MIQE: Guidelines for the Design and Publication of a Reliable Real-time PCR Assay

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Abstract

The capacity to amplify and detect trace amounts of nucleic acids has made the polymerase chain reaction (PCR) the most formidable molecular technology in use today. Its versatility and scope was further broadened first with the development of reverse transcription (RT)-PCR, which opened up the entire RNA field to thorough exploration and then, most conspicuously, with its evolution into real-time quantitative PCR (qPCR). Speed, simplicity, specificity, wide linear dynamic range, multiplexing and high-throughput potential, reduced contamination risk, simplified detection and data analysis procedures as well as availability of increasingly affordable instrumentation and reduced reagent cost have made qPCR the molecular method of choice when quantifying nucleic acids. Detection of pathogens, SNP analyses and quantification of RNA, even real-time analysis of gene expression *in vivo* have become routine applications and constant enhancements of chemistries, enzymes, mastermixes and instruments continue to extend the scope of qPCR technology by promising added benefits such as extremely short assay times measured in minutes, low reagent usage and exceptionally rapid heating/cooling rates. The whole process is driven by the insatiable demand for ever-more specific, sensitive, convenient and cost-effective protocols.

However, it has also become clear that variable pre-assay conditions, poor assay design and incorrect data analysis have resulted in the regular publication of data that are often inconsistent, inaccurate and often simply wrong. The problem is exacerbated by a lack of transparency of reporting, with the details of technical information wholly inadequate for the purpose of assessing the validity of reported qPCR data. This has serious consequences for basic research, reducing the potential for translating findings into valuable applications and potentially devastating implications for clinical practice. In response, guidelines proposing a minimum standard for the provision of information for qPCR experiments ('MIQE') have been launched. These aim to establish a standard for accurate and reliable qPCR experimental design as well as recommendations to ensure comprehensive reporting of technical detail, indispensable conditions for the maturing of qPCR into a robust, accurate and reliable nucleic acid quantification technology.

Introduction

Quantitative real-time PCR (qPCR) has become a ubiquitous, mainstream technology widely referred to as the gold standard for nucleic acid detection and quantification in

basic research as well as for clinical applications (Bustin, 2010). Awkwardly, the remarkable transparency and practical simplicity of qPCR has been replaced by numerous conceptual, reagent, protocol and analysis alternatives that continue to yield quantitative results, but require a detailed understanding of the methodology as well as the methods involved to allow an assessment of the data validity. In its simplest form, qPCR is extensively used as a low throughput method for the detection or quantification of limited numbers of individual SNPs or RNAs in relatively few samples or for corroborating fluorescent microarray analyses, as it complements their relatively limited sensitivity, and dynamic range. However, its power and versatility have propelled a growing enthusiasm towards more complex applications. Novel chemistries, both fluorescence – and non-fluorescence-based, fast cyclers with rapid ramping and cooling capability and able to process thousands of assays at the same time, and fast reagents allowing minimal cycling times have combined with advances in automation technology to convert qPCR into a very high-throughput technology generating results in very short time. One of the most remarkable technical developments is the integration of qPCR with nanotechnology. The emergence of high-throughput nanolitre qPCR (Morrison, 1995) and microfluidic digital PCR (Zhang and Xing, 2007) is coupled to advances such as the integration of DNA purification, concentration and qPCR into a real time micro-PCR chip (Min *et al.*, 2011) and the development of innovative, portable PCR instruments (Qiu *et al.*, 2011). Amongst many novel applications, qPCR is used for the rapid and reliable detection of biological threat agents (Christensen *et al.*, 2006), custom-made commercial detection systems are available for the detection of clinical pathogens (Rossney *et al.*, 2008; Novak-Weekley *et al.*, 2010; Zidaric *et al.*, 2011), genetically modified organisms (Dorries *et al.*, 2010) and a combination of nanotechnology and electrochemical qPCR (Yeung *et al.*, 2008) has resulted in the development of hand-held devices that allow local and immediate assays for point-of-care testing applications (Won *et al.*, 2011). This has channelled the technology from a research setting, where, in general, qPCR assays are conceived, designed, performed and controlled by experienced researchers into a wider arena, with high volume 'black box' assays performed as rapidly as possible by less proficient staff.

Current qPCR assay design and reporting are flawed

Unsurprisingly, the ubiquity of the assay itself, combined with abundant choices of methodology, methods and applications, has given rise to numerous protocol alternatives with the potential to produce conflicting data (Bustin and Nolan, 2004; Bustin, 2008a,b; Murphy and Bustin, 2009). Any latent tendency towards inconsistency is exacerbated by the significant probability of variability associated with the numerous steps that make up a qPCR assay (Bustin, 2010) (Fig. 11.1). Pre-analysis variability derives from issues such as poorly defined sample selection or handling, patchy nucleic acid quality, inconsistent use of controls, poor assay design as well as non-existent optimization and validation. Inconsistencies with post-qPCR data processing are caused by a lack of quality control of the post-qPCR data, especially unsuitable methods of normalization, misguided data analysis procedures and challenges associated with applying the correct statistical methods (Hellemans *et al.*, 2007).

For RT-qPCR, in particular, these problems are critical and have been well documented over many years (Bustin, 2000, 2002, 2004; Bustin and Nolan, 2004; Bustin *et al.*, 2005; Nolan *et al.*, 2006a). Poorly designed, validated and executed assays may, and indeed do,

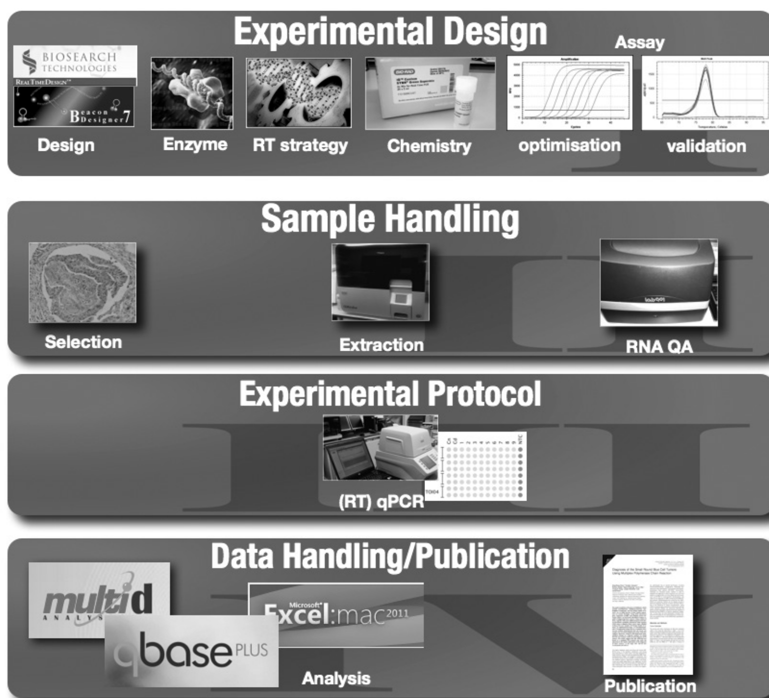

Figure 11.1 RT-qPCR assay. A complete RT-qPCR assay consists of four key steps: (1) Experimental design which includes in silico and empirical validation of each assay. (2) Consistent sample handling and quality assessment. (3) The RT-qPCR steps themselves. (4) Data analysis and publication.

generate a wealth of measurements, but the results can be highly variable and thus inaccurate, ultimately reducing the precision of the measurement or, worse, introducing biases leading to statistically positive but incorrect results. Hence the persistent appeal of developing a set of standard and objective quality control measures (Bustin and Mueller, 2005, 2006; Bustin, 2006, 2008a,b,d, 2010; Murphy *et al.*, 2007; Murphy and Bustin, 2009). The most reliable means of judging the plausibility of a body of work is to study the 'Materials and Methods' section for the relevant information, particularly that relating to the latter aspect of the experimental protocol. A recent survey confirms that this section is avidly read by researchers (Bustin, 2010), but a second survey shows that the detail of the information provided is often wholly inadequate (Huggett and Bustin, 2011). Unfortunately, not only are many assays designed poorly but also many publications utilizing qPCR technology provide insufficient information to allow a reader to assess the assay and evaluate the validity of conclusions derived from the qPCR data (Bustin, 2010).

Common omissions that could easily be remedied include simple things like the accession numbers for mRNA or genomic sequences or specifics of the reverse transcription step (Huggett and Bustin, 2011). The latter is especially important, since cDNA priming method and choice of RT have long been known to have a significant impact on results (Stahlberg *et al.*, 2004a,b). Even if the claim is that the experimental protocol recommended by the manufacturer was followed, experience shows that individual researchers frequently

introduce subtle, yet consequential variations. Several other pieces of key information are often omitted:

1 Detailed sample information: It remains a remarkable feature of many publications utilizing qPCR technology that very basic information with respect to the samples under investigation is not published. This is of particular importance when considering gene expression analyses from tissue biopsies, where sample selection, acquisition, handling and storage can significantly affect quantification results. Since it should be taken for granted that the researchers have gathered as much information as possible about their samples, the release of that information should add no additional burden to the exacting task of gathering data for publication. Furthermore, it is also necessary to provide details of sample processing procedures, since sample pass through numerous preparative steps prior to the qPCR assay, every one of which can introduce additional variability (Hammerle-Fickinger *et al.*, 2009; Tichopad *et al.*, 2009).
2 Sample quality: it is accepted that RNA integrity and quality assessment is essential for reliable quantification of RNA using RT-qPCR assays (Perez-Novo *et al.*, 2005; Fleige and Pfaffl, 2006; Fleige *et al.*, 2006) and a recent, comprehensive study demonstrates that compromised RNA quality has a measurable impact on the significance of differential expression of prognostic marker genes between cancer patient risk groups, and on risk classification performance using a multigene signature (Vermeulen *et al.*, 2011). The availability of microfluidics-based devices for nucleic acid quality assessment allows automated, rapid and standardized quality assessment of very small amount of total RNA with quality metrics such as the RIN (Agilent), RQI (BioRad) or SDV (lab901) to represent the level of degradation in a sample. However, it must be remembered that assessing the integrity of rRNA does not necessarily equate with assessing mRNA integrity, and specifically did not in the study by Vermeulen *et al.* (2011), hence the suggestion of introducing a 3′:5′ mRNA-specific integrity assessment (Nolan *et al.*, 2006a) as a more suitable alternative. Importantly, inhibition of reverse-transcription or PCR should be checked by dilution of the sample or use of a universal inhibition assay such as SPUD (Nolan *et al.*, 2006b). Yet very few publications mention the term RNA quality (Huggett and Bustin, 2011).
3 Normalization: normalization strategies are aimed at selecting stable and minimally regulated reference genes and identifying the optimal number of reference genes for the calculation of a normalization factor. This reduces technical variability and increases confidence in data by controlling for experimental error introduced during the multistage process required to extract and process the RNA. Different strategies are not mutually exclusive and the general recommendation is to match sample size, RNA quantity and protocol around three validated internal controls for the final normalization (Huggett *et al.*, 2005). Certainly, there is abundant published evidence that the use of a single reference gene to show small changes in target copy numbers generates unreliable data (Tricarico *et al.*, 2002; Dheda *et al.*, 2004). However, most papers normalize target gene copy numbers against single, invalidated reference genes, even though there are several published methods clearly detailing methods for selection of appropriate reference genes sets (Andersen *et al.*, 2004; Pfaffl *et al.*, 2004; Vandesompele *et al.*, 2002). When this is combined with the reporting of small changes (e.g. differences of less than threefold) it is impossible to conclude whether findings are

differences in expression of the gene of interest, the references gene or a combination of both; consequently statistically significant measurement bias can be introduced leading to incorrect findings (Dheda *et al.*, 2005).

4 PCR efficiency: relative expression levels of mRNA of genes of interest (GOI) are frequently reported relative to those of one or, ideally, several reference genes using the comparative *Cq* method (Livak and Schmittgen, 2001). This involves normalizing the expression of GOI against the internal reference gene(s) *within* two samples, one of which acts as control (ΔCq), followed by a comparison of the normalized difference in expression *between* the two samples ($\Delta\Delta Cq$). In order to be reliable, this requires constant doubling of amplicon with each cycle for each of the targets, or equal amplification efficiency at the very least. However, actual efficiency can range between 60% and 100%, depending on assay quality, sample and target characteristics, reagents as well as instrument variability. Hence small differences between GOI and reference genes can lead to huge differences in relative expression ratios and generate distorted results. Again it is remarkable that not only is this a well-known fact, but that there are a number of efficiency-corrected relative quantification models that provide an efficient and reliable means of quantifying nucleic acids (Pfaffl, 2001; Peirson *et al.*, 2003). They depend on the calculation of PCR efficiency for every target and incorporate this information into relative expression algorithms. Popular software packages such as Genex (Bergkvist *et al.*, 2010), qBase (Hellemans *et al.*, 2007) and REST (Relative Expression Software Tool) (Pfaffl *et al.*, 2002) all make use of such models.

Since all of this information is essential to assay performance, omitting it in a publication makes it impossible to judge the technical quality of published data.

One of the most egregious examples of the enormous implications for the health and lives of individuals that result from inappropriate use of this technology is provided by the use of RT-qPCR data that purported to demonstrate the presence of measles virus (MV) RNA in children with developmental disorders (Uhlmann *et al.*, 2002). It provided sustenance to the controversy surrounding the triple measles mumps and rubella (MMR) virus vaccine, as the data were interpreted as providing evidence for a link between MMR, gut pathology and autism. However, a detailed analysis of the raw data underlying that report carried out by one of the authors (SAB) acting as an expert witness to the UK High Court and the US Vaccine Court, revealed that these data were obtained amongst a catalogue of mistakes, inaccuracies and inappropriate analysis methods as well as contamination and poor assay performance (Bustin, 2008c). A reanalysis of the data concluded that the assay had been detecting DNA and since MV is an RNA-only virus, the RT-qPCR data had been erroneously interpreted, a conclusion confirmed elsewhere (Afzal *et al.*, 2006; D'Souza *et al.*, 2006; Hornig *et al.*, 2008).

MIQE

A growing consensus has been developing around the need to improve published information with relevant experimental detail that covers every aspect important to the qPCR assay itself, as well as issues relating to pre – and post-assay parameters. Specifically, it became clear that there is a requirement for a set of recommendations that can be used by journal reviewers, who need to be able to evaluate the reliability of the experimental protocols and ensure the

inclusion of all essential information in the final publication. This need was addressed by the 'Minimum Information for Publication of Quantitative Real-Time PCR Experiments' (MIQE) guidelines (Bustin *et al.*, 2009), with a recent amendment clarifying the disclosure of primer sequences (Bustin *et al.*, 2011). The last year has seen a rapid expansion in the number of researchers aware of the existence of these guidelines (Fig. 11.2) as well as an increasing number of citations of the original publication in the per-reviewed literature (Fig. 11.3). There even is an iPAD/iPOD/iPhone app available from the iTunes store.

The ultimate goal of the MIQE guidelines is to make all technical aspects of the qPCR assay sufficiently transparent, so that the reader of a publication can take technical excellence for granted and focus on the biological relevance of the data. MIQE is made up from nine sections that list 85 parameters that constitute the minimum information required to allow potential reproduction as well as unambiguous quality assessment of a qPCR-based experiment. The nine key areas are 'Experimental design, sample, nucleic acids, reverse transcription, target, primers and probes, assay details, PCR cycling and data analysis'. At first sight, these look overwhelming, arduous and over-exacting. In practice, it is clear that most, if not all of these parameters describe information that would be obtained as a matter of course during the experimental design, optimization and validation stages. Importantly, there is a clear hierarchy with some parameters, labelled 'E' (essential) in the published guidelines, indispensable for an adequate description of the qPCR assay, whereas other components, labelled 'D' (desirable) more peripheral, yet constituting an effective foundation for the realization of best practice protocols. There is increasing recognition that the MIQE guidelines provide the basis for much-needed standardization as well as encourage the publication of essential information that should be accessible to reviewer and reader. Of course, these parameters are not set in stone and are open for discussion; indeed, a core set of sections is being implemented by the BMC group of open-access journals (Bustin *et al.*, 2010).

The most controversial aspects of the original MIQE guidelines related to the proposal

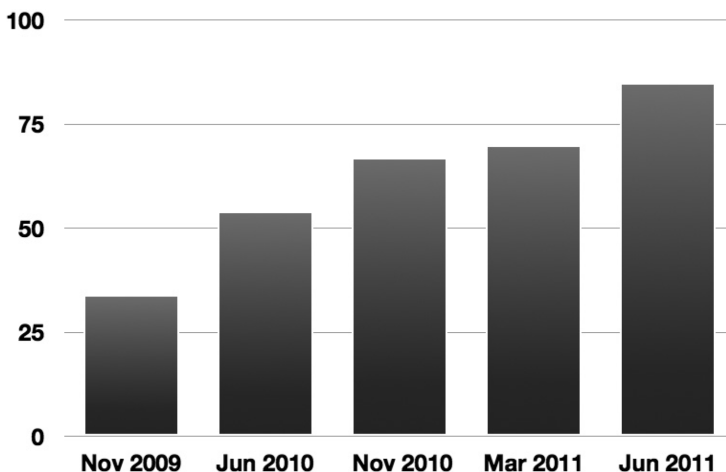

Figure 11.2 MIQE awareness. Attendees of qPCR meetings (2009: qPCR USA, San Francisco; 2010: Gothenburg and qPCR USA; 2011: Freising and Prague) were asked whether they had heard of the MIQE guidelines. The graph shows the percentage of 'yes' responses.

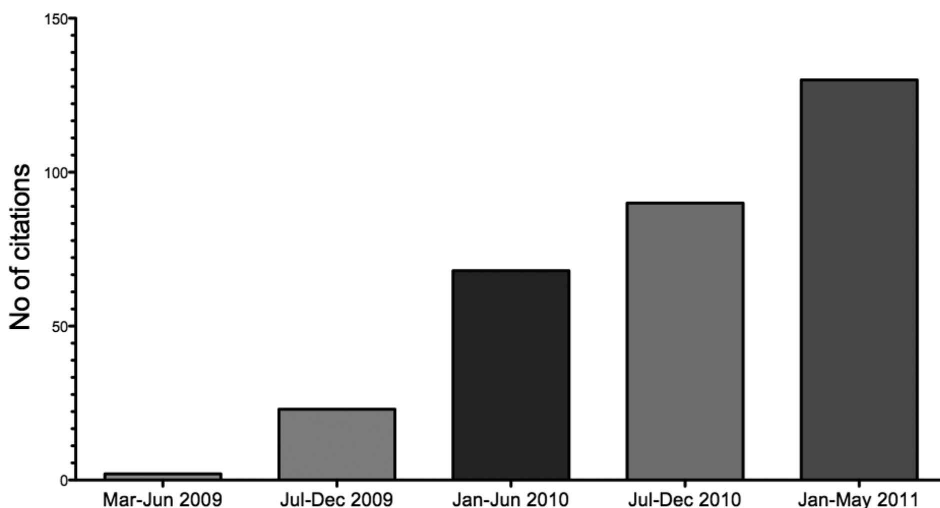

Figure 11.3 Citations of the MIQE paper in the peer-reviewed literature. The number of citations were obtained from Web of Knowledge (www.webofknowledge.com) and stood at 453 at the end of November 2011.

that publications must divulge the sequences of any primers used and especially should also report the sequences of any probes. The rationale behind releasing the primer sequences was straightforward: an experiment cannot be reproduced if one of the principal reagents is unavailable. Lack of access to a probe sequence, on the other hand, does not preclude analysis of the specificity, efficiency and sensitivity of an assay; however, for completeness' sake it is but a small step to take for most researchers. Many commercial qPCR assays are not supplied with the primer/probe sequences, since most vendors consider this commercially sensitive information; usually there are also no details provided on empirical validation of each individual assay. Publications utilizing such assays could not satisfy the original MIQE requirements, placing limits on a universal acceptance of MIQE. Consequently, an amendment of the original guidelines now requires either primer sequences or a clearly defined amplicon context sequence. This guidance was issued based on the assessment that in the absence of full primer sequence disclosure it is possible to achieve an adequate level of transparency, but only if there is an appropriate level of background information and disclosure of validation results on the qPCR assay. Consequently, if primer sequences are not disclosed, a MIQE-compliant publication should institute the same validation criteria used for assays reporting primer/probe sequences. Specifically, when reporting a precise fold-change for a transcript it remains an essential requirement that the PCR efficiency, analytical sensitivity and specificity of each individual assay be determined. This information should be verified by the investigator for the actual assay that is being reported using the conditions and personnel in their laboratory and not extrapolated from commercial assays validated by the vendors.

The implementation of appropriate statistical methodologies for data handling and processing is an essential complement to any improvements introduced to the practical workflow. Obviously, there are a large number of statistical tools that can be used to address

and minimize the variability discussed above and amongst many, specific studies have been published that look at the identification and handling of outliers and precision associated with calibration curves (Burns *et al.*, 2005), the relative merits of obtaining *Cq*s from the threshold method or sigmoidal functions (Rutledge and Cote, 2003; Rutledge, 2004; Rutledge and Stewart, 2008) and limits of detection modelled from sample replication and *Cq* values (Burns and Valdivia, 2008). Furthermore, tools have become available that allow management and analysis of qPCR data (Muller *et al.*, 2002; Jin *et al.*, 2006; Hellemans *et al.*, 2007; Gallup and Ackermann, 2008; Ritz and Spiess, 2008). However, since there are no guidelines or universally accepted standards for data handling and interpretation, the use of multiple statistical tools adds to variability and discordance. Hence MIQE guidelines propose the disclosure of key statistical features associated with a qPCR assay, as well as reporting of what software was used to analyse results.

Appropriate statistical modelling and analysis for the interpretation of qPCR data is of particular importance for clinical applications, where false-positive or negative results can have serious implications. A systematic evaluation of the various qPCR data analysis methods has shown that they differ substantially in their performance (Karlen *et al.*, 2007); hence MIQE guidelines specify the importance of providing detailed information on the methods of data analysis and confidence estimation, especially identifying the statistical methods used to evaluate variances. Since expanding sample size can increase the power of a statistical test, technical repeats are a much favoured and reported sign of qPCR virility. Whilst these do help reduce measurement error, technical repeats simply provide a commentary on the researchers', or their robots' ability to pipette accurately. Technical replicates are needed when optimizing a PCR reaction to ensure that the assay is optimum. However the high precision of an optimal qPCR assay (typically less than 10% coefficient of variation) means that when this is replicated, far from increasing the reliability of results, it can distort the statistics of determining confidence in experimental data. Furthermore if technical replicates are to be included when conducting RT-qPCR it is essential that the more variable reverse transcription reaction is replicated (a process that is automatic with one step RT-qPCR), as a frequent conceptual error is to perform a single reverse transcription and replicate the PCR step on the cDNA sample. This action has surely led to numerous publications of very precise yet totally biased results.

Appropriate biological replication is essential if findings are meant to be valid in the context of a conceptually large population from which the subjects were sampled, rather than only for the particular individuals considered in the experiment (Mehta *et al.*, 2004). Since biological variability is larger than technical variation, increasing biological replication usually translates into more effective gains in power. However, increasing sample size generally leads to added cost and increased time for performing the experiments. In addition, some biological replication cannot be increased, e.g. when comparing large numbers of healthy individuals with a limited number of patients with a particular disease.

It is important to note that the need for appropriate biological replication and other aspects of the MIQE recommendations do not reflect the deliberations of a handful of purists frequenting the whitest of ivory towers. MIQE matters because it is more important that findings truly reflect what is being investigated than it is to publish a cell or nature paper. When, as is often the case, high impact publications are the sole aim of a study a poor design can lead to the embarrassing process of a retraction.

Conclusions

qPCR and RT-qPCR are powerful technologies that have enabled many of the advances made in our understanding of basic biological and disease processes; both are also increasingly used for clinical diagnostic purposes. However, the combination of ease of use and lack of rigorous standards of practice has resulted in widespread misinterpretation of data and consequent publication of erroneous conclusions. Any solution to the challenge of how to make PCR-based assays more reliable requires both an appreciation and an understanding of numerous attributes that include biological concepts, statistics, mathematical modelling, technical know-how and a willingness to share this knowledge. This range of fundamental variables must be addressed by guidelines that permit a shift of focus from questions regarding the technological relevance underlying a publication's conclusion to the actual biological or diagnostic issues being addressed. MIQE constitutes a reference framework for communication within the research community, instrument and reagent manufacturers and publishers that promises to deliver guidelines that promote transparency of experiments and confidence in results and conclusions that advance, rather than impede our knowledge.

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