Critical Factors for Successful Real-Time PCR

New

Slope = –3.59

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Introduction

Real-time PCR and RT-PCR are highly sensitive techniques enabling amplification and quantification of a specific nucleic acid sequence with detection of the PCR product in real time. Quantification of DNA, cDNA, or RNA targets can be easily achieved by determination of the cycle when the PCR product can first be detected. This is in contrast with endpoint detection in conventional PCR, which does not enable accurate quantification of nucleic acids. Real-time PCR is highly suited for a wide range of applications, such as gene expression analysis, determination of viral load, detection of genetically modified organisms (GMOs), SNP genotyping, and allelic discrimination.

In this guide, we provide information on the basic principles of real-time PCR, terms used in real-time PCR, and factors influencing the performance of real-time PCR assays. In addition, we describe the advantages and disadvantages of different reaction chemistries as well as providing answers to frequently asked questions and guidelines for successful results. Examples of the spectrum of research currently being carried out are also included. We extend our thanks to those who have contributed to this project and hope that it may provide a useful guide to successful real-time PCR for researchers everywhere.

Detection of PCR products in real-time

Real-time PCR and RT-PCR allow accurate quantification of starting amounts of DNA, cDNA, and RNA targets. Fluorescence is measured during each cycle, which greatly increases the dynamic range of the reaction, since the amount of fluorescence is proportional to the amount of PCR product. PCR products can be detected using either fluorescent dyes that bind to double-stranded DNA or fluorescently labeled sequence-specific probes.

SYBR Green I

SYBR® Green I binds all double-stranded DNA molecules, emitting a fluorescent signal of a defined wavelength on binding (Figure 1). The excitation and emission maxima of SYBR Green I are at 494 nm and 521 nm, respectively, and are compatible for use with any real-time cycler. Detection takes place in the extension step of real-time PCR. Signal intensity increases with increasing cycle number due to the accumulation of PCR product. Use of fluorescent dyes enables analysis of many different targets without having to synthesize target-specific labeled probes. However, nonspecific PCR products and primer-dimers will also contribute to the fluorescent signal. Therefore, high PCR specificity is required when using SYBR Green I.

Fluorescently labeled sequence-specific probes

Fluorescently labeled probes provide a highly sensitive and specific method of detection, as only the desired PCR product is detected. However, PCR specificity is also important when using sequence-specific probes. Amplification artifacts, such as nonspecific PCR products and primer-dimers may also be produced, which can result in reduced yields of the desired PCR product. Competition between the specific product and reaction artifacts can compromise assay sensitivity and efficiency. The following section discusses different probe chemistries.
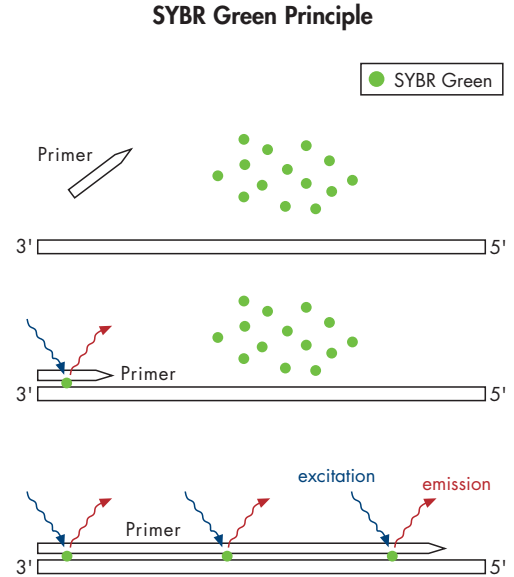
SYBR Green Principle

Figure 1 Principle of SYBR Green I based detection of PCR products in real-time PCR.
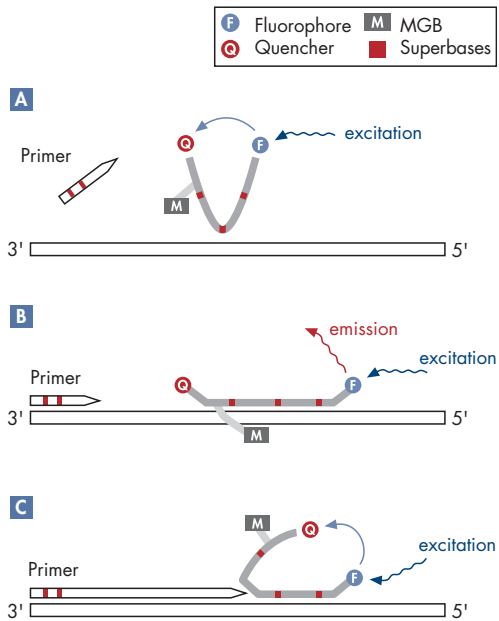
QuantiProbe™ Principle

Figure 2 Principle of QuantiProbes in quantitative, real-time PCR. **A** When not bound to its target sequence, the QuantiProbe forms a random structure in solution. The proximity of the fluorescent reporter with the quencher prevents the reporter from fluorescing. **B** During the PCR annealing step, the QuantiProbe hybridizes to its target sequence. This separates the fluorescent dye and quencher resulting in a fluorescent signal. The amount of signal is proportional to the amount of target sequence, and is measured in real time to allow quantification of the amount of target sequence. **C** During the extension step of PCR, the probe is displaced from the target sequence, bringing the fluorophore and quencher into closer proximity, resulting in quenching of fluorescence.

TaqMan Probe Principle
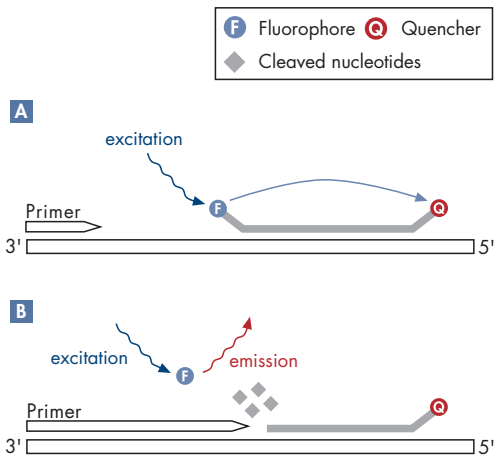
QuantiProbes

QuantiProbes, included in QuantiTect® Gene Expression Assays and QuantiTect Custom Assays (see pages 38–40), are sequence-specific fluorescently labeled probes with a fluorophore at the 3' end, and a nonfluorescent quencher and minor groove binder at the 5' end (Figure 2). MGB™ technology leads to increased probe stability and allows the use of shorter probes. QuantiProbes form a random structure in solution, which facilitates efficient quenching of the fluorescent signal. When the QuantiProbe hybridizes to its target sequence during the PCR annealing step, the fluorophore and quencher separate and a fluorescent signal is generated. The fluorescent signal is directly proportional to the amount of PCR product present in the reaction at a given time point, enabling sensitive and accurate quantification of target sequences. The MGB prevents hydrolysis of the QuantiProbe by the 5' → 3' exonuclease activity of *Taq* DNA polymerase, and the probe is simply displaced from the template during the extension step of PCR.

Modified bases known as Superbases, included in the QuantiProbe and primers, together with the MGB at the 5' end of the QuantiProbe, allow successful detection of target sequences that may not have worked well in previous real-time PCR assays, such as AT-rich or GC-rich sequences. Superbases are analogs of the corresponding naturally occurring bases. SuperA™ and SuperT™ form strong bonds with their unmodified complementary bases in the target sequence. The stability of the base pair is comparable to that of a GC base pair. SuperA in the QuantiProbe forms three hydrogen bonds with its complementary T base in the target sequence. In contrast, SuperT in the QuantiProbe forms only two hydrogen bonds with the unmodified A base in the target sequence, but stability of the base pair is increased by improved base stacking. SuperG™ inhibits the formation of tetrad aggregates from a run of several Gs in a row and enables probe design for GC-rich sequences. The modified bases, together with the MGB, enable shorter probes to be used due to the increased T_m. This allows use of primers and probe at predefined sequences, such as splice sites, while still delivering optimal real-time PCR performance — something that may not be possible with other dual-labeled probes or FRET probes.

Figure 3 Principle of TaqMan probes in quantitative, real-time PCR. **A** Both the TaqMan probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the fluorescent reporter to the quencher prevents the reporter from fluorescing. **B** During the PCR extension step, *Taq* DNA polymerase extends the primer. When the enzyme reaches the probe, its 5' → 3' exonuclease activity cleaves the fluorescent reporter from the probe. The fluorescent signal from the free reporter is measured.

TaqMan probes

TaqMan® probes, are sequence-specific oligonucleotide probes carrying a fluorophore and a quencher dye (Figure 3). The fluorophore is attached at the 5' end of the probe and the quencher dye is located at the 3' end. During the combined annealing/extension phase of PCR, the probe is cleaved by the 5' → 3' exonuclease activity of *Taq* DNA polymerase, separating the fluorophore and the quencher dyes. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

FRET probes

PCR with fluorescence resonance energy transfer (FRET) probes, such as LightCycler® hybridization probes, uses two labeled oligonucleotide probes that bind to the PCR product in a head-to-tail fashion (Figure 4). When the two probes bind, their fluorophores come into close proximity, allowing energy transfer from a donor to an acceptor fluorophore. Therefore, fluorescence is detected during the annealing phase of PCR and is proportional to the amount of PCR product. FRET probes usually carry dyes that are only compatible for use on the LightCycler machine. As the FRET system uses two primers and two probes, good design of the primers and probes is critical for successful results.

Molecular Beacons

Molecular Beacons are dual-labeled probes with a fluorophore attached at the 5' end and a quencher dye attached at the 3' end. The probes are designed so that the ends are complementary. When the probe is in solution, the two ends of the probe hybridize and form a stem-loop structure with the fluorophore and quencher in close proximity (Figure 5). This efficiently quenches the fluorescent signal. When the probe binds to the target sequence, the stem opens and the fluorophore and quencher separate. This generates a fluorescent signal in the annealing step that is proportional to the amount of PCR product. With Molecular Beacons, successful probe design can be difficult.

FRET Probe Principle

Figure 4 Principle of FRET probes in quantitative, real-time PCR. **A** When not bound to their target sequence, no fluorescent signal from the acceptor fluorophore is detected. **B** During the PCR annealing step, both FRET probes hybridize to the target sequence. This brings the donor and acceptor fluorophore into close proximity, allowing energy transfer between the fluorophores and resulting in a fluorescent signal from the acceptor fluorophore that is detected. The amount of signal is proportional to the amount of target sequence, and is measured in real time to allow quantification of the amount of target sequence. **C** During the extension step of PCR, the probes are displaced from the target sequence and the acceptor fluorophore is no longer able to generate a fluorescent signal.

Molecular Beacon Principle

Figure 5 Schematic diagram of the principle of Molecular Beacons in quantitative, real-time PCR. **A** When not bound to its target sequence, the Molecular Beacon forms a hairpin structure. The proximity of the fluorescent reporter with the quencher prevents the reporter from fluorescing. **B** During the PCR annealing step, the Molecular Beacon probe hybridizes to its target sequence. This separates the fluorescent dye and reporter, resulting in a fluorescent signal. The amount of signal is proportional to the amount of target sequence, and is measured in real time to allow quantification of the amount of target sequence. **C** During the extension step of PCR, the probe is displaced from the target sequence, bringing the fluorophore and quencher into closer proximity, resulting in quenching of fluorescence.

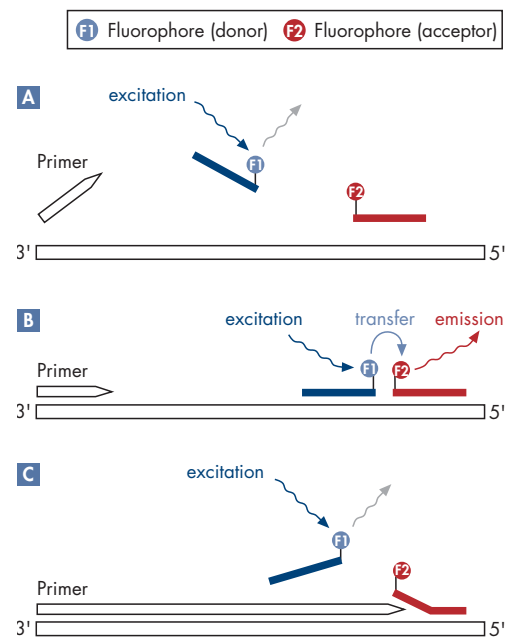**Table 1. Dyes Commonly Used for Quantitative, Real-Time PCR**

Dye	Excitation maximum (nm)	Emission maximum (nm)*
Fluorescein	490	513
Oregon Green	492	517
FAM	494	518
SYBR Green I	494	521
TET	521	538
JOE	520	548
VIC	538	552
Yakima Yellow™	526	552
HEX	535	553
Cy®3	552	570
TAMRA	560	582
Cy3.5	588	604
ROX	587	607
Texas Red	596	615
LightCycler-Red 640 (LC640)	625	640
Cy5	643	667
Cy5.5	683	707

* Emission spectra may vary depending on the buffer conditions.

Dyes used for fluorogenic probes in real-time PCR

Except for SYBR Green I detection, which uses a double-stranded DNA binding molecule, all methods for quantitative, real-time PCR are based on fluorescently labeled probes. The wide variety of fluorescent dyes available makes real-time multiplex PCR possible, providing the dyes are compatible with the excitation and detection criteria of the real-time cycler used. The emission spectra of the chosen dyes must also be sufficiently distinct from one another. For information on excitation and emission spectra for commonly used fluorescent dyes, see Table 1.

? **My sample does not give a fluorescent signal. How can I decide whether this is because the PCR did not work or because the target is not expressed?**

Use a control sample in which the gene of interest is definitely expressed. PCR products that span the region to be amplified in the real-time experiment can also be used as a positive control. Check by agarose gel electrophoresis that the amplification reaction was successful. The quality of the starting template and the integrity of the reagents can be determined by amplifying a housekeeping gene, such as GAPDH or HPRT.

? **What are Superbases and the MGB for?**

Superbases are analogs of the naturally occurring bases. SuperA and SuperT stabilize probe and primer binding within AT-rich regions. SuperG inhibits the formation of tetrad aggregates from a run of several Gs in a row in the primers and probe. The MGB stabilizes the template–probe hybrid and, compared with conventional probes, allows the use of shorter probe sequences. The combination of Superbases with the MGB enables design of real-time RT-PCR assays at pre-defined sequences — even for difficult templates.

? **Do I need to calibrate my real-time cycler if I want to use Yakima Yellow?**

The emission maximum of Yakima Yellow (552 nm) is almost identical to that of the fluorescent dye VIC. Therefore, the channel and filter settings for VIC can also be used for Yakima Yellow.

Methods in real-time PCR

Two-step and one-step RT-PCR

For analysis of gene expression levels or viral load, the RNA first needs to be transcribed into cDNA using a reverse transcriptase. Reverse transcriptases are enzymes generally derived from RNA-containing retroviruses. RT-PCR can take place in a two-step or one-step reaction. With two-step RT-PCR, the RNA is first reverse transcribed into cDNA using oligo-dT primers, random oligomers, or gene-specific primers. An aliquot of the reverse-transcription reaction is then subsequently added to the real-time PCR. In two-step RT-PCR, it is possible to choose between different types of RT primers, depending on experimental needs. Use of oligo-dT primers or random oligomers for reverse transcription means that several different transcripts can be analyzed by PCR from one RT reaction. In addition, precious RNA samples can be immediately transcribed into more stable cDNA for later use and long-term storage.

In one-step RT-PCR — also referred to as one-tube RT-PCR — both reverse transcription and amplification take place in the same tube, with reverse transcription preceding PCR. This is possible due to specialized reaction chemistries and cycling protocols (see pages 23–27). The fast procedure enables rapid processing of multiple samples and is easy to automate. The reduced number of handling steps results in high reproducibility from sample to sample and minimizes the risk of contamination since less manipulation is required. The advantages of each method are given below.

Two-step RT-PCR

- Multiple PCRs from one RT reaction
- Flexibility with RT primer choice
- Enables long-term storage of cDNA

One-step RT-PCR

- Easy handling
- Fast procedure
- High reproducibility
- Low contamination risk

DNA as template in real-time PCR

In contrast with RNA, which requires conversion into cDNA, purified genomic DNA or plasmid DNA can be directly used as starting template in real-time PCR. Not only can genomic DNA be quantified in real-time PCR, for example, in detection of bacterial DNA or GMOs, but it can also be used for qualitative analysis, such as single nucleotide polymorphism (SNP) detection. SNP analysis involves the detection of single nucleotide changes using two probes labeled with different fluorophores. One probe is specific for the wild-type allele, the other for the mutant allele (Figure 6). Real-time PCR is highly suited for the detection of small sequence differences, such as SNPs and viral variants.

Reliable SNP Genotyping with Small Amounts of Template
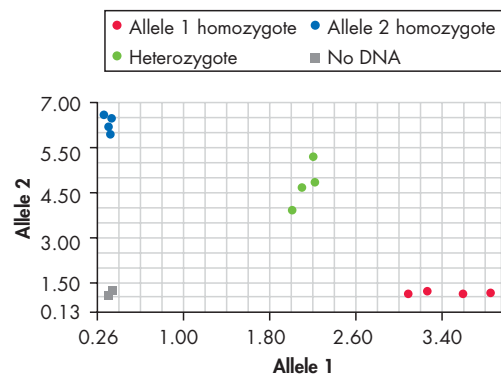
Figure 6 Real-time PCR was carried out using the QuantiTect Probe PCR Kit and dual-labeled (TaqMan) probes for the CPY2D6*4 SNP, using 2 ng or 5 ng DNA template. The allelic discrimination plots clearly indicate the homozygotes for each allele and the heterozygotes.

Quenched FRET assay enables detection of single base mutations

Quenched FRET assays are similar to FRET assays except that the decrease in energy of the donor fluorophore is measured instead of the increase in energy of the acceptor fluorophore. FRET probes were designed to detect the single base mutations H63D and S65C. The QuantiTect Probe PCR Kit enabled highly sensitive detection of wild type and mutant sequences (Figure 7).
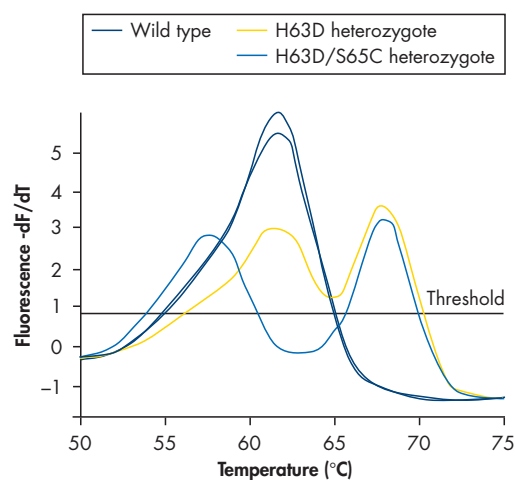
Figure 7 Following real-time PCR using the QuantiTect Probe PCR Kit on the Rotor-Gene™ system, amplification products were subjected to melting curve analysis.

(Data kindly provided by T. Kaiser, Corbett Research, Australia.)

Basic terms used in real-time PCR

A number of basic terms are used in real-time PCR analyses and are briefly described in the following section.

Data are displayed as sigmoidal-shaped amplification plots (when using a linear scale), in which the fluorescence is plotted against the number of cycles (Figure 8).

Baseline and threshold settings

Before gene expression levels can be quantified in real-time PCR studies, the raw data must be analyzed and baseline and threshold values set. When different probes are used in a single experiment, for example, when analyzing several genes in parallel or when using probes carrying different reporter dyes, the baseline and threshold setting must be adjusted for each probe. Furthermore, analysis of different PCR products from a single experiment using SYBR Green I detection requires baseline and threshold adjustments for each individual assay. Guidelines are given below for data analysis. For more information on data analysis, refer to the recommendations provided by the manufacturer of the real-time cycler.

Baseline: The baseline is the noise level in early cycles, typically measured between cycles 3 and 15, where there is no detectable increase in fluorescence due to amplification products. The number of cycles used to calculate the baseline can be changed and should be reduced if high template amounts are used or if the expression level of the target gene is high (Figure 9A). To set the baseline, view the fluorescence data in the linear scale amplification plot. Set the baseline so that growth of the amplification plot begins at a cycle number greater than the highest baseline cycle number (Figure 9B). The baseline needs to be set individually for each target sequence. The average fluorescence value detected within the early cycles is subtracted from the fluorescence value obtained from amplification products.

Background: This refers to nonspecific fluorescence in the reaction, for example, due to inefficient quenching of the fluorophore or the presence of large amounts of double-stranded DNA template when using SYBR Green I. The background component of the signal is mathematically removed by the software algorithm of the real-time cycler.

Reporter signal: Fluorescent signal that is generated during real-time PCR by either SYBR Green I or by a fluorescently labeled sequence-specific probe.

Typical Amplification Plot
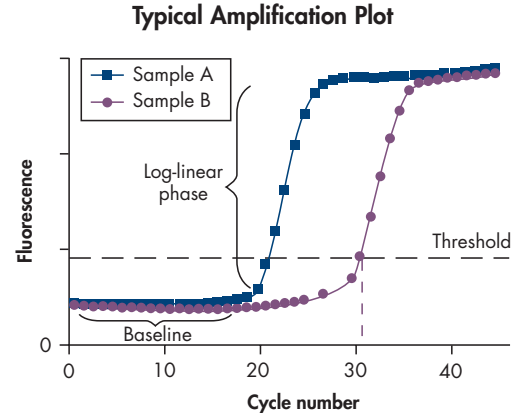
Figure 8 Amplification plots showing increases in fluorescence from 2 samples (A and B). Sample A contains a higher amount of starting template than sample B.

Correct Baseline and Threshold Settings are Important for Accurate Quantification
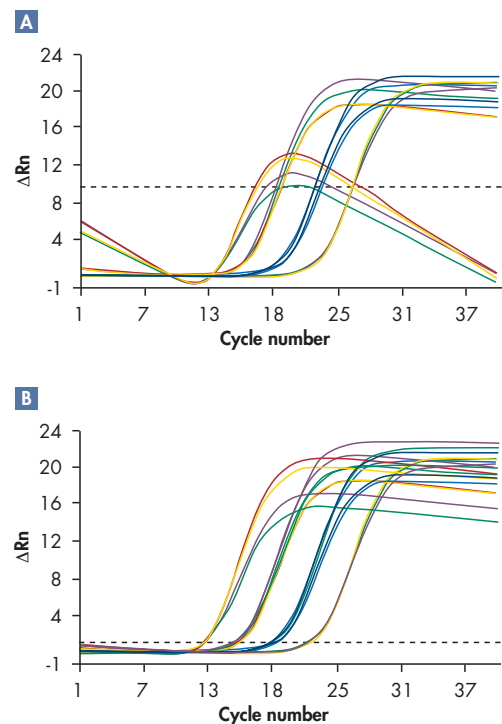
Figure 9 A Amplification product becomes detectable within the baseline setting of cycles 6 to 15 and generates a wavy curve with the highest template amount. B Setting the baseline within cycles 6 to 13 eliminates the wavy curve. The threshold is set at the beginning of the detectable log-linear phase of amplification.

Normalized reporter signal (Rn): This is the emission intensity of the reporter dye divided by the emission intensity of the passive reference dye measured in each cycle.

Passive reference dye: On some real-time PCR instruments, the fluorescent dye ROX serves as an internal reference for normalization of the fluorescent signal. It allows correction of well-to-well variation due to pipetting inaccuracies and fluorescence fluctuations. Its presence does not interfere with real-time PCR assays, since it is not involved in PCR and has an emission spectrum completely different from fluorescent dyes commonly used for probes.

Threshold: The threshold is adjusted to a value above the background and significantly below the plateau of an amplification plot. It must be placed within the linear region of the amplification curve, which represents the detectable log-linear range of the PCR. The threshold value should be set within the logarithmic amplification plot view to enable easy identification of the log-linear phase of the PCR. If several targets are used in the real-time experiment, the threshold must be set for each target.

Threshold cycle (C_T) or crossing point: This is the cycle at which the amplification plot crosses the threshold, i.e., at which there is a significant detectable increase in fluorescence. The C_T serves as a tool for calculation of the starting template amount in each sample (see pages 14–22 for more information).

ΔC_T value: The ΔC_T value describes the difference between the C_T value of the target gene and the C_T value of the corresponding endogenous reference gene, such as a housekeeping gene:

$\Delta C_T = C_T$ (target gene) – C_T (endogenous reference gene)

$\Delta\Delta C_T$ value: The $\Delta\Delta C_T$ value describes the difference between the average ΔC_T value of the sample of interest (e.g., stimulated cells) and the average ΔC_T value of a reference sample (e.g., unstimulated cells). The reference sample is also known as the calibrator sample and all other samples will be normalized to this when performing relative quantification:

$\Delta\Delta C_T$ = average ΔC_T (sample of interest) – average ΔC_T (reference sample)

For more information on ΔC_T and $\Delta\Delta C_T$ see "Quantification of target amounts", pages 14–22.

Endogenous reference gene: This is a gene whose expression level should not differ between samples, such as a housekeeping or maintenance gene. Comparison of the C_T value of a target gene with that of the endogenous reference gene allows the gene expression level of the target gene to be normalized to the amount of input RNA or cDNA (see ΔC_T value). This is done without determining the exact amount of template used in the reaction. The use of an endogenous reference gene corrects for variation in RNA content, variation in reverse-transcription efficiency, possible RNA degradation or presence of inhibitors in the RNA sample, variation in nucleic acid recovery, and differences in sample handling. Genes commonly used as references are shown in Table 2.

Table 2. Housekeeping Genes Commonly Used as Endogenous References

Gene name	Relative expression level*
18S ribosomal RNA	++++
Glyceraldehyde-3-phosphate dehydrogenase	+++
β-Actin, cytoplasmic	+++
β-2-microglobulin	+++
Phosphoglycerate kinase 2	+++
Peptidylprolyl isomerase A (cyclophilin A)	+++
Ribosomal protein, large, PO	+++
Hypoxanthine phosphoribosyl transferase 1	++
β-Glucuronidase	+
TATA box binding protein	+
Transferrin receptor	+

* "+" indicates relative abundance of the transcripts.

Calibrator sample: This is a reference sample used in relative quantification. For example, RNA isolated from a cell line or tissue, to which all other samples are compared to determine the relative expression level of a gene. The calibrator sample usually has a stable expression ratio of target gene to endogenous reference gene.

No template control (NTC): A control reaction that contains all essential components of the amplification reaction except the template. This enables detection of contamination.

RT minus control: RNA preparations may contain residual genomic DNA, which may be detected in real-time PCR if assays are not designed to only detect and amplify RNA (see pages 36–37). DNA contamination can be detected by performing a control reaction in which no reverse transcription takes place.

Standard: Sample of known concentration or copy number used to construct a standard curve.

Standard curve: To generate a standard curve, C_T values/crossing points of different standard dilutions are plotted against the logarithm of input amount of standard material. The standard curve is commonly generated using a dilution series of at least 5 different concentrations of the standard. Each standard curve should be checked for validity, with the value for the slope falling between –3.3 to –3.8. Standards are ideally measured in triplicate for each concentration. Standards which give a slope differing greatly from these values should be discarded.

Efficiency and slope: The slope of a standard curve provides an indication of the efficiency of the real-time PCR. A slope of –3.322 means that the PCR has an efficiency of 1, or 100%, and the amount of PCR product doubles during each cycle. A slope of less than –3.322 (e.g., –3.8) is indicative of a reaction efficiency <1. Generally, most amplification reactions do not reach 100% efficiency due to experimental limitations. A slope of greater than –3.322 (e.g., –3.0) indicates a PCR efficiency, which appears to be greater than 100%. This can occur when values are measured in the non-linear phase of the reaction or it can indicate the presence of inhibitors in the reaction (see page 24).
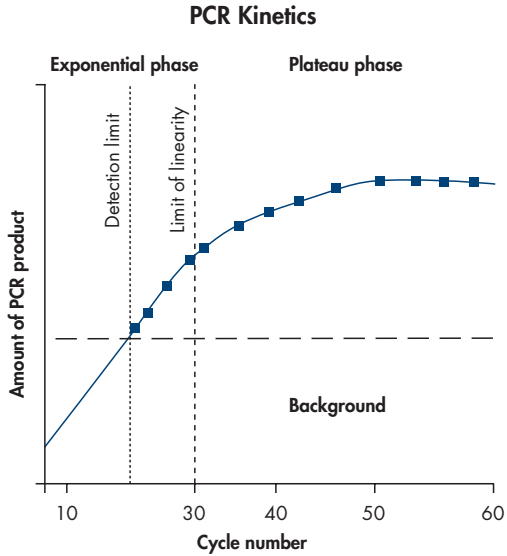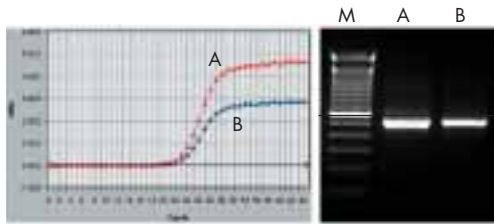
PCR Kinetics

Figure 10 Reaction kinetics in PCR.

Problems Associated with Endpoint Detection

A **Same starting amount**
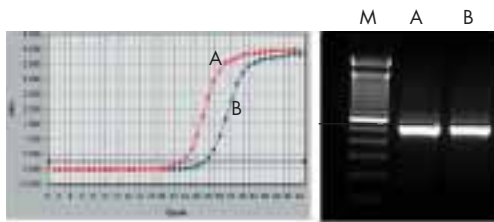

B **Different starting amount**

Figure 11 Two-step RT-PCR was carried out with **A** the same amount and **B** different amounts of template RNA.
M: markers.

Quantification of target amounts

With PCR, minute amounts of starting template DNA or cDNA can be amplified enabling detection of a target sequence. If PCR products are analyzed by endpoint analysis, quantification is not possible as most reactions will already have reached the plateau phase of amplification. During this phase, no significant increase in PCR product amount takes place (Figure 10). This is mainly due to depletion of PCR components and renaturation of PCR product strands caused by the high concentration of endproducts, which prevents further primer annealing.

If identical template amounts are used, this may not necessarily result in identical yields of PCR products (Figure 11A). Equally, if different amounts of template are used, the yield of PCR products may be similar making quantification impossible (Figure 11B).

Real-time PCR overcomes this problem by determining the actual amount of PCR product present at a given cycle, indicated by the intensity of fluorescence. The fluorescence generated by SYBR Green I or fluorescently labeled probes is indicative of the amount of PCR product in the reaction, providing the reaction is in the log-linear or so-called exponential phase (see Figure 10) and providing that amplification proceeds with a comparable efficiency for all samples. By selecting the threshold within the log-linear phase for all samples, it is possible to calculate the actual amount of initial starting molecules since the fluorescence intensity is directly proportional to the amount of PCR product in the exponential phase.

Quantification

Target nucleic acids can be quantified using either absolute quantification or relative quantification. Absolute quantification determines the absolute amount of target (expressed as copy number or concentration), whereas relative quantification determines the ratio between the amount of target and an endogenous reference molecule, usually a suitable housekeeping gene. This normalized value can then be used to compare, for example, differential gene expression in different samples.

Absolute quantification

Use of external standards enables the expression level of a gene to be given as an absolute copy number. For gene expression analysis, the most accurate standards are RNA molecules of known copy number or concentration. Depending on the sequence and structure of the target and the efficiency of reverse transcription, only a proportion of the target RNA in the RNA sample will be reverse transcribed. The cDNA generated during reverse transcription then serves as template in the subsequent real-time PCR. The use of RNA standards takes into account the variable

efficiency of reverse transcription as well as the possible presence of reverse-transcription inhibitors, which may be in the RNA sample. Brief guidelines for the generation of RNA standards and determination of RNA concentration are given in "Appendix B: Determination of PCR Efficiency and Quantification of Gene Expression Levels" in the *QuantiTect Gene Expression Assay Handbook*.

A standard curve (plot of C_T values/crossing points of different standard dilutions against log of amount of standard) is generated using a dilution series of at least 5 different concentrations of the standards (Figure 12). The amount of unknown target should fall within the range tested. Amplification of the standard dilution series and of the target sequence is carried out in separate wells. C_T values of standard samples are determined. Then, C_T values of the unknown samples are compared with the standard curve to determine the amount of target in the unknown sample (Figure 12).

It is important to select an appropriate standard for the type of nucleic acid to be quantified. The copy number or concentration of the nucleic acids used as standards must be known. In addition, standards should have the following features:

■ Primer and probe binding sites identical to the target to be quantified

■ Sequence between primer binding sites identical or highly similar to target sequence

■ Sequences upstream and downstream from the amplified sequence identical or similar to "natural" target

■ Equivalent amplification efficiencies of standard and target molecules

Relative quantification

Alternatively, gene expression levels can be calculated by determining the ratio between the amount of a target gene and an endogenous reference gene that is in all samples. This ratio is then compared between different samples. Usually, housekeeping or maintenance genes are chosen as an endogenous reference. The target and reference gene are amplified from the same sample, either separately or in the same reaction (duplex real-time PCR). The normalized value is determined for each sample and can be used to compare, for example, differential expression of a gene in different tissues. However, the expression level of the endogenous reference gene must not vary under different experimental conditions or in different states of the tissue (e.g., "stimulated" versus "unstimulated" samples). When gene expression levels are compared between samples, the expression level of the target is referred to as being, for example, 100-fold higher in stimulated cells than in unstimulated cells. The quantification procedure differs depending on whether the target and the endogenous reference gene are amplified with comparable or different efficiencies.
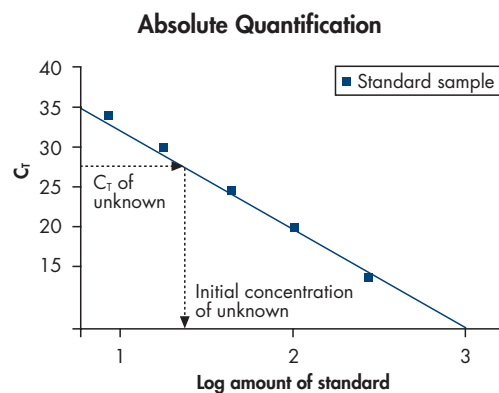
Figure 12 Typical standard curve showing determination of concentration of sample of interest.
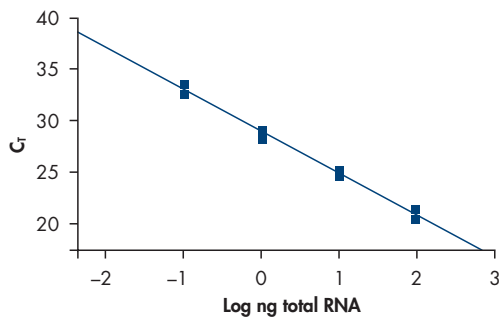
Figure 13 RNA was stabilized and isolated from 10 mg mouse liver and kidney using the RNeasy® Protect Mini Kit. Real-time RT-PCR was carried out using the QuantiTect Probe RT-PCR Kit and the amounts of RNA template shown. Amplification was performed on the DNA Engine Opticon system using primers and probe specific for the mouse β-actin gene.

Use of β-actin as a reference gene in real-time RT-PCR

β-actin is a so-called housekeeping gene, whose expression remains constant under a wide variety of physiological conditions. For this reason, β-actin is commonly used as a standard in quantitative, real-time PCR. One-step real-time RT-PCR of the mouse β-actin transcript was carried out on the DNA Engine Opticon® system (Figure 13). The results showed that β-actin was expressed at similar levels in liver and kidney. This makes it suitable for use as an endogenous reference in this experiment.

Table 3. C_T Values Obtained for β-Actin in Kidney and Liver

| | Amount of RNA | | | |
Sample	100 ng	10 ng	1 ng	0.1 ng
Kidney	21.32	25.29	29.07	32.81
Liver	21.92	25.01	28.77	32.58

Determining amplification efficiencies

The amplification efficiency of two genes (target A and target B) can be compared by preparing a dilution series for both genes from a reference RNA or cDNA sample. Each dilution series is then amplified in real-time one-step or two-step RT-PCR and the C_T values obtained are used to construct standard curves for target A and target B. The amplification efficiency (E) for each target can be calculated according to the following equation:

$$E = 10^{(-1/S)} - 1, \text{ where S is the slope of the standard curve.}$$

To compare the amplification efficiencies of the two target sequences, the C_T values of target A are subtracted from the C_T values of target B. The difference in C_T values is then plotted against the logarithm of the template amount (Figure 14). If the slope of the resulting straight line is <0.1, amplification efficiencies are comparable.

Different amplification efficiencies

Amplification efficiencies of the target gene and the endogenous reference gene are usually different since efficiency of primer annealing, GC-content of the sequences to be amplified, and PCR product size usually varies between the two genes. In this case, a standard curve needs to be prepared for the target gene as well as for the endogenous reference gene, for example, using total RNA prepared from a reference cell line (calibrator or reference sample). Due to differences in PCR efficiency, the resulting standard curves will not be parallel and the differences in C_T values of the target and the reference will not be constant when the template amounts are varied (Figure 15).

Efficiency Comparison
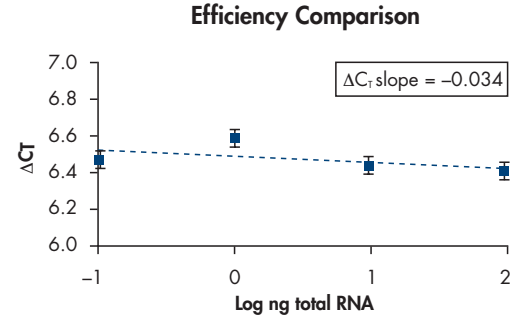
Figure 14 C_T values were determined for the tumor necrosis factor alpha (TNF-α) gene and the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) using RNA isolated from human leukocytes. Real-time RT-PCR was performed using the QuantiTect Probe RT-PCR Kit and the QuantiTect Hs_TNF Assay or the QuantiTect Hs_HPRT1 Assay. The difference in C_T values was plotted against log template amount. The difference in PCR efficiency was determined by calculating the slope of the line.

Different PCR Efficiency
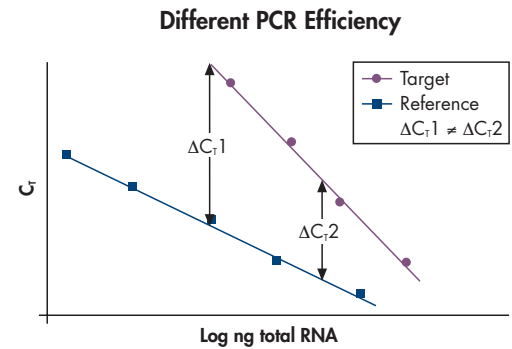
Figure 15 Typical standard curve showing amplification of two targets with different PCR efficiencies.

Table 4. Data used to Generate Standard Curves

Template amount (ng)	Log template amount (ng)	C_T (IL8)	C_T (B2M)
100	2	19.65	20.74
10	1	23.01	23.96
1	0	26.55	27.43
0.1	–1	30.55	30.85
0.01	–2	34.01	
0.001	–3	37.41	
Slope		–3.59	–3.38
PCR efficiency		0.90	0.98

Relative Quantification with Different PCR Efficiencies

A — IL8 standard curve

Slope = –3.59

C_T vs Log ng total RNA

B — B2M standard curve

Slope = –3.38

C_T vs Log ng total RNA

Figure 16 Standard curves were generated for interleukin 8 (IL8) and the housekeeping gene β-2-microglobulin (B2M) using total RNA from human leukocytes. RT-PCR was performed using the corresponding QuantiTect Gene Expression Assay with the QuantiTect Probe RT-PCR Kit.

Guidelines for relative quantification with different amplification efficiencies

- Choose an appropriate endogenous reference gene (e.g., β-2 microglobulin or peptidylprolyl isomerase A) whose expression level does not change under the experimental conditions or between different tissues

- Prepare a dilution series (e.g., fivefold or tenfold dilutions) of a cDNA or RNA control sample to construct standard curves for target and reference

- Perform real-time PCR/RT-PCR

- Determine C_T values for the standards (Table 4) and samples of interest

- Construct standard curves for both target and reference by plotting C_T values (Y-axis) against the log of template amount or dilution (X-axis) (Figure 16)

- Calculate amount of target and reference in samples of interest using C_T value and corresponding standard curve (see Table 5)

- To calculate the normalized amount of target, divide the amount of target by the amount of reference (if replicate reactions were performed, use average value)

- Set the normalized target amount of one sample to 1 (calibrator sample) and compare relative expression level of target gene in samples of interest by dividing the normalized target amounts by the value of the calibrator

Table 5. Quantification and Normalization of IL8 and B2M Expression Levels

Sample	IL8 C_T	B2M C_T	Amount of IL8 RNA (ng)*	Amount of B2M RNA (ng)	Normalized amount of IL8 RNA (ng)*	Ratio
Untreated (calibrator sample)	37.01	18.83	1.40E-03	346	4.05E-06	1.0
PMA	29.43	18.59	1.80E-01	417	4.32E-04	106.7
LTA	34.43	18.59	7.20E-03	417	1.73E-05	4.3

Jurkat cells were untreated or treated with either phorbol 12-myristate acetate (PMA) or lipoteichoic acid (LTA). Total RNA was isolated and after real-time RT-PCR, the relative amounts of target and reference RNA were determined using the appropriate standard curve (see Figure 16).

* 1.00E-03 ng = 1 pg

Comparable amplification efficiencies

If the amplification efficiencies of target and endogenous reference genes are comparable, one standard curve for the reference gene is sufficient. The differences in C_T values of the target and endogenous reference gene will be constant w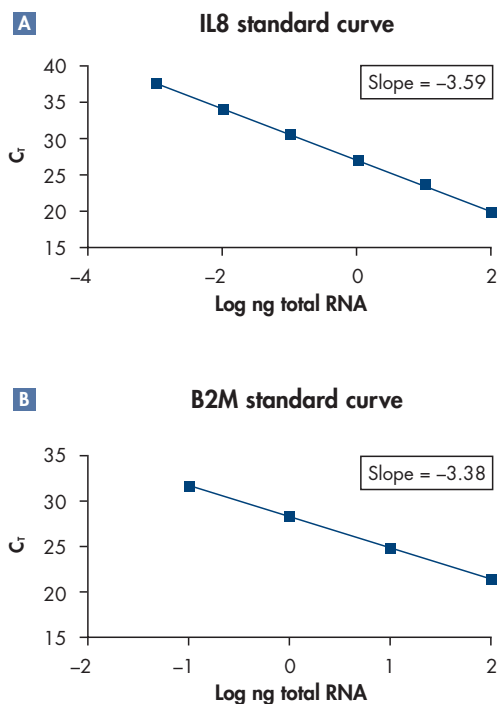hen the amounts of template are varied (Figure 17). Unknown amounts of target in the sample are calculated by comparing the C_T values with the standard curve of the reference sample as described below.

Guidelines for relative quantification with comparable amplification efficiencies

■ Choose an appropriate endogenous reference gene (e.g., β-2-microglobulin or peptidylprolyl isomerase A) whose expression level does not change under the experimental conditions or between different tissues

■ Prepare a dilution series (e.g., fivefold or tenfold dilutions) of a cDNA or RNA control sample to construct a standard curve for the endogenous reference gene only

■ Perform real-time PCR/RT-PCR

■ Determine C_T values for the standards and samples of interest

■ Construct a standard curve for the endogenous reference gene by plotting C_T values (Y-axis) against the log of template amount or dilution (X-axis)

■ Calculate amount of target and reference in samples of interest using C_T value and standard curve

■ To calculate the normalized amount of target, divide the amount of target by the amount of reference (if replicate reactions were performed, use average value)

■ Set the normalized target amount of one sample to 1 (calibrator sample) and compare relative expression level of target gene in samples of interest by dividing the normalized target amounts by the value of the calibrator
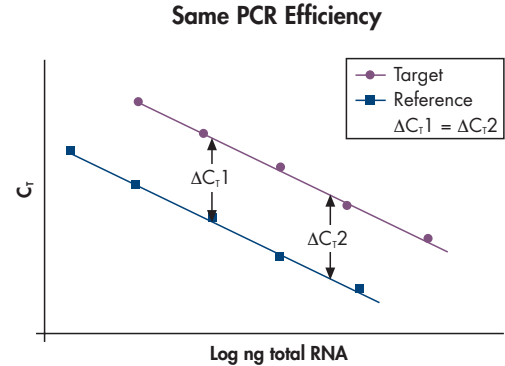
Same PCR Efficiency

Figure 17 Typical standard curve showing amplification of two targets with similar PCR efficiencies.

Comparative method or $\Delta\Delta C_T$ method of relative quantification

An alternative approach is the comparative or $\Delta\Delta C_T$ method, which relies on direct comparison of C_T values. The preparation of standard curves is only required to determine amplification efficiencies of target and endogenous reference gene in an initial experiment. In all subsequent experiments, no standard curve is required for quantification of the target sequence. If amplification efficiencies are comparable, amounts of target are simply calculated by using C_T values as described below.

First of all, the ΔC_T value for each sample is determined by calculating the difference between the C_T value of the target gene and the C_T value of the endogenous reference gene. This is determined for each unknown sample as well as for the calibrator sample.

ΔC_T (sample) = C_T target gene – C_T reference gene

ΔC_T (calibrator) = C_T target gene – C_T reference gene

Next, the $\Delta\Delta C_T$ value for each sample is determined by subtracting the ΔC_T value of the calibrator from the ΔC_T value of the sample.

$\Delta\Delta C_T = \Delta C_T$ (sample) – ΔC_T (calibrator)

If the PCR efficiencies of the target gene and endogenous reference gene are comparable, the normalized level of target gene expression is calculated by using the formula:

Normalized target gene expression level in sample = $2^{-\Delta\Delta C_T}$; see Table 7.

However, if the PCR efficiency is not the same between the target gene and endogenous reference gene, this method of quantification may lead to inaccurate estimation of gene expression levels. The error is a function of the PCR efficiency and the cycle number and can be calculated according to the formula:

Error (%) = $[(2^n/(1+E)^n) \times 100)] - 100$, where E is the efficiency of PCR and n the cycle number.

Therefore, if the PCR efficiency is only 0.9 instead of 1.0, the resulting error at a threshold cycle of 25 will be 261%. The calculated expression level will be 3.6-fold less than the actual value. Therefore, the $\Delta\Delta C_T$ method should only be chosen if the PCR efficiency of target gene and endogenous reference gene are the same, or if the difference in expression levels is sufficiently high to tolerate the resulting error. However, errors can be corrected by using efficiency-corrected calculation programs, such as the Relative Expression Software Tool (REST©) (1).

Reference

1. Pfaffl, M.W., Horgan, G.W., and Dempfle, L. (2002). Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 2002 May 1; 30(9): E36

Guidelines for relative quantification using $\Delta\Delta C_T$ method

■ Perform validation experiment to determine PCR efficiency for target and reference as described on page 17 and shown in Figure 18

■ Perform real-time RT-PCR for target and reference with RNA derived from different samples

■ Determine ΔC_T value by subtracting endogenous reference gene C_T value from the target gene C_T value for each sample (Table 6)

■ Define calibrator sample and determine $\Delta\Delta C_T$ value by subtracting the calibrator ΔC_T value from ΔC_T value of each sample (Table 7)

■ Calculate normalized level of target expression relative to calibrator by using the formula $2^{-\Delta\Delta C_T}$

Table 6. Data used to Generate Standard Curves

Template amount (ng)	Log template amount	C_T (TNF)	C_T (HPRT)	ΔC_T (C_T TNF − C_T HPRT)
100	2	29.28	22.88	6.40
10	1	32.52	26.09	6.43
1	0	35.80	29.22	6.58
0.1	−1	39.40	32.93	6.47

Table 7. Calculation of TNF Expression Levels in Jurkat Cells Using the $\Delta\Delta C_T$ Method

Sample	Average C_T TNF	Average C_T HPRT	ΔC_T TNF − HPRT	$\Delta\Delta C_T$ ΔC_T − ΔC_T calibrator	Expression of TNF compared to calibrator ($2^{-\Delta\Delta C_T}$)
Untreated (calibrator sample)	36.5	22.8	13.7	0	1
PMA	31.0	23.1	7.9	−5.8	55.7

Jurkat cells were untreated or treated with PMA. Total RNA was isolated and after real-time RT-PCR the relative amounts of target and reference RNA were determined.

Comparison of PCR Efficiency for TNF-α and HPRT Genes
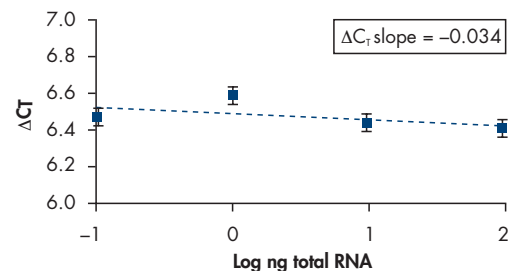
Figure 18 C_T values were determined for the tumor necrosis factor alpha (TNF-α) gene and the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) using RNA isolated from human leukocytes. Real-time RT-PCR was performed using the QuantiTect Probe RT-PCR Kit and the QuantiTect Hs_TNF Assay or the QuantiTect Hs_HPRT1 Assay. The difference in C_T values was plotted against log template amount. The difference in PCR efficiency was determined by calculating the slope of the line.

Endogenous reference genes

For relative quantification, it is important to choose a suitable gene to use as a reference. The expression level of the reference gene must not vary under experimental conditions, or in different states of the same tissue or cell line (e.g., "disease" versus "normal" samples). The expression level of the reference RNA should also be approximately the same as the RNA under study. Reference RNA commonly used for relative quantification includes β-actin, β-2-microglobulin, peptidylprolyl isomerase A, and GAPDH mRNAs, and also 18S rRNA. β-actin mRNA is ubiquitously expressed and was one of the first RNAs to be used as a reference sequence. However, its transcription levels may vary and the presence of pseudogenes may mean that genomic DNA is detected during real-time PCR, leading to inaccuracies in quantification. GAPDH is a housekeeping gene commonly used as a reference for quantification of gene expression. GAPDH mRNA levels may vary between individuals, at different stages of the cell cycle, and following treatment with different

drugs making it unsuitable as a reference in some systems. As 18S rRNA is not an mRNA, its expression levels in the cell may not accurately reflect the cellular mRNA population. Therefore, a combination of genes may provide the most reliable reference for quantification studies. A comprehensive list of housekeeping genes is available from QIAGEN as functionally validated QuantiTect Gene Expression Assays for real-time PCR at www.qiagen.com/goto/assays .

? Which controls are necessary in a real-time PCR experiment?

To detect contamination, an NTC must be included in all quantification experiments. If possible, a positive control such as nucleic acid from an established cell line or plasmid DNA should be used — particularly when amplifying a new target sequence — to check that the primer–probe combination works. For gene expression analysis, an RT minus control should be included. If performing one-step RT-PCR, reverse transcriptase should not be added to the reaction. In two-step RT-PCR, the RT minus control should contain all components except for reverse transcriptase. Reverse transcription therefore cannot take place and the only template available is contaminating DNA.

? Why do replicates sometimes have different plateau heights?

The plateau phase of PCR is where the reaction is no longer in log-linear growth and the height of the plateau indicates the yield of PCR product. Identical amounts of starting template will not always result in identical yields of PCR product.

? What should I use as a standard for absolute quantification?

For quantification of RNA, we strongly recommend using RNA molecules as standards. Use of in vitro transcripts as standards takes into account the variable efficiency of the RT reaction. An alternative to the use of in vitro transcripts as RNA standards is the use of a defined RNA preparation (e.g., from a cell line or virus preparation), for which the absolute concentration of the target has already been determined. For quantification of DNA, several types of DNA can be used, such as plasmids, PCR products, or even genomic DNA. For more information, see the *QuantiTect Handbooks*, or go to www.qiagen.com .

Guidelines for successful real-time RT-PCR: reverse transcription

1. Choice of RT primers

RT-PCR can be performed either as a one-step or two-step reaction. In one-step RT-PCR, also known as one-tube or single-tube RT-PCR, reverse transcription and PCR are performed sequentially in the same tube without any interruption. The downstream PCR primer is also the primer for reverse transcription. Therefore, one-step RT-PCR is always performed with gene-specific primers. Alternatively, the two reactions can be separated, both temporally and physically, in two-step RT-PCR. In two-step RT-PCR, three types of primers can be used for reverse transcription — oligo-dT primers (13–18mers), random oligomers (such as hexamers, octamers, or nonamers), or gene-specific primers (Table 8). If oligo-dT primers are used, only mRNAs will be reverse transcribed starting from the poly-A tail at the 3' end. Random oligomers will enable reverse transcription from the entire RNA population, including ribosomal RNA, transfer RNA, and small nuclear RNAs. Since reverse transcription is initiated from several positions within the RNA molecule, this will lead to relatively short cDNA molecules. In comparison, gene-specific primers allow reverse transcription of a specific transcript.

Table 8. Suitability of Primer Types for RT-PCR

Application	Recommended type of primer
RT-PCR of specific transcript:	Gene-specific primer gives highest selectivity and only the RNA molecule of choice will be reverse transcribed
RT-PCR of long amplicon:	Oligo-dT or gene-specific primers
RT-PCR of an amplicon within long transcript:	Gene-specific primers, random oligomers, or a mixture of oligo-dT primers and random nonamers (see page 24) are recommended so that cDNA covering the complete transcript is produced

Effect of RT Primer Choice on RT-PCR

A Amplicon 2 kb from 3' end of template
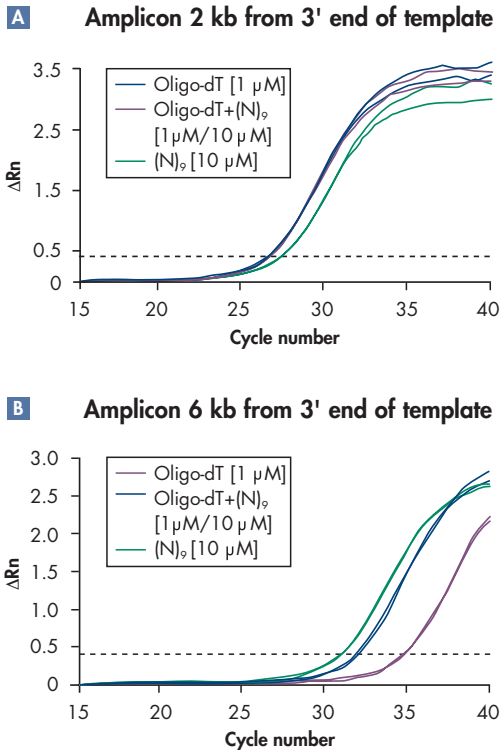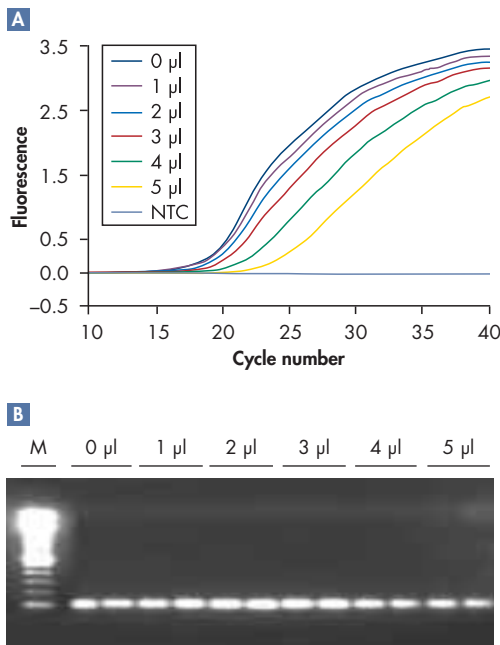

B Amplicon 6 kb from 3' end of template

Figure 19 Two-step RT-PCR was carried out using the QuantiTect SYBR Green PCR Kit and the primer combinations shown. The amplicon was **A** 2 kb from the 3' end or **B** 6 kb from the 3' end of the template RNA.

Inhibition of Real-Time PCR by Addition of RT Reaction

A

B

2. Universal priming method for real-time, two-step RT-PCR

To amplify several targets from one RNA population, we recommend using a mixture of oligo-dT primers and random nonamers. This enables successful amplification and detection of PCR product regardless of transcript length and amplicon position. To examine the effect of primers on RT-PCR, two regions within a 10 kb transcript were selected for amplification in real-time, two-step RT-PCR using SYBR Green I for detection of the PCR products. Each amplicon was approximately 150 bp in length. For the amplicon 2 kb away from the 3' end of the transcript, the best C_T values were obtained using either oligo-dT primers alone or a mixture of oligo-dT primers and random nonamers (Figure 19A). However, when the amplicon was 6 kb away from the 3' end of the transcript, random nonamers provided the best C_T value closely followed by the mixture of oligo-dT primers and random nonamers (Figure 19B). The use of oligo-dT primers alone resulted in detection of the PCR product at a later stage in PCR, or higher C_T. We recommend using a mixture of random nonamers and oligo-dT primers in a ratio of 10 µM:1 µM for real-time, two-step RT-PCR. Random nonamers proved to be better than random hexamers or dodecamers.

3. Effect of RT volume added to two-step RT-PCR

In two step RT-PCR, RNA is first transcribed into cDNA, using random oligomers, oligo-dT primers, or gene-specific primers. Following reverse transcription, an aliquot of the RT reaction is added to the real-time PCR. However, in addition to the cDNA template salts, dNTPs, and RT enzyme are also added. The RT reaction buffer, which has a different salt composition to that of the real-time PCR buffer, can significantly affect the performance of the real-time PCR. However, if 10% or less of the final volume of the real-time PCR is added from the RT reaction, performance should not be significantly affected. Increasing the volume of the RT reaction added to more than 10% of the final PCR volume can dramatically inhibit the real-time PCR (Figure 20). Addition of 3 µl of RT reaction to a 20 µl PCR, which is equal to 15% of the final volume, can lead to significant inhibition of the real-time PCR (Figure 20A). The individual real-time PCRs were also analyzed by agarose gel electrophoresis (Figure 20B), providing further evidence that large volumes of RT reaction affect the sensitivity and linearity of PCR. We recommend testing dilutions of the RT reaction in real-time PCR to check the linearity of the assay. This helps to eliminate any inhibitory effects due to addition of an aliquot of the RT reaction, which might affect accurate quantification of transcript levels.

Figure 20 Real-time PCR was carried out using plasmid DNA as template. The volumes of RT reaction (without template RNA) indicated above were added to the PCR to determine their effect on amplification. **A** Amplification plot **B** Analysis of real-time reactions by agarose gel electrophoresis.

4. Choice of reverse transcriptase

QIAGEN Omniscript® and Sensiscript® Reverse Transcriptases provide efficient and sensitive reverse transcription of any template. Omniscript Reverse Transcriptase is suitable for reverse transcription using any amount of RNA from 50 ng to 2 µg per reaction and Sensiscript Reverse Transcriptase is designed for reverse transcription with less than 50 ng RNA, including carrier RNA. The unique RT buffer system promotes highly specific primer binding and guarantees accurate and sensitive results in real-time RT-PCR. Nonspecific primer binding can lead to a number of problems with the efficiency of RT and RT-PCR processes, and with the interpretation of results. Nonspecific products produced in the reverse transcription reaction can compete with specific products in PCR, decreasing the sensitivity of the reaction leading to inaccurate quantification. Both Omniscript and Sensiscript Reverse Transcriptase provide high yields of cDNA, and nonspecific products are reduced to a minimum. When Omniscript Reverse Transcriptase was used in quantitative RT-PCR on the LightCycler system, subsequent melting curve analysis showed a distinct peak for the specific product with a characteristic melting point of 85.5°C (Figure 21A). In comparison, a commonly used MMLV RNase H⁻ RT gave a peak of similar magnitude to nonspecific background, indicating the correspondingly lower specificity and sensitivity of this enzyme (Figure 21B). Nonspecific products generated in the RT reaction were amplified in the PCR.

RNA secondary structure can affect RT-PCR results in a number of ways. During reverse transcription in both one-step or two-step RT-PCR, regions of RNA with complex secondary structure can cause the reverse transcriptase to stop or dissociate from the RNA template (Table 9A). The truncated cDNAs, missing the downstream primer-binding site, are then not amplified during PCR. Alternatively, the RT can skip over looped-out regions of RNA. This causes the looped-out region to be excluded from the reverse-transcribed cDNA. In the PCR step, these cDNAs with internal deletions are amplified and appear as shortened PCR products (Table 9B and Figure 21B). Ideally, the reverse transcriptase should not be affected by RNA secondary structure (Table 9C) and should be capable of reverse transcribing any template, without the need for optimization in one-step or two-step RT-PCR. Omniscript and Sensiscript Reverse Transcriptases have a particularly high affinity for RNA. Their high affinity for RNA means that the enzymes are closely associated with the template RNA. This enables read-through of templates with complex secondary structure or high GC content, producing full-length cDNA.

Melting-Curve Analysis Demonstrating Higher Specificity of Omniscript RT
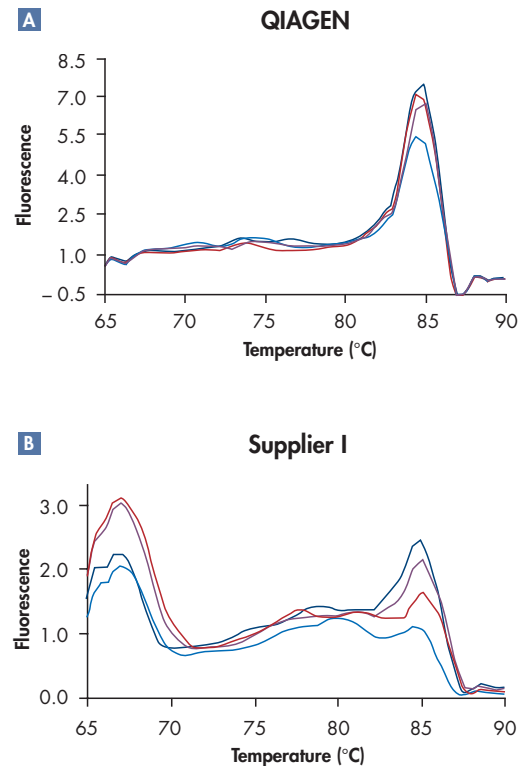
Figure 21 Melting-curve analysis of interleukin-1 RT-PCR products was carried out on the LightCycler system using SYBR Green I as a fluorescent label. RT-PCR was carried out with serial dilutions of total RNA and **A** Omniscript Reverse Transcriptase (**QIAGEN**) in the RT step or **B** an MMLV RNase H⁻ RT (**Supplier I**).

RNase H Required for GC-Rich Target

A Omniscript RT — M 37 42 50 °C

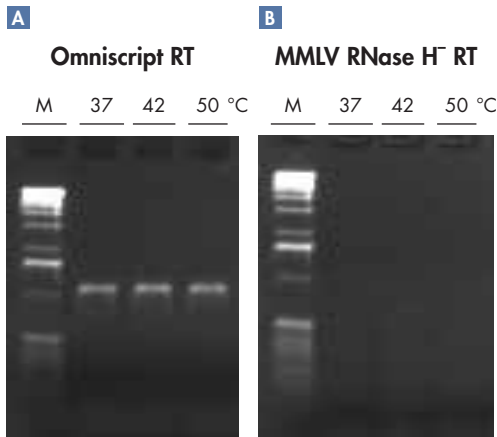**B** MMLV RNase H⁻ RT — M 37 42 50 °C

Figure 22 RT-PCR was carried out with total RNA from maize leaves using the indicated RT reaction temperatures. A 1.2 kb fragment of the maize gl2 transcript was amplified. **A** RT was carried out using the standard Omniscript RT protocol at the standard (37°C) and higher reaction temperatures as indicated. **B** RT was carried out using the standard MMLV RNase H⁻ RT protocol (Supplier I) at the standard (42°C) and other reaction temperatures as indicated. This standard protocol was carried out with the required preliminary denaturation step but without the RNase H digest step.

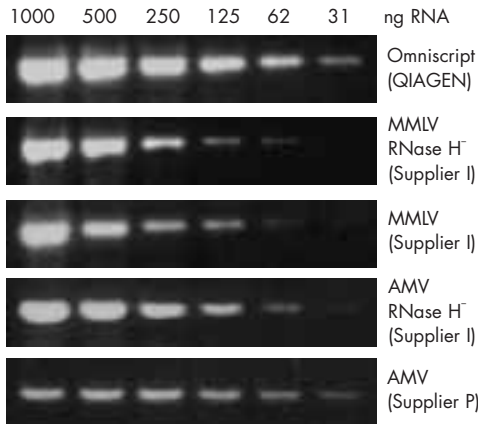## Superior Sensitivity and Dynamic Range of Omniscript RT

1000 500 250 125 62 31 ng RNA

Omniscript (QIAGEN)

MMLV RNase H⁻ (Supplier I)

MMLV (Supplier I)

AMV RNase H⁻ (Supplier I)

AMV (Supplier P)

Figure 23 Reverse transcription was carried out with different reverse transcriptases according to suppliers' specifications, using the indicated amounts of total RNA from HeLa cells. 1/20 of the reverse-transcription reaction was used in a 25-cycle PCR amplification with QIAGEN *Taq* DNA Polymerase. A 1.7 kb β-actin fragment was amplified.

References

1. Omniscript and Sensiscript RT Kits for highly efficient reverse transcription. QIAGEN News 1999 No. 2,1.

2. Tacke, E. et al (1995) Transposon tagging of the maize *Glossy2* locus with the transposable element *En/Spm*. Plant J. **8**, 907.

Table 9. Effects of Complex Secondary Structure on RT-PCR: RT Effects

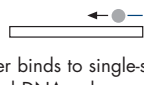| Cause	Effect on cDNA	RT-PCR result
A RT stops or dissociates at RNA region with complex secondary structure	Truncated cDNA products	cDNA products missing primer-binding site not amplified
B RT skips RNA region with complex secondary structure	Shortened cDNA products with internal deletions	Shortened PCR products with internal deletions
C RT reads through	Full-length cDNA products	Full-length RT-PCR products

With high GC content, the tight association of RNA:DNA hybrids can interfere with primer binding during PCR and prevent DNA polymerases from progressing (Table 10A). RNase H removes RNA in RNA:DNA hybrids to allow primer binding and second-strand DNA synthesis (Table 10B).

With an MMLV RNase H⁻ RT, the additional RNase H digest has been previously shown to improve RT-PCR yield (1) and to be required for amplification of some sequences, even as short as 157 bp (2).

Omniscript RT provides optimal RNase H activity during the RT step and there is no need for an additional enzyme digest step. For RT-PCR with difficult, high GC content as well as less complex templates, Omniscript RT provides highly sensitive and specific RT-PCR (Figure 22).

Since RT-PCR quantification of RNA is based on amplification of cDNAs, the amount of cDNA produced by the reverse transcriptase must accurately represent original amounts to enable accurate quantification. In comparison with other reverse transcriptases, Omniscript Reverse Transcriptase consistently gives higher sensitivity and a linear response, and can be used with any amount of RNA from 50 ng to 2 µg per reaction (Figure 23).

Table 10. Effects of High GC Content on RT-PCR: PCR Effects

Cause	Effect on PCR	RT-PCR result
A RNA:DNA hybrids with high GC content do not dissociate easily	Primer binding and enzyme progression obstructed	No amplification
B RNase H removes RNA in RNA:DNA hybrids	Primer binds to single-stranded cDNA and DNA polymerase proceeds	Amplification

5. One-step RT-PCR

In one-step RT-PCR, reaction setup and sample handling are easy. Primers, probe, and template RNA are added to the reaction mix and the cycling program is started. In one-step RT-PCR, gene-specific primers are used. This method enables fast processing of multiple samples and the procedure can be easily automated. The reduced number of handling steps increases reproducibility from sample to sample, and the risk of contamination is minimized. However, one of the main problems associated with one-step RT-PCR is the inhibitory effect that reverse transcriptases can have on the PCR step. This can lead to increased C_T values and reduced sensitivity and specificity when compared with two-step RT-PCR. QuantiTect RT-PCR Kits have a patented additive in the reaction buffer that overcomes the problem of PCR inhibition. In addition, other buffer components allow reverse transcription to take place at high temperatures (50–60°C). The optimized ratio of RT enzymes to HotStarTaq® DNA Polymerase enables highly sensitive and efficient RT-PCR, comparable to two-step RT PCR — with both SYBR Green I and sequence-specific probes (Figure 24).

High-Sensitivity Two-Step and One-Step RT-PCR

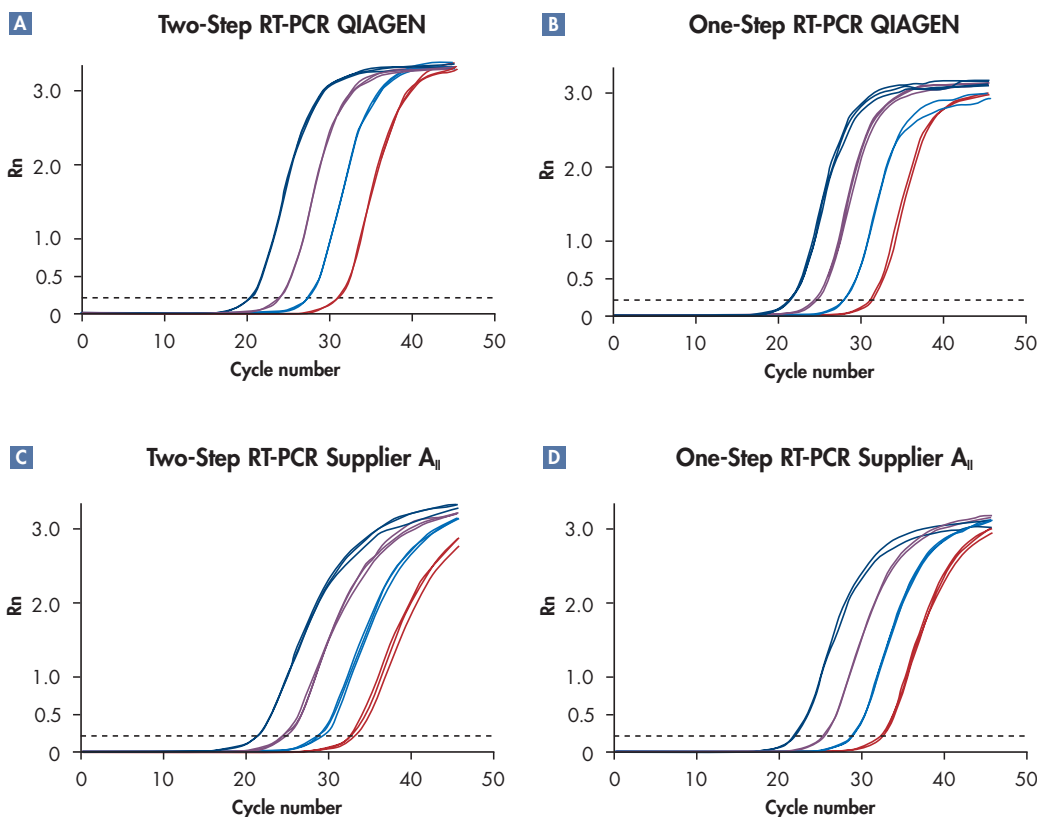**Figure 24** HSP89 expression levels were analyzed using [A] QuantiTect Probe PCR or [B] RT-PCR Kits (**QIAGEN**) or [C] real-time PCR and [D] RT-PCR kits from Supplier A_II (**Supplier A_II**). Amplification reactions were carried out according to manufacturers' recommendations.

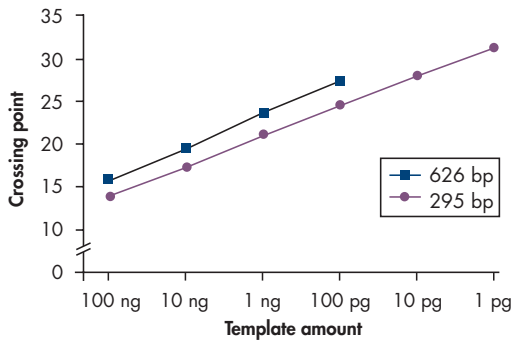## Improved PCR Efficiency and Sensitivity with Shorter Amplicons

Figure 25 Quantitative, real-time one-step RT-PCR of a 295 bp fragment and a 625 bp fragment of the β-actin gene was carried out using the QuantiTect SYBR Green RT-PCR Kit on the LightCycler system. Reactions were performed using varying amounts of template RNA, as indicated. Crossing-point values are shown for each reaction. No crossing-point values are given for amplification of the longer fragment with low amounts of template (1 pg and 10 pg) since these reactions produced primer-dimers and nonspecific products in addition to the specific RT-PCR product.

Guidelines for successful real-time PCR: amplification

PCR is both a thermodynamic and an enzymatic process. Successful real-time PCR requires amplification and detection under optimal conditions and each reaction component can affect the result. The annealing step is critical for high PCR specificity. When primers anneal to the template with high specificity, this leads to high yields of specific PCR products and increases the sensitivity of the amplification reaction. However, due to the high primer concentration in the reaction, primers will also hybridize to non-complimentary sequences with mismatches. If the primers anneal to the template sequence with low specificity, amplification of nonspecific PCR products and primer-dimers may occur. Competition in the amplification reaction between these artifacts and the desired PCR product may reduce the yield of the specific product, thereby reducing the sensitivity and linear range of the real-time reaction.

Low PCR specificity can significantly affect quantitative PCR particularly when using SYBR Green I for detection. As SYBR Green I binds to any double-stranded DNA sequence, primer-dimers and other nonspecific PCR products will generate a fluorescent signal. This reduces the overall sensitivity of the assay and also leads to inaccurate quantification of the transcript of interest.

Factors critical for high specificity in PCR include primer design and the reaction chemistry used.

1. Primer design

Prerequisites for successful RT-PCR include design of optimal primer pairs for each reaction, use of appropriate primer concentration, and correct storage of primer and probe solutions. For efficient amplification in real-time RT-PCR, primers should be designed so that the size of the amplicon is ideally <150 bp, enabling comparison of amplification reactions for different targets. Amplification efficiency, represented by the C_T values, and sensitivity of the reaction drop significantly with increasing amplicon size (Figure 25).

It is particularly important to minimize nonspecific primer annealing so that high yields of specific PCR product are obtained. The annealing step in PCR is affected by both primer design and primer concentration. Guidelines are given in Table 11 and 12, respectively.

However, even when primers are designed using specialized software, specificity can still be affected particularly when amplifying minute amounts of starting template or when detecting genes that are expressed at low level. These problems can be minimized by using an appropriate reaction chemistry.

Table 11. Primer Design for Real-Time PCR

Sequence:	■ Length of PCR product should ideally be less than 150 bp
	■ Avoid complementary sequences within and between primers and probes
	■ Avoid mismatches
	■ Avoid a 3'-end T as this has a greater tolerance of mismatch
Length:	■ 18–30 nucleotides
GC content:	■ 40–60%
T_m: (simplified)	■ $T_m = 2°C \times (A+T) + 4°C \times (C+G)$

Table 12. Primer Concentrations in One-Step and Two-Step RT-PCR

	Primer concentration	
QuantiTect Kit	Block cyclers	LightCycler
QuantiTect Probe RT-PCR Kit	0.4 µM	1 µM
QuantiTect Probe PCR Kit	0.4 µM	0.5 µM
QuantiTect SYBR Green RT-PCR Kit	0.5 µM	1 µM
QuantiTect SYBR Green PCR Kit	0.3 µM	0.5 µM

2. Effect of cations and hot start on real-time PCR specificity

Cations, especially Mg^{2+}, critically influence the melting behavior of DNA and therefore also affect the hybridization of primers to the target template. K^+ and Mg^{2+} ions bind to the negatively charged phosphate groups on the backbone of the DNA. This weakens the electrorepulsive forces between the target DNA and primer, and stabilizes the primer–template complex (Figure 26). QIAGEN PCR Buffer, provided with all QIAGEN PCR enzymes and kits, has been developed to eliminate the need for optimization of individual primer–template systems, saving time and money. The balanced combination of KCl and $(NH_4)_2SO_4$ in the buffer promotes specific primer annealing, maximizing yields of specific PCR product. Real-time PCR specificity can also be increased by using a hot start. This reduces amplification of nonspecific products, background, and primer-dimer formation in every PCR cycle. HotStarTaq DNA Polymerase, a modified form of QIAGEN *Taq* DNA Polymerase, provides high specificity in PCR. The stringent hot start in combination with unique QIAGEN PCR Buffer results in the highest PCR specificity compared with other hot-start enzymes (Figure 27).

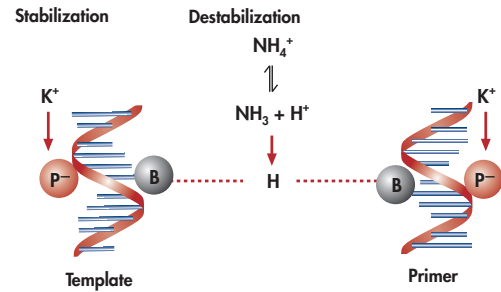### Effect of Unique PCR Buffer Supplied with QIAGEN PCR Enzymes

Figure 26 K^+ binds to the phosphate groups (**P**) on the DNA backbone, which thereby stabilizes the annealing of the primers to the template. NH_4^+, which exists both as the ammonium ion and as ammonia under thermal-cycling conditions, can interact with the hydrogen bonds between the bases (**B**), destabilizing principally the weak hydrogen bonds at mismatched bases. The combined effect of the two cations maintains the high ratio of specific to nonspecific primer–template binding over a wide temperature range.

Different Hot-Start Methods
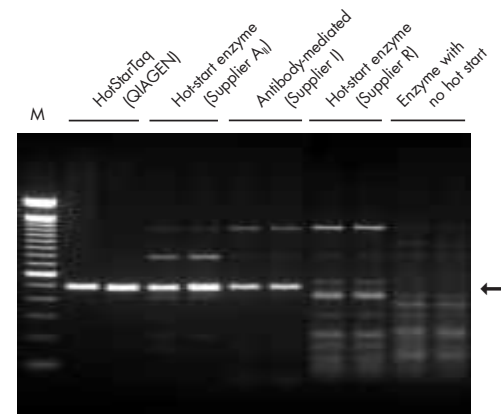
Figure 27 A 497 bp fragment was amplified from 50 copies of an HIV-*pol*-gene construct which had been added to 1 µg human genomic DNA. Different hot-start enzymes were employed: HotStarTaq DNA Polymerase from QIAGEN; hot-start enzymes from Suppliers A$_{II}$ and R; *Taq*–antibody mixture from Supplier I; or an enzyme with no hot start. Arrow indicates the specific PCR product. Equal volumes of the reaction were analyzed on a 2% agarose gel. **M**: markers.

QIAGEN offers two reaction chemistries for real-time RT-PCR — QuantiTect Probe PCR and RT-PCR Kits for use with sequence-specific probes, and QuantiTect SYBR Green PCR and RT-PCR Kits for use with SYBR Green. Reduced specificity is often a major problem when using SYBR Green I for quantitative, real-time PCR and RT-PCR. All QuantiTect Kits contain HotStarTaq DNA Polymerase, which enables a hot start. A hot start increases reaction specificity because it prevents the formation of primer-dimers and nonspecific products during reaction setup and the initial heating step. In comparison with kits from other suppliers, QuantiTect SYBR Green Kits show improved PCR specificity when using SYBR Green (Figure 28).

Low sensitivity can be a problem when using SYBR Green I since fluorescence generated by nonspecific PCR products, such as primer-dimers contributes to the overall signal. This effect reduces the range of the linear relationship between the number of cycles needed to detect the PCR product and the initial template amount. Quantification of low-copy-number templates is therefore often unreliable. In contrast to a SYBR Green PCR Kit from Supplier A_{II}, the QuantiTect SYBR Green Kit provides highly specific amplification, allowing the accurate quantification of as few as 5 copies of template (Figure 29). Sensitivity can also be a problem when using fluorescently labeled probes. See "Probe-based detection in real-time PCR", page 32 for more information.
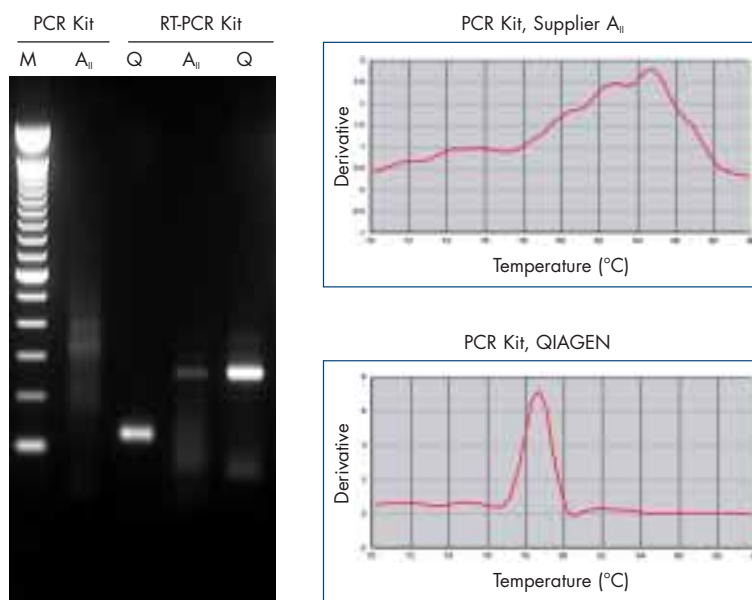
Highly Specific Real-Time PCR

Figure 28 Agarose gel electrophoresis and melting curve analyses of reactions carried out using the ABI PRISM® 7700 and the GeneAmp® 5700 Detection Systems. PCR products were amplified from 5 copies of a plasmid containing a fragment of the CFTR gene, using a PCR kit from Supplier A_{II} (**A_{II}**) or the QuantiTect SYBR Green PCR Kit from QIAGEN (**Q**). RT-PCR products were amplified from the low-density lipoprotein receptor (LDLR) transcript in 100 ng HeLa RNA, using an RT-PCR kit from Supplier A_{II} (**A_{II}**) or the QuantiTect SYBR Green RT-PCR Kit from QIAGEN (**Q**). **M**: markers.

Sensitivity of SYBR Green Real-Time PCR

Supplier A_{II}
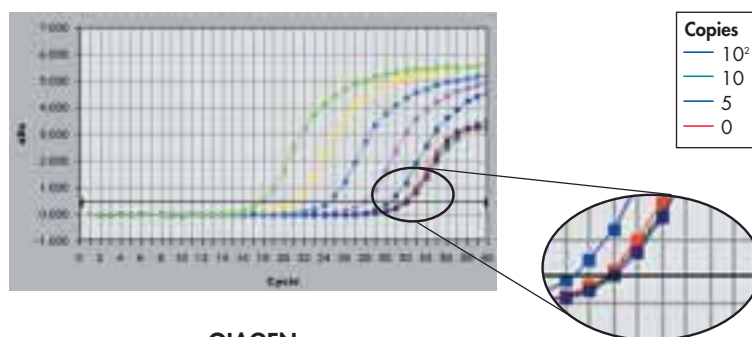
QIAGEN

Figure 29 1 x 10⁶ to 5 copies of a plasmid containing a fragment of the human CFTR gene were amplified in a background of 50 ng genomic DNA isolated from maize (maize does not contain the CFTR gene). Reactions were carried out on the ABI PRISM 7700 Detection System, using a SYBR Green PCR kit with a hot-start enzyme (**Supplier A_{II}**) or the QuantiTect SYBR Green PCR Kit (**QIAGEN**).

Highly specific detection of NKG2C

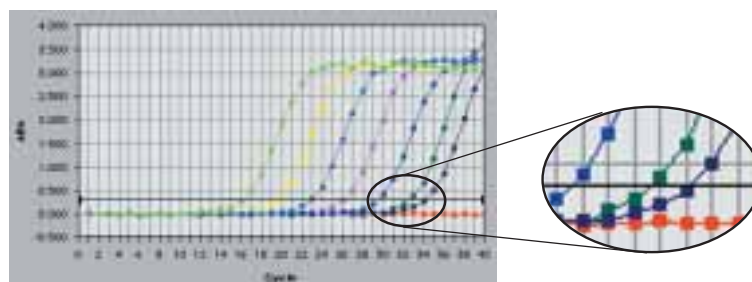NKG2 belongs to a family of lectin-like receptors that form heterodimers with CD94 on the surface of natural killer cells. The NKG2 gene family encodes several highly similar proteins, which are involved in both activation and inhibition of natural killer cells. The QuantiTect SYBR Green PCR Kit was used to detect NKG2C, which has ≥95% homology to the NKG2A gene. Even with 10 times more NKG2A template in the real-time reaction, the QuantiTect SYBR Green PCR Kit enabled highly specific detection of the NKG2A cDNA (Figure 30 and Table 13).

Table 13. High Specificity Using the QuantiTect SYBR Green PCR Kit

Template amount/copies		
NKG2A	NKG2C	Crossing point
10^6	–	43
10^6	10^5	28
10^5	–	–
10^5	10^4	32
10^4	–	–
10^4	10^3	35
10^3	–	–
10^3	10^2	39

Figure 30 Reactions were carried out using the amounts of template shown and primers specific for the NKG2C gene. Real-time PCR was performed using the QuantiTect SYBR Green PCR Kit with the LightCycler system. After real-time PCR, samples were analyzed by agarose gel electrophoresis. **A**: NKG2A, **C**: NKG2C.

(Data kindly provided by L. Arlettaz, Transplantation Immunity Unit, University Hospital Geneva, Switzerland.)
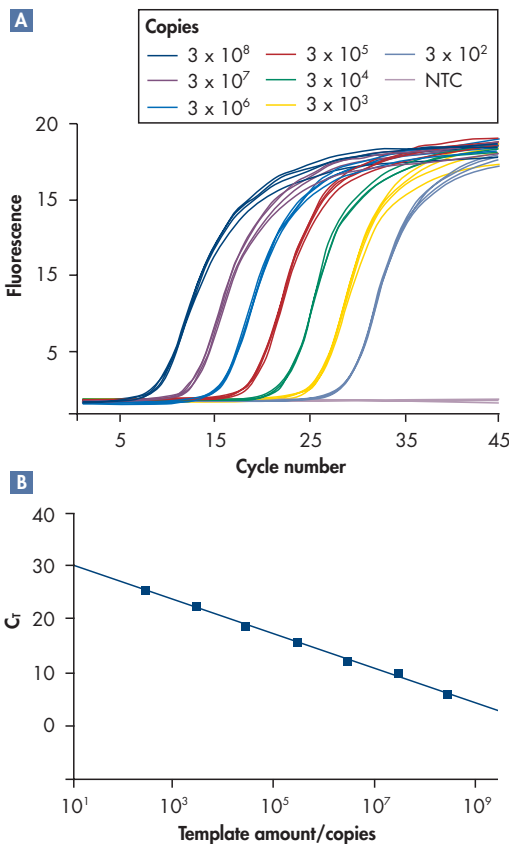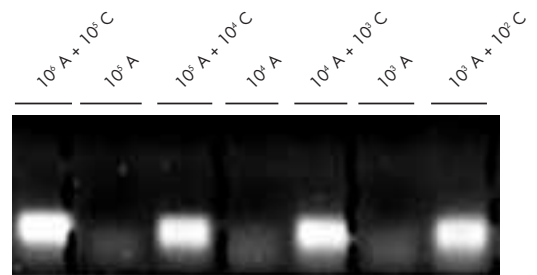
Analysis of a low-copy-number template using the QuantiTect SYBR Green PCR Kit

Sensitivity in real-time PCR can be a problem, particularly when using a limited amount of starting material. Nonspecific PCR products, such as primer-dimers can be formed due to the high ratio of primers to the small amount of template. Accordingly, the PCR must be extremely sensitive and specific to allow accurate detection of low template amounts. The QuantiTect SYBR Green PCR Kit was used with the Rotor-Gene system to successfully amplify a region of DNA from a typical DNA virus. As few as 3×10^2 copies of template DNA could be detected (Figure 31A). The data obtained showed high linearity over the wide range of template amount used (Figure 31B).

Figure 31 DNA from a typical DNA virus was serially diluted to give the template amounts shown. **A** Real-time PCR was performed using the QuantiTect SYBR Green PCR Kit with the Rotor-Gene system. **B** The C_T values from part A were used to make a standard curve.

(Data kindly provided by T. Kaiser, Corbett Research, Australia.)

Improved PCR Efficiency and Specificity

Template amount
- ■ 100 ng   ■ 100 pg   ■ 10 ng
- ■ 10 pg   ■ 1 ng   ■ NTC
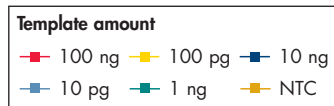
QIAGEN

Supplier A$_{II}$

Figure 32 Mouse Bcl-2 cDNA was amplified using a dilution series of 100 ng to 10 pg cDNA and the QuantiTect Mm_Bcl-2 Gene Expression Assay. Reactions were carried out on the ABI PRISM 7700 Detection System, using a probe kit (**Supplier A$_{II}$**) or the QuantiTect Probe PCR Kit (**QIAGEN**).
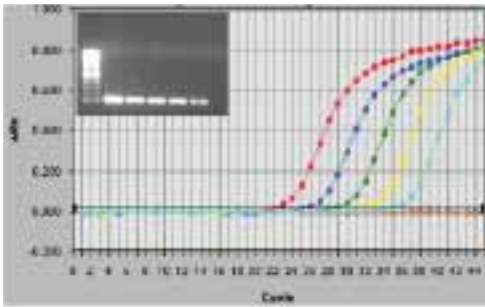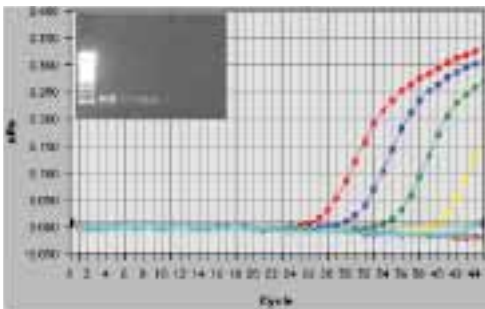
3. Probe-based detection in real-time PCR

Sequence-specific probes provide a highly sensitive method of detection in real-time PCR. When probes bind to their target sequence during real-time PCR, this results in detectable fluorescence that is directly proportional to the amount of accumulated PCR product. In contrast to SYBR Green I, primer-dimers and other nonspecific PCR products will not generate a fluorescent signal, however, PCR results will still be affected by nonspecific amplification. Nonspecific products can compromise reaction sensitivity and efficiency by competition between the desired PCR product and the nonspecific product. This can reduce the dynamic range of the PCR and low template amounts and/or genes expressed at low level may not be detected. The amplification efficiency, calculated from the increase of detectable fluorescence in ratio to the template amount, is lower. This is often indicated by non-uniform C_T distances between dilutions of the template.

Improved PCR specificity leads to higher PCR efficiency, sensitivity, and dynamic range (Figure 32). Expression levels of the mouse apoptosis gene Bcl-2 (B-cell leukemia/lymphoma 2) were monitored using either the QuantiTect Probe PCR Kit or a real-time PCR kit from Supplier A$_{II}$. The kit from Supplier A$_{II}$ allowed detection of 100 pg of template cDNA. In contrast, the QuantiTect Probe PCR Kit enabled sensitive detection of just 10 pg of template cDNA.

The increased sensitivity of the QuantiTect Probe PCR Kit is also reflected by lower C_T values. At the highest template amount, Mouse Bcl-2 could be detected more than 3 cycles earlier with the QuantiTect Probe PCR Kit, increasing the dynamic range of the reaction. C_T values obtained with the QuantiTect Probe PCR Kit maintain a uniform distance over the whole dilution series giving a PCR efficiency of 98%, whereas with the reaction chemistry from Supplier A$_{II}$ C_T distances increase as the amount of template is reduced. This decreases the PCR efficiency to 89% for template amounts from 100 to 1 ng and to 61% for the whole dilution series.

Application data

Highly sensitive detection of ochratoxin-synthesizing fungi using real-time PCR

Ochratoxin A is a mycotoxin produced by certain types of fungi, which are commonly found in cereals. Ochratoxin A is highly toxic and poses a considerable threat to human health. Consequently, barley and malt need to be routinely checked for fungal contamination. Screening of foodstuffs for contamination requires sensitive and accurate techniques. A real-time PCR assay using the QuantiTect Probe PCR Kit with the LightCycler system was established to detect DNA purified from an *Aspergillus ochraceus* culture. A range of template amounts was tested, from 40,000 pg to 10 pg of *A. ochraceus* DNA. As little as 10 pg of *A. ochraceus* DNA could be successfully detected (Figure 33A). Barley seeds were then tested for contamination with *A. ochraceus*. The QuantiTect Probe PCR Kit enabled highly sensitive detection of as little as 0.01 pg of *A. ochraceus* DNA (Figure 33B).
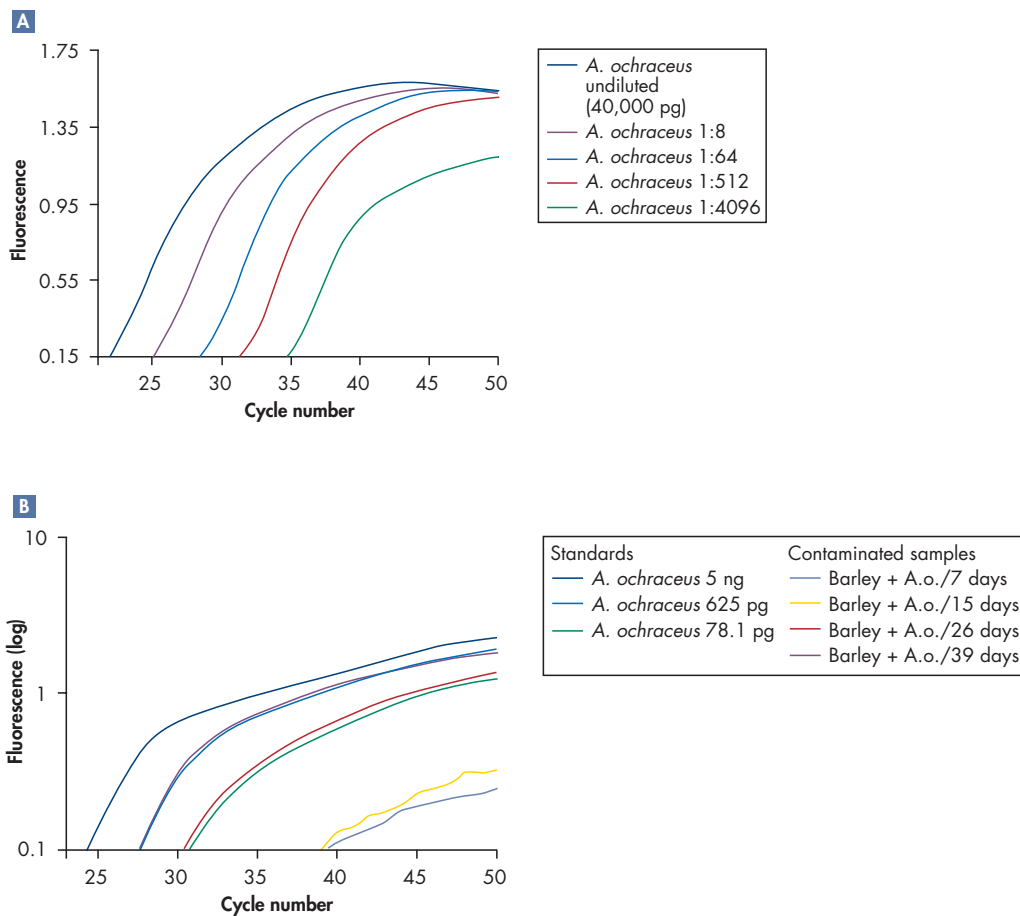
Figure 33 Real-time PCR was carried out using the QuantiTect Probe PCR Kit, primers, and LightCycler hybridization probes specific for *A. ochraceus*. **A** DNA purified from an *A. ochraceus* culture was quantified and diluted in steps of 1:8 before analysis using the LightCycler system. The initial template amount was 40,000 pg. **B** Levels of *A. ochraceus* contamination in barley seeds were analyzed following inoculation with a liquid culture of *A. ochraceus*. Aliquots were taken at the time intervals shown and flour prepared from the samples. Fungal DNA was isolated from the flour and quantified using the LightCycler system.

(Data kindly provided by M. Voetz, Research and Teaching Institute for Brewing, Berlin, Germany.)

Application data

Reliable real-time PCR results without optimization

Developing quantitative, real-time PCR and RT-PCR assays often requires extensive and time-consuming optimization procedures, such as adjusting primer concentrations, Mg^{2+} concentration, and amount of enzyme. QuantiTect Probe protocols are optimized for use on any real-time cycler, which greatly minimizes the need to adjust reaction parameters. The QuantiTect Probe PCR Kit was used to detect *Mycoplasma pneumoniae* DNA from a positive control sample and dilutions of the control sample up to 1:10,000 (Figure 34). Time-consuming optimization steps were not necessary.
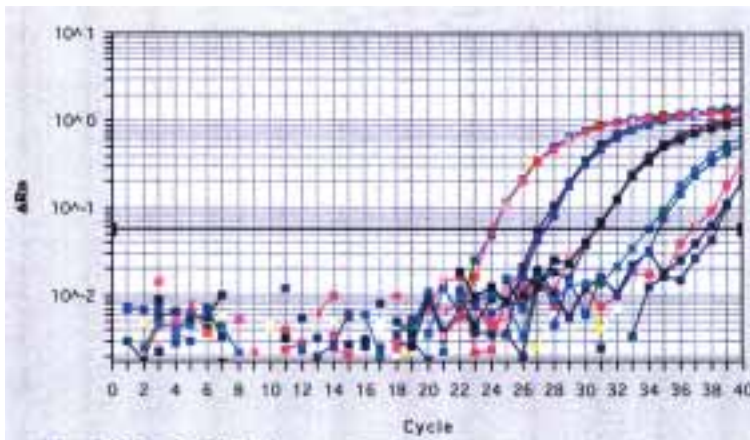
Figure 34 DNA from a *Mycoplasma pneumoniae* positive control sample was used in real-time PCR with the QuantiTect Probe PCR Kit either undiluted, or diluted 1:10, 1:100, 1:1000, or 1:10,000. Amplification reactions were performed using the ABI™ PRISM 7700 Sequence Detection System.

(Data kindly provided by M.R. de Gasperis and M.D. Caione, Laboratory of Virology, Ospedale Bambin Gesù, Rome, Italy.)

High linearity and reproducibility using the QuantiTect Probe PCR Kit

Prerequisites for accurate quantification include high PCR specificity and sensitivity and high linearity over a range of template amounts. A fragment of the bcl-2 gene was amplified from a genomic DNA dilution series using gene-specific primers and dual-labeled probe with the QuantiTect Probe PCR Kit. Highly reproducible results were obtained for each dilution in the series and high linearity was maintained over the range of template amounts (Figure 35).
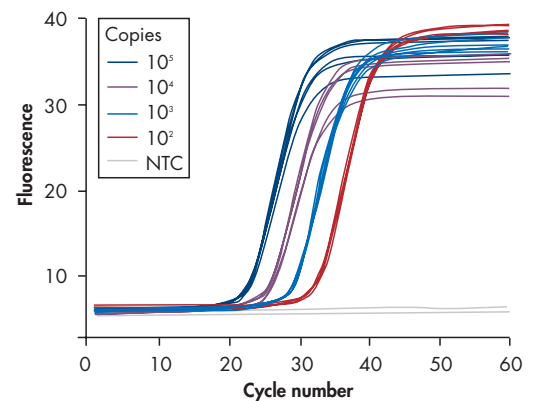
Figure 35 Real-time PCR of a fragment of the bcl-2 gene was carried out using the Rotor-Gene system. The number of copies of template DNA used are shown and six reactions were set up for each dilution.

(Data kindly provided by T. Kaiser, Corbett Research, Australia.)

? How many copies of template can I detect?

This depends on the amplification reagents used and on successful primer design. With QuantiTect Probe and SYBR Green Kits, <10 copies of template can be detected. When functionally validated QuantiTect Gene Expression Assays are used together with the QuantiTect Probe PCR Kit in two-step RT-PCR, 10 copies of template cDNA can be detected.

? How can I avoid the formation of primer-dimers?

We recommend using a hot start to PCR to prevent primer-dimer formation. In addition, a PCR buffer should be used that promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. All QuantiTect Kits provide a built-in hot start and contain a PCR buffer specifically adapted for quantitative PCR analysis. In addition, complementary sequences within and between the primers and probe should be avoided.

? I see several peaks in the melting curve of my SYBR Green I experiment. What does that mean?

There can be several reasons for this. In addition to the specific PCR product, there may also be primer-dimers in the reaction. The T_m of primer-dimers is generally lower than that of the specific PCR product. A shoulder in the curve, often above the T_m of the specific PCR product, indicates nonspecific amplification. Two distinct melting peaks could indicate the simultaneous amplification of cDNA and contaminating genomic DNA or the primers might have annealed to two different targets with identical primer binding sites, (e.g., two members of a homologous gene family).

? What reaction volume should I use?

QuantiTect Kits for real-time PCR and RT-PCR have been optimized for a final reaction volume of 50 μl for use in a 96-well plate in block cyclers or 20 μl for use in a 384-well plate and LightCycler capillaries. We strongly recommend using the primer and probe concentrations, reaction volumes, and amount of starting template given in the QuantiTect handbooks. However, volumes may be reduced to 20 μl and 10 μl, respectively.

? Can I set up the reactions and store the plates containing all reaction components for later use?

With QuantiTect Probe and SYBR Green PCR and RT-PCR Kits, reaction plates can be stored for several hours at 4°C. To avoid bleaching of probes or SYBR Green I, plates should be stored protected from light. Reactions set up using QuantiTect Probe and SYBR Green PCR Kits can be stored overnight at –20°C. The effect of storage over an extended period cannot be predicted since factors such as type and quality of the probe, quality of the template, and the temperature consistency of the freezer may affect experimental results. We do not recommend freezing reactions set up using QuantiTect RT-PCR Kits since the reverse transcriptases are sensitive to freezing when in an aqueous environment.

? **How can I tell if I have primer-dimers in my reaction?**

If using SYBR Green I in quantitative PCR, the cycling program should always be followed by melting curve analysis. Primer-dimers will appear as a peak with a T_m (usually between 70°C and 80°C) that is less than the T_m of the specific product. A detectable PCR product in the NTC usually indicates the presence of primer-dimers. If you see only one peak in all samples including the NTC, you can run an agarose gel to check whether primer-dimers have formed or to determine if the product is from nucleic acid contamination of the amplification reagents.

? **How long should the amplicon be if I am using SYBR Green I detection?**

For accurate quantification using SYBR Green I, the amplicon should be no longer than 150 bp. In general, the shorter the amplicon, the higher the amplification efficiency will be.

? **How should I store primers and probes?**

Dissolve lyophilized primers and probes in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) and store in small aliquots at –20°C. Repeated freeze–thaw cycles should be avoided since they may lead to primer degradation. To avoid bleaching of the fluorophore, fluorescently labeled probes should be stored protected from light.

4. Genomic DNA in real-time RT-PCR

Contaminating genomic DNA in RNA samples may also be detected in real-time PCR leading to inaccurate quantification of the transcript of interest. Depending on the method used for RNA purification and in particular what type of tissue or starting material was used for RNA purification, genomic DNA can remain in the RNA sample. Even if the probe is designed to cross an exon/exon boundary, genomic DNA may still be detected due to the presence of pseudogenes. Pseudogenes are nonfunctional copies of the respective gene that are highly homologous to their functional counterpart, but usually lack introns. Detection of DNA can be avoided by removing contaminating DNA with a DNase I digestion step. This may be necessary if pseudogenes exist, if the gene of interest is a single-exon gene, if the organism being studied produces transcripts lacking introns (e.g., bacteria), or if sequence information is not available on intron/exon boundaries. This can be performed by on-column digestion when using the RNeasy purification procedure (see appendix, page 42). However, if the intron/exon boundaries of a gene are known, appropriate primer and probe design can prevent amplification of sequences from genomic DNA (Figure 36).

The probe can be designed to cross the exon/exon boundary (Figure 36B). This enables detection of RNA only but co-amplification of contaminating genomic DNA may still occur. The forward and reverse primers anneal to different exons and the probe hybridizes up- and downstream of the splice site between the two exons. If the intron is large, no PCR product will be amplified. However, if the exons are only separated by a small intron, this can result in co-amplification of products from the genomic DNA sequence. The product from genomic DNA will not be detected since the probe cannot bind, however, amplification efficiency and sensitivity will be reduced.

The alternative approach involves designing a primer across an exon/exon boundary, enabling selective amplification and detection of the PCR product from mRNA or cDNA sequences (Figure 36A). The primer crossing the splice-site cannot bind to genomic DNA therefore preventing amplification of a PCR product from the genomic sequence. This approach was used to develop functionally validated QuantiTect Gene Expression Assays.

No Co-amplification of Genomic DNA
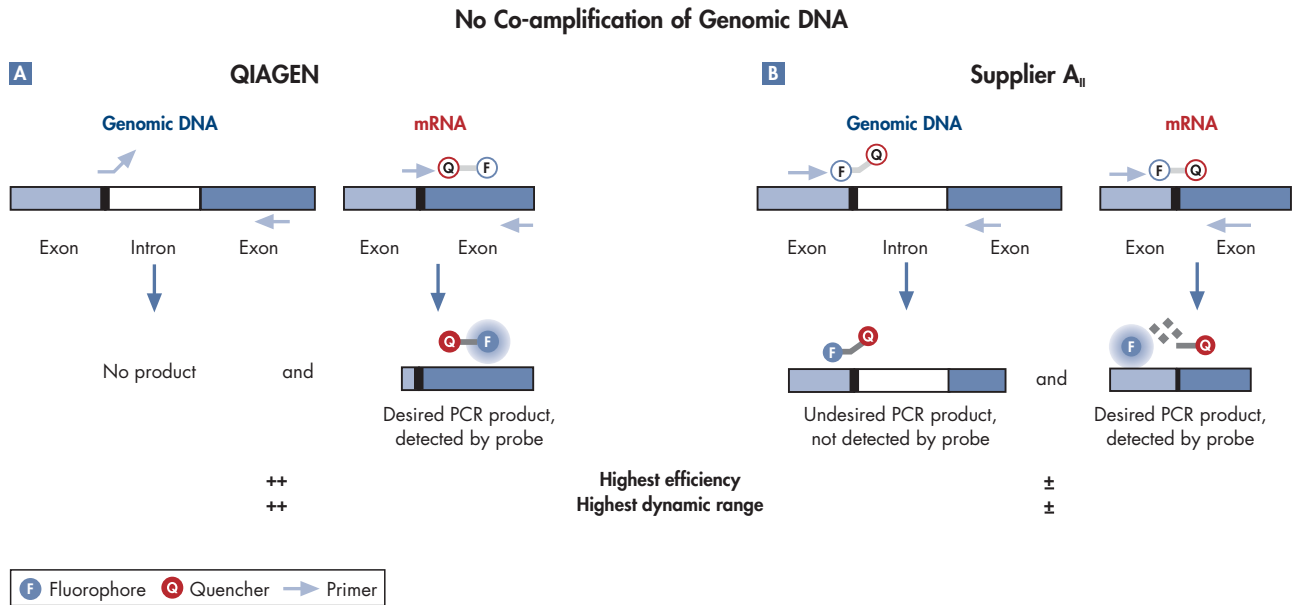

Figure 36 Primer/probe design to eliminate signals from contaminating genomic DNA. Comparison of primer design for **A** QuantiTect Gene Expression Assays (**QIAGEN**). In contrast to Supplier A$_{II}$, primers in QuantiTect Gene Expression Assays are designed to cross exon/exon boundaries. **B** Pre-designed gene expression assays from Supplier A$_{II}$ (**Supplier A$_{II}$**).

QuantiTect Gene Expression Assays

New QuantiTect Gene Expression Assays are functionally validated primer–probe sets that are ready to use in quantitative, real-time RT-PCR on any real-time cycler. Optimal results are guaranteed, and assays are available for both human and mouse genes. QIAGEN provides an expanding range of QuantiTect Gene Expression Assays for commonly analyzed genes and QuantiTect Custom Assays (see page 39) for any gene of choice. QuantiTect Gene Expression Assays, comprising two gene-specific primers and a dual-labeled QuantiProbe (see page 6), are provided in a convenient 10x Assay Mix. Assays have been optimized and validated to provide maximum sensitivity and a wide dynamic range. Each assay is validated together with QuantiTect Probe Kits to ensure high PCR efficiency and accurate quantification of as few as 10 copies of template (Table 14).

In contrast to other commercial assays, primers are designed to cross exon/exon boundaries where possible, enabling amplification and detection of RNA sequences only (Figure 36, page 37). This prevents co-amplification of genomic DNA, which can compromise assay sensitivity and efficiency by competition between the desired PCR product and the product derived from genomic DNA. Unique oligonucleotide technology makes this possible through the use of Superbases. See "QuantiProbes", page 6 for more information.

? **Can I use QuantiTect Gene Expression Assays for validation of siRNA experiments?**

Yes. We have seen that 24 hours or later after transfection with siRNA from QIAGEN, the reduction in transcript level could be reliably detected with QuantiTect Gene Expression Assays, both with assays encompassing the cleavage site as well as with assays located up- and downstream of it.

? **Can I use QuantiTect Gene Expression Assays and assays using TaqMan probes in the same run?**

No. QuantiTect Gene Expression Assays are used in a 3-step cycling protocol with data acquisition during the annealing step, whereas TaqMan probes require a 2-step cycling protocol with data acquisition during the combined annealing/extension step.

Table 14. Comparison of Gene Expression Assays

Specifications	QIAGEN	Supplier A[II]
Sensitivity tested down to 10 copies of template	✓	–
Tested for high PCR efficiency	✓	–
Tested on range of real-time cyclers	✓	–
Tested in one-step RT-PCR	✓	–
Tested in two-step RT-PCR	✓	✓
Available for human gene sequences	✓	✓
Available for mouse gene sequences	✓	✓

QuantiTect Custom Assays

Designing primer–probe sets for real-time PCR can be difficult and often requires a considerable degree of experience. In addition, further time-consuming optimization procedures, such as primer and probe titration experiments are often required for optimal real-time PCR results. Free, Web-based software at www.qiagen.com/goto/assays makes it easy to design custom assays that are ready to use on any real-time cycler — even when designing primer or probe sequences that cross splice sites. Each QuantiTect Custom Assay consists of one QuantiProbe and a mix containing forward and reverse primers; both are supplied as 20x concentrates.

QuantiProbes and primers frequently contain Superbases. This enables the use of primers and probe at predefined sequences, such as splice sites, while still delivering optimal real-time PCR performance. The combination of Superbases together with the MGB located at the 5' end of the QuantiProbe allows greater flexibility in assay design and is highly suitable for amplification of difficult sequences.

QuantiTect Custom Assays are designed for use with QuantiTect Probe Kits and can be used in both one-step and two-step RT-PCR. Optimized protocols, including instrument-specific settings, are available for any real-time cycler at www.qiagen.com/goto/assays or from your local QIAGEN Technical Service Department.

Easy-to-use QuantiProbe Design Software

When using traditional programs to design real-time PCR assays, there are many tedious design steps involved and it is often not possible to place primers and probes within certain regions, for example, splice sites. In contrast, the easy-to-use QuantiProbe Design Software enables fast and efficient design of QuantiTect Custom Assays. The amount of time required to design a new assay ranges from less than 20 seconds, for easy sequences, to a few minutes, for the most difficult sequences, saving a lot of time in the front-end steps of assay design. To design an assay, the gene sequence ID number or gene sequence is simply entered into the software. The software has two modes: Express Design Mode, for quick and easy design of reliable assays using default design parameters, and Custom Design Mode, for assay design using user-defined design parameters (Figure 37). In Express Design Mode, multiple sequences can be entered, enabling high-throughput design. The sequences are automatically processed one after another by the software, minimizing the amount of hands-on time required for the design process.

Easy-to-use QuantiProbe Design Software

Figure 37 Screenshots of the QuantiProbe Design Software. **A** Select the design method and enter the target sequence. **B** The software designs the optimal primer–probe combination.

Two different methods can be chosen for both design modes: Gene Expression Method for cDNA detection or Splice Site Method for RNA only detection. QuantiTect Custom Assays designed by the Gene Expression Method target cDNA, where no splice sites are available (e.g., open reading frames from lower organisms and genes from higher organisms with unknown exon structure or without introns). If assays are required to detect RNA only, either a genomic DNA or cDNA sequence can be entered and splice sites will be targeted for design. It is also possible to select a specific splice site for the design process. The software first designs the forward and reverse primers, of which at least one crosses the splice site, and then designs the QuantiProbe that is compatible with this pair of primers. The incorporation of Superbases into primer and QuantiProbe sequences increases the likelihood of finding an assay, even for very difficult sequences. The detection of specific transcripts from splice variants, gene families, or mutated genes often requires more complex design procedures. The "exclude region" function in both Gene Expression Method and Splice Site Method allows as many regions of a sequence to be excluded from the design process as desired. Only those primers and probes will be considered in the design process, which cannot hybridize to the excluded sequences. These assays distinguish between highly similar sequences with stretches of sequence differences. Annotating a "probe targeting window" in the Gene Expression Method allows discrimination between sequences with minor differences, for example, transcripts from different mutants as the QuantiProbe completely covers the desired region.

? **Can I make a master mix containing QuantiProbes, primers, and all other reaction components and store it for later use?**

QuantiProbes and primers can be stored in a reaction master mix with the QuantiTect Probe PCR Master Mix for more than 4 weeks at both –20°C and 4°C. The reaction master mix must be stored protected from light to avoid bleaching of the fluorescent probe. We do not recommend storing QuantiProbes and primers as a reaction master mix together with the QuantiTect Probe RT-PCR Master Mix and the QuantiTect RT Mix as the reverse transcriptases are sensitive to freezing in an aqueous environment.

? **Can I combine the QuantiProbe and primers or should I store them separately?**

For added convenience, the 20x Primer Mix and 20x QuantiProbe can be combined in a 1:1 volume ratio. This gives a 10x primer–probe mix that is ready to use. We recommend storing this primer–probe mix in aliquots to avoid multiple freeze–thaw cycles.

Hook-Shaped Amplification Plot

Figure 38 Amplification plot showing apparent decrease in yield of PCR product towards the end of PCR.

? **My amplification plots are hook-shaped. Why is that?**

During the late phase of PCR, as a lot of PCR product has been generated, there is strong competition between hybridization of the probe to the target strand and re-association of the two complementary product strands. For some primer–probe combinations, re-association occurs more quickly than probe hybridization towards the end of the PCR. Therefore, the yield of PCR product seems to decrease (Figure 38). However, for accurate quantification, fluorescence data are measured during the log-linear phase of the reaction, i.e., during the initial increase of fluorescence, before this phenomenon occurs.
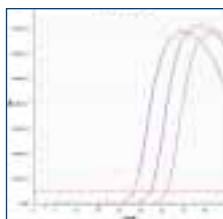
? **Can I use the QuantiProbe Design Software to design assays to detect DNA?**

Yes. Simply select Gene Expression for the "Design Mode".

? **The quality of my assays seems to decrease over time. What could that be due to?**

Make sure that primers, probes, and amplification reagents are stored correctly. Avoid multiple freeze–thaw cycles for primers and probes. Check the performance of your real-time instrument as some instruments require the halogen lamp to be frequently replaced. Lasers must also be replaced occasionally.

Appendix

RNA template preparation and stabilization

Since PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, EDTA, and other chemical solvents than single-step enzyme-catalyzed reactions. Purity of nucleic acid templates is particularly important for real-time PCR, since contaminants can interfere with fluorescence detection. QIAGEN offers a complete range of nucleic acid purification systems, ensuring the highest-quality templates for RT-PCR. Phenol and other contaminants can be efficiently removed from RNA preps using the RNeasy MinElute Cleanup Kit to clean up and concentrate RNA for sensitive assays. QIAGEN offers a range of RNeasy Kits for isolation of high-quality RNA from a wide range of sample types (Figure 39). Evaluation of the purified RNA is important to determine the yield, purity, and quality of the purified RNA. RNA purity and integrity are important factors for successful gene expression assays.

Isolation of RNA from whole blood

The PAXgene™ Blood RNA system is an integrated and standardized system for collection and stabilization of whole blood specimens and isolation of cellular RNA. Blood is collected into PAXgene Blood RNA Tubes and RNA is isolated using the PAXgene Blood RNA Kit. PAXgene Blood RNA Tubes contain a proprietary blend of reagents that bring about immediate stabilization of RNA. This blend prevents the drastic changes in the cellular expression profiles that normally take place in vitro, after blood collection. The resulting RNA accurately represents the expression profile in vivo and is suitable for use in a range of downstream applications.

RNeasy Protect system

The RNeasy Protect system provides a complete solution for RNA protection and isolation, from sample harvesting to pure RNA, in one kit. Proven RNeasy silica-gel–membrane technology, combined with the RNA stabilizing properties of RNA*later*™ RNA Stabilization Reagent, allows purification of high-quality, intact RNA. This process efficiently preserves the expression profile of the sample at the time of harvesting to ensure reliable gene expression analysis.

Determining concentration and purity of nucleic acids

The concentration of RNA should be determined by measuring the absorption at 260 nm (A_{260}) in a spectrophotometer. For accuracy, absorbance readings at 260 nm should fall between 0.15 and 1.0. Brief guides to spectrophotometric and molar conversion values for different nucleic acid templates are listed in Tables 15 and 16, page 43.

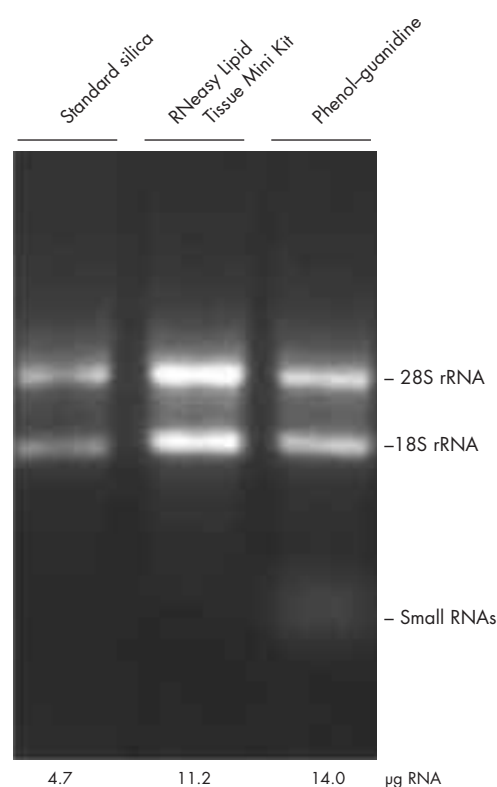**High Yields of RNA without Phenol Carryover**

Figure 39 RNA was isolated from 10 mg rat brain tissue using the RNeasy Lipid Tissue Mini Kit, a standard silica-gel–membrane procedure, or a phenol–guanidine based reagent, following suppliers' instructions. Formaldehyde agarose gel analysis shows high yields of RNA using the RNeasy Lipid Tissue Mini Kit. Using the RNeasy and other silica-based method, small RNAs (such as 5.8S rRNA, 5S rRNA, and tRNAs) are selectively excluded.

Alternatively, RNA can be quantified using fluorescent dyes that bind specifically to RNA. Measurement of the fluorescent signal on a fluorimeter enables RNA quantification.

Note that absorbance measurements cannot discriminate between DNA and RNA. Depending on the method used for template preparation, RNA may be contaminated with DNA, and this will result in misleadingly high A_{260} values. It is particularly important to bear this in mind when quantifying RNA concentration by measuring the absorbance at 260 nm.

The ratio between the absorbance values at 260 nm and 280 nm gives an estimate of the purity of RNA. To determine nucleic acid purity, we recommend measuring absorbance in 10 mM Tris·Cl,* pH 7.5. Pure RNA has A_{260}/A_{280} ratios of 1.8–2.0 and 1.9–2.1[†] respectively. Lower ratios indicate the presence of contaminants such as proteins.

Table 15. Spectrophotometric Conversions for Nucleic Acid Templates

1 A_{260} unit[†]	Concentration (µg/ml)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

[†] Absorbance at 260 nm = 1; 1 cm detection path

Table 16. Molar Conversions for Nucleic Acid Templates

Nucleic acid	Size	pmol/µg	Molecules/µg
1 kb DNA	1000 bp	1.52	9.1×10^{11}
pUC19 DNA	2686 bp	0.57	3.4×10^{11}
pTZ18R DNA	2870 bp	0.54	3.2×10^{11}
pBluescript® II DNA	2961 bp	0.52	3.1×10^{11}
Lambda DNA	48,502 bp	0.03	1.8×10^{10}
Typical mRNA	1930 nt	1.67	1.0×10^{12}
Genomic DNA			
Escherichia coli	4.7×10^{6}	3.0×10^{-4}	$1.8 \times 10^{8[§]}$
Drosophila melanogaster	$1.4 \times 10^{8[‡]}$	1.1×10^{-5}	$6.6 \times 10^{5[§]}$
Mus musculus (mouse)	$2.7 \times 10^{9[‡]}$	5.7×10^{-7}	$3.4 \times 10^{5[§]}$
Homo sapiens (human)	$3.3 \times 10^{9[‡]}$	4.7×10^{-7}	$2.8 \times 10^{5[§]}$

[‡] Base pairs in haploid genome.

[§] For single-copy genes

Storage of RNA

Purified RNA should be stored at –20°C or –70°C, in RNase-free water.* Diluted solutions of nucleic acids (e.g., dilution series used as standards) should be stored in aliquots and thawed once only. We recommend storage of aliquots in siliconized tubes where possible. This avoids adsorption of the nucleic acid to the tube walls, which would reduce the concentration of nucleic acid in solution.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

[†] Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

? How much template can I use in the reaction and what is the maximum volume of template that can be used?

Even when detecting low-abundance targets, we recommend using no more than 500 ng template RNA or cDNA. Generally, 1–100 ng template should be sufficient and for abundant transcripts as little as 1 pg can be used. Template purity is important if large volumes of low concentration template are to be added to the reaction. Using DNA or RNA purified with QIAGEN products, the template can contribute up to 40% of the final reaction volume as long as the recommended template amounts are not exceeded. If cDNA from an RT reaction is used as template, the volume of undiluted RT reaction added should not exceed 10% of the final PCR volume.

? How does the method used to prepare the DNA or RNA template influence the real-time PCR?

The purity of the starting template can have a tremendous effect on the quantification results, since contaminants such as proteins, phenol/chloroform, salts, EDTA, and other chemical solvents can interfere with reverse transcription, amplification, and fluorescence detection. For sensitive applications, we recommend spiking a sample to be purified with an exogenous control, such as an in vitro transcript, before the purification procedure. The amplification efficiency can then be compared to a positive control to monitor for the presence of inhibitors acquired during the sample preparation process. QIAGEN offers a complete range of nucleic acid purification systems, ensuring the highest-quality templates for real-time PCR.

Ordering Information

Product	Contents	Cat. no.
QuantiTect SYBR Green PCR and RT-PCR Kits — for quantitative, real-time PCR and RT-PCR using SYBR Green		
QuantiTect SYBR Green PCR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect SYBR Green PCR Master Mix (providing a final concentration of 2.5 mM $MgCl_2$), 2 x 2.0 ml RNase-free water	204143
QuantiTect SYBR Green RT-PCR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect SYBR Green RT-PCR Master Mix (providing a final concentration of 2.5 mM $MgCl_2$), 100 µl QuantiTect RT Mix, 2 x 2.0 ml RNase-free water	204243
QuantiTect Probe PCR and RT-PCR Kits — for quantitative, real-time PCR and RT-PCR using sequence-specific probes		
QuantiTect Probe PCR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe PCR Master Mix (providing a final concentration of 4 mM $MgCl_2$), 2 x 2.0 ml RNase-free water	204343
QuantiTect Probe RT-PCR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe RT-PCR Master Mix (providing a final concentration of 4 mM $MgCl_2$), 100 µl QuantiTect RT Mix, 2 x 2.0 ml RNase-free water	204443
QuantiTect Gene Expression Assays — for validated, ready-to-use, real-time RT-PCR assays		
QuantiTect Gene Expression Assays	For 100 x 50 µl reactions (96-well plate or single tubes) or 250 x 20 µl reactions (384-well plate or single capillaries): 1 x 0.5 ml 10x QuantiTect Assay Mix	Varies
QuantiTect Custom Assays — for ready-to-use, real-time RT-PCR assays for customer-defined sequences		
QuantiTect Custom Assay (500)*	For 500 x 50 µl reactions (96-well plate or single tubes) or 1250 x 20 µl reactions (384-well plate or single capillaries): 1 x 1.25 ml 20x Primer Mix, 1 x 1.25 ml 20x QuantiProbe	–
Omniscript RT Kit — for reverse transcription using ≥50 ng RNA		
Omniscript RT Kit (50)*	For 50 reverse-transcription reactions: 200 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (containing 5 mM each dNTP), RNase-free water	205111
Sensiscript RT Kit — for reverse transcription using <50 ng RNA		
Sensiscript RT Kit (50)*	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (containing 5 mM each dNTP), RNase-free water	205211

* Larger kit sizes and/or different formats available; please inquire.

Ordering Information

Product	Contents	Cat. no.
QIAGEN OneStep RT-PCR Kit — for fast and efficient one-step RT-PCR		
QIAGEN OneStep RT-PCR Kit (25)*	For 25 reactions: QIAGEN OneStep RT-PCR Enzyme Mix, 5x QIAGEN OneStep RT-PCR Buffer (containing 12.5 mM $MgCl_2$), dNTP Mix (containing 10 mM each dNTP), 5x Q-Solution, RNase-free water	210210
HotStarTaq DNA Polymerase — for highly specific hot-start PCR		
HotStarTaq DNA Polymerase (250 U)*	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer (containing 15 mM $MgCl_2$), 5x Q-Solution, 25 mM $MgCl_2$	203203
RNeasy Mini Kit — for purification of total RNA from animal cells or tissues, yeast, or bacteria		
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
RNeasy Protect Mini Kit — for RNA*later* stabilization and RNeasy purification of total RNA from animal tissues		
RNeasy Protect Mini Kit (50)*	RNA*later* RNA Stabilization Reagent (50 ml), 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74124
PAXgene Blood RNA System — for blood collection, and RNA stabilization and isolation		
PAXgene Blood RNA Kit (50)	For 50 RNA preps: 50 PAXgene RNA Spin Columns, buffers, proteinase K, and processing tubes; to be used with PAXgene Blood RNA Tubes (cat. no. 762115 US and Canada; 762125 all other countries)	762134
PAXgene Blood RNA Tubes (100)	100 blood collection tubes. To be used in conjunction with the PAXgene Blood RNA Kit (50).	762115
DNeasy Tissue Kit — for isolation of up to 40 µg genomic DNA from animal tissues and cells, yeast, or bacteria		
DNeasy Tissue Kit (50)*	50 DNeasy Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69504
QIAamp® DNA Blood Mini Kit — for isolation of genomic, mitochondrial, or viral DNA from blood and related body fluids		
QIAamp DNA Blood Mini Kit (50)*	For 50 DNA minipreps: 50 QIAamp Mini Spin Columns, QIAGEN Protease, Reagents, Buffers, Collection Tubes (2 ml)	51104

* Larger kit sizes and/or different formats available; please inquire.

Real-Time PCR Applications

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