Technical Note: Performance Comparison Between the EverGreen Universal qPCR Master Mix and Equivalent Competitor Master Mixes

Introduction

Recent years have seen a clear shift in molecular biology applications towards real-time PCR, owing to its high sensitivity, specificity, and turnaround time. However, suboptimal PCR conditions lead to erroneous results [1]. Chemically, PCR is a reaction between oligonucleotides primers and dNTPs in the presence of a DNA polymerase, cofactors, stabilizers, enhancers, and a DNA template (the sample) to yield a PCR product/amplicon. Many commercially available PCR master mixes are comprised of all of these components except the sample and primers, such as our EverGreen Universal qPCR Master Mix (ref number PCR5505), providing a convenient solution for the end user. Therefore, the combination of efficient primers, pure sample, and an optimized master mix are essential for obtaining accurate, reproducible results.

In this technical note we used previously validated samples and PCR primers to assess the performance of our EverGreen master mix, a dye-based real-time PCR master mix, and two equivalent master mixes from other vendors. The assessment parameters and their passing criteria are listed in table1, and we discuss them in detail in the next sections.

In all experiments we made sure that the only variable was the master mix. All other variables were kept the same (samples, primers, plastics, experimenter, etc.). Also, to ensure that any difference in performance was not due to inter-run variability, comparative studies were run in parallel on the same plate. The three master mixes had the same PCR cycling recommendations from the manufacturers which made them ideal candidates for this study (3 minutes at 95°C, then 40 cycles of 95°C for 15 seconds and 55-65°C for 1min/kb).

Table1: Master mix performance assessment parameters, description of how each parameter is tested, and the passing criteria for each test.

Assessment Parameter	Test Description	Passing Criterion
Template specificity	Melting curve analysis of different PCR products	Single melting peaks
PCR efficiency	Amplification of a standard curve (relative concentration range 1X–256X)	Efficiency of 90–110% is widely acceptable [2-4]
Long target amplification	Amplification of a 1.5kb-long target	Robust amplification curve
Low-input sample amplification	Amplification of a sample that was diluted 1,000-fold	Robust amplification curve

Template specificity

First, we set out to compare the ability of each master mix to amplify specific human targets without interference from homologous targets. For this, we designed primers specific for the B2M, IKBKG, and NDRG2 genes, which produce amplicons ranging in size from 57-94bp. These primers were used to amplify a human cDNA sample, in triplicate reactions, with the aid of either of the 3 master mixes in parallel (on the same plate).

A specific PCR reaction should yield a single PCR product, which is reflected in the presence of a single melting peak in a melt curve analysis on a real time PCR machine. Our EverGreen produced a single, clean melting peak in all triplicates of the 3 assays, indicating specific amplification of the PCR targets (figure1). On

the other hand, although the Prom master mix produced a single, clean melting peak in all 3 assays, it had a weak amplification (low melting peak) in 2 of the NDRG2 replicates, failing to amplify the third. The Vazm master mix produced a single, specific melting curve only in the B2M triplicates, having two or more peaks in all replicates of the other 2 assays.

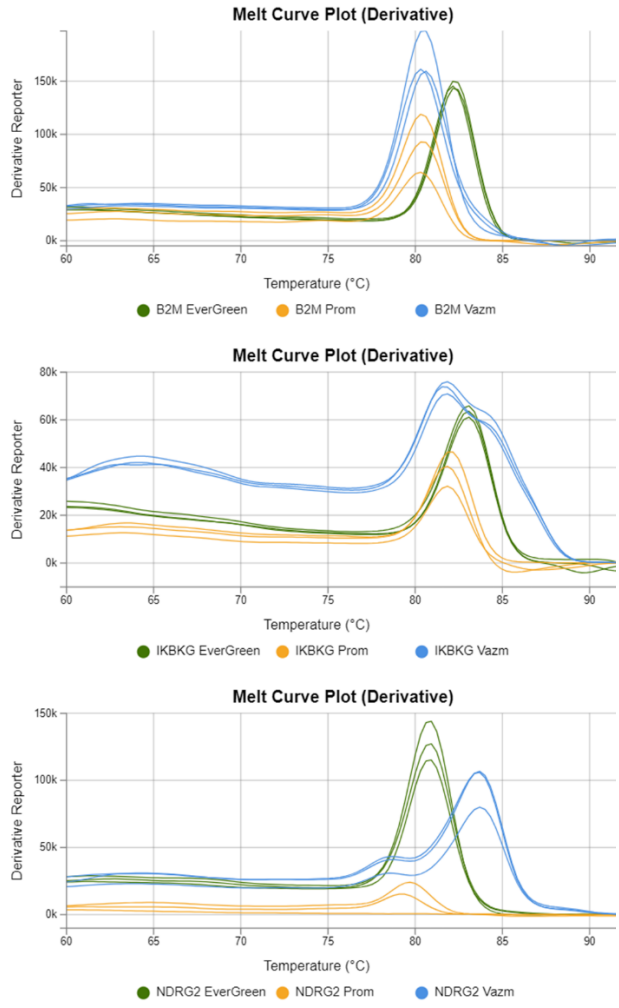

***Figure1**: Melting peaks for the B2M (upper panel), IKBKG (middle panel), and NDRG2 (bottom panel) assays amplified with our EverGreen, Prom, or Vazm master mixes.*

PCR efficiency

The theoretical efficiency of PCR is 100%, which indicates perfect doubling of the template after each cycle. However, a PCR efficiency in the range of 90-110% is widely acceptable [2-4]. To examine the ability of the three master mixes to support an efficient, consistent PCR performance, a 5-point, 4X serial dilution was constructed from a human WBC cDNA sample (1X to 256X standard curve). The master mixes were used to amplify the B2M gene in the serial dilution series in triplicate reactions and PCR efficiency and R^2 were calculated from the plot of log relative concentration against Cq value (figure2). Our Evergreen had a PCR efficiency of 95.6% with an impressive R^2=1, indicating a robust, consistent amplification. Similarly, the Vazm master mix had a PCR efficiency of 104.0% and an R^2=0.994, indicating a robust, consistent amplification. However, the Prom master mix had an efficiency of only 43% with R^2 of 0.925. This was obviously due to its lowered ability to amplify the 256X dilution, so it was omitted from the analysis to improve linearity (figure2e). Indeed, R^2 improved to 0.990, but PCR efficiency was still at a low 64.1%.

Amplification of different product sizes

We used two universal 16S rRNA assays to amplify, in triplicate reactions, either a ~580bp or a ~1,500bp segments of the S. typhimurium genome (ATCC 14028) using either of the 3 master mixes (figure3). All 3 master mixes were able to amplify the 580bp bacterial segment with similar threshold cycles, but, unlike our EverGreen and the Vazm master mixes, the Prom master mix failed to amplify the longer ~1,500bp segment in any of the 3 replicate.

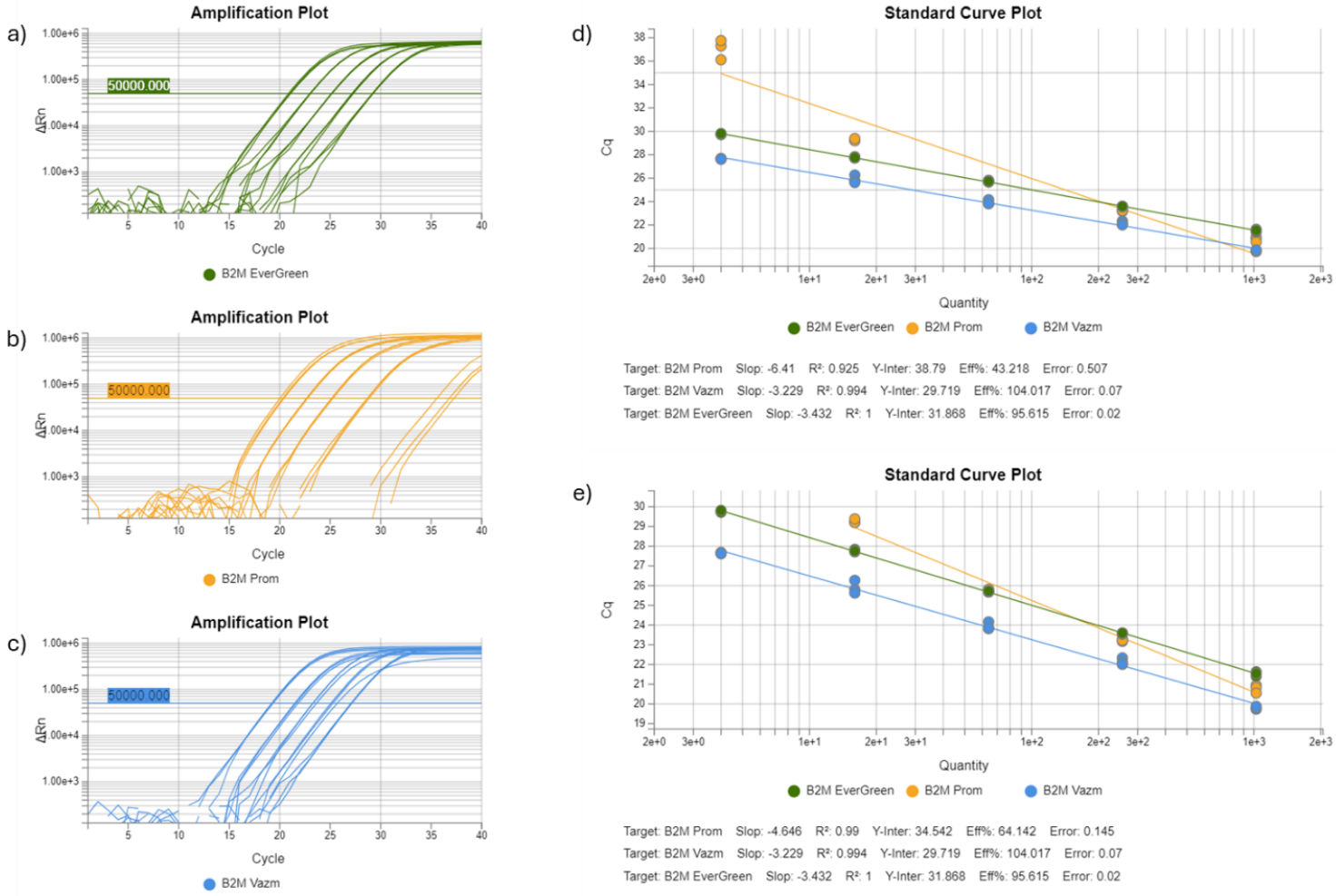

***Figure2**: A 4X serial dilution of a human WBC cDNA sample (relative concentration 1X to 256X) was used to amplify the human B2M gene with either the EverGreen (a), Prom (b), or the Vazm (c) master mixes. Threshold lines and their values are shown. PCR efficiency and R^2 are calculated from plotting the log of relative quantity against Cq value (d). The plot was redrawn after removing the last dilution, 256X, for the Prom master mix (e) to improve linearity.*

Amplification of low input sample

To test the ability of the master mixes to amplify low input samples, the human WBC cDNA sample used previously was diluted 1,000-fold and the human B2M gene was amplified in triplicate reactions using either of the three master mixes. Both our EverGreen and Vazm master mixes were able to amplify the diluted sample at Cq's of 32 and 30, respectively, but the Prom master mix failed to amplify it (data not shown).

Compatibility with the different Real-Time PCR machines

Some real-time PCR machines require the passive reference dye ROX for fluorescence signal normalization and some do not. We purposefully ran the experiments on either the QuantStudio™ 5 (requires ROX) or the CFX Opus 96 (does not require ROX) machines to show that our master mix is versatile when it comes to the type of machine used, regardless of whether ROX is required by the machine.

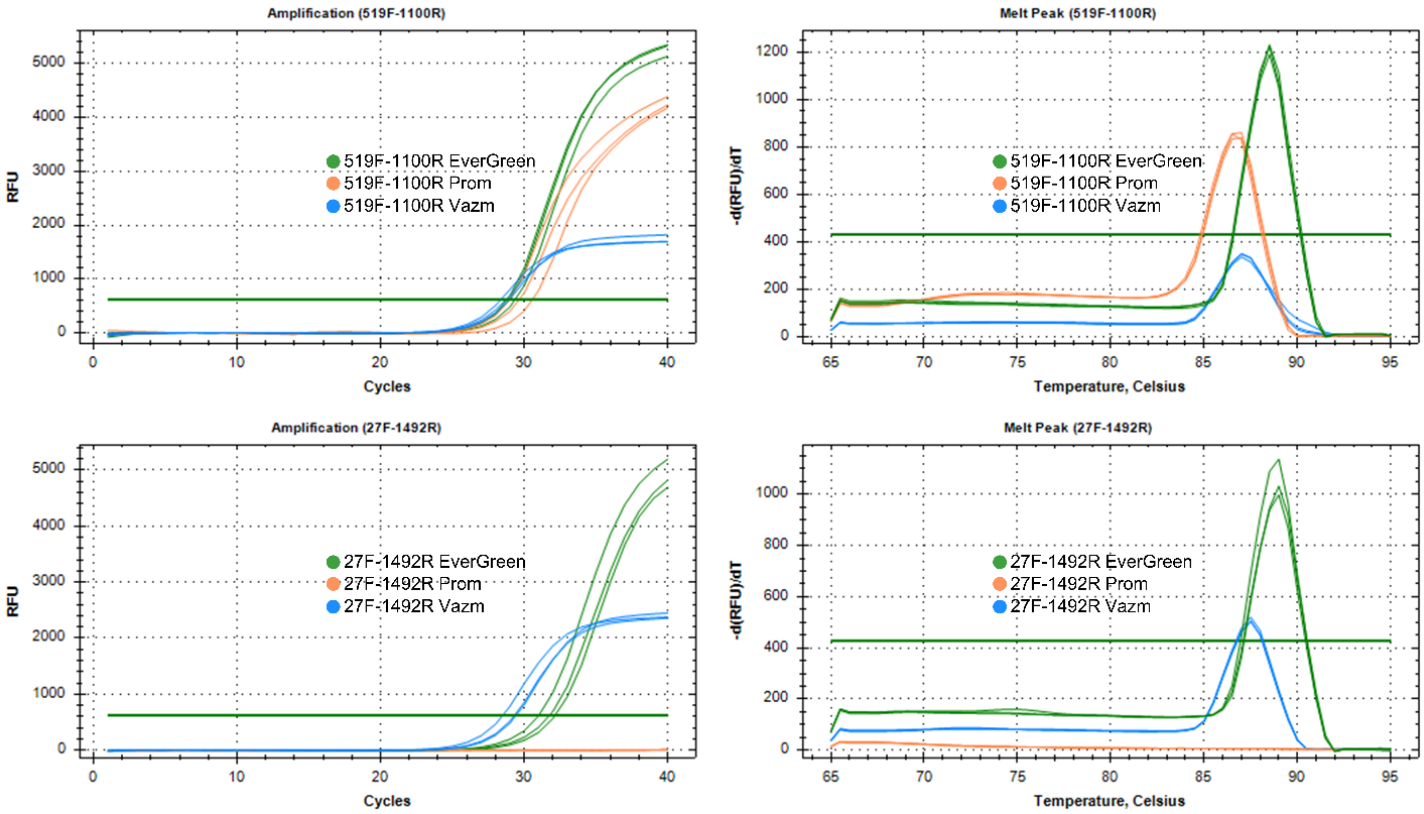

***Figure3**: Amplification of either a ~580bp (upper panel) or a ~1,500bp (lower panel) segments of the S. typhimurium genome using the EverGreen, Prom, and Vazm master mixes. Both amplification (left panel) and melting (right panel) curves are shown.*

Summary

We compiled a series of well controlled experiments with well-defined passing criteria to assess the performance of our EverGreen Universal qPCR Master Mix along with two equivalent master mixes from other suppliers. The master mixes were tested for whether they can support template-specific PCR reactions, efficient PCR assays, amplification of long targets, and amplification of low input samples. Our EverGreen master mix passed all of these parameters successfully, unlike the competitor master mixes that had to sacrifice the success of one or more parameters for the sake of the others (table2).

Taken together, these studies illustrate that our EverGreen is an efficient, robust, versatile master mix that can support a wide range of real-time qPCR applications in any commercially available PCR machine, making it a truly universal real-time qPCR master mix.

Table2: Summary of the performance of each master mix in terms of the 4 parameters outlined in table1. A passed parameter is indicated by a check mark (✓) and a failed parameter by a cross mark (✗).

	EverGreen	Prom	Vazm
Template specificity	✓	✓	✗
PCR efficiency	✓	✗	✓
Long target amplification	✓	✗	✓
Low-input sample amplification	✓	✗	✓

References

1. Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry, 55(4), 611–622.
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3. Geraci-Yee, S., Allam, B., & Collier, J. L. (2022). Keeping up with advances in qPCR pathogen detection: an example for QPX disease in hard clams. Diseases of aquatic organisms, 148, 127–144.
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