

# The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

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**BACKGROUND:** Currently, a lack of consensus exists on how best to perform and interpret quantitative real-time PCR (qPCR) experiments. The problem is exacerbated by a lack of sufficient experimental detail in many publications, which impedes a reader's ability to evaluate critically the quality of the results presented or to repeat the experiments.

**CONTENT:** The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines target the reliability of results to help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency. MIQE is a set of guidelines that describe the minimum information necessary for evaluating qPCR experiments. Included is a checklist to accompany the initial submission of a manuscript to the publisher. By providing all relevant experimental conditions and assay characteristics, reviewers can assess the validity of the protocols used. Full disclosure of all reagents, sequences, and analysis methods is necessary to enable other investigators to reproduce results. MIQE details should be published either in abbreviated form or as an online supplement.

**SUMMARY:** Following these guidelines will encourage better experimental practice, allowing more reliable and unequivocal interpretation of qPCR results.

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The fluorescence-based quantitative real-time PCR (qPCR)<sup>15</sup> (1–3), with its capacity to detect and measure minute amounts of nucleic acids in a wide range of samples from numerous sources, is the enabling technology par excellence of molecular diagnostics, life sciences, agriculture, and medicine (4, 5). Its conceptual and practical simplicity, together with its combination of speed, sensitivity, and specificity in a homogeneous assay, have made it the touchstone for nucleic acid quantification. In addition to its use as a research tool, many diagnostic applications have been developed, including microbial quantification, gene dosage determination, identification of transgenes in genetically modified foods, risk assessment of cancer recurrence, and applications for forensic use (6–11).

This popularity is reflected in the prodigious number of publications reporting qPCR data, which invariably use diverse reagents, protocols, analysis methods, and reporting formats. This remarkable lack of consensus on how best to perform qPCR experiments has the adverse consequence of perpetuating a string of serious shortcomings that encumber its status as an independent yardstick (12). Technical deficiencies that affect assay performance include the following: (a) inadequate sample storage, preparation, and nucleic acid quality, yielding highly variable results; (b) poor choice of reverse-transcription primers and primers and probes for the PCR, leading to inefficient and less-than-robust assay performance;

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<sup>15</sup> Nonstandard abbreviations: qPCR, quantitative real-time PCR; MIQE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments; RT-qPCR, reverse transcription–qPCR; FRET, fluorescence resonance energy transfer; C<sub>q</sub>, quantification cycle, previously known as the threshold cycle (C<sub>t</sub>), crossing point (C<sub>p</sub>), or take-off point (TOP); RDML, Real-Time PCR Data Markup Language; LOD, limit of detection; NTC, no-template control.

and (c) inappropriate data and statistical analyses, generating results that can be highly misleading. Consequently, there is the real danger of the scientific literature being corrupted with a multitude of publications reporting inadequate and conflicting results (13). The publication (14) and retraction (15) of a *Science* “Breakthrough of the Year 2005” report provides a disquieting warning. The problem is exacerbated by the lack of information that characterizes most reports of studies that have used this technology, with many publications not providing sufficient experimental detail to permit the reader to critically evaluate the quality of the results presented or to repeat the experiments. Specifically, information about sample acquisition and handling, RNA quality and integrity, reverse-transcription details, PCR efficiencies, and analysis parameters are frequently omitted, whereas sample normalization is habitually carried out against single reference genes without adequate justification.

The aim of this document is to provide authors, reviewers, and editors specifications for the minimum information, set out in Table 1, that must be reported for a qPCR experiment to ensure its relevance, accuracy, correct interpretation, and repeatability. MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments, pronounced *mykee*) is modeled on similar guidelines drawn up for DNA microarray analysis (16), proteomics experiments (17), genome sequence specification (18), and those under discussion for RNA interference work (19, 20) and metabolomics (21), all of which are initiatives coordinated under the umbrella of MIBBI (Minimum Information for Biological and Biomedical Investigations, <http://www.mibbi.org>) (22). Compulsory inclusion of a common reporting language to allow data sharing is not proposed, although it is envisaged that a future update of these guidelines could include such a recommendation. Rather, these guidelines target the reliability of results to help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency. They should be read in conjunction with recent publications that deal in depth with the issue of qPCR standardization (23–26).

## 1. Nomenclature

A few terms require standardization to ensure clarification:

1.1 We propose that the abbreviation qPCR be used for quantitative real-time PCR and that RT-qPCR be used for reverse transcription–qPCR. Applying the abbreviation RT-PCR to qPCR causes confu-

sion and is inconsistent with its use for conventional (legacy) reverse transcription–PCR.

- 1.2 Genes used for normalization should be referred to as *reference genes*, not as *housekeeping genes*.
- 1.3 TaqMan probes should be referred to as *hydrolysis probes*.
- 1.4 The term *FRET probe* (fluorescence resonance energy transfer probe) refers to a generic mechanism in which emission/quenching relies on the interaction between the electron-excitation states of 2 fluorescent dye molecules. LightCycler-type probes should be referred to as *dual hybridization probes*.
- 1.5 The *Oxford English Dictionary* lists only *quantification*, not *quantitation*; therefore, the former is the proper word.
- 1.6 The nomenclature describing the fractional PCR cycle used for quantification is inconsistent, with *threshold cycle* ( $C_T$ ), *crossing point* ( $C_P$ ), and *take-off point* (TOP) currently used in the literature. These terms all refer to the same value from the real-time instrument and were coined by competing manufacturers of real-time instruments for reasons of product differentiation, not scientific accuracy or clarity. We propose the use of *quantification cycle* ( $C_q$ ), according to the RDML (Real-Time PCR Data Markup Language) data standard (<http://www.rdml.org>) (27).

## 2. Conceptual Considerations

To explain and justify the guidelines, we find it useful to review a number of key issues surrounding qPCR experiments:

2.1 *Analytical sensitivity* refers to the minimum number of copies in a sample that can be measured accurately with an assay, whereas *clinical sensitivity* is the percentage of individuals with a given disorder whom the assay identifies as positive for that condition. Typically, sensitivity is expressed as the *limit of detection* (LOD), which is the concentration that can be detected with reasonable certainty (95% probability is commonly used) with a given analytical procedure. The most sensitive LOD theoretically possible is 3 copies per PCR (28), assuming a Poisson distribution, a 95% chance of including at least 1 copy in the PCR, and single-copy detection. Experimental procedures typically include sample-processing steps (i.e., extraction) and, when required, reverse transcription. If the volume changes and the efficiencies of these steps are accounted for, the most sensitive LOD theoretically possible can be expressed in units relevant to the experiment, such as copies per nanogram of tissue. Experimental results less than the theoretic-

Table 1. MIQE checklist for authors, reviewers, and editors.<sup>a</sup>

Item to check	Importance	Item to check	Importance
Experimental design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D <sup>d</sup>
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	qPCR protocol	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg <sup>2+</sup> , and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for FFPE <sup>b</sup> samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	qPCR validation	
Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A <sub>260</sub> /A <sub>280</sub> )	D	Specificity (gel, sequence, melt, or digest)	E
Yield	D	For SYBR Green I, C <sub>q</sub> of the NTC	E
RNA integrity: method/instrument	E	Calibration curves with slope and y intercept	E
RIN/RQI or C <sub>q</sub> of 3' and 5' transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	CI <sub>s</sub> for PCR efficiency or SE	D
Inhibition testing (C <sub>q</sub> dilutions, spike, or other)	E	r <sup>2</sup> of calibration curve	E
Reverse transcription		Linear dynamic range	E
Complete reaction conditions	E	C <sub>q</sub> variation at LOD	E
Amount of RNA and reaction volume	E	CI <sub>s</sub> throughout range	D
Priming oligonucleotide (if using GSP) and concentration	E	Evidence for LOD	E
Reverse transcriptase and concentration	E	If multiplex, efficiency and LOD of each assay	E
Temperature and time	E	Data analysis	
Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
C <sub>q</sub> s with and without reverse transcription	D <sup>c</sup>	Method of C <sub>q</sub> determination	E
Storage conditions of cDNA	D	Outlier identification and disposition	E
qPCR target information		Results for NTCs	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Description of normalization method	E
Location of amplicon	D	Number and concordance of biological replicates	D
Amplicon length	E	Number and stage (reverse transcription or qPCR) of technical replicates	E
In silico specificity screen (BLAST, and so on)	E	Repeatability (intraassay variation)	E
Pseudogenes, retropseudogenes, or other homologs?	D	Reproducibility (interassay variation, CV)	D
Sequence alignment	D	Power analysis	D
Secondary structure analysis of amplicon	D	Statistical methods for results significance	E
Location of each primer by exon or intron (if applicable)	E	Software (source, version)	E
What splice variants are targeted?	E	C <sub>q</sub> or raw data submission with RDML	D

<sup>a</sup> All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from RTPrimerDB, information on qPCR target, oligonucleotides, protocols, and validation is available from that source.

<sup>b</sup> FFPE, formalin-fixed, paraffin-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deoxynucleoside triphosphate.

<sup>c</sup> Assessing the absence of DNA with a no-reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as DNA free, inclusion of a no-reverse transcription control is desirable but no longer essential.

<sup>d</sup> Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it cannot be an essential requirement. Use of such assays is discouraged.

cally possible LOD should never be reported. It also follows that results of “0” are meaningless and misleading. LOD estimates in qPCR analyses are complicated by the logarithmic nature of  $C_q$ , because  $C_q$  is undefined when the template concentration is zero. Appropriate determination and modeling of the LOD in the qPCR is the focus of continued research (26).

- 2.2 *Analytical specificity* refers to the qPCR assay detecting the appropriate target sequence rather than other, nonspecific targets also present in a sample. *Diagnostic specificity* is the percentage of individuals without a given condition whom the assay identifies as negative for that condition.
- 2.3 *Accuracy* refers to the difference between experimentally measured and actual concentrations, presented as fold changes or copy number estimates.
- 2.4 *Repeatability* (short-term precision or intraassay variance) refers to the precision and robustness of the assay with the same samples repeatedly analyzed in the same assay. It may be expressed as the SD for the  $C_q$  variance. Alternatively, the SD or the CV for copy number or concentration variance may be used. CVs should not be used with  $C_q$ s, however (29).
- 2.5 *Reproducibility* (long-term precision or interassay variance) refers to the variation in results between runs or between different laboratories and is typically expressed as the SD or CV of copy numbers or concentrations.  $C_q$  values generated from different runs are subject to inherent interrun variation (30); hence, reporting interrun  $C_q$  variation is not appropriate.

Publications describing mRNA concentrations for target genes should make it clear precisely what the targets are. The transcripts of most human genes and many genes in other multicellular organisms are alternatively spliced (31, 32), and these splicing variants specify alternative protein isoforms, with variation in splicing patterns reported in different tissues or at different developmental stages. Consequently, single exon-based RT-qPCR assays may detect a number of splice variants, whereas intron-spanning primers may be more selective but may miss some splice variants altogether. Most recently, autosomal nonimprinted genes that display allelic imbalance in their expression have been described (33). Taken together, these findings imply that use of an RT-qPCR assay that simply targets one or at most 2 exons of an mRNA is no longer sufficient to describe the expression level of a particular gene. Consequently, sequence information for primers must be provided together with an assessment of their specificity with respect to known splice variants and

single-nucleotide polymorphism positions documented in transcript and single-nucleotide polymorphism databases. For primer sets selected from the RTprimerDB database (34, 35), this is easily done by consulting the RTprimerDB Web site (<http://www.rtpriimerdb.org>), which contains all the relevant information. For commercial assays, lot information and the providers' experimental validation criteria are required. The reporting of results for nonvalidated commercial assays and assays that have been validated only in silico are strongly discouraged.

It must be remembered that detection of the presence of an mRNA provides no information on whether that mRNA will be translated into a protein or, indeed, whether a functional protein is translated at all.

Immunohistochemistry, western blotting, or other protein-quantification methods are not always able to corroborate quantitative cellular mRNA data. It is now well established that there is frequently a lack of concordance between mRNA- and protein-concentration data (36), which is particularly true for mRNAs that specify proteins that are part of multi-function protein complexes (37). Finally, it has become clear that knowledge of the presence and function of specific microRNAs is as important to understanding gene expression as being able to quantify the mRNA species (38).

It is also necessary to be aware that most quantitative RNA data are not absolute, but relative. Thus, the reference genes or materials used for standardization are critical, and any assessment of the validity of an RT-qPCR experiment must also consider the appropriateness of the relative-quantification reference. Therefore, the development of universal reference DNA and RNA calibration materials, although very helpful (39, 40), will not be a universal panacea (41, 42).

Much of the variance in reported expression values produced in RT-qPCR experiments is not simply due to variation in experimental protocols but is caused by corrections applied by various data-processing algorithms, each of which makes its own assumptions about the data. Consequently, although qPCR has frequently been proclaimed a touchstone or a gold standard, in practice this “standard” is a variable one, and the reporting of results requires considerable sophistication of analysis and interpretation (43).

### 3. Research vs Diagnostic Applications

Applications of qPCR technology can be broadly divided into research and diagnostic applications. Research applications usually analyze a wide range of targets with a fairly low throughput and many different sample types. The main parameters that need to be addressed relate to assay analytical sensitivity

and specificity, which in this context refer to how many target copies the assay can detect and whether the no-template controls (NTCs) are reliably negative, respectively.

In contrast, diagnostic applications usually analyze a limited number of targets, but require high-throughput protocols that are targeted at only a few sample types. Although all of the considerations that apply to research applications also apply to diagnostic assays, clinical-diagnostic assays have a number of additional requirements that need to be considered. These requirements include information on analytical sensitivity and specificity that in this context refers to how often the assay returns a positive result when a target is present and how often it is negative in the absence of the target. Furthermore, the accuracy and precision within and between laboratories is often monitored by external QC programs. Additional clinical laboratory requirements include criteria for generating reportable results, whether repeated measurements are made on samples, data on the resolution of false-positive/false-negative data, and the similarity of results from multiple laboratories that use the same and different technologies. Thus far, only a couple of interlaboratory comparisons have been performed, and both of these studies emphasized the need for standardization of qPCR diagnostic assays (44, 45). Another interlaboratory exercise is planned within the European Framework 7 project: SPIDIA (Standardisation and Improvement of Generic Pre-analytical Tools and Procedures for In-Vitro Diagnostics; <http://www.spidia.eu>).

#### 4. Sample Acquisition, Handling, and Preparation

Sample acquisition constitutes the first potential source of experimental variability, especially for experiments targeting RNA, because mRNA profiles are easily perturbed by sample-collection and -processing methods. There is some suggestion that fresh tissue can be stored on ice without major effects on RNA quality and concentration (46), but although this supposition may be true for some mRNAs and tissues, it may not be universally applicable. Hence, it is better to be cautious. Consequently, it is important to report in detail where the tissue sample was obtained and whether it was processed immediately. If the sample was not processed immediately, it is necessary to report how it was preserved and how long and under what conditions it was stored.

A brief description of the sample is also essential. For example, microscopical examination of a tumor biopsy will reveal what percentage of the biopsy is made up of tumor cells, and this information should be reported.

Nucleic acid extraction is a second critical step. Extraction efficiency depends on adequate homogenization, the type of sample (e.g., in situ tissue vs log-phase cultured cells), target density, physiological status (e.g., healthy, cancerous, or necrotic), genetic complexity, and the amount of biomass processed. Therefore, it is necessary that details of the nucleic acid-extraction method be provided and that the methods used for measuring nucleic acid concentration and assessing its quality be described. Such details are particularly crucial for RNA extracted from fresh frozen laser-microdissected biopsy samples, because variations in tissue-preparation procedures have a substantial effect on both RNA yield and quality (47).

#### 5. QC of Nucleic Acids

##### 5.1. RNA SAMPLES

Quantification of RNA in the extracted samples is important, because it is advisable that approximately the same amounts of RNA be used when comparing different samples. There are several quantification procedures in common use, however, including spectrophotometry (NanoDrop; Thermo Scientific), microfluidic analysis (Agilent Technologies' Bioanalyzer, Bio-Rad Laboratories' Experion), capillary gel electrophoresis (Qiagen's QIAxcel), or fluorescent dye detection (Ambion/Applied Biosystems' RiboGreen). The methods produce different results, making it unwise to try to compare data obtained with the different methods (48). The preferred method for quantifying RNA uses fluorescent RNA-binding dyes (e.g., RiboGreen), which are best for detecting low target concentrations. In any case, it is advisable to measure all samples with a single method only and to report this information.

It is also important to test for and report the extent of genomic-DNA contamination and to record the threshold cutoff criteria for the amounts of such contamination that are tolerable. It is essential to report whether the RNA sample has been treated with DNase (including the type of DNase used and the reaction conditions) and to report the results from a comparison of  $C_q$ s obtained with positive and no-reverse transcription controls for each nucleic acid target.

It is also essential to document the quality assessment of RNA templates. The only situation in which this requirement does not apply is when the quantity of total RNA extracted is too low to permit quality assessment. This situation arises when RNA is extracted from single cells, plasma, other cell-free body fluids, some laser-captured samples, or clarified tissue culture medium. It also applies in cases in which extraction and RT-qPCR steps are performed as a continuous, single-tube experiment. Key information to report includes data on RNA quantity, integrity, and the absence of

reverse transcription or PCR inhibitors. It is worth remembering that RNA degrades markedly *in vivo*, owing to the natural regulation of mRNAs in response to environmental stimuli (49). This source of RNA degradation is beyond the control of the researcher; one of its manifestations is that even high-quality RNA samples can show differential degradation of individual mRNAs.

The  $A_{260}/A_{280}$  ratio must be measured in a buffer at neutral pH, but such measurement is not sufficient if the nucleic acid is to be used for quantitative analysis, especially when the aim is to measure minor differences (<10-fold) in cellular mRNA concentrations. The absorbance ratio does provide an indication of RNA purity, because the presence of DNA or residual phenol alters the ratio. Instead, one should provide gel electrophoresis evidence at the least or, better yet, results from a microfluidics-based rRNA analysis (50) or a reference gene/target gene 3':5' integrity assay (51). The advantage of the use of a Bioanalyzer/Experion system to calculate an RNA integrity number or an RNA quality indicator number is that these measures provide quantitative information about the general state of the RNA sample. It is important to bear in mind, however, that these numbers relate to rRNA quality and cannot be expected to be an absolute measure of quality. Use of a 3':5' assay requires that the PCR efficiencies of both assays be virtually identical (51) and not be subject to differential inhibition. This assay also necessitates the establishment of a threshold criterion that delineates the RNA quality sufficient to yield reliable results. Ideally, the assay should target a panel of "integrity reference genes," probably without introns, with a 3':5' threshold ratio of approximately 0.2–5. Clearly, further work is required to generate a universally applicable, cost-effective, and simple protocol for assessing RNA integrity.

Inhibition of reverse-transcription activity or PCR should be checked by dilution of the sample (preferably) or use of a universal inhibition assay such as SPUD (52, 53). If the RNA sample is shown to be partially degraded, it is essential that this information be reported, because the assay's sensitivity for detecting a low-level transcript may be reduced and relative differences in the degradation of transcripts may produce incorrect target ratios.

## 5.2. DNA SAMPLES

In general, degradation is much less of an issue with DNA; however, it is important to be able to assess the extent of DNA degradation for forensic applications, *i.e.*, in cases in which harsh environmental conditions at scenes of crimes or mass disasters or at sites involving missing-person cases may have degraded the chemical structure of DNA. The small amplicon size of qPCR

assays helps to minimize assay-related problems, but methods have been developed that provide a quantitative measurement of DNA quality (54) and should be considered for such specialized purposes.

The potential for inhibition is a more generally applicable variable that must be addressed in a publication, and it is important to ensure that no inhibitors copurified with the DNA will distort results, *e.g.*, pathogen detection and their quantification (55). Although such approaches such as spiking samples with positive controls (52) can be used to detect inhibition, different PCR reactions may not be equally susceptible to inhibition by substances copurified in nucleic acid extracts (56, 57). Consequently, it is better to routinely use dilutions of nucleic acids to demonstrate that observed decreases in  $C_q$ s or copy numbers are consistent with the anticipated result and to report these data.

## 6. Reverse Transcription

The reverse-transcription step introduces substantial variation into an RT-qPCR assay (58, 59). Hence, it is essential that a detailed description of the protocol and reagents used to convert RNA into cDNA be provided. This documentation must include the amount of RNA reverse-transcribed, priming strategy, enzyme type, volume, temperature, and duration of the reverse-transcription step. It is recommended that the reverse-transcription step be carried out in duplicate or triplicate and that the total RNA concentration be the same in every sample (58).

## 7. qPCR

The following information must be provided for qPCR assays: database accession numbers of each target and reference gene, the exon locations of each primer and any probe, the sequences and concentrations of each oligonucleotide, including the identities, positions, and linkages of any dyes and/or modified bases. Also required are the concentration and identity of the polymerase, the amount of template (DNA or cDNA) in each reaction, the  $Mg^{2+}$  concentration, the exact chemical compositions of the buffer (salts, pH, additives), and the reaction volume. The investigators must also identify the instrument they used and document all of the PCR cycling conditions. Because the consumables used affect thermal cycling, it is necessary to identify the use of single tubes, strips, or plates, and their manufacturers. The degree of transparency of the plasticware used, *e.g.*, white or clear, is also important, because different plastics exhibit substantial differences in fluorescence reflection and sensitivity (60). When plates are used, the method of sealing (heat bonding

vs adhesives) can affect the evaporation of samples at the plate perimeter and should therefore be documented.

Because PCR efficiency is highly dependent on the primers used, their sequences must be published. This requirement is perfectly feasible even with commercial primers, because there is a precedent for companies to make their primer and probe sequences available ([http://www.primersdesign.co.uk/research\\_with\\_integrity.asp](http://www.primersdesign.co.uk/research_with_integrity.asp)).

In addition, submission to public databases such as RTprimerDB is strongly encouraged; over time, these databases could become universal clearinghouses.

#### 7.1. SECONDARY STRUCTURE

The structure of the nucleic acid target (e.g., stem and loop secondary RNA structure) has a substantial impact on the efficiency of reverse transcription and the PCR. Therefore, the positions of primers, probes, and PCR amplicons must take the folding of RNA templates into consideration. Sequences should be checked with nucleic acid–folding software, e.g., mfold for DNA (<http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi>) or RNA (<http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1-2.3.cgi>). Ideally, the folding structures should be made available to reviewers.

#### 7.2. SPECIFICITY

In silico tools such as BLAST or equivalent specificity searches are useful for assay design. Any appreciable homology to pseudogenes or other unexpected targets should be documented and provided as aligned sequences for review; however, specificity must be validated empirically with direct experimental evidence (electrophoresis gel, melting profile, DNA sequencing, amplicon size, and/or restriction enzyme digestion).

Algorithms for predicting an oligonucleotide's melting temperature ( $T_m$ ) are useful for initial design, but the practical optimum temperature for annealing must be determined experimentally. Although primer optimization has become unfashionable, it is clear that poor annealing optimization has a large effect on assay quality (51). A marked presence of primer dimers produces a lower PCR efficiency in probe-based assays and may generate false positives in assays based on SYBR Green I. Some evidence for primer optimization should be provided to reviewers, ideally in the form of annealing temperature or  $Mg^{2+}$  gradients, and be presented as  $C_q$  values, plots of fluorescence vs cycle number, and/or melting curves (61).

#### 7.3. CONTROLS AND QUANTIFICATION CALIBRATORS

In addition to the no–reverse transcription control in RT–qPCR assays mentioned above, additional controls

and/or quantification calibrators are required for all qPCR reactions. NTCs detect PCR contamination when probes are used and can also distinguish unintended amplification products (e.g., primer dimers) from the intended PCR products in SYBR Green I reactions. NTCs should be included on each plate or batch of samples, and conditions for data rejection be established. For example, NTCs with  $C_{qs} \geq 40$  could be ignored if the  $C_q$  for the lowest concentration unknown is 35.

Positive controls in the form of nucleic acids extracted from experimental samples are useful for monitoring assay variation over time and are essential when calibration curves are not performed in each run.

Quantification calibrators may be purified target molecules, such as synthetic RNA or DNA oligonucleotides spanning the complete PCR amplicon, plasmid DNA constructs, cDNA cloned into plasmids, RNA transcribed in vitro, reference RNA pools, RNA or DNA from specific biological samples, or internationally recognized biological standards (as they become available). Dilutions should be carried out into defined concentrations of carrier tRNA (yeast or *Escherichia coli* at 10–100 ng/ $\mu$ L). For detection of human pathogens, calibrators can be diluted into negative control sample RNA or DNA, or they can be diluted into healthy human plasma, after which lysis may be carried out in the presence of carrier tRNA. Serial dilutions of a particular template can be prepared as stock solutions that resist several freeze–thaw cycles. A fresh batch should be prepared when a  $C_q$  shift of 0.5–1.0 is detected. Alternatively, solutions for calibration curves can be stored for a week at 4 °C and then discarded.

For diagnostic assays, the qPCR should include an independently verified calibrator, if available, that lies within the linear interval of the assay. Positive and negative extraction controls are also recommended.

#### 7.4. ASSAY PERFORMANCE

The following assay performance characteristics must be determined: PCR efficiency, linear dynamic range, LOD, and precision.

**7.4.1. PCR efficiency.** Robust and precise qPCR assays are usually correlated with high PCR efficiency. PCR efficiency is particularly important when reporting mRNA concentrations for target genes relative to those of reference genes. The  $\Delta\Delta C_q$  method is one of the most popular means of determining differences in concentrations between samples and is based on normalization with a single reference gene. The difference in  $C_q$  values ( $\Delta C_q$ ) between the target gene and the reference gene is calculated, and the  $\Delta C_{qs}$  of the different samples are compared directly. The 2 genes must be amplified with comparable efficiencies for this com-

parison to be accurate. The most popular method is not necessarily the most appropriate, however, and alternative, more generalized quantitative models have been developed to correct for differences in amplification efficiency (62) and to allow the use of multiple reference genes (30).

PCR amplification efficiency must be established by means of calibration curves, because such calibration provides a simple, rapid, and reproducible indication of the mean PCR efficiency, the analytical sensitivity, and the robustness of the assay. Amplification efficiency should be determined from the slope of the log-linear portion of the calibration curve. Specifically, PCR efficiency =  $10^{-1/\text{slope}} - 1$ , when the logarithm of the initial template concentration (the independent variable) is plotted on the  $x$  axis and  $C_q$  (the dependent variable) is plotted on the  $y$  axis. The theoretical maximum of 1.00 (or 100%) indicates that the amount of product doubles with each cycle. Ideally, the CIs or SEs of the means of estimated PCR efficiencies are reported from replicated calibration curves.

Calibration curves for each quantified target must be included with the submitted manuscript so that this information can be made available to the reviewers; slopes and  $y$  intercepts derived from these calibration curves must be included with the publication. Differences in PCR efficiency will produce calibration curves with different slopes. As a consequence, differences between the  $C_q$  values of the targets and the references will not remain constant as template amounts are varied, and calculations of relative concentrations will be inaccurate, yielding misleading results.

$C_q$  values  $>40$  are suspect because of the implied low efficiency and generally should not be reported; however, the use of such arbitrary  $C_q$  cutoffs is not ideal, because they may be either too low (eliminating valid results) or too high (increasing false-positive results) (26).

**7.4.2. Linear dynamic range.** The dynamic range over which a reaction is linear (the highest to the lowest quantifiable copy number established by means of a calibration curve) must be described. Depending on the template used for generating calibration curves, the dynamic range should cover at least 3 orders of magnitude and ideally should extend to 5 or 6  $\log_{10}$  concentrations. The calibration curve's linear interval must include the interval for the target nucleic acids being quantified. Because lower limits of quantification are usually poorly defined, the variation at the lowest concentration claimed to be within the linear interval should be determined. Correlation coefficients ( $r^2$  values) must be reported, and, ideally, CIs should be provided through the entire linear dynamic range.

**7.4.3. LOD.** The LOD is defined as the lowest concentration at which 95% of the positive samples are detected. In other words, within a group of replicates containing the target at concentrations at the LOD, no more than 5% failed reactions should occur. Low-copy PCRs are stochastically limited, and LODs of  $<3$  copies per PCR are not possible. If multiple reactions are performed, however, accurate quantification of lower concentrations can be obtained via digital PCR (29, 63, 64). Indeed, concentration calibrators can be checked by limiting dilution to show that the percentage of failed and successful reactions follows a Poisson distribution.

**7.4.4. Precision.** There are many explanations for variation in qPCR results, including temperature differences affecting the completion of annealing and/or denaturation, concentration differences introduced by pipetting errors, and stochastic variation. Precision in qPCR typically varies with concentration, decreasing with the copy number. Ideally, intraassay variation (repeatability) should be displayed in figures as SD error bars or as CIs on calibration curves with replicate samples. CVs should not be used with  $C_q$ s (29) but can be used to express the variance in copy numbers or concentrations. This technical variation should be distinguished from biological variation. Biological replicates can directly address the statistical significance of differences in qPCR results between groups or treatments. For diagnostic assays, it may also be necessary to report interassay precision (reproducibility) between sites and different operators.

#### 7.5. MULTIPLEX qPCR

The ability to multiplex greatly expands the power of qPCR analysis (65, 66), particularly when applied to the simultaneous detection of point mutations or polymorphisms (67). Multiplexing requires the presentation of evidence demonstrating that accurate quantification of multiple targets in a single tube is not impaired, i.e., that assay efficiency and the LOD are the same as when the assays are run in uniplex fashion. This concern is of particular importance when targets of appreciably lower abundance are coamplified with highly abundant targets.

## 8. Data Analysis

Data analysis includes an examination of the raw data, an evaluation of their quality and reliability, and the generation of reportable results. Various data-collection and -processing strategies have been described, and a systematic evaluation has revealed that qPCR data-analysis methods differ substantially in their performance (68).



Detailed information on the methods of data analysis and confidence estimation is necessary, together with specification of the software used. The methods of identifying outliers and the disposition of such data must be specified. Documenting assay precision requires identification of the statistical methods used to evaluate variances (e.g., 95% CIs) and presentation of the corresponding concentrations or  $C_q$  values. Such information should include both repeatability and reproducibility data, if available. As discussed above, reporting of CVs for  $C_q$ s is inappropriate (29), because  $C_q$ s will always be lower (and therefore potentially misleading) than CVs calculated for copy numbers. Information must be provided on the methods used for assessing accuracy, including the statistical significance of reported differences between groups.

#### 8.1. NORMALIZATION

Normalization is an essential component of a reliable qPCR assay because this process controls for variations in extraction yield, reverse-transcription yield, and efficiency of amplification, thus enabling comparisons of mRNA concentrations across different samples. The use of reference genes as internal controls is the most common method for normalizing cellular mRNA data; however, although the use of reference genes is commonly accepted as the most appropriate normalization strategy (69), their utility must be experimentally validated for particular tissues or cell types and specific experimental designs. Unfortunately, although there is an increased awareness of the importance of systematic validation and although the potentially highly misleading effects of the use of inappropriate reference genes for normalization are widely known, these considerations also are still widely disregarded (70). Consequently, many molecular analyses still contain qPCR data that are poorly normalized.

Normalization involves reporting the ratios of the mRNA concentrations of the genes of interest to those of the reference genes. Reference gene mRNAs should be stably expressed, and their abundances should show strong correlation with the total amounts of mRNA present in the samples.

Normalization against a single reference gene is not acceptable unless the investigators present clear evidence for the reviewers that confirms its invariant expression under the experimental conditions described. The optimal number and choice of reference genes must be experimentally determined and the method reported (71–73).

#### 8.2. VARIABILITY

The inherent variability of biological systems may rival or exceed experimental differences between groups. This variation is often observed when many biological

replicates are used to increase the statistical significance of the experiment. Although differences between biological replicates may be large, sufficient numbers may allow smaller experimental differences to be discerned. A recent publication provides a textbook example for handling such data and how to salvage biologically meaningful data from assays subject to high biological variation (74). Many factors contribute to experimental variation and influence the number of biological replicates necessary to achieve a given statistical power. Consequently, power analysis is useful for determining the number of samples necessary for valid conclusions.

#### 8.3. QUALITATIVE ANALYSIS

The use of the PCR to detect merely the presence of a nucleic acid template, rather than to quantify it accurately, is referred to as qualitative PCR, which is widely used in pathogen diagnostics. The problem with qualitative/quantitative stratification of PCR methods is that an accurate yes/no answer requires information about the low-end sensitivity of the PCR assay. Consequently, even a qualitative assay should provide information about the assay's performance characteristics, especially with respect to the points discussed in sections 7.4.2. and 7.4.3.

### Conclusions

The necessity for ensuring quality-assurance measures for qPCR and RT-qPCR assays is well recognized (25, 44, 75–86). The main difference between qPCR and conventional (legacy) PCR assays is the expectation of the former's potential to quantify target nucleic acids accurately. This difference must be clearly recognized, and one cannot assume that legacy PCR assays can translate directly into the qPCR format. Table 1 provides a checklist for authors preparing a report of a qPCR study. Items deemed essential (E) are required to allow reviewers to evaluate the work and other investigators to reproduce it. Items considered desirable (D) are also important and should be included if possible, but they may not be available in all cases. Certainly, it is important to apply common sense: Compliance with all items on the checklist is not necessary for initial screening of expression signatures targeting hundreds of targets. Once a more limited set of targets (fewer than 20) has been identified, however, assay performance should be described as detailed by the checklist, which is hosted on <http://www.rdml.org/miqe/>.

In summary, the purpose of these guidelines is 3-fold:

1. To enable authors to design and report qPCR experiments that have greater inherent value.

2. To allow reviewers and editors to measure the technical quality of submitted manuscripts against an established yardstick.
3. To facilitate easier replication of experiments described in published studies that follow these guidelines.

As a consequence, investigations that use this widely applied technology will produce data that are more uniform, more comparable, and, ultimately, more reliable.

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