The Importance of Quality Control During qPCR Data Analysis

Introduction

Since its introduction in 1993, qPCR has paved its way towards one of the most popular techniques in modern molecular biology [1]. Despite its apparent simplicity, which makes qPCR such an attractive technology for many researchers, final results are often compromised due to unsound experimental design, a lack of quality control, improper data analysis, or a combination of these. To address the concerns that have been raised about the quality of published qPCR-based research, specialists in the qPCR field have introduced the MIQE guidelines for publication of qPCR-based results [2]. The main purpose of this initiative is to make qPCR-based research transparent, but the MIQE guidelines may also serve as a practical framework to obtain high-quality results. Within the guidelines, quality control at each step of the qPCR workflow, from experimental design to data analysis, is brought to the attention as a necessity to ensure trustworthy results.

Numerous papers have been written about assay and sample quality control [3], but less attention has been spent on quality control on post-qPCR data. This article summarizes recommendations for this latter type of quality control including: detection of abnormal amplification, inspection of melting curves, control on PCR replicate variation, assessment of positive and negative control samples, determination of reference gene expression stability, and evaluation of deviating sample normalization factors.

Amplification Plots

Typical qPCR amplification plots contain four distinct regions: (1) a region in which fluorescence does not come above the background during the first cycles of the PCR, followed by (2) a small region in which exponential amplification can be detected, immediately continued by (3) a region with decreasing amplification efficiency, ultimately leading to (4) the plateau phase at the end of the reaction. The shape of these sigmoidal curves is affected by the amplification efficiency of a specific assay that should be the same for all samples to make basic quantification models valid [4]. Samples that contain inhibitors may be detected by comparing the amplification plots across all samples for a given assay, provided that the degree of inhibition is sufficiently large to result in a noticeable effect on the amplification plot. This comparison may be done visually by looking for plots that are less steep, and often result in lower plateau phase levels (Fig. 1). More recently, algorithms have been developed to objectively (and more sensitively) identify such abnormal amplification reactions [5, 6].

Melt Curves

Intercalating dyes are not sequence specific, but they allow the generation of melt curves by including a dissociation run at the end of the qPCR program. The double-stranded PCR product is melted during the gradual heating steps, which goes hand-in-hand with the release of the intercalating dye and a reduction of the fluorescent signal. When plotting the decrease in fluorescence as a function of increasing temperature (-dF/dT), an amplicon-specific melt peak is generated.
Overall, four different types of melt peaks can be distinguished. A specific PCR reaction is characterized by a single sharp peak with a melting temperature (Tm) that depends on the amplicon’s length, GC content, and PCR reactions components (Fig. 2a). Nonspecific PCR reactions, on the other hand, will usually generate one or multiple extra peaks or a single broader peak, depending on the number of nonspecific products and the variation in Tm (Fig. 2d). Primer dimers may generate an additional broader and more flattened peak at lower temperature and are usually more pronounced in reactions with low template concentrations and no template controls (NTC) (Fig. 2b). Sometimes a specific amplification reaction may show a so-called shoulder peak, an additional melt peak very close in Tm to the main peak. This occurs with amplicons that comprise multiple melting domains with varying GC content, which are more likely to occur in longer amplicons (Fig. 2c). Nonspecific amplification reactions may be better distinguished from reactions with primer dimers or from amplicons with multiple melting domains by testing the PCR products on agarose gels or capillary systems (Fig. 2e).

**Controls**

Several types of control samples constitute a prerequisite in each qPCR-based analysis, in particular for (clinical) tests that fall under quality assurance programs.

As for all PCR-based assays, no template controls (NTCs) are indispensable in detecting the presence of contaminating DNA. An amplification signal in the NTC sample indicates a potential contamination issue or the formation of primer dimers. Such signals may be ignored as long as the difference in Cq value between the NTC and the sample with the highest Cq value is sufficiently large. For example, a Cq value difference of 5 corresponds to a fold difference of about 32, indicating that approximately 3% of the signal in the samples may be caused by unwanted signals, which is well below the technical error on PCR replicates. Smaller differences between the NTC and the unknown samples should be avoided.

When primer dimers are detected the experimenter should check the Cq values of NTC and samples of interest. If the difference in Cq value is small, the signal in your sample may mainly reflect primer dimers rather than a specific signal for the target of interest. Therefore, results can only be trusted if the contribution of the primer dimer signal is marginal (e.g., a difference in Cq > 5) (see section ‘Controls’). To minimize primer dimers, it is recommended to use primers at a lower concentration (typically 150-250 nM). If the primer dimer signal remains problematic, assays should be further optimized or redesigned.

Melt curve analysis is performed at the end of the PCR program, typically after reaching the plateau phase. Therefore, there is no absolute correlation between the height of the melt peaks and the Cq values. A lower melt curve peak may result from samples with low expression in which the plateau phase was not yet reached, but a lower curve may also indicate a plateau phase with a lower fluorescent signal in comparison to other samples. The latter situation is suggestive for the presence of inhibiting substances within the reaction.

**FIGURE 2**

Ideally, PCR products should show a single sharp peak on -dF/dT plots (a). Other patterns may result from the presence of primer dimers (b), the occurrence of multiple melting domains in a single specific PCR product (c), or nonspecific amplification (d). When tested by size separation (e), these products will show respectively a single clean band, an extra vague and rather broad band at low molecular weight, a single clean band, and 2 or more distinct bands.

A second type of control is the minus reverse transcription control (-RTC) to assure the absence of contaminating gDNA for qPCR-based gene expression analysis. To date, the most efficient strategy to remove contaminating DNA is to perform an enzymatic DNase treatment. Such treatment is not 100% effective and a careful check for the absence of DNA should be performed through qPCR analysis of a DNA target on the crude RNA, also referred to as the minus reverse transcription control. An amplification signal in the -RTC indicates a potential contamination with residual gDNA or the formation of primer dimers. Such signals can be ignored as long as the difference in Cq value between the -RTC and the sample with the highest Cq value is sufficiently large.

Specific for qPCR-based copy number analysis is the inclusion of reference samples with a known copy number (i.e., with normal copy numbers or with deletions or duplications). During calculations and result interpretation, these control samples can be used as a reference point (or calibrator) for the determination of copy numbers [7]. The inclusion of multiple control samples will result in more accurate results. The latter is analogous to the use of multiple reference genes during normalization, which also generates more reliable results. Reference samples can also be included for qPCR-based expression analysis. These can be biological controls against which up- or down-regulation is tested, or true negative or positive controls in which the target is not or is expressed, respectively.

**Dilution Controls**

When working with probes, two additional types of controls can be included, namely no amplification controls (NAC) and internal amplification controls (IAC). An NAC contains all reaction components and the template (gDNA or cDNA), except for the polymerase. An amplification signal indicates auto-fluorescence of the probe, which may be attributed to probe degradation. An IAC serves to exclude false-negative results due to inhibition by substances originating from the sample or due to other flaws during the qPCR reaction [8]. IACs are of utmost importance when using samples that are prone to inhibition, thus posing a risk for false-negative results [8-10]. An IAC is a DNA sequence that is spiked into the reaction and that is co-amplified with the target. The absence of an amplification signal for an IAC denotes a false negative result, which can be attributed to inhibition if an amplification signal can be observed on a dilution of the same sample, or to an error in the reaction composition.

Dilution controls may serve as a good alternative to IACs to detect inhibition when working with intercalating dyes. The effect of inhibiting substances becomes less pronounced by diluting a sample. Therefore, a diluted sample can be included in an experiment as a control to detect inhibition. The difference in Cq value between a diluted and an undiluted sample should correlate with the dilution factor, which will not be the case if inhibiting substances are present. The inclusion of dilution controls in each experiment comes with a higher cost and implies a tradeoff between cost per sample and the need for this kind of quality control.

**PCR Replicates**

PCR replicates are often included in qPCR experiments to increase the precision of the measurements and to obtain confidence in the results. Both are directly related to the number of PCR replicates. Larger number of replicates (≥2) are often used when high confidence is required (e.g., in a diagnostic setting) or when high precision is needed to
allow detection of small differences. On the other hand, large screenings in which overall expression profiles, rather than individual data points, are of importance or studies with a sufficiently large group of samples may not need repeated measurements [11].

The quality of PCR replicates is evaluated in terms of its variation; expressed as 1) delta-Cq values (ΔCq, difference between the replicate with the highest Cq value and that with the lowest Cq value), or 2) standard deviations (SD). Both measures are equally well suited to evaluate PCR replicate variability, and are in fact mathematically linked to each other: SD = | ΔCq | / CF with CF as conversion factor (Table 1). In fact, the standard deviation increases 0.1 units per 0.141 cycle difference between duplicated reactions; similarly, the standard deviation increases 0.071 units per 0.1 cycle difference between duplicated reactions. Hence, if you want to flag bad duplicates that differ by more than 0.2 standard deviations, you need to use a 0.282 cycle difference. For triplicates, a standard deviation threshold of 0.1 means that the highest and lowest Cq value can only differ by maximum 0.2 cycles from each other and by 0.1 cycles from the middle point.

A general idea about the overall quality of PCR replicates within an experiment can be obtained by plotting the cumulative distribution of ΔCq (or SD) values, or by evaluating the percentage of replicates for which ΔCq (or SD) falls below a predefined threshold. No absolute reference values can be given for these thresholds since they depend on the required precision. However, a maximum ΔCq of 0.50 (SD of 0.35) could be a good starting point for the evaluation of duplicate qPCR reactions.

If low-quality PCR replicates are detected, one may consider cleaning up the data, i.e., removing bad replicates to retain only high-quality data. Despite the potential benefit of data clean up, it is important to be very cautious not to tune data to ‘more pleasing’ results. There has to be a good objective reason to remove specific replicates. Outliers (e.g., detected with Grubb’s test), known pipetting errors and flawed reactions that can be recognized based on abnormal amplification plots or melt curves (see above) may objectively pinpoint the failed reaction. All too often, it is impossible to recognize the bad replicate, leaving the experimenter with two options: (1) removal of all bad replicate data points to make sure that all remaining results are of good quality, or (2) retention of all replicates while doing proper error propagation to make sure that the final errors on the results properly reflect the lower quality of the underlying data.

### Reference Gene Stability

In the past, many approaches for normalization of gene expression data have been proposed. Among these, the inclusion of reference genes (in the past referred to as housekeeping genes) is by far the most popular method for normalization of qPCR data. All technical variations during sample preparation affect reference genes and genes of interest equally, making it the preferred method. However, this approach is not completely assumption free: reference gene-based normalization is only correct if the reference genes are stably expressed across all samples being compared. This assumption must be tested and validated...
before results can be trusted, geNorm pilot studies in which the stability for a series of candidate reference genes is evaluated in a representative set of samples [12] are the preferred way to determine the best set and required number of reference genes to be used (Figure 3).

Despite the validation of reference genes in such pilot studies, it remains important to confirm the stability of the selected reference genes in the actual experiments containing all samples being evaluated. Stability of reference genes can be determined by calculating their geNorm M value (M) or their coefficient of variation on the normalized relative quantities (CV). These values can then be compared against empirically determined thresholds for acceptable stability. For normalization of genomic DNA copy numbers, very low numbers should be obtained (M < 0.2, CV < 10%) [7]. Acceptable values in gene expression studies depend on the heterogeneity of the sample set. For homogeneous sample sets like cell cultures of the same cell type, M should be lower than 0.5 and the CV should be below 25%. For more heterogeneous sample sets (comparison between different cell types, biopsies, cancer material, …) it is often quite impossible to obtain the previously stated stability measures, but it is advisable to aim for M < 1 and CV < 50% [13].

It is important to note that the evaluation procedure described above can only be performed if multiple reference genes have been used for normalization. Also, the geNorm algorithm only works on datasets without missing data, i.e., all reference genes should have expression values for all samples. A recent improvement of this algorithm can deal with missing data and provides users with an expert interpretation of their geNorm pilot experiment (Fig. 3).

There are several reasons why reference gene quality control may fail (reveal high M and CV values) in a given experiment despite successful validation of these genes in a pilot experiment. Bad technical performance of qPCR measurements may introduce significant amounts of variation, resulting in lower stability. Also, the biological variation may be larger because of, for example, new culture conditions, new
can be treated as a measure for the level of technical variation between runs. Because this technical variation may be gene specific, IRCs must be included for all assays that are spread across multiple runs. Importantly, because inter-run calibration affects all results, one has to make sure that the data for these IRCs are of the highest quality possible. Also, the use of dedicated software for this data processing is highly recommended to avoid calculation errors and to perform proper error propagation.

Similar to the above described normalization procedure, quality control on inter-run calibration is only possible when more than one inter-run calibrator has been included in the experiment. All inter-run calibrators should equally measure the technical variation between runs. If one inter-run calibrator returns a substantially different interpretation of this technical variation, as compared to the others, the quality of the data underlying this inter-run calibrator may not be what it ought to be. Exclusion of an inter-run calibrator is perfectly valid as long as it is excluded from all runs for that particular gene.

Conclusions

In this paper, we summarized the different quality control steps while handling post-qPCR data. We state that it is essential to evaluate the quality of the post-qPCR data and to discard data points that do not meet the predefined criteria prior to drawing valid conclusions. User-friendly data analysis software is an invaluable tool within this process. These tools help applying rigorous MIQE-compliant procedures and guide the experimenter to highest quality results.

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1. What, in your opinion, is currently the single largest trend with respect to qPCR?

MP: The largest lasting and still ongoing trend is to increase the throughput by minimizing the single reaction volume. The development of high throughput platforms, like the Fluidigm, Biotrove or the new Roche cycler with 1536-reaction documents this trend.

AS: High throughput expression profiling of samples with low RNA/DNA content.

RP: The largest and most exciting trend with qPCR is that it is becoming a truly mainstream technology. The hardware has become more and more affordable meaning that the technology is available to even more people. The introduction of new categories of machine means that the technology is available to everyone from individual researchers right through to high throughput pharmaceutical users.

KE: The single largest trend is the push to increase the throughput of the qPCR process. Not only are newer instruments capable of analyzing more samples in a single experiment, but the cycle time of the instruments running the experiment has also been improved. In addition, complementary automation technologies have improved setup times and now allow for near continuous operation of the instrument without human intervention.

JG: The move to digital qPCR and general trend of miniaturization of reaction volumes. This needs to be balanced with the ability to maintain repeatability and sensitivity.

JV: There has been a major focus on fast qPCR. Suppliers came with fast PCR instruments and fast reagents, contributing to reducing time to results. Importantly, it is often neglected that other factors contribute to getting faster results. Proper experiment design can not only save a lot of time, but also reagents. In Helemans et al., Genome Biology, 2007, we demonstrated that a plate lay-out based on maximizing the number of samples analysed in a given plate (so-called sample maximization approach) has many advantages. In keeping with this, it is not required to repeat the reference genes on each plate (provided that not all genes of interest can be measured in the same plate). Furthermore, using higher capacity PCR instruments (384-well plates and beyond) and designing qPCR assays with higher annealing temperature also contribute to faster turnaround times. Last but not least, by making sure that you can fully trust your results (by ascertaining proper normalization and correct data analysis), you will not lose precious time by following-up false positive results.

SM: With an unprecedented amount of data generated by high-throughput applications such as GWAS and microarrays, life science researchers have a wealth of new gene information to validate and more accurately characterize. Real-Time PCR is the perfect tool to enable these numerous validation studies thanks to its adapted throughput, high precision (with the ability to detect 2 fold changes with a confidence of more than 99%), great sensitivity and low cost per reaction. These validation studies will surely lead to new pathway discovery, as well as new drug development and diagnostic applications. And the versatility of this gold-standard method leads to workflow process improvements in additional applications such as food safety, environment testing or forensic tests.

2. How, in your mind, has the landscape of qPCR research shifted from when the technique was first introduced to how it is being implemented today?

MP: The entire qPCR field and in detail the quantitative transcriptomics has been developed dramatically during the last decade. In the early days of RT-qPCR (end of the 90-ies) we had major problems by reproducing the quantitative mRNA results from day to day. Most inventions were initially made in the field of the qPCR method itself, by standardizing extraction methods, stabilizing RNA, optimizing kits, master-mixes, and cycling machines.

Later on the research fields and interests changed - from pure mRNA analytics to quantitative microRNA analytics event for pri-, pre- and mature microRNA transcripts. We all started by using an “absolute quantification” by measuring mRNA concentrations and quantities in any biological matrix. Later we moved to “relative quantification”
and were more interested in the mRNA expression changes. Efficiency corrected models were developed by using multiple reference genes to reduce technical variability and to produce more valid reliable results. Later on advanced quantification and data analysis model were developed by using robust statistical models to get information about the statistical significance of the gene expression changes. Today we can say we have enough tools in hand to work on small physiological gene expression changes and report them in a MIQE compliant way.

**AS:** From the beginning most qPCR research aimed to show the proof of principles of the technique for different applications. Today, qPCR is one of the most utilized and standardized techniques in laboratories. It is used as a precise and robust tool to facilitate all kind of molecular work.

**MK:** When the technique was introduced challenges were related to the actual qPCR measurement, i.e., assay design, dye and probe development, instrument performance etc. Today the qPCR is robust and challenges are rather the preanalytical steps (see www.spidiareu) and post-processing of the data, i.e., experimental design, data analysis and data mining (see: www.qpcrforum.com).

**RP:** qPCR is no longer the specialist tool it used to be. Everyone is using it which is great but there is a downside too. A lot of the qPCR data that is produced is simply meaningless because of the poor standards that many laboratories employ. It’s crucial that good practice and the MIQE guidelines in particular are promoted globally to ensure that the extraordinary amount of real-time PCR data produced today is truly useful.

**KE:** The technique was originally designed for the quantification of gene expression; however, qPCR is now capable of performing a large range of assays. Beyond the original gene expression assay, scientists now commonly use qPCR for techniques such as probe-based SNP genotyping, HRM analysis, and plus/minus assays.

**JG:** Like any other technique, qPCR used to be a tool for the elite researcher when first launched. The landscape has shifted now in that there are more instruments available on the market and more and more labs have one (as opposed to using a shared facility)

**JV:** When qPCR was first introduced, most attention was spent to evaluate and improve the overall performance of the real-time PCR instrument and the polymerase chain reaction itself. Nowadays, these 2 elements are no longer an issue; there is a wide availability of high quality instruments and reagents. Instead, the last couple of years, researchers started to scrutinize the entire workflow of qPCR and came up with important quality control at each step, including sample preparation, assay validation and data analysis.

**SM:** About 15 years ago, only a single vendor offered Real-Time PCR solutions. Now multiple vendors offer a wide selection of instruments and reagents that are reliable and robust.

The main advantage to the scientific community is that healthy competition among vendors has made qPCR technology more accessible to life science researchers. Using the new Eco Real-Time PCR system from Illumina as an example, it is now possible to get a high performance instrument capable of multiplexing and High Resolution Melt technology for as low as $13,900. This is about a fourth of comparable Real-Time PCR instruments. With the trend towards easy-to-use software and reliable reagents, more scientists than ever can use this gold-standard technology in their own labs to generate accurate data.

### 3. What country/region, in your opinion, is currently leading the way in qPCR technology and why?

**MP:** Today qPCR is used worldwide and new technologies/applications are continuously coming. Most researchers using qPCR are specialist in specific fields. Northwest Europe is one region with many excellent qPCR researchers, who are in the forefront of the field. They have contributed with many basic publications about the potentials and limitations of qPCR.

**MK:** Europe is leading the development of qPCR applications, while US is leading the technological development. Development of applications in Europe is catalyzed by less stringent separation of clinical diagnostics and research. In most European countries it is not too complicated to introduce new applications into diagnostics as research tools and often even having them validated by expert laboratories. There are also major efforts to stimulate development and introduction of novel medical and diagnostic applications through various European commission programs (http://ec.europa.eu/research). In US venture capital is more abundant.

**RP:** Much of the new IP and ideas of course continue to come from the US whilst China will not doubt play its part in driving down costs of kits and hardware over time. But much of the best practice and most exciting research is occurring in the UK and Europe. In particular, a major contribution has been made in developing methods for accurate normalising real time PCR data culminating in the geNorm and NormFinder approaches. Also the best data processing platform for real time PCR ([qBasePLUS]) has also been pioneered by Biogazelle in Belgium. Many of the other key opinion leaders and best practice drivers who contributed to the MIQE guidelines are based in the UK and Europe which promotes a very high standard of qPCR execution within the region. Our company is a prime example of an organization with qPCR expertise at the heart of it. We are founded on academic expertise which is born out of the high baseline level of expertise that exists in our region.

**KE:** The technology is now being used world wide and, as such, we see significant contributions from around the globe. Based on the quantity of contributed work, the United States is probably the one country that can be called a leader in this technology area.

**JG:** The USA is leading the way. I think that funding initiatives from the NIH for programs around next gen sequencing as well as the government-sponsored need for better detection mechanisms for non-clinical diagnostic purposes (partly spawned from the need to detect bio-pathogens post 9/11). This investment of public money has led to more focused research programs in nucleic acid detection

**JV:** From a product development perspective, the USA is probably leading the way: not necessarily in terms of quality, but definitely in terms of number of companies active in this field. From an application perspective, there also appear to be regional differences. In Europe for example, more people use DNA binding dyes as detection chemistry; in the USA, more people use probe based detection methods. It also seems that more qPCR conferences, workshops and courses are organized in Europe, where the qPCR community might be a lot more active.

**SM:** Most recently Europe has led the advances of Real-Time PCR by its early adoption of the High Resolution Melting (HRM) analysis technology. This technology offers a new option to scientists for performing affordable genotyping studies without the need to rely on more expensive option such as alleleic discrimination using hydrolysis probes. This technology is now expanding worldwide thanks to an increasing number of publications.

### 4. If things progress as they have the past five years, what can we expect in the next five years, with respect to qPCR?

**MP:** I am working now with qRT-PCR for 12 years and must tell the qPCR community they should apply the already developed tools in a correct way. The complexity of the qPCR community is following the MIQE guidelines. This will help those most to generate good and valid results. There are lots of hot and problematic areas where the broad expression profiling community still has to evolve, especially in the Pre-PCR field. Tissue sampling, tissue storage and a proper extraction still bears a lot of errors and introduce, if performed wrong, a huge technical noise in the expression results. From queries on conference and questionnaires at workshop we know that some research labs are still overstrained doing proper expression profiling. Too
many steps are involved in the entire workflow and too many errors can be made. I hope that people learn to adapt the already generated knowledge in a proper way in their research fields.

Of course we need further innovative developments to drive the frontiers forward. Especially in miniaturization, e.g. nano-fluidics and solitary-cell analysis, I see in future an interesting and growing market. Circulating tumor cells or the analysis of migrated cells in any body fluids seems very promising in molecular diagnostics. It will help to understand tumor biology, cell physiology, and cell-to-cell communication in high throughput manner.

A further field will drive the research – non-coding small RNAs. Up to now the microRNA with its pri- and pre-microRNA precursors and the regulation of the transcriptome by mature-microRNAs are in the focus. But the family of the non-coding small RNAs is growing fast and will open a totally new field of applications for quantitative transcriptome analytics.

AS: Increased high throughput, improved pre-steps and refined tools for data handling, standardization and interpretation.

MK: The trend is towards working with less complex samples and high throughput. In particular we expect much more profiling work, using multiple markers and exploiting the correlation between the markers in the interpretation of the data. We also expect integration of the various kinds of molecular markers in the biological interpretations, so called multimodal analysis. In particular, epigenomics markers are likely to make an impact in future diagnostic applications.

RP: Every lab in every biology research institution will have its own qPCR machine. In fact I would not be surprised to find the majority of post-doctoral level researchers having their own personally dedicated machine. Of course there will be growth in large scale high throughput procedures but the growth in the number of individual users is set to boom.

KE: The increases in assay throughput and the improved flexibility of the technology will facilitate the increased use of qPCR in genome-wide and transcriptome-wide screens. In addition, qPCR will become an increasingly important tool to help verify the results of other screening approaches, including GWAS, RNAi knockdown experiments, and whole-genome and whole-transcriptome sequencing studies.

JG: More and more qPCR diagnostic tests for disease markers. This will require higher throughput for large screening studies as well as personalized systems to run the assays in small hospital-like environments.

JV: Reaction miniaturization (down to 500 to even 10 nl), increased number of parallel reactions in one qPCR run (up to 1536, 3072, 5184, 9216 reactions and beyond) and sample pre-amplification methods will further revolutionize the way we do qPCR based screenings. It is expected that entire transcriptomes will be measured by qPCR, with superior sensitivity and specificity, and much faster time to results compared to microarray technology. The relatively simple workflow and straightforward data analysis also put qPCR in a favorable position compared to massively parallel sequencing technologies.

SM: Real-Time PCR will be more widely adopted in virtually any life sciences lab and be located on all lab bench tops next to other traditional lab equipment, such as centrifuge, gel box and plate reader.

It may completely replace traditional PCR thermocyclers as the price is now so compelling.

5. In your opinion, what research field is benefitting most from the implementation of qPCR and why?

MP: Since the volume and the necessary sample amount are getting smaller and smaller all minimal invasive diagnostic applications benefit most. Today man can analyze in a drop of blood already multiple hundreds genetic or transcriptomic markers by qPCR.

Further the single-cell diagnostic e.g. circulating tumor cells (CTCs) will help to understand tumor biology and the mechanism of metastasis.

AS: Hard to say, since there are so many useful applications of qPCR. However, the diagnostic field has benefited a lot of the implementation of qPCR. To mention some aspects, qPCR compared to most other comparable techniques is more sensitive, has a larger dynamic range, can be automatized and handle more samples, use lower amount of biological material, save time and has reduced cost per sample.

MK: The area that benefits most from qPCR today is arguably molecular diagnostics of infectious diseases. qPCR is just so much more sensitive, specific, reliable and faster than alternative techniques for this purpose, and it is becoming affordable for routine laboratories. Major expansion is expected in 2012, when key patents expire making this huge market accessible to many new suppliers of qPCR tests and also instrument manufacturers. In research cell biology, including stem cell research, cancer research and developmental research is benefitting greatly from qPCR and in particular from the forthcoming new qPCR applications, including single cell expression profiling and digital PCR.

JP: It’s hard to say which area is benefitting most: the real excitement lies in the fact is that they all are. Drug discovery is one area to benefit but the list is endless: academic/diagnostics/genomics/food/veterinary/. Real time PCR is both powerful and versatile and new applications of it are constantly entering the market. For this reason the technology will continue to be deployed at the front line of many complex drug discovery and research problems.

KE: Obviously, qPCR has revolutionized any area of science where it is necessary or important to measure the concentration or amount of RNA or DNA. However, one field that is really benefitting from the use of qPCR is clinical microbiology. The technology allows for the rapid detection of bacterial or viral infections at their earliest stages, helping to prevent and treat illness much sooner than prior technologies would allow. The technology has also helped to profile and understand the emergence and spread of new variants of common illnesses such as the flu.

JG: I am not sure that any one field is benefitting from qPCR. I think that qPCR is part of the whole resurgence of PCR and nucleic acid detection on the back of technologies such as Next Gen sequencing and RNAI (as well as array work) which requires some sort of detection chemistry for validation.

JV: When it comes to research, virtually all fields benefit from qPCR technology. While gene expression analysis is probably the most popular qPCR application, any application with limited amounts of sample material (e.g. fine needle biopsies, single cell studies, etc.) or necessity to get fast answers could potentially benefit from qPCR. Importantly, outside the research field, qPCR is becoming more widely used in molecular diagnostics, e.g. pathogen detection, assessment of copy number changes and profiling of disease gene panels (gene expression signatures).

SM: Researcher in the field of environment, animal and plant sciences have a harder time accessing large funding compared to scientists in the human or clinical research fields. As Real-Time PCR gets more affordable it will become more accessible and enable faster time to data than current technologies.

6. What impact is being seen following the introduction of the MiQE guidelines?

MP: MiQE guidelines were established to describe the minimum information necessary for evaluating qPCR experiments. They should provide a catalogue for authors, reviewers and editors to measure the technical quality of submitted manuscripts.

The guidelines establish a clear framework to conduct quantitative RT-qPCR experiments and to support experimental transparency and increase reproducibility between laboratories worldwide. For me the most important outcome of the MiQE guidelines is to increase reliability of generated results and to help to insure the integrity of scientific work, with major focus on biological relevance.

AS: Even if it goes slowly, more researchers are today aware about minimum/necessary requirements that are needed to report reliable qPCR data. I think/hope that researcher will start using the MiQE guidelines as a tool to improve their own experimental design.

MK: Researchers have become much more aware of the complications associated with accurate nucleic acid analysis based on qPCR, which will improve the quality and reliability of published data. At TATAA Biocenter we see a major increase in the interest of our Priority Customer Service, where we advise clients in setting up reliable and cost optimized
qPCR studies, and we also see major interest for our MIQE compliant qPCR courses on quality control and experimental design (http://www.tataa.com/Courses/Courses.html). Users are learning that many studies are too small leading to inconclusive results and occasionally too large resulting in unnecessary spending to reach desired conclusions. We also see that qPCR symposia (www.qpcrsymposium.com, www.qpcrsymposium.eu) attract more and more crowd, particularly sessions related to MIQE guidelines, quality control and standardization.

RP: Not as much impact as I would like to have seen! The MIQE guidelines have caused a bit of a stir. Some individuals and organizations have reacted defensively to them which is inevitable but at least the subject of good practice is being discussed in those places as a result. The principles within the guidelines have a robust scientific foundation which I do believe would be of great benefit if they were universally adhered to. However there is some scope for further guidance on how to implement them in a practical and cost effective way. I think that debate would facilitate a wider acceptance of the guidelines. Editorial bodies as well as individuals need to protect the integrity of published data by grasping the benefits that good practice vs bad practice can bring. My hope is that the MIQE guidelines will come to be seen as a watershed moment in the way the technology is used and data reported. That would be fantastic step forward for this technology and the global research community as a whole.

KE: We have seen scientists who perform qPCR enthusiastically embrace the MIQE guidelines. As such, we are receiving an increase in requests for information that will allow researchers to comply with these guidelines. Wherever possible, Sigma-Aldrich responds to these requests with the information necessary.

JG: People are becoming more aware of the need to pay attention to these guidelines if they are to get a publication. In addition more of the vendors in the qPCR space are making sure that their products/services are MIQE-compliant.

JV: Interestingly, users and companies appear to readily adopt the nomenclature guidelines. For example, many speakers at conferences now refer to Cq values instead of Ct, and reference genes instead of housekeeping genes. There is also a lot of attention to the RDML data format for exchange and storage of qPCR data. Biogazelle’s qbasePLUS was the first real-time PCR data analysis software that is MIQE compliant and that offers RDML import and export functionality. Other third party software companies and real-time PCR instrument suppliers are currently making their software RDML compatible. Unfortunately, when it comes to following and reporting the laboratory guidelines, the voluntary uptake rate by the user community needs improvement. The MIQE authors are trying to spread the word as much as possible, but also journal editors and reviewers have a great responsibility. In analogy to the MIAME guidelines, it would be great if MIQE was to be imposed by the journal before a manuscript can be considered in which qPCR results contribute to the conclusions of the study.

SM: The MIQE guidelines have been embraced by many Real-Time PCR instrument providers. This is validation that such guidelines were needed in this growing field to ensure that qPCR data are produced in a rational way.

Adopting these guidelines is still a challenge to some scientists due to the large amount of info that is required. Researchers are gradually becoming more comfortable with the guidelines as they see their utility in designing future experiments. We have a quick checklist on our illumina.com/ecoqpcr website that is aimed at facilitating the use of the MIQE guidelines.

These guidelines will continue to evolve and ultimately be adopted by the growing Real-Time PCR scientific community.

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