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Special Report

# MIQE 2.0: Revision of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines

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**BACKGROUND:** In 2009, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines established standards for the design, execution, and reporting of quantitative PCR (qPCR) in research. The expansion of qPCR into numerous new domains has driven the development of new reagents, methods, consumables, and instruments, requiring revisions to best practices that are tailored to the evolving complexities of contemporary qPCR applications.

**CONTENT:** Transparent, clear, and comprehensive description and reporting of all experimental details are necessary to ensure the repeatability and reproducibility of qPCR results. These revised MIQE guidelines reflect recent advances in qPCR technology, offering clear recommendations for sample handling, assay design, and validation, along with guidance on qPCR data analysis. Instrument manufacturers are encouraged to enable the export of raw data to facilitate thorough analyses and re-evaluation by manuscript reviewers and interested researchers. The guidelines emphasize that quantification cycle (Cq) values should be converted into efficiency-corrected target quantities and reported with prediction intervals, along with detection limits and dynamic ranges for each target, based on the chosen quantification method. Additionally, best practices for normalization and quality control are outlined and reporting requirements have been clarified and streamlined. The aim is to encourage researchers to provide all necessary information without undue burden, thereby promoting more rigorous and reproducible qPCR research.

**SUMMARY:** Building on the collaborative efforts of an international team of researchers, we present updates, simplifications, and new recommendations to the original MIQE guidelines, designed to maintain their relevance and applicability in the context of emerging technologies and evolving qPCR applications.

## Introduction

The quantitative real-time polymerase chain reaction (qPCR) and reverse transcription-qPCR (RT-qPCR) (1) continue to be widely used in health and life science research (2). Applications have expanded significantly in recent years to include diverse fields such as clinical diagnostics, agriculture, environmental monitoring, forensic science, and regulatory testing. However, the increasing reliance on qPCR in real-world scenarios amplifies the impact of inaccurate or inconsistent results, with potentially severe consequences such as misdiagnoses, wrongful convictions, and compromised public health measures. Consequently, it has become opportune to revise the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (3) to accommodate the growing complexity of qPCR applications.

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A typical qPCR work flow is difficult to define because sample type and method for nucleic acid preparation depend on the intended application, which also influences assay design and choice of applicable analysis. In addition, results can be acquired using a wide range of reagents, instruments, and protocols. This latitude, along with the speed and ease of acquiring large amounts of data, has resulted in hundreds of thousands of peer-reviewed publications dealing with topics as diverse as ancient biology (4) and gene expression analysis on the International Space Station (5). Unfortunately, this heterogeneity also led to the widespread publication of data that can be confusing at best, and misleading at worst (6).

The MIQE guidelines established a standardized set of recommendations for designing, optimizing, validating, analyzing, and reporting of qPCR results. Adherence to these guidelines has enhanced the quality of results within individual laboratories and enabled more reliable comparisons of data between laboratories, even when different experimental protocols are employed (7). Transparent reporting improves the accuracy of results, validates interpretations, and strengthens the impact of conclusions. It also helps identify potential sources of variation, distinguishing genuinely observed biological relevance from methodological inconsistencies (8). However, despite encouraging investigators to adhere to MIQE, many publications using qPCR continue to lack transparency and employ inappropriate methods (9–12).

Since the MIQE guidelines were published, many advances in qPCR reagents, methods, consumables, and instruments have emerged, improving the limit of detection (LOD), precision, and throughput. For example, there have been significant developments in qPCR primer design (13), along with a greater variety and availability of oligonucleotide modifications. Assay validation has been better defined (14), reference and control materials are becoming available (15, 16), and gene expression profiles from single cells (17) and extracellular vesicles (18) are being reported. An exciting development is "extreme PCR", in which amplification is completed in less than 1 min (19, 20). When ultrafast heating and cooling is combined with increased primer and polymerase concentrations, a PCR cycle can take less than 1 s without compromising specificity, sensitivity, or yield. If this method can be standardized and validated, the transformative implications for on-site analysis, such as point-of-care diagnostics, microbial identification, and rapid pathogen detection will be dramatic (21).

This rapid pace of technological advancement requires updates to the MIQE guidelines to ensure they continue to reflect best practices, much like the updated digital MIQE guidelines (22, 23). MIQE 2.0 aims to provide clear, carefully considered recommendations that both support researchers and advance scientific progress. Effective guidelines should ensure the consistent reporting of key minimum information while promoting creativity and flexibility in experimental and study design. In collaborative projects involving multiple scientists and laboratories, standardized protocols play an important role in ensuring consistency across complementary experiments and partnering laboratories. Although the original MIQE guidelines were not intended as rigid, mandatory rules, they have served as the foundation for ISO20395:2019, which specifies requirements for evaluating nucleic acid quantification methods (https://www.iso.org/standard/67893.html) and have been incorporated into the recommendations for method development and validation of qPCR assays in support of gene therapy drug development (24).

Building on this foundation, MIQE 2.0 addresses critical factors that influence experimental accuracy, consistency, and reproducibility. These include: (*a*) sources of variability, (*b*) assay design, (*c*) sample storage and nucleic acid preparation, (*d*) reverse transcription and template dilution, (*e*) qPCR protocol, (*f*) PCR efficiency, (*g*) melting curve analysis, (*h*) data processing and analysis, and (*i*) the use of appropriate controls. By focusing on these elements, MIQE 2.0 provides researchers with an updated framework for generating reliable and biologically meaningful qPCR data (Fig. 1).

## Sources of Variability

RT-qPCR and qPCR experiments are susceptible to variability and errors that fall into three main categories: biological, protocol, and technical (10, 25, 26). Minimizing these errors requires a rigorous, step-by-step approach to assay planning, optimization, standardization, validation, and reporting (27).

### BIOLOGICAL VARIABILITY

This includes both the variation inherent to the study subject and the variance introduced by factors such as individual sample differences, including genomic sequence variation. When comparing treated and untreated groups, biological noise often increases in the treated group due to both intrinsic differences between individuals and their distinct responses to treatment. The degree of variance depends greatly on the studied subjects, and although it cannot be entirely controlled, it can be reduced by establishing stringent inclusion and exclusion criteria to increase the homogeneity of the study cohorts without loss of group-specific characteristics. However, ensuring comparable levels of homogeneity between the treated and untreated groups is essential to mitigate bias. In many cases biological noise limits the power of the analyses (28). It can be managed by

1. **Expanded Scope & Relevance**

• MIQE 2.0 extends the original guidelines, providing expanded recommendations for both reporting and performing qPCR experiments across a broader range of applications.

• Greater emphasis is placed on ensuring reproducibility across different laboratories and platforms, enhancing result comparability.

2. **Refined Definitions & Experimental Validation of Limits**

• MIQE 2.0 introduces more rigorous definitions for LoD, LLoQ, and ULoQ, requiring experimental validation and reporting with confidence intervals.

3. **Strengthened Controls & Quality Assurance**

• MIQE 2.0 recommends multiple negative controls (NTC & NRTC) and the use of exogenous spike-ins per experiment to monitor NTC stability over time, RNA degradation, RT yield, PCR inhibition, sample preparation variability, and competitive bias in multiplex assays.

4. **Expanded Recommendations for Sample Integrity & Storage**

• Greater focus on RNA degradation monitoring and mitigation, including best practices for blood and tissue storage and minimization of freeze-thaw cycles.

5. **Enhanced qPCR Efficiency Standards & Transparency**

• MIQE 2.0 strongly recommends determining and reporting PCR efficiencies with confidence intervals and introduces strategies for correcting efficiency biases to improve reproducibility.

• Instrument manufacturers are encouraged to enable raw data export for greater transparency and independent verification.

• MIQE 2.0 promotes digital repositories for data sharing, supporting deposition of raw fluorescence data and analysis scripts.

6. **More Rigorous Data Analysis, Normalization & Reporting**

• Stronger recommendations for data normalization, baseline correction, and outlier detection in qPCR analysis.

• Cq values and fold-change estimates should not be reported alone but should be accompanied by efficiency-corrected target quantities and appropriate measures of uncertainty.

• Absolute quantification using calibration curves should include prediction intervals and dynamic range estimates.

• Geometric mean expression normalization is emphasized as the preferred method to minimize sampling and RT variation when using reference genes.

7. **Integration of New & Emerging Technologies**

• MIQE 2.0 acknowledges the challenges posed by emerging technologies, including extreme PCR, machine learning applications, direct RT-qPCR workflows in clinical and point-of-care settings, and long-term biobanking standardization.

**Fig. 1. Summary of revisions and updates from previous guidelines (3).**

increasing the number of individuals, refining the experimental design, normalizing with appropriate controls and applying appropriate statistical analyses to evaluate the impact of variability.

#### PROTOCOL VARIABILITY

Protocol variability, or differences in standard procedures, arises from different reagents, instruments, and data analysis methods. Reagent variation includes differences in the choice and concentration of primers and probes, enzymes, and master mix components [such as $Mg^{2+}$, pH, monovalent cations, and deoxynucleotide triphosphates (dNTPs)], all of which can lead to variable PCR efficiencies. qPCR instruments themselves vary in temperature accuracy, ramping rates, plate temperature homogeneity, and precision of temperature and fluorescence acquisition. Choices related to temperatures and times for sample preparation, reverse transcription (RT), and PCR cycling also affect results. Additionally, variability in data analysis methods, such as background subtraction algorithms, calculations of Cq values, quantification thresholds, outlier detection and exclusion, handling of missing values, diagnostic cutoffs for qualitative assays, the use of standard curves, and choice of reference genes for quantitative assays, can systematically affect reported outcomes. These procedural variations contribute to the discordance observed in data from different laboratories.

#### TECHNICAL VARIABILITY

It is present throughout the experimental work flow and can arise from various sources, including sample storage and processing, pipetting, mixing of components, deviations from recommended time and temperature protocols, and other operator-dependent variables. The RT step, in particular, is an important source of variability for quantitative analyses of RNA samples (29). PCR efficiency can vary between assays (30) and sample heterogeneity, particularly when analyzing solid tissue or single cells, can also confound differences amongst subjects or groups (28).

To reduce protocol and technical variability, it is important to implement appropriate controls, quality assurance measures, standardized protocols, and rigorous experimental design and data analysis (31, 32). At low target copy numbers, technical variability is primarily influenced by the unavoidable Poisson sampling distribution. To mitigate this and increase accuracy, researchers can increase the number of technical replicates, concentrate samples, or analyze larger volumes. Errors in qPCR can be categorized as random (affecting precision) or systematic (affecting trueness), with both contributing to the total error, which defines accuracy. Accuracy represents how closely a measurement aligns with the true value and is determined by trueness, the average of repeated measurements relative to the true value and precision, which is the variability within repeated measurements. Two key aspects of precision include (*a*) repeatability, the variation observed within replicate measurements from the same biological sample within a single experiment (intra-assay precision) and (*b*) reproducibility, the variation observed across different days, operators, instruments, or laboratories (inter-assay precision). In addition, robustness must also be considered. Robustness refers to the consistency of results from a single assay when run under varying conditions, such as changes in reagent concentrations, primer or probe batches, or annealing temperatures. Ensuring robustness is important for reliable and reproducible qPCR results.

In summary, variation in sampling, genomic sequence, PCR inhibitors, PCR efficiency, threshold settings, PCR artifacts, pipetting, instruments, operators, and analysis methods can lead to highly variable qPCR results. This variability is particularly pronounced when results are reported as Cq, ΔCq, or ΔΔCq values, which complicates interpretation (33). Therefore, a key objective of these guidelines is to highlight technical errors and protocol differences and thereby reduce their impact, allowing for a more accurate assessment of biological variability within and between experiments and laboratories.

## Assay Design

Primers provide the sequence specificity of PCR assays, and this specificity is a key criterion for assay robustness and analytical sensitivity (13). The sequence and positioning of primers, as well as their concentrations, are crucial factors that determine an assay's PCR efficiency and affect its LOD. Generic recommendations for primer design include using the nearest-neighbor method to determine the melting temperature ($T_m$) of each primer, with the $T_m$s of both primers close to each other ($\Delta T < 3$ °C), while aiming for lengths of 18 to 24 bases and GC percentages of 40% to 60%, avoiding stable secondary structures, and minimizing primer interactions that result in dimers. Adjustment for mono- and divalent ion concentrations in the reaction mix is important for accurate $T_m$ calculation. If unknown, 50 mM $Na^+$ and 3 mM $Mg^{2+}$ are good starting points. Additionally, avoidance of repeat sequences maximizes specificity as there is a correlation between the rate of PCR failure and the number of alternative binding sites in the background DNA (34). Fluorescent signals in qPCR can be generated using either DNA binding dyes or probes and result in similar amplification curves, although for some chemistries and inputs, a correction of Cq values is required (see Baseline Fluorescence Correction section) (35). While probes offer additional specificity, dyes may facilitate optimization due

to their lower cost. However, when dyes are used, performing a melting curve analysis after amplification is essential to identify nonspecific products.

Template sequence variation must be carefully considered to ensure that desired targets are specifically amplified while avoiding amplification of unintended targets. In microbiology, this is typically achieved through sequence alignment of different isolates to identify suitable sequence regions that exclude closely related microbes. In genetics, distinguishing between pseudogenes, paralogues, and active genes is necessary. Pseudogenes should also be considered in expression profiling as processed ones usually lack introns, and residual genomic DNA may be amplified even when using intron spanning primers. It is also necessary to take alternative splicing into account during design assays. While accession number annotated sequences provide consensus sequences and information on alternative splicing, it is important to review sequence variation at potential primer and probe sites using variant databases or genome browsers such as the University of California Santa Cruz genome browser (https://genome.ucsc.edu), Ensembl genome database/browser (https://www.ensembl.org), and NIH Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Variation at the 3′ end of primers will likely hinder amplification, whereas mismatches near the 5′ end are generally more tolerated (36). When target sequence variation is high, shorter primers or probes may offer better match potential and binding affinity can be maintained with nucleotide analogues.

Designing primer pairs requires careful attention to avoid interactions, such as primer dimers, which can arise when primers partly anneal to each other. Thermodynamic analysis, using tools that evaluate parameters like $T_m$ and free energy (ΔG) include but are not limited to programs such as Primer 3Plus (https://www.primer3plus.com/), OligoAnalyzer (https://www.idtdna.com/pages/tools/oligoanalyzer), PrimerROC (http://www.primer-dimer.com/roc/), Dinamelt (http://www.unafold.org/Dinamelt/applications/hybridization-of-two-different-strands-of-dna-or-rna.php), and mFold (http://www.unafold.org/). Use of these tools can predict and minimize these undesired interactions, ensuring optimal primer performance. Ensuring that multiple primers anneal concurrently involves matching their nearest-neighbor $T_m$ estimates. However, annealing temperature optimization is still necessary, as master mix components can influence the $T_m$ in ways that are often variable and not well documented (37).

The distance between primers determines the amplicon length, which typically ranges from 50 to 200 base pairs (bps). Shorter amplicons are generally more robust and are particularly useful for analyzing degraded nucleic acids from formalin-fixed tissues, liquid biopsies, forensic samples, and archaeological specimens. They are also easier to genotype using high-resolution melting (HRM), although distinguishing primer dimers becomes more challenging. Longer amplicons, on the other hand, may exhibit multiple melting domains, which can enhance HRM characterization. It is crucial to check the secondary structure of the amplicon at primer and probe annealing sites to avoid potential inhibition of primer annealing or probe detection. Similarly, when using gene-specific priming for RT, any secondary structures in RNA at the primer binding site should be avoided to ensure efficient RT.

Criteria for designing assays should be tailored to the specific application, and the recommendations provided here should not constrain innovative design approaches. The easiest and most straightforward recommendation is to design exon–exon spanning primers and probes, and/or amplicons that are intron-spanning to avoid detection of genomic sequences when aiming at specific RNA transcripts. Assays with low amplicon $T_m$s can benefit from using lower denaturation temperatures to minimize the risk of amplifying high $T_m$ artifacts (38, 39). Additionally, instruments with rapid temperature control capabilities may allow for shorter polymerase extension times to prevent the generation of unintended long products. The versatility of PCR encourages flexibility in assay design. A practical approach for new assay development is to design 2 or 3 alternative forward and reverse primers with matched $T_m$s located in the same region, different combinations of which can be tested simultaneously under the same conditions to find the optimal primer pair (13).

## Sample Storage and Nucleic Acid Preparation

Appropriate storage of biological samples is essential for maintaining nucleic acid integrity and ensuring accurate qPCR results since nucleic acids can degrade through enzymatic, chemical, or physical means (40). RNA, being more labile than DNA, requires particularly stringent storage conditions. Tissue samples or cell pellets should be rapidly frozen and stored in the vapor phase of liquid nitrogen (−180°C) or in a −80°C freezer. Alternative preservation methods include high-salt solutions or lysis buffers. In case of blood samples, collection media should be used that lyse the cells and preserve the biological RNA profiles, as live cells respond rapidly to altered environmental conditions. While additives in certain collection tubes, such as EDTA and citrate, can profoundly affect expression profiles, numerous alternative tubes are available on the market that stabilize whole blood for downstream RNA or DNA processing. Alternatively, when red blood cell RNA is not required, peripheral blood mononuclear cells can be isolated and stored using differential centrifugation. The preanalytical

process is an important component of qPCR work flows and was studied by the SPIDIA consortium (www.spidia.eu), which contributed to the work behind many industry guidelines and standards.

Nucleic acid integrity can vary with tissue type, storage conditions, and extraction protocols. It is important to report the extraction method and yield, as inadequate homogenization, extraction, or DNase treatment of RNA isolates can lead to underestimation or overestimation of target concentrations. Additionally, different extraction methods can selectively enrich or deplete nucleic acid fragments of specific sizes. Extracted RNA should be stored at the lowest possible temperature to minimize hydrolysis. RNA can be stored at −20°C for days, at −80°C for months (41) and is stable for at least 5 years in the vapor phase of liquid nitrogen (42). While DNA is more robust than RNA, its quality is also influenced by storage conditions (43, 44). To prevent degradation, multiple freeze–thaw cycles should be avoided (45, 46) by storing RNA and DNA in small aliquots. When storing dilute samples (<1 ng/μL), there is a risk of nucleic acids adhering to the container walls, potentially leading to loss of material. To ensure accurate quantification, concentrations should be checked after thawing.

Nucleic acid quality control is vital for accurate RT as well as qPCR. Quality control procedures should assess nucleic acid integrity, the absence of inhibitors, and concentration. Simple spectrophotometry can be used to assess nucleic acid concentration, while size ladders can evaluate integrity by highlighting fragment distribution. Fluorometry with DNA or RNA binding dyes offers great sensitivity but lacks information on contamination and integrity. It is advisable to combine dye staining with electrophoresis to assess size, quantity, and integrity, although this still does not reveal contamination. Knowing the concentration of DNA or RNA allows estimation of genome (typically 7 pg per mammalian genome) or transcriptome (typically 10 to 30 pg total RNA per mammalian cell) equivalents. Using 5 ng of RNA equivalents (170 to 500 transcriptome equivalents) is generally sufficient to quantify most mammalian transcripts, although rare transcripts in plant and environmental testing will have different requirements and can require significantly more nucleic acid (47).

The COVID-19 pandemic highlighted the need for rapid, scalable diagnostic methods. The adoption of direct RT-qPCR using crude samples was a notable development, as it reduced contamination risks associated with traditional purification methods, preserved limited biological material, and benefitted from recently developed robust enzyme formulations resistant to inhibitors. Clearly, this shift towards more efficient, streamlined nucleic acid analysis methodologies has significant implications for diagnostics and environmental monitoring (48–51). However, challenges remain in achieving robust and accurate diagnostic results. These challenges include variability in RNA quantity and quality, which can compromise both the qualitative and quantitative reliability of the assays. To balance the need for speed with the demands for accuracy and reproducibility, further optimization and validation of direct RT-qPCR work flows are essential.

## RT and Template Dilution

The yield and specificity of RT reactions have a major and generally disregarded effect on the accuracy of RT-qPCR results. Both are affected by the quality and quantity of RNA input (52), secondary structure and palindromes, priming method (53, 54), reverse transcriptase enzyme (29, 53, 55), and reaction conditions. The abundance of RNA is especially relevant when using one-step RT-qPCR protocols that combine the RT and qPCR reactions in a single tube, as the yield of the RT step can be below the sensitivity of the qPCR assay (56, 57). Too much RNA, on the other hand, can result in inaccurate quantification of some transcripts (58).

RT may not be a linear process (59), requiring RNA rather than DNA standards to accurately estimate dynamic range and RNA copy numbers. RT yield also varies among targets (60). Synthetic RNA standards can be used as controls, although their performance may differ from the natural target RNA. To minimize systematic variation, it is important to maintain consistent RNA input, priming strategy, enzymes, and reaction conditions throughout an experiment. However, random variation, such as pipetting errors during setting up the reactions or making dilutions, will still occur. Priming strategies should be tailored to the RNA target, for example, employing qPCR compatible specific reverse primers, anchored polyT primers, or random primers for mRNAs or two-tailed PCR for micro RNAs (miRNAs) (61, 62).

In research laboratories, it is common practice to dilute copy DNA (cDNA) after the RT step to mitigate interference from RT reagents or, depending on target abundance, to ensure that the PCR target input is within the assay's dynamic range. Typically, cDNA samples are assessed with a panel of target and reference genes. It is crucial to recognize that dilution can influence quantitative results and to account for variability introduced by RNA or cDNA dilution (63). This is less relevant in clinical settings, where direct use of samples post-RT is more common due to the prevalence of one-step RT-PCR and qualitative assays, where a lower limit of detection is the primary concern.

## Quantitative PCR Protocol

Optimization and validation of qPCR conditions are crucial for a reliable qPCR work flow. Key parameters

include amplification efficiency, linearity, dynamic range, LOD, and limit of quantification (LOQ) (30, 64). The lower limit of quantification (LLOQ) is defined as the smallest amount of nucleic acid that can be reliably quantified, meeting specified criteria for accuracy and precision within a given assay. In contrast, the upper limit of quantification (ULOQ) is the highest amount that can be accurately measured without signal saturation or loss of linearity. The LOD lies below the LLOQ and indicates the smallest amount of target that can be reliably detected with, typically, 95% certainty but does not allow precise quantification. Reporting these values is imperative for evaluating the reliability of quantitative results (65).

Optimizing PCR conditions involves assessing factors such as denaturation and annealing temperatures and cycling times to maximize amplification efficiency and specificity. This can be achieved by using the temperature gradient feature available on many instruments or by testing a range of temperatures. The choice of polymerase, buffer, and reagents can have a significant impact on qPCR results. Data obtained with reagents from one manufacturer's kit can be difficult to reproduce with another's reactants, even under the same reaction conditions (66, 67). Therefore, optimization and validation of assays must be repeated when changing kits or suppliers and verified when changing batches. If using custom buffers, attention must be paid to buffer components that affect annealing temperature and amplification efficiency (68). It is advisable to store reaction components in aliquots at −20°C or according to the manufacturer's instructions and to revalidate assays when new batches of reagents, primers, or probes are introduced.

While a two-temperature PCR setup with a combined annealing/extension phase at 60°C to 65°C is used for simplicity (13), separate annealing and extension temperatures may improve specificity and may be necessary for longer amplicons or when rapid temperature control allows shorter cycle times. In addition, specificity can be adjusted by varying $Mg^{2+}$ (1.5 to 4 mM) or primer (100 to 800 nM) concentrations. Additives such as dimethyl sulphoxide (DMSO), glycerol, or betaine can help lower amplicon melting temperatures for high GC-content products that are challenging to denature. Switching to $K^+$-free buffer can help to amplify guanine-rich sequences that are capable of forming quadruplexes.

## PCR Efficiency

Quantification by qPCR relies on a linear relationship between the logarithm of the initial target quantity and the observed fractional Cq derived from the fluorescence amplification curve. This linear relationship, which is evident during the exponential phase of PCR, enables the calculation of assay amplification efficiency and sensitivity (69). However, this relationship becomes nonlinear during the transition into the plateau phase, where the fluorescence no longer increases linearly with product quantity and levels off as the reaction reaches its maximum product yield due to factors such as reagent depletion, amplicon re-annealing and the saturation of detection systems (i.e., the plateau phase). A variety of approaches are employed to evaluate PCR efficiency and ensure accurate quantification throughout the exponential phase (30, 70).

### EFFICIENCY VALUES DERIVED FROM A STANDARD CURVE BASED ON A DILUTION SERIES

The efficiency and dynamic range of a qPCR assay are commonly evaluated by generating standard curves using a series of dilutions of a standard with a known amount of amplicon-specific DNA or RNA (2, 71). Ideally, this standard has been accurately quantified, for example by digital PCR (72), and represents the target analyte in both sequence and matrix context. The slope of the linear regression of a plot of Cq ($y$-axis) vs the log of the target concentration ($x$-axis) is determined by the amplification efficiency (E), which is calculated as $\%E = 100*(-1 + 10^{(-1/\text{slope})})$. The standard curve should ideally include a minimum of 3 technical replicates for each step of the dilution series, prepared by separate reaction mixes and processed in separate wells. This series should cover 4 to 5 orders of magnitude of template concentration (37). Reported data points should be limited to those within the linear range of the standard curve between the LLOQ and the ULOQ. Confidence intervals should also be reported to indicate the precision of the estimate of PCR efficiency.

A major issue with standard curves is that the $x$-axis represents the log of the intended dilution. Consequently, calibration errors in pipetting during serial dilution can introduce systematic errors, such as slopes below −3.32, which indicate a PCR efficiency >100% (30). These systematic errors may represent small differences on the log scale but can lead to appreciable differences where small linear differences are of interest. Random errors can also arise from inconsistent pipetting, and wide confidence intervals may result from a low number of replicate reactions per dilution (73).

### EFFICIENCY VALUES DETERMINED FROM THE AMPLIFICATION CURVES OF INDIVIDUAL REACTIONS

PCR efficiency can also be determined from individual amplification curves. Different approaches are available for fitting fluorescence vs cycle number data, including exponential, polynomial, or sigmoidal models for linear fluorescence data, or a straight-line model for log-transformed fluorescence data (69, 70, 74–77). In the latter approach,

the slope of data points in the exponential phase, which is between the baseline noise (ground phase) and the beginning of the transition to the plateau phase (i.e., transition phase), directly reflects the PCR efficiency of an individual reaction (%E = 100*(−1 + $10^{slope}$)). Comparisons between these methods have shown similar results (70). For standardized laboratory experiments, the mean efficiency extracted from individual curves, using a common quantification threshold across both standards and unknown samples, provides the most sensitive and precise efficiency-corrected target quantification. However, for clinical samples with variable levels of PCR inhibition, it may be more appropriate to derive PCR efficiency from individual reactions (30) provided that the amplification curve data accurately reflect any efficiency differences caused by the presence of inhibitors. The same consideration applies to multiplex reactions if targets are present at highly divergent concentrations and may lead to inaccurate quantification. In addition, the user must ensure that multiplexed results are the same as those obtained by singleplex analyses. If not, quantitative multiplexing may not be possible.

#### PCR INHIBITION

PCR inhibitors affect the calculated PCR efficiency in both the standard curve and individual amplification curve approaches. In the standard curve method, diluting the sample also dilutes the inhibitor, thus lowering Cq values at higher dilutions resulting in artificially higher calculated efficiencies (73). In contrast, efficiency values derived from individual amplification curves tend to be lower when inhibitors are present or when mismatches occur near the 3′ end of the primers (36). Comparison to the efficiency of control targets, or analysis of Cq values from diluted samples, can be used to identify inhibited samples. Identifying samples with aberrant PCR efficiency values is especially important in qPCR-based point-of-care diagnostics, where outlier tests can then detect samples with high concentrations of inhibitors (30). A PCR assay with overall reduced efficiency typically exhibits lower sensitivity (78).

## Data Processing and Analysis

#### MELTING CURVE ANALYSIS

For dye-based assays, the amplification curves (cycle number vs fluorescence plots) do not differentiate between the intended product and any off-target amplicons amplified by the same primers with typically similar annealing and PCR efficiencies. Consequently, the identity of different products cannot be inferred from the shape of their amplification curves (79). However, melting curve analysis of PCR amplicons, generated in the presence of double-stranded DNA-binding dyes, can provide information about the uniqueness and specificity of the PCR product. During this analysis, the reaction is heated at the end of the PCR, while fluorescence is continuously monitored. As the temperature exceeds the amplicon's $T_m$, the amplicon denatures, causing a sharp drop in fluorescence.

Although an amplicon's $T_m$ depends on the reaction mixture composition, the heating ramp speed, and the calibration of the qPCR instrument, the $T_m$ is specific to individual amplicons, as it is determined by their length, GC content, and sequence composition (38, 79). When the amplification and melting procedures are standardized, the $T_m$ of a product can be established in a pilot experiment with a positive control and used for reference in subsequent experiments. Alternatively, software tools like "uMELT Quartz" (https://dna-utah.org/umelt/quartz/) can predict amplicon $T_m$ as well as the shape of melting curves (80). Amplicons with unbalanced GC content may melt in blocks, resulting in multiple peaks that can be useful for further characterization. Certain "saturation dyes" (e.g., LCGreen™) are more sensitive to detecting multiple peaks than others (e.g., SYBR™ Green I), that struggle to detect low-$T_m$ products when multiple peaks are present due to dye redistribution during melting (79). Single-stranded DNA is generally not detected when using double-strand–specific DNA dyes. A final polymerase extension step is not necessary before melting analysis because product reannealing is rapid upon cooling after denaturation. While some secondary structures may be detected, their $T_m$s are typically low and do not interfere with amplicon melting. For example, snapback primers or unlabeled probes can be utilized for genotyping at $T_m$s lower than the amplicon (38). The impact of asymmetric PCR on quantification depends on the degree of primer concentration asymmetry. If the exponential phase is limited, efficiency and Cq determination can be compromised.

Melting curve analysis is more sensitive than size fractionation on agarose gels. However, just as 2 different PCR products can have the same size, they can also share the same $T_m$. Therefore, a single peak at the correct $T_m$ does not guarantee identification of the intended amplicon. Clinical assays often use amplicon-specific fluorescent probes alongside target-specific primers to address this limitation. Nonetheless, as instrument resolution improves, the ability to distinguish different products using melting curves increases. Given its simplicity, melting curve analysis is often used in place of gels for PCR optimization, not least because it reduces the hazard of contaminating the laboratory. Melting curve analysis has a range of additional applications, including relative quantification by competitive PCR (38).

#### ACCURACY OF qPCR RESULTS

Accurate qPCR results are particularly important when conclusions are translated into clinical practice (26).

The observed Cq value depends on several factors, including the sample processing method, fluorescence acquisition, and data analysis. Important analysis steps include the following:

1. How baseline fluorescence is subtracted.
2. How and whether a quantification threshold is used.
3. Whether thresholds are consistent across runs or determined individually for each run.
4. Whether a smoothing algorithm is applied to calculate the Cq.

Additionally, the way Cq values are converted into target quantities or fold changes in gene expression due to experimental treatments significantly influences the accuracy of qPCR results (33, 81). When analyzing RNA, RT produces a single-stranded cDNA. During PCR, the first cycle then produces double-stranded DNA, rather than amplifying. Hence, one cycle should be subtracted when converting Cq values to quantities of mRNA/cDNA (2). This removes the systematic shift of one cycle between Cq values between reactions starting with DNA or cDNA for fluorescent dyes and cDNA targeting probes. However, subtraction is not necessary if a fluorescent probe is used that targets the original mRNA sequence (35).

*Original (Raw) Fluorescence Acquisition.* Fluorescence is a relative measure, and all qPCR instruments adjust analog signals using electronic offsets and gains prior to digitization. This adjustment eliminates most background fluorescence, which is independent of the PCR monitoring chemistry but includes autofluorescence from the optical system and consumables (82, 83). Since there is no absolute zero for fluorescence in qPCR, early cycle fluorescence is used to define a baseline, which may not be linear, before amplification is detected.

Raw fluorescence data should be collected and made available upon request. Ideally, these data should be appended as a supplement or deposited in data repositories. Raw data, which cannot be reconstructed from Cq values or baseline-corrected data, allow for re-analysis with new tools or for addressing different research questions. Two vendor-independent file formats for qPCR data exchange are currently available:

1. The *Real-Time PCR Data Essential Spreadsheet Format* (RDES) (84), which includes sample identification and raw fluorescence data, has as a simple row vs column export format.
2. The *Real-Time PCR Data Markup Language* (RDML) (85), a more comprehensive XML-based format that contains all necessary information for further analysis.

Both formats can be generated and analyzed using free software like RDML-Tools (86). Instrument manufacturers should implement one of these options to enable raw fluorescence data export. Until this is provided, we encourage investigators to supply qPCR data as supplementary files in accessible formats, such as Excel.

*Baseline Fluorescence Correction.* Baseline fluorescence, which arises from the PCR monitoring chemistry independent of amplification, must be subtracted from each reaction individually. Older qPCR instruments calculated baseline fluorescence as the mean value over a user-defined range of early cycles. Modern instruments, however, typically calculate baseline fluorescence as a trendline through the ground-phase fluorescence values and subtract the respective value of the extrapolated trendline from the measured fluorescence value at each cycle.

Upward- or downward-sloping baselines can significantly affect the shape and position of amplification curves, influencing the readout of Cq values and calculated PCR efficiency (69). When raw fluorescence data are available, errors from improper baseline correction can be addressed (86). Some commercial probe-based kits have high baseline fluorescence, which compromises the dynamic range and resolution of fluorescence acquisition. With DNA-binding dyes, baseline fluorescence is partly influenced by template and primer concentrations, suggesting that reducing concentrations may improve fluorescence detection as long as PCR efficiency is maintained (69). Similarly, probes with inefficient quenching may exhibit high baseline fluorescence, and lowering their concentrations can be beneficial, provided end point fluorescence levels are still acceptable.

*Quality Control.* Quality control in qPCR is vital and involves assessing both amplification and melting curves to confirm target-specific amplification, effective background fluorescence subtraction, and the application of reasonable baseline and threshold settings (86–88). These evaluations ensure that amplification is specific and free of nonspecific products or primer dimers. In some cases, manual adjustment of the early cycles selected to define the baseline is required to exclude anomalous readings and include enough cycles to get the best estimate. Baseline-corrected amplification plots, typically plotted with log[fluorescence] on the *y*-axis and cycles on the *x*-axis, help in visualizing the exponential amplification phase, which is crucial for accurate quantification.

When standard curves are used for quantification, it is necessary to report both confidence intervals and prediction intervals of the Cq vs log concentration plots. The confidence interval reflects the uncertainty in the estimated slope of the standard curve, which is used to calculate PCR efficiency. On the other hand, the prediction interval provides a measure of uncertainty

for individual target quantities derived from the observed Cq value through interpolation on the standard curve. Since the prediction interval accounts for variation in individual measurements, it is broader than the confidence interval and, therefore, the correct measure for the precision of the reported target quantity.

*Data Analysis.* Numerous methods exist for analyzing qPCR data, and they are not always compatible (33). Therefore, it is important to report which approach is used. The Cq values obtained from qPCR are intermediate data and meaningful results require further processing depending on the experiments' purpose. qPCR experiments are designed for various objectives, such as the following:

1. Determining the target quantity within each reaction or biological sample.
2. Normalizing target expression per sample (target expression/reference expression).
3. Calculating the fold-difference in normalized expression between experimental and control groups.

The intermediate Cq value is a measure of the input target concentration but influenced by both PCR efficiency and the quantification threshold. Consequently, each result requires careful handling of the Cq, PCR efficiency value, and standardized threshold setting. However, many publications assume a 100% PCR efficiency or require readers to make this assumption when interpreting Cq, ΔCq, or ΔΔCq values (33).

*Target quantity per gene.* In qPCR studies, especially in clinical diagnostics, it is common to report only Cq values per reaction or, with technical replicates, the mean Cq per sample. However, it is important to understand that Cq values are exponentially related to the amount of the target nucleic acid (17). Therefore, the arithmetic average of Cq values reflects the geometric mean of the target quantities, not the arithmetic mean (32, 89). Further statistical tests on Cq values should be avoided because these values are not efficiency-corrected and are not normalized. Consequently, Cq values should be converted to target quantities before analysis (33).

Target quantities are usually calculated from Cq values by interpolation using a calibration curve. This curve is created from a dilution series of a known standard and this approach is commonly referred to as "absolute quantification." However, the accuracy of the results depends on the accuracy of the standard and the precision of the dilution series. An alternative method that does not require a dilution series calculates target quantities using the Cq value, the quantification threshold, and the PCR efficiency. This results in an efficiency-corrected target quantity, expressed in fluorescence units (69). These fluorescence units can then be converted to absolute copy numbers by comparing them to a single known standard, which is typically run in multiple replicates (30, 90). This approach is simpler and reduces the risk of errors associated with preparing a dilution series.

*Normalized expression per sample.* In gene expression analysis, normalization of qPCR data using reference genes is another basic step for supporting precise measurements. This procedure helps correct technical biases that arise from differences in nucleic acid input across reactions (91–93). However, inconsistencies in how normalization is performed—such as variations in the number, expression stability, or abundance of selected reference genes—can introduce additional errors and variability into the results (94, 95). To achieve robust normalization, it is crucial to carefully justify the selection and number of reference genes. This requires demonstrating the stability of their expression levels, as these may vary depending on factors such as tissue type, experimental conditions, or disease states.

The most common method for calculating expression ratios uses the $2^{-\Delta Cq}$ formula, where ΔCq is the difference between the Cq values of the target gene and the reference gene. Although the authors of this influential paper proposed a test to determine whether PCR efficiencies are similar enough to disregard their difference (96), this requirement is generally overlooked. Crucially, assuming a 100% PCR efficiency introduces a Cq-dependent bias in the normalized expression when amplification efficiencies differ between target and reference genes. For technical replicates and multiple reference genes, the ΔCq calculation uses mean Cq values of replicate reactions averaged over reference genes. Consequently, there is loss of information about absolute expression levels of genes, making it harder to detect deviating reactions and possibly biologically meaningful expression differences (30).

An alternative approach to normalization is to calculate target/reference expression ratios per sample using the target quantities per reaction. This method accounts for multiple reference genes, gene-specific amplification efficiencies, and the associated errors across all measured parameters throughout the calculation process (89). Since the expression levels of different reference genes can vary widely—sometimes by several orders of magnitude—using the geometric mean of their expression ensures that each gene contributes proportionally to the normalization. This minimizes the risk of bias from any single reference gene dominating the calculation (91).

*Fold-difference between groups.* The same assumption of 100% amplification efficiencies for both genes of interest and reference genes significantly affects the

calculation of fold differences between treated and control samples using the formula $2^{-\Delta\Delta Cq}$ (96). Although the overall bias in this method is somewhat reduced compared to the ΔCq approach because biases for treated and control samples are partly in the same direction, we discourage the use of the ΔΔCq approach (33). When expression levels are markedly affected by experimental conditions, the reported fold differences remain biased, leading to potentially misleading conclusions (30). While an efficiency-corrected version of the equation can mitigate efficiency-related bias (97), the ΔΔCq approach still suffers from critical limitations. It poses challenges in handling technical replicates and multiple reference genes, and it exacerbates issues inherent to the ΔCq method, such as the loss of absolute expression information. Furthermore, by averaging Cq values within experimental groups, the ΔΔCq approach obscures biological variation, making it difficult to perform robust statistical comparisons (98). For these reasons, we strongly recommend against relying on the ΔΔCq approach in quantitative analysis.

Therefore, fold differences between groups should be calculated from mean normalized gene expression quantities in the treated and control groups as: $\frac{mean\left(\frac{GOI}{RG}\right)treated}{mean\left(\frac{GOI}{RG}\right)control}$ where GOI is the expression of a gene of interest and RG is the geometric mean of the expression of reference genes (89). To assess treatment effects, statistical analysis should be performed with the normalized data per sample before averaging per group.

*LOD and LLOQ.* For each assay LLOQ and LOD should be determined and reported, as they provide crucial information on assay sensitivity and accuracy (64). These determinations are not required if low target expression levels are not an issue. In molecular diagnostics, both parameters are important for regulatory compliance and serve as acceptance criteria for assay validation studies that demonstrate the accuracy of the method and ensure compliance with regulatory standards. The LLOQ and LOD contribute to assay optimization but should not be used as cutoffs for reporting negative findings in qualitative assays. A positive reaction in a validated qualitative assay in the absence of contamination or probe degradation indicates that the target is present and should be reported, irrespective of the Cq value. Notably, the Poisson sampling distribution dictates that for any given qPCR the LOD cannot be below 3 target copies (3). Similarly, variability increases below 10 copies per reaction, the LLOQ of a good assay. At a PCR efficiency of 90%, such an LLOQ is reached at 35 cycles, and LOD at 37 cycles. For 70% efficiency, the respective Cq values are 42 and 45 cycles (33).

## Controls

Appropriate controls are necessary in qPCR experiments to differentiate true biological signals from technical artifacts. These controls ensure the reliability, specificity, and sensitivity of the assay. Indispensable controls include positive, negative, no-template, and no-reverse transcriptase (no-RT) controls.

### POSITIVE CONTROLS

Positive controls contain the target nucleic acid sequence and serve as a benchmark for evaluating the performance of qPCR-based assays. Ideally, positive controls should consist of genomic nucleic acid samples from the target organism in the same matrix as the clinical sample or experimental condition. However, this may not always be feasible (e.g., during the early stages of the COVID-19 pandemic), particularly when high mutation rates complicate access to reliable control sample sources (99).

As an alternative, synthetic double-stranded DNA fragments or RNA molecules are often used. These can be easily synthesized, updated to match newly emerging mutations, and optimized to detect rare variants that may be challenging to detect in natural samples. Synthetic nucleic acids should be stabilized with carrier molecules, such as (transfer RNA) tRNA at 5 ng/μL, and, when possible, added to the same matrix used in the experiment (39). Including positive controls at various concentrations allows for the assessment of RT and qPCR amplification efficiencies, as well as the determination of the LOD and LOQ. This establishes the sensitivity and linear dynamic range of the assay. A comparison of amplification curves from positive controls and unknown samples on a log[fluorescence] axis permits the detection of potential inhibitors or suboptimal reaction conditions. Positive controls also provide reference points for quality control, aid in threshold settings, and help with the interpretation of results.

### NEGATIVE CONTROLS

Negative controls contain carrier nucleic acid but lack the specific target sequence, and they are needed to confirm PCR assay specificity. An amplification signal in a negative control suggests contamination or nonspecific priming and amplification, and in probe-based assays, it can also indicate unintended probe hybridization or degradation. Negative controls are especially valuable in detecting subtle genetic differences, such as distinguishing between wild-type and variant alleles. In these cases, the wild-type DNA (used as the negative control) may be nearly identical to the variant DNA, increasing the risk of nonspecific amplification. Likewise, when targeting small noncoding RNAs, such as small interfering RNAs or viral miRNAs absent in healthy subjects, background

nucleic acids in matrix-matched negative controls may closely resemble those in potentially positive samples. Including negative controls thus strengthens the reliability of results by ensuring that observed signals are specific to the intended target. When a negative control shows amplification, an unknown sample should be considered positive only if its Cq precedes that of the negative control by at least 5 cycles.

#### NO-TEMPLATE CONTROLS

No-template controls (NTCs) are prepared by replacing the sample nucleic acid with water or buffer, ensuring that no template is present. Their primary purpose is to detect contamination from reagents or the laboratory environment, which can result in false positives. If amplification occurs in the NTC, this could indicate reagent contamination or cross-contamination between samples. Depending on the severity of the contamination, results from the entire run may be invalidated, or caution should be taken when interpreting results from unknown samples.

Artifacts can also generate signals in NTCs. In dye-based qPCR assays, these artifacts are usually distinguishable from true products by analyzing the melting curves (39). However, in diagnostic assays, if the NTC shows a detectable increase in probe fluorescence, the results from the run should not be reported, and the analysis should be repeated.

#### NO-RT CONTROLS

In RT-qPCR assays, it is necessary to include a no-RT control, which omits the reverse transcriptase enzyme but contains the same nucleic acids as the experimental sample. This control checks for the presence of contaminating genomic DNA, which could be mistakenly amplified and lead to false positives. Without RT, only DNA (if present) will be amplified, while true RNA targets will not yield any signal. This control is crucial in confirming that the observed amplification is from RNA rather than contaminating DNA. To evaluate whether the primers can amplify random targets in genomic DNA, pure genomic DNA of the species analyzed can be run alongside. An alternative is an assay that amplifies non-transcribed genomic DNA, hence measuring genomic DNA background in a cDNA sample. Combined with a control measurement on a genomic DNA standard, RT-qPCR data can be corrected for genomic DNA background (100).

#### MULTIPLEX (IN-SAMPLE) CONTROLS

In clinical testing, it is common to incorporate internal controls within the same reaction as the target(s) using multiplex qPCR. While these in-sample controls do not replace external positive and negative controls, they offer real-time evidence that the sample and reagents are sufficient for amplification. The internal control should be the same type of nucleic acid (DNA or RNA) as the target and is added at a concentration 3 to 10 times above the LOD.

In infectious disease testing, human reference genes or an additional external template, preferably added before sample preparation, can serve as the internal control. This ensures that the entire work flow, from extraction to amplification, is functioning correctly. Detection of the internal control is essential to validate a negative test result, ensuring that the absence of a pathogen signal is not due to technical failure. The one exception is in cases of high pathogen load, where competition for reagents may prevent amplification of the internal control. In such instances, the failure to detect the internal control is acceptable for a positive test result, as the pathogen signal remains the primary diagnostic indicator.

The control and target(s) are usually differentiated by using probes with distinct fluorescent labels to allow simultaneous detection without cross-interference. A validated multiplex reaction should have the same result as the individual singleplex reactions.

## Conclusions

1. The MIQE 2.0 guidelines address the limitations of the original version and provide a comprehensive framework for the publication of qPCR results along with practical recommendations for their implementation.
2. Developed through expert consensus, these revised guidelines offer improved recommendations for sample handling, experimental design, assay validation, quality control, data analysis, and reporting.
3. The revised guidelines emphasize efficiency-corrected data analysis, appropriate statistical methods, consistent reporting of technical replicates, and the inclusion of positive and negative controls. These measures are important for minimizing bias and increasing the rigor of qPCR experiments in quantitative applications.
4. Implementation of the MIQE 2.0 guidelines across diverse fields, including basic research, diagnostic, forensic, veterinary, environmental, and agricultural tasks, will promote consistency in experimental practices and facilitate the reliability and interpretation of reported results.
5. MIQE 2.0 encourages the use of digital resources and data repositories for sharing experimental details, raw fluorescence data, and analysis scripts. This fosters collaboration, allows for independent verification of results and enhances the transparency of research output.

**Table 1. MIQE 2.0 checklist for authors, reviewers, and editors (this table can also be downloaded in editable format as online Supplemental Table 1).**

| | Provided[a] | Description/ Justification[b] |
|---|---|---|
| **1. REAGENT PREPARATION** | | |
| Bioinformatics tools and versions and settings used to design assays | | |
| Official gene symbol, species, and sequence accession number | | |
| Location of amplicon | | |
| Amplicon length | | |
| Primer and probe sequences[c] | | |
| Location and identity of any modifications | | |
| Manufacturer of oligonucleotides | | |
| Details of optimization performed | | |
| **2. SAMPLE PREPARATION** | | |
| Detailed description of sample types and numbers | | |
| Sampling procedure (including time to storage) | | |
| Sample aliquoting, storage conditions and duration | | |
| Description of extraction method including amount of sample processed | | |
| Source and amount of spike-in nucleic acids added | | |
| Volume of elution buffer used to elute/resuspend nucleic acids | | |
| Number of extraction replicates | | |
| Extraction blanks and percent yield included | | |
| Method to evaluate quality and quantity of nucleic acids | | |
| Storage conditions: temperature, concentration, duration, buffer, aliquots | | |
| Clear description of dilution steps used to prepare working template solution | | |
| Template modification (digestion, sonication, pre-amplification, DNAse treatment etc.) | | |
| Purification after modification | | |
| **3. REVERSE TRANSCRIPTION[d]** | | |
| cDNA priming method and primer concentration | | |
| One- or two-step protocol (include reaction details for two-step) | | |
| Amount of RNA used per reaction | | |
| Detailed reaction components and conditions | | |
| Estimated copies measured with and without addition of RT[e] | | |
| Manufacturer of reagents, catalog number, and lot number | | |
| Storage of cDNA: temperature, concentration/dilution, duration, buffer, aliquots | | |
| **4. qPCR PROTOCOL** | | |
| Template treatment (initial heating or chemical denaturation) | | |
| Primer and probe concentration in the reaction and source | | |
| Polymerase identity and concentration, $Mg^{2+}$ and dNTP concentrations[f] | | |
| Buffer/kit (manufacturer, catalog number and lot number) | | |
| Complete thermocycling parameters including reaction volume | | |
| Manufacturer and type of qPCR instrument | | |

*Continued*

**Table 1. (continued)**

| | Provided[a] | Description/ Justification[b] |
|---|---|---|
| **5. DATA ANALYSIS** | | |
| Storage and submission of raw fluorescence data using RDES[g] or RDML[h] | | |
| Identity of standards (synthetic, plasmid, genomic, IVT[i], mRNA etc.) and method of quantification | | |
| Method of baseline correction and Cq determination | | |
| qPCR analysis program (source, version) | | |
| Details of positive and negative controls | | |
| Frequency and Cq of negative controls | | |
| Examples of positive and negative results | | |
| PCR efficiency estimation and method for its determination | | |
| Method of target quantity calculation[j] | | |
| Description of replicates | | |
| Repeatability (intra-experiment variation) | | |
| Reproducibility (inter-experiment/user/lab etc. variation) | | |
| Limit of detection calculated? | | |
| Dynamic range (limits of quantification) | | |
| Method of validation of reference genes | | |
| Description of normalization method/calculation of normalized expression | | |
| Statistical methods used for analysis | | |
| Choice of significance level and calculation of statistical power | | |
| Specificity (when measuring rare mutations, pathogen sequences etc.) | | |

[a]Authors should insert "Yes" or "No".
[b]If "Yes", specify the location of the information in the article or include the information here. If "No", outline the rationale for omission.
[c]Disclosure of the primer and probe sequences is highly desirable and strongly encouraged. However, when commercial pre-designed assay vendors do not release this information, assay context sequences must be submitted.
[d]This section and parts of Section 5 may not apply depending on the experiment.
[e]Assessing the absence of DNA using a no RT assay (or where RT has been inactivated) is important when first extracting RNA. Once the sample has been validated as DNA-free, inclusion of a no-RT control is desirable, but no longer essential.
[f]Details of reaction components are highly desirable, however not always provided by commercial vendors. Inclusion of reagent manufacturer, catalog and batch number as well as assay context sequences is necessary where component reagent details are not available.
[g]Real-time PCR Data Essential Spreadsheet Format (84).
[h]Real-Time PCR Data Markup Language (85).
[i]In vitro transcribed.
[j]Efficiency-corrected target quantity calculation is necessary.

6. While the guidelines provide a robust and adaptable framework, ongoing refinement will be necessary to address emerging technologies and advancements in qPCR methods. This will require continued engagement and collaboration within the scientific community.
7. We recommend that laboratories adopt the accompanying recommendations (see online supplemental file "MIQE 2.0 Recommendations") as part of routine research practices. The MIQE 2.0 checklist (Table 1) can be used to guide experiment planning and the review of manuscripts. The same checklist can be downloaded in editable format (online Supplemental Table 1). Journals, funding agencies, and other stakeholders should provide incentives for researchers to adopt and follow these guidelines. For example, the checklist can be incorporated into the submission process of journals, as is already the case with some journals, e.g., *Clinical Chemistry*, or funding agencies could encourage the submission of grant proposals that include a MIQE-inspired detailing of the proposed experimental work flow.

Collectively, the adoption of MIQE 2.0 will improve the overall quality and transparency of qPCR research, enhancing its credibility and impact across the scientific community. By fostering consistency, rigor, and reproducibility, these guidelines will strengthen the foundation of qPCR applications and ensure their continued relevance in an evolving scientific landscape.

## Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

**Nonstandard Abbreviations:** MIQE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments; qPCR, quantitative PCR; Cq, quantification cycle (formerly known as Ct or Cp); RT-qPCR, reverse transcription quantitative PCR; LOD, limit of detection; RT, reverse transcription; $T_m$ (melting temperature—the temperature at which 50% of nucleic acid duplexes are single stranded); LLOQ, lower limit of quantification.

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