QUANTIFICATION OF MICRO-ORGANISMS: NOT HUMAN, NOT SIMPLE, NOT QUICK

RUNNING TITLE: MICROBIAL QUANTIFICATION BY REAL-TIME PCR

IAN M. MACKAY[1,3], STEPHEN A. BUSTIN[2], JOSÉ MANUEL ANDRADE[5], MIKAEL KUBISTA[4,5], THEO P SLOOTS[1,3,6]

[1]Queensland Paediatric Infectious Diseases Laboratory, Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital, Brisbane, Australia
[2]Centre for Academic Surgery, Institute of Cell and Molecular Science, Barts and the London Queen Mary's School of Medicine and Dentistry, University of London London E1 1BB UK
[3]Clinical Medical Virology Centre, University of Queensland, Brisbane, Australia
[4]TATAA Biocenter, Medicinargatan 8A, 413 46 Göteborg, Sweden.
[5]Dept of Analytical Chemistry, University of A Coruna, A Zapateira s/n, E-15071 A Coruna, Spain.
[6]Division of Microbiology, Queensland health pathology service, Royal Brisbane Hospitals Campus, Queensland, Australia

Corresponding Author	Ian Mackay
Phone:	+61 3636 8716
Fax:	+61 3636 1401
eMail:	ian.mackay@uq.edu.au
Mail:	Qpid Laboratory CMVC, University of Queensland & SASVRC, Royal Children's Hospital, Herston Rd Herston, Queensland 4029 AUSTRALIA

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Abstract

The majority of real-time PCR applications in microbiology are for qualitative (resulting in a yes or no answer) detection of a virus, bacterium, fungus or parasite. In terms of disease relevance, the importance of quantitative PCR (qPCR) to microbiology has been proven, however it is less clear just how punctilious the clinical microbiology laboratory must be to produce relevant quantitative results. Despite a decade of qPCR experience, commercial development of applications is limited and many of our approaches remain entrenched among the PCR techniques used to monitor human mRNA levels rather than addressing adequately the diverse needs of the microbiology field. Real-time PCR has permeated every aspect of microbiology, but its applications have particular value in the clinical microbiology laboratory where the speed, sensitivity, reproducibility and accuracy of this tool help to produce robust data in a clinically relevant timeframe. Other areas within microbiology have also gained from the use of real-time PCR; gene therapy has found benefit from qPCR applications that monitor the production, replication and administration of viral vectors used to transport therapeutic genes into host cells or tissues. Studies of the host's response to microbial replication suggest a vision of the future wherein patient specimens may be used to provide an indication not only of the type of micro-organism present and its replicative status, but the stage of disease and the type of immune response underway. To make such vision reality, we must first discuss and reach consensus on the best, microbiology-specific qPCR approaches to permit the production of comparable microbial load data. This process must include the development of clear definitions associating microbial load with clinical outcome, the production of more reference materials, the development of more quality assessment schemes and of commercial kits. It may be that we find the perfect estimate of micro-organism numbers is not as important as reproducible and clinically relevant data. The increased identification of newly emergent or previously unknown endemic pathogens demands that we must strive harder than ever to expand our understanding of infectious diseases, and for that we need reliable results from reliable tools.

Introduction

The majority of real-time PCR applications in microbiology are for qualitative (resulting in a yes or no answer) detection of a virus, bacterium, fungus or parasite. However the technique also permits quantification of target nucleic acids for both pure research purposes and for a myriad of applications in the clinical microbiology laboratory. While much more user-friendly than earlier conventional competitive quantitative PCR (qPCR), quantification by real-time PCR retains many time-consuming requirements that must be addressed in the context of microbiology in order to produce valid data. A direct relationship is assumed to exist between the amount of microbial nucleic acid and the number of micro-organisms in a patient's specimen; minimising variability while correcting for that which remains can strengthen this association (Ferré, 1992).

Real-time PCR has simplified our ability to visualise the impact of each assay variable, thus permitting improved optimisation, standardisation and normalisation of qPCR assays. It is then disappointing that despite a decade of qPCR experience, commercial development of applications is limited and many of our approaches remain entrenched in the techniques used to monitor human mRNA levels, rather than adequately addressing the diverse needs of the microbiology field.

Why is it important to quantify micro-organisms?

The most clinically important reasons for quantifying an infecting micro-organism are to permit the medical practitioner rapid insight into the efficacy of an ongoing antimicrobial therapy and to identify when treatment should commence. Should a treatment prove ineffective, as identified by stable or increasing microbial concentration or "load", then the clinician has rapid access to objective data which may aid the selection of an improved therapeutic course or the adaptation of the current approach, thus tailoring treatment to best suit the microbiological and clinical picture.

Another application that benefits from quantifying microbial load data is the monitoring of patients undergoing organ transplantation who are subject to extensive immunosuppression. Treatment of this group can be quickly deployed in response to change in the status of low-level latent or persistent infections. Quantification also has the potential to clarify the extent of virus-host interactions and to propose roles for pathogenicity (Clementi, 2000; Limaye et al., 2000). In addition, the microbial load can frequently be related to disease progression in immune-competent subjects for example by detecting a pathogen increase preceding clinical symptoms. However, early doubts were cast upon the simplicity of the assumptions made

about this relationship (Watzinger et al., 2004). Relying solely upon qPCR has again been called into question for the quantification of HIV-1 because of discrepancies between results from nucleic acid and other diagnostic approaches (Rodríguez et al., 2006).

Collection and testing of serial patient samples has an important role for all of these qPCR applications because compared to testing a single sample, it allows the gathering of more robust data and can reveal clearly visible trends caused by changes in microbial load over time. In the research laboratory, qPCR is used to corroborate microarray data and determine the *in vitro* propagation of virus during attempted isolation using cell culture. The latter role is especially important for discriminating viral replication from residual inoculum when working with molecularly identified viruses that produce little or no cytopathic effect and/or for which serological reagents do not exist (Schildgen et al., 2006).

While the importance of qPCR seems apparent, the conditions required to produce relevant quantitative results in the clinical microbiology laboratory are less well defined. How pedantic do we have to be? Historically, *in vitro* culture has provided a clear-cut qualitative method indicating success or failure. Culture can be adapted to quantify the microbe but it is a time-consuming process that likely under-represents the number of micro-organisms in the original specimen. The serological methods introduced the "equivocal" result and made it commonplace, complicating the diagnosis of infectious disease. Serology is generally employed to quantify the host's antibody response to an invading micro-organism. DNA techniques produce data that can be either clear-cut or equivocal, depending on the micro-organisms and clinical situation. Perhaps the question of pedantry can be addressed by also asking whether the mere presence of an invading micro-organism is suitably diagnostic or whether the amount of the putative pathogen is a better indication of clinical outcome. Consensus on the answer to this question for many micro-organisms could go a long way toward simplifying the development of qPCR techniques for microbiology.

What's already on the shelf?

Several benefits arise from employing commercial kits for qPCR in the clinical microbiology laboratory; principal among these is doing away with incompatible in-house methods and the resulting improvement in data comparability between laboratories around the world. The introduction of quantitative molecular testing was first commercialised for only a handful of micro-organisms by providers including Roche Diagnostics, Bayer Diagnostics and bioMérieux (formerly Organon Teknika; listed in (Schutten and Niesters, 2001; Niesters, 2002; Watzinger et al., 2006)). Targets included *Chlamydia trachomatis*, cytomegalovirus (CMV), hepatitis B and C viruses (HBV and HCV) and the human immunodeficiency virus-1

(HIV-1). These analyte specific reagents (ASRs) have generally been upgraded alongside developments in laboratory systems and PCR detection formats. ASR kits are also available from artus™ (now produced by QIAGEN™) with additional targets including parvovirus B19, influenza A virus, influenza B virus, enteroviruses, West Nile virus, orthopoxviruses, dengue viruses, additional human herpesviruses (herpes simplex viruses (HSV) 1 and 2 and varicella zoster virus), the SARS coronavirus, *Bacillus anthracis* virulence plasmids, *Borrelia burgdorferi* sensu lato, *Salmonella* serovars, *Listeria monocytogenes*, *Mycobacterium tuberculosis* complex and the malaria parasites *Plasmodium falciparum, P.vivax*, *P.ovale* and *P.malariae*. Despite of a description to the contrary, not all of these kits include a quantitative capacity; some contain neither a quantification calibrator nor the protocol required to perform true quantification.

Change the paradigm, change the field

There are still some glaring omissions from the list of kits and from qPCR studies in the research literature. Why are many common microbial infectious agents not the subject of comprehensive investigation by real-time qPCR? The answers are manifold and includes the real and perceived technical difficulties to develop these assays, the new costs, the lack of commercial support and the ingrained belief that quantification is only for "special" micro-organisms. On this last point there is no question that the quantification of some micro-organisms is clinically unwarranted (although there are always reasons for research studies); the mere presence of Ebola virus or variola virus infection is sufficient to enact a clinical regimen of support and treatment because persistent or subclinical infections are not common features of infection. However for other targets, interpreting the qPCR data may be extremely difficult because of the often innocuous presence of the organism in the human host, e.g. *Escherichia coli*, *Streptococcus pneumoniae*, *Neisseria* spp., HSV or CMV. Our understanding of the remaining microbial targets which have rarely if at all been the subject of qPCR studies could benefit from good quality quantitative research studies to better understand the role each pathogen plays, especially in illnesses not commonly associated with infection and whether they circulate among asymptomatic populations. Several respiratory viruses including the picornaviruses fall into this category since they are sometimes detected in patients lacking overt clinical signs of illness. Micro-organisms causing treatable infections are generally acknowledged as the most worthy qPCR targets, leaving many of the most common (and frequent) viral pathogens that are serviced by relatively few therapeutic options, without any commercial alternative for quantification. It will be important for future studies to challenge infectious disease paradigms using real-time qPCR to ensure our beliefs hold up to close scrutiny.

How does real-time PCR improve upon previous qPCR methods?

Real-time PCR offers some immediate and significant improvements over previous conventional PCR methods for the quantification of micro-organisms. Principally the closed tube or "homogeneous" nature of real-time PCR has been a boon to the clinical microbiology laboratory by dramatically reducing the risk that amplicon will contaminate subsequent assays. The technique can also be used to amplify efficiently and representatively an enormous range of template concentrations without affecting assay efficiency, making it a dramatic improvement over earlier qPCR methods. This "dynamic range" extends to at least eight $\log_{10}$ of template molecules (Ryncarz et al., 1999; Abe et al., 1999).

The versatility of real-time qPCR is made possible because its fluorescence data are collected from the linear phase of the exponential PCR. In this region conditions of DNA duplication are optimal, rather than in the end-point or "plateau" phase where the amount of amplicon present has little relationship with the original template concentration. End-point amplicon quantities are especially affected by conditions of saturation by inhibitory PCR by-products and specific double-stranded DNA (amplicon). The fractional cycle at which fluorescence crosses a defined y-axis value (see Chapter 1 for more detail) is known as the threshold cycle (C_T). The C_T values from amplification of an unknown can be compared to similar data generated by the amplification of a dilution series of standards or calibrators after such data are presented as a standard or calibration curve.

Collecting data from the linear phase of the reaction also makes possible the detection of an equally broad dynamic range of amplicon; the lack of such scope was a significant cause for additional amplicon handling among methods predating real-time PCR (Fig.1). Dilution of an amplicon is no longer required before detecting it nor is repetition of an assay using a diluted template required because a preliminary result fell above the upper limits of the detection assay. Both of these problems were common to conventional end-point qPCR assays because the detection formats could not encompass amplicon from high template loads whilst maintaining an adequate template sensitivity (Kawai et al., 1999; Schaade et al., 2000; Weinberger et al., 2000; Brechtbuehl et al., 2001). However, the reliance on C_T data evokes one detrimental outcome: the impact from inhibition is enhanced when compared with an end-point quantification approach. Poorly optimised conditions or amplification inhibitors (other than those due to the amplification process itself) delay amplification which has a greater impact on the C_T values than on the final amount of amplicon since final amounts often remain unchanged after 30 to 45 cycles of PCR.

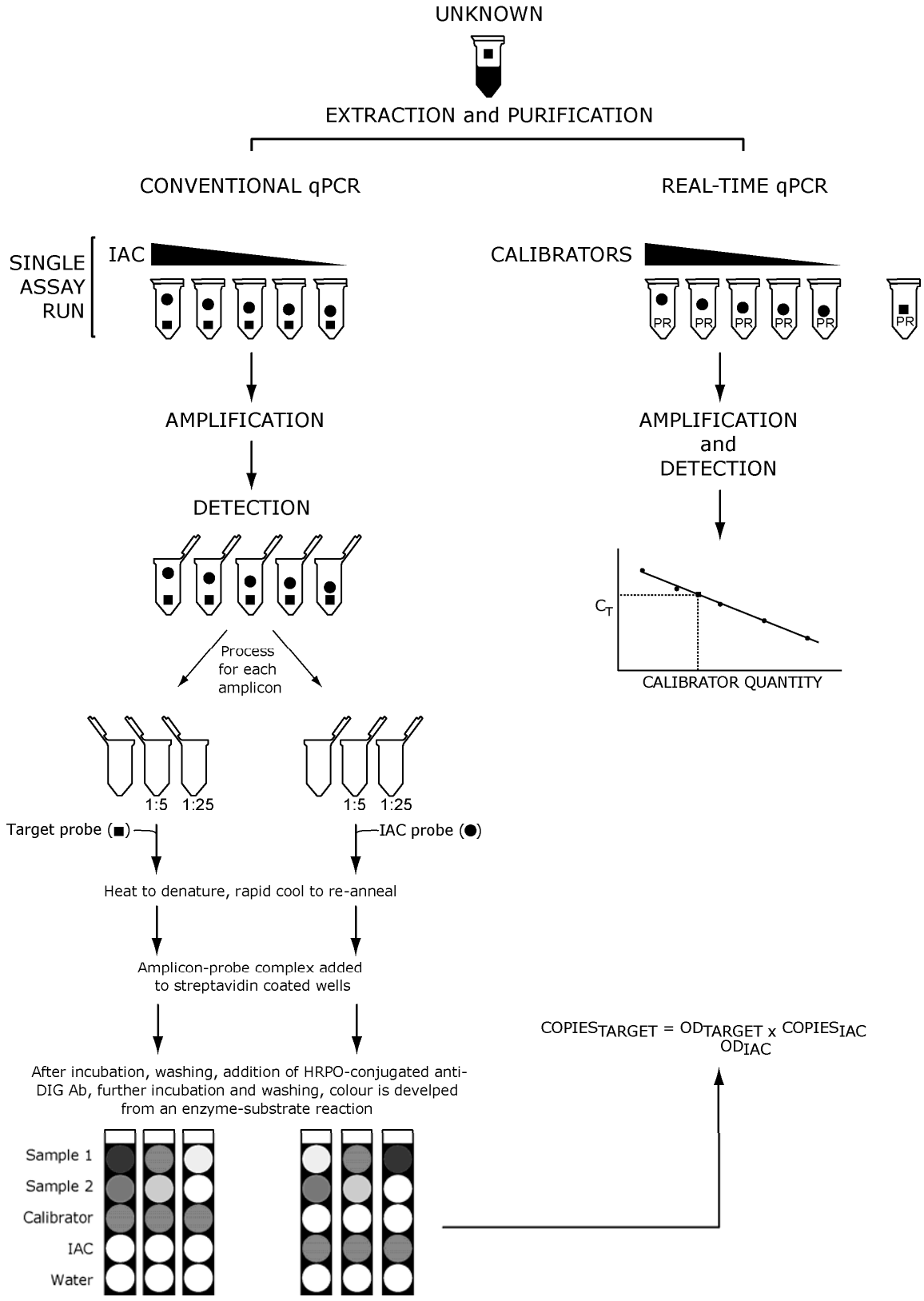


Fig.1.

A simplified comparison of the complexities of conventional, competitive qPCR versus real-time qPCR methods. Competitive qPCR required the amplification of a fixed amount of target (filled circle) and a dilution series of a characterised competitive internal amplification control (IAC; filled square). The two molecules must then be

discriminated after the PCR is completed. Using a PCR-ELISA approach, the highest amplicon dilution that gives an optical density (OD) within a pre-established range provides the data for the following equation. Antibody, Ab; digoxigenin, DIG; horse-radish peroxidise, HRPO; passive reference, PR; quantitative PCR, qPCR

Real-time PCR can also demonstrate low inter-assay and intra-assay variability and since it is a PCR method, it is also extremely sensitive (Abe et al., 1999; Locatelli et al., 2000; Schutten et al., 2000). However, one could argue that only the latter is an innate feature of real-time PCR and that the remainder requires suitable reference materials, regular quality control and protocols that are well developed and strictly adhered to. Nonetheless real-time PCR permits template quantification, of some sort, from a wide variety of sample types which contain a large yet unpredictable range of template concentrations, as is often the case in samples collected for microbiological investigations. It is a superior tool well suited to the quick decision making required in a clinical situation since time-to-result or "turnaround time" is reduced (Locatelli et al., 2000; Tanaka et al., 2000).

Before real-time PCR, the most objective and accurate approach to quantification was the analysis, following co-amplification (two templates amplified in the same vessel; Fig.1), of a competing nucleic acid template of known concentration along with the "wild-type" target nucleic acid of unknown concentration (Gilliland et al., 1990; Siebert and Larrick, 1992; Clementi et al., 1995; Orlando et al., 1998). While the molecule of known concentration is not always employed as a competitor its amplification absolutely depends on the same reagents as the microbial template. Since there are generally more similarities than differences we employ a single title for these molecules which helps simplify further discussion. We refer to this molecule as an internal amplification control (IAC; discussed below) however it has been used in several roles and is known by many names (including internal control, internal standard, competitive standard, internal calibrator or mimic) indicating the distinguished history of an IAC in the PCR detection of micro-organisms (Wang et al., 1989; Niesters, 2002).

The IAC was the driving force behind the development of competitive qPCR and worked most efficiently in this role when it was of almost identical sequence to the assay's main target (Pannetier et al., 1993). When both the IAC and unknown target were amplified together for the purpose of qPCR, the amount of the unknown template was determined from a comparison of signals. This approach commonly required several reactions containing a range of IAC concentrations to accommodate the detrimental effects of excessive competition if one template was more abundant than the other. Each reaction contained a different amount of IAC but a constant volume of the unknown template. Alternatively one

could perform a pre-qPCR reaction to obtain a crude estimate of the template load and use only one or two similar IAC concentrations.

Despite comparison of data generated either by absolute calibration curves, relative calibration curves or C_T values producing similar results (Johnson et al., 2000), competitive PCR has not been extensively examined for quantification using a real-time PCR format (Halford, 1999; Halford et al., 1999). The absence of software with the ability to automatically calculate the concentration of an unknown by comparing the signals generated during co-amplification makes the development of real-time competitive qPCR applications difficult (Kleiber et al., 2000; Pfaffl et al., 2002). In the only application published to date, competitive amplification successfully defined the amount of nodavirus RNA in cell culture samples (Grove et al., 2006). The method was reportedly less sensitive than a noncompetitive version (presumably because of competitive inhibition), required the use of four aliquots of the sample extract and did not monitor reverse transcriptase (RT) or amplification efficiencies. An earlier experimental approach employed a wild-type and two co-extracted, co-amplified heterologous plasmid templates employing an algorithm to determine the number of target copies (Gruber et al., 2001). While this study did not include a normalisation step, the dual template amplification correlated over four $\log_{10}$ of template. The second IAC could not be simultaneously detected by the software of the time.

While not surprising, the point must be reiterated that PCR data cannot be related to microbial viability unless preceded by a biological isolation or enrichment step (Josefsen et al., 2004; Nitsche et al., 2006). While real-time qPCR is useful to monitor changes in the levels of a micro-organism's genome or gene products, it must be used in concert with biological methods, at least during the developmental process, if one wants to assign biological relevance to the results.

Optimisation and consistency are critical for obtaining reproducible results yet real-time RT-PCR assays are capable of significantly less variation than conventional RT-PCR protocols which can be subject to significant error (Souazé et al., 1996). In principle, quantification of RNA by real-time RT-qPCR is straightforward: the more copies of an RNA molecule there are at the beginning of the assay, the fewer cycles of amplification are required to generate a specific number of products (Fig.2). Therefore, the C_T is inversely correlated to the amount of RNA in the original sample.

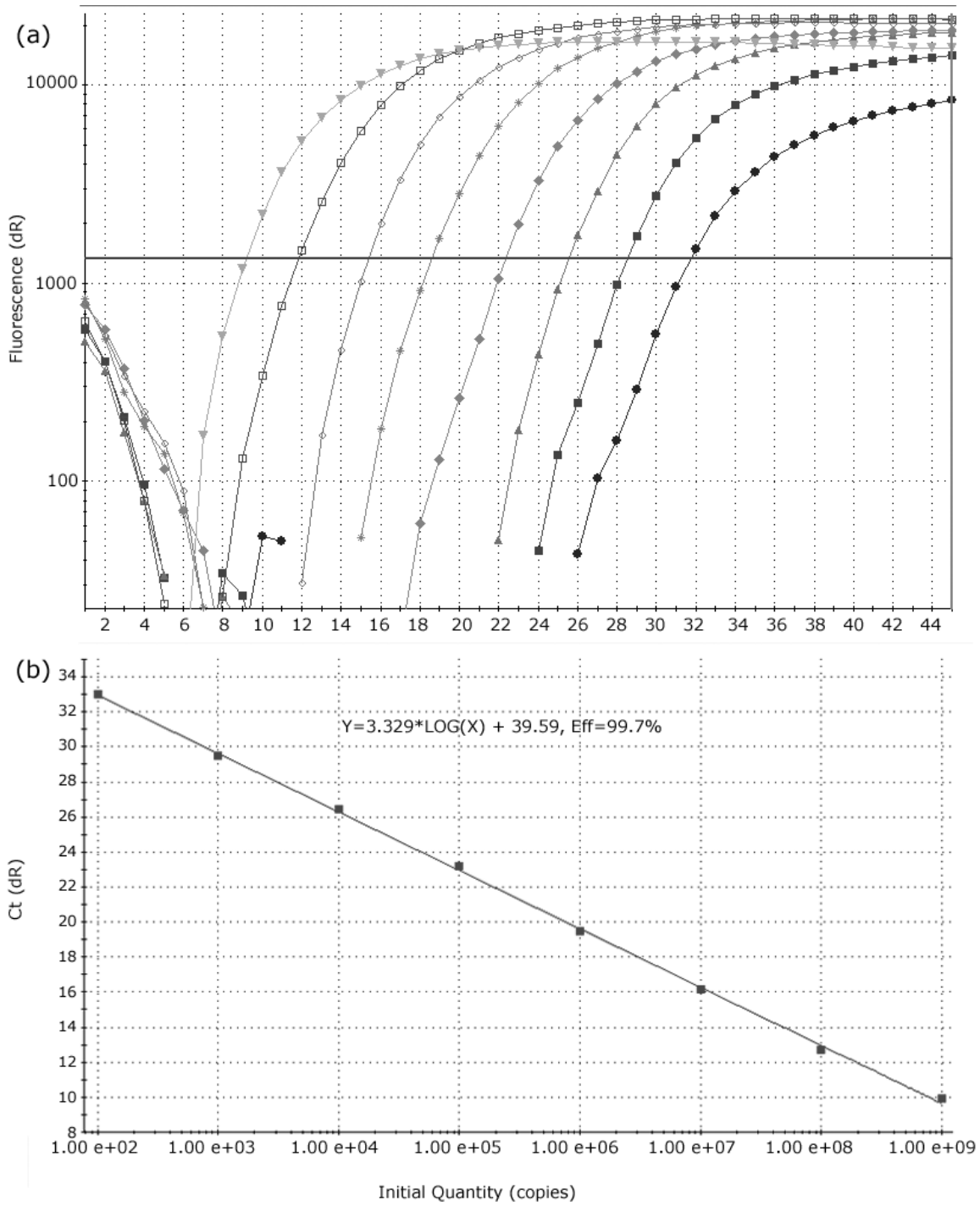


Fig.2. (a) Amplification plot for a standard curve. Sense-strand oligodeoxynucleotides were serially diluted from $1x10^9$ down to $1x10^2$ molecules and subjected to real-time RT-PCR assays on a Stratagene MX3005P. The software calculates the threshold cycle (C_T), which is defined as the number of PCR cycles where the fluorescence generated from the amplification plot crosses a defined fluorescence threshold (bold horizontal line). It depends on the sensitivity of the detection system and can vary significantly depending on assay-specific background levels. This provides several orders of linear dynamic range, in this case seven logs. The graph of normalised fluorescence vs. cycle number during PCR has three stages. Initially, the fluorescent signal is below the

detection limit of the sequence detector. In the second stage, the signal can be detected as it continues to increase in direct proportion to the increase in the products of PCR. As PCR product continues to increase, the ratio of polymerase to PCR product decreases, product ceases to grow exponentially and the signal is roughly linear and finally reaches a plateau. (b) A plot of C_T against the log of initial oligonucleotide copy number results in a straight line that is linear over at least seven orders of magnitude and linear regression analysis permits the calculation of the copy number of any unknown target relative to that standard curve. The standard curve also reveals the amplification efficiency of the reaction (slope) and gives some indication of its sensitivity (y-intercept).

The potential to quantify nucleic acids using PCR has proven most popular in the field of gene expression or transcriptome analysis (PhorTech, 2003) but it has also been employed in microbiology since the 1990s (Zhang et al., 1991). Because comprehensive lists of applications in microbiology have recently been published elsewhere (Espy et al., 2006; Watzinger et al., 2006), the remainder of this chapter will critically examine the impact of RT on qPCR assay performance, describe the mathematical approaches employed to obtain quantitative data, discuss the role of quality control and assurance, identify some sources of reference material and review two uncommon uses of real-time PCR quantification in the multi-disciplinary field of microbiology.

Calibrating the process of microbial quantification

Although the terminology is often confused, real-time PCR does not inherently imply qPCR. Thus no discussion of nucleic acid quantification can ensue without first mentioning the necessary inclusion of the correct positive and negative controls to determine assay function and the relevant reference standards (ideally of biological origin as per terminology employed by the International Organisation for Standardisation) or calibrators (derived from a standard and by far the more commonly used molecule for in-house qPCR) required to generate accurate data. The externally amplified calibration curve (see Fig 1 and 2) relies upon titration and amplification of an identical template, performed within the same experimental run but in separate vessels to the unknown target. However, the calibration curve can itself serve as a source of error when inter-tube fluorescence variation is not controlled. This, as with similar omissions throughout the qPCR process, is usually a trade-off between two realities: (i) obtaining some form of quantitative data in a clinically useful timeframe (while under the stresses of a high throughput laboratory environment) and (ii) the need to address multiple sources of error to achieve suitable quantitative data.

Suitability is usually guided by the laboratory or its clients and sometimes by participation in quality assessment programs. Clinical relevance may ultimately be a better guide.

No matter what calibrators and controls are used to determine the presence of inhibitors, monitor successful amplification or generate quantitative data, it is imperative to accurately determine their concentration to ensure that they are used at suitable levels. A spectrophotometer alone is inadequate for quantifying these molecules; however in combination with an experimental and statistical analysis, the reliability of the data can be greatly enhanced (Taswell, 1981; Glasel, 1995; Bagnarelli et al., 1995; Rodrigo et al., 1997; Wang and Spadoro, 1998; Sykes et al., 1998). Determination of small *in vitro* synthesised single-stranded DNA or RNA molecule concentrations may require the aid of a fluorescence-based signal amplication system such as PicoGreen® or RiboGreen® (Invitrogen).

When employed, calibrator and control templates should be handled as identically as possible to the test samples. Practically this means that the sample matrix (for example serum, urine or bronchoalveolar lavage) should be used to provide the "background" into which the calibrator is added prior to nucleic acid extraction and purification. In some cases it is not possible to obtain matrix in a standardised form from negative samples e.g. precise collection a nasal swab or bronchoalveolar lavage is extremely difficult; one may weigh the swab after collection although this cannot discriminate between patient cells and absorbed fluids (Maggi et al., 2003).

Careful development and implementation of calibrators will permit better standardisation and ensure that comparison between assay results and testing locations is possible (Niesters, 2001). Without question, the reliability of qPCR methods is intimately associated with the choice and quality of the assay controls (Alexandre et al., 1998; Celi et al., 2000).

The mathematical mechanics of quantification

Unfortunately for those of us less interested in this aspect of qPCR, mathematics make the method. One cannot escape that useful qPCR data requires some understanding of how the results are obtained. In every PCR cycle the nucleic acid template is copied, ideally giving rise to a doubling of the number of template molecules in the reaction vessel. The doubling process is described by (Kubista et al., 2006):

$$N = N^0 2^x \quad \textbf{(1)}$$

N is the number of template molecules after X amplification cycles and N^0 is the initial number of double stranded template molecules. This equation is based on four assumptions discussed below.

Assumption 1: Initial template is double-stranded

Eukaryotic and most prokaryotic genomes are double stranded. But many viral genomes are single-stranded. Also, when analysing RNA, reverse transcriptase protocols produce single-stranded cDNA for PCR. The single stranded DNA is then converted into double-stranded DNA in the first PCR cycle, thus true amplification starts in cycle two. Hence, when template is single-stranded the doubling process is described by (Kubista et al., 2006):

$$N = N^0 2^{(x-1)} \quad \textbf{(2)}$$

As before, N is the number of double-stranded molecules present after X cycles, and N^0 is the initial number of template molecules, but in this case they are single-stranded. The correction for this extra cycle, which has been missed in many publications, often cancels out, as we will see below. However, in some quantification the omission can introduce a two-fold error.

Assumption 2: PCR efficiency is 100%; both strands of all template molecules are copied into full length products each cycle.

Experience shows that PCR efficiency almost never reaches 100%. Even the complete copying of both strands of short, purified amplicon templates rarely exceeds an efficiency of 95%. This reduced efficiency, in the absence of inhibition, is due to inefficient primer hybridisation, template folding and probe and dye interference. It can sometimes be improved by redesigning the oligonucleotides to reduce mispriming or formation of folded structures, by increasing primer and/or template concentrations to push equilibrium towards the primed state (although, this can also cause primers to form primer-dimer products; (Lind et al., 2006)), by adjusting annealing temperature, buffer conditions (in particular concentrations of K^+ and Mg^{2+}), thermostable DNA polymerase concentration, by changing or reducing the length of the amplicon or reducing the dye or probe concentration (Kubista et al., 2001; Bengtsson et al., 2003). In biological samples the PCR efficiency is further reduced by concomitants in the sample matrix which have not been removed during the

nucleic acid extraction process (Akane et al., 1994; Al-Soud and Rådström, 1998; Al-Soud et al., 2000). In particular, matrices containing high amounts of lipids tend to be inhibitory. The following equation accounts for PCR inhibition:

$$N = N^0\left(1+E_{ts}\right)^{(x-1)} \quad \textbf{(3)}$$

E_{ts} is the PCR efficiency and it has a value $0 \leq E_{ts} \leq 1$. E_{ts} has two subscripts indicating that it depends on both the target (t) assay and on the sample (s). E_{ts} can be estimated *in situ* using the methods of serial dilutions of or standard additions to the test sample itself (Ståhlberg et al., 2003). A standard or calibrator curve is produced from the data acquired and the PCR efficiency is estimated. Since the calibrator data are all based on the same single test sample these approaches give the best estimate of its PCR efficiency. The standard additions approach uses the specimen under investigation to provide the matrix for the calibrator or standard. The sample is divided into aliquots that are spiked with increasing amounts of the standard in pure form. One aliquot is left without standard (Massart et al., 1997). The method has some limitations including a lack of evidence that the added standard will behave the same way as the natural target, increased use of precious sample and the costs of extra reagents and time. Nonetheless, it is presently the best method we have to prepare standards that resemble the true sample matrix. The method of serial dilutions is analogous. The test sample is diluted serially, for example 4-fold, and extracts from the dilutions are analysed by (RT) real-time PCR. A standard curve is produced based on the data and the PCR efficiency for the particular sample is estimated. Another possibility to estimate E_{ts} is to add purified template to target-free sample matrix (i.e., a negative sample) and use it to construct a calibration curve.

For practical purposes it is helpful to factorise E_{ts} into a contribution that is target-specific, E_t, and one related to sample matrix-specific inhibition, E_s:

$$E_{ts} = E_t \times E_s \quad \textbf{(4)}$$

The target specific contribution E_t is obtained from a calibration curve based on purified template in target-free matrix. E_s depends on the sample and can be estimated using an alien spike or endogenous control (see below). Sometimes an average value of $\overline{E_t}$ is assumed for all samples.

Assumption 3: PCR efficiency is constant throughout the amplification process.

The equations above assume that PCR efficiency is constant during the course of the reactions. This may not be the case. If the original template is long e.g. genomic DNA, it may form folded structures that amplify poorly. If the template is supercoiled, which is the case for plasmids, mitochondria, bacterial genomes and circular viruses then PCR efficiency is significantly lower than for the short template molecules that are produced during the course of the PCR (Nogva and Rudi, 2004). The effect of the length and structure of the initial template can be reduced by pre-analytical treatment of the sample with nucleases to remove supercoils or to excise the target region. Another possibility is to compare with calibrators based on the same starting material.

Assumption 4: Fluorescence is proportional to the amount of template

In real-time PCR we are not measuring the number of amplicons directly, but rather we measure the intensity of the emitted fluorescence as it increases with the number of amplicons formed. This fluorescence depends on the dye or probe used, the amplified sequence (Nygren et al., 1998), the length of the amplicon (longer amplicons associate with more intercalating dye), the optical properties of the instrument (filters, lamp etc), the data acquisition temperature and the instrument settings (gain etc). Further, although the fluorescence generally increases along with the number of amplicons formed, it is not strictly a direct relationship. Indeed, during the first cycles we do not detect any fluorescence increase because the signal is indistinguishable against the fluorescent background. But not even in the phase where we can observe increasing fluorescence can we assume that fluorescence intensity is strictly proportional to the number of amplicons present (Nygren et al., 1998). Binding of a probe to template is governed by thermodynamic principles and the fraction of probe bound depends on the concentrations of both the probe and amplicon. When using degradable probes, such as TaqMan® and Qzyme™, the active probe concentration decreases during the course of the reaction, leading to less efficient probing as the reaction proceeds. Dye binding shows cooperative effects at low binding ratios as well as self quenching at high binding ratios. Both phenomena give rise to non-linear behaviour.

$$I = k'(N_t) \times N_t = k'(N_t) \times N_t^0 \left(1 + E_{ts}\right)^{(x-1)} \qquad \textbf{(5)}$$

The factor $k'\left(N_t\right)$ describes the dependence of fluorescence intensity on the number of amplicons in the sample; it is generally unknown, which complicates analysis of PCR response curves. However, in real-time PCR one typically compares the number of amplification cycles required to reach a certain fluorescence threshold level so the unknown

factor is not a problem, since at the point of comparison all samples contain the same number of fluorescent molecules. For ease of reading we have used CT to denote the C_T value described previously. With X = CT, $k'(N_t) = k'$, the intensity is now independent of N, and we obtain a value for every sample A, B, C etc.

$$I = k_t' \times N_t = k_t' \times N_{tA}^0 (1 + E_{tA})^{(CT_{tA} - 1)} = k_t' \times N_{tB}^0 (1 + E_{tB})^{(CT_{tB} - 1)} = ... \quad \textbf{(6)}$$

This can be rearranged to give the relative initial number of single stranded DNA target molecules in two samples:

$$\frac{N_{tA}^0}{N_{tB}^0} = \frac{(1 + E_{tB})^{(CT_{tB} - 1)}}{(1 + E_{tA})^{(CT_{tA} - 1)}} \quad \textbf{(7)}$$

Assuming that the sample matrix is relatively constant among the samples, we can replace the sample specific efficiencies with an average target specific efficiency $\overline{E_t}$:

$$\frac{N_{tA}^0}{N_{tB}^0} = \frac{(1 + E_{tB})^{(CT_{tB} - 1)}}{(1 + E_{tA})^{(CT_{tA} - 1)}} \approx (1 + \overline{E_t})^{(CT_{tB} - CT_{tA})} \quad \textbf{(8)}$$

With this assumption the "-1" in the exponentials cancel out. Hence, expression is valid for both single and double-stranded targets.

Setting N_t^0 = 1 and $CT_t = CT_t$ (sc) (CT for a single copy sample) we obtain:

$$N_t^0 = (1 + \overline{E_t})^{(CT_t(sc) - CT_t)} \quad \textbf{(9)}$$

Taking the logarithm of both sides and rearranging gives:

$$CT_t = CT_t(sc) - \frac{1}{\log(1 + \overline{E_s})} \log(N_t^0) \quad \textbf{(10)}$$

This equation is the basis for quantification using an external calibration curve otherwise known as absolute quantification. But the name is misleading since concentrations are not determined in an absolute sense. Instead, concentrations of test samples are estimated by comparison to those of calibrator samples. Plotting the threshold values (CT_t) versus the logarithm of the initial number of template molecules in calibrator samples should give a straight line (Fig 2.) with:

$$slope_t = -\frac{1}{\log\left(1+\overline{E}_t\right)} \qquad \textbf{(11)}$$

Inserting $\overline{E}_t$ = 1 we find that a 100% efficient PCR gives rise to a slope of -3.32. From the slope the mean PCR efficiency is calculated as:

$$\overline{E}_t = 10^{-\frac{1}{slope_t}} - 1 \qquad \textbf{(12)}$$

The slope is determined by linear regression. Most software for linear regression will also calculate the standard error of the slope:

$$SE\left(slope\right) = \sqrt{\frac{\sum_{i=1}^{n}\left(CT_{measured,i} - CT_{predicted,i}\right)^2}{n-2}} \Bigg/ \sqrt{\sum_{i=1}^{n}\left(\log\left(N_i^0\right) - \overline{\log\left(N^0\right)}\right)^2} \qquad \textbf{(13)}$$

In Microsoft® Excel® the LINEST function in array mode calculates the standard error of slope. The standard error of the PCR efficiency is obtained from the SE(slope) by error propagation based on truncated Taylor expansion:

$$SE\left(\overline{E}_t\right) = SE\left(slope\right) \times \frac{\ln 10\left(1+\overline{E}_t\right)}{slope^2} \qquad \textbf{(14)}$$

The 95% confidence interval for the PCR efficiency is then given by a Student's t-test with n-2 degrees of freedom:

$$\overline{E}_t \pm t_{95\%,2-tails,,n-2} \times SE\left(\overline{E}_t\right) \qquad \textbf{(15)}$$

The standard error and confidence interval for CT values and log concentrations can be calculated by standard error propagation or by the Fieller's theorem (Fieller, 1940; Verderio

et al., 2004). If the concentrations of the calibrators are available in copy numbers, the intercept determines the CT of a single copy sample:

$$intercept = CT(sc) \quad \textbf{(16)}$$

Calibrator concentrations can also be given in relative values, such as dilution factors of a stock. The concentrations of the test samples will then be obtained in the same relative measure. Using relative concentrations for calibrators only affects the intercept. The PCR efficiency is still obtained from the slope by equation 12.

Microbial quantification: relatively speaking

Determining the amount of template by PCR can be achieved in two ways: relatively or 'absolutely'. 'Absolute' quantification strategies claim to determine the exact number of nucleic acid targets present in the sample and can express this in relation to a specific unit that is easily interpreted by both scientists and medical practitioners and comparable across different real-time PCR instruments and between laboratories. Such denominators include unit volume of serum, plasma, whole blood, urine or cerebrospinal fluid, the number of cells in blood or the mass of tissue or genomic nucleic acid (Freeman et al., 1999; Pfaffl et al., 2002). The decision about which denominator to use should preferably be made in consultation with one's peers to improve the chance of producing compatible results. Absolute quantification is a valuable tool when there is only a single sample available from a patient or when viral load data are to be compared to a predetermined, clinically relevant threshold value or range. Absolute quantification is the more commonly reported approach utilised by the microbiology laboratory and is easier for DNA analysis because calibrator samples are readily prepared based on purified target DNA diluted is suitable sample matrix. RNA quantification is more complex in an absolute sense because proper usage requires RNA calibrators, which are more cumbersome to generate. Calibration curves based on DNA do not account for any variations in cDNA synthesis yields. An alternative strategy is relative quantification. In relative quantification the amount of target RNA in a sample is measured relative to a second RNA, which serves as the reference. It can be an ERG transcript either from the host or from the same micro-organism, or an RNA spike. Both references would control for variation in reverse transcription efficiency, while the former also controls for variations in the overall expression level. The relative expression of the target RNA to the reference RNA reflects the degree of microbial transcription and is the measure compared among samples.

Equation 8 showed the relative expression of one RNA target in two samples. The relative expression of two RNA targets in one sample a sample is given instead by (Kubista et al., 2006):

$$\frac{N_{aA}^{0}}{N_{bA}^{0}} = K_{RS}\frac{\eta_{bA}\left(1+E_{bA}\right)^{(CT_{bA}-1)}}{\eta_{aA}\left(1+E_{aA}\right)^{(CT_{aA}-1)}} \quad \textbf{(16)}$$

Where indices *a* and *b* refer to RNA molecules and *A* indicates the sample, η is the RT efficiency, which depends on the RNA and may also vary from sample to sample. It also depends on the RT protocol used, including the primers and the reverse transcriptase. K_{RS} is the relative sensitivity of the two qPCR assays (Ståhlberg et al., 2004a). Clearly, measuring the relative expression of two targets in a sample requires determining a number of parameters. Usually these methods are employed for the expression of two human genes to identify how much the target gene's expression varies from the reference gene. The same equations apply to the measurement of a viral RNA target relative to an RNA spike used as control, or relative to an endogenous RNA reference such as ribosomal RNA or a validated ERG.

$$\frac{\frac{N_{aA}^{0}}{N_{bA}^{0}}}{\frac{N_{aB}^{0}}{N_{bB}^{0}}} = \frac{\frac{\eta_{bA}\left(1+E_{bA}\right)^{(CT_{bA}-1)}}{\eta_{aA}\left(1+E_{aA}\right)^{(CT_{aA}-1)}}}{\frac{\eta_{bB}\left(1+E_{bB}\right)^{(CT_{bB}-1)}}{\eta_{aB}\left(1+E_{aB}\right)^{(CT_{aB}-1)}}} \quad \textbf{(17)}$$

In equation 17 the expression K_{RS} cancels. If the two samples are reverse transcribed using the same protocols, and the sample to sample variation is small, the relative RT yields for the two RNA molecules in the two samples should be the same (Ståhlberg et al., 2004a).

$$\frac{\eta_{bA}}{\eta_{aA}} = \frac{\eta_{bB}}{\eta_{aB}} \quad \textbf{(18)}$$

Further, we assume that the degree of PCR inhibition in the two samples is the same. Then the sample specific PCR efficiencies can be replaced with averages values.

$$\frac{\frac{N_{aA}^0}{N_{bA}^0}}{\frac{N_{aB}^0}{N_{bB}^0}} = \frac{\frac{\left(1+\overline{E}_b\right)^{(CT_{bA}-1)}}{\left(1+\overline{E}_a\right)^{(CT_{aA}-1)}}}{\frac{\left(1+\overline{E}_b\right)^{(CT_{bB}-1)}}{\left(1+\overline{E}_a\right)^{(CT_{aB}-1)}}} \qquad \textbf{(19)}$$

This simplifies to:

$$\frac{\frac{N_{aA}^0}{N_{bA}^0}}{\frac{N_{aB}^0}{N_{bB}^0}} = \frac{\left(1+\overline{E}_b\right)^{(CT_{bA}-CT_{bB})}}{\left(1+\overline{E}_a\right)^{(CT_{aA}-CT_{bB})}} = \frac{\left(1+\overline{E}_a\right)^{(CT_{aB}-CT_{aA})}}{\left(1+\overline{E}_b\right)^{(CT_{bB}-CT_{bA})}} \qquad \textbf{(20)}$$

This is the expression usually recommended to calculate the relative amounts of two RNA molecules in two samples. It assumes that:

1. For every RNA assay the PCR efficiency is the same in all samples
2. The ratio of the RT yields of the two RNAs is the same in all samples

Assigning "a" to the microbial RNA target (tar), b to the reference (ref), and A to the test sample and B to the control sample, we obtain:

$$\frac{\frac{N_{tar,test}^0}{N_{ref,test}^0}}{\frac{N_{tar,control}^0}{N_{ref,control}^0}} = \frac{\left(1+\overline{E}_{tar}\right)^{\Delta CT_{tar}(control-test)}}{\left(1+\overline{E}_{ref}\right)^{\Delta CT_{ref}(control-test)}} = R \qquad \textbf{(21)}$$

This expression takes into account different efficiencies between test and control samples (Sagner et al., 2004), with efficiencies defined as $0 \leq E \leq 1$ (Pfaffl, 2001). The four CT values are measured for the two RNA molecules in the two samples and the average PCR efficiencies for the two assays are estimated from calibration curves. The expression ratio controls for reverse transcription so DNA calibrator curves can be used. The ratio "R" is the relative expression of target RNA in the two samples using the RNA reference as normaliser.

If the PCR efficiencies for the two assays are equal, we obtain:

$$R = \left(1+\overline{E}\right)^{\Delta CT_{GOI}(control-test)-\Delta CT_{RG}(control-test)} = \left(1+\overline{E}\right)^{-\Delta\Delta CT} \quad \textbf{(22)}$$

This is the ΔΔCT method. Finally, if only very rough estimates of relative expression are needed, 100% PCR efficiency can be assumed. This gives:

$$R = \left(1+\overline{E}\right)^{-\Delta\Delta CT} = 2^{-\Delta\Delta CT} \quad \textbf{(23)}$$

Earlier we learnt that a calibration curve with an appropriately added IAC (sometimes called a "spike") is a good approach to assay for microbial and cellular DNA, while a calibration curve combined with an exogenous control is a good approach to assay for RNA targets. The underlying assumption in these approaches is that PCR efficiency does not vary appreciably among samples such that we can assume a fixed PCR efficiency for the target nucleic acids. If this assumption is wrong we may obtain quite erroneous data. Indeed, a study of lymphoma based on serial dilutions showed that several samples would be misclassified if a constant PCR efficiency was assumed (Ståhlberg et al., 2003). How can we recognise if there is a problem with PCR efficiencies?

The development of a real-time PCR signal over time (Fig.2), also called the real-time PCR response curve, contains information about PCR efficiency (eq. 5). Although the efficiency is difficult to estimate from the response curve, its shape can be used for quality control. The method of kinetic outlier detection (KOD) compares the response curves of samples and identifies those with anomalous responses (Bar et al., 2003). These will have PCR efficiencies that deviate significantly from normal and their C_T values are likely to be erroneous. These samples should be rerun and if they retain a deviant PCR efficiency they must either be discarded or re-analysed by serial dilutions or standard additions to account for their anomalous efficiencies. A web-based solution for KOD will soon be available at LabonNet (kttp://www.labonnet.com).

Micro-organisms are not human transcripts; defining the differences

For a number of reasons qPCR data can be difficult to replicate. Real-time qPCR assays must include the capacity to correct for variations caused by a range of factors; without this capacity qPCR data are at best more appropriately described as being semi-quantitative and at worst they produce biologically irrelevant results (Tricarico et al., 2002; Dheda et al., 2005). Whether such a claim is overly meticulous is debatable. However the continued

production of data which cannot be compared between laboratories except in the broadest sense should give pause for thought; poor reproducibility will stymie attempts to collaborate in research studies and diagnostic quality assurance programs and confound attempts to improve our understanding of the interaction between micro-organism and host (Hoorfar et al., 2003). To this day it is rare to find well-controlled real-time qPCR assays among those in the clinical microbiology literature. That could quickly change if pressure was brought to bear by the editorial staff and peer reviewers of scientific journals (Mikovits et al., 1998). In the interim it would be useful to identify what is actually required to perform robust real-time qPCR in the clinical microbiology laboratory, under realistic conditions.

Some real-time PCR systems have already overcome the simplest technical variable, fluorescence changes caused by reaction mix composition, mix volume variation or because of non-specific quenching. One approach has been to include a non-participating, or "passive", internal reference molecule incorporating a 6-carboxy-N,N,N',N'-tetramethylrhodamine (ROX) fluorophore. This doesn't require a separate amplification since the reference is present in the reaction buffer and its signal is used to indicate volume variations and non-specific fluorescence quenching. The corrected or "normalised" values (not to be mistaken for normalisation of qPCR data using a reference or calibrating template), obtained from a ratio of the emission intensity of the fluorophore and ROX, are termed RQ+. To further control amplification fluctuations, the fluorescence from a "no-template" control reaction (RQ-) is subtracted from RQ+ for each sample, resulting in the ΔRQ value that indicates the magnitude of the reference signal generated for the given PCR (Gelmini et al., 1997). This approach embodies important criteria for the design of a PCR control to be employed for correcting unwanted variation or error; it can perform its job within the same tube as the template under study, it is relatively simple to design and implement and there is no interference with enzymes or oligonucleotides. The passive reference does not determine template quality, enzyme inhibition nor account for variation in nucleic acid amount so these issues must be addressed during a pre-analytical phase. If the issues are suitably addressed however, a passive reference provides the ideal approach to account for inter-tube variation of the sort that can occur between unknowns and the calibrator dilutions of an external calibration curve.

Important steps to consider before quantifying: The pre-analysis phase

Coming to terms with the complexities of performing high-quality real-time qPCR is a particular and daunting challenge for the diagnostic clinical microbiology community. The largest single adjustment is to understand that these assays cannot be performed as simply as qualitative real-time PCRs. A number of pre-analysis steps are required to ensure that the

template is adequately prepared for quantification and this sets quantitative molecular methods apart from the increasingly high-throughput methods that populate the clinical microbiology laboratory today.

In addition to minimising the usual technical variables including staff competence, equipment performance and upkeep, reagent quality and batch variation and use of sample replicates, molecular quantification of a micro-organism relies upon the use of measures to account for:

1. Variation in template amount between samples
2. Poor or variable quality template (due to variable purification, mis-handling or poor storage)
3. The presence of inhibitors (of both fluorescence and amplification)
4. Variation in the performance of RNA dependent DNA polymerases
5. Variation in the performance of DNA dependent DNA polymerases
6. Variation in amplification between sample and calibrators

In addition to causing acute infections, viruses are the underlying cause of a significant proportion of human cancers. They are also pose a major bioterrorism threat despite requiring specific expertise to produce, engineer, and disseminate in weaponised form. In particular, RNA viruses comprise the most abundant group of human, animal and plant pathogens with new, highly pathogenic viruses such as Avian influenza continually emerging. Newly identified endemic RNA viruses also comprise a growing population of human pathogens that are poorly characterised by qPCR. We will therefore dwell, and frequently refer to, RNA virus targets in the following sections. However the approaches we discuss are often compatible with the quantification of DNA templates from viruses, bacteria, fungi and parasites.

Along with technical variation, the first three points in the previous list should be addressed before performing any quantification. We advocate the following considerations for qPCR to ensure optimal assay reproducibility:

- The quantity of RNA should be determined using a single method for all assays and the amount used for the RT step must be kept as constant as possible. This is often difficult for a microbial target since its genome or gene transcript may be a small and variable fraction of the total nucleic acid present.

- An assessment of the quality of any RNA preparation used for quantification is essential. In particular information should be provided regarding the absence of inhibitors in each sample and the integrity of the RNA.
- The cDNA priming method must be consistent, with specific priming the preferred option, together with as short an incubation time as possible and as high a reaction temperature as practical.
- Calibration curve data should be analysed so the amplification efficiency and sensitivity of every PCR assay can be reported and to confirm that the quantification of any unknowns has been carried out within the dynamic range of that assay.
- A common IAC should be used in every reaction of the qPCR to permit correction for inter-tube amplification variation.
- When reporting data as a relative change, the C_T range of target detection should be quoted, since this allows the reader to gauge the likely accuracy and reliability of the quantitative data, particularly if the mRNA levels of the targets are widely different.
- Any signal detected in the negative controls should be reported and qualified by melt curve (if possible) or other analysis.
- Normalisation of qPCR (see below) must be transparent and reference genes, if used for comparison, must be validated for each experimental set-up, clinical condition and matrix type and appropriate data should be included in every publication.

A more detailed discussion of these and other considerations can be found in print (Bustin, 2004) and online (http://www.gene-quantification.info/).

<u>The amount and quality of nucleic acid templates.</u>

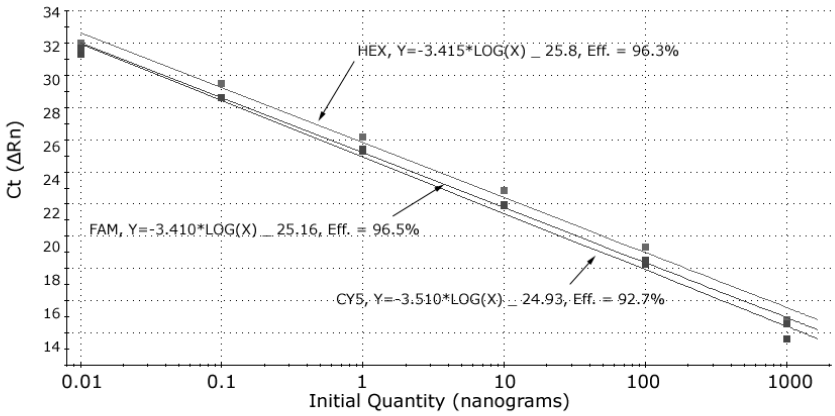
Many viruses and other micro-organisms contain a DNA genome and these pathogens are increasingly detected alongside RNA viruses within the same sample where they may collaborate in contributing to disease (Jennings et al., 2004; Arden et al., 2006). Thus, rather than preparing separate DNA and RNA extracts, it is preferable for the clinical microbiology laboratory to use a purification method capable of efficiently preparing both classes of nucleic acids in order to maximise the usefulness of the precious patient specimen e.g. when detecting enterovirus and *Neisseria meningitidis* from a small volume paediatric sample.

The importance of preparing intact microbial RNA for generating reproducible, quantitative data cannot be overstated. Since most of the RNA isolation procedures take place in a strong denaturant which protects the template, appropriate tissue handling including collection,

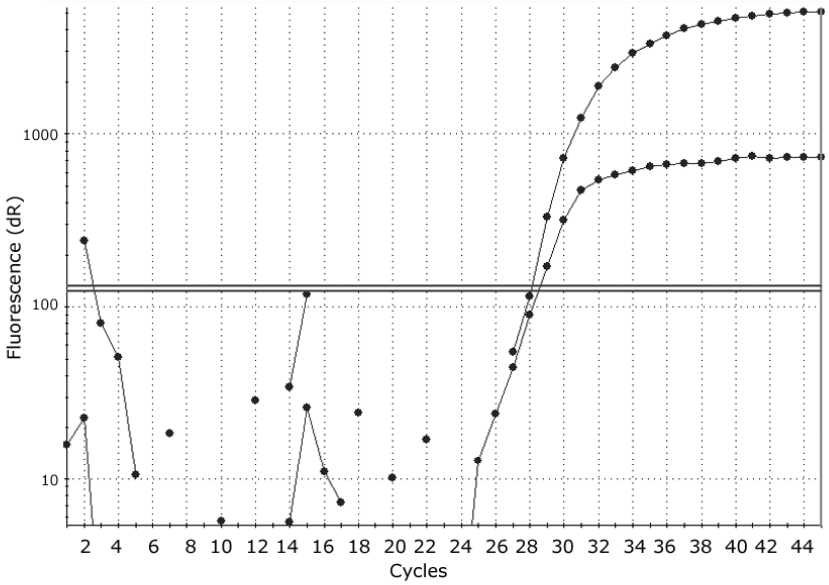
transport and storage prior to its extraction is essential to avoid template loss at this early stage.

Similarly, the importance of quantification and quality assessment of the RNA template for qPCR applications cannot be overstated yet these are usually overlooked by applications in microbiology. The most commonly used method, absorbance measurement at OD_{260} and analysis of $OD_{260/280}$ in a spectrophotometer, is insufficiently accurate. In addition, this method can waste significant amounts of precious RNA. The most accurate and preferred means of quantification uses an RNA-binding dye, RiboGreen; however, this does not provide qualitative information. There are two other options: first, LabChip® technology allows the rapid analysis of very small quantities of RNA. It is based on microfluidics technology, and a LabChip cassette consists of a series of interconnected microchannels and reservoirs embedded in a palm-sized chip architecture. Migrations through the channels are monitored and controlled using a bench top instrument such as the Agilent 2100 Bioanalyser or the Bio-Rad Experion. The RNA LabChip accurately quantifies as little as 50 pg of RNA and calculates the ratio of 28S:18S rRNA in the sample to provide a simultaneous assessment for each sample, and by inference for the quality of the microbial nucleic acids. Results can be viewed as gel-like images, electropherogrammes or in tabular formats. However, this analysis relates to rRNA and provides no information with respect to mRNA integrity. Integrity can be determined using the second option, the 3':5' assay. This assay is modelled on the standard approach adopted by microarray users (Auer et al., 2003) and measures the integrity of GAPDH mRNA, which is taken as representative of the integrity of all mRNAs in a given RNA sample. It synthesises cDNA from the 3' end of the RNA and then calculates the ratio of abundance of amplicons located at the 3' and 5' ends, respectively (Fig.3). A ratio of >5 suggests RNA degradation (Hands et al., 2007)

(a)


HEX, Y=-3.415*LOG(X) _ 25.8, Eff. = 96.3%
FAM, Y=-3.410*LOG(X) _ 25.16, Eff. = 96.5%
CY5, Y=-3.510*LOG(X) _ 24.93, Eff. = 92.7%
Ct (ΔRn)
Initial Quantity (nanograms)

(b)


Fluorescence (dR)
Cycles

(c)

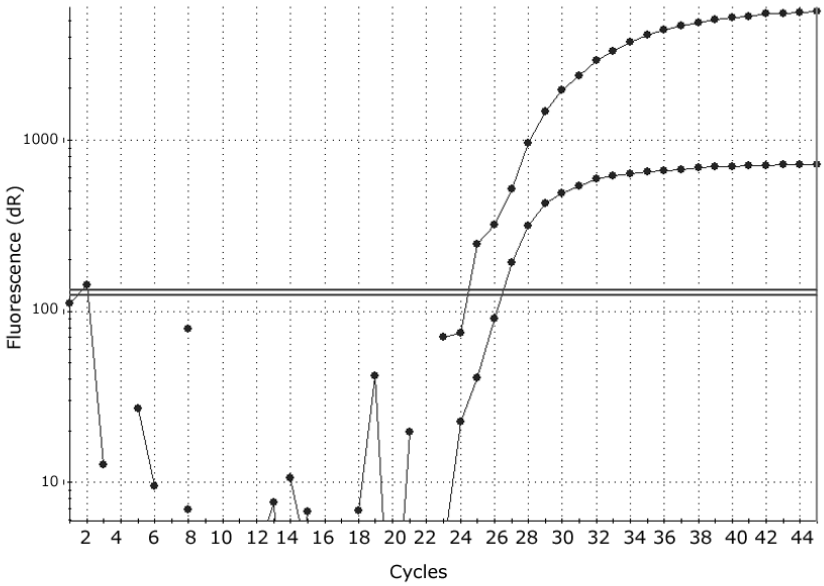

Fluorescence (dR)
Cycles

Fig.3. Integrity of RNA. Following reverse transcription with oligo-dT, three sets of primers and probe target amplicons at different positions (5', centre and 3') on the RNA, here GAPDH mRNA. The ratio of amplicons reflects the relative success of the oligo-dT primed RT to proceed along the entire length of the transcript. Clearly, the progress of the RT enzyme past the 5' amplicon is dependent on the intactness of the mRNA, with the enzyme unable to reach it if the mRNA is degraded. (a) When run in a multiplex reaction, it is apparent from the slopes that the amplification efficiencies of the three reactions are approximately the same, with similar y-intercepts indicating roughly similar sensitivity. (b) For clarity, results from only two amplicons are shown. The amplification plots suggest that this RNA preparation is of very high quality and fully intact. (c) The amplification plots of a moderately degraded RNA, showing a ΔCt of 4.

Inhibition, inefficiency and the internal amplification control

As introduced earlier, the IAC is a molecule that is co-amplified with the unknown target. IACs are today most frequently used to indicate the occurrence of false negative reactions or any reduction in amplification efficiency due to inhibition. We will refer to an IAC that has been engineered to bind the same primers but is discriminated by an oligoprobe that hybridises to a unique sequence within the amplicon, as an homologous IAC (Mulder et al., 1994; Tarnuzzer et al., 1996; Alexandre et al., 1998; Aberham et al., 2001; Stöcher et al., 2002) whereas one that consists of a completely different sequence from that of the microbial target we call an heterologous IAC; the latter is our preferred approach for microbiology (Templeton et al., 2003; Templeton et al., 2005; Scheltinga et al., 2005). When an IAC is added into the sample prior to template extraction and purification (termed an extraction or full-process IAC) or just prior to amplification (amplification IAC), it is described as an exogenous IAC, since it does not occur naturally within the sample matrix but is still co-amplified within the same reaction vessel. An endogenous, heterologous IAC occurs naturally within the sample; these are not engineered molecules and thus require separate primers for amplification. Endogenous IACs are most commonly known as "housekeeping" or endogenous reference genes (ERGs) and they can also be used to measure purification efficiency and/or identify inhibition. Of particular concern is the possibility that, since its concentration cannot be controlled from patient sample to patient sample, an overwhelming amount may be co-purified and detrimentally compete with the microbial template for amplification.

It is also possible to control for template loss during purification, which otherwise goes unnoticed. In this application it is preferable to add the IAC to the lysis buffer rather than

the crude sample itself to avoid degradation of the IAC molecule, unless the IAC has been protected or 'armoured' with a cell coat (e.g. MS-2 RNA; (Pasloske et al., 1998)). Since the purification efficiency can differ between different tissue types it is also best to limit a quantitative study, or the use of a kit, to the same type of tissue and employ the same method extraction.

If the exogenous IAC is only used to monitor inhibition, it should be added to the master mix, minimising pipetting error incurred by addition to each separate vessel. In general, inhibition does not cause problems for amplification of calibrators or reference standards since they are more carefully prepared. The unidentified presence of inhibitors in some biological samples will result in an underestimation of the nucleic acid levels in the test samples (Ståhlberg et al., 2005). Inhibition unrelated to the amplification process itself can have an impact at four main points along the laboratory process of obtaining qPCR data (adapted from (Wilson, 1997)): inhibitors can interfere with cell lysis, degrade nucleic acids, act directly and detrimentally on the reverse transcriptase and partially or completely interfere with amplification or fluorescent detection. Consequently, inhibitors, such as reagents used during nucleic acid extraction or co-purified components from the biological sample, can therefore significantly reduce the sensitivity and alter the kinetics of qPCR assays, rendering quantitative data invalid (Cone et al., 1992; Wilson, 1997; Perch-Nielsen et al., 2003; Guy et al., 2003; Suñen et al., 2004; Lefevre et al., 2004; Rådström et al., 2004; Jiang et al., 2005). Obviously, inhibitors are likely to distort any comparative quantitative data. However, a survey of practices at this time has revealed that only 6% of researchers test their nucleic acid samples for the presence of inhibitors (Bustin, 2005).

There are various methods that can assess the presence of inhibitors within biological samples but they all generally require a dedicated amplification step; for qPCR this occurs in the pre-analysis phase. As discussed earlier, the simplest method is to carry out a serial dilution of the sample spanning a large concentration range although the effect of some inhibitors can be difficult to remove this way (Kubista et al., 2006). Studies have shown that the degree of PCR inhibition can vary substantially among similar biological samples and that inhibition may affect different assays in different ways (Ståhlberg et al., 2003). Different polymerases also react differently to inhibitors and it is worth checking different matrix extracts for inhibition using several different thermostable DNA polymerases to identify the one most efficient at amplifying the template. This then raises the question of how many patients from each type of disease, and from normal populations, should be tested before we can be satisfied that we have exposed our reagents and protocols to every possible form of inhibition? Comprehensive studies are needed to address this question for the benefit of all clinical microbiology laboratories using real-time qPCR. When using a full-process IAC, signal

due to amplification of the IAC must be reproducible and reach a predetermined C_T value or, preferably, range when co-amplified with the template under investigation (Pasloske et al., 1998; Reiss and Rutz, 1999; Smith et al., 2003; Burggraf and Olgemöller, 2004). Delay in the C_T value indicates the presence of inhibition, poor quality template or low recovery of template (Levy, 1999). By comparing that value to the value obtained from a similar matrix sample containing only the IAC one can determine if template loss or the presence and extent of inhibition (Niesters, 2004). Of course the pedant could also question the validity of this approach since there is no mention of a calibrator to control for inter-tube amplification variability although replicates are often used to address this aspect. The presence of partial inhibition is not usually a significant problem for uniplex qualitative real-time PCR but becomes a much more important factor for multiplex assays and for quantification. Any reduction in signal during quantification will result in an underestimation of the amount of micro-organism present. The IAC used during pre-analysis must not significantly interfere with the detection of small quantities of the microbial template if it is to determine the extent, not just the presence, of inhibition therefore its use must be carefully evaluated. Such an IAC must also play no interfering role during the analysis phase of qPCR testing.

When the IAC was employed as a competitive template for conventional qPCR (Chehab et al., 1987) it was carefully added to ensure it would not overwhelmingly and detrimentally compete for reagents with the wild-type template (Brightwell et al., 1998; Rosenstraus et al., 1998). A major caveat for the use of an IAC as a competitor was that it had, or should have had, identical amplification efficiency to the template under investigation (Zimmermann and Manhalter, 1996). To achieve this extensive sequence similarity was required between the microbial and IAC templates which subsequently led to the formation of heteroduplex amplicons (Zimmermann and Manhalter, 1996). These mixed DNA hybrids consisted of one strand from the IAC DNA and one from the intended target amplicon; they reduced the sensitivity of detection and the efficiency of amplification for the intended target and made discrimination from the unknown sequence impossible by classical methods such as restriction enzyme digestion and agarose gel electrophoresis (Freeman et al., 1999; Pierce et al., 2005). An ideal competitive IAC should be of the same length and GC content as the intended target but differ from any microbial target sequence in all other regions apart from the oligonucleotide hybridisation sites. Unfortunately the current use of very small amplicons may leave practically no unique sequence to reduce heteroduplex formation. Thus it is more practical to employ an heterologous IAC, ensuring that amplification efficiency is as close to the assay's microbial target template as possible.

It is useful to add the pre-analysis IAC to samples that will be stored or transported at or just after collection to monitor any degradation of the sampled material. This is much more

important for RNA than for DNA, since RNA is the less stable nucleic acid. Practically this is a very difficult proposition for clinical microbiology samples since the spike would usually have to be added by non-laboratory personnel in the hospital, clinic, collecting centre or general practice. Pre-addition of a stabilised IAC to specimen tubes could be the solution.

Biological material derived from cultured micro-organisms should be avoided as a calibrator or an IAC without completely understanding and accounting for technical issues due to this source of template. For example, *in vitro* virus culture suffers from the production of defective interfering particles that may contain template but be non-infectious, or alternatively may lack template but retain the capability of receptor attachment and thus contribute to a gold standard biological detection system such as a plaque-forming or indirect immunofluorescent assay, falsely elevating the viral particle count (discussed for respiratory viruses in Chapter 8).

It is also important that the type of IAC nucleic acid is compatible with the microbial target. For analysis of DNA, a DNA-based calibration curve and IAC is a reliable combination. For analysis of RNA, an RNA-based calibration curve and IAC is the best choice. It is also possible to use a DNA-based calibration curve, when quantifying the amount of cDNA produced by the RT (Ståhlberg et al., 2004a), in combination with an RNA IAC that controls for extraction and RT yields. A combination of an RNA-based calibration curve with a DNA IAC is not very useful, since DNA and RNA purifies very differently. An RNA IAC should share any important sequence features with the microbial RNA target that may affect stability. A polyA-tail and a 5' cap that protect from degradation should be present for mRNA targets while other features may be more important in IACs used alongside viral genomic RNA targets.

The IAC should have the same secondary (double vs. single strand) and tertiary (supercoiled) structures as the intended target. A naturally supercoiled plasmid, for example, is not a good extraction control for genomic or viral DNA. Most recently a simple qPCR-based assay has been described that consists of a plant-derived universal exogenous IAC template sequence, primers and a detecting probe (Nolan et al., 2006) and there are now heterologous IAC systems commercially available e.g. from Applied Biosystems.

Reverse transcription: The Achilles heel of quantification

A real-time reverse transcription qPCR (RT-qPCR) assay consists of three steps: (1) the conversion of RNA into cDNA using an RNA-dependent DNA polymerase, (2) the

amplification of the cDNA using the PCR and (3) the detection and quantification of amplification products in real-time (Gibson et al., 1996).

Although seemingly a small step, the conversion of RNA into an amplifiable cDNA template is, in practice, an important contributor to the variability observed in any RT-qPCR experiments (Kolls et al., 1993; Bustin et al., 2005). Quite apart from biological variability, there are significant technical issues. One major drawback of the RT step is its lack of standardisation, which means its variable efficiency can continue uncontrolled, resulting in inaccurate quantification. RT yields can vary up to 100-fold with the choice of enzyme and this variation is target-dependent (Ståhlberg et al., 2004b). Furthermore, results also depend on the priming strategy and the concentration of RNA (Bustin and Nolan, 2004; Ståhlberg et al., 2004a). Hence it is essential to at least use the same enzyme, priming strategy, and experimental conditions, if quantitative (or qualitative) RNA measurements are to be comparable between laboratories. Furthermore, the choice of smaller amplicons is a preferred workaround for degradation of longer RNA templates (Fleige and Pfaffl, 2006).

Real-time RT-qPCR assays can be performed either as a one tube, single RT and PCR enzyme method or a separate RT and PCR enzyme technique using one or two tubes (Fig.4). A single enzyme such as *Tth* polymerase is able to function both as an RNA- and DNA-dependent DNA polymerase and can be used for the one tube method without secondary additions to the reaction mix (Cusi et al., 1994; Juhasz et al., 1996). Its main advantages are the reduced hands-on time and potential for contamination. Its main disadvantage is that since all reagents are added to the reaction tube at the beginning of the reaction, it is not possible to optimise the two reactions separately. Furthermore, this reaction is characterised by extensive accumulation of primer dimers, which may obscure the true results in quantitative assays (Vandesompele et al., 2002a).

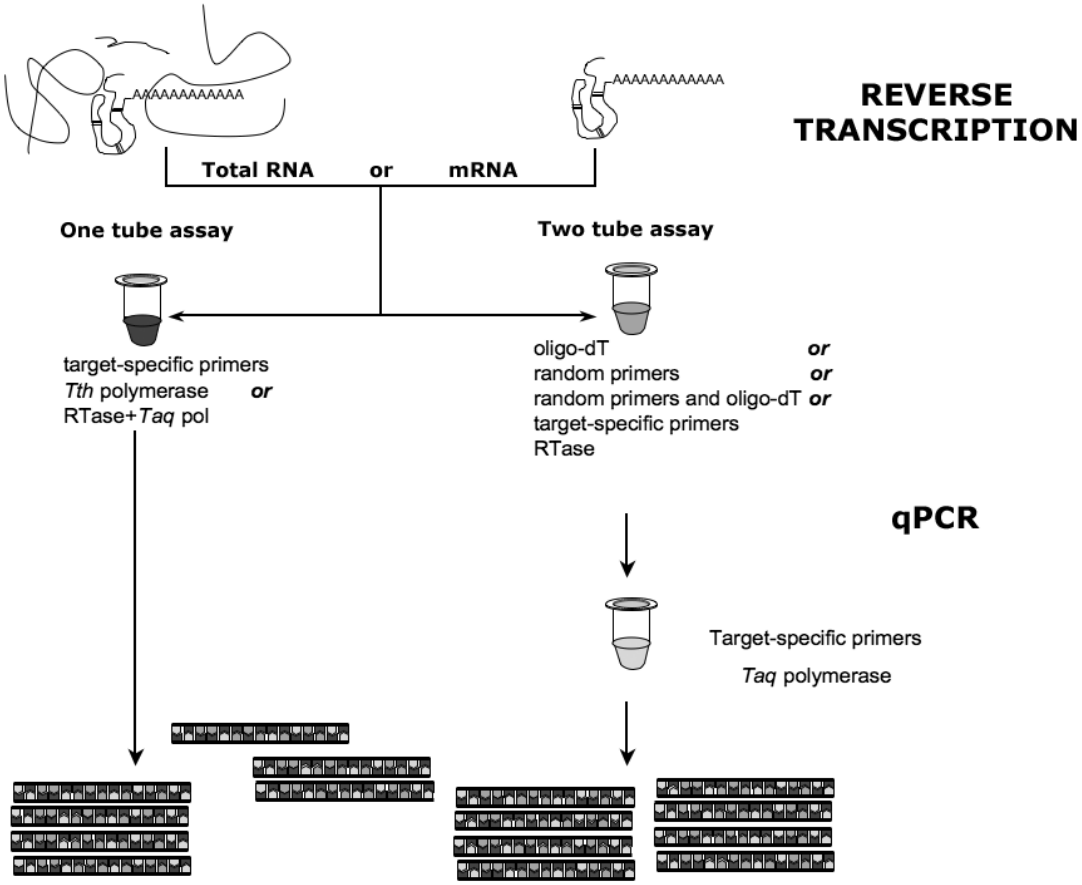


Fig.4. RT-qPCR protocols. Most assays targeting viruses will use total RNA and target-specific priming. In contrast, most assays targeting non-viral targets tend to use random priming or a combination of random and oligo-dT priming.

In the separate techniques RT and PCR enzymes can be used either in "coupled" (single tube) or "uncoupled" (two tube) reactions. In the coupled method the reverse transcriptase synthesises cDNA in the presence of either gene-specific or oligo-dT primers. Following the RT reaction, PCR buffer, a thermostable DNA polymerase, and gene-specific primers are added and the PCR is performed in the same tube. In the uncoupled alternative the reverse transcriptase synthesises cDNA in a first tube, under optimal conditions, using random, oligo-dT or gene-specific primers. An aliquot from the first reaction is then transferred to another tube containing the thermostable DNA polymerase, DNA polymerase buffer, and PCR primers and the PCR is carried out under conditions that are optimal for the DNA polymerase. Interassay variation of two enzyme protocols can be very small when carried out properly, with correlation coefficients ranging between 0.974 and 0.988 (Vandesompele et al., 2002a). The disadvantages of this approach, particularly for the high throughput clinical microbiology laboratory are that it takes longer, there are additional opportunities for contamination and the reverse transcriptase can inhibit subsequent PCR assays even after

inactivation, resulting in an overestimation of amplification efficiency and target quantification, particularly for rarely expressed transcripts, with the yield of amplified RNA increased up to 20-fold (Suslov and Steindler, 2005).

Random priming or oligo-dT priming both allow a pool of cDNA to be produced during a single reaction. However, it has been shown that priming using random hexamer primers does not result in equal efficiencies of RT for all targets in the sample and that there is not a linear correlation between input target amount and cDNA yield when specific targets are measured (Bustin and Nolan, 2004; Lacey et al., 2005). A recent comparison of the efficiency of RT priming by random primers of varying lengths showed that 15-nucleotide-long random oligonucleotides consistently yielded at least twice the amount of cDNA as random hexamers (Stangegaard et al., 2006). The 15-mers were more efficient at priming, resulting in RT of >80% of the template, while random hexamers induced RT of only 40%. Not surprisingly, this resulted in the detection of one order of magnitude more genes in whole transcriptome DNA microarray experiments.

Oligo-dT primers should only be used with intact RNA and generally only with those micro-organisms known to have a polyA tract associated with the genome or transcript. Even then the cDNA molecules may be truncated, since the RT enzyme cannot proceed efficiently through highly structured regions. Accordingly, oligo-dT-primed assays should be targeted towards the 3' end of the transcript. Oligo-dT priming is not recommended when using RNA extracted from paraffin tissue sections, since formalin fixation results in the loss of the polyA tails on mRNA (Lewis and Maughan, 2004).

Gene-specific primers are the most specific and most sensitive method for converting mRNA into cDNA (Lekanne Deprez et al., 2002) and are the recommended choice when RNA quantity is not a limiting factor. Even if the amount of RNA is a limiting factor, a recent report demonstrates the use of specific primers for the reliable and specific amplification of 72 genes from limiting amounts of RNA using a multiplexed tandem PCR approach (Stanley and Szewczuk, 2005). Nevertheless, as with random priming there may be differences in the efficiencies at which individual RT reactions occur. These variations must be controlled by reference of the PCR data generated from an unknown sample to that from a calibrator sample (when using $\Delta\Delta C_t$ analysis) or to a calibration curve. Specific priming of RNA dilutions results in a linear response of target cDNA yield (Bustin and Nolan, 2004) and so a further advantage of using this priming method is that the efficiency of the combined RT-qPCR reaction can be confirmed by analysis of the slope of the calibration curve. However the extent of the linearity is entirely dependent on the level of conservation between the oligonucleotide and its target site. The inclusion of a calibrator sample or calibration curve in

every assay is an important control for measuring inter-assay variability that may occur when multiple samples are run on different assays.

Nucleotide target sequence variation

Sequence variation has always been and will most likely always remain an issue of significant concern when developing PCR assays but it is particularly troublesome when it plays a role in quantitative assays. Even quantitative kits targeting DNA viruses can be subject to problems caused by sequence variation highlighting the importance of kit comparisons (Ruiz et al., 2005).

In particular, the low efficiency of proofreading and post-replicative repair activities of viral RNA polymerases results in relatively high mutation rates, causing potential problems for an assay that relies on exquisite sequence specificity. Furthermore, populations of RNA viruses are extremely heterogeneous existing as quasispecies in which RNA viral genomes are statistically defined but individually indeterminate (see Chapter 8 for more detail). This has raised some concerns about the detrimental effect of primer-template mismatches on the polymerisation efficiency and problems associated with geographically disparate reference sequences (Mullan et al., 2004) but is of equal concern from season to season in the same location. In our experience, amplifying the same nucleic acid extract using subtlety different primer pairs quite usefully demonstrates that sequence variation is common. Each primer pair may preferentially amplify different quasispecies demonstrating just how problematic the design of useful probes can be.

While some degree of sequence mismatch can be tolerated by primers and even by some fluorogenic probe chemistries during qualitative real-time PCR, any mismatch is considered undesirable for quantitative purposes. Depending on its position, it can lead to destabilisation of primer or oligoprobe binding to sample templates and may result in reduced amplification efficiency. This reduction is not reflected by the perfect match between oligonucleotides and the templates used to produce calibration curves, and creates disparity. A practical outcome for the clinical laboratory is that some virus lineages and sublineages will appear to have higher or lower viral loads than others due to better or worse hybridisation efficiency, respectively. One approach to combat the impact of microbial variation is to narrow down the genetic identify of the micro-organism using qualitative assays, during the pre-analysis phase of the qPCR process (Watzinger et al., 2006). This approach also avoids the loss of specificity and sensitivity that can result from using degenerate primers (which are essentially a mixture of primers which vary at one position) or pan-micro-organism primers designed to detect all members of a species, serovar or genus.

For example, the extreme sequence heterogeneity of HIV-1 poses a significant challenge to its efficient detection with nucleic acid-based assays. Not only must the primers bind to regions that are conserved and not subject to rapid mutation, but also the probes used for specific chemistry assays must not hybridise to variable sequences. This has led to the development of assays using an intercalating dye e.g. SYBR®-Green, that rely on the use of melting curves to ensure that the obtained signal is specific (Ruelle et al., 2004). These assays are better than branched-chain DNA assays in determining viral load (Leutenegger et al., 2001), especially during the monitoring of HAART treatment (Gibellini et al., 2004) and have a superior linearity over a wide concentration range (Klein et al., 2003) however we do not recommend their general use for quantification.

Normalisation of real-time qPCR data: The analysis phase

All PCR assay results are subject to variability caused by technical as well as biological variation. Once technical variability (e.g. pipetting, instrumental performance and reagent quality) is recognised and kept to a minimum the chances of identifying clinically relevant changes in nucleic acid levels are improved. Apart from sequence variation, normalisation of amplification data addresses perhaps the most important biological variable in need of control. However, it is difficult to identify how best to address this variation for microbial qPCR applications. Perhaps this is why normalisation is commonly overlooked by the clinical microbiology laboratory or why approaches are often selected from the human gene expression literature. It is essential that microbiologically relevant approaches to normalisation are identified in order to produce robust real-time qPCR data for this field.

Data normalisation is essentially the process of correcting deviations from expected performance caused by the amount and quality of template amplified. The results are more meaningful if they have not been influenced by inhibitors or degraded or lost template so it is essential to account for these during the pre-analytical phase. Normalisation is also required to correct for changes in enzyme function principally indicated by amplification performance. Further contributing to the poor uptake of normalisation is the often confusing use of the word. Normalisation is also used to describe the adjustment of real-time PCR fluorescence data to account for amplification-independent fluctuations, or to define a component of the quality control process (Watzinger et al., 2006).

Variable sample collection is another significant cause of error in many aspects of diagnostic microbiology, as is variation within the sample itself exemplified by changes in the cell

content or the amount of extracellular fluid between samples from the same patient during the course of an infectious process or during an immunosuppressive or cytotoxic treatment regimen. Sampling and sample variation are particular obstacles to the design of a successful real-time PCR normalisation strategy for the clinical microbiology laboratory.

Normalising to an ERG is the most common approach for real-time qPCR in human gene transcription studies and is currently the preferred option so long as it is carried out correctly (Goossens et al., 2005). This strategy can express the target gene's quantity relative to that of one or, preferably, more ERGs. For human transcriptome studies this approach benefits from the fact that all the steps of the final qPCR measurement are controlled. The procedure is simplified as both the gene of interest and the ERGs are co-amplified using real-time RT-PCR; this is more reliable than adding material from an external source. However, it is essential that reference gene expression in the target tissue is carefully analysed (Perez-Novo et al., 2005) so that only targets exhibiting a minimum of variability are employed (Vandesompele et al., 2002b). Ideally transcription of an ERG target should be minimally regulated and exhibit a constant and cell cycle-independent basal level of transcription (Selvey et al., 2001). The current gold standard combines the evaluation of a panel of several reference genes (e.g. available commercially http://www.tataa.com or http://www.primerdesign.co.uk) together with a method for selecting reference genes with the most stable expression e.g. GeNorm (Vandesompele et al., 2002b), Bestkeeper (Pfaffl et al., 2004), Normfinder (Andersen et al., 2004) or GenEx (http://www.multid.se). In this way dramatically up and down regulated genes can be identified under the conditions expected within the host tissues and their detrimental variability avoided (Radonic et al., 2004; Radonic et al., 2005). ERGs have been successfully used to quantify gene expression using RT-PCR and to monitor template integrity after its purification since their preparation is also identical to that of the microbial template (Chehab et al., 1987). Studies have shown that an 18S rRNA target meets these criteria for some applications (Thellin et al., 1999; Selvey et al., 2001). This is not the case for other genes including β-actin; unfortunately their remains widespread, especially for human gene transcript analyses (Härtel et al., 1999).

A very important question raised by the assumptions above, and for which there is currently no definitive answer, is how can we be certain that "ideal" ERG targets remain minimally regulated throughout the course of often lengthy and complicated infectious event; let alone between hosts? No single gene expresses consistently among all tissues despite promising candidate targets (Radonic et al., 2004). The RNA polymerase gene displays low variation over many tissues, even during mitogenic stimulation of a T-cell line. RNA polymerase mRNA encodes the principle enzyme used in mRNA transcription and is therefore part of a self regulating cycle. It may be assumed that this gene is expressed steadily and independently

of the cell state. However authentication is still required for each situation, a daunting task for applications in microbiology.

Specific problems normalising data to quantify micro-organisms

Correcting the data is particularly useful when conducting studies to compare microbial levels in serial specimens collected from the same subject over time. By comparing normalised data, one can better identify a change in delta CT values and then correlate it with a change in the level of a micro-organism irrespective of sample or subject. Similarly, real-time qPCR studies of latent or persistent viral infection can better demonstrate reactivation of the micro-organism.

As mentioned, most of the work on real-time qPCR normalisation has been conducted for human gene expression studies and consequently, on RNA templates. Nonetheless similar approaches to the ones discussed below should function just as well for DNA templates although using samples previously extracted with a method that claims specificity for RNA may result in lower DNA yields. However, it remains unclear how useful currently popular gene transcription normalisation strategies are for the quantification of micro-organisms.

Normalisation against input RNA amount is a feasible and, if validated, acceptable option (Bustin, 2002). However, as for gene expression studies there are problems comparing a microbial target to a marker that proliferates at a different rate, since this will affect the mRNA/rRNA ratio. Making this more difficult is the presence of micro-organisms in many different environments, including those with little background RNA (virus alone contributes little nucleic acid mass; a single human cell contains approximately 7pg of nucleic acid, matched in mass by approximately 10^6 rhinovirus virions) or among patients with underlying disorders that may affect the expression levels of ERGs. There may also be one or more pathogen-specific effects on host cell expression all of which create problems for the production of reliable data.

Comparing the target signal against genomic DNA is particularly useful when performing real-time RT-qPCR analyses on RNA obtained from minute samples such as those obtained from laser capture microdissection. The main problems here are that (1) differential stability of DNA and RNA may distort quantification, (2) sample cannot be DNase-treated, (3) that, whilst being an internal control, there is no equivalent RT step and (4) genomic DNA may be a source of variation if the gene of interest is disproportionately represented compared to other genes because of target unrelated reasons such as tumour growth.

Normalising directly to a sample's cellular content is too uncertain for broad application. For example in patients with HIV-AIDS or those with malignant haematopoietic disorders, peripheral blood cell counts may be lowered or raised respectively, compared to otherwise healthy subjects infected by the micro-organism under investigation. In these instances the number of extracellular target microbes per cell would be falsely raised or lowered (Huggett et al., 2005). Another example is the detection of intracellular viruses or malarial parasites; in our previous example their numbers may appear falsely lowered or raised respectively. A differential cell count is advisable to avoid, or at least indicate, the possibility of such problems. Similarly, certain tissue conditions such as fibrosis or malignancy may increase the number of cells, but perhaps not in favour of those supporting the growth of the micro-organism under investigation therefore resulting in falsely lowered normalised microbial load values (Huggett et al., 2006).

A useful compromise for normalisation of qPCR data obtained from the clinical microbiology laboratory is to employ a second distinguishable, accurately quantified, synthetic, heterologous, IAC added to each extract and to an aliquot of extract obtained from the same matrix type for treatment in parallel (Fig.5). If poor extraction efficiency or inhibition was identified during pre-analysis, this would need to be addressed before qPCR could be undertaken (discussed earlier). Of particular benefit is the ability to quality control the large scale production of synthetic IACs and distribute them among clinical microbiology and research laboratories, perhaps one day leading to the development of commercial versions (Smith et al., 2003). An IAC used in this way could not account for differences between the relative amounts of intra- and extracellular virus or nucleic acid but then in practical terms, no approach can.

If one agrees that no existing pre-analytical method can accurately identify the DNA or RNA content attributable to the micro-organism and that values determined using current strategies more likely reflect the host's inconsistent cellular content and variable transcriptional status, then one must also agree that there is no perfect way to normalise qPCR data in microbiology. However, employing IACs that are unaffected by host-related variation is much closer to an ideal approach. Agreement on these points would go some way to aligning our expectations with the reality of diagnostic clinical microbiology, rather than the unachievable approaches handed down from human transcription studies.

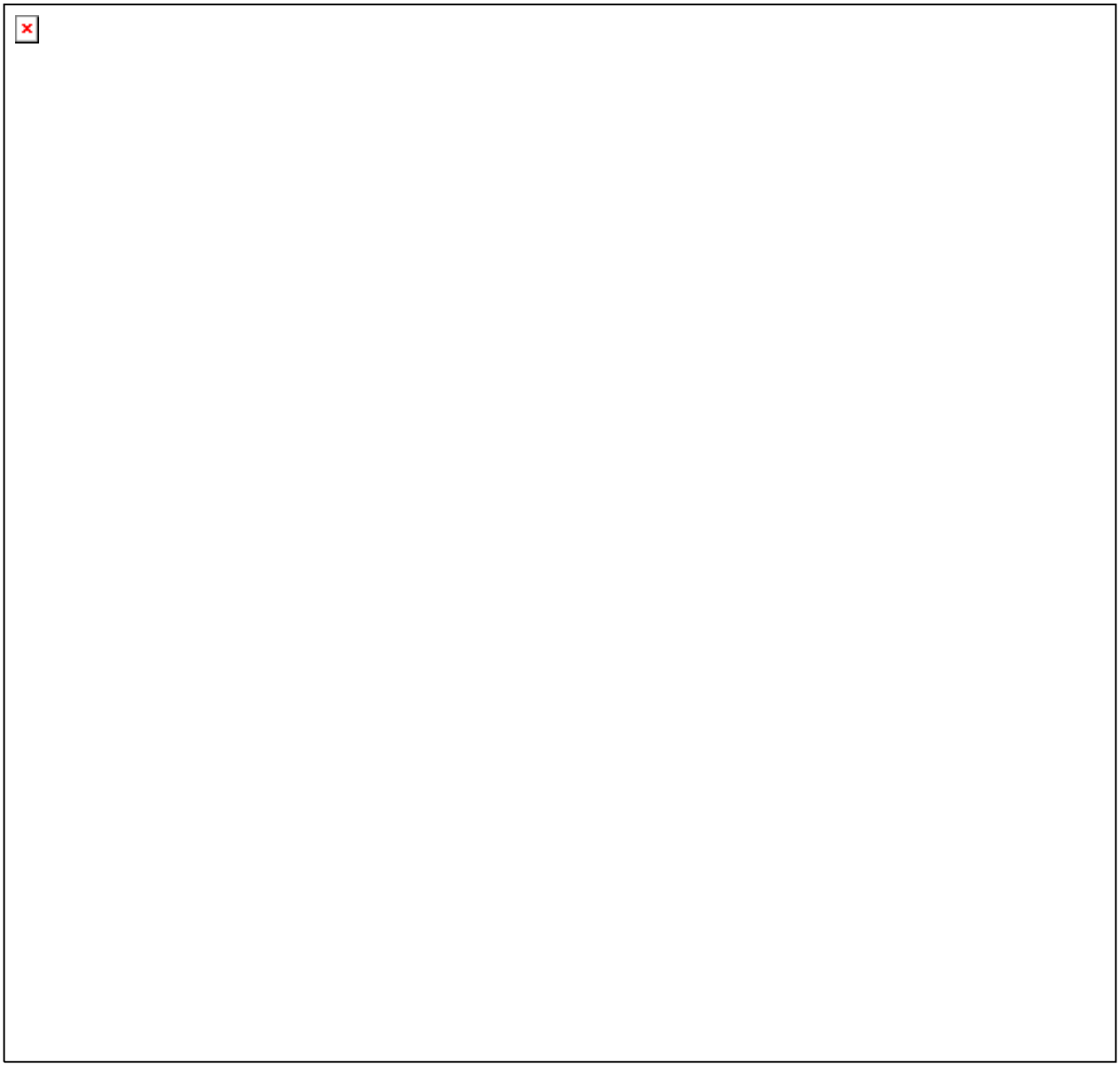


Fig.5. A schematic representation of the steps required during the pre-analysis and analysis phase of real-time qPCR. The addition of an heterologous IAC (IAC 1;filled square) along with the chaotropic extraction reagent is recommended to identify extraction efficiency and the presence of inhibiting substances after purification. This requires a "pre-analysis" real-time PCR assay to examine the yield of IAC 1. Once inhibitor free nucleic acids have been efficiently extracted, the quality of the potential microbial template (filled circle) should be examined as described in the text. Only then should one consider the qPCR assay or "analysis" phase. A 2nd heterologous IAC is added to a pre-determined amount of each unknown template preparation and the signal from the co-amplified IAC is used to normalise the qPCR data. A reference standard or calibrator dilution series is prepared and amplified alongside the unknown(s). A passive reference (PR; filled diamond) is also included in every tube to account for inter-tube variation caused by fluorescence inhibition or pipetting error.

The corrected data then permits one to determine the amount of unknown in the extract by interpolation from the calibration curve.

A disadvantage often attached to the use of synthetic, heterologous IACs is the need to carefully choose and laboriously clone the molecule and possibly transcribe RNA from the DNA versions *in vitro* (Huggett et al., 2005). Also the claim is often made that such a molecule will not perform the same way during extraction when compared to deriving the intended target from an intracellular source but there seems to be little or no definitive evidence to support the existence of such a difference. The first reason is false economy; it does take time to choose and clone a synthetic target, but a heterologous target has the potential to be used for all assays whereas a panel of suitable ERGs must be identified for every tissue, disease, target and experimental condition; this workload far surpasses that for a cloning and *in vitro* transcription protocol.

Quantification occurs close to home

The diagnostic needs of medical practitioners and researchers must often be met by the local research or diagnostic microbiology laboratories because there are relatively few kits available for quantifying micro-organisms by PCR and few internationally validated reference standards or calibrators. However these sources are commonly perceived to produce assays which are less well developed than commercial versions making in-house assay accreditation extremely difficult. In practice this perception may be far from the truth and anecdotal evidence provides many tales of poorly performing commercial kits. In the absence of adequate commercial support or a relevant quality assurance program, a need exists for different laboratories who quantify the same targets to come together to construct suitable reagents for standardising the performance of their assays. It should be possible to employ a cloning strategy to incorporate suitably conserved micro-organism sequences of interest into simple, encapsidated particles (Chapter 4 and (Pasloske et al., 1998)) for use as co-amplified DNA or RNA controls. Surely, in this age of rapid communication and comprehensive protocols for the transport of biological materials, some key reference microbiology laboratories could develop and disseminate this material among their peers with a sharing of costs, in order to at least develop proof-of-concept quality assurance programs for more targets. Success in such endeavours may be the lure that attracts commercial interests to become more actively involved in producing the reagents on a larger scale and under best practice methods.

Emerging roles for real-time PCR quantification

Among the many real-time qPCR applications in microbiology, some are less frequently considered than others. These also suffer from the issues we have discussed above and because they may have a larger role in clinical microbiology in the future, now is a good time to consider these less common applications as part of an overall process of improving real-time qPCR for microbiological applications.

Viral gene therapy

Gene therapy aims to introduce genetic material to an ill patient for therapeutic purposes with the goal of improving the quality of life; ideally providing a permanent cure for the target illness (Pfeifer and Verma, 2001). Genes are most commonly delivered to patient tissues in modified virus vectors (http://www.wiley.co.uk/genetherapy/clinical/). While still a young science burdened by many significant hurdles, one could envisage that the successful development of viral gene therapy protocols could result in their routine therapeutic use which would require the services of the high-throughput clinical microbiology laboratory. In addition to the use of real-time qPCR to quantify vector concentration in the research laboratory, diagnostic screening applications could be routinely used to determine how much virus is present in treated patients over time, to screen for the emergence of replication competent viruses due to recombination or mutation and to monitor the presence, integrity and activity of the transferred gene (transgene) or its regulatory elements. Traditional methods to detect the efficiency of viral vector-mediated gene transfer into a target cell or tissue (transduction) and to monitor the gene's integrity and transcriptional activity include histochemistry (Blömer et al., 1997), immunofluorescent microscopy (Chen et al., 2002) and Southern or Northern blot hybridisation (Woo et al., 2006). However, these techniques are lengthy, often insensitive and they have a low sample throughput. Therefore rapid and reliable methods to quantify targets are essential (Gautheret et al., 1996; Tang et al., 1998; Andreadis et al., 2000; Delenda and Gaillard, 2005).

Studies have recently identified that analysis of DNA, specifically by real-time PCR using TaqMan probes, is as much as 10^3 to 10^4-fold more sensitive than monitoring a green fluorescent protein (GFP) reporter transgene by FACS analysis or determining RNA concentration from culture supernatants, when assessing the titre of a lentiviral vector (Scherr et al., 2001; Sastry et al., 2002). However the question then arises: how does one determine whether particles are infectious (Delenda and Gaillard, 2005)? Because this answer remains unknown, the detection of proviral DNA has become a useful surrogate

measure for retrovirus-mediated gene transfer since these vectors have then at least transduced the target cell. Real-time PCR has also been used to detect integration of retroviral vectors (Martin-Rendon et al., 2002). This approach is reliant upon a correlation between a positive signal and vector integration following serial passage of the host cells and identifies the presence of infectious vector particles. Passaging the cells of a human host is not feasible but more importantly, this approach can not confirm that the signal supposedly of indicating the presence of proviral DNA is not derived from DNA retained within infected cells in some other non-integrated form (Gerard et al., 1996). While PCR detection does not prove integration of the retrovector, identifying transduction is useful to show that a packaged vector preparation is infectious.

The predominantly TaqMan-based real-time PCR studies used for quantification in gene therapy applications, as well as our own earlier conventional competitive quantitative PCR approaches either chose target sequences that were common to the vector class e.g. the common packaging or regulatory signal of a retrovirus vector (Gerard et al., 1996; Sastry et al., 2002; Lizeé et al., 2003), strong stop retroviral cDNA (Scherr et al., 2001) or the transgene e.g. *gfp* or neomycin phosphotransferase (Sanburn and Cornetta, 1999; Klein et al., 2000; Mackay et al., 2001) or its promoter sequence (Martin-Rendon et al., 2002)). In this way, variants of the vector backbone could be monitored using the same quantitative assay without re-development for different disease targets. Virus-specific sequences have also been used to monitor the biodistribution of viral vectors within the subject's organs (Hackett et al., 2000). This approach identified that the majority of an intravenous preparation of adenovector was deposited within the lung. Whilst others have reported success using viral RNA templates derived from culture supernatants, the use of a DNA target avoids introducing additional handling and standardisation requirements. However one must be very careful to avoid, or at least account for, contamination with DNA from the original plasmid components left over from the preparation phase of a viral vector; DNase treatments are suggested to at least remove any extracellular contaminants (Scherr et al., 2001).

Conventional methods which analyse transgene function such as FACS rely on a fluorescent reporter; therefore they have limited use for testing therapeutically treated patients. On these occasions ELISA methods may indicate the presence of an expression product if a suitably specific antiserum is available. Such reagents are often unavailable commercially or prone to problems with background and cross-reaction. Furthermore, promoter strength and choice of biological host system can significantly influence the apparent number of vector particles (Bowers et al., 2000). Real-time qPCR is a more objective method and it has proven itself capable of reliable vector detection (Deffrasnes et al., 2005).

Microbial load and the host response to infection: The future of pathogenicity?

The application of real-time PCR is beginning to provide evidence to support paradigm shifts in the way we conceptualise the interplay between microbe and host as well as in the way we describe these interactions (Casadevall and Pirofski, 2003). Pathogen-centric views have dogmatically defined virulence as the result of microbial factors, when in fact the role of the host can be clearly shown to exert a significant influence (Mitchell, 1998; Casadevall and Pirofski, 2001). Increasingly, the old view is being challenged by our growing understanding that changing host immunity is as integral to the course of an infectious disease as the microbe present in the infected host. This poses the question 'what is a pathogen'? (Casadevall and Pirofski, 2002). Microarray technologies present answers from the host's perspective providing a comprehensive snap-shot of the state of the host or invading microbe's transcriptome however arrays do not permit reliable quantification of the target change over a broad dynamic range (Lockhart and Winzeler, 2000; Lucchini et al., 2001; Clewley, 2004). Real-time PCR is the method of choice for validating and further characterising experimental data generated by microarrays and it will be at this level that we may be able to answer the pathogenicity question. Considerably more has been published on the role of real-time PCR in the basic detection of cytokine transcripts from stored or *in vitro* stimulated blood cells than as an indicator of microbial impact (Kruse et al., 1997; Härtel et al., 1999; Blaschke et al., 2000; Stordeur et al., 2002). This is set to be a growth area for future real-time PCR applications and perhaps an addition to the repertoire of the clinical microbiology laboratory of the future.

Cytokines play an important role in the innate mammalian immune response, where they mediate the communication with and recruitment of leukocytes and the removal of invading micro-organisms. However, it is the host's molecular pattern recognition systems operating via specialised receptors that trigger many of the events leading to cytokine induction post-infection (Strieter et al., 2003). Real-time PCR has identified critical mRNA from these receptors and from cytokine genes as a *de facto* indicator of protein production, (Overbergh et al., 1999; Giulietti et al., 2001; Hein et al., 2001; Overbergh et al., 2003). While this is not a perfect relationship insofar as protein levels are modified by more than simply transcript abundance, studies have also shown a good correlation between the transcriptome and the proteome for many genes (Balnaves et al., 1995). Additionally, a molecular approach is valuable because it is often impossible to detect the expressed product in tissue samples due to the low amounts present and short half-lives of cytokine proteins (Stordeur et al., 2002; Broberg et al., 2003). Nonetheless, transcriptional studies using real-time PCR are enhanced by accompanying protein expression data.

Well-controlled quantitative real-time PCR experiments have permitted the differentiation and quantification of IFN-α subclasses as a measure of the innate immune response to infectious or inactivated virus in humans (Löseke et al., 2003). The probe or SYBR Green I-based real-time determination of expression of human IL-4, IFN-γ or mouse IL-12p35, 12p40, 23p19 and IFN-γ cytokines has also been successfully monitored in murine models of microbial immunopathogenesis (Deng et al., 2003; Broberg et al., 2003). In both studies mice were used to provide a laboratory model of infection and immunopathogenesis for human viral pathogens or to study the use of quantitative real-time PCR on practical samples. β-actin was used to normalise the data. Relative quantification by TaqMan-based real-time PCR and glyceraldehyde 3-phosphate dehydrogenase normalisation was employed by Song *et al* to examine the direct impact of cytokines on viral gene expression and the effect of a human herpesvirus-8 (HHV-8) cytokine homologue (vIL-6) on co-infecting viruses (Song et al., 2002). This study demonstrated both interplay between pathogens and the host, indicating the complex nature of microbial infections and identified a potential therapeutic strategy to combat HIV and HHV-8 co-infection. In another study, a SYBR-Green I-based RT-qPCR approach was employed to demonstrate that IL-8 production was upregulated after *Chlamydia trachomatis* protein production commenced within infected HeLa cells (Buchholz and Stephens, 2006). This study aimed to discern the mechanisms of IL-8 production since this cytokine is chronically produced in *C.trachomatis* infections and it is also associated with inflammation-based tissue damage, a hallmark of *Chlamydia*-based disease.

Zhao and colleagues described the use of quantitative TaqMan-based real-time PCR to confirm the results of microarray analysis of *in vitro* adenovirus-infected HeLa cultures (Zhao et al., 2003). Adenovirus infection downregulated several cytokine genes and genes associated with growth arrest. Some stress response genes were upregulated as was an apoptosis inhibitor. Interestingly, β-actin was not differentially expressed and so was used to normalise the data. SYBR-Green I or TaqMan applications have validated microarray data indicating that parvovirus H-1 NS1 gene transcript accumulation could be correlated with the nonapoptotic death of an infected cell line and that a deletion mutant of African swine fever virus was unable to replicate effectively in infected macrophages, possibly due to a relatively enhanced type I interferon (IFN) response compared to that induced by a parental virus (Afonso et al., 2004; Li et al., 2005). These data have also correlated with protein or Northern blot analyses, respectively. TaqMan-based real-time relative RT-qPCR and ELISA were used to show an impaired type I interferon response resulted from HRV-16 infection of *in vitro* cultured primary bronchial epithelial cells obtained from patients with asthma (Wark et al., 2005). Since a type I IFN deficiency in asthmatics facilitates virus replication, there

may be a therapeutic role for IFN-β in moderating virus-induced exacerbations of asthma. SYBR Green I based real-time PCR once again confirmed microarray data which suggested the human polyomavirus JCV induces a regulatory immune response in transfected and infected cells (Verma et al., 2006). In turn, Western blot data corroborated the PCR results for two of the upregulated genes. Among immunocompetent hosts this cytokine response may constrain the progression of more serious disease and perhaps permit a long term latency and persistent infection. Another respiratory pathogen, *Bordetella pertussis* was investigated by Ishibashi and colleagues using SYBR-Green I-based real-time PCR and they showed that a fimbrial structure on the surface of the bacterium upregulated intercellular adhesion molecule-1 expression whilst the presence of pertussis toxin downregulated its expression thus inhibiting a putative antibacterial mechanism (Ishibashi and Nishikawa, 2002). Once again β-actin was used to normalise these data.

Using a SYBR Green I real-time RT-qPCR, Nelson and colleagues were able to propose that by upregulating expression of siderocalin, a potent bacteriostatic effector, *Streptococcus pneumoniae* and *Haemophilus influenzae* could limit the growth of susceptible competitor species of bacteria (Nelson et al., 2005). On a larger scale, SYBR Green I-based real-time RT-qPCR has been used to validate microarray data for 17 genes expressed in response to SARS-CoV infection of Vero E6 cells, providing insight into the pathophysiology of SARS (Leong et al., 2005).

Despite an increase in the number of published studies employing real-time qPCR to investigate the impact of a micro-organism on its host, there are still few studies looking at microbial determinants, or virulence factors, at the same time as the host's response. Furthermore these approaches also fail to account for multiple virulence factors (McClelland et al., 2005; McClelland et al., 2006) which is an area for future development.

Quality and real-time qPCR

Not only does the lack of commercially available kits remain a major problem for quantification of micro-organisms, but the absence of standardisation among existing in-house assays results in the production of incomparable data (Kao et al., 2005). These weaknesses and others we have outlined throughout the entire chapter mean that many qPCR approaches are not fit to deliver upon the their stated intent, in particular true absolute quantification of the micro-organism in a patient specimen.

The implementation of quantitative molecular techniques in the 1990s (Piatak Jr. et al., 1993) confronted us then as it does now with questions related to the accurate and precise detection of different micro-organisms, the best approach to standardise these quantitative test systems, how to monitor performance of the calibrators, the relevance of extreme sensitivity and whether a test system developed in-house is less reliable than one available commercially. Questions aside, the quality of data firstly relies upon our continual vigilance over routine technical PCR variables including the technical competence of the operator, ongoing operator training, consistent thermal cycler performance, protocol variations, use of reaction replicates, the method of oligonucleotide selection, oligonucleotide manufacturing quality and the storage and batch variation of all qPCR materials. Quality also depends on variables addressed during the pre-analysis phase which include the amount of nucleic acid tested, the quality of the template and the presence of inhibitors. Together with the variability of the RT-step and the subjectivity of data analysis there are clearly many ways to confuse quantitative results (Bustin, 2002). Consequently, it is essential to minimise variability and maximise reproducibility by quality controlling every component of the RT-qPCR assay and adhering to common guidelines for data analysis (Hands et al., 2007). Additional complexity is added to the development of robust qPCR protocols by the different approaches to developing and implementing standards or calibrators. Comparative studies should be performed to clearly identify the advantages and disadvantages of using intact microbes versus naked nucleic acid versus encapsidated pseudoviral targets.

Commercial sources of certified reference materials and calibrators include the National Institute for Biological Standards and Control (NIBSC, UK; http://www.nibsc.ac.uk/), BBI diagnostics (http://www.seracare.com/index.htm, USA), BioRad laboratories and ZeptoMetrix corporation (http://www.zeptometrix.com/nattrol.htm) and include parvovirus B19, hepatitis A virus (HAV), HBV, HCV, HIV, West Nile virus, cytomegalovirus (CMV), human papillomavirus (HPV), *Chlamydia trachomatis* or *Neisseria gonorrhoeae* materials for qualitative, quantitative or genotyping nucleic acid testing assays.

Quality control (QC) systems measure data integrity, correctness and completeness, address errors and document the entire process. To be fit for its stated purpose laboratory data must enable a user to make technically and administratively correct decisions (Kubista et al., 2006). Though simple in concept, QC is not an easy regimen to apply in daily work because most clients of the clinical microbiology laboratory do not specify what they expect to find and to what level of accuracy they require the laboratory to operate. Clients often only ask for "best measurement", which is more a concept than an absolute since the accuracy of a measurement is improved by replicates that average out technical variability. Of course extra testing comes at a cost. It follows then that obtaining data of the highest possible

accuracy and precision may not always be required to satisfy a client, but may be essential to perform well in proficiency studies. In proficiency studies the technical competence of the laboratory is tested and measures of accuracy and precision can be derived. Despite this fundamental ambiguity, one very basic idea is clear: a good result should be correct. Correctness relies upon both precision and accuracy.

The importance of analytical precision is not in question although most discussions are concerned with how the replicates should be performed; in the same session, on different days, by different operators, etc. In most cases this is solved by specifying whether the precision calculations were made in a repeatable (repetitions were made in one working session using the same reagents, instruments, by the same staff, etc.) or reproducible (repetitions were made by changing many experimental conditions) manner, or even using an intermediate level of precision (repetitions were made changing only some experimental conditions).

Accuracy is much more difficult to calculate, if possible at all, since it is the degree to which the measured value and the "true" value agree. The main problem here is that "true" values can not be determined. So, how can we address this fundamental problem? There are three possibilities: using certified reference materials, comparing results with Reference Methods, and participating in inter-laboratory exercises or quality assurance (QA) programs. The two first options are either not yet available and we can presently only rely on inter-laboratory exercises.

Quality assurance aids the production of comparable data by formally assessing quality controlled laboratory methods, the suitability of the standard operating procedures, organisation and management of the laboratory and result auditing. Within Europe an energetic culture of activities exist to review PCR procedures. The largest provider of European nucleic acid based quality control schemes groups is QCMD (Quality Control for Molecular Diagnostics, http://www.qcmd.org/). Established in 2001, QCMD is endorsed by the European Society for Clinical Microbiology and Infectious Disease (ESCMID). Other groups conducting quality assurance programs include the European Network for Diagnostics of Imported Viral Diseases (ENIVD; http://www.enivd.org), the United Kingdom National External Quality Assessment Service (UKNEQAS; http://www.ukneqas.org.uk), the Institute for Standardisation and Documentation in the Medical Laboratory (INSTAND; http://www.instand-ev.de) the Royal College of Pathologists Australasia (RCPA; http://www.rcpaqap.com.au) and the National Serology Reference Laboratory, Australia which also provides nucleic acid testing quality assurance programs (NRL; http://www.nrl.gov.au).

There are six principles that a quality assurance program should address (EURACHEM working group, 1998):

1. Analytical measurements should be made to satisfy an agreed requirement i.e. the client should specify what is required and this should be on par with the quality of peer laboratories.
2. Analytical measurements should be made using methods and equipment which are themselves certified as fit for the purpose.
3. Staff making analytical measurements should be both qualified and competent to undertake the task.
4. There should be a regular independent quality assessment of the technical performance of the laboratory, based on predetermined requirements.
5. Analytical measurements made in one location should be consistent with those made elsewhere.
6. Organisations making analytical measurements should have well defined QC and QA procedures.

Most of these principles relate to method validation, which is beyond the scope of this brief discussion. Below we will further consider inter-laboratory exercises.

There are three main types of inter-laboratory exercise (Lawn and Thompson, 1997):

1. Collaborative trials intended to study an analytical procedure in depth. The participating laboratories must strictly follow the guidelines and written procedures provided by the organising body. There should be minimum freedom to introduce variability, because the aim is to identify any bias, figures of merit, etc. for a particular method, or perhaps even to certify it.
2. Certification exercises aiming to verify one or more targets in a given sample matrix. Here, freedom is exercised regarding the choice of analytical method, procedure, instrument, etc. The main requirement is that the participating laboratories have experience working with the target and/or the matrix.
3. Proficiency testing is a collaborative exercise to evaluate how laboratories perform i.e. to evaluate their technical competence and compare that with others. Participating laboratories can select the analytical procedure for the purpose. They receive an unknown sample to be analysed and they report results back to the organisation that is in charge of the study.

The goal of proficiency testing is to obtain a regular, objective, and independent assessment of the accuracy of an analytical laboratory's performance using routine test samples and to promote improvements in their operating procedures (Lawn and Thompson, 1997).

Two issues should be stressed. Proficiency testing is not a one-off event. To be useful several exercises are required. In fact, the first exercise is often a disaster for some of the participating laboratories. Three exercises per year are usually recommended (Lawn and Thompson, 1997). Routine is the magic word in these exercises. It is well known that many managers and analysts pamper inter-laboratory test samples. This produces results that do not represent routine operations, but rather how well a laboratory can perform under unrealistically ideal conditions. If a laboratory really is interested in finding out how good their routine is, these samples must be handled as would any other sample.

Simple statistical evaluations of the results include removing outlier samples and outlier laboratories and autoscaling the reported data (so-called z-scores). Autoscaling consists on subtracting the mean of the overall set of values and dividing by the standard deviation (SD). Based on the Gaussian distribution laboratories reporting data outside mean +/- 3SD (99% confidence interval) have performed poorly, while laboratories reporting within +/- 2SD (95% confidence intervals) performed well. The final general meeting between the laboratory managers should look for and explain major differences and identify sources of error. By the way, never throw away any valuable leftover test samples; although it is not a certified reference material, it is a valuable calibrator for the continued control of one's own assays and laboratory performance.

In 2006 a report of the first European proficiency inter-laboratory test on nucleic acid analysis and real-time PCR was presented from the EU framework 6 program (Ramsden et al., 2006). 137 laboratories from 29 member states participated. The laboratories were provided plasmid calibrators containing the ABL proto-oncogene, and primers and TaqMan probes for the detection of ABL. They were also provided three cloned cDNA samples; two samples contained cells resuspended in RNAlater®. They were asked to:

1. Construct a calibration curve based on the ABL plasmid calibrators provided
2. Estimate ABL cDNA copy numbers in the three cloned samples by real-time PCR
3. Optionally perform RNA extraction and RT and measure cDNA levels by real-time PCR to estimate the amounts of ABL RNA in the resuspended cells.

For the pre-extracted samples more than 95% of the participants showed valid performance, while for the quantification of cells, valid performance was limited to 40 to 50 % of the

participants. This very large discrepancy can most likely be explained by the large variations in performance of RT protocols. While all laboratories used the same real-time PCR reagents and protocol (provided by the organisers) to quantify cDNA levels, they used in-house methods for RNA extraction and purification and RT to analysing the cells. The very large gene specific variation among reverse transcriptases and RT priming methods (Ståhlberg et al., 2004b) cannot be controlled using reference genes, IACs and DNA based calibrators. The only way to obtain comparable inter-laboratory results when using different reverse transcriptase protocols is to use exogenous calibrators based on RNA.

The above discussion and the European EQUAL project calls for guidelines and standard operating procedures for real-time PCR based diagnostics. In US the FDA is preparing guidance for both industry and FDA staff on the best use of nucleic acid based *in vitro* diagnostic devices for detection of microbial pathogens (http://www.fda.gov/dockets/ecomments). In Europe directives for CE marking for *in vitro* diagnostic medical devices were laid in 1998 (2006). Nonetheless the need for better quality real-time PCR assays encompassing more target micro-organisms in clinical microbiology is real but remains poorly addressed. Published QA studies, commonly focus on the same targets as those developed for commercial kits and describe up to a third of participants performing inadequate human enterovirus, HSV, HCV or respiratory virus PCR detection with particular problems detecting low template loads (van Vliet et al., 2001; Schirm et al., 2002; Schloss et al., 2003; Templeton et al., 2006). A common feature of these publications is the use of a wide range of commercial and in-house PCR formats making determination of the best assay type for each target, or in general, difficult to assess although in-house methods have been flagged as performing with less reliability than commercial approaches (Verkooyen et al., 2003). While the main purpose of a quality assurance program is to examine the capability of participants to provide reliable results, method selection is an important factor for improved performance that can only be interpreted when such a diverse range of users come together as occurs during QA programs. It is apparent that regular participation in QA schemes improves the proficiency of PCR testing in microbiology (Mancini et al., 2004) so it is up to the end-users to create the environment for improvement to all aspects of assay quality so that real-time qPCR can reach its true potential.

Future Trends

A natural progression of real-time qPCR technology and its role in clinical microbiology would seem to be quantification of micro-organisms in parallel with the immune responses they elicit; yet this area remains largely unexplored. Additional areas that will hopefully receive

more attention in the future include improved collaboration among those using qPCR for under-characterised micro-organisms, the production of more reference materials and calibrators, the production of more commercial real-time qPCR kits for the diagnostic laboratory and the overall need to identify simple, relevant and realistic goals for qPCR in the clinical microbiology laboratory.

Conclusions

Real-time technology has revolutionised the use of PCR assays and permitted the simple, speedy, sensitive and specific generation of quantitative data. Furthermore, the amount of data generated lends itself to analysis using statistics, which increases the confidence in reporting quantitative results. The development of standardised experimental protocols and designs that are rigorously controlled still remains an important challenge. Overcoming this challenge will permit meaningful global comparisons of microbial load data.

While real-time PCR can detect two-fold differences in the amount of DNA (Bubner et al., 2004; Pal et al., 2006), the technique struggles to produce equivalent results from an RNA template due to issues relating to our current inability to directly detect small quantities of RNA. Practically, two-fold differences in the amount of a micro-organism may not prove to be clinically relevant. If realistic thresholds could be identified for each micro-organism then we could use these to add value to our results. If the amount of target micro-organism is below the threshold then the laboratory could report with confidence that infection is not associated with illness. Similarly, a sub-threshold microbial load may indicate successful antimicrobial therapy whereas a rise above threshold may signal reactivation of latent or persistent infection, heralding a poor clinical outcome. Validated thresholds may be one way we can relax some of the technical constraints we are currently bound by while we unproductively attempt to develop near-perfect quantitative assays.

Currently we apply real-time qPCR methods better suited to the study of human transcription and we usually expect them to provide robust data for what is an entirely different set of problems; those of complex infectious disease processes. If we could modify our approach and openly discuss the variables that set clinical microbiology apart, then perhaps we may produce real-time qPCR applications providing more realistic and robust results that benefit a greater number of patient outcomes around the world. While such applications may not produce the perfect estimate of micro-organism numbers, they will lessen the likelihood of irreproducible and clinically irrelevant data. The major implication of inaccurate qPCR assays is a slowing of our understanding of infectious disease aetiology. Since we live in a time when the discovery of newly emergent or previously unknown

endemic pathogens is increasing in frequency, we must strive harder than ever before to expand our understanding of infectious diseases, and for that we need reliable results from trustworthy tools.

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Web Sites

European Network for Diagnostics of Imported Viral Diseases (ENIVD)
http://www.enivd.org/
A collaboration of scientists from university medical centres, country health departments, and hospitals all over Europe working to improve diagnosis of "imported" viral

Tataa Biocenter
http://www.tataa.com
The Center conducts commissioned research and training within molecular diagnostics and gene expression analysis using real-time PCR technology to specifically quantify nucleic acids

Quality Control for Molecular Diagnostics (QCMD)
http://www.qcmd.org/
QCMD specialises in the standardisation and Quality Control for molecular diagnostics and genomic technologies

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