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DOI 10.1007/s00769-011-0769-y

REVIEW

Standardisation and reporting for nucleic acid quantification

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Received: 11 January 2011 / Accepted: 3 March 2011

Abstract The real-time quantitative polymerase chain reaction (qPCR) is probably the most common molecular technique in use today, having become the method of choice for nucleic acid detection and quantification and underpinning applications ranging from basic research through biotechnology and forensic applications to clinical diagnostics. This key technology relies on fluorescence to detect and quantify nucleic acid amplification products, and its homogeneous assay format has transformed legacy polymerase chain reaction (PCR) from a low-throughput qualitative gel-based technique to a frequently automated, rapid, high-throughput quantitative technology. However, the enormous range of protocols together with frequently inappropriate pre-assay conditions, poor assay design and unsuitable data analysis methodologies are impeding its status as a mature, 'gold standard' technology. This, combined with inconsistent and insufficient reporting procedures, has resulted in the widespread publication of data that can be misleading, in particular when this technology is used to quantify cellular mRNA or miRNA levels by RT-qPCR. This affects the integrity of the scientific literature, with consequences for not only basic research, but with potentially major implications for the potential development of molecular diagnostic and prognostic monitoring tools. These issues have been addressed by a set of guidelines that propose a minimum standard for the provision of information for qPCR experiments ('MIQE'). MIQE aims to systematise current variable qPCR methods into a more consistent format that will encourage detailed auditing of experimental detail, data analysis and reporting principles. General implementation of these guidelines is an important requisite for the maturing of qPCR into a robust, accurate and reliable nucleic acid quantification technology.

Keywords Real-time polymerase chain reaction · DNA · RNA · Pathogen · Quantification · FRET

Presented at the conference "Future of Reference Materials", November 2010, Geel, Belgium.

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Introduction

The real-time fluorescence-based quantitative polymerase chain reaction (qPCR) is the most ubiquitous molecular technology in use today and is most commonly used for the detection and quantification of nucleic acids [1] and more recently of proteins [2]. Its major advantage is that its detection of the fluorescence released during or following the amplification of specific DNA sequences eliminates the need for the post-PCR processing inherent in the separate tube-based amplification and gel-based analysis steps of the original PCR reaction. The technology utilises the quantitative relationship between the amount of target initially present in a sample and the amount of PCR product at any given cycle number and is characterised by the quantification cycle (C_q), which defines the time point when amplification of a PCR product is first detected

Published online: 19 March 2011

(Fig. 1). Hence, the C_q represents a detection threshold for the instrument and is dependent on the starting template copy number as well as the efficiency of PCR amplification. The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number.

Detection chemistries

There are two major approaches to generating the fluorescence required to quantify amplified nucleic acids:

1. The simplest method uses a fluorescent dye, e.g. SYBR Green, which binds specifically to double-stranded DNA [3]. The unbound dye exhibits little fluorescence in solution, but during elongation increasing amounts of dye bind to the nascent double-stranded DNA. When monitored in real-time, this results in an increase in the fluorescence signal that can be observed during the polymerisation step Fig. 2a). Although this approach is no more specific than conventional PCR, the PCR product can be verified by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon [4] (Fig. 2b). A characteristic melting peak at the amplicon's melting temperatures (T_m) will usually distinguish it from amplification artefacts that melt at lower temperatures in broader peaks. It is important to note that multiple dye molecules bind to a single amplified molecule; consequently, the amount of fluorescent signal generated is dependent on the mass of double-stranded DNA produced in the reaction, with longer products generating more signal than shorter ones.
2. More specificity is provided by approaches that rely on the hybridisation of fluorescence-labelled oligonucleotides to the correct amplicon: a fluorescent signal is only generated if the amplicon-specific probe hybridises to its complementary target. Depending on the chemistry used, the increase in fluorescence may be reported at the annealing step, when the probe is hybridised or at the end of the polymerisation step, when probe hydrolysis has resulted in the physical separation of fluorophore and quencher. The most popular method uses single hydrolysis probes and is popularly known as 'TaqMan' [5]; other commonly used methods, especially widely used for diagnostic assays, use two hybridisation probes [6] or structured probes ('Molecular Beacons') [7] to maximise specificity, with additional alternative chemistries on offer [1].

Potential problems

A key attraction of qPCR technology is its apparent simplicity: an assay consisting of combining oligonucleotides,

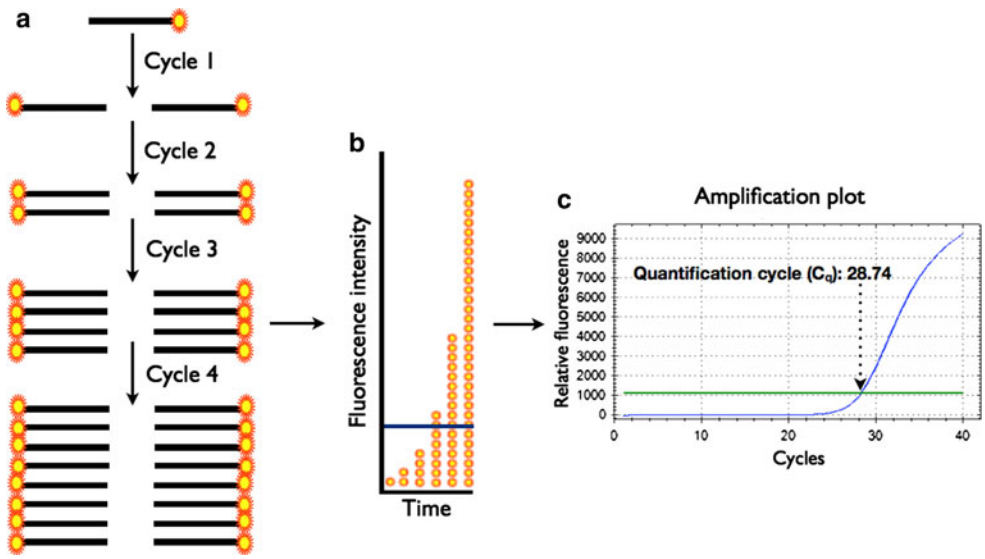

Fig. 1 qPCR methodology. **a** Under ideal conditions, every cycle of the PCR reaction results in the doubling of the amplified DNA target. qPCR assays quantify the amount of amplified product through the detection and quantification of a fluorescent dye. Upon excitation that dye emits a signal that increases in intensity in direct proportion to the amount of amplified PCR product. **b** A *plot* of fluorescence intensity versus time allows shows the exponential nature of the accumulation of PCR product. *The horizontal line* indicates the position of the threshold below which the instrument is unable to detect fluorescence above background. **c** A *plot* of increased fluorescence against cycle number generates the characteristic 'amplification plot'. During the initial cycles of PCR, the fluorescence signal remains unchanged as it is below the detection threshold of the detection instrument; this defines the baseline for that amplification *plot*. Accumulated PCR product is detected by an increase in fluorescence above the baseline. The quantification cycle (C_q) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold and so allows the quantification of the amount of target DNA

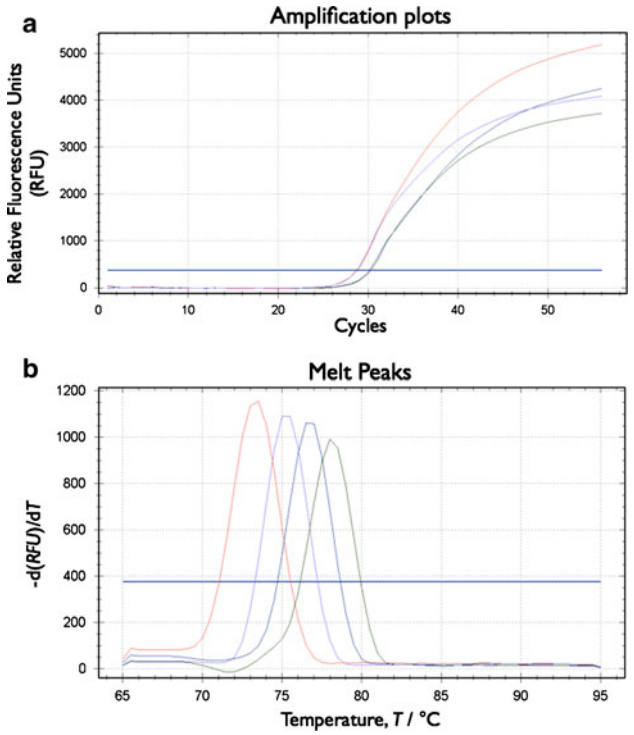

Fig. 2 Discrimination of PCR amplicons detected using SYBR Green by melt curve analysis. **a** Characteristic amplification plots obtained from four different qPCR assays carried out simultaneously. **b** After the PCR, samples are heated from 65 to 95 °C. When the temperature reaches the T_m of the different PCR amplicons, there is a steep decrease in fluorescence as the product denatures to single strands that no longer bind SYBR Green I Dye. This is visualised most easily on a plot of the negative derivative of fluorescence with respect to temperature($-dF/dT$) versus temperature. This generates 'melting peaks' in temperature regions of steep fluorescence decrease. The centre of the melting peak is the T_m of the melted DNA product melted and is different for the four amplicons

PCR enzyme and buffer with a nucleic acid template to produce a qPCR reaction is perceived as undemanding. This practical simplicity is complemented by the absence of any requirement for post-assay handling, as well as the development of user-friendly data analysis software that makes data generation and visualisation in the shape of amplification plots remarkably simple. Indeed, qPCR is often described as a mature technology and as the 'gold standard' for nucleic acid quantification. Whilst it is true that the technology is capable of exquisite sensitivity and specificity, coupled with high reproducibility and accuracy, it is essential to understand that qPCR assays are made up of numerous, often divergent protocols that use different instruments, enzymes, buffers and non-identical targets. Furthermore, today's biomedical testing is resulting in sample sizes becoming smaller and smaller, driving the need to measure samples with ever-lower detection limits. These problems have been known and publicised for a long time [8–12] and it has gradually become clear that it is essential to develop a set of standard and objective quality control measures that allow the design and publication of reliable qPCR assay data [13–21]. The problem with poorly designed assays is that whilst they generate measurements, the results become highly variable, inaccurate and ultimately meaningless. This is illustrated in Fig. 3, which compares two assays: the first one is a highly efficient assay that is characterised by a uniform efficiency over a wide dynamic range. Regardless of where the threshold is drawn (Fig. 3a and b), the relative copy numbers of (in this case) mRNA are very similar since the slopes of the amplification plots are very similar. In contrast, the second assay shows amplification plots characteristic of a poor assay, with slopes and recorded fluorescence levels widely different. C_qs and calculated copy numbers vary widely, and lower copy number targets are not reliably detectable (Fig. 3c and d).

A properly conducted qPCR experiment requires extensive assay and sample validation and optimisation as well as appropriate analysis. Unfortunately, not only are many assays not designed well but many publication utilising qPCR technology provide insufficient information to allow a reader to assess the assay and evaluate the validity of conclusions derived from the qPCR data [21]. For example, although it is accepted that RNA integrity and quality assessment is essential for reliable quantification of RNA using reverse transcription (RT)-qPCR assays [22–25], very few publications even mention the term RNA quality. Furthermore, appropriate normalisation strategies are essential, as they control for experimental error introduced during the multistage process required to extract and process the RNA. Different strategies are not mutually exclusive, and the general recommendation is to match sample size, RNA quantity and use around three validated internal controls for the final normalisation [26]. Certainly there is abundant published evidence that the use of a single reference gene to show small changes in target copy numbers generates unreliable data [27, 28]. However, most papers normalise target gene copy numbers against single, unvalidated reference genes, even though there are several published methods clearly detailing methods for selection of appropriate sets of reference genes [29–31]. When this is combined with the reporting of small changes (e.g. differences of less than threefold), it is impossible to conclude whether findings are differences in expression of the gene of interest, the references gene or a combination of both; consequently, statistically significant measurement bias can be introduced leading to incorrect findings [32]. Other common omissions include providing adequate information on amplicons, primers and probes, details of the reverse transcription step and the efficiency of the PCR reaction (Fig. 4). Since this information is essential to assay performance, it becomes impossible to judge the technical

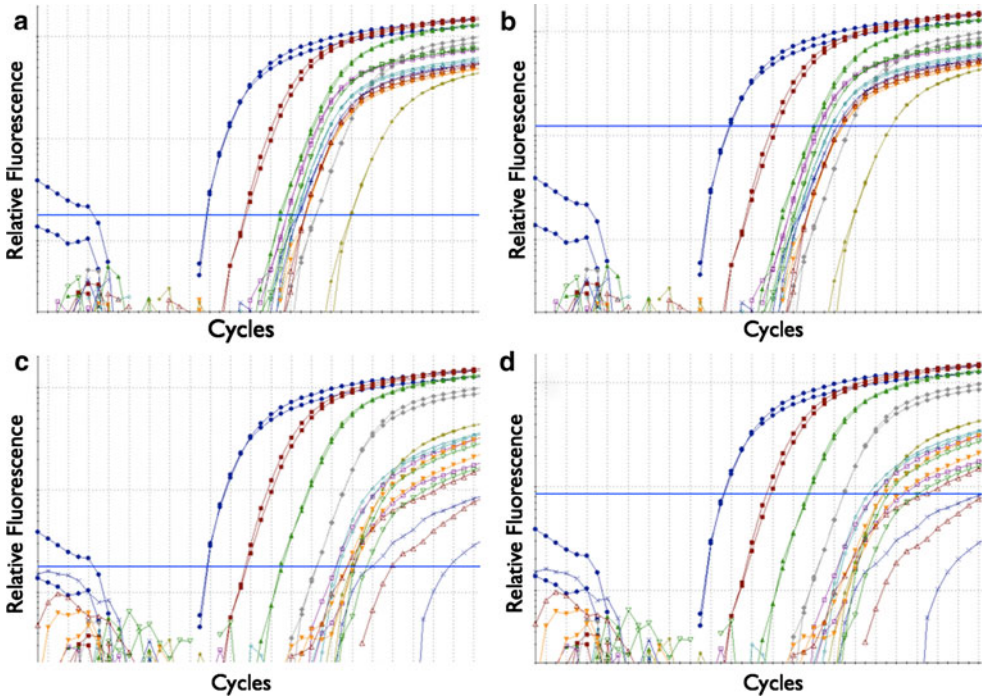

Fig. 3 Effect of poor qPCR on reliability of results. **a** Amplification plots generated by a well-designed efficient assay. The slopes of the amplification *plots* are virtually parallel over a wide dynamic range (C_q*s* 17–32). **b** If the threshold is moved upwards, comparable copy numbers are generated that differ by a maximum of 25%. **c** A poorly designed assay is characterised by slopes that vary considerably, depending on the amount of target DNA present and by highly variable increases in relative fluorescence. **d** If the threshold is moved upward, widely different copy numbers are generated, with some samples now negative (i.e. the increased fluorescence is below the threshold line). Clearly, this renders relative quantification pointless

	Science	Cell	BioMed Central
Sample Quality	0	0	6
RT details	5	1	8
Assay Optimisation	0	2	2
PCR efficiency	1	0	6
Normalisation >1 RG	0	1	4
RG validation	0	0	6
	n=17	n=15	n=16

Fig. 4 Analysis of peer-reviewed publications utilising qPCR and published in 2010. Clearly, the amount of critical information provided is inadequate for the purpose of evaluating the validity of any qPCR results

quality of published data, which should be one of the corner stones of peer-reviewed publication of laboratory research.

One of the most egregious examples of the enormous implications for the health and lives of individuals that result from inappropriate use of this technology is provided by the use of RT-qPCR data that purported to demonstrate the presence of measles virus (MV) RNA in children with developmental disorders [33]. It provided sustenance to the controversy surrounding the triple measles mumps and rubella (MMR) virus vaccine, as the data were interpreted as providing hard evidence for a link between MMR, gut pathology and autism. However, a detailed analysis of the raw data underlying that report carried out by one of the authors (SAB) acting as an expert witness to the UK High Court and the US Vaccine Court revealed that these data were obtained amongst a catalogue of mistakes, inaccuracies and inappropriate analysis methods as well as contamination and poor assay performance [34]. A reanalysis of the data concluded that the assay had been detecting DNA and since MV is an RNA-only virus, the RT-qPCR data had been erroneously interpreted. A recent paper that included two of the authors from the initial report was unable to reproduce the original findings and concluded that there was no link between autism and enteropathy [35]. Astonishingly, despite this and other evidence [36, 37], the authors of the original report have not retracted their previous findings, in spite of the immense repercussions their initial report has had upon public health.

There is an increasing consensus that there is an urgent need to strengthen published information with relevant experimental detail. This requirement for a set of recommendations that can be used by journal reviewers who need to be able to evaluate the reliability of the experimental protocols and ensure the inclusion of all essential information in the final publication was addressed by the 'Minimum Information for Publication of Quantitative Real-Time PCR Experiments' (MIQE) guidelines [38], with a recent amendment regarding the disclosure of primer sequences [39].

MIQE guidelines

MIQE constitutes a set of publication guidelines for researchers, journal reviewers and editors that lists the

minimum information required to allow potential reproduction as well as unambiguous quality assessment of a qPCR-based experiment. MIQE is modelled on analogous rules drawn up for DNA microarray analysis [40], proteomics experiments [41], genome sequence specification [42] and those under discussion for RNAi work [43] and metabolomics [44], initiatives coordinated under the auspices of MIBBI, Minimum Information for Biological and Biomedical Investigations (www.mibbi.org) [45]. The four key areas of standardisation that define any qPCR experiment are study design, technical detail, analysis methods and statistics. MIQE addresses these under a set of captions that describe a large number of individual elements: 'Experimental design, sample, nucleic acids, reverse transcription, target, primers and probes, assay details, PCR cycling and data analysis'. At first sight, these look daunting, arduous and over-exacting. In practice, it is clear that most, if not all of these parameters describe information that would be obtained as a matter of course during the experimental design, optimisation and validation stages. Importantly, there is a clear hierarchy with some parameters, labelled 'E' (essential) in the published guidelines, indispensable for an adequate description of the qPCR assay, whereas other components, labelled 'D' (desirable) more peripheral, yet constituting an effective foundation for the realisation of best practice protocols. There is increasing recognition that the MIQE guidelines provide the basis for much needed standardisation as well as encourage the publication of essential information that should be accessible to reviewer and reader. Of course, these parameters are not set in stone and are open for discussion; indeed, a core set of sections is being implemented by the BMC group of open-access journals [46].

Conclusions

qPCR is a formidable enabling technology that has facilitated many of the advances made in our understanding of basic biological and disease processes. 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. Any solution to the challenge of how to make PCR-based assays more reliable requires both an appreciation and an understanding of numerous attributes that include biological concepts, statistics, mathematical modelling, technical know-how and a willingness to share that information. This range of fundamental variables must be addressed by guidelines that permit a shift of focus from questions regarding the technological relevance underlying a publication's conclusion to the actual biological or diagnostic issues being addressed.

MIQE constitutes a reference framework for communication within the research community, instrument and reagent manufacturers and publishers. Systematic adherence to these recommendations provides structured principles for any qPCR experiment and promotes transparency of experiments. It enables authors to design and report qPCR experiments with greater inherent value, allows journal reviewers and editors to measure the technical quality of submitted manuscripts against an established yardstick and, of most practical importance, results in the publication of papers that will be much easier to replicate [21].

This will deliver a significant boost to the quality of data published and increase the confidence in results and conclusions that advance, rather than impede our knowledge.

Acknowledgments SAB wishes to thank the charity Bowel and Cancer Research for their support.

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