The need for transparency and good practices in the qPCR literature

Two surveys of over 1,700 publications whose authors use quantitative real-time PCR (qPCR) reveal a lack of transparent and comprehensive reporting of essential technical information. Reporting standards are significantly improved in publications that cite the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, although such publications are still vastly outnumbered by those that do not.

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Fluorescence-based qPCR is without doubt the premier molecular enabling technology for the detection and quantification of nucleic acids[1]. Its status is reflected in the rapid growth in the number of publications that use this technology, a trend driven by the growing awareness of newly discovered cell regulatory mechanisms, the continued search for diagnostic and prognostic biomarkers and concerns about bioterrorism[2]. To obtain consistent and biologically relevant qPCR measurements, researchers must complete a number of complex technical steps, adequately address a range of quality-control issues, use appropriate instrument settings to generate accurate amplification plots, and select the relevant statistical approach for analyzing their data. Finally, experimental details need to be reported in a transparent manner that permits replication of the experiment and quality assessment of the qPCR results.

The MIQE guidelines[3] aim "to encourage better experimental practice and more transparent reporting, resulting in more reliable, comparable and unequivocal interpretation of qPCR results"[4]. They are a response to the considerable misgivings with which many researchers perceive the quality of published qPCR data. That unease comes as a surprise to those who incorrectly believe that the conceptual simplicity and accessibility of qPCR translates into an equally uncomplicated experimental procedure.

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In reality, it is very easy to publish qPCR results that are meaningless[5]. Without transparency for optimization, validation and quality-control procedures, it is impossible for the reader of a publication to distinguish a reliable from a biased result or technical variation. This is particularly true for protocols aimed at quantifying RNA targets using reverse transcription qPCR (RT-qPCR), for which the relevance of the results is critically dependent on sampling procedure, sample properties, template quality and analysis procedures in addition to any relevant qPCR parameters[6].

The problems associated with deciphering the validity of molecular data are demonstrated clearly by the publication of two conflicting reports regarding the potential for *STK33*, which encodes a serine/threonine kinase, as a candidate drug target for tumors expressing mutant *KRAS*[7,8]—an inconsistency characteristic of much medical research[9]. We examined the respective methods sections of the two conflicting papers to try to determine which of the two conclusions is likely to be the correct one. The original publication[7] makes no mention of how RNA was prepared, quality assessed or reverse transcribed. The RT-qPCR section provides no information about experimental conditions. Furthermore, normalization was carried out using a single reference gene, without any evidence that it was validated for this study or that the efficiency of its amplification was determined. The information provided by the second publication again provides no information regarding RNA quality. Strangely, for a publication attempting to reproduce published RT-qPCR data, mRNA expression levels were quantified instead by a branched DNA assay. The absence of technical information detailing the RT-qPCR or branched DNA methods in either paper is typical of many publications and makes it impossible to establish which of the conclusions is the correct one. This was effectively expressed in a recent *Nature* Editorial[10], which emphasized the responsibility of journals "to exert sufficient scrutiny over the results that they publish" and to "publish enough information for other researchers to assess results properly."

The MIQE guidelines address the principal criteria that determine the quality of qPCR and RT-qPCR–based data. A reliable assessment of published data relies entirely on transparent reporting of those variables. Some of these parameters are more critical than others, and there are four categories that are absolutely fundamental: RNA quality, reverse transcription conditions, PCR assay details and data analysis methodology (**Fig. 1**). It is essential that reviewers and readers of scientific publications have access to this information. The online supplement has now become ubiquitous, removing the only possible argument against authors providing detailed technical information.

We have undertaken two large surveys of the peer-reviewed literature to investigate whether the reporting of qPCR-based data is sufficiently transparent to allow assessment and reproduction of the results. The surveys looked at the key parameters mentioned above and scored publications according to

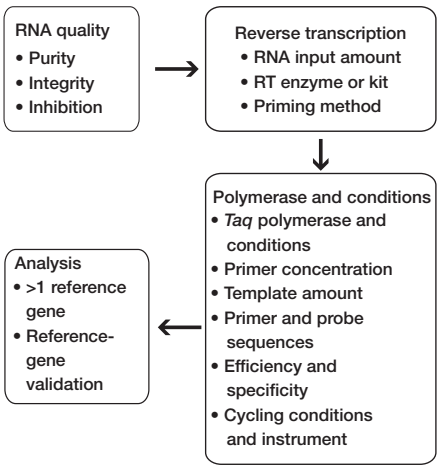

Figure 1 | Key parameters that determine the quality of qPCR data.

whether the necessary information was provided. The absence of information on some criteria—for example, RNA purity, integrity or inhibition—does not necessarily mean that the authors did not look at those parameters. They may simply have not reported their data. However, lack of information regarding PCR efficiency in experiments that compare the expression of a series of target mRNAs is a serious omission because small differences in this parameter can result in substantial shifts to the quantification cycle[11]. Similarly, the use of a single, unvalidated reference gene when normalizing experiments that attempt to demonstrate small differences in mRNA abundance has been shown to lead to unreliable conclusions, especially when used with tissue samples[12,13].

Survey from 2009 to 2011

The first survey covered an analysis of papers published in the years 2009–2011 (**Supplementary Data**). Each survey participant was instructed to choose 20 publications from any journal (**Supplementary Note 1**), although fewer than 20 papers were assessed for four of the journals, and more than 20 were assessed for eight of the journals (**Supplementary Table 1**). This resulted in an evaluation of 80 journals with impact factors (IFs) ranging from 1.9 to 32.2. There was no selection based on compliance or expected compliance or with any prior knowledge of compliance/non-compliance with the MIQE criteria. The only guideline was that participants would choose papers of interest to their area of research. Similarly, the journals were chosen at random from a subset that met the participants' interest and whose full-text versions were accessible to the participants. Fourteen key MIQE criteria were selected for analysis (**Supplementary Table 2**). The data were stratified into three groups according to the IFs of the journals: IF < 5, 5 ≤ IF < 10 and IF ≥ 10. Although all the journals gave authors the option of providing supplementary information, the participant use of this option was significantly positively associated with IF (**Supplementary Fig. 1**). In contrast, the higher the IF of a journal, the less information about the RT-qPCR assays was provided, as assessed by compliance with the fourteen MIQE criteria (ANOVA $P < 0.0001$; **Fig. 2a**). This translated into a negative correlation between the amount of relevant qPCR-specific technical information included in a publication and the IF of the publishing journal (**Fig. 2b** and **Supplementary Tables 3** and **4**).

It has been demonstrated that RNA purity, integrity and inhibitors affect the validity of any method aimed at quantifying RNA targets[14] and that monitoring RNA quality is of critical importance for obtaining meaningful and reliable gene expression data and for ensuring reproducibility of results[15]. Hence, the second focus of this survey was to investigate reporting of RNA purity and integrity. The aim was not to identify which methods were used to assess RNA quality parameters; instead, the requirement was just to find a mention of analysis of these criteria. Reporting of either parameter was exceedingly poor, and there was a significant negative correlation with IF (**Supplementary Figs. 2** and **3**).

The selection of appropriate reference genes for data normalization is one of the essential steps in the experimental design phase of a project. Normalization is required to minimize inherent technical or experimentally induced variation and confounding sample-specific variation, allowing accurate quantification of biological changes[6]. Although the advantage of using multiple, validated reference genes was demonstrated as early as 2002 (ref. 13), a survey carried out in 2005 on qPCR user practice revealed the widespread use of single, unvalidated reference genes[16], an approach that has been demonstrated to cause biased results[17]. Our survey reveals minimal change in the intervening 5 years (**Supplementary Figs. 4** and **5**): most publications included in this survey performed badly, with publications following best practice being rare. Furthermore, high-IF journals performed significantly worse than journals with lower IF: whereas 28% of journals in this survey with IF <5 did not have a single paper that used a validated reference gene, this portion rose to 73% in high-IF journals (**Supplementary Fig. 6**; $P = 0.012$, Fisher's exact test).

This leads to the conclusion that the qPCR data underlying the vast majority of publications reporting use of this technique are, at the very least, inadequately reported and that the peer review process allows the publication of incomplete experimental protocols, yielding results that are difficult to evaluate independently.

MIQE developments since 2009

The most enthusiastic early adopters of the MIQE guidelines were qPCR instrument manufacturers and reagent suppliers, who have provided their own specialists with extensive training and have been instrumental in bringing the guidelines to

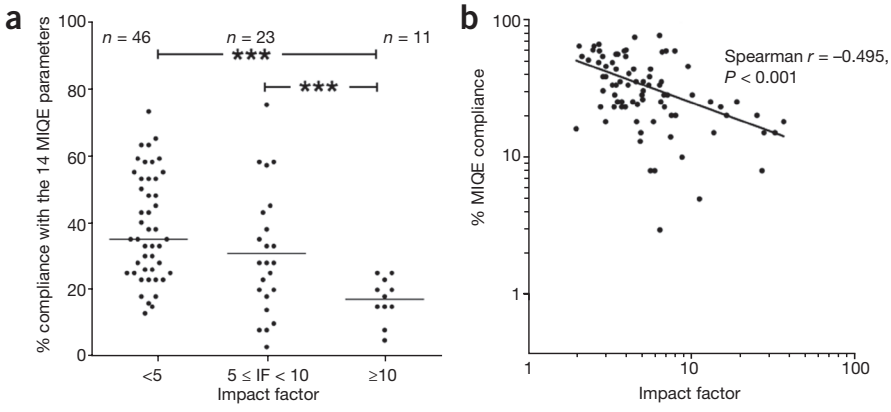

Figure 2 | Relationship between compliance with MIQE guidelines and journal impact factor (IF). (**a**) Each data point represents a journal and denotes the median compliance of the individual papers ($n = 1,623$) with all 14 MIQE parameters. The black horizontal bars indicate the overall medians. ***$P < 0.0001$. (**b**) Percent compliance versus IF.

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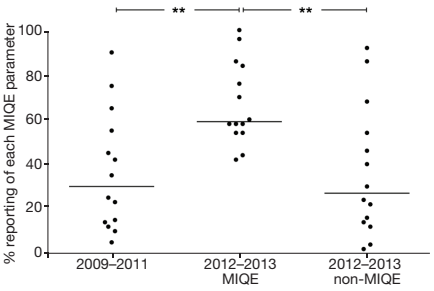

Figure 3 | MIQE impact on reporting transparency. Each data point represents 1 of the 14 MIQE parameters assessed (n = 1,623 papers for 2009–2011; n = 50 for papers citing MIQE in the 2013–2013 survey, and n = 50 papers not citing MIQE in the 2012–2013 survey). The horizontal bar indicates median compliance levels. Data pass both the D'Agostino-Pearson and Shapiro-Wilk normality tests. $^{**}P$ = 0.0027.

the attention of a worldwide audience by sponsoring workshops, seminar series and Internet webinars. This has resulted in the curious situation wherein most companies' technical specialists are more expert at performing qPCR experiments than their customers at the academic bench. In addition, there have been academia-led workshops, such as the successive European Molecular Biology Laboratory (EMBL) master courses on MIQE and the qPCR symposia series held every 2 years at the Technical University Munich in Freising-Weihenstephan or in the San Francisco Bay Area. Specialist qPCR analysis software has been developed, making it relatively straightforward to comply with MIQE requirements for experimental setup, assay optimization and appropriate data analysis. There has also been a *Science*/AAAS webinar entitled "The Future of qPCR: Best practices, Standardization, and the MIQE Guidelines" (http://webinar.sciencemag.org/webinar/archive/future-qpcr/). There have been editorials in *BMC Molecular Biology*[18], *The Veterinary Journal*[19] and the *International Journal of Molecular Sciences*[20] promoting the idea of the submission of comprehensive experimental protocols. *Nucleic Acids Research*, *PeerJ*, *Molecular Medicine*, *European Urology*, *Journal of Clinical Microbiology*, *Journal of Molecular Medicine* and *Reproduction, Fertility, and Development* have recommended—and *Clinical Chemistry* has mandated—adherence to the essential MIQE parameters. In addition, *Nature* journals have removed length limits on Online Methods, which should also encourage the publication of more detailed methods.

This intensive promotion of the guidelines has begun have an impact on the awareness of the research community that there is a need for appropriate quality-control reporting for qPCR experiments. The original MIQE publication is the fifth-most-cited publication in *Clinical Chemistry*, having been cited over 1,800 times, with more than 600 citations between January and September 2013. Papers citing the MIQE guidelines are still a minority, but a PubMed search for the terms "'real-time reverse transcription PCR' or RT-qPCR or qRT-PCR" and cross-referencing with the Web of Knowledge for MIQE-citing papers showed that around 5% of qPCR-based papers from 2011 cite the MIQE guidelines, which increased to 7% for 2012 and 11% so far for 2013 (χ^2 $P < 0.0001$; **Supplementary Table 5**). Nevertheless, it is uncertain whether researchers citing the guidelines do so because they are convinced of their relevance or because they feel peer pressure to do so.

Survey from 2012 to 2013

To investigate whether citation of the MIQE guidelines and improved transparency of reporting are correlated, we conducted a second survey covering papers published in the years 2012 and 2013, analyzing 178 publications from three categories: those that cite the MIQE guidelines, those that do not and, as a separate group, those published in three high-IF journals (**Supplementary Note 2**). An addendum to the MIQE guidelines discusses the use of predesigned commercial assays, for which primer and probe sequences are not disclosed, and how to report adequately their location and validation to make studies using these assays as MIQE compliant as possible[21]. Hence, we further subdivided the publications in the survey (MIQE and non-MIQE) into those that used commercial assays and those that did not. No other selection or preselection criteria were used (**Supplementary Data**).

The most notable conclusion from this survey is that the MIQE guidelines are having a significant impact on the quality of data reporting in 2012–2013 publications that use qPCR. There was a consistent and significant increase in the comprehensiveness of reporting of the 14 parameters by papers citing the MIQE guidelines, especially with regard to RNA quality, PCR efficiency and data normalization procedures (ANOVA P = 0.0027), with respect to all analyzed qPCR papers from 2009–2011 (**Fig. 3** and **Supplementary Table 6**). In contrast, there was no improvement (P = 0.9371) in the transparency of reporting, relative to the 2009–2011 publications, in the 2012–2013 publications whose authors did not cite the MIQE guidelines. A good example of how to report methods and results is shown in supplementary table S1 of a recent publication[22].

The significance of this finding was maintained when publications reporting the use of commercial assays and either citing or not citing the MIQE guidelines were compared (paired t-test P = 0.0169; **Fig. 4** and **Supplementary Table 7**). Fewer parameters were analyzed because not all 14 criteria are relevant for commercial assays, which include PCR arrays. However, it is encouraging to note that many researchers who are aware of the MIQE guidelines go to considerable lengths to acquire more information about the commercial assays they are using than is automatically provided by the manufacturers. Interestingly, this extends to the information provided about the sample itself: around 50% of MIQE-citing papers provided information about RNA purity and integrity, compared with fewer than 20% of those papers not citing the guidelines.

The respective median IFs of the surveyed journals containing papers that cite (2.91, range = 1–18.04) or do not cite (3.31, range = 0.11–24.76) MIQE in 2012–2013 were not significantly different (Mann-Whitney test P = 0.43; **Supplementary Fig. 7**). However, when we analyzed compliance with the MIQE guidelines from papers published in three high-IF journals (*Nature*, *Science* and *Cell*), with no selection for citation of MIQE, the results

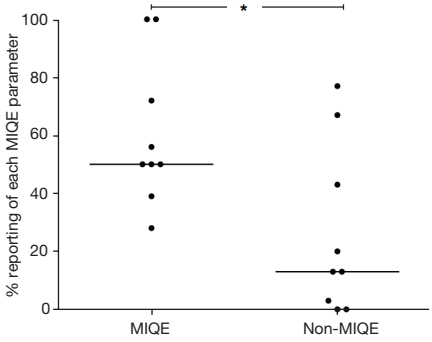

Figure 4 | MIQE impact on commercial assays used in 2012–2013 publications. Nine relevant MIQE parameters were compared between publications citing (n = 18) and those not citing (n = 30) the MIQE guidelines. Each data point represents one of the nine parameters assessed; the horizontal bar indicates median compliance levels. Data pass both the D'Agostino-Pearson and Shapiro-Wilk normality tests. $^{*}P$ = 0.0169.

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BOX 1 PRINCIPAL CONCLUSIONS

- The amount of essential technical detail on qPCR experiments reported in most papers is inadequate despite the provision of online supplements. The higher the impact factor of the journal, the less information is provided (2009–2011 survey).
- Very few papers published from 2009 to 2011 reporting use of RT-qPCR provide any information about RNA purity or integrity.
- Normalization procedures in papers for both surveys (2009–2011 and 2012–2013) are inadequate and therefore likely to generate questionable results.
- The transparency of experimental reporting is significantly improved in papers citing the MIQE guidelines. However, these papers are still vastly outnumbered by those that do not cite the guidelines, which continue to report inadequate experimental procedures.
- Researchers that use commercial assays and cite MIQE provide more comprehensive experimental details than those who use commercial assays and do not cite MIQE.

indicated that the quality and completeness of reporting in these journals was significantly lower than that of publications selected for analysis on the basis of MIQE citation (Mann-Whitney test $P < 0.0001$; **Supplementary Fig. 8**), consistent with the findings of a previous, small survey[23]. This is particularly disappointing when we consider that every one of these papers makes use of the online supplement and often includes detailed additional information on many other aspects of the techniques used.

Conclusions

qPCR is probably the most widely used technique in molecular biology, but a widespread lack of transparency, standardization and assay quality control precludes it from being a 'gold standard'. The results of our surveys suggest that the quality of reported qPCR data cannot be evaluated in a high percentage of publications owing to a lack of transparent reporting of technical and quality-control details, and this deficiency makes it difficult to assess the biological or clinical relevance of the results (**Box 1**). Unfortunately, peer review and publication *per se* confers a certain stamp of approval on a paper that makes it very difficult to contradict its conclusion, even if rebutted by other publications[24].

A prescient and courageous review concluded that progress in clinical research is hindered by the lack of relevance and congruence of *in vitro* and animal models to human disease[25]. The findings of our investigation suggest an additional reason for the many contradictory results that have been published over the years: the inappropriate application of an extremely powerful and 'simple' technology, exacerbated by poor standards of reporting of its technical details. The results of our more recent survey give some reason to hope that this has started to change. Nevertheless, it is also apparent that even those papers that cite the MIQE guidelines do not necessarily contain all essential technical information.

The quantitative concepts introduced by qPCR challenge the thinking of molecular biologists rooted in qualitative analysis. Biologists in general may not be used to observing tight standards and guidelines and often consider exact definitions of assay conditions to be of minor importance. However, much of modern biology has become quantitative, and qPCR acts as a bridge into the brave new world of systems biology–based studies, where quality control and validation are essential criteria. Implementation of the MIQE guidelines, or at least the most essential ones, in publication guidelines will help qPCR fulfill that role. Even if awareness of the MIQE guidelines increasingly penetrates the collective consciousness of the research community, there remains the problem of a huge body of literature that reports conclusions that may be meaningless and will cause research resources to be wasted. For now, we conclude that the integrity of the scientific literature that depends upon qPCR data is severely challenged and that the MIQE guidelines are useful for improving these data. We call upon journal editors to implement more stringent quality-control measures for publication.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/nmeth.2697).

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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