

# Reagent Comparison Guide for Real-Time PCR



## Introduction

With the introduction of the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines for publishing real-time PCR results (Bustin et al. 2009), it has become increasingly important to ensure that data being generated from these experiments are fully “validated” for acceptable performance. As a strong supporter of the **MIQE guidelines (Taylor et al. 2011)** and a leading manufacturer of real-time PCR instruments and consumables, Bio-Rad strives to help researchers make informed decisions about the wide variety of reagents offered on the market today.

This guide is intended to help researchers design effective reagent comparisons and maximize the quality of data generated in their real-time PCR experiments.

## Reagent and Assay Comparison Matrix



| Comparison                                  | Reagents to Use    |                     |                         |
|---|--------------------|---------------------|-------------------------|
|   | cDNA Synthesis Kit | Supermix/Master Mix | qPCR Assay              |
| cDNA synthesis kits only                    | Bio-Rad            | Same                | Same                    |
|   | Other vendor       |                     |                         |
| Supermix/master mix only                    | Same               | Bio-Rad             | Same                    |
|   |                    | Other vendor        |                         |
| qPCR assays only                            | Same               | Same                | Bio-Rad PrimePCR assays |
|   |                    |                     | Other vendor            |
| cDNA synthesis kits and supermix/master mix | Bio-Rad            | Bio-Rad             | Same                    |
|   | Other vendor       | Other vendor        |                         |
| Supermix/master mix and qPCR assays         | Same               | Bio-Rad             | Bio-Rad PrimePCR assays |
|   |                    | Other vendor        | Other vendor            |

# Important Considerations for Reagents Comparison

It is not uncommon for a reagents comparison to be performed using a single assay and/or data point and for conclusions to be drawn from quantification cycle (C<sub>q</sub>) and standard deviation (SD) values from that single experimental run. Unfortunately, C<sub>q</sub> values are only relative numbers within a given experiment and alone are not indicative of reagent or assay performance. Through proper experimental design, one can investigate multiple performance characteristics that, in addition to C<sub>q</sub> values, will help determine the overall performance of various reagents. An appropriate reagents comparison should allow one to test the assay parameters below in experiments that include serial dilutions and melt curve analysis (for SYBR<sup>®</sup> Green assays). Only after consideration of all these performance characteristics can one make an effective judgment of reagent performance.

## Efficiency

The term “efficiency” can refer to performance characteristics for both reverse transcription (RT) and quantitative PCR (qPCR). RT efficiency refers to how accurately the RT enzyme can represent the number of RNA molecules as cDNA. For example, if a particular sample contains 1,000 RNA transcripts for a given gene, an RT reaction with 100% efficiency will generate exactly 1,000 cDNA templates. Unfortunately, many RT enzymes exhibit a bias for accurate representation of high- or low-copy transcripts, which can impact the quantitation of expressed genes. qPCR efficiency refers to how effectively the PCR polymerase duplicates the template DNA during every cycle in a concentration-independent manner (100% efficiency being ideal). For both RT and qPCR, as efficiencies decrease, the accuracy of quantification also decreases. RT efficiency is determined from analysis of a serial dilution of RNA (typically 1 µg), followed by multiple RT reactions and qPCR. qPCR efficiency is determined from analysis of a serial dilution of cDNA amount (typically from 1 µg RNA). Thus, RT and qPCR efficiencies cannot be measured simultaneously through a single RT-qPCR reaction and must be measured independently. It is important that all RT-qPCR or qPCR assays are validated for acceptable efficiencies if accurate relative or absolute quantification is desired.

## Linearity

For a given serial dilution (standard curve) of RNA or DNA, the coefficient of determination (R<sup>2</sup>) value refers to how well the experimentally defined C<sub>q</sub> values correlate with the dilution series (or how well the points “fit the line”). For quality assays and results, the R<sup>2</sup> value should be ≥0.980. When the R<sup>2</sup> is <0.980, it is due in most cases to outlier data points or pipetting variability.

## Dynamic Range

Dynamic range is the range of input template (between the highest and lowest input RNA or DNA) for which acceptable linearity (R<sup>2</sup> is ≥0.980) and efficiency (preferably between 90 and 110%) are observed. For qPCR experiment results, the only valid C<sub>q</sub> values are those that fall into the validated dynamic range.

## Sensitivity

The lowest amount of starting material that can be reliably detected using qPCR is a measure of sensitivity. It is the lowest point of the serial dilution where replicate reproducibility is high and the linearity of the standard curve is unaffected (R<sup>2</sup> is ≥0.980). Sensitivity is assay specific and dependent on sample type and quality. It is not the y-intercept of the C<sub>q</sub> vs. concentration graph and should be determined only empirically, not through extrapolation.

## Specificity

The ability of a qPCR assay to generate a single specific and expected amplicon that can be verified using melt curve analysis (for SYBR<sup>®</sup> Green) is important for accurate quantification. When using probe-based assays, such as TaqMan, fluorescence detection and specificity are conferred through use of the probe; however, amplification specificity is not verified and can be confirmed only through additional gel and/or sequencing analysis.

## Reproducibility

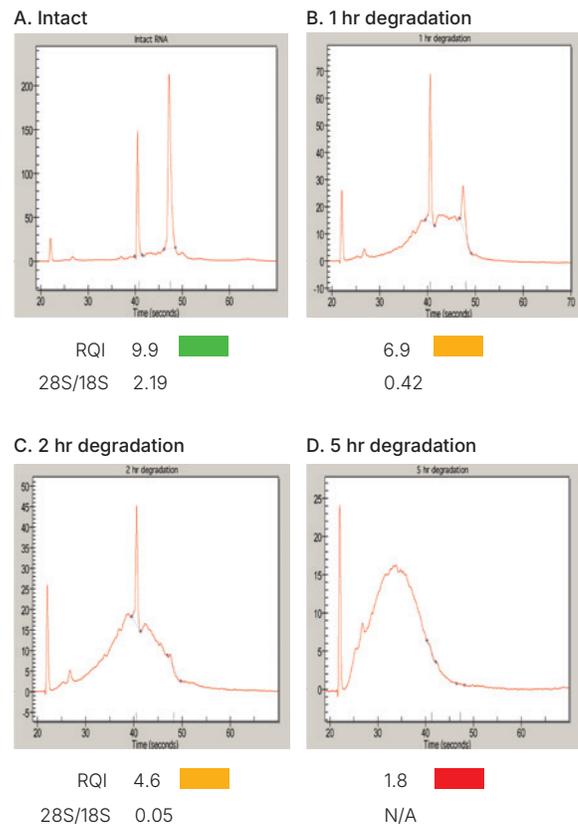
Numerous factors contribute to variability in qPCR. Variability is measured as the SD between replicates and can occur in the form of the following: biological replicates (biological variability), technical replicates (technical variability), or differences between identical reactions on different plate runs (inter-run or instrument variability). In this reagents comparison, we will be able to assess technical variability between replicate wells. Often, reagents will exhibit different variability characteristics at the lowest and highest input template amounts. Reduced technical variability can also decrease the number of biological replicates required for determining statistically significant fold changes.

# Preparation of the Samples

## RNA Samples

- Isolate RNA using the appropriate method for the given sample type (for example, **Aurum Total RNA Mini Kit** for cell lines; **Aurum Total RNA Fatty and Fibrous Tissue Kit** for tissue samples)
- Compare the expected yield to the actual yield to ensure the isolation method yielded the appropriate RNA concentrations (for example, 5–30 pg/cell, 0.1–4 µg/mg tissue). When the yield is less than expected, this may lead to less than optimal qPCR data results due to less than ideal quality samples as a result of a less than optimal sample preparation workflow
- When the RNA will be used for RT-qPCR, it is recommended that you treat the sample with DNase to remove residual contaminating DNA. DNase treatment is also a good idea when isolating RNA from tissues that are high in DNA because the excess DNA may affect downstream applications
- Store the RNA in an appropriate solution:
  - 0.1 mM EDTA (in DEPC-treated ultrapure water)
  - TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0)
- Store the RNA at –80°C in single-use aliquots
- Assess the RNA quality for integrity and purity
  - Use the **Bio-Rad Experion Automated Electrophoresis System** to evaluate the integrity of the RNA sample. When using multiple samples in the comparison, ensure that the RQI numbers are similar to ensure accurate qPCR results
  - Use an agarose gel to assess RNA integrity if the above systems are not available. Apply the same analysis concepts. High-quality RNA will yield 2 clean peaks, 18S and 28S (Figure 1A). Degraded RNA will appear as a smear on the gel
  - To assess purity, evaluate the following spectrophotometer readings:
    - $A_{260}/A_{280} > 2.0$  for pure RNA
    - $A_{260}/A_{230} \sim 2.0$  for pure RNA

**Note:** Lower ratios are indicative of contaminants from salts, carbohydrates, peptides, proteins, phenols, and guanidine thiocyanate.



**Fig. 1. Experion RNA quality indicator.** A, high-quality RNA is indicated by the presence of the 18S and 28S peaks and a flat baseline; B, as RNA degrades, 28S degrades first and the baseline becomes noisier; C–D, further degradation.

# Preparation of the Samples

## DNA Samples

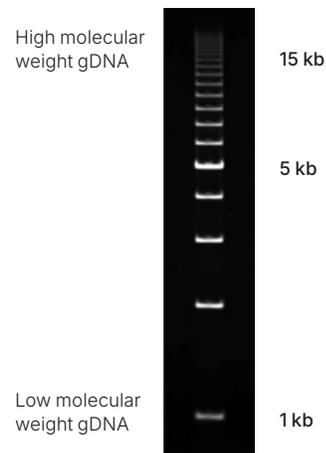
- Isolate DNA using the appropriate method for the given sample type (for example, column purification for cell lines; phenol/chloroform/column purification for tissue samples)
- Store the DNA in an appropriate solution:
  - 0.1 mM EDTA (in DEPC-treated ultrapure water)
  - TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
- Store the DNA at  $-80^{\circ}\text{C}$  in single-use aliquots
- Assess DNA quality with an agarose gel (Figure 2); a single band indicates high-integrity DNA, whereas a smear indicates degraded DNA
- Assess the DNA purity using a spectrophotometer for the following:
  - $A_{260}/A_{230} > 1.5$  (lower ratios may be attributed to carryover guanidine and/or inhibitors like humic acid and organics)
  - $A_{260}/A_{280} 1.7\text{--}2.0$  (lower ratios are indicative of contaminants from salts, carbohydrates, peptides, proteins, phenols, and guanidine thiocyanate; higher ratios may be indicative of RNA contamination)

### Tips for DNA Samples

- Heat treating DNA may be required prior to qPCR to relax strong secondary structure
- Using a restriction digest enzyme may be required for select qPCR applications, such as copy number variation, to increase the signal-to-noise ratio

## Plasmid Samples

- Prepare plasmids using an appropriate method
- Store the stock plasmid in an appropriate solution, such as TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
- Store the plasmid at  $-80^{\circ}\text{C}$  in single-use aliquots
  - Storing concentrated plasmids helps to ensure integrity because diluted plasmids tend to degrade in storage. Adding a carrier (for example, glycogen or tRNA) to the plasmid helps preserve integrity and produces greater precision of qPCR data where sensitivity is desired
- Assess plasmid quality with an agarose gel; a single band indicates high-integrity plasmid, whereas a smear indicates degraded plasmid or excess enzymatic activity
- Assess the plasmid purity using a spectrophotometer for the following:
  - $A_{260}/A_{280} 1.7\text{--}1.9$  (lower ratios are indicative of contaminants from salts, carbohydrates, peptides, proteins, phenols, and guanidine thiocyanate; higher ratios may be indicative of RNA contamination)



**Fig. 2. Electrophoresis of genomic DNA (gDNA).** Ensuring high molecular weight gDNA enables optimal qPCR data; highly fragmented gDNA may lead to less than optimal qPCR data.

# Preparation of the Assays

## Predesigned Assays

There are a few important considerations to note when comparing assays between vendors, mainly, the design and validation. While some vendors simply use limited bioinformatics pipelines, **Bio-Rad PrimePCR assays** for real-time PCR are expertly designed and wet-lab validated to ensure optimal assay performance. Wet-lab validation of every assay provides confidence in results while eliminating the time-consuming assay design and optimization steps. These differences will affect the qPCR data quality and results.

In addition, it is important to ensure that the assays in the comparison are targeting the same loci (for example, for *GAPDH*, both assays should target the exonic region 12:6643166–6643352). When different isoforms (splice variants) are targeted, as in the example below, the gene expression data may vary greatly and invalidate the comparison due to incorrect assay choices, not performance.

### Example

*VEGF* 120/121 isoforms are predominantly expressed in human tissues and cells. However, *VEGF* 205/206 isoforms are rare variants. If assay #1 in the comparison targets 120/121 and assay #2 in the comparison targets 205/206, there may be significant differences in the qPCR data, thus resulting in an invalid comparison.

## Custom Assays

When using custom-designed assays, several important considerations should be noted:

- Biological significance (for example, correct isoform/splice variant chosen)
- Sequence quality/secondary structure—evaluate using web-based tools to understand the complexity of the structure because it can impact the reaction performance
- Sequence length—use the entire gene sequence or specific region of interest for the bioinformatics steps to ensure a well-designed assay can be completed
- Masking the sequence—use web-based masking tools to mask low-complexity and repetitive regions, avoiding assay design in these regions
- Uniqueness of the sequence (BLAST or BLAT)—ensure no homology exists to avoid mispriming events
- Uniqueness of the assay (in silico PCR—a method to “blast” the primers against the genome of interest to validate primer design specificity)

## Assay Design Considerations

Although there are numerous assay design software tools available, it is best to use a tool that will provide a complete assessment of the sequence and primer considerations. Note the default settings in the software and ensure they are set correctly (for example, salt conditions and oligo and amplicon sizes). We recommend the use of Beacon Designer software for a complete and thorough evaluation of the sequence and assay design.

### Some Key Design Attributes

- For optimal PCR efficiency, design the amplicon size between 70 and 150 bp (<70 bp may be needed for degraded or formalin-fixed paraffin-embedded template)
- Maintain primer lengths between 18–22 bp for good specificity and binding abilities
- Annealing temperatures ( $T_a$ ) between 58–62°C are optimal (greater range can be obtained using Bio-Rad's Sso7d-based supermixes); a higher  $T_a$  may result in less binding efficiency and a lower  $T_a$  may result in less specificity
- GC content 40–60%
- Avoiding primer secondary structures reduces potential primer-dimer issues
- No more than 2 GCs in the last 5 bases on the 3' end of the primer avoids mispriming

# Reverse Transcription Comparison

## STEP 1A

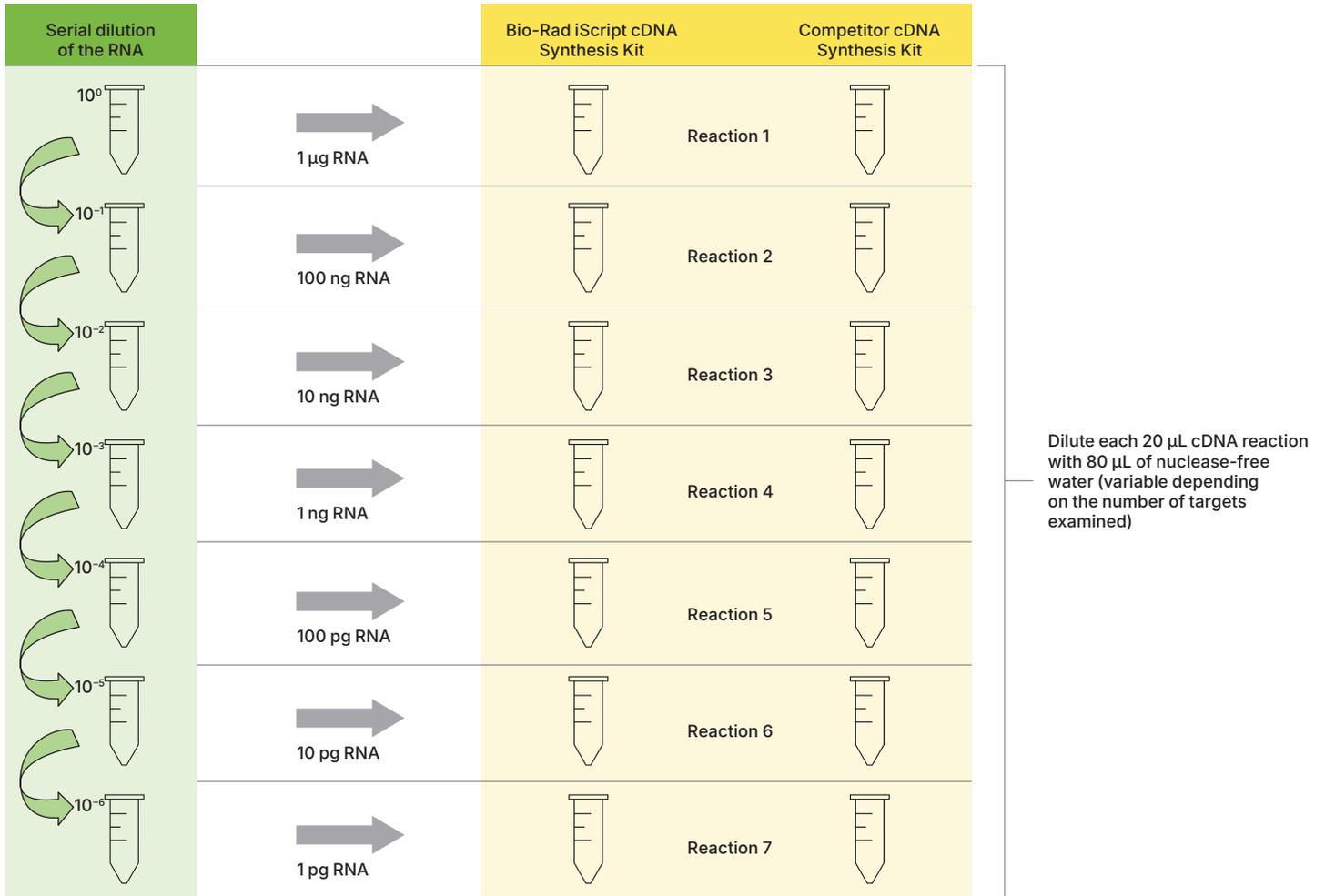
### Preparing the RNA Serial Dilution

A serial dilution of a single RNA source or pooled RNA sample is required to prepare the cDNA synthesis reactions for the experiment. Comparing two cDNA synthesis kits requires an adequate amount of RNA; adjust concentrations and volumes accordingly.

## STEP 1B

### Preparing the cDNA Synthesis Reactions

Perform RT using 20  $\mu$ L reactions. Transfer the RNA, as shown in the diagram, to the respective reaction tubes. For example, transfer 1  $\mu$ g of RNA to each Reaction 1 tube. Repeat transferring RNA to the remaining reaction tubes.



### Tips

- Always use the same thermal cycler for the comparison to ensure uniformity throughout the evaluation
- A 10-fold dilution series is recommended to cover the most logs of dynamic range; however, depending on the expression level of the gene(s) evaluated and total RNA available, this can be reduced to a 5-fold dilution series
- The starting input RNA concentration may be adjusted to suit the total RNA concentration available

# Reverse Transcription Comparison

## Preparing the qPCR Reaction

Now that you have your RNA serial dilutions synthesized into cDNA, it is time to prepare the qPCR reaction.

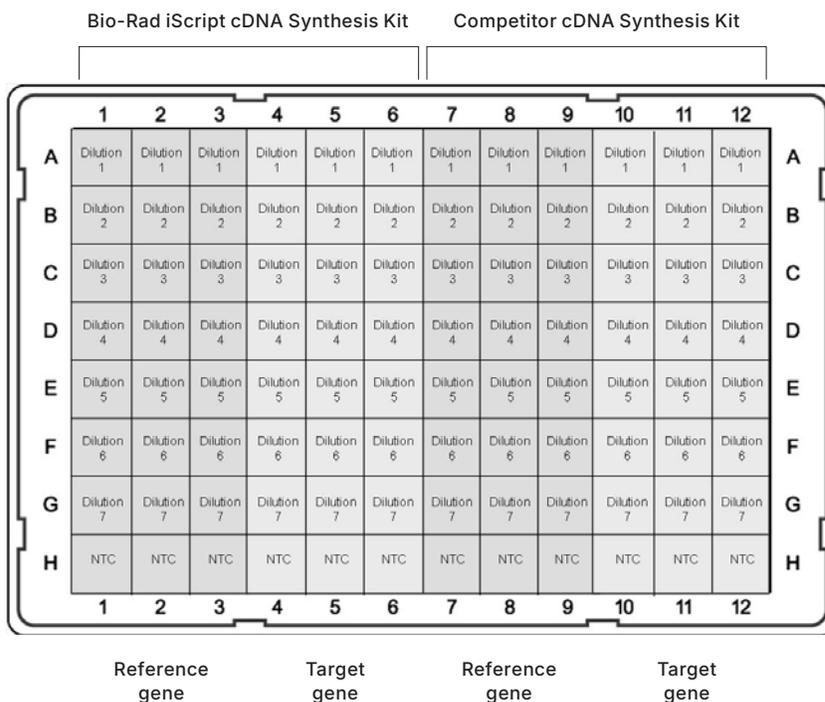
1. To prepare a master mix for each gene, label two 1.5 mL tubes: (R) reference and (T) target. To each tube, add 500  $\mu$ L of 2x supermix, 250  $\mu$ L of nuclease-free water, and 250  $\mu$ L of primers. This will give you more than enough for 48 wells.

**Note:** Volumes shown assume 20  $\mu$ L for 96-well instruments. For 384-well instruments, volumes can be halved to achieve a final reaction volume of 10  $\mu$ L.

2. Touch vortex and then touch spin for each master mix.
3. Pipet 15  $\mu$ L of the master mix into the corresponding wells.
4. Add 5  $\mu$ L of the appropriate diluted cDNA to the corresponding wells.
5. Seal the plate well and centrifuge for 1 min at 1,000 rpm.
6. Cycle as recommended by the manufacturer for the supermix.
7. Analyze the data as outlined on page 9.

### Tips

- To increase data precision, prepare individual master mixes for each sample by combining 45  $\mu$ L of the qPCR supermix, nuclease-free water, and primers mixture with 15  $\mu$ L of the template. Then pipet 20  $\mu$ L to the respective wells on the plate
- Although performing the comparison with a minimum of two assays is adequate (reference gene and target gene), we strongly recommend the use of four assays—reference, low, medium, and high expressing genes—for the most robust dynamic range and performance evaluation. This will require the use of two plates, one for each cDNA synthesis kit



# Reverse Transcription Comparison

## Analyzing the Data

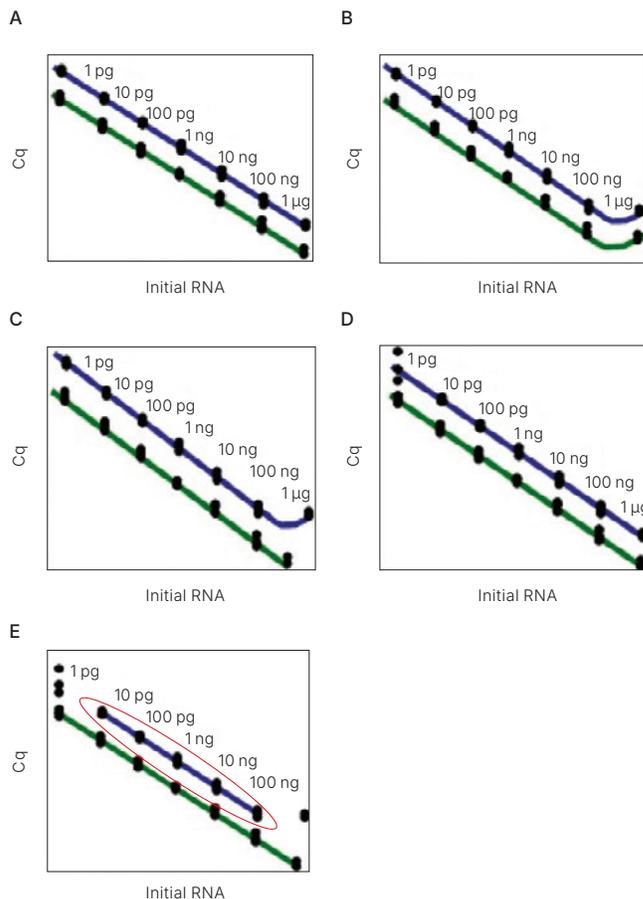
Once the qPCR reaction is complete, it is time to evaluate the results. As you evaluate the standard curves, the following key attributes will enable you to determine the best performing reverse transcription kit.

**Dynamic range:** This range is defined by the upper and lower input amounts of RNA into the RT reaction where a linear response exists. The larger the range, the broader the concentration of RNA that may be used in the cDNA synthesis reaction. Cq values obtained outside of the range are invalid; however, this information is not known unless dynamic range testing is completed.

**Linearity:** The  $R^2$  statistic for the serial dilution (standard curve) should be as close to 1.000 as possible. In most cases, when the  $R^2$  statistic is  $<0.98$ , it is due to a single outlier data point. When this point is removed from consideration, the linearity of the curve returns to near 1.000 with improvement in efficiency.

**RT inhibition:** At the most concentrated points, it is not uncommon for the RT reaction to be either inhibited due to carryover contaminants from the sample preparation step, or for the RT reaction to become oversaturated with RNA due to an input greater than the specifications for the kit (Figure 3B). The range in which an issue is noted must be removed from the final dynamic range definition.

**Sensitivity:** At the most dilute points, the SD of the technical replicates often increases due to the Monte Carlo effect (Figure 3D). When pipetting low concentrations, it is very difficult to evenly distribute molecules into each replicate well. As a result, inaccurate qPCR data obtained at these points, and the affected range, must be removed from the final dynamic range definition.



**Fig. 3. Representative standard curves of reagents and assay performance.** **A**, two reagents with equivalent performance, even though one reagent exhibits earlier Cq values; **B**, both reagents are either saturated at the 1  $\mu$ g data point or the RT reaction is inhibited; **C**, one reagent has a broader dynamic range than the other reagent; **D**, variability for one RT kit is unacceptably high at the lowest concentration (1 pg) and should not be considered part of the dynamic range; **E**, concentration points that define the dynamic range from one RT kit after rejecting the variant (1 pg) data and the saturated/inhibited 1  $\mu$ g data point are circled; the effective dynamic range is 10 pg–100 ng, which indicates a less optimal cDNA synthesis kit compared with the other. Cq, quantification cycle.

# qPCR Supermix Comparison

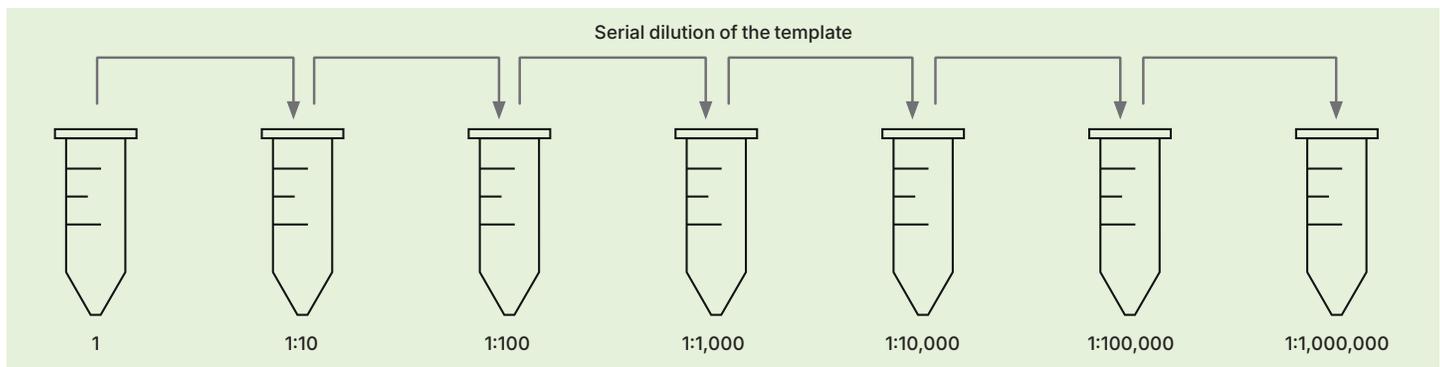
## Preparing the Template Serial Dilution

A serial dilution of the cDNA, gDNA, or plasmid template is required to prepare the qPCR supermix comparison. When comparing two or more supermixes, ensure an adequate supply of template and volume is available. A tenfold dilution series is recommended to cover the most logs of dynamic range; however, depending on the expression level of the gene(s) evaluated and the total template amount available, this can be reduced to a fivefold dilution series.

### Calculating the volume required per dilution for the qPCR reactions

- Sample volume per well = 5  $\mu$ L
- Number of assays = 4
- Number of technical replicates = 3
- Number of supermixes = 2
- Amount of excess for pipet error = 15  $\mu$ L

$(5 \times 4 \times 3 \times 2) + 15 = 135 \mu\text{L}$  total volume required per dilution point



# qPCR Supermix Comparison

## Preparing the qPCR Supermix Reactions

To evaluate the performance of a Bio-Rad qPCR supermix compared to your current master mix, **we recommend preparing two plates** for evaluation using standard curves. This is because of the differences in polymerase activation times (for example, Bio-Rad qPCR supermixes require only 30 seconds to 3 minutes).

1. Dilute your template as outlined on page 10, then proceed to the next step.
2. For each supermix and for each gene, label a 1.5 mL tube: (R) reference, (L) low expressor, (M) medium expressor, and (H) high expressor. To each tube, add 250  $\mu$ L of 2x supermix, 125  $\mu$ L of nuclease-free water, and 125  $\mu$ L of primers. This will give you more than enough for 24 wells.

**Note:** Volumes shown assume 20  $\mu$ L for 96-well instruments. For 384-well instruments, volumes can be halved to achieve a final reaction volume of 10  $\mu$ L.

3. Touch vortex and then touch spin for each master mix.
4. Pipet 15  $\mu$ L of the master mix into the corresponding wells.
5. Add 5  $\mu$ L of the appropriate diluted cDNA to the corresponding wells.
6. Seal the plate well and centrifuge for 1 min at 1,000 rpm.
7. Cycle as recommended by the manufacturer for the supermix/master mix.
8. Analyze the data as outlined on pages 13–14.

### Tips

- To increase data precision, prepare individual master mixes for each sample by combining 45  $\mu$ L of the qPCR supermix, nuclease-free water, and primers mixture with 15  $\mu$ L of the template. Then pipet 20  $\mu$ L to the respective wells on the plate
- Although performing the comparison with a minimum of two assays is adequate (reference gene and target gene), we strongly recommend the use of four assays—reference, low, medium, and high expressing genes—for the most robust dynamic range and performance evaluation

Recommended Plate Layout

|   |                |            |            |               |            |            |                  |            |            |                |            |            |   |
|---|----------------|------------|------------|---------------|------------|------------|------------------|------------|------------|----------------|------------|------------|---|
|   | 1              | 2          | 3          | 4             | 5          | 6          | 7                | 8          | 9          | 10             | 11         | 12         |   |
| A | Dilution 1     | Dilution 1 | Dilution 1 | Dilution 1    | Dilution 1 | Dilution 1 | Dilution 1       | Dilution 1 | Dilution 1 | Dilution 1     | Dilution 1 | Dilution 1 | A |
| B | Dilution 2     | Dilution 2 | Dilution 2 | Dilution 2    | Dilution 2 | Dilution 2 | Dilution 2       | Dilution 2 | Dilution 2 | Dilution 2     | Dilution 2 | Dilution 2 | B |
| C | Dilution 3     | Dilution 3 | Dilution 3 | Dilution 3    | Dilution 3 | Dilution 3 | Dilution 3       | Dilution 3 | Dilution 3 | Dilution 3     | Dilution 3 | Dilution 3 | C |
| D | Dilution 4     | Dilution 4 | Dilution 4 | Dilution 4    | Dilution 4 | Dilution 4 | Dilution 4       | Dilution 4 | Dilution 4 | Dilution 4     | Dilution 4 | Dilution 4 | D |
| E | Dilution 5     | Dilution 5 | Dilution 5 | Dilution 5    | Dilution 5 | Dilution 5 | Dilution 5       | Dilution 5 | Dilution 5 | Dilution 5     | Dilution 5 | Dilution 5 | E |
| F | Dilution 6     | Dilution 6 | Dilution 6 | Dilution 6    | Dilution 6 | Dilution 6 | Dilution 6       | Dilution 6 | Dilution 6 | Dilution 6     | Dilution 6 | Dilution 6 | F |
| G | Dilution 7     | Dilution 7 | Dilution 7 | Dilution 7    | Dilution 7 | Dilution 7 | Dilution 7       | Dilution 7 | Dilution 7 | Dilution 7     | Dilution 7 | Dilution 7 | G |
| H | NTC            | NTC        | NTC        | NTC           | NTC        | NTC        | NTC              | NTC        | NTC        | NTC            | NTC        | NTC        | H |
|   | 1              | 2          | 3          | 4             | 5          | 6          | 7                | 8          | 9          | 10             | 11         | 12         |   |
|   | Reference gene |            |            | Low expressor |            |            | Medium expressor |            |            | High expressor |            |            |   |

# qPCR Supermix Comparison

## Preparing the qPCR Supermix Reactions

To evaluate the performance of a **Bio-Rad PrimePCR assay** compared to your current assay, **we recommend preparing one or two plates** for evaluation using standard curves based on the number of assays in the evaluation.

1. Dilute your template as outlined on page 10, then proceed to the next step.
  2. To prepare a Bio-Rad supermix (or your current master mix) for each gene, label two 1.5 mL tubes: (R) reference, (L) low expressor, (M) medium expressor, and (H) high expressor. To each tube, add 250  $\mu$ L of 2x supermix, 125  $\mu$ L of nuclease-free water, and 125  $\mu$ L of primers. This will give you more than enough for 24 wells.
- Note:** Volumes shown assume 20  $\mu$ L for 96-well instruments. For 384-well instruments, volumes can be halved to achieve a final reaction volume of 10  $\mu$ L.
3. Touch vortex and then touch spin for each master mix.
  4. Pipet 15  $\mu$ L of the master mix into the corresponding wells.
  5. Add 5  $\mu$ L of the appropriate diluted cDNA to the corresponding wells.
  6. Seal the plate well and centrifuge for 1 min at 1,000 rpm.
  7. Cycle as recommended by the manufacturer for the supermix/ master mix.
  8. Analyze the data as outlined on pages 13–14.

### Tips

- To increase data precision, prepare individual master mixes for each sample by combining 45  $\mu$ L of the qPCR supermix, nuclease-free water, and primers mixture with 15  $\mu$ L of the template. Then pipet 20  $\mu$ L to the respective wells on the plate
- Although performing the comparison with a minimum of two assays is adequate (reference gene and target gene), we strongly recommend the use of four assays—reference, low, medium, and high expressing genes—for the most robust dynamic range and performance evaluation

Recommended Plate Layout

|   |                |            |            |               |            |            |                  |            |            |                |            |            |   |
|---|----------------|------------|------------|---------------|------------|------------|------------------|------------|------------|----------------|------------|------------|---|
|   | 1              | 2          | 3          | 4             | 5          | 6          | 7                | 8          | 9          | 10             | 11         | 12         |   |
| A | Dilution 1     | Dilution 1 | Dilution 1 | Dilution 1    | Dilution 1 | Dilution 1 | Dilution 1       | Dilution 1 | Dilution 1 | Dilution 1     | Dilution 1 | Dilution 1 | A |
| B | Dilution 2     | Dilution 2 | Dilution 2 | Dilution 2    | Dilution 2 | Dilution 2 | Dilution 2       | Dilution 2 | Dilution 2 | Dilution 2     | Dilution 2 | Dilution 2 | B |
| C | Dilution 3     | Dilution 3 | Dilution 3 | Dilution 3    | Dilution 3 | Dilution 3 | Dilution 3       | Dilution 3 | Dilution 3 | Dilution 3     | Dilution 3 | Dilution 3 | C |
| D | Dilution 4     | Dilution 4 | Dilution 4 | Dilution 4    | Dilution 4 | Dilution 4 | Dilution 4       | Dilution 4 | Dilution 4 | Dilution 4     | Dilution 4 | Dilution 4 | D |
| E | Dilution 5     | Dilution 5 | Dilution 5 | Dilution 5    | Dilution 5 | Dilution 5 | Dilution 5       | Dilution 5 | Dilution 5 | Dilution 5     | Dilution 5 | Dilution 5 | E |
| F | Dilution 6     | Dilution 6 | Dilution 6 | Dilution 6    | Dilution 6 | Dilution 6 | Dilution 6       | Dilution 6 | Dilution 6 | Dilution 6     | Dilution 6 | Dilution 6 | F |
| G | Dilution 7     | Dilution 7 | Dilution 7 | Dilution 7    | Dilution 7 | Dilution 7 | Dilution 7       | Dilution 7 | Dilution 7 | Dilution 7     | Dilution 7 | Dilution 7 | G |
| H | NTC            | NTC        | NTC        | NTC           | NTC        | NTC        | NTC              | NTC        | NTC        | NTC            | NTC        | NTC        | H |
|   | 1              | 2          | 3          | 4             | 5          | 6          | 7                | 8          | 9          | 10             | 11         | 12         |   |
|   | Reference gene |            |            | Low expressor |            |            | Medium expressor |            |            | High expressor |            |            |   |

# Data Analysis

As you begin to analyze the data, it is important to note the following key points regarding Cq or threshold cycle ( $C_T$ ) values:

- The threshold setting can significantly alter the Cq value when using the single threshold method for Cq determination
- A Cq value by itself is relatively meaningless but becomes valuable once it is converted to a quantity value (absolute quantification) or used in  $\Delta Cq$  or  $\Delta\Delta Cq$  analysis (relative quantification)
- Supermix composition (for example, salts, pH level, reference dyes) and threshold determination will affect raw Cq values
- Understanding the PCR efficiency is critical to understanding a given Cq value. For example, compare the Cq value of 20 using supermix X with 80% efficiency vs. a Cq value of 22 using supermix Y with 100% efficiency. The Cq value from supermix X may appear to be better because it is 2 cycles sooner, but the lower efficiency of this assay with supermix X indicates less than optimal qPCR data will be obtained

Assay performance criteria you can determine using your analysis software include:

- Slope of the standard curve line
- R<sup>2</sup> for the entire dynamic range of input template
- Standard deviation between technical replicates
- Amplification specificity using melt curve analysis (for SYBR® Green)

## Data Exclusion and Variability

It is generally accepted that the expected deviation between technical replicates should be within 0.5 Cq. If this is not the case, outliers may be removed. If there are no clear outliers, the entire concentration point may be excluded. Determine the technical variability between replicates using SD. Compare the SD at each point for reagent X vs. reagent Y.

### Efficiency

Calculate efficiency using the software or the following equation:

$E = (10^{[-1/m]} - 1) \times 100$ . A PCR efficiency result between 90 and 110% efficiency (slope values from -3.6 to -3.1) is preferred.

### Linearity

Calculate the R<sup>2</sup> statistic for each standard curve using the qPCR analysis software. An R<sup>2</sup>  $\geq 0.980$  is acceptable. However, if the R<sup>2</sup> is  $< 0.980$ , remove data points as needed. For example, in Figures 4–5 on page 14, the linearity is affected by the lowest template concentrations. When these points are removed, the linearity of the curves will return to near 1.000.

### Dynamic Range

Determine the highest and lowest concentrations where linearity (R<sup>2</sup>) and efficiency are within acceptable ranges, as stated above. Some examples of standard curves and dynamic range calculations are provided on page 14 (Figures 4–5).

### Sensitivity

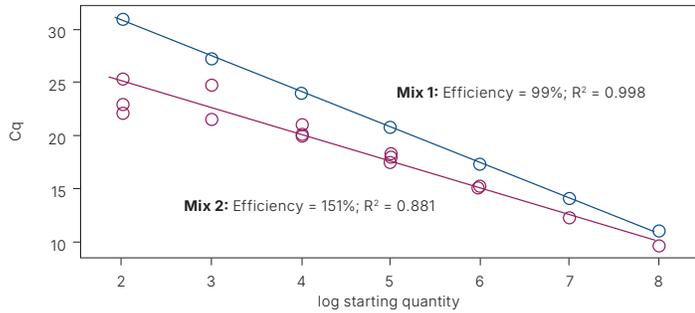
Determine the lowest concentration of the serial dilution where replicate reproducibility is high and the R<sup>2</sup> of the standard curve is  $\geq 0.980$ .

### Specificity

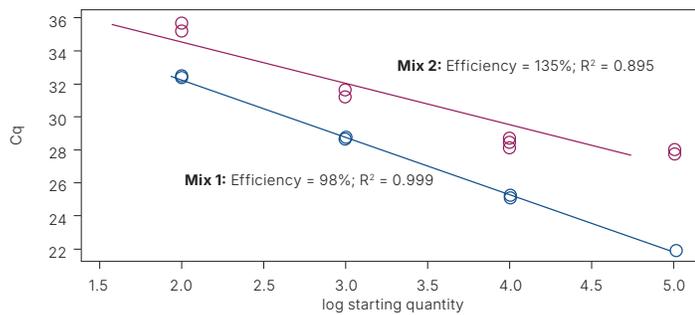
Evaluate the melt profiles when using SYBR®-based assays (Figure 6, page 14). For probe-based assays, gel analysis is required to observe a single band (one PCR product). However, because of the excessive primers provided in some predesigned assays, additional smaller band(s) may be noted as primer-dimers.

# Data Analysis

## Linearity and Dynamic Range



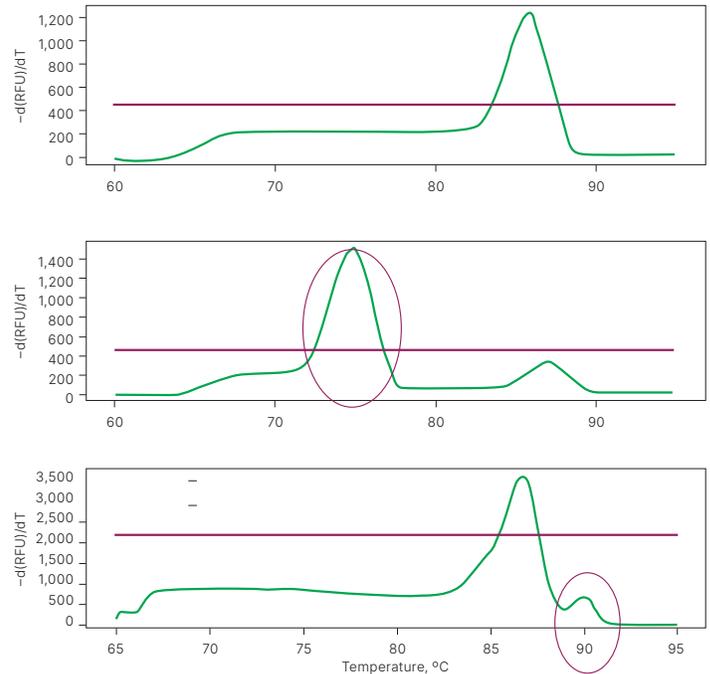
**Fig. 4. qPCR supermix comparison.** Comparison of two different supermixes targeting the same gene loci using the same template source and assay. Both reactions cover six logs of dynamic range; however, mix 2 demonstrated a higher PCR efficiency of 151%. Mix 1 performed at a 99% PCR efficiency with an  $R^2$  value of 0.998. The optimal mix is mix 1 because of its better PCR efficiency and  $R^2$  values. Cq, quantification cycle.



**Fig. 5. qPCR assay comparison.** Comparison of two different qPCR assays for the same target using the same template source and supermix/master mix. Both reactions cover three logs of dynamic range; however, mix 2 demonstrated a higher PCR efficiency of 135%. Mix 1 performed at a 98% PCR efficiency with an  $R^2$  value of 0.999. The optimal mix is mix 1 because of its better PCR efficiency and  $R^2$  values. Cq, quantification cycle.

## Specificity

For SYBR® Green assays, amplification specificity can be verified by examining the melt curve profile. Sample melt curves are shown in Figure 6. When using probe-based qPCR, specificity can be determined only by agarose gel electrophoresis and/or sequence validation.



**Fig. 6. Melt curve profiles.** A, a single well-defined peak indicates a single specific product. B, two or more peaks indicate poor specificity. A typical primer-dimer (in red) is due to excess final primer concentrations. Primer-dimers typically amplify in the mid-70°C range. C, two or more peaks indicate poor specificity. A typical mispriming (in red) is shown.

# Data Record

| Gene      | Efficiency, % | Linearity, R <sup>2</sup> | Dynamic Range, concentration | Sensitivity, concentration | Specificity, Y/N |
|-----------|---------------|---------------------------|------------------------------|----------------------------|------------------|
| <b>1</b>  |               |                           |                              |                            |                  |
| Reagent A |               |                           |                              |                            |                  |
| Reagent B |               |                           |                              |                            |                  |
| <b>2</b>  |               |                           |                              |                            |                  |
| Reagent A |               |                           |                              |                            |                  |
| Reagent B |               |                           |                              |                            |                  |
| <b>3</b>  |               |                           |                              |                            |                  |
| Reagent A |               |                           |                              |                            |                  |
| Reagent B |               |                           |                              |                            |                  |
| <b>4</b>  |               |                           |                              |                            |                  |
| Reagent A |               |                           |                              |                            |                  |
| Reagent B |               |                           |                              |                            |                  |

### Interpretation of Results

It is important to use more than Cq and SD values to draw proper conclusions from a reagent comparison. Along with these values, use the data compiled in the sample table above to perform a thorough analysis and performance comparison between reagents. For assistance with interpreting your results or for general questions regarding the use of this guide, please contact Bio-Rad Technical Services at 1-800-424-6723.

## References

Bustin SA et al. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55, 611–622.

Taylor S et al. (2011). A practical approach to RT-qPCR—publishing data that conform to the MIQE guidelines. *Bio-Rad Bulletin* 5859.

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