ApplicationNote

Quantitative PCR Using SYBR® Green JumpStart™ Taq ReadyMix™: Lights Your Way to Quantitative PCR and RT-PCR

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Introduction

Quantitative PCR (qPCR) has developed into a popular technique since being pioneered by Higuchi et al. in the early 1990s.[1] Two basic chemistries, the keys to making this technique extremely sensitive, have been used in qPCR systems. The first, a probe-based system, utilizes a sequence-specific probe for quantitation of the template of interest. In the second method, intercalating dyes fluoresce only when bound to double-stranded products generated by PCR. The design of highly specific primers and optimized reagents insure sensitive quantitation of the target amplicon. Quantitative PCR relies on identification of the first cycle that gives a signal over the background. Calculation of this threshold cycle (C_T) makes quantitation much more precise than end-point analyses.

Fluorescence-based chemistries coupled with instrumentation suitable for amplification, detection and quantification of DNA have made qPCR an especially powerful technique. Many qPCR instruments are currently available from a variety of vendors, however, most follow two basic formats – either a thermal block or a capillary-based system. Although each system has its own unique detection method, both types of systems are closed-tube and require no manipulation following amplification. Sigma-Aldrich has tested reagents in both a thermal block system as well as one that is capillary based. Data from both instruments will be shown in this report.

The sensitivity and precision of qPCR, due to the fluorescent chemistries as well as instrumentation, allows this technology to be compatible in innumerable applications.[2] Gene detection, measurement of transcription levels and allelic genotyping are some of the applications for which qPCR has become a vital technique.This report focuses on the use of SYBR® Green I dye with quantitative PCR. Accurate quantitation can be obtained over a large range of initial template amounts if conditions are optimal. SYBR Green JumpStart™ Taq ReadyMix™ has been shown to be linear over a 10^8-fold range of template concentrations, sensitive down to 10 copies and performs as well as competitive mixes while providing the convenience of a ReadyMix (master mix). Inclusion of anti-*Taq* antibody suppresses nonspecific product and allows SYBR Green I dye to intercalate solely into the template of interest.

Materials and Methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated. SYBR Green I dye was received from Molecular Probes (Eugene, OR) and pBAC-2cp was obtained from Novagen Corporation (Madison, WI).

DNA Templates

pBAC-2cp DNA was initially quantified spectrophotometrically at 260 nm.

Quantitative PCR Conditions

PCR experiments were conducted in the ABI PRISM™ 7700 Sequence Detection System (Perkin Elmer/Applied Biosystems, Foster City, CA) using SYBR Green JumpStart Taq ReadyMix (2X; Product Code S 4438) contains: 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 7 mM $MgCl_2$, 0.4 mM each dNTP (dATP, dCTP, dGTP, TTP), stabilizers, 0.05 unit/µl Taq DNA Polymerase, JumpStart Taq antibody, and SYBR Green I). All reactions were performed in 50-µl reaction volumes that contained 1X SYBR Green JumpStart Taq ReadyMix, 1X internal reference dye, 400 nM each of plasmid-specific primers, and various concentrations of template DNA.

Sensitivity experiments were performed using pBAC-2cp as a template, and plasmid-specific primers designed to produce a 311 bp amplicon. The PCR was initiated with a 1-minute denaturation step at 95 °C. Initial denaturation was followed by 40 cycles at 95 °C for 15 seconds, 1-minute annealing at 60 °C, and 1-minute extension at 72 °C. Cycling was followed by a 4 °C hold. The template was diluted ten-fold past zero copies. Analyses of data were accomplished using the ABI PRISM 7700 Sequence Detection Software.

Quantitative RT-PCR (qRT-PCR) reactions were performed on the Roche LightCycler (Roche Molecular Biochemicals, Indianapolis, IN). RNA was extracted from ~10^5 cells using the GenElute™ Mammalian Total RNA Miniprep Kit (Product Code RTN-10). Reactions were conducted in 20-µl reaction volumes and contained 0.2 µM each primer, 0.2 units eAMV, 1X SYBR Green RT-PCR ReadyMix (to be released in the near future, and 2 µl (4 % total yield) total RNA. The reactions were incubated at 48 °C for 20 minutes, then denatured at 94 °C for one minute, and finally subjected to 45 cycles of 95 °C for 0 (zero) seconds, 55 °C for 7 seconds, and 72 °C for 13 seconds.

Molecular Biology

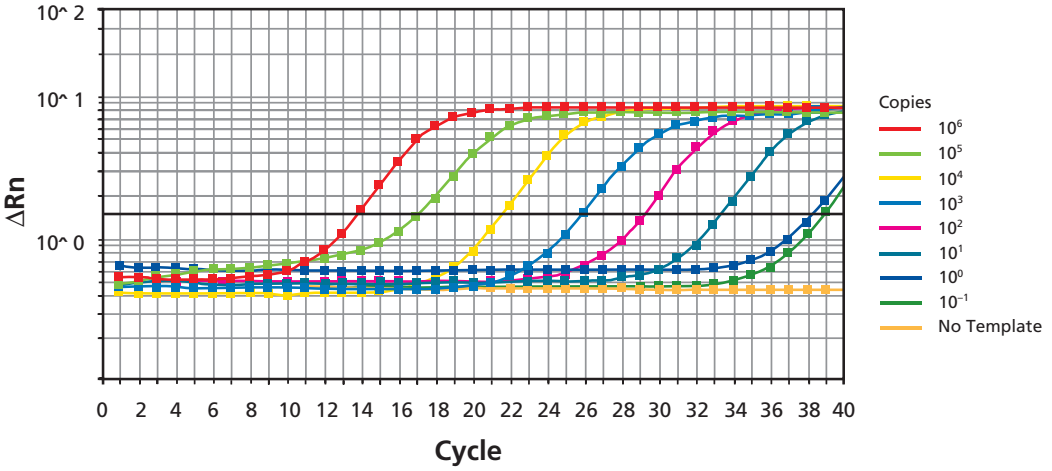


Figure 1. Amplification Plot showing 10- fold dilutions of pBac-2cp. *SYBR Green JumpStart Taq ReadyMix was added to dilutions of pBac-2cp template and plasmid specific primers. Initial template copy number was 10^6 and diluted 10-fold in subsequent wells. 10^{-1} signifies a 10-fold dilution of the previous well. Specific primers were designed to produce a 311 base pair amplicon. Reactions were carried out and analyses were done on the ABI Prism 7700 Sequence Detection System.*

Results and Discussion

SYBR Green JumpStart Taq ReadyMix produces accurate results over a large linear range

The linear range over which SYBR Green JumpStart Taq ReadyMix is useful was explored in a series of experiments in which several different templates were analyzed. A representative experiment is shown in Figure 1, and the analysis of this set of reactions is in Figure 2. SYBR Green JumpStart Taq ReadyMix has been shown to produce results that are linear over 8 logs of initial template concentrations. In addition, the data show that the use of SYBR Green JumpStart Taq ReadyMix allows accurate quantification of as few as ten copies. Results are reproducible over a wide range of starting template amounts.

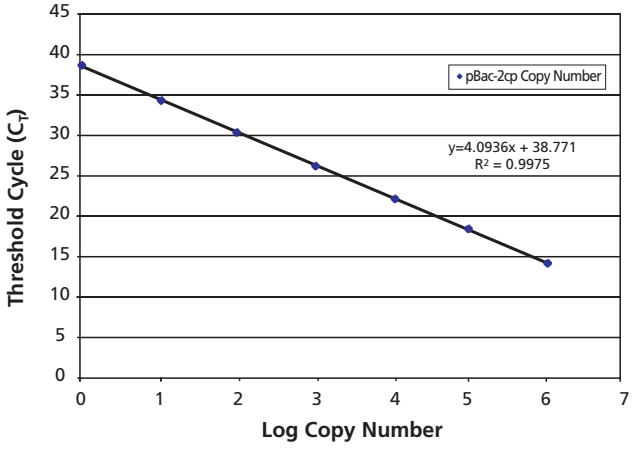


Figure 2. Linearity of pBac-2cp dilutions. *Ten-fold dilutions of the template are linear over 7 logs.*

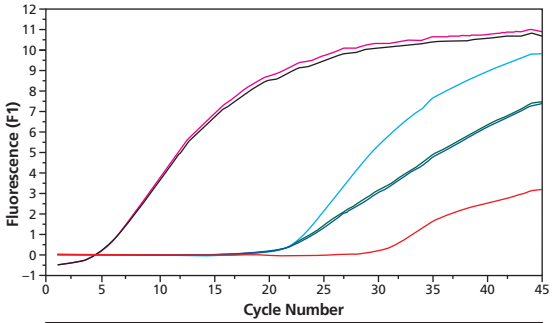
Low specificity is often a major drawback when using SYBR Green I in quantitative PCR reactions. Non-specific products formed in the PCR reaction contribute to the fluorescent signal and produce C_T readings that appear much earlier in the cycling process than should actually be seen. The inclusion of a hot start mechanism in the qPCR reduces non-specific product information and produces more accurate readings.

Additionally, an internal reference dye is important in quantitative PCR for the normalization of reporter dye signal. It becomes increasingly important when two or more reporter dyes are present in the PCR reaction. Normalization produces less error in the final data analysis and keeps high C_Ts more accurate.

Sigma's reference dye is included as a separate component in the SYBR Green qPCR system, allowing the user to decide when to add the reference. With an emission maximum of 605 nm, the dye is far removed from the reporter signal and will not interfere with its fluorescence.

Effect of Media on expression of a target protein

SYBR Green I dye can be used in quantitative PCR as well as in applications for both one-step and two-step RT-PCR. Use of the intercalator for qRT-PCR is widespread. A representative example shows how the technique can be used to evaluate the effect of media on expression of a target protein. Human cells were tested for expression levels of 18S ribosomal RNA (a control reaction) and P63 mRNA, the target of interest. The amplification plot shown in Figure 3 demonstrates the expression levels of the two targets. Analysis of the crossing points shows that the 18S controls have lower threshold values therefore showing higher expression level than the target of interest P63 mRNA. The data also show that the product generated by the RT-negative controls yield a genomic DNA-based signal but at a much-reduced level than the reactions that include reverse transcriptase. In addition, the melt curve analysis confirms specific product formation and emphasizes the reduction of primer dimers by JumpStart Taq (Figure 4). These conclusions were reinforced by agarose gel electrophoresis (data not shown). As shown in this application, the speed and flexibility of the LightCycler/SYBR Green dye system allow for results in minimal time without compromising accuracy and precision.



	Threshold cycle (C_T)	T_m °C
18S primers, + reverse transcriptase	5.972	82.71
18S primers, + reverse transcriptase	6.393	82.71
18S primers, – reverse transcriptase	23.24	82.71
P63 primers, + reverse transcriptase	23.93	82.24
P63 primers, + reverse transcriptase	23.66	82.24
P63 primers, – reverse transcriptase	33.15	81.48

Figure 3. *Amplification plot verifying expression levels in 18S control and P63 target template.*

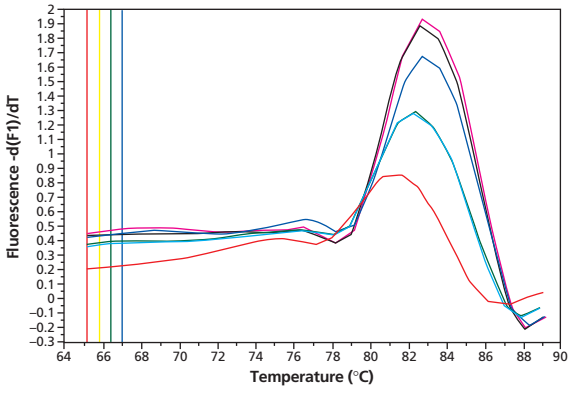


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Figure 4. *Melt curve analysis on the LightCycler shows separation of specific products while suppressing primer dimer.*

Summary

The advantages of Sigma's SYBR Green JumpStart Taq ReadyMix can be readily seen when performing high throughput qPCR. Quantitative RT-PCR with Sigma's SYBR Green RT-PCR ReadyMix shows the same high quality results. Ease of use combined with high sensitivity and broad linear range, combined with improved specificity from JumpStart Taq antibody make the SYBR Green ReadyMixes ideal for qPCR and qRT-PCR. Addition of an internal reference dye increases accuracy by signal normalization over multiple cycles and conditions. Furthermore, SYBR Green I dye can be used to quantify a wide variety of templates in many applications.

Acknowledgements

I acknowledge my colleagues at Sigma-Aldrich R&D, especially Ernie Mueller, for providing the SYBR Green RT-PCR data.

References

1. Higuchi, R., Dollinger, G., Walsh, P. S. and Griffith, R., Simultaneous amplification and detection of specific DNA sequences. Biotechnology (NY), **10,** 413-417 (1992).

2. Bustin, S.A., Absolute quantification of mRNA using real-time reverse transcription polymerase. Journal of Molecular Endocrinology, **25,** 169-193 (2000).

About the Author
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ORDERING INFORMATION

Product Code	Product Description	Unit
S 4438	SYBR® Green JumpStart™ Taq ReadyMix™	100 reactions
		500 reactions

SUGGESTED LITERATURE

Q 4379	Quantitative PCR Protocols (1999)	1 each
R 5277	RT-PCR Protocols (2002)	1 each