TECHNICAL MANUAL

Guidelines for a Successful qPCR Master Mix Comparison

Instructions for Use of Products
A6001, A6002, A6101 and A6102

11/16
TM498

Guidelines for a Successful qPCR Master Mix Comparison

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this guide: techserv@promega.com

1. Description

Real-time quantitative PCR (qPCR) is a powerful tool to detect and quantify nucleic acids. By incorporating fluorescently labeled probes or fluorescent double-stranded DNA (dsDNA)-binding dyes into the PCR, product formation can be monitored following each PCR cycle. GoTaq® qPCR Master Mixes for dye-based or probe-based detection are optimized for fast and reproducible qPCR assays. In this guide, we outline some of the most important considerations for comparing the performance of your assay using either GoTaq® qPCR Master Mix with BRYT® Green Dye or GoTaq® Probe qPCR Master Mix to that of your current qPCR reagent.

Testing previously-optimized qPCR assays with a new qPCR master mix requires careful experimental design to compare 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 performed as well as how to analyze the data.

1. Description (continued)

These guidelines were developed to provide a means to quickly compare qPCR master mix performance within two plates and two instrument runs as outlined in Figure 1. Assay specificity, repeatability, linearity, sensitivity and efficiency must be evaluated to compare performance of both dye- and probe-based qPCR reagents. Throughout this document the terms qPCR reagent, qPCR master mix and GoTaq® qPCR Master Mix will be used to refer generally to products for both dye and probe-based detection of amplification products, except in cases where dye or probe-based detection is specifically indicated. A more rigorous comparison of qPCR reagent performance would require a significant investment of time to first determine the primer (and probe, if applicable) concentrations and thermal cycling protocol that gives the best performance for each assay and reagent independently, followed by a comparison of two qPCR reagents under each of their respective optimal assay conditions.

What to Expect:

- **Add passive reference dye as appropriate for your real-time instrument.** Some instruments require passive reference dye for signal normalization. The GoTaq® products use CXR, which can be directly substituted for ROX™ passive reference dye. GoTaq® qPCR Master Mix is formulated with a low concentration of CXR that is appropriate for instruments that require no or low concentrations of passive reference dye. To allow for maximum fluorophore multiplexing capability, the GoTaq® Probe qPCR Master Mix does not contain CXR. A tube of CXR passive reference dye is provided with both the dye and probe GoTaq® qPCR Master Mixes so that reactions may be supplemented with the concentration of passive reference dye required by your real-time PCR instrument. Please refer to the instrument manufacturer's documentation for the amount of passive reference dye that is required for optimal assay performance.
- **Melt temperatures of PCR amplification product(s) may shift.** The PCR product melting temperature is influenced by the sequence and length of the PCR product, as well as the salts and additives in the master mix. The particular composition of the qPCR reagents being compared may result in different melt temperatures for the same amplification product.
- **Optimum primer annealing temperatures may be different in different qPCR master mixes.** As with product melt temperatures, the salts and additives in qPCR master mixes may influence primer annealing temperature. For assays already in use, the existing thermal cycling conditions are typically a good starting point, but low amplification efficiency may indicate the need for optimization.
- **Each qPCR master mix should be compared on individual plates and in separate instrument runs.** Amplification on separate plates allows for the run parameters to be adjusted according to the required differences in the thermal cycling protocols (e.g., hot-start activation time). Additionally, it ensures that the data with each qPCR master mix are collected independently to avoid any influence of the other qPCR master mix on signal gain adjustments applied plate-wide by the instrument software.
- **Baseline and threshold settings are affected by both the assay and the master mix.** When comparing two master mixes, ensure that the software analyzes each assay independently (by indicating unique target names or by omitting the wells containing data for other assays). Do not apply a universal manual threshold when testing new master mixes.

- **A thorough assessment of qPCR reagent performance requires that assays with each reagent be tested over a range of DNA concentrations.** Comparing C_q with two different qPCR master mixes using only a single-input DNA concentration does not provide sufficient information to look at important factors of qPCR reagent performance. Specifically, reagent linearity (as demonstrated in Figure 6), sensitivity and efficiency can only be assessed if the reagent performance in an assay is tested over a range of DNA input concentrations. Therefore, it is important to follow the recommendation of this guide to look at the performance over a range of input DNA concentrations.
- **Moving a threshold within the exponential phase of amplification will alter the C_q.** C_q is a factor of how a specific analysis software defines the threshold and is therefore a somewhat arbitrary value. For valid quantitation, the fluorescence threshold can be set at any point within the exponential phase of amplification, as long as the threshold is significantly above background. This means that C_q is a relative measure that cannot be used as an indicator of sensitivity for comparing two qPCR master mixes.

1. Description (continued)

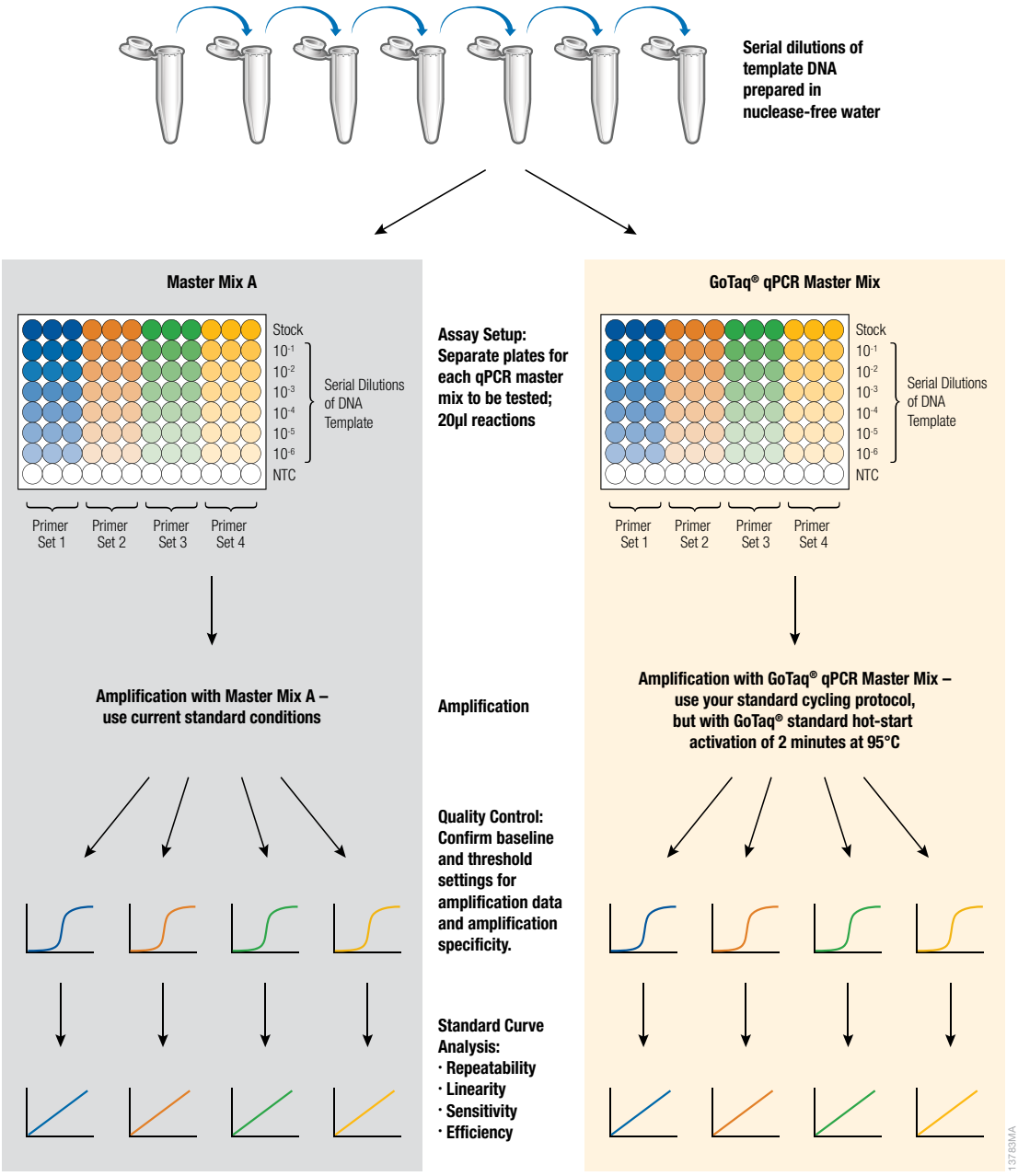

Figure 1. Outline of steps to perform a qPCR reagent comparison.

2. Experimental Setup for Comparing qPCR Master Mixes

1. Prepare a serial dilution of the template DNA. Start with a high-purity stock of DNA template for your dilutions. For high-complexity eukaryotic DNA templates, a total of 100ng DNA per reaction is an appropriate starting concentration. 1:10 or 1:5 serial-dilutions are recommended.

   Note: The formula below can be used to determine how much volume of each DNA dilution should be prepared, assuming a 5µl template volume. We recommend adding a larger template volume, such as 5µl of template per reaction, to minimize replicate variability due to pipetting, but you may choose to add a lower volume of template to each reaction, according to your preference

   (**5**µl template DNA added per reaction) × (**2** qPCR master mixes to be tested) × (**X** number of primer sets to be tested) × (**3** technical replicates) × (**1.3**) = (total volume of each DNA dilution required)

2. Thaw the GoTaq® qPCR Master Mix and the Nuclease-Free Water. Do not thaw the master mix at elevated temperatures (i.e., above room temperature).

3. Briefly vortex the GoTaq® qPCR Master Mix for 3–5 seconds to mix.

4. Determine the number of reactions to be set up. This should include negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does require using a small amount of extra reagent, it ensures that you will have enough qPCR master mix for all samples.

   Notes:

   1. The reagent composition for a 20µl reaction volume is shown in Table 1. Component volumes may be scaled for larger or smaller reaction volumes.
   2. The reaction concentrations of primers and hydrolysis probes (if applicable) should be optimized for each primer and hydrolysis probe combination.

5. Prepare the reaction mix (without the template DNA) by combining the GoTaq® qPCR Master Mix, PCR primers, hydrolysis probe (if applicable) and Nuclease-Free Water as shown in Table 1. Vortex briefly to mix.

6. Add the appropriate volume of reaction mix (without the template DNA) to each PCR tube or to each well of an optical grade PCR plate appropriate for your instrument. Figure 2 shows a recommended plate configuration for testing multiple assays over a range of template concentrations.

7. Add DNA template to the sample reactions.

8. Seal the tubes or optical plates; centrifuge briefly to collect the contents of the wells at the bottom. The samples are ready for thermal cycling. Protect from extended light exposure or elevated temperatures before cycling.

9. Follow the standard optimized thermal cycling protocol for amplification with your current qPCR master mix. For cycling with GoTaq® qPCR Master Mix, use the same protocol, except for initial hot start activation. GoTaq® Hot Start Polymerase **requires** initial hot start activation for 2 minutes at 95°C.

   Note: For experiments with ds DNA binding dye-based detection of qPCR products, include a dissociation (melt) step after thermal cycling. The dissociation step allows for melt analysis to be performed to assess the specificity of the qPCR assay.

2. Experimental Setup for Comparing qPCR Master Mixes (continued)

Table 1. Preparation of reaction mix.

Component	Volume per 20µl[1] Reaction	Final Concentration	Typical Reaction Concentration Dye	Typical Reaction Concentration Probe
GoTaq® qPCR Master Mix (2X)[2]	10µl	1X	1X	1X
Forward Primer[3]	_____µl	Typically 200nM—1µM	500nM	900nM
Reverse Primer[3]	_____µl	Typically 200nM—1µM	500nM	900nM
Hydrolysis Probe (if applicable)[3]	_____µl	Typically 100nM—300nM	N/A	250nM
Template DNA	2—5µl	≤250ng	≤100ng	≤100ng
Nuclease-Free Water	_____µl	To a 20µl total reaction volume		

[1]An example 20µl reaction is shown, but reactions can be scaled from 5—50µl, depending on plate capacity.
[2]CXR Reference Dye should be added to the master mix if passive reference normalization is required by your real-time thermal cycler.
[3]Primers and probe(s) may be added individually, or as a mix. Primers and probes for multiple targets may be included for multiplex reactions.

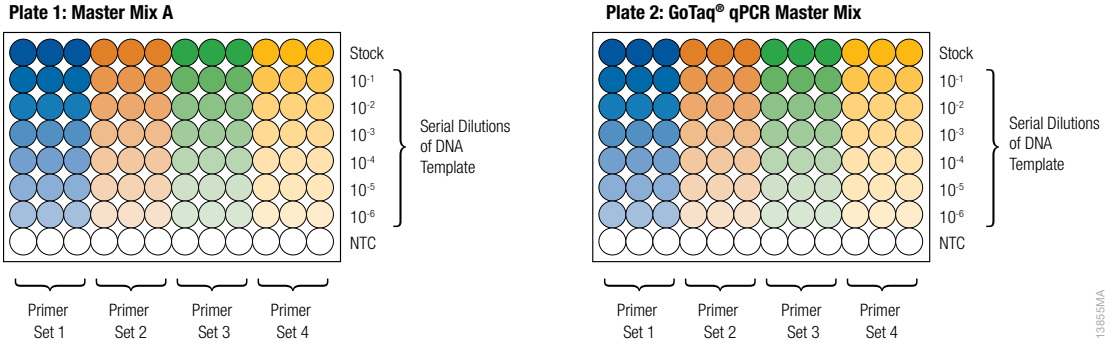

Figure 2. Recommended plate setup for testing two qPCR master mixes with up to four primer sets targeting different amplicons. For each qPCR master mix tested, a separate plate should be used. Each DNA template concentration will be tested in triplicate for each combination of qPCR master mix and primer/probe set.

Table 2. Thermal cycling conditions for your current qPCR Master Mix and GoTaq® qPCR Master Mix.

	# Cycles	Cycling Program with Current qPCR Master Mix	Cycling Program with GoTaq® qPCR Master Mix
Hot-Start Activation	1	A°C for X minutes	**95°C for 2 minutes**
Denaturation	40	B°C for Y seconds	B°C for Y seconds
Annealing/Extension		D°C for Z seconds	D°C for Z seconds
Dissociation (optional)	1	60-95°C	60-95°C

Note: A, B and D represent the temperature for the hot-start activation, denaturation and annealing/extension steps, respectively, in the thermal cycling protocol you use with your current qPCR master mix. X, Y and Z correspond to the length of each of these steps. For GoTaq® qPCR Master Mix, the hot-start activation step should not be altered. However, use the same temperature and length of time for the denaturation and annealing/extension steps for assays with both reagents.

3. Analysis of qPCR Amplification Data Quality

In this section, we will discuss how to assess the quality of the amplification data generated with the experimental setup described above. Since data analysis software varies widely depending on the instrument manufacturer, we do not include instructions for use of a specific manufacturer's software. If you are in need of guidance for use of the software, you should either contact the instrument manufacturer or Promega's Technical Services group (**techserv@promega.com**; 1-800-356-9526).

Key terms:

- The term *assay* in this section refers to a set of reactions containing a specific combination of primer set, including probe (if applicable) and qPCR master mix. Each assay will be analyzed separately for data quality.
- The *baseline window* is the range of amplification cycles during which there is no detectable amplification, resulting in little change in fluorescence.
- The *threshold* is typically defined as the fluorescence signal equivalent to 10 times the standard deviation around the mean of the baseline signal for all of the wells containing a single assay. It is the fluorescence signal that can be reliably detected above the noise of the background. Some software algorithms will do additional data transformation to set the threshold.
- The *quantification cycle* (C_q) is the amplification cycle at which the fluorescence signal detected reaches the threshold in a specific well. The C_q is inversely related to the amount of starting template in the reaction.

1. If data were generated for multiple assays on one plate, ensure that you independently analyze the dataset for each. Exclude wells in which different assays were performed and those containing the NTC reaction.

   Notes:

   1. Although NTC reactions are not used in the data analysis, these reactions give important information about reaction specificity and can be useful for diagnosing issues with reaction setup.
   2. For independent analysis of each assay, you may use the software options to include only the wells containing the reactions for one assay and omit other wells containing different assays from the analysis. In some analysis software, the wells for a particular assay can be grouped by setting unique target names for each assay or by establishing well groups for each assay.

3. Analysis of qPCR Amplification Data Quality (continued)

2. In the data analysis settings, ensure that the options for automatic threshold and baseline window determination are selected, even if you use a manual threshold with typical assay use.

   Note: The baseline window and threshold value are intrinsic to the assay, and these values should be determined independently for each assay. It is best practice to use the automatic threshold and baseline settings for both your standard assays and assays with GoTaq® qPCR Master Mix when doing a reagent comparison.

3. On a linear plot of the fluorescence signal versus cycle number, confirm that the baseline signal looks flat as shown in Figure 3, Panel A. The baseline window should encompass multiple cycles. In our experience, a baseline window consisting of a minimum of 8 cycles yields enough data for appropriate baseline correction. Generally, the first two cycles are not included in the baseline window, as the data obtained during these initial cycles tend to have high variability.

4. Check to confirm that the line defining the threshold falls within the exponential phase of amplification (Figure 4). On the semi-log plot (log *fluorescence signal* versus cycle number) the threshold should cross within the portion of the amplification curves that appears linear (Figure 4.A).

   Note: In general, it is not instructive to compare the overall shape of the amplification curves at cycles beyond the threshold. As amplification product accumulates, there may be other activities in the reaction that will affect the rate of amplification and thus affect the overall shape of the amplification curve. For example, the fluorescence signal may or may not appear to reach a signal plateau within 40 cycles, depending on whether reaction components become limiting in each assay being compared. However, these differences later in amplification do not affect the quantification in the earlier amplification cycles.

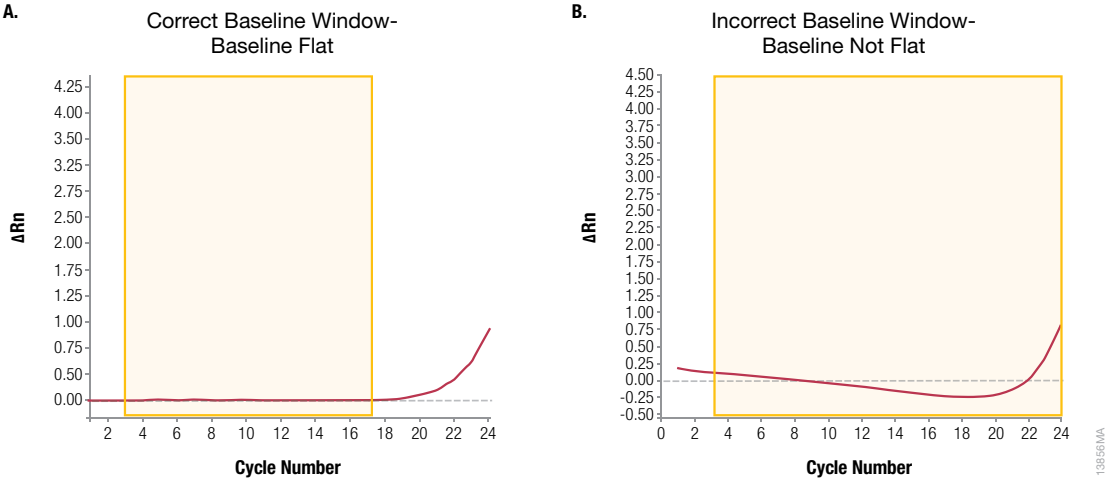

Figure 3: Perform a baseline check. The baseline window in each panel is indicated by orange shading. **Panel A** shows an example of an appropriately set baseline window, in which the baseline appears flat around zero. **Panel B** shows an example of a poorly set baseline window. The end of the baseline window in this example occurs after cycles in which amplification is detected, causing the baseline to dip below zero.

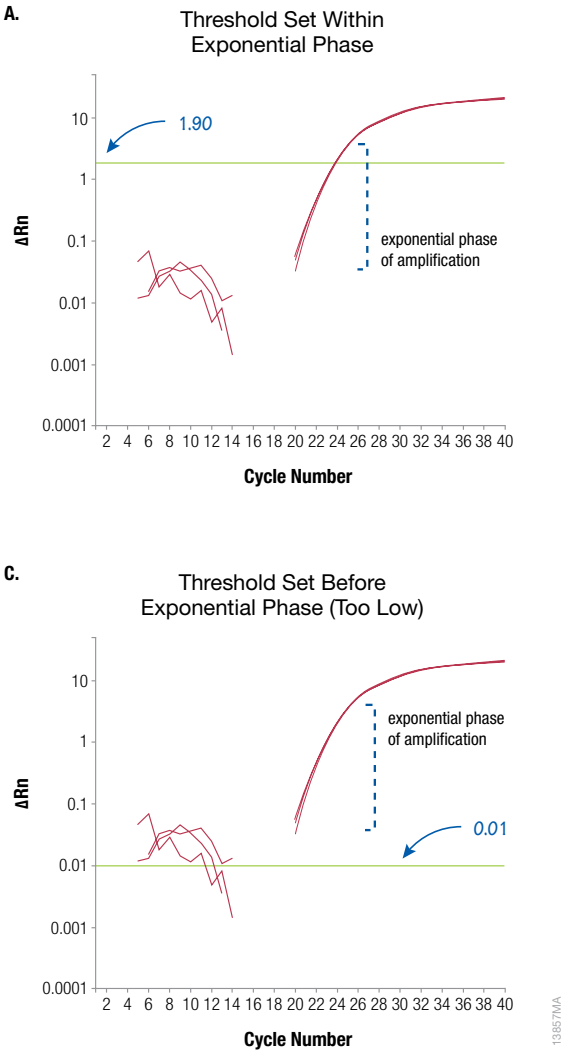

Figure 4. Perform a threshold check. On a semi-log plot of the log *fluorescence signal* versus cycle number confirm that the threshold falls within the exponential phase of the amplification curve. Quantification is most robust during the exponential phase. When plotted on a semi-log scale, the exponential phase of amplification appears linear.

4. Determining Which qPCR Master Mix Works Best

Once you have confirmed the quality of the amplification data generated, you can assess the specificity, repeatability, linearity, sensitivity and efficiency of the qPCR master mix and primer/probe combinations that you have tested. Always keep in mind that it is best practice to perform a full optimization of primer and probe concentrations and annealing/extension temperature in the thermal cycling conditions before using an assay routinely. This section will include example data from a qPCR reagent comparison (shown in Figures 5 and 6) for the discussion of each factor of qPCR reagent performance.

4.A. Specificity of qPCR Amplification

Check the NTC reactions to ensure that no amplification is observed; a C_q value should not be determined for these NTC reactions. 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. C_q values earlier than cycle 38 may be indicative of primer-dimer artifact or reagent contamination.

dsDNA Dye Systems

If using a dsDNA dye binding detection method, the post-amplification melt curve analysis can provide additional information regarding the specificity of your qPCR assay. For reactions where a single product is expected, the melt curve should show a single, well-defined peak, as in Figure 5, Panel A. The melting temperature of the same amplification product may be different between master mixes, but should be consistent for a single master mix. Reactions for a single master mix that show an irregular melt profile likely contain non-specific amplification products and should be excluded from the analysis.

Hydrolysis Probe-Based Systems

With hydrolysis probe-based detection, the accumulation of fluorescent signal is irreversible, and melt analysis is not possible. To check whether multiple amplification products are generated in the amplification reaction, you can run post-amplification analysis by gel electrophoresis. Theoretically, the probe increases specificity for the *detection* of the desired product; however, it does not alter the specificity of the *amplification*. Amplification of multiple products in a probe-based detection qPCR assay is not always problematic, unless the secondary products are generated at high levels. In this case, the secondary products can compete with the target for amplification by the polymerase, decreasing the qPCR efficiency for the product detected by the probe.

Note: To minimize the risk of contamination, we recommend designating separate zones in the lab, as well as a separate set of pipettes, for the handling of qPCR reagents versus the post-amplification analysis, such as running gel electrophoresis.

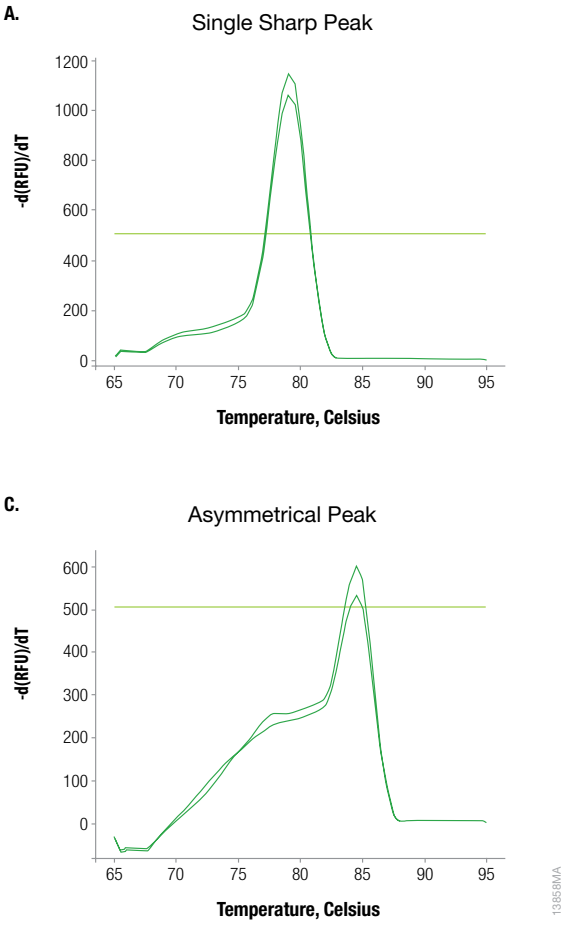

Figure 5. Specific reactions with dsDNA dye based detection of amplification products have a single peak in the melt curve. The negative first derivative of the change in fluorescence signal versus temperature is plotted. **Panel A.** The melt curve analysis gives a single, sharp and symmetrical peak indicative of a specific amplification reaction. **Panels B and C.** For reactions where a single product is expected, additional peaks in the melting curve represent non-specific species generated in your reaction.

4.B. Repeatability of a qPCR Assay

Calculate the average and the standard deviation for the C_q of the three technical replicates of each DNA concentration and assay. Reagents and experimental technique (including pipetting accuracy) can affect standard deviation, particularly at the high and low ends of the concentration range. Lower standard deviation at the extremes of the standard curve may indicate a robust reagent with a broad linear range. For most applications, standard deviations of <0.5 cycles are acceptable.

Note: If variability of the C_q at all DNA input concentrations is high, more attention to experimental technique may be required. Check the calibration of your pipette and ensure you are following proper pipetting practices. Make sure to thoroughly mix the primers and qPCR master mixes before preparing the reaction mixes. Finally, make sure to mix the DNA serial dilutions and reaction mixes thoroughly before pipetting into the amplification plate.

4.C. qPCR Assay Linearity

Most qPCR instrument software includes an option to review standard curve data fitted with a linear regression, as long as the concentrations of the standards have been indicated in the appropriate wells. From this graph, the assay linearity can be determined from the coefficient of determination (r^2). If your instrument software does not have an option to fit the data with a standard curve, you can perform linear regression on the data plotted as C_q versus log *input concentration* using spreadsheet software. Points at the high and low end of the template concentration range may not be within the linear range of the assay, and so the assay linear range should be defined.

1. Determine if the r^2 value over the entire standard curve is ≥0.98 (1).
2. If not, analyze the plotted data to determine whether the highest or lowest template concentrations deviate substantially from the plotted line. If any of these points deviate from the plotted line, exclude them from the analysis. The assay is not linear at that concentration.
3. If the r^2 value over the remaining standards (after exclusion of those outside the linear range) is now ≥0.98, the assay is linear over the remaining concentrations.

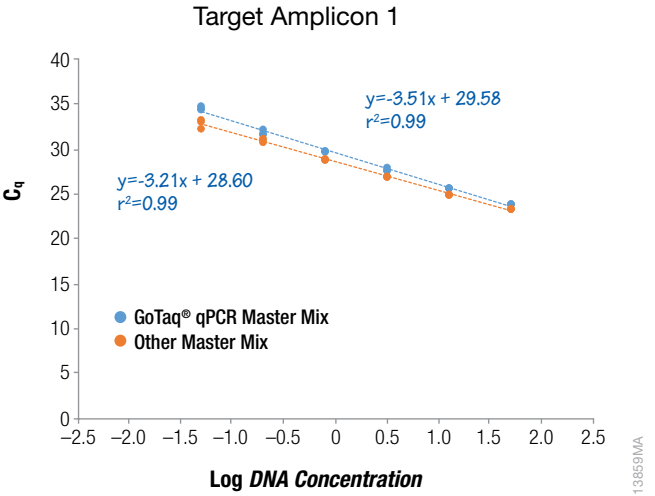

Figure 6: Comparison of the linear regression for two target amplicons in a qPCR assay. A single target was amplified from gDNA using GoTaq® qPCR Master Mix and another qPCR master mix over a range of gDNA concentrations. The data were plotted as average C_q versus log *DNA concentration*. For the target amplified, no data points were excluded from the linear regression analysis due to a negative impact on the coefficient of determination (r^2). All of the gDNA concentrations tested were within the linear dynamic range for GoTaq® qPCR Master Mix and the other qPCR master mix.

4.D. qPCR 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 template concentration included for linear regression analysis. A more thorough analysis across a narrow range of concentration at the low end of the standard curve is required to determine sensitivity in terms of limit of detection (LOD) for presence/absence or limit of quantitation (LOQ) for how many copies can be reliably quantified.

4.E. Reaction Efficiency

The efficiency of a qPCR assay can be determined from the slope of the linear regression according to the following equation and is automatically calculated in most software:

$$Efficiency = -1 + 10^{\left(-\frac{1}{slope}\right)}$$

In a theoretically perfect qPCR assay, the efficiency would be 100%. The efficiency may be calculated to be greater than 100%, as mathematical methods are approximations. In practice, efficiency values within the range of 90 to 110% are acceptable (1).

5. The Final Decision: A Side-by-Side Comparison of qPCR Master Mix Performance

Now that you have individually assessed each factor of qPCR performance, you can compare the reagents side-by-side using the Comparison Checklist in Section 6. The product that has the greatest number of checkmarks may be performing better for your assay. Keep in mind that plate-to-plate and user variation can affect the results of this type of comparison. If you are a confident and experienced user of the qPCR technique and the difference in reagents is substantial, then a single plate may allow you to determine the better qPCR reagent for your assay. However, if you are a novice user, you may want to perform the assays on replicate plates to look at the reproducibility of your results over multiple runs.

It is worth emphasizing that the intention of this guide is to provide a means to quickly compare qPCR master mix performance within two plates and two instrument runs. A thorough analysis of reagent performance would involve multiple plates to test the reagents over a range of DNA concentrations with high, medium and low primer concentrations (e.g., 250, 500 and 750nM primer) and annealing/extension temperatures (i.e., ±2°C).

If you need any additional assistance with data analysis or choosing a qPCR master mix that will suit your needs, please contact Promega Technical Services (**techserv@promega.com**, 1-800-356-9526; please check our homepage for your local branch or distributor contact).

6. Comparison Checklist

Below is an example checklist to use as a tool in a comparison of qPCR reagents. 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 corresponding column in each case that the qPCR master mix fits the indicated criteria. If the two qPCR reagents are equivalent for a specific criterion, put a check mark in both columns. The qPCR master mix that has the most checkmarks performs best for your assay of interest.

		GoTaq® qPCR MM	Other qPCR MM
Target 1:	**Specificity** Was there no or very late amplification in the NTC reaction wells? If you are using dye-based qPCR for amplification of a single target, did you observe a single peak in the melt analysis?		
	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%?		
Total Checkmarks			
		GoTaq® qPCR MM	**Other qPCR MM**
Target 2:	**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?		
	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%?		
Total Checkmarks			

7. 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.

8. Additional Resources

PubHub Articles:

- Is qPCR For You?
- FAQ for RT-qPCR

qPCR Webinars:

- Introduction to Real-Time PCR: Basic Principles and Chemistries
- Optimize Your qPCR and RT-qPCR Assays with Careful Planning and Design
- Maximize Your Reverse Transcription-qPCR (RT-qPCR) Assays

Application Notes:

- Real-Time PCR: Considerations for Comparing Reagent Performance
- Real-Time PCR: Guidelines for a Comparison of Reagent Performance

Promega Connections Blog:

- Harnessing qPCR and RT-qPCR in Your Laboratory
- qPCR: The Very Basics