Real-time quantitative PCR – opportunities and pitfalls

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Stephen Bustin obtained his PhD from Trinity College, University of Dublin in molecular genetics. His research interests centre around investigating the process of invasion and metastasis in colorectal cancer, in particular the role played by metastasis-associated oncogenes, signaling cascades and suppressor genes. His specific aim is to identify metastasis-associated expression signatures that will allow the inclusion of molecular parameters into clinical tumour staging, thus improving prognostic accuracy for individual cancer patients. The ultimate aim is the identification of specific sub-groups of patients that will benefit from new, individualised molecular therapeutic strategies. His laboratory continues to operate at the forefront of technological development in nucleic acid quantification. He is an internationally acknowledged leader in this area.

The emergence of next generation sequencing technology has brought the prospect of digital analyses closer, technology that will allow not just the quantification of nucleic acids, but will result in the fine-tuning of this information with respect to tissue- and cell-specific transcription, the identification of new transcriptional units, e.g. the detection of new splice variants and their overall correlation with genomic elements. Until that time, the real-time quantitative polymerase chain reaction (qPCR) continues as the enabling technology par excellence offering an unrivalled combination of simplicity, cost-efficiency, accuracy and availability, with application in every area of life sciences and medicine[1]. Its sensitivity, specificity, and wide linear dynamic range makes qPCR today's method of choice for any research and diagnostic application that aims to detect and measure nucleic acids[2].

This process has been helped by several recent developments that have combined technology to improve qPCR assay performance and accuracy and simplify data analysis:

- the introduction of less expensive, optimised reagents that make reaction assembly simpler and more consistent;
- the development of more intuitive analysis software by both instrument manufacturers as well as by third party developers to help with assay setup and project management e.g. Prexcel (available from eag@iastate.edu) or BioGazelle's qBase Plus (www.biogazelle.com)[3];
- the introduction of advanced algorithms that allow more accurate quantification[4,5,6,7,8,9,10,11].
- The extension of the technology into novel areas such as high throughput, nanoliter qPCR[12]. Specifically, microfluidic digital PCR is an exciting new development that extends the scope of qPCR technology[13]. Miniaturisation offers several potential advantages such as short assay time, low reagent usage and rapid heating/cooling rates, as well as integration of multiple processing modules to further reduce size and power consumption. Heating rates of

175ºC and cooling rates of 125ºC have been achieved[14], albeit at high cost and reaction volumes as low as as 0.45 nl have been reported[15]. Potential problems are that as the sample volume is decreased, sample solution can easily evaporate and that amplification is increasingly prone to biochemical surface absorption problems at the chamber/channel walls due to the increasing surface-to-volume ratio. Furthermore, nucleic acid contamination in PCR chips can generate false positive results, a problem addressed by the use of a disposable PCR chip[16]. Clearly, there is a huge potential in clinical diagnostics for the combination of PCR microfluidic chips and qPCR, as long as the technology can accommodate a wide range of crude biological samples as analytical targets[17,18]. Another recent development has been termed qPCR tomography[19] and combines the use of laser microdissection with qPCR analysis to obtain expression profiles from within single cells.

- The launch of less expensive, but more robust and reliable qPCR thermal cyclers, e.g. Corbett's Rotor-Gene 6000 (www.corbettlifescience.com) or BioRad's CFX (www.biorad.com) models. The introduction of qPCR instruments using a 384-well format (e.g. BoRad's CFX and Roche's Lightcycler 480 (http://www.roche.com/prod_diag_lc-480.htm) facilitates high assay throughput and, together with support for faster assay conditions, enables shorter assay times. High-density array-based formats are being developed, allowing the parallel screening of hundreds of targets at vastly reduced reagent cost. Of course, this needs to be balanced with potantial problems related to reduced assay sensitivity and accuracy of target quantification.

Clearly, from a technical point of view there are numerous developments that are strengthening qPCR technology and are permitting its application to an extremely broad range of applications. However, it is worth reflecting on these applications, and pausing to reflect on the appropriateness and biological relevance of many of the reported findings.

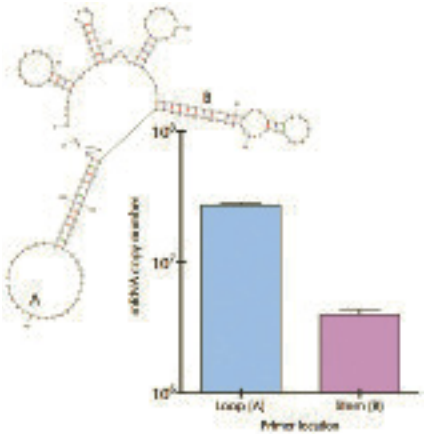

Figure 1: The importance of primer design targeting absence of a secondary structure. Two assays were designed to detect GAPDH mRNA, with identical forward primers, but with one reverse primer targeting a loop structure (A) and the other one the stem (B). Although assays using either primer gave quantitative results, assay A resulted in significantly higher apparent copy numbers than assay B. The increased sensitivity of primer set A is particularly important when attempting to amplify targets from limited amounts of sample.

Targeting of DNA: qPCR

qPCR is at its most successful when used for the detection and discrimination of DNA, e.g. pathogens, translocations, mutations or SNP analyses. This is because sample handling, template preparation, assay protocols and data interpretation are all relatively straightforward. The main limitations are associated with assay design in general, and primer design in particular. It is now well established that amplification efficiency, and hence assay sensitivity, is directly associated with good primer design (see Figure 1). In practice this means designing several primer combinations for each target and testing the various permutations to obtain the optimal combination. Although this has been well documented and is easily and cheaply implemented, it is still an aspect of assay design that is all too frequently neglected. This results in many primers being used that have not been optimised, both in terms of design as well as reaction conditions. An increasingly practical alternative to assay design from scratch is the interrogation of qPCR primer and probe databases, especially if the assay targets human or mouse sequences. For example, RTPrimerDB (http://medgen.ugent.be/rtprimerdb) is a curated database of validated primers for use in real-time PCR[20] and contains nearly 4,000 primer sets, 2,900 of which target human sequences, with the vast majority useful for mRNA quantification experiments; 2,400 primer sets make use of SYBR Green I, most of the others utilise TaqMan chemistry. It provides a freely accessible data retrieval system and an *in silico* assay evaluation pipeline for all of these qPCR assays. Specifically, assay reports contain gene information, oligonucleotide sequences, reaction conditions), publication information, users' experimental evaluation feedback and submitter's contact details. Additional information is provided on the alignment of primer and probe sequences on known transcript variants of a gene, along with SNP positions and peptide domain information. Importantly, the secondary structure of the amplicon sequence is predicted, as this has been reported to impact the efficiency of the PCR. If appropriate, its use helps prevent time-consuming primer design and experimental optimisation, and introduces a certain level of uniformity and standardisation among different laboratories.

Inhibition of the PCR assay by factors co-purified during nucleic acid extraction can be an important factor contributing to poor assay performance. This is illustrated in the results of the SPUD inhibition assay[21] shown in Figure 2 on page 21, which shows that inhibitors remain present in DNA extracted from human stool, despite extensive purification following the instructions of the kit manufacturer. It is well worth testing every sample for the presence of inhibitors that can affect different targets, enzymes and assays in different ways.

Targeting of RNA: RT-qPCR

RT-qPCR is widely used for the detection of cellular mRNA[22], micro RNAs[23] as well as pathogens[24]. However, there are biological as well as technical limitations to its use that make its use problematic[25]. This

is particularly so if the aim is to determine differences in expression patterns between single cells. Variability arises from the uncertainty caused by fluctuations in the amplificaton of very low numbers of target molecules as well as differences in mRNA levels between identical cells caused by the stochastic nature of transcription in mammalian cells[26,27,28].

1. Biological variability

A significant contribution to variability in gene expression studies, and one that constitutes '*baseline*' variability, arises from the fact that cellular mRNA levels are meant to be variable; after all, mRNA is constantly synthesised, localised, translated and degraded in response to extracellular signals. *In vivo* RNA 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[29]. This is likely to result in significant natural variability of mRNA levels between genes expressed in different tissues and individuals. Consequently, different biopsies from the same individual as well as biopsies from different individuals will reveal a basic set of variable mRNA levels that must be taken into account when interpreting changes in mRNA levels. Each biopsy will be subject to sample-specific as *'in vivo'* degradation over which the investigator has no control.

Another biological variable concerns gene splicing, 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. This affects most human genes[30] and plays an important role in human pathologies, including cancer[31] and 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- or treatment-specific adjustments between different isoforms.

The interpretation of mRNA quantification data is further complicated by the widespread differences in allelic expression among autosomal non-imprinted genes in animals[32] as well as plants[33]. This suggests that it is not sufficient just to quantify mRNA expression, but that it is important to determine precisely which allele is being expressed. This is particularly so since allelic imbalance and allele-specific expression patterns are associated with disease risk[34,35] poses further problems for. 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[25].

2. Technical variability

There are numerous reasons for technical variability although, unlike biological variability, these can be adressed by the use of appropriate standing operating procedures. The most obvious causes are inconsistent sample selection, handling, and RNA isolation. For example, a comparison of RNA levels between cancer samples must take into account the complexity and heterogeneity of tissue biopsies and may reqire the use of microdisected samples for maximum accuracy. Crucially, the accuracy of gene expression profiling is highly dependent on RNA quality, both in terms of its integrity as well as in terms of the lack of inhibitors co-purified during the extraction procedures[21,36,37]. The instability of RNA and its sensitivity to degradation introduced during storage or the extraction of the RNA are well-known. Whilst these comments may seem obvious, their implications have never been explored in detail. Unfortunately, not sufficient attention is paid to the analysis of RNA quality and a 2005 survey of the working practices of 100 experienced qPCR users revealed that around half did not adequately quality assess their RNA[38]. A recent survey of papers published in 2007/08 revealed that >60% of papers not even mentioning mRNA quality[25]. This area requires urgent attention, and proposals for adequate RNA integrity testing have been put forward[36].

The conversion of mRNA to cDNA is probably is a highly variable step in the quantification process. RT-qPCR gene expression measurements are comparable only when the same priming strategy and reaction conditions are used in all experiments and the samples contain the same total amount of RNA[39]. Furthermore, reverse transcription yields vary considerably with the choice of reverse transcriptase and variation is target gene dependent[40]. Similarly, the mechanism of cDNA priming has a significant effect on the outcome of any quantification experiment, since gene-specific priming,

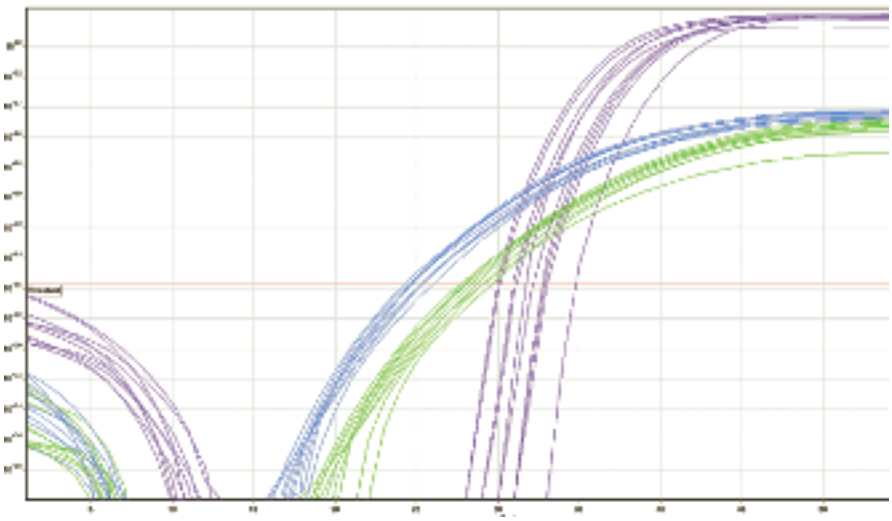

Figure 2: Effects of inhibitors of qPCR analysis. Unreliable dilution of target DNA (purple amplification plots) can be explained by the demonstration of inhibitors present in the purified DNA sample. A SPUD assay was used to show that the threshold cycles (Ct) are significantly higher in the presence of DNA extracted from human stool (green amplification plot) when compared with a water-only control (blue amplification plots).

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[41]. 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.

Proper normalisation of gene expression data between different samples generated in the same laboratory, or generated in different geographical regions using a single platform or multiple platforms is essential for obtaining accurate gene expression data[42]. Unfortunately, data normalisation continues to be used in an inappropriate manner, with a high proportion of papers still reporting expression patterns of target genes normalised against a single, unvalidated reference gene. This issue is equally relevant, but all too frequently unappreciated, when assessing miRNA levels[43]. 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[44].

Conclusions

Technological advances mean that there is an ever-increasing choice of platforms, chemistries, protocols as well as applications and targets for qPCR analysis. This is exciting and is generating a vast amount of data in, amongst others, basic research, medical, agricultural, microbiological and forensic applications. However, it is clear that a high percentage of publications utilising qPCR technology, and especially those aiming to profile cellular RNA levels, report poorly designed, executed and interpretated experiments and results[41]. Considerations of mRNA transcription, *in vivo* stability, regulation by miRNAs, tissue-specificity of splice variants, allele-specific differences in expression, the lack of concordance between most mRNAs and their specified proteins, the critical importance of post-translational modifications and questions of tissue heterogeneity all describe serious issues that are not being addressed in an adequate manner[25].

Trust in the accuracy and integrity of the scientific literature is an essential prerequisite for maintaining scientific excellence and advancing knowledge. This calls for urgent action by researchers, reviewers and editors who need to agree a basic set of quality criteria and adhere to elementary procedures that result in the publication of reliable and reproducible data. Such a list must include delineating minimum quality standards for template preparation, validation and consistent use of cDNA priming methods, enzymes, protocols and, equally critically, appropriate analysis of data. Furthermore, 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. In addition, it is vital that data acquisition, analysis and reporting become more transparent. Consequently, it is necessary for the editors of scientific and biomedical publications to issue prescriptive checklists specifying the key information to be included 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. It is important that data acquisition, analysis and reporting are transparent, thus enabling reinterpretation of data by others and helping to guarantee compliance with quality standards. Therefore, following the example of the MIAME (Minimum Information About A Microarray Experiment) guidelines adopted for microarray data, guidelines specifying the Minimal Information about qPCR experiments (MIqPCR) have been proposed. 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/). A MIqPCR compliant RDML file should contain all measured data as well as information about the samples and targets being analysed. This 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 reliable exchange of annotated qPCR data between authors, peer reviewers, journals and readers.

Ultimately, these approaches need to be combined with more prosaic biological considerations, so that results are not a reflection of technical inadequacies and biological artifacts, but truly start describing actual differences in expression profiles between cells, tissues, individuals, disease states and treatment responses. Unfortunately, we are still far removed from this state, with a lot of intellectual and capital investment in technological development that drives research whose results can be fundamentally flawed. It will require a significant amount of courage, and a sea-change in attitude from the research community to deal with this quagmire.

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