EXPERT REVIEWS

# Reliability of real-time reverse-transcription PCR in clinical diagnostics: gold standard or substandard?

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Molecular diagnostics is one of the major growth areas of modern medicine, with real-time PCR established as a qualitative and quantitative technology that is rapid, accurate and sensitive. The sequencing of the human genome, comprehensive genomic, mRNA and miRNA expression profiling of numerous cancer types, the ongoing identification of disease-associated polymorphisms and the expanding availability of genomic sequence information for human pathogens has opened the door to a wide range of translational applications for this technology. Consequently, novel real-time PCR assays have been developed for diagnosis and prognosis, treatment monitoring, transplant biology and pathogen detection, as well as more controversial uses such as lifestyle genotyping. However, this technology is still troubled by significant technical deficiencies. Hence its often-improper use as a clinical tool has important public health implications, most recently demonstrated through its association with the measles, mumps and rubella vaccine/autism controversy. This serves as a timely reminder of the indispensable requirement for careful experimental design, validation and analysis.

**Keywords:** bacteria • cancer • diagnosis • miRNA • mRNA • pathogen • PCR • prognosis • reverse transcription • transplant • virus

The real-time, reverse transcription (RT), quantitative (q)PCR enables rapid identification, screening, classification and monitoring of RNA targets and is established as the current method of choice for the accurate detection of RNA [1]. Its ability to quantify cellular mRNA [2] and miRNA [3], as well as genomic RNA from viral pathogens has made it the ideal technology for the development of assays that address important clinical issues; for example, early diagnosis of cancers [4,5], graft rejection and side effects of nonspecific immunosuppression in transplant biology [6], accurate pathogen detection [7], therapeutic molecular intervention [8] and many others [9]. RT-qPCR assays are well suited to the clinical environment, given their speed, which is typically measured in tens of minutes; convenience, especially the elimination of post-amplification processing; high throughput; and potential for reliable target quantification, typically over a huge dynamic range down to single copy numbers [10].

A comparison with other technologies used in routine diagnostic pathology practice suggests that RT-qPCR is as reliable as legacy techniques and is frequently more cost effective and less time consuming [11]. In addition, continuous improvement and enhancement of the technology is resulting in a stream of novel applications [12,13] and there is no doubt that the awareness of its diagnostic potential is becoming ever more widespread (Figure 1). Conversely, two of the main obstacles impeding a more extensive adoption of RT-qPCR assays for clinical use are concerns over assay quality assessment and standardization, both of which affect reproducibility. This is of particular importance when monitoring viral load; for example, HIV patients are tested over long periods of time at, potentially, many different laboratories. Measurements must therefore be consistent and assay results have to be directly comparable with previous tests.

The combination of molecular diagnostics with therapeutics constitutes a key component of integrated healthcare. The recent past has seen

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significant contributions of RT-qPCR technology in three particular areas of clinical interest: diagnostic and prognostic applications in cancers, transplant biology and pathogen detection.

This review aims to place this progress into the context of a mounting realization that the practical implementation of RT-qPCR technology requires considerable changes to the way such assays are carried out and reported.

## Diagnostic & prognostic applications

The RT-qPCR assays are widely used to corroborate disease-associated expression signatures derived from microassays [14]. Furthermore, qPCR technology is well suited to translating microarray-derived profiles into accurate and quantitative, clinically useful assays. Such applications hold immense promise and are expanding all the time [15–18]. Cancer is one of the most significant diseases in modern society; hence it is not surprising that RT-qPCR technology is being applied at every stage of the treatment process, ranging from early detection through molecular staging and monitoring of targeted oncology therapies.

### *Leukemias*

The usefulness of RT-qPCR assays is arguably most evident when it is utilized for prognostication of hematological malignancies. These are caused by characteristic, recurrent chromosomal translocations and rearrangements that generate oncogene fusion transcripts. Such aberrations determine the biological behavior of the leukemia subtypes, hence their detection and quantification by RT-qPCR is clinically relevant. However, RT-qPCR is not only useful for diagnosis and prognostication, since the exquisite specificity of the assay may assist with the selection of the appropriate therapy and help monitor response during therapy.

A recent RT-qPCR assay involving a 17-gene predictor can stratify adult acute myeloid leukemia patients into favorable versus intermediate/unfavorable cytogenetic groups with 95% specificity [19]. A SYBR Green I multiplex assay has identified a distinct expression signature that enables accurate treatment stratification and prognostication of childhood acute lymphoblastic leukemia patients [20]. By contrast, quantification of a single marker, cyclin D1 mRNA, discriminates mantle cell lymphoma from other B-cell lymphoproliferative disorders [21]. Treatment monitoring by RT-qPCR can also provide clinically relevant information: some chronic myeloid leukemia patients do not respond to imatinib, whereas others lose an initial response. High pretreatment expression of imatinib uptake transporter mRNA is directly correlated with chronic myeloid leukemia progression-free and overall survival [22].

### *Solid cancers*

In comparison with leukemia, the impact of RT-qPCR assays on the assessment of prognosis in solid tumors has been comparatively modest [23–27]. There are at least two explanations for this: the complexity and multiplicity of alterations underlying both tumorigenesis and metastasis; and the related lack of appropriate tumor-specific diagnostic biomarkers.

Early detection is essential for reducing cancer mortality and RT-qPCR could be extremely useful in providing an effective early diagnostic assay. A recent report describes the feasibility of expression profiling colorectal cancer cells isolated from feces, and demonstrates that several genes, including *MMP7*, *MYBL2*, *PTGS2* and *TP53*, are expressed at significantly higher levels by colorectal cancer patients than healthy volunteers [28]. The main drawback with this approach is that the meaning of significantly higher or lower must be clearly defined and standardized, since technical variability (see later) can distort interpatient, interassay and interlaboratory comparisons.

The usefulness of using RT-qPCR assays for prognostic purposes following diagnosis of a solid cancer remains unclear [24]. There continues to be a constant flow of publications utilizing RT-qPCR assays to describe individual molecular markers with supposed prognostic significance in a variety of tumors (e.g., pituitary [5], thyroid [29], breast [30], brain [31] and colorectal [32] cancers, as well as melanomas [33]). Leaving aside the question of whether a single marker can be predictive for such complex sets of diseases as solid cancers, the poor understanding of the process of their malignant transformation, progression and metastasis remains a serious problem, even if predictive expression signatures have been identified.

One example of this challenge is melanoma, whose incidence is increasing at a rate faster than any other cancer. The technique of sentinel lymph node biopsy, which is the first lymph node reached by metastasizing cancer cells, is a highly efficient method of staging this cancer, and the presence or absence of metastases in the sentinel lymph node is an important prognostic factor. However, the incidence of false negativity is a considerable problem and has a major impact on patient survival outcome [34]. A recently described multimarker RT-qPCR analysis detected occult metastatic melanoma in sentinal nodes that were identified as negative by histopathological and immunohistochemistry analysis, resulting in the upstaging of four of 33 such patients [35]. Nevertheless, the clinical and biological significance of this molecular upstaging is not clear and it is important to remember that the clinical significance of melanoma cells detected by RT-qPCR has not been validated. It may be that melanoma cells identified by RT-qPCR may not be capable of growing into viable metastases or, alternatively, the metastases may be viable but undergo regression. Indeed, two recent studies, one of which was a prospective multi-institutional study involving 1446 patients with 30 months median follow-up, detected no difference in disease-free survival, distant disease-free survival and overall survival between RT-qPCR positive and negative patients [36,37].

The value of detecting mRNAs in other solid cancers remains unclear, mainly because the relevance of the mRNAs being selected as targets for the assays is in doubt. In colorectal cancer, huge efforts have been made to identify biomarkers useful for its molecular staging and treatment monitoring. The validity of reports ascribing prognostic significance to altered expression levels of genes specifying proteins such as β2 microglobulin [38], activator protein-4 [39], PDGF-BB [40], MMP-2 [41] and many others remain, at best, unconfirmed. An important reason for

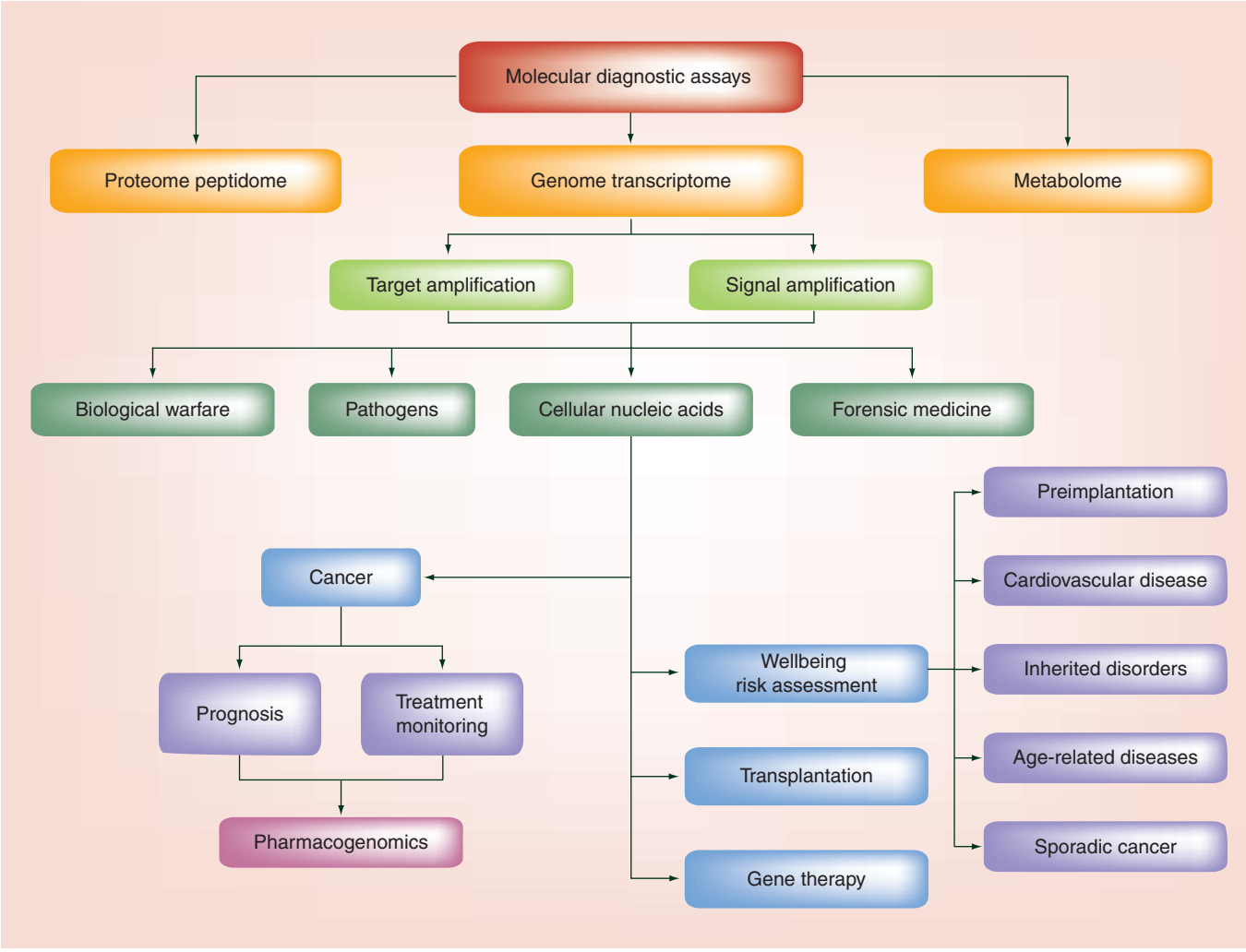


**Figure 1. Complexity of molecular diagnostic assays.** Numerous targets are available for a wide range of molecular assays. Real-time, reverse transcription, quantitative PCR is useful for applications associated with biological warfare, pathogen (especially RNA virus) detection, expression analysis (cellular nucleic acids) and forensic medicine. Expression analysis covers a wide range of uses, ranging from prophylactic and diagnostic assays to prognostic applications in cancers.

this uncertainty is that none of these markers are tumor specific; instead they are described as tissue specific, which leaves their detection open to illegitimate transcription and other problems, discussed previously [42].

In prostate cancer, RT-qPCR assays offer the potential for non-invasive screening, again assuming that appropriate biomarkers can be identified. The current method, screening for serum prostate-specific antigen (PSA), has significant drawbacks since PSA is often elevated in benign conditions and biopsy-detectable prostate cancers occur even in patients with low PSA levels. This equivalence is confirmed by RT-qPCR assays that reveal a low frequency of positive results in patients with prostatic cancer and a high frequency of positive results in those with benign prostatic hyperplasia [43]. However, four promising prostate cancer biomarkers present in urine that provide a specificity and positive predictive value of greater than 75% have been identified recently [44]. This combination, which includes *PCA3*, is more accurate than *PCA3* on its own, probably because the use of multiple markers addresses the problem of heterogeneity of tumor evolution. Even more exciting is the discovery of recurrent gene fusions in a majority of prostate cancers [45], not least because it raises the possibility that similar genomic aberrations occur in other solid cancers. At a stroke, this would replace the uncertainty of detecting and quantifying tissue-specific markers with ones that identify specific and recurrent genetic alterations, especially for solid tumors that are amenable to noninvasive procedures, for example, colorectal cancer.

Whilst many studies report novel biomarkers, few independently validate them. This makes a recent study of non-small-cell lung cancer stand out: it compared the expression of a number of mRNAs as predictors of disease outcome in chemonaive non-small-cell lung cancer patients who had undergone surgical resection [46]. This study showed that, together with disease stage, high levels of *BRCA1* mRNA expression was significantly correlated with overall survival. Importantly, this was corroborated using an independent cohort of 58 patients. Clinically, this could be very significant since *BRCA1* expression is associated with differential sensitivity to cisplatin and

antimicrotubule drugs. Hence, *BRCA1* mRNA expression may enable more targeted administration of adjuvant antimicrotubule-based chemotherapy, especially in stage IB, where the role of adjuvant chemotherapy has not been clearly demonstrated.

### *miRNA*

Micro-RNAs provide another explanation for the uncertain usefulness of current mRNA targets as prognostic biomarkers. These form a class of small noncoding RNAs with post-transcriptional regulatory functions that reduce or silence the expression of particular genes by interfering with their mRNA. Recent evidence from translational studies has provided an increasingly detailed portrait of the involvement of these small regulatory molecules in human cancers [47]. Many miRNAs are aberrantly expressed [48] and thus appear to play a significant role in carcinogenesis [49]. A recent landmark study compared miRNA expression profiles in colon tumor and adjacent normal tissue and identified five miRNAs that were present in much greater amounts in colon cancer tumors than in normal tissues [50]. One miRNA in particular, miR-21, appears to be highly informative: comparisons of tumor stage, miR-21 expression and clinical outcome in patients with stage II or III colon cancer revealed associations between high levels of miR-21 and poor survival, poor therapeutic outcome and, in patients who experienced a disease relapse, more rapid recurrence. These data suggest that miRNAs may be useful as prognostic tools in colon cancer as well as serving as useful drug development targets. In breast cancer, overexpression of the same miRNA is correlated with advanced tumor stage, lymph node metastasis and poor survival of the patients [51], while miRNAs 221/222 play a role in the development of resistance to tamoxifen [52]. Conversely, expression of specific miRNAs may be lost as cancer cells develop metastatic potential. In an elegant set of experiments utilizing RT-qPCR, the authors showed that restoring the expression of miR-126 reduces overall tumor growth and proliferation, whereas miR-335, which regulates a set of genes whose collective expression in a large cohort of human tumors is associated with risk of distal metastasis, inhibits metastatic cell invasion. Therefore, it is likely that both miR-126 and -335 miRNAs are metastasis suppressors in human breast cancer [52]. In lung cancer, high hsa-mir-155 and low hsa-let-7a-2 expression correlates with poor survival [53], whereas in gastric cancer let-7 miRNA is a negative regulator of the high mobility group A2 (HMGA2) nonhistone chromosomal protein, whose expression correlates with tumor invasiveness and is an independent prognostic factor [54]. Let-7 miRNA is downregulated in a number of cancers, including colorectal cancer [55]. Other reports suggest that specific miRNA expression patterns exist in some leukemias [56] and that some miRNAs are correlated with survival [57,58]. The behavior of cancers cannot be accurately predicted based on mRNA signatures alone, as discussed earlier. It is therefore interesting to speculate that the additional complementary information provided by miRNA profiling is critical for the accurate prognostic expression profiling of cancers.

As a further step, the potential usefulness of a miRNA-based therapy in cancer is now being investigated since miRNAs involved in specific networks, such as the apoptotic pathway, the human epidermal growth factor (HER) family-driven or estrogen receptor-mediated signaling influence the response to chemotherapy or to targeted therapies [59]. Consequently, prospective studies must now determine if a particular miRNA or a small number of miRNAs can also predict a patient's response to specific therapies. It is also important to note that the question of appropriate normalization for the interpretation of miRNA quantification data remains to be addressed in an acceptable manner.

## Transplant biology

### *Graft rejection*

A major challenge in transplantation medicine is controlling the very strong immune responses to foreign antigens that are responsible for graft rejection. Although contemporary immunosuppressive drugs are efficient inhibitors of acute graft rejection, this process continues to be a common complication of solid-organ transplantation. Successful treatment of graft acute cellular rejection (ACR) depends on an accurate early diagnosis. The current gold-standard method for establishing a diagnosis of ACR is histological examination of biopsy samples. This may be complemented by RT-qPCR assays that target mRNAs associated with graft rejection and allograft vasculopathy. For example, increased VEGF expression is strongly associated with severe ACR and cardiac-related death [60]. Interestingly, proinflammatory cytokine expression that might be expected to have an adverse effect appears not to correlate with cellular rejection [61]. However, histological or transcript analysis of biopsies requires invasive procedures to obtain adequate tissue samples. Importantly, RT-qPCR analysis promises to provide new noninvasive strategies to monitor graft rejection among transplant recipients. Acute rejection following lung transplantation is currently diagnosed with the use of invasive transbronchial lung biopsies. Recently, an acute rejection-associated signature characterized by increased T-cell, CD8 cytotoxic cell and neutrophil gene expression has been identified in bronchoalveolar lavage cells. If confirmed, this signature may enable the development of rapid PCR-based assays of gene expression for clinical acute rejection diagnosis [62].

### *Infections*

The use of increasingly potent immunosuppressive agents has increased patients' susceptibility to opportunistic infections and the monitoring of systemic infection in transplant recipients remains a challenge. Human cytomegalovirus (HCMV) infection remains one of the most exigent infectious complications in solid organ transplant recipients, as it is associated with an increased predisposition to allograft rejection, accelerated hepatitis C recurrence and other opportunistic infections, as well as reduced overall patient and allograft survival [63]. Unfortunately, HCMV diagnostic tests are not well standardized and, to date, there is no gold-standard test to detect HCMV disease [64]. The use of RT-qPCR assays to detect viral mRNAs may be useful for monitoring the dynamics of HCMV infection, since the detection of late transcripts may better reflect active HCMV replication, dissemination and disease *in vivo*. A RT-qPCR assay that detects the HCMV has been described [65], but it appears to be

less sensitive than qPCR assays targeting viral DNA and less useful at detecting drug resistance mutations [66]. Nevertheless, these limitations can be tackled using improved assay design and modification of experimental protocols.

Other viral pathogens have been associated with infections in transplant patients. The pathogenic role of the Epstein–Barr virus (EBV) is poorly understood but appears to be the cause of a productive infection resulting in EBV-associated post-transplant lymphoproliferative disorder. A recent RT-qPCR assay did not find any difference in broncheoalveolar lavages from transplant recipients and controls [67], and increased viral load in transplant recipients is not necessarily predictive of post-transplant lymphoproliferative disorder [68]. This issue is being addressed by the development of a RT-qPCR assay that provides additional information by differentiating between latent and productive EBV infection [69]. Another virus, human polyomavirus BK, causes nephropathy and hemorrhagic cystitis in kidney and bone marrow transplant patients, respectively. Successful treatment with the anitiviral cidofovir has been reported, although the relevance of this intervention is unclear since polyomaviruses do not encode a viral DNA polymerase. It has now been shown that while treatment with cidofovir inhibits viral replication, viral inhibition is only partial, with some cells being completely refractory [70]. This finding raises important issues with respect to current treatment strategies and the development of future drugs.

The utility of RT-qPCR technology for monitoring transplant patients has also been demonstrated by the development of assays identifying the four genetic lineages of human metapneumovirus, a recently discovered paramyxovirus that is known to cause respiratory tract infections in immunocompromized individuals. Human metapneumovirus was detected in 4.3% of immunosuppressed lung transplant recipients, making it the most prevalent etiologic agent detected in patients with respiratory symptoms [71].

Invasive fungal infections (IFIs) are a major problem affecting up to 50% of bone marrow transplant patients and 5–20% of solid organ transplant recipients. Early identification of IFIs in transplant patients is therefore essential, as a delayed or missed diagnosis of IFI results in increased rates of respiratory morbidity and mortality. However, diagnosis of most IFIs, especially invasive aspergillosis, is difficult because classic tests have low sensitivity and specificity, and radiology often provides nonspecific and transient results. Although a range of RT-qPCR assays has been described that provide greater diagnostic sensitivity and specificity [72], they have not yet been standardized and hence have not been introduced into clinical practice.

## Pathogen detection

The previous section demonstrates that RT-qPCR technology is ideally suited to rapid and accurate quantification of pathogens. Consequently, it has become widely used for their detection, both in research and diagnostic settings, and the past year has seen some important developments. Nevertheless, RT-qPCR assays cannot detect novel or unexpected viruses. Furthermore, the diversity of potential pathogens that elicit similar clinical symptoms and diseases can make the application of individual RNA-based diagnostic assays both complex and expensive. Even multiplex qPCRs are limited to tens of candidate pathogens and may be confounded if viral evolution results in mutations at the primer binding site.

### *Viruses*

RNA viruses make up the most abundant group of human pathogens in man. A recently described multiplex RT-qPCR assay enables the simultaneous identification and quantification of HIV type 1, and hepatitis B and C viruses in donor plasma specimens [73]. This innovative method consists of target-specific forward primers separately immobilized inside individual polyacrylamide gel pads, while reverse primers are free in the surrounding solution. The complete spacial isolation of the primers makes it possible to use a single nonspecific DNA binding dye to detect all of the multiplex qPCR reaction products simultaneously and independently. Both the sensitivity and specificity of the assay, based on 132 blood specimens analyzed, were 100% and the dynamic range of the quantitative analysis covered a six-order interval ranging from $10^2$ to $10^6$ genome equivalents per assay. Another approach utilizes locked nucleic acids to identify all subtypes of HIV-1 in a single assay [74]. These advances simplify treatment monitoring and minimize the need for invasive procedures. Similarly, improved assays for the SARS-coronavirus [75] and influenza virus [76,77] have appeared in anticipation of future pandemics.

### *Bacteria*

Although most bacterial assays target DNA, there are a few reports of RT-qPCR assays targeting bacterial pathogens. One is designed to address improved food safety by measuring the responses and adaptations of *Escherichia coli* O157:H7 to antimicrobial agents. By quantifying the expression of the pathogen's multiple antibiotic resistance operon, the RT-qPCR enables investigations into increased antimicrobial resistance in bacterial pathogens [78]. However, the complexity of RNA extraction, combined with the difficulties in maintaining RNA quality makes it unlikely that RNA-based PCR assays will be useful for the routine diagnostic detection of bacterial pathogens. Instead they will find their niche in complementary studies investigating molecular mechanisms underlying bacterial pathology.

## Problems

Despite the huge number of reports endorsing the use of RT-qPCR in clinical settings and describing significant advances to the understanding of a whole range of diseases, it has become increasingly clear that there are significant biological as well as technical limitations that make the use such a sensitive and, potentially, easily contaminated assay challenging in clinical settings [23]. It is therefore essential that RT-qPCR assays target appropriate biomarkers, use suitably validated assay conditions and are properly analyzed.

As discussed later and in previous publications [1,9, 23–25,79], the reality is that RT-qPCR results are frequently derived from experimental designs characterized by badly chosen target choice, inappropriate tissue compartments and data analyses.

### *Biological variability*

This is of particular significance for experiments designed to assess cellular mRNA levels, for example in response to treatment. Cellular mRNA levels are meant to be variable: mRNA is synthesized, spliced, localized, translated and degraded in response to extracellular signals. This *in vivo* degradation constitutes baseline variability and results in significant natural variation of mRNA levels between genes expressed in different tissues or at different times, even within the same individual. It is sample specific and outside the investigator's control, but must be taken into account when interpreting changes in mRNA copy numbers.

Another biological variable concerns gene splicing, a post-transcriptional modification in which a single gene can specify multiple proteins, enabling the synthesis of protein isoforms that are structurally and functionally distinct. This affects most human genes [80], plays an important role in human pathologies, including cancer [81], and generates significant problems with the interpretation of RT-qPCR and microarray data, since the relative abundance of splice variants can change without any change in overall mRNA expression levels, signifying cell-, tissue- or treatment-specific adjustments between different isoforms. This issue is addressed by very few publications and is likely to be the cause of many discordant results obtained using assays that target different variants of the same gene.

The interpretation of mRNA quantification data is further complicated by the widespread differences in allelic expression among autosomal nonimprinted genes in animals [82] as well as in plants [83]. Since allelic imbalance and allele-specific expression patterns are associated with disease risk [84,85], it is no longer sufficient to quantify mRNA expression, but it is necessary to determine precisely which allele is being expressed. One implication of this is that, rather than avoiding SNPs when designing primers, it may be necessary to include them as part of an overall assay design strategy in order to be able to quantify allele-specific expression accurately [23].

It is now clear that there are significant differences in gene expression patterns between individual cells [86], even within apparently homogenous cell cultures. Gene expression is stochastic [87], hence the contribution of individual cells to an overall expression profile is variable and can be significantly different from it. This raises a whole range of new questions, which are outside the scope of this review. However, it is clear that appropriate analysis and interpretation of expression profiles from single cells requires the development of dedicated experimental protocols and analytical procedures [88].

### *Technical variability*

Technical limitations are exemplified by inappropriate experimental design, poor assay efficiency, invalid controls or improper data analysis. These problems are clearly illustrated by the retraction of a paper with *Science Magazine* 'Breakthrough of the Year' status [89] because of incorrect RT-qPCR data analysis [90]. Other publications report impossibly high amplification efficiencies and apply incorrect statistical analyses that call into question the reliability and relevance of any the conclusions based upon these assays [91]. One of the most astonishing examples of the enormous implications for the health and lives of individuals that result from inappropriate use of this technology is provided by the controversy surrounding the triple MMR virus vaccine. RT-qPCR data appeared to demonstrate the presence of measles virus RNA in children with developmental disorders, which was interpreted as providing hard scientific evidence for a link between MMR, gut pathology and autism [92]. However, a detailed analysis of the raw data underlying that report carried out by one of the authors acting as an expert witness to the UK High Court and the US Vaccine Court, revealed a catalogue of mistakes, inaccuracies and inappropriate analysis methods as well as contamination and poor assay performance [93]. The assay had been detecting DNA and since the measles virus is an RNA-only virus, the RT-qPCR data had been erroneously interpreted. Interestingly, a recent paper that included two of the authors from the initial report, was unable to reproduce the original findings and concluded that there was no link between autism and enteropathy [94]. Astonishingly, despite this and other evidence [95,96], the authors of the 2002 report have not retracted their original findings, in spite of the immense repercussions their original report has had on public health.

Fortunately, unlike biological variability, technical limitations can be addressed by the use of appropriate standard operating procedures, optimized assays and appropriate data analysis. The most immediate causes for technical inadequacy are inconsistent sample selection, handling and RNA isolation. For example, a comparison of RNA levels between cancer samples must take into account the complexity and heterogeneity of tissue biopsies and may require the use of microdissected samples for maximum accuracy. Crucially, the accuracy of gene-expression profiling is highly dependent on RNA quality, both in terms of its integrity as well as in terms of the lack of inhibitors copurified during extraction procedures [97–99]. The instability of RNA and its sensitivity to degradation introduced during storage or the extraction of the RNA are well known. Whilst these comments may seem obvious, their implications have never been explored in detail. Unfortunately, insufficient attention is paid to the analysis of RNA quality: a recent survey of papers published in 2007–2008 revealed that more than 60% of papers do not even mention mRNA quality [23]. This area requires urgent attention and proposals for adequate RNA integrity testing have been put forward [98].

The conversion of mRNA to cDNA is a highly variable step in the quantification process. RT-qPCR gene expression measurements are comparable only when the same priming strategy and reaction conditions are used in all experiments and the samples contain the same total amount of RNA [100]. Furthermore, reverse transcription yields vary considerably with the choice of reverse transcriptase and variation is target gene-dependent [101]. Similarly, the mechanism of cDNA priming has a significant effect upon the outcome of any quantification experiment, since gene-specific priming, random priming and oligo-dT all

produce diverse results that are distinct for different mRNA targets. The choice of primer location on the target mRNA can also yield significantly different results, as mRNA adopts a tight secondary structure that is characterized by extensive intrastrand base pairing, resulting in stem-loop structures [102]. If reverse transcription primers are designed to target stems rather than loops, or if the amplicon can adopt secondary structures, the efficiency of the RT step can be significantly compromised. Characteristically, this results in nonquantitative and nonreproducible results.

Proper normalization of gene expression data between different samples generated in the same laboratory, or generated in different geographical regions using a single platform or multiple platforms is essential for obtaining accurate gene expression data [103,104]. Unfortunately, although there is an increased awareness of the importance of systematic validation and the potentially highly misleading effects of using inappropriate reference genes (RG) for normalization, this issue continues to be widely disregarded [105]. Consequently, qPCR data are still poorly normalized in many molecular analyses. Normalization involves reporting the concentration ratios of genes of interest to RG mRNAs. RG mRNAs should be stably expressed, with their abundance strongly correlated to the total amounts of mRNA present in each sample. Unfortunately there are no universal RGs. The optimal number and choice of RG must be experimentally determined and the method reported [106]; normalization against a single RG is not acceptable. This issue is equally relevant, but all too frequently unappreciated, when assessing miRNA levels [107]. Inappropriate experimental designs, improper analyses, subjective interpretation of RT-qPCR data, variability of microarray results depending on the choice of analysis algorithms all combine to compromise the interpretation and confident application of quantitative, mRNA-targeted data [9].

The more widespread acknowledgement of these problems has resulted in an initiative aimed at improving the reliability of qPCR data. Specifically, proposals have been submitted that draw up specifications for the minimum information for the publication of quantitative PCR experiments (MIQE) [106]. MIQE is modeled on similar guidelines drawn up for DNA microarray analysis [108], proteomics experiments [109], genome sequence specification [110], and those under discussion for RNAi work [111,112] and metabolomics [113], initiatives coordinated under the umbrella of Minimum Information for Biological and Biomedical Investigations (MIBBI) [114,201]. These guidelines will improve the relevance, accuracy, correct interpretation and repeatability of qPCR data, and thus help ensure the integrity of the scientific literature, promote consistency between laboratories and increase experimental transparency.

## Conclusions & expert commentary

Technological advances mean that there is an ever-increasing choice of platforms, chemistries, protocols as well as applications and targets for qPCR analysis. This is exciting and is generating a vast amount of data in basic research, medical, agricultural, microbiological and forensic applications. However, there is increasing concern that many publications utilizing RT-qPCR technology and especially those aiming to profile cellular RNA levels, report poorly designed, executed and interpreted experiments and results. Considerations of mRNA transcription, *in vivo* stability, regulation by miRNAs, tissue specificity of splice variants, allele-specific differences in expression, the lack of concordance between most mRNAs and their specified proteins, the critical importance of post-translational modifications and questions of tissue heterogeneity all describe serious issues that are not being addressed in an adequate manner. Trust in the accuracy and integrity of the scientific literature is an essential prerequisite for maintaining scientific excellence and advancing knowledge. This calls for urgent action by researchers, reviewers and editors who need to agree a basic set of quality criteria and adhere to elementary procedures that result in the publication of reliable and reproducible data. Such a list must include a delineation of minimum quality standards for template preparation, validation and consistent use of cDNA priming methods, enzymes, protocols and, equally critical, appropriate analysis of data. Furthermore, it is entirely unacceptable that most publications do not address the critical issue of RNA quality assessment. The increasing use of automated RNA preparation procedures coupled to its amplification and detection minimizes the risk of contamination, but does not necessarily address the issue of RNA quality control. It is equally unacceptable that data are not normalized in an appropriate manner. In addition, it is vital that data acquisition, analysis and reporting become more transparent. Consequently, it is necessary for the editors of scientific and biomedical publications to issue prescriptive checklists specifying the key information to be included when reporting experimental data and a proposed standard, MIQE, will be published shortly [106]. Ultimately, these approaches need to be combined with basic biological considerations, so that results are not a reflection of technical inadequacies and biological artifacts, but truly start to describe actual differences in expression profiles between cells, tissues, individuals, disease states and treatment responses. Unfortunately, we are still far removed from this state, with a lot of intellectual and capital investment in technological development that drives research whose results can be fundamentally flawed. It will require a significant amount of courage as well as a significant change in attitude from the research community to deal with this problem.

## Five-year view

Increased adherence to standardized protocols and rigorous enforcement of minimum reporting standards will improve the quality of RT-qPCR assays. A combination of robust RT-qPCR assays and more relevant biomarkers in solid cancers based on genomic rearrangement will revolutionize our ability to identify cancers accurately and, it is hoped, at an earlier stage. Furthermore, an association between oncogene fusion and tumor behavior will extend the assay's utility to rational therapeutic targeting. Whilst improved assay quality will also make RT-qPCR assays more consistent and reliable for the detection of pathogens,

5 years from now, next-generation sequencing technologies will have become established and may even be cost effective. Next-generation sequencing has significant advantages for pathogen diagnosis over nucleic acid amplification-based technologies, since it:

- Provides an unbiased parallel high-throughput method for the direct diagnosis of viral infections;
- Does not require viral genome information and thus can be utilized for novel or unexpected viral infections;
- Enables the simultaneous genetic characterization of the pathogens [115].

For the moment, cost is a major concern, but in 5 years time this will be less of an issue. Nevertheless, RT-qPCR will continue to excel at providing a rapid, cost-effective and reliable assay for the detection and quantification of RNA.

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## Key issues

- Reverse transcription quantitative PCR is the current method of choice for the accurate detection of RNA.
- Reverse transcription quantitative PCR is increasingly used for clinical diagnostic and prognostic applications.
- Results interpretation requires attention to biological and technical variability.
- There are concerns over assay design, execution, data analysis and interpretation that interfere with assay quality.
- Specifications for the minimum information for the publication of quantitative PCR experiments have been drawn up.
- Despite the emergence of next-generation sequencing technology, reverse transcription quantitative PCR will continue to excel at providing a rapid, cost-effective and reliable assay for the detection of RNA.

## References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 Bustin SA. Real-time quantitative PCR-opportunities and pitfalls. *Eur. Pharm. Rev.* 4, 18–23 (2008).

2 Pignot G, Bieche I, Vacher S *et al.* Large-scale real-time reverse transcription-PCR approach of angiogenic pathways in human transitional cell carcinoma of the bladder: identification of VEGFA as a major independent prognostic marker. *Eur. Urol.* DOI:10.1016/j.eururo.2008.05.027 (2008) (Epub ahead of print).

3 Schmittgen TD, Lee EJ, Jiang J *et al.* Real-time PCR quantification of precursor and mature microRNA. *Methods* 44(1), 31–38 (2008).

4 Rizzi F, Belloni L, Crafa P *et al.* A novel gene signature for molecular diagnosis of human prostate cancer by RT-qPCR. *PLoS ONE* 3(10), e3617 (2008).

5 Evans CO, Moreno CS, Zhan X *et al.* Molecular pathogenesis of human prolactinomas identified by gene expression profiling, RT-qPCR, and proteomic analyses. *Pituitary* 11(3), 231–245 (2008).

6 Mischitelli M, Fioriti D, Anzivino E *et al.* BKV QPCR detection and infection monitoring in renal transplant recipients. *New Microbiologica* 30(3), 271–274 (2007).

7 Lu X, Holloway B, Dare RK *et al.* Real-time reverse transcription-PCR assay for comprehensive detection of human rhinoviruses. *J. Clin. Microbiol.* 46(2), 533–539 (2008).

8 Kammula US, Serrano OK. Use of high throughput qPCR screening to rapidly clone low frequency tumour specific T-cells from peripheral blood for adoptive immunotherapy. *J. Transl. Med.* 6, 60 (2008).

9 Bustin SA, Mueller R. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clin. Sci. (Lond.)* 109(4), 365–379 (2005).

10 Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 25(2), 169–193 (2000).

•• **Most highly cited quantitative PCR reference.**

11 Capizzi E, Gruppioni E, Grigioni AD *et al.* Real Time RT-PCR approach for the evaluation of ERBB2 overexpression in breast cancer archival samples: a comparative study with FISH, SISH, and immunohistochemistry. *Diagn. Mol. Pathol.* 17(4), 220–226 (2008).

12 Rogerson L, Darby S, Jabbar T *et al.* Application of transcript profiling in formalin-fixed paraffin-embedded diagnostic prostate cancer needle biopsies. *BJU Int.* 102(3), 364–370 (2008).

13 Doleshal M, Magotra AA, Choudhury B *et al.* Evaluation and validation of total RNA extraction methods for microRNA expression analyses in formalin-fixed, paraffin-embedded tissues. *J. Mol. Diagn.* 10(3), 203–211 (2008).

14 Yachida S, Sakamoto M, Imaida K *et al.* p27 is overexpressed in very early stages of hepatocarcinogenesis. *Cancer Sci.* 99(11), 2152–2159 (2008).

15 Yamada Y, Arao T, Gotoda T *et al.* Identification of prognostic biomarkers in gastric cancer using endoscopic biopsy samples. *Cancer Sci.* 99(11), 2193–2199 (2008).

16 Fu S, Zhao H, Shi J *et al.* Peripheral arterial occlusive disease: global gene expression analyses suggest a major role for immune and inflammatory responses. *BMC Genomics* 9, 369 (2008).

17 Cheng CF, Fan J, Bandyopahdhay B *et al.* Profiling motility signal-specific genes in primary human keratinocytes. *J. Invest. Dermatol.* 128(8), 1981–1990 (2008).

18 Sorensen LK, Havemose-Poulsen A, Sonder SU *et al.* Blood cell gene expression profiling in subjects with aggressive periodontitis and chronic arthritis. *J. Periodontol.* 79(3), 477–485 (2008).

19 Andreeff M, Ruvolo V, Gadgil S *et al.* HOX expression patterns identify a common signature for favorable AML. *Leukemia* 22(11), 2041–2047 (2008).

20 Ibrahim K, Daud SS, Seah YL *et al.* Rapid detection of prognostically important childhood acute lymphoblastic leukemia chimeric transcripts using multiplex SYBR green real-time reverse transcription PCR. *Ann. Clin. Lab. Sci.* 38(4), 338–343 (2008).

21 Brizova H, Kalinova M, Krskova L *et al.* Quantitative measurement of cyclin D1 mRNA a potent diagnostic tool to separate mantle cell lymphoma from other B-cell lymphoproliferative disorders. *Diagn. Mol. Pathol.* 17(1), 39–50 (2008).

22 Wang L, Giannoudis A, Lane S *et al.* Expression of the uptake drug transporter hOCT1 is an important clinical determinant of the response to imatinib in chronic myeloid leukemia. *Clin. Pharmacol. Ther.* 83(2), 258–264 (2008).

23 Bustin S. Molecular medicine, gene-expression profiling and molecular diagnostics: putting the cart before the horse. *Biomark. Med.* 2, 201–207 (2008).

24 Murphy J, Dorudi S, Bustin SA. Molecular staging of colorectal cancer: new paradigm or waste of time? *Expert Opin. Med. Diagn.* 1(1), 31–45 (2007).

25 Bustin SA, Mueller R. Real-time reverse transcription PCR and the detection of occult disease in colorectal cancer. *Mol. Aspects Med.* 27, 192–223 (2006).

26 Sotiriou C, Piccart MJ. Taking gene-expression profiling to the clinic: when will molecular signatures become relevant to patient care? *Nat. Rev. Cancer* 7(7), 545–553 (2007).

27 Koscielny S. Critical review of microarray-based prognostic tests and trials in breast cancer. *Curr. Opin. Obstet. Gynecol.* 20(1), 47–50 (2008).

28 Koga Y, Yasunaga M, Moriya Y *et al.* Detection of colorectal cancer cells from feces using quantitative real-time RT-PCR for colorectal cancer diagnosis. *Cancer Sci.* 99(10), 1977–1983 (2008).

29 Prasad NB, Somervell H, Tufano RP *et al.* Identification of genes differentially expressed in benign versus malignant thyroid tumors. *Clin. Cancer Res.* 14(11), 3327–3337 (2008).

30 Li J, Zhang N, Song LB *et al.* Astrocyte elevated gene-1 is a novel prognostic marker for breast cancer progression and overall patient survival. *Clin. Cancer Res.* 14(11), 3319–3326 (2008).

31 Reddy SP, Britto R, Vinnakota K *et al.* Novel glioblastoma markers with diagnostic and prognostic value identified through transcriptome analysis. *Clin. Cancer Res.* 14(10), 2978–2987 (2008).

32 Motoyama K, Tanaka F, Kosaka Y *et al.* Clinical significance of *BMP7* in human colorectal cancer. *Ann. Surg. Oncol.* 15(5), 1530–1537 (2008).

33 Riker AI, Enkemann SA, Fodstad O *et al.* The gene expression profiles of primary and metastatic melanoma yields a transition point of tumor progression and metastasis. *BMC Med. Genomics* 1, 13 (2008).

34 Caraco C, Marone U, Celentano E *et al.* Impact of false-negative sentinel lymph node biopsy on survival in patients with cutaneous melanoma. *Ann. Surg. Oncol.* 14(9), 2662–2667 (2007).

35 Karim RZ, Scolyer RA, Li W *et al.* False negative sentinel lymph node biopsies in melanoma may result from deficiencies in nuclear medicine, surgery, or pathology. *Ann. Surg.* 247(6), 1003–1010 (2008).

36 Scoggins CR, Ross MI, Reintgen DS *et al.* Prospective multi-institutional study of reverse transcriptase polymerase chain reaction for molecular staging of melanoma. *J. Clin. Oncol.* 24(18), 2849–2857 (2006).

37 Temple CL, Snell LJ, Powser SM *et al.* Clinical significance of the RT-PCR positive sentinel node in melanoma. *J. Surg. Oncol.* 95(7), 546–554 (2007).

38 Shrout J, Yousefzadeh M, Dodd A *et al.* β(2)microglobulin mRNA expression levels are prognostic for lymph node metastasis in colorectal cancer patients. *Br. J. Cancer* 98(12), 1999–2005 (2008).

39 Cao J, Tang M, Li WL *et al.* Upregulation of activator protein-4 in human colorectal cancer with metastasis. *Int. J. Surg. Pathol.* 17(1), 16–21 (2009).

40 Nakamura Y, Tanaka F, Yoshikawa Y *et al.* PDGF-BB is a novel prognostic factor in colorectal cancer. *Ann. Surg. Oncol.* 15(8), 2129–2136 (2008).

41 Oshima T, Kunisaki C, Yoshihara K *et al.* Clinicopathological significance of the gene expression of matrix metalloproteinases and reversion-inducing cysteine-rich protein with Kazal motifs in patients with colorectal cancer: *MMP-2* gene expression is a useful predictor of liver metastasis from colorectal cancer. *Oncol. Rep.* 19(5), 1285–1291 (2008).

42 Bustin SA, Dorudi S. Molecular assessment of tumour stage and disease recurrence using PCR- based assays. *Mol. Med. Today* 4(9), 389–396 (1998).

43 Barbero G, Destefanis P, Procida S *et al.* Highly specific detection of prostate-specific antigen-positive cells in the blood of patients with prostate cancer or benign prostatic hyperplasia, using a real-time reverse-transcription-polymerase chain reaction method with improved sensitivity. *BJU Int.* 102(11), 1566–1572 (2008).

44 Laxman B, Morris DS, Yu J *et al.* A first-generation multiplex biomarker analysis of urine for the early detection of prostate cancer. *Cancer Res.* 68(3), 645–649 (2008).

45 Kumar-Sinha C, Tomlins SA, Chinnaiyan AM. Recurrent gene fusions in prostate cancer. *Nat. Rev. Cancer* 8(7), 497–511 (2008).

•• **Exciting description of arguably the most promising biomarkers for a solid tumor.**

46 Rosell R, Skrzypski M, Jassem E *et al.* *BRCA1*: a novel prognostic factor in resected non-small-cell lung cancer. *PLoS ONE* 2(11), e1129 (2007).

• **Elegantly carried out and validated study.**

47 Sassen S, Miska EA, Caldas C. MicroRNA: implications for cancer. *Virchows Arch.* 452(1), 1–10 (2008).

48 Ahmed FE, Role of miRNA in carcinogenesis and biomarker selection: a methodological view. *Expert Rev. Mol. Diagn.* 7(5), 569–603 (2007).

49 Yang L, Belaguli N, Berger DH. MicroRNA and colorectal cancer. *World J. Surg.* DOI:10.1007/s00268-008-9865-5 (2009) (Epub ahead of print).

50 Schepeler, T., Reinert JT, Ostenfeld MS *et al.* Diagnostic and prognostic microRNAs in stage II colon cancer. *Cancer Res.* 68(15), 6416–6424 (2008).

51 Yan LX, Huang XF, Shao Q *et al.* MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA* 14(11), 2348–2360 (2008).

52 Miller TE, Ghoshal K, Ramaswamy B *et al.* MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1. *J. Biol. Chem.* 283(44), 29897–29903 (2008).

53 Yanaihara N, Caplen N, Bowman E *et al.* Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* 9(3), 189–198 (2006).

•• **Association between miRNA and prognosis.**

54 Motoyama K, Inoue H, Nakamura Y *et al.* Clinical significance of high mobility group A2 in human gastric cancer and its relationship to let-7 microRNA family. *Clin. Cancer Res.* 14(8), 2334–2340 (2008).

55 Fang WJ, Lin CZ, Zhang HH *et al.* Detection of let-7a microRNA by real-time PCR in colorectal cancer: a single-centre experience from China. *J. Int. Med. Res.* 35(5), 716–723 (2007).

56 Isken F, Steffen B, Merk S *et al.* Identification of acute myeloid leukaemia associated microRNA expression patterns. *Br. J. Haematol.* 140(2), 153–161 (2008).

57 Garzon R, Volinia S, Liu CG *et al.* MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood* 111(6), 3183–3189 (2008).

58 Garzon R, Garofalo M, Martelli MP *et al.* Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proc. Natl Acad. Sci. USA* 105(10), 3945–3950 (2008).

59 Iorio MV, Casalini P, Tagliabue E *et al.* MicroRNA profiling as a tool to understand prognosis, therapy response and resistance in breast cancer. *Eur. J. Cancer* 44(18), 2753–2759 (2008).

60 Bayliss J, Maguire JA, Bailey M *et al.* Increased vascular endothelial growth factor mRNA in endomyocardial biopsies from allografts demonstrating severe acute rejection: a longitudinal study. *Transpl. Immunol.* 18(3), 264–274 (2008).

61 Breinholt JP, Vallejo JG, Gates CM *et al.* Myocardial pro-inflammatory cytokine expression and cellular rejection in pediatric heart transplant recipients. *J. Heart Lung Transplant.* 27(3), 317–324 (2008).

62 Patil J, Lande JD, Li N *et al.* Bronchoalveolar lavage cell gene expression in acute lung rejection: development of a diagnostic classifier. *Transplantation* 85(2), 224–231 (2008).

63 Perez-Sola MJ, Caston JJ, R. Solana *et al.* Indirect effects of cytomegalovirus infection in solid organ transplant recipients. *Enferm. Infecc. Microbiol. Clin.* 26(1), 38–47 (2008).

64 Kim DJ, Kim SJ, Park J *et al.* Real-time PCR assay compared with antigenemia assay for detecting cytomegalovirus infection in kidney transplant recipients. *Transplant. Proc.* 39(5), 1458–1460 (2007).

65 Meyer-Koenig U, Romberg I, Schneider K *et al.* Diagnostic value of reverse transcription-PCR for detection of cytomegalovirus pp67 in samples from solid-organ transplant recipients. *J. Clin. Microbiol.* 44(9), 3394–3396 (2006).

66 Gohring K, Mikeler E, Jahn G *et al.* Rapid semiquantitative real-time PCR for the detection of human cytomegalovirus *UL97* mutations conferring ganciclovir resistance. *Antivir. Ther.* 13(3), 461–466 (2008).

67 Costa C, Elia M, Astegiano S *et al.* Quantitative detection of Epstein–Barr virus in bronchoalveolar lavage from transplant and nontransplant patients. *Transplantation* 86(10), 1389–1394 (2008).

68 Tysarowski A, Fabisiewicz A, Paszkiewicz-Kozik E *et al.* Usefulness of real-time PCR in long-term follow-up of follicular lymphoma patients. *Acta Biochimica Polonica* 54(1), 135–142 (2007).

69 Zawilinska B, Kosinska A, Lenart M *et al.* Detection of specific lytic and latent transcripts can help to predict the status of Epstein–Barr virus infection in transplant recipients with high virus load. *Acta Biochimica Polonica* 55(4), 693–699 (2008).

70 Bernhoff E, Gutteberg TJ, Sandvik K *et al.* Cidofovir inhibits polyomavirus BK replication in human renal tubular cells downstream of viral early gene expression. *Am. J. Transplant.* 8(7), 1413–1422 (2008).

71 Ljubin-Sternak S, Santak M, Cepin-Bogovic J *et al.* Detection of genetic lineages of human metapneumovirus in Croatia during the winter season 2005/2006. *J. Med. Virol.* 80(7), 1282–1287 (2008).

72 Einsele H, Loeffler J. Contribution of new diagnostic approaches to antifungal treatment plans in high-risk haematology patients. *Clin. Microbiol. Infect.* 14(Suppl. 4), 37–45 (2008).

73 Khodakov DA, Zakharova NV, Gryadunov DA *et al.* An oligonucleotide microarray for multiplex real-time PCR identification of HIV-1, HBV, and HCV. *Biotechniques* 44(2), 241–246, 248 (2008).

74 Abe M, Klett C, Wieland E *et al.* Two-step real-time pcr quantification of all subtypes of human immunodeficiency virus type 1 by an in-house method using locked nucleic acid-based probes. *Folia Medica* 50(3), 5–13 (2008).

75 Peiris JS, Poon LL. Detection of SARS coronavirus in humans and animals by conventional and quantitative (real time) reverse transcription polymerase chain reactions. *Methods Mol. Biol.* 454, 1–12 (2008).

76 Yu XF, Pan JC, Ye R *et al.* Preparation of armored RNA as a control for multiplex real-time reverse transcription-PCR detection of influenza virus and severe acute respiratory syndrome coronavirus. *J. Clin. Microbiol.* 46(3), 837–841 (2008).

77 Wang W, Ren P, Sek M *et al.* Design of multiplexed detection assays for the diagnosis of human pathogenic avian influenza A subtypes by SmartCycler real-time reverse transcription-PCR. *J. Clin. Microbiol.* 47(1), 86–92 (2009).

78 Critzer FJ, Dsouza DH, Golden DA. Transcription analysis of *stx1*, *marA*, and *eaeA* genes in Escherichia coli O157:H7 treated with sodium benzoate. *J. Food Prot.* 71(7), 1469–1474 (2008).

79 Bustin SA. Nucleic acid quantification and disease outcome prediction in colorectal cancer. *Per. Med.* 3(2), 207–216 (2006).

80 Ben-Dov C, Hartmann B, Lundgren J, Valcárcel J. Genome-wide analysis of alternative pre-mRNA splicing. *J. Biol. Chem.* 283(3), 1229–1233 (2008).

•• **Most human genes are spliced.**

Pettigrew CA, Brown MA. Pre-mRNA splicing aberrations and cancer. *Front. Biosci.* 13, 1090–1105 (2008).

81 Lo HS, Wang Z, Hu Y *et al.* Allelic variation in gene expression is common in the human genome. *Genome Res.* 13(8), 1855–1862 (2003).

• **Much ignored description of the importance of quantifying the appriopriate allele.**

82 Springer NM, Stupar RM. Allele-specific expression patterns reveal biases and embryo-specific parent-of-origin effects in hybrid maize. *Plant Cell* 19(8), 2391–2402 (2007).

83 Meyer KB, Maia AT, O'Reilly M *et al.* Allele-specific up-regulation of *FGFR2* increases susceptibility to breast cancer. *PLoS Biol.* 6(5), e108 (2008).

84 Chen X, Weaver J, Bove BA *et al.* Allelic imbalance in *BRCA1* and *BRCA2* gene expression is associated with an increased breast cancer risk. *Hum. Mol. Genet.* 17(9), 1336–1348 (2008).

85 Maheshri N, O'Shea EK. Living with noisy genes: how cells function reliably with inherent variability in gene expression. *Annu. Rev. Biophys. Biomol. Struct.* 36, 413–434 (2007).

86 Raj A, Peskin CS, Tranchina D *et al.* Stochastic mRNA synthesis in mammalian cells. *PLoS Biol.* 4(10), e309 (2006).

87 Bengtsson M, Hemberg M, Rorsman P *et al.* Quantification of mRNA in single cells and modelling of RT-qPCR induced noise. *BMC Mol. Biol.* 9, 63 (2008).

88 Huang T, Bohlenius H, Eriksson S *et al.* The mRNA of the Arabidopsis gene FT moves from leaf to shoot apex and induces flowering. *Science* 309(5741), 1694–1696 (2005).

89 Bohlenius H, Eriksson S, Parcy F *et al.* Retraction. *Science* 316(5823), 367 (2007).

90 Garson JA, Huggett JF, Bustin SA *et al.* Unreliable real-time pcr analysis of human endogenous retrovirus-W (HERV-W) RNA expression and DNA copy number in multiple sclerosis. *AIDS Res. Hum. Retroviruses* (2009) (In Press).

91 Uhlmann V, Martin CM, Sheils O *et al.* Potential viral pathogenic mechanism for new variant inflammatory bowel disease. *Mol. Pathol.* 55(2), 84–90 (2002).

92 Bustin SA. RT-qPCR and molecular diagnostics: no evidence for measles virus in the GI tract of autistic children *Eur. Pharm. Rev. Dig.* 1(1), 11–16 (2008).

• **Detailed description of poor experimental practice with significant implication for public health.**

93 Hornig M, Briese T, Buie T *et al.* Lack of association between measles virus vaccine and autism with enteropathy: a case–control study. *PLoS ONE* 3(9), e3140 (2008).

94 Afzal MA, Ozoemena LC, O'Hare A *et al.* Absence of detectable measles virus genome sequence in blood of autistic children who have had their MMR vaccination during the routine childhood immunization schedule of UK. *J. Med. Virol.* 78(5), 623–630 (2006).

95 D'Souza Y, Dionne S, Seidman EG *et al.* No evidence of persisting measles virus in the intestinal tissues of patients with inflammatory bowel disease. *Gut* 56(6), 886–888 (2007).

96 Nolan T, Hands RE, Ogunkolade BW *et al.* SPUD: a qPCR assay for the detection of inhibitors in nucleic acid preparations. *Anal. Biochem.* 351, 308–310 (2006).

97 Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* 1(3), 1559–1582 (2006).

• **Detailed protocols for optimizing reverse-transcription quantitative PCR assays.**

98 Gallup JM, Ackermann MR. Addressing fluorogenic real-time qPCR inhibition using the novel custom Excel file system 'FocusField2–6GallupqPCRSet-upTool-001' to attain consistently high fidelity qPCR reactions. *Biol. Proced. Online* 8, 87–152 (2006).

99 Stahlberg A, Hakansson J, Xian X *et al.* Properties of the reverse transcription reaction in mRNA quantification. *Clin. Chem.* 50(3), 509–515 (2004).

100 Stahlberg A, Kubista M, Pfaffl M. Comparison of reverse transcriptases in gene expression analysis. *Clin. Chem.* 50(9), 1678–1680 (2004).

101 Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J. Biomol. Tech.* 15(3), 155–166 (2004).

102 Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* 29(1), 23–39 (2002).

103 Vandesompele JK, Preter DE, Pattyn F *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3(7), 0034.1–0034.11 (2002).

•• **First usable and most cited method for mRNA normalization.**

104 Gutierrez L, Mauriat M, Guenin S *et al.* The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. *Plant Biotechnol. J.* 6(6), 609–618 (2008).

• **Clear description of the problems associated with inappropriate normalization.**

105 Bustin SA, Benes V, Garson J *et al.* The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* (2009) (In Press).

•• **Standardized guidelines for publication of quantitative PCR experiments.**

106 Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA* 14(5), 844–852 (2008).

•• **First clear demonstration of the importance of proper data normalization for miRNA quantification.**

107 Brazma AP, Hingamp J. Quackenbush *et al.* Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat. Genet.* 29(4), 365–371 (2001).

108 Taylor CF, Paton NW, Lilley KS *et al.* The minimum information about a proteomics experiment (MIAPE). *Nat. Biotechnol.* 25(8), 887–893 (2007).

109 Field D, Garrity G, Gray T *et al.* The minimum information about a genome sequence (MIGS) specification. *Nat. Biotechnol.* 26(5), 541–547 (2008).

110 Echeverri CJ, Beachy PA, B. Baum *et al.* Minimizing the risk of reporting false positives in large-scale RNAi screens. *Nat. Methods* 3(10), 777–779 (2006).

111 Haney SA. Increasing the robustness and validity of RNAi screens. *Pharmacogenomics* 8(8), 1037–1049 (2007).

112 Sansone SA, Fan T, Goodacre R *et al.* The metabolomics standards initiative. *Nat. Biotechnol.* 25(8), 846–848 (2007).

113 Taylor CF, Field D, Sansone SA *et al.* Promoting coherent minimum reporting guidelines for biological and biomedical investigations: the MIBBI project. *Nat. Biotechnol.* 26(8), 889–896 (2008).

114 Nakamura S, Yang C-S, Sakon N *et al.* Direct metagenomic detection of viral pathogens in nasal and fecal specimens using an unbiased high-throughput sequencing approach. *PLoS ONE* 4(1), e4219 (2009).

•• **Detailed description of the potential of next-generation sequencing technology.**


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