DNA Integrity Number (DIN) For the Assessment of Genomic DNA Samples in Real-Time Quantitative PCR (qPCR) Experiments

Application Note

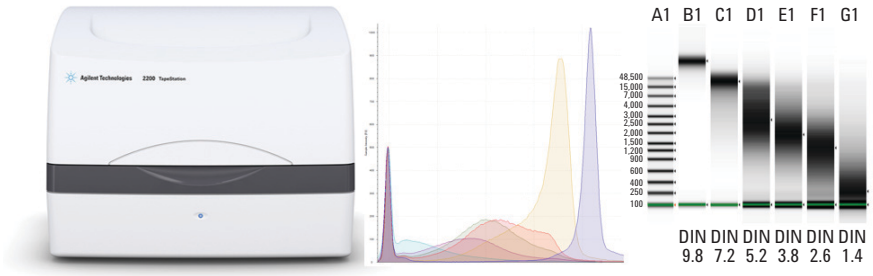
Nucleic Acid Analysis

Author

Arunkumar Padmanaban
Agilent Technologies, Inc.
Bangalore, India

Abstract

Real-time quantitative PCR (qPCR) is an indispensable molecular biology tool in the post-genome era. QPCR assays have become a robust and ideal tool for several applications such as clinical diagnostics, gene expression studies, biomarker discovery, and more. Using an intact genomic DNA (gDNA) template is crucial for generating meaningful qPCR data. Therefore, assessment of the gDNA integrity is important. With the Agilent 2200 TapeStation system, the Agilent Genomic DNA ScreenTape assay, and the DNA Integrity Number (DIN)[1], the assessment of gDNA integrity has become easy, reliable, and objective. In this study, we demonstrate the application of the Genomic DNA ScreenTape assay to assess the integrity of gDNA samples before qPCR reactions. The impact of gDNA sample integrity on target amplification, quantitation cycle (Cq), and target quantitation is assessed using DIN as a QC criteria.



Agilent Technologies

Introduction

Real-time quantitative PCR (qPCR) is the method of choice for targeted gene quantitation, due to its large dynamic range and high sensitivity. The initial sample integrity is an important factor that affects gene quantitation in qPCR experiments[2]. The quality control of the starting material is crucial to achieve reproducible and meaningful data. The Agilent 2200 TapeStation system with the Agilent Genomic DNA ScreenTape assay offers an automated, quantitative, and qualitative analysis of genomic DNA (gDNA) samples. The Agilent 2200 TapeStation Analysis Software (version A.01.05 and higher) assesses the integrity of gDNA samples and assigns the DNA Integrity Number (DIN), a numerical measure of the gDNA integrity. This value ranges from 1 to 10, where 1 indicates highly degraded gDNA and 10 represents highly intact gDNA[3].

This study demonstrates the importance of sample integrity assessment prior to qPCR. Intact gDNA and artificially degraded gDNA samples were used as templates to amplify three genes of interest using a SYBR green-based qPCR method. The qPCR parameters were then analyzed and compared. Finally, the obtained qPCR data were correlated to the DIN quality assessment to demonstrate the impact of sample integrity on the quality of the qPCR data.

Methods

Material

2200 TapeStation system (p/n G2964AA) with the 2200 TapeStation Analysis Software [revision A01.05 (SR1)], Genomic DNA ScreenTape (p/n 5067-5365) and Genomic DNA Reagents (p/n 5067-5366), AriaMx Real-time PCR System (p/n G8830A) with AriaMx Software (revision 1.0), RNase Free DNase I (p/n 600031), Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (p/n 600882) were obtained from Agilent Technologies Inc. (Santa Clara, CA, USA).

A NanoDrop 1000 spectrophotometer and a Qubit 1.0 Fluorometer with a Qubit dsDNA BR assay kit was purchased from Thermo Scientific (Waltham, MA, USA). Primers were designed using Primer-BLAST, and ordered from Sigma-Aldrich (St Louis, MO, USA). Male Human genomic DNA (G1471) was purchased from Promega (Madison, WI, USA), and used as the sample template for all experiments.

Sample preparation

To generate gDNA of different integrity, highly intact gDNA was digested using DNase I for different time points. In brief, 2 µg of intact gDNA was mixed with 1 unit of DNase I enzyme in a reaction buffer with 40 mM *Tris*-HCl (pH 7.5), 6 mM $MgCl_2$, and 2 mM $CaCl_2$ in a final volume of 25 µL. The mixture was then incubated at 37 °C for 1, 3, 5, 10, and 15 minutes. The digestion was stopped by the addition of 5 mM EDTA (final concentration), and incubation at 75 °C for 10 minutes, followed by storage on ice. The control sample was treated accordingly, but without the addition of the enzyme.

QC analysis

After digestion, the gDNA samples were quantified using UV measurement or a fluorescent dsDNA assay following the manufacturer guidelines. In addition, the samples were quantified and qualified using the 2200 TapeStation system[4].

DNA analysis was performed using the 2200 TapeStation system in combination with the Genomic DNA ScreenTape assay, according to the manufacturer's instructions.

Real-time quantitative PCR amplification

Primer sets for three different genes were used in this study (Table 1). A six-point calibration curve with fivefold dilutions was prepared using high integrity gDNA with total load ranging from 8 pg to 25 ng. All digested gDNA samples were normalized to 2 ng/µL by dilution based on UV quantitation, resulting in a final template load of 4 ng. The qPCR reaction mix was prepared using Agilent Brilliant III Ultra-Fast SYBR Green QPCR Master Mix in a final reaction volume of 20 µL with a primer concentration of 250 nM. ROX was used as a passive reference dye at a concentration of 30 nM. A fast thermal cycle with a hot start of 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 5 seconds, 60 °C for 10 seconds, with measurement at 60 °C was used. A dissociation curve was generated, using a thermal profile of 95 °C for 30 seconds, 65 °C for 30 seconds, and 95 °C for 30 seconds with continuous fluorescent measurement along the ramp from 60 °C to 95 °C. All standards and samples were run in triplicate with a no-template control (NTC).

Table 1. Primer sequence, Ensembl gene ID, and theoretical amplicon sizes.

Gene	Ensembl gene ID	Primers	Primer sequence	Amplicon size (bp)
ZFPY	ENSG00000067646	ZFPY forward	5′-AGAAAGCACATGCGAATCCAT-3′	313
		ZFPY reverse	5′-CGCACATCTCACACTTATGAGGA-3′	
PGBD4	ENSG00000182405	PGBD4 forward	5′-GCTCCATGAAATGGTCAGCTC-3′	250
		PGBD4 reverse	5′-TCGTCATTGTCAGTGTCTTTCC-3′	
GUSB	ENSG00000169919	GUSB forward	5′-GTTCCCATGACCAGGCTTCA-3′	228
		GUSB reverse	5′-GAGAGGATGGGAGGGTGTCT-3′	

Results and Discussion

The impact of gDNA integrity on downstream qPCR amplification was studied by using artificially degraded gDNA samples as starting material.

Genomic DNA integrity analysis

The gDNA samples, DNase digested for different time points, were analyzed in triplicate using the 2200 TapeStation system and the Genomic DNA ScreenTape assay. Figure 1A shows the obtained gel image with the DIN on the bottom of each gel lane. The gel image clearly shows a shift in DNA size and distribution with increasing degradation of the gDNA over time. Highly intact gDNA samples migrated as a well-defined peak above the largest ladder peak (48,500 bp). With increasing degradation, the main peak shifted towards smaller sizes. Highly degraded gDNA migrated as a broad peak with sizes below 2,000 bp. This shift was also observed in the electropherogram overlay (Figure 1B). This observation is also reflected in the DIN values shown in Figure 1A. The sample with the highest DNA integrity has the highest DIN values, and increasing DNase incubation times results in a decreasing DIN.

Figure 2 summarizes the DIN obtained for the gDNA samples with different integrity, as shown in Figure 1B, and further demonstrates the excellent reproducibility of the method.

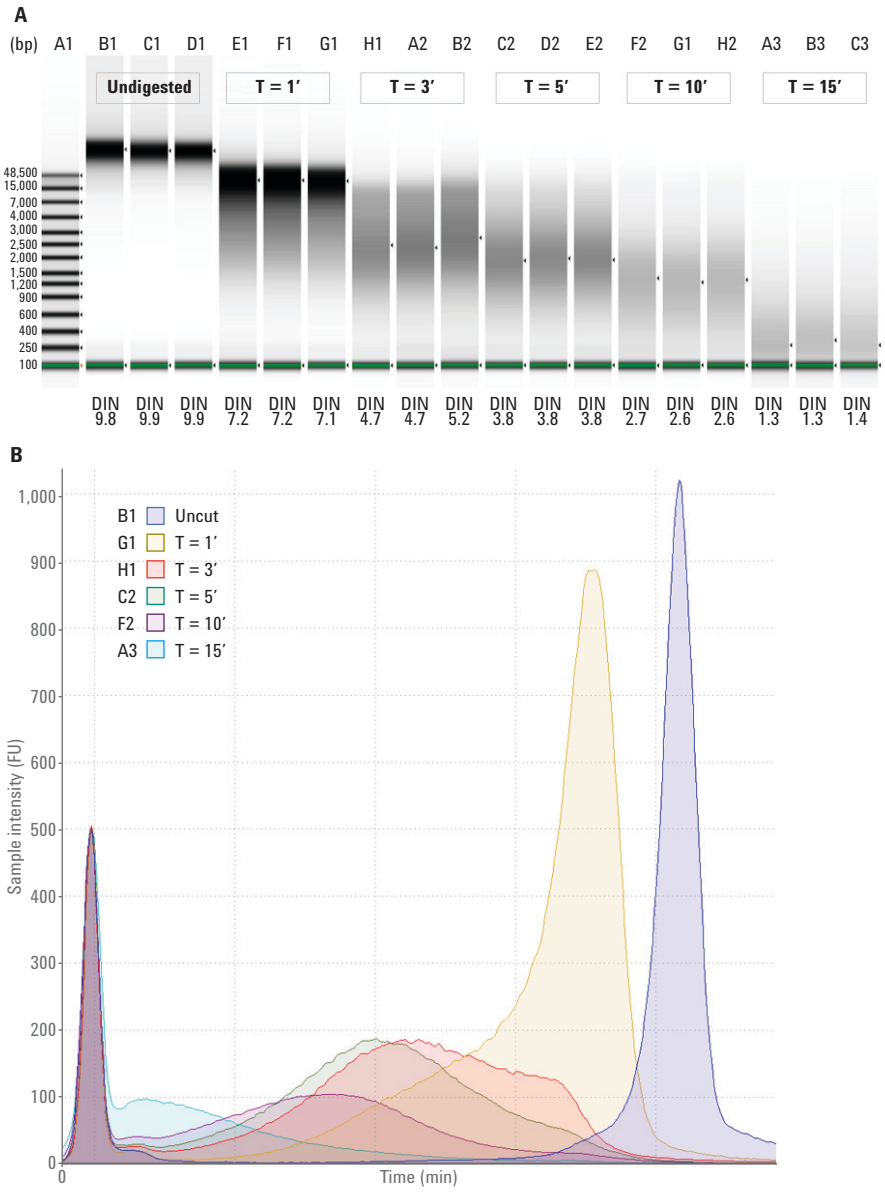


Figure 1. gDNA of varying integrity due to DNase incubation of highly intact gDNA for different time points (T = 1 minute to 15 minutes) was analyzed with the Agilent Genomic DNA ScreenTape assay. The gel image is shown in (A) with DIN, indicating the gDNA integrity of each sample. B) shows an electropherogram overlay of gDNA samples treated with DNase for different times.

Genomic DNA quantitation analysis

Genomic DNA samples were quantified using the 2200 TapeStation system, NanoDrop, and Qubit (Figure 3). In addition to the integrity assessment, the Genomic DNA ScreenTape assay quantifies the sample in a single run. The Genomic DNA ScreenTape assay uses a fluorescent dye specific for double-stranded DNA, and is not biased by other contaminants such as enzymes or salts in the sample. As expected, comparable quantitation values were obtained using the 2200 TapeStation and the Qubit systems. Conversely, quantitation by NanoDrop UV measurement was largely affected by sample contaminants such as single-stranded DNA, oligonucleotides, salts, and buffer components.

Quantitative PCR analysis

To ensure a uniform DNA template input, the samples were diluted to 2 ng/µL based on NanoDrop quantitation values. UV-based normalization was taken into consideration to include double-stranded DNA, single-stranded DNA, and oligonucleotides from the digestion steps, thus ensuring uniform DNA load. The samples were again quantified after dilution using NanoDrop to ensure that equal concentrations were subjected to the qPCR reactions.

The qPCR reactions were designed using MIQE guidelines as reference[5]. The six-point calibration curve with fivefold dilution was prepared and evaluated for primer specificity, slope of the standard curve (R^2), PCR efficiency, quantitation cycle (Cq), and NTC contamination.

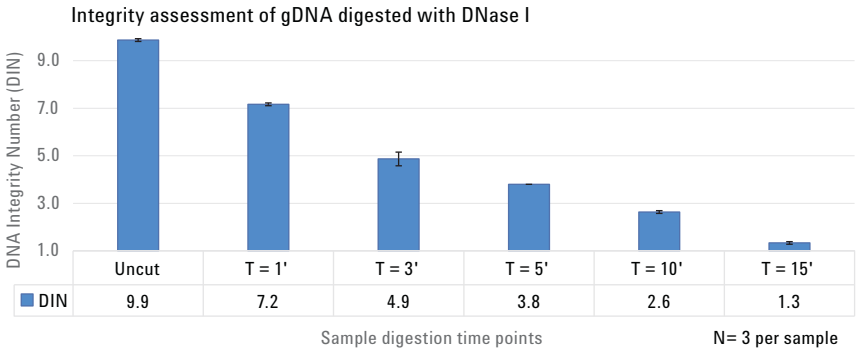


Figure 2. Average DIN for the gDNA samples shown in Figure 1. Error bars indicate standard deviation with n = 3.

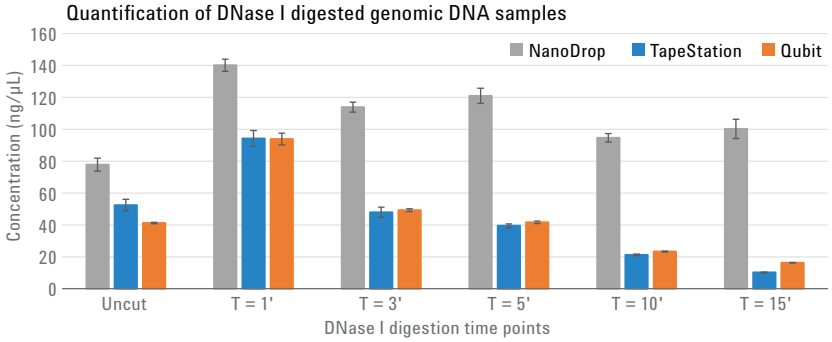


Figure 3. Quantitation of digested gDNA samples using NanoDrop, an Agilent 2200 TapeStation system and a Qubit Fluorometer (n = 3 per sample).

The calibration curve yielded a PCR efficiency of 97.51 %, 119.07 %, and 92.44 % with an R^2 of 0.999, 0.977, and 0.996 for the three tested genes ZFPY, PGBD4, and GUSB, respectively (Figure 4).

A qPCR standard curve with an R^2 greater than 0.980 is generally considered acceptable. The melt curve of the standards showed a single peak suggesting sequence specificity of the primers (Figure 5).

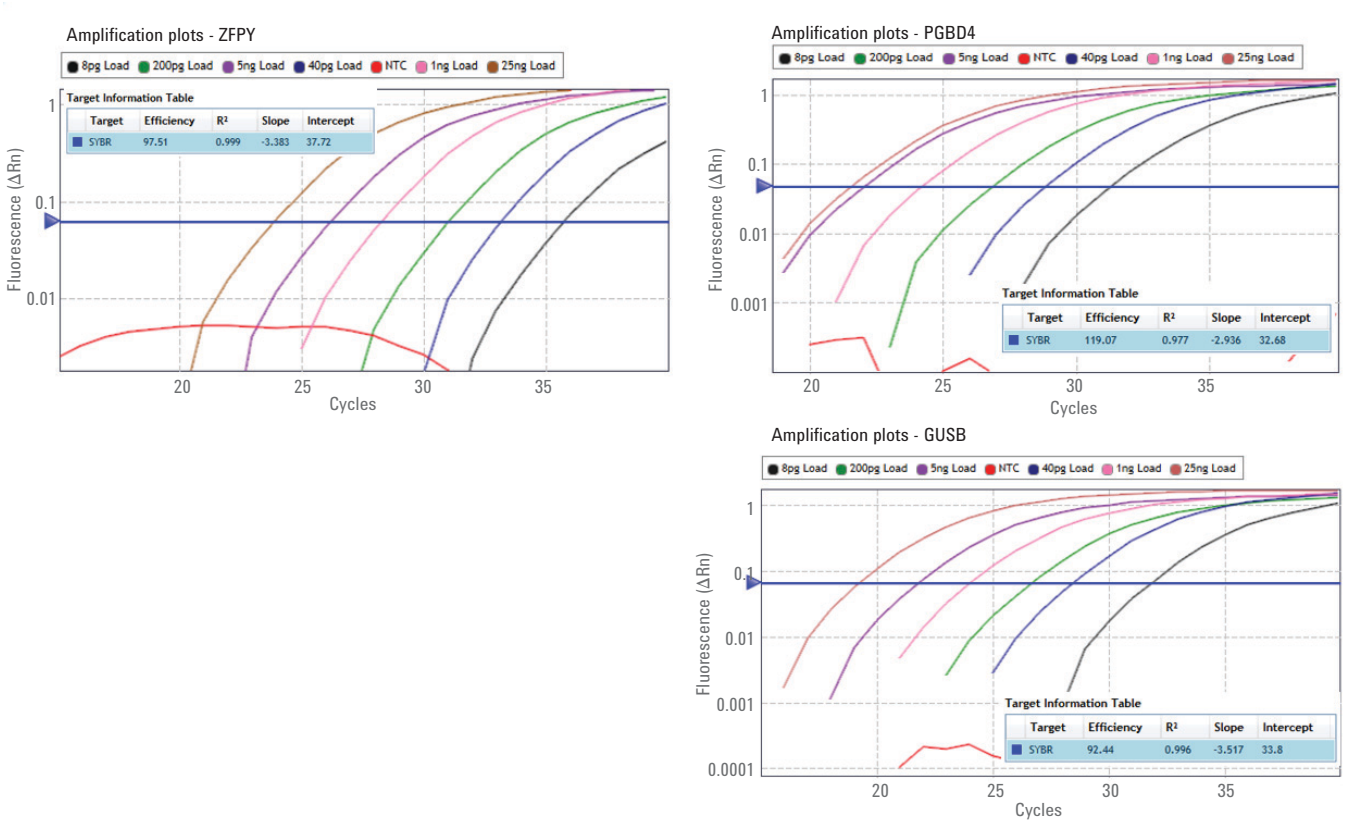


Figure 4. Six point standard curve (8 pg to 25 ng) for the three tested genes, ZFPY, PGBD4, and GUSB.

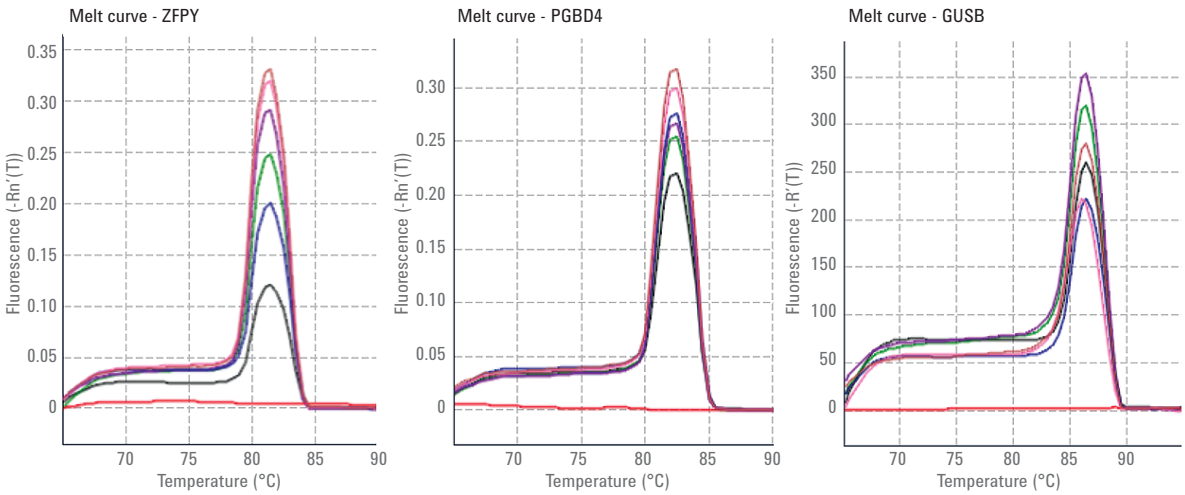


Figure 5. Melt curve for the three tested genes, ZFPY, PGBD4, and GUSB.

Finally, the gDNA samples with different integrity were analyzed for target quantitation. Quantified values were normalized to the NanoDrop template input. The quantitation of the target from each sample was calculated against the highly intact gDNA sample, and the accuracy is presented in Figure 6.

The bar graph shows a differential quantitation of the target, which clearly depends on the integrity of the template. All three primers show a similar trend in decreasing quantitation of the target compared to the intact gDNA template of DIN 9.9. The samples with DIN 4.9 and DIN 3.8 gave similar quantitation percentages compared to the control. DIN 1.3 showed least target quantitation, as most of the template was degraded and the target sequence is not available.

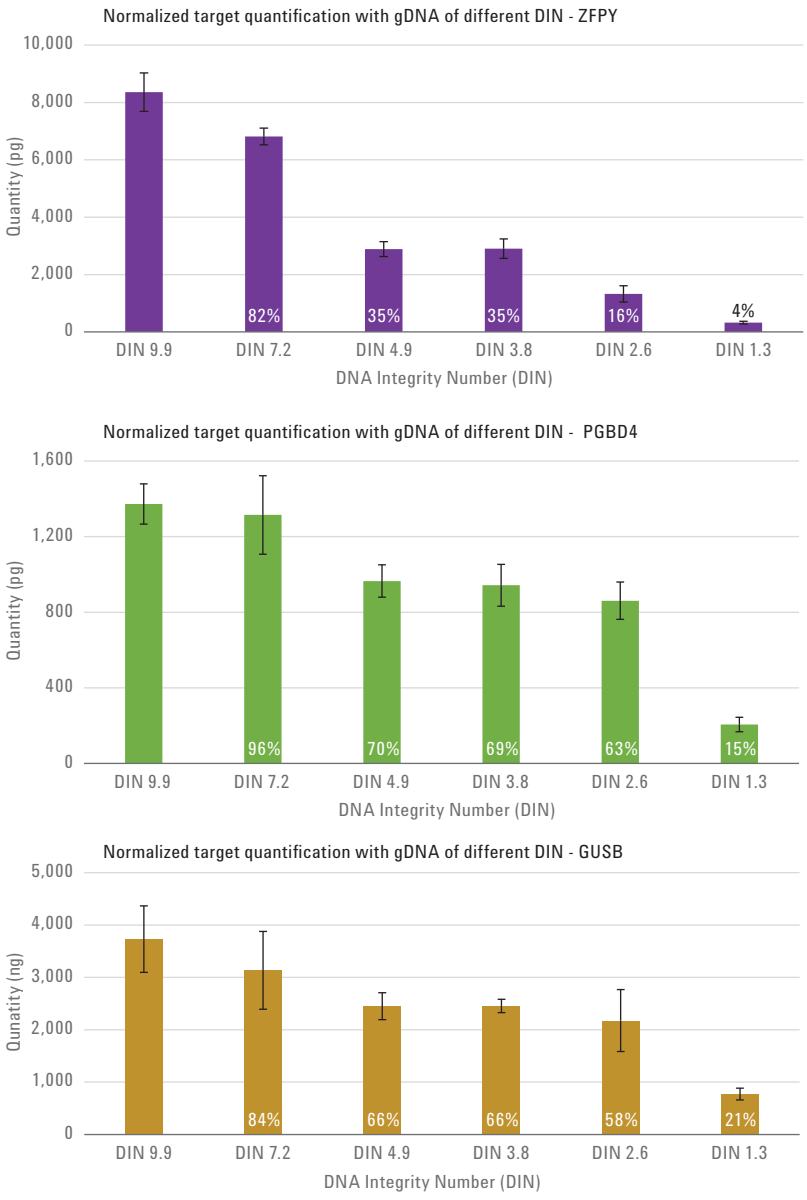


Figure 6. Quantification of target and the relative percentage of target amplification compared to intact gDNA template of DIN 9.9 for ZFPY, PGBD4, and GUSB, respectively.

Conclusions

The Agilent 2200 TapeStation system with the Agilent Genomic DNA ScreenTape assay offers an automated quality control using DIN as a quantitative measure of gDNA integrity. In this study, artificially degraded gDNA samples were used for subsequent qPCR amplification, demonstrating that

- qPCR amplification and quantitation is highly influenced by gDNA template integrity.
- gDNA with DIN below 7 shows a considerable loss in the target sequence, thereby under quantifying the target. The extent of sequence loss varies between different genes.
- DIN highly correlates with the key qPCR parameters like quantitation cycle (Cq), target quantitation and efficiency of the amplification.
- DIN can be used to determine and validate a sample integrity threshold level for specific downstream qPCR experiments.

References

1. Gassmann, M.; McHoull, B. DNA Integrity Number (DIN) with the Agilent 2200 TapeStation System and the Agilent Genomic DNA ScreenTape Assay. *Agilent Technologies*, publication number 5991-5258EN, **2014**.
2. Brisco, M. J.; *et al.* Incorporation of measurement of DNA integrity into qPCR assays. *BioTechniques* **2010**, *49(6)*, pp. 893-7.
3. Nguyet, K.; *et al.* Integrating the DNA Integrity Number (DIN) to Assess Genomic DNA (gDNA) Quality Control Using the Agilent 2200 TapeStation System. *Agilent Technologies*, publication number 5991-5442EN, **2014**.
4. Agilent Genomic DNA ScreenTape System Quick Guide. *Agilent Technologies*, publication number G2964-90040, rev.C. **2014**.
5. Bustin A. S. Definitive qPCR. A Practical Guide, **2015**.

www.agilent.com/genomics/tapestation

Published in the USA, November 1, 2015
5991-6368EN

Agilent Technologies