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 TaqMan® probe–based assays, in which each assay has a specific probe labeled with a unique fluorescent dye, resulting in different observed colors 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 validated, 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 (dNTPs, enzymes, endogenous controls, etc.)
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 validated and often require optimization. Depending on the targets being analyzed and the samples being used, reaction optimization and validation 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 are 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 TaqMan® PreAmp Master Mix is a suitable option, particularly if many targets need to be analyzed.

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

Primer Design for Duplex PCR

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

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

- A primer should not contain bases that are complementary to other bases 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 www.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 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 www.ncbi.nlm.nih.gov/blast.

Optimization of a Duplex Reaction

Before optimization of a duplex assay, consider the following:

1. What are the relative expression levels of the targets that will be detected?
2. What target will be used as an endogenous control?
3. What is the magnitude of the fold differences that are expected?

Since both assays are amplified in the same tube, they compete for the same reagents (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 Expression Levels 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 abundantly expressed 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.

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 validated as described below. However, if validation 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.

Primer-Limiting Assays

Applied Biosystems provides common endogenous control assays for human, mouse, and rat that are available primer-limited and labeled with the VIC® dye, which is recommended for multiplexing with FAM™ dye. These assays can be located at www.appliedbiosystems.com by searching for 'PDAR' (Pre-Developed Assay Reagents). If the required endogenous control target is available, you can begin validating your duplex PCR. However, if it is not, you must primer-limit the assay yourself. The goal of primer-limiting the assay is to find the primer concentration that gives the lowest (earliest) possible C_t value for the more abundant target without distorting the C_t value of the less abundant target. Limiting the primer concentration for the more abundant target has the effect of lowering its ΔRn (Figure 1); however, the C_t should remain unchanged under primer-limited conditions. A sample should be assayed using decreasing amounts of primer in order to determine the optimal primer concentration for each assay.

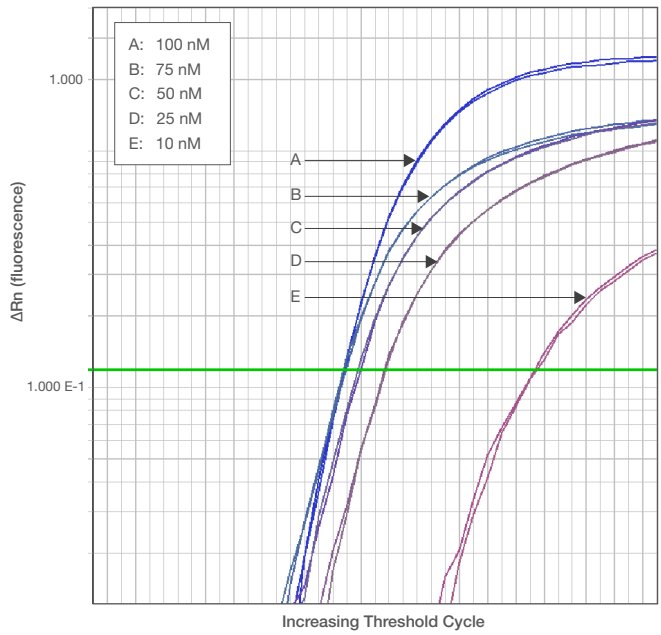

Figure 1. Determining Primer Limit for 18S rRNA. Total RNA (100 ng; Stratagene) was reverse transcribed with the Applied Biosystems® High Capacity RNA-to-cDNA™ Kit and amplified using the indicated primer concentrations in 10 µL reactions. The same C_t is obtained at primer concentrations of 100 and 75 nM, with a lower ΔRn observed at 75 nM. There is an increase in C_t when primer concentrations of 50, 25, and 10 nM are used. As C_t was unchanged at a primer concentration of 75 nM, this was selected as the minimum concentration for amplifying 18S rRNA in a duplex reaction. Amplification reactions were performed on an Applied Biosystems® 7900HT Fast Real-Time PCR System.

Validating Duplex Reactions

Make a dilution series of the sample containing 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.

Table 2. Summary of Experiment Setup for Validating Duplex Reactions.

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

1. 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 expression levels of the target(s) of interest may vary widely between samples.

2. Dynamic Range

Take a careful look at the standard curve, ensuring that there is a good fit of the line to all the standard points, and that the correlation coefficient (R^2) of the line is 0.99 or higher. 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* (Publication 136AP01-01) at www.appliedbioystems.com for guidance on how to evaluate an experiment.

3. 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.

4. Precision

Multiplexing should eliminate differences in relative expression 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.

When small differences in relative expression are being investigated, lower precision may cause these differences to be statistically insignificant and will require more replicates to be run. The value of duplexing is reduced 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.

5. 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 in order 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 to perform multiplex PCR with more than two targets. In fact, there are several Applied Biosystems® multiplex assays focused mainly on human identification or pathogen detection applications, which are suitable for use on a range of platforms. A typical multiplex PCR to detect three targets using the Quantifiler® Duo DNA Quantification Kit on an Applied Biosystems® real-time PCR instrument is shown in Figure 3.

The recommendations for performing multiplex PCR with more than two targets are the same as for a standard duplex reaction. It is still important to take into consideration the relative abundance within the samples of each of the targets and, if necessary, to consider primer-limiting one or all of the assays. It is also important to validate the multiplex results using the process described above to ensure that there is no competition for reagents within the reaction. Note that 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.

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

- Consider using TaqMan® MGB probes rather than the TAMRA™ probes. TAMRA™ dye is used as the quencher for the TAMRA™ probes; however, as TAMRA™ is a fluorescent dye, there is one less filter available for detection (NED™/TAMRA™ filter). MGB quenchers do not emit fluorescence, making possible the use of additional reporter probes labeled with NED™.
- Consider the spectral properties of each dye (intensity of fluorescence, spectral overlap with other dye) within your reaction and aim for the order of amplification for each experiment you perform. That is, organize the assays such that the internal positive control, or the assay containing NED™ dye, exhibits the lowest C_t values, followed by the assay containing the FAM™, VIC®, or Cy®5 dye. Ensure that the internal control assay is primer-limited to prevent it from competing with the true targets for reagents.

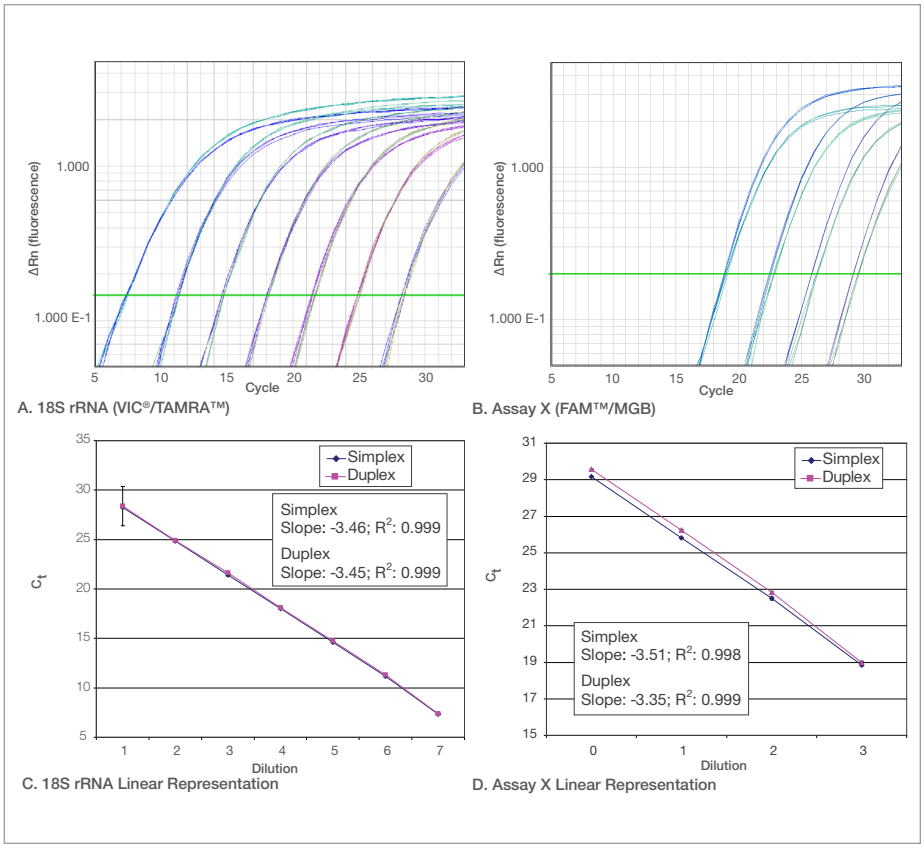

Figure 2. Validation of a Duplex Reaction of 18S rRNA and Assay X. Seven points of a 10-fold dilution series of template were analyzed by simplex or duplex real-time PCR for 18S rRNA (VIC®/TAMRA™ probe; 75 nM primer concentration) (**A** and **C**) and Assay X (FAM™/MGB probe; 200 nM primer concentration) (**B** and **D**) on the Applied Biosystems® 7900HT Real-Time PCR Instrument. The duplex reactions for 18S rRNA showed exactly the same efficiency as the single reactions, as shown by the standard curve and R^2 values in C. There is inhibition of Assay X when it is run as a duplex, resulting in higher C_t values and different R^2 values, as seen in **B** and **D**.

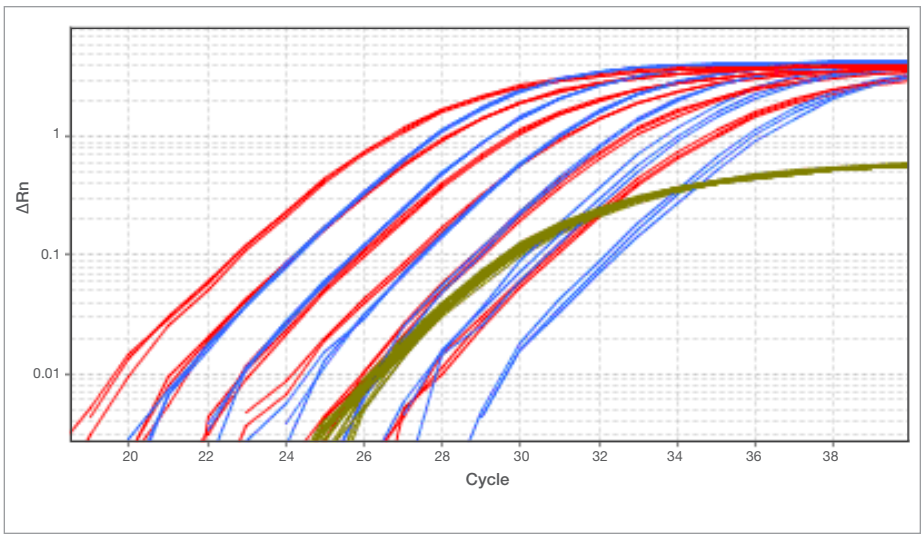

Figure 3. Triplex Real-Time PCR Using the Quantifiler® Duo DNA Quantification Kit and an Applied Biosystems® Real-Time PCR Instrument. The Quantifiler® Duo DNA Quantification Kit contains all the necessary reagents for the amplification, detection, and quantitation of a human-specific DNA target (red lines) and a human male-specific DNA target (blue lines), as well as an internal positive PCR control (green lines). A TaqMan® MGB probe labeled with VIC® dye is used to detect the amplified human target sequence, a TaqMan® MGB probe labeled with FAM™ dye is used to detect the human male amplified target sequence, and a TaqMan® MGB probe labeled with NED™ dye is used for the internal positive control.

Table 3. Compatibility and Recommendations for Applied Biosystems® Real-Time PCR Instruments.

Instrument	Number of Filters and Dyes Available	Passive Reference Dye *	Dyes Available for Multiplexing			
StepOne™	3	ROX™	FAM™	VIC®		
StepOnePlus™	4	ROX™	FAM™	VIC®	NED™	
7300	4	ROX™	FAM™	VIC®	NED™	
7500	5	ROX™	FAM™	VIC®	NED™	Cy®5
7500 Fast	5	ROX™	FAM™	VIC®	NED™	Cy®5
7900HT Fast	4	ROX™	FAM™	VIC®	NED™	

* ROX™ dye is recommended for use as a passive reference.

Choice of Reagents

The choice of master mix used for multiplex PCR can greatly influence the data obtained. Applied Biosystems® Gene Expression Master Mix is designed specifically for use in multiplex reactions (Figure 4). Two synthetic templates, target A and target B, were used to compare the effect of master mixes on duplex PCR. Each reaction comprised a dilution series (1:10) of target A and a fixed concentration of target B, for which the reaction was primer-limited. Gene Expression Master Mix performs better than Universal Master Mix for duplex PCR.

The Value of Multiplex PCR

Careful consideration must be given to performing multiplex PCR, which may be the ideal approach for high-throughput, routine assays. For such assays, the time and cost associated with validation will be offset by the overall cost savings of running a well-optimized multiplex reaction. Once the decision to multiplex is made, strict attention must be paid to primer design, choice of master mix, and optimizing and validating reactions. Most targets can be analyzed by duplex PCR with careful primer design and appropriate choice of master mix. However, the value in multiplexing higher numbers of targets needs careful assessment. The time and expense required to optimize and validate 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.

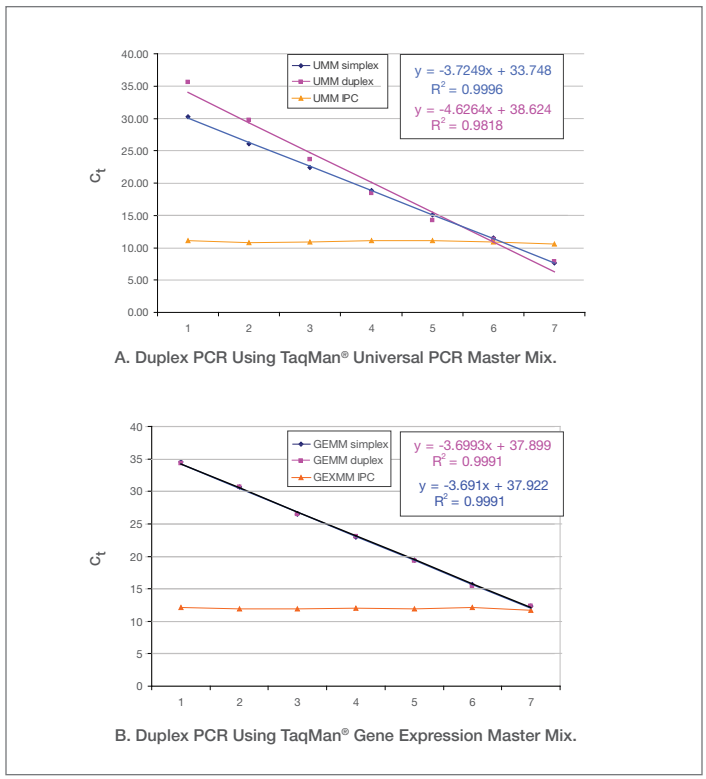

Figure 4. Reagent Choice Influences Results Obtained by Duplex PCR. Duplicate single and duplex real-time PCR assays were performed on samples containing two synthetic templates, target A and target B, using **(A)** Applied Biosystems® Universal Master Mix or **(B)** Applied Biosystems® Gene Expression Master Mix. A dilution series of target A (lowest dilution: 12 copies) was analyzed with a fixed concentration of target B. The assay for target B was primer-limited. There was excellent correlation between the single and duplex reactions when Gene Expression Master Mix was used.

Reference

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

ORDERING INFORMATION

Description	Size	Part Number
TaqMan® Gene Expression Master Mix, 1 Mini-Pack	1 x 1 mL (40 rxns)	4370048
TaqMan® Gene Expression Master Mix, 1-Pack	1 x 5 mL (200 rxns)	4369016
TaqMan® Gene Expression Master Mix, 5-Pack	5 x 5 mL (1,000 rxns)	4369510
TaqMan® Gene Expression Master Mix, 1 Bulk Pack	1 x 50 mL (2,000 rxns)	4370074
TaqMan® Gene Expression Master Mix Protocol	1 protocol	4371135

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