRT-PCR performance," *Mol. Aspects Med. 2-3, 126-139*, **2006**

3.
"The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments," *Clin.Chem. 4, 611-622*, **2009**

4.
"mRNA and microRNA quality control for RT-qPCR analysis," *Methods*, **2010**

5.
"RNA degradation compromises the reliability of microRNA expression profiling," *BMC.Biotechnol. 1, 102*, **2009**

6.
"Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes," *Genome Biol. 7, RESEARCH0034*, **2002**

7.
"Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets," *Cancer Res. 15, 5245-5250*, **2004**

8.
"A novel and universal method for microRNA RT-qPCR data normalization" *Genome Biol. 6, R64*, **2009**

www.agilent.com/chem/2100-rna

Agilent Technologies