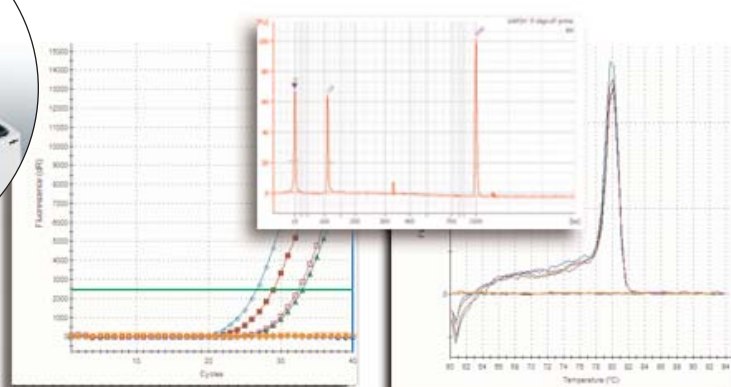


Optimizing real-time quantitative PCR experiments with the Agilent 2100 bioanalyzer

Application Note

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Abstract

This Application Note describes the benefits of the Agilent 2100 bioanalyzer in real-time quantitative PCR (QPCR) experiments. The results show that the Agilent 2100 bioanalyzer is an indispensable tool to:

- ensure experimental success by verifying RNA template quality prior to QPCR experiments
- improve the assay design and validation process by monitoring size and purity of the QPCR amplicons.

Agilent Equipment:

Agilent 2100 bioanalyzer
Stratagene Mx3005P QPCR system

Application Area:

Gene Expression



Agilent Technologies

Introduction

Real-time quantitative PCR (QPCR) is a highly sensitive method to assess gene expression changes in biological systems. As for all experimental designs, high quality starting material is essential for the success of the experiment. In microarray applications, validation of RNA template quality using the Agilent 2100 bioanalyzer with its associated RNA integrity number (RIN) algorithm is a widely accepted and commonly used technique to ensure best possible results.^{1,2} Especially RIN has been established as the standard tool for assessing RNA integrity.^{3,4}

There are various mechanisms by which RNA can be degraded either at the 5' or 3' end. Not knowing the extent of possible degradation can lead to false negative results or misinterpretation of data if the amplicon falls into a degraded region. Therefore, the degradation level of RNA samples is an important parameter to monitor when designing primers and probes for QPCR. An amplicon biased towards the mRNA ends or a longer amplicon (>100 bp) might fail to amplify if the RNA is highly degraded as in archival samples of FFPE tissue. Knowing of the template quality allows to accommodate the amplicon design and reverse transcription (RT) priming strategy, ensuring experimental success.

In addition to RNA quality control, an important part of the assay design process in QPCR is the validation of the newly designed set of primers and probes. This is usually done by checking primer binding specificity using a SYBR Green chemistry analyzing melt curve data.

The sensitivity of the assay is validated by assessing the linear dynamic range using a standard curve over a wide range of concentrations of a positive control. However, due to the properties of SYBR Green, the resolution of a melt curve is limited. This can make it challenging to identify specificity if the product melts at similar temperatures as primer dimers. In addition, the melt curve analysis can not show the size of the amplicon generated.

Here we show how the Agilent 2100 bioanalyzer can be used to assess the level of degradation of the RNA starting material. In addition, to overcome the limitations of a SYBR Green based melt curve analysis, sizes and purity of all amplicons were assessed with the DNA 1000 assay on the Agilent 2100 bioanalyzer.

Materials and methods

RNA purification

Total RNA was purified from 5×10^6 HEK cells using Stratagene's Absolutely RNA Miniprep Kit . All experimental steps were conducted according to the manufacturer's description including an on-column DNase digest. The RNA was eluted in 30 μ L pre-warmed elution buffer.

RNA quantification

1 μ L of the RNA was measured using the Agilent 2100 bioanalyzer and a NanoDrop spectrophotometer. The RNA was diluted 1:5 for quality control.

RNA quality control

1 μ L of the total RNA and of the RNA degradation reactions were analyzed with the RNA 6000 Nano

assay on the Agilent 2100 bioanalyzer to assess the level of degradation as indicated by the RIN number.

RNA degradation

4 μ g of RNA in a total volume of 12 μ L were used for each time-point of the degradation. The RNA was thermally degraded using different incubation periods at 70 °C. 3 μ L of the degradation reaction were immediately used in a subsequent RT reaction.

Reverse transcription

1 μ g of total RNA from HEK cells was reverse transcribed using Stratagene's AffinityScript QPCR cDNA Synthesis Kit at 55 °C for 30 minutes in a 20 μ L reaction. The cDNA was diluted to 100 μ L before use in real-time quantitative PCR.

Real-time quantitative PCR

All QPCR experiments were performed on Stratagene's Mx3005P QPCR system.

Primers:

GAPDH (Ensembl gene ID ENSG00000111640):
GAPDH 5' forward 5'-TCGGAGT-CAACGGATTTGGTTCG-3'
GAPDH 5' reverse 5'-TAAACCATGTAGTTGAGGTCAATGAAGG-3'
Amplicon size 118 bp

GAPDH 3' forward 5'-AAGCT-CATTTCTGCTGATGACAACG-3'
GAPDH 3' reverse 5'-TCTTC-CTCTTGCTCTTGTCTGG-3'
Amplicon size 126 bp

HPRT1 (Ensembl gene ID ENSG00000165704):
HPRT1 5' forward 5'-CGTCGT-GATTAGTGATGATGAACCAG-3'

HPRT1 5' reverse 5'-AGCAA-GACGTTTCAGTCCTGTCC-3'
Amplicon size 130 bp

HPRT1 3' forward 5'-TTCAGGGATTTGAATCAT-GTTTGTGTC-3'
HPRT1 3' reverse 5'-GCGATGT-CAATAGGACTCCAGATG-3'
Amplicon size 114 bp

YWHAZ (Ensembl gene ID
ENSG00000164924):
YWHAZ 5' forward 5'-TTGAGACG-GAGCTAAGAGATATCTGC-3'
YWHAZ 5' reverse 5'-GCAAC-CTCAGCCAAGTAACGG-3'
Amplicon size 142 bp

YWHAZ 3' forward 5'-CTTC-CTTTGCTTGCATCCCACAG-3'
YWHAZ 3' reverse 5'-GACAAT-GACAGACCATTTCAGGATAGG-3'
Amplicon size 128 bp

Primers were designed to span exon junctions where possible with the exception of YWHAZ where the 5' assay has the primers in exon 2 and 3 with a 23.6 kb intron in between and the 3' assay is located in exon 6.

Primers were used at 100 nM final concentration in the PCR.

Thermoprofile:

Taq activation: 95 °C 10 minutes
Cycling: 95 °C 20 s, 60 °C 20 s, 72 °C 20 s for 40 cycles with measurement on 60 °C

Melt curve

analysis: 95 °C 30 s, 60 °C 30 s, 95 °C 30 s with continuous measurement along the ramp from 60 °C to 95 °C.

Reactions were carried out in 20 µL using Stratagene's Brilliant II SYBR Green QPCR mastermix.

DNA amplicon analysis:

To assess size and purity of QPCR amplicons, 1 µl of the QPCR reactions was analyzed with the DNA 1000 assay on the Agilent 2100 bioanalyzer according to manufacturer's instructions.

Results and discussion

QPCR assay validation

To validate the specificity of the primer design and to determine the best possible RT priming strategy, QPCR and bioanalyzer tests were conducted for 3 genes (GAPDH,

HPRT1 and YWHAZ) by using high quality RNA (RIN = 10) as template. The purified intact RNA (figure 1) was reverse transcribed with either oligo-dT or random priming. As the results for all three genes were highly similar, QPCR and bioanalyzer validation results for GAPDH are exemplarily shown in figure 2. Oligo-dT primed cDNA gave earlier Ct values compared to random priming. In addition, all No Reverse Transcriptase (NoRT) controls were negative indicating no amplification from potential genomic DNA contamination. Analyses of the QPCR amplicons on the Agilent 2100 bioanalyzer showed a slight deviation of amplicon sizes from the expected size (sizing results for GAPDH amplicons are exemplarily shown in

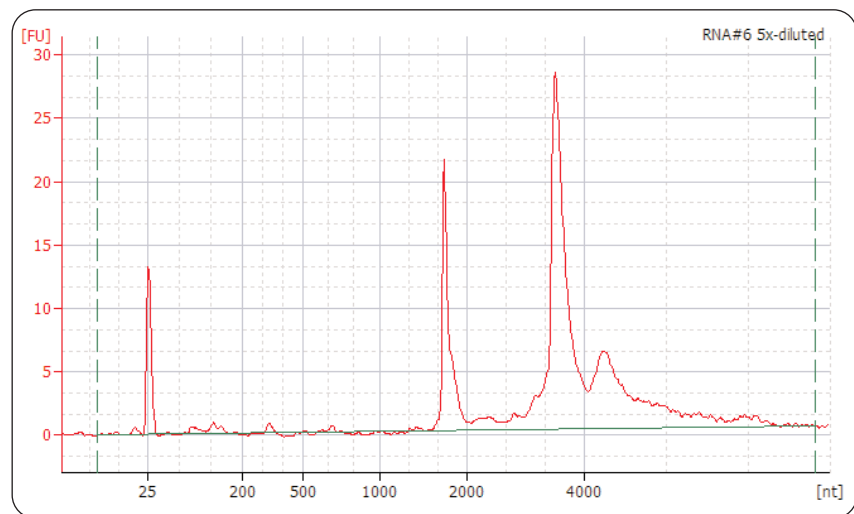


Figure 1
Quality control of purified total RNA. Total RNA was purified from 5×10^6 HEK cells and analyzed on the 2100 bioanalyzer. The value of 10 obtained for the RIN shows the very high integrity of the purified sample.

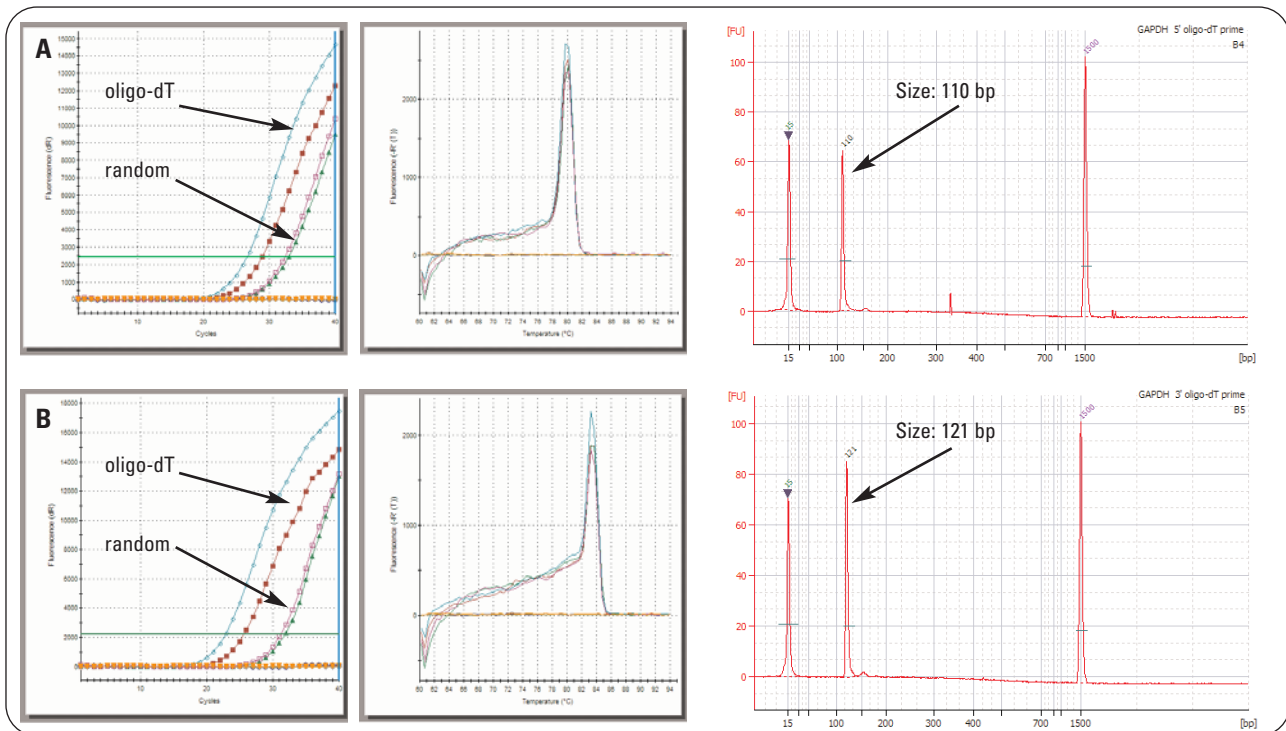


Figure 2
Assay Validation: Amplification plots, melt curve and bioanalyzer analysis of amplicons for GAPDH. A) 5'-assay, B) 3'-assay.

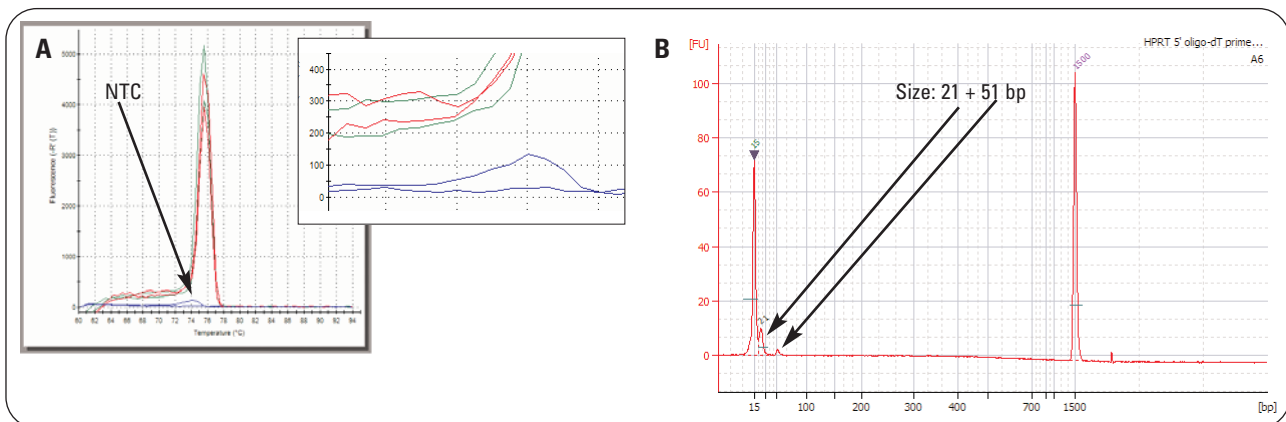


Figure 3
Validation of positive NTC results. Melt curve and bioanalyzer analysis of HPRT1 5'-amplicons. Enlargement of melt curve clearly shows a product in the NTC control with similar T_m to the positive control. Bioanalyzer analysis shows two minor peaks of 21 and 51 bp pointing to primer dimer formation.

figure 2). Reasons could be that 1 μ L of the amplification reaction was used directly for the analysis including SYBR Green and salts, which could introduce a skew. Usually, no-template-controls (NTC) assess contamination and potential

primer dimer formation. This is always recommended to perform since a small amount of contaminating template can lead to amplification. For the HPRT1 5' assay one of the NTC was positive, showing a peak in the melt curve at a similar

melting temperature compared to the positive control (figure 3A). To verify that no contamination of the well has occurred, the NTC was analyzed using the DNA 1000 assay on the Agilent 2100 bioanalyzer (figure 3B). The run showed that two minor

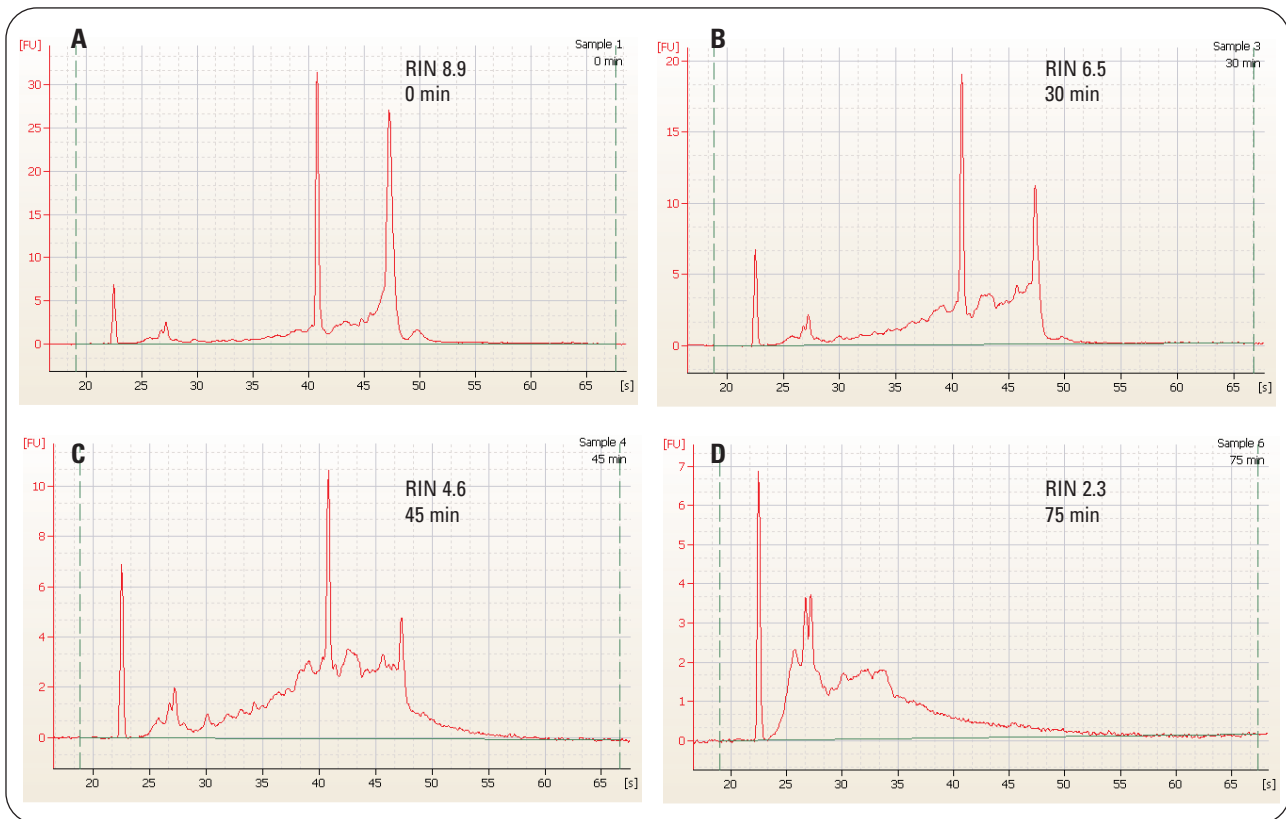


Figure 4
Analysis of thermally degraded RNA samples. Total RNA was degraded at 70 °C for varying time and analyzed on the Agilent 2100 bionalyzer. The resulting electropherograms and RIN numbers after 0, 30, 45, 75 minutes of incubation are shown respectively in A, B, C, and D.

peaks could be detected at 21 and 51 bp which are most probably related to primer and primer dimers. This highlights the high information content obtained by the Agilent 2100 bionalyzer and the poor discrimination capabilities of a SYBR Green based melt curve.

Monitoring the impact of RNA degradation on QPCR experiments

To evaluate the effect of RNA degradation in a QPCR experiment, we used a set of thermally degraded RNA and monitored the level of degradation on the Agilent 2100 bionalyzer. The degraded

RNA samples were used to detect the amount of 3 different mRNA targets (GAPDH, HPRT1 and YWHAZ). To further investigate the degradation effect on QPCR results, amplicons were designed targeting both the 5' as well as 3' end of the respective mRNAs.

The untreated high quality total RNA was subjected to thermal degradation at 70 °C for various times between 0 and 75 minutes. The equivalent volume to 1 µg of RNA was immediately used in a RT reaction. The degradation state of each sample was analyzed with the RNA Nano assay on the

Agilent 2100 bioanalyzer. The samples used in this experiment ranged from intact RNA (RIN 8.9) to highly degraded (RIN 2.3) (figure 4).

For each of the above samples QPCR was carried out to quantify the amount of 5' and 3' ends of the messengers for GAPDH, HPRT1 and YWHAZ. Relative quantities of the respective cDNA copies towards intact RNA (RIN 8.9) were calculated. A significant difference in the relative quantities is observed for all samples with RIN 4.6 and below. The most dramatic changes occur below a RIN of 4.6.

In addition, for highly degraded RNA (RIN 2.3) the extent of degradation differs for 5' and 3' end and seems to be gene specific (figure 5). The data shows clearly that under the conditions used there is a differential degradation of 5' and 3' end of mRNA resulting in a faster loss of 5' vs 3' end both for GAPDH and HPRT1. YWHAZ showed a slightly different behavior in having a preferential loss of the 3' end at highly degraded stages.

The presented data indicates that the integrity of RNA templates can influence the outcome of a QPCR experiment. Furthermore, depending on the position of the amplicon major differences of the calculated fold-change in a comparative quantification can occur.

Conclusion

In QPCR assay validation it is essential to have a more accurate validation of specificity than provided by a simple melt curve analysis. This is especially important if negative controls show amplification products with highly similar melting temperatures. Analyses of the QPCR validation amplicons on the Agilent 2100 bioanalyzer allowed discrimination of primer dimers from target amplicons which could not be achieved by standard melt point analysis

Due to the differential degradation of the 5' and 3' end of mRNA, the position of the amplicon can have a strong influence on the

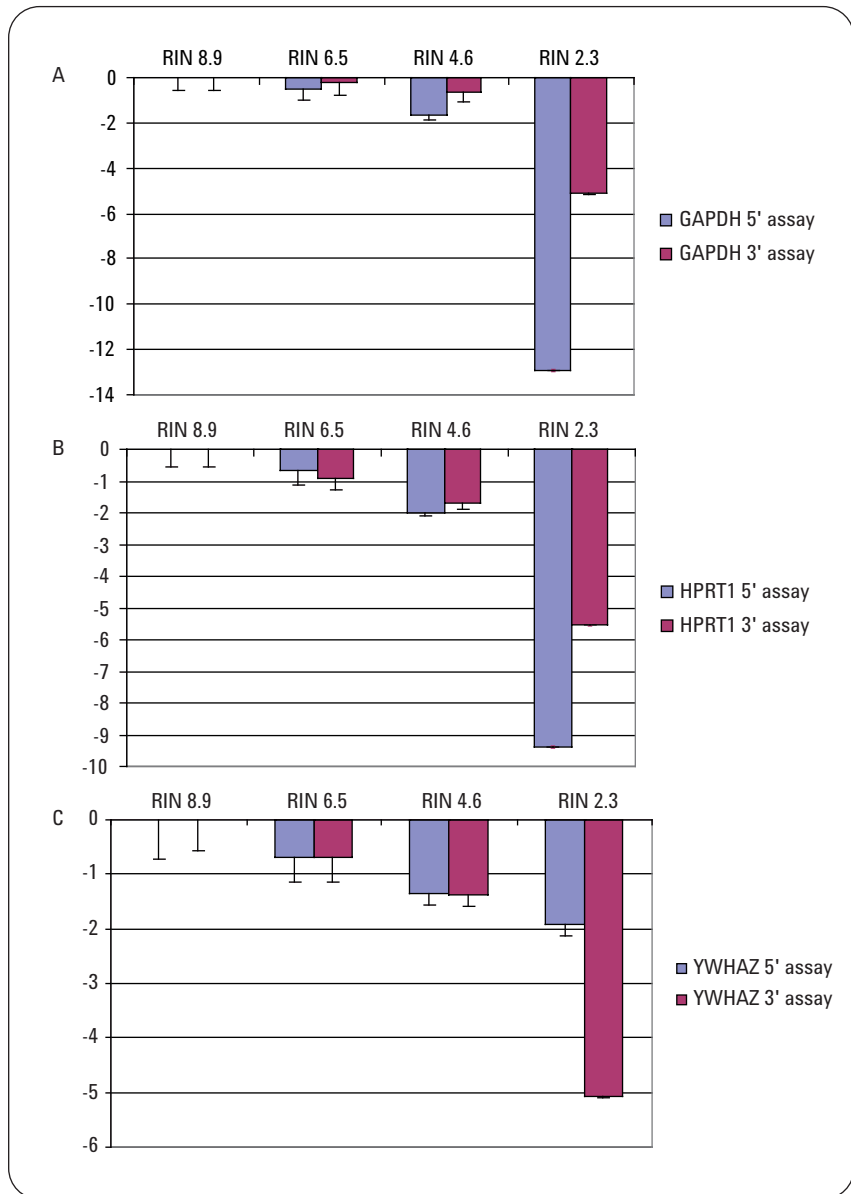


Figure 5
Relative quantities of amplicons positioned at the 5' or 3' end of three target genes. QPCR results derived from differentially degraded RNA templates compared with highly intact RNA (RIN 8.9). The Y-axis scale is logarithmic to base 2.
A) GAPDH, B) HPRT1, C) YWHAZ. Blue: 5'-assay; violet: 3'-assay

results. The results of our experiments indicate that the amount and directionality of degradation are highly gene-dependent and that the most pronounced effects appears below a RIN of 4.6. These data highlights the importance of a highly reproducible RNA integrity assessment for QPCR assay design and subsequent routine measurement.

The data presented show how the Agilent 2100 bioanalyzer was successfully implemented in a QPCR workflow to monitor essential experimental parameters as template quality and assay specificity.

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