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Guest Editorial

Are MIQE Guidelines Being Adhered to in qPCR Investigations in Photobiomodulation Experiments?

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The polymerase chain reaction (PCR) is an important and reliable technology for research and diagnostic analysis, and is a quick and easy method of enzymatically synthesizing and amplifying unlimited copies of specific DNA sequences in a few short hours. From its inception, PCR has matured over the years from a laborious, time-consuming, and gel-based technique to an automated, high throughput, rapid quantitative technique. This technique, which formed the cornerstone of the human genome project, was only developed 20 years ago. The technique as we know it originates from research conducted in the 1980s at Cetus Corporation in California.

The story begins in 1983 with Kary Mullis, PhD, who came up with the idea while driving one day.[1] The novelty and potential impact of the idea was developed and the theory became a reality. In 1985, the technology was presented for the first time and the first set of results was published later that year in *Science*.[2] In 1993, Mullis was awarded the Nobel Prize for chemistry.[1]

Initially, the technique was slow and arduous, and required the manual transfer of tubes between water baths set at different temperatures, and the DNA polymerase first used (isolated from *Escherichia coli*) was inactivated during DNA denaturation and had to be manually replaced at the start of each cycle. As the years passed, the technology developed and significant advances were made with the discovery of *Taq* polymerase (isolated from *Thermus aquaticus*), which was able to withstand the high temperatures required during DNA denaturation, and the development of the closed thermal cycler by PerkinElmer.[1] These developments have led to the streamlining and shortening of the process, and minimizing the number of steps that require human interaction. Today, the technology has evolved into a quantitative, fluorescence-based real-time, quantitative PCR (qPCR).

qPCR is currently the gold standard and global mainstay for the quantification of microRNA and messenger RNA (mRNA); however, there is a downside to the technique that is related to the adaptations to the methodology and inconsistent publication of technical information. As a consequence, qPCR can become an inadequately standardized and inconsistent technique.[3] The lack of agreement on how best to perform and interpret qPCR experiments and insufficient experimental detail led to the development of the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines in 2009, which is aimed at ensuring integrity and reliability of publications, promote consistency between different research groups, and increase experimental transparency.[4]

MIQE is a set of guidelines describing the minimum information that should be reported on when publishing qPCR experiments. These guidelines cover aspects related to sample acquisition, experimental design and validation, and data analysis.[5] These guidelines are easily accessible and available from the MIQE gene-quantification info website (http://miqe.gene-quantification.info).

Three main causes for variable qPCR results have been identified, and include biological variability, technical variability, and experimental design.[3] Biological variability is more difficult to control, and is determined by natural genotypic and phenotypic variation between individuals, cells, and tissues. qPCR is dependent on the organism, tissue type, and time of experiment (time of extraction of nucleic acid post-treatment), and thus conclusions should be placed into context. Technical aspects that affect qPCR performance and variability include sample type, isolation, storage, handling, and preparation; replicate numbers (biological versus technical replicates); nucleic acid quality, quantity, and purity; choice of reverse transcription (RT) primers and probes; assay design; methods of normalization; and data and statistical analysis.[3,4] These technical aspects are all in the hands of the researchers, and the numerous steps involved in qPCR allow for the introduction of assay discrepancies and errors.

When it comes to reporting on assay optimization, one should include database accession numbers (as there can be variants for the same gene), amplicon size, primer sequence, and probe sequence (including any modified bases), position, and dye linkage. A problem with supplying this information is introduced by companies who supply ready-made primers, probes, and assays. For self-designed primers, the design software should be stated and primer specificity (using BLAST) should be validated. Primer optimization, including $MgCl_2$ concentration and annealing temperature, and priming conditions should be provided. There should also be evidence of intra- and interassay precision, which are measures of repeatability and reproducibility, respectively.[3,4]

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Explicit details of RT (if applicable) should be given as cDNA priming method and choice of RT can have severe and significant impact on qPCR results. The amount of total RNA that is reverse transcribed should be the same for all samples, and RT replicates should be included to improve reliability (technical repeats). A number of controls should be built in and include DNA contamination control (no reverse transcriptase control), which only needs to be run once per sample; no template control, which is pertinent in determining PCR contamination, and must be performed with each experiment and PCR run; positive controls, which are performed as target gene-specific dilution curves; and no amplification controls, which are included when making use of probes to monitor probe degradation.[3] Experimental layout and design must also be carefully looked at. It is better to run as many samples in the same PCR run as opposed to running multiple genes on the same run. This minimizes the risk of inter-run variation between samples. If it is not possible to run all samples at the same time, then an identical sample that is run in both/all runs must be included (inter-run calibrator).

An essential and important component of any reliable qPCR assay includes the normalization of results against reference genes. Normalization compensates for variation pre-PCR (mRNA extraction and cDNA synthesis) and during PCR itself. Unvalidated reference gene usage is a major source of experimental error, and is widely overlooked. Identification and validation of stable reference genes (whose expression levels remain unaffected by experimental factors) is such an important and pertinent step in RT-qPCR, yet reference gene validation seems to be rarely carried out.

Seeing as no single biological gene is stably expressed between cell types, even the same cell types, and experiments/conditions, reference genes must be validated experimentally for tissue or cell type, and to ensure amplification efficiencies of target and reference genes are similar under each treatment and control condition.[5,6] Normalization is typically performed against multiple reference genes (not fewer than three), unless a single reference gene has been fully validated.[3,5] Thus, initial experiments should determine the most stable reference genes in each specific experiment or biological setting.

This subset of stably expressed reference genes is then used to calculate a normalization factor based on the geometric mean of the most stable reference genes.[6] There are several mathematical and statistical algorithms that should be utilized when determining suitable and stable reference genes. Four such common approaches include NormFinder, GeNorm, BestKeeper, and the comparative delta Ct (threshold cycle, or as per MIQE guidelines the Cq or quantification cycle).[6]

qPCR results may be expressed based on absolute or relative values. In the absolute method, the exact sample copy number of the gene of interest is read off a standard curve prepared with serial dilutions of known concentrations of the test sequence. In the relative method, most commonly used in photobiomodulation (PBM) experiments, a standard curve is used to determine reaction efficiency, whereas normalized expression levels are expressed as ratios in relation to a control/calibrator.[7] When it comes to data and statistical analysis, specialist software should be utilized and specified in the publication. The most common model for data analysis of qPCR results is the $\Delta\Delta$Cq (otherwise known as $\Delta\Delta$Ct), which relies on selection of appropriate reference genes.[5] MIQE guidelines recommend the disclosure of all statistical features and software utilized in qPCR data analysis.

In conclusion, one of the most trustworthy ways to judge the credibility of any research is to carefully scrutinize the materials and methods section and examine the results of the article for uniformity and reliability with the conclusions that are derived from the data.[3] This is not possible if all the technical aspects to the technique are not given. This is not limited to qPCR, but goes for all techniques described in any original research article. There is a growing tendency for publications to contain insufficient technical information and detail for reproduction of the work. This makes it difficult, and sometimes near impossible, for reviewers, editors, and readers to adequately judge and analyze an article, especially when it comes to qPCR experiments in PBM. MIQE guidelines comprise a reference framework for researchers to adequately design and report qPCR experiments and have a greater impact as to the accuracy, validity, and uniformity of the results, and to facilitate easier replication of experiments by different research groups. Are your qPCR experiments adhering to the MIQE guidelines?

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