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Standardization of qPCR and RT-qPCR

New Guidelines Seek to Promote Accurate Interpretation of Data and Reliable Results

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The perceived ease of use of real-time quantitative PCR (qPCR) and reverse transcription PCR (RT-qPCR) technology has revolutionized life science research. Its effectiveness at amplification and quantification of low levels of nucleic acids has driven the emergence of numerous applications, including cellular mRNA and miRNA quantification, biomarker discovery and validation, microbial quantification, cancer risk assessment, gene dosage determination, and detection of extremely low copy targets for

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forensic investigations.

This, in turn, has resulted in an abundance of publications utilizing qPCR data obtained with diverse reagents, protocols, analysis methods, and reporting formats. Unfortunately, few papers report in detail how these results were obtained. This lack of clarity and transparency has led to concern in the research community over the reliability of qPCR data interpretation

and the real danger of the scientific literature being corrupted with publications reporting erroneous and conflicting results.

This has already occurred in some cases, resulting, for example, in retraction of a *Science* “Breakthrough of the Year 2005” report¹. Now that qPCR has come of age, standardization is needed to ensure its validity, prompting the recent formulation of guidelines to increase experimental transparency, promote consistency between laborato-

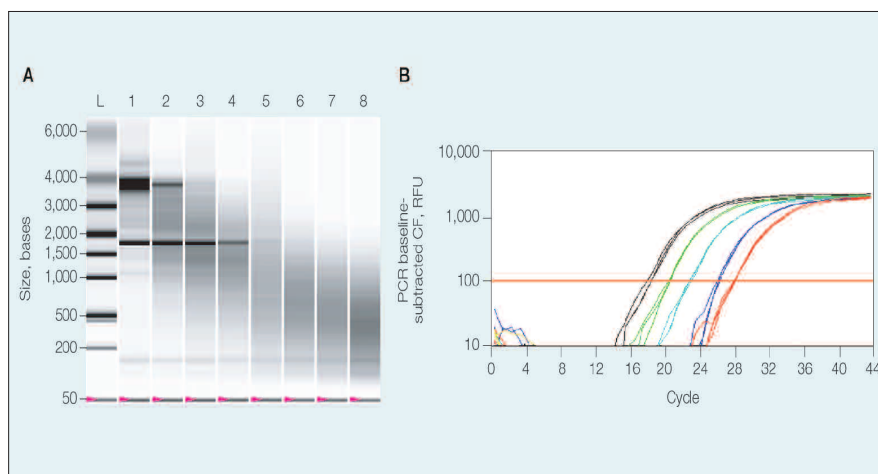


Figure 1. Time course of degradation of liver carcinoma RNA: Samples of liver carcinoma total RNA were incubated at 90°C in TE buffer in 1-hour increments from 0 (A, lane 1) to 7 hours (A, lane 8). Aliquots (50 ng) were then analyzed on the Experion system. (A) Simulated gel view showing the distribution of RNA lengths as a function of degradation time, relative to a sizing ladder. (B) RT-qPCR traces obtained using the RNA samples degraded for various lengths of time and primers for a GAPDH reference gene amplicon. No degradation (black); 1 hour (green); 3 hours (turquoise); 5 hours (blue); 7 hours (red).
Data courtesy of Bio-Rad Laboratories

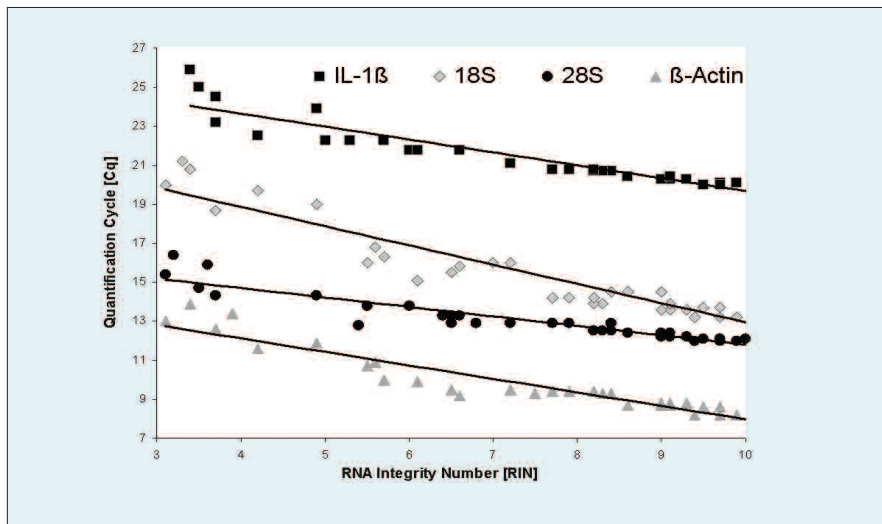


Figure 2. Quantitative RT-PCR results using total RNA of varying quality and real-time amplification of four genes from bovine white blood cells: The Cq values can vary more than six cycles, depending on the RIN value of the starting RNA, and the rate (slope) of that variation can change with the target gene.
Data courtesy of Michael W. Pfaffl

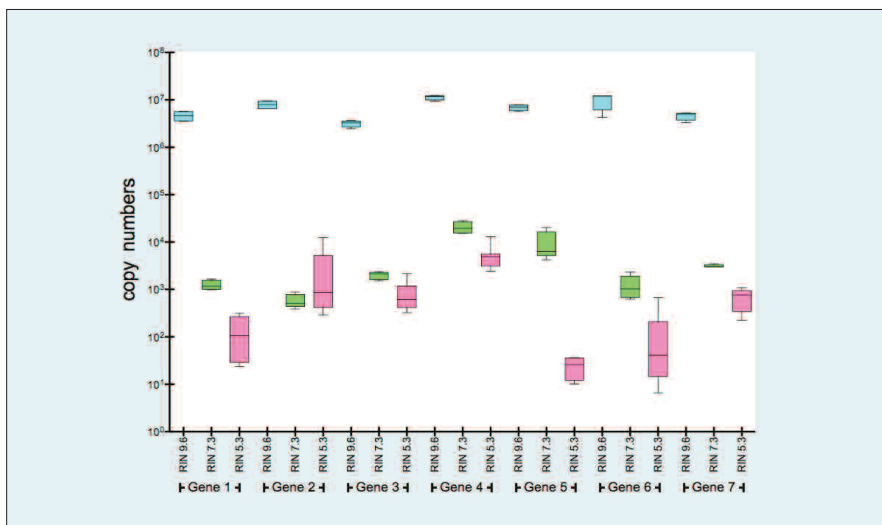


Figure 3. Seven target genes in human colorectal cancer tissue were reverse transcribed using three RNA preparations with RIN values of 9.6 (blue), 7.3 (green), and 5.3 (pink), respectively, and amplicon-specific standard curves were used to calculate copy numbers for each preparation. In each case, the 9.6 RIN samples gave the highest copy numbers, and the 5.3 RIN samples produced the lowest copy numbers. However, whereas with target genes 1 and 5 there is a good correlation between RIN value and calculated copy number, further degradation of genes 2 and 3 in the 5.3 RIN samples does not result in further reduction in calculated copy number.
Data courtesy of Stephen A. Bustin

ries, and therefore, help assure the publication of valid conclusions².

Guidelines Proposed

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (MIQE; www.rdml.org/miqe) aim to provide a yardstick for the quality assessment of a publication. The MIQE guidelines define the

minimum information required for evaluation of qPCR results, and they include a checklist to be included in the initial submission of a manuscript to a publisher.

MIQE is modeled on similar guidelines such as MIAME (Minimal Information about a Microarray Experiment), developed several years ago³ and MIAPE (Minimal Information about a Proteomics Experiment)⁴. All of these

are initiatives developed under the umbrella of the MIBBI (Minimum Information for Biological and Biomedical Investigations) standardization body, which has the goal of unifying all of the standardization guidelines for biological and biomedical research.

In addition, the Real-Time PCR Data Markup Language (RDML; www.rdml.org) has been developed by a consortium to enable the straightforward exchange of qPCR data and related information between qPCR instruments and third-party data analysis software, between colleagues, and with journals or public data repositories⁵.

Increased Confidence in Results

Increased experimental transparency constitutes a key advantage of adopting a relevant and comprehensive set of standards for qPCR. The MIQE guidelines stipulate full disclosure of reagents, protocols, and analysis methods, thus establishing that qPCR data meets a minimal set of standards. This increases confidence in its validity by ensuring that data meets a uniform quality benchmark before it is submitted for publication, rather than discovering flaws in the data after it is published.

The guidelines could eventually become a “quality label” denoting the data in a conforming publication as being of high quality. In addition, once data is provided in the RDML format, it can be carefully analyzed by the research community to assure that it supports the conclusions of the study.

The MIQE guidelines are in their initial formulation and are expected to be in continual evolution. The consortium is asking for feedback from the research community, commercial suppliers of qPCR products, and scientific journals

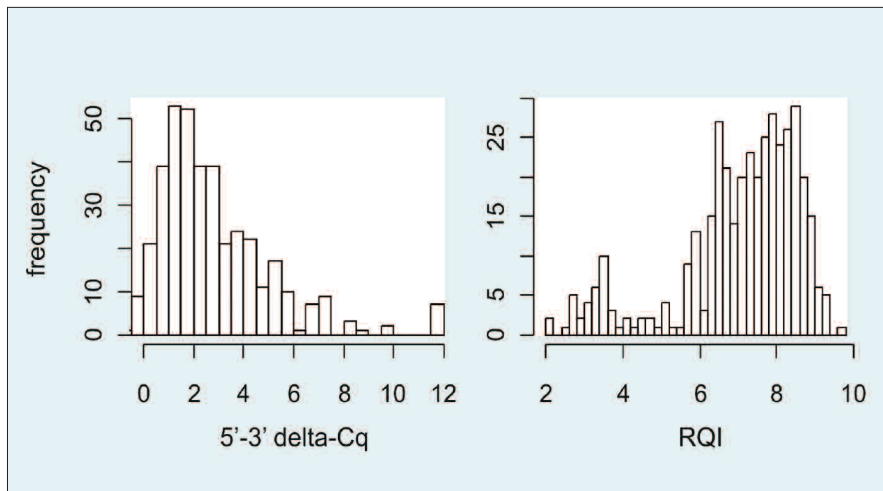


Figure 4. RNA quality assessment of more than 700 neuroblastoma tumor samples: The difference in Cq for qPCR reactions performed using amplicons from the 5' and 3' ends of the target gene (5'-3'-delta-Cq) was determined for each sample, along with the RNA quality indicator (RQI) derived from microfluidic electrophoresis analysis on the Experion system. The frequency (number of samples) of each 5'-3'-delta-Cq or RQI value in the population was then calculated and plotted.
Data courtesy of Jo Vandesompele

to assure that they have optimal utility.

While a first look at the MIQE guidelines might lead researchers to assume that they will require significant additional effort and slow down the publication process, it is important to note that many of them are discretionary, with only those having the most impact on data quality being mandatory.

Most can be adopted quickly, because they are obvious—for example reporting the quantification cycle (Cq), previously variously referred to as C_t, C_p, or TOP, noting gene accession numbers, defining amplicon locations, and calculating qPCR efficiency. Adoption of the mandatory guidelines as a first strategy assures that key parameters affecting data quality are being addressed immediately and will have a swift impact on confidence levels in the data and the conclusions drawn from it.

Focus on pre-PCR

For RT-qPCR experiments, an additional focus on the pre-PCR steps is essential as these can be a major source of error. The guidelines address sam-

pling, RNA stabilization, storage, and quality-assurance procedures. For example, degraded RNA has a substantial impact on qPCR results and the conclusions drawn from them. Significant degradation of target RNA can result in a Cq that is artificially high, leading to an underestimation of its concentration and copy number (Figure 1).

This effect of RNA quality on RT-qPCR results can be dramatic, with a difference of more than six cycles in the

Cq values of intact and heavily degraded and intact RNA samples (Figure 2). Differential sensitivity of mRNA to degradation can have an enormous impact on the interpretation of qPCR results, as different reference genes appear to be suitable for normalization in degraded versus intact RNA samples (Table).

Normalizing RT-qPCR results to reference genes without knowledge of the degradation status of the RNA could thus lead to incorrect conclusions⁶. In addition, the degree of dependence can vary from one sample to another, emphasizing the need to understand the relationship between RNA quality and the results in any given study (Figure 3).

RNA Integrity

Consequently, quality control of the RNA starting material is an essential checkpoint addressed by the guidelines. These require that RNA integrity be measured by manual gel electrophoresis at the very least, but preferably by microfluidics-based RNA analysis using a system such as the Experion (Bio-Rad Laboratories) or the Bioanalyzer 2100 (Agilent Technologies). This technique

Candidate Reference Genes Ranked in Order of Expression Stability*

Step**	Chronic Rhinosinusitis		Nasal Polyposis	
	Degraded RNA	Intact RNA	Degraded RNA	Intact RNA
1	HPRT1	GAPDH	HPRT1	YWHAZ
2	YWHAZ	YWHAZ	ACTB	B2M
3	B2M	RPL13A	RPL13A	RPL13A
4	TBP	B2M	GAPDH	UBC
5	RPL13A	UBC	TBP	GAPDH
6	UBC	HPRT1	YWHAZ	HMBS
7	ACTB	TBP	HMBS	HPRT1
8	GAPDH	ACTB	SDHA	SDHA
9	HMBS-SDHA	HMBS-SDHA	B2M-UBC	ACTB-TBP

* As determined by geNorm, increasing from top to bottom in two diseases dichotomized with respect to RNA integrity; the two most stable genes in each sample series, for example ACTB and TBP in intact RNA from nasal polyposis tissue, cannot be ranked in order because of the required use of gene ratios for gene stability. Some discrepant results are apparent when comparing intact vs. degraded samples (highlighted in color).

** The step number at which the least stable gene is excluded

Data courtesy of Jo Vandesompele

is fast, highly standardized, uses a small amount of total RNA, and is automated, including the data analysis.

Each system uses a quality index scale to represent the level of degradation in a sample. The Agilent RNA Integrity Number (RIN) is a number from 1 to 10 derived by an algorithm that takes into account eight portions of the electrophoretic trace. The Bio-Rad RNA Quality Indicator (RQI), also a number from 1 to 10, is generated by an algorithm that takes into account three portions of the electrophoretic trace and then compares the electropherogram of RNA samples to a series of standard degraded RNA samples.

The two quality indices are correlated and can generally be used interchangeably as reliable indicators of RNA quality⁷. The PCR-based 3':5' mRNA integrity assay⁸ constitutes an additional RNA quality-assessment tool, although its practical usefulness remains to be determined.

Ideally, a threshold that delineates the quality of RNA required to produce reliable results should be established for each study. For example, in a study of more than 700 biopsies

from neuroblastoma patients, the average values for samples with acceptable quality were a difference in Cq of 2.36 for the 3':5' assay and an Experion-derived RQI of 7.4, while both of these indices show a wide distribution of values across the samples (*Figure 4*)⁹.

A general guideline has also been suggested that recommends an RIN of at least 5 to obtain reliable RT-qPCR results¹⁰. Finally, inhibition of reverse-transcription or PCR should be checked by dilution of the sample or use of a universal inhibition assay such as SPUD¹¹.

Conclusion

qPCR and RT-qPCR are powerful enabling technologies that impel the advances made in our understanding of basic biological and disease processes as well as underpin the field of molecular diagnostics. 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.

The RNA quality issue highlighted in

this article is just one example of the many crucial parameters that must be addressed by guidelines that shift the focus of concern from questions regarding the technological relevance underlying a publication's conclusion to the actual biological or diagnostic issues being addressed. MIQE and RDML have initiated a dialogue in the research community that should result in guidelines that promote absolute transparency of experiments, high confidence in results, and valid conclusions that continue to advance our understanding. **GEN**

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