Real-Time qPCR: Guidelines for a Comparison of Reagent Performance

Sarah Teter, PhD, and Leta Steffen, PhD, Promega Corporation

Introduction

Testing previously optimized qPCR assays with a new qPCR master mix requires careful experimental design to look at several factors of reagent performance including assay specificity, repeatability, linearity, sensitivity and efficiency. Each of these qPCR assay performance indicators will be discussed with respect to how the qPCR master mix comparison experiments should be set up and how to analyze the data to assess each of these key factors of qPCR assays.

qPCR assay plate setup

1. **Prepare serial dilutions.** Start with a high-purity stock of DNA template and prepare 6 dilutions. 1:10 or 1:5 serial-dilutions are recommended. For high-complexity eukaryotic DNA templates, 100ng DNA per reaction is an appropriate starting concentration.
2. **Prepare bulk reaction mixes with your current qPCR reagent and GoTaq® qPCR Master Mix.** Combine 2X qPCR master mix, primers (and probe, if applicable), and water to prepare a sufficient volume of reaction mix for 24 20µl reactions + 10% excess for pipetting error. Use your standard optimized primer/probe concentrations in the reaction mixes prepared with your current qPCR reagent and GoTaq® qPCR Master Mix. Add passive reference dye as required.
3. **Set up the assay plate.** Add the reaction mix and template to the plate for a final reaction volume of 20µl (e.g. 15µl of reaction mix + 5µl template). Each template concentration should be tested in triplicate. Seal the plate and centrifuge briefly to collect the liquid at the bottom of wells.
4. **Amplify reactions with the appropriate thermal cycling conditions.** Use your optimized denaturation and annealing/extension cycling with your current qPCR reagent and with GoTaq® qPCR Master Mix. Do not change the Hot-Start Activation step for the GoTaq® qPCR Master Mix Cycling Protocol; initial activation at 95°C for 2 minutes is required. Include a dissociation/melt curve step in assays with double-stranded DNA (dsDNA) binding dye-based amplification detection.

Analysis of qPCR amplification data quality

1. **Ensure that no amplification is observed in no template control (NTC) reactions.** Check that the NTC reactions have no C_q value determined. Otherwise, very high C_q values (>38 cycles) may be acceptable, as long as they are not within 3 cycles of the reactions containing your lowest template concentrations.
2. **Analyze the data for each assay separately.** For each analysis, include a subset of wells containing one assay while excluding the data from wells in which different assays were performed. Also, exclude the wells containing the NTC.
3. **Use automatic baseline and threshold settings.** Select the option for automatic baseline window and threshold settings in the software, even if you use a manual threshold with typical assay use.
4. **Confirm that the appropriate baseline window was set for the data.** On a linear plot of fluorescence signal versus cycle number, confirm that the baseline signal looks flat within the window defined by the analysis software.
5. **Confirm that the appropriate threshold was determined for data.** The line defining the threshold must fall within the exponential phase of amplification across all reactions for a specific assay. On the semi-log plot (log(*fluorescence signal*) versus cycle number) the threshold should cross within the portion of each amplification curve that appears straight.

Determining which qPCR master mix works best

Once you have confirmed the quality of the amplification data that you have generated, you can look at the specificity, repeatability, linearity, sensitivity and efficiency of the qPCR master mix and primer/probe combinations that you have tested.

1. **Reaction specificity.** If using a qPCR master mix with dsDNA binding dye, a single peak in the melt curve analysis indicates specificity. For probe-based detection of amplification, the probe should impart specificity for the amplicon of interest. NTC reactions should show no amplification, late amplification, or primer artifact (low Tm) amplification only.
2. **Assay repeatability.** Repeatability can be assessed from the standard deviation around the C_q for the technical replicates. ±0.5 C_q is a reasonable cutoff for high standard deviation. A data point should be excluded if the standard deviation of $C_q > 0.5$.
3. **Assay Linearity.** The coefficient of determination, r^2, for the fit of the linear regression equation fitted to the semi-log plot, C_q vs. log(*input concentration*), should be >0.98. Points at the extreme ends of the concentration range which throw off the fit of the linear regression equation ($r^2 < 0.98$ if point is included) are excluded as outside the linear range of the assay.
4. **Assay sensitivity.** The sensitivity is the lowest template concentration at which a C_q 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.
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: $Efficiency = -1 + 10^{(-1/slope)}$.

Tool for comparing qPCR reagents used with your assay

This table is a rubric for assessing qPCR assay performance based on assay specificity, repeatability, linearity, sensitivity and efficiency. Place a check mark in the appropriate column corresponding to the qPCR master mix for each criterion. The qPCR master mix that has the most checkmarks performs best for your assay of interest.

Put a checkmark in the corresponding column in each case that the qPCR reagent fits the indicated criteria for specificity, repeatability, linearity, sensitivity, or efficiency. If the two qPCR reagents are equivalent for a specific criterion, put a checkmark in both columns.		**GoTaq® qPCR MM**	**Other qPCR MM**
Target Name:	**Specificity** Was there no or very late amplification in the NTC reaction? If you are using dye-based qPCR for amplification of a single target, did you observe a single peak in the melt analysis? (Not a criterion for probe-based assays.)		
	Repeatability Which product has the fewest standard curve points excluded for high standard deviation ($\geq 0.5C_q$)?		
	Linearity Is the r^2 of the linear regression equation ≥ 0.98?		
	Sensitivity Which product has the best assay sensitivity? (The lowest DNA concentration included in the linear range should not negatively impact the r^2, and the standard deviation of the C_q with this concentration of DNA should be ≤ 0.5.)		
	Efficiency Is qPCR assay efficiency between 90–110%?		
		GoTaq® qPCR MM	**Other qPCR MM**
Total Checkmarks			

Reference:

1. 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 Resources:

Refer to *GoTaq® qPCR Master Mix Technical Manual* #TM318, *GoTaq® Probe qPCR Master Mix Technical Manual* #TM378 and *Guidelines for a Successful qPCR Master Mix Comparison* #TM498 for more details.