

The State of RT-Quantitative PCR: Firsthand Observations of Implementation of Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE)

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Key Words

RT-quantitative PCR · Quantitative PCR · Minimum information for the publication of quantitative real-time PCR experiments (MIQE) · Experimental design

Abstract

In the past decade, the techniques of quantitative PCR (qPCR) and reverse transcription (RT)-qPCR have become accessible to virtually all research labs, producing valuable data for peer-reviewed publications and supporting exciting research conclusions. However, the experimental design and validation processes applied to the associated projects are the result of historical biases adopted by individual labs that have evolved and changed since the inception of the techniques and associated technologies. This has resulted in wide variability in the quality, reproducibility and interpretability of published data as a direct result of how each lab has designed their RT-qPCR experiments. The ‘minimum information for the publication of quantitative real-time PCR experiments’ (MIQE) was published to provide the scientific community with a consistent workflow and key considerations to perform qPCR experiments. We use specific examples to highlight the serious negative ramifications for data quality when the MIQE guidelines are not applied and include a summary of good and poor practices for RT-qPCR.

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A Method for Consistency

The application of inconsistent experimental design and techniques to quantitative PCR (qPCR) experiments has resulted in the publication of artifactual qPCR data with potentially misleading conclusions [Bustin 2010; Bustin et al. 2009b], leading to the retraction of high-profile papers [Böhlenius et al. 2007; Retraction, 2010]. This situation is further revealed in the Materials and Methods sections of many publications, where it is evident that primers and/or probes were not validated or the associated sequences were not reviewed for competing sequence homology [Wang et al., 2012]. Finally, a large number of published articles with findings that hinge on reverse transcription (RT)-qPCR data report that normalization was performed using a single reference gene untested for stability such as GAPDH, β -actin, tubulin or 18S RNA [Barber et al., 2005; Jacob et al., 2013; Rhinn et al., 2008; Schmittgen and Zakrajsek, 2000; Thellin et al., 1999; Yang et al., 2012].

Teaching and practicing qPCR according to a well-defined methodology that will ensure quality data has been a central theme in recent years and especially since the inception of the ‘minimum information for the publication of quantitative real-time PCR experiments’ (MIQE) guidelines [Taylor et al., 2010]. Adherence to key components of a robust experimental design, including best practices in sample preparation, extraction and storage,

Table 1. Producing excellent qPCR data: do's

Step	Description	Theme	Substeps	What to do
1	Sample extraction and storage	quickly and reproducibly	tissue removal	stagger treatments for consistent treatment times
				reproducibly sacrifice individual animals, dissect tissue and flash-freeze in liquid nitrogen for cell culture, wash plates, add lysis buffer from RNA extraction kit directly to the plate and generate a stable homogenate by scraping cells and pipetting up and down, then freeze
2	RNA extraction	batch samples, RNase-free, fully frozen, use a kit	preparing the bench	use RNase-free tips, tubes on a clean bench, pipettes and gloves that have been cleaned with an RNase cleaning product such as ZAP or RNase Away
			removing samples from the -80°C freezer	remove a batch of samples to extract at the same time; all samples should be kept on dry ice or dry ice mixed with ethanol until RNA extraction
			RNA extraction procedure	use a kit-based method to extract RNA such as Bio-Rad's Aurum TM kits; grind tissue to a powder in a mortar containing liquid nitrogen and then quickly add the frozen powder to the lysis buffer from the kit
3	Testing the RNA samples	purity and quality	purity test to limit protein contaminants	test the DNase-digested, column-purified RNA samples on a spectrophotometer to ensure an $\text{OD}_{260/280}$ of 1.8 or higher
			quality test to avoid using degraded RNA	after the purity test, run the RNA samples on a formaldehyde denaturing agarose gel (28S/18S ribosomal RNA ratio of at least 1) or if samples are precious and limiting on an automated electrophoresis instrument like the Experion TM to ensure that they are not degraded (RQI number of at least 7.0)
4	Reverse transcription	use same input RNA	normalize concentrations	normalize all extracted RNA samples to the same approximate concentration and then add the same volume to the RT reaction
			use a good kit	use a good RT kit such as iScript TM that contains a robust enzyme mix over a broad dynamic range of RNA concentrations
5	Primer design	specific primers without secondary structure	Primer Blast and UNAFold	design primers to produce amplicons in the 80- to 200-bp range with annealing temperatures at 60°C using Primer Blast and then UNAFold
6	Primer validation	annealing temperature, gels and standard curves	thermal gradient	a thermal gradient-enabled qPCR machine provides a quick and fast option to test 8 annealing temperatures in a single experiment using a 10-fold diluted, pooled cDNA sample from all the treatment conditions
			run qPCR product on a gel	running a gel and potentially sequencing the amplicon is good practice during primer validation to ensure primer specificity
			standard curve to test efficiency, dynamic range and determine dilution of unknowns	for each primer pair, perform an 8-point dilution series of a pooled cDNA sample from across the treatment conditions as follows: high expressors, 8-fold; medium expressors, 4-fold; low expressors, 2-fold
7	Reference gene validation	source literature and test with GeNorm and NormFinder	source literature for stable reference genes	use Google Scholar and search for 'qPCR reference gene genorm [your organism/tissue of interest]'; derive a list of 6 – 10 targets from the literature to test their stability in your samples
			test the 6 potential targets using GeNorm and pick the 2 or 3 most stable for normalization	an initial qPCR experiment with the validated reference gene primers and selected cDNA samples from each treatment should be run; CFX Manager from Bio-Rad incorporates GeNorm to determine the most stable targets that vary minimally in their expression over the treatment conditions

RQI = RNA Quality Indicator.

RNA isolation and purification, RT, qPCR with validated primers and normalization with stable reference targets, will eliminate erroneous data.

To address the challenge of obtaining precise, reproducible and accurate results from qPCR experiments, a group of scientists came together in 2009 to develop a set of guidelines known as MIQE [Bustin et al., 2009a]. MIQE guidelines were the community's first attempt to map out the methodology and key validation criteria required to perform qPCR experiments. Since their publication, a number of papers have supported the need for a consistent and rigorous methodology to ensure the publication of accurate results [De Keyser et al., 2013; Dooms et al., 2013; Lanoix et al., 2012; Taylor et al., 2010, 2011].

Given their framework for generating robust qPCR data, it is surprising that the MIQE standards have not been embraced more widely in practice. Since 2010, more than 23,000 papers featuring qPCR data have been published, but only approximately 5% of these cite the MIQE guidelines (Google Scholar search for 'qPCR' after 2010). This low citation rate suggests that the vast majority of labs have either not been informed of the guidelines, have chosen to ignore them or believe that they do not apply to their experiments based on historical knowledge and biases that may date back to the early days of the technique.

MIQE: A Defined Methodology for Reliable, Consistent Data

The best practices for gene expression experiments as outlined by the MIQE criteria provide a simple and practical road map for scientists to navigate through the design of RT-qPCR experiments to obtain the highest-quality data and avoid common pitfalls in experimental design and execution (table 1). Alternatively, by skipping key steps from the MIQE guidelines, data will likely still be generated but can result in irreproducible and incorrect conclusions (table 2).

Sample Extraction and Storage: Freeze Tissue Immediately after Sample Extraction and Lyse Cells Directly in the Plate

The methods for cell and tissue culture sample extraction may vary significantly from lab to lab. With adherent cells, some groups first trypsinize, scrape the plate and transfer the cells to tubes. This is followed by centrifugation to pellet the cells and RNA extraction. Other researchers wash the cells on the plate, add RNA extraction

buffer directly to the plate and then scrape the plate to form a stable homogenate. For tissue samples, some labs surgically remove tissue from animals and weigh the samples at room temperature. They then slice the tissue and transfer it into tubes, all at room temperature, and finally freeze the samples at -80°C . Other labs immediately flash-freeze the animal tissue in liquid nitrogen, transfer it into tubes on dry ice and store it at -80°C . These different sample extraction and storage methods can yield vastly different results because the transcriptome is affected by each sample manipulation and can change very quickly in response to chemical and environmental treatments [Huang et al., 2013; Viertler et al., 2012]. Rigorous and reproducible methodology is achieved by halting transcription as soon as possible after sample collection. This ensures that differences recorded between bioreplicates in response to experimental treatments are due to treatment and not an artifact of sample handling.

RNA Isolation: Maintain the Cold Chain with Frozen Samples prior to RNA Extraction

Keeping tissue frozen until homogenization in a solution containing RNase inhibitors ensures consistent results by preventing inconsistent thawing of samples, which leads to differential RNA degradation [Botling et al., 2009; Huang et al., 2013; Kirschner et al., 2013]. Some labs remove tissue samples from -80°C storage, transport them on blue ice and then proceed to homogenize samples and extract the RNA using a wide variety of techniques, during which the samples may begin to thaw prior to RNA extraction. Others place the sample tubes on dry ice and then grind the tissue to a powder in a mortar under liquid nitrogen before adding the RNA extraction buffer. There are many methodologies, reagents and instrument technologies for tissue disruption and homogenization for both protein and nucleic acid extraction. The goal is to convert the sample into a uniform, stable homogenate in a highly reproducible manner while preventing as much as possible any degradation and transcriptional changes from the -80°C freezer through homogenization.

RNA Purification and Analysis: Test RNA Sample Purity and Quality

After RNA extraction, most labs measure the optical density (OD)_{260/280} and OD_{260/230} to quantify the amount of total RNA and to ensure the sample meets

Table 2. Producing excellent qPCR data: don'ts

Step	Description	What to avoid	Data ramifications
1	Sample extraction and storage	sacrificing animals at the same time before tissue extraction	poor biological variability and large error bars from transcriptional variability caused by inaccurate treatment times in mortality and variability in timing of removal of tissue from deceased animals
		trypsinizing, collecting and centrifuging cells before adding lysis buffer	erroneous gene expression data caused by excessive handling of cells prior to homogenization
2	RNA extraction	using regular tips or tubes that have been exposed to the lab	rapid digestion of extracted RNA leading to variable and high Cq values
		placing samples on blue ice, which results in thawing and exposure to RNase prior to RNA extraction	lysis of tissue prior to RNA extraction from freeze to thaw cycle causing high variability in degradation between samples
		homogenizing tissue at room temperature with a procedure yielding nonuniform homogenate	risk of variability in RNA extraction, potential partial digestion of RNA and lower yield potentially resulting in variable results and Cq values
3	Testing the RNA samples	accepting OD _{260/280} values below 1.8	variable and higher Cq values from protein contaminant inhibition of the RT and qPCR reactions
		not testing the RNA quality	degraded RNA samples will result in artifactual and uninterpretable results giving large variability in Cq values between samples
4	Reverse transcription	adding different RNA amounts to the RT reaction	wide variability in Cq values caused by differences in the RT reaction from variable RNA input can dramatically alter the resulting cDNA
5	Primer design	using primer sequences from the literature or websites without validation	it is not uncommon that published primer sequences are either incorrect sequences or correct sequences for the wrong target; check all sequences using software tools to save weeks of troubleshooting
6	Primer validation	running qPCR experiments at predicted annealing temperatures from primer design software without wet chemistry validation	samples used for qPCR may contain contaminants and chemicals that deviate from the predicted annealing temperature from software resulting in high and potentially variable Cq and artifactual data
		assuming that a single melt curve peak means that the predicted product has been amplified	months of wasted work studying an artifact
		assuming primers are validated with good efficiency and diluting all samples by a standard dilution (i.e. 10-fold) for all targets	if samples are not diluted such that they amplify with Cq values in the efficient range of the standard curve, the gene expression data produced can be an artifact of nonefficient amplification
7	Reference gene validation	trusting without independent validation a peer recommended reference gene target	since reference gene targets are used to normalize the data between samples, the resulting normalized, relative gene expression data will be directly affected by changes in the regulation of the reference gene as opposed to the target gene
		using the usual suspects as reference gene targets including GAPDH, β -actin, tubulin and 18S RNA without confirming stability	

the minimal purity criteria with respect to protein and chemical contamination (minimum acceptable OD values of 1.8 and 2.0, respectively). Samples with lower OD values typically contain higher levels of contaminants that can inhibit both the RT and qPCR reactions, resulting in artificially high and variable quantification cycle (C_q) values and imprecise quantification. In our experience, a test that most labs do not perform is an RNA quality assessment to ensure that samples are not degraded, as RNA degradation can occur even when the utmost care is taken with sample handling [Huang et al., 2013]. RNA sample quality can be measured by visualizing extracted fragment sizes on a denaturing formaldehyde-agarose gel or by using more sensitive and precise instrumentation such as the Experion™ automated electrophoresis system from Bio-Rad or the Bioanalyzer™ from Agilent. As with purity, RNA sample quality is directly correlated with altered C_q values, where a degraded sample can give significantly higher C_q values than an intact sample [Huang et al., 2013; Taylor et al., 2011]. Furthermore, RNA quality has been shown to directly affect reference gene variability and the significance of differential gene expression data [Vermeulen et al., 2011].

RT: Normalize Input RNA

The RT reaction is a key step in sample processing. RT priming strategy, dynamic range and RT enzyme type are all important to ensure mRNA expression levels are accurately represented in the resulting cDNA [Jacob et al., 2013]. Transcription of both low- and high-expression targets, and thus a wide linear dynamic range for the RT step, is required for accurate representation of these expression levels in the final data. Performing a serial dilution of the input RNA to determine the linear dynamic range of reverse transcribed cDNA will reveal the amount of RNA required for the RT step to ensure consistent coverage of all targets in the sample. Care should be taken to normalize the amount of input RNA for RT with consistency in kit selection and protocol to ensure that all RNA samples are treated similarly. If different amounts of input RNA are used between samples, variable levels of contaminants can be introduced that may inhibit the RT reaction in an unpredictable manner, resulting in variable RNA coverage and cDNA output. The resulting gene expression results are often uninterpretable; therefore, care should be taken to ensure consistent loading of

RNA. The hallmarks of a good RT kit include a mix of random hexamers and oligo dTs to obtain the best coverage of the RNA with high fidelity and robust reverse transcriptase containing RNase H to digest the copied template as the transcript is transcribed. A single-step kit in which RNA is added to a single RT mix containing a combination of the RT and hot-start qPCR mixes in one reaction can help minimize technical variability among samples.

Primer Validation: Always Validate Primer Sequences

Many researchers do not validate their primers because the sequences were sourced directly from peer-reviewed literature, obtained from prior lab members or directly from vendors as ‘off-the-shelf’ assays. This practice presumes that the scientists who originally published the qPCR data correctly validated their primers in the same cells and/or tissues as in the current study set, but this may not be the case [Wang et al., 2012]. Many ‘off-the-shelf’ assays have only been designed *in silico* and are often not provided with any validation data, which may preclude MIQE compliance [Bustin et al., 2011]. Rather than make this presumption, labs should validate all primers – including those used by previous authors and vendors – for primer concentration, annealing temperature, specificity and efficiency, with further validation for linear dynamic range with a standard curve from a representative sample [Mikeska and Dobrovic, 2009; Taylor et al., 2010, 2011]. Ideally, validation should be performed on a qPCR instrument that is enabled with thermal gradient capability and has the sensitivity to detect 10 or fewer copies of the target sequence. The ramifications of poorly validated primers for annealing temperature and efficiency with standard curves are inaccurate C_q values and gene expression results leading to incorrect and even opposite conclusions [Opel et al., 2010]. Because primers require independent validation for each sample type (for example, brain versus heart tissue) and also for each RNA extraction method (such as TRIzol versus a kit-based method), an 8-point standard curve of the appropriate fold dilution of cDNA using a good-quality qPCR supermix is recommended. Only thorough primer validation will ensure that the qPCR reaction conditions are optimal for a given sample set and that the samples are diluted such that reaction efficiency is optimal for each target.

Reference Gene Selection: Choose a Target by Testing Stability between Experimental Conditions

Since the release of the MIQE guidelines, a number of published articles have described the effect of improper reference gene selection on the final data [Barber et al., 2005; Jacob et al., 2013; Lanoix et al., 2012; Rhinn et al., 2008; Schmittgen and Zakrajsek, 2000; Taylor et al., 2011; Thellin et al., 1999; Yang et al., 2012]. Normalization of qPCR data with a poorly selected reference gene can dramatically alter the final results to the extent that opposite conclusions can be obtained when compared to results with normalization with stable reference genes. Many labs performing qPCR on a regular basis have only normalized samples to a single, unvalidated reference gene that they have used for all qPCR projects over many years. The list of potential reference genes has increasingly been chosen from publications referring to the tools GeNorm and NormFinder for reference gene stability. A compilation of at least 6–10 separate reference gene candidate primer pairs should be validated as described in the previous section and then tested for stability in samples derived from each of the experimental

conditions using GeNorm, NormFinder and BestKeeper [Jacob et al., 2013; Lanoix et al., 2012]. The result of poor reference gene selection for the final data and conclusions is now well documented and has called into question the validity of publications that have only used a single unvalidated reference gene [Barber et al., 2005; Jacob et al., 2013; Rhinn et al., 2008; Schmittgen and Zakrajsek, 2000; Thellin et al., 1999; Williams, 2012; Yang et al., 2012].

Conclusions

Although some labs continue to argue that MIQE criteria are simply ‘guidelines’ that do not necessarily need to be followed, there are very good reasons to adopt the best practices outlined here as well as other elements of the guidelines. While some forethought is required for planning an MIQE-guided experiment, the benefits of following these guidelines help ensure robust, reliable and reproducible gene expression results for publication and provide the confidence that the data, interpretations and conclusions will hold up to reader scrutiny.

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