

Analysis of mRNA Expression by Real-Time PCR

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Abstract

The last few years have seen the transformation of the fluorescence-based real-time reverse transcription polymerase chain reaction (RT-PCR) from an experimental tool into a mainstream scientific technology. Assays are simple to perform, capable of high throughput, and combine high sensitivity with exquisite specificity. The technology is evolving rapidly with the introduction of new enzymes, chemistries and instrumentation and has become the “Gold Standard” for a huge range of applications in basic research, molecular medicine, and biotechnology. Nevertheless, there are considerable pitfalls associated with this technique and successful quantification of mRNA levels depends on a clear understanding of the practical problems and careful experimental design, application and validation.

Introduction

The conventional reverse transcription polymerase chain reaction (RT-PCR) (Simpson *et al.*, 1988; Vrieling *et al.*, 1988) is unrivalled as a sensitive assay for the rapid, inexpensive and simple detection of RNA. Importantly, the ability to amplify several templates in a single reaction (multiplexing) (Edwards *et al.*, 1994) has made it a truly high throughput assay (Willey *et al.*, 1998).

However, for a long time quantitative RT-PCR was perceived as a qualitative assay capable of answering yes/no questions only, since even minor variations in reaction components and thermal cycling conditions can greatly affect the yield of any amplified product (Wu *et al.*, 1991). However, the publication of several ground-breaking reports addressing the fundamental theoretical and practical issues associated with quantification (Kelley *et al.*, 1993; Nedelman *et al.*, 1992; Raeymaekers, 1993; Siebert *et al.*, 1992) led to a more general recognition of the inherent quantitative capacity of the PCR assay (Halford *et al.*, 1999). This development was advanced by the obvious need for quantitative data, *e.g.* for measuring viral load in HIV patients (Kappes *et al.*, 1995), monitoring of occult disease in cancer (Bustin *et al.*, 1998) or examining the genetic basis for individual variation in response to therapeutics through pharmacogenomics (Jung *et al.*, 2000).

Conventional quantitative protocols rely either on competitive techniques where known amounts of RT-PCR-amplifiable competitors are spiked into the RNA samples prior to the RT step (Freeman *et al.*, 1999) or on non-competitive methods where the target is co-amplified with a second RNA molecule with which it shares neither the primer recognition sites nor any internal sequence (Reischl *et al.*, 1999). However, the lack of standardisation produces strikingly inconsistent results (Apfalter *et al.*, 2001; Mahony *et al.*, 2000; Smieja *et al.*, 2001), with variable reproducibility (Henley *et al.*, 1996; Souzae *et al.*, 1996), false positive rates as high as 28% (Johnson *et al.*, 1999) and error rates ranging between 10% and 60% dependent on the analysis method (Souzae *et al.*, 1996; Zhang *et al.*, 1997a; Zhang *et al.*, 1997b). Equally as important, conventional quantification protocols

are, without exception, labour- and reagent-intensive (Raeymaekers, 1993), with post-PCR processing particularly likely to result in subjective interpretation of results.

The real-time fluorescence-based quantitative RT-PCR (qRT-PCR) combines the amplification and analysis steps of the PCR reaction, thereby eliminating the need for post-PCR processing. It does this by monitoring the amount of DNA produced during each PCR cycle and its sensitivity, specificity and wide dynamic range have revolutionised the approach to PCR-based quantification of RNA, making it the method of choice for quantitating steady-state mRNA levels (Bustin, 2000). The method is based on the principle that there is a quantitative

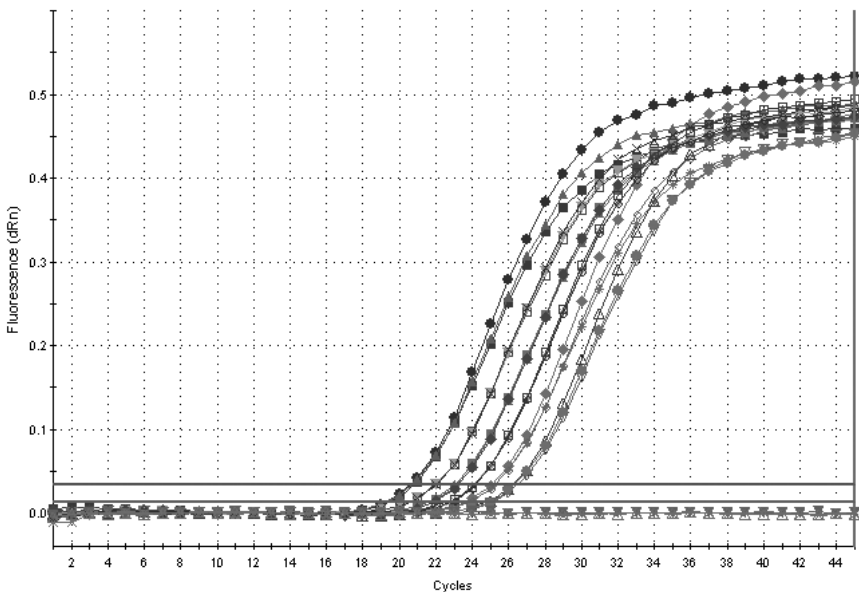


Figure 1. Determination of CT. First, the levels of background fluorescence are established for a particular run. Next, platform-specific algorithms are used to define a fluorescence background threshold. Finally, the algorithm searches the data from each sample for a point that exceeds the baseline. The cycle at which this point occurs is defined as the CT. It is dependent on the starting template copy number, the efficiency of PCR amplification, efficiency of cleavage or hybridization of the fluorogenic probe and the sensitivity of detection. The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number.

relationship between the amount of target RNA present at the start of the assay and the amount of product amplified during its exponential phase. The crucial conceptual innovation and the key to understanding quantification by qRT-PCR is the threshold cycle (CT) (Figure 1).

The development of robust chemistries (as discussed in chapter 3) and the cost-reduction caused by the introduction of several competing combined thermal cycler/fluorimeters has converted qRT-PCR into a simple and relatively affordable assay and has transformed it from an experimental tool into a mainstream scientific technology (Ginzinger, 2002). Its convenience (Ke *et al.*, 2000a), detection range (Gerard *et al.*, 1998), low inter-assay variation (Wall S.J. *et al.*, 2002) and reproducibility (Max *et al.*, 2001) have made it the method of choice for the quantification of mRNA expression (Bustin, 2000; Bustin, 2002). Currently its only real drawback is the substantial monetary investment in instrumentation and reagents that is required to carry out an assay.

RNA Purification

A cell's ability to adjust its mRNA copy numbers in response to environmental changes is a crucial element in the complex regulation of gene expression. In addition, a wide spectrum of pathological processes is associated with changes in gene expression at the mRNA level. These alterations are regulated by numerous endogenous and exogenous stimuli and are the result of at least two processes: (1) increased or reduced transcription caused by highly complex combinatorial control mechanisms (Hill *et al.*, 1995) and (2) changes to mRNA stability, with the degradation rates of different mRNAs varying over a broad range (Ross, 1995).

Therefore, extraction and analysis of cellular RNA provides a snapshot of steady-state mRNA levels and the preparation of the RNA is arguably the most important determinant of a meaningful qRT-PCR experiment. This is tricky as (1) the original expression profile must be conserved, (2) RNA must be of the highest quality, (3) it should be free of DNA and (4) no inhibitors of the RT or PCR steps must be co-purified. The

first problem is that the sources of mRNA are disparate and include not just undemanding *in vitro* material such as tissue culture cells, but *in vivo* biopsies obtained during endoscopy, post-surgery, post-mortem and from archival materials. Naked RNA is extremely susceptible to degradation by endogenous ribonucleases (RNases) 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 (Lee *et al.*, 1997). A second problem for experiments quantitating mRNA levels is that mRNA expression profiles can change rapidly after cells or tissue samples have been collected, but before they have been frozen. This is a particular problem with cells extracted from whole blood (Keilholz *et al.*, 1998) and mRNA profiles can change over several orders of magnitude even in the short time it can take from collecting the blood to processing it in the laboratory. Causes are RNA degradation, the induction of certain genes, *e.g.* Cox-2, as well as the method of RNA preparation (de Vries *et al.*, 2000). Furthermore, blood is notorious for containing numerous inhibitors of the PCR reaction (Akane *et al.*, 1994; Al Soud *et al.*, 2001; Fredricks *et al.*, 1998). Therefore, appropriate sample acquisition has a major influence on the quality of the RNA and subsequently on any result of qRT-PCR assays (Vlems *et al.*, 2002) and once a biological sample has been obtained, immediate stabilization of RNA is the most important consideration (Madejon *et al.*, 2000).

Recent reports suggest that RNA extracted from formalin-fixed, paraffin-embedded archival materials can be successfully quantitated by real-time RT-PCR assays. Between 60 % (Mizuno *et al.*, 1998) and 84% (100% of samples less than 10 years old) (Coombs *et al.*, 1999) of templates can be amplified by RT-PCR, and it is possible to quantitate mRNA expression levels (Cohen *et al.*, 2002; Godfrey *et al.*, 2000; Specht *et al.*, 2001; Stanta *et al.*, 1998a; Stanta *et al.*, 1998b), even after immunohistochemical staining (Fink *et al.*, 2000). This is exciting, since retrospective analysis of archival tissue could enable the correlation of molecular findings with the patient's response to treatment and eventual clinical outcome. Real-time RT-PCR assays, with their amplicon lengths of around 100 bp, are ideally placed to

amplify the usually degraded RNA from these archival samples whose average size is 200-250 nucleotides (Bock *et al.*, 2001; Lehmann *et al.*, 2001).

Just as real-time chemistries have revolutionized the PCR reaction, so the development of the Agilent Bioanalyzer together with the RNA 6000 LabChip has revolutionized the ability to analyze RNA samples for quality. This technology is without doubt the method of choice for analyzing RNA preparations destined to be used in qRT-PCR assays. The Agilent 2100 Bioanalyzer is a small bench top system that integrates sample detection, quantification, and quality assessment. It does this by using a combination of microfluidics, capillary electrophoresis and fluorescent dye that binds to nucleic acid. Size and mass information is generated by the fluorescence of the nucleic acid molecules as they migrate through the channels of the chip. The instrument software quantitates unknown RNA samples by comparing their peak areas to the combined area of the six reference RNAs. It has a wide dynamic range and can quantitate as little as 5 ng/μl, although it is most accurate at concentrations above 50 ng/μl. An electropherogram of fluorescence versus time and a virtual gel image are generated, and the software assesses RNA quality by using the areas under the 28S and 18S rRNA peaks to calculate their ratios. The analysis of a total RNA sample from two human colon biopsies is shown in Figure 2.

qRT-PCR Assay

A successful qRT-PCR experiment is made up of several steps that must be considered carefully before starting the experiment. Variability of any one can result in unreliable quantification and render the assay meaningless.

mRNA or Total RNA

First, a decision must be made whether to use mRNA or total RNA as the target for the RT-PCR assay. There is some suggestion that starting with mRNA results in better sensitivity (Burchill *et al.*, 1999) but any

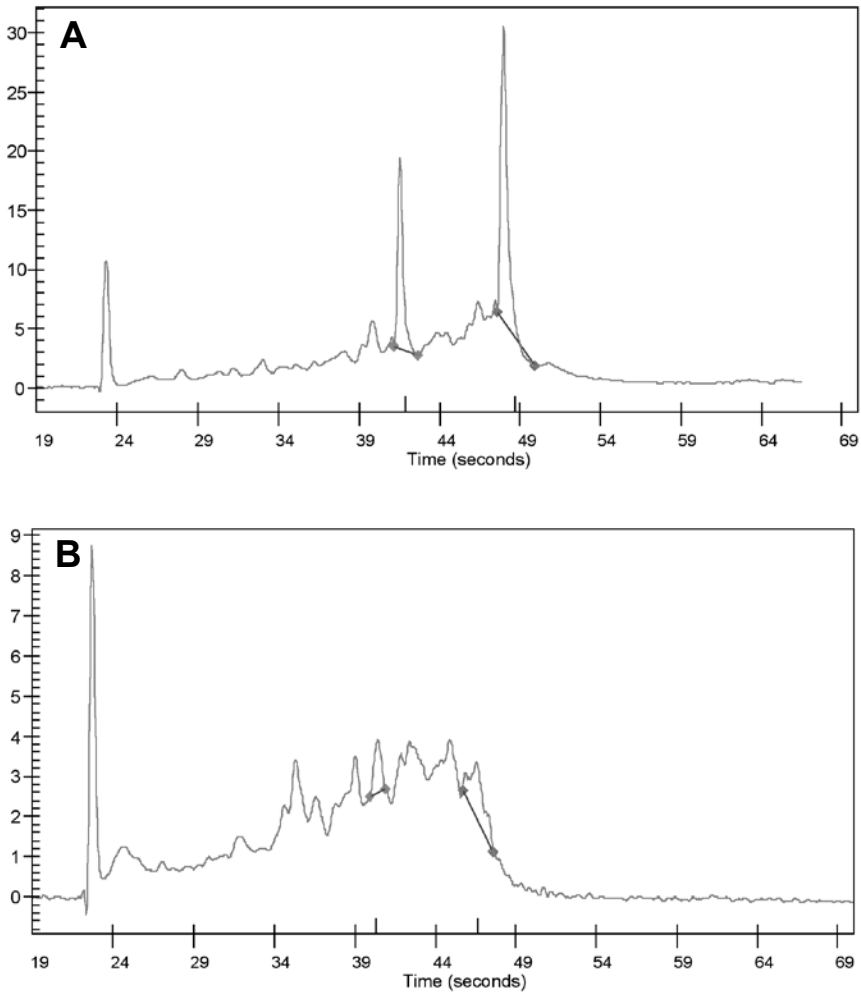


Figure 2. Electropherogram generated using the Agilent 2100 Bioanalyzer. A: An example of a high quality RNA preparation with distinct 28S and 18S peak ratios. The amount of small RNA (peak 1), which can include 5.8S and 5S ribosomal peaks and transfer RNA, is highly dependent on the preparation protocol. B: An example of a poor RNA preparation, with lots of additional peaks indicative of the presence of degraded RNA.

inhibitory effects of background RNA are noticeable only when very small numbers of target RNA template (<10) are present (Curry *et al.*, 2002). There are several reasons for preferring the use of total RNA: (1) purification of mRNA involves an additional step and any increased

sensitivity may be cancelled by the possible loss of material, (2) not all mRNA molecules have polyA tails and (3) the concentration of mRNA may be insufficient to allow proper quality assessment. Therefore on balance, it is advisable to use total RNA, as the ultimate sensitivity of the qRT-PCR assay is likely to be determined by factors other than whether mRNA or total RNA was used in the first place.

Regardless of whether total RNA or mRNA is used, sample acquisition and purification of the RNA mark the initial step of every qRT-PCR assay. It cannot be over-emphasized that the quality of the template is arguably *the* most important determinant of the reproducibility and biological relevance of subsequent qRT-PCR results. Any problems that affect reproducibility are likely to have originated here (Bomjen *et al.*, 1996). Many samples, especially biopsies of human tissue, are unique, hence a wasted nucleic acid preparation means that the opportunity to record data from that sample is irretrievably lost. Consequently it is prudent to expend extensive efforts on getting every stage of this process absolutely right, starting with consistency when collecting, transporting and storing samples, continuing with rigorous adherence to extraction protocols and remaining vigilant when taking samples out of storage for analysis.

cDNA Priming

Priming of the cDNA reaction from the RNA template can be done using random primers, oligo-dT, or target-specific primers and the choice of primer can cause marked variation in calculated mRNA copy numbers (Zhang *et al.*, 1999).

Random Primers

This method is by definition, non-specific, but yields the most cDNA and is most useful for transcripts with significant secondary structure. However, since transcripts originate from multiple points along the transcript, more than one cDNA transcript is produced per original target. Furthermore, the majority of cDNA synthesis is done from total RNA

will be ribosomal RNA-derived and may compete with a target that is present at very low levels. The T_m of random primers and oligo-dT is low, hence neither can be used with thermostable RT enzymes without a low temperature pre-incubation step.

Oligo-dT

cDNA synthesis using oligo-dT is more specific than random priming, as it will not result in 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 tail. In addition, oligo-dT priming requires very high quality RNA that is full length, hence is not a good choice for priming from RNA that is likely to be fragmented, such as that obtained from formalin-fixed 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.

Target-Specific Primers

Target-specific primers synthesise the most specific cDNA and provide the most sensitive method of quantification (Lekanne Deprez *et al.*, 2002). Their main disadvantage is that they require separate priming reactions for each target, which is wasteful if only limited amounts of RNA are available.

Choice of Enzyme

Viral RTs have a relatively high error rate, and have a strong tendency to produce truncated cDNA products because of pausing (Bebenek *et al.*, 1989). Their lack of thermal stability is a particular problem as the significant secondary structure of many mRNA molecules requires their denaturation for efficient reverse transcription (Buell *et al.*, 1978; Kotewicz *et al.*, 1988; Shimomaye *et al.*, 1989). Therefore, extensive efforts have been directed at improving these enzymes and

finding new ones. Consequently, there are many enzymes to choose from (Table 1) and they have differing abilities to copy small amounts of template and transcribe RNA secondary structures accurately. For example, increasing the reaction temperature to 50°C (Freeman *et al.*, 1996) or 55°C (Malboeuf *et al.*, 2001), depending on the RT, enhances both the accuracy and the specificity of retroviral RTs as not only is the intra- and intermolecular base pairing reduced but false priming is also minimised.

Other strategies for dealing with RNA structure include supplementing the RT reaction with dimethyl sulphoxide (DMSO), which disrupts

Table 1. Reverse transcriptase enzymes.

Enzyme	Supplier	T _{opt} (°C)	RNaseH	Features
AMV-RT	Various	37	+++	
MMLV-RT	Various	45	+	
Omniscript	Qiagen	37	+	Not AMV- or MMLV-derived
Sensiscript	Qiagen	37	+	Not AMV- or MMLV-derived
Powerscript	Clontech	42	-	
Superscript II	Invitrogen	42-50	-	
StrataScript	Stratagene	42	-	Point mutation in RNaseH domain of MMLV-RT
ImProm-II	Promega	55	-	
MMLV PM	Promega	42-55	-	Point mutation in RNaseH domain of MMLV-RT
<i>Tfl</i> pol	Promega	60-72	-	one tube/one enzyme RT-PCR
<i>Tth</i> pol	Various	60-72	-	one tube/one enzyme RT-PCR
Expand RT	Roche	42	-	Point mutation in RNaseH domain of MMLV-RT
RevertAid	Fermentas	42-45	-	Point mutation in RNaseH domain of MMLV-RT
Thermoscript	Invitrogen	50-65	-	

base-pairing and can increase the yield during amplification. Sodium pyrophosphate (<4mM) may also be included in the RT step of two-step assays that use Avian myeloblastosis virus (AMV) RT to overcome RNA secondary structure and increases the yield of first-strand cDNA. The pyrophosphate slows the DNA polymerisation rate of the enzyme and is presumed to make it less susceptible to disassociating from the template at regions of strong secondary structure. However, this is not a recommended approach if using Moloney murine leukemia virus (MMLV)-RT since this enzyme has an increased sensitivity to pyrophosphate.

AMV RT is more robust and exhibits greater processivity than MMLV RT (Brooks *et al.*, 1995) and retains significant polymerisation activity up to 55°C (Freeman *et al.*, 1996), although it is usually used at 42°C in two-step RT-PCR. Its error rate is 4.9×10^{-4} based on misincorporation studies (Ricchetti *et al.*, 1990). Life Technologies offers the ThermoScript™ RT, a recombinant, genetically engineered AMV RNaseH^{ve} RT with increased thermal stability compared with standard AMV-RT. This allows reactions to be run at temperatures up to 65°C to generate long cDNA transcripts. Native MMLV-RT has significantly less RNaseH activity than native AMV-RT (Gerard *et al.*, 1997), but is less thermostable and is typically used at 37°C. The error rate is slightly higher than that of AMV-RT (Ricchetti *et al.*, 1990) and several modified MMLV-RT-derived enzymes have been introduced that offer higher efficiency and improved synthesis of cDNA, *e.g.* PowerScript™ RT (Clontech), SuperScript™ II (Invitrogen) and THERMO-RT™ (Display Systems Biotech Inc).

In addition, there are several DNA-dependent DNA polymerases, *Tth* (Myers *et al.*, 1991), *Tfl* (Harrell *et al.*, 1994) and *BcaBEST*™ (Takara), that exhibit both RNA- and DNA-dependent polymerisation activities in the presence of Mn²⁺. All three are genuinely thermostable enzymes. In practical terms, only the primer T_m limits the temperature at which the reverse transcription step can be carried out. The sensitivity of target detection using *Tth* polymerase can be extremely high, and the detection of low abundance mRNA from single cells has been reported (Chiocchia *et al.*, 1997). These enzymes do not have 3-5' exonuclease

activities, hence their error rates are comparatively high (but no higher than those of the viral RTs).

Amplification Strategies

The RT-PCR can be performed as a one enzyme, one tube procedure (sometimes described as one-step) or a two-enzyme procedure, either as a one tube or a two-tube reaction assay. Single-tube RT-PCR procedures, whether one or two enzyme-based, are the techniques of choice in most clinical and high throughput laboratories because they are simple to use and offer a reduced risk of cross-contamination (Aatsinki *et al.*, 1994; Goblet *et al.*, 1989; Mallet *et al.*, 1995; Wang *et al.*, 1992). In two-tube RT-PCR, first-strand synthesis is performed in a small reaction volume, and an aliquot is then added to a PCR reaction for amplification of a specific target. However, the RT enzyme itself (Fehlmann *et al.*, 1993) and certain RNase inhibitors (Lau *et al.*, 1993) can inhibit the PCR step.

Two Enzyme Procedures: Separate RT and PCR Enzymes

In “uncoupled” two tube, two-enzyme procedures first-strand cDNA synthesis is performed in the first tube, under optimal conditions, using random, oligo-dT or sequence-specific primers. An aliquot of the RT reaction is then transferred to another tube, which contains the thermostable DNA polymerase, DNA polymerase buffer, and PCR primers, for PCR carried out under optimal conditions for the DNA polymerase. In “continuous” one tube, two-enzyme procedures the reverse transcriptase produces first-strand cDNA in the presence of Mg^{2+} , high concentrations of dNTPs, and either target-specific or oligo-dT primers. Following the RT reaction, an optimized PCR buffer (without Mg^{2+}), a thermostable DNA polymerase, and target-specific primers are added to the tube and PCR is performed. Alternatively, a single buffer can be used for both steps, obviating the need to open the tubes between the two steps. Interassay variation of two step RT-PCR assays can be very small when carried out properly, with correlation coefficients ranging between 0.974 and 0.988 (Vandesompele *et al.*,

2002a). The disadvantages of this approach are:

1. In two enzyme/one tube assays a template switching activity of viral RTs can generate artefacts during transcription (Mader *et al.*, 2001). This does not occur with bacterial polymerases.
2. Since in two enzyme/one tube assays all reagents are added to the reaction tube at the beginning of the reaction, it is not possible to separately optimize the two reactions.
3. Two enzyme/two tube reactions involve a significant amount of additional effort and present additional opportunities for contamination.

Single RT and PCR Enzyme Assays

A single enzyme able to function both as an RNA- and DNA-dependent DNA polymerase can be used in one tube/one enzyme procedures that are performed in a single tube without secondary additions to the reaction mix. However, there are several disadvantages to this approach:

1. Since all reagents are added to the reaction tube at the beginning of the reaction, it is not possible to separately optimize the two reactions.
2. The one tube/one enzyme assay is about 10-fold less sensitive than an uncoupled assay, due possibly to the less efficient RT activity of *Tth* polymerase (Cusi *et al.*, 1994; Easton *et al.*, 1994). However, it should be noted that one manufacturer (Promega) claims a one enzyme continuous assay is more sensitive than an uncoupled assay.
3. A direct comparison of one enzyme and two enzyme continuous assays has revealed more consistent sample-to-sample results with the latter.
4. This assay requires the use of significantly more units of DNA polymerase which can drive up the cost of the single enzyme assay.
5. The most thorough study comparing one step and two step reaction conditions using SYBR Green chemistry found that the one step

reaction was characterised by extensive accumulation of primer dimers, which could obscure the true results in quantitative assays (Vandesompele *et al.*, 2002a).

Inhibitors

The co-purification of inhibitors of the RT-PCR reaction during template preparation can present a serious problem to accurate and reproducible quantification of mRNA levels (Cone *et al.*, 1992). 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) (Wilson, 1997). In addition, factors like DNA fragmentation (Golenberg *et al.*, 1996; Pikaart *et al.*, 1993) and the presence of residual anti-coagulant heparin (Beutler *et al.*, 1990) or proteinase K-digested haem compounds like haemoglobin (Akane *et al.*, 1994) or myoglobin (Belec *et al.*, 1998) will negatively affect PCR efficiency. The problem with this type of inhibitor is that it makes the comparison of qPCR results from different patients, or different samples from the same patient impossible as it results in different amplification efficiencies and hence CTs of the same target from different patients. Worryingly, laboratory plastic ware has been identified as one potential source of PCR inhibitors (Chen *et al.*, 1994). It is also important to remember that reagents can have a significant effect on assay reproducibility, with lot-to-lot variation an essential consideration (Burgos *et al.*, 2002) and that DNA amplification products themselves can inhibit the polymerase (Kainz, 2000).

Different polymerases will react differently to inhibitors and it is worthwhile checking each template preparation for inhibition and testing several polymerases for their efficiency at amplifying the template. Sequence-dependent PCR amplification bias has been observed and results in incorrect or ambiguous quantification (Shanmugam *et al.*, 1993) and can even result in the preferential amplification of one allele over another (Weissensteiner *et al.*, 1996).

AmpliTaq Gold and the *Taq* polymerases are totally inhibited in the presence of 0.004% (v/v) blood, whereas HotTub (*Tfl*) from *Thermus flavus*, *Pwo*, *rTth*, and *Tfl* DNA polymerases are able to amplify DNA in the presence of 20% (v/v) blood without reduced amplification sensitivity; the DNA polymerase from *Thermotoga maritima* (Ultma) appears to be the most susceptible to a wide range of PCR inhibitors. HotTub and *Tth* are the most resistant to the inhibitory effect of K⁺ and Na⁺ (Al Soud *et al.*, 1998) and biological samples (Poddar *et al.*, 1998; Wiedbrauk *et al.*, 1995). 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. One thing to bear in mind is that some enzymes are in fact enzyme mixes that combine a highly processive enzyme with a proof-reading enzyme to balance fidelity and yield (Barnes, 1994). Finally, contamination of the PCR assay is an ever present danger (Kwok *et al.*, 1989).

Additives

Chemical additives have been reported as improving the reliability of “tricky” PCR assays (Pomp *et al.*, 1991). Just as there are numerous potential causes of any PCR problem, there are numerous reagents that are supposed to solve those problems. The list includes non-ionic detergents such as Tween 20 (Demeke *et al.*, 1992), additives such as glycerol and formamide (Comey *et al.*, 1991), the proteins bacteriophage T4 Gene 32 protein, gelatine and bovine serum albumin (Comey *et al.*, 1991, Kreader, 1996), tetramethylammonium chloride (TMAC) (Chevet *et al.*, 1995), the sugars sucrose and trehalose (Louwrier *et al.*, 2001) and even ethidium bromide (Hall *et al.*, 1995). These additives may stabilise the polymerase, enhance its processivity, disrupt template secondary structure and decrease melting temperature. There are commercially available additives such as Stratagene’s Taq Extender™ PCR Additive and PerfectMatch® PCR Enhancer, CLONTECH’s GC-Melt™ and Novagen’s NovaTaq™ PCR Master mix. Three factors are important if additives are to be useful: high potency, high specificity and a wide effective range (Chakrabarti *et al.*, 2001), but, unfortunately, there is no consensus with respect to their effectiveness. Different additives have significantly different effects

with different targets and, it requires empirical evidence, rather than theoretical deliberations, to choose the most appropriate one. In some amplifications, specificity is reported to be improved by formamide, but not DMSO (Sarkar *et al.*, 1990), whereas in others DMSO was more effective than formamide (Varadaraj *et al.*, 1994). Therefore, it is best to test a wide variety of potential additives and to choose the one that is the most effective in one's own particular assay.

The most common additive is DMSO (Winship, 1989), which is added at a concentration of 2-10 % and several other sulfoxides have been identified as novel enhancers of high GC target amplification (Chakrabarti *et al.*, 2002). DMSO decreases secondary structure, lowers the melting temperature of the primers, can suppress recombinant molecules forming during the PCR (Shammas *et al.*, 2001a) and works with *Tth* polymerase (Sidhu *et al.*, 1996). On the other hand, it inhibits *Taq* polymerase activity at concentrations higher than 10 %.

Trehalose may increase the enzymatic activity of MMLV-RT at 60°C (Carninci *et al.*, 1998) and the specificity of oligonucleotide-dT priming by Superscript II (Mizuno *et al.*, 1999), probably by increasing their thermal stability (Hottiger *et al.*, 1994). Another stabilising agent is betaine (N, N, N-trimethylglycine) (Henke *et al.*, 1997; Weissensteiner *et al.*, 1996), which is sometimes used in combination with DMSO (Baskaran *et al.*, 1996) or trehalose (Spiess *et al.*, 2002) and is present in a number of commercially available PCR enhancement kits. Betaine also increases the thermal stability of proteins (Santoro *et al.*, 1992), may prevent non-specific interaction of polymerase and template and reduce or even eliminate the base pair composition dependence of DNA thermal melting transitions by equalizing the contributions of GC and AT base pairs (Rees *et al.*, 1993). The contradictory effect obtained with such additives is underlined by a comparison of the amplification efficiency of GC rich templates in the presence of betaine with that of DMSO, glycerol, trehalose, or TMAC. The results showed that only betaine had any appreciable effect (McDowell *et al.*, 1998). Betaine also increases the tolerance of the RT-PCR assay to inhibition by heparin (Weissensteiner *et al.*, 1996) and reduces recombination events (Shammas *et al.*, 2001a; Shammas *et al.*, 2001b). Another problem with these additives is that the various reports recommend different optimal

concentrations. This probably suggests that the effects are template and amplicon dependent and that careful titration and optimisation of their concentration prior to any assay is required for these additives to have any positive effects. This is especially so because adding too much has an inhibitory effect (Spiess *et al.*, 2002). Therefore, if the RT-PCR assay turns out to be a difficult one, it might be more productive to properly optimise the RT-PCR assay from the start, together with a possible re-design of primers and probes.

PCR Optimisation Strategies

Optimisation prior to carrying out the real-time PCR assay with precious samples will generate data that reflect on the quality of the assay design and produces valuable information to indicate where problems may lie. By using the minimum primer and probe concentration to give the best assay conditions it is often possible to reduce the concentration of oligonucleotides included in the assays and so increase the assay's specificity as well as saving costs. The simple steps detailed below are designed to be as labour, time and cost efficient as possible. Note that successful multiplex reactions depend critically on assay optimisation.

The optimisation process requires the use of a suitable template that specifies the target under investigation. This can be from any convenient source, and commonly used templates are a linear plasmid DNA containing the amplicon, a purified PCR fragment, genomic DNA (*only intron-less genes*) or cDNA. Optimisation of primers should be carried out using SYBR Green I. This allows the design of several alternative assays around the sequence of interest and the testing of several primer combinations before purchasing an internal oligonucleotide probe. Since SYBR Green I is a convenient and inexpensive approach, it provides reassurance that the primers are functioning and, with a melting curve, a measure of the quality of the reaction. Primer dimer products may be formed and will be visualized using melting profiles. Although primer dimer products are not detected using labelled internal oligonucleotide probes, substantial non-specific product formation could inhibit the PCR reaction and result in reduced

efficiency. For this reason it is imperative to select primer pairs that produce little or no non-specific products in the PCR reaction.

Primer Optimisation

1. Dilute probe and primers to 100 μM for stock storage, aliquot and store at -70°C
2. Dilute primers to 10 μM for working solutions (store at -20°C)
3. Dilute probe to 5 μM for working solution (store at -20°C)
4. Select template DNA that can be used for optimisation

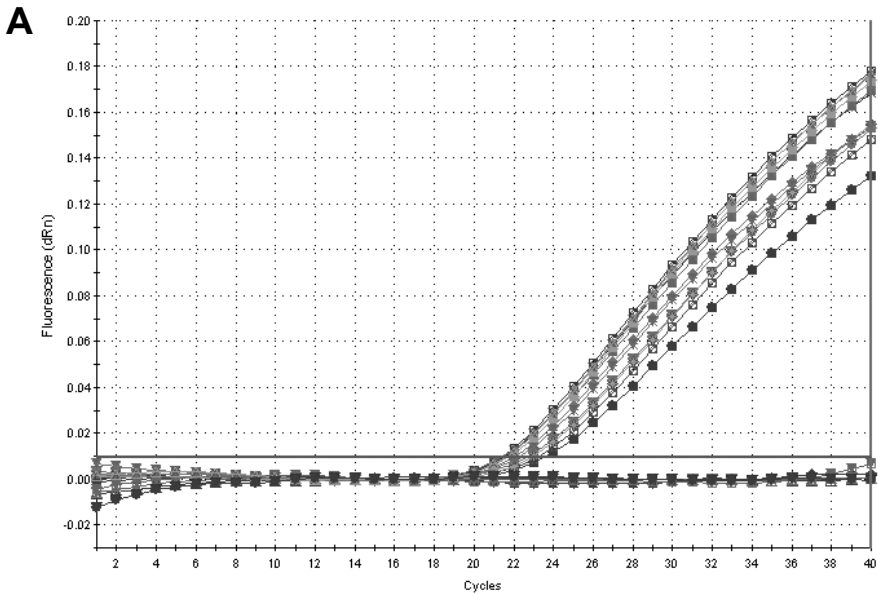
The optimal concentration of primers can span a surprisingly wide range from 50 nM to 900 nM and so a matrix of reactions encompassing this range is performed. The following optimisation reactions are run in duplicate:

1. Set up a matrix of 0.5 ml tubes as shown on Table 2. Each tube will eventually hold reaction mix to produce 2 x 25 μl duplicate optimisation reactions. These will then be divided into individual 25 μl reactions.
2. Make a reaction master mix by adding the reagents in the order shown in Table 3, mix gently and then finally add *Taq* polymerase and mix gently again. Add 38.4 μl of the reaction mix to each primer/water set-up tube in the matrix. Mix gently and then aliquot 25 μl of this into each of two instrument compatible microfuge tubes. Cap carefully, label tubes (if required), spin briefly, gently wipe the surface of each tube and ease into the PCR block noting the orientation of the tubes.
3. Perform a qPCR assay and melting analysis as described in Table 3. SYBR Green I data can be collected at either the extension stage or the latter half of the annealing stage.

To select the optimal primer pairs, examine the amplification plots and select the combination that give the lowest CT value. An example of a primer optimisation matrix is shown in Figure 3.

Table 2. Primer Optimisation Matrix set-up. R= Reverse (primer final concentration) F= Forward (primer final concentration). NOTE: all 50 nM final concentration primers use 1µM primer stocks, all other concentrations use 5 µM primer stocks.

	µl		µl		µl		µl		µl
F (50nM) 1µM stock	3	F (50nM) 1µM stock	3	F (50nM) 1µM stock	3	F (50nM) 1µM stock	3	F (50nM) 1µM stock	3
R (50nM) 1µM stock	3	R (100nM) 5µM stock	1.2	R (300nM) 5µM stock	3.6	R (600nM) 5µM stock	7.2	R (900nM) 5µM stock	10.8
ddH ₂ O	15.6	ddH ₂ O	21.4	ddH ₂ O	15	ddH ₂ O	11.4	ddH ₂ O	7.8
F (100nM) 5µM stock	1.2	F (100nM) 5µM stock	1.2	F (100nM) 5µM stock	1.2	F (100nM) 5µM stock	1.2	F (100nM) 5µM stock	1.2
R (50nM) 1µM stock	3	R (100nM) 5µM stock	1.2	R (300nM) 5µM stock	3.6	R (600nM) 5µM stock	7.2	R (900nM) 5µM stock	10.8
ddH ₂ O	17.4	ddH ₂ O	19.2	ddH ₂ O	16.8	ddH ₂ O	13.2	ddH ₂ O	9.6
F (300nM) 5µM stock	3.6	F (300nM) 5µM stock	3.6	F (300nM) 5µM stock	3.6	F (300nM) 5µM stock	3.6	F (300nM) 5µM stock	3.6
R (50nM) 1µM stock	3	R (100nM) 5µM stock	1.2	R (300nM) 5µM stock	3.6	R (600nM) 5µM stock	7.2	R (900nM) 5µM stock	10.8
ddH ₂ O	15	ddH ₂ O	16.8	ddH ₂ O	14.4	ddH ₂ O	10.8	ddH ₂ O	7.2
F (600nM) 5µM stock	7.2	F (600nM) 5µM stock	7.2	F (600nM) 5µM stock	7.2	F (600nM) 5µM stock	7.2	F (600nM) 5µM stock	7.2
R (50nM) 1µM stock	3	R (100nM) 5µM stock	1.2	R (300nM) 5µM stock	3.6	R (600nM) 5µM stock	7.2	R (900nM) 5µM stock	10.8
ddH ₂ O	11.4	ddH ₂ O	13.2	ddH ₂ O	10.8	ddH ₂ O	7.2	ddH ₂ O	3.6
F (900nM) 5µM stock	10.8	F (900nM) 5µM stock	10.8	F (900nM) 5µM stock	10.8	F (900nM) 5µM stock	10.8	F (900nM) 5µM stock	10.8
R (50nM) 1µM stock	3	R (100nM) 5µM stock	1.2	R (300nM) 5µM stock	3.6	R (600nM) 5µM stock	7.2	R (900nM) 5µM stock	10.8
ddH ₂ O	7.8	ddH ₂ O	9.6	ddH ₂ O	7.2	ddH ₂ O	3.6	ddH ₂ O	0
NTC 1 F(50nM) 1µM stock R (50nM) 1µM stock	3 3							NTC 2 F (900nM) 5µM stock R (900nM) 5µM stock	10.8 10.8
ddH ₂ O	15.6							ddH ₂ O	0



B

Forward primer concentration nM	Reverse primer concentration nM			
	100	300	600	900
100	25.01			
100		24.49		
100			23.77	
100				24.23
300	23.13			
300		23.1		
300			22.62	
300				22.58
600	23.14			
600		22.82		
600			23.13	
600				22.68
900	No Ct			
900		No Ct		
900			No Ct	
900				No Ct
ntc	No Ct			

Figure 3. Optimisation of primer pairs. A: Amplification plot of primer optimization assays. The corresponding CTs are presented in B: At 900nM concentrations of forward primer all reactions are inhibited. C: The lowest CT (22.58) and highest ΔRn results from a combination of 300 nM forward primer and 900 nM reverse primer.

C

Primer Optimisation Matrix

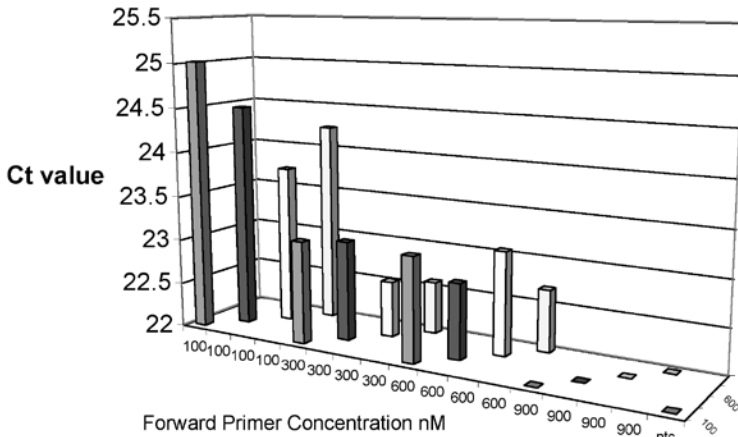


Table 3. Primer optimisation protocol for SYBR Green I reaction. This protocol uses the Stratagene Brilliant SYBR Green I Core reagent kits.

Reagent	Volume	Volume
Master mix	1 x 60 μ l reaction (μ l)	27 x 60 μ l
[Primers in matrix]	[21.6]	[583.2]
ddH ₂ O	TBD*	TBD*
buffer (10x)	6	162
MgCl ₂ (50 mM) (2.5 mM final concentration)	3	81
dNTP (20 mM) (0.8 mM final)	2.4	64.8
Rox diluted 1:200	0.9	24.3
Sybr Green I (dilute 1/5000)	0.6	16.2
Taq (5 U/ μ l)	0.6	16.2
Template (aim for 10 ⁴ to 10 ⁶ copies)	TBD*	TBD*

Final volume 1620

*TBD, to be determined. The volume of water to be added will depend upon the concentration of the template.

Remember to remove the reaction mix for the NTC before adding template and add water to compensate.

Perform a qPCR assay:

1 cycle: 95°C 10:00

40 cycles: 95°C 00:30

Annealing temp 01:00 Collect data 72°C 00:30 Collect data

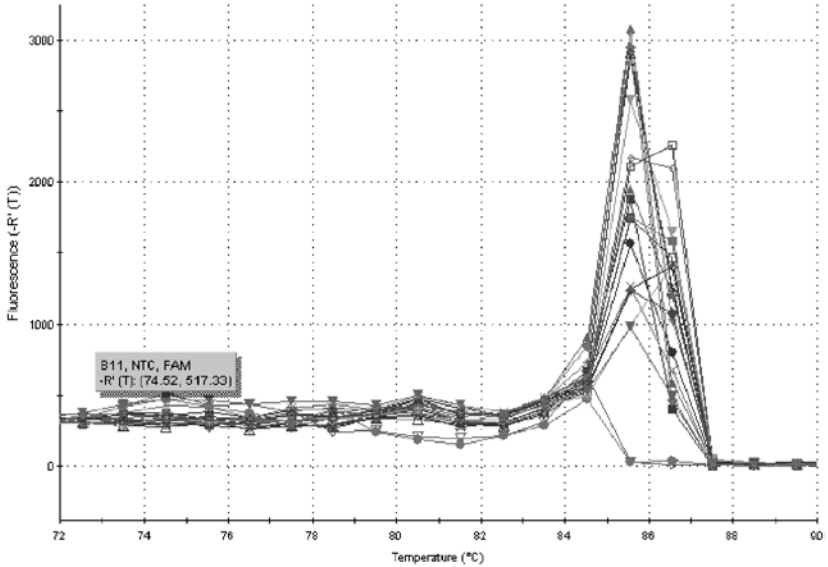
Followed by a melting profile:

1 cycle: 95°C 01:00

41 cycles: Collect data

starting at 55°C hold for 00:30 and repeat incrementing temperature by 1°C per cycle.

A



B

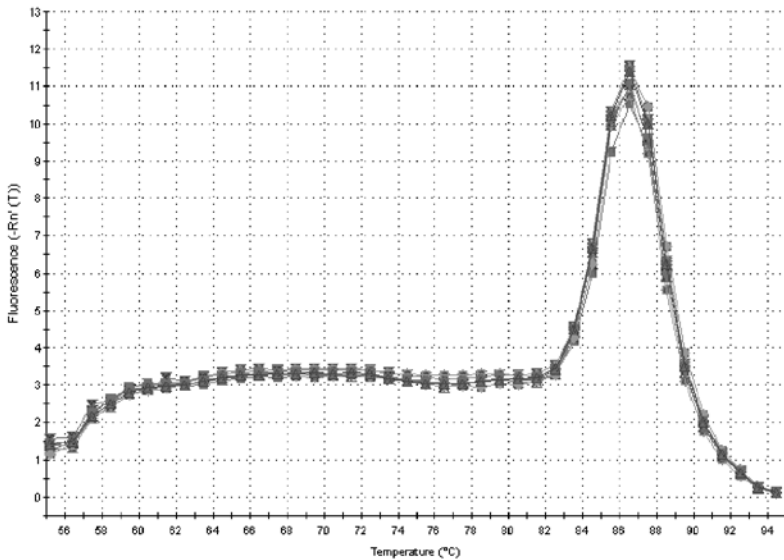


Figure 4. SYBR Green I Melting Curve Data. A. The first derivative view, $-R'(T)$ with respect to temperature provides a clear view of the rate of SYBR Green I loss and the temperature range over which this occurs. For this example a view of that data between 72°C and 90°C will be used. The small peak at 74.5°C is more than likely due to primer dimer product formation since this is the only peak to occur in the NTC sample. The main peaks occur around 85.5°C though there are some with a distinctly different profile and a peak at 86.5°C. These distinct profiles represent different products in the final PCR product. B shows an optimized dissociation curve.

In order to perform a melting curve all products from the PCR are held at 95°C to ensure that they are fully separated and then cooled to ensure complete hybridisation. These duplex molecules are then subjected to incubations at increments of temperature. The period of hold and the temperature incremental steps influence the stringency of the data, a longer hold with smaller temperature steps results in a more stringent definition of the melting profile of the product.

The raw data from a melting profile are presented as a first derivative plot of fluorescence units against temperature (Figure 4A) which provides a clear view of the rate of SYBR Green I loss and the temperature range over which this occurs. The small peaks at 74.5°C and 80.5°C are most likely to be due to primer dimer product and non-specific product formation since these are the only peaks to occur in the NTC sample. The main peaks occur at around 85.55°C though there are some reactions with a distinctly different profile and a further peak at 86.55°C. These distinct profiles represent different products in the final PCR product and are clear evidence that this reaction is sub-optimal. Once optimised, the assay loses the additional peaks and now has a single melting profile indicating a single product (Figure 4B).

PCR Amplification Efficiency

Having chosen the optimum concentration for the primers it is important to determine the efficiency of the qPCR reaction. The standard curve is an excellent tool for examining the quality of the overall qPCR assay. This is particularly important if multiple reactions are to be combined in multiplex or if the experiment will be run in the absence of a standard curve and a comparative quantification determination (or $\Delta\Delta C_t$) is to be used. In a perfect world, every amplicon will be replicated perfectly to generate a second copy during every amplification cycle. Assuming that a labelled probe binds to every single amplicon, each amplicon produced will result in one fluorophore molecule being released at each cycle. Hence the critical question concerns the minimum number of molecules of fluorophore that can be detected. If this were one, it would detect one molecule at cycle one. If it were eight molecules that the instrument could detect, it would detect one template input molecule

at cycle four and if it could detect only 512 molecules of label, it would take ten cycles to detect a single template input molecule. For most instruments a ballpark figure of 10^{11} free FAM molecules can be detected, with reasonable confidence, above combined background noise. It will require 38 cycles of a perfect serial duplication PCR reaction to produce 1.4×10^{11} copies from an initial single template copy. Therefore, a plot of CT versus the log of the copy number of a utopian qPCR assay has a gradient (slope) of -3.3 and a y-intercept of around 38. For the purposes of this discussion it is worthy of note that the R^2 is indicative of the fit of each data point to the line. A number of factors influence this value; the accuracy of the dilution series, pipetting reproducibility for each data point, contamination or non-specific fluorescence detection, lack of uniformity on the thermal block. With this in mind the gradient, intercept and R^2 values can be used as indicators of the efficiency and sensitivity of the specific qPCR assay (see below).

One Enzyme/One Tube TaqMan RT-PCR Protocol

Preparations

- Maintain a dedicated set of micro pipettes and use filter barrier tips for all qRT-PCR reactions. They must be calibrated regularly, especially pipettes dispensing 10, 2 or 1 μ l
- Use RNase free water, aliquot (20ml) and store at -20°C .
- Always aliquot all reaction components and use fresh aliquots if product is detected in no template control (NTC) or contamination is suspected.
- Two NTC reactions, one prepared at the beginning of the assay before any DNA is dispensed, and one at the end should always be performed, to confirm the absence of contamination.
- Defrost all reagents on ice prior to making up reaction mixes. Avoid exposing fluorescent probes and SYBR Green I to light (wrap in tin foil).
- Perform the qRT-PCR as soon as possible.

Primers and Probes

Forward and reverse primers (10 μ M each) can be mixed together and should be stored alongside the probes (5 μ M) in aliquots at -70 C.

RT-PCR Enzyme

Tth DNA Polymerase 2.5 U/ μ l

RT-PCR Solutions

- 5 x *Tth* RT-PCR buffer (250 mM Bicine, 575 mM potassium acetate, 0.05 mM EDTA, 300 nM Passive Reference ROX, 40% (w/v) glycerol, pH 8.2),
- 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 20 mM dUTP, 25 mM Mn(OAc)₂

The 5x *Tth* buffer contains a passive reference dye that does not participate in the 5'-nuclease assay. It provides an internal reference to which the reporter dye signal is normalised during data analysis. This corrects for fluorescent fluctuations resulting from pipetting errors of the master mix. It does not, of course, correct for pipetting errors of the template. Division of the emission intensity of the reporter dye by the emission intensity of the passive reference generates the normalised reporter (ΔR_n). A graph of ΔR_n versus cycle number has three stages. Initially ΔR_n appears as a flat line because the fluorescent signal is below the detection threshold of the fluorimeter. In the second stage, the signal becomes detectable and increases exponentially. Eventually, a third plateau stage is reached where the ΔR_n is linear (Figure 1).

A major advantage of collecting data during every cycle of the PCR reaction is that this provides additional information about the reaction that is most useful when problems arise. All real-time instruments permit a cycle-by-cycle analysis of the raw fluorescence data to show how fluorescence changes over time. The multicomponent view displays the complete spectral contribution of each dye and the

background component in a particular well over the duration of the whole run. Problems with the probe, background or even electricity supply are readily detectable.

Preparation of Master Mix

Preparation of a master mix reduces the number of pipetting steps and reagent transfers and so increases the reproducibility of the results. Mix buffer, dNTPs, Mn(OAc)₂, primer and probes by briefly vortexing, followed by centrifugation to collect residual liquid from the top and sides of the tubes. Mix enzyme by inverting the tube several times, followed by brief centrifugation. Make the master mix by combining the reagents in the order shown on Table 4, mix gently by repeatedly pipetting up and down (making sure there are no bubbles) and finally add *Tth* polymerase and mix gently again.

Table 4. One enzyme/one tube reagents and amplification set-up.

Reagent	Volume/25 μ l reaction (μ l)	Final concentration
5X RT-PCR buffer	5.0	1X
25mM Mn(OAc) ₂	3.0	3 mM
10mM dATP	0.75	300 μ M
10mM dCTP	0.75	300 μ M
10mM dGTP	0.75	300 μ M
20mM dUTP	0.75	600 μ M
10 μ M Forward Primer	0.5	200 nM
10 μ M Reverse Primer	0.5	200 nM
5 μ M Probe	0.5	100 nM
<i>Tth</i> DNA Pol (2.5 U/ μ L)	1.0	0.1 U/ μ l
RNase free water	to 20	

Perform a qRT-PCR protocol of:

	Step	Time	Temperature	Action
1 cycle:	RT	3 min	60°C	
40 cycles:	Denaturation	15 sec	92°C	
	Annealing/extension	45 sec	62°C	Collect data

Preparation of Standard Curve

The first time an assay is run, a full standard curve experiment must be carried out. Subsequently, only 5 dilution points need to be included with every run.

1. Prepare duplicate 10-fold serial dilutions of the DNA or RNA to be used to generate the standard curve.
2. The dilutions should cover the range 1×10^1 - 1×10^8 starting copy numbers. CTs for each dilution point will be determined in triplicate; hence each standard curve will be made up of a total of six CTs per dilution point.
3. Aliquot 60 μ l of the master mixes into adjacent wells of a 96-well reaction plate. Twelve wells should be reserved for no template controls.
2. Add 15 μ l from each of the dilution points to individual sample wells and aspirate using the micropipette tip.
3. To each of the three no template control wells add 15 μ l of water.
4. Transfer 25 μ l of master mix/template and master mix/water from each well to the empty wells in the two rows below to generate triplicate samples.
5. Cap the wells and ensure that they are properly sealed. It is crucial to ensure that the lids are tightly sealed as the small volumes in which the assays are carried out mean that product is easily lost to evaporation.
6. Transfer the plate to the thermal cycler and perform the RT-PCR reaction as detailed in Table 4.

Template Reaction

1. Aliquot 40 μ l of the master mix into the adjacent wells of every other row of a 96-well reaction plate. Four wells should be reserved for no template controls (NTC) and ten wells for the standard curve.
2. Add 10 μ l of RNA template to individual unknown sample wells and aspirate using the micropipette tip.
3. To each of the two NTC wells 10 μ l of water.
4. Transfer 25 μ l of master mix/template and master mix/water from

- each well to the empty well in the row below to generate duplicate samples.
5. Cap the wells.
 6. Take the ten-fold serial dilutions of the standard 10^2 - 10^6 copies of amplicon and add 10 μ l to each of the five wells containing the master mix.
 7. Aspirate using the micropipette tip and transfer 25 μ l to each adjacent empty well.
 8. Cap the tubes and ensure that they are properly closed.
 9. Transfer the plate to the thermal cycler and perform the RT-PCR assay (Table 4).
 10. Analyze the results (Figure 5).

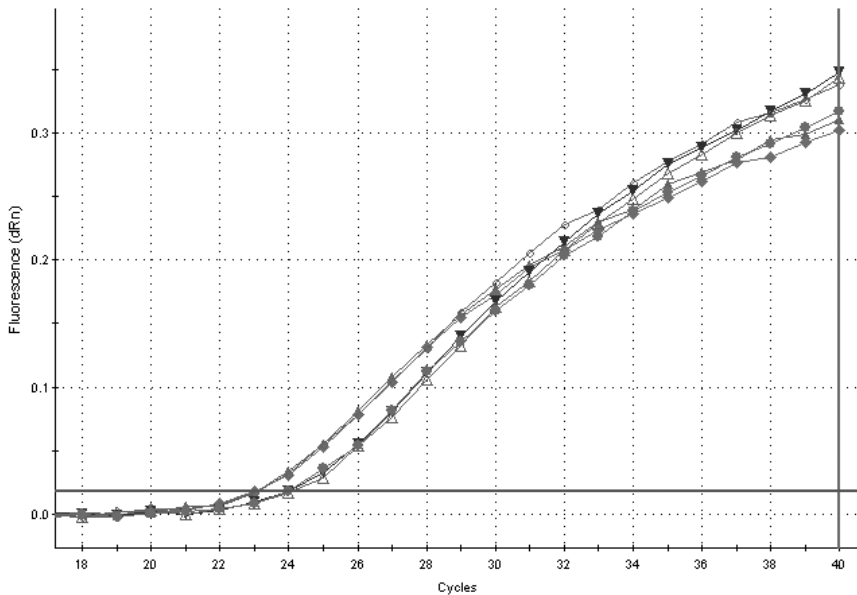


Figure 5. Amplification plots of two templates. Total RNA prepared from colorectal cancer biopsies was analysed by a one enzyme/one tube qRT-PCR assay. Each template was analyzed in triplicate and CTs were converted to copy numbers using an amplicon-specific standard curve coupled to normalization to total RNA quantitated using Ribogreen. The high reproducibility of the assay is apparent, with the 1 CT difference translating into $5,000 \pm 400$ copies of mRNA/ μ g total RNA and $10,000 \pm 750$ copies of mRNA/ μ g total RNA.

Two Enzyme/Two Tube TaqMan RT-PCR Protocol

Enzymes

- MMLV-RT 50 U/ μ l
- AmpliTaq Gold 5 U/ μ l

The correct choice of DNA polymerase is important as not all possess the 5'-3' nuclease activity necessary for the cleavage of the fluorogenic hybridisation probe bound to its target amplicon (Kreuzer *et al.*, 2000).

Solutions

The two-step RT-PCR reaction requires two reaction mixes:

- RT Reaction Mix
- PCR Reaction Mix

1. RT step

- 2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 mM dTTP,
- 50 μ M Oligo d(T)₁₆ or 10 μ M sequence-specific reverse primer
- 10x RT buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.3
- 25 mM MgCl₂

2. PCR step

- 10x TaqMan Buffer 500 mM KCl, 0.1 mM EDTA, 100 mM Tris-HCl, pH 8.3, and 600 nM Passive Reference
- 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 20 mM dUTP,
- 25 mM MgCl₂

Preparation of Master Mixes

Mix the individual ingredients (*except for enzymes*) for the separate RT and PCR steps by briefly vortexing, followed by centrifugation to

Table 5. Two enzyme/two tube reagents and amplification set-up.

RT-Step		
Reagent	Volume/12.5 μ l reaction (μ l)	Final concentration
10 x RT buffer	1.25	1x
25 mM MgCl ₂	2.75	5.5 mM
2.5 mM dNTP mix	2.50	500 μ M each
10 μ M specific primer*	0.25	200 nM
50 U/ μ l MMLV-RT	0.3	1.25 U/ μ l
RNase-free water	2.95	

Place at 48 °C for 30 min. If using oligo-dT₁₆ primer, a 10 min step at 25 °C is required to maximize primer/template annealing.

* If using oligo-dT₁₆ primer, the final concentration should be 2.5 μ M, *i.e.* use 0.63 μ l

PCR-Step		
Reagent	Volume/25 μ l reaction (μ l)	Final concentration
10 x PCR buffer	2.50	1X
25 mM MgCl ₂	5.5	5.5 mM
10 mM dATP	0.5	200 μ M
10 mM dCTP	0.5	200 μ M
10 mM dGTP	0.5	200 μ M
20 mM dUTP	0.5	400 μ M
10 μ M Forward Primer	0.5	200 nM
10 μ M Reverse Primer	0.5	200 nM
5 μ M Probe	0.5	100 nM
AmpliTaq Gold (5 U/ μ L)	0.125	0.025 U/ μ l
RNase free water	18.88	
cDNA template	2.5	

Perform a PCR protocol of:

	Step	Time	Temperature	Action
1 cycle:	Activation	10min	95°C	
40 cycles:	Denaturation	15 sec	95°C	
	Annealing/extension	45 sec	62°C	Collect data

collect residual liquid from the top and sides of the tubes. Mix enzyme by inverting the tube several times, followed by brief centrifugation. Make the master mix by combining the reagents in the order shown on Table 5, mix gently by repeatedly pipetting up and down (making sure there are no bubbles) and finally add MMLV-RT and mix gently again. For SYBR Green I assays optimisation for specificity is essential. Therefore, $MgCl_2$ (3 mM instead of 5 mM) and primer (20-50 nM instead of 200 nM) concentrations are lower than in standard assays.

Preparation of Standard Curve

The first time an assay is run, a full standard curve experiment must be carried out. Subsequently, only 5 dilution points need to be included with every run.

1. Prepare duplicate 10-fold serial dilutions of the DNA or RNA to be used to generate the standard curve
2. The dilutions should cover the range 1×10^1 - 1×10^8 starting copy numbers. CTs for each dilution point will be determined in triplicate, hence each standard curve will be made up of a total of six CTs per dilution point.

RT-Step

3. Prepare the RT reaction mix as described in Table 5. Each assay requires 10 μ l of RT-mix and 2.5 μ l of RNA. Hence RTs carried out on eight duplicate dilution series will require $10 \times 8 \times 2 = 160$ μ l of RT mix. Two NTCs require an additional 20 μ l. Therefore, to be on the safe side, 200 μ l should be prepared.
4. Aliquot 10 μ l of the master mixes into a microfuge tube.
5. Add 2.5 μ l from each of the dilution points to individual tubes and aspirate using the micropipette tip.
6. To each of the two NTC tubes add 2.5 μ l of water.
7. Cap the tubes and ensure that they are properly sealed.
8. Transfer the plate to a thermal cycler and perform the RT-step reaction as detailed in Table 5.

PCR Step

9. Prepare the PCR Reaction Mix as described in Table 5. Each assay requires 22.5 µl of PCR mix and 2.5 µl of cDNA. Hence PCR assays carried out on eight duplicate dilution series in triplicate will require $22.5 \times 8 \times 2 \times 3 = 1080$ µl of PCR mix. Six NTCs require an additional 135 µl. Therefore, to be on the safe side, 1.3 ml should be prepared.
10. Aliquot 60 µl of the master mixes into the adjacent wells of every other row of a 96-well reaction plate.
11. Add 15 µl from each of the dilution points to individual wells and aspirate using the micropipette tip.
12. To each of the two NTC wells add 15 µl of water.
13. Transfer 25µl of master mix/template and master mix/water from each well to the empty wells in the two rows below to generate triplicate samples.
14. Cap the wells and ensure that they are properly sealed. It is crucial to ensure that the lids are tightly sealed as the small volumes in which the assays are carried out mean that product is easily lost to evaporation.
15. Transfer the plate to the thermal cycler and perform the PCR reaction as detailed in Table 5.

Unknowns Reaction

RT Step

1. Prepare the RT reaction mix as described in Table 5. Each assay requires 10 µl of RT-mix and 2.5 µl of RNA. The exact amount of RT master mix required will depend on the number of assays. Two NTCs require an additional 20 µl.
2. Aliquot 10 µl of the master mixes into a microfuge tube.
3. Add 2.5 µl (5-200 ng RNA) to individual tubes and aspirate using the micropipette tip.
4. To each of the two NTC tubes add 2.5 µl of water.
5. Cap the tubes and ensure that they are properly sealed.
6. Transfer the plate to a thermal cycler and perform the RT step reaction as detailed in Table 5.

PCR Step

7. Prepare the PCR master mix as described in Table 5. Each assay requires 22.5 μl of PCR master mix and 2.5 μl of cDNA. The exact amount of PCR master mix required will depend on the number of assays. Allow for four NTC wells
8. Aliquot 45 μl of the PCR master mixes into the adjacent wells of every other row of a 96-well reaction plate.
9. Add 5 μl from each cDNA preparation to individual wells and aspirate using the micropipette tip.
10. To each of two NTC wells add 5 μl of sham cDNA, to a further two NTC wells add 5 μl H₂O.
11. Transfer 25 μl of master mix/template and master mix/water from each well to the empty wells in the row below to generate duplicate samples.
12. Cap the tubes and ensure that they are properly sealed.
13. Transfer the plate to a real-time thermal cycler and perform the PCR step as detailed in Table 5.

Trouble Shooting

Two positive controls should always be included, one for the RT step and one for the PCR step. The controls should be RNA or DNA that undergo RT-PCR or PCR, respectively. If no fluorescence is detected with the RT control, but the PCR control is normal, then there was a problem with the RT step. If neither result in fluorescence, then the problem was with the PCR.

RT Step

- Check that all reagents were mixed properly and have been added at the correct concentration.
- If using random primers (*not advisable!*) or oligo-dT, was a 10 min step at 25°C step included?
- If using specific primers, was the reverse primer used?
- Was the temperature of the RT reaction too high? If using 37°C

or 42°C, the temperature can be increased to overcome secondary structure problems. However, this will reduce the activity of the RT.

- If the target is very GC rich or is known to form extensive secondary structures, denature RNA/primer mix (*without enzyme*) for 5 min at 75°C.
- For such templates it may also be useful to increase the RT reaction time
- Reduce/increase the amount of RNA.
- Use 5–20 U of an RNase inhibitor, especially if the assay contains low amounts of RNA (less than 10 ng).

PCR Step

Observation: No increase in fluorescence with cycling.

Possible causes:

- SYBR Green I: Ensure the correct dilution of SYBR Green I was used. Is the SYBR Green I concentration too high? Once SYBR Green I has been diluted it goes off very quickly and should be kept in the dark at 5°C for no more than one week. Dilution into a solution of DMSO may stabilize SYBR Green I, but may also affect the PCR assay. One major consideration when storing working solutions of SYBR stains is that there is a significant increase in the pH of Tris buffers when stored at 4°C versus room temperature. If buffers were prepared at pH 8.0 at room temperature, then the pH will increase to 8.5 at 4°C. This increased pH is beyond the range at which SYBR stains are most stable.
- TaqMan: The probe is not binding to the target efficiently because the annealing temperature is too high. Verify the calculated T_m using appropriate software. Note that Primer Express T_m s can be significantly different than T_m s calculated using other software packages.
- Molecular Beacon/Scorpion: The probe is not binding to the target efficiently because the loop portion (Molecular Beacon) or the probe (Scorpion) is not completely complementary to the target.

Perform a melting curve analysis to determine if the probe binds to a perfectly complementary target. Make sure the Scorpion probe sequence is complementary to the newly synthesised strand. The assay medium may contain insufficient salt for MB stems to form.

- A reagent is missing from the PCR reaction, repeat the PCR.
- The MgCl_2 concentration is not optimal. Increase it in 0.5 mM increments.
- Hot start DNA polymerase was not activated. Ensure that the 10 minute. incubation at 95°C was performed as part of the cycling parameters.
- Ensure the annealing and extension times are sufficient. Check the length of the amplicon and increase the extension time if necessary.
- The probe is not binding to the target efficiently because the PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
- The probe is not binding to the target efficiently because the Mg^{2+} concentration is too low. Perform a Mg^{2+} titration to optimise the concentration.
- The probe has a non-functioning fluorophore. Verify that the fluorophore functions by detecting an increase in fluorescence in the denaturation step of thermal cycling or at high temperatures in a melting curve analysis. If there is no increase in fluorescence, re-synthesise the probe.
- The reaction is not optimised and no or insufficient product is formed. Verify formation of enough specific product by gel electrophoresis.
- Ensure that not more than 10% (v/v) cDNA was used in the PCR step.

Analysis

Quantitative RT-PCR data can be expressed relative to an internal standard (“relative quantification”) (Fink *et al.*, 1998) or an external standard curve (“absolute” (Bustin, 2000) or “standard curve quantification” (Ginzinger, 2002)).

Relative Quantification

Relative quantification determines the changes in steady-state mRNA levels of a gene relative to the levels of an internal control RNA (Fink *et al.*, 1998). This reference is usually a housekeeping (HK) gene that is either co-amplified in the same tube or amplified in a separate tube. Therefore, relative quantification does not require standards with known concentrations and all samples are expressed as an n-fold difference relative to the HK gene and the number of target gene copies are normalised to the HK gene. In theory, this should be superior to and far more convenient than absolute quantification. This is because the result is a ratio, hence RNA concentration is irrelevant and there are several mathematical models that calculate the relative expression ratio, some of which correct for differences in amplification efficiency (Liu *et al.*, 2002a; Liu *et al.*, 2002b; Meijerink *et al.*, 2001; Peccoud *et al.*, 1996; Pfaffl, 2001; Pfaffl *et al.*, 2002; Soong *et al.*, 2001) while some do not (Livak *et al.*, 2001; Winer *et al.*, 1999).

The two main problems with relative quantification are that (1) this approach tends to introduce a significant statistical bias that results in misleading biological interpretation (Hocquette *et al.*, 2002). This is particularly true when there are vast differences in the expression levels of target and normaliser or when the target gene is expressed at very low levels when the relationship between the two may not be linear; (2) it is difficult to find a HK gene whose expression is constant and against which the target gene copy numbers can be normalized during the experimental conditions. This is particularly so for *in vivo* biopsies.

GAPDH, β -actin (Kreuzer *et al.*, 1999), histone H3 (Kelley *et al.*, 1993), ribosomal highly basic 23-kDa protein (Jesnowski *et al.*, 2002), cyclophilin (Haendler *et al.*, 1987), β -2-microglobulin (Lupberger *et al.*, 2002) and porphobilinogen deaminase (Fink *et al.*, 1998) are commonly used normalisers for relative quantification. GAPDH was a curious choice, since its gene product is well known to play a role in membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA export, DNA replication, DNA repair, apoptosis, age-related neurodegenerative disease, prostate cancer and viral

pathogenesis (Sirover, 1999). β -actin should also not be used to normalize qRT-PCR data (Weisinger *et al.*, 1999) as its mRNA levels 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). Matrigel, which is widely used for cell attachment and to induce cell differentiation, adversely affects β -actin mRNA levels (Selvey *et al.*, 2001). A recent systematic analysis and comparison of their usefulness on *in vivo* tissue biopsies has concluded that a single housekeeping gene should not be used for normalisation (Tricarico *et al.*, 2002). The recent demonstration of the effectiveness of normalisation against the geometric mean of multiple carefully selected HK genes is interesting (Vandesompele *et al.*, 2002b). However, this method requires extensive practical validation to identify a combination of reference genes appropriate for every individual experiment, something that is not at all trivial. In addition, as the choice of HK gene panel is tissue or cell-dependent, this is not a universal method. If HK genes are to be used, they must be validated for the specific experimental setup and it is probably necessary to choose more than one, as was done for example for expression profiling of T-helper cell differentiation (Hamalainen *et al.*, 2001).

Absolute Quantification

Absolute quantification provides a more accurate and reliable, albeit more labour-intensive method for the quantification of nucleic acids (Ke *et al.*, 2000b). CTs obtained from an unknown sample are compared to CTs generated from a series of samples of known concentration or copy number. Results can be expressed as copy number per unit mass, *e.g.* μg total RNA. The expression of a target nucleic acid is usually compared across many samples, often from different individuals and sometimes from different tissues. Since small differences in nucleic acid input can lead to large differences in PCR product yield, the amount of starting material must be quantitated with rigorous accuracy to normalize sample data and correct for tube-to-tube differences. Therefore, if absolute quantification is to be accurate, it needs to take that variability into account.

The initial dilutions to use for the standard curve should encompass as large a range as possible and so for optimisation purposes a three-fold to ten-fold dilution series over five to seven orders of magnitude usually serves best. Each sample should be run in triplicate. An example of an ideal standard curve is shown on Figure 6A. A three-fold serial dilution was used and the gradient of -3.358 and 98.5% efficiency indicates that this is a well optimised assay and suitable for application to experimental samples and quantification. In contrast a less than ideal example is shown on Figure 6B. This standard curve was constructed from a two-fold serial dilution of template. The gradient of -4.458 and efficiency 67.6% indicates that the reaction is very inefficient. It would not be advisable to use this assay for quantitative studies and would require a process of trouble shooting and possible redesign before proceeding.

The steps involved in constructing a standard curve are very straightforward:

1. Five to six serial 10-fold dilutions of known concentration or copy number are prepared from whatever standard is being used, *e.g.* amplicon-specific sense-strand oligonucleotides, T7-transcribed RNA, linear plasmid DNA etc.
2. Serial dilutions are analyzed by qRT-PCR in separate sample wells but within the same run and the resulting CTs are recorded.
3. A plot of CT versus logarithm of concentration or the copy number results in a straight line, the standard curve, which is the linear regression line through the data points.
4. The number of target gene copies can then be extrapolated from the standard curve equation: $\text{Copy number} = (\text{CT}) - (\text{intercept}) / \text{slope}$. The replicate readings should be sufficiently close (<0.5 CTs) to indicate a valid analysis. The limits depend on the concentration of the template in the sample. If the readings are too far apart then the sample should be retested.

A standard curve provides several vital pieces of information:

- A high R^2 value (>0.98) indicates that there is near perfect correlation between CT and copy number. Such R^2 values are

common for most good, carefully constructed standard curves.

- The slope of the standard curve can be used to determine the efficiency of the PCR reaction by the following equation: Efficiency = $[10^{(-1/slope)}]-1$. In the above example, the efficiency of amplification is 99.5%.
- The y-intercept is less reproducible than the slope, but gives some indication of how sensitive the assay is.

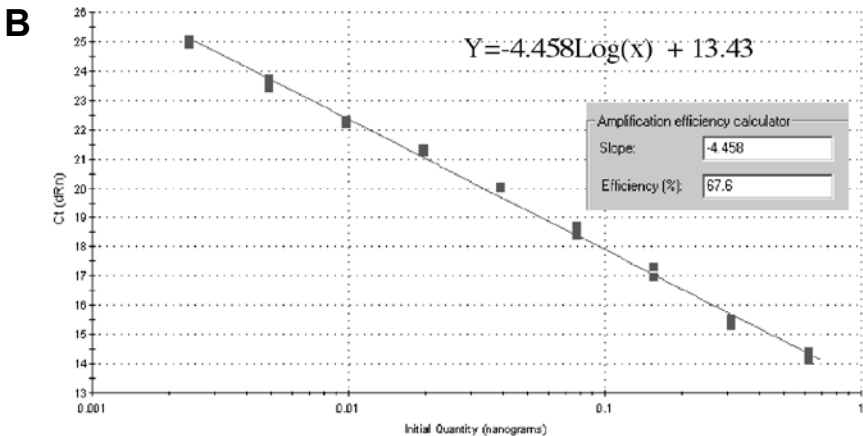
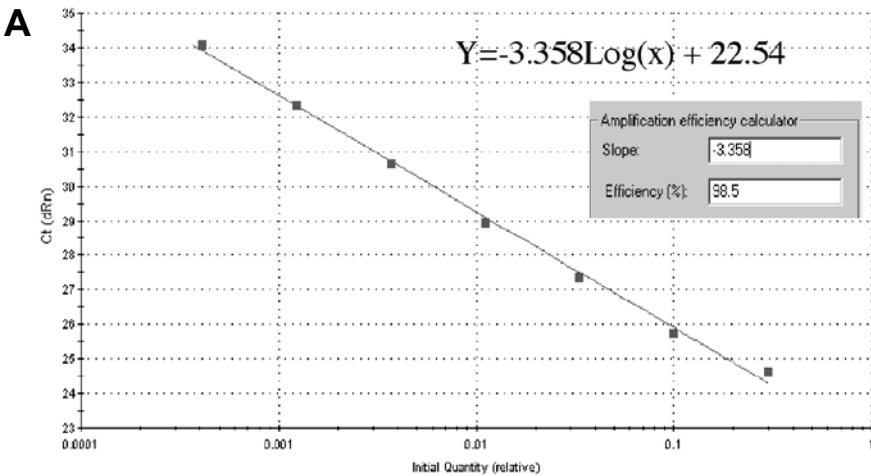


Figure 6. A: Optimised standard curve. B: Poor standard curve.

The main disadvantage of using an external standard is that it cannot provide a control for detecting inhibitors of the PCR reaction. This requires the addition of an internal control template that could be amplified and any variation from the expected CT would suggest the presence of inhibitors. Inclusion of such an internal control generates more confidence in negative results where no template is detectable.

Normalisation

Data normalisation, while a vital aspect of experimental design (Karge *et al.*, 1998), remains a real problem for absolute quantification (Thellin *et al.*, 1999), especially when the samples are from *in vivo* biopsies obtained from different individuals. Ideally, an internal standard should be used which is expressed at a constant level among different tissues, at all stages of development, and which should be unaffected by any 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 expression level in all of these situations (Haberhausen *et al.*, 1998), various HK gene, rRNA and total RNA are most commonly used to normalize gene expression patterns.

Tissue Culture

Experiments involving tissue culture cells that are being subjected to certain treatments, with mRNA levels measured before and after treatment can be normalised against cell numbers, with mRNA levels expressed as copy numbers per cell. Assuming that cells are counted accurately, this will generate precise, accurate and meaningful results. Alternatively, it may be not necessary to carry out absolute quantification experiments as is likely that one of the many HK genes proposed as internal standards will be suitable as a normaliser for relative quantification. Indeed, this is one of the few experimental designs where relative quantification may be acceptable. However, note that cellular subpopulations of the same pathological origin can be highly heterogeneous (Goidin *et al.*, 2001) and that careful

consideration of the appropriate HK genes is crucial. The mRNA levels of the target are reported as a value relative to the average mRNA levels of the selection of HK genes.

Nucleated Blood Cells (NBC)

Counting NBCs and expressing mRNA copy numbers per cell is the simplest way of reporting mRNA levels. However, this may not be accurate, since blood is made up of numerous subpopulations of cells of different lineage at different stages of differentiation, and differences in mRNA expression patterns are likely to be masked by this variability, a problem exacerbated when attempting to compare mRNA levels between different individuals. Quantification relative to a lineage-specific marker may be appropriate: Primers and fluorescent probes have been reported for numerous subtypes of NBC, *e.g.* CD45 (pan-NBC), CD3 (T-lymphocyte), CD19 (B-lymphocyte), CD14 (monocyte), and CD66 (granulocyte), and the specificity of quantification by real-time RT-PCR compares well with flow cytometric analysis of enriched cell populations (Pennington *et al.*, 2001). Normalisation against total RNA is also possible, as there is relatively little variation in the amount of total RNA per NBC (Bustin, 2000). However, normalisation against total RNA does not overcome the problem of variable subpopulations leading to inappropriate quantification and conclusions.

Solid Tissue Biopsies

Biopsies contain numerous cell types in variable proportions and there is no easy way of sorting or counting them without affecting the expression profile of the sample. Cancers in particular contain not just normal and cancer cells, but there may be several subclones of cancer cells together with stromal, immune and vascular cells (Baisse *et al.*, 2001). This variability means that while it is acceptable to generate qualitative results, there must be a question mark over quantitative data. The use of relative quantification is suspect because the mRNA levels of potential normalisers are not known and their levels may be altered locally by tissue-specific factors. Normalisation against

cell number is a useful option only for cells obtained from Laser Capture Microdissection (LCM), as it establishes a direct quantitative relationship between the target mRNA copy number and the cells from which the RNA was derived. Normalisation against total cellular RNA (Bustin, 2000) has been shown to produce quantitative results that are biologically meaningful (Tricarico *et al.*, 2002), but is crucially dependent on accurate quantification of the RNA which may not be very reliable. Messenger RNA levels can be normalised against total genomic DNA, with copy numbers expressed as absolute numbers per unit of DNA. Ribosomal RNA (rRNA) has been proposed as an alternative normaliser (Bhatia *et al.*, 1994; Zhong *et al.*, 1999) as rRNA levels vary less under conditions that affect the expression of mRNAs (Barbu *et al.*, 1989; Goidin *et al.*, 2001; Schmittgen *et al.*, 2000). However, rRNA levels are also affected by biological factors and drugs (Spanakis, 1993) and they vary significantly in haemopoietic subpopulations (Raaijmakers *et al.*, 2002) and in both normal and cancer biopsies taken from different individuals (Tricarico *et al.*, 2002). Furthermore, the vast difference in expression levels between rRNA and any target gene can result in misleading quantification data. Normalisation against HK genes runs into the same problems as discussed above with respect to relative quantification and cannot be recommended for RNA extracted from *in vivo* biopsies.

Quantification of MMP2 and MMP9 mRNA in Colorectal Cancers

The mRNA levels of two metalloproteinases, MMP2 and MMP9 were quantitated in 39 paired normal and adjacent colorectal cancer tissue biopsies. The probe and primer sequences are shown in Table 6. Two amplicon-specific standard curves were generated as described above (Figure 7) and used to quantitate MMP2 and MMP9 copy numbers in the colorectal biopsies. mRNA copy numbers were normalised against total RNA that was quality assessed using the Agilent 2100 Bioanalyzer and quantitated using Ribogreen assays. The results (Figure 8) suggest that (1) there is a wide range of mRNA levels (greater than 4 orders of magnitude) between different individuals; (2) MMP2 mRNA levels

Table 6. MMP primer and probe sequences.

MMP2	F:	5' -CGCTCAGATCCGTGGTGAG-3'
	R:	5' -TGTCACGTGGCGTCACAGT-3'
	Probe:	5' -TTCTTCTTCAAGGACCGGTTTCATTTGGC-3'
MMP9	F:	5' -CCCTGGAGACCTGAGAACCA-3'
	R:	5' -CCCGAGTGTAACCATAGCGG-3'
	Probe:	5' -CCGACAGGCAGCTGGCAGAGGAAT-3'

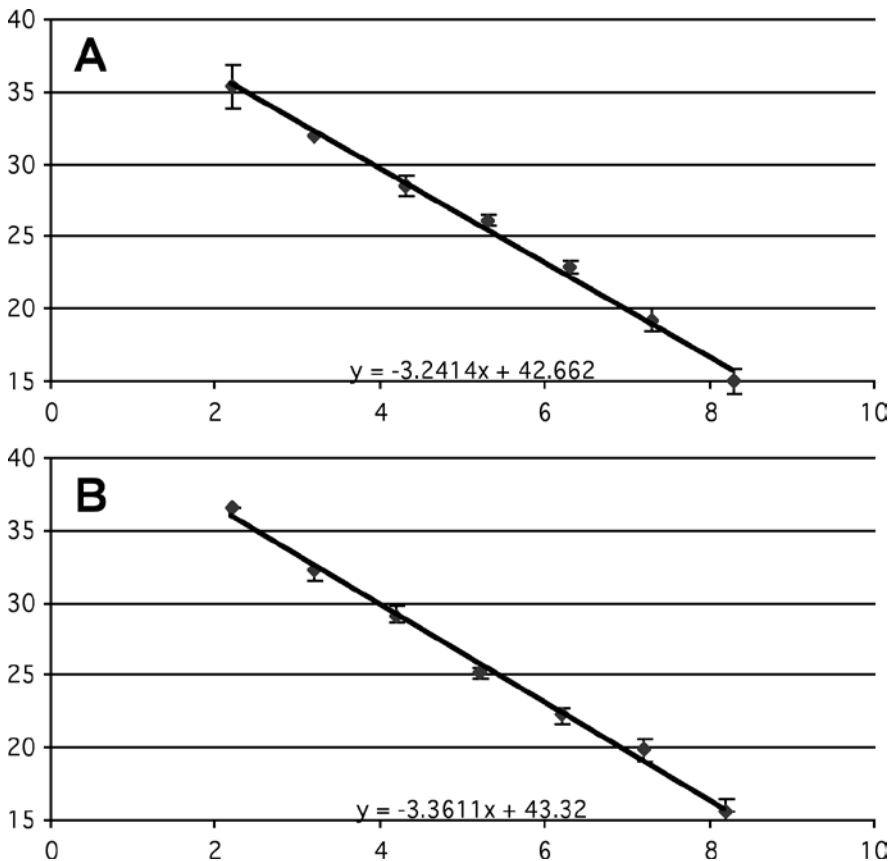


Figure 7. Standard curves for A: MMP2 and B: MMP9. Note that the y-intercept of both standard curves is >40, suggesting that the primer/probe combination is not optimised for sensitivity. However, the slopes indicate that the amplification efficiencies are close to 100%.

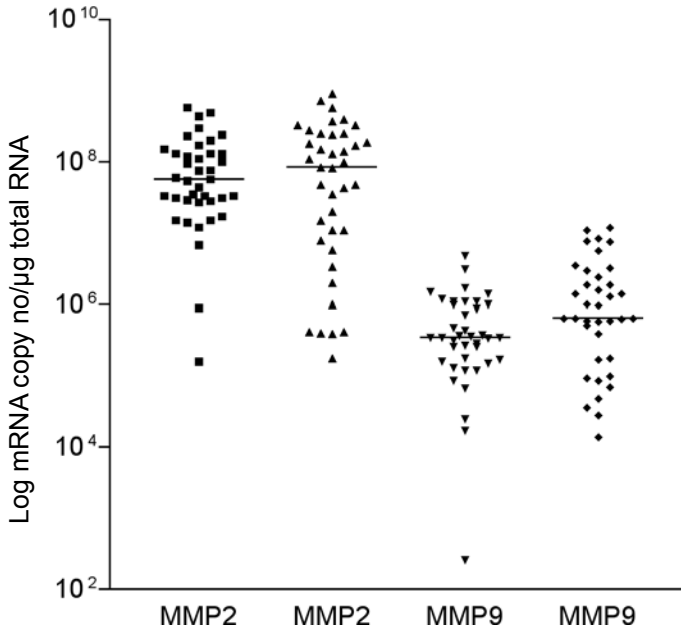


Figure 8. Comparison of MMP2 and MMP9 mRNA copy numbers in adjacent normal and paired colorectal cancer samples.

are higher than those of MMP9 and (3) there is no difference in their expression levels between adjacent normal and cancer tissue.

Conclusions

There can be little doubt that the rapid and easy acquisition of quantitative mRNA expression data by real-time fluorescence-based RT-PCR has placed this technology at the centre of a revolution that is allowing researchers to probe cellular expression profiles with a very high degree of precision. However, the huge variability of samples and templates, the wide range of enzymes, primer, amplicon and chemistry combinations makes a comparison between results obtained in different laboratories difficult. The aim of this chapter was to delineate a series of steps that must be taken if the vast amount of quantitative data generated by qRT-PCR assays is to be not just precise but also biologically relevant.

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