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REVIEW

Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis

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ABSTRACT

qRT-PCR (real-time reverse transcription-PCR) has become the benchmark for the detection and quantification of RNA targets and is being utilized increasingly in novel clinical diagnostic assays. Quantitative results obtained by this technology are not only more informative than qualitative data, but simplify assay standardization and quality management. qRT-PCR assays are most established for the detection of viral load and therapy monitoring, and the development of SARS (severe acute respiratory syndrome)-associated coronavirus qRT-PCR assays provide a textbook example of the value of this technology for clinical diagnostics. The widespread use of qRT-PCR assays for diagnosis and the detection of disease-specific prognostic markers in leukaemia patients provide further examples of their usefulness. Their value for the detection of disease-associated mRNA expressed by circulating tumour cells in patients with solid malignancies is far less apparent, and the clinical significance of results obtained from such tests remains unclear. This is because of conceptual reservations as well as technical limitations that can interfere with the diagnostic specificity of qRT-PCR assays. Therefore, although it is evident that qRT-PCR assay has become a useful and important technology in the clinical diagnostic laboratory, it must be used appropriately and it is essential to be aware of its limitations if it is to fulfil its potential.

INTRODUCTION

qRT-PCR [real-time RT (reverse transcription)-PCR] [1] has become the standard for the detection and quantification of RNA targets [2] and is firmly established as a mainstream research technology [3]. Its potential for high-throughput, together with regular introduction of enhanced or novel chemistries, more reliable instrumentation and improved protocols, has also seen the development of qRT-PCR-based clinical diagnostic assays [4–6]. Although not necessarily more sensitive than conventional RT-PCR [7], qRT-PCR assays have several significant advantages [8]: (i) they use fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR, and the combination of the DNA amplification and detection steps into one homogeneous assay obviates the requirement for post-PCR processing; (ii) their wide dynamic range allows the analysis of samples differing in target abundance by orders of magnitude; (iii) there is little inter-assay variation, which helps generate reliable and reproducible results; and (iv) fluorescence-based qRT-PCR realizes the inherent quantitative capacity of PCR-based assays [9], making it a quantitative rather than a qualitative assay. This is important as there is an obvious need in molecular medicine for quantitative data, e.g. for

Key words: cancer, colon, leukaemia, occult disease, pathogen, real-time reverse transcription-PCR (qRT-PCR), virus detection.

Abbreviations: APL, acute promyelocytic leukaemia; CEA, carcinoembryonic antigen; C_t, threshold cycle; CV, coefficient of variance; ds, double stranded; FFPE, formalin-fixed paraffin-embedded; IPCR, immunopolymerase chain reaction; LN, lymph nodes; MRD, minimal residual disease; PIV, parainfluenza virus types; qRT-PCR, real-time RT-PCR; RNase, ribonuclease; RSV, respiratory syncytial virus; RT, reverse transcription; SARS, severe acute respiratory syndrome; SCT, stem cell transplantation; ss, single-stranded; T_m, melting temperature.

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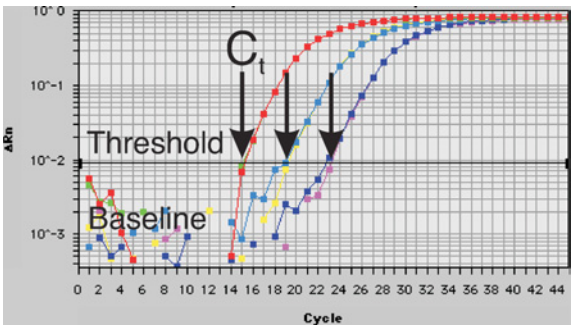

Figure 1 Characteristics of qRT-PCR amplification curves, which plot fluorescence signal versus cycle number

The curves for three samples, run in duplicate, are shown. C_t values are indicated by arrows and represent the cycle fractions where the instrument can first reliably detect fluorescence derived from the amplification reaction. The fluorescence signal during the initial cycles of the PCR is below the instrument's detection threshold and defines the baseline for the amplification plot. An increase in fluorescence above the threshold indicates the detection of accumulated PCR product. The key parameter C_t is defined as the fractional cycle number from clinical samples at which the fluorescence passes a fixed threshold chosen either by the instrument or by the operator. A plot of the log of initial target copy number for a set of standards versus C_t is a straight line. The amount of target in an unknown sample is quantified by measuring the C_t and using the standard curve to determine starting copy number.

measuring viral load or monitoring of response to therapy in haematological malignancies.

In theory, RT-PCR differs from PCR only by the addition of a preliminary step, the initial conversion of RNA into a DNA template by an RNA-dependent DNA polymerase (reverse transcriptase). In practice, this additional procedure results in a much more fragile and variable assay [10,11]. Nevertheless, despite the problems associated with this technique, its promise has resulted in a concerted effort to develop diagnostic assays that make use of the assay's strengths, whilst trying to circumvent its pitfalls [12].

WHAT IS qRT-PCR?

The principle of qRT-PCR assays is straightforward: following the RT of RNA into cDNA, it requires a suitable detection chemistry to report the presence of PCR products, an instrument to monitor the amplification in real-time and appropriate software for quantitative analysis [13]. qRT-PCRs are characterized by the point in time during cycling when amplification of a PCR product is first detected (Figure 1). The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

Detection chemistries can be either probe- or non-probe based, also referred to as 'specific' and 'non-specific' respectively. The most widely used non-probe-based chemistry detects the binding of SYBR Green I to ds (double-stranded) DNA [14]. In solution, the unbound dye exhibits little fluorescence; during the PCR assay, increasing amounts of dye bind to the nascent ds DNA. When monitored in real-time, this results in an increase in the fluorescence signal as the polymerization proceeds. The PCR product can be verified by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon [15] (Figure 2). An important advantage of non-probe-based chemistries is that in most

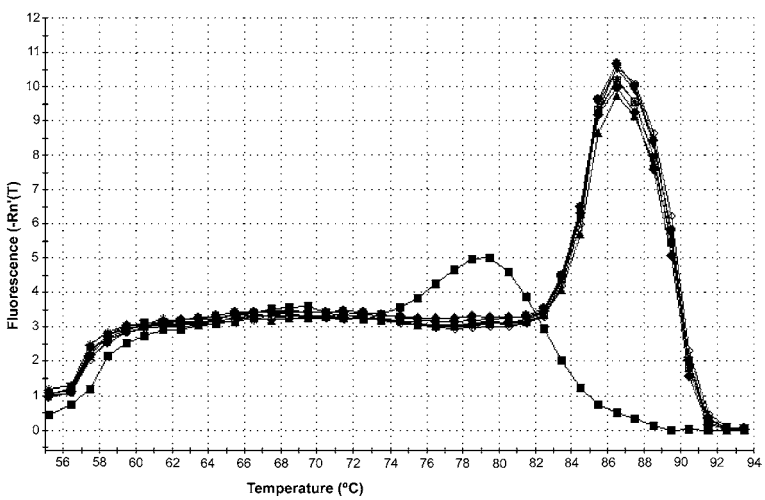

Figure 2 Dissociation curves are useful for determining the presence of multiple species in the sample

Every PCR product melts at a characteristic temperature, its T_m (melting temperature). A characteristic melting peak at the amplicon's T_m will usually distinguish it from amplification artifacts that melt at lower temperatures in broader peaks. In the PCR, these are typically primer–dimer artifacts or non-specific amplicons. For standard analysis, samples are first melted at 95 °C, and then equilibrated at 60 °C before being slowly re-heated (dissociated) back to 95 °C. The centre of each peak reflects T_m. The dissociation curves show typical primer–dimer formation. The specific product is shown with a T_m of 87 °C, whereas the primer–dimer (■) has a characteristically lower T_m of 79 °C. Primer–dimers will be most prevalent in NTC ('no template control') wells and sample wells containing low concentrations of template.

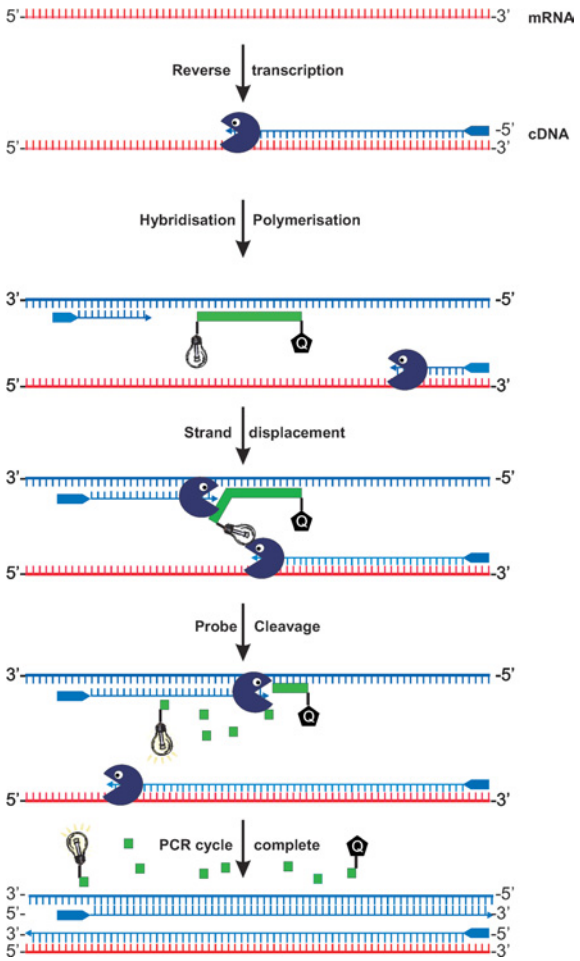

Figure 3 The principle of the 5′ nuclease (*Taq*Man) assay

The RT step synthesizes a cDNA copy of the RNA template. After denaturation, primers and probe anneal to their targets. The probe contains a reporter dye at the 5′ end and a quencher (Q) at its 3′ end. During the polymerization step, the 5′ nuclease activity of the *Taq* polymerase displaces and cleaves the probe. This physically separates the reporter dye and quencher dyes, resulting in reporter fluorescence. The increase in signal is directly proportional to the number of molecules released during that cycle. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.

instances optimized conventional RT-PCR assays can be converted immediately into real-time assays [16]. An important disadvantage is that their specificity remains dependent on the specificity of the primers.

Probe-based chemistries make use of amplicon-specific fluorescent probes and a fluorescent signal is only generated if the probe hybridizes with its complementary target (Figure 3). Therefore probe-based chemistries introduce an additional level of specificity, in effect combining RT-PCR with a validation step previously carried out separately after the PCR.

Multiplexing permits the detection and differentiation of multiple targets, e.g. infectious agents or biomarkers, in a single tube at the same time. Although SYBR Green has been used for the simultaneous detection of enteric [17] and noroviruses [18], its scope is rather limited, not least because the preferential binding of SYBR Green to specific DNA sequences during amplification interferes with PCR kinetics [19]. Other non-specific chemistries [20,21] are not any better at multiplexing than SYBR Green [22], but a recently developed non-probe-based chemistry promises to simplify multiplexing. It uses isoguanosine (iG) and isocytosine (iC) [23], which can pair only with each other. Upstream (sense) PCR primers are tagged at their 5′ ends with different fluorophores plus a single iso-dC (5′-methylisocytosine). A quencher-labelled iso-dG (2′-deoxyisoguanosine) is added to the amplification master mix and its incorporation opposite iso-dC results in increasing fluorescence quenching during amplification [24]. This technology is available as 'Plexor' from Promega. For multiplexing with probe-based chemistries, probes are labelled with different fluorophores. There are three oligonucleotides per target, which can result in non-specific interactions between the primers and probes, and this demands careful assay design and reagent selection [25]. At the moment, quadruplex (quintuplex on the ABI 7500 and Stratagene MX3005P) assays are possible, but stretch the limits of the technology's capabilities [26]. Nevertheless, improved chemistries are constantly being developed and validated and it is clear that multiplex qRT-PCR will have an important role to play in clinical diagnostic assays [27].

It is also possible to carry out nested qPCR (quantitative PCR) assays [28] that consist of two separate amplification reactions primed by two sets of primers: the first reaction primed by two external primers is followed by a second reaction that uses two internal primers and a hybridization probe. Nested PCR assays can provide higher analytical sensitivity [29], and have been successfully applied to the early detection of the SARS (severe acute respiratory syndrome) virus [29,30]. However, increased susceptibility to contamination is an ever-present problem and the actual sensitivity and specificity of this method remains to be evaluated with studies involving a larger number of clinical specimens.

QUANTIFICATION STRATEGIES

Quantification can either be relative to an external standard curve or to one or more co-amplified internal control mRNAs [2]. The former is based on the use of a dilution series of an external standard, which can be used to generate a standard curve of C_t (threshold cycle) against initial target copy number [31]. The copy numbers of unknown samples can be calculated from the linear regression of that standard curve, with the *y*-intercept giving the sensitivity and the slope giving the amplification efficiency. Standard curves can be constructed from PCR fragments, *in vitro* T7-transcribed

sense RNA transcripts, single-stranded sense-strand synthetic oligodeoxyribonucleotides or from commercially available universal reference RNAs [32]. This strategy is most obviously useful for quantifying viral or tumour load in body fluids. The accuracy of absolute quantification depends entirely on the accuracy of the standards. In general, standard curves are highly reproducible and allow the generation of specific and reproducible results. However, external standards cannot detect or compensate for inhibitors that may be present in the samples.

Quantification relative to internal standards compares the C_t values from target RNAs to those of one or more internal reference genes and results are expressed as ratios of the target-specific signal to the internal reference. This produces a corrected relative value for the target-specific RNA product that can be compared between samples and allows an estimate of the relative expression of target mRNA in those samples. It is crucial that the amplification efficiencies of target and reference are similar, since this directly affects the accuracy of any calculated expression result. Several models have been published that use different algorithms to correct for efficiency and claim to allow a more reliable estimation of the real expression ratio [33,34]. However, since the expression of most reference genes is regulated and their levels usually vary significantly with treatment or between individuals, relative quantification can be misleading [35]. Furthermore, if the relative levels of the reference and target RNAs vary by orders of magnitude, during amplification the former may have entered its plateau phase by the time a C_t for the target becomes apparent. Unless compensated for, this may interfere with the accurate quantification of the target RNA.

QUALITY CONTROL ISSUES

Optimization and consistency are as critical for obtaining reproducible results using qRT-PCR as they are for conventional methods [36]. qRT-PCR assays are significantly less variable than conventional RT-PCR protocols [37–39], which are subject to significant error [40,41]. However, since reproducibility is influenced by parameters such as distribution statistics [42], qRT-PCR data are less reproducible when working with very low copy numbers due to the stochastic sampling effects [43]. This emphasizes the importance of repetitive testing in clinical samples and one of the strengths of these assays is the ease with which it is possible to determine multiple C_t values for every sample, which encourages replicate determinations of the same sample and permits the application of statistical analyses to the quantification procedure.

Like any clinical diagnostic assay, qRT-PCR must be properly validated [44] and meet the criteria expected of any laboratory test applied in clinical medicine: (i) standardization of the test across different laboratories; (ii) reproducibility of positive and negative predictive values; and (iii) reliable sensitivity and specificity. This involves the establishment of a set of quality protocols, the use of appropriate positive- and negative-control samples, and suitable analysis and reporting guidelines. Standardization is all-important [5,45] and generates the robust and reliable results that are of critical importance for the management of patients entered into multicentre therapeutic trials [46].

A successful clinical diagnostic qRT-PCR assay requires careful consideration of several issues. (i) Optimal sample quality is a prerequisite to generate valid quantitative data [47]. Hence sample collection, preparation and transport and RNA extraction methods are all critical parameters in test performance and must be optimized. In general, extraction of RNA from blood and serum is relatively straightforward, whereas there are significant problems associated with the extraction of RNA from solid tumours, faeces and semen samples. The main problems are that RNA is easily degraded and that it is easy to co-purify inhibitors of the RT or PCR steps which will generate inconsistent results. (ii) Primer selection, especially of the reverse primer used in the RT-step, is critical since it affects the sensitivity of the RT-PCR assay [48]. The structure of the RNA target at the primer-binding site must be taken into account, as this affects the accessibility of the target to the primers. mRNA displays extensive secondary structure and selection of a primer binding site in a ds target site that is folded will result in a very inefficient assay. For RNA viruses there is the additional problem of different viral serotypes resulting in sequence variability and it may be necessary to use a nested RT-PCR assay with universal primers that bind to target sequences which are shared by all the serotypes, followed by a serotype-specific primer pair [49]. (iii) Regular calibration of the real-time instrument is crucial for obtaining consistent and accurate results. C_t is neither absolute nor invariant, but varies between assays carried out on different days with different reagents or on different instruments. This is because C_t depends on the instrument's threshold setting, which depends on background fluorescence, which in turn varies with different probes, chemistries, instruments and assay protocols. Therefore samples should not be compared by C_t values [11], but they should be converted into and reported as target copy numbers. (iv) Analytical sensitivity and specificity are critical parameters of any diagnostic qRT-PCR assay. Analytical sensitivity refers to the smallest number of RNA molecules that can be detected and distinguished from a zero result and is best calculated using a standard curve which defines the range of the assay. It is inappropriate to report results that lie significantly outside the upper and lower concentration of target defined by the standard curve. Analytical specificity is determined by identifying the percentage of

samples without the target sequence that also generate a negative result. (v) There must be agreement between replicates within and between runs of the assay, as this provides important information about the reliability of the assay. Repeatability is measured as the amount of agreement between replicates tested in different runs in the same laboratory. Reproducibility is determined in several laboratories using the identical assay (protocol, reagents and controls). It is important to maintain the internal quality control by monitoring the assay for both parameters. If the assay is to be applied in another geographical region and/or population, it might be necessary to revalidate it under the new conditions as mutations or polymorphisms within the primer sites, especially at their 3′ ends, will affect the performance of the assay and render the established validation no longer valid. It is also advisable to regularly sequence the selected genomic regions in the national isolates of the infectious agents. This is especially true for the primer sites to ensure that they remain stable, so that the validation of the assay cannot be questioned. (vi) False-positive results may arise either from product carryover from positive samples or, more commonly, from cross-contamination by PCR products from earlier experiments [50]. It is critically important to include negative controls, i.e. samples that are as similar to the test samples as possible but exclude the target. It is also advisable to include a no RT control that excludes the reverse transcriptase to exclude signal generation from contaminating DNA. Since false-negative results in an optimized assay are mostly due to inhibitory effects and/or pipetting errors, it is important to always include a positive control with any qRT-PCR assay [51], ideally in the form of a standard curve. In addition, all samples should be tested for inhibition using a simple 'alien' assay [11] and any RNA preparations showing inhibition must be repurified.

A recent report [52] suggests that the variability of calibration curves is strongly influenced by the large variability of the measurements below 100 copies of a target gene. The authors [52] propose reducing the variance of the standard curve by not running the 1 and 10 copy standards and instead adding additional dilutions between 10 and 100 copies to the standard curve. The problem with this approach is that the highest dilutions provide information about the variability of the assay at those very low target copy numbers. qRT-PCR facilitates the inclusion of exact sensitivity controls on a per sample basis [53] and, clearly, PCR findings, positive or negative, are questionable if they are not supported by the associated data demonstrating the overall sensitivity of the assay applied [44]. Therefore it is acceptable to omit the highest dilutions if the target copy numbers are well above that threshold number. However, if the assay's specification is to detect 1–50 target copies, the standard curve must include the 1 and 10 copy dilutions. The authors also state that the reduced variance of the modified standard curve makes it unnecessary to construct a calibration curve with each run and that running of the samples in duplicate is unnecessary. Running a standard curve with each assay immediately reveals any problem with that particular run and increases confidence when reporting negative results. The same argument applies to running samples in duplicate or, preferably, in triplicate and in this qRT-PCR is no different from any clinical diagnostic assay.

APPLICATIONS

Viral pathogens

RNA viruses constitute the most abundant group of pathogens in man, animals and plants and are classified according to their RNA genome. The low efficiency of proofreading and post-replicative repair activities of viral RNA polymerases results in high mutation rates, causing potential problems for an assay that relies on exquisite sequence specificity. Furthermore, populations of RNA viruses are extremely heterogeneous and RNA viral genomes are statistically defined but individually indeterminate. This has raised some concerns about the detrimental effect of primer–template mismatches on the polymerization efficiency and problems associated with geographically disparate reference sequences [54]. Nevertheless, the ability of qRT-PCR to generate accurate quantitative data has had a huge impact on the study of viral agents of infectious disease [55] and is helping to clarify disputed infectious disease processes and demonstrate links between specific viral sequences and patient clinical symptoms [56]. However, the lack of commercially available validated reagent kits for most viruses remains a major problem, as does the absence of standardization of the existing tests [57].

Negative-strand RNA viruses

The genome of negative-stranded RNA viruses consists of ss (single-stranded) 'antisense' RNA. These are transcribed into sense-strand mRNA by RNA-dependent RNA polymerases supplied by the viruses. This group includes the measles and mumps viruses and various viruses targeting the respiratory tract. Influenza virus types A and B, RSV (respiratory syncytial virus) and PIV (parainfluenza virus) types 1–4 cause respiratory infections of the upper and lower respiratory tract in infants and young children and are important causes of severe lower respiratory tract disease in elderly and immunocompromised patients, with significant morbidity and mortality. Since these respiratory viral pathogens cause very similar clinical symptoms, differential diagnosis of the pathogens is required. Although multiplex conventional RT-PCR assays have been developed, they tend to be less sensitive than individual assays [56] and require extensive post-PCR manipulation. For example,

a recent report [58] describes the detection of the seven viruses in a combined RT-PCR/enzyme-linked amplicon hybridization assay; however, post-PCR processing involves a hybridization reaction, incubations, washes and spectrophotometric analysis. In contrast, a novel qRT-PCR assay consists of two multiplex reactions, one detecting influenza A and B and RSV, the other one PIV 1–4, and can generate a result within 6 h. The assays display 100 % specificity and detected virus in an additional 23 % of samples [59]. Other real-time assays can differentiate between influenza A subtypes [60,61] and RSV A and B [62] facilitating diagnosis, patient management and strain identification for vaccine production. A recent report [63] describes the specific detection of mumps virus not only in typical, but also in suspected cases which show only symptoms of meningitis or encephalitis.

Positive-strand RNA viruses

The genome of positive-stranded RNA viruses consists of ss 'sense' RNA and specifies its own RNA-dependent RNA polymerase. The RNA is translated by the host and then catalyses the synthesis of large numbers of antisense replicative intermediates that, in turn, serve as templates for the synthesis of a large number of mRNA molecules. This group includes the entero-, rhino- and corona-viruses. The SARS outbreak provides a textbook example of the importance and usefulness of qRT-PCR assays. SARS emerged in southern China in November 2002 and within months the disease had spread globally, affecting over 8000 patients in 29 countries with 774 fatalities. Although useful in the identification of the SARS virus, conventional RT-PCR assays were insufficiently sensitive to provide a diagnosis in the first few days after disease onset, the period during which laboratory diagnosis is most relevant for patient care. qRT-PCR-based diagnostic assays for the associated coronavirus were rapidly developed [64,65] and improved to result in an assay with 100 % specificity in samples collected from day 1–3 of disease onset [66,67]. The assay's potential for high throughput was also of critical importance in areas with outbreaks of SARS in which large numbers of specimens had to be tested. In addition, the ability to quantify helped elucidate the pathogenesis of the disease, showing that, unlike other respiratory viral infections, viral load and rates of positivity of SARS coronavirus in the upper respiratory tract increased progressively to peak at around day 10 after disease onset [68]. Viral load was highest in specimens of the lower respiratory tract and was higher in nasopharyngeal aspirate than in throat swabs [69]. Faecal samples had very high viral loads towards the end of the first week of illness, and were the specimen of choice during the second week of disease [70].

The qRT-PCR assay also provides critical prognostic information for clinical management. It revealed that high viral load in the nasopharyngeal aspirate was associated with the occurrence of diarrhoea [71], the requirement for intensive care [72] and was an independent predictor of mortality [71]. qRT-PCR assays can now be used routinely to exclude SARS-associated coronavirus in patients hospitalized with respiratory symptoms even in the presence of other respiratory viruses [73]. qRT-PCR assays have been designed that are specific for other positive-strand RNA viruses. A qRT-PCR assay developed for the clinical diagnosis of viral meningitis detects enterovirus in cerebrospinal fluid [74] and is significantly more sensitive than viral culture [75]. Another assay targets noroviruses, one of the most common aetiological agents of outbreaks of acute gastroenteritis [76]. This assay is four orders of magnitude more sensitive than a conventional RT-PCR assay with the same primer sets and as sensitive as nested conventional RT-PCR [77].

ds RNA viruses

A third group of viruses has a segmented ds RNA genome, together with a RNA-dependent RNA polymerase that transcribes each of the ds RNA molecules into an mRNA. This group includes human rotaviruses, the most important aetiological agents of severe diarrhoeal illness of infants and young children worldwide. The classical clinical presentation of rotaviral infection is fever and vomiting, followed by non-bloody diarrhoea, which can lead to severe and potentially life-threatening dehydration. qRT-PCR assays based on SYBR Green [78] and *Taq*Man [79] chemistries have been developed and validated and are beginning to reveal the virus's epidemiology and pathogenesis [80].

Retroviruses

Viruses in this group, which include HIV and HTLV (human T-cell leukaemia virus), contain RNA-dependent DNA polymerases that copy their RNA genome into DNA, which integrates into the host genome. The hosts' normal transcription generates viral mRNA which is either translated or packaged into new virus particles. The concentration of HIV-1 RNA in the plasma of HIV-infected individuals is an important predictor of disease outcome and a marker of antiretroviral drug efficacy, allowing a specific analysis of treatment failure caused by emerging resistance to specific anti-retroviral compounds. The most sensitive FDA (Food and Drug Administration)-approved tests for HIV-1 RNA in plasma have detection limits of 50 copies/ml [81,82] and treatment is generally considered successful if the level of viraemia can be reduced to below this level. However, reduction of the HIV-1 RNA load to < 50 copies/ml does not guarantee long-term success, and a rebound of drug resistance can occur, implying that HIV-1 replication and evolution is continuing while patients are receiving

therapy. Many patients whose levels of viraemia are suppressed to < 50 copies/ml have persistently detectable HIV-1 RNA [83]. Consequently, the development of qRT-PCR assays that can detect and quantify HIV-1 RNA in plasma down to 1 copy/ml promises a significant increase in the accuracy and clinical relevance of HIV testing [84].

The extreme sequence heterogeneity of HIV-1 poses a significant challenge to the 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 hybridize to mutating sequences. This has led to the development of non-specific SYBR-Green-based assays that rely on the use of melting curves to ensure that the obtained signal is specific [85]. These assays are better than branched-chain DNA assays in determining viral load [86], especially during the monitoring of HAART (highly active anti-retroviral therapy) treatment [87], and have a superior linearity over a wide concentration range [88]. The imminent release of the iso-C/G chemistry (see above) promises to enhance the performance of non-probe-dependent chemistries.

A multiplex real-time quantitative RT-PCR assay was developed for simultaneous detection, identification and quantification of HBV (hepatitis B virus), HCV (hepatitis C virus) and HIV-1 in plasma or serum samples. Genomic amplification of one virus was unaffected by the simultaneous amplification of the other two. Although competition between HCV and HIV-1 amplifications slightly affected the yield of HIV-1 amplification, quantification of a single virus was possible [89].

The IPCR (immunopolymerase chain reaction) assay combines the molecular recognition of antibodies with the high amplification capability of PCR [90], and real-time IPCR assays have been developed for the detection of cellular proteins such as VEGF (vascular endothelial growth factor), which is elevated in some cancers [91], and gliadins in patients with coeliac disease [92]. The procedure is similar to conventional ELISAs but allows for more sensitive detection. Instead of an enzyme, a DNA molecule is linked to the detection antibody and serves as a template for PCR. A real-time IPCR assay has been reported for the detection of HIV [93]. This assay detects the p24 protein of the virus, rather than viral nucleic acid. Since each virus particle contains thousands of molecules of p24, there is a greater amount of target to detect.

Disease-specific marker detection

Overall leukaemia-free survival rates in adult AL (acute leukaemia) depend on the diagnostic subtype of AL. These subtypes display significant differences in their clinical features, treatment responses, relapse sites and kinetics. Risk models are continuously amended with new prognostic factors, one of which is the evaluation of MRD (minimal residual disease), and the ability of qRT-PCR to quantify MRD has resulted in its widespread adaptation for this purpose.

CML (chronic myeloid leukaemia) is characterized by the presence of the Philadelphia chromosome, a reciprocal t(9;22) translocation that transposes the c-abl oncogene from chromosome 9q34 to the *BCR* gene on chromosome 22q11. The rearranged *BCR-ABL* gene is transcribed into a chimaeric mRNA, which in turn is translated into a 210 kDa fusion protein (p210) that mediates myeloid proliferation and transformation. Detection of this distinctive cytogenetic abnormality is the most important prognostic parameter for the assessment of complete remission and long-term survival. However, the sensitivity of cytogenetic analysis is low and, by the time disease is detectable, clinical relapse may be inevitable.

This has resulted in the development of RT-PCR-based monitoring methods that can detect all break points and can be performed on peripheral blood specimens [94]. The most comprehensive validation of a qRT-PCR assay for clinical diagnostic use tested 372 clinical specimens and 50 peripheral blood samples from patients not known to have any myeloproliferative disorders. The assay was shown to be 100 % specific, and able to detect a single copy of a target spiked into negative RNA. The between-run reproducibility had a CV (coefficient of variance) of 12.3 %, and within-run reproducibility had a CV of 13.8 % [95], helping replace conventional RT-PCR assays, even nested ones [96], as the most sensitive and reliable method for molecular follow-up [97,98].

Its capacity for accurate quantification is a significant advantage of qRT-PCR assays, since mere detection of MRD does not permit reliable prediction of the course of disease in individual patients [99], whereas its quantification correlates with disease stage [100]. Consequently, monitoring of the dynamics of residual disease in CML patients by serial qRT-PCR performed at regular intervals predicts impending clinical relapse in patients who are still in haematological and cytogenetic remission and is useful in guiding clinical therapeutic decisions [101,102].

Detection of disease-specific translocations is also at the core of strategies aimed at assessing the response of individual patients to therapeutic intervention [103]. Conventional RT-PCR results reporting on treatment response in patients who had undergone allogeneic-matched sibling SCT (stem cell transplantation) were ambiguous, with some reports identifying subgroups of patients at risk of disease recurrence, and others detecting no association between PCR positivity and subsequent relapse [104,105]. Conversely, qRT-PCR assays quantifying levels of BCR-ABL RNA showed no such ambiguity and were clearly able to identify patients likely to relapse after bone marrow transplant [106–109],

making this technology the method of choice for early therapeutic decision making [110].

qRT-PCR is also penetrating clinical diagnostic assays designed to detect other haematological malignancies. The presence of a transcript specifying a BCR–ABL fusion protein of 190 kDa in ALL (acute lymphoblastic leukaemia) usually indicates poor prognosis and warrants haematopoietic SCT [111]. A simple multiplex assay distinguishing between the transcript variants has been described recently [112]. APL (acute promyelocytic leukaemia) is characterized by a reciprocal translocation between chromosomes 15 and 17, resulting in the creation of a hybrid gene (*PML/RAR*) that is thought to mediate leukaemogenesis. A multiplex qRT-PCR assay has been designed that is capable of quantifying and simultaneously identifying transcript variants. Three different PML/RAR fusion transcripts forms have been identified in APL and a treatment response may depend upon which fusion transcript form they express [113].

Disease-associated detection

In contrast with haematological malignancies, solid tumours are not characterized by universal molecular markers. Nevertheless, 'molecular staging' using RT-PCR to amplify tissue-specific mRNA has long been viewed as a possible solution for increasing the accuracy of post-operative staging and has been evaluated extensively in the molecular assessment of tumour stage and disease recurrence [114,115]. However, there are significant technical and conceptual limitations [116,117] that hold back its adaptation into clinical practice [118] and it continues to constitute a 'proof of principle' rather than robust and reliable clinical assay [119]. The introduction of significant variability by the RT step makes it problematic to delineate universal biologically relevant quantitative 'cut-off' points [10] and the lack of standardized protocols results in lack of reproducibility between laboratories [120]. However, most crucially, there are no disease-specific markers uniquely associated with any solid cancer. Hence the use of tissue-specific markers, which are presumed to detect the presence of cancer cells in patients' blood, bone marrow or LN (lymph nodes). Unfortunately, it is unclear just how tissue-specific these markers are [116].

Colorectal cancer provides a good paradigm of the difficulties encountered when attempting to use molecular techniques as diagnostic assays [119]. Although the detection of tumour invasion in draining LN by histopathological staging is a poor prognostic factor, there is considerable prognostic heterogeneity within each tumour stage [121]. Achieving accurate stratification of individuals into prognostic groups within a given stage is assuming increasing importance with the recent emergence of more effective adjuvant chemotherapy protocols that have had a positive impact on patient survival [122].

There have been numerous studies reporting the detection of mRNA markers such as CEA (carcinoembryonic antigen), cytokeratins (ck), mucins, CD44 and GCC (guanylate cyclase C) in different tissue compartments and attempting to assess their prognostic significance [123]. One report [124] suggests that it is possible to distinguish histologically positive LN from histologically negative ones by counting the number of CEA-expressing cells. However, there was significant overlap between the two groups and cell numbers were calculated relative to a CEA-expressing cell line, ignoring inter-sample or inter-patient variation of CEA mRNA levels. A second study [125] calculated CEA copy numbers in LN relative to 18S RNA levels, and used cut-off levels to suggest that high CEA mRNA levels might be predictive of distant recurrence. A third study [126] also concluded that quantification of CEA mRNA levels in LN from patients with advanced colorectal cancer yielded prognostic information. Unfortunately, quantifying the amount of an mRNA value does not allow the calculation of the number of circulating tumour cells, since the expression of most genes varies by several orders of magnitude between tumours in different individuals and often varies in the tumour of the same individual [127]. Also, none of these authors discussed how to implement a relative quantification assay in practice.

In complete contrast, studies using both conventional [128] and real-time [116] RT-PCR reported the detection of CEA mRNA in up to 85% of control LN, with significant overlap of CEA copy numbers between histologically involved and uninvolved LN. There was no correlation between CEA copy numbers and prognosis, suggesting that a CEA-based assay is unable to identify patients at risk of distant disease recurrence. At least there is a rationale for attempting to detect occult disease in LN: histological detection of occult disease during staging is an important prognostic indicator. This is not the case for blood. Nevertheless, some reports suggest that CEA mRNA levels in the blood of colorectal cancer patients are associated with disease stage [129] and may be of prognostic value [130,131]. These contrast with others that question its specificity and suggest that peripheral blood is not a suitable compartment for detection of tumour cells [117,120,128,132], or advocate analysis of yet another tissue compartment [133]. Similar contradictory results have been reported for other tissue-specific markers [119]. This discordance is typical and, when results are analysed in detail, there is little agreement on the specificity of the various markers, and there is a significant percentage of patients that test positive for the marker in question, yet survive for 5 years, or do not test positive for the marker, yet die within 5 years [116,117]. Characteristically, the usual conclusion is that the respective markers have not yet been evaluated sufficiently to recommend their inclusion in a clinical assay [134]. This is not surprising when considering

that blood, bone marrow or LN sampling represents a single snapshot of a complex and dynamic process and that few of the large number of cancer cells shed from a primary tumour ever form metastatic tumours [135]. Consequently, despite this vast effort, PCR-based techniques have still not been validated clinically in prospective studies and the presence of circulating tumour cells cannot be considered a reliable prognostic indicator.

This suggests a conceptual flaw underlying the attempts to use RT-PCR assays to allow prediction of successful distant metastasis, as it is based on a simplistic view of the biology and kinetics of tumour cell traffic through the lymphatic and systemic circulation and subsequent metastasis development. Instead RT-PCR may simply be detecting cells of no biological significance [136] and variability in survival within each staging category probably reflects not only the inaccuracy of detecting occult residual disease, but also a lack of understanding of the sequestration, release and subsequent trafficking of the tumour cell in both the lymphatic and systemic circulation. None of the qRT-PCR assays address the question of the biological relevance of detecting tumour cells in blood or LN and do not provide any information about their metastatic potential or take into account the role of patient genotype in allowing or suppressing metastasis. Animal models suggest that only 0.01 % of cells circulating in the blood ultimately develop into a metastatic site [137], and in humans the likelihood of tumour cells seeding to become metastases is also very low [138]. Furthermore, the genotype of LN metastases differs from that of the main clone in the primary tumour in >50 % of patients, with a significant minority displaying a genotype not detected in the primary tumour at all [138]. In addition, humans themselves are genetically polymorphic, and the outcome of metastasis depends on the interplay of tumour cells with various host factors, including the organ microenvironment, which can influence the biology of cancer growth, angiogenesis and metastasis [139]. Therefore it is not surprising that the detection of occult disease is unlikely to have prognostic value.

RT-PCR-ASSOCIATED PROBLEMS

There are several technical causes that can result in ambiguous data, especially when targeting disease-associated markers [11].

RNA purification

Clinical samples from which RNA is extracted tend to be disparate and include blood and other body fluids as well as solid biopsies obtained during endoscopy, post-surgery, post-mortem and from archival materials. Naked RNA is extremely susceptible to degradation by endogenous RNases (ribonucleases) that are present in all living cells. Therefore the key to the successful isolation of high-quality RNA and to the reliable and meaningful comparison of qRT-PCR data is to ensure that neither endogenous nor exogenous RNases are introduced during the extraction procedure [140].

Recent reports suggest that RNA extracted from FFPE (formalin-fixed paraffin-embedded) archival materials can be successfully quantified by qRT-PCR assays. Between 60 % [141] and 84 % (100 % of samples less than 10 years old) [142] of templates can be amplified by RT-PCR, and it is possible to quantify mRNA expression levels [143]. Formalin-fixed tissues are ideal for retrospective clinical studies of disease mechanisms and, as the use of PCR technology has become more common in molecular testing, it has enhanced the clinical utility of these tissues. This is important, since such studies have the potential to enable the correlation of molecular findings with the patient's response to treatment and eventual clinical outcome. qRT-PCR assays, with their amplicon lengths of below 100 bp, are ideally placed to amplify the usually degraded RNA from these archival samples whose average size is 200–250 nucleotides. However, care must be taken when interpreting the results obtained from archival material, as gene expression profiles from FFPE samples do not correlate exactly with the profiles generated from the corresponding frozen samples ($r^2 = 0.69$) [144]. The same authors report that, although 64 genes were differentially expressed in matching fresh-frozen normal colon and cancer samples, only 38 were in the corresponding FFPE samples. Furthermore, only 28 of these genes were in common. Thus any results obtained using FFPE samples require independent experimental determination [145], but also may underestimate or report misleading changes in gene expression patterns.

cDNA synthesis

Priming of the cDNA reaction from the RNA template can be carried out using random primers, oligo(dT), a mixture of both or target-specific primers. The choice of primer can cause marked variation in calculated mRNA copy numbers [146] and results obtained using the different methods are not comparable [147]. It is also little appreciated that cDNA synthesis can be primed efficiently without addition of any primer at all [148].

Random primers

This method yields the most cDNA but, since transcripts originate from multiple points along the transcript, more than one cDNA transcript is produced per original target. Furthermore, the majority of cDNA synthesized from total RNA will be ribosomal RNA-derived and may compete with a target that is present at very low levels. As the T_m of random primers is low, they cannot be used with thermostable RT enzymes without a low temperature

pre-incubation step. This is the least satisfactory method of synthesizing cDNA.

oligo(dT)

cDNA synthesis using oligo(dT) is more specific than random priming, as it will not result in the priming from rRNA. It is the best method to use when the aim is to obtain a faithful cDNA representation of the mRNA pool, although it will not prime any RNAs that lack a polyA$^+$ (polyadenylated) tail. In addition, oligo(dT) priming requires very high-quality RNA that is full-length, and hence is not a good choice for priming from RNA that is likely to be fragmented, such as that obtained from FFPE archival material. Furthermore, the RT may fail to reach the primer/probe-binding site if secondary structures exist or if the primer/probe-binding site is at the extreme 5′ end of a long mRNA. It is possible to mix random primers and oligo(dT); however, this may exacerbate the problems of accurate quantification, as the variable priming of the random oligonucleotides is likely to introduce variability.

Target-specific primers

Target-specific primers synthesize the most specific cDNA and provide the most sensitive method of quantification [149]. Their main disadvantage is that they require separate priming reactions for each target, which is wasteful if only limited amounts of RNA are available.

Inhibitors

The co-purification of inhibitors of the RT-PCR during template preparation can present a serious problem to accurate and reproducible quantification of mRNA levels [150]. Common inhibitors include various components of body fluids and reagents encountered in clinical and forensic science (e.g. haemoglobin and urea), food constituents (e.g. organic and phenolic compounds, and fats) and environmental compounds (e.g. humic acids and heavy metals) [151]. In addition, factors such as DNA fragmentation [152] and the presence of residual anticoagulant heparin [153] or proteinase K-digested haem compounds such as haemoglobin [154] will negatively affect PCR efficiency. The problem with this type of inhibitor is that it makes the comparison of qRT-PCR results from different patients or different samples from the same patient impossible as it results in different amplification efficiencies and hence C_t values of the same target from different patients. Worryingly, laboratory plasticware has been identified as one potential source of PCR inhibitors [155]. It is also important to remember that reagents can have a significant effect on assay reproducibility, with lot-to-lot variation an essential consideration [156]. Different polymerases display variable sensitivity to the presence of inhibitors such as blood, ions or biological samples [157–159]. Thus the PCR-inhibiting effect of various components in biological samples can, to some extent, be eliminated by the use of the appropriate thermostable DNA polymerase.

Other considerations

The variability of RT-PCR results obtained from identical samples assayed in different laboratories continues to be a problem [160]. The single most likely source of data variation is due to variability introduced by the person carrying out the experiment. Since there are so many steps involved in going from a tissue sample to a quantitative result, it is not surprising that this is so. Other sources of variability are the reagents, especially the probe and RT, and it is essential to include appropriate controls with every run.

The recent introduction of robots capable of extracting RNA from tissue samples and pipetting very small volumes promises to address two bottlenecks, those of template preparation and the dispensing of reagents. It should also reduce the variability and contamination observed when different operators prepare multiple templates [161].

SUMMARY AND CONCLUSION

Some technologies arrive at a time that is ‘just right’, and advances in chemistries and instrumentation make qRT-PCR a technology whose time has come [162]. This is reflected in the increasingly important role it plays in clinical diagnosis, in particular when used for the assessment of viral load and the analysis of disease-specific translocation products in haematological malignancies. It is also clear that appropriate application, quality control and standardization are issues that must be addressed and it is vital to consider each stage of the experimental protocol, starting with the laboratory set-up, proceeding through sample acquisition and template preparation, and the RT and PCR steps. Only if every one of these stages is properly validated is it possible to obtain reliable quantitative data. Of course, choice of chemistries, primers and probes, and instruments must be appropriate to whatever is being quantified. Finally, data must be interpreted, and this remains a real problem. Significant technical problems remain, mainly associated with the conversion into cDNA and subsequent amplification of the RNA template.

These problems hint at a next generation of assays that address the problems that are inherent to any RT-PCR. Developments in microfluidics are already allowing the amplification of target nucleic acid from nanolitres of sample [163]. However, the ideal assay would eliminate the need to amplify a target and it is likely that real-time RT-PCR will eventually be replaced by methods capable of direct analysis of single biological molecules. One such single-molecule fluorescence detection technology is fluorescence correlation spectroscopy, which

detects expression by hybridizing two dye-labelled DNA probes to a selected target molecule, which can be either DNA or RNA in solution. The subsequent dual-colour cross-correlation analysis allows the quantification of the biomolecule of interest in absolute numbers down to target concentrations of less than 10^{-12} M [164]. Combined with single molecule sequencing [165], this will eventually permit RNA identification as well as distortion-free quantification. That is the future. For now, qRT-PCR assays, when carried out appropriately, are the method of choice for RNA detection and quantification.

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