

# Multiplex qPCR with JumpStart™ Taq ReadyMix™ for Quantitative PCR

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## INTRODUCTION

Multiplex qPCR is a technique that simultaneously amplifies two or more target sequences in a single reaction. Analysis of multiple targets per sample allows for conservation of limited starting materials, the ability to run an internal control (i.e. a housekeeping gene in a gene expression assay), and increased sample throughput. Multiplexing is generally more complicated than simply adding additional primers and probes to a singleplex qPCR. Additional consideration must be given to the selection of primers, probes, cycling conditions, and reaction formulation.

## METHODS

### Preparation, Primer/Probe Set Selections

In multiplex qPCR, as in a singleplex qPCR, it is critical to start with quality primer and probe sets. Each set used in a multiplex reaction should be designed and evaluated for optimum reaction metrics. Quality primers can be designed by using one of several commercially available software programs. Sigma-Genosys utilizes Premier Biosoft Beacon Designer™ in its probe assay development service. A freeware alternative for primer design is Primer3. For multiplex qPCR, it is suggested that primers with nearly identical melting temperatures ( $T_m$ ), 45-60% GC content, and no sequence homology with other primers be used.<sup>1</sup> After primer design, empirical testing of each primer set is recommended. Metrics to consider when evaluating an individual primer set are: single target specificity, qPCR efficiency, and dynamic range. To confirm that each selected primer set amplifies one product, perform qPCR and assay by agarose gel electrophoresis.

Optimal individual primer sets are a key foundation to multiplex qPCR as individual amplicon dynamic ranges and efficiencies may deteriorate when combined into a multiplex reaction. An example of dynamic range deterioration from 6 to 3 logs is shown in Figure 1. Efficiency is a measure of the amplification yield after each PCR cycle and must be determined empirically. Efficiency and dynamic range are derived by plotting the log of known template concentration vs. Ct. The slope of the standard curve is proportional to the efficiency with  $-3.32$  being 100% efficient. In practice, a slope between  $-3.50$  and  $-3.14$  is typically acceptable. The dynamic range is generally described as the range of concentrations for which the slope of the standard curve remains linear with an  $R_2$  value greater than 0.98. The best multiplex reactions will be achieved using primers that have high individual efficiencies and broad dynamic ranges.

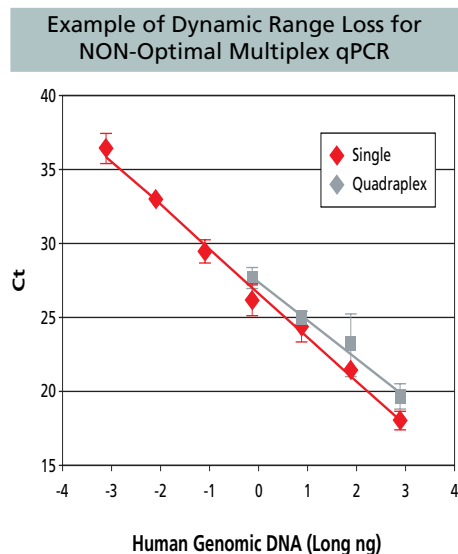
Primer/probe sets affording the same efficiency in individual and multiplex reactions are highly desirable for multiplex qPCR. Often it is necessary to design and evaluate several primer/probe sets before finding the best set for a particular target.<sup>2,3</sup>

In addition to carefully selecting the primer/probe sequences, it is important to choose a combination of fluorophores that has compatible excitation and emission spectra. Your real-time qPCR instrument's capabilities (filters) are critical to keep in mind when selecting fluorophore combinations for multiplexing.

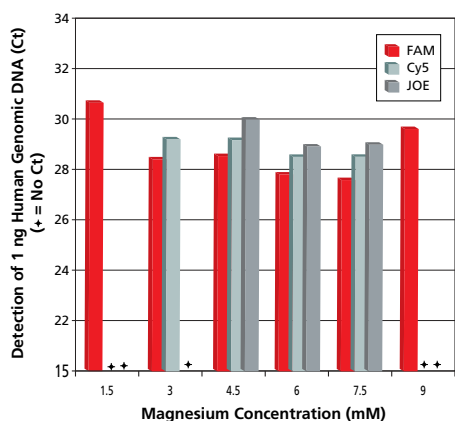
### The Multiplex Reaction Formulation

The nucleotides, enzyme, and buffer concentrations in Sigma's JumpStart Taq ReadyMix for Quantitative PCR, Catalog Number D7440, have been optimized for a wide variety of singleplex qPCR applications. To tailor the mix for multiplex qPCR, an elevated magnesium level is needed. A final concentration of 6 mM  $Mg^{2+}$  is appropriate for most multiplex reactions. Figure 2 demonstrates the improved sensitivity from supplementing magnesium levels above the standard 1.5 mM. In this example, some multiplex products were not even detected using 1.5 mM  $MgCl_2$ .

Magnesium supplementation can be achieved by following the instructions in the JumpStart Taq ReadyMix for Quantitative PCR Technical Bulletin, Catalog Number D7440. For a 20  $\mu$ L multiplex reaction use the standard 10  $\mu$ L of JumpStart Taq ReadyMix, Catalog Number P2893,



**Figure 1.** FAM Primer/probe dilution series completed as individual PCR and quadruplex PCR. Primers/probe sets Cy5, FAM, HEX, and Texas Red were amplified together in JumpStart Taq ReadyMix for Quantitative PCR (P2893) adjusted to a 6 mM  $MgCl_2$  final concentration. The 20  $\mu$ L reactions were run with primers and probes at 200 nM and 100 nM, respectively.

Effect of MgCl<sub>2</sub> on Multiplex qPCR

**Figure 2.** Multiplex amplification of three primer/probe sets at various magnesium chloride concentrations demonstrating the need for supplementation. Primers/probe sets FAM, Cy5, and JOE were amplified together in JumpStart Taq ReadyMix (P2893) with increasing MgCl<sub>2</sub> concentration. The 20  $\mu$ L reactions were run with primers and probes at 200 nM and 100 nM, respectively. Detection at lower Ct is optimal.

primers, probe, template, reference dye (if necessary) and add 3.6  $\mu$ L of the provided 25 mM MgCl<sub>2</sub>, Catalog Number M8787. Mix and place in a thermocycler. In some cases, it may be necessary to increase the magnesium level even further. Typically the optimum working level is between 5 and 20 mM. Should reaction volume become a concern, use 1 M MgCl<sub>2</sub>, Catalog Number M1028, diluted to 100 mM in place of the 25 mM MgCl<sub>2</sub>.

If the magnesium level has changed since primer specificity was verified, re-verification of specificity with the final reaction conditions is suggested.

## Basic Optimization of the Multiplex Reaction – Primer/Probe Concentration

An accurate comparison between multiplex targets is dependent on all targets in the reaction having similar efficiencies. After the initial primer/probe set selection, multiplex qPCR optimization should focus on optimizing the target product reaction efficiencies, including specificity. One way to do this is to increase the primer concentration for the poorly amplified target, but one must be mindful that non-specific amplicons are also more likely to be generated. The most robust and accurate multiplex will have absolute specificity across the testing range and efficiencies near theoretical.

Increasing other reagent concentrations such as polymerase, dNTPs or salts usually do not result in significant differences in PCR efficiency, although there are exceptions. One caution is that increased dNTP concentrations will require increased magnesium, as the polymerase uses these substrates as a dNTP•Mg complex. A free magnesium concentration of 0.5-1.0 mM is necessary to support PCR. Levels of dNTP that exceed Mg<sup>2+</sup> will poison the reaction.

Specific reactions with unequal reaction efficiencies can be accommodated in two ways. The best way is to generate a standard curve for each target under multiplex conditions and mathematically correct for the reduced PCR amplification. The alternative is to make all primer/probe set efficiencies equal by decreasing the relative primer/probe set concentrations of the efficient reaction(s).<sup>1,3-5</sup> This latter course should only be used as a last resort, as it reduces the sensitivity and linear range of the multiplex assay.

## REFERENCES

1. Markoulatos, P., et al; Multiplex polymerase chain reaction: a practical approach. *Clin Lab Anal*; **16**: 47-51 (2002).
2. Henegariu, O., et al; Multiplex PCR: Critical Parameters and Step-by-step Protocol., *BioTechniques* **23**: 504-11 (1997).
3. Schoske, R., et al; Multiplex PCR design strategy used for the simultaneous amplification of 10 Y chromosome short tandem repeat (STR) loci. *Anal Bioanal Chem*, **375**: 333-43 (2003).
4. Elnifro, E.M., et al; Multiplex PCR: Optimization and Application in Diagnostic Virology. *Clinical Microbiology Reviews*, **13**: 559-570 (2000).
5. Bustin, S. A., Ed. A-Z of Qualitative PCR; International University Line: La Jolla, CA, 2004.

## PRODUCT INFORMATION

Cat. No.	Description
<b>D7440</b>	JumpStart Taq ReadyMix for Quantitative PCR
<b>P2893</b>	JumpStart Taq ReadyMix
<b>M8787</b>	Magnesium chloride solution, PCR Reagent, 25 mM
<b>M1028</b>	Magnesium chloride solution, for molecular biology, 1.00 M

D7440 is a complete quantitative PCR Kit, containing a ReadyMix, magnesium and a bulletin with qPCR references. P2893 is the ReadyMix component of the Kit, Catalog Number D7440, and will require additional magnesium and optimization expertise.