

REVIEW

Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays

S A Bustin

Academic Department of Surgery, St Bartholomew's and the Royal London School of Medicine and Dentistry, Queen Mary and Westfield College, London E1 1BB, UK; Email: s.a.bustin@mds.qmw.ac.uk

ABSTRACT

The reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive method for the detection of low-abundance mRNA, often obtained from limited tissue samples. However, it is a complex technique, there are substantial problems associated with its true sensitivity, reproducibility and specificity and, as a quantitative method, it suffers from the problems inherent in PCR. The recent introduction of fluorescence-based kinetic RT-PCR procedures significantly simplifies the process of producing reproducible quantification of mRNAs and promises to overcome these limitations. Nevertheless, their successful application depends on a clear understanding of the practical problems, and

careful experimental design, application and validation remain essential for accurate quantitative measurements of transcription. This review discusses the technical aspects involved, contrasts conventional and kinetic RT-PCR methods for quantitating gene expression and compares the different kinetic RT-PCR systems. It illustrates the usefulness of these assays by demonstrating the significantly different levels of transcription between individuals of the housekeeping gene family, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH).

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INTRODUCTION

Why mRNA quantification?

Many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to quantitate transcription levels of specific genes has always been central to any research into gene function (Zamorano *et al.* 1996). More recently, the emergence of molecular medicine has resulted in the increased use of techniques able to quantitate levels of RNA in clinical diagnostics. Such applications are wide-ranging and include procedures designed to quantify the regulation and expression of drug resistance markers in tumour cells (Ramachandran & Melnick 1999), monitor responses to chemotherapy (Desjardin *et al.* 1999), measure the biodistribution and of transcription of gene-encoded therapeutics (Fairman *et al.* 1999), provide a molecular assessment of tumour stage (Bustin & Dorudi 1998), detect circulating tumour cells in

cancer patients (Ghossein & Rosai 1996), and detect bacterial (Hill 1996) and viral (Holodniy 1994) pathogens.

Four methods are in common use for the quantification of transcription: northern blotting and *in situ* hybridisation (Parker & Barnes 1999), RNase protection assays (Hod 1992, Saccomanno *et al.* 1992) and the reverse transcription polymerase chain reaction (RT-PCR) (Weis *et al.* 1992). A fifth method, cDNA arrays, is still limited in its use by cost considerations (Bucher 1999). Northern analysis is the only method providing information about mRNA size, alternative splicing and the integrity of RNA samples. The RNase protection assay is most useful for mapping transcript initiation and termination sites and intron/exon boundaries, and for discriminating between related mRNAs of similar size, which would migrate at similar positions on a northern blot. *In situ* hybridisation is the most complex method of all, but is the only one that allows localisation of transcripts to specific cells within a tissue. The main limitation of these three

techniques is their comparatively low sensitivity (Melton *et al.* 1984).

RT-PCR is an *in vitro* method for enzymatically amplifying defined sequences of RNA (Rappolee *et al.* 1988) and permits the analysis of different samples from as little as one cell in the same experiment. It is the most sensitive and the most flexible of the quantification methods (Wang & Brown 1999) and can be used to compare the levels of mRNAs in different sample populations, to characterise patterns of mRNA expression, to discriminate between closely related mRNAs, and to analyse RNA structure. RT-PCR can also circumvent time-consuming and technically demanding cloning steps and generate reagents, such as full-length complementary DNA (cDNA) inserts for cloning (Borson *et al.* 1992), or arbitrarily primed enhanced sequence tag cDNA libraries (Neto *et al.* 1997).

The focus on maximising sensitivity has led to the development of ever-more complex procedures. Semi-nested (Wasserman *et al.* 1999), nested (Israeli *et al.* 1994, Soeth *et al.* 1996), and even three-step nested (Funaki *et al.* 1997, 1998) RT-PCR techniques increase the sensitivity, but compromise the specificity of the reaction (Henke *et al.* 1997). They increase the likelihood of contamination and of detection of false positives (Kwok & Higuchi 1989) and are unable to distinguish genuine, low-level transcription from illegitimate transcription (Chelly *et al.* 1989).

Basic considerations

The RT-PCR is a complex assay (Fig. 1A) and all physical and chemical components of the reaction are interdependent. They must be considered carefully when optimising the specificity, sensitivity, reproducibility or fidelity of the reaction. Successful performance of the RT-PCR depends on a clear understanding of the primary aim of the assay. Specificity, sensitivity and reproducibility will be the most important qualitative characteristics for clinical diagnostic uses. Conversely, if the purpose of the experiment is mutation analysis, cloning or expression, fidelity will be the prime consideration.

As RNA cannot serve as a template for PCR, the first step in an RT-PCR assay is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction. Usually, this involves the use of dedicated RNA- and DNA-dependent DNA polymerases, either in separate ('two-enzyme/two-tube') or in single ('two-enzyme/one-tube') reactions, as the use of dedicated enzymes with different properties

allows for the customisation of individual RT-PCR assays. Separation of the RT and PCR steps has the advantage of generating a stable cDNA pool that can be stored virtually indefinitely. Alternatively, a single polymerase able to function both as an RNA and DNA-dependent DNA polymerase can be used in a 'one-enzyme/one-tube' reaction and minimises hands-on time in addition to the risk of contamination.

Reverse transcription

RNA transcripts can exhibit significant secondary structure that affects the ability of the RNA-dependent DNA polymerase (reverse transcriptase, RT) to generate transcripts (Buell *et al.* 1978). This can affect RT-PCR quantification and should be minimised when comparing or quantifying diverse mRNA populations (Shimomaye & Salvato 1989). The two commonly used RTs are avian myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT). AMV-RT is more robust than MMLV-RT (Brooks *et al.* 1995), retains significant polymerisation activity up to 55 °C (Freeman *et al.* 1996) and can help eliminate problems associated with RNA secondary structure. In contrast, MMLV-RT and engineered derivatives (Kotewicz *et al.* 1988) have significantly less RNase H activity than AMV-RT (Gerard *et al.* 1997). As even reduced RNase H activity can interfere with the synthesis of long amplicons (DeStefano *et al.* 1991), MMLV-RT may be a better choice if the aim of the experiment is the amplification of full-length cDNA molecules.

The RT step can be primed using specific primers, random hexamers or oligo-dT primers, and the choice of primers requires careful consideration. The use of mRNA-specific primers decreases background priming, whereas the use of random and oligo-dT primers maximises the number of mRNA molecules that can be analysed from a small sample of RNA. Primers can cause marked variation in calculated mRNA copy numbers and, compared with specific downstream primers, random hexamers can overestimate mRNA copy numbers by up to 19-fold (Zhang & Byrne 1999).

Polymerase chain reaction

In an analogous manner, the choice of thermostable DNA-dependent DNA polymerases is determined by the aims of the experiment. There is a wide choice of enzymes that differ in their processivity, fidelity, thermal stability and ability to read

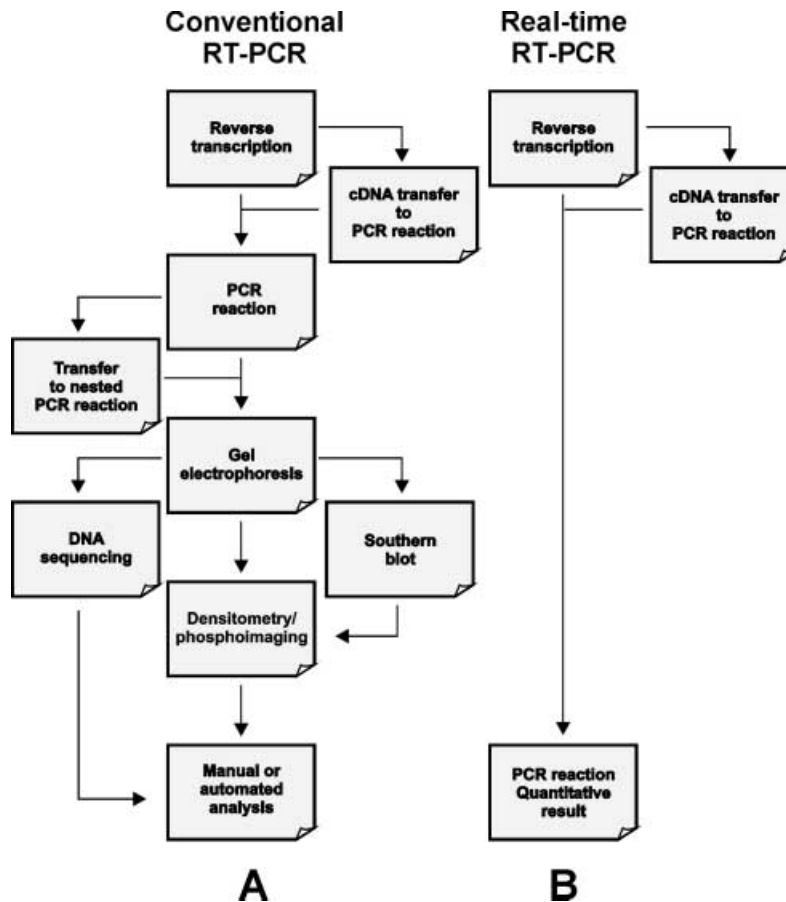


FIGURE 1. RT-PCR quantification. (A) Conventional RT-PCR. The reaction can be carried out either with reverse transcriptase in a separate reaction, followed by transfer of some of the cDNA into a second reaction for the PCR, or as a combined one-tube reaction with a heat-stable DNA-dependent polymerase. The former may be more sensitive and has the advantage of generating a cDNA reservoir that can be re-used repeatedly to carry out further PCR reactions. The latter has the advantages of speed, simplicity and less chance of contamination. Maximum sensitivity is achieved using a nested PCR reaction; however, this is frequently at the cost of reduced specificity. Gel electrophoresis is common to all procedures, but Southern blotting and/or DNA sequencing may be required to confirm the identity of the amplicon. A final step involves some method for quantitating the bands on the gel. (B) Real-time RT-PCR. The reverse transcription reaction is carried out as described above. All subsequent steps are carried out by the thermal cycler/detector and a quantitative result is processed by computer.

modified triphosphates such as deoxyuridine and deoxyinosine in the template strand (Adams & Kelly 1994, Perler *et al.* 1996). The most commonly used enzyme, Taq DNA polymerase, has a 5'-3' nuclease activity but lacks a 3'-5' proofreading exonuclease activity. This makes its use less advisable when fidelity is the main consideration; instead, proofreading exonucleases such as Vent and Deep Vent (New England Biolabs, Beverly, MA,

USA) or Pfu (Stratagene, La Jolla, CA, USA) should be used (Cline *et al.* 1996), although their Michaelis constants ($K_m(\text{DNA})$) can differ by more than two orders of magnitude from that of Taq polymerase, which has a direct effect on the effective primer annealing temperature. As the specificity of the PCR step is crucially dependent on the primers (Zhang & Byrne 1999), reaction conditions must be re-optimised every time a different enzyme is used

and results obtained may not be directly comparable. Furthermore, the proofreading activity can lead to primer degradation ('nibbling') from the 3' end. The rate of degradation is dependent on the length of the primer: a 60 mer is degraded much faster than a 30 mer. This can be counteracted by synthesising primers with phosphorothioate linkages on the last two 3' bases, as this linkage renders the primers extendable but not degradable (de Noronha & Mullins 1992).

In addition, Mg^{2+} and dNTP concentrations require strict control, because Mg^{2+} affects enzyme activity, and imbalanced dNTP mixtures will reduce polymerase fidelity (Eckert & Kunkel 1991). In addition, Mg^{2+} increases the melting temperature (T_m) of double-stranded DNA, and forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognises. Therefore, high concentrations of dNTPs interfere with polymerase activity and affect primer annealing by reducing free Mg^{2+} . In addition, the K_m (dNTP) values of different polymerases vary significantly and must be taken into consideration when planning an RT-PCR assay.

One-enzyme RT-PCR

In situations in which convenience is the main priority or the target RNA contains extensive secondary structure, it is advisable to use a single tube ('one-enzyme/one-tube') system utilising *Thermus thermophilus* (Tth) polymerase, a DNA polymerase with intrinsic RT, but no RNase H, activity (Myers & Gelfand 1991). This assay uses bicine buffers containing Mn^{2+} ions that are compatible with both RT and subsequent PCR (Chiocchia & Smith 1997). This permits the RT reaction to be carried out at increased temperatures using primers with significantly higher T_m , while reducing both hands-on time and the likelihood of introducing contaminants into reaction mixtures. There is also evidence that RT-PCR reactions using Tth polymerase may be more robust and resistant to inhibitors present in biological specimen (Poddar *et al.* 1998). The main drawbacks of this system are that: (i) because the sensitivity of Tth polymerase is two orders of magnitude lower than that of Taq polymerase (Grabko *et al.* 1996), this system is not as sensitive as two-enzyme procedures (Cusi *et al.* 1994, Leutenegger *et al.* 1999); (ii) the presence of Mn^{2+} ions reduces the fidelity of nucleotide incorporation (Beckman *et al.* 1985, Fromant *et al.* 1995); (iii) like Taq polymerase, Tth polymerase lacks a 3'-5' exonuclease proofreading activity; and (iv) first-strand synthesis must be initiated from specific primers, rather than using random primers.

We have found that reducing the denaturation temperature to 92 °C can improve the sensitivity of the assay, probably because Tth polymerase is somewhat less thermostable than Taq polymerase. However, for some templates, especially if they are long or G/C rich, this reduction may result in lower amplification efficiencies, as incompletely melted DNA will mask primer and probe binding sites.

QUANTIFICATION

RT-PCR quantification strategies

Quantification of mRNA can be by semi-quantitative or quantitative methods (Ferre 1992). Semi-quantitative RT-PCR results are typically generated by frequent sampling of the RT-PCR reaction mixture followed by dot-blot analysis (Montgomery & Dallman 1997). This additional complexity does not lend itself to easy automation and, as the name implies, this method is at best semi-quantitative and its use should be avoided.

Quantitative procedures use non-competitive or, more commonly, competitive RT-PCR techniques (Wang *et al.* 1989, Vanden Heuvel *et al.* 1993). They depend on the spiking into the RNA samples before RT of known amounts of RT-PCR-amplifiable competitors that, ideally, should be synthetic RNA molecules. Non-competitive RT-PCR refers to the co-amplification of the target with a second RNA molecule under reagent concentrations and conditions so that there is no competition between target and standard, and with which it shares neither the primer recognition sites nor any internal sequence (Reischl & Kochanowski 1999). However, this means that quantification can be unreliable, as the efficiencies of both the RT and PCR steps are likely to vary even when monitoring relative changes of transcription within a sample (Ferre 1992). In addition, as standard RNA is added in a linear fashion within one order of magnitude of the target, non-competitive RT-PCR requires some approximate knowledge of target amounts (Freeman *et al.* 1999).

In competitive RT-PCR, the internal standard shares the same primer recognition and internal sequences with the primary target, leading to competition for reagents. Because they are virtually identical, both should be amplified with the same efficiency and their amplicons can be distinguished by the addition of a restriction enzyme site to the standard (Gilliland *et al.* 1990, Becker-Andre 1993) or by varying its size (Gilliland *et al.* 1992, Scheuermann & Bauer 1993). A series of PCR tubes containing the target are spiked with serial dilutions

of known copy numbers of the internal standard. The greater the concentration of the internal standard, the more likely it is that the primers will bind and amplify it, rather than the target. After gel electrophoresis, a comparison of the intensities of ethidium-bromide-stained standard and target amplicons allows target quantification (Raeymaekers 1999). However, this may still not result in absolute quantification because, for example, stable differences in amplification efficiency between target and competitor will remain undetected (Raeymaekers 1993). There have been numerous attempts to enhance the accuracy of quantification by improving RNA isolation (Fernandez *et al.* 1997), reaction conditions (Corey *et al.* 1997), and detection methods (Borson *et al.* 1998, Hayward *et al.* 1998, Marchand *et al.* 1999). In spite of, or perhaps because of, this, competitive quantification procedures are, without exception, labour- and reagent-intensive.

The minimum error in competitive RT-PCR reactions is approximately 10%, even when competitor and target are present in equal amounts (Souaze *et al.* 1996), and it is usually significantly greater. There are several reasons for this. (i) The amplification efficiencies of exogenous competitor, which must be synthesised for each gene being quantified, are not always equivalent. (ii) Competitor RNA must be transcribed *in vitro*, which adds an additional enzymatic step; furthermore, once transcribed it may degrade during long-term storage. (iii) Heteroduplex formation between the nearly identical standard and target can result in variable sensitivity and accuracy (Henley *et al.* 1996); use of DNA competitors with primer binding sites flanking unrelated internal sequences (mimics) (Siebert & Larrick 1993) eliminates that problem, but causes new ones. (iv) The quantification of competitor fragments generated by restriction digestion requires the dilution of PCR products before digestion, introducing one source of error; a second source of error is the restriction digest itself, as it may not completely digest the competitor. (v) Finally, the technique is not amenable to high throughput because, typically, for each unknown target a large series of reactions with different competitor:target ratios has to be run (Raeymaekers 1993); therefore, conventional RT-PCR-based procedures are not suitable for routine use, especially when several targets from many samples are being quantified.

Instrumentation

The application of fluorescence techniques to the RT-PCR, together with suitable instrumentation

capable of combining amplification, detection and quantification, has led to the development of kinetic RT-PCR methodologies that are revolutionising the possibilities for quantitating nucleic acids (Orlando *et al.* 1998). There are three competing instruments on the market; all three are run as closed-tube systems and quantification requires no post-amplification manipulation. This avoids problems of contamination, results in short turn-around times for data acquisition and analysis and minimises hands-on time (Fig. 1B). The entire process, starting at the RT and ending with full quantification, is automated (Fig. 2), which makes these instruments ideally suited for high-throughput screening applications (Martell *et al.* 1999, Wattjes *et al.* 2000).

The ABI Prism 7700 (Perkin-Elmer–Applied Biosystems, Foster City, CA, USA) contains a built-in thermal cycler with 96-well positions, and is able to detect fluorescence between 500 nm and 660 nm. Fluorescence is induced during the RT-PCR by distributing laser light to all 96 samples contained in thin-walled reaction tubes via a multiplexed array of optical fibres. The resulting fluorescent emission returns via the fibres and is directed to a spectrograph with a charge-coupled device (CCD) camera. Because each well is irradiated sequentially, the dimensions of the CCD array can be used for spectral resolution of the fluorescent light. This instrument can be used for assays based on DNA-binding dyes, molecular beacons and hydrolysis probes. RT-PCR reactions typically take 2 h to complete. Its description by the manufacturer as a real-time technique is not strictly correct as, unlike competing systems, the progress of the RT-PCR reaction can currently be analysed only after termination of the amplification run.

The Lightcycler (Roche Molecular Biochemicals, Mannheim, Germany) performs the RT-PCR in small-volume glass capillary tubes, contained within a rotor-like carousel, that are heated and cooled in an airstream (Wittwer *et al.* 1989). The carousel is rotated past a blue light-emitting diode, and fluorescence is read by three photodetection diodes with different wavelength filters that allow the use of spectrally distinct fluorescent probes (Wittwer *et al.* 1997b). The performance of the Lightcycler in terms of sensitivity and specificity is equal to that of Taqman (Nitsche *et al.* 1999). In addition to being able to use assays based on DNA-binding dyes, hydrolysis probes and molecular beacons, it can be used for dual hybridisation probes. Up to 32 reactions are typically carried out in 5–20 µl volumes (Wittwer *et al.* 1990) and RT-PCR reactions are completed in less than 20 min (Wittwer & Garling 1991). The Lightcycler a truly

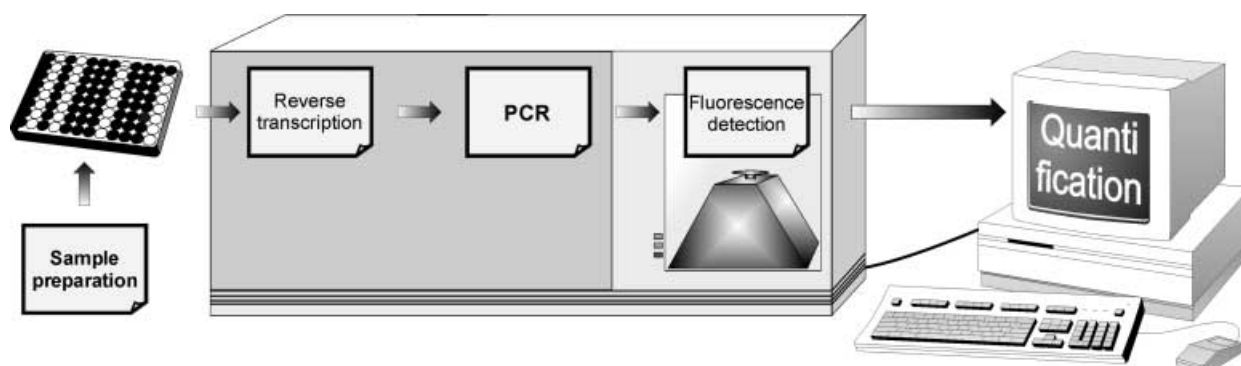


FIGURE 2. The principle of Taqman RT-PCR. Amplification is performed in closed, optical tubes of a 96-well microplate that is placed in a combined thermal cycler/detector, the ABI 7700. A laser is directed to each of the 96 sample wells, and the fluorescence emission data for each sample are collected once every few seconds as the PCR products are being generated. The fluorescence is spectrally analysed, allowing the simultaneous use of more than one spectrally distinct fluorescent probe. The starting copy number is determined by monitoring when PCR product is first detected – the higher the starting copy number of the target, the sooner a significant increase in fluorescence is observed. The data are fed to a computer, which analyses and displays the results, eliminating the need for post-PCR processing.

‘real-time’ technique, and the fluorescence readings taken at every cycle of the PCR reaction are displayed immediately after each measurement, allowing amplification runs to be terminated or extended, as appropriate, during individual runs.

Biorad Instruments have recently launched an optical module that fits their standard thermal cycler and transforms it into a real-time RT-PCR system. This instrument is capable of generating and detecting a wider range of excitation frequencies than either the ABI 7700 or the Lightcycler. At present, it can monitor up to four different fluorescent reporters at any one time and can be used for any one of the alternative fluorescent RT-PCR strategies. Furthermore, unlike the ABI 7700 which scans its 96 samples sequentially, this instrument can scan up to 96 samples simultaneously, with a sampling frequency that can be defined by the user.

Chemistries

There are currently four competing techniques available that detect amplified product with about the same sensitivity (Wittwer *et al.* 1997a). They use fluorescent dyes and combine the processes of amplification and detection of an RNA target to permit the monitoring of PCR reactions in real-time during the PCR; their high sensitivity eliminates the need for a second-round amplification, and decreases opportunities for generating false-positive results (Morris *et al.* 1996). The simplest method uses fluorescent dyes that bind specifically to double-stranded-DNA. The other three rely on the

hybridisation of fluorescence-labelled probes to the correct amplicon. The methods differ in their specificity, although at later amplification cycles all can show artefacts that do not correlate to specific product accumulation. As amplicon detection in the molecular beacon, hydrolysis and hybridisation probe assays depends on successful hybridisation of the probe, these RT-PCR procedures obviate the need for post-PCR Southern analysis or sequencing to confirm the identity of the amplicon.

Molecular beacons

Molecular beacons (Stratagene) are DNA hybridisation probes that form a stem-and-loop structure; the loop portion of the molecule is complementary to the target nucleic acid molecule and the stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence (Tyagi & Kramer 1996) (Fig. 3). A fluorescent marker is attached to the end of one arm and a quencher is attached to the end of the other arm. The quencher is a non-fluorescent chromophore that dissipates the energy that it receives from the fluorophore as heat. In solution, free molecular beacons adopt a hairpin structure and the stem keeps the arms in close proximity, resulting in efficient quenching of the fluorophore. When molecular beacons encounter a complementary target at the annealing temperature, they undergo a conformational transition that forces the stem apart and results in the formation of a probe/target hybrid that is longer and more stable than the stem (Bonnet *et al.* 1999). This separates the fluorophore and the quencher, leading to the

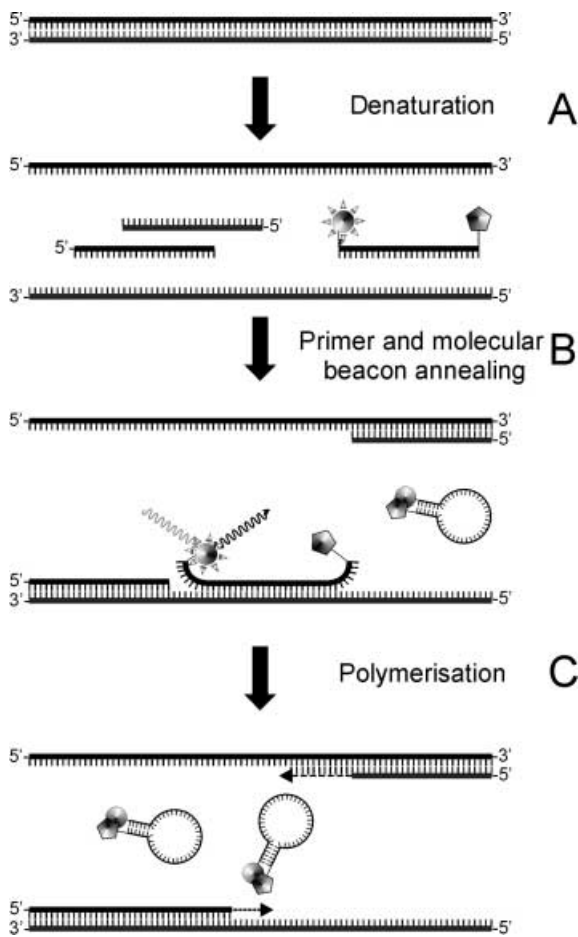


FIGURE 3. The molecular beacons assay. Molecular beacons are designed to be complementary to a sequence in the middle of the expected amplicon. The RT step has been omitted. (A) During the denaturation step, the molecular beacons assume a random coil configuration and fluoresce. (B) As the temperature is decreased to allow annealing of the primers, stem hybrids form rapidly, preventing fluorescence. However, in the presence of target, molecular beacons also bind to the amplicons and generate fluorescence. (C) When the temperature is increased for the primer extension step, the molecular beacons dissociate from their targets, and fluorescence is again quenched. A new hybridisation takes place in the annealing step of every cycle, and the intensity of the resulting fluorescence indicates the amount of accumulated amplicon at the end of the previous cycle.

restoration of fluorescence, which can be detected, whereas any free molecular beacons remain closed and non-fluorescent. If the target DNA sequence does not exactly match the molecular beacon sequence, hybridisation and fluorescence will not occur. This is because the thermodynamic properties of the molecular beacons favour the formation

of a hairpin form rather than continued hybridisation to a less than perfectly matched target sequence. Molecular beacons require the use of a spectrofluorimetric thermal cycler for real-time detection, but can also be used in an endpoint assay using a fluorimeter. The specificity of this system is such that it can readily detect single nucleotide differences (Giesendorf *et al.* 1998, Marras *et al.* 1999), something that can be difficult to achieve with the Taqman system.

Molecular beacons can also be used in non-PCR amplification assays that utilise isothermal nucleic acid amplification schemes (Guatelli *et al.* 1990, Tyagi *et al.* 1996) such as strand-displacement amplification (Walker *et al.* 1992), nucleic acid sequence-based amplification (Leone *et al.* 1998), and rolling-circle amplification (Lizardi *et al.* 1998).

The main drawback with molecular beacons is associated with the design of the hybridisation probe. Optimal design of the molecular beacon stem is crucial, as the molecular beacon may fold into alternate conformations that do not place the fluorophore in the immediate vicinity of the quencher, resulting in a sub-population that is not quenched well and in large background signals. Alternatively, if the stem of a molecular beacon is too strong, it can interfere with the hybridisation and the fluorescence of the beacon annealed to its target may be incompletely restored. Therefore, accurate thermal denaturation profiles to determine their melting characteristics must be established for each molecular beacon and different target sequences will have to be matched with different stem sequences.

DNA-binding dyes

This method (Fig. 4A) involves detection of the binding of a fluorescent dye (SYBR Green) to DNA (Morrison *et al.* 1998). The unbound dye exhibits little fluorescence in solution, but during elongation increasing amounts of dye bind to the nascent double-stranded DNA. When monitored in real-time, this results in an increase in the fluorescence signal that can be observed during the polymerisation step, and that falls off when the DNA is denatured. Consequently, fluorescence measurements at the end of the elongation step of every PCR cycle are performed to monitor the increasing amount of amplified DNA. This method obviates the need for target-specific fluorescent probes, but its specificity is determined entirely by its primers. As the presence of any double-stranded DNA generates fluorescence, this assay is no more specific than conventional RT-PCR. However, additional specificity and RT-PCR product verification can be

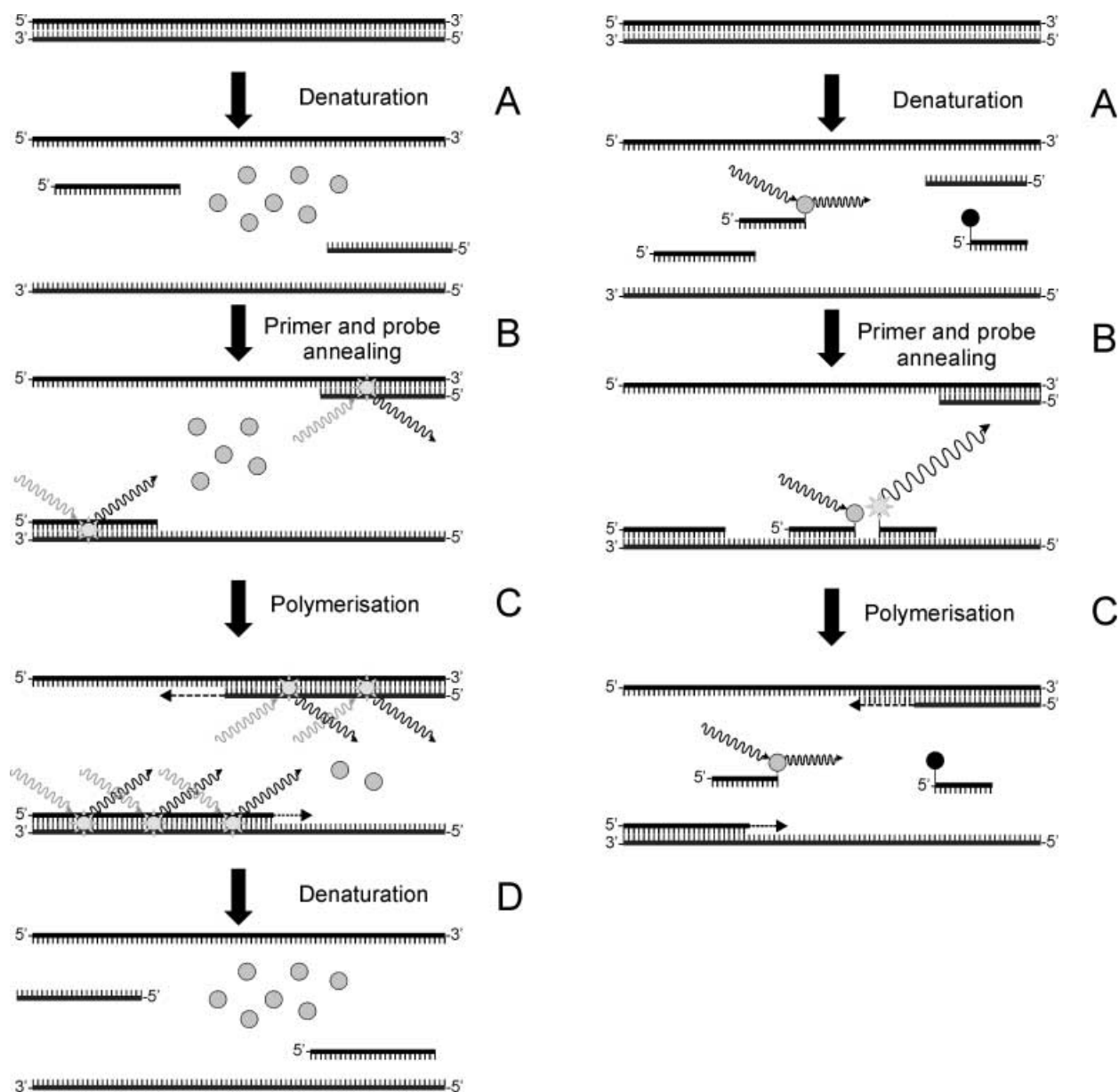


FIGURE 4. The Lightcycler assay. *Left:* Dye incorporation method. (A) During denaturation, unbound SYBR Green I dye exhibits little fluorescence. (B) At the annealing temperature, a few dye molecules bind to the double-stranded primer/target, resulting in light emission upon excitation. (C) During the polymerisation step, more and more dye molecules bind to the newly synthesised DNA, and the increase in fluorescence can be monitored in real-time. (D) On denaturation, the dye molecules are released and the fluorescence signal returns to background. *Right:* Hybridisation probe method. The RT step has been omitted. (A) During the denaturation step, both hybridisation probes remain in solution and separate. Any emission from fluorescein is at 530 nm, and is disregarded by the detector. (B) During the annealing step, the probes hybridise in a head-to-tail arrangement, the two dyes come in close proximity and the emitted energy excites the second dye, which emits red fluorescent light at a longer wavelength. (C) At the polymerisation temperature, both probes return into solution and any emissions from fluorescein are ignored.

achieved by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon (Ririe *et al.* 1997). This is done by slowly increasing the temperature above the T_m of the amplicon and measuring the fluorescence. As the T_m of the amplicon depends markedly on its nucleotide composition, it is possible to identify the signal obtained from the correct product. A characteristic melting peak at the melting temperature (T_m) of the amplicon will distinguish it from amplification artefacts that melt at lower temperatures in broader peaks. A second drawback of this procedure is that multiple dye molecules bind to a single amplified molecule and, consequently, the amount of signal generated after irradiation is dependent on the mass of double-stranded DNA produced in the reaction. Assuming the same amplification efficiencies, amplification of a longer product will generate more signal than a shorter one. If amplification efficiencies are different, quantification will be even more inaccurate. This is in contrast to the use of fluorogenic probes (see below), in which the signal from a single fluorophore is observed for each amplified molecule synthesised, regardless of its length.

Hybridisation probes

This method uses two hybridisation probes to maximise specificity (Wittwer *et al.* 1997a) (Fig. 4B). One of the probes carries at its 3' end a fluorescein donor, which emits green fluorescent light when excited by the Lightcycler's light source. Its emission spectrum overlaps the excitation spectrum of an acceptor fluorophore that is attached to the 5' end of the second probe. This probe must be blocked at its 3' end to prevent its extension during the annealing step. Excitation of the donor results in fluorescence resonance energy transfer to the acceptor and the emission of red fluorescent light. In solution, the two dyes are apart, and because the energy transfer depends on the spacing between the two dye molecules, only background fluorescence is emitted by the donor. After the denaturation step, both probes hybridise to their target sequence in a head-to-tail arrangement during the annealing step. This brings the two dyes in close proximity to one another and the fluorescein can transfer its energy at high efficiency. The intensity of the light of longer wavelength emitted by the second dye is measured, with increasing amounts of measured fluorescence proportional to the amount of DNA synthesised during the PCR reaction. A fluorescent signal is detected only as a result of two independent probes hybridising to their correct target sequence. This increases

specificity and generates additional flexibility for probe design. Furthermore, as the probes are not hydrolysed, fluorescence is reversible and allows the generation of melting curves (see below).

Hydrolysis probes

The Taqman assay (Perkin-Elmer–Applied Biosystems) utilises the 5'-nuclease activity of the DNA polymerase to hydrolyse a hybridisation probe bound to its target amplicon. It usually utilises either Taq or Tth polymerase, but any enzyme with an equivalent 5'-nuclease activity properties (e.g. Tfl) can be used (Gut *et al.* 1999). After the reverse transcription step (Fig. 5A), successful quantification requires the annealing of three oligonucleotides to the DNA. Two template-specific primers define the endpoints of the amplicon and provide the first level of specificity (Fig. 5B). Problems associated with DNA contamination are minimised by choosing primers that span at least one intron of the genomic sequence. The additional specificity of this assay is provided by the use of a third oligonucleotide probe that hybridises to the amplicon during the annealing/extension phase of the PCR (Fig. 5C). The probe contains a fluorescent reporter dye at its 5' end, the emission spectrum of which is quenched by a second fluorescent dye at its 3' end. If no amplicon complementary to the probe is amplified during the PCR, the probe remains unbound. As the 5'-exonuclease activity of Taq and Tth polymerase is double-strand-specific (Heid *et al.* 1996), unbound probe remains intact and no reporter fluorescence is detected. Conversely, if the correct amplicon has been amplified, the probe can hybridise to that amplicon after the denaturation step. It remains hybridised while the polymerase extends the primers until it reaches the hybridised probe, when it displaces its 5' end to hold it in a forked structure. The enzyme continues to move from the now free end to the bifurcation of the duplex, where cleavage takes place (Lyamichev *et al.* 1993). This separates the reporter and quencher dyes and releases quenching of reporter fluorescence emission (Fig. 5D,E). The largest fluorescence signal is obtained when the two labels are at the extreme 5' and 3' ends of the probe, probably because of more efficient cleavage by the polymerase (Livak *et al.* 1995).

As the polymerase will cleave the probe only while it remains hybridised to its complementary strand, the temperature conditions of the polymerisation phase of the PCR must be adjusted to ensure probe binding. Most probes have a T_m of around 70 °C; therefore, the Taqman system uses a combined annealing and polymerisation step at

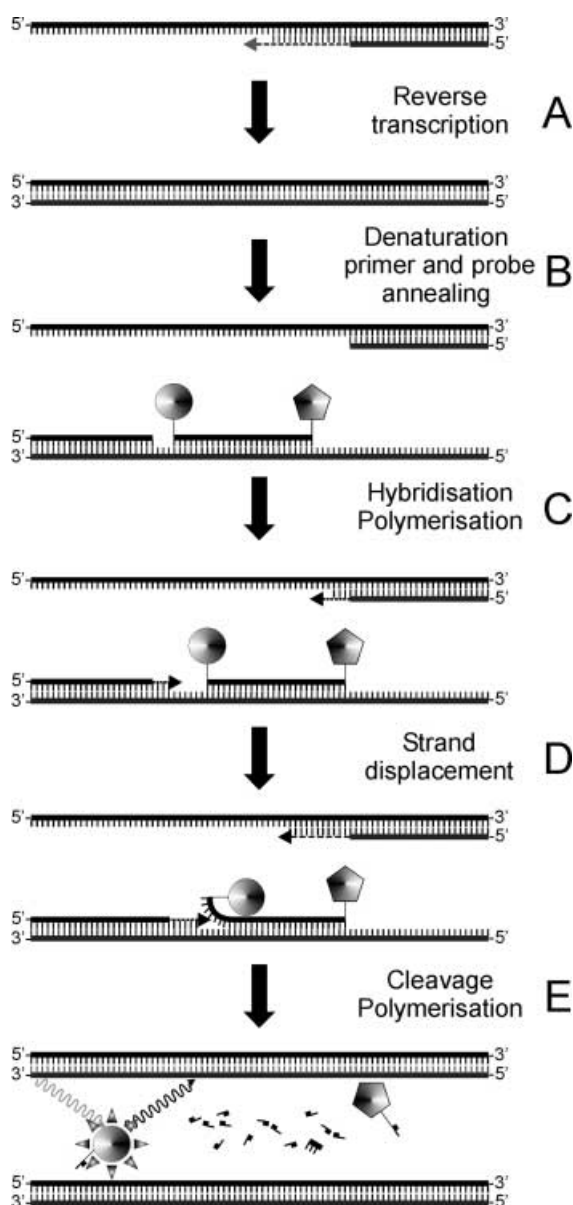


FIGURE 5. The Taqman assay. Three parameters affect the performance of the 5'-nuclease assay: the quenching of the intact probe, its hybridisation efficiency and the efficiency of cleavage by the polymerase. (A) The RT step synthesises a cDNA copy of the mRNA. (B) After denaturation, primers and probe anneal. The proximity of the two dyes quenches the signal from the fluorescent dye at the 5' end of the probe. (C) Polymerisation proceeds at the same temperature as the annealing step. (D) The polymerase displaces and hydrolyses the labelled probe. (E) The fluorescent dye is released from its proximity to the quencher, and fluorescence is detected. This signal is directly proportional to the number of molecules present at the end of the previous or beginning of the current cycle.

60–62 °C. This ensures that the probe remains bound to its target during the primer extension step. It also ensures maximum 5'–3' exonuclease activity of the Taq and Tth DNA polymerases (Tomblin *et al.* 1996). However, it also reduces the processivity of the polymerases, which are required to function at sub-optimal temperatures. For longer amplicons, this may require a longer annealing/polymerisation step and/or increased Mn^{2+} or Mg^{2+} concentration to stabilise probe binding. It also makes this system potentially less efficient and flexible than other real-time systems, especially when mutation-specific RT-PCR is attempted.

Amplicon design

The optimal amplicon length for real-time RT-PCR is less than 100 bp, and we aim to generate amplicons no longer than 80 bp, but have used the Taqman system to detect amplicons as long as 400 bp (Bustin *et al.* 1999). Shorter amplicons amplify more efficiently than longer ones and are more tolerant of reaction conditions. This is because they are more likely to be denatured during the 92–95 °C step of the PCR, allowing the probes and primers to compete more effectively for binding to their complementary targets. As the extension rate of Taq polymerase is between 30 and 70 bases/second (Jeffreys *et al.* 1988), it also means that polymerisation times as short as 15 s are sufficient to replicate the amplicon, making amplification of genomic DNA contaminants less likely and reducing the time it takes to complete the assay. The length of primers and probe required to generate a specific product defines the minimum practical length of the amplicon, which in our hands is 63 bp (Bustin *et al.* 1999).

Primer design

Primers should bind to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA. If the intron/exon boundaries are unknown, or when targeting an intron-less gene, it is necessary to treat the RNA sample with RNase-free DNase. Use of Mn^{2+} rather than Mg^{2+} minimises any problems caused by amplification of re-annealed DNA fragments (Bauer *et al.* 1997). Primer (and probe) selection is based on estimated T_m , the desire for small amplicon size and the location of the probe. They must be designed to very exacting specifications and the Taqman system provides its own primer/probe design program, Primer Express. The current version usually generates appropriate primer/probe sets, but it contains several bugs that can make

primer design difficult, and requires manual fine-tuning. There are several alternative software tools available, with the most useful ones calculating T_m thermodynamically using the nearest-neighbour algorithm (Rychlik *et al.* 1990) – for example, OLIGO (Molecular Biology Insights, Cascade, CO, USA, <http://oligo.net>) or the online oligonucleotide T_m calculator (<http://alces.med.umn.edu/rawtm.html>). Recently, a program has been developed for T_m calculations of matched and mismatched probes designed to run on Microsoft Excel (Schutz & von Ahsen 1999) and is available for academic users as freeware from <http://server1.medikc.med.uni-goettingen.de/meltcalc.htm>. The optimal length for single-stranded primers is about 15–20 bases, and the G/C content should be between 20% and 70%. Their T_m should not differ by more than 1–2 °C, with minimum and maximum T_m of 58 °C and 60 °C, respectively, for Taqman primers. Molecular beacons- and hybridisation probe-associated primers can have a wider range of T_m , but the T_m of any one pair should be similar. Primers are generally used in the 50–200 nM range. Too great a primer concentration may promote mispriming and accumulation of non-specific product. Too low a primer concentration is probably less of a problem for real-time assays, as target copy numbers will have been calculated well before the primer supply is exhausted. Non-specific priming can be minimised by selecting primers that have only one or two G/Cs within the last five nucleotides at the 3' end. This relative instability at their 3' ends makes them less likely to hybridise transiently, and available for non-specific extension by the DNA polymerase.

Probe design

The optimum concentration of fluorogenic probes depends on background fluorescence in addition to primer concentration. The fluorescence of non-hybridised molecular beacons and hybridisation probes is quenched more efficiently than is the fluorescence of uncleaved hydrolysis probes, resulting in significantly reduced background fluorescence and enhanced ability to distinguish the fluorescence of hybridised molecular beacon and hybridisation probes. Probe concentration is usually in the 100 nM range and they must compete for binding to their target with the amplification primers, which are present in a greater concentration. As amplification primers are extended as soon as they bind to their targets, the hybridisation target sequence is rapidly masked with newly synthesised DNA. Therefore, the T_m of the probes must be significantly greater (approximately 10 °C)

than that of the primers, to ensure that they hybridise before the primers. The fluorescence of hybridisation probes is directly correlated to probe hybridisation, hence they must remain hybridised during the annealing step. Conversely, the fluorescence of exonuclease probes is correlated with probe hydrolysis, and they must be available for cleavage during the polymerisation step, to allow fluorescence measurement.

An additional requirement of the two-hybridisation-probe system is that the target sequence should be located towards the 3' end of the amplicon, close to the reverse primer-binding site on the opposite strand. As fluorescence is measured during the annealing phase, this arrangement allows time for the measurement to be made before the probes are displaced by the polymerase. Paired probes used for quantification should have equal T_m , whereas for mutational analysis the T_m of the sensor probe should be less than that of the anchor probe. This ensures that the sensor probe controls the generation of the fluorescent signal.

Most of our probes are about 30 bases in length, and we aim for a G/C content of around 50%. This ensures good specificity and a T_m high enough to be useful under PCR conditions. It also minimises the potential for inter- or intramolecular complementarity, which can interfere with the PCR reaction. The probes should not overlap with, or have sequence complementarity with either of the primers, and Taqman probes should not contain a G at their 5' ends, because such an arrangement quenches reporter fluorescence, even after cleavage.

Threshold cycle

The concept of the threshold cycle (C_t) is at the heart of accurate and reproducible quantification using fluorescence-based RT-PCR (Higuchi *et al.* 1993). Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background (Gibson *et al.* 1996). This point is defined as the C_t , and will always occur during the exponential phase of amplification. Therefore, quantification is not affected by any reaction components becoming limited in the plateau phase, which results in a systematic bias against the more abundant templates and makes any quantification based on measurements of overall product yield intrinsically unreliable.

The reporter signal is normalised to the fluorescence of an internal reference dye (Taqman) or between the three dyes (Lightcycler), to allow for corrections in fluorescent fluctuations caused by changes in concentration or volume, and a C_t value is reported for each sample. This value can be translated into a quantitative result by constructing a standard curve.

Standard curves

Standard curves using fluorescence are easily generated, thanks to the linear response over a large dynamic range. Quantification of mRNA transcription can be either relative or absolute. Relative quantification determines the changes in steady-state transcription of a gene and is often adequate. A relative standard consists of a sample, the calibrator, which is used to create a dilution series with arbitrary units. The calibrator can be any nucleic acid, as long as its concentration and length of amplicon are known. During the RT-PCR assay, the target C_t is compared directly with the calibrator C_t and is recorded as containing either more or less mRNA. In contrast, absolute quantification of transcription allows the precise determination of copy number per cell, total RNA concentration, or unit mass of tissue. It requires the construction of an absolute standard curve for each individual amplicon to ensure accurate reverse transcription and PCR amplification profiles.

There are two ways to prepare an absolute standard curve. The first method involves subcloning the amplicon behind a T7 or SP6 RNA polymerase promoter in a plasmid vector. An *in vitro*-transcribed sense RNA transcript is generated, the sample is digested with RNase-free DNase and the RNA is quantitated accurately. It is important to ensure that the standard RNA template is a single, pure species free from DNA contamination. We take triplicate measurements of its concentration on a spectrophotometer such as the Genequant II (Amersham Pharmacia Biotech, Little Chalfont, UK) and convert the absorbance to a 'target copy number per μg RNA'. The standard curve is generated by performing three independent serial dilutions of the RNA standard and assaying each dilution in duplicate, together with positive and negative control reactions. To maximise accuracy, the dilutions should be made over the range of copy numbers that include the amount of target mRNA expected in the experimental RNA samples. The high and low C_t values are discarded to correct for pipetting error and the remaining four values are averaged to give the final C_t value for that dilution. The C_t value is inversely proportional to

the log of the initial copy number (Higuchi *et al.* 1993). Therefore, a standard curve is generated by plotting the C_t values, with 95% confidence intervals, against the logarithm of the initial copy numbers. The copy numbers of experimental RNAs can be calculated after real-time amplification from the linear regression of that standard curve.

We have found that the second method – use of single-stranded sense-strand oligodeoxynucleotides – is an acceptable alternative to the T7-RNA polymerase-generated standard amplicon. We have compared the sensitivity (y intercept) and amplification efficiency (slope) of a standard amplicon prepared from T7-transcribed RNA and a sense oligodeoxynucleotide and found them to be virtually identical. In contrast, the sensitivity of a double-stranded DNA amplicon standard is increased, although the amplification efficiency is unchanged (Overbergh *et al.* 1999). The advantage of using a single-stranded sense oligodeoxynucleotide is that it significantly simplifies the process of obtaining a standard curve for amplicons up to 100 bp, which encompasses most real-time RT-PCR amplicons.

We have constructed standard curves for many target amplicons and have detected differences in both their amplification efficiencies and their sensitivities. This is illustrated by the standard curves for eIF4E (74 bp), GAPDH (226 bp) and Cox-2 (69 bp) shown in Fig. 6. The sensitivity of the eIF4E RT-PCR (y intercept 39.88) is significantly greater than that of the other two, whereas its amplification efficiency (slope – 2.8666) is similar to that of GAPDH (– 3.0635) and better than that of Cox-2 (– 3.893). In our hands, the most sensitive primer/probe combination, detecting the 86 bp guanylyl cyclase C amplicon, can reliably detect as few as 13 copies of mRNA (Bustin *et al.* 1999). Conversely, the least sensitive combination could detect only 1×10^4 or more copies of BRF-1 mRNA, an amplicon of 76 bp (Bustin & McKay 1999). In most cases, the lower detection limit is around 50–500 copies of target mRNA.

Reproducibility

The RT-PCR reaction is fraught with hazards (Freeman *et al.* 1999) and is characterised by significant variation and non-reproducibility, even with identical samples between different laboratories (Keilholz *et al.* 1998). Its reproducibility is inevitably compromised by the variable efficiency of the RT itself and the need for two sequential enzymatic steps (Bishop *et al.* 1997). The exponential nature of PCR amplification, together with the small quantities of target molecules, means that trivial variations in reaction components and

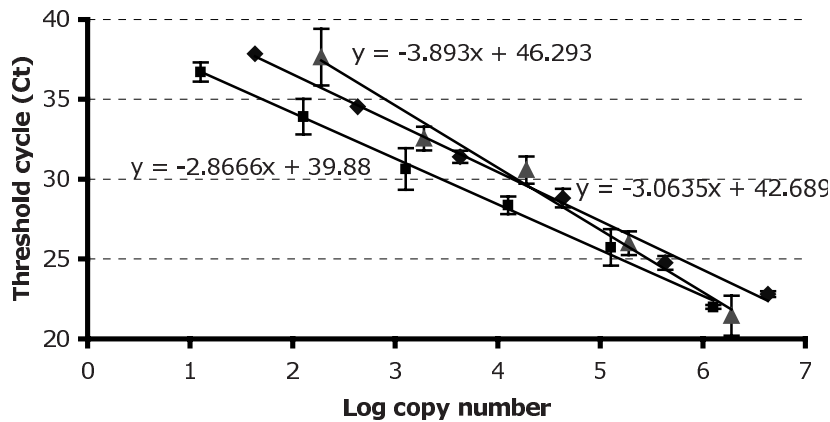


FIGURE 6. Standard curves for GAPDH (◆), eIF4E (■) and Cox-2 (▲) amplicons in 'one-enzyme/one-tube' reactions. The plot of the log of initial target copy number versus C_t is a straight line. Amplicons lengths are 226 bp, 74 bp and 69 bp respectively. A 5-min reverse transcription step at 60 °C was followed by 40 cycles of a 20-s denaturation step at 92 °C and a 30-s annealing/polymerisation step at 62 °C. Tth polymerase can add approximately 40–50 bases per second at 62 °C. The error bars indicate the 95% confidence intervals. The smaller the C_t value (y intercept) in the regression equation, the greater the sensitivity of the RT-PCR reaction.

thermal cycling conditions and mispriming events during the early stages of the PCR can greatly influence the final yield of the amplified product (Wu *et al.* 1991). This has created considerable uncertainty about the usefulness and reliability of the RT-PCR assay (Sokoloff *et al.* 1996), especially when used as a routine clinical diagnostic assay (Gala *et al.* 1998, de la Taille *et al.* 1999). It is a particular problem when cancer patients are screened for the presence of micrometastatic disease (Dingemans *et al.* 1997, Zippelius *et al.* 1997, Henke & Loening 1998) and severely limits its usefulness for prognosis and patient surveillance (Lambrechts *et al.* 1998). Clearly, there is an urgent requirement for stringent quality control (Keilholz 1998), standardisation of the sample acquisition (Jung *et al.* 1997) and processing processes (Schittek *et al.* 1999), the RT-PCR procedure (Raj *et al.* 1998) and the detection of the RT-PCR products (Hughes & Goldman 1990).

Optimisation and consistency are as critical for obtaining reproducible results using real-time RT-PCR as they are for conventional methods. However, there can be no doubt that real-time RT-PCR assays are significantly less variable than any conventional RT-PCR procedure. Where measured, the coefficient of variation for C_t data has been shown to be very low, at less than 2% for the Taqman (Heid *et al.* 1996, Gerard *et al.* 1998) and as little as 0.4% for the Lightcycler (Wittwer *et al.* 1999a), which is significantly better than the 14% reported for conventional

RT-PCR (Zhang *et al.* 1997). In our experience using Taqman, the variability between duplicates and triplicates within the same run, and between different runs, is usually between 0 and 5%.

Reproducibility is influenced by parameters such as distribution statistics (Poisson's law) (de Vries *et al.* 1999), and C_t data are less reproducible when working with very low copy numbers, because of the stochastic effects in the quantification of small numbers of target molecules (Peccoud & Jacob 1996). Particle distribution statistics predict that it will require a much greater number of replicates to differentiate five from 10 copies of RNA than for the differentiation of 5000 from 10 000 copies. Of course, this emphasises the importance of repetitive testing in clinical samples. We have seen some evidence that C_t values obtained from short amplicons show greater reproducibility than those obtained from long ones. It is also likely that amplification efficiency influences reproducibility. Regardless, 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.

Normalisation

RT-PCR-specific errors in the quantification of mRNA transcripts are easily compounded by any

variation in the amount of starting material between samples. This is especially relevant when the samples have been obtained from different individuals, and will result in the misinterpretation of the expression profiles of the target genes. Consequently, the question of what constitutes an appropriate standard arises (Thellin *et al.* 1999), and constitutes an important aspect of experimental design.

The accepted method for minimising these errors and correcting for sample-to-sample variation is to amplify, simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values can be normalised (Karge *et al.* 1998). The ideal internal standard should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by the experimental treatment. In addition, an endogenous control should also be expressed at roughly the same level as the RNA under study. In the absence of any one single RNA with a constant level of expression in all these situations (Haberhausen *et al.* 1998, Thellin *et al.* 1999), three RNAs are most commonly used to normalise patterns of gene expression. The mRNAs specifying the housekeeping genes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and β -actin and are ribosomal RNAs (rRNA), although other mRNAs have been used occasionally – for example histone H3 (Kelley *et al.* 1993) and cyclophilin (Haendler *et al.* 1987).

β -Actin

β -Actin mRNA is expressed at moderately abundant levels in most cell types and encodes a ubiquitous cytoskeleton protein. It was one of the first RNAs to be used as an internal standard, and it is still advocated as a quantitative reference for RT-PCR assays (Kreuzer *et al.* 1999). This is despite widespread evidence that its levels of transcription can vary widely in response to experimental manipulation in human breast epithelial cells (Spanakis 1993), and blastomeres (Krussel *et al.* 1998), as well as in various porcine tissues (Foss *et al.* 1998) and canine myocardium (Carlyle *et al.* 1996). In addition, the presence of pseudogenes interferes with the interpretation of results (Dirnhofer *et al.* 1995, Raff *et al.* 1997, Mutimer *et al.* 1998) and primers commonly used for detecting β -actin mRNA amplify DNA also (Dakhama *et al.* 1996).

GAPDH

The RNA encoding GAPDH is a ubiquitously expressed, moderately abundant message. It is

frequently used as an endogenous control for quantitative RT-PCR analysis because, in some experimental systems, its expression is constant at different times and after experimental manipulation (Edwards & Denhardt 1985, Winer *et al.* 1999). However, there is overwhelming evidence suggesting that its use as an internal standard is inappropriate (Oliveira *et al.* 1999, Thellin *et al.* 1999). GAPDH concentrations vary significantly between different individuals (Bustin *et al.* 1999), during pregnancy (Cale *et al.* 1997), with developmental stage (Puissant *et al.* 1994, Calvo *et al.* 1997), during the cell cycle (Mansour *et al.* 1993) and after the addition of the tumour promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (Spanakis 1993), dexamethasone (Oikarinen *et al.* 1991) and carbon tetrachloride (Goldsworthy *et al.* 1993). Insulin stimulates GAPDH transcription (Rolland *et al.* 1995, Barroso *et al.* 1999) through multiple insulin-responsive elements in its promoter (Alexander *et al.* 1988, Nasrin *et al.* 1990), at least one of which is recognised by C/EBP (Alexander-Bridges *et al.* 1992). Similarly, the calcium ionophore A23187 induces GAPDH transcription through specific motifs in its promoter (Chao *et al.* 1990). Growth hormone (Freyschuss *et al.* 1994), vitamin D (Desprez *et al.* 1992), oxidative stress (Ito *et al.* 1996), hypoxia (Graven *et al.* 1994, Zhong & Simons 1999), manganese (Hazell *et al.* 1999) and the tumour suppressor TP53 (Chen *et al.* 1999), have all been shown to activate its transcription, which is also inducible in endothelial cells (Rimarachin *et al.* 1992) and during apoptosis (Ishitani *et al.* 1997). Furthermore, it has been shown to be upregulated in cancer – for example in rat hepatomas (Chang *et al.* 1998), malignant murine cell lines (Bhatia *et al.* 1994) and human prostate carcinoma (Ripple & Wilding 1995). Conversely, food deprivation (Yamada *et al.* 1997) and retinoic acid (Barroso *et al.* 1999) downregulate GAPDH transcription in the gut and in adipocytes, respectively. Finally, numerous transcription regulatory domains have been identified in the yeast GAPDH promoter, again suggesting that this gene is subject to complex transcriptional regulation (Yagi *et al.* 1994).

We have previously used real-time RT-PCR to demonstrate that there is wide variation in the levels of transcription of GAPDH mRNA in nucleated blood cells, between individuals and between samples taken from the same individual at different time points (Bustin *et al.* 1999). An analysis of colon epithelial samples taken from an additional 51 patients corroborates these earlier findings (Fig. 7). Copy numbers ranged from 7.9×10^4 to 9.6×10^9 , with a median of 3.4×10^8 copies of GAPDH mRNA/ μ g total RNA. This confirms that the use of

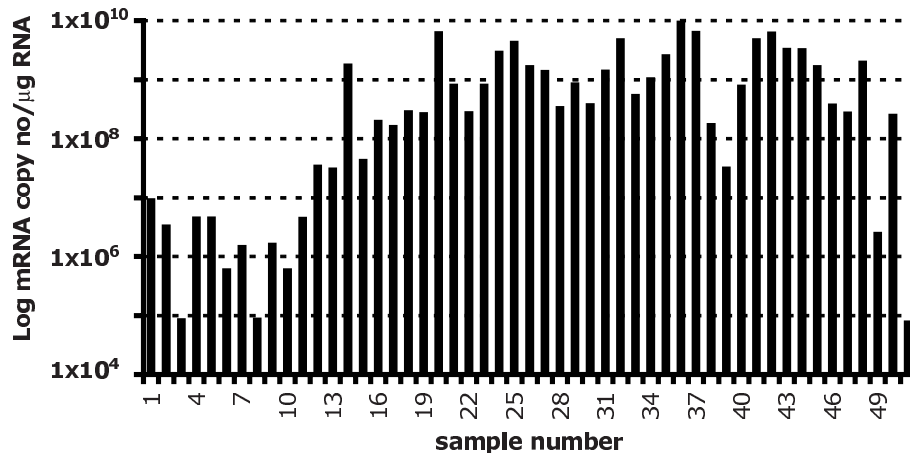


FIGURE 7. GAPDH mRNA levels in normal colon epithelium from 51 individuals. Fifty nanograms total RNA from normal colon epithelium was analysed by quantitative RT-PCR analysis using the primers, probe and one-enzyme/one-tube reaction conditions described above. mRNA copy numbers were calculated from the GAPDH standard curve generated in Fig. 6.

GAPDH to normalise RNA levels in experiments that involve using mRNA from different individuals is inaccurate and should be discouraged. In light of all this evidence, it is a mystery why GAPDH continues to find favour as an internal standard.

rRNA

rRNAs, which constitute 85–90% of total cellular RNA, are useful internal controls, as the various rRNA transcripts are generated by a distinct polymerase (Paule & White 2000) and their levels are less likely to vary under conditions that affect the expression of mRNAs (Barbu & Dautry 1989). Indeed, they have been shown to be more reliable than either of the housekeeping genes in rat livers (de Leeuw *et al.* 1989), human skin fibroblasts (Mansur *et al.* 1993) and human (Zhong & Simons 1999) and mouse (Bhatia *et al.* 1994) malignant cell lines. However, rRNA transcription can be affected by biological factors and drugs (Spanakis 1993) and the variation in levels of transcription between samples taken from different individuals has not been quantified. In addition, there are two other drawbacks to its use: rRNA cannot be used for normalisation when quantitating targets that have been enriched for mRNA because it is lost during mRNA purification, and rRNA is expressed at much greater levels than target mRNAs.

Alternatives

Clearly, this raises questions about how to compare copy numbers between samples obtained from

different individuals or from different tissues. For RNA extracted from tissue culture cells or from blood – for example when haematological malignancies are under investigation – target gene copy numbers can be readily expressed as copy numbers/cell. This is not possible when the RNA has been extracted from solid tissue or cancers. Therefore, it is necessary to find another means of normalising their copy numbers. Until we identify a more appropriate measure, in our laboratory we relate all copy numbers to total RNA concentration, which we measure accurately using a Genequant II spectrophotometer (Amersham Pharmacia Biotech). Copy numbers of target genes are then recorded as copy number per μg total RNA. There are obvious limitations to this approach. Total RNA levels may be increased in highly proliferating cells and this will affect the accuracy of any comparison of absolute copy numbers between normal and tumour cells. As rRNA makes up the bulk of total RNA, the reservations expressed earlier also apply. In addition, the accuracy of the quantification will be critically dependent on the accuracy of RNA measurement. The major disadvantages of the A_{260} method are the large relative contribution that proteins and free nucleotides make to the signal, the inability to distinguish between DNA and RNA, the interference caused by contaminants commonly found in nucleic acid preparations, and the relative insensitivity of the assay. However, the use of fluorescent nucleic acid stains – for example RiboGreen RNA quantitation (Molecular Probes, Eugene, OR, USA) – alleviates many of these problems and can detect as little as 5 ng/ml RNA (Jones *et al.* 1998).

In our hands, there is good agreement between levels of transcription of various mRNAs calculated on the basis of total RNA concentration and published data using other techniques, such as immunocytochemical detection of their translation products. Nevertheless, it must be clearly stated that none of the methods used for standardisation between samples is completely satisfactory, and individual experimental conditions will dictate what the most appropriate normaliser should be. Clearly, the ability to achieve absolute quantification of target nucleic acids makes the search for a relevant and universal means of comparing and normalising between samples very urgent.

APPLICATIONS

Quantification

Straightforward quantification is easily accomplished by any of the methods described above, and there is probably little to choose between them in terms of their sensitivity, although hybridisation-based assays are more specific than those dependent on DNA-binding dyes and there is some evidence that molecular beacons are a little more specific than linear probes (Tyagi *et al.* 1998). The Taqman assay has been used to quantitate transcription from individual cells (Fink *et al.* 1998) and paraffin-embedded tissue (Sheils & Sweeney 1999), to investigate tissue-distribution proteins associated with signal transduction (Bustin & McKay 1999), for cytokine mRNA profiling from fresh (Overbergh *et al.* 1999) and stored frozen cells (Kruse *et al.* 1997) and for quantitating the RNA component of human telomerase (Yajima *et al.* 1998). Its potential applications in medical diagnostics are wide-ranging and it has been used to screen serum for the diagnosis of genetic diseases (Kaplan *et al.* 1992), the presence of viral (Morris *et al.* 1996, Berger *et al.* 1998, Mercier *et al.* 1999) and bacterial (Norton & Batt 1999) pathogens, detection of colorectal (Bustin *et al.* 1999) and melanoma (de Vries *et al.* 1999) cells in patients' blood, and quantification of *c-myc* (Bieche *et al.* 1999a) or ERBB2 (Bieche *et al.* 1999b) transcription in breast cancer.

Molecular beacons have been used to detect RNAs from a single cell (Steuerwald *et al.* 1999) and from within living cells (Matsuo 1998, Sokol *et al.* 1998) and for other standard research (Lewin *et al.* 1999) and diagnostic (Arnold *et al.* 1999) RT-PCR applications.

The range of applications for the Lightcycler are similarly wide, and include detection of minimal residual disease (Eckert *et al.* 2000), cancer cells in

the peritoneal cavity of patients with gastrointestinal and gynaecological malignancies (Nakanishi *et al.* 1999) and pathogens (Mathews *et al.* 1999), and monitoring delivery, expression and maintenance of transgenes (Thorpe & Porteous 1999).

Mutation/allele detection

The flexibility of the Lightcycler design currently gives it an advantage over the Taqman system for the detection of mutations or allelic variants and it has been used extensively in molecular diagnostics (Aslanidis *et al.* 1999, Nakao *et al.* 2000). Two alternative procedures depending on melting-curve analysis have been developed. The first uses a sense primer internally labelled with an acceptor dye together with a donor-labelled probe that is completely complementary to the wild-type sense strand (Lay & Wittwer 1997, Bernard *et al.* 1998a). After asymmetric amplification, the probe is annealed to the extension product of the labelled primer. This brings the fluorophores into contact close enough for resonance energy transfer to occur. As the temperature increases, the probe melts from its target, decreasing the resonance energy transfer. If there is no mismatch, melting occurs at a characteristic temperature that is greater than if a single base mutation is present (Bernard *et al.* 1998b, Cane *et al.* 1999, Aoshima *et al.* 2000).

The second procedure is similar, but uses two hybridisation probes (Bernard *et al.* 1999, Bollhalder *et al.* 1999). One, the sensor, covers the site of the mutation, and the other one, the anchor, is designed to be longer, with a greater T_m and generates the fluorescent signal. The probe hybridisation status is monitored continuously as the temperature is slowly increased and characteristic, sequence-specific probe melting curves are generated. A single base mismatch under the probe decreases the T_m by as little as 3 °C for G–T and as much as 10 °C for A–C mismatches. The recent introduction of peptide nucleic acids (PNA) (Corey 1997) offers additional scope for this technology. These DNA mimics can bind DNA independently of salt concentration and demonstrate significantly greater specificity in binding to complementary DNA, with the effect that a PNA–DNA mismatch is more destabilising than a DNA–DNA mismatch.

The Taqman system has also been used for allelic discrimination by using probes labelled with different fluorescence reporter dyes (Lee *et al.* 1993, Livak 1999). The assay is performed under competitive conditions with both probes present in the same tube. Under the correct conditions, there is preferential binding of the exact-match probe, which blocks hybridisation of the mismatched

probe. In addition, the mismatch itself has a disruptive effect on hybridisation and speeds up the complete dissociation from the template. Both effects result in inefficient cleavage of the mismatch probe. Consequently, an increase in either fluorescent signal indicates homozygosity, whereas an increase in both signals indicates heterozygosity. A recent improvement involves the use of probes containing 5-propyne-2'-deoxyuridine in place of thymidine, which increases the T_m relative to standard Taqman probes and is useful when amplifying sequences with high A/T content. Nevertheless, the need to carry out the PCR reaction between 60 °C and 62 °C makes it more difficult to achieve perfect discrimination between mismatch and exact-match probes. This is of particular relevance when attempting mutant allele-specific-type amplifications from samples in which only a minority of the templates will contain the targeted mutation – for example detecting mutant K-ras in the blood of patients with colorectal cancer.

Multiplex RT-PCR

Detailed diagnostic and scientific analyses are often restricted by the limited availability of biological material. Multiplex RT-PCR is a time- and reagent-saving amplification technique in which multiple primer sets are used to amplify multiple specific targets simultaneously (Edwards & Gibbs 1994) from the same sample. In principle, all real-time detection systems are capable of simultaneous detection of multiple fluorescent dyes. However, successful multiplexing is never trivial, and requires careful consideration about the suitability of both chemistry and instrumentation. One limitation arises from the limited availability of multiple fluorescent reporters with good spectral resolution. Both the hydrolysis and hybridisation probe methodologies require the interaction of two different fluorescent dyes per target, and each probe or probe set requires a different pair of fluorophores. Consequently, fewer probes can be distinguished from each other in a multiplex assay. Molecular beacons, in contrast, use a universal, non-fluorescent quencher and require only a single fluorophore per probe. Therefore, use of molecular beacons has the potential to be used to identify more targets per reaction. A second limitation is that the use of fluorophores is dependent on the excitation and detection characteristics of the individual spectrofluorimetric thermal cyclers. The ABI 7700 uses a laser with a fixed excitation wavelength, which limits the fluorophores to those that can be efficiently excited by its blue laser, preventing the

use of fluorophores that fluoresce in the far-red wavelengths. In addition, it can detect fluorescence only between 500 nm and 660 nm. The three-channel fluorimeter design of the Lightcycler restricts it to detecting a maximum of three fluorescent dyes simultaneously, whereas the Biorad instrument has the potential to excite and detect emission from the widest range of fluorophores; however, the latter has been launched only recently.

There are relatively few reports of multiplexing using any of these systems. The Taqman system has been used successfully to quantitate up to three (Sharma *et al.* 1999, Shin *et al.* 1999) targets, with no apparent reduction in sensitivity compared with the individual reactions (Moody *et al.* 2000). We have tried to use the Taqman procedures for K-ras codon 12 mutant-specific multiplexing, but have had inconsistent results. The non-fluorescent quencher used by molecular beacons allows the quantification of four pathogenic retroviruses simultaneously in blood (Vet *et al.* 1999) and the Lightcycler has been used to detect two independent targets simultaneously (Bernard *et al.* 1999, Lee *et al.* 1999a, von Ahnen *et al.* 2000).

The potential for combining several dyes with instrumentation that allows synchronous scanning of excitation and emission wavelengths has been demonstrated with the detection of six PCR products in one reaction (Lee *et al.* 1999b). In addition, the recent introduction of the Biorad instrument with a variable-wavelength light source and a high-resolution detector should allow the use of a wider range of fluorophores and the accommodation of more probes in the same assay.

The final difficulty is associated with limitations caused by mutual interference of multiple sets of PCR primers, which can reduce the dynamic range of the sensitivity and make quantification unreliable. Furthermore, the efficiency of multiplex detection is significantly affected by the extension time and the concentrations of dNTPs, primers and MgCl₂ (Seoh *et al.* 1998, James 1999). Vastly different levels of target mRNAs will cause quantification problems even for real-time detection, as the exponential phase of amplification of the less abundant mRNA will not overlap with that of the highly abundant target. Therefore, in practice, if accurate quantification is the main aim, it is probably best to limit multiplexing to the detection of two or three transcripts.

Detection of mRNA splice variants

Alternative splicing of RNAs is an important component of cell-specific gene regulation that contributes to the fine-tuning of gene expression

and the functional diversification of proteins (Lopez 1998). It permits an additional tissue-specific level of regulation, as the mRNA can be transcribed from the same promoter, but in each tissue the RNA transcript can be spliced in a particular way to produce a different mRNA and hence protein. Alternative RNA splicing is widespread, with the Alternative Splicing Database (ASD) currently listing 1922 protein and 2486 DNA sequences (Dralyuk *et al.* 2000). The combined amplification/hybridisation approach of real-time RT-PCR assays is ideally suited for the detection and quantification of known splice variants. For example, hormonal regulation of gene expression can occur through the direct regulation of mRNA splicing (Chew 1997). The role of these alternatively spliced products in normal physiology has been difficult to determine, partly because detection of alternative transcripts using conventional procedures is time-consuming (Winkler *et al.* 1999) and their quantification, although reasonably accurate, requires extensive additional processing (Robinson *et al.* 1997), as discussed previously. The specificity of the real-time RT-PCR assay allows the detection of multiple mRNA splice variants, as they can be distinguished reliably by a combination of exon-specific primers and an appropriate probe (Kafert *et al.* 1999) (Fig. 8). Probes designed to hybridise to specific exon splice junctions will detect and allow quantification of individual splice variants (Fig. 8A,C,D). Alternatively, probes designed to hybridise wholly within an exon will result in the more general identification of all mRNAs defined by the exon-specific upstream and downstream primer sequences (Fig. 8B). Therefore, judicious arrangement of primer/probe combinations allows maximum information about the presence, and the levels of, any number of mRNA splice variants to be gathered with a minimum of complexity and trouble. Importantly, because the results obtained are ratios of one splice variant to another, the problems of normalisation become irrelevant.

Cost considerations

In technical terms, there is very little difference between the hybridisation-dependent real-time RT-PCR systems and they easily outperform conventional RT-PCR assays. Their main problem is the requirement for dedicated, sophisticated spectrofluorimetric thermal cyclers, which are expensive to purchase and maintain. The ABI 7700 Prism is significantly more expensive to purchase than either the Lightcycler or Biorad's instrument, and all three are more likely to be part of a core facility than on the bench in every laboratory.

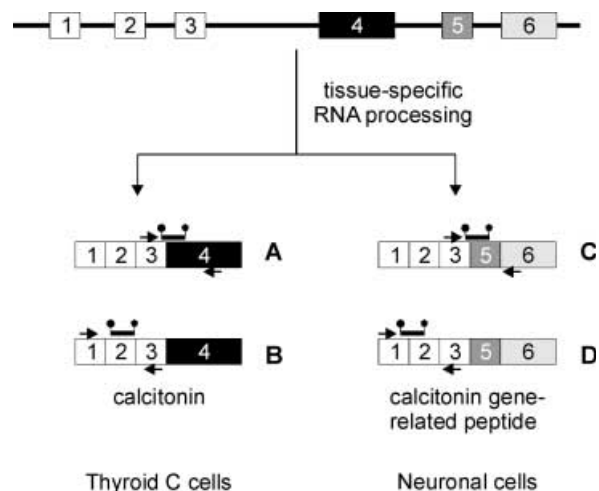


FIGURE 8. Detection of splice variants of the calcitonin/calcitonin gene-related peptide gene. This gene undergoes alternative mRNA processing in thyroid C cells and in neuronal cells to generate two mRNA molecules specifying two distinct peptide hormones. (A,C) The two splice variants can be distinguished easily through the use of splice-junction-specific probes. This permits simple comparison between the relative levels of transcription of the two splice variants. (B,D) The levels of either mRNAs, or any other possible variants, can be quantified by designing a primer/probe set that amplifies the common 5' end of the mRNA. Again, the probe can be designed to hybridise either to a splice junction or to one exon only.

However, as the opportunities for using quantitative RT-PCR in a wide variety of settings continue to expand, it is likely that more cost-effective solutions will be developed that will make this assay more easily accessible.

DNA dye-binding assays are the cheapest option for performing kinetic RT-PCR assays, as they do not require an amplicon-specific probe. Hybridisation analyses require a separate primer set, together with fluorescent-labelled probe for each amplicon under investigation, which can make gene expression profiling experiments costly. In addition, assays carried out on the ABI 7700 have an absolute requirement for a reference dye in the reaction mixture. That reaction buffer can be obtained only as part of a complete kit, resulting in additional expense. In combination, all this adds significantly to the costs of performing quantitative real-time RT-PCR assays. However, kinetic RT-PCR primers and probes are designed according to very strict criteria, with narrowly defined divalent cation and T_m ranges. Together with the reliability and ease of these assays, this reduces the need to waste reagents for assay optimisation. Furthermore, their

specificity obviates the need to sequence or use Southern blotting to confirm the identity of the amplicons, and produces significant saving on researchers' time. We have designed and used Taqman primer/probe sets for more than 100 mRNA targets, and have achieved acceptable amplification every time, without any need for optimisation. The ability to obtain immediate results also compensates for the increased consumables cost incurred, as does the ease with which numerous templates can be quantitated in a very short period.

CONCLUSIONS

Kinetic, or real-time, fluorescence RT-PCR significantly simplifies and accelerates the process of producing reproducible quantification of mRNAs. With the use of appropriate standard curves, absolute copy numbers of mRNA can easily be calculated. Several chemistries are available and their combination of amplification with simultaneous hybridisation means that the high sensitivity of the assay is not compromised by concerns about its specificity. In addition, kinetic RT-PCR assays are readily standardised, making the comparison of results from different laboratories easier and more reliable. Therefore, real-time RT-PCR must be the method of choice for any experiments requiring sensitive, specific and reproducible quantification of mRNA.

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