

qPCR: Guidelines for Validating a Multiplex Assay

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Introduction

Quantifying expression of multiple target genes in the same qPCR reaction well, also known as multiplexing, has many advantages. Multiplexing can save setup time, reduce cost and minimize the amount of sample consumed in each experiment. However, there are some important considerations and additional work that must be done up front to make sure you get the most out of your multiplex assay, especially when validating one for the first time.

When validating a multiplex assay, you need to make sure that several essential factors of assay performance are unaffected by the presence of other primer/probe sets. Those factors include quantification cycle (C_q) values, linearity, sensitivity and efficiency for each target. Interference from other amplifications occurring within the reaction well can lead to poor or biased amplification and skew quantification results. This guide will take you step-by-step through the process of validating a qPCR multiplex assay to provide optimal results.

qPCR setup for multiplex validation

1. **Prepare a standard curve template.** Start with a representative nucleic acid standard of a known concentration, and prepare a dilution series. Prepare at least six serial dilutions of 1:10 or 1:5. Always include appropriate controls, such as no template and no reverse transcriptase (for RT-qPCR).
2. **Prepare reaction mixes with primer/probe sets individually and in multiplex.** Combine 2X qPCR master mix, primers, probes, and water to prepare a sufficient volume of reaction mix for your standard curve and controls in triplicate plus a 10% excess for pipetting error for each condition. For a duplex reaction, make up one mix with assay 1, one mix with assay 2, and another mix with assays 1 and 2 combined. For multiplex reactions with greater than two targets, it is easiest to interpret results and identify problem primer/probe sets if each target is added sequentially to the multiplex reaction.
3. **Set up the assay plate.** Add the reaction mix and template to the plate (e.g., 15 μ l of reaction mix + 5 μ l of template or water for the no-template control). Each template concentration should be tested in triplicate for each condition. Seal the plate and centrifuge briefly to collect the liquid at the bottom of the wells.
4. **Amplify reactions with the appropriate thermal cycling conditions.** You should determine optimal denaturation and annealing/extension cycling conditions by designing primers with similar melting temperatures (T_m) and testing singleplex reactions prior to this validation. A good starting point is the recommended standard cycling conditions for your qPCR reagent.

Comparing performance of multiplex and singleplex reactions

To validate your multiplex reaction, you must compare the C_q values, assay reproducibility, linearity, sensitivity and efficiency for single versus multiplex reactions with each primer/probe combination. If any of these five factors is significantly affected by multiplexing or if both singleplex and multiplex reactions do not perform as expected, further optimization or primer/probe changes may be necessary.

1. **C_q values.** Singleplex and multiplex reactions should be within one C_q value for each dilution. Use a single threshold across singleplex and multiplex reactions being compared.
2. **Assay reproducibility.** Standard deviations of C_q values greater than 0.5 also could indicate problems with your multiplex reaction and should be examined further.
3. **Assay linearity.** The coefficient of determination (r^2) for the fit of the linear regression equation fitted to the semi-log plot should be greater than 0.98 for single and multiplex reaction according to MIQE guidelines (1).
4. **Assay sensitivity.** The sensitivity is the lowest template concentration at which a C_q value can be reliably determined. Information about the assay sensitivity can be inferred from the lowest data point included in the standard curve with a good fit of the linear regression equation. Sensitivity between multiplex and singleplex reaction should be the same.
5. **Reaction efficiency.** The efficiency for the qPCR assay can be determined from the slope of the linear regression line according to the following equation and is automatically calculated in most software:
Efficiency = $(10^{(-1/\text{slope})}) * 100$. Ideally efficiency should be between 90–110% (1).

Tool for comparing qPCR reagents used with your assay

This table is a rubric for assessing qPCR assay performance based on assay specificity, reproducibility, linearity, sensitivity and efficiency. Place a check mark in the appropriate column corresponding to when the multiplex reaction for a target fits the criterion when compared to singleplex reaction. Perform the comparison below for each target of interest.

Table 1. Rubric for assessing qPCR assay performance.

Check if your multiplex reaction for this target fits the following criteria when compared to the singleplex reaction.		✓
Target Name:	C_q Values Are C_q values between multiplex and singleplex reactions within one C_q ?	
	Reproducibility Are standard deviations within each assay below $0.5C_q$?	
	Linearity Is the r^2 value greater than 0.98?	
	Sensitivity Is the lowest DNA concentration included in the linear range of the assay the same with single versus multiplex reaction?	
	Efficiency Is efficiency for both reactions between 90–110%	



Example of multiplex validation using GoTaq® Probe 1-Step RT-qPCR System

A five-point standard curve (200–0.02ng) with total human colon RNA was used for amplification with each of four different primer/probe sets in separate reactions as a singleplex reaction, or all together in one reaction as a quadruplex reaction. Each condition was run in quadruplicate, and C_q , r^2 and efficiencies were calculated for each condition. C_q values versus RNA input amount was graphed for each primer and probe set (Figure 1). Efficiency and r^2 values were calculated for singleplex and quadruplex reactions (Table 2). Standard curves, efficiencies and r^2 values were not changed with a quadruplex reaction when compared to a singleplex reaction. If following the rubric above, this multiplex would pass all criteria listed.

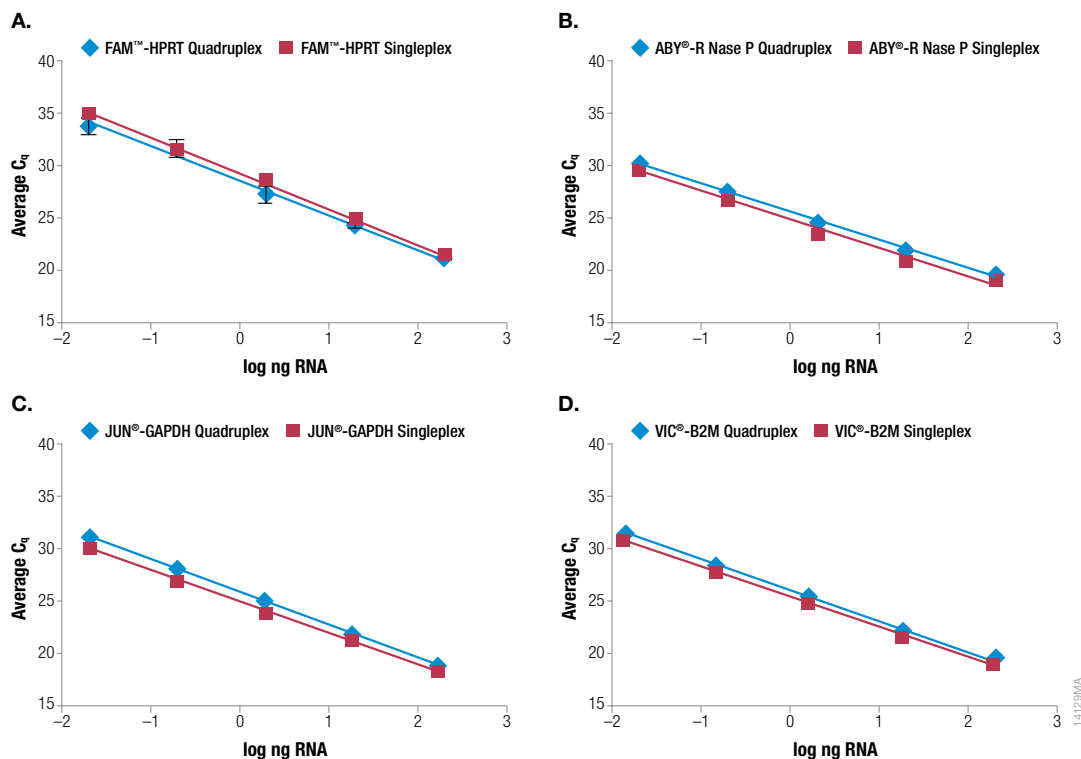


Figure 1. C_q values from RT-qPCR using the GoTaq® Probe 1-Step RT-qPCR System with a five-point standard curve of total human colon RNA. FAM™-HPRT primer/probe set (Panel A), ABY®-RNase P primer/probe set (Panel B), JUN®-GAPDH primer/probe set (Panel C) and VIC®-B2M primer/probe set (Panel D) were used for multiplex or singleplex reactions.

Table 2. PCR efficiency and coefficient of correlation for singleplex or multiplex RT-qPCR using GoTaq® Probe 1-Step RT-qPCR System with total human colon RNA.

	PCR Efficiency		Coefficient of Determination (r^2)	
	Singleplex	Quadruplex	Singleplex	Quadruplex
VIC®-B2M	98.81%	92.42%	0.999	0.999
FAM™-HPRT	98.22%	103.79%	0.999	0.996
ABY®-RNase P	106.22%	107.39%	0.995	0.996
JUN®-GAPDH	97.90%	91.58%	0.999	0.999

Reference

- Bustin, S.A. *et al.* (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem.* **55**, 1–12.

Additional Resource

Refer to GoTaq® Probe qPCR Master Mix Technical Manual #TM378 for more details.

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