Factors influencing multiplex real-time PCR

Introduction

Multiplex PCR is the simultaneous amplification of more than one target sequence in a single reaction [1]. Specifically, duplex PCR is the amplification of two target sequences in one reaction, triplex PCR is the amplification of three targets, and so on. Multiplex real-time PCR is possible using Applied Biosystems™ TaqMan® Assays, in which each assay has a specific probe labeled with a unique fluorescent dye, resulting in a different emission spectrum for each assay. Real-time PCR instruments can discriminate between the different dyes. The signal from each dye is used to separately quantitate the amount of each target. Duplex PCR, which has several advantages over individual reactions (Table 1), is routinely performed in many research labs. However, setting up reliable multiplex PCR can be a challenge, as the results need to be verified and, in some situations, optimization of the reaction conditions may be necessary.

Table 1. Key benefits of multiplexing.

Benefit	Advantages of multiplexing
Cost savings	Fewer reactions are run, conserving expensive reagents (e.g., dNTPs, enzymes, endogenous controls)
Preservation of limited samples	Where sample amount is limited, multiplexing allows more targets to be analyzed using a single aliquot of sample material
Reliability (e.g., reduced effect of pipetting errors)	Data quality can be improved because the target of interest is normalized to the endogenous control within the same aliquot of the sample

Challenges involved in multiplex real-time PCR

Multiplex assays must always be verified and often require optimization. Depending on the targets being analyzed and the samples being used, reaction optimization and verification could range from a simple, straightforward exercise to a costly, time-consuming endeavor. The cost and time involved increase with the number of targets to be investigated. It is absolutely essential that results obtained from multiplex reactions be verified to confirm that the same results would be obtained if the reactions were performed individually. Where sample amounts are extremely limited, preamplification using the Applied Biosystems™ TaqMan® PreAmp Master Mix is a suitable option, particularly if many targets need to be analyzed.

This application note outlines the optimization and verification of duplex PCR and provides recommendations for multiplex reactions with a greater number of targets.

Assay choice for multiplex PCR

All TaqMan Assays, whether chosen from our database of predesigned assays or generated using our custom assay design tools, have been designed using our genome-aided probe and primer design pipeline. Our design pipeline optimizes design parameters like GC content, T_m, and amplicon length to ensure that all of our assays have consistent and high amplification efficiency. In addition, by utilizing a bioinformatic screening of potential assay designs, we ensure that the designs that we deliver have a high degree of specificity for the sequence that they target. In a multiplexed reaction, having both consistent efficiency and high specificity across all targets is crucial to eliminating bias from the quantitation results.

We have used this pipeline to design over 2.8 million predesigned Applied Biosystems™ TaqMan® Gene Expression Assays covering over 30 species and pathogens. Each of our predesigned TaqMan Gene Expression Assays is available in FAM™ dye, VIC™ dye, and primer-limited VIC dye formats, enabling duplex PCR from any combination of assays in our catalog. For targets that are not covered by an assay in our predesigned assay catalog, we additionally offer a Custom TaqMan Assay Design Tool that can be used to create an orderable assay from any acceptable input sequence. These custom assays are also offered in the same dye formats, and can be freely used in combination with any predesigned assay. This tool can be accessed at **thermofisher.com/order/custom-genomic-products/tools/cadt/**.

For multiplex applications beyond a duplex, alternative fluorescent dyes with alternative quenchers must be used. In addition, extra care should be taken to minimize the potential for interaction between any of the component oligos in the assays. Our Specialty Oligos team can assist with converting any predesigned or custom assay to a different dye–quencher combination, and can additionally assist with checking assay combinations for multiplex compatibility. The Specialty Oligos team can be contacted via email at **Specialty_Oligos@thermofisher.com**.

Applied Biosystems™ Primer Express™ Software is another option for real-time PCR primer and probe design. Primer Express Software is a flexible, easy-to-use program that has been developed specifically for use with Applied Biosystems™ real-time PCR instruments. The software is capable of automated or manual primer and probe design.

When designing and choosing multiple PCR primers for multiplex assays, it is important to consider the following:

- A primer should not contain base sequences that are complementary to other base sequences within the primer (self-complementary), nor should it have complementarity to other primers. Complementarity at the 3′ ends should especially be avoided to minimize the formation of artifact products, often called primer-dimers or primer-oligomers.

  Tip: Use the free AutoDimer software to check your primers. This tool can be accessed at **cstl.nist.gov/strbase/AutoDimerHomepage/AutoDimerProgramHomepage.htm**.

- A 40–60% GC content is recommended for all primers, avoiding long stretches of any one base. Also, primers should not hybridize to regions of secondary structure within the target, as these structures tend to have a higher melting point than the primer.

- The primer T_m should be the same for all primers used in the multiplex reaction.

- The length of the amplicon should be 50–150 bp for optimal PCR efficiency. Primers that generate a longer amplicon may result in poor amplification efficiency. If longer amplicons cannot be avoided, it may be necessary to optimize the thermal cycling protocol and reaction components.

- The primer should be specific for the target. Perform a BLAST™ search at **ncbi.nlm.nih.gov/blast**.

Optimization of a duplex reaction

Before optimization of a duplex assay, consider the following:

- What are the relative levels of the targets that will be detected?
- What target will be used as an endogenous control?
- What are the magnitudes of the fold differences that are expected?

Since both assays are amplified in the same tube, they compete for the same reaction components (dNTPs and polymerase). It is important that this competition be minimized. The two assays can also inhibit each other through interactions among the four primers, the two probes, the targets or amplicons, or any combination of these. The goal of multiplex PCR is to accurately quantitate the amount of each target present, without interference from competition or inhibition between assays.

Relative abundance of the targets to be duplexed

There are three possible scenarios for the relative expression levels of the two targets:

1. One of the targets—typically the endogenous control—is always expressed at much higher levels than the other.
2. The relative expression levels are about the same.
3. Either target can be more abundant than the other in any given sample.

Case 1—one target is always more abundant

When duplex PCR is performed on a sample in which one target is more abundant, the greater starting quantity of the more abundant target causes the assay for that target to perform better than the other from the start, using up the dNTPs in the reaction, and leaving little for the other assay. This problem is overcome by limiting the amount of primer for the more abundant target. As a result, the primers for that assay are used up rapidly, leading the reaction to plateau early and leaving sufficient dNTPs for the amplification of the less abundant target. All of the cataloged and custom-made TaqMan Assays can be purchased in a primer-limited (PL) format with the VIC dye.

Case 2—targets are of similar abundance

Generally, in this situation, neither assay needs to be primer-limited. The assays can simply be run as single or duplex reactions and verified as described below. However, if verification fails, it may be necessary to primer-limit one or both assays to enable the duplexing to perform better.

Case 3—either target may be more abundant

If either target could be more abundant than the other, depending on the samples being investigated, then both assays need to be primer-limited.

Endogenous control assays

We offer common endogenous control assays for all of the species represented in our TaqMan Assay database, with each assay available in the full range of FAM dye, VIC dye, and primer-limited VIC dye formats. These assays can be located at **thermofisher.com/taqmancontrols**. The ideal endogenous control should have a constant RNA transcription level under different experimental conditions and be sufficiently abundant across different tissues and cell types. Although any gene that is stably expressed under the defined experimental conditions can serve as a normalization gene, the selection is most commonly made from constitutively expressed mRNA housekeeping genes, or ribosomal RNAs such as 18S rRNA. Please refer to the document **"Using TaqMan Endogenous Control Assays to select an endogenous control for experimental studies"** for further guidance on selecting an appropriate control for your experiments.

Verifying duplex reactions

Make a dilution series of the sample, with seven 10-fold dilutions in triplicate. Run each assay individually and in duplex using each dilution in the series. The setup is summarized in Table 2.

General guidelines

Ensure that the dynamic range of the standard curve is broad enough to encompass most of the experimental samples, bearing in mind that the levels of the target(s) of interest may vary widely between samples.

Dynamic range

Take a careful look at the standard curve, ensuring that there is a good fit of the line to all of the points, and that the correlation coefficient (R^2) of the line is at least 0.99. A lower R^2 value indicates that some of the dilutions (usually the lowest, highest, or both) do not sit on the standard curve. Identify the samples that do not sit on the curve and, using the well inspector, omit them, and reanalyze. Please refer to the document "Real-Time PCR: Understanding C_t" at **thermofisher.com/understandingct** for guidance on how to evaluate an experiment.

Relative quantities

Using the dilution series, calculate the relative abundance of the target in a representative sample. The values obtained using the single reactions need to match those obtained from duplexing. Substantial differences between the numbers indicate that there is significant interference between the two reactions, rendering the duplexing data unreliable. If this occurs, it is recommended that you choose an alternative endogenous control assay or a different assay for your target of interest, or run the samples as single reactions.

Precision

Multiplexing should eliminate differences in relative abundance that occur as a result of pipetting errors. Therefore, a C_t variation greater than 3% indicates that other factors, such as competition or inhibition in the duplex reaction, are contributing to the lack of precision.

Table 2. Summary of experimental setup for verifying duplex reactions.

Assay	Reactions	Number of wells or tubes
Target A or endogenous control (primer-limited) using sample of known concentration	Triplicate singleplex reactions using 7 points of a 10-fold dilution series	21
Target B (same sample with known concentration)	Triplicate singleplex reactions using 7 points of a 10-fold dilution series	21
Duplex: target A (primer-limited) and target B	Triplicate duplex reactions using 7 points of a 10-fold dilution series	21
Representative samples: target A	Triplicate singleplex reactions	3
Representative samples: target B	Triplicate singleplex reactions	3
Representative samples (targets A and B, duplex)	Triplicate duplex reactions	3
No-template control (NTC), for each target	Triplicate singleplex reactions and duplex reactions	9

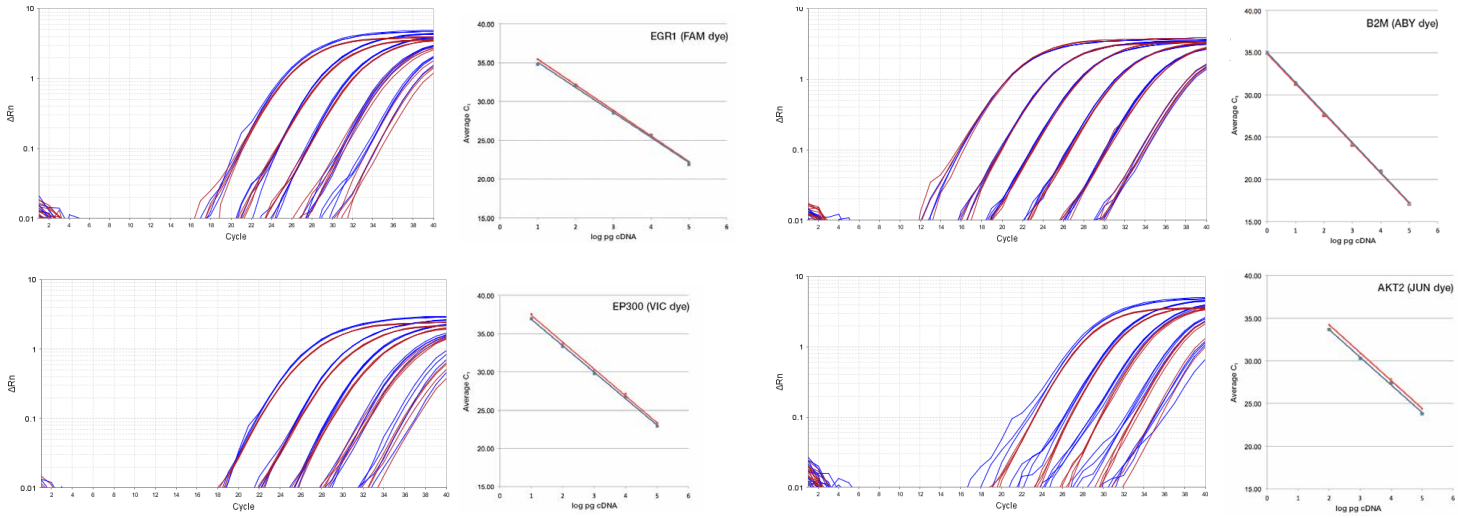
When small differences in relative abundance are being investigated, lower precision may cause these differences to be statistically insignificant and will require more replicates to be run. If statistically significant data are obtained by running 3 replicate assays as single reactions but 4 or 5 replicates are required to achieve the same level of statistical significance when the reactions are run in duplex, the value of duplexing is reduced..

Standard deviation

Determine the standard deviations of samples assayed as single and duplex reactions. If the standard deviation is higher in the duplex reactions but the differences being measured are small, more replicates may be required to obtain statistically significant data. However, this increases the cost and reduces the value of duplexing. In this case, you may find that running assays as single reactions is cheaper, easier, and more convenient than duplexing.

Multiplexing with more than two targets

It is also possible, under carefully optimized conditions, to perform multiplex PCR with more than two targets. This can provide huge savings in costs, reagents, and time, but the experiments are more complex and verification becomes more time-consuming. When interrogating 3 or 4 targets in the same well, there is even more competition for shared reagents than in a duplex reaction, and the potential for unwanted interactions between primers and probes increases. Thus, it is necessary to verify that similar results are achieved in the multiplex reaction as in the singleplex reaction (Figure 1). The previously outlined guidelines for performing a duplex reaction should be followed for a multiplex reaction.



	PCR efficiency (%)		Coefficient of correlation (R^2)	
	Singleplex	4-plex	Singleplex	4-plex
EGR1 (FAM dye)	101.21	104.18	0.997	0.996
EP300 (VIC dye)	92.57	94.54	0.996	0.994
B2M (ABY dye)	91.04	91.54	0.999	0.999
AKT2 (JUN dye)	102.35	103.38	0.993	0.995

Figure 1. Example of verification of a multiplex reaction. An *EGR1* assay using FAM dye, *EP300* assay using VIC dye, *B2M* assay using ABY dye, and AKT2 assay using JUN dye were analyzed in singleplex and 4-plex reactions. Amplification was performed using a serial dilution of reference colon cDNA from 20,000 pg to 2 pg per 10 µL reaction. In all four amplification plots, blue represents 4-plex reactions and red represents singleplex reactions. The table summarizes the PCR efficiency and coefficients of correlation for the assays.

In a multiplex reaction with 2 or more targets, you can utilize TaqMan Assays for the genes of interest, with each assay probe labeled with a different dye. Our range of dyes now includes Applied Biosystems™ ABY™ and JUN™ dyes, whose fluorescence emission spectra peak at 580 nm (yellow) and 617 nm (orange-red), respectively, so they can be used in conjunction with FAM and VIC dyes. Applied Biosystems™ TaqMan® QSY™ probes were developed for optimal high-level multiplexing, with best performance in 3- and 4-plex reactions. The QSY quencher used in these probes is not fluorescent, which is a feature shared with the nonfluorescent quencher (NFQ) used in MGB-NFQ probes. However, the QSY quencher is not conjugated to a minor groove binder (MGB) moiety. Due to the lack of the MGB moiety, QSY probe sequences are not interchangeable with MGB probe sequences, and must be redesigned. If you are currently using predesigned TaqMan Assays containing MGB-NFQ probes (also referred to as MGB probes), you can continue to use these assays in a multiplex reaction. However, to ensure successful amplification, the multiplex reaction should not have more than two MGB-containing probes. To convert an MGB probe–containing TaqMan Assay to a QSY probe–containing assay, please contact our Specialty Oligos team at **Specialty_Oligos@thermofisher.com** to have assays redesigned.

Bear the following general recommendations in mind when you plan an experiment to carry out multiplex reactions for more than two targets:

- A multiplex reaction can contain up to eight primers and four probes (to produce four amplicons), so it is good practice to minimize conditions that result in primer-dimer formation or other unfavorable interactions. Our Specialty Oligos team can assist with checking for these interactions as part of a request to redesign your assays.
- The ABY and JUN dyes have been selected to function optimally with FAM and VIC dyes. If alternative dyes are needed, be sure to choose dyes with little to no overlap in their emission spectra. You will also want to match dye intensity with target abundance by pairing the brightest dye (e.g., FAM) with low-abundance targets, and the dimmest dye (e.g., VIC) with high-abundance targets (e.g., an internal positive control).
- Because all of the assays are amplified in the same tube, they compete for the same reaction components (dNTPs, Mg^{2+}, and polymerase). The more targets that are assayed in a multiplex reaction, the more likely it is that there will be competition for reagents and inhibition between assays. Primer-limited assays should be used when appropriate, and master mixes specifically designed for performing multiplex PCR should be used to offset the effect of competition for reagents.

Choosing the right master mix

The choice of master mix used for multiplex PCR can greatly influence the data obtained. Applied Biosystems™ TaqMan® Fast Advanced Master Mix has been optimized and verified for duplexing while providing best-in-class performance for sensitivity, accuracy, dynamic range, and specificity. TaqMan Fast Advanced Master Mix contains ROX™ dye as a passive reference dye, so it is not suitable for multiplexing using dyes such as JUN dye that fluoresce in a similar range. For higher-level multiplexing, we have developed Mustang Purple™ dye, a passive reference dye with a fluorescence peak of 654 nm that will not interfere with dyes in the yellow or orange-red filter channels (Table 3). Applied Biosystems™ TaqMan® Multiplex Master Mix has been formulated using Mustang Purple dye, and has been optimized and verified for running 4-plex assays, with excellent concordance of results in singleplex and multiplex reactions.

The value of multiplex PCR

The choice to perform multiplex PCR should be considered carefully, and it may be the ideal approach for high-throughput, routine assays. For such assays, the time and cost associated with verification will be offset by the overall cost savings of running a well-optimized multiplex reaction. Once the decision to multiplex is made, attention must be paid to primer design, choice of master mix, and optimizing and verifying reactions. Most targets can be analyzed by duplex PCR with careful primer design and an appropriate choice of master mix. However, the value of multiplexing higher numbers of targets needs careful assessment. The time and expense required to optimize and verify the multiplex PCR data to ensure they are the same as would have been obtained using single reactions may outweigh the potential benefits of the resulting multiplex design.

Table 3. Dye recommendations for Applied Biosystems real-time PCR instruments.

Instrument	Number of filters and dyes available	Passive reference dye	Dyes recommended for multiplexing			
StepOne	3	ROX	FAM	VIC		
StepOnePlus	4	ROX	FAM	VIC	ABY	
QuantStudio 3	4	ROX	FAM	VIC	ABY	
7500 or 7500 Fast	5	ROX, Mustang Purple*	FAM	VIC	ABY	JUN
QuantStudio 6 or 6 Pro	5	ROX, Mustang Purple*	FAM	VIC	ABY	JUN
QuantStudio 5, 7 Flex, 7 Pro, or 12K Flex	6	ROX, Mustang Purple*	FAM	VIC	ABY	JUN

* Mustang Purple dye is recommended for use as a passive reference dye when multiplexing with JUN dye.

Ordering information

Product	Dye	Size	Number of 20 µL reactions	Concentration	Cat. No.
Predesigned assays					
TaqMan Gene Expression Assay	FAM	Extra small	75	20X	4453320* or 4448892
		Small	250		4331182*
		Small	360		4351372
		Medium	750		4351370
		Large	2,900	60X	4351368
	VIC	Small	360	20X	4448489
		Medium	750		4448490
		Large	2,900	60X	4448491
TaqMan Gene Expression Assay, Primer-Limited (PL)	VIC	Small	360	20X	4448484
		Medium	750		4448485
		Large	2,900	60X	4448486
TaqMan Non-coding RNA Assay	FAM	Small	360	20X	4426961
		Medium	750		4426962
		Large	2,900	60X	4426963

* This product is inventoried.

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Ordering information (continued)

Product	Dye	Size	Number of 20 µL reactions	Concentration	Cat. No.
Custom assays					
Custom Plus TaqMan RNA Assay	FAM	Small	360	20X	4441114
		Medium	750		4441117
		Large	2,900	60X	4441118
	VIC	Small	360	20X	4448514
		Medium	750		4448515
		Large	2,900	60X	4448516
Custom Plus TaqMan RNA Assay, Primer-Limited (PL)	VIC	Small	360	20X	4448511
		Medium	750		4448512
		Large	2,900	60X	4448513
Custom TaqMan Gene Expression Assay	FAM	Small	360	20X	4331348
		Medium	750		4332078
		Large	2,900	60X	4332079
	VIC	Small	360	20X	4448508
		Medium	750		4448509
		Large	2,900	60X	4448510
Custom TaqMan Gene Expression Assay, Primer-Limited (PL)	VIC	Small	360	20X	4448487
		Medium	750		4448488
		Large	2,900	60X	4448492

Ordering information

Product	Size	Cat. No.
Master mixes		
TaqMan Fast Advanced Master Mix	1 x 1 mL	4444556
	1 x 5 mL	4444557
	2 x 5 mL	4444963
	5 x 5 mL	4444964
	10 x 5 mL	4444965
	1 x 50 mL	4444558
TaqMan Multiplex Master Mix	1 x 1 mL	4461881
	1 x 5 mL	4461882
	2 x 5 mL	4461884
	5 x 5 mL	4484262
	10 x 5 mL	4484263
	1 x 50 mL	4486295

Reference

1. Henegariu O, Heerema NA, Dlouhy SR et al. (1997) Multiplex PCR: Critical parameters and step-by-step protocol. *Biotechniques* 21:504–511.

Find out more at **thermofisher.com/multiplexqpcr**

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