How to Avoid the Generation of Nonspecific Amplification When Using TaqMan Assays-on-Demand Gene Expression Products

Data Sheet

In validating Brilliant III Ultra-Fast QPCR and QRT-PCR Master Mixes over many targets and a broad template dilution range, some TaqMan 'Assays-on Demand' (AOD) generated primer-dimers with a competitor's fast QPCR master mix. Such PCR artifacts were not observed with the Brilliant III Ultra-Fast reagents, suggesting the novel hot start technology of Brilliant III prevents generation of non-specific secondary products, providing the user with a greater degree of confidence in their results.

Introduction

A common concern for real-time PCR users is reactions that produce low amplification efficiency. A reaction with low amplification efficiency will result in low reproducibility of replicates, later or delayed Cts for lower DNA input amounts, and lower limits of detection. Pre-designed assays (like AOD) are a convenient method for testing expression levels of known genes. Some AOD targets can give rise to amplification of secondary non-specific PCR products leading to erroneous data analysis and misinterpretation of expression profiles due to decreased sensitivity of detection. The results suggest that having a more robust chemistry and superior hot start technology, as employed in Brilliant III Ultra-Fast, can improve the specificity of amplification for these problematic primer and probe sets.

Agilent Technologies

Experimental Methods

Total RNA from Stratagene Universal Human Reference RNA (cat. # 740000) was reverse transcribed using a mix of oligo(dT) and random primers (AffinityScript QPCR cDNA Synthesis kit; cat. # 600559) to generate cDNA. PCR assays (20µl) contained 1x primer/probe mix (GUS or TBP Assays-on-Demand) and cDNA concentrations ranging from 100-0.01 ng per reaction. Thermal cycling was performed on the ABI StepOnePlus Real-Time PCR system (see Figures 1 & 3 for details of cycling parameters for Brilliant III Ultra-Fast QPCR (cat. # 600880) Master Mix and Competitor A master mix). The resulting PCR products (1 µL) were evaluated on an Agilent Bioanalyzer 2100 instrument using a DNA 1000 Chip (cat. # 5067-1504).

Results

Brilliant III Ultra-Fast QPCR Master Mix was compared against a commercially available master mix (Competitor A) on two TaqMan AOD gene expression products (GUS and TBP) over a 4-log dilution range (100, 10, 1, 0.1, 0.01 ng/Rxn). Figures 1 & 3 (A &B) show the amplification plots and standard curves of the two fast QPCR master mixes with the GUS and TBP gene expression products respectively. The results indicated a significant difference in amplification efficiencies between the two master mixes on both AOD targets, suggesting further analysis was required to determine the nature of the disparity.

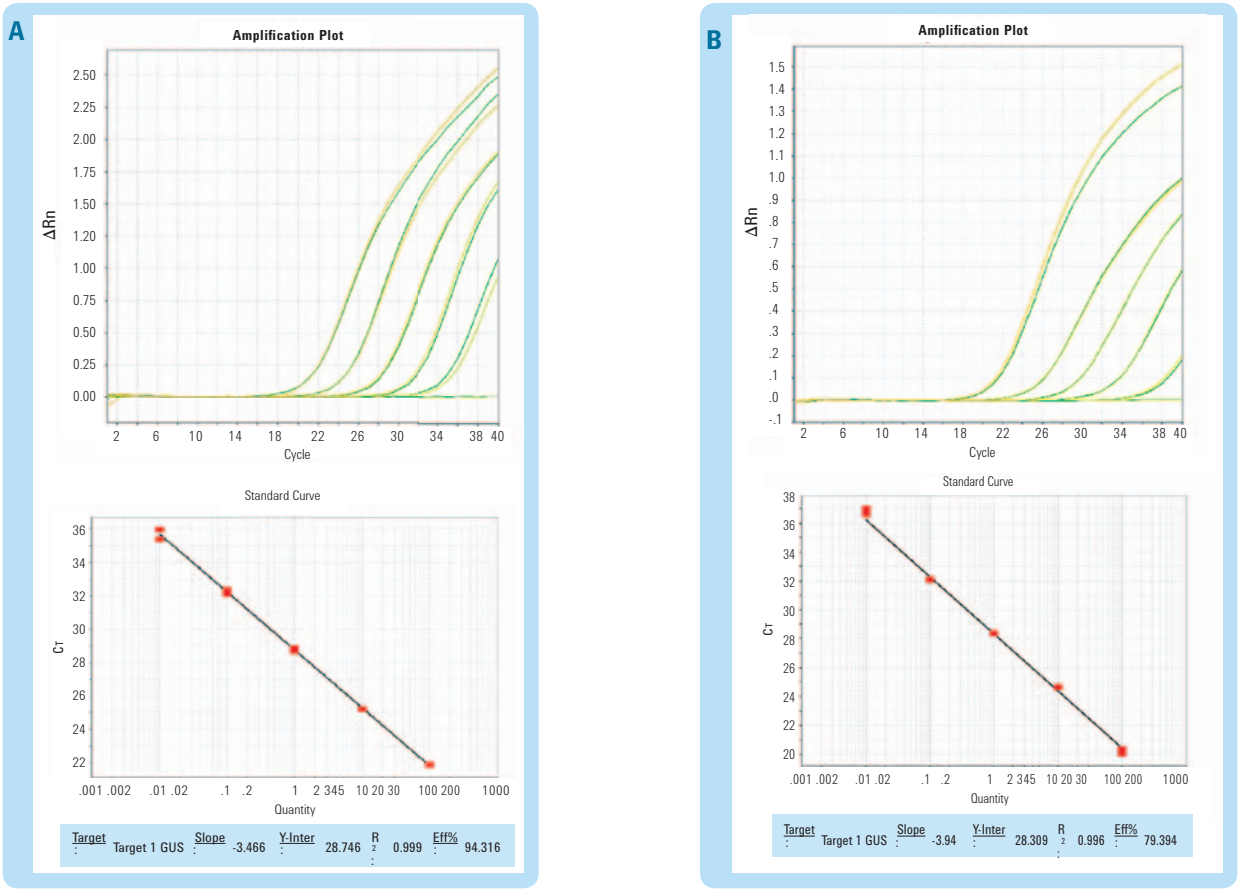
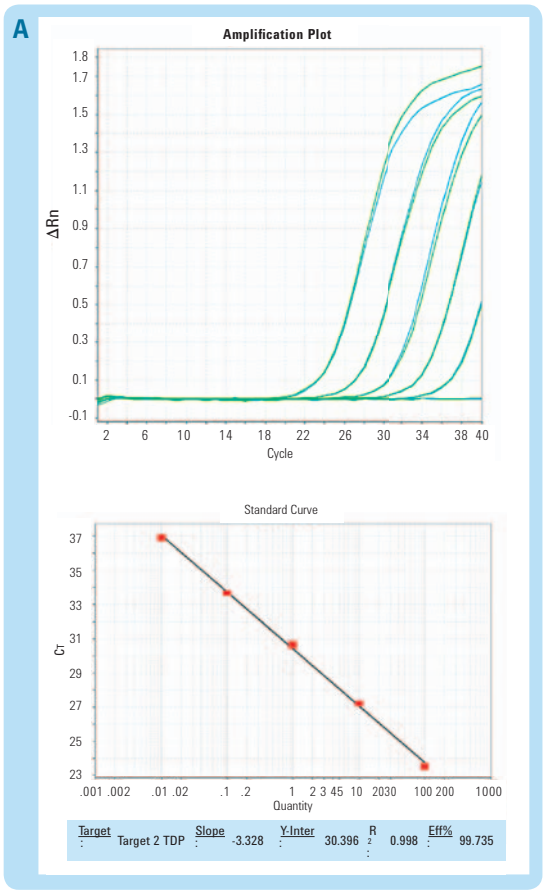


Figure 1.
Amplification plots and corresponding standard curve of GUS primer/probe set (ABI Assays-On-Demand) using (A) Brilliant III Ultra-Fast Master Mix and (B) using Competitor A master mix on the StepOnePlus PCR system.
Reactions (20ul) contained 1x primer/probe and human universal cDNA ranging from 100-0.01 ng/Rxn. Thermal cycling conditions for Brilliant III Ultra-Fast Master Mix were 3min at 95°C initial denaturation followed by 40 cycles of 5sec at 95°C and 10sec at 60°C. Thermal cycling conditions for Competitor A master mix were 20sec at 95°C initial denaturation followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C. The standard curves span four orders of magnitude resulting in an Rsq of 0.999 and amplification efficiency of 94.3% for the Brilliant III Ultra-Fast Master Mix and an Rsq of 0.996 and amplification efficiency of 79.4% for Competitor A master mix.



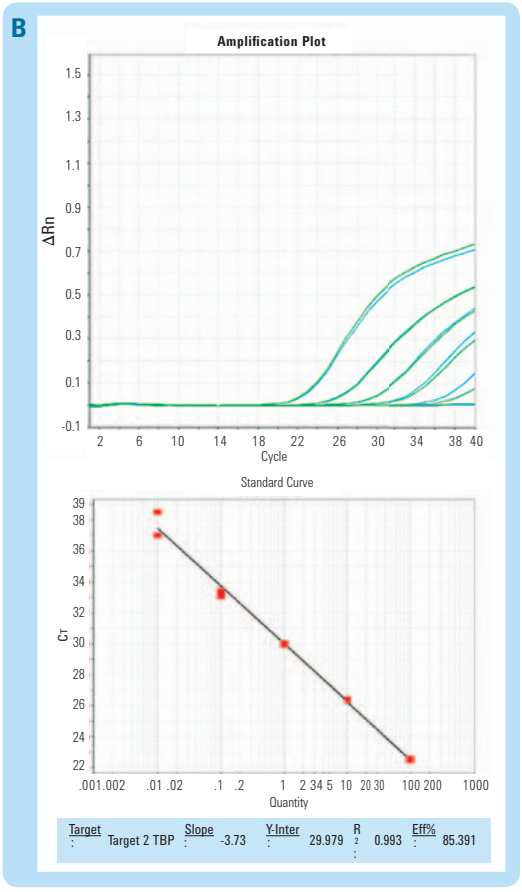


Figure 3.
Amplification plots and corresponding standard curve of TBP primer/probe set (ABI Assays-On-Demand) using (A) Brilliant III Ultra-Fast master mix and (B) using Competitor A master mix on the StepOnePlus PCR system.
Reactions (20ul) contained 1x primer/probe and human universal cDNA ranging from 100-0.01 ng/Rxn. Thermal cycling conditions for Brilliant III Ultra-Fast master mix were 3min at 95°C initial denaturation followed by 40 cycles of 5sec at 95°C and 10sec at 60°C. Thermal cycling conditions for Competitor A master mix were 20sec at 95°C initial denaturation followed by 40 cycles of 1sec at 95°C and 20sec at 60°C. The standard curves span four orders of magnitude resulting in an Rsq of 0.998 and amplification efficiency of 99.7% for the Brilliant III Ultra-Fast master mix and an Rsq of 0.993 and amplification efficiency of 85.4% for Competitor A master mix.

In order to investigate the cause for the amplification efficiency discrepancy, all amplification products were run on an Agilent Bioanalyzer (Figures 2 & 4). Non-specific PCR products of low molecular weight (primer-dimers) were observed for Competitor A's fast master mix with both AOD targets. Not surprisingly, the amount of these non-specific products increases as the amount of input cDNA decreases. Such products were not visible in amplicons generated by Brilliant III Ultra-Fast QPCR Master Mix. Primer-dimer formation in a reaction competes with the specific product amplification and thereby reduces the amplification efficiency and reduces the assay limit of detection and dynamic range.

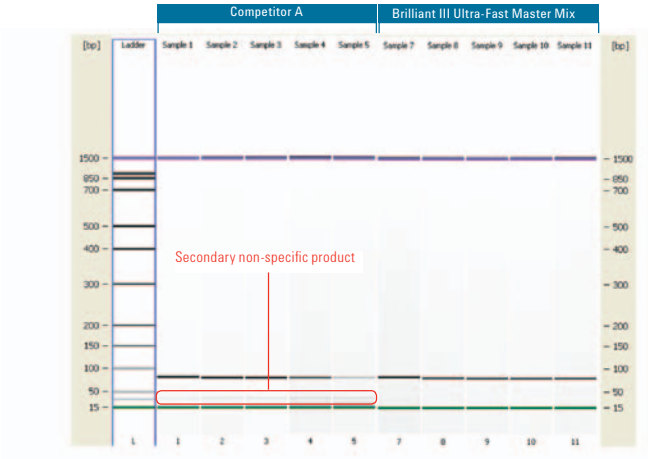


Figure 2.
Trace of GUS qPCR products generated using Competitor A master mix (left) or Brilliant III Ultra-Fast Master Mix (right) run on Agilent 2100 Bioanalyzer.
Presence of primer-dimers/non specific amplification is evident when Competitor A master mix is used for amplification.

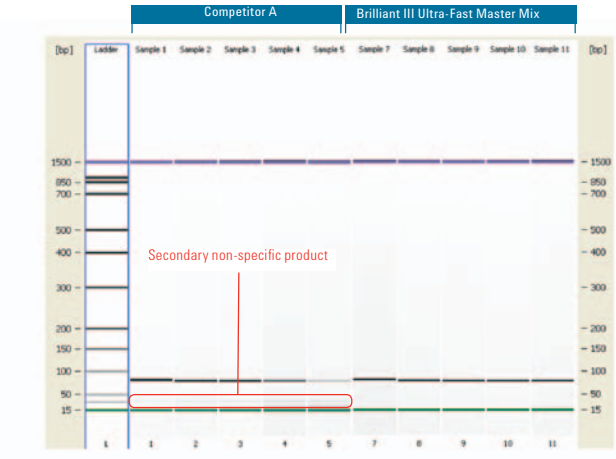


Figure 4.
Trace of TBP qPCR products generated using Competitor A (left) or Brilliant III Ultra-Fast Master Mix (right) run on Agilent 2100 Bioanalyzer.
Presence of primer-dimers/non specific amplification is evident when Competitor A master mix is used for amplification.

Conclusion

Non-specific amplification and primer-dimer formation can greatly reduce amplification efficiency of the target gene since they compete for reaction components during amplification, resulting in later Cts, lower sensitivity, decreased replicate reproducibility, and reduced dynamic range. These performance attributes may drastically impact the end result. Pre-designed assays, such as AOD, have the potential to generate primer-dimers and throw into doubt the validity of the analysis. The data presented here illustrates the importance of the QPCR master mix on the final result. The novel *Taq* mutant and new hot start technology of Brilliant III Ultra-Fast Master Mixes reduces the need for more extensive assay validation and provides more reliable and consistent data across a wider range of different assays.

The versatile Brilliant III Ultra-Fast QPCR reagents offer the greatest flexibility in master mixes delivering superior performance across an array of quantitative applications on any Real-Time PCR platform.

Ordering Information

Description	Quantity	Reactions*	Catalog No.
Brilliant III Ultra-Fast QPCR Master Mix for ABI StepOnePlus and BioRad CFX96	2 x 2 ml	400	600880
Brilliant III Ultra-Fast QPCR Master Mix for ABI StepOnePlus and BioRad CFX (10 pack)	20 x 2 ml	4000	600881
Brilliant III Ultra-Fast QRT-PCR Master Mix for ABI StepOnePlus and BioRad CFX96	2 x 2 ml	400	600884
Brilliant III Ultra-Fast QRT-PCR Master Mix for ABI StepOnePlus and BioRad CFX96 (10 pack)	20 x 2 ml	4000	600885
Brilliant III Ultra-Fast SYBR Green QPCR Master Mix for ABI StepOnePlus and BioRad CFX96	2 x 2 ml	400	600882
Brilliant III Ultra-Fast SYBR Green QPCR Master Mix for ABI StepOnePlus and BioRad CFX (10 pack)	20 x 2 ml	4000	600883
Brilliant III Ultra-Fast SYBR Green QRT-PCR Master Mix for ABI StepOnePlus and BioRad CFX96	2 x 2 ml	400	600886
Brilliant III Ultra-Fast SYBR Green QRT-PCR Master Mix for ABI StepOnePlus and BioRad CFX96 (10 pack)	20 x 2 ml	4000	600887

*assumes 20 µl reaction volume

Learn more about Brilliant III Ultra-Fast QPCR Master Mixes and how they can improve your quantification on any Real-Time PCR instrument at www.stratagene.com/brilliant3.

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