



## Why the need for qPCR publication guidelines?—The case for MIQE

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### ABSTRACT

The polymerase chain reaction (PCR) has matured from a labour- and time-intensive, low throughput qualitative gel-based technique to an easily automated, rapid, high throughput quantitative technology. Real-time quantitative PCR (qPCR) has become the benchmark technology for the detection and quantification of nucleic acids in a research, diagnostic, forensic and biotechnology setting. However, ill-assorted pre-assay conditions, poor assay design and inappropriate data analysis methodologies have resulted in the recurrent publication of data that are at best inconsistent and at worst irrelevant and even misleading. Furthermore, there is a lamentable lack of transparency of reporting, with the “Materials and Methods” sections of many publications, especially those with high impact factors, not fit for the purpose of evaluating the quality of any reported qPCR data. This poses a challenge to the integrity of the scientific literature, with serious consequences not just for basic research, but potentially calamitous implications for drug development and disease monitoring. These issues are being addressed by a set of guidelines that propose a minimum standard for the provision of information for qPCR experiments (“MIQE”). MIQE aims to restructure to-day’s free-for-all qPCR methods into a more consistent format that will encourage detailed auditing of experimental detail, data analysis and reporting principles. General implementation of these guidelines is an important requisite for the maturing of qPCR into a robust, accurate and reliable nucleic acid quantification technology.

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### 1. Introduction

The polymerase chain reaction (PCR) stands apart from the body of key technologies that have instituted present-day life sciences. “Legacy” PCR has made a pre-eminent contribution to modern biology, medicine, agriculture and biotechnology, whilst its evolution into quantitative, fluorescence-based real-time PCR (qPCR) has rendered ubiquitous the technology’s scope, convenience and reach. The conceptual transparency of the PCR is remarkable: repeated heating and cooling cycles of a mixture of DNA, specific oligonucleotides, DNA polymerase and dNTPs results in exquisitely sensitive, exponential amplification of a unique DNA target. The practical simplicity of qPCR is equally striking: addition of a single additional reagent (a dye or dye-labelled probe) allows the monitoring of that amplification and quantification of its products in real time. qPCR has become the gold standard for the quantification of miRNAs and other regulatory RNAs, cellular mRNA and mRNA splice variants. It is widely used for end-point SNP and real-time high resolution melt analyses and has become ensconced as the benchmark for clinical prognosis and pathogen detection [1–3].

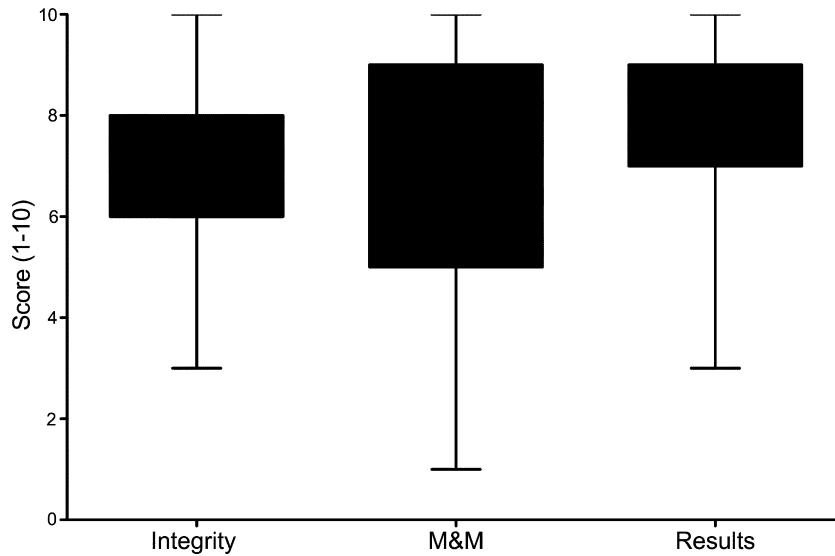
Invariably, this popularity has a downside: the original, simple protocol has spawned countless adaptations that, given the expo-

ponential nature of PCR amplification, have resulted in protocols that have lead to the habitual publication of discordant and contradictory data. Since most publications limit the provision of technical information to a minimum, it is often challenging to clarify which conclusions are based on appropriate data and which are not [4–7].

As a consequence of these variables, qPCR has become an inadequately standardised, complex and, frequently, inconsistent technique that invites the publication of flawed conclusions. The extent to which the scientific literature has become unreliable is not widely appreciated, with most researchers in a recent survey rating its integrity as high, although a substantial minority expressed their grave reservations (Fig. 1). 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 relating to experimental detail and to examine the “Results” section for consistency with the conclusions derived from the data. The same recent survey confirmed that both sections are avidly read by researchers, although there is a wide range in the assiduity with which both sections are considered (Fig. 1). The publication of the MIQE guidelines [8], which suggest a minimum set of information that researchers should provide for their qPCR data, has sparked a substantial interest in a more realistic assessment of the value of qPCR for the analysis of gene expression as well as a diagnostic tool.

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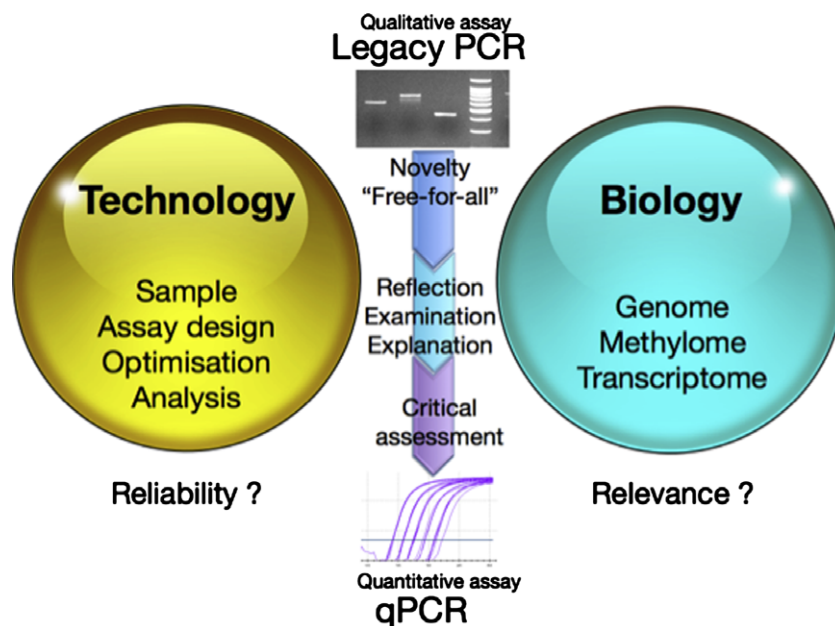
**Fig. 1.** Scientific literature readers' habits. This survey was carried out in October 2009 amongst 41 individuals (post-graduate students, post-docs and academics) at two UK Universities (QMUL and Manchester) and in November 2009 amongst 50 participants of the 3rd qPCR USA meeting in San Francisco. The first question was "How reliable do you think the scientific literature is?" (1: not; 10: very). The second question was "How carefully do you study the Materials and Methods section?" (1: not; 10: very) and the third "How carefully do you study the Results section?". In each case the answer could range from 1 (not) to 10 (very).

## 2. The evolution of PCR

Since first mooted [9], PCR has evolved from a labour- and time-intensive qualitative technique that relied on the visual interpretation of stained gels to detect the presence of amplification products into to-days simple, rapid and quantitative qPCR, which uses precision optics and DNA-binding fluorescent dyes or fluorescent labels to monitor amplification in real-time. This progression was accompanied by prodigious advances in our understanding of the underlying technology as well as the biology it describes (Fig. 2).

The early years of qPCR assay design, implementation and data interpretation were not governed by accepted rules, as there were

none (the "Wild West" scenario). However, a rising dissatisfaction with the way experiments were being carried out and reported led to escalating calls for a reassessment and comprehensive examination of the validity of qPCR results and a recognition for the need for transparent explanation of any conclusions derived from PCR-based assays [10–14]. A readiness to confront these issues has contributed to the growing appreciation that qPCR data and their interpretation can be (and often are) meaningless and downright misleading. Consequently, since the core qPCR assay itself has become a routine technology and is unlikely to be technically bettered, the challenges now centre around (i) tackling the numerous limitations of the pre- and post-assay workflows and



**Fig. 2.** PCR evolution. The evolution of legacy, gel-based PCR, established as a powerful method for the qualitative detection of nucleic acids into the quantitative qPCR assay of to-day proceeded through a protracted phase of trial and error. This scrutiny resulted in a more detailed understanding of both the technological as well as biological limitations and challenges of this technology. The serious question marks surrounding both the reliability and relevance of qPCR data contributed heavily to the development of the MIQE guidelines.

(ii) providing sufficient information about experimental detail to permit an objective assessment of the validity of results and consequent conclusions.

Other methods, especially next generation sequencing (NGS) technology, are becoming more prevalent and provide a new approach to the quantification of RNA by utilising a digital readout of gene expression levels using DNA sequencing [15,16]. Not only is it becoming possible to detect and quantify reliably low-abundance transcripts, but NGS allows the identification of sequence changes, novel splice variants and fusion transcripts that would otherwise escape detection [17]. Clearly, once costs are controlled and bioinformatics bottlenecks removed, this technology will supplant qPCR for many applications. Nevertheless, it is also clear that the relative simplicity and portability of qPCR-based assays will continue to be in demand for a long while yet; hence constant improvements to the PCR envelope are both exigent and indispensable if PCR is to continue to retain its relevance after 25 years as the foremost technology in molecular biology.

### 3. Causes of variability

There are three obvious causes for the large number of variable, even contradictory results obtained by PCR-based assays and published in the peer-reviewed scientific literature (Fig. 3). They are rather obvious and, one would like to think, well understood.

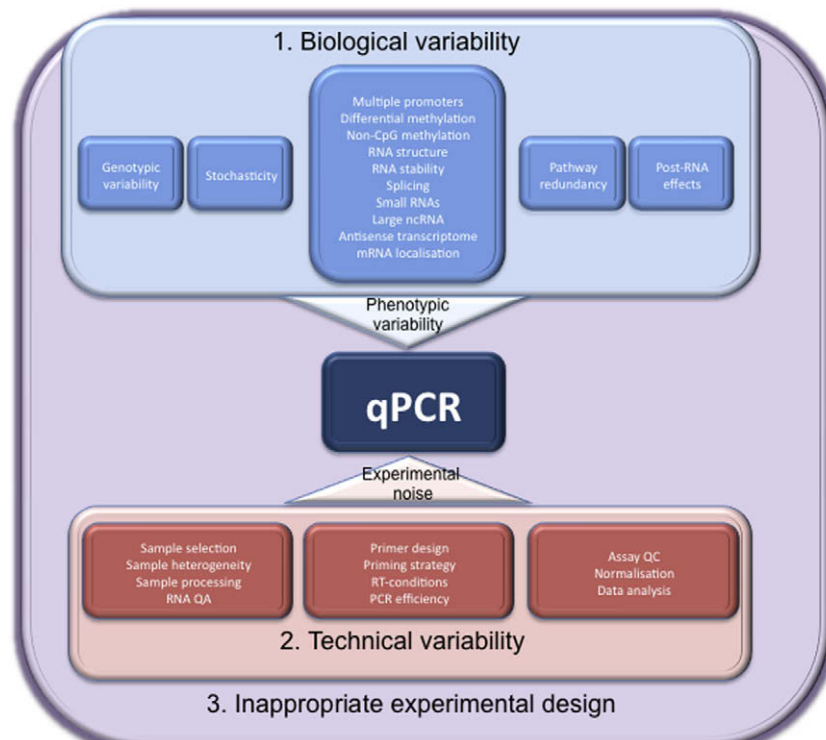
- A. *Biological variability*: the first cause is the evident reality that biology describes variability; hence experiments will never yield identical results.
- B. *Technical variability*: the second reason derives from measurement errors that define technical variability. It describes the noise introduced into the assay and is inherent in any molecular technology.

C. *Inappropriate experimental design*: the third explanation relates to inappropriate underlying assumptions generating results that exist in isolation, often are biologically or clinically of little consequence, may have negligible translational relevance and frequently are wholly wrong.

#### 3.1. Biological variability

Biological variability is a hallmark of life and embraces an eclectic combination of distinctive components defined by the natural genotypic and phenotypic variation among individuals. Cells and tissues constitute dynamic systems characterised by complex and variable behaviour patterns subject to spatial and temporal heterogeneity. Genetic variation is evident at several levels, and includes polymorphisms, copy number variation, alternative splicing, post-transcriptional and post-translational regulation and epigenetic modifications. Phenotypic variation is strongly affected by environmental interaction by and within the individual organisms and derives from factors such as age, stage of life cycle and reproductive cycle, gender, time of day or year and nutritional status. The definition of such heterogeneity and the quantification of its effects remains a difficult task, since it is hard to identify every component giving rise to heterogeneity and to associate experimental measures in a noisy natural environment [18]. As a consequence, qPCR-derived gene expression results are usually organism, tissue- and time-dependent; hence it is important that any conclusions must be qualified by placing them into specific experimental contexts.

There is another essential, yet frequently overlooked, source of biological variability. The analysis of gene expression patterns in single cells has demonstrated that biological complexity is a reflection not just of genetic variability, but also of the intrinsic stochastic kinetic noise of biochemical reactions [19]. This implies inherent stochastic heterogeneity between cells, with the result



**Fig. 3.** Parameters affecting qPCR data and conclusions. Biological and technical variability are considerably higher for *in vivo* biopsies or environmentally sampled organisms compared to cells or organisms studied in the laboratory under standardised experimental conditions. Together with inappropriate experimental design they are the major confounding factors affecting pertinent conclusions of qPCR data.

that the dynamic behaviour of a single cell is not exactly reproducible. Indeed it is now clear that there are significant differences in gene expression patterns between individual cells [20], even within apparently homogenous cell cultures. These are influenced by the interactions between regulatory molecules and DNA and results in the production of large bursts of mRNA transcripts followed by an increase of protein molecules. Consequently, stochasticity in gene expression [21] results in genetically identical cells exposed to the same environmental conditions showing significant variation in mRNA [22,23] and protein [24] expression patterns resulting in marked differences in phenotypic characteristics [25,26]. Indeed, the physical location of mRNAs within a single cell can vary and result in differential expression within that cell [27]. Furthermore, biological networks display a remarkable degree of robustness, with built-in redundancy preserving their functioning under variations of biochemical parameters, different environmental conditions or even different levels of their components [28]. Taken together, these different effects escalate the noise, variability and heterogeneity of biological systems and require the development of dedicated experimental protocols and analytical procedures [29] as well as incorporation into more realistic statistical models to allow biologically relevant data interpretation [30].

This rising awareness of the ever-increasing complexity of biological mechanisms underlying the regulation of gene expression is moving our horizons away from a description of single biomolecules and their interaction with other individual molecules towards a quantitative description of complex biological systems involving the interaction of many components. It is no longer sufficient to describe changes in the levels of an mRNA, either in “absolute” terms or relative to those of a number of reference genes. Consequently, as our comprehension of the underlying biology continues to both expand and surprise, we move from evaluating gene expression based on changes in transcription that affect relative expression levels of mRNA to understanding that such changes are but one effect amongst many others that include at the RNA level alone a requirement to consider RNA structure and its relation to molecular interactions [31], splicing [32,33], small RNAs [34,35] and their post-translational regulation [36], large noncoding RNAs [37], the antisense transcriptome [38] as well as mRNA localisation [39]. This does not even touch on the numerous co- and post-translational modifications that can be transient, permanent or subject to alteration during the lifespan of proteins. Together with re-folding, multimerisation and localisation this considerably increases the information content and functional repertoire of proteins, so generating variability in proteins that extends far beyond that provided by the genetic code.

The interpretation of mRNA quantification data is further complicated by the discovery of novel methylation patterns that result in increased transcription [40] as well as widespread differences in allelic expression among autosomal non-imprinted genes in animals [41] and plants [42]. Since methylation, allelic imbalance and allele-specific expression patterns are associated with disease risk [43,44], it may be no longer sufficient to quantify a gene's mRNA expression, but it is becoming necessary to determine precisely which allele is being expressed. One implication of this is that, rather than avoiding SNPs when designing primers, it may be necessary to include them as part of an overall assay design strategy in order to be able to quantify allele-specific expression accurately [6].

### 3.2. Technical variability


The ubiquity of qPCR has resulted in a proliferation of protocols that vary at every stage of the workflow and are a major source of discordance between published conclusions. The key to minimising technical variability is meticulous attention to sample isolation,

storage and preparation, numbers of replicates, assay design and execution as well as methods of normalisation and statistical analysis. The combination of protocol profusion and a multitude of steps within each protocol provides ample scope for the introduction of inconsistencies. These frustrate the ability to discern true differential expression between experimental groups as the power of statistical tests is a function of sample size, the differences among experimental groups and the variability of the system [45]. Since sample sizes are usually relatively small, the resulting statistical tests have low power, fold change estimates have low precision and there is an increased likelihood of obtaining false-negative results.

Technical disparity describes a broad spectrum of inconsistencies. The key to resolving any discordance is provided by assiduous reporting of and careful attention to experimental detail [46]. Discrepancies may simply be a function of the reality that every experiment is different and is carried out by separate individuals. Hence even though a target, e.g. a particular mRNA, may be nominally the same, experimental samples, nucleic acid extraction methods and primer location are likely to differ. Furthermore, experimental protocols become customised by different laboratories, e.g. in the choice of cDNA synthesis strategy, cycling conditions or data analysis. These modifications inevitably lead to differences; however they are directly under the control of the investigator and can be resolved if sufficient experimental details are provided. Other inconsistencies are inherent to qPCR technology and arise because the investigator has a choice of (i) distinct instruments based on different technologies, (ii) a wide range of enzymes that are further refined by manufacturer-specific reaction buffers and (iii) data analysis software based on different statistical methodology. The investigator can choose and mix these components, but overall details of their performance remain dependent on the reagent or software supplier.

Discordance can be the result of improved understanding and consequent application of qPCR technology. Whilst this may lead to superior assay design, it may also contradict earlier results achieved using less sophisticated experimental design. Examples include changes in primer design criteria [47–49], increased awareness of the need to analyse PCR efficiency [50–52], critical contributions of varying RT-conditions [53,54], effects of variable RNA quality [55] and integrity [56–58] issues, the role normalisation plays in data interpretation [59–61] and many others. Whilst these criteria can generate data that are at odds with previous reports, the provision of adequate information within a publication should allow the reader to account for and clarify any discrepancies.

Regardless of whether a result leads to a “positive” or “negative” conclusion, it is crucial that a manuscript reports all relevant aspects of the experimental workflow in detail. This assertion should be non-contentious, yet it accentuates a startling and avoidable problem encountered in real-life: that of inadequate information provided by authors to guide the reader. It is staggering that the vast majority of publications do not provide sufficient information in their “Materials and Methods” sections to allow the reader to infer whether the published data support the conclusions in that publication. An analysis of four papers randomly chosen from a PubMed search containing the keywords “RT-qPCR”, “gene expression” and “2009” uncovered that not one provided all the critical information required to allow an assessment of the technical quality of that publication (Fig. 4). An extreme example of the standard of information provided by high impact factor journals comes from Nature Medicine published in October 2009. Here the online experimental information is compressed to: “We extracted RNA with Trizol (Invitrogen) and performed quantitative PCR using LightCycler 1.5 Carousel and LightCycler FastStart DNA Master<sup>PLUS</sup> SYBR Green I (Roche). Primer sequences are available upon request” [62].



Publication Parameter	A	B	C	D
RNA QA	Agilent	gel	×	Agilent
Primers probes	Assay on demand	✓	✓	✓
RT	✓	✓	✓	✓
cDNA priming	oligo-dT	×	random hexamers	×
Temp/vol/time	✓/✓/×	✓/✓/×	×/✓/×	×/×/×
PCR efficiency	×	×	×	×
Normalisation	single, unvalidated RG	single, unvalidated RG	unvalidated 18S rRNA	single, unvalidated RG
Biological replicates	×	×	×	×

**Fig. 4.** Analysis of four randomly chosen 2009 qPCR publications. The Materials and Methods sections of four publications were analysed for information content. A red cross denotes no information provided, a green tick denotes information provided.

More typically, a publication will state that “RNA was isolated with Trizol reagent”, but provide no information on RNA quality assessment. Whilst the supplier of a reverse transcriptase is now usually named, cDNA priming conditions often are not. The quantification of target genes relative to, with luck, multiple reference genes using the  $\Delta\Delta C_q$  method is frequently used to report relative expression levels without any mention of respective qPCR efficiencies. On the other hand, a significant number of publications still normalise against a single, unvalidated reference gene and the importance of biological, as opposed to excessive use of technical replicates, is still widely ignored. Ironically, when some experimental detail is provided, this often reveals a complete lack of understanding of the qPCR, with one particularly egregious publication comparing relative expression of target genes with apparent amplification efficiencies of 440% and 175% and quoting coefficients of variation based on  $C_{q,s}$ , not copy numbers [63].

### 3.3. Inappropriate experimental design

This third source of variability is prompted by experimental designs employing false assumptions, deficient technologies, inappropriate sampling procedures, inconsistent use of controls, incorrect methods of normalisation, unsound data analysis procedures and misdirected statistical methods [5,6,64]. Furthermore, whilst the requirement for first-class quality control at every step of a qPCR assay has been clearly recognised as its Achilles heel, on publication this element of the experimental protocol is frequently not afforded the prominence it deserves. Although these issues have been discussed extensively, they have either not penetrated the general consciousness of the scientific community or they are being ignored [7,65].

Some questions require heroic assumptions about the accuracy of sampling or expression profiling. For example, is an mRNA expression profile obtained from a single whole tumour biopsy really able to predict that tumour's behaviour? Is a twofold difference in mRNA levels really biologically significant? Others go to the very heart of the assumptions underlying modern molecular medicine. How valid is the assumption of congruence between *in vitro* and animal models of disease and the corresponding human condition? Any notion of consistency may well be invalid for most models [66], and the use of diverse and unsuited model

systems simply serves to generate even further disparity of results. In consequence, the conclusions reported by individual publications may well be justified, but relate only to very specific conditions, with very little translational relevance to actual reality.

A scanning of the scientific literature quickly reveals numerous instances of publications reporting one observation, e.g. the up regulation of a gene in response to treatment or the association of a pathogen with a particular disease, and being contradicted by a string of publications reporting the opposite result. This is of particular importance when the aim is to extend qualitative legacy PCR into quantitative qPCR for diagnostic purposes [67–70]. Currently, there is a tangible problem with the translation of qPCR technology into clinical practice [71–73]. For example, some reports suggest that carcinoembryonic antigen (CEA) mRNA levels in the blood of colorectal cancer patients is associated with disease stage [74] and may be of prognostic value [75,76]. Another report suggests that detection of CEA in tumour drainage, but not peripheral blood has prognostic significance [77]. On the other hand, since white blood cells appear to express a splice variant of CEA that hinders detection of tumour cell cDNA in whole blood samples [78], sufficient specificity may require the use of immunobeads to selectively enrich for tumour cells prior to the PCR [79]. Yet other reports question the specificity of CEA altogether and suggest that peripheral blood is not a suitable compartment for detection of tumour cells [80–83]. Since these results are irreconcilable, the inference must be that one or the other are based on inappropriate experimental design.

In principle, given sufficiently comprehensive information, any reader comparing discordant published results should be able to discern which ones are likely to be caused by flaws in experimental design, execution or interpretation. In practice, this essential detail is usually not available and detailed examination of discordant data is either not possible or requires the investment of an inordinate amount of effort. The penalties of the corruption of the scientific literature are wide reaching. Whilst it might be arguable that the consequences of an erroneous reporting of a gene's up or down regulation might be disruptive for research, but limited to inconvenience, time and money wasting for other researchers and damage to the authors' reputation by the subsequent retraction of that publication [84,85], the consequences for publications describing results important for diagnostic/prognostic purposes are much more severe.

The role qPCR played in upholding speculation about a link between the measles mumps and rubella vaccine, autism and bowel disease is highly instructive, horrifying and scandalous. A peer-reviewed publication in 2002 used RT-qPCR technology to claim that measles virus RNA was absent in gut biopsies from normal children but had been detected in a majority of children with autism and gastrointestinal disease [86]. As published, this finding appeared to provide molecular evidence for the speculation that there was a link between the triple vaccine against measles, mumps, and rubella (MMR) and autism. That paper, together with the RT-qPCR data underlying its conclusions, was deconstructed as part of the 'omnibus autism proceedings' in the US Court of Federal Claims in Washington, DC, in 2007. At that trial the reliability of the qPCR data was seriously challenged, revealing a remarkable trail of inappropriate laboratory practices such as positive no template controls, discordant replicates and non-reproducible results, none of which were detectable by reading the published paper [87]. The conclusion was that DNA contamination was the likely cause of most, if not all, positive results [88]. Furthermore, the data were never independently reproduced [89–91]. All lingering doubt evaporated with the publication from a multi-centre group of authors that refuted any association between persistent measles virus RNA in the gut and autism [92]. Astonishingly, this publication includes the two main authors of the original paper, and despite publishing evidence that contradicts their own, they have not retracted their original paper. The evidence presented at the 2007 trial was used in two further trials that concluded that there was no credible link between the MMR vaccine and autism and, in 2009 three appeal judges concurred with the three original judgements. Not surprisingly, the distorted findings of the original publication have caused untold distress to thousands of parents and have likely contributed to the dramatic decline in MMR vaccination in a number of countries, with measles virus now endemic in the UK.

#### 4. Peer-reviewed publications

Research can have an impact only if it is disseminated to and absorbed by an audience and used as a stepping-stone for further research. The primary vehicle for communicating science is the research paper published in the peer-reviewed literature, which as a whole is widely respected (Fig. 1). Literature databases contain the accumulated scientific knowledge that provides a repository for all original research that underpins and helps adjudicate scientific debate. Accordingly it is paramount that protocols, results and analysis methods of any investigation are described in such a way that a knowledgeable reader with appropriate experience should be able to reproduce published results. I would like to term this rule the "prime directive" of publication. Peer review is meant to provide a quality assessment step that ensures that these fundamental criteria are met. However, referees assessing a publication cannot be expert in all the techniques underlying the result; hence they may not be cognisant of the details that denote a reliable qPCR assay and that are requisite to allow a reader to judge the validity of any conclusions. The previously discussed MMR publication provides a clear example of the failure of the peer-review system, with the likely explanation that the reviewers were not sufficiently versed in RT-qPCR technology to scrutinise that manuscript appropriately. Furthermore, and this is true especially of the "Premier League" of journals, technological scrutiny is regarded as far less important than conceptual deliberation and is often relegated from the published article. Hence it is ironic that information relating to experimental design and interpretation of results is frequently more reliable when published in lower impact factor journals.

Unfortunately, the prime directive of scientific publishing has become undermined in too many instances by to-day's huge pressure to publish at all cost. Tenured positions, promotions, grant

applications and individuals' perceptions of their worth depend on a constant stream of publications. As a consequence, the literature is full of publications with uncertain value, relying on woolly thinking and sketchy biology, buttressed by dubious methods. The subject is usually not deliberate misrepresentation or falsification; instead, the focus is on concerns such as poor or absent quality controls, over-reliance on statistics and the use of inappropriate technical methods or statistical methodologies coupled to inadequate reporting of results. Interestingly, there is an ongoing, albeit low-key, debate about this very issue and it has been suggested that research claims, especially in the medical literature, are more likely to be false than true [93]. Indeed, for several independent studies of equal power, the probability of a research finding being true declines with increasing number of studies. The author explains this by commenting on the phenomenon that research findings on "hot" topics can generate major excitement and publicity, only to be followed by contradictory results that provoke severe disappointment. He suggests that the publication of alternating extreme research claims and refutations is the result of many groups needing to publish their numerous data as rapidly as possible to beat the competition and that publication of a "negative" result becomes attractive if it contradicts the publication of a "positive" result in a prestigious journal [94]. This highlights the need for genuine, adequately powered replication studies characterised by full transparency and non-selective reporting of research results [95]. It also places an important onus on reviewers and journal editors to encourage a publication environment that is comfortable with uncertainties and encourages authors to discuss biases, study limitations and confounding factors [96]. Nevertheless, although heterogeneity of results can be valuable, as it may generate a pool of novel data that reflect potentially genuine, biological variability, it is equally likely that this heterogeneity is a reflection of inappropriate experimental or statistical detail and so serves to obscure the underlying biological message. Hence, whilst there may be some circumstances that merit tolerating a potentially wrong finding in acceptance of a research hypothesis [97], one has to be careful to distinguish such embracing of uncertainty from results that are plainly wrong.

Ultimately, it is essential that any other, competent laboratory can replicate a published qPCR-based finding. As discussed above, the difficulties of using different instruments, software, reagents, plates or seals can lead to often underestimated run-to-run differences that need to be compensated in order to make data comparable [98]. Whilst a strategy to detect and correct inter-experimental variation has been published recently [98], this still pre-supposes that variables such as samples and sample handling, template quality, targets and RT or PCR strategies are known and comparable.

#### 5. The MIQE guidelines

The above narrative has established the following framework: (i) experimental protocols enable research and define its outcome; (ii) publication of experimental protocols enables others to assess the caliber of that research and, if they so desire, attempt to reproduce the data; (iii) to-day's publications consist of a number of methods that, combined, allow conclusions to be drawn from a variety of complementary results; (iv) qPCR is but one of these methods and, inherently, no more important or controversial than any of the others likely to have been utilised. So why is there a need for a set of qPCR data publication guidelines? qPCR is frequently used as a "gold standard" to corroborate data obtained using other techniques, e.g. microarrays. This makes it a reference technique, and requires the application of superior standards to ensure its validity. Unfortunately, the coalescence of increased complexity of experimental methods together with steadily more

diversity of reagent and data analysis practices has resulted in an unsettled, inconsistent and subjective technology that is ill-suited to its status as a benchmark technology. Hence the principal aim of these guidelines is to encourage transparency of protocols, data analysis and conclusions so as to allow straightforward reproducibility or, at the very least, simple assessment of a publication's technical merit (Fig. 5).

A secondary aim is to provide a practical framework to help advance a punctilious implementation of a qPCR assay, although MIQE is certainly not designed to ordain how experiments must be carried out. Notwithstanding that, in general, researchers routinely deliberate on the elements of best practice, the outcome of these deliberations is rarely made explicit in their publications [99]. A consequence of the objective to provide a practical framework is the aspiration that the guidelines will drive progress towards “best practice” protocols, here defined as the theoretical and experimental approach that is most likely to lead to a biologically/clinically relevant qPCR result. Currently, the main barriers to the adoption of best practice are a lack of impetus to make the changes required by their adoption, a lack of knowledge and skills required to do so and, perhaps of greatest consequence, a lack of consensus about what constitutes best practice.

The MIQE guidelines propose such a consensus, offer the motivation for their implementation and provide guidance with respect to knowledge and skills. Of course, it is important to acknowledge that no practice can be best for everyone or in every situation, and no best practice remains best forever, since new enzymes, chemistries and analysis methods will continue to appear. Hence these guidelines must be seen as an evolving set of rules, with the flexibility to adjust to new developments. Furthermore, the current guidelines provide few detailed recommendations for experiments utilising multiplex assays or that target miRNA, SNP analysis or high resolution melts. These will need to be incorporated into an updated, Mk II version of MIQE.

Finally, the guidelines will also assist in supporting communication between researchers, especially when the recommendations with respect to a common qPCR language are implemented [100].

### 5.1. MIQE key issues

The four key areas of standardisation that define any qPCR experiment are (i) study design, (ii) technical detail, (iii) analysis methods and (iv) statistics. MIQE addresses these under a set of nine captions that describe a large number of individual elements (Fig. 6). At first sight, these look daunting, 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

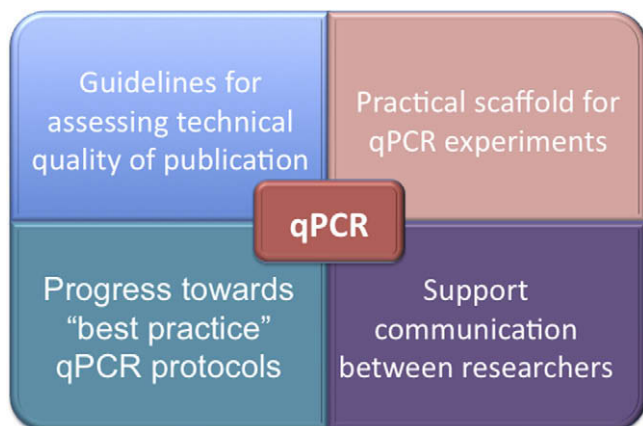


Fig. 5. Aims of the MIQE guidelines.

during the experimental design, optimisation and validation stages. Importantly, there is a clear hierarchy with some parameters, labelled E (essential) in the published guidelines, indispensable for attaining the ambition of the main aim, whereas other components, labelled D (desirable) more peripheral, yet constituting an effective foundation for the realisation of best practice protocols. Of course, these parameters are not set in stone and are open for discussion. Nonetheless, there is bedrock of requisite information that should be accessible to reviewer and reader and which is outlined below.

### 5.2. Detailed sample information

It remains a remarkable feature of many publications utilising 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 researcher(s) have gathered as much information as possible about their samples, the release of that information should add no additional burden to the wearisome task of gathering data for publication. Furthermore, it is also essential to provide details of sample processing procedures, since any sample has to pass through a number of preparative steps prior to the qPCR assay, every one of which can introduce additional variability [101,102].

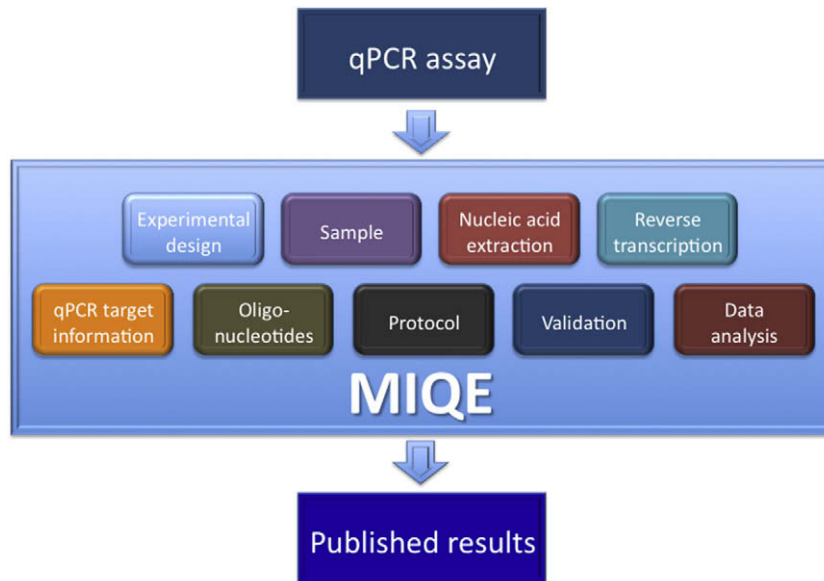
### 5.3. Basic (RT)-qPCR quality control metrics

The most essential details provide information about the quantity and quality of extracted nucleic acid, qPCR efficiency, evidence for specificity, details of limits of detection and details of control reactions. The use of degraded RNA vastly increases variability; it can generate  $C_q$ s that are falsely high, leading to an underestimation of target concentration and copy number. This effect of RNA quality on RT-qPCR results can be very pronounced, and have an enormous influence on the interpretation of qPCR results [56,57,103]. Although at least one model claims to be able to correct gene expression measured on highly degraded RNA [104], even if true this cannot replace a detailed evaluation of and striving for high RNA quality. The availability of microfluidics-based devices for nucleic acid quality assessment allows automated, rapid and standardised 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, hence the suggestion of introducing a 3':5' mRNA-specific integrity assessment [105]. 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 [55].

### 5.4. Target information/oligonucleotides

A search of GenBank for many nucleic acid sequences will reveal a number of variants, making it not always easy to deduce which particular pathogen, DNA or RNA has been targeted during the course of an experiment. Providing an accession number is the most basic, yet frequently neglected piece of information that must be published. Whilst not essential, it is considerate to provide additional target information, as this makes life easier for the harassed reviewer and interested reader.

In many ways the most controversial aspects of the MIQE guidelines relate to the proposal that publications must divulge the sequences of any primers used and especially should also report



**Fig. 6.** MIQE components. The qPCR assay is separated into nine major components that contain detailed information on pre- and post-assay parameters as well as comprehensive documentation of the experimental protocol.

the sequences of any probes. The rationale behind releasing the primer sequences is very 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. The problem lies with companies that supply ready-made assays that researchers purchase and use off the shelf. This point has been the subject of considerable discussion at many meetings, and can be debated endlessly. The key question is this: can reagent companies be trusted to design, validate and then empirically optimise assays and should other researchers, who may record different results, be forced to purchase such assays in order to trouble-shoot discrepant results? In my opinion, and this may not reflect the judgement of the MIQE co-authors, the answer must be no, and I believe there is no room for compromise here. If a commercial assay is well designed and utilises reagent manufacturer-specific modifications (e.g. MGB moieties, LNA or Pentabases), it is very unlikely that there will be a mass movement to have these assays synthesised elsewhere, especially since cost would not be significantly different. The manufacturers should understand that the impetus behind releasing all relevant information about a commercial assay is not a desire to poach an assay and manufacture it elsewhere, but the need to have as much information as possible available about a specific assay.

##### 5.5. Details of RT, if used, and qPCR efficiency

Since cDNA priming method and choice of RT have a significant impact on results [53,54], the extraordinarily sparse reporting of RT protocols makes the reproduction of published work problematic. It is essential that a publication describes in detail what cDNA priming method was used, with exact experimental conditions. 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.

The most commonly used models for the analysis of qPCR data use either the  $\Delta\Delta Cq$  [106] or the more generalised efficiency calibrated model [51] and updates or variations continue to be introduced [107,108]. However, confidence interval and statistical

significance considerations are still not accorded high enough priority [109] and many publications establishing relative mRNA expression levels never seem to have ensured that amplification efficiencies of target and reference genes are similar under treatment and control conditions. Indeed, there is a tremendous reluctance to use dilution curves to test the amplification efficiencies of individual assays, even though this method remains by far the easiest, most transparent and informative method for determining amplification efficiency as well as the relative gene expression abundance. Furthermore, dilution curves also provide convenient positive controls, can act as inhibition controls, and help define the dynamic range and the limits of detection all at the same time. Ideally such a dilution curve should be run with each sample, as all these parameters could (and probably do) vary between samples.

##### 5.6. Justification for normalisation procedure

It is disappointing to continue to see a large proportion of publications reporting relative expression of target genes relative to that of a single, unvalidated reference gene. The requirement for the establishment of appropriate normalisation parameters is an unavoidable and essential aspect of any qPCR assay [60]. The main argument of cost, preciousness of material or time-consuming obstacles cannot compensate for the fact that if results based on inappropriate normalisation are allowed to be published, quantification data may be wrong and everyone suffers. On the other hand, the question of which normalisation procedure is the most appropriate one remains a valid one. However, new reference targets and strategies are currently being identified that will make generalised normalisation much easier and so should remove any last obstacles from universally acceptable normalisation procedures.

##### 5.7. Replicates, repeats and statistical power

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 minimise 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 [110], the relative merits of obtaining  $C_q$ s from the threshold method or sigmoidal functions [111–113] and limits of detection modelled from sample replication and  $C_q$  values [114]. Furthermore, tools have become available that allow management and analysis of qPCR data [115–119]. 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 disastrous implications. A systematic evaluation of the various qPCR data analysis methods has shown that they differ substantially in their performance [120]; 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. Furthermore, far from increasing the reliability of results, technical replicates distort the statistics of determining confidence in experimental data. Hence 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 [121]. 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.

## 6. Conclusions

qPCR and RT-qPCR are powerful enabling technologies that have driven 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 intelligence. 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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