# qPCR Assay Quality assessment

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by Stephen Bustion on

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**Guidelines for minimum information required for publication of qPCR data are currently being assembled and will be published in Clinical Chemistry.**

qPCR quality assessment relates mainly to the reverse transcription -qPCR (RT-qPCR) variant of the technology. This is widely used to measure pathogen as well as cellular RNA copy numbers; the former, given appropriate standard operating procedures and technical expertise, is fairly straightforward. The latter can be highly problematic. For both types of assay, however, RNA quality is a major consideraton.

Quality assessment is a big fat elephant sitting in the room: everyone knows what needs to be done, but most researchers do not follow basic quality control guidelines. This serves to undermine the integrity of the scientific literature to such an extent, that a high proportion of publications are reporting technical or analytic artifacts.

Incredibly, many researchers are not bothered by this; indeed some have been heard to remark that they can't be bothered assessing RNA quality, worrying about reverse transcription or determining what normalisdation strategy to follow. However, efforts are underway to establish a checklist for journal editors and reviewers, with the aim of introducing a minimum standard of assay reporting.

## What are the problems?

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PCR inhibition assessment generally depends on the assumption that inhibitors affect all PCR reactions to the same extent; i.e. that the reaction of interest and the control reaction are equally susceptible to inhibition. However, it appears that when co-purified inhibitors are assessed in different PCR reactions, differential inhibition is observed and susceptibility to inhibition is highly variable between reactions. This has serious implications for all PCR-based gene expression studies, including the relatively new PCR array method, and for both qualitative and quantitative PCR-based molecular diagnostic assays, suggesting that careful consideration should be given to inhibition compatibility when conducting PCR analyses. Clearly, it is not safe to assume that different PCR reactions are equally susceptible to inhibition by substances co-purified in nucleic acid extracts.

Reference: Huggett JF, Novak T, Garson JA, Green C, Morris-Jones SD, Miller RF, Zumla A. Differential susceptibility of PCR reactions to inhibitors: an important and unrecognised phenomenon. BMC Res Notes 2008;1:70.

1. Inappropriate sample selection, coupled with the complexity and heterogeneity of any tissue biopy, especially from cancer and inconsistent handling procedures, results in variability and inaccurate mRNA quantification. In addition, there can be two sources of error: (i) sampling error, ie even if epithelial cells are being collected, the cell type within the epithelial population may have a different distribution compared with the collected population' (ii) measurement error, which depends on the quality of instruments, reagents and operator.

2. The conversion of mRNA to cDNA is a major stumbling block and arguably is the single most variable step in the whole quantification procedure. It is well known, although not well publicised, that different reverse transcriptases have significantly different efficiencies of reverse transcription, and that these are target-dependent (1,2). Similarly, the mechanism of cDNA priming has a significant effect on the outcome of any quantification experiment, since gene-specific priming, random priming and oligo-dT all produce diverse results that are distinct for different mRNA targets. The choice of primer location on the target mRNA also can yield significantly different results, as mRNA adopts a tight secondary structure characterised by extensive intra-strand base pairing resulting in stem-loop structures (3). If reverse transcription primers are designed to target stems, rather than loops, or if the amplicon can adopt secondary structures, the efficiency of the RT step is significantly compromised. Characteristically, this results in non-quantitative and non-reproducible results.

3. The accuracy of gene expression profiling is highly dependent on mRNA quality (4,5). Unfortunately, this is an area that is distinguished by a prevalent lack of concern. A 2005 survey of the working practices of 100 experienced qPCR users revealed that attending a worryingly high 37% did not quality assess their RNA, with a further 4% using absorbance ratios which even then were known to be inadequate for quantification of mRNA (6). A survey of BMC publications in 2007/08 reveals that we have regressed since then, with >60% of papers not even mentioning mRNA quality and a substantial 10% continuing to rely on absorbance ratio measurements. Even when RNA quality is assessed, it is evaluated using either gel electrophoresis or microfluidics-based systems; this approach fails to take into account that such measurements only look at ribosomal RNA without relating the results to mRNA integrity, which is, after all, the real target of interest.

4. Splicing is a post-transcriptional modification in which a single gene can specify multiple proteins, allowing the synthesis of protein isoforms that are structurally and functionally distinct. Gene splicing affects most human genes (7) and plays an important role in human pathologies, including cancer (8). This generates significant problems with the interpretation of RT-qPCR and microarray data, since presence or, indeed significant changes in mRNA levels may reflect cell-, tissue- ot treatment-specific adjustments between different isoforms.

5. The increased realisation that allelic imbalance and allele-specific expression patterns are associated with increased disease risk (9,10) poses further problems for the interpretation of mRNA quantification data. Rather than avoiding SNPs when designing primers, it may be necessary to include them as part of an overall assay design strategy so as to be able to quantitate allele-specific expression accurately.

6. It is worth emphasising that in vivo mRNA is subject to constant degradation by complex interactions of deadenylation and decapping enzyme complexes as well as 3'-5, 5'-3' exonucleases as well as endonucleases (11). This is likely to result in significant natural variability of mRNA levels between genes expressed in different tissues and individuals. This is in addition to any degradation introduced during the extraction of the RNA from tissue samples or during storage. Whilst these comments may seem obvious, their implications have never been explored.

7. Normalisation, known to be an essential component of proper data analysis (12), continues to be used in an inappropriate manner particularly in RT-qPCR applications, with a high proportion of papers still reporting expression patterns of target genes normalised against a single, unvalidated reference gene .

8. Inappropriate experimental designs, improper analyses, subjective interpretation of RT-qPCR data, variability of microarray results depending on the choice of analysis algorithms all combine to compromise the interpretation and confident application of quantitative, mRNA-targeted data (13).

The consequence of these, and other poor standards, is that a large number of publications report data that are at best unreliable, at worst misleading, with a dramatic and damaging effect on the integrity of the scientific literature. For example, a paper published in Science and named as a "breakthrough of the year", has had to be withdrawn, because its results could not be repeated (14). More seriously, a paper using RT-qPCR technology and purporting to confirm an association between the presence of measles virus and gut pathology in children with developmental disorder (15) was used to claim a link between the MMR vaccine and autism (16). However, the data were significantly flawed as the RT-qPCR assay was applied in an inappropriate manner (ftp://autism.uscfc.uscourts.gov/autism/cedillo.html).

## What is the solution?

First, it is essential to step back and concentrate on getting the basic technical problems sorted out. This includes enforcing minimum quality standards for template preparation, validation and consistent use of cDNA priming methods, enzymes, protocols and, equally critically, appropriate analysis of data.

Second, it is entirely unacceptable that most publications do not address the critical issue of RNA quality assessment. It is equally unacceptable that data are not normalised in an appropriate manner. Third

Third, it is essential that data acquisition, analysis and reporting become more transparent. Consequently, it is essential for the editors of scientific and biomedical publications to issue prescriptive checklists specifying the key information to include when reporting experimental results. There are significant efforts underway to organise such 'minimum information' checklists, with the "Minimum information for biological and biomedical investigations" (MIBBI) project offering a common portal aimed at promoting gradual data integration (http://mibbi.sourceforge.net).

Another development concerns the problems associated with attempting to share qPCR data between different laboratories and users. A new initiative, the "Real-time PCR Data Markup Language" (RDML) describes a structured and universal data standard for exchanging qPCR data (http://www.rdml.org/). Together with the accompanying guidelines for Minimal Information (MIqPCR), the data standard will contain sufficient information to understand the experimental setup, re-analyse the data and interpret the results. This is of particular importance for transparent exchange of annotated qPCR data between authors, peer reviewers, journals and readers.

Those intimately familiar with the molecular technologies underlying the advances proclaimed by the highest impact factor journals, then taken up by the popular press and finally shaping peoples' expectations are only too familiar with their serious shortcomings. Unfortunately, it seems that very few researchers are willing to listen and even fewer are willing to change their modi operandi. It really is time to put the horse before the cart, and stop being blinded with ever-more technology.

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**Update on 21 January 2010**

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## MIQE, the guidelines for minimim information required for publication of qPCR data have published in Clinical Chemistry.

The real-time polymerase chain reaction uses fluorescent reporter dyes to combine DNA amplification and detection steps in a single tube format. The increase in fluorescent signal recorded during the assay is proportional to the amount of DNA synthesised during each amplification cycle. Individual reactions are characterised by the cycle fraction at which fluorescence first rises above a defined background fluorescence, a parameter previously known as the threshold cycle (Ct) or crossing point (Cp), now standardised by MIQE as the quantification cycle (Cq). Consequently, the lower the Cq, the more abundant the initial target. This correlation permits accurate quantification of target molecules over a wide dynamic range, while retaining the sensitivity and specificity of conventional end-point PCR assays. The homogeneous format eliminates the need for post-amplification manipulation and significantly reduces hands-on time and the risk of contamination. MIQE abbreviates real-time PCR to qPCR, with reverse transcription PCR abbreviated to RT-qPCR.

**There are three main chemistries in general use:**

* **DNA binding dyes**, such as SYBR-Green, which fluoresce upon light excitation when bound to double stranded DNA. These are cheap, easily added to legacy assays and amplification products can be verified by the use of melt curves. They can lack specificity and fluorescence varies with amplicon length. In general, they are one Cq or so more sensitive than probe-based assays. Their main drawback is that the NTCs often come up around Cqs of 36+, although melt curves can often distinguish genuine ampolification from nom-specific noise.

* **Fluorophores attached to primers**, e.g. Invitrogen's Lux or Promega's Plexor primers. These are relatively inexpensive and amplification products can be verified by melt curves. Specificity depends on the primers and specific, usually company-specific design software needs to be used for optimal performance. This is not necessarily a bad thing (indeed the Plexor software is very useful), but it is not always possible to change primer design parameters.

* **Probe based methods**, e.g. hydrolysis (TaqMan), Scorpions or Molecular Beacons. These are the most specific, as products are only detected if the probes hybridise to the appropriate amplification products. There are many variations on this theme, with melt curve analysis possible for some chemistries. Their main disadvantages are cost, complexity and occasional fragility of probe synthesis, especially when incorporating DNA analogues. There are potential problems associated with the fact that probe-based assays do not report primer dimers that can interfere with the efficiency of the amplification reaction. Hence establishing the efficiency of any assay is an important component of assay design.

qPCR targeting DNA is a robust assay, with assay quality determined mainly by PCR primer quality. Its derivate, RT-qPCR, which targets RNA, on the other hand, is much less robust, as the obligatory conversion of RNA into cDNA can be highly variable.

### qPCR QUALITY ASSESSMENT

Reliable quantification requires consideration of each step of the qPCR assay. The issue of quality control is discussed on the QUALITY ASSESSMENT page.

# qPCR Assay Quality assessment


Prof Stephen Bustin
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Last updated on 21 January 2010

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## *Further reading*


- The MIQE Guidelines: Minimum Information for Publication of Quantitative Real- Time PCR Experiments (Link »)
- Pattyn, F., Robrecht, P., Speleman, F., De Paepe A. & Vandesompele, J. (2006). RTPrimerDB: the Real-Time PCR primer and probe database, major update 2006. Nucleic Acids Research, 34(Database issue): D684- 688
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## *Selected links*

- qPCR Data Markup Language
- qPCR Primer & Probe Database
- Realtime PCR
- Minimum information for biological and biomedical investigators