

Real-Time Reverse Transcription Quantitative PCR (RT-qPCR) Methodological Standards and Reporting Practices

Stephen A. Bustin ^{a,*} and Carl T. Wittwer^b

BACKGROUND: Real-time reverse transcription quantitative PCR (RT-qPCR) is utilized in many areas of the life sciences, diagnostics, and forensics, yet concerns about methodological quality and reporting transparency persist. Diagnostic testing during the recent pandemic brought those concerns into the public domain. The Minimum Information for Publication of Quantitative PCR Experiments (MIQE) guidelines, introduced in 2009 and updated in 2025, were intended to standardize assay design and reporting, but their impact has been modest.

CONTENT: We assessed trends in RT-qPCR methodological reporting between 2007 and 2025 using PubMed Central searches and manual evaluation of 355 full-text articles from 2019 and 2024. Parameters analyzed included RNA integrity assessment, oligonucleotide sequence disclosure, reference gene validation, PCR efficiency reporting, and MIQE citation. In addition, targeted cohorts of 50 “reference gene” and 50 “PCR efficiency” publications from 2024/25 were evaluated. Results were compared across timepoints, geographic regions, and MIQE-citing vs non-citing studies.

SUMMARY: Reporting of core parameters remained low or declined. Between 2019 and 2024, RNA integrity reporting fell (22% to 11%), reference gene validation was rare (13% to 5%), and PCR efficiency reporting collapsed (13% to 1%). MIQE-citing papers in 2024 showed better adherence (31% RNA integrity, 47% reference gene validation, and 40% PCR efficiency) but still omitted essential details. Asia now dominates RT-qPCR output by volume, while Europe contributes most MIQE-citing studies. Targeted cohorts reported more methodological information, yet many still failed to meet basic standards. These findings confirm that incomplete experimental design

and reporting continue to undermine reproducibility and robustness of RT-qPCR assays.

Introduction

Real-time reverse transcription quantitative PCR (RT-qPCR) is arguably the most widely applied molecular technology, underpinning gene expression studies, diagnostic testing, forensic investigations, and diverse applications across the life sciences. During the COVID-19 testing surge, this laboratory method became a frontline clinical assay, with results directly influencing patient care, public health measures, and national policy (1). For the first time, the limitations of qPCR moved beyond the scientific community and into public awareness, as questions over false positives, false negatives, and assay comparability gained widespread attention (2–5).

These concerns echo long-standing issues with reproducibility, incomplete reporting, and variable methodological rigor that pre-date COVID-19 (6–8) and compromise confidence in qPCR-derived data (9). Misuse of the technology has real public health consequences, most notably in the now discredited study claiming to detect measles virus in intestinal biopsies from children with autism. That study, based on flawed RT-qPCR methods, contributed to widespread misinformation about the measles–mumps–rubella (MMR) vaccine and fuelled vaccine hesitancy (10). In response to such misuse and to broader concerns about inconsistent experimental quality and incomplete reporting, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were introduced in 2009 (11). They were intended to improve both technical performance and methodological transparency, thereby reducing misinterpretation and enhancing reproducibility (12, 13). However, uptake has been inconsistent, and key quality-assessment parameters are often omitted, as previously documented in a large-scale 2013 analysis of MIQE compliance (14). Subsequent surveys (15–18) and the present study provide further snapshots showing that little substantive progress has occurred. Whilst these studies provide valuable snapshots of practice at single timepoints, they have not assessed whether

^aMedical Technology Research Centre, Faculty of Health, Education, Medicine and Social Care, Anglia Ruskin University, Chelmsford, United Kingdom; ^bSchool of Medicine, University of Utah, Salt Lake City, UT, United States.

*Address correspondence to this author at: MTRC, Michael Salmon Building, Bishop Hall Lane, Chelmsford, Essex CM1 1SQ, United Kingdom. E-mail stephen.bustin@aru.ac.uk.

Received August 30, 2025; accepted November 7, 2025.
<https://doi.org/10.1093/clinchem/hvaf176>

heightened visibility during the COVID-19 testing surge has led to measurable improvements in methodological quality across the broader literature.

The global diagnostic expansion during the COVID-19 pandemic offered a natural experiment to ask whether heightened scrutiny improved RT-qPCR practice. To address this, we conducted a longitudinal analysis of RT-qPCR publications from 2007 to 2025, quantifying trends in the reporting of RNA integrity, oligonucleotide sequences, reference gene validation, PCR efficiency, and MIQE citation. We then performed a detailed manual assessment of 355 full-text articles from 2019 and 2024, analyzed the geographical distribution of publications, and evaluated methodological rigour and MIQE compliance. Finally, to assess whether heightened visibility influenced methodological rigor, we examined whether MIQE-citing papers published in 2024 showed improved adherence to best practice compared with the general (non-MIQE-citing) cohort from 2019.

Methods

SEARCH STRATEGY FOR PUBLICATION TRENDS (2007 TO 2025)

We conducted 5 broad searches in the PubMed Central (PMC) database (<https://pmc.ncbi.nlm.nih.gov>) to evaluate publication trends between 2007 and 2025. The first search recorded the total number of publications containing any of the terms “RT-qPCR,” “real-time RT-PCR,” or “qRT-PCR” (Search 1). Papers containing more than one search term may appear in multiple parameter counts, as we did not perform cross-term deduplication using a combined Boolean query. Each parameter was analyzed independently and expressed as a proportion of all RT-qPCR publications for that year. This approach avoids undercounting multi-term papers and does not distort the reported frequencies or observed trends.

Subsequent searches combined these terms with “RNA integrity” (Search 2), “reference genes” (Search 3), or “PCR efficiency” (Search 4), and recorded raw publication counts without adjustment for overlap. A fifth search used the standalone term “MIQE.”

CORE DATA SET ASSEMBLY FOR 2019 AND 2024/5

Three core sets of full-length, primary research articles were randomly retrieved from the PMC database. Reviews, commentaries, and preprints were excluded. Two sets used the term “RT-qPCR,” restricted to publications from January 1 to December 31 in either 2019 or 2024. The third set used the term “MIQE,” restricted to 2024 to 2025. To avoid overrepresentation of reference gene studies, any paper with “reference gene” or “housekeeping gene” in the title was excluded. The resulting 255 papers (3 groups of 85) comprised

RT-qPCR studies published before and after the COVID-19 pandemic, as well as MIQE-citing studies published in its aftermath.

TARGETED SUBSETS FOR REFERENCE GENE AND PCR EFFICIENCY REPORTING

Two additional targeted searches were carried out for 2024 to evaluate whether technical focus was associated with improved methodological reporting. The first subset comprised 50 papers with “reference gene” in the title, selected at random. The second included 50 papers retrieved using the keyword “PCR efficiency.” Both subsets were evaluated using the same scoring framework applied to the core data set.

SCORING CRITERIA

Each paper was evaluated for 5 predefined parameters. Each parameter was scored as 1 if clearly fulfilled, and 0 otherwise. Mentions without supporting methods or data were scored 0. The parameters were as follows:

1. MIQE citation: whether the study explicitly cited the MIQE guidelines.
2. RNA integrity assessment: whether RNA quality was assessed using electrophoretic methods (e.g., agarose gel or automated platforms). Studies reporting only A260/280 ratios were scored 0.
3. Primer and probe disclosure: whether full primer and, where applicable, probe sequences were reported. Commercial assays without sequence disclosure were scored 0.
4. Reference gene validation: whether multiple candidate genes were evaluated using expression stability tools such as BestKeeper (19) or geNorm (20). Vague mentions of normalization or use of a single default gene were scored 0.
5. PCR efficiency reporting: whether numerical efficiency values were determined and reported.

Detailed publication lists, individual methodological scores, and source URLs are provided in the [Supplementary Material](#) for the manually evaluated 2019 and 2024/25 cohorts.

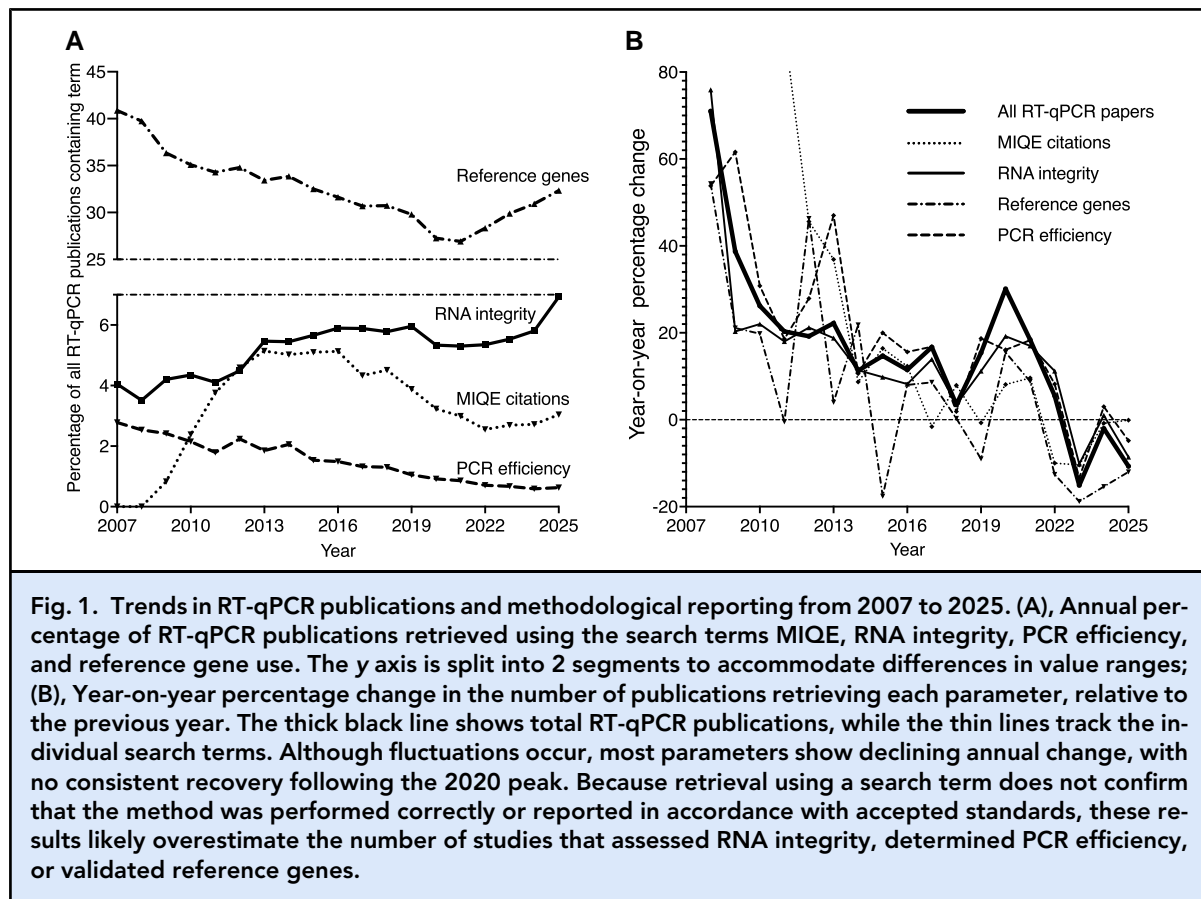
GEOGRAPHIC AND JOURNAL METRICS

Geographic region was assigned based on the first author’s institutional affiliation and categorized as follows: United States; Americas (excluding the United States; including Canada, Mexico, Brazil, and Colombia); Europe; Asia (China, Japan, and South Korea); and other (including Australia, New Zealand, and South Africa).

Journal impact factor (IF) values were retrieved from publisher websites in June 2025.

DATA EXTRACTION AND STATISTICAL ANALYSIS

Screening and scoring were performed manually. Statistical analyses were conducted using GraphPad Prism for Mac



(v10.5). Differences in proportions were evaluated using two-tailed z -tests for independent samples. Calculations were cross-checked in Excel using the z -statistic and the NORM.S.DIST function to obtain two-tailed P values.

χ^2 tests were also performed where appropriate to compare categorical distributions across multiple groups, particularly in geographic analyses. z -Tests were used for direct pairwise comparisons of proportions (e.g., MIQE vs non-MIQE; reference gene vs PCR efficiency cohorts), as they provide a more precise test of differences between 2 independent proportions. χ^2 Tests were reserved for comparisons involving more than 2 categories, where z -tests are not applicable. The Fisher exact test was used once, for the 50-paper reference gene and PCR efficiency cohorts, where expected counts were small.

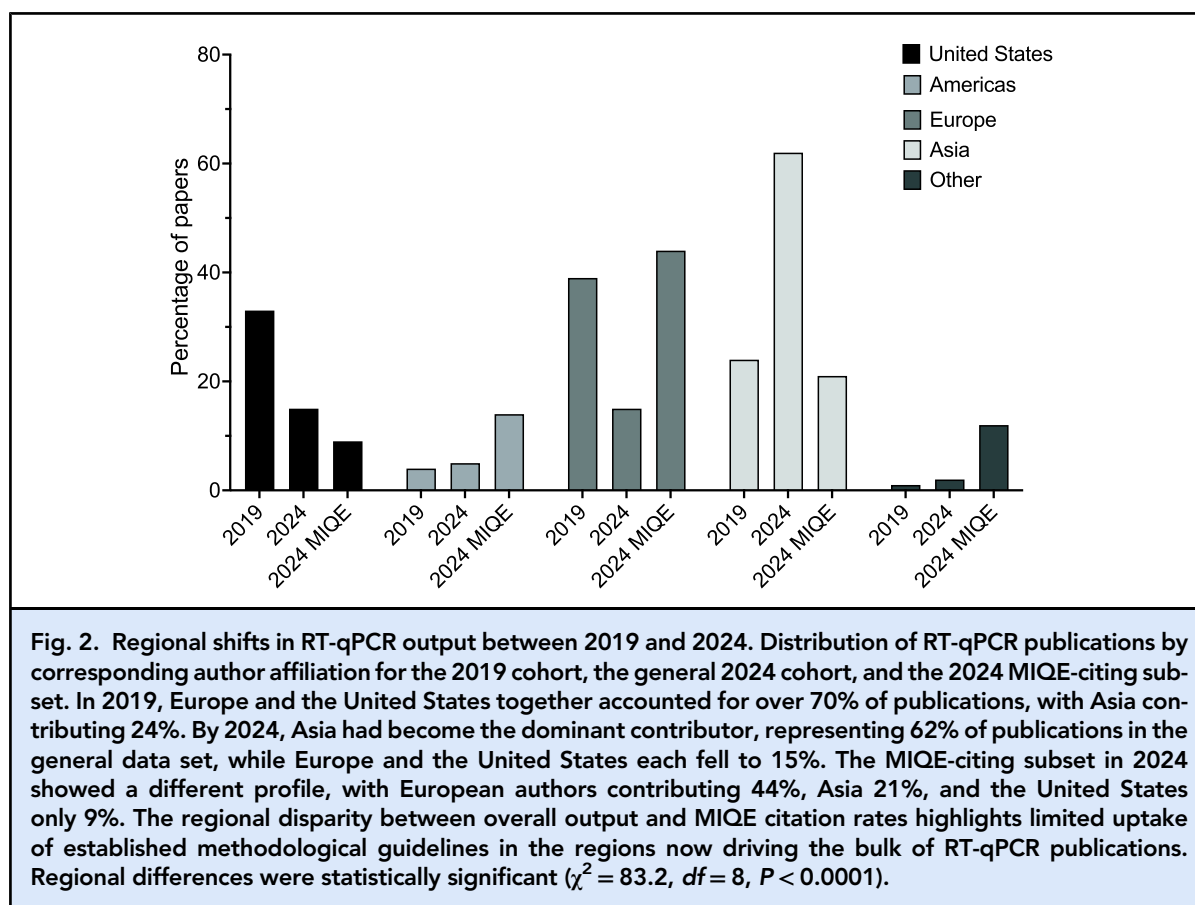
Results

PUBLICATION TRENDS AND METHODOLOGICAL MENTIONS (2007 TO 2025)

To assess whether methodological reporting has changed over time, we analyzed RT-qPCR publications

indexed in PMC from 2007 to 2025. The total number of RT-qPCR publications indexed in PMC rose from 3340 in 2007 to 56 545 in 2022, followed by a modest decline to 49 733 in 2025. Given the sharp increase in RT-qPCR usage during the pandemic, we examined whether this expansion influenced the adoption of reporting standards. Four parameters were assessed: MIQE citation, RNA integrity, PCR efficiency, and reference gene use. Two complementary analyses were performed: (a) the annual percentage of RT-qPCR-related publications retrieved using each search term, and (b) the year-on-year percentage change in the number of publications retrieved by each parameter (Fig. 1).

Because retrieval using a search term does not confirm that the method was performed correctly or in compliance with accepted standards, these values are likely to overestimate the true frequency of methodological assessment and reporting. MIQE citation peaked between 2014 and 2016 at approximately 5%, followed by a gradual decline in subsequent years (Fig. 1A). References to RNA integrity increased modestly from 4% (146/3340) in 2007 to 6% (2975/34 845) by



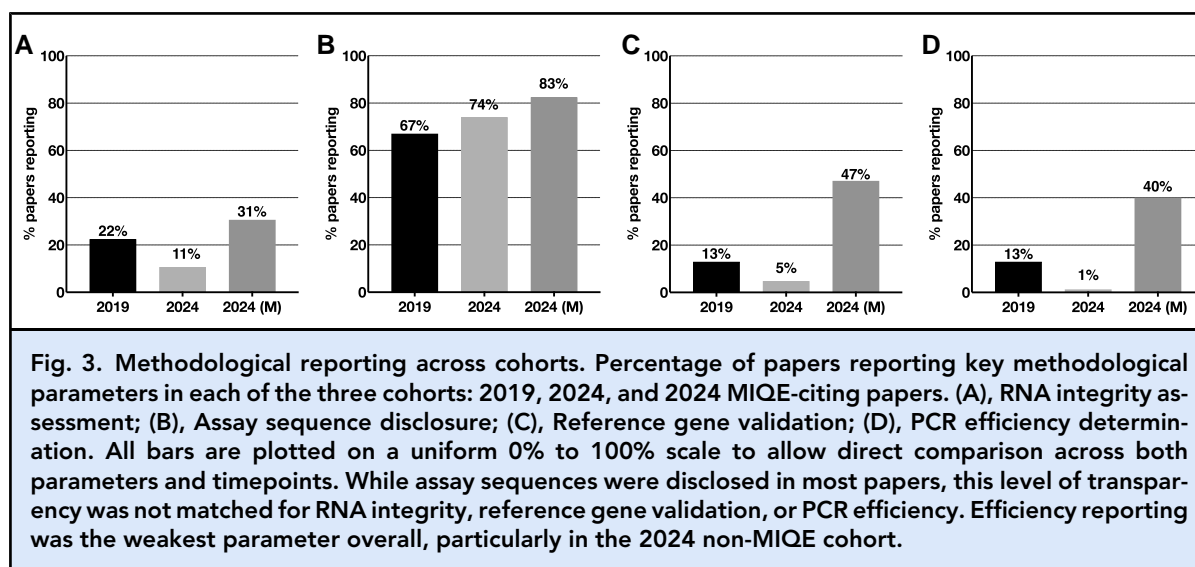
2019, before declining to 5% (2856/53 846) in 2021 and increasing to 7% (3446/49 733) by 2025 ($P = 0.041$, two-proportion z -test for 2007 vs 2019). Mentions of “PCR efficiency” fell significantly from 3% (100/3340) in 2007 to <1% (313/49 733) in 2025 ($P < 0.001$). “Reference-gene” use decreased from 40% (1336/3340) prior to 2009 to 31% (17 529/56 545) in 2019 and 32% (16 080/49 733) in 2025, a net decline of about 8% ($P < 0.001$).

The year-on-year analysis (Fig. 1B) shows that although the absolute number of RT-qPCR publications continued to increase until 2022, the rate of growth declined over time, except for a transient increase during the pandemic years. Because Fig. 1B depicts year-on-year percentage change rather than absolute frequency, values can remain positive even when long-term trends are declining. For example, the proportion of papers mentioning “PCR efficiency” decreased steadily over the study period (Fig. 1A), but occasional years of slower decline or small transient increases produce brief positive changes in Fig. 1B. This slowing was mirrored by all four parameters, each showing diminishing year-on-year gains that eventually became negative. The correspondence between these patterns suggests

no consistent recovery following the temporary 2020 to 2021 surge, with methodological engagement declining thereafter.

REGIONAL SHIFTS IN RT-QPCR OUTPUT BETWEEN 2019 AND 2024

An analysis of corresponding author affiliations reveals substantial regional shifts in RT-qPCR research output between 2019 and 2024 (Fig. 2). In 2019, publications were distributed across Europe (39%), the United States (33%), Asia (24%), the Americas excluding the United States (4%), and “other” regions (1%). By 2024, Asia had become the dominant contributor, accounting for 62% of the general data set compared with 24% in 2019. This expansion was accompanied by marked declines in Europe and the United States, each falling to 15% of publications. The 2024 MIQE-citing subset showed a different pattern: European authors accounted for 44% of papers, Asia 21%, and the Americas excluding the United States 14%, while the United States contributed only 9%. These regional differences were statistically significant ($\chi^2 = 83.2$, $df = 8$, $P < 0.0001$). Asia’s increased share of global output, driven



predominantly by China, was not matched by proportionate MIQE citation. This discrepancy highlights a growing gap between output and adherence to reporting standards, with potential implications for the reliability of diagnostic assay development and validation.

We extracted the journal IFs for all papers and compared their distributions across the three cohorts (2019, 2024, and 2024 MIQE-citing). As the data were not normally distributed, a nonparametric Kruskal–Wallis test was used to compare median IFs. No significant differences were observed between the groups ($H=0.48$, $P=0.785$), indicating that journal rank, as indexed by IF, did not account for differences in methodological reporting. Papers were also categorized by whether they appeared in journals with IFs above or below a pragmatic threshold of 5. Two-proportion z -tests, which evaluate binary differences between independent samples, showed no significant differences between groups ($P>0.05$), indicating that MIQE citation was not associated with journal IF.

CHANGES IN REPORTING OF METHODOLOGICAL PARAMETERS

We next assessed the reporting of 4 methodological parameters across the three cohorts published in 2019, 2024, and 2024 citing MIQE, as shown in Fig. 3.

RNA Integrity. Reporting of RNA integrity declined from 22% (19/85) in 2019 to 11% (9/85) in 2024 (two-proportion z -test, $P=0.039$). Among the 2024 MIQE-citing papers, reporting was significantly higher at 31% (26/85) compared with non-MIQE papers ($P=0.0013$; Fig. 3A).

Assay Sequence Disclosure. Reporting rates were consistently high across all cohorts. The few papers scored as

“not reporting” had used commercial assays for which primer and probe sequences were unavailable. Although most provided manufacturer and catalog details, none included amplicon context information (such as target exon, transcript, or genomic location). Because MIQE stipulates that such contextual details are essential to permit assay interpretation and replication, these papers were scored as not reporting. This indicates that genuine sequence transparency, either through direct disclosure or adequate contextual information is good but remains incomplete. (Figure 3B).

Reference Gene Validation. Reporting declined from 13% (11/85) in 2019 to 5% (4/85) in 2024 (two-proportion z -test, $P=0.12$), without year-to-year improvement. In the 2024 MIQE-citing cohort, however, reporting was substantially higher at 47% (40/85) ($z=6.62$, $P<0.0001$; Fig. 3C).

PCR Efficiency. Reporting fell from 13% (11/85) in 2019 to 1% (1/85) in 2024 (two-proportion z -test, $P=0.004$), indicating a significant decline. In the 2024 MIQE-citing group, 40% (34/85) of papers reported PCR efficiency, representing a marked improvement compared with the non-MIQE cohort ($z=6.36$, $P<0.0001$; Fig. 3D).

These results show that the 2024 MIQE-citing papers reported methodological details more consistently across all four parameters. However, this improvement was not reflected in the broader 2024 cohort, where reporting of core technical details remained low, highlighting persistent gaps in RT-qPCR methodological transparency.

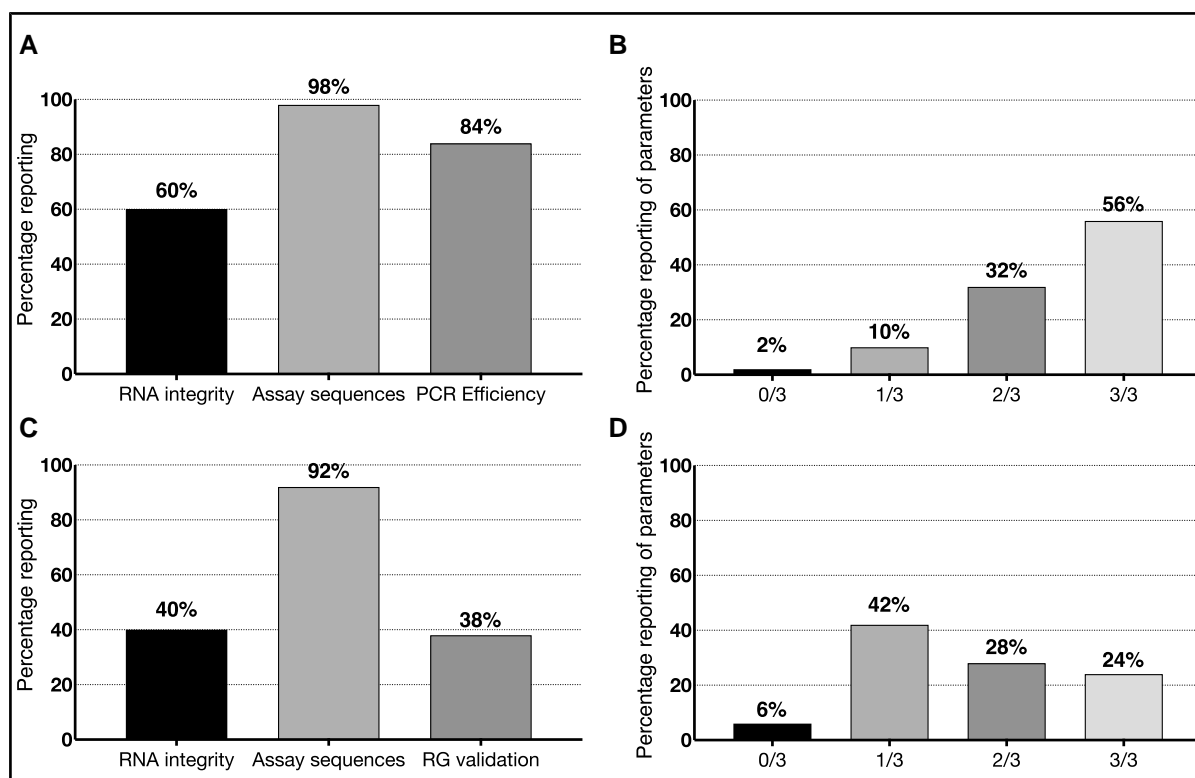


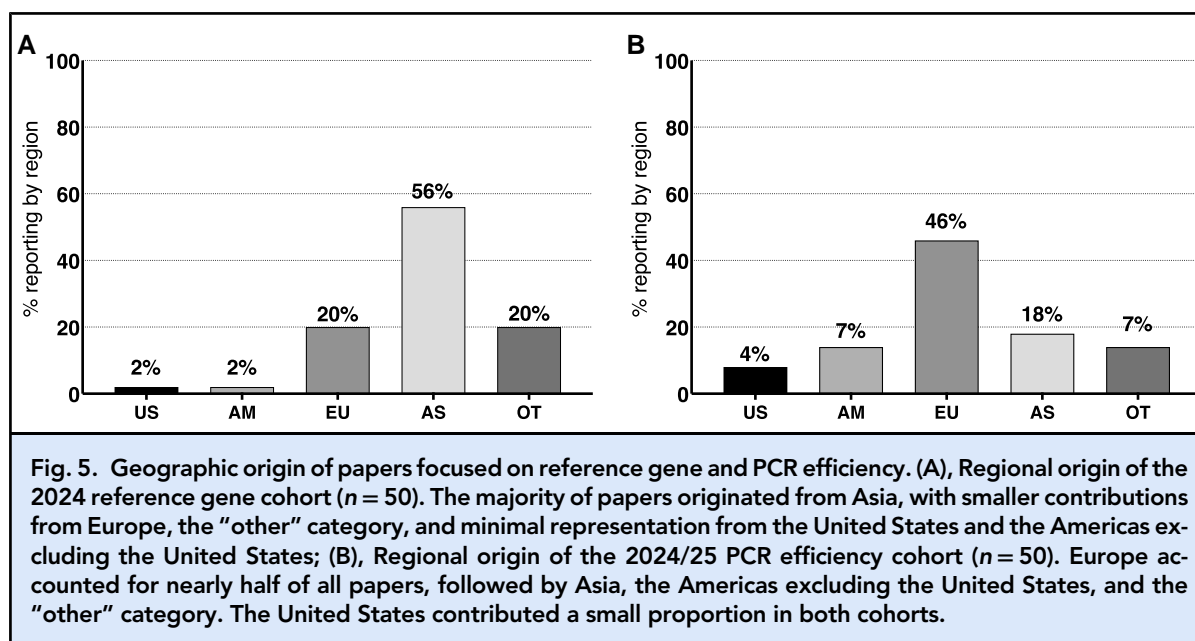
Fig. 4. Methodological reporting focused on reference gene and PCR efficiency. (A), Percentage of 2024 reference gene papers ($n = 50$) reporting RNA integrity, PCR efficiency, and assay sequence disclosure; (B), Methodological completeness in the reference gene cohort, calculated as the proportion of papers reporting all three parameters (RNA integrity, PCR efficiency, and assay sequences), two, one, or none (reference gene validation was present by definition and excluded from scoring); (C), Percentage of PCR efficiency papers ($n = 50$) reporting RNA integrity, reference gene validation, and assay sequence disclosure; (D), Methodological completeness in the PCR efficiency cohort, calculated as the proportion of papers reporting all three parameters (RNA integrity, reference gene validation, and assay sequences), two, one, or none (PCR efficiency was present by definition and excluded from scoring). In both cohorts, assay sequence disclosure was high, but the reference gene cohort reported RNA integrity and PCR efficiency more frequently, and a significantly greater proportion reported all three assessed parameters (56% vs 24%, $z = 3.27$, $P = 0.0011$).

TARGETED ANALYSIS OF REFERENCE GENE AND PCR EFFICIENCY PAPERS

Because reference gene validation is critical for both the discovery and diagnostic phases of RT-qPCR, we examined whether studies explicitly addressing reference gene selection adhered more closely to key technical standards. We identified 50 publications from 2024 that included the terms “RT-qPCR” and “reference gene” in the title (Fig. 4). This cohort showed markedly higher reporting rates for RNA integrity and PCR efficiency than were observed in any other group analyzed. RNA integrity was reported in 60% of papers and PCR efficiency in 84%, while assay sequences were disclosed in 98% (Fig. 4A). Within this set, 34 papers cited MIQE

and 16 did not. MIQE-citing papers reported slightly higher rates of RNA integrity (65% vs 50%), PCR efficiency (88% vs 75%), and assay sequence disclosure (100% vs 97%), but none of these differences were statistically significant (Fisher exact test, all $P > 0.2$). Both MIQE and non-MIQE studies therefore outperformed the broader literature, suggesting that the technical orientation of reference gene papers, rather than MIQE citation, drove more complete methodological reporting.

When methodological completeness was assessed by counting the number of parameters reported (RNA integrity, PCR efficiency, and assay sequences; reference gene validation was present by definition), 28 papers (56%) reported all three, 16 (32%) reported two, 5



(10%) reported one, and one (2%) reported none (Fig. 4B). This distribution confirmed that although reference gene-focused studies were more detailed than the general literature, almost half still failed to report all three key parameters.

A final 50 papers were selected from a search of 2024/25 publications that used the terms “RT-qPCR” and “PCR efficiency.” Of these, 20 (40%) reported RNA integrity, 46 (92%) disclosed assay sequences, and 19 (38%) reported validated reference genes (Fig. 4C). When methodological completeness was assessed by counting the number of parameters reported (RNA integrity, assay sequences, and reference gene validation; PCR efficiency was excluded as it was present by definition), 12 papers (24%) reported all three, 14 (28%) reported two, 21 (42%) reported one, and 3 (6%) reported none (Fig. 4D).

Compared to the reference gene cohort with 28 of 50 papers (56%) reporting all three relevant parameters, only 12 of 50 (24%) in the PCR efficiency cohort did so ($z = 3.27$, $P = 0.0011$). This suggests that studies focused on reference gene selection tend to adhere more closely to methodological best practice than studies focused on PCR efficiency. RT-qPCR assays rely on both appropriate reference gene selection and evaluation of PCR efficiency for accurate quantification of pathogen load or gene expression changes. Studies on reference gene validation and efficiency assessments often focus on technical issues rather than extensive experimental research. Such work is critical in diagnostics because inadequate normalization or

poorly characterized amplification performance can undermine the reliability of clinical results.

GEOGRAPHIC VARIATION IN TARGETED COHORTS

The geographic distribution of papers diverged from previous trends (Fig. 5A). Within the reference gene cohort, MIQE-citing ($n = 34$) and non-MIQE ($n = 16$) studies showed similar patterns with no significant differences between them. Analyzing the combined data set of 50 papers, the majority originated from Asia (28/50, 56%), with smaller contributions from Europe (10/50, 20%), “other” regions (10/50, 20%), and only isolated representation from the United States (1/50, 2%) and the Americas outside the United States (1/50, 2%). Compared with the 2024 MIQE cohort of 85 papers (Fig. 2), which was dominated by European output (44%) with far fewer contributions from Asia (21%), the shift was striking. A χ^2 test confirmed that the distribution of reference gene studies differed significantly from that of the 2024 MIQE cohort ($\chi^2 = 25.0$, $df = 4$, $P < 0.0001$). This change reflects Asia’s emergence as the predominant contributor to RT-qPCR reference gene studies, while Europe and the United States now account for a much smaller proportion.

In the PCR efficiency cohort, Europe was the most frequent contributor (46%), followed by Asia (18%), the Americas excluding the United States (7%), and the “other” category (7%). The United States again contributed only a small proportion (4%) (Fig. 5B). Although the sample sizes are modest, the differences suggest underlying regional variability in methodological focus and publication patterns.

Discussion

The reliability of molecular methods, including qPCR-based conclusions, is compromised by inconsistent design and incomplete reporting, which continue to undermine reproducibility in biomedical research and public trust in diagnostic science (17, 21). This study provides current, empirical evidence that those concerns remain justified. The recent publication of the MIQE 2.0 guidelines (11) prompted us to reassess the technical and reporting standards in contemporary RT-qPCR studies, with a focus on MIQE as well as RNA integrity (22–24), reference genes (20, 25), and PCR efficiency (26, 27), 3 parameters critical for diagnostic reliability.

MIQE citation rose rapidly after 2009 but declined after 2014, returning to near-2011 levels by 2025 (Fig. 1A). RNA integrity reporting showed modest improvement, while PCR efficiency reporting declined steadily. None of the four parameters exceeded a 7% retrieval rate in the general literature. Reference gene mentions dropped until 2021, followed by a minor recovery. Figure 1B shows that citation, RNA integrity, and efficiency follow similar downward trends, suggesting that initial awareness did not translate into sustained practice. Pandemic-era fluctuations were brief interruptions, not inflection points, and recent patterns indicate a continued retreat from methodological engagement.

To assess whether the pandemic-era surge in RT-qPCR publications masked any underlying shifts in practice, we compared 3 cohorts: a pre-pandemic baseline from 2019, a general post-pandemic sample from 2024, and a targeted 2024 subset of MIQE-citing papers. The rise of Asia as the dominant contributor to RT-qPCR output in 2024 marks a clear geographic realignment (Fig. 2). In 2019, Europe and the United States together accounted for over 70% of publications. By 2024 Asia, driven predominantly by China, was 62% of the total, while Europe and the United States had each fallen to 15%. However, this expansion was not accompanied by proportional methodological engagement. The majority of MIQE-citing papers continued to originate from Europe, while the United States contributed just 9%. The regional disparity between overall output and MIQE citation rates highlights limited uptake of established methodological guidelines in the regions now driving the bulk of RT-qPCR publications. Regional differences were statistically significant ($\chi^2 = 83.2$, $df = 8$, $P < 0.0001$).

To better understand whether recent shifts have influenced methodological rigor, we compared the same three cohorts for reporting of RNA integrity, assay sequences, reference gene validation, and PCR efficiency.

RNA integrity reporting fell significantly between 2019 and 2024 ($P = 0.039$), and although MIQE-citing papers showed higher reporting rates (31%), this was only modestly above the 2019 general literature (22%) (Fig. 3A). Assay sequence disclosure was high across all groups (Fig. 3B), but reference gene validation remained low. In 2024, only 5% of non-MIQE papers reported validation, down from 13% in 2019, while only 46% of MIQE-citing papers included any validation (Fig. 3C). PCR efficiency reporting was nearly absent in non-MIQE studies (1%) and reported in less than half (40%) of MIQE-citing papers (Fig. 3D).

These omissions are not minor technicalities. RNA integrity, reference gene validation, and PCR efficiency act as 3 interdependent safeguards. They confirm the quality of the input material, the accuracy of the normalization strategy, and the quantitative reliability of expression changes. Failure to document any one of these weakens both the reliability of research findings and the validity of diagnostic results, regardless of how well the others are addressed.

Figure 4 illustrates a critical distinction: methodological rigor is not uniformly poor across RT-qPCR studies. When technical focus is explicit, as it is in papers dedicated to reference gene selection, reporting standards improve but remain patchy (Fig. 4A). This suggests that omissions in the general literature are not solely due to lack of awareness but may also reflect prioritization. When rigor is central to the research question, authors tend to perform and report accordingly. The reference-gene cohort reported all three parameters in 56% of papers, compared with 47% in the MIQE-citing (Fig. 4B). Nearly half of the reference-gene papers did not cite MIQE, yet their reporting was no different from those that did ($P = 0.31$). This indicates that technical focus alone does not guarantee adherence to best practice and that awareness of methodological principles does not consistently translate into complete reporting.

In contrast, the PCR efficiency cohort performed markedly worse, despite addressing a parameter that directly influences quantitative accuracy (Fig. 4C). Only 40% of these papers reported RNA integrity, and just 38% validated their reference genes. While assay sequence disclosure was high (92%), this is arguably the least diagnostically consequential of the three parameters and often the easiest to report. That only 24% of these PCR efficiency papers reported all three contextual parameters (Fig. 4D) reinforces the conclusion that technical focus does not guarantee methodological completeness. In fact, the PCR efficiency cohort performed significantly worse than the reference gene cohort ($z = 3.27$, $P = 0.0011$), despite targeting a parameter with direct consequences for diagnostic quantification. This gap underscores that even within technically themed

publications, reporting is often narrow in scope and disconnected from the broader requirements of assay validation.

This is not just a theoretical problem. Unknown or uncorrected PCR efficiencies can skew fold-change calculations by 20% to 40% or more. One study demonstrated that even modest inaccuracies in efficiency estimation can substantially distort calculated expression ratios, particularly when comparing transcripts with large expression differences (28). A recent modelling analysis showed that at a ΔC_q of 4, fold-change estimates vary from 11.3 (92% efficiency) to 23.0 (110%)—a 40% deviation in either direction from the ideal 16-fold value (29). If RNA integrity is also unverified, these distortions are compounded. The fact that such fundamental issues remain underreported even in efficiency-focused literature points to a systemic weakness in the way methodological rigor is conceived and operationalized in RT-qPCR studies.

Fig. 5 shows a clear geographic shift in methodologically focused RT-qPCR studies. The reference gene cohort was dominated by papers from Asia, with little contribution from Europe, the United States, or “other” regions. In contrast, the PCR efficiency cohort was led by Europe, with fewer papers from Asia and minimal input from elsewhere.

These patterns suggest that while Asia’s output is increasing, reporting quality remains variable. Europe retains strength in technical areas like PCR efficiency, but performance was inconsistent across both cohorts. Regional shifts in focus do not equate to uniform adherence to best practice.

The regional patterns likely stem from a complex interplay of editorial expectations, reporting norms, and research culture, rather than differences in assay capability. However, the practical consequence is the same. Variable adherence to best practices weakens the comparability of RT-qPCR results across regions. In diagnostics, this matters. Assays validated under differing or undocumented quality parameters may perform inconsistently when transferred or replicated elsewhere. If methodological reporting remains regionally fragmented, so too will diagnostic confidence.

Ultimately, Fig. 5 reinforces a central message of this study: improving RT-qPCR reliability is not solely a technical task. It requires harmonized standards across regions to ensure that diagnostic accuracy does not depend on geography. Despite renewed visibility for qPCR during the COVID-19 testing expansion, there has been no improvement in adherence to these principles. The decline in methodological engagement observed between 2007 and 2019 has persisted. For diagnostic applications, this means that many studies and the assays they underpin risk producing unreliable results. Without a sustained commitment to transparent

and complete reporting, methodological weaknesses will continue to erode both scientific credibility and clinical confidence in RT-qPCR data.

These findings suggest that regional and field-specific variation in methodological rigor reflects broader systemic pressures rather than purely technical limitations. RT-qPCR is widely regarded as a routine, low-complexity assay, fostering procedural complacency and the assumption that standard protocols ensure reliability. Publication pressure, short funding cycles, and performance metrics that reward output over reproducibility discourage detailed validation and transparent reporting. MIQE is often cited perfunctorily rather than implemented, and editorial enforcement remains weak: reviewers seldom assess methodological adequacy, and journals rarely require compliance documentation. Such patterns align with broader analyses of reproducibility failures across biomedical research, which link declining rigor to incentive structures, editorial inertia, and misplaced confidence in “mature” technologies (30).

Correcting these trends requires coordinated action. Journals should reinstate concise, method-specific checklists and instruct reviewers to assess compliance. Funders and institutions should embed reproducibility training into research assessment and career progression. Practical reinforcement could include integration of MIQE-derived modules within electronic laboratory notebooks or data repositories. RT-qPCR’s apparent simplicity conceals significant analytical nuance, and restoring confidence in its outputs will depend on rebuilding the culture of methodological transparency that originally defined the technology’s reliability.

Conclusions

Accurate diagnostic RT-qPCR depends, among other factors, on 3 interdependent methodological safeguards: RNA integrity assessment, reference gene validation, and PCR efficiency determination. Together, these parameters confirm input quality, validate normalization, and underpin reliable quantitative interpretation. Neglect of any one undermines diagnostic accuracy, regardless of compliance with the others.

This study shows that omissions at these checkpoints are frequent, often occurring together, even in publications that cite MIQE or focus on assay optimization. The resulting data may appear precise, yet rest on unstable methodological foundations, increasing the risk of misleading diagnostic conclusions. Despite the renewed attention to qPCR during the recent period of global testing expansion, adherence to these basic principles has not improved, and in some areas has declined. For diagnostics, this means that many reported assays

cannot be assumed to be robust or reproducible without independent verification. Sustained improvements will require not just guideline citation, but consistent, transparent application of the underlying principles in both research and clinical contexts.

LIMITATIONS

This study relied on keyword-based searches of PMC to identify references to RNA integrity, reference genes, and PCR efficiency. Because PMC indexes only the full text of open-access publications, the data set may not be fully representative of all RT-qPCR papers. Open-access journals often have less restrictive formatting and more variable editorial standards, which could bias the observed frequencies of methodological reporting in either direction. Nevertheless, manual review of 355 full-text articles confirmed the same directional trends seen in the automated analysis, suggesting that our estimates reflect genuine reporting patterns across the broader literature.

Not all papers in the data set were diagnostic in focus; however, all reported RT-qPCR data intended to support biological or clinical interpretation. The key issue is therefore not the study type but the lack of essential methodological detail, which prevents independent verification and limits reproducibility across both research and diagnostic contexts.

These limitations do not weaken our conclusions. On the contrary, they reinforce the central concern. The difficulty of locating key methodological information—whether due to inconsistent terminology, incomplete reporting, or inaccessible formatting—is itself symptomatic of the broader transparency problem. Reporting opacity is not a peripheral inconvenience; it is integral to the problem of reproducibility. Given that our approach likely underestimates the true scale of these deficiencies, the case for improving methodological clarity, transparency, and compliance is, if anything, understated.

Supplemental Material

Supplemental material containing the URLs for all papers analyzed in this study and the scores recorded for each one is available at [Clinical Chemistry](#) online.

Nonstandard Abbreviations: RT-qPCR, real-time reverse transcription PCR; MIQE, Minimum Information for Publication of Quantitative PCR Experiments; qPCR, quantitative PCR; MMR, measles–mumps–rubella; PMC, PubMed Central; IF, impact factor; C_q, quantification cycle.

Author Contributions: *The corresponding author takes full responsibility that all authors on this publication have met the following required criteria of eligibility for authorship: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved. Nobody who qualifies for authorship has been omitted from the list.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form.*

Research Funding: None declared.

Disclosures: The authors declare that S.A. Bustin and C.T. Wittwer were the lead and senior authors, respectively, of the original MIQE (2009) and MIQE 2.0 (2025) guidelines. S.A. Bustin serves as Section Editor-in-Chief of the *International Journal of Molecular Sciences (IJMS)* (Molecular Diagnostics) and declares no financial or commercial relationships with companies that develop or market PCR reagents, instruments, or analysis software. C.T. Wittwer receives royalties from the University of Utah through licenses to bioMérieux/BioFire from patents related to melting analysis and fast nucleic acid amplification; consulting fees from CoDiagnostics, Magic LifeScience, PathogenDx, and InBiome; received the Coulter lecture award 2024 from the International Society for Laboratory Hematology (ISLH) and Norman P. Kubasik Lectureship Award 2025 from ADLM New York Upstate Local Section; is listed on issued patents (High resolution DNA melting analysis [expires in 2025] and Extreme PCR); is on advisory boards for CoDiagnostics and InBiome; has equity interest in Crestwood Technology, CoDiagnostics, PathogenDx, Magic LifeScience, and Nusantics; and is an Associate Editor of *Clinical Chemistry* (ADLM).

References

- Dutta D, Naiyer S, Mansuri S, Soni N, Singh V, Bhat KH, et al. COVID-19 diagnosis: a comprehensive review of the RT-qPCR method for detection of SARS-CoV-2. *Diagnostics (Basel)* 2022;12:1503.
- Alcoba-Florez J, Gil-Campesino H, Artola DG, González-Montelongo R, Valenzuela-Fernández A, Ciuffreda L, et al. Sensitivity of different RT-qPCR solutions for SARS-CoV-2 detection. *Int J Infect Dis* 2020;99:190–2.
- Bivins A, Kaya D, Bibby K, Simpson SL, Bustin SA, Shanks OC, et al. Variability in RT-qPCR assay parameters indicates unreliable SARS-CoV-2 RNA quantification for wastewater surveillance. *Water Res* 2021; 203:117516.
- Wernike K, Keller M, Conraths FJ, Mettenleiter TC, Groschup MH, Beer M. Pitfalls in SARS-CoV-2 PCR diagnostics. *Transbound Emerg Dis* 2021;68:253–7.
- Bevan I, Stage Baxter M, Stagg HR, Street A. Knowledge, attitudes, and behavior related to COVID-19 testing: a rapid scoping review. *Diagnostics (Basel)* 2021;11:1685.
- Baker M. Is there a reproducibility crisis? *Nature* 2016;533:452–4.
- Baker M, Dolgin E. Reproducibility project yields muddy results. *Nature* 2017;541: 269–70.
- Ioannidis JPA. Why most published research findings are false. *PLoS Med* 2005; 2:e124.
- Taylor SC, Mrkusich EM. The state of RT-quantitative PCR: firsthand observations of implementation of minimum information for the publication of quantitative real-time PCR experiments (MIQE). *J Mol Microbiol Biotechnol* 2014;24:46–52.
- Bustin SA. The MMR vaccine, measles virus, and autism—a cautionary tale. In: Bustin SA, editor. *The PCR revolution: basic technologies and applications*. Cambridge (UK): Cambridge University Press; 2009. p. 229–42.
- Bustin SA, Ruijter JM, van den Hoff MJB, Kubista M, Pfaffl MW, Shipley GL, et al. MIQE 2.0: revision of the minimum information for publication of quantitative real-

- time PCR experiments guidelines. *Clin Chem* 2025;71:634–651. doi:10.1093/clinchem/hvaf043.
12. Taylor S, Wakem M, Dijkman G, Alsarraj M, Nguyen M. A practical approach to RT-qPCR-publishing data that conform to the MIQE guidelines. *Methods* 2010;50: S1–5.
 13. Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J. The ultimate qPCR experiment: producing publication quality, reproducible data the first time. *Trends Biotechnol* 2019;37:761–74.
 14. Bustin SA, Benes V, Garson J, Hellemans J, Huggett J, Kubista M, et al. The need for transparency and good practices in the qPCR literature. *Nat Methods* 2013;10: 1063–7.
 15. Dijkstra JR, van Kempen LC, Nagtegaal ID, Bustin SA. Critical appraisal of quantitative PCR results in colorectal cancer research: can we rely on published qPCR results? *Mol Oncol* 2014;8:813–8.
 16. Abdel Nour AM, Azhar E, Damanhoury G, Bustin SA. Five years MIQE guidelines: the case of the Arabian countries. *PLoS One* 2014;9:e88266.
 17. Bustin S, Nolan T. Talking the talk, but not walking the walk: RT-qPCR as a paradigm for the lack of reproducibility in molecular research. *Eur J Clin Invest* 2017;47:756–74.
 18. Bustin SA. Improving the quality of quantitative polymerase chain reaction experiments: 15 years of MIQE. *Mol Aspects Med* 2024;96:101249.
 19. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnol Lett* 2004;26:509–15.
 20. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3: 0034.1–0034.11.
 21. Gannot G, Cutting MA, Fischer DJ, Hsu LJ. Reproducibility and transparency in biomedical sciences. *Oral Dis* 2017;23:813–6.
 22. Fleige S, Walf V, Huch S, Prgomet C, Sehm J, Pfaffl MW. Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. *Biotechnol Lett* 2006;28:1601–13.
 23. Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. *Mol Aspects Med* 2006;27:126–39.
 24. Vermeulen J, De Preter K, Lefever S, Nuytens J, De Vloed F, Derveaux S, et al. Measurable impact of RNA quality on gene expression results from quantitative PCR. *Nucleic Acids Res* 2011;39:e63.
 25. D'haene B, Mestdagh P, Hellemans J, Vandesompele J. miRNA expression profiling: from reference genes to global mean normalization. *Methods Mol Biol* 2012;822:261–72.
 26. Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, van den Hoff MJB, et al. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res* 2009; 37:e45.
 27. Ruijter JM, Barnewall RJ, Marsh IB, Szentirmay AN, Quinn JC, van Houdt R, et al. Efficiency correction is required for accurate quantitative PCR analysis and reporting. *Clin Chem* 2021;67:829–42.
 28. Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. How good is a PCR efficiency estimate: recommendations for precise and robust qPCR efficiency assessments. *Biomol Detect Quantif* 2015;3:9–16.
 29. Bustin SA, Kirvell S, Nolan T, Mueller R, Shipley GL. When two-fold is not enough: quantifying uncertainty in low-copy qPCR. *Int J Mol Sci* 2025;26:7796.
 30. Bustin SA, Wittwer CT. Fragile methods, fractured trust: rethinking scientific responsibility. *Methods* 2025;242:54–61.