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Mini-review

A practical approach to RT-qPCR—Publishing data that conform to the MIQE guidelines ☆,☆☆

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A B S T R A C T

Given the highly dynamic nature of mRNA transcription and the potential variables introduced in sample handling and in the downstream processing steps (Garson et al. (2009) [4]), a standardized approach to each step of the RT-qPCR workflow is critical for reliable and reproducible results. The MIQE provides this approach with a checklist that contains 85 parameters to assure quality results that will meet the acceptance criteria of any journal (Bustin et al. (2009) [1]).

In this paper we demonstrate how to apply the MIQE guidelines (www.rdml.org/miqe) to establish a solid experimental approach.

1. Introduction

Real-time quantitative PCR (qPCR) has become a definitive technique for quantitating differences in gene expression levels between samples. Over the past 10 years, the popularity of this method has grown exponentially, with the publication of well over 25,000 papers from diverse fields of science, including agricultural, environmental, industrial, and medical research, making reference to RT (reverse transcription)-qPCR data.

One of the central factors that has stimulated this impressive growth is the increased demand from journal review panels for the use of RT-qPCR to support phenotypic observations with quantitative, molecular data. Furthermore, gene expression analysis is now being used to support protein expression data from proteomics-based assays. Since no strict guidelines have been established, researchers have generally designed their experiments based on information gathered from disparate sources, which has resulted in data of variable quality.

In an effort to assist the scientific community in producing consistent, high quality data from qPCR experiments, the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines have been recently published [1]. This has been followed by the development of an XML-based real-time PCR data markup language (RDML) for the consistent reporting of real-time PCR data by the RDML consortium [10]. This consortium is active in the development of appropriate and standardized terminology, guidelines on minimum information for biological and biomedical investigations, and a flexible and universal data file structure with tools to create, process, and validate RDML files. All relevant information about the RDML project is available at www.rdml.org.

The ultimate goal of RDML and MIQE is to establish a clear framework within which to conduct RT-qPCR experiments and to provide guidelines for reviewers and editors to use in the evaluation of the technical quality of submitted manuscripts against an established yardstick. As a consequence, investigations that use this widely applied methodology will produce data that are more consistent, more comparable, and ultimately more reliable.

2. Experimental design

Proper experimental design is the key to any gene expression study. Since mRNA transcription can be sensitive to external stimuli that are unrelated to the processes studied, it is important to work under tightly controlled and well-defined conditions. Taking the time to define experimental procedures, control groups, type and number of replicates, experimental conditions, and sample handling methods within each group is essential to minimize variability (Table 1). Each of these parameters should be carefully recorded prior to conducting gene expression experiments to assure good biological reproducibility for published data.

3. RNA extraction

If samples must be collected over a period of time or in too large a number to process immediately, they should be stored in appro-

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Table 1
RT-qPCR experimental design and sample management.

Experiment design	Control groups	Replicates	Experiment conditions	Sample handling
Disease or treatment groups	Time course study (i.e., $t = 0$)	Biological (different sample per well)	Growth conditions (media and time or OD)	Precise time to harvest cells or tissue
Target genes implicated	Normal vs. disease (i.e., normal)	Technical (same sample per well)	Days of embryonic development	Sample extraction method
Potential reference genes	Untreated vs. drug treated (i.e., untreated)	—	Amount per mass of drug or compound	Preservation method and time
Number of data points to draw statistically significant conclusions	—	—	Sex, phenotype	Thaw and homogenization procedure
—	—	—	Incubation time	Total RNA extraction procedure

This table summarizes the workflow of a typical RT-qPCR experiment from experimental design to defining control groups, replicates, and experimental conditions to the detailed procedures for sample handling. This ultimately assures that the key steps in RT-qPCR data production lead to high quality, reproducible, and publishable data.

priate conditions (frozen at −80 °C and/or in RNA storage solution) until use. To minimize handling time during the RNA extraction procedure, it is recommended that samples be processed in relatively small batches of 10–20. The RNA extraction procedure should include a DNase I treatment step to remove any contaminating genomic DNA.

4. RNA quality control

Ensuring that only RNA of high purity (no contaminants) and high integrity (not degraded) is used is one of the most critical points in the RT-qPCR experimental workflow. Impurities in the RNA sample may lead to inhibition of the RT and PCR can lead to varying and incorrect quantification results [3,5]. Since sample purity and integrity are not related, both should be assessed to ascertain that the RNA sample meets minimal acceptance criteria for the downstream workflow.

Purity of the sample with respect to protein contamination can be assessed spectrophotometrically by measuring the $OD_{260/280}$ ratio in a pH neutral buffer. An $OD_{260/280}$ of 1.8–2.0 indicates good quality RNA that is devoid of protein and phenol contamination (Fig. 1B). However, no RNA integrity information can be obtained from a spectrophotometric reading. RNA integrity can be assessed using several methods. The traditional method is visual inspection after electrophoresis on a formaldehyde agarose gel in the presence of a fluorescent dye such as ethidium bromide. Observation of two sharp bands for the large and the small subunit ribosomal RNAs (rRNA) with the intensity of the larger band being about twice that of the smaller band is indicative of intact RNA. While this method is inexpensive, the interpretation of the data is mostly subjective and requires approximately 200 ng of total RNA, which may be difficult if the sample is only available in limited quantities. This method can be refined by quantifying the intensity of the rRNA bands using an imager with densitometry scanning. While the values of the 28S/18S ratio can vary between different tissues or cell types, a ratio between 1 and 2 is indicative of an intact RNA sample.

A major improvement in RNA integrity analysis came with the introduction of microfluidics-based electrophoresis systems [9] such as the Experion™ automated electrophoresis system (Bio-Rad) that combines integrity and concentration quantification of RNA in a single step from nanogram or picogram quantities (Fig. 1A and C). In addition to generating a virtual gel, an electropherogram, and calculating the 28S/18S ratio, the Experion system's software automatically calculates and reports an RNA quality indicator (RQI) value (Fig. 1C) that reflects the integrity of the RNA sample based on several criteria [2] of the electropherogram. The RQI value ranging from 1 (degraded) to 10 (intact) provides an objective assessment of the integrity of the RNA sample and it can be used to screen out degraded samples.

By assuring consistency in both purity and quality across all RNA samples, variability between biological replicates can be reduced (Fig. 1D) [9]. When a batch of RNA samples has successfully met the standards of quality control, we recommend their immediate use in RT-qPCR experiments, or their conversion into much more stable cDNA by reverse transcription to preserve RNA integrity after the quality check.

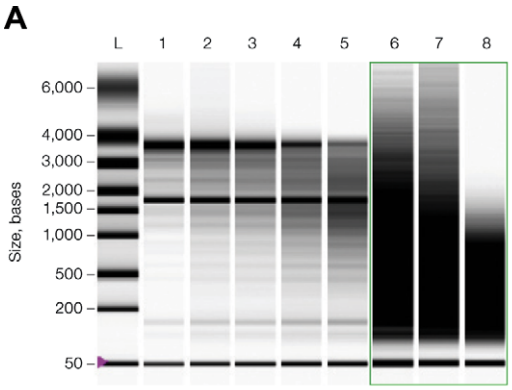
5. Reverse transcription

Given the prevalence of RNase in the environment, we recommend performing the reverse transcription of total RNA samples to cDNA immediately following the quality control assessment. This will avoid the risk of RNA samples degradation from multiple freeze/thaws before conversion to cDNA. For the RT step, the key is to assure consistent and complete coverage of the transcribed genome in the extracted RNA sample. Some genes are very long, but the associated RT products for these sequences cannot be as large, especially if the RT primers anneal only at the ends of each mRNA. By annealing primers at the end of the mRNA plus at random points within each mRNA sequence a good sampling of the population of each gene is obtained. This method, which is more representative than just annealing at the ends or at random sites within each transcript, provides better coverage of the transcribed genome.

A reverse transcription buffer should contain a mix of primers that are random in sequence allowing for a better sampling of the mRNA; RNase H, the enzyme that specifically degrades RNA in DNA/RNA duplexes; a specific and sensitive reverse transcriptase with broad dynamic range for RNA amounts from 1 pig to 1 pg and; a simple and fast protocol. We recommend that the same amount of total RNA be used and that reaction time be maintained for reverse transcription for all experimental samples to minimize variability between biological replicates. Reverse transcribed RNA can be stored frozen at −20 °C or −80 °C until use and diluted before use in the downstream qPCR.

6. Primer and amplicon design

Both primer design and careful choice of target sequence are essential to ensure specific and efficient amplification of the products. Target sequences should be unique, 75–150 bp long with a GC content between 50% and 60%, and should not contain secondary structures. It is recommended that primers should have a GC content of 50–60% and a melting temperature of 55–65 °C. Long G or C



B

	Sample	$A_{260/280}$*	$A_{260/230}$*
1	Control—no heat	1.90	2.44
2	3 min @ 90°C	1.93	2.40
3	5 min @ 90°C	2.06	2.37
4	10 min @ 90°C	2.03	2.37
5	15 min @ 90°C	2.02	2.31
6	1 hr @ 90°C	1.99	2.18
7	2 hr @ 90°C	2.00	2.32
8	4 hr @ 90°C	1.89	2.23

* Generally accepted ratios ($A_{260/280}$ and $A_{260/230}$) for good quality RNA are >1.8.

C

Well ID	Sample Name	RNA Area	RNA Concentration (ng/µl)	Ratio [28S/18S]	RQI	RQI Classification	RQI Alert
1	Control - no heat	278.23	102.60	1.60	9.8		
2	3 min @ 90C	317.12	116.94	1.23	9.2		
3	5 min @90C	306.89	113.17	0.89	8.1		
4	10 min @ 90C	257.56	94.98	0.50	6.5		
5	15 min @ 90C	247.31	91.20	0.15	5.9		
6	1 hour @ 90C	200.94	74.10	0.46	2.2		
7	2 hour @ 90C	252.37	93.07	0.81	2.0		
8	4 hour @ 90C	274.16	101.10	0.00	1.8		

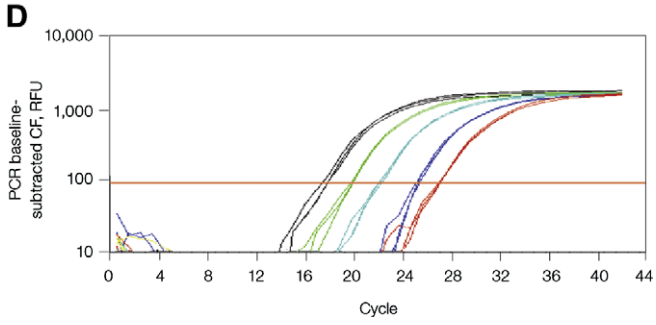


Fig. 1. Analysis of RNA purity and integrity. This figure shows an example of RNA purity and integrity analysis using spectrophotometry and Bio-Rad's Experion automated electrophoresis system. Mouse liver total RNA samples were subjected to degradation by heating at 90 °C for different periods of time and analyzed for both purity and integrity. Green boxes highlight the fact that the total RNA sample appears by absorbance readings alone (>1.8) to be of sufficient quality, whereas RQI measurement on the Experion system clearly shows degraded sample with low RQI values. (A) Virtual gel image generated by the Experion software showing various degradation levels of the RNA samples and a progressively decreasing intensity of the 18S rRNA band; (B) the OD_{260}/OD_{280} ratio measured for all samples on the Nanodrop spectrophotometer is between 1.8 and 2.0 indicating that the samples are devoid of protein contamination; (C) summary of RNA integrity analysis using the Experion software. The 28S/18S rRNA ratio and the RQI indicate a decreasing integrity of the heat treated RNA samples. A color classification allows for easy identification of the samples that are not adequate for qPCR; (D) example of qPCR analysis of *GAPDH* expression performed on degraded samples [5]. The profiles show increasing C_q with the degraded samples. No degradation (—); 1 h degradation (—); 3 h degradation (—); 5 h degradation (—); 7 h degradation (—).

stretches in the primer should be avoided, but it is recommended to have G or C at the end of the primers.

A number of programs are available to help design primer pairs and pick target sequences. We recommend designing oligonucleotides using Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHomeAd), a program developed by NCBI that uses the algorithm Primer3 [12]. Primer sequences are compared (blasted) to the user-selected databases to ensure they are unique and specific for the gene of interest. The program MFOLD (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) can then be used to analyze the amplicons for potential secondary structures that may prevent efficient amplification [14]. Ideally, two sets of oligonucleotides should then be ordered and tested for their performance in a qPCR.

7. qPCR validation

A validated qPCR assay is one that has been assessed for the optimal range of primer annealing temperatures, reaction efficiency, and specificity using a standard set of samples [1]. This will assure that the reaction conditions, buffers, and primers have been optimized and that the cDNA samples are not contaminated with inhibitors of Taq polymerase. Bio-Rad has created a practical web resource (www.bio-rad.com/genomics/pcrsupport) for qPCR design and validation.

7.1. Determination of annealing temperature, melt curve analysis, gel analysis of amplicon, and no template control

A critical step in a PCR is the annealing of the primers to their target sequences. It has to be performed at the right temperature for the primers to anneal efficiently to their targets, while preventing nonspecific annealing and primer–dimer formation. The fastest way to determine optimal annealing temperature is to use a ther-

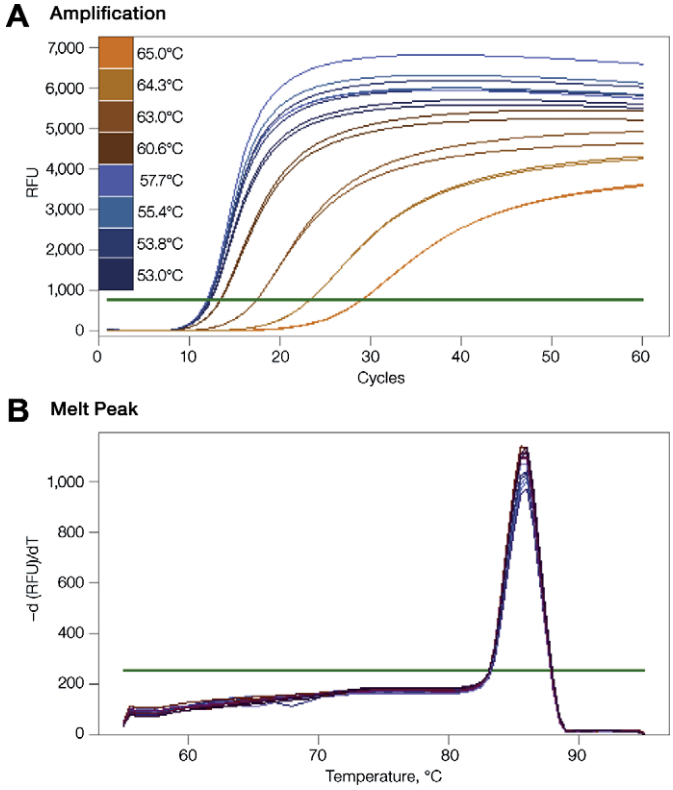


Fig. 2. Validation of qPCR primers. (A) qPCR is performed at a range of annealing temperatures using a thermal gradient block. Amplification profiles indicate that the most efficient amplification occurs at the four lowest annealing temperatures between 53 °C and 57.7 °C where the curves are at the lowest C_q; (B) a single peak on the melt curve analysis indicates a single PCR product.

mal cycler equipped with a temperature gradient feature. A range of temperatures around the calculated T_m of the primers should be tested (Fig. 2A).

It is important to check the specificity of the reaction by analyzing the PCR product. A melt curve analysis, performed at the end of the PCR cycles, will confirm specificity of primer annealing. The melt curve should display a single sharp peak (Fig. 2B). In addition, samples should be run on an agarose gel or polyacrylamide gel to confirm that the amplicon is of the expected size. Alternatively, an automated electrophoresis system such as the Experion automated electrophoresis system can be used.

A duplicate, no template control (NTC) reaction should be included in every run for each primer pair to test buffers and solutions for DNA contamination and to assess for primer–dimers.

7.2. Establishment of a standard curve (to evaluate PCR efficiency)

The efficiency of a PCR is a measure of the rate at which the polymerase converts the reagents (dNTPs, oligonucleotides, and template cDNA) to amplicon. The maximum increase of amplicon per cycle is 2-fold representing a reaction that is 100% efficient. It is important to measure reaction efficiency as it is indicative of problems with the qPCR that can cause artifactual results. Low-efficiency reactions (<90%) may be caused by contaminating Taq inhibitors, high or suboptimal annealing temperature, old or inactive Taq, poorly designed primers, or amplicons with secondary structures. High reaction efficiency (>110%) is generally the result of primer–dimers or nonspecific amplicons. The most common causes of both high and low reaction efficiencies are poorly calibrated pipettes or poor pipetting technique.

A standard curve is generally used to determine the reaction efficiency for any qPCR. The template for this typically is a sample of cDNA or spiked plasmid cDNA in a sample extract. We recommend initially producing a 10-fold dilution series over eight points starting from the most concentrated cDNA sample, to ensure the standard curve covers all potential template concentrations that may be encountered during the study (i.e., broad dynamic range). For each dilution, a standard qPCR protocol should be performed in triplicate for all the primer pairs to be used in the experiment and C_q values determined. The standard curve is constructed by plotting the log of the starting quantity of the template against the C_q values obtained. The equation of the linear regression line, along with Pearson's correlation coefficient (r) or the coefficient of determination (r^2), can then be used to evaluate whether the qPCR assay is optimized.

Ideally, the dilution series will produce amplification curves with tight technical replicates that are evenly spaced. If perfect doubling occurs with each amplification cycle, the spacing of the fluorescence curves will be determined by the equation 2^n = dilution factor, where n is the number of cycles between curves at the fluorescence threshold (in other words, the difference between the C_q values of the curves). For example, with a 10-fold serial dilution of DNA, $2^n = 10$. Therefore, $n = 3.32$, and the C_q values should be separated by 3.32 cycles. Evenly spaced amplification curves will produce a linear standard curve with the goal of 90–110% reaction efficiency.

The r or r^2 value of a standard curve represents how well the experimental data fit the regression line, that is, how linear the data are. Linearity, in turn, gives a measure of the variability across assay replicates and whether the amplification efficiency is the same for different starting template copy numbers. A significant difference in observed C_q values between replicates will lower the r or r^2 value. An r with an absolute value >0.990 or an r^2 value >0.980 is desirable for RT-qPCRs. Deletion of points at both ends of the standard curve may be required to obtain an acceptable slope (efficiency) and r^2 value. This will ultimately define the dynamic range of cDNA concentration for each primer pair with respect to sample dilution.

7.3. qPCR reagents, instrument, and analysis program

There are a wide variety of commercial qPCR reagent kits available. We recommend a saturating dye that provides much better sensitivity than SYBR® Green.

A large number of qPCR detection instruments are commercially available. Sample volumes with a range of 10–50 μl in standard 96-well format, 0.2 ml, low-profile thermal cycler plates, strips, or tubes from any manufacturer.

All qPCR instruments are packaged with data analysis software and we recommend that a good software package include the following features:

(1) Flexibility to enter plate setup information such that well identifiers can be loaded and edited before, during, or after the run.
(2) Ability to group wells from multiple experiments on one plate.
(3) Built-in gene expression analysis that can normalize data to multiple reference genes [13] and individual reaction efficiencies [11].
(4) The ability to combine multiple plates of experimental data into a large gene study.

8. Choice of reference genes

In RT-qPCR experiments, reference genes are used as controls to normalize the data by correcting for differences in quantities of cDNA used as a template [6,8,13]. A perfect reference gene is therefore one that does not exhibit changes in expression between samples from various experimental conditions or time points. Reference genes must therefore be carefully selected based on experimental data and we recommend the following protocol:

(1) Extract the total RNA from at least one or two samples from each experimental condition or time point and confirm their purity and quality (see Steps 2 and 3 above).
(2) Normalize the sample concentration and perform reverse transcription PCR (see Step 4) from the same volume of each sample.
(3) Perform the qPCR experiment using the same volume of each cDNA sample as a template.
(4) Use the geNorm method [7] to calculate the target stability between the different conditions (available at medgen.ugent.be/genorm/).

A good reference gene should have an M value below 0.5 or 1 in homogeneous and heterogeneous samples set, respectively [13]. geNorm also helps in the selection of the optimal number of reference genes.

Typically, between 3 and 5 good reference are required to achieve the most accurate normalization [13].

9. Experimental reproducibility

There are two sources of variability in a gene expression experiment that may affect the results:

(1) Biological variability which is due to inherent differences in gene expression levels between individual organisms, tissues, or cell culture samples.

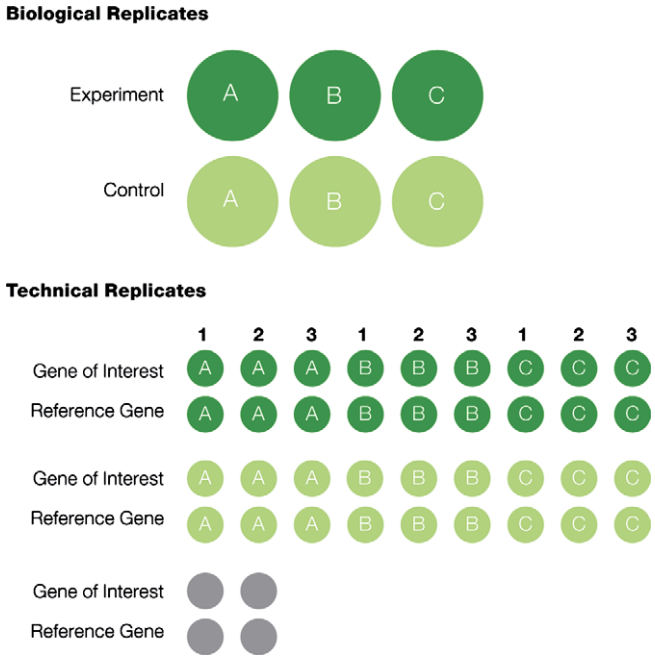


Fig. 3. Experimental replicates. All experimens should be designed with a combination of biological and technical replicates. Zhis illustrates a simple experiment with triplicate biological samples from control and treatment/experimental conditions. For each biological sample, three technical replicates are recommended for the gene of interest as well as for the reference gene(s). This results in a total of at least 36 samples plus the duplicate NTC for a total of >40 wells.

(2) Technical variability in the experimental process itself which is typically associated with pipetting, poorly calibrated pipettes, and sample quality and quantity.

To mitigate the effect of biological and technical variability, it is generally accepted that at least three biological and two technical replicates per biological replicate be performed for each experiment (Fig. 3). If the experiment compares gene expression levels between control and treated samples, the three biological replicates should be samples that were treated in separate and independent experiments.

10. Conclusions

RT-qPCR is the method of choice for gene expression analysis because of its high sensitivity from samples of very low RNA concentrations. However, in order to assure accurate and reproducible, quantitative data, strict standard operating procedures should be followed. All experimental details and controls should be accurately reported when publishing gene expression experiments. This will allow proper assessment of the data by the scientific community, and enable informed comparison of expression data between labs and between experiments.

In summary the key steps for most RT-qPCR experiments include:

(1) Experimental design with appropriate number of biological replicates and proper control samples.
(2) Sample procurement which requires adherence to strict experimental protocols for acquisition, processing, and storage to assure reproducibility and minimize standard deviations between replicates.
(3) Quality control of RNA for purity and integrity.
(4) Reverse transcription to convert the total RNA to cDNA.
(5) qPCR experiments with proper primer design, choice of target sequence and reference genes, and technical replicates.
(6) The MIQE guidelines were written to provide all the parameters that should be met to publish acceptable results from qPCR experiments. Here we have shown how the most important items in the MIQE checklist can be addressed in practice to assure high quality results.

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