Rapid qPCR Using Agilent Brilliant III Ultra-Fast SYBR Green QPCR Master Mix

Application Note

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Abstract

Standard PCR hardware is capable of rapid cycling; however, qPCR cycling is limited by the time it takes for the DNA polymerase to anneal to the target template and extend the amplicon. To optimize qPCR cycle time, the performance of Agilent Brilliant III Ultra-Fast SYBR Green QPCR Master Mix was measured while testing two different sets of cycling parameters. These parameters include a shortened denaturation step, and a shortened combined annealing and elongation step. As a result of these tests, it was determined that the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix achieves high performance and reproducibility, with lower cycle times.

Introduction

While PCR hardware capable of very fast cycling has been available for a number of years, qPCR assay speed has been limited by the time required for the DNA polymerase in the master mix to anneal to the target template and extend the amplicon. Some qPCR master mix manufacturers recommend a three-step cycling protocol, with annealing and extension as different steps, whereas others combine these into a single step within a two-step cycling protocol. The two-step protocol offers an advantage in that the ramp time between annealing and extension is omitted thereby saving a few seconds per cycle, but the combined step is often 30 – 60 seconds long to allow sufficient time for the enzyme to copy the template.

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Material and Methods

For RNA extraction, RT112, T24, and HFL1 cells were grown to confluence in six-well plates, and 1 mL QIAzol (QIAGEN) was used to lyse the cells in each well. The lysate was mixed with 200 µL chloroform, shaken by hand for 15 seconds, incubated on the bench for 5 minutes, and then centrifuged at 10,000 g for 15 minutes at 4 °C to separate the phases. The aqueous phase was transferred to a fresh 1.5 mL tube, mixed with 1 volume of 70 % ethanol and loaded onto an RNeasy column (QIAGEN). The remaining steps were performed according to the manufacturer's instructions. RNA integrity was assessed using an Agilent Bioanalyzer (RIN >9.5) and RNA purity and concentration were determined using a NanoDrop 1000 ($A_{260/280}$ >2.0, $A_{260/230}$ >1.8) (Thermo Fisher Scientific Inc.). One µg of RNA was reverse transcribed to cDNA using a Quantitect reverse-transcription kit (QIAGEN) which included a genomic DNA wipeout step. The completed RT reaction was diluted 10-fold with tRNA in water (5 µg/mL). Genomic DNA was extracted from HFL1 cells using a DNeasy kit according to the manufacturer's instructions and purity checked using a NanoDrop 1000 ($A_{260/280}$ >1.8, $A_{260/230}$ >1.7).

Brilliant III Ultra-Fast SYBR Green QPCR Master Mix performance was tested for two sets of cycling parameters using a Rotor-Gene Q (QIAGEN). The first set comprised our control conditions and the second set comprised the test conditions, which consisted of a shortened denaturation step and greatly shortened combined annealing and elongation step (Table 1). In both conditions, following 40 cycles, the temperature was ramped from 65 °C to 95 °C at 1 °C per step for melt curve analysis.

Table 1. qPCR cycling conditions used.

	Control Conditions	Test Conditions
Initial denaturation (min)	5	3
Denaturation (s)	10	5
Annealing (s)	20	1*
Extension (s)	10	1*
Total run time excluding melt (min)	64	39

* Combined annealing/extension step of 1 s.
Both conditions used 40 cycles.

A master mix of forward and reverse primers at 500 nmol/L each, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix and nuclease-free water was prepared, and 8 µL were dispensed using a Corbett CAS1200 automated liquid handling system into 100 µL Rotor-Gene reaction tubes. Two µL of purified PCR product (qPCR standard) containing 10^7 to 10^1 copies, 2 µL of diluted cDNA from the three human cell lines or 10 ng human genomic DNA in 2 µL, were added as templates in a total reaction volume of 10 µL. The no-template control was tRNA 5 µg/mL. For amplification of cDNA (human) and qPCR standards (rat and human) eight validated assays (qStandard, UK) were selected to include a range of amplicon lengths from 60 to 468 bp. Two additional assays (qStandard, UK) capable of amplifying human genomic DNA were also included. Details are reported in Table 2. After test conditions were compared with the control conditions, known copy number standards for a further 124 assays (qStandard, UK) were run under the test conditions. These assays varied in amplicon % GC content (up to 68 %) and amplicon length (up to 900 bp).

The effects of four established PCR inhibitors on Brilliant III Ultra-Fast SYBR Green QPCR Master Mix performance were evaluated. These inhibitors included three that can originate from the sample itself (hemoglobin, collagen, and urea) and one that can result from the nucleic acid purification process (phenol). Either water or inhibitor (four concentrations) were added to four human fibroblast cDNA samples starting at the highest concentration previously reported to inhibit PCR or at a concentration that would be readily detectable in an RNA sample (for example, visible presence of blood/hemoglobin, phenolic odor). Reactions were run in duplicate under the test conditions and copy numbers were calculated.

Results and Discussion

The qStandard assays used were previously validated under our three-step control conditions to ensure assays with high specificity (that is, single DNA dissociation peak on melt analysis and single band of the expected size by agarose gel analysis), high efficiency, and linearity from 10^7 to 10^1 copies. The run under control conditions, excluding the melt step, takes 64 minutes.

As reduced run times would provide a significant time saving, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix performance was tested under conditions that reduced the run time, excluding melting time, to only 39 minutes. Most qStandard assays are designed to give products from 60 to 200 bp, but a few assays fall outside this range; two of these were included as extremes under which Brilliant III Ultra-Fast SYBR Green QPCR Master Mix performance could be tested beyond the limits of the typical range of amplicon length in qPCR.

Known copy number standards for assays were amplified with Brilliant III Ultra-Fast SYBR Green QPCR Master Mix under both control and test conditions. For all eight assays, standards amplified as well under test conditions as they did under standard conditions; standard curves were linear from 10^7 to 10^1 copies and assay efficiency remained high, >90 % (Table 2).

Table 2. Efficiencies and R^2 values of copy number standard curves for eight assays with amplicon lengths from 60 to 468 bp. All primers span an intron.

			Standard curve R^2		Standard curve efficiency	
Assay	Species	Amplicon length	Control condition	Test condition	Control condition	Test condition
1	rat	60	0.99985	0.99989	1.01	1.04
2	rat	78	0.99926	0.99868	0.97	0.97
3	rat	112	0.99988	0.99960	0.99	1.05
4	rat	146	0.99816	0.99916	0.94	0.91
5	rat	171	0.99843	0.99995	1.02	1.00
6	rat	193	0.99923	0.99745	0.95	1.00
7	rat	310	0.99700	0.99912	0.94	0.97
8	*H.sapiens*	468	0.99977	0.99935	0.99	1.01

Table 3. Genes used to evaluate amplification of genomic DNA using Agilent Brilliant III Ultra-Fast SYBR QPCR Master Mix.

Gene	Species	Fwd sequence	Rev sequence	Amplicon length
COL10A1	*H.sapiens*	ttgagaaactcggcatttcctt	acctcctggatgtttcctagaag	81
RUNX2	*H.sapiens*	tgacaccaccaggccaat	ggtggaggattccaatgaag	69

These primers are not intron spanning.

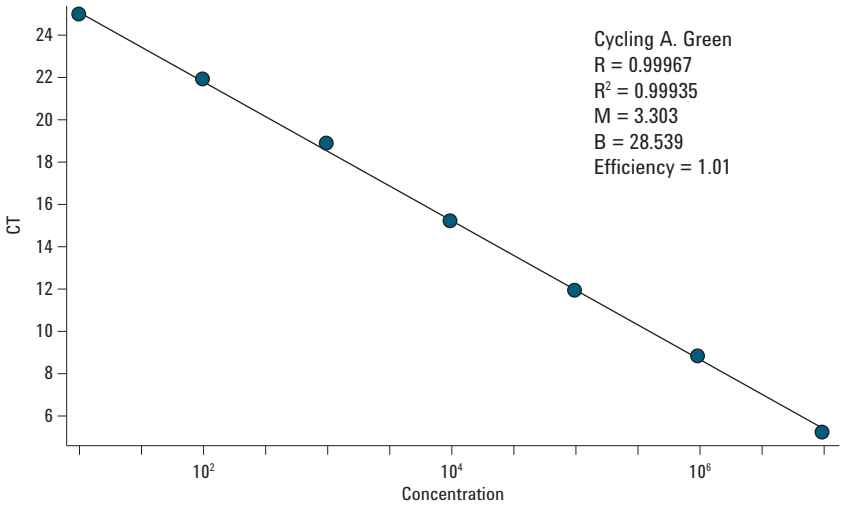

Figure 1. Standard curve for assay 8 (EEF1E1) copy number standards under test conditions.

For the longest amplicon, assay 8 (eukaryotic translation elongation factor 1 epsilon 1, EFF1E1 468 bp) Brilliant III Ultra-Fast SYBR Green QPCR Master Mix performed just as well under test conditions as control conditions; standards amplified with high efficiency (99 %) and the standard curve was linear (Figure 1; R^2 =0.9994) between 10^7 and 10^1 copies (Figure 2).

A further experiment was run to determine how Brilliant III Ultra-Fast SYBR Green QPCR Master Mix performed when amplifying a single target in a complex biological sample, such as cDNA or genomic DNA, which may contain tens of thousands of potential templates. The longest amplicon, (EEF1E1) was amplified under test conditions using cDNA from the different cell lines and Brilliant III Ultra-Fast SYBR Green QPCR Master Mix. In cDNA obtained from all three cell lines, a single product at 468 bp was generated by agarose gel analysis (Figure 3).

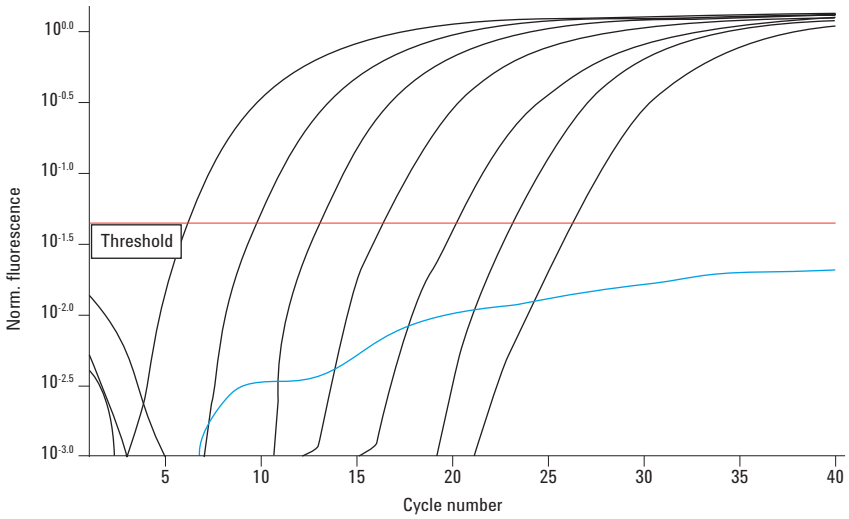

Figure 2. Amplification curves for assay 8 (EEF1E1) copy number standards (black lines) from 10^7 to 10^1 copies per reaction under test conditions. Blue line = no-template control. Y axis shows log fluorescence, X-axis shows cycle number.

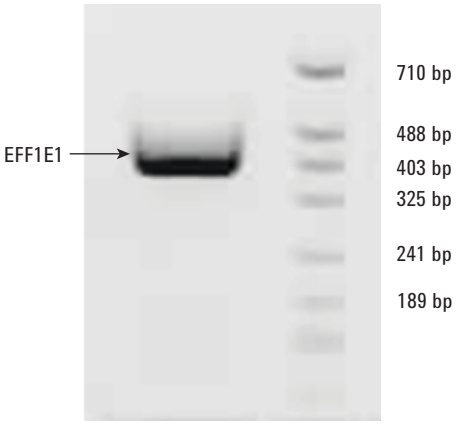

Figure 3. Agarose gel analysis of the qPCR product generated by EFF1E1 primers from R112 cell cDNA.

This product showed a single peak on melt analysis after amplification (Figure 4), and EFF1E1 amplified from cDNA with the same efficiency as purified known copy number standards (Figure 5). Replicate amplifications of each cell line cDNA gave EEF1E1 copy numbers with low variability (Table 4). Coefficients of variation were 4.4 – 8.1 %.

It may be concluded that Brilliant III Ultra-Fast SYBR Green QPCR Master Mix amplifies a single target with high efficiency in a complex cDNA sample.

To investigate the performance of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix under test conditions with genomic DNA as a target, two validated assays in which the primers were designed within the same exon were selected. The standard curves for these two assays (COL10A1 and RUNX2) also demonstrated high efficiency and linearity; COL10A1 efficiency = 102 %, R^2 = 0.9998 and for RUNX2 efficiency = 103 %, R^2 = 0.99966. Genomic DNA from HFL1 cells was used as a template and similar copy numbers were observed for both genes (Table 5). With genomic DNA as a template, different gene assays should yield the same copy number as long as assay efficiency is identical, and the gene is not duplicated or deleted. Therefore, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix also performed well with a genomic DNA sample as a template.

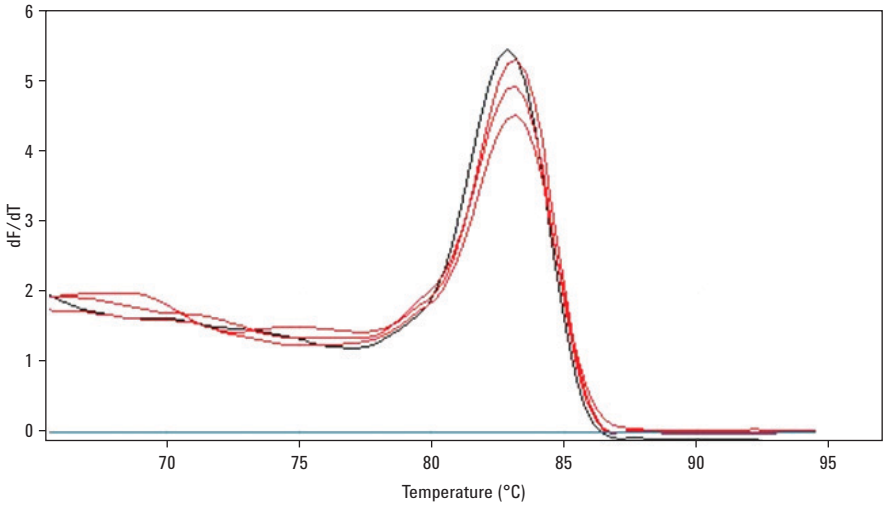

Figure 4. Melting analysis of the EFF1E1 qPCR product generated from standard (black), from cDNA obtained three human cell lines (RT112, T24 and HFL1 cells (all red), and an NTC control (no peak). Melting peak is 82.8 – 83.0 °C.

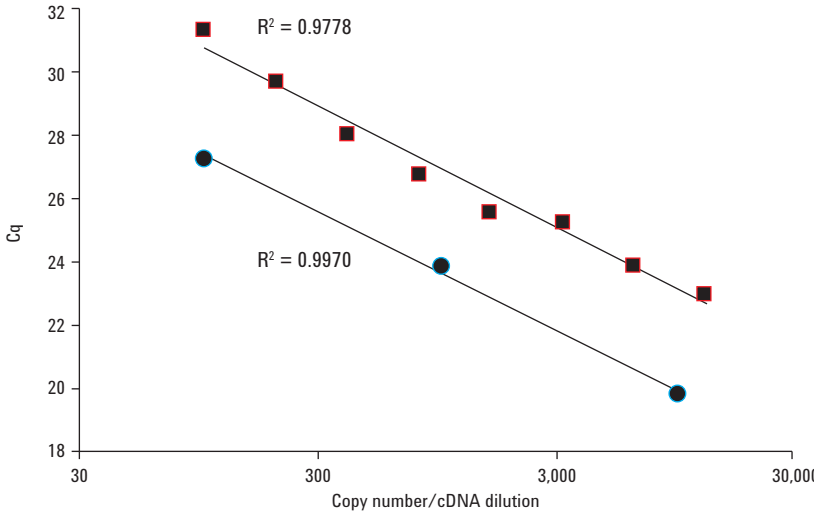

Figure 5. Comparison of standard curve for EEF1E1 copy number standards (circles) and doubling dilutions of human fibroblast cDNA (squares) under test conditions.

Table 4. Cq and calculated copy number for EEF1E in five replicates of cDNA from three different cell lines.

cDNA source	RT112 cells		T24 cells		HFL1 cells	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
Cq	17.218	0.115	17.368	0.096	17.968	0.065
Copy number	5514	450	4970	317	3298	145

Once the repeatability of the test conditions was established, we tested Brilliant III Ultra-Fast SYBR Green QPCR Master Mix in a large number of assays using known copy number standards (>95 % of assays from qStandard, Figure 6). Assay efficiency was not comprised by high amplicon % GC. In order to determine the maximum length of amplicon that could be amplified with the 1 second annealing/extension step, we included three assays with PCR products of 608, 613, and 900 bp. Efficiency declined with these very large amplicons and so we extended the annealing/extension time to 5 seconds. Under these conditions, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix could amplify all three long PCR products with high efficiency (100 – 104 %, R^2>0.997).

Table 5. Cq and calculated copy number for two genes in five replicates of 10 ng of genomic DNA from HFL1 cells.

Gene symbol	COL10A1		RUNX2	
	mean	**SD**	**mean**	**SD**
Cq	22.312	0.068	21.250	0.033
Copy number	912	45	1098	36

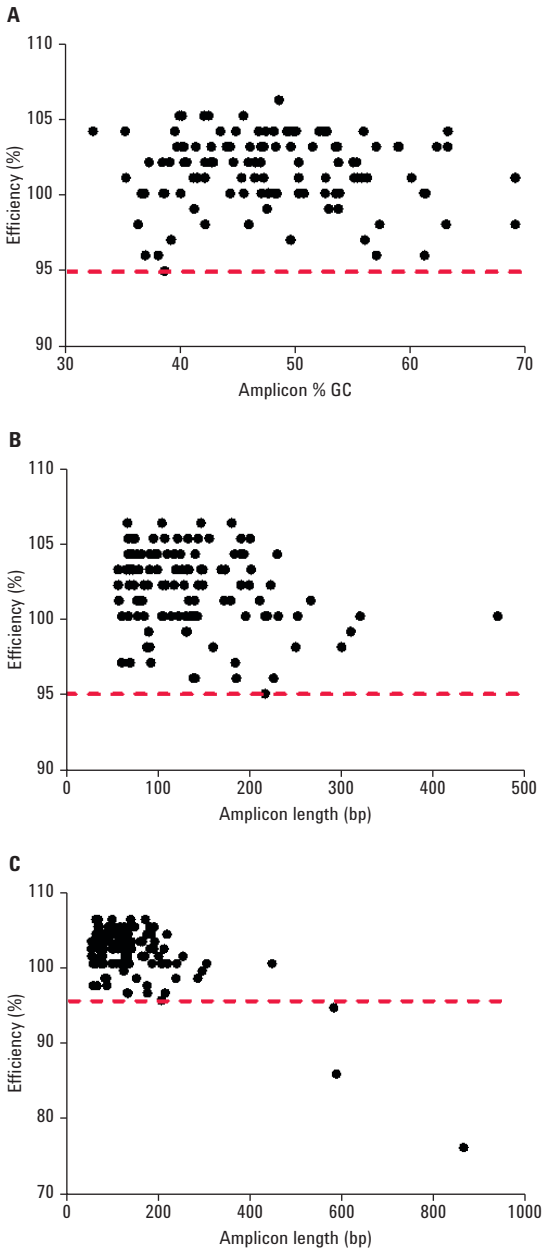

Figure 6. Efficiency of amplification for known copy number standards using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix under test conditions in 144 assays with different amplicon lengths and % GC: assays with amplicon CG content from 33 – 68 % (A), assays with amplicons up to 468 bp (B), and assays including >600 bp (C) amplicons. The red dotted line indicates the threshold at which we consider an assay to be satisfactory.

The effects of known PCR inhibitors on Brilliant III Ultra-Fast SYBR Green QPCR Master Mix performance were investigated using an assay for human beta-actin that was susceptible to inhibition (Figure 7). Phenol at concentrations up to 1 % v/v in the cDNA reaction had no discernable effect on copy numbers in the four samples tested. At 2 % v/v phenol the Cq was delayed, the slope of the amplification curve was notably less steep and copy numbers decreased by approximately 50 – 70 % of the uninhibited values. A similar effect was observed in samples treated with hemoglobin – inhibition occurred only at a concentration that was visible as a brown discoloration in the RNA sample. Urea present at concentrations observed in normal concentrated urine and up to 1 µg of collagen failed to inhibit the qPCR reactions.

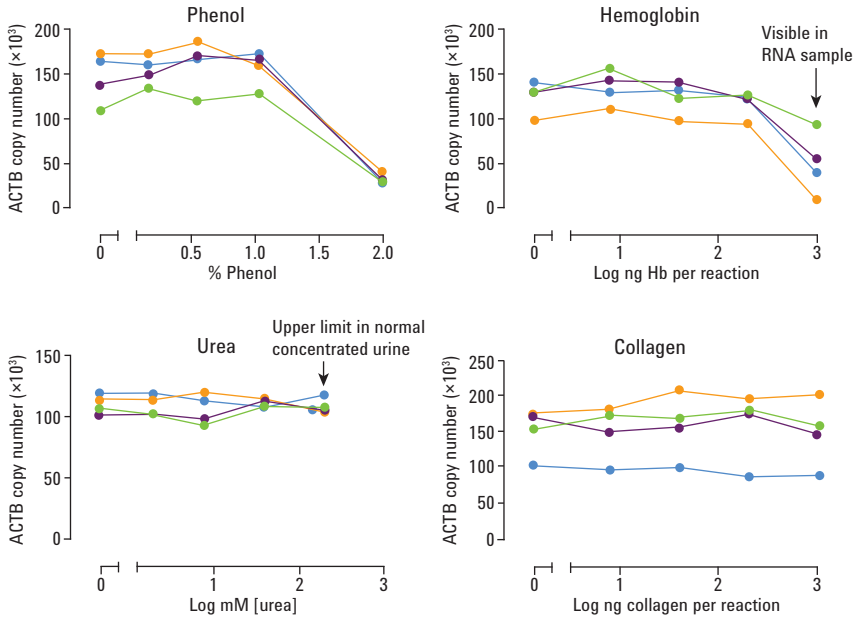

Figure 7. The effects of four known qPCR inhibitors on Brilliant III Ultra-Fast SYBR Green QPCR Master Mix performance. Four human fibroblast cDNA samples (colored data points) were spiked with increasing concentrations of inhibitor and subjected to qPCR for ACTB under the test conditions.

Conclusions

The Brilliant III Ultra-Fast SYBR Green QPCR Master Mix performed extremely well using ultra fast qPCR cycling conditions (5 seconds denaturation and 1 second combined annealing/extension). This cycling protocol saved 25 minutes on each qPCR run, even on a fast, air-driven cycler. Amplification efficiency, tested on purified PCR standards from eight different genes, averaged 99.3 %. Assay linearity was also exceptional with an average correlation coefficient (R^2) of 0.99915 over a 10-fold dilution series range of 10^7 to 10^1 copies.

This same outstanding performance was manifest for qPCR assays generating products of varying sizes from 60 bp to 468 bp. There was no evidence of a decline in qPCR assay linearity or efficiency as the size of the product amplified increased (up to 468 bp). As a result, a 1-second combined annealing/extension step was sufficient to generate a specific 468 bp product and allow development of a sensitive, selective qPCR assay for this gene.

The Brilliant III Ultra-Fast SYBR Green QPCR Master Mix was also shown to amplify the 468 bp product efficiently, reliably, and selectively from complex templates such as cDNA from three different cell lines which contain many thousands of potential targets. Also, when genomic DNA was used as a template, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix demonstrated comparable performance.

Of 124 new assays (designed by qStandard) that we subsequently tested, we observed no failures with Brilliant III Ultra-Fast SYBR Green QPCR Master Mix; even for high GC content amplicons (68 %). For very long amplicons, >600 bp, increasing the combined annealing/extension step to just 5 seconds enabled Brilliant III Ultra-Fast SYBR Green QPCR Master Mix to amplify standards with high efficiency and linearity under these rather challenging conditions.

Finally, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix exhibited excellent resistance to four common PCR inhibitors. Inhibition was only observed under conditions that would not occur in normal laboratory practice; 2 % v/v phenol is detectable by smell and 3 µg of hemoglobin would be visible in a nucleic acid sample. We do not suggest that the use of impure samples is acceptable in qPCR, as the effects of these inhibitors on pre-PCR procedures, for example reverse transcription of RNA, were not tested in this study. However, amplification of genomic DNA from blood with hemoglobin contamination that is not visible in the RNA sample, may be achieved reliably with Brilliant III Ultra-Fast SYBR Green QPCR Master Mix.

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Published in the USA, June 18, 2012
5991-0372EN

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