

FailSafe™ PROBES Real-Time PCR Optimization Kit Greatly Improves Multiplex Performance

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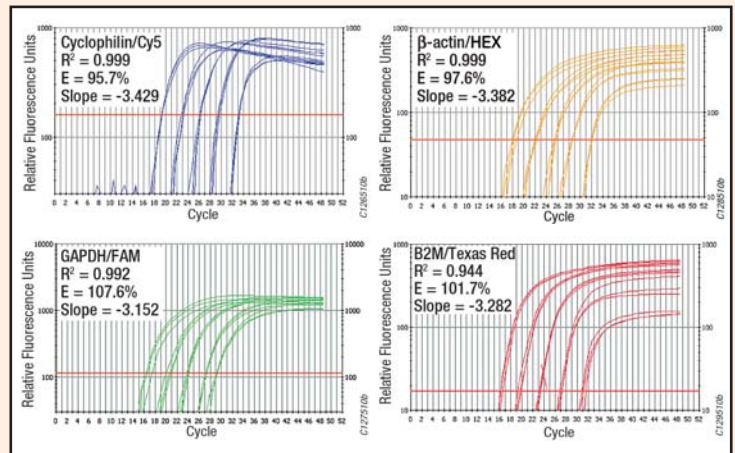
Introduction

Real-time quantitative PCR (qPCR) using fluorescently labeled nucleic acid probes, primers, or DNA-binding dyes, has become the method of choice for the detection and quantification of gene sequence targets.¹ qPCR has numerous benefits, but also presents many challenges, especially if the data obtained are to be technically accurate and biologically relevant. Multiplex qPCR is the simultaneous amplification of multiple targets with different fluorescent reporter dyes within a single reaction tube. This offers a number of advantages over qPCR of a single target gene in a single reaction (singleplex qPCR), including savings in time and reagents. Furthermore, in situations where there is limited template availability, multiplex qPCR allows the user to save precious sample while still detecting multiple targets.

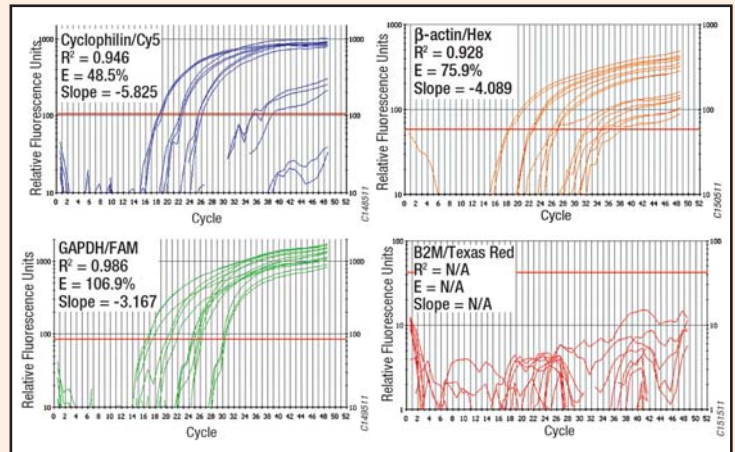
Multiplex qPCR requires careful experimental design and optimization so that the multiple sets of primers and probes used in each reaction vessel will not interact and compete for reagents. This interaction can negatively affect the amplification efficiency of one or more of the gene targets. For successful multiplex qPCR, a number of factors must be considered, including primer and probe design, fluorophore selection, and optimal concentrations of all reaction components, i.e., primers, probes, Taq DNA polymerase, dNTPs, MgCl₂ and PCR enhancers.

Multiplex qPCR optimization can be greatly simplified and expedited by using EPICENTRE Biotechnologies' FailSafe™ PROBES Real-Time PCR Optimization Kit.^a This system includes all the reaction components needed for optimal qPCR including a unique blend of thermostable DNA polymerases and a set of eight carefully selected 2X PCR PreMixes, and the FailSafe™ PCR Enhancer with Betaine.^b Simply add your template, primers, and labeled probes to the enzyme blend and each of the eight 2X PreMixes, cycle using standard qPCR reaction conditions, and select the PreMix that provides the best multiplex

A
FailSafe™
4-plex qPCR using
10 pg to 100 ng
HeLa cDNA



B
Supplier A
4-plex qPCR using
10 pg to 100 ng
HeLa cDNA



C
Supplier Q
4-plex qPCR using
10 pg to 100 ng
HeLa cDNA

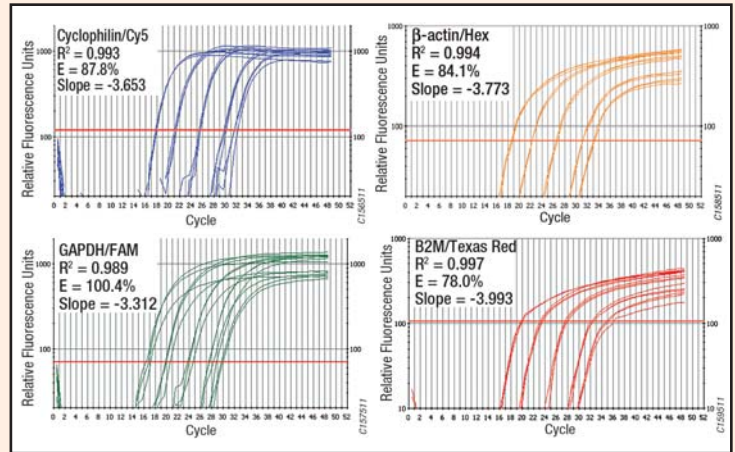


FIG 1. The superior dynamic range and amplification efficiency of the FailSafe™ PROBES Real-Time PCR Optimization Kit when compared to kits from suppliers A and Q. Multiplex qPCR was performed on four gene targets from four 10-fold serial dilutions of HeLa cDNA ranging from 100 ng to 10 pg. **Panel A**, Quantification graphs of FailSafe PROBES Optimization Kit amplification. **Panel B**, Quantification graphs of Supplier A qPCR Master Mix amplification. **Panel C**, Quantification graphs of Supplier Q qPCR multiplex Master Mix amplification.

Supplier	Singleplex			Duplex		
	Range of copies amplified	R ²	PCR Efficiency	Range of copies amplified	R ²	PCR Efficiency
FailSafe™	10-10 ⁷ copies	1.000	99.2%	10-10 ⁷ copies	0.999	100.2%
Supplier A	10-10 ⁷ copies	0.999	91.6%	10 ⁵ -10 ⁷ copies	0.997*	73.4%*
Supplier Q	10-10 ⁷ copies	0.996	106.7%	10 ⁴ -10 ⁷ copies	0.998**	104.8%**

*Values calculated based on 3-log dynamic range

**Values calculated based on 4-log dynamic range

Table 1. Superior amplification efficiency, higher sensitivity and wider dynamic range in singleplex and duplex qPCR reactions using the FailSafe™ PROBES Real-Time PCR System. Singleplex or duplex PCR using from 10 to 10⁷ copies of β -actin was performed in the presence of 10⁶ copies of GAPDH using the FailSafe PROBES System and kits from suppliers A and Q.

qPCR results. The kit is compatible with all qPCR instrument platforms and all types of fluorescently labeled, sequence-specific probes.

Superior dynamic range and excellent PCR efficiency of FailSafe™ PROBES multiplex PCR reactions when compared to two other suppliers

Methods and Results

Four gene targets, β -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-2-microglobulin (B2M), and cyclophilin A (peptidylprolyl isomerase A) were amplified simultaneously in the same PCR reaction tube. Four-plex (4-plex) qPCR was carried out using 10-fold serial dilutions of HeLa cDNA (100 ng to 10 pg cDNA), with all dilutions being done in triplicate. Probes for β -actin, GAPDH, B2M, and cyclophilin were 5'-labeled with the fluorophores HEX™, FAM™, Texas Red®, and Cy™5, respectively. Each 25 μ l 4-plex reaction contained the following components: 12.5 pmoles of each forward and reverse primer, 100 nmoles of each probe, 1X FailSafe™ PROBES PreMix P8, 1 U FailSafe PCR Enzyme Mix, and various amounts of HeLa cDNA. The reactions using the kits from suppliers A and Q were performed according to the manufacturers' recommended protocols. All reactions were set up at room temperature. Unlike suppliers A and Q, no hot-start reactivation of the DNA polymerase was needed for the FailSafe reactions. Real-time PCR experiments were performed on a Bio-Rad iCycler iQ® Real-Time PCR Detection System for 50 cycles of 15 seconds at 95°C and 90 seconds at 60°C. FIG 1 shows that EPICENTRE's FailSafe PROBES Real-Time PCR Optimization Kit produces superior PCR efficiency and dynamic range when compared to the kits from manufacturers A and Q.

Excellent efficiency, sensitivity and dynamic range when co-amplifying two targets with largely different copy numbers

Multiplex qPCR is most difficult when the target genes of interest are expressed at different levels. This is because competition for reagents can often lead to the high copy number target being preferentially amplified over the low copy number target. The following data compare singleplex and duplex qPCR of two gene targets present at drastically different copy numbers.

Duplex qPCR was carried out using from 10 to 10⁷ copies of the β -actin gene target in the presence of 10⁶ copies of the GAPDH gene target. Probes for β -actin and GAPDH were 5'-labeled with HEX and FAM, respectively. Singleplex reactions using the same copy numbers of β -actin and GAPDH target templates were also carried out. qPCR conditions were the same as those for the 4-plex assays described above, except that the annealing/extension time was reduced to 60 seconds, and FailSafe™ PROBES PreMix P3 was used with the FailSafe reactions.

In the singleplex qPCR reactions, all three kits yielded excellent efficiencies and dynamic ranges for β -actin and GAPDH (Table 1). However, EPICENTRE's FailSafe PROBES PCR System was 10³-10⁴ fold more sensitive in the duplex qPCR of β -actin than kits from suppliers A and Q. The FailSafe PROBES System also demonstrated the most consistent and replicable amplification graphs for GAPDH.

Verification of multiplex PCR performance

Multiplex qPCR data are only valid if they demonstrate the same amplification profile for a transcript as displayed in a singleplex reaction. Ideally, the threshold cycle (C_T) values between the two reactions should not differ by more than 1 cycle, which indicates that multiplexing does not alter the amplification of each gene target. Optimization is often required to achieve such similar amplification profiles.

Three gene targets (GAPDH, β -actin, and 18S rRNA) were amplified simultaneously in the same reaction tube (see FIG 2). qPCR data from the three-plex (3-plex) reactions were compared to the corresponding singleplex reactions of these three genes alone. Probes for GAPDH, β -actin, and 18S rRNA were 5'-labeled with FAM, HEX, and Cy5, respectively. One nanogram of HeLa cDNA was used as the PCR template. All reactions for both 3-plex and singleplex PCR were carried out in quadruplicate. qPCR experiments were performed on an Applied Biosystems 7500 Fast Real-Time System using the same conditions as described for the 4-plex assays. ROX™ was used as a normalizing dye for the reactions.

The data in FIG 2 demonstrate a perfect match between multiplex and singleplex qPCR results, suggesting that the FailSafe

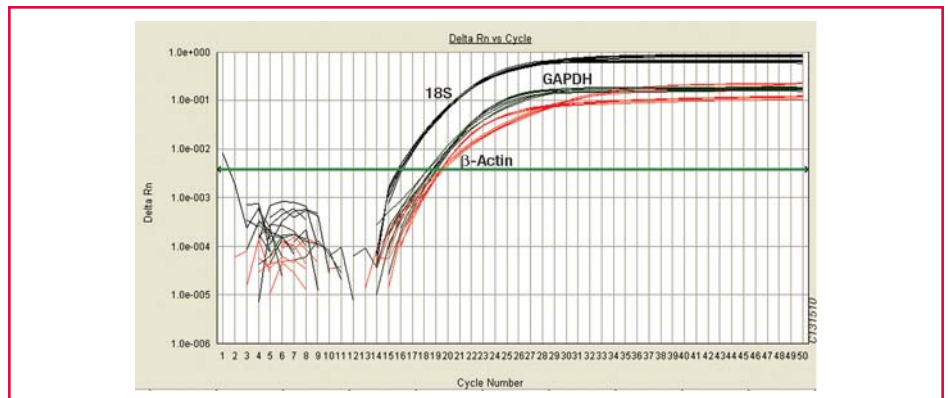


FIG 2. Performance verification of FailSafe™ multiplex qPCR data with corresponding singleplex data. 3-plex and singleplex data were superimposed on the same graph for all three gene targets. The C_T values match perfectly for all quadruplicates.

PROBES Systems are suitable for multiplex qPCR without compromising the sensitivity and specificity of singleplex PCR of a chosen gene target.

Optimization using FailSafe™ PROBES Real-Time PCR Optimization Kit

In order to effectively optimize multiplex qPCR reactions, the ultimate goal is to select the conditions that provide the fastest C_T values, the best PCR efficiency and widest dynamic range. However, it is time consuming and costly to examine each reaction component by performing a standard curve analysis for every set of primers/probe(s). The following data demonstrate the result of choosing the optimal FailSafe PROBES PreMix for a duplex qPCR when comparing C_T values obtained versus a complete standard curve analysis.

A duplex qPCR reaction was designed for single nucleotide polymorphism (SNP) analysis of the APOE (apolipoprotein E) gene. The detection of different SNP variations offered an excellent test of qPCR amplification efficiency since the target sequences are essentially identical. In addition, because the target gene was a heterozygote for the SNP, the two variations would also have identical starting copy numbers. The two probes were 5'-labeled with FAM and VIC™. Eight FailSafe PROBES qPCR PreMixes along with master mixes from suppliers A and Q were tested to select the reagents that

Supplier	FAM Channel			VIC Channel		
	C _T from 20 ng genomic DNA	R ²	PCR Efficiency	C _T from 20 ng genomic DNA	R ²	PCR Efficiency
FailSafe™ PreMix P7	31.5	.999	100.6%	31.2	0.996	93.0%
Supplier A	34.2	0.991	71.9%	35.7	0.972	141.2%
Supplier Q	35.2	0.993	136.5%	37.8	0.979	172.8%

Table 3. Standard curve analysis comparison between FailSafe™ PROBES PreMix P7 and master mixes from suppliers A and Q for duplex qPCR of the APOE gene.

provided the fastest C_T values for each target sequence. Twenty nanograms of human genomic DNA were used in each 25 µl reaction for optimization. Real-time PCR was performed on a Bio-Rad iCycler iQ Real-Time PCR Detection System; cycling conditions were set at 95°C for 2 minutes followed by 50 cycles of 95°C for 10 seconds, and 60°C for 1 minute, all reactions were performed according to individual manufacturer's recommended protocols. As indicated in FIG 3 and Table 3, FailSafe PROBES PreMix P7 provided the fastest C_T values for both the FAM and VIC channel. Amplification using master mixes from suppliers A and Q were ~3-6 C_T cycles slower than those obtained with FailSafe.

Standard curves for both target sequences were also generated using six 2-fold serial dilutions. Reaction components and cycling conditions were the same as previously described. As shown in Table 3, FailSafe demonstrated the best linearity and PCR efficiency.

Standard curve analysis is the most reliable way to determine the success of a multiplex qPCR reaction. To save time and sample, it is also possible to compare C_T values to determine the most favorable reaction conditions, then follow up with a standard curve analysis to verify the choice of FailSafe™ PreMix. The FailSafe™ PROBES Real-Time PCR Optimization Kit makes multiplex PCR optimization reliable, simple, accurate, and fast as shown by the data presented in this report.

Conclusion

The many challenges of a successful multiplex qPCR reaction often require careful optimization to achieve accurate data quantification. In these experiments, the FailSafe PROBES System demonstrated the best PCR efficiency, widest dynamic range, and highest sensitivity in a straightforward, reproducible manner. Additionally, the FailSafe System dis-

played excellent consistency between multiplex and corresponding singleplex qPCR reactions, thus verifying the functional performance of the multiplex PCR assays.

Reference

1. Bustin, S.A. et al., (2005) *J. Mol. Endocrinol.* 34(3), 597.

www.EpiBio.com/failsafeprob.es.asp

FailSafe™ PROBES Real-Time PCR Optimization Kit
 FSP51048 48 25µl Reactions
 Contents: FailSafe™ PCR Enzyme Blend, 8 FailSafe™ PROBES Real-Time PCR 2X PreMixes, Passive Reference Dye, and Stabilizer.

FailSafe™ PROBES Real-Time PCR PreMix-Choice Kit
 FSP51200 200 25 µl Reactions
 FSP5101K 5 X 200 25 µl Reactions
 Contents: FailSafe™ PCR Enzyme Blend, Passive Reference Dye, and stabilizer.
 FSP51200 includes any 2 of the 8 different FailSafe™ PROBES Real-Time PCR 2X PreMixes (your choice). FSP5101K includes any 10 of the 8 different FailSafe™ PROBES Real-Time PCR 2X PreMixes (your choice).

^aEPICENTRE's PCR products are sold under licensing arrangements with F. Hoffmann-La Roche Ltd., Roche Molecular Systems, Inc., and Applied Biosystems. The products containing a thermostable DNA polymerase are accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) and RT-PCR for life science research in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Applied Biosystems or as purchased, i.e., an authorized thermal cycler.

^bThe use of Betaine for DNA Polymerase Reactions, including, but not limited to use for PCR or DNA Sequencing, is covered by U.S. Patent No. 6,270,962, European Patent No. 0742838, German Patent No. DE4411588C1, and other issued or pending applications in the U.S. and other countries that are either assigned or exclusively licensed to EPICENTRE. These products are accompanied by a limited non-exclusive license for the purchaser to use the purchased products solely for life science research. Contact EPICENTRE for information on licenses for uses in diagnostics or other fields.

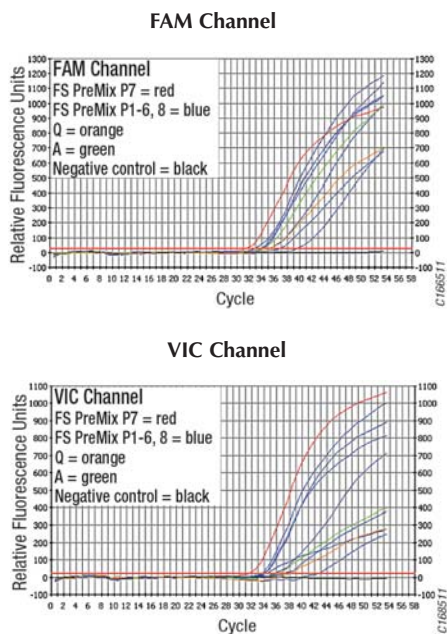


FIG 3. C_T value comparison among 8 FailSafe™ PROBES PreMixes and master mixes from suppliers A and Q for duplex qPCR of the APOE gene.