RT-PCR Optimization Strategies

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Abstract

PCR technology is based on a simple principle; an enzymatic reaction that increases the amount of nucleic acids initially present in a sample. This powerful method makes it possible to detect specific mRNA transcripts in any biological sample by the application of RT-PCR. The RT-PCR quantitative analysis workflow has several steps, each of which is crucial to the success of the experiment. It starts with a sampling step, followed by nucleic acid extraction and stabilization, cDNA synthesis and finally the qPCR where the mRNA quantification takes place. PCR itself is quite a stable reaction with reproducibility between 2–5% but the number and nature of the pre-PCR steps mean that there are many sources of experimental variance in the workflow. Reliable data can be produced only when the experimental variance is minimized, so the sources of noise must be identified and optimized for each step of each experiment. Typically, the pre-PCR steps are often neglected and optimization is done for PCR reaction only.

In this chapter the optimization of the whole RT-PCR workflow will be discussed and recommendations to reduce experimental variance are shown to produce more reproducible and reliable results are put forward.

Pre-PCR steps and optimization of RNA quality

In RT-qPCR experiments a special focus should be on the pre-PCR steps, because they can be major sources of error, especially during tissue sampling, RNA storage, RNA extraction, and quality assurance procedures. High RNA integrity guarantees valid mRNA and microRNA quantification, but fragmented RNA has a substantial impact on qPCR results and the conclusions drawn from them.

The validity and accuracy of gene expression evaluation by RT-qPCR is known to be profoundly influenced by the quality, the purity and integrity, of the starting RNA (Fleige *et al.*, 2006). Low-quality RNA is highly likely to compromise the results of downstream applications which are often labour-intensive, time-consuming and highly expensive (Raeymarkers, 1993, Imbeaud *et al.*, 2005). Thus, for any taking steps to obtain and preserve high quality, intact and not fragmented RNA, is essential.

This is especially true in clinical applications for which tissues are obtained via surgery or biopsy or after laser micro–dissection and limited sample material is available. It is well known that RNA is sensitive to degradation by inadequate sample handling, stabilization, or storage as well as by postmortem processes (Perez-Novo *et al.*, 2005). RNA extraction

from adipose, connecting or collagen-rich tissues often provides samples of a lower yield and quality, which invariably contain partly degraded RNA sub-fractions (our unpublished results). To circumvent this, particular steps during the tissue handling procedure have to be carefully controlled in order to preserve the purity, quality and integrity of the RNA material and reliable RNA quality analysis is necessary prior to quantitative analysis (Bustin and Nolan, 2004). Thus, the ability to quickly assess RNA quality in small samples has become increasingly important (Becker *et al.*, 2010; Fleige *et al.*, 2006).

The quantity and quality of purified RNA varies from sample to sample and, due to the inherent instability of RNA, tends to decline during storage. Especially long mRNAs (up to multiple kilobases) are very sensitive to degradation and will be easily cleaved by nucleases or sheared during the purification process. This can happen through cleavage by endogenous RNAses or exogenous RNAses introduced by handling with tissue samples or RNA extracts (Pfaffl and Fleige, 2006).

All steps of the RNA workflow including tissue sampling and storage, RNA stabilization, storage, extraction, dilution, and quantity and quality checks must be carried out with great care. In order to be considered as 'high quality', RNA samples must fulfil the following criteria (Bustin and Nolan, 2004; Pfaffl, 2005):

- Be free of RNAses.
- Be free of protein (verified at absorbance 260 nm/280 nm).
- Be free of genomic DNA.
- Total RNA should be not fragmented (28S/18S rRNA ratio should be roughly between 1.8 and 2.0) and the RIN/RQI should be at least 5 (Fleige *et al.*, 2006; Becker *et al.*, 2010).
- Be free of enzymatic inhibitors (verified at absorbance 260 nm/230 nm or 260 nm/320 nm) in later RT and PCR reaction. This is strongly dependent on the purification or clean-up methods employed and on the experience of the laboratory personal. Inhibitors can be tested by SPUD assay (Nolan *et al.*, 2006).
- Be free of any substances that complex essential reaction co-factors (e.g. Mg^{2+} or Mn^{2+}, or any nucleases which might degrade RNA).

Further problems can arise during tissue sampling before the tissue is fixed and stored safely in RNase stabilizers [e.g. PAXgene™ (PreAnalytix), LeukoLock (Applied Biosystems), RNALater (Ambion/Qiagen), or PrepProtect (Miltenyi Biotec)]. It is often very challenging to decrease the sampling time to a minimum within the framework of clinical routine sampling to prevent this. RNA quality may also be impaired in samples stored for long periods or under sub-optimal conditions, e.g. in wrong buffer solution or at elevated temperatures (Schoor *et al.*, 2003; Schroeder *et al.*, 2006).

To verify RNA quality, purity and integrity in a standardized manner, we recommend lab-on-chip automated capillary electrophoresis before any RNA based analysis. Various instruments are commercially available to accomplish this, e.g. 2100 Bioanalyzer (Agilent Technologies) and Experion (Bio-Rad). Both of these devices are sensitive, give highly reproducible data and are suitable for reliable quality control of RNAs over a wide concentration and integrity range (Pfaffl *et al.*, 2008).

Overall RNA quality can be determined using an electropherogram and a virtual RNA gel image. From this the ratio of both major ribosomal RNA (rRNA) 28S and 18S

subunits can be calculated. Although this serves as a RNA quality marker it is reported, that the 28S/18S rRNA ratio is not a reliable marker for total RNA integrity (Fleige *et al.*, 2006; Pfaffl *et al.*, 2008). Instead the standard RNA integrity and quality markers the RNA integrity number (RIN) and the RNA quality index (RQI) should be used. To simplify the assessment of RNA integrity for the researcher these are typically calculated by the instrument software. A RQI/RIN value of 1 represents completely fragmented and degraded RNA, while a value of 10 represents intact RNA (Schroeder *et al.*, 2006). Previous studies have shown that a RIN/RQI of at least five is sufficient for obtaining reliable PCR results (Fleige and Pfaffl, 2006) and in a recent study, we demonstrated that this threshold value seems also sufficient for quantitative microRNA analysis (Becker *et al.*, 2010).

Significant degradation of target RNA can result in an artificially high quantification cycle (Cq) value, leading to an underestimation of its concentration and the initial copy number. This can be exacerbated by differential sensitivities of mRNAs to degradation and cause an enormous impact on the interpretation of qPCR results since different reference genes appear to be suitable for normalization in degraded versus intact mRNA or microRNA samples. In addition, the degree of dependence can vary between samples, emphasizing the need to understand the relationship between RNA quality and the results in any given study.

MicroRNA is affected to a lesser degree than mRNA, presumably because its small size reduces the ability of RNA nucleases to attack it. Nevertheless, the verification of mRNA and microRNA integrity is recommended prior to any quantitative RNA analysis, although the application of an appropriate normalization method can partly reduce the impact of degradation in qRT-PCR experiments (Fleige *et al.*, 2006; Becker *et al.*, 2010).

PCR detection chemistry

The market for PCR reagents is constantly increasing. Providers now offer a portfolio of chemistries for the whole experimental set-up, from sampling to reverse transcription to final PCR reaction. Chemical composition and concentrations are well established in the offered kits, therefore laborious optimization procedures at the bench can be avoided. But the downside of pre-optimized kits is their limited flexibility in allowing the user to optimize the reaction in order to overcome poor sample quality or eliminate non-specific PCR products. Nevertheless, even with these kits, there are options available for the user to influence the experiment and its performance and these are detailed in this section.

DNA polymerase

The DNA polymerase is the 'workhorse' in the PCR reaction and choice of polymerase type can have a profound effect on the success of a reaction. The first enzyme used for PCR was DNA polymerase I isolated from *E. coli* but today many different DNA polymerases boasting a variety of features, are commercially available.

The basic DNA synthesis process, through the Mg^{2+}-dependent 5′ to 3′ incorporation of free dNTPs to build new complementary second DNA strand, is common to all of the DNA polymerases. But for accurate DNA synthesis, and thus low error-rate PCR amplification, it is essential that the enzyme has a 3′–5′ exonuclease (proofreading) activity, which decreases the rate of incorporation false paired bases.

The first available thermostable DNA polymerase was the *Taq* polymerase isolated from *Thermus aquaticus* which is thermostable but lacks any proofreading activity. For higher conversion rates and long strand synthesis T4 and T7 polymerases from *E. coli* phage T4 and T7 are commonly used, although these are not thermostable. *Pwo* (*Pyrococcus woresei*) and *Pfu* (*Pyrococcus furiosus*), on the other hand, are thermostable polymerases that exhibit good PCR amplification fidelity and proofreading activity, which makes them generally good choices for qRT-PCR. For some special applications like amplifying GC-rich templates, polymerase mixes are often used to combine the positive characteristics of the different enzyme types (Table 5.1) (Al-Soud *et al.*, 1998, Eckert *et al.*, 1991, 1993).

'Hot start' polymerases, which were developed to increase specificity and convenience of the PCR reaction, are another tool worth considering. Hot start polymerases are produced by reversibly blocking the polymerase through chemical modification, a wax-barrier or a *Taq* polymerase-specific antibody. The polymerase is then mostly unblocked at the first high temperature DNA denaturation step at 92–95°C, then further enzyme is activated at the denaturation step of each subsequent cycle. The blocking of the polymerase early on in the reaction prevents the synthesis of non-specific products at lower temperatures during the PCR set-up and early stages. Thus, performing hot-start PCR reduces non-specific amplification and increases specific PCR product target yield (Lebedev *et al.*, 2008).

Polymerase fidelity is described in terms of errors per nucleotide polymerized and varies among the different enzyme types from a rate of 10^{-3} to 10^{-6}. The fidelity of the chosen DNA polymerase under difficult and changing conditions, for example in tissue extracts with varying compositions and inhibitor levels is a very important consideration. In the field of SNP analysis and genotyping the fidelity of amplification is especially important. Thus, in such applications, enzymes with a proofreading activity are highly recommended (Dietrich *et al.*, 2002, Tanguy *et al.*, 2004, Griffith *et al.*, 2007). It has been experimentally shown that low magnesium and dNTP concentrations, a pH 6.0–6.5 and lower cycle

Table 5.1 Overview of different polymerase types

Polymerase type		3′–5′ activity	Temperature optimum	Special features
Taq	*Thermus aquaticus*	no	72–95°C	High fidelity
T4	Coli-Phagen T4	yes		High fidelity
T7	Coli-Phagen T4	yes		High fidelity
Pfu	*Pyroccocus furiosus*	yes	72–74°C	Thermostable; good proofreading
Pwo	*Pyrococcus woesei*	yes	72°C	Thermostable, good proofreading, blunt-End cloning
Tfl	*Thermus flavus*	yes	74°C	thermostable
Phi29	*Bacillus subtilis* phage	yes		Whole genome amplification

numbers improve polymerase fidelity (Eckert *et al.*, 1991, 1998), providing further scope for improving PCR accuracy where needed.

DNA polymerase activity is affected by residual tissue components, such as bile salts, complex polysaccharides (faeces), haemoglobin (blood), proteinases (milk) and urea (urine). Other inhibitors can be retrieved from a contamination out of the extraction procedure by phenols or other protein detergents, which degrade or inhibit any enzyme activity in RT and PCR reactions. Different DNA polymerases show varying sensitivities to these substances so pre-screening various polymerases to identify the optimal choice for a given reaction is recommended (Al-Soud *et al.*, 1998). Special applications need specific enzymes, e.g. GC-rich PCR can be done with an enzyme mix of *Pwo and Taq* polymerase. For long-range PCR special dNTP kits and enzyme mixes exist and for cloning of PCR product a combination of *Taq* and *Pwo* polymerase is helpful.

General recommendations

- Use hot start enzymes for PCR reactions.
- Be aware that RNA integrity and inhibitors can influence polymerase activity.
- Enzymes with proofreading activity are recommended.
- Fidelity is improved with low magnesium and low dNTP concentrations.
- Choose the enzyme depending on your experimental conditions.

Reverse transcription

In the reverse transcription (RT) step the RNA template is reverse transcribed into complementary DNA (cDNA), which will later be amplified in the PCR. The components required in the RT reaction are the reverse transcriptase enzyme, a reverse primer and an optimized buffer containing Mg^{2+} and/or Mn^{2+} ions and free dNTPs. The optimization of the RT step often is disregarded in favour PCR optimization but since the error accumulation in any experimental set-up is higher in the first quantification steps (sampling, extraction and RT) these steps deserve closer attention (Tichopad *et al.*, 2009).

Reverse transcriptase

Reverse transcriptase (RT) is an RNA-dependent DNA polymerase that transcribes single-stranded RNA into cDNA. All known reverse transcriptases are encoded by retroviruses and *in vivo* their role is to copy the viral RNA genome into DNA prior to its integration into host cells. The first isolated RT enzymes were AMV, from avian myeloblastosis virus, and MMLV, from Moloney murine leukaemia virus. A downside to their application in RT-PCR is that, when present at high concentrations, both of these enzymes inhibit PCR due to their RNase H domain which cleaves the DNA–RNA hybrids. However in many commercially available enzymes this domain is often inactivated by a point mutation, called RNAse H^{minus} (Berger *et al.*, 1983; Hawkins *et al.*, 2003). To get an integer and long cDNA strand out of the RT reaction set-up, the use of RNAse H^{minus} is recommended in addition to ensure that the starting RNA is fully intact and exhibits a good RIN or RQI number.

Thanks to the development of thermotolerant RT enzymes that are stable at elevated temperatures (e.g. SuperScript III; Invitrogen), RT is nowadays performed at reaction temperatures of up to 65 °C. This melts any secondary stem-and-loop RNA structure, giving the reverse primer better access to template RNA (Stahlberg *et al.*, 2004a).

So which RT enzyme is the best for your RT reaction? Which enzyme will give best RT yield and fully reverse transcribe your starting RNA? Unfortunately, studies show that there is no universal RT enzyme which always shows best results in all mRNA reverse transcribed. All RT enzymes show different RT yields and sensitivities to distinct mRNA secondary structures. Consequently, the results of any quantitative RT-PCR (qRT-PCR) experiment will depend largely on the RT efficiency, and less so on the PCR efficiency (Tichopad *et al.*, 2009). This is especially true for genes that are expected to show a lower expression in the sample need high RT yields (Bustin, 2000; Stahlberg *et al.*, 2004a).

The 'best RT enzyme' gives the highest cDNA yield, fully reverse transcribes all RNAs investigated and introduces no bias or error in the cDNA pool (Stahlberg *et al.*, 2004b). The only way to find the best RT enzyme for a given study is to do extensive testing in the optimization phase.

Nevertheless, some general recommendations for the selection of a good RT enzyme can be made:

- RT enzymes without RNAse H (=RNAse H$^-$) activity are recommended, especially in samples with low RNA quantity and quality.
- Experimental evaluation of RT reagents in a pre-PCR optimization study is recommended.
- Use high-quality RNA in the RT reaction.
- Use a temperature-stable RT enzyme.
- Heat samples at 60°C before RT to minimize secondary RNA structure.

RT priming strategies

The RT step is one source of variability in a qRT-PCR experiment and for each enzyme the specific reaction conditions has to be optimized. Salt contamination, alcohol, phenol, and other inhibitors carried over from the RNA isolation process can affect the apparent RT efficiency (Wong *et al.*, 1998; Freeman *et al.*, 1996). Another source of variability is the choice of priming method used to initiate cDNA synthesis, which can be either target gene specific or non-specific. In general three different priming strategies exist for the RT step:

- gene-specific priming;
- oligo-dT or poly-dT priming;
- random priming – using hexamer, octamer or decamer primers.

The main differences in the priming strategies are the product family after reverse transcription and the lengths of the resultant cDNAs. Target gene specific priming works well in conjunction with elevated RT-reaction temperatures up to 60°C to eliminate secondary RNA structure and spurious transcripts. Here only one cDNA complementary to the target gene mRNA is synthesized.

Non-specific primers such as random hexamer, octamer or decamer primer mixes can be used to circumvent high inter-assay variations between biological samples during RT. These random primers will anneal at various positions of the single-stranded RNA and give multiple starting points for the reverse transcriptase. Therefore fragments of difference length will be produced from one starting mRNA. The entire RNA sample is effectively

reverse transcribed, and thus a total cDNA pool of all present RNA fragments is synthesized, including the highly abundant ribosomal (rRNA) subunits, 5S, 18S and 28S and the tRNA family. Therefore the RT yield using random primers is very high and theoretically 'all genes' can be measured in later PCR.

Similarly, poly-T oligonucleotides (consisting solely of 16–25 deoxythymidine residues) can anneal to the polyadenylated 3' (poly-A) tail found on most mRNAs (Bustin, 2000). This facilitates the specific reverse transcription of the entire mRNA pool in the sample, although it should be noted that some mRNAs, such as the ubiquitin message, have no poly-A tail so would not be transcribed in such an approach.

cDNA pools synthesized with non-specific or oligo-dT primers can later be split into a number of different target-specific PCR assays. This maximizes the number of genes that can be assayed from a single cDNA pool derived from one total RNA sample. Therefore the gene expression results are comparable between the applied split assays, at least within the same cDNA pool.

It is reported that reverse transcription can take place without any primers, because any RNA fragments present in the extract can be used as primers in the RT reaction (Stahlberg *et al.*, 2004b). However, the yields of such reactions are very low, so the use of RT primers is always recommended.

Gene specific priming is used in one-step RT-PCR, because no separate RT step is required in the procedure. To combine the benefits of the different RT priming strategies, a mix of oligo-dT and random primers is recommended (Table 5.2). Primers can be mixed equally (1:1), so a high specificity of the oligo-dT and the high RT yield of the random primers is guaranteed (Zhang *et al.*, 1999).

Finally, the different priming strategies can be ranked in order of RT efficiency and cDNA yield as follows (unpublished results):

mixture of poly-dT and random primers > random primers > poly-dT primer ≥ gene-specific primer.

Table 5.2 Overview of RT priming strategies

Primers	Processed RNAs	RT yield	Special application
Oligo-dT	mRNA, full-length products	Low	Gene expression analysis
Random primers	mRNA and rRNA and small RNA, fragmented products	High	Multiple applications
Gene specific primers	One RNA = one specific gene sequence	High but only one measurable gene	One-step PCR
Primer mixes	mRNA and rRNA and small RNA, fragmented products	Very high	Gene expression analysis, multiple applications

RT protocol

The variety of RT available protocols, optimized for the sensitivities of the different RT enzymes, provide additional opportunities for the user to optimize their reactions. Generally used parameters are RNA concentrations between 100 and 1000 ng total RNA per reaction, total reaction times of 10–60 min and performance of the RT step at anywhere between 37°C and 60°C, depending on the thermostability of the reverse transcriptase enzyme. A post-reaction denaturation step at 95°C for several minutes is generally included to degrade any remaining template RNA.

For special applications like single-cell expression analysis, where only a few picograms of total RNA are available for each biological sample, a pre-amplification step between the RT and PCR reaction is effective (own unpublished results). Applying 10 cycles of pre-amplification can increase the cDNA by 1000-fold ($2^{10} = 1024$, own results), but is reported to introduce a bias in the generated cDNA pool that skews the quantitative PCR result (Ng *et al.*, 2005; Mestdagh *et al.*, 2008). To avoid this, a very simple method to decrease standard deviation between the samples should be employed. This involves is to setting up the experimental replicates as early as possible; ideally in sampling step but at the latest in the RT step. A very common error is to use one replicate in the RT and take duplicates or triplicates for the PCR reaction, resulting in large deviations in the replicates because the main technical variance belongs to the RT step and not to the qPCR step. Placing replicates already in the RT step can help to increase technical variance of the whole experiment. Choosing the triplicates in the RT step and doing single analysis in the PCR reduces the deviations of the replicates significantly (Stahlberg *et al.*, 2004b; Tichopad *et al.*, 2009; Hammerle-Fickinger *et al.*, 2009).

General recommendations

- RNA should be heated up to 65°C for 5 min before the RT reaction to open secondary RNA structures and allow a better annealing of the primers.
- RT enzyme should be the last substance given to the mastermix (keep the enzyme on ice as long as possible).
- Chose the replicates as early as possible to decrease error accumulation; best at extraction step or in the RT step – not in the PCR step.
- Common RT protocols are based on 100 ng to 1 µg total RNA.

Intercalating fluorophore dyes

Intercalating fluorescent dyes are commonly used for the real-time detection of the newly generated double-stranded PCR product. Sometimes these dyes are called 'non-specific dyes' because they bind to all double stranded DNA (dsDNA) molecules, including unwanted products like primer dimers or genomic DNA. But this name can be misleading because it has nothing to do with the specificity in the PCR reaction. The specificity of the generated PCR product is only determined by the binding abilities of the primer pair. The dye helps to detect the newly synthesized product and to make it visible for the fluorescence acquisition system. During PCR the increase of amplified product is directly proportional to the increase of the fluorescence signal.

The first-generation of dyes used for PCR analysis were fluorophores, such as ethidium bromide (EtBr), YOHO, YOPRO-1, or Hoechst 33258 (Katz *et al.*, 1990), which

intercalate with nucleic acids, an effect that is widely used in agarose gel electrophoresis. When exposed to ultraviolet light, EtBr will fluoresce with an orange colour and can be visualized by eye or a CCD camera, allowing the visualization of nucleic acids bands on a gel. EtBr is still used in post-PCR gel analysis because it is one of the cheapest dyes available. EtBr's disadvantages are its high toxicity and low fluorescence enhancement of DNA; high concentrations of nucleic acids (5 ng DNA in single band, 25–50 ng in polydispersed samples) are needed for accurate visualization (Ahn *et al.*, 1996). The second generation of intercalating dyes such as PicoGreen (a DNA binding dye) and RiboGreen (an RNA binding dye) are used in conventional PCR for DNA quantification (Molecular Probes, Invitrogen). These come with the added advantage of having little background noise since the unbound dye has virtually no fluorescence. By the binding to double stranded DNA (dsDNA) the fluorescence is enhanced by 20-fold, therefore low amounts down to 0.25 ng DNA can be measured. This makes these dyes much more sensitive than EtBr (Ahn *et al.*, 1996).

For real-time PCR, the most popular second generation intercalating dye is SYBR Green I (Molecular Probes, Invitrogen). SYBR Green I binds to the minor groove of dsDNA, which results in a 30-fold increase in the fluorescence signal. The free unbound SYBR Green I shows very low background noise. On the downside, high SYBR Green I concentrations inhibit the polymerase reaction and therefore diminish the PCR efficiency (Wittwer *et al.*, 1997).

A third generation of saturated intercalating dyes was introduced to the market in parallel with the development of high-resolution melting (HRM) curve analysis and genotyping. Examples include EvaGreen (Biotium), SYTO 9 and other members of the family of SYTO dyes (Molecular Probes, Invitrogen), LC Green (Idaho Technology), BEBO/BOXTO and Chromofy (TATAA Biocenter, Sweden). These dyes are non-toxic to the polymerase and exhibit greater stability at higher temperatures than earlier dyes, allowing higher concentrations to be used in reaction mixes, resulting in an increase in sensitivity of detection. In addition to their increased sensitivity, the main benefits of these dyes are their low running costs and ease-of-handling in the primer design and assay set-up. Problems can arise with these dyes when primer design and assay quality are poor, in terms of unwanted PCR products or primer dimers. Each double-stranded DNA molecule in the assay will generate a signal, generated by primer dimer formation or genomic DNA. Therefore the PCR product's specificity should be checked by including a melting curve analysis in the PCR protocol, its length verified by agarose gel electrophoresis and its sequence checked by sequencing.

The alternative to dye-based PCR detection chemistries are probe-based systems, which can be separated into single probe and primer-probe based systems.

Primer-probe systems

Primer-probe based systems utilize a specifically designed PCR primer with a covalently attached fluorophore to facilitate product detection (e.g. scorpion primer). The background signal, from the fluorophores of unincorporated primers, is kept to a minimum by a quencher dye. During amplification, the distance between fluorophore and quencher is increased, so that the signal of the reporter fluorophore can be detected. Quencher dyes which themselves are not fluorescent (e.g. black whole quenchers, BHQ) are recommended

because the background noise during real-time detection is acceptable low, e.g. as applied in hydrolysis probes. These probes have a very high binding specificity, but detailed attention must be paid to their design. The number of different primer-probe systems supplied by the various vendors are too numerous to list here, but a few prominent examples include LNA probes (Exiqon) and scorpion primer.

Hydrolysis probes

Structurally, probes can be linear or non-linear. Linear probes have no secondary structure so often have better hybridization efficiency and their design is easier because no complementary sequences need to be considered.

The first available, and best known, linear probes are hydrolysis (or nuclease) probes, (e.g. TaqMan probes, Applied Biosystems), which are still the most popular probes used in research and diagnostic applications. TaqMan probes have a fluorophore dye (typically FAM) and a quencher (BHQ) that inhibits the signal of the fluorophore. In the elongation phase the DNA polymerase exonuclease activity separates the fluorophore from the quencher and so the fluorescence signal can be detected.

Hydrolysis probes can be used in both singleplex and in multiplex PCR. For multiplex PCR the probes require different fluorescent labels, to allow the various signals to be distinguished by the instrument (Bustin *et al.*, 2004). Advantages of TaqMan probe systems are their high binding specificity and the multiplexing capabilities. However they are more expensive and primer/probe design is more difficult compared to intercalating dyes. With probes it is also impossible to check for primer dimers or unwanted artefacts in the reaction because the probe only detects the specific product (Shipley, 2006).

Oligo-probes

Second most popular among the linear probes are oligo-probe systems such as Hyb-Probes (Roche Diagnostics), which are often used in multiplex PCR and SNP detection and have become the method of choice for the LightCycler systems (Roche Diagnostics). Such systems consist of two probes that are designed to bind adjacent to one another. One probe, the so called anchor probe, carries the donor dye located at the 3′-end. The second probe, called the reported probe, carries the acceptor dye at the 5′-end. In the annealing phase the energy of the donor dye is transferred to the acceptor dye which is activated by a so called fluorescence energy transfer (FRET) mechanism (Förster, 1948). The signal of the acceptor dye is detected; the donor signal only emits background fluorescence. With HybProbes the generation of melting curves and SNP analysis is possible (Read *et al.*, 2001).

Non-linear probes

Molecular beacons are non-linear probes because of the hairpin structure, with a complementary stem sequence and a loop sequence complementary to the target. A fluorophore and a quencher dye are covalently attached at the 3′- and 5′-ends of the probes (Tyagi *et al.*, 1998). The quencher, 4,4′-dimethylamino-phenylazo-benzene (DABCYL), differs from the description of TaqMan chemistry in that it is non-fluorescent and so does not produce any background signal at all. The tight stem structure of the probe allows a very efficient quenching of the fluorophore dye. In the presence of a complementary sequence, i.e. the

primer binding sites, the beacon probe will hybridize to the template and, shift into an open configuration. This increases the distance between the fluorophore and the quencher, allowing the emitted light of the fluorophore to be detected. The occurrence of a mismatch between a beacon probe and its target has a greater destabilizing effect on the duplex than the introduction of an equivalent mismatch between the target and a linear oligo-probe because the hairpin structure provides a highly stable alternate conformation. Therefore, beacons show more specificity than the more common linear oligo-probes making them ideal candidates for detecting SNPs (Tyagi *et al.*, 1998). Molecular beacons can be used in numerous applications like multiplex PCR, SNP analysis, and detection of enzymatic cleavage (Bustin *et al.*, 2004).

Stabilizers or chemical changes in the probe DNA backbone are used to keep melt temperature requirements constant but reduce the required length, in order increase binding affinity and specificity of the probe to the amplicon. Examples for stabilizers are:

- Minor groove-binders (MGB, Applied Biosystems), which are added to one end of the linear probe to increase the binding affinity, for example this is done in TaqMan Probes for SNP detection.
- Locked nucleic acids (LNA, Exiqon; see Fig. 5.2) which are inserted at every 5th–8th base position in the probe sequence and are used for microRNA detection (Exiqon), SNP and allelic discrimination assays. An optimized set of LNA probes is commercially available as Universal Probe Library (Roche Diagnostics). Here primer and probe design is performed automatically according to the selected target and reference gene sequence and pre-optimized kits are used for mRNA quantification.
- Peptide nucleic acids (PNA) (Paulasova and Pellestor, 2004), which have a peptide sequence with an attached fluorescence dye. PNA molecules do not have a negatively charged backbone facilitating stronger annealing to the template strand (Bustin *et al.*, 2000; Shipley *et al.*, 2006).

Comparison of detection chemistries

When comparing signals from the different chemistries, the destruction of nuclease oligo-probes continues despite a plateau in product accumulation whereas intercalating dye fluorescence in the no template control (NTC) generally increases non-specifically during later cycles. This is due to primer dimer which (can) accumulate in late cycles (> cycle 35). Adjacent oligo-probe fluorescence begins to decrease as the rate of collision between the growing numbers of complementary amplicon strands increases favouring the formation of double-stranded DNA over the hybridization of oligo-probe to its target DNA strand. Reported in the literature as 'hook effect' some oligo-probes are consumed by sequence-related nuclease activity of the used polymerases (Mackay *et al.*, 2002).

All three oligo-probe chemistries, SYBR Green I, TaqMan hydrolysis probes and HybProbes are capable of detecting amplified PCR product with approximately the same sensitivity and quantification range. Generally the sensitivity of SYBR Green I assays depends strongly on the limitation by unspecific product formation due to inefficient primer design. The usage of TaqMan hydrolysis probes or HybProbes assays offer higher specificity, at least in the quantification of the generated PCR product (Wittwer *et al.*, 1997).

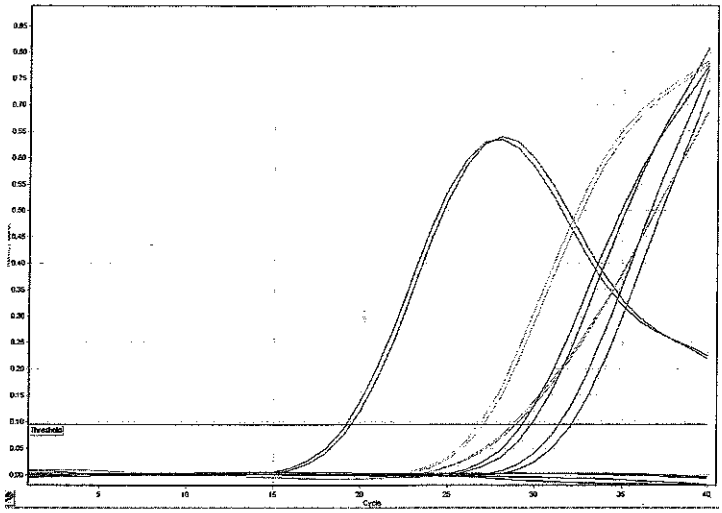

Figure 5.1 Example of hook effect in sample with highest starting amount.

General recommendations

- All detection chemistries are comparable in sensitivity and specificity.
- Use black whole quencher as quencher in probe systems.
- Using intercalating dyes always check specificity of the product by melt curve analysis and gel electrophoresis.
- Saturating dyes give better resolution in melting curve analysis (HRM is possible).

PCR cofactors and additives

PCR buffer and dNTPs

The optimal PCR buffer composition is essential for the fidelity and efficiency of the PCR reaction. Magnesium is the central cofactor for the DNA polymerase so Mg^{2+} ions should be included in the buffer to give a final concentration in the reaction between 1 and 5 mM. Magnesium is a chelating cation which can inhibit the PCR reaction and form complexes that reduce PCR fidelity (Wiedbrauk *et al.*, 1995), therefore excess magnesium ($\geq$ 7.5 mM) should be avoided.

dNTPs are essential for the synthesis of the new dsDNA strain in PCR but since they bind magnesium ions, their total concentration should be lower, ideally 4-fold, than that of the magnesium so dNTP concentrations between 0.2 and 0.8 mM are recommended (Shipley, 2006).

The cations potassium and ammonium are also essential components of the PCR buffer. Pre-optimized kits contain a mix of potassium chloride (KCl) and ammonium sulfate $(NH_4)_2SO_4$ in Tris-HCl. Potassium interacts with the phosphate ion of the single DNA strand and stabilizes the structure for better primer annealing, while ammonium can bind the hydrogen between the bases, decreasing the probability of mispriming and primer dimer formation (Qiagen News, 1996).

PCR enhancers

The amplification capacity of PCR can be significantly decreased by inhibitory compounds found in the sample, such as haem from blood or uric acid from urine or lactoferrin from milk (Al-Soud *et al.*, 2001; Wiedbrauk *et al.*, 2001). Some tissues like fatty tissue or gastro intestinal fluids can themselves cause problems in PCR efficiency (unpublished results).

These inhibitors can often be removed, at least partly, in the sample preparation and extraction steps, but are not always totally eliminated and additional purification steps can be very time-consuming and have potential for loss of target nucleic acid. The influence of PCR inhibitors can be seen in the slope of the real-time PCR fluorescence history plot, in the PCR efficiency or in the variability of the technical replicates in RT and PCR. To check for active inhibitors in the sample extract, we recommend performing inhibition testing assays, e.g. the SPUD assay (Nolan *et al.*, 2006).

The use of PCR enhancers can help to decrease the effect of inhibitors in the reaction by complexing them, annulling their effects and positively influencing the reaction fidelity. Enhancers can have varying effects in different biological samples or tissues and they can be used to increase reaction yield for delicate PCR products or in special PCR applications. Table 5.3 shows some of the most commonly used enhancers and their features.

General recommendations

- Magnesium concentrations have to be higher than dNTP concentrations in the PCR reaction.
- A mix of potassium and ammonium ions in the PCR buffer should be preferred.
- RNA extraction should be performed with care and should contain no inhibitors.
- Working with tissues which have natural inhibitors, PCR enhancers should be used.
- Use a SPUD assay (Nolan *et al.*, 2006) to check for inhibitors.

Optimization of the amplification protocol

In the early days of block PCR amplification the optimization of PCR protocols was a central part of the technology. It was highly correlated with the quality of the result and the length of the annealing and elongation steps which were in the focus of the optimization strategy. Today the PCR protocol is more standardized and reagents for RT and PCR reaction can be bought as ready-to-use kits, with already optimized reagent concentrations and validated temperature profiles. On one hand this saves time for the users because optimization becomes a minor endeavour but on the other, where optimization is required it is more difficult. Nevertheless, increasing PCR specificity and efficiency by changing the protocol is still possible, and potential approaches are described in this chapter.

Original approaches to optimize the PCR protocol centred on obtaining maximal product yield and getting rid of unwanted primer dimer formations. A typical PCR protocol starts with a DNA denaturation step at 95°C for some minutes to fully denature the starting DNA followed by 30–40 iterative PCR cycles, each with a short denaturation step (at least 5 s at 95°C), a primer annealing step at a primer-specific annealing temperature (between 55 and 60°C) and a step at 68–75°C for the elongation of the new strand.

Today, however, two-step protocols (denaturation and annealing step), three-step (denaturation, annealing and elongation step) or four-step protocols (denaturation, annealing, elongation step and elevated temperature acquisition step at 80–85°C) are

Table 5.3 List of enhancers for PCR reaction

Enhancers	Function	Applications	Concentration	References
BSA	Reduces sample binding to reaction vessel Binds inhibitors	Light Cycler systems LC 1.0, LC 1.5 and LC 2.0	1–400 ng/µl	Al-Soud *et al.* (2001), Kreader (1996)
DMSO	Reduces temperature differences between primer DNA	GC-rich and long-range PCR	2–10%	
Betaine	Reduces sequence-dependent temperature differences		0.5–2 M	
Glycerol	Stabilizes DNA polymerase			
TMAC	Eliminates non-specific priming Increases yield and specificity of PCR		15–100 mM	Wood *et al.*, (1985), Chevet *et al.* (1995),
Formamide	Improve the specificity of PCR		1–5%	Gelfand (1989), Sarkar *et al.* (1990)
Triton X-40 Triton X-100 NP-40	Prevents aggregation of polymerase May increase yield but also increase non-specific amplification		0.01–1%	
Tween 20	Reduces non-specific interactions			
T4 Gen 32 Protein	Reduces temperature differences between primer-DNA		100–150 ng/µl	Kreader (1996)

performed. Two- and three-step protocols, either with or without an additional elongation step, are the most popular and commonly used for probe based detection systems. The longer annealing step performed at 60°C should always be as short as possible, to reduce formation of primer dimers, but as long as necessary to make a specific annealing possible. In older publications four-step PCR protocols with fluorescence acquisition at elevated

temperatures (80–85°C) were performed to circumvent any detection of generated primer dimers (Roche Diagnostics, 1999). Primer-dimers will still be in the reaction and possibly affect whole assay efficiency. In the early days of qPCR using SYBR Green I detection an additional fourth step was included in the protocol to circumvent the quantification of unwanted primer dimers. Existing primer dimers were melted at elevated temperature acquisition 80–85°C and therefore become invisible in quantification (Pfaffl, 2001).

Today it is very common to perform fast, and yet still quantitative RT-qPCR assays. Fast PCR is the combination between a reliable fast processing polymerase mix and suitable instruments and consumables. In the past, reaction speed was limited by the performance of the Peltier elements used for block heating and cooling. But with the development of new fast PCR block technologies, using either silver block (realplex Eppendorf), honey comb block (Bio-Rad CFX series) or new conductive materials in LightCycler 480 (Roche Diagnostics) or 7500 and 7900 fast cyclers (Applied Biosystems) they are also applicable on block cyclers. The platforms exhibit heating rates up to 6°C/s and cooling rates 4°C/s and show good block homogeneity. But the real fast systems are the good old fashioned rotary systems, e.g. LightCycler family (1.0, 1.5 and 2.0) or the Rotor-Gene family (Corbett Research Rotor-Gene 3000 or 6000; Qiagen Rotor-Gene Q). These show optimal temperature uniformity over all samples, very quick heating and cooling up to 10°C/s and make fast PCR possible since they have been introduced to the market (Swerdlow *et al.*, 1997; own findings). For more information on instruments, see Chapter 6.

Today the focus of the protocol optimization is directed towards the primer annealing step. The annealing temperature depends primarily on the primer sequence and has to be optimized for every analysed primer pair. Generally the predicted melt temperature calculated by the primer design program is not the optimal run temperature. Therefore it is recommended that the annealing temperature should be optimized in a gradient PCR

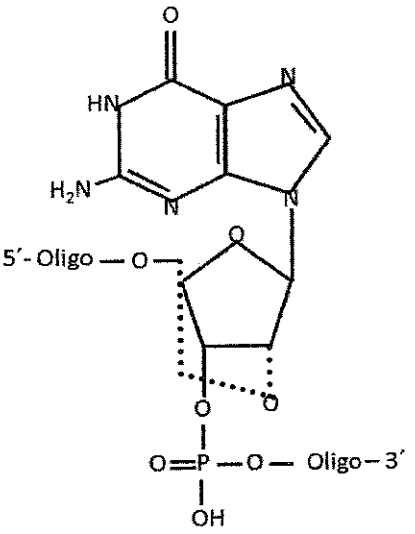

Figure 5.2 LNA probe with 'locked' ring (dotted lines).

experiment (see next section on 'optimization of primers'). The primer annealing also depends on the composition and ionic strength of the buffer system and on the binding affinities of the employed consumables. Whether a three-step protocol is really needed depends on the dye used and on the amplicon length. Second generation dyes were processed with an extra elongation step to allow inclusion of the dye in the new synthesized dsDNA. Nowadays, third generation intercalating dyes can be used with a two-step protocol, including a longer annealing phase, that was already standard for probe assays. Long-range PCR with an amplification of 5–40 kb fragments still needs an additional elongation phase; between 10–30 min so that DNA polymerase has the opportunity to synthesize the new strand (Roche Diagnostics, 2006).

Optimizing the temperature profile can be done by using a RT-PCR, a combined RT and PCR reaction, or by separate RT and PCR reactions. Combined RT-PCR is often used in gene expression studies, to measure various biological samples, and because the RT step is part of the PCR protocol, the possible error accumulation of the RT reaction is definitely reduced (Tichopad *et al.*, 2009). Here PCR primers are also taken in the RT reaction step, so no additional RT primers are necessary. This is cost savings but also means that only one single gene expression can be measured in each PCR run.

General recommendations

- Often two-step protocols are sufficient even with intercalating dyes.
- Perform a stringent protocol or perform fast PCR: annealing temperature as high as possible and the protocol as fast as possible.
- Samples with low starting amounts, e.g. single cells, often need more than 35 cycles for adequate amplification.

Optimization of primers

Primer design requires the sequence of the gene of interest and suitable primer design software that can calculate possible primer pairs for the detection system. In theory this is simple but often the calculated primer pairs do not work in PCR. Therefore optimization of the primer design or the amplification protocol is necessary.

Primer design is the most critical step in PCR analysis, because it influences the specificity, efficiency and accuracy of the assay so it should be done carefully to avoid any primer dimers or unwanted reaction artefacts. Initially this may be laborious and time-consuming, but after failure an additional primer pair redesign and ordering becomes very fretful.

First step is to find the correct sequence for the gene to be quantified. Several databases for gene sequence retrieval are available, the most popular being NCBI (http://www.ncbi.nlm.nih.gov/) and ENSEMBL (http://www.ensembl.org). Specialized databases for bacteria and virus sequences are also available (http://www.ebi.ac.uk/genomes/). As more and more gene sequences are identified and published this can become very complex and confusing because each gene of interest my have several sequence variations posted, for example sequences from DNA or RNA, from different isoforms, splice variants, SNPs, mutations, partial sequences (CDS, introns, exons, 5′- or 3′-sequences) or from sequence variations from different breeds. Therefore it can take some time to find the actual sequence that has to be amplified. It helps to know the abbreviation of the gene name, the accession number, and to look for the gene or transcript information.

After the correct sequence is identified it can then be uploaded into primer design software. Software for both primer and probe design are available, either as freeware, or, more commonly, as a proprietary package bundled with an instrument, e.g. PrimerExpress (Applied Biosystems). The most popular freeware package is Primer 3 (Rozen and Skaletsky, 2000) but many more, too numerous to list in this article are available and a summary can be found here http://primers.gene-quantification.info.

All software can calculate primer pairs from a given gene sequence following predefined parameters. Most important parameters are: primer melting temperature, primer GC content, primer length, product length, GC content of product, self priming potential of primer hetero- and homodimers. The calculated melting temperature (T_m) is especially important for PCR because this should also be the optimal annealing temperature of the primer in the reaction. Development of primer binding algorithms to calculate the primer Tm began in the 1960s and various additional models have been built on these original efforts (Breslauer *et al.*, 1968; Owzarzy *et al.*, 2004; Santa Lucia *et al.*, 1996, 2001; Schildkraut *et al.*, 1965). In Primer 3 the user can choose between these models to calculate optimal T_m. A list of the available models is shown in Table 5.4.

It is very common to design primer pairs at the same T_m for different genes to allow amplification of these different genes using identical amplification protocols. Such designs do not relate to the real T_m and annealing behaviour of the primers, but instead represent a best-fit comprise for the group of targets. By definition, at the optimal T_m half of the primers will be annealed, so in these best-fit primer groups, different amounts of primers will be bound to the each target depending on the actual T_m of the individual target sequence. This becomes very important in multiplex real-time PCR set-up where multiple amplicons are compared within one reaction set-up so similar amplification efficiencies for the measured genes are essential to make them fully comparable. Therefore it is essential that all used primer pairs have an equal concentration binding to the DNA template strands. To calculate similar hybridization behaviours it would be more useful to design primers with similar Gibbs free energy (ΔG°) instead of similar T_m (Zucker *et al.*, 1999). The free energy is based on the enthalpy and the entropy of the reaction that describes the annealing behaviour of the primer to the target strand. Freely available software applets for primer design,

Table 5.4 Summary of research groups developing algorithms for T_m calculation

Model	Calculation of T_m	
2 × 4 rule	4°C × (number G/C bases) + 2°C × (number A/T bases)	
Breslauer (1968)	Nearest-neighbor	For short oligos (8 bp), 1 M Na
Santa Lucia (1996)	Nearest neighbour + end interactions	1 M Na
Santa Lucia (1998)	Nearest neighbour + end interactions + salt correction	Salt concentration ~1.5 M Na
Schildkraut (1965)	Salt correction	
Owczarzy (2004)	Nearest neighbour + end interactions	DNA dumbbells and polymers, short oligos, 25 mM Na

such as Netprimer (Premier Biosoft), can calculate the free energy of the designed primer pairs. We recommend that primers should be optimized for both T_m and $\Delta G°$ between the genes of interest in such experiments.

There is no need for the primer concentrations to be balanced, but they should be high enough to allow sufficient sensitivity, specificity and fidelity within the assay. High primer concentrations increase the risk of primer-dimer formation. A range between 200 and 600 nM is recommended as optimal. In multiplex PCR the adjustment of each primer concentration becomes more difficult and it is recommended that the primer concentration of the genes with lower expression levels is increased so that similar PCR efficiencies are reached and a comparison of the multiplexed products is possible (Persson *et al.*, 2005). In any case, following all recommendations it is possible to optimize primers to meet all of the above-mentioned parameters and experience shows that they perform with reproducible and high PCR efficiency (Allawi *et al.*, 1998; Santa Lucia, 2001).

General recommendations

- Select target sequence with care.
- Use the same model for calculation of T_m in primer design software when several genes are designed.
- Avoid secondary structures within the primer sequence.
- Avoid self-complementarity, especially at the 3′ end of primer pairs.
- Try to design primers with similar T_m and $\Delta G°$.

Consumables

Consumables are not a crucial factor in quantification process but still an important one and the correct choice will depend on sample number and the PCR instrumentation employed. Some instruments require specific plastic consumables, others glassware but for most of the qPCR formats, especially with plate based formats, various types of consumables can be used. The consumables have to be taken in combination with the ability of the hardware, e.g. in performing a fast PCR protocol.

A range of PCR consumables is available for PCR analysis to suit the sample number and reaction volumes required. Choices include:

- single tubes with caps;
- strip tubes with strip caps;
- plates with various sealing films;
- special consumables, e.g. LightCycler capillaries (Roche Diagnostics), Rotor-Gene Q reaction tubes (Qiagen), or Smartcycler reaction tubes (Cepheid).

When performing fully quantitative real-time PCR it is always recommended to calibrate the instrument according to different dye spectra with whatever consumables you intend to use in your analysis. Some real-time instruments can even be calibrated for a range of different consumables and calibration plates and standardized kits are in generally available from the instrument manufacturer (Applied Biosystems; Bio-Rad). The calibration of the instrument can be seen as additional optimization and standardization step, to increase specificity and sensitivity of the fluorescence measurement, for real-time PCR data and

should be repeated annually, according to the rules of good laboratory practice (GLP or MIQE guidelines; Bustin *et al.*, 2009). In the LightCycler capillary cyclers, a colour compensation kit can be applied to calibrate platform, glass capillaries, fluorescence acquisition unit and software (Roche Diagnostics).

High-volume 0.2 ml tubes are often taken for block PCR cycling with limited number of samples and 8-strips with caps are used when sample number is low (< 96 samples). Tubes and 8-strips need higher reaction volumes, generally between 15–50 µl to avoid the upper dead volume, although new cap styles exist that minimize the free volume and therefore minimize evaporation rate (Eppendorf). Using single tubes and 8-strips saves on consumables because vial number can be optimized according to the sample number.

As well as the standard 96-well plate, high-throughput 384 well-plates, and even 1536-well plates are now available (Roche Diagnostics). Because the dimension of the plate is standardized, the well volume changes in proportion to number of wells. In 96-well plates 10–25 µl, in 384-well 3–10 µl, and in 1536-well only 0.5–2 µl is used as total volume for a single PCR run. This helps to save chemicals and costs.

To avoid evaporation, plates can be closed with caps or, most commonly, self-adhesive or heat-sealing films or foils. Heat-sealing avoids possible reaction mix evaporation during the PCR run, which can happen using sticky adhesive films if they are old and have lost their stickiness or if the instrument lid pressure is insufficient. Problems with lid pressure can also be avoided if new instruments with high lid pressures are used (Bio-Rad CFX series; Roche LC 480). To access the PCR products in heat sealed vessels the film can either be pierced with the pipette tip or, depending on the sealing system, the films can be removed entirely (Reiter and Pfaffl, 2008).

Users can also choose between transparent and white plates. Transparent plates allow better control of the pipetting process and better fluorescence readout (BioNews, 2009, Eppendorf). This is important e.g. when robot pipetting has to be checked or when the PCR product has to be processed after PCR reaction. Transparent consumables are also indispensable in real-time instruments to allow scanning through the tube (from bottom to top) as it is the case in the Rotor-Gene (Corbett Research or Qiagen) and the Light-Cycler system family (Roche Diagnostics). White plates definitely decrease the detection level of the dye because of the higher fluorescence reflection (BioNews, 2008, Eppendorf) and therefore quantification at lower starting concentrations is possible. Furthermore, white plates could decrease the variance of PCR replicates. Significant differences in PCR efficiency between transparent and white plates have not been detected. In high-throughput analysis or samples with low concentration, a better fluorescence signal can help to increase sensitivity and specificity (Reiter and Pfaffl, 2008).

General recommendations

- White plates show higher fluorescence.
- Heat sealing reduces evaporation and border effects.
- Consumables have influence on data variability.
- High-throughput plates help to save reaction mastermix.

Conclusion

The previous decade has been characterized by important methodological advances that have made quantitative PCR more sensitive, less variable and therefore more valid and reliable. As described in the past chapter, most advances were implemented in the optimization of the PCR method itself, but pre-PCR steps are still highly variable and introduce a noise in the quantification results. Clearly, we are still only half way in terms of having the optimal reaction composition or the optimal quantification work flow (Pfaffl, 2010). The developed MIQE guidelines will help to improve future experiments, but researchers must apply these instructions to get more valid and 'true' quantification results (Bustin *et al.*, 2009).

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