

A Systematic Approach to Optimize Real-Time Quantitative RT-qPCR Experiments with the Agilent 2200 TapeStation System

Application Note

Nucleic Acid Analysis

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Abstract

This Application Note describes and demonstrates the efficiency of the Agilent 2200 TapeStation system and the RNA ScreenTape assay in RT-qPCR workflows for assessing the integrity of the initial total RNA template. This template is crucial for the successful amplification of the target genes. Moreover, the D1000 ScreenTape assay helps assess the amplified products for sizing and eliminate false positive results. The data presented demonstrate that the 2200 TapeStation system is an ideal quality control platform for RT-qPCR workflow solutions. The Application Note further describes the advantages of presenting the qPCR results in accordance with the MIQE guidelines.



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Introduction

RT-qPCR is a complex assay and must be carefully optimized for specificity, sensitivity, and reproducibility. In addition to RNA quality, several experimental variables affect the outcome of RT-qPCR assay performance and may lead to false positive results. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)² guidelines were put forth in 2009 to develop robust experimental design and evaluate the results. The MIQE guidelines help achieve high quality data by designing and reporting experimental conditions that yield reproducible results. This Application Note demonstrates the successful application of the 2200 TapeStation system to optimize real-time quantitative PCR experiments by recapitulating the experimental outline of a previous study with an Agilent 2100 Bioanalyzer¹.

The experimental design for this study followed the guidelines and considerations of the MIQE guidelines. The sample extraction and handling procedure, quality control of total RNA, details of commercial kits and reagents, primer sequences, location of the target genes, and qPCR specificity with standard curve and melt analysis are reported at the appropriate sections of the RT-qPCR experimental workflow. The MIQE guidelines direct special attention to the

importance of the initial quality control of total RNA. Studies have shown that the integrity of the starting material is a crucial factor determining the success of the qPCR assay in amplifying the desired product³. The RNA integrity number equivalent (RIN^e) provides an instant and objective evaluation of total RNA degradation for the 2200 TapeStation system. RIN^e is directly comparable in assessing RNA integrity to the widely accepted and highly cited RNA Integrity Number (RIN) of the Agilent 2100 Bioanalyzer system⁴.

Materials and Methods

RNA purification and analysis

HEK293 cells (ATCC) were grown in MEM media with 10 % FBS, 4 mM L-glutamine, and 1 % strep/penicillin antibiotics, and left to reach 90 % confluence. The cells were then trypsinized and collected as pellets containing 1×10^6 cells, and immediately frozen at -80°C until use. Total RNA was extracted using the Agilent Total RNA Isolation Minikit (p/n 5185-6000) following the manufacturer guidelines. The extracted samples were then analyzed for integrity using the RNA ScreenTape and RNA 6000 Nano assays with 2200 TapeStation and 2100 Bioanalyzer systems respectively following manufacturer guidelines. The samples were also parallel analyzed using NanoDrop spectrophotometer.

RNA degradation

Two vials of high integrity total RNA samples containing 1 μg each were incubated at 90°C for different amounts of time (5 and 10 minutes) to generate RNA with different qualities. Following the degradation, the samples were immediately incubated on ice to stop further degradation. A 1 μL amount was taken for analysis using the 2200 TapeStation and 2100 Bioanalyzer systems. The samples were then diluted 1:5, and 2 μL were used in subsequent RT reactions.

Reverse transcription

A five-point standard curve was generated using total RNA at 0.001, 0.01, 0.1, 1, and 10 ng as final load template amounts. The total RNA was reverse transcribed using the Agilent AffinityScript QPCR cDNA Synthesis Kit (p/n 600559) following the manufacturer guidelines in a 20 μL reaction volume (25°C for 5 minutes, 42°C for 15 minutes, 95°C for 5 minutes) using an Agilent SureCycler 8800 Thermal Cycler. Each standard was run in triplicate. A 4 μL amount of the cDNA was used directly in subsequent qPCR reactions.

Real-time quantitative PCR

Primers were obtained from Sigma-Aldrich as HPLC purified oligonucleotides. Previously published primer sequences were used in this study¹. Primers were designed to target both 5' and 3' splice variants of two genes: hypoxanthine phosphoribosyl transferase-1 (HPRT1) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ). A summary of the primer sequence, gene ID, and amplicon sizes are presented in Table 1. The qPCR reactions were carried out using Agilent Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (600882) in a final reaction volume of 20 µL, and a final primer concentration of 200 nM. ROX was used as a passive reference dye at a final concentration of 30 nM. A fast thermal cycle of 95 °C

for 10 minutes, 40 cycles of 95 °C for 20 seconds, 60 °C for 20 seconds with measurement at 60 °C was used. A dissociation curve was generated using a thermal profile of 95 °C for 30 seconds, 60 °C for 30 seconds, and 95 °C for 30 seconds with continuous fluorescent measurement along the ramp from 60 °C to 95 °C. Each standard was run in triplicate, with no reverse transcriptase (NRT) and no template control (NTC).

All primers span exon-exon junctions with exception of YWHAZ, where the 5' assay has the primers in exons 2 and 3 with a 23.6 kb intron in between. All qPCR experiments were performed on an Agilent Mx3005P qPCR system.

DNA amplicon analysis

The amplified products were analyzed using the D1000 ScreenTape and DNA 1000 assays with the 2200 TapeStation and 2100 Bioanalyzer systems for size assessment.

Data analysis

The qPCR data obtained from the Mx3005P system was analyzed using MxPro software and exported to MS Excel for presentation. The quantification cycle (C_q) was calculated based on baseline corrected normalized fluorescence (dRn). If not stated otherwise, all samples and standards were run in triplicate. The data is presented as mean \pm S.D.

Table 1. Details of the primers used in the study.

Gene	Ensembl gene ID	Primers	Primer sequence	Amplicon size (bp)
HPRT1	ENSG00000165704	HPRT1 5' forward	5'-CGTCGTGATTAGTGATGATGAACCAG-3'	130
		HPRT1 5' reverse	5'-AGCAAGACGTTCAGTCTGTCC- 3'	
		HPRT1 3' forward	5'- TTCAGGGATTGAATCATGTTGTGTC- 3'	114
		HPRT1 3' reverse	5'-GCGATGTCAATAGGACTCCAGATG-3'	
YWHAZ	ENSG00000164924	YWHAZ 5' forward	5'-TTGAGACGGAGCTAAGAGATATCTGC- 3'	142
		YWHAZ 5' reverse	5'-GCAACCTCAGCCAAGTAACGG-3'	
		YWHAZ 3' forward	5'-CTTCCTTGCTTGCATCCCACAG-3'	128
		YWHAZ 3' reverse	5'-GACAATGACAGACCATTCAAGGATAGG-3'	

Results and Discussion

Total RNA integrity analysis

The extraction procedure, sample processing, and sample integrity are essential information to be documented in qPCR. This ensures that only the highest quality samples are used in the study to obtain meaningful results. Sample integrity was determined using the 2200 TapeStation and 2100 Bioanalyzer systems before the qPCR validation. Total RNA was analyzed in replicates of six. The gel images and electropherogram traces showed two intact peaks (28S and 18S) with no degradation products, confirming intact RNA (Figures 1 and 2). The integrity numbers for the samples were expressed as average RIN^e and RIN of 10 and 9.7 by the 2200 TapeStation and 2100 Bioanalyzer systems respectively. Additionally, purity analysis on the extracted RNA by the NanoDrop spectrophotometer gave an absorbance ratio (A260/A280) of 2.0 for all samples, confirming the absence of contaminants and average yields of 25 µg per pellet. The samples were quantified as 111.8 ng/µL (26 µg per pellet) and 99.5 ng/µL (23 µg per pellet) by the 2200 TapeStation and 2100 Bioanalyzer systems respectively.

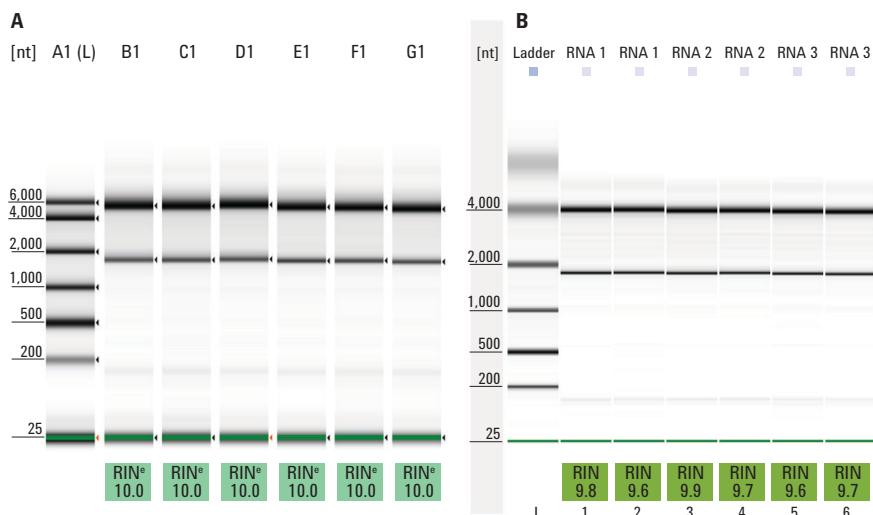


Figure 1. Gel image of total RNA analysis carried out by the Agilent 2200 TapeStation system (A) and Agilent 2100 Bioanalyzer system (B) with an average RIN^e and RIN of 10 and 9.7 respectively.

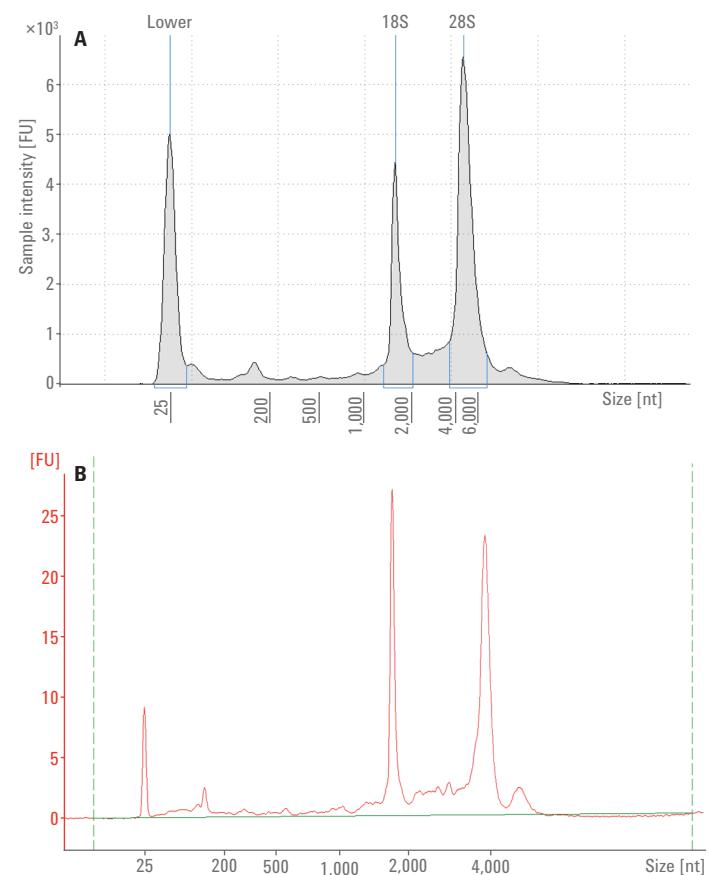


Figure 2. Electropherogram trace of the total RNA from the Agilent 2200 TapeStation system (A) and Agilent 2100 Bioanalyzer system (B).

QPCR validation and amplicon analysis

The primer specificity was validated following the MIQE guidelines by generating a five-point standard curve with 10 fold dilutions with each standard run in triplicate. The slope of the standard curve (R^2), PCR efficiency, C_q , and dissociation curve of the controls were evaluated. Primer validation for specificity was carried out using intact total RNA with RIN^e above 9.0 as template.

A five-point standard curve with total RNA input load ranging from 0.001 ng to 10 ng was generated for each primer pair. The RNA was reverse transcribed into cDNA using oligo (dT) primer. The synthesized cDNA was used as template for qPCR amplifications. NRT was used to indicate genomic DNA (gDNA) contamination and NTC was used as negative control.

Figure 3 shows an amplification plot and dissociation curve data from YWHAZ-5' and HPRT-3' to illustrate the results. The flat line for NRT shows that there is no gDNA carryover from the Agilent total RNA isolation mini kit, and the NTC shows that the reaction is free from cross contaminations. The dissociation curve of YWHAZ-5' and HPRT1-3' shows a single peak with a T_m of 80.3 °C and 79 °C for all samples across the concentration range. The dissociation curve of the NRT and NTC also shows no presence of amplicons or primer dimers.

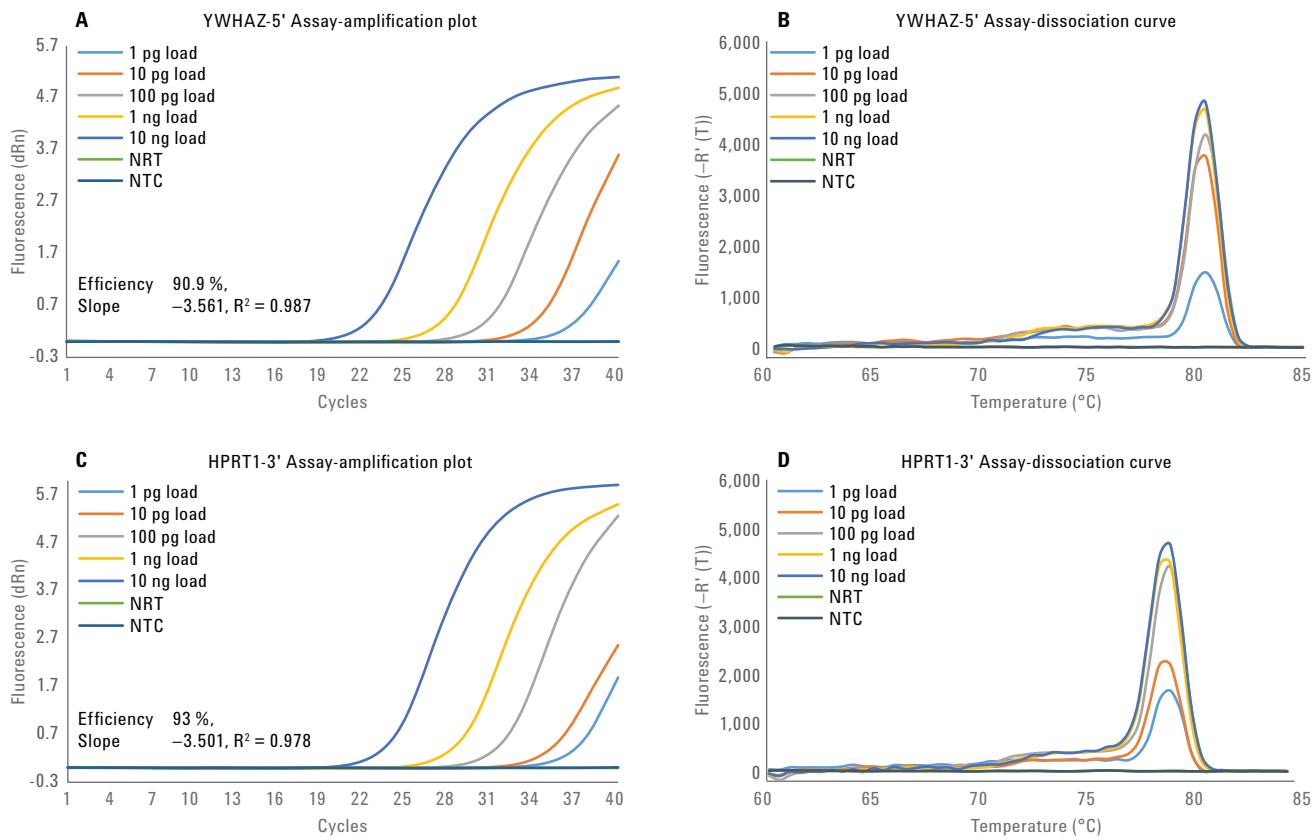


Figure 3. Amplification plots (A and C) and dissociation curve (B and D) data from YWHAZ-5' and HPRT1-3' primer pairs, respectively.

The amplicon sizes were further analyzed using the 2200 TapeStation and 2100 Bioanalyzer systems with the D1000 ScreenTape and DNA 1000 assay. The sizing data from both systems were compared to the theoretical sizes, and are presented in Figure 4 with a positive correlation between both systems with a sizing accuracy of $\pm 5\%$. Typical electropherogram traces presented in Figure 5 show an overlay of YWHAZ-5' products from 0.001 ng load with average size of 140 bp, NRT, and NTC controls from the 2200 TapeStation and 2100 Bioanalyzer systems respectively.

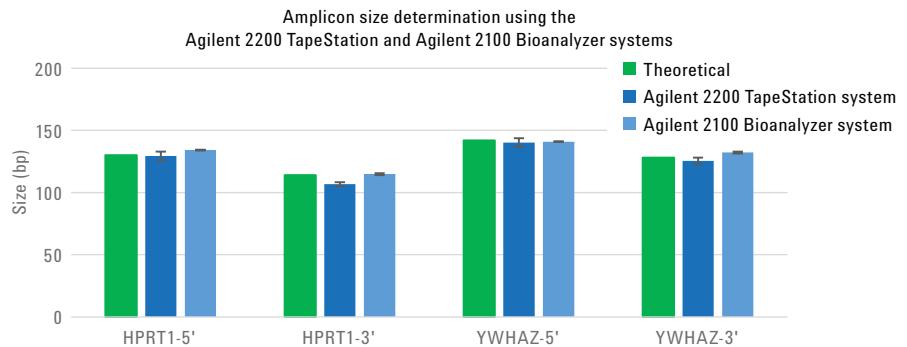


Figure 4. Amplicon sizing data from the Agilent 2200 TapeStation (dark blue) and Agilent 2100 Bioanalyzer (light blue) systems. The theoretical sizes are shown in green. Error bars indicate standard deviation.

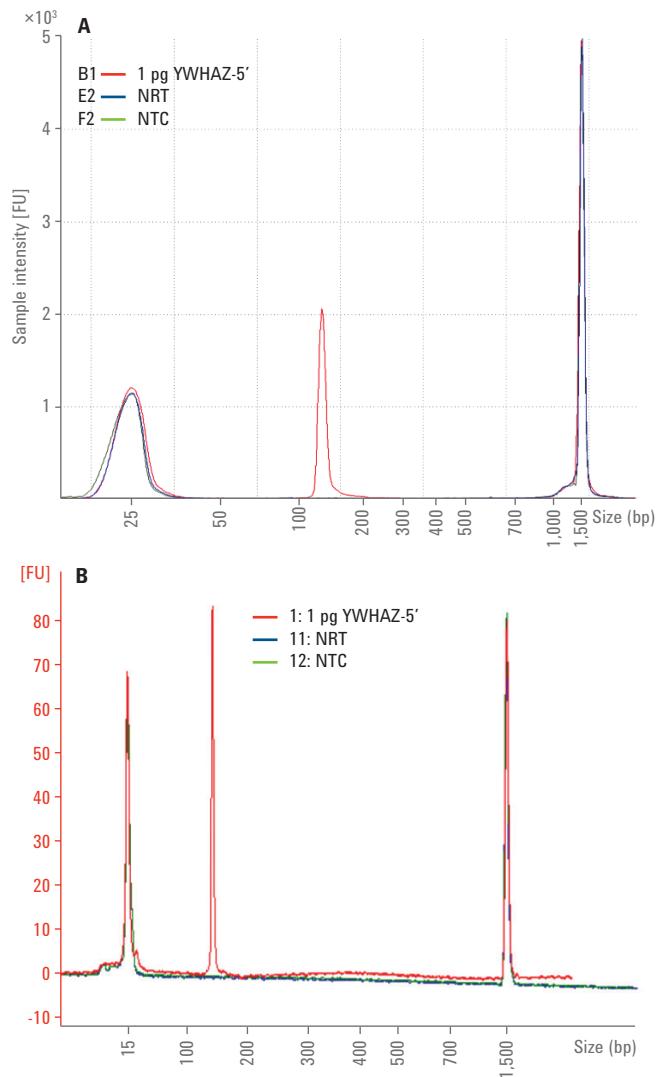


Figure 5. Electropherogram trace overlay from the YWHAZ-5' assay, showing amplicon data from a 1 pg load (red), NRT (blue), and NTC (green) using the Agilent 2200 TapeStation system (A) and Agilent 2100 Bioanalyzer system (B).

Monitoring the impact of RNA degradation on qPCR experiments

The importance of using only high integrity RNA samples for RT-qPCR experiments as recommended by MIQE guidelines was shown by carrying out RT-qPCR amplification using heat-degraded RNA as a template. The differentially heat-degraded RNA and untreated RNA were analyzed using the 2200 TapeStation and 2100 Bioanalyzer systems and designated as high, medium, and low integrity based on RIN^e. The samples were analyzed using the 2200 TapeStation and 2100 Bioanalyzer

systems and designated as high, medium, and low integrity based on RIN^e.

Figure 6 shows gel images from the 2200 TapeStation and 2100 Bioanalyzer systems showing RNA with three different levels of degradation and their respective integrity number. An electropherogram trace overlay of the three RNA samples from the 2200 TapeStation system is presented in Figure 7A.

qPCR amplifications were carried out using different integrities of total RNA. The amplification plots are shown in

Figure 7B. The C_d was determined for all primer pairs. The C_d value of the untreated and highly intact RNA sample was taken as a reference. The relative C_d difference of the treated RNA templates was calculated, and is presented in Figure 8. The figure suggests that the 5' end of the HPRT1 gene is more susceptible to degradation compared to the respective 3' end. YWHAZ shows a similar level of impact for both 5' and 3' end mRNA transcripts. These results clearly show that differential degradation is specific to the respective gene products.

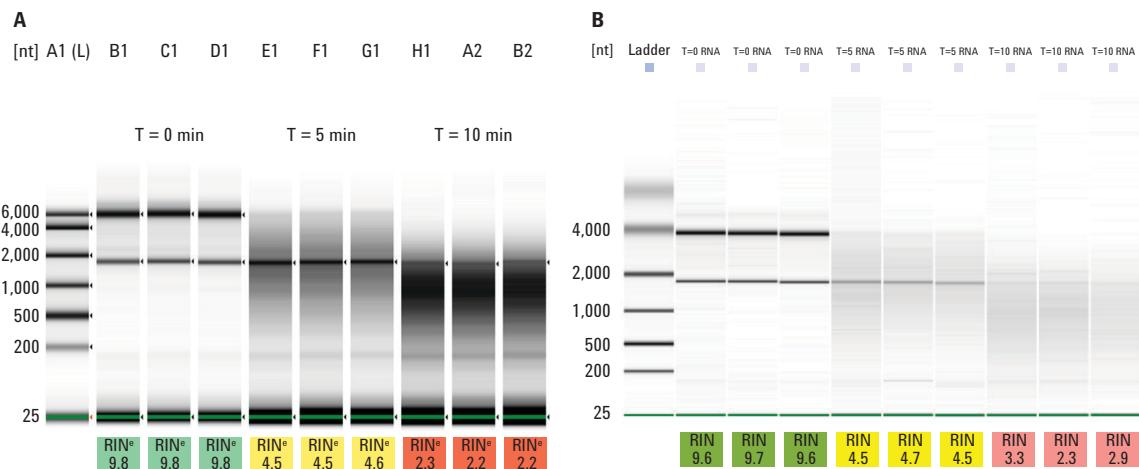


Figure 6. Gel image showing the respective RNA integrity numbers of untreated and heat degraded RNA samples. RIN^e for the Agilent 2200 TapeStation (A), and RIN for the Agilent 2100 Bioanalyzer (B) systems.

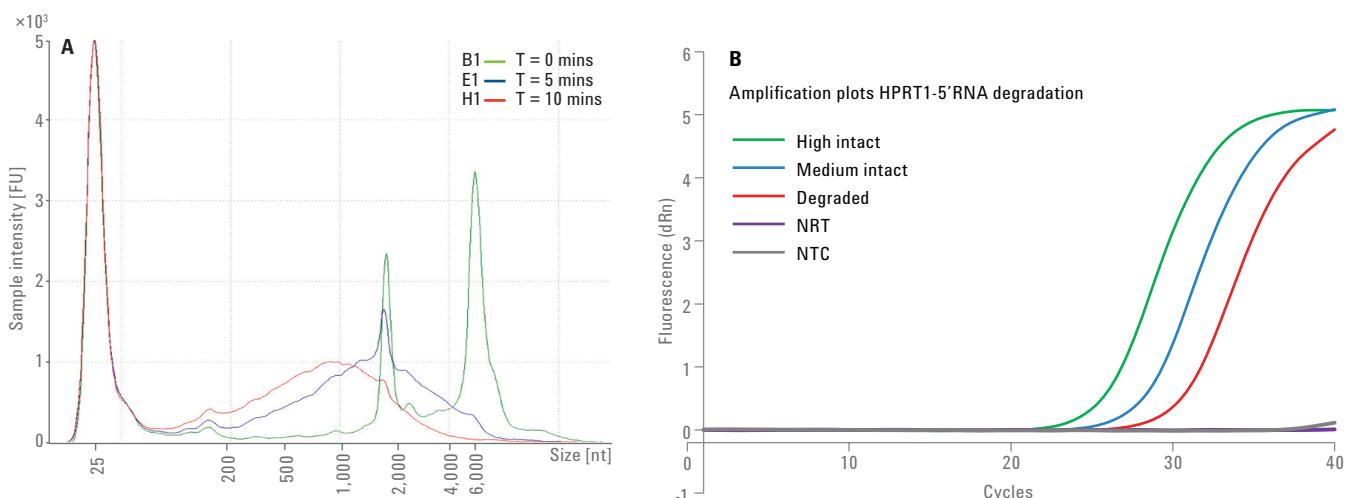


Figure 7. A) An Agilent 2200 TapeStation system electropherogram trace overlay for RNA samples that are highly intact (green), medium level of degradation (blue), and highly degraded (red). B) Amplification plot obtained from the RNA of different integrity using HPRT1-3' primers.

Conclusions

The performance of the RNA ScreenTape assay using the Agilent 2200 TapeStation system in assessing the integrity of total RNA was compared with the Agilent 2100 Bioanalyzer system and shown to be equal. Following the experimental outline of a previous study, the impact of the RNA integrity on the qPCR application was assessed by artificial degradation of total RNA samples¹. The results from this study recapitulate and confirm the previous findings that RNA integrity impacts the amplification of the target gene, and that directionality is gene dependent. Thus, this study demonstrates that the RNA ScreenTape assay can be reliably used in determining the integrity of total RNA samples. In addition to RNA quality control, the 2200 TapeStation system can be used for subsequent sizing analysis of PCR products and provide an ideal system to fit into any qPCR workflow in accordance with the MIQE guidelines.

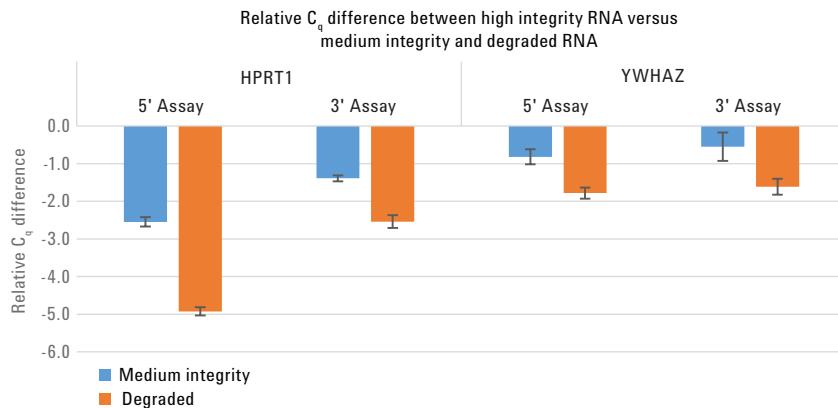


Figure 8. Relative C_q for different degradation stages of template RNA.

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