Real-time, fluorescence-based quantitative PCR: a snapshot of current procedures and preferences

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Real-time fluorescence-based quantitative PCR has become established as the benchmark technology for the quantification of nucleic acids, offering an immense choice of protocols, chemistries and instruments. However, whilst there are comparatively few technical problems associated with DNA-targeted quantitative PCR, this is not the case for real-time reverse transcription PCR assays, and there is considerable uncertainty regarding biological or clinical relevance of many real-time reverse transcription PCR results. A survey of working practices of nearly 100 delegates carried out prior to the *Third qPCR Symposium* held in London, UK, April 25–26, 2005, reveals some of the reasons underlying the variability of reported real-time reverse transcription PCR results. Specifically, the survey reveals extensive interlaboratory variation in assay design, validation and analysis that, together with other dubious practices, are the likely cause for the publication of variable results. These results emphasize the urgent need for the establishment of best practice guidelines for this technology, particularly in the context of its mounting adaptation as a high-throughput clinical diagnostic assay.

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Real-time, fluorescence-based quantitative (q)PCR has become ubiquitous in biological, medical, veterinary, biotechnology and forensic research [1–3]. However, significant problems remain, particularly with real-time reverse transcription (qRT)-PCR assays, and these impose significant limitations on the use of this technology [4–7]. In particular, it has been suggested that the lack of agreed standards, diversity of protocols, instruments and analysis methods as well as nonexistent guidelines for the reporting of results requires qRT-PCR results to be treated with caution [8]. This technical variability is coupled with significant biologic variability, particularly when applied to the detection of RNA from *in vivo* biopsies that are characterized by significant heterogeneity [9]. Nonetheless, to date, there has been no survey of just how variable protocols are and how researchers go about obtaining and analyzing their qPCR results.

The *Third qPCR Symposium* held in London, UK, April 25–26, 2005, was designed as a discussion forum with the aim of dissecting the individual steps leading from sample acquisition to reporting the results of real-time qPCR assays. A detailed questionnaire was sent to all participants and the answers provided offer the first independent and comprehensive snapshot of current practices by 93 laboratories from 21 countries, mainly European but also from Australia, Brazil, Canada and the USA, carrying out qPCR assays.

Participants

Most of the participants came from academic laboratories (65%), 16% were from industry and 19% came from diagnostic laboratories. 40% of delegates work exclusively with RNA, 49% quantitate both DNA and RNA and only 11% work exclusively with DNA. This suggests that the results of this survey are most applicable to qPCR work carried out for expression analysis (i.e., quantification of either cellular mRNA or viral pathogen RNA levels). Most respondents were very experienced with this technology, with 44% running more than 30 (range 30–400) assays, 42%

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between ten and 30 and only 14% running fewer than ten assays a month. Interestingly, although 63% of respondents work with tissue biopsies, only 13% use microdissection to ensure their analysis is carried out on defined samples. This is a very important issue, since RNA extracted from a whole-tissue biopsy represents a composite of more than one distinct source [10–12]. Tissues are complex 3D structures and the cell subpopulation of interest (e.g., malignant cells in tumors) may constitute only a small fraction of the total tissue volume. Consequently, analysis of RNA extracted from a whole cancer (e.g., a colorectal tissue biopsy) will result in expression profiles reflecting the proportion of stromal contamination in the sample, rather than underlying tumor (epithelial cell) biology. Such analysis would record an average gene expression and may mask significant differences in tumor-specific gene expression patterns. Indeed, a case can be made that even the analysis of microdissected tissue may not be sufficient, as it does not allow the evaluation of any variation in gene expression between individual cells [13,14]. At the very least, inappropriate sample selection is likely to increase the variability of any qPCR results, and at worst could be misleading and be of no biological or clinical interest. Clearly, it is essential that quantification of mRNA levels from tissue biopsies should be carried out only on microdissected tissue.

Preanalysis/assay design

It has become increasingly clear that assay standardization is very important if results are to be comparable between different laboratories [15–17]. Ideally, this would involve a series of agreed standard operating procedures (SOPs) that would allow investigators to comply with specific protocols, thus ensuring consistency, control and repeatability of results. It was therefore disconcerting that only 51% of researchers follow SOPs in their laboratories. Surprisingly, 33% of diagnostic laboratories do not have SOPs in place. Not unexpectedly, there was a correlation between experience and adherence to an SOP. A total of 66% of experienced researchers from laboratories carrying out more than 30 (range 30–400) qPCR assays per month followed SOPs, whereas only 40% from laboratories carrying out over ten assays per month did so. Again, it is not surprising that the absence of SOPs will increase the number of variable results recorded within a laboratory and make it more difficult to repeat those results elsewhere.

The most widely used chemistry remains the 5´-nuclease assay (72%), with SYBR® Green I chemistry used by 50% of respondents. All other chemistries, including Roche's dual hybridization probes, are used by less than 15% of researchers. The increasing sophistication of real-time instrumentation combined with significant advances in chemistries is beginning to make multiplexing a more feasible proposition. This is reflected in the 29% of participants that regularly multiplex up to four fluorescent dyes. Nonetheless, it is clear that multiplexing is still not trivial and requires three oligonucleotides per amplicon (two primers, one probe), resulting in the need to optimize 12 oligonucleotides for a quadruplex reaction. However, this issue is being addressed by continuing advances and recently developed nonprobe-based chemistry promises to simplify multiplexing by omitting the probe. It makes use of the specific properties of isoguanosine (iG) and isocytosine (iC) [18], which have the polarity of their hydrogen bonding reversed and so can pair only with each other, not with their natural counterparts. Upstream (sense) PCR primers contain a single isodeoxy(d)C at their 5´ ends, together with a different fluorophore for each target amplicon. A quencher is attached to the iso-dG, which is added to the amplification master mix. During the amplification, the quencher-labeled dG is incorporated opposite the iso-dC, and this results in fluorescence quenching. The more target is amplified, the more the initial fluorescence is quenched, and the amount of quenching is directly proportional to the amount of target being amplified [19]. This chemistry is commercially available as Plexor™ by Promega.

Most researchers (90%) design their own primers, and Applied Biosystems' (ABI) Primer Express and web-based free programs (e.g., Primer 3) are the most popular. However, there is cause for concern with respect to the quality controls implemented by researchers to ensure that the primers have the required specificity. Although 94% validate their primers using BLAST searches, only 6% validate the resulting amplicon, either by sequencing or restriction mapping. Although 75% check for primer-dimers, 80% of those rely on their primer design programs to highlight potential problems, with only 20% using gels or melt curves to confirm the absence of actual primer-dimers. Disconcertingly, only 36% optimize the amplification conditions (Mg^{2+} and primer concentrations) for their primers using wet chemistry, and 58% check to determine whether folding of the mRNA might interfere with primer access during the reverse transcription step (e.g., using Mfold [101]). These results show that the majority of qPCR assays are not necessarily run under optimal conditions and the sensitivity and specificity of many assays have not been properly validated. This could have a significant impact in diagnostic situations where clinical decisions depend on the sensitivity and reliability of the qPCR assay.

Sample/template preparation

Most researchers interested in analyzing cellular RNA extract total RNA (91%). The most commonly used method involves spin columns (50%) or a combination of spin columns and other reagents (21%). Interestingly, 8% still use home-made reagents and protocols, approximately the same percentage as those who use Trizol/RNAzol (13%) and magnetic beads (8%). The main reason quoted by the 9% extracting mRNA is to ensure maximum sensitivity of the qRT-PCR assay. The finding that 37% of laboratories do not quality assess their RNA was as unexpected as it is disconcerting. Obviously, quality assessment is essential if negative results (e.g., when detecting viral pathogens) are to be credible or observed differences in mRNA levels (e.g., after treatment) are to be meaningful. Those who do quality assess their RNA mainly use the Agilent Technologies Bioanalyzer or gels (50%), with 4% relying on $A_{260:280}$ and 9%

assessing the 3´:5´ ratio of reference genes. Equally worryingly, following purification, 58% of researchers that store their RNA do not aliquot it, 80% do not periodically check its integrity and 94% of all respondents do not use an inhibition assay as a routine step in their assessment of RNA quality. It is well known that repeated freeze/thaw cycles affect the integrity of the RNA, and repeated opening of a stock RNA will increase the likelihood of its contamination. It is particularly important to assess any RNA extracted from formalin-fixed archival material for inhibition of the qPCR assay, since technical rather than real biological reasons could underlie any differences in RNA levels [8].

Most researchers quantitate their RNA, but 14% do not. The most popular method remains measuring the absorbance at 260 nm in a spectrophotometer (40%), followed by Nanodrop (18%), Agilent (8%) and Ribogreen (5%). A total of 15% of laboratories rely on a combination of methods. However, results from a comparative study carried out during the European Molecular Biology Organization (EMBO) qPCR course run in Heidelberg, Germany in 2003 showed that there are significant differences in quantification results recorded by these different methods (FIGURE 1). The conclusion from these data is that it is best not to compare data between preparations quantitated using different methods and that it is important to choose one method of quantification and then stick with it.

The overwhelming dominance of ABI in the instrumentation market is clearly on the wane, although 40% of delegates still use one of several ABI instruments. The old warhorse, the 7700, remains the most popular ABI instrument at 16%, with the high-throughput 7900 being used by 13% of delegates. The remaining 60% of the market is split between seven manufacturers, with Stratagene (15%), Roche (13%), Corbett (11%) and BioRad (10%) sharing similar market shares.

Reverse transcription & PCR

Most researchers are very conservative in their use of enzymes, with 86% always using the same reverse transcriptase (RT) and 80% the same Taq polymerases in their assays. This is probably wise, since there are significant differences in the performances of the different RT [20] as well as Taq [21] enzymes. RT priming strategies, on the other hand, are highly diverse. Random priming is by far the most popular choice (43%), followed by oligo-dT (21%), random primers plus oligo-dT (18%) and specific primers (18%). This variability is important, as results obtained using different priming strategies are not comparable [22] and can be nonlinear, particularly when combined with different amounts of starting material [8]. These results are slightly different from a survey carried out last year, which showed oligo-dT to be the most popular priming method (40%), followed by random priming (30%), specific priming (20%) and random priming plus oligo-dT (10%) [9]. One reason for the differences, particularly with respect to the relative popularities of the random and oligo-dT priming methods, may be that the previous survey was carried out largely in the USA, whereas most respondents to the current questionnaire came from Europe.

A minority of researchers work with extremely small amounts of initial target and preamplify their mRNA (12%). The amount of RNA used in the RT reaction by the majority (88%) was surprisingly variable, ranging from less than 10 ng up to 10 µm and most respondents using a still surprisingly high 1 µm total RNA (FIGURE 2). The most authoritative source recommends using between 5 and 250 ng RNA [23] and the efficiency of reverse transcribing 10 µm of RNA must be subject to significant doubt.

Despite the lack of primer optimization, most researchers claim to achieve highly efficient PCR assays, with 70% reporting amplification efficiencies of between 90 and 110%, and only 5% reported less than 80% efficieny. Most respondents

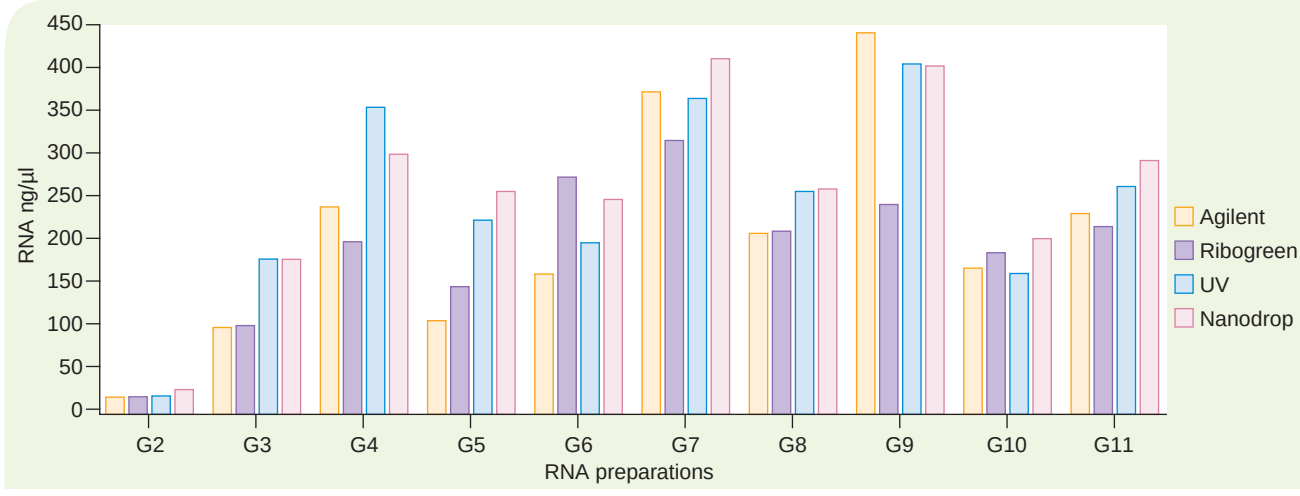

Figure 1. RNA was extracted by different groups of students (G2–11) from the same number of tissue culture cells and quantitated. Three points are notable: there is significant variation in the amount of RNA extracted by the different groups; the quantification of RNA within groups is highly variable; and there is no obvious correlation between the different quantification methods.
UV: Ultraviolet.

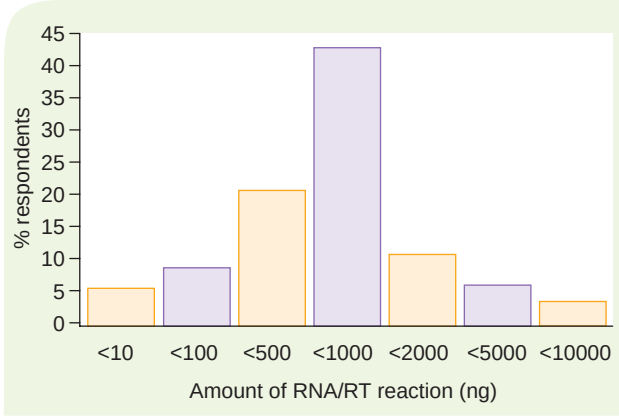

Figure 2. Significantly different amounts of RNA are used for complementary DNA synthesis. Most protocols recommend the use of no more than approximately 250 ng RNA per reaction.
RT: Reverse transcriptase.

(73%) monitor amplification efficiency using standard curves with every assay, with complementary DNA (39%) by far the most popular template for generating the serial dilutions. Only 15% used RNA, either an amplicon-specific T7 transcript (6%) or total RNA (e.g., Stratagene's Universal RNA; 9%). It would be preferable if RNA were used, since standard curves obtained using DNA can be significantly different from those obtained using RNA [8].

Only 17% of respondents prepare their own PCR reagents, with the remainder relying on commercial kits. ABI still command the most loyalty (35%), with Roche (10%) and several other suppliers competing for the remainder of the market. The main problem with relying on manufacturers' kits is that the end user has no control over the additives included in the master mixes. Therefore, any change of formulation by the manufacturer may result in variable results being recorded. Furthermore, it makes troubleshooting more difficult, as it is not possible to systematically substitute and add different components of the mastermix. Therefore, highly experienced researchers tend to use their own mastermixes, whereas those running only occasional assays rely on manufacturers' kits.

Normalization

Biologically meaningful quantification of RNA requires accurate and relevant normalization to some standard, ideally internal, as RT-PCR-specific errors in the quantification of mRNA transcripts are easily compounded by any variation in the amount of starting material between samples. This is particularly relevant when dealing with *in vivo* samples that have been obtained from different individuals or when comparing samples from different tissues. Consequently, the question of appropriate standardization arises and constitutes one of the most critical aspects of experimental design. Unfortunately, normalization of qPCR assays remains an unsolved problem, with none of the proposed methods gaining universal approval. Nevertheless, there has been agreement that normalization against a single reference gene should be strongly discouraged [24–33]. In the last few years, several software tools have become available that permit relatively straightforward selection and use of appropriate reference genes and there really is no excuse for not carefully selecting and applying internal references [29,30,34,35]. Nonetheless, 53% of respondents still use a single reference gene and, even more worryingly, 62% of those do not validate that gene. Only 30% of researchers use the recommended three reference genes, and all of those validated them as suggested. The most popular validation tool is geNorm [102], which is used by 24% of those validating their reference genes. However, it is abundantly clear that the vast majority of researchers continue to report expression data using a single, unvalidated reference gene and that, consequently, many data will be unreliable at the very least.

Data analysis & reporting

Data analysis does not mean simply scanning the experimental report generated by the real-time instrument. Most respondents (85%) have now realized this and examine the amplification plots from every individual well to confirm that a positive threshold cycle (C_t) is the result of genuine amplification, rather than some artifact. Furthermore, 53% will change the instrument-suggested threshold, to ensure the crossing point falls within the exponential part of the amplification curve. A total of 96% of researchers run replicates, and out of those, 32% run duplicates, 62% run triplicates and the remainder run up to six replicates. However, 44% of respondents do not repeat their assay, surely a fundamental way of confirming any result, positive or negative. Of the 56% that do a repeat run, 35% repeat their assay once or twice, 10% more than twice (up to ten times) and 11% replied that it 'depends', although they did not specify on what. Most researchers determine the credibility of their assays by relying on a minimal difference in C_t between replicates: differences of less than 0.5 (13%) or 1 (17%), or some percentage variation (12%) are the most commonly used methods. However, 52% provided no answer or replied 'in development', with 6% citing 'gut feeling' or 'eyeballing'.

One of the most important controls in any biologic assay is the negative control that reveals the presence of contamination. In the case of qPCR assays, this is the no template control (NTC), which should be negative. Although 92% of researchers include NTCs with their assays, 8% do not. Of the 92% that include NTC, 59% would not use any data from that run, 25% would do so if the difference in C_t between their unknown sample and the NTC was greater than four C_ts, and a surprising 16% will use their data even if the NTC is positive. However, one might argue the acceptability of a positive result that differs by four or five C_ts from a positive NTC [8], it is disconcerting that a significant minority of users are prepared to use data from a contaminated run.

There is near universal agreement that any publication should reveal the primer sequence (95%), slightly less so for the probe (85% of those using a probe). Surprisingly, publication of the assay's sensitivity and amplification efficiency is not considered necessary by 69 and 31% of respondents, respectively. The

same 50% of respondents consider publishing RNA quality and integrity and coefficients of variation of their assays. They are also contained within the 58% that consider publishing the dynamic range of their assays.

Conclusion

These are the first published results from an independent questionnaire answered by nearly 100 researchers from academia, industry and diagnostic laboratories. The answers confirm the suspicion that many contradictory results are caused by the huge range of protocols employed to carry out qPCR assays. However, it is also clear that many qPCR assays are carried out inappropriately at the design stage, the assay itself and during data analysis. These data confirm the urgent need for agreed standards, operating procedures and best practice analysis methods. Until that happens, data obtained from qPCR assays must be continued to be viewed with some degree of doubt.

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