SsoFast™ EvaGreen® Supermix Stability for Automated qPCR

Yan Wang and Lisa Cho, Bio-Rad Laboratories, Inc., Life Science Group, Gene Expression Division, 2000 Alfred Nobel Drive, Hercules, CA 94547 USA

Introduction

Quantitative PCR (qPCR) is a powerful technique for quantitation, mutation analysis, copy number variation, and genotyping studies. Many of these applications increasingly require high-throughput experiments utilizing automated real-time PCR systems. Automated real-time PCR systems typically integrate a real-time PCR instrument with a plate stacker and a bar code reader, allowing multiple plates to be sequentially run without user intervention. Unlike manual workflows, assay plates in automated systems are stored at room temperature in the plate stacker for extended periods while the other plates in the queue are processed. Therefore, a requirement for these workflows is that the reaction mix must be stable at room temperature for extended periods of time.

Hot-start PCR is often used for automated applications because of its ability to prevent nonspecific amplification at room temperature, which is typically well below the optimal annealing temperature for the primers, ensuring consistency across all plates of a run. SsoFast EvaGreen supermix contains an antibody-mediated hot-start polymerase. At room temperature, the antibody-bound polymerase is inactive; upon heating to 95°C the antibody dissociates, activating the polymerase. For compatibility with an automated system, the antibody-polymerase complex in SsoFast EvaGreen supermix must remain stable at room temperature for the duration of the run to ensure that the polymerase activity is sufficiently inhibited to prevent nonspecific amplification.

We evaluated the stability and compatibility of SsoFast EvaGreen supermix for automation applications using two assays: (1) a qPCR assay that is prone to primer-dimer amplification was used to evaluate the stability of the hot-start function of the SsoFast EvaGreen supermix; (2) a human genomic DNA (hDNA) assay and a cDNA qPCR assay were used to assess the stability of the SsoFast EvaGreen supermix for use in quantitation assays over different concentration ranges.

Experimental plates were incubated at room temperature for 48 hours prior to the run, and the results were compared to control plates which were run immediately after reaction setup. Similar efficiency, dynamic range, specificity, and threshold cycle (C_T) values were observed, indicating that SsoFast EvaGreen supermix is suitable for automated real-time PCR applications.

Methods

Assay Design

The hDNA assay (TIP116) was designed to be prone to primer-dimer amplification by using a reverse primer (TIP116R) with 3' self-complementarity (Figure 1A). The primers amplify a 116 bp region of the human *THYMU2* gene. The primers were purchased from Integrated DNA Technologies, Inc. (IDT), and hDNA was purchased from Clontech Laboratories, Inc.

The cDNA assay (18S_68) was designed as a more representative assay without a strong tendency to form primer-dimers. The primers (Figure 1B) amplify a 68 bp section of cDNA corresponding to the human 18S rRNA gene. The cDNA was generated using iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Inc.) from HeLa total RNA (Life Technologies Corporation).

A

TIP116F: 5' TATGCCTCCACCAAGGAGAGC 3'

TIP116R: 5' TTGGAGAGGATCGGCTGAGC 3'

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5' TTGGAGAGGATCGGCTGAGC 3'
                | | |  | | |
    3' CGAGTCGGCTAGGAGAGGTT 5'
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B

18S1: 5' GAGGGAGCCTGAGAAACGG 3'
18S2: 5' GTCGGGAGTGGGTAATTTGC 3'

Fig. 1. Primer sequences for qPCR assays. A, TIP116 hDNA assay primers and expected primer-dimer; **B**, 18S_68 cDNA assay primers.

Hot-Start Stability Test

The reaction mix was prepared on ice using 500 nM of each TIP116 primer and 2.5 pg/µl of hDNA template in 1x SsoFast EvaGreen supermix (Bio-Rad).

To disrupt the antibody-polymerase interactions and remove the hot-start function of the supermix, 50 µl of reaction mix were dispensed in quadruplicate in a 96-well PCR plate (Bio-Rad) and heated to 95°C for 1 min. Twenty µl of the heat-inactivated reaction mix and 20 µl of unheated control reaction mix were dispensed side by side in quadruplicate into two 96-well PCR plates. The plates were sealed with optically clear seals (Microseal® 'B' adhesive seals, Bio-Rad), and spun briefly. The control plate was run immediately while the experimental plate was incubated at 24–26°C for 48 hr prior to qPCR. The 48-hour time point was chosen to ensure that the incubation time exceeds the expected room temperature exposure of reaction plates used for a typical automated qPCR run.

Functional Stability Tests

The reaction mix was prepared on ice using the corresponding primers and DNA templates with the following concentrations: 500 nM each primer and 50 ng–50 pg of hDNA or 10 ng–10 fg of cDNA in 1x SsoFast EvaGreen supermix.

The reaction mix (20 µl) was dispensed in triplicates or quadruplicates into white qPCR plates (Bio-Rad), sealed with Microseal 'B' adhesive seals, and spun briefly. Control plates (0 hr) were run immediately. Experimental plates were incubated at room temperature (24–26°C) for 48 hr prior to qPCR.

Amplification Protocol

Plates were run on a CFX96™ real-time PCR detection system (Bio-Rad). The TIP116 hDNA PCR protocol was 95°C for 2 min, followed by 45 cycles of 95°C for 10 sec and 60°C for 10 sec. Amplification was followed by a standard melt curve from 65 to 95°C, in increments of 0.5°C, holding for 2 sec at each step. The 18S_68 cDNA PCR protocol was 95°C for 30 sec, followed by 45 cycles of 95°C for 10 sec and 60°C for 10 sec. Amplification was followed by a standard melt curve as noted above.

Data Analysis

All data were analyzed using CFX Manager™ software, version 1.5 (Bio-Rad). For standard curves, auto-threshold was used. For C_T value comparison, threshold was manually set at 4% of the corresponding maximum raw fluorescence unit (RFU) value.

Results and Discussion

Hot-Start Stability Assay

Because of its high tendency to form primer-dimers, the TIP116 hDNA assay was expected to be very sensitive in monitoring any potential loss of hot-start function in a qPCR reaction mix. To verify that this assay is sensitive enough to monitor the hot-start stability of SsoFast EvaGreen supermix, the hot-start function was removed from the supermix by heating prior to the room temperature incubation and the qPCR run. At 0 hours, the reactions lacking hot-start function showed identical specificity to the reactions with intact hot-start function, with both yielding melt curves indicating a single amplification product (Figure 2A). After 48 hours of incubation at room temperature, the reactions lacking hot-start function showed significant primer-dimer amplification, whereas the reactions with intact hot-start function showed a single amplification product similar to the 0-hour plates (Figure 2B). This demonstrates that the TIP116 hDNA assay can detect loss of hot-start function. The detection of a single melt peak corresponding to a specific amplification product also demonstrates that the hot-start function of SsoFast EvaGreen supermix withstands 48 hours of room temperature incubation.

Human DNA Quantitation Assay

The TIP116 assay was used to evaluate the qPCR functional stability of SsoFast EvaGreen supermix. Serial dilutions of genomic DNA from 50 ng to 50 pg were amplified in a volume of 20 µl using the TIP116 primers, and the results were compared between the control plate (0 hours) and the experimental plate (48 hours). As shown in Figure 3, incubation at room temperature for 48 hours had no impact on qPCR performance with respect to dynamic range, amplification efficiency, and specificity.

Complementary DNA Quantitation Assay

The 18S_68 assay was selected as a well-designed representative assay to further assess the functional stability of SsoFast EvaGreen supermix for a wider dynamic range of quantitation from cDNA. A tenfold serial dilution of cDNA ranging from 10 ng to 10 fg in a volume of 20 µl was used as the template, and the results were compared between plates incubated at room temperature and the plate run immediately after they were prepared. Consistent results were observed between the two experimental conditions in dynamic range, amplification efficiency (Figure 4), and specificity (Figure 5). Moreover, there were few to no differences in C_T values between the incubated and the non-incubated plates across the cDNA dilution range (Table 1). The results confirm that SsoFast EvaGreen supermix maintains full functionality after prolonged incubation at room temperature.

Table 1. Comparison of C_T values for 18S_68 cDNA assay at 0 hours and 48 hours.

Template, ng	10	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
C_T (0 hr)	10.1	13.6	16.8	20.1	23.4	26.9	30.1
C_T (48 hr)	10.1	13.5	16.8	20.1	23.5	27.0	30.4
ΔC_T (0–48 hr)	0.0	0.1	0.0	0.0	–0.1	–0.1	–0.3

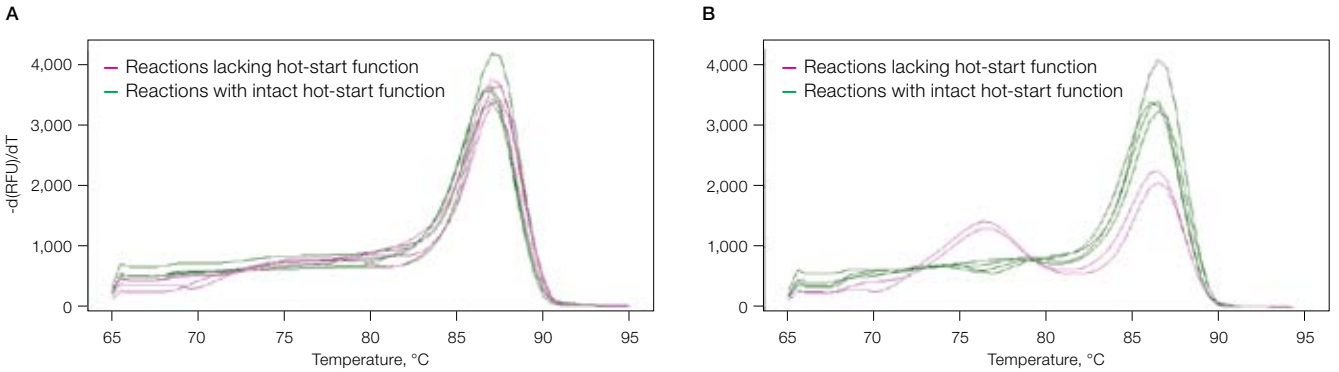

Fig. 2. Comparison of amplification specificity of qPCR reactions with or without incubation. A, control plate run immediately after preparation; **B**, plate run after 48 hr incubation at room temperature.

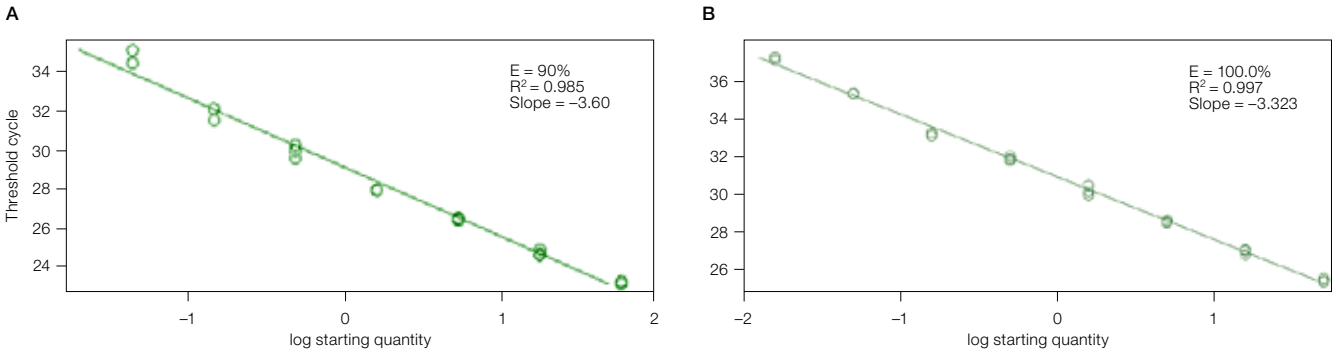

Fig. 3. Comparison of TIP116 hDNA assay performance with and without incubation. Standard curves were constructed using serial dilutions of hDNA. **A**, control plate run immediately after preparation; **B**, plate run after 48 hr incubation at room temperature.

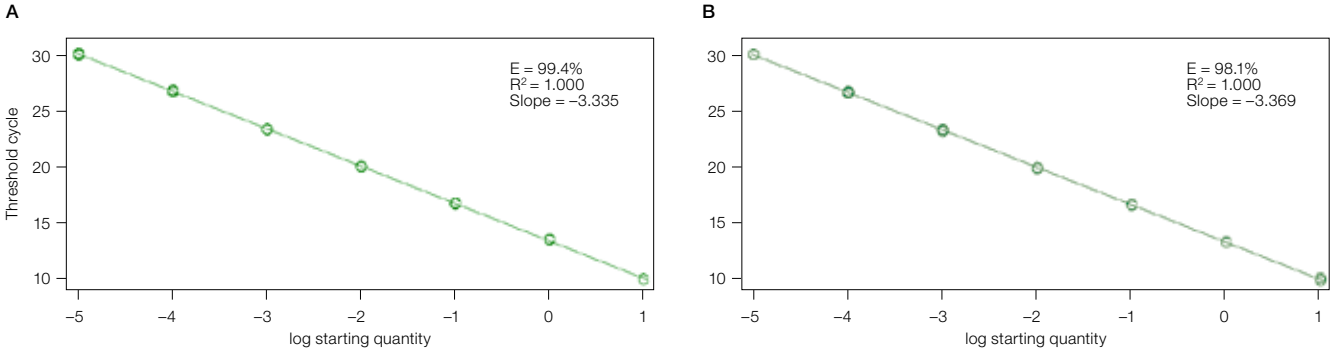

Fig. 4. Comparison of 18S_68 cDNA assay performance with and without incubation. Standard curves were constructed using serial dilutions of human 18S rRNA cDNA. **A**, control plate run immediately after preparation; **B**, plate run after 48 hr incubation at room temperature.

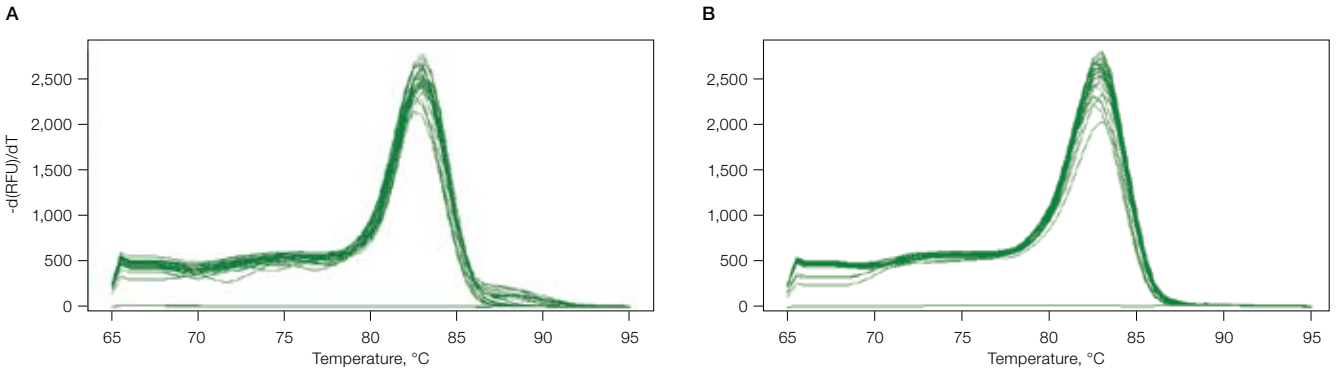

Fig. 5. Amplification specificity of the 18S_68 cDNA assay. Melt-curve analysis was performed on a serial dilution of human 18S rRNA cDNA. **A**, control plate used immediately after preparation; **B**, plate used after 48 hr incubation at room temperature.

Conclusions

In automated qPCR assays involving multiple plates, it is important to achieve consistent amplification of target genes across all plates of an experiment to ensure reliable quantitation of the targets and meaningful comparison of the results. Selection of a high-performance supermix with stable hot-start function together with proper primer design is essential to avoid amplifying primer-dimers and other nonspecific products (Boeckman et al. 2001). The findings of this study demonstrate that SsoFast EvaGreen supermix can be used to achieve highly consistent results over a large dynamic range of input templates with an automated run lasting up to 48 hours.

Reference

Boeckman F et al. (2001). Real-time PCR: general considerations. Bio-Rad Bulletin 2593.

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