# Key Considerations in qPCR Assay Controls

By Nish Kumar, Bio-Rad Laboratories, Inc.

## Introduction

Quantitative PCR (qPCR) is a widely utilized technique for the semi-quantitative study of gene expression. However, its accuracy and reproducibility depend significantly on the proper use of assay controls. This brief e-book delves into the distinct types of assay controls crucial for ensuring reliable qPCR results. It guides users on the design and application of controls, including the DNA contamination control, positive PCR control, RNA quality assessment, reverse transcription control, and on the selection and validation of reference genes with the use of prevalidated assays.

## The Different Types of Assay Controls Used in qPCR

Different types of assay controls are used for qPCR experiments, including synthetic DNA templates, which are designed to facilitate the assessment of key experimental factors impacting your qPCR results (Table 1).

### DNA Contamination Control Assay

Use the PrimePCR DNA Contamination Control Probe Assay to determine whether genomic DNA (gDNA) is present in a sample at a level that may affect PCR results. This assay may also be used to compare relative levels of gDNA contamination in different samples to determine if PCR results may be affected.

### Positive PCR Control Assay

Use the PrimePCR Positive Control Probe Assay to qualitatively assess the performance of a PCR reaction associated with a single sample. This assay may also be used to compare the relative performance of PCR reactions associated with different samples.

### RNA Quality Assay

Use the PrimePCR RNA Quality Probe Assay to determine whether RNA integrity may adversely affect PCR results for a single sample. This assay may also be used to compare relative RNA integrity among different samples to determine how PCR results might be affected.

### Reverse Transcription Control Assay

Use the PrimePCR Reverse Transcription Control Probe Assay to qualitatively assess the performance of the reverse transcription reaction associated with a single sample. This assay may also be used to compare the relative performance of the reverse transcription reactions associated with different samples.

**Table 1. Real-time PCR assay controls.** Accurate and reproducible qPCR studies of gene expression require the use of the assay controls listed. PrimePCR controls are available for both qPCR and Droplet Digital™ PCR (ddPCR™) if performing assays with human, mouse, or rat samples.

| PrimePCR Control | Purpose | How |
|---|---|---|
| PCR control assay | Assesses performance of qPCR reaction with sample | Primers and complementary template are added to a reaction with sample |
| Reverse transcription control assay | Assesses performance of reverse transcription reaction | RNA template is added to cDNA synthesis reaction — then transcript is detected with primers |
| DNA contamination control assay | Detects genomic DNA presence in sample | Primers that only detect a genomic DNA sequence in model organism |
| RNA quality assay | Checks RNA integrity in sample | Two primer sets that target the same transcript — higher concentration of the shorter product (RQ1) indicates degraded RNA |
| No-template control | Identifies sample contamination from user error | Water is added in place of the RNA template from a reaction |

## Identifying Appropriate Reference Genes for Expression Analysis

In relative gene expression analysis, reference genes are utilized to normalize reverse transcription qPCR (RT-qPCR) results. This normalization accounts for variations in input messenger RNA (mRNA) amount, RNA integrity, or cDNA sample loading across samples. To ensure accurate quantification, include one or more reference genes exhibiting constant expression levels under the experimental conditions.

### Reference Gene Assays

To streamline reference gene selection, Bio-Rad offers PCR primers for a set of commonly used reference genes. These assays can be used individually and easily screened using our preplated 96-well and 384-well reference panels, or they can be added to custom-designed plates.

To ensure proper normalization of RNA samples in a gene expression experiment, use one or more stable, validated reference genes. The reference genes should maintain consistent expression across all samples in the project regardless of treatment, source, or extraction method. The number used and specific choice of reference genes are key factors in determining the magnitude of change in expression that can be detected. For example, to detect large expression changes (greater than fourfold), a single reference gene may be sufficient. For smaller changes (less than fourfold), use multiple well-validated reference genes to prevent confounding effects produced by fluctuations in basal or uninduced expression in any single reference gene.

Researchers can validate reference genes for stability using the process shown in Figure 1, described in detail in the following section and summarized in Table 2.

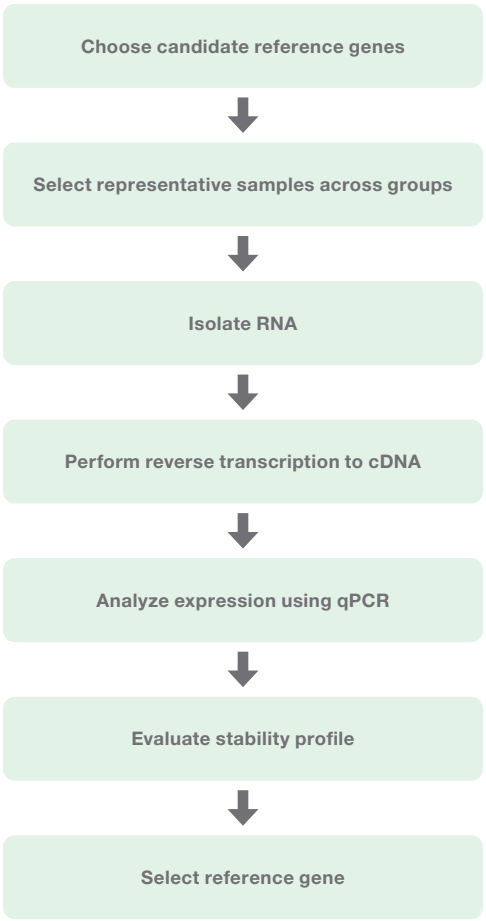


**Fig. 1. Procedure for validating stability of candidate reference genes.**

## Steps to Identify and Validate Reference Genes for qPCR

Researchers often follow these steps to identify and validate reference genes (summarized in Table 2):

1. Choose candidate reference genes. Select a list of candidate reference genes using published references as guidance. Use at least five reference genes for evaluation. For your convenience, Bio-Rad offers human, mouse, and rat preplated reference gene arrays using our validated and optimized PrimePCR Assays. Each reference gene array contains 14 reference genes commonly used in gene expression studies. The 96-well plate can accommodate up to 6 unique samples (or 3 samples in duplicate or 2 samples in triplicate), whereas the 384-well plate can accommodate 24 unique samples (or 12 samples in duplicate, 8 samples in triplicate).
2. Select representative samples across the groups. Select samples to represent all the conditions to be used in the study (for example, treatments, tissues, and time courses), ensuring that all variables within the sample groups are evaluated.
3. Isolate the RNA. Obtain RNA from the samples and treat with DNase using the same protocol for all samples. Quantify and normalize the RNA to the same concentration.
4. Perform reverse transcription to cDNA. Perform a reverse transcription reaction for each sample using the same kit, column, and concentration. Dilute the cDNA, as needed, treating each sample the same to ensure no differences in volume and concentration from sample to sample from the initial input RNA.
5. Analyze gene expression using qPCR. Perform a qPCR experiment using the samples and reference gene array or your selected reference gene assays.
6. Evaluate the stability profile to select your reference gene. CFX Maestro Software enables you to easily select an appropriate reference gene and analyze its stability with the Reference Gene Selection tool, found in the Gene Study module. The Reference Gene Selection tool identifies the tested reference genes and categorizes them as Ideal, Acceptable, or Unstable based on their stability profile:
   - Ideal reference genes are stable and represent minimal variation across tested samples
   - Acceptable reference genes are not ideally stable and represent moderate variation across tested samples. Use these reference genes in analysis only if no Ideal reference genes are present
   - Unstable reference genes may lead to excessive variation across tested samples

**Table 2. Summary of the reference gene selection process.** Normalization of gene expression requires the selection of stable reference genes for the assay's experimental conditions. Selection of candidate genes from published sources, experimental design to represent study conditions, careful preparation of RNA from samples, and identification of stable reference genes suitable for assay conditions are all required before performing gene expression analysis.

| Reference Gene Selection | Purpose | How |
|---|---|---|
| Choose candidate list | Select candidate list (at least five for evaluation) | Use published reference genes as a guide to start |
| Experiment design | Evaluate all variables within the sample groups | Select samples to represent all the conditions to be used in the study (for example, treatments, tissues, and time courses) |
| Sample preparation | Isolate and reverse transcribe RNA | Obtain RNA and treat with DNase and perform reverse transcription. Quantify and normalize to the same concentration |
| Analyze gene expression | Compare gene expression between samples and reference gene list | Perform a qPCR experiment using the samples and the reference gene array or your selected reference gene assays |
| Evaluate stability profile | Identify stable (Ideal) reference genes for use | Analyze reference gene stability with the Reference Gene Selection tool — Ideal, Acceptable, or Unstable based on their stability profile |

Given the importance of reference gene selection for the accuracy of gene expression analysis, studies can fail to choose appropriate reference genes if they rely on housekeeping genes as their primary option. The following section describes why this is a problem for gene expression accuracy.

## Housekeeping Genes Make Poor Reference Genes in Expression Analysis

Accurate normalization of gene expression levels is an absolute prerequisite for reliable results, especially when the biological significance of a subtle difference in gene expression is being studied. Based on the need for stability of expression, housekeeping genes typically make poor reference genes for gene expression analysis. Early studies of gene expression used *GAPD*, *ACTB*, 18S, and 28S rRNA as single control genes for normalization in more than 90% of cases (Vandesompele et al. 2002), and still, there are reports of their erroneous use today (Hounkpe et al. 2021). Total RNA and rRNA levels are not appropriate reference genes because of the observed imbalance between rRNA and mRNA fractions.

As described below, housekeeping gene expression of *GAPD* and *ACTB* can vary considerably. Given that the experimental conclusions are highly dependent on the choice of reference gene, improper selection may lead to inaccurate results.

### Problems Associated with the Use of the Housekeeping Genes *GAPD* and *ACTB* in Expression Analysis

**Variable expression:** The expression levels of *GAPD* and *ACTB* can vary significantly under different experimental conditions or treatments, or in different tissues. This variability can introduce inaccuracies when these genes are used for normalization.

**Regulation by experimental conditions:** Housekeeping genes like *GAPD* and *ACTB* can be regulated by the same experimental conditions that are being studied, leading to changes in their expression levels. This undermines their reliability as stable references.

**Cell type–specific expression:** *GAPD* and *ACTB* expression can vary between different cell types, making these genes unsuitable for experiments involving multiple tissue types or cell lines.

**Stress response:** *GAPD* and *ACTB* can be upregulated or downregulated in response to cellular stress, such as hypoxia, drug treatment, or disease states, which can confound the results.

## Using a Stability Plot to Confirm Reference Gene Selection

The purpose of normalization is to remove the sampling differences (such as RNA quantity and quality) to identify real gene-specific variation. For proper internal control genes, this variation should be minimal or none and shown by analysis of a reference gene stability plot like Figure 2.

CFX Maestro Software for the CFX Opus Real-Time PCR Systems can be used to identify the most stable control genes in each set of tissue samples and to determine the optimal number of genes required for reliable normalization of qPCR data. The strategy presented in this software analysis tool can be applied to any number or type of genes or tissues and should allow more accurate gene expression profiling. More accurate profiling is of utmost importance for studying the biological significance of subtle expression differences and for executing confirmatory and/or extended analysis of microarray results by means of qPCR.

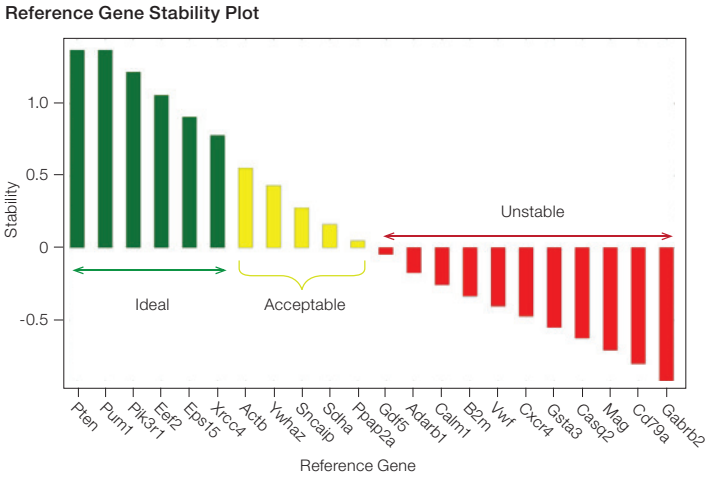


**Fig. 2. A reference gene stability plot.** To confirm the stability of candidate reference genes, run a preplated panel of several reference genes to identify the most suitable ones for your experimental condition. For users who lack access to CFX Maestro Software, tools like NormFinder, geNorm, and BestKeeper are also reported to help evaluate reference gene stability (Song et al. 2022). Most stable reference genes (■); moderately stable (■); not stable (■).

## Conclusion

Assay controls play a critical role in achieving accurate and reproducible results in qPCR gene expression studies. Key elements of the qPCR process include implementing the DNA contamination control for detecting genomic DNA contamination, a positive control for assessing PCR performance, and an RNA quality control for evaluating RNA integrity. Additionally, the reverse transcription control assay is vital for monitoring the efficiency of reverse transcription reactions.

Finally, selecting stable reference genes is required for normalization, and the strategies provided here for their validation and usage can aid in ensuring consistent and precise gene expression analysis. Utilizing these strategies can ultimately mitigate technical variability and enhance the reliability of the qPCR data obtained.

## References

Hounkpe BW et al. (2021). HRT Atlas v1.0 database: redefining human and mouse housekeeping genes and candidate reference transcripts by mining massive RNA-seq datasets. Nucleic Acids Res 49, D947–D955.

Song J et al. (2022). Identification and validation of stable reference genes for quantitative real time PCR in different minipig tissues at developmental stages. BMC Genomics 23, 585.

Vandesompele J et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3, research0034.1.

Visit **bio-rad.com/PrimePCR** for more information.



BIO-RAD

***Bio-Rad Laboratories, Inc.***

*Life Science Group*

***Website*** *bio-rad.com* ***USA*** *1 800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *00 800 00 24 67 23* ***Belgium*** *00 800 00 24 67 23* ***Brazil*** *4003 0399*
***Canada*** *1 905 364 3435* ***China*** *86 21 6169 8500* ***Czech Republic*** *00 800 00 24 67 23* ***Denmark*** *00 800 00 24 67 23* ***Finland*** *00 800 00 24 67 23*
***France*** *00 800 00 24 67 23* ***Germany*** *00 800 00 24 67 23* ***Hong Kong*** *852 2789 3300* ***Hungary*** *00 800 00 24 67 23* ***India*** *91 124 4029300* ***Israel*** *0 3 9636050*
***Italy*** *00 800 00 24 67 23* ***Japan*** *81 3 6361 7000* ***Korea*** *82 080 007 7373* ***Luxembourg*** *00 800 00 24 67 23* ***Mexico*** *52 555 488 7670*
***The Netherlands*** *00 800 00 24 67 23* ***New Zealand*** *64 9 415 2280* ***Norway*** *00 800 00 24 67 23* ***Poland*** *00 800 00 24 67 23* ***Portugal*** *00 800 00 24 67 23*
***Russian Federation*** *00 800 00 24 67 23* ***Singapore*** *65 6415 3188* ***South Africa*** *00 800 00 24 67 23* ***Spain*** *00 800 00 24 67 23* ***Sweden*** *00 800 00 24 67 23*
***Switzerland*** *00 800 00 24 67 23* ***Taiwan*** *886 2 2578 7189* ***Thailand*** *66 2 651 8311* ***United Arab Emirates*** *36 1 459 6150* ***United Kingdom*** *00 800 00 24 67 23*

*Bulletin 3683 Ver A US/EG* *24-0374 0724 Sig 0124*