Rapid SYBR® Green qPCR on the Illumina Eco™ Real-Time PCR System

KAPABIOSYSTEMS

Fast real-time PCR is a demanding application that requires consistent and reproducible results from difficult amplicons and low-volume reactions.

The combination of the Illumina Eco™ Real-Time PCR System and KAPA SYBR® FAST qPCR Kits from Kapa Biosystems provides an industry-leading solution for low volume, fast real-time PCR without compromising reaction efficiency, specificity and reproducibility.

Introduction

The number of applications utilizing real-time PCR (qPCR) is expanding rapidly. The improved accuracy of results relative to end-point PCR, multiplexing capability, reduced risk of amplicon contamination, and streamlined time-to-results from closed-tube qPCR assays are factors contributing to the growth of real-time PCR. Common qPCR applications include: gene expression profiling, genotyping, copy number detection, validation of siRNA knockdown, miRNA assays, bacterial and viral load determination, ChIP qPCR, and next-generation sequencing library quantification.

The specificity and efficiency of qPCR depend upon precise temperature control during the denaturation and annealing steps. For the highest accuracy, the temperature must remain uniform across the entire heating block to ensure all samples are processed equally. Standard SYBR® Green I-based qPCR cycling protocols take between 1 hr to 2 hrs, depending on the ramp rate and data acquisition times of the specific qPCR instrument used (Figure 1). Faster cycling protocols (~45 min) that do not compromise reaction efficiency typically require instrumentation with increased thermal ramp rates, uniform heating across the block, and specially formulated qPCR reagents.

The Illumina Eco™ Real-Time PCR System is based on a proprietary thermal system that enables highly uniform temperature control and fast ramp rates. To achieve accurate temperature control, the Eco™ thermal system incorporates a precisely electroformed hollow silver block that is heated and cooled by a single Peltier device. During PCR cycling, conductive fluid circulates rapidly throughout the hollow chamber, transferring heat from the Peltier device evenly across the entire block. This unique design virtually eliminates thermal non-uniformity and block edge effects, providing a new level of thermal performance of ± 0.1 °C well-to-well uniformity across a 48-well plate. The result is higher qPCR performance, including tighter replicates and improved reaction efficiency and specificity.

Wild-type *Taq* DNA polymerase is theoretically capable of a 30 second combined annealing and extension time required for short qPCR amplicons. However, inhibition due to SYBR® Green I dye, steric hindrance from templates with high GC or AT content, and lower enzyme activity at 60 °C can result in compromised reaction efficiencies or even reaction failure when fast qPCR cycling protocols are used in conjunction with reagents containing wild-type *Taq* DNA polymerase.

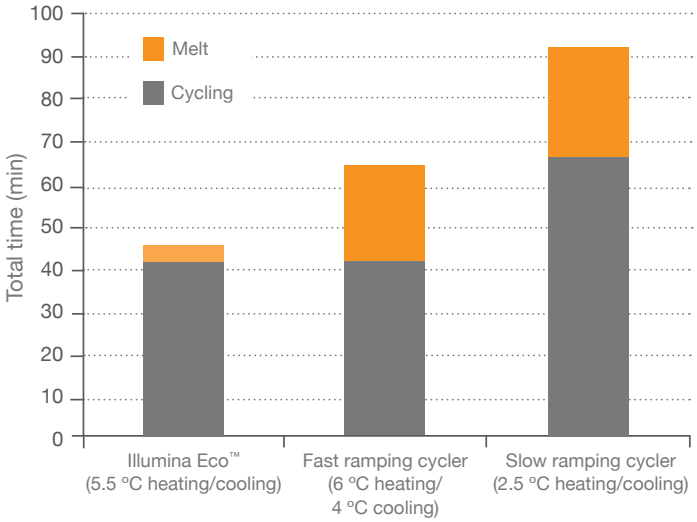

Figure 1: Total qPCR run time of Illumina Eco™ Real-Time PCR system in comparison to instruments with slow and fast ramp rates. The same fast qPCR cycling and melting protocol was run on all three instruments (40 cycles of 5 sec at 95 °C and 30 sec at 60°C, 60 °C - 95 °C melt). The slow ramping thermocycler has an average ramp rate of 2.5 °C/sec while the fast ramping thermocycler has an average ramp rate of 6 °C/sec heating and 4 °C/sec cooling. The Illumina Eco™ Real-Time PCR instrument has an average ramp rate of 5.5 °C/sec. In addition, the Eco™ supports continuous data collection in a single channel during melt analysis, resulting in increased data points and considerably reduced melt run times.

In contrast, the KAPA SYBR® FAST qPCR Kit contains a second-generation DNA polymerase with an intrinsic ability to synthesize DNA much faster than wild-type *Taq* DNA polymerase. The KAPA SYBR® DNA Polymerase was engineered via molecular evolution

in the presence of elevated concentrations of SYBR® Green I dye, resulting in an enzyme that exhibits less SYBR® Green inhibition and a faster extension rate than wild-type *Taq* DNA polymerase. High signal strength, which is particularly useful when performing low-volume qPCR, is achieved by an increased concentration of SYBR® Green I dye in KAPA SYBR® FAST master mix. KAPA SYBR® FAST qPCR Kits are supplied with an antibody-based hot start, enabling a short initial activation hold time at 95 °C. PCR protocols using KAPA SYBR® DNA Polymerase are primarily based on reduced extension times that allow for a significant reduction in PCR cycling time without the risk of compromising reaction performance.

PCR reaction setup and cycling conditions

Primers were designed using Beacon Designer™ 7 to amplify AT- and GC-rich regions on human genomic DNA (hgDNA). The hDMD primer set amplified a 76.9% AT-rich target amplicon; the ss48422848 primer set amplified a balanced AT/GC target amplicon (51%) while the hKCNG1 primer set amplified a 78% GC-rich target amplicon.

Amplification of these targets was performed using either a standard or fast cycling protocol in 10 µl reaction volumes. These reactions were set-up in 48-well Eco™ plates with optical Eco™ adhesive seals using the Illumina Eco™ Real-Time PCR System. The KAPA SYBR® FAST 10 µl reaction set-up is shown in Table 1.

Table 1: KAPA SYBR® FAST qPCR Kit reaction setup

Reaction component	Final conc.	Per 10 µl reaction[1]
PCR grade water	–	Up to 10.0 µl
2X KAPA SYBR® FAST qPCR Master Mix	1X	5.0 µl
Forward primer (10 µM total)	0.2 µM	0.2 µl
Reverse primer (10 µM total)	0.2 µM	0.2 µl
hgDNA template	15 ng - 15 pg	1.0 µl

[1] Reaction volumes between 5 µl and 20 µl are recommended for the Illumina Eco™.

Table 2: Standard cycling parameters for the Illumina Eco™

Cycling step		Temperature and time	
Initial denaturation		5 min at 95 °C	Hold
Denaturation		15 sec at 95 °C	x 40 cycles
Annealing/Extension[1]		60 sec at optimal Ta	
Melt	Melt	95 °C	15 sec
	Anneal	60 °C	15 sec
	Melt	95 °C	15 sec

[1] The optimal annealing temperature (Ta) of each primer set varied. The Ta for the AT-rich and balanced amplicons was 60 °C. The Ta for the GC-rich amplicon was 67 °C.

Table 3: Fast cycling parameters for the Illumina Eco™

Cycling step		Temperature and time	
Initial denaturation		5 min at 95 °C	Hold
Denaturation[1]		5 - 15 sec at 95 °C	x 40 cycles
Annealing/Extension[2]		30 sec at optimal Ta	
Melt	Melt	95 °C	15 sec
	Anneal	60 °C	15 sec
	Melt	95 °C	15 sec

[1] The cycling denaturation time was extended to 15 sec for the GC-rich region.
[2] The optimal annealing temperature (Ta) of each primer set varied. The Ta for the AT-rich and balanced amplicons was 60 °C. The Ta for the GC-rich amplicon was 67 °C.

Effect of reaction volume on total fluorescence and robustness

Reduced qPCR running costs, particularly in combination with increased throughput, are always desirable. KAPA SYBR® FAST qPCR Kit delivers high levels of fluorescence and consistent C_q values, even when used at extremely low reagent volumes. This is illustrated when four different reaction volumes (2.5 µl, 5 µl, 10 µl and 20 µl) were used to amplify ss48422848, a 150 bp target amplicon with a balanced AT/GC content, using a standard cycling protocol (Table 2). A 1X master mix containing KAPA SYBR® FAST Master Mix, primers and template was prepared and the reaction volumes mentioned were aliquoted into PCR wells in replicates of six. As the reaction volume was halved, the resulting C_q values reflected the expected 2-fold change in copy number even at the lowest reaction volume (20 µl = C_q 20.9, 10 µl = C_q 22.0, 5 µl = C_q 23.0, 2.5 µl = C_q 24.1).

A decrease in the reaction volume resulted in a lower background subtracted fluorescence. The lowest reaction volume still provided a robust signal due to the presence of a high concentration of SYBR® Green I dye in KAPA SYBR® FAST qPCR Master Mix (Figure 2).

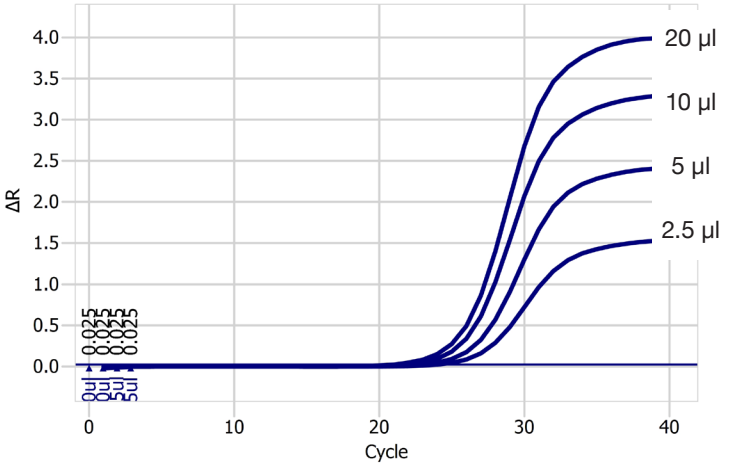

Figure 2: Decrease in background subtracted fluorescence with a decrease in reaction volume. A uniform decrease in background subtracted fluorescence is obtained with a uniform decrease in volume. Consistent amplification and a robust signal is still obtained at 2.5 µl which is the lowest reaction volume recommended.

Effect of different cycling protocols on efficiency and specificity across a range of AT- and GC-rich target amplicons

To demonstrate the ability to perform fast qPCR cycling on AT and GC rich target amplicons, without compromising performance, a set of four 10-fold serial dilutions of hgDNA was amplified using either a standard or fast cycling protocol. The base pair distribution of each amplicon is displayed as a GC% plot (Figure 4, top row). The resulting log amplification plots and melt curves for reactions performed using the standard protocol and fast protocol (Figure 4, middle row and lower row respectively) indicate that both the specificity and efficiency of the qPCR reactions have not been compromised as a result of using fast cycling protocols relative to standard cycling protocols (for a description of each protocol see Table 2 and 3).

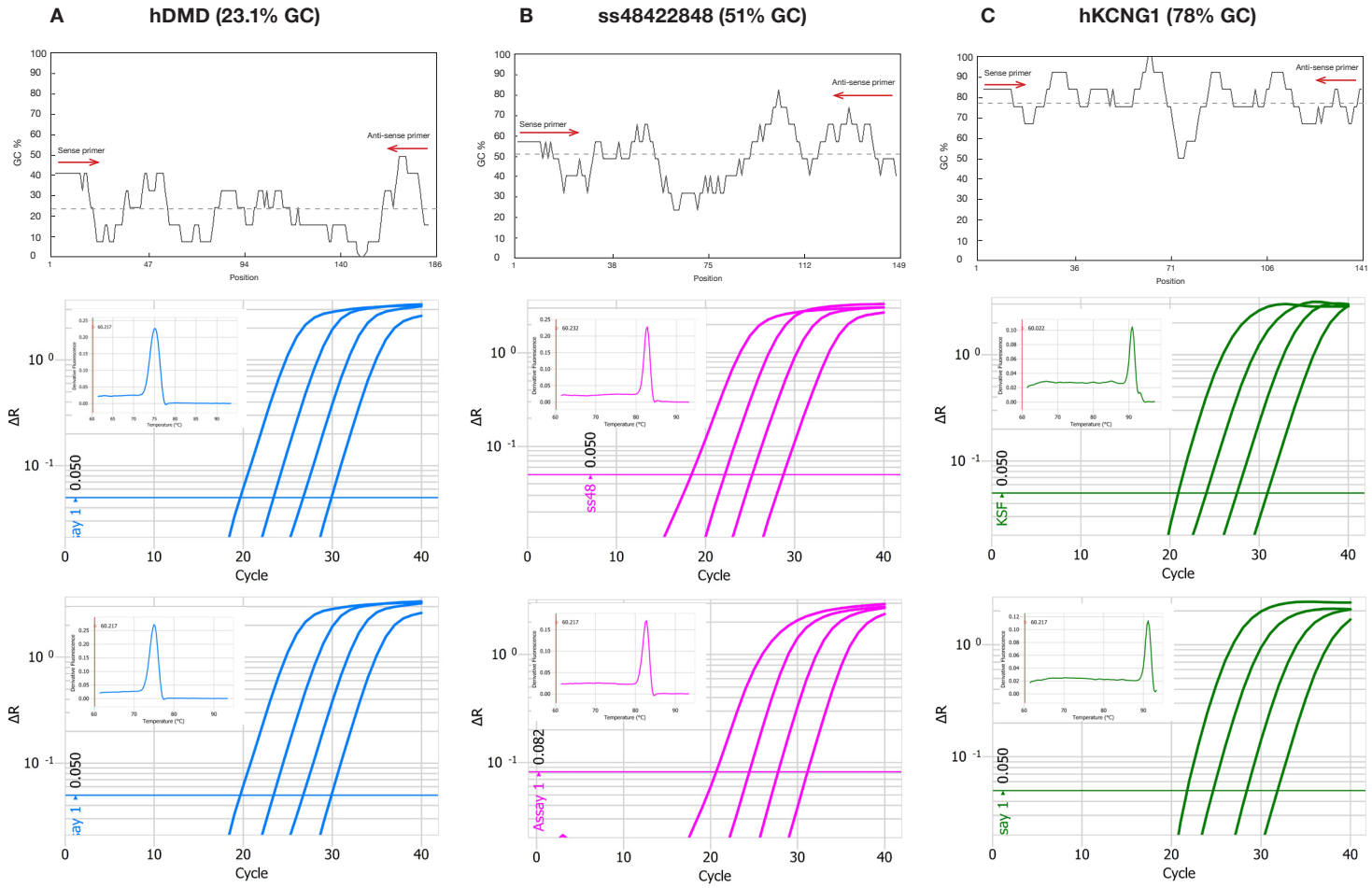

Figure 4: Effect of different cycling protocols on efficiency and specificity across a range of AT- and GC-rich target amplicons. hDMD, ss48422848 and hKCNG1 targets were amplified from a set of four 10-fold serial dilutions of hgDNA in 10 µl reactions using standard (middle row) and fast cycling protocols (lower row). The average GC content of each target amplicon is shown as the GC% plots (top row). The average GC% is indicated as a hatched line and primer binding sites are shown as red arrows. Reactions were set-up in replicates of three. Column A shows the amplification data for the AT-rich target amplicon (hDMD) which resulted in an efficiency of 96% for both the standard and fast protocols. Column B shows data obtained from the balanced AT/GC target amplicon (ss48422848). The efficiency obtained from the standard and fast cycling protocols were 96% and 93% respectively. Column C shows data obtained from the GC-rich target amplicon (hKCNG1). The efficiency obtained from the standard and fast cycling protocols were 99% and 96% respectively.

Conclusion

High-performance, fast qPCR on AT- and GC-rich targets requires a real-time instrument capable of extremely fast ramp rates with excellent temperature uniformity across all samples, and a qPCR reagent containing a DNA polymerase capable of synthesizing DNA significantly faster than wild-type *Taq* DNA polymerase. The combination of the Illumina Eco™ Real-Time PCR system and KAPA SYBR® FAST qPCR Kits provides an industry-leading solution for high-performance, fast qPCR without compromising reaction efficiency, specificity and reproducibility. In addition, the ability to perform low-volume qPCR can reduce running costs while maintaining high quality results.

For more technical support please contact **support@kapabiosystems.com**. For ordering information please contact **sales@kapabiosystems.com** or visit **www.kapabiosystems.com**.