Critical Factors for Successful Real-Time PCR

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Sample & Assay Technologies

Contents

1. 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 determining the cycle when the PCR product can first be detected. This is in contrast with end-point 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. We also cover the latest technologies in real-time PCR, including multiplex analysis, fast cycling, direct analysis of cell lysates, and whole transcriptome amplification. In addition, we describe the advantages and disadvantages of different reaction chemistries as well as provide 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.

2. 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.

2.1 SYBR® Green

The fluorescent dye 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, allowing use of the dye 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 SYBR Green 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.

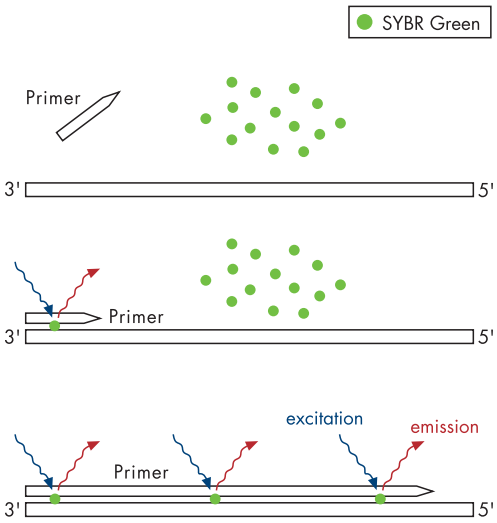


Figure 1. SYBR Green principle. Principle of SYBR Green-based detection of PCR products in real-time PCR.

2.2 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 for reaction components can compromise assay sensitivity and efficiency. The following sections discuss different probe chemistries.

2.2.1 TaqMan® probes

TaqMan probes are sequence-specific oligonucleotide probes carrying a fluorophore and a quencher moiety (Figure 2). The fluorophore is attached at the 5' end of the probe and the quencher moiety 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 moiety. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product. Examples of quencher moieties include TAMRA fluorescent dye and Black Hole Quencher® (BHQ®) nonfluorescent dyes.

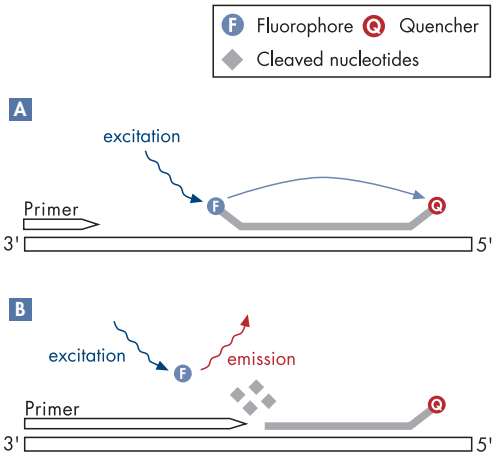


Figure 2. TaqMan probe principle. A Both the TaqMan probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the quencher to the fluorophore strongly reduces the fluorescence emitted by the fluorophore. B During the PCR extension step, *Taq* DNA polymerase extends the primer. When the enzyme reaches the probe, its 5' → 3' exonuclease activity cleaves the fluorophore from the probe. The fluorescent signal from the free fluorophore is measured. The signal is proportional to the amount of accumulated PCR product.

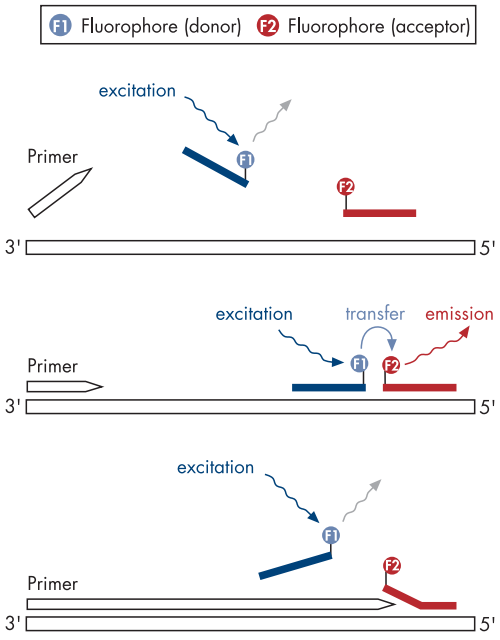


Figure 3. FRET probe principle. A When not bound to the target sequence, no fluorescent signal from the acceptor fluorophore is detected. B During the PCR annealing step, both FRET probes hybridize to the target sequence. This brings the donor and acceptor fluorophores into close proximity, allowing 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.

2.2.2 FRET probes

PCR with fluorescence resonance energy transfer (FRET) probes, such as LightCycler® hybridization probes, uses 2 labeled oligonucleotide probes that bind to the PCR product in a head-to-tail fashion (Figure 3). When the 2 probes bind, their fluorophores come into close proximity, allowing energy transfer from a donor fluorophore 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 with LightCycler and Rotor-Gene® instruments. As the FRET system uses 2 primers and 2 probes, good design of the primers and probes is critical for successful results.

2.2.3 Dyes used for fluorogenic probes in real-time PCR

For real-time PCR with sequence-specific probes, various fluorescent dyes are available, each with its own excitation and emission maxima (Table 1 and Figure 4). The wide variety of dyes makes multiplex, real-time PCR possible (detection of 2 or more amplicons in the same reaction), provided the dyes are compatible with the excitation and detection capabilities of the real-time cycler used, and the emission spectra of the chosen dyes are sufficiently distinct from one another. Therefore, when carrying out multiplex PCR, it is best practice to use dyes with the widest channel separation possible to avoid any potential signal crosstalk.

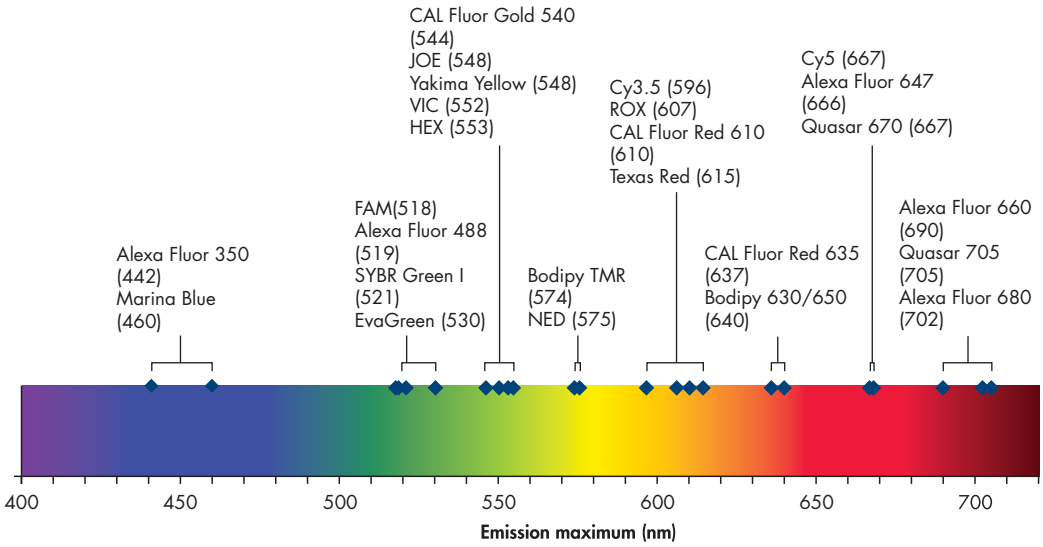


Figure 4. Emission maximum of selected reporter dyes. The emission maximum (nm) of selected reporter dyes are displayed in parentheses. Emission maximum may vary depending on buffer conditions. Other dyes with similar wavelengths may not be suitable for multiplex assays due to low fluorescence and/or stability.

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
Bodipy® TMR	544	574
NED	546	575
TAMRA	560	582
Cy3.5	588	604
ROX	587	607
Texas Red®	596	615
LightCycler Red 640 (LC640)	625	640
Bodipy 630/650	625	640
Alexa Fluor® 647	650	666
Cy5	643	667
Alexa Fluor 660	663	690
Cy5.5	683	707

* Emission spectra may vary depending on the buffer conditions.

? 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 or the target sequence is present. 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, or by amplifying the cloned target sequence, either in the form of in vitro transcribed RNA or plasmid DNA.

? Do I need to calibrate my real-time cycler in order to detect reporter dyes?

Some real-time cyclers require you to perform a calibration procedure for each reporter dye. Check whether the reporter dyes you selected for your assays are part of the standard set of dyes already calibrated on your instrument. If they are not, perform a calibration procedure for each dye before using them for the first time. For details about calibration, refer to the user manual supplied with your cycler.

3. Methods in real-time PCR

DNA template (e.g., genomic DNA or plasmid DNA) can be directly used as starting template in real-time PCR. Real-time PCR can be used to quantify genomic DNA, for example, in detection of bacterial DNA or GMOs, and can also be used for qualitative analysis, such as single nucleotide polymorphism (SNP) detection.

RNA template is used for analysis of gene expression levels or viral load of RNA viruses. Real-time RT-PCR is carried out, where the RNA first needs to be transcribed into cDNA using a reverse transcriptase prior to PCR. Reverse transcriptases are enzymes generally derived from RNA-containing retroviruses. Real-time RT-PCR can be either a two-step or a one-step procedure, as described in section 3.1.

Application data

Using real-time PCR for SNP genotyping

SNP analysis involves the detection of single nucleotide changes using 2 probes labeled with different fluorophores. One probe is specific for the wild-type allele, the other for the mutant allele (Figure 5). Real-time PCR is highly suited for the detection of small sequence differences, such as SNPs and viral variants.

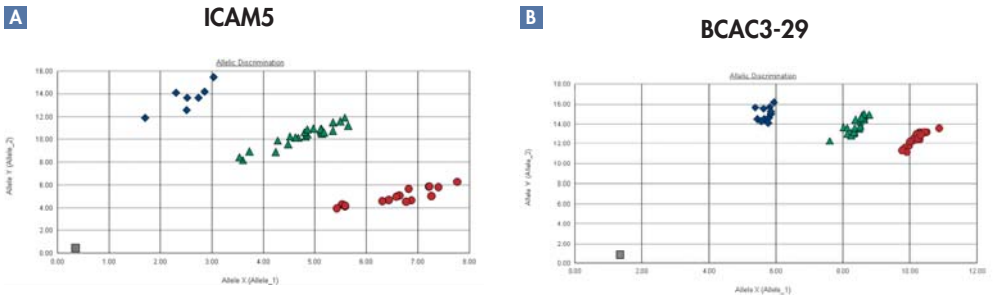


Figure 5. Fast and reliable SNP genotyping. Real-time PCR with genomic DNA template and subsequent end-point allelic discrimination was carried out on the Applied Biosystems® 7500 Fast System using the QuantiFast® Probe PCR +ROX Vial Kit and TaqMan probes for detecting SNPs in A ICAM5 (intercellular adhesion molecule) and B BCAC3-29. The allelic discrimination plots clearly indicate the homozygotes for each allele (● **Allele X** or ◆ **Allele Y**) and the heterozygotes (▲ **Both**). No template control is indicated by ■. The PCR run time was about 35 minutes. (Data kindly provided by Peter Schuermann, Department of Gynecology and Obstetrics, Hannover Medical School, Hannover, Germany.)

3.1 Two-step and one-step RT-PCR

Real-time RT-PCR can take place in a two-step or one-step reaction (Figure 6 and Table 2). 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 added to the real-time 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 a single 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 real-time PCR take place in the same tube, with reverse transcription preceding PCR. This is possible due to specialized reaction chemistries and cycling protocols (see section 8.4, page 36).

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.

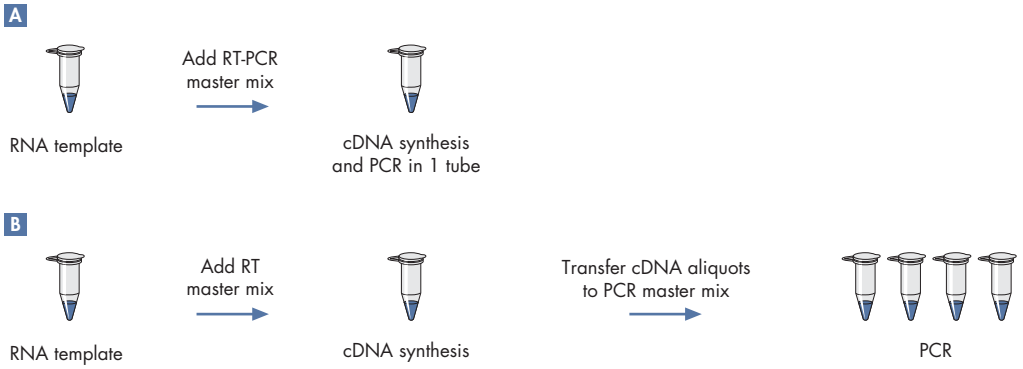


Figure 6. Comparison of two-step and one-step RT-PCR. A In one-step RT-PCR, reverse transcription and PCR take place sequentially in the same tube. B In two-step RT-PCR, cDNA is synthesized in 1 tube, and aliquots of the cDNA are transferred to other tubes for PCR.

Table 2. Advantages of different RT-PCR procedures

Procedure	Advantages
Two-step RT-PCR	■ Multiple PCRs from a single 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

3.2 Multiplex PCR and RT-PCR

In multiplex, real-time PCR, several genomic DNA targets are quantified simultaneously in the same reaction. Multiplex, real-time RT-PCR is a similar method, allowing simultaneous quantification of several RNA targets in the same reaction. The procedure can be performed either as two-step RT-PCR or as one-step RT-PCR (see section 3.1).

Multiplex PCR and RT-PCR offer many advantages for applications such as gene expression analysis, viral load monitoring, and genotyping. The target gene(s) as well as an internal control are coamplified in the same reaction, eliminating the well-to-well variability that would occur if separate amplification reactions were carried out. The internal control can be either an endogenous gene that does not vary in expression between different samples (e.g., a housekeeping gene; see Table 7, page 24) or an exogenous nucleic acid. For viral load monitoring, the use of an exogenous nucleic acid as internal control allows the following parameters to be checked: the success of sample preparation, the absence of inhibitors, and the success of PCR. Multiplex analysis ensures high precision in relative gene quantification, where the amount of a target gene is normalized to the amount of a control reference gene. Quantification of multiple genes in a single reaction also reduces reagent costs, conserves precious sample material, and allows increased throughput.

Multiplex PCR and RT-PCR are made possible by the use of sequence-specific probes that are each labeled with a distinct fluorescent dye and an appropriate quencher moiety. This means that the emission maxima of the dyes must be clearly separated and must not overlap with each other (see Figure 4 on page 6). In addition, reactions must be carried out on an appropriate real-time cycler that supports multiplex analysis (i.e., the excitation and detection of several non-overlapping dyes in the same well or tube). To view successful multiplex data from a wide range of real-time cyclers, visit www.qiagen.com/multiplex.

3.3 Real-time RT-PCR using cell lysates

Real-time RT-PCR is an ideal tool for cell assays that require accurate analysis of gene expression, for example, in experiments to validate gene knockdown after transfection of siRNAs. However, high-throughput assays are difficult to achieve, since the purification of RNA from large numbers of cultured-cell samples involves both time and effort. This bottleneck can be overcome by eliminating the RNA purification steps and using cell lysates directly in real-time RT-PCR. However, the method for cell lysis needs to be carefully optimized so that the lysates provide similar performance in real-time RT-PCR as pure RNA templates. The method should preserve the gene expression profile and also prevent cellular and buffer components from interfering with amplification and detection. A reliable method for real-time RT-PCR direct from cells is presented at www.qiagen.com/FastLane.

3.4 Fast PCR and RT-PCR

Most new real-time cyclers are installed with thermal-cycling modules that provide high ramping rates (i.e., fast heating and cooling capacities). This technology shortens the time to switch from one temperature to another, allowing faster run times in real-time PCR. Further and more significant time savings can be achieved by reducing the duration of the denaturation, annealing, and extension steps in each PCR cycle as well as shortening the time required for activation of the hot-start DNA polymerase. When reducing these PCR parameters, care should be taken to avoid compromising the specificity and sensitivity of real-time PCR. Examples of fast PCR without compromising performance can be viewed for various different real-time cyclers at www.qiagen.com/fastPCR.

3.5 Whole genome amplification

The small amount of genomic DNA in precious samples limits the number of real-time PCR analyses that can be carried out. This limitation can be overcome by whole genome amplification (WGA), a method which allows amplification of all genomic DNA targets in a sample. Unbiased and accurate amplification of whole genomes can be achieved with Multiple Displacement Amplification (MDA). For details, visit www.qiagen.com/WGA.

3.6 Whole transcriptome amplification

Whole transcriptome amplification (WTA) allows amplification of entire transcriptomes from very small amounts of RNA, enabling unlimited analyses by real-time RT-PCR. WTA of RNA samples can be achieved by reverse transcription and cDNA ligation prior to MDA (see page 28).

Application data

Single-base detection using quenched FRET assays

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).

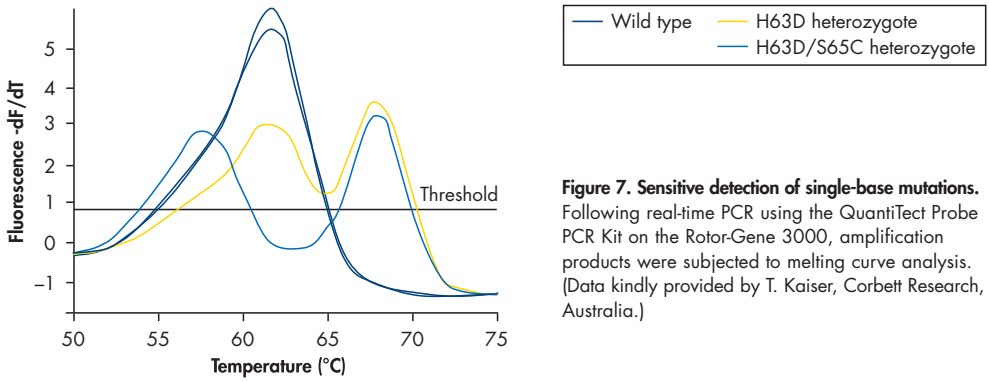


Figure 7. Sensitive detection of single-base mutations. Following real-time PCR using the QuantiTect Probe PCR Kit on the Rotor-Gene 3000, amplification products were subjected to melting curve analysis. (Data kindly provided by T. Kaiser, Corbett Research, Australia.)

4. Basic terms used in real-time PCR

Before levels of nucleic acid target can be quantified in real-time PCR, the raw data must be analyzed and baseline and threshold values set. When different probes are used in a single experiment (e.g., when analyzing several genes in parallel or when using probes carrying different reporter dyes), the baseline and threshold settings must be adjusted for each probe. Furthermore, analysis of different PCR products from a single experiment using SYBR Green detection requires baseline and threshold adjustments for each individual assay. Basic terms used in data analysis are given below. For more information on data analysis, refer to the recommendations from the manufacturer of your real-time cycler. 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).

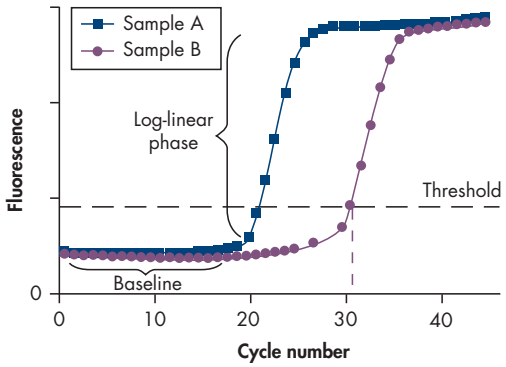


Figure 8. Typical amplification plot. Amplification plots showing increases in fluorescence from 2 samples (A and B). Sample A contains a higher amount of starting template than sample B. The Y-axis is on a linear scale.

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. Recent versions of software for various real-time cyclers allow automatic, optimized baseline settings for individual samples.

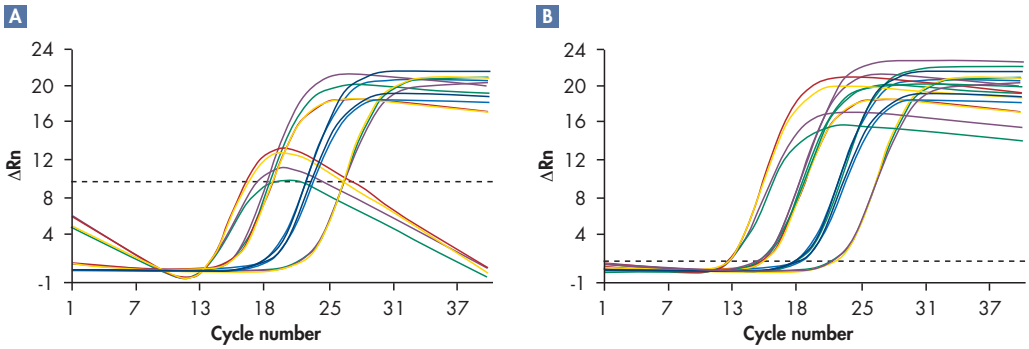


Figure 9. Correct baseline and threshold settings are important for accurate quantification. A Amplification product becomes detectable within the baseline setting of cycles 6 to 15 and generates a wavy curve with the highest template amount. B Setting the baseline within cycles 6 to 13 eliminates the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

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. 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 or a fluorescently labeled sequence-specific probe.

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 cyclers, 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, well position, 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 (Cp): The cycle at which the amplification plot crosses the threshold (i.e., there is a significant detectable increase in fluorescence). C_T can be a fractional number and allows calculation of the starting template amount (see section 5, page 15).

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, and is used to normalize for the amount of template used:

$$\Delta C_T = C_T \text{ (target gene)} - C_T \text{ (endogenous reference gene)}$$

$\Delta\Delta C_T$ 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:

$$\Delta\Delta C_T = \text{average } \Delta C_T \text{ (sample of interest)} - \text{average } \Delta C_T \text{ (reference sample)}$$

For more information on ΔC_T and $\Delta\Delta C_T$, see section 5, page 15.

Endogenous reference gene: This is a gene whose expression level should not differ between samples, such as a housekeeping gene (1). Comparing the C_T value of a target gene with that of the endogenous reference gene allows normalization of the expression level of the target gene to the amount of input RNA or cDNA (see ΔC_T value). The exact amount of template in the reaction is not determined. An endogenous reference gene corrects for possible RNA degradation or presence of inhibitors in the RNA sample, and for variation in RNA content, reverse-transcription efficiency, nucleic acid recovery, and sample handling. See page 24 for a list of housekeeping genes. ▶

Internal control: This is a control sequence that is amplified in the same reaction as the target sequence and detected with a different probe (i.e., duplex PCR is carried out). An internal control is often used to rule out failure of amplification in cases where the target sequence is not detected.

Calibrator sample: This is a reference sample used in relative quantification (e.g., RNA purified 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 can be any sample, but is usually a control (e.g., an untreated sample or a sample from time zero of the experiment).

Positive control: This is a control reaction using a known amount of template. A positive control is usually used to check that the primer set or primer–probe set works.

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

No RT control: RNA preparations may contain residual genomic DNA, which may be detected in real-time RT-PCR if assays are not designed to detect and amplify RNA sequences only (see section 9.7, page 52). DNA contamination can be detected by performing a no RT control reaction in which no reverse transcriptase is added.

Standard: This is a 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 PCR 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 nonlinear phase of the reaction or it can indicate the presence of inhibitors in the reaction (see section 8.2, page 33).

The efficiency of a real-time PCR assay can be calculated by analyzing a template dilution series, plotting the C_T values against the log template amount, and determining the slope of the resulting standard curve. From the slope (S), efficiency can be calculated using the following formula:

$$\text{PCR efficiency (\%)} = (10^{(-1/S)} - 1) \times 100$$

Reference

1. Thellin, O. et al. (1999). Housekeeping genes as internal standards: use and limits. J. Biotechnol. **75**, 291.

5. 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 end-point analysis, quantification is not possible as most reactions will already have reached the plateau phase of amplification. During this phase, no significant increase in the amount of PCR product 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 end products, 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 or fluorescently labeled probes is indicative of the amount of PCR product in the reaction, provided the reaction is in the log-linear or so-called exponential phase (see Figure 10) and provided 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.

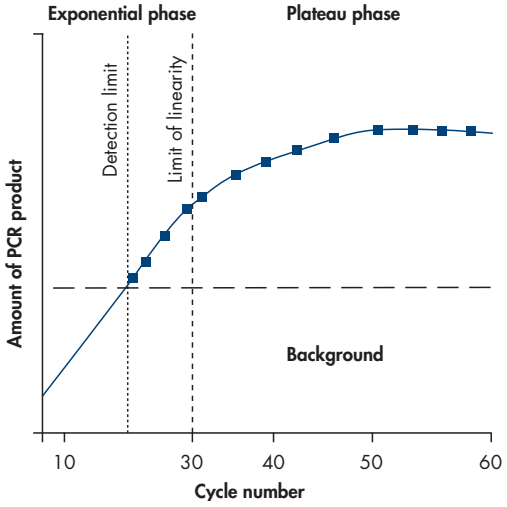


Figure 10. PCR kinetics. Reaction kinetics in PCR. The Y-axis is on a log scale.

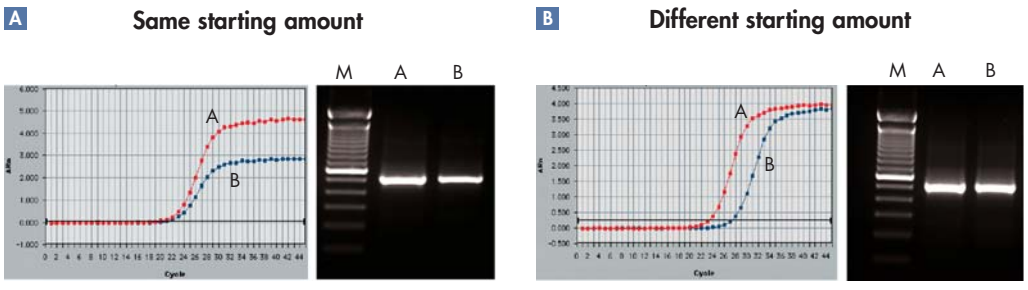


Figure 11. Problems associated with end-point detection. Two-step RT-PCR was carried out with the A same amount and B different amounts of template RNA. The Y-axis is on a linear scale. **M**: markers.

5.1 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 the amount of a control (e.g., 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.

5.2 Absolute quantification

Use of external standards enables the 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. Brief guidelines for preparing RNA standards are given in section 5.2.1, page 17.

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 standard (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. The C_T values of the standard samples are determined. Then, the C_T value of the unknown sample is compared with the standard curve to determine the amount of target in the unknown sample (Figure 12).

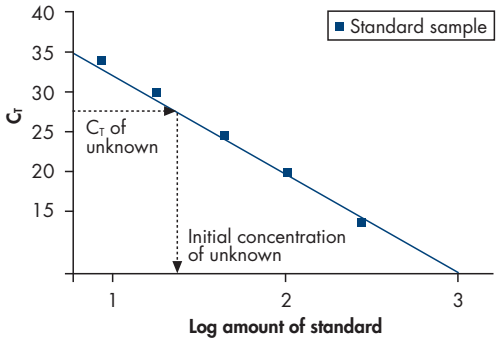


Figure 12. Absolute quantification. Typical standard curve showing determination of concentration of sample of interest.

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 the target sequence
- Sequences upstream and downstream from the amplified sequence identical or similar to the "natural" target
- Equivalent amplification efficiencies of standard and target molecules

5.2.1 RNA standards for absolute quantification

RNA standards can be created by cloning part or all of the transcript of interest into a standard cloning vector. The insert can be generated by RT-PCR from total RNA or mRNA, or by PCR from cDNA. The cloning vector must contain an RNA polymerase promoter such as T7, SP6, or T3. Ensure that in vitro transcription of the insert leads to generation of the sense transcript. After in vitro transcription, plasmid DNA must be removed completely with RNase-free DNase, since residual plasmid DNA will lead to errors in spectrophotometric determination of RNA concentration and will also serve as a template in the subsequent PCR. Furthermore, ensure that the RNA used as a standard does not contain any degradation products or aberrant transcripts by checking that it migrates as a single band in gel or capillary electrophoresis.

After determination of RNA concentration by spectrophotometry (see section 6.1.2, page 26), the copy number of standard RNA molecules can be calculated using the following formula:

(X g/µl RNA / [transcript length in nucleotides x 340]) x 6.022×10^{23} = Y molecules/µl

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.

5.2.2 DNA standards for absolute quantification

Plasmid DNA: The most convenient way to create a DNA standard is to clone a PCR product into a standard vector. Advantages of this method are that large amounts of standard can be produced, its identity can be verified by sequencing, and the DNA can easily be quantified by spectrophotometry. Plasmid standards should be linearized upstream or downstream of the target sequence, rather than using supercoiled plasmid for amplification. This is because the amplification efficiency of a linearized plasmid often differs from that of the supercoiled conformation and more closely simulates the amplification efficiency of genomic DNA or cDNA.

After spectrophotometric determination of plasmid DNA concentration, the copy number of standard DNA molecules can be calculated using the following formula:

(X g/µl DNA / [plasmid length in basepairs x 660]) x 6.022×10^{23} = Y molecules/µl

PCR fragment: A PCR product containing the target sequence can also be used as a DNA standard. We recommend including at least 20 bp upstream and downstream of the primer binding sites of the amplicons. Copy number is calculated using the formula for plasmid DNA (see above), replacing "plasmid length" with the length of the PCR product.

Genomic DNA: If the target of interest is present in only 1 copy per haploid genome and amplification of pseudogenes and/or closely related sequences can be excluded, genomic DNA can also be used as a DNA standard for absolute quantification. The copy number of the target present in the genomic DNA can be directly calculated if the genome size of the organism is known. For example, the genome size (haploid) of *Mus musculus* is 2.7×10^9 bp, a molecular weight of 1.78×10^{12} Daltons.

1.78×10^{12} g of genomic DNA corresponds to 6.022×10^{23} copies of a single-copy gene.

1 µg of genomic DNA corresponds to 3.4×10^5 copies of a single-copy gene.

5.3 Relative quantification

In relative quantification, the ratio between the amounts of a target gene and a control gene (e.g., an endogenous reference gene present in all samples) is determined. This ratio is then compared between different samples. In gene expression analysis, housekeeping or maintenance genes are usually 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, for example, to compare differential expression of a gene in different tissues or to compare gene expression between siRNA-transfected cells and untransfected cells. 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.

5.3.1 Determining amplification efficiencies

The amplification efficiency of 2 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:

$E = 10^{(-1/S)} - 1$ (S = slope of the standard curve)

To compare the amplification efficiencies of the 2 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 13). If the slope of the resulting straight line is <0.1, amplification efficiencies are comparable.

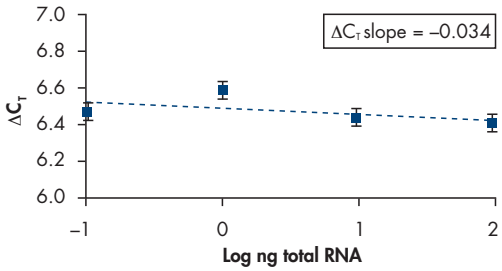


Figure 13. Efficiency comparison. C_T values were determined for the tumor necrosis factor alpha (TNF) gene and the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) using RNA purified from human leukocytes. Real-time RT-PCR was performed using the QuantiTect Probe RT-PCR Kit and gene-specific primers and probe. 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.

5.3.2 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 vary between the 2 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 14).

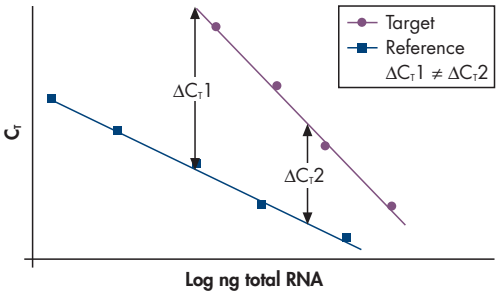


Figure 14. Different PCR efficiencies. Typical standard curves showing amplification of 2 targets with different PCR efficiencies.

5.3.3 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 the target and reference
- Perform real-time PCR/RT-PCR
- Determine the C_T values for the standards (Table 3) and the samples of interest
- Construct standard curves for both the target and reference by plotting C_T values (Y-axis) against the log of template amount or dilution (X-axis) (Figure 15)
- Calculate the amount of target and reference in the samples of interest using their C_T values and the corresponding standard curve (Table 4)
- To calculate the normalized amount of target, divide the amount of target by the amount of reference (if replicate reactions were performed, use the average value)
- Define the calibrator sample and compare the relative expression level of the target gene in the samples of interest by dividing the normalized target amounts by the value of the calibrator

Table 3. 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

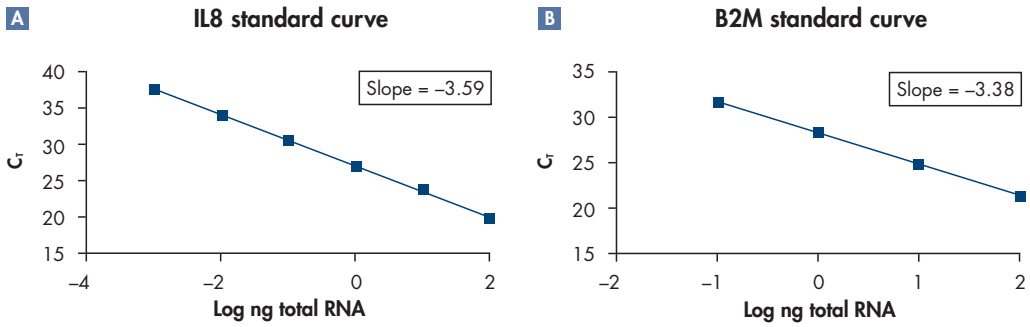


Figure 15. Relative quantification with different PCR efficiencies. Standard curves were generated for interleukin 8 (IL8) and the housekeeping gene β-2-microglobulin (B2M) using total RNA from human leukocytes. Real-time RT-PCR was performed using gene-specific primers and probe with the QuantiTect Probe RT-PCR Kit.

Table 4. 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	1.40×10^{-3}	346	4.05×10^{-6}	1.0
PMA	29.43	18.59	1.80×10^{-1}	417	4.32×10^{-4}	106.7
LTA	34.43	18.59	7.20×10^{-3}	417	1.73×10^{-5}	4.3

Jurkat cells were untreated or treated with either phorbol 12-myristate acetate (PMA) or lipoteichoic acid (LTA). Total RNA was purified and, after real-time RT-PCR, the relative amounts of target and reference RNA were determined using the appropriate standard curve (see Figure 15).

5.3.4 Comparable amplification efficiencies

If the amplification efficiencies of the target gene and the endogenous reference gene are comparable, one standard curve for the reference gene is sufficient. The differences in C_T values of the target and the reference will be constant when the amounts of template are varied (Figure 16). The amounts of target and reference in an unknown sample are calculated by comparing the C_T values with the standard curve for the reference gene as described below.

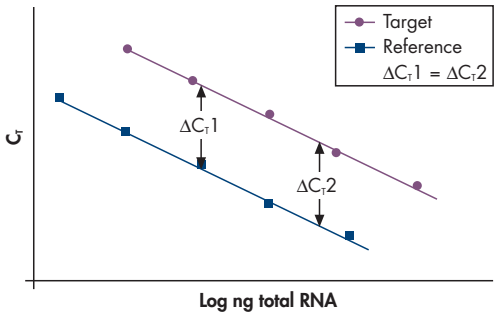


Figure 16. Same PCR efficiencies. Typical standard curves showing amplification of 2 targets with similar PCR efficiencies.

5.3.5 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 the C_T values for the standards and the 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 the amount of target and reference in the samples of interest using their C_T values and the 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 the average value)
- Define the calibrator sample and compare the relative expression level of the target gene in the samples of interest by dividing the normalized target amounts by the value of the calibrator

5.3.6 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 the amplification efficiencies of the target and endogenous reference genes 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 6)

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:

$$\text{Error (\%)} = [(2^n/(1+E)^n) \times 100)] - 100$$ (E = efficiency of PCR; n = 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.

Note: The $\Delta\Delta C_T$ method should only be chosen if the PCR efficiency of the 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).

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

- Perform a validation experiment to determine the PCR efficiency for the target and reference as described in section 5.3.1, page 18 and shown in Figure 17
- Perform real-time RT-PCR for the target and reference with RNA derived from different samples
- Determine the ΔC_T value by subtracting the endogenous reference gene C_T value from the target gene C_T value for each sample (Table 5)
- Define the calibrator sample and determine the $\Delta\Delta C_T$ value by subtracting the calibrator ΔC_T value from the ΔC_T value of each sample (Table 6)
- Calculate the normalized level of target expression relative to the calibrator by using the formula $2^{-\Delta\Delta C_T}$

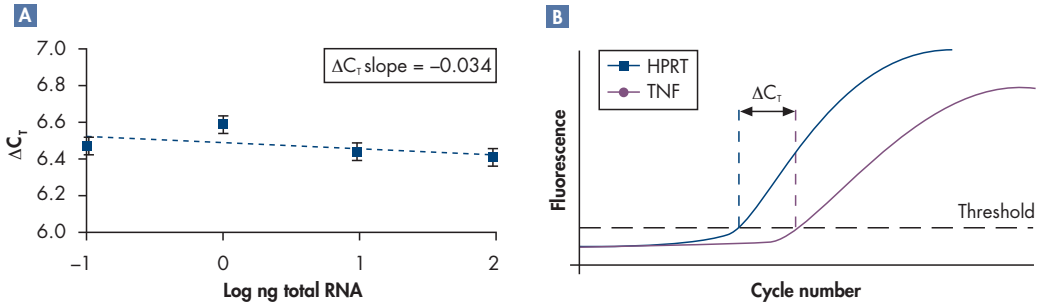


Figure 17. Comparison of PCR efficiency for TNF and HPRT genes. A C_T values were determined for the tumor necrosis factor alpha (TNF) gene and the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) using RNA purified from human leukocytes. Real-time RT-PCR was performed using the QuantiTect Probe RT-PCR Kit and gene-specific primers and probe. 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. B Illustration showing how the ΔC_T value at one template amount is determined. ΔC_T is the C_T value for HPRT subtracted from the C_T value for TNF.

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 Res. **30**, e36.

Table 5. 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 6. Calculation of TNF expression levels in Jurkat cells using the $\Delta\Delta C_T$ method

Sample	C_T TNF (average)	C_T HPRT (average)	ΔC_T (C_T TNF – C_T 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 purified and, after real-time RT-PCR, the relative amounts of target and reference RNA were determined.

5.3.8 Endogenous reference genes

For relative quantification of gene expression, it is important to choose a suitable gene to use as a reference (Table 7). 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 GAPDH 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 range of bioinformatically validated assays for housekeeping genes is available from QIAGEN. These are QuantiTect Primer Assays, which are primer sets for SYBR Green-based real-time RT-PCR and can be ordered at www.qiagen.com/GeneGlobe.

Table 7. Housekeeping genes commonly used as endogenous references

	Gene symbol		Relative expression level*	
Gene	**Human**	**Mouse**	**Human**	**Mouse**
18S ribosomal RNA	RRN18S	Rn18s	++++	++++
Actin, beta	ACTB	Actb	+++	+++
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Gapdh	+++	+++
Phosphoglycerate kinase 1	PGK1	Pgk1	+++	++
Peptidylprolyl isomerase A	PPIA	Ppia	+++	+++
Ribosomal protein L13a	RPL13A	Rpl13a	+++	+++
Ribosomal protein, large, P0	RPLP0		+++	
Acidic ribosomal phosphoprotein PO		Arbp		+++
Beta-2-microglobulin	B2M	B2m	++ – +++	++ – +++
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	YWHAZ	Ywhaz	++ – +++	+
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	SDHA	Sdha	++	+
Transferrin receptor	TFRC	Tfrc	++	+
Aminolevulinate, delta-, synthase 1	ALAS1	Alas1	+	+
Glucuronidase, beta	GUSB	Gusb	+	+
Hydroxymethylbilane synthase	HMBS	Hmbs	+	++ – +++
Hypoxanthine phosphoribosyltransferase 1	HPRT1	Hprt1	+	+
TATA box binding protein	TBP	Tbp	+	+
Tubulin, beta	TUBB		+	
Tubulin, beta 4		Tubb4		+

* "+" indicates relative abundance of the transcripts.

5.4 Controls

5.4.1 No-template control

A no-template control (NTC) allows detection of contamination of the PCR reagents. An NTC reaction contains all real-time PCR components except the template. Detection of fluorescence in an NTC reaction indicates the presence of contaminating nucleic acids.

5.4.2 Positive control

A positive control may be necessary, for example, when amplifying a new target sequence to confirm whether the primer set or primer–probe set works. A positive control can be an absolute standard, which is a nucleic acid template of known copy number that provides quantitative information. Absolute standards, such as a nucleic acid from an established cell line, a plasmid containing cloned sequences, or in vitro transcribed RNA, are commercially available or can be generated in the lab. A positive control can also be a known positive sample, which is usually a substitute for an absolute standard and used only to test for the presence or absence of a target.

5.4.3 No RT control

A no RT control, where real-time RT-PCR is carried out without reverse transcriptase, should be included when performing gene expression analysis.

For viral load monitoring, a no RT control may be necessary, depending on the sample type and the life cycle of the virus species detected. Since reverse transcription cannot take place, a no RT control reaction allows detection of contaminating DNA, such as DNA from viral sequences integrated into the host genome. Contaminating DNA in RNA samples can be removed by DNase treatment before starting RT-PCR.

5.4.4 Internal, positive control

An internal, positive control can be used to test for the presence of PCR inhibitors. A duplex reaction is carried out, where the target sequence is amplified with 1 primer–probe set, and a control sequence (i.e., the internal, positive control) is amplified with a different primer–probe set. The internal, positive control should be at a high enough copy number for accurate detection. If the internal, positive control is detected, but the target sequence is not, then this indicates that the amplification reaction was successful and that the target sequence is absent (or at too low a copy number to be detected).

? 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 (see Figure 11, page 15).

6. Guidelines for preparation of template, primers, and probes

Success in real-time PCR and RT-PCR depends on the purity and integrity of the template, primers, and probes used. A template can be DNA or RNA purified from the biological sample being analyzed, or a known amount of DNA or RNA to be used as a standard or positive control. Primers and probes are DNA oligonucleotides which are typically purchased from a commercial supplier.

6.1 Purification of nucleic acid templates

Since PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, and EDTA 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 that provide pure, high-quality templates for PCR and RT-PCR. These include QIAprep® Kits for purification of plasmid DNA, QIAamp® and DNeasy® Kits for purification of genomic DNA, RNeasy® Kits for purification of total RNA, and the PAXgene® Blood RNA System for stabilization and purification of RNA from blood. Phenol and other contaminants can be efficiently removed from crude RNA preps using the RNeasy MinElute® Cleanup Kit to clean up and concentrate RNA for sensitive assays. Details about QIAGEN kits for nucleic acid purification can be found at www.qiagen.com.

6.1.1 Determining the integrity of RNA templates

The integrity and size distribution of total RNA can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining or by using a capillary electrophoresis system such as the QIAxcel (www.qiagen.com/goto/QIAxcel). The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification (e.g., RNA integrity and quality is significantly reduced in formalin-fixed, paraffin-embedded tissue samples).

6.1.2 Determining the concentration and purity of nucleic acid templates

The concentration of the DNA or RNA template should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (Table 8). For accuracy, absorbance readings at 260 nm should fall between 0.15 and 1.0. Alternatively, RNA can be quantified using fluorescent dyes that bind specifically to RNA. Measurement of the fluorescent signal on a fluorimeter enables RNA quantification.

Table 8. 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; measurement in water.

Note that absorbance measurements at 260 nm cannot discriminate between DNA and RNA. Depending on the method used for template preparation, the purified DNA may be contaminated with RNA, or the purified RNA may be contaminated with DNA. If this is the case, the A_{260} value will be too high and lead to inaccurate quantification. The ratio between the absorbance values at 260 nm and 280 nm (A_{260}/A_{280}) gives an estimate of the purity of the DNA or RNA template. As this ratio is influenced considerably by pH, absorbance measurements should be made in 10 mM Tris·Cl, pH 7.5. In this buffer, pure DNA has an A_{260}/A_{280} ratio of 1.8–2.0, and pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1 (values of up to 2.3 are routinely obtained for pure RNA with some spectrophotometers). Lower ratios indicate the presence of contaminants such as proteins.

6.1.3 Storage of nucleic acid templates

Purified DNA should be stored at –20°C or –70°C under slightly basic conditions (e.g., Tris·Cl, pH 8.0), as acidic conditions can cause depurination of DNA. 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 dedicated tubes that prevent adherence of nucleic acids to the tube walls. Otherwise, the concentration of nucleic acids in solution may be reduced. QIAGEN offers QuantiTect Nucleic Acid Dilution Buffer (supplied with QuantiTect Virus Kits), which provides reliable dilution and storage of nucleic acid standards, preventing adhesion on plastic surfaces.

6.2 Handling and storing primers and probes

For optimal results in real-time PCR and RT-PCR, primers and probes should be purchased from an established oligonucleotide manufacturer. Upon receipt, the lyophilized primers and probes should be resuspended, and their concentrations checked by spectrophotometry. Lyophilized primers and probes should be dissolved in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) to provide a stock solution of 100 µM, and divided into small aliquots to avoid repeated freezing and thawing.

? How much template can I use in the reaction and what is the maximum template volume?

Even when detecting low-abundance targets, we recommend using no more than 100 ng template RNA or cDNA. Generally, 1–100 ng template will 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, and EDTA 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. ▶

7. Guidelines for successful whole transcriptome amplification

When only nanogram amounts of an RNA sample are available, the number of real-time RT-PCR analyses that can be performed is limited. This problem can be resolved by whole transcriptome amplification (WTA). With this technology, all mRNA transcripts in an RNA sample are replicated to provide microgram amounts of cDNA template, enough for unlimited real-time PCR analysis and stable archiving.

7.1 WTA techniques

To ensure reliable results in real-time PCR, it is essential that the WTA method provides unbiased and accurate amplification of the whole transcriptome. This means that the sequence and the relative abundance of each transcript should be preserved after WTA, otherwise false results in gene expression analysis will occur. The method used by the QuantiTect Whole Transcriptome Kit to prepare amplified cDNA dedicated for use in real-time PCR consists of the following steps: reverse transcription, ligation, and amplification using Multiple Displacement Amplification (MDA) technology (Figure 18). As reverse transcription takes place using a mix of random and oligo-dT primers, a cDNA library covering all transcript sequences, including both 5' and 3' regions, is prepared (Figure 19). Subsequent ligation of the cDNA followed by MDA using a uniquely processive DNA polymerase generates amplified cDNA that preserves the transcript representation of the original RNA sample (Figure 20). This is critical for accurate gene expression analysis.

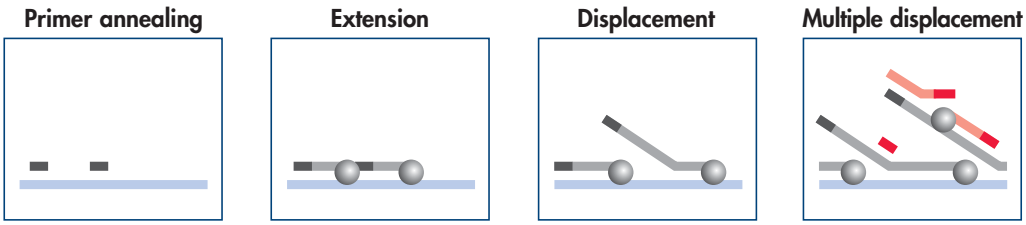


Figure 18. Schematic representation of whole transcriptome amplification. cDNA is first synthesized from template RNA and then ligated (not shown). REPLI-g® DNA polymerase moves along the cDNA template strand, displacing the complementary strand. The displaced strand becomes a template for replication, allowing high yields of cDNA to be generated.

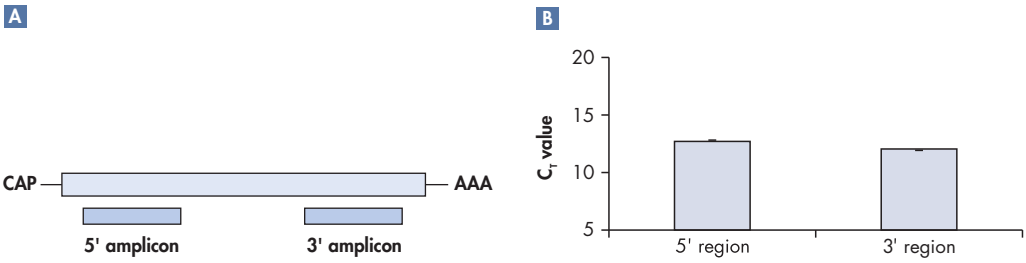


Figure 19. Equal amplification of 5' and 3' regions. A Schematic diagram showing the location of the 2 amplicons with respect to the transcript. B Total RNA (1 ng) was amplified using the QuantiTect Whole Transcriptome Kit. This was followed by real-time PCR using 10 ng cDNA, primers specific for β-actin, and the QuantiTect SYBR Green PCR Kit. Amplicons corresponding to the 5' and 3' regions of the β-actin transcript were detected with similar C_T values, indicating that the QuantiTect Whole Transcriptome Kit provided equal amplification of all transcript regions.

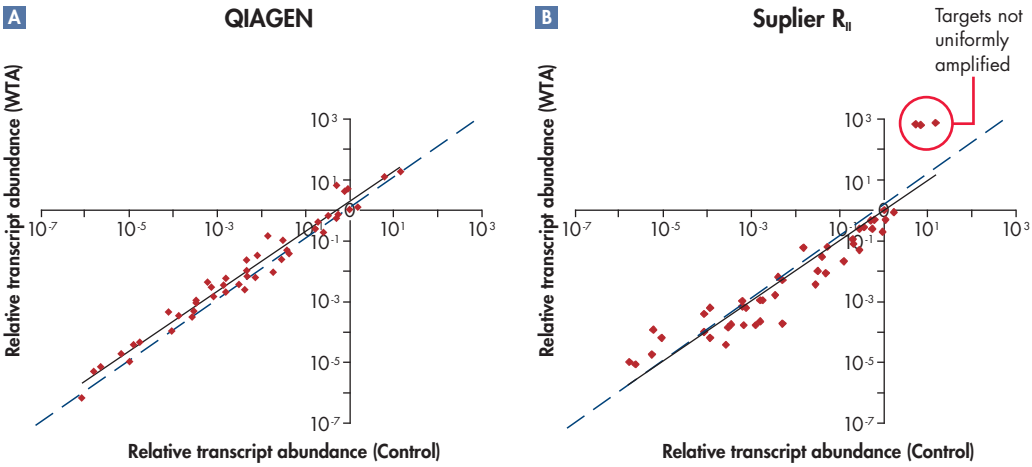


Figure 20. Preservation of transcript profile. A Total RNA (10 ng) from HeLa cells, leukocytes, or whole blood was amplified using the QuantiTect Whole Transcriptome Kit for 8 hours (**WTA**). As a control, RNA was reverse transcribed without amplification using the Sensiscript® RT Kit (**Control**). The expression of 15 different genes (including NFKB, IL8, CASP8, TP53, and GAPDH) in the 3 samples (HeLa cells, leukocytes, and blood) was then analyzed by real-time PCR, providing 45 independent analyses. Relative transcript levels (normalization to β-actin) from the WTA and Control samples were plotted against each other. The blue dotted line indicates 100% correlation between the 2 data sets (while the solid line indicates actual correlation). As the data points are close to this dotted line, this indicates that amplification with the QuantiTect Whole Transcriptome Kit preserves the gene expression profile of the RNA template. B The experiment was repeated using a kit from Supplier R_{II} for whole transcriptome amplification. Some targets were not uniformly amplified, as some data points lay outside the dotted line that indicates 100% correlation.

7.2 The importance of RNA quality

The efficiency of WTA depends on the quality and quantity of the starting RNA template. It is therefore important to use intact RNA. High amounts of carrier RNA, which may be used in procedures for purification of very low amounts of target RNA, may affect the specific amplification of transcript sequences. To ensure reproducible and efficient WTA, we recommend purifying RNA template using silica-membrane technology. RNeasy Kits, the PAXgene Blood RNA Kit, and the QIAamp RNA Blood Mini Kit are based on this technology. High amounts of carrier RNA (>20 ng) in the RNA purification procedure should be avoided. However, total RNA purified with carrier RNA using the RNeasy Micro Kit or RNeasy Plus Micro Kit performs well in WTA using the QuantiTect Whole Transcriptome Kit.

7.3 Working with degraded RNA

RNA from certain sources, such as formalin-fixed, paraffin-embedded (FFPE) tissue sections, may be degraded and not be suitable for WTA. For efficient RNA amplification with the QuantiTect Whole Transcriptome Kit, RNA should not be heavily degraded (i.e., RNA should be longer than 500 nucleotides). The method cannot be used to amplify RNAs substantially smaller than mRNA molecules, such as tRNAs or miRNAs.

7.4 Amplification from low cell numbers

When carrying out WTA, it is important to consider both the amount of starting material (i.e., the number of cells or the amount of RNA) and the copy number of the transcripts of interest. Table 9 and Figure 21 show the relationship between the amount of starting material and transcript representation (note that this is only a guide: the number of transcripts per given amount of starting material can vary). In starting material where the copy number of a transcript is 10 or less ▶

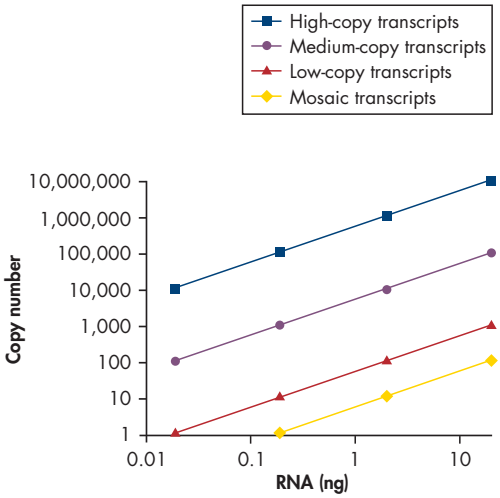


Figure 21. Transcript representation in different RNA amounts. Relationship between amount of starting RNA and copy number of high-copy, medium-copy, low-copy, and mosaic transcripts.

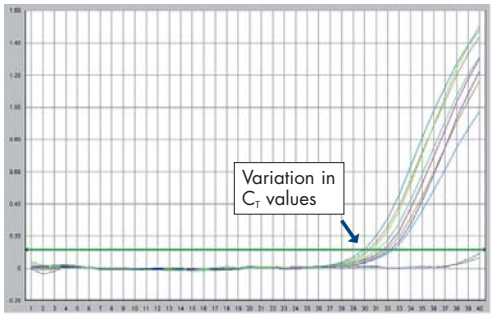


Figure 22. Stochastic problems when analyzing a low-copy transcript. Eight replicate RNA samples (1 ng each) were amplified using the QuantiTect Whole Transcriptome Kit. Real-time PCR analysis of NFκB transcript was then performed using 10 ng amplified cDNA and the QuantiFast Probe PCR Kit. The resulting C_T values were in the range of 30–32.5. This significant variation occurred because the stochastic variation in low-copy NFκB transcript in the replicate RNA samples is amplified, resulting in widely differing amounts of NFκB cDNA.

(highlighted in bold in Table 9), stochastic problems will occur (i.e., the unequal distribution of a very low number of transcripts in a highly dilute solution). This may result in underrepresentation of the low-copy transcript at the start of WTA. Special consideration should be given to mosaic transcripts, which are derived from genes that are expressed only in a subset of cells in tissues. Since these transcripts are not present in every cell, they will not be accurately represented in low amounts of starting material (i.e., 1–10^2 cells).

Reliable WTA depends on the copy number of the transcripts. If the recommended amount of starting material (i.e., 10 ng intact RNA) is amplified with the QuantiTect Whole Transcriptome Kit, all transcripts will be accurately represented after amplification. 10 ng of RNA corresponds to about 500 cells, and even low-copy transcripts are well represented in this RNA amount. Using lower amounts of RNA or a very limited number of cells means that the starting material could have a partial representation or an absence of low-copy transcripts (Figure 22 provides an example).

Table 9. Transcript representation in different cell amounts

	10^3 cells*	10^2 cells†	10 cells‡	1 cell§
Amount of RNA (ng)	20	2	0.2	0.02
No. of high-copy transcripts	10^7	10^6	10^5	10^4
No. of medium-copy transcripts	10^5	10^4	10^3	10^2
No. of low-copy transcripts	10^3	10^2	**10**	**1**
No. of mosaics transcripts	10^2	**10**	**1**	**0**

* Complete representation of all transcripts.

† Stochastic problems for mosaic transcripts.

‡ Stochastic problems for low-copy and mosaic transcripts.

§ Stochastic problems for low-copy transcripts and loss of mosaic transcripts.

8. Guidelines for successful reverse transcription

When performing real-time RT-PCR, the primers and the enzyme for reverse transcription must be carefully chosen. The primers should allow reverse transcription of all targets of interest, and the reverse transcriptase should yield cDNA amounts that accurately represent the original RNA amounts to ensure accurate quantification. In addition, the effects of the components of the RT reaction on subsequent real-time PCR must be minimized.

8.1 Choice of RT primers

The choice of primers for reverse transcription depends on whether one-step or two-step RT-PCR is being carried out (see section 3.1, page 9). In one-step RT-PCR, the downstream PCR primer is also the primer for reverse transcription. Therefore, one-step RT-PCR is always performed with gene-specific primers. In two-step RT-PCR, 3 types of primers, and mixtures thereof, can be used for reverse transcription: oligo-dT primers (13–18mers), random oligomers (such as hexamers, octamers, or nonamers), or gene-specific primers (Table 10). 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 10. 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 section 8.1.1) are recommended so that cDNA covering the complete transcript is produced

8.1.1 Universal priming method for real-time two-step RT-PCR

A universal priming method for the RT step of real-time two-step RT-PCR should allow amplification and detection of any PCR product regardless of transcript length and amplicon position, and achieve this with high sensitivity and reproducibility. To determine the optimal primers for reverse transcription, 2 regions within a 10-kb transcript were analyzed by real-time two-step RT-PCR. Each amplicon was approximately 150 bp in length. For the amplicon 2 kb away from the 3' end of the transcript, the lowest C_T value was obtained using either oligo-dT primers alone or a mixture of oligo-dT primers and random nonamers (Figure 23A). However, when the amplicon was 6 kb away from the 3' end of the transcript, random nonamers provided the lowest C_T value, closely followed by the mixture of oligo-dT primers and random nonamers (Figure 23B). The use of oligo-dT primers alone resulted in a much higher C_T value, indicating transcript detection was significantly less sensitive. Random nonamers proved to be better than random hexamers or dodecamers (data not shown; the performance of random nonamers varies depending on the reverse transcriptase used). ▶

This relationship between choice of RT primers and sensitivity of real-time RT-PCR has been observed with many different transcripts and RT-PCR systems. Therefore, when quantifying several targets from one RNA population, we recommend using a mixture of oligo-dT primers and random nonamers for reverse transcription. The QuantiTect Reverse Transcription Kit, which is specially designed to synthesize cDNA for use in real-time PCR, contains an optimized primer mix consisting of a unique blend of oligo-dT and random primers (Figure 24).

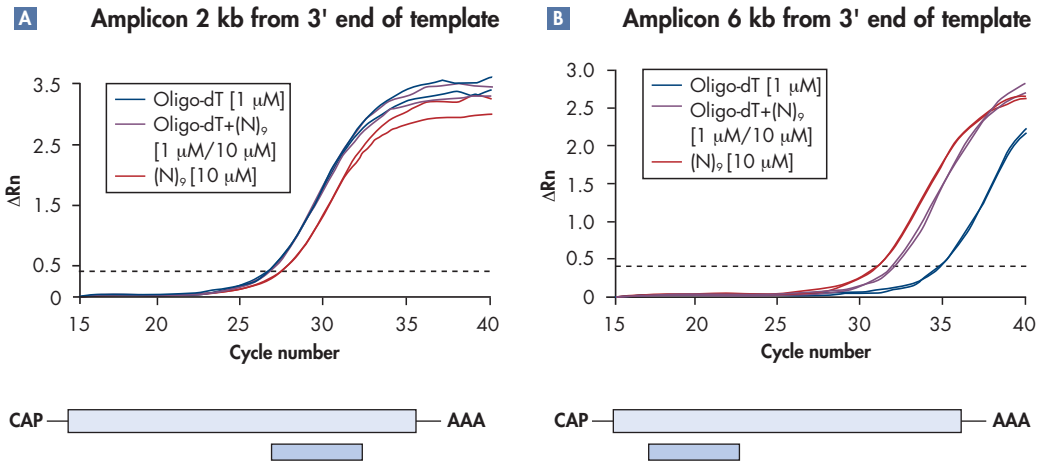


Figure 23. Effect of RT primer choice on RT-PCR. Two-step RT-PCR was carried out using the QuantiTect SYBR Green PCR Kit and the primer combinations shown. The amplicon was A 2 kb from the 3' end or B 6 kb from the 3' end of the template RNA.

Application data

Highly sensitive real-time two-step RT-PCR

In contrast with other reverse transcriptases, the QuantiTect Reverse Transcription Kit provides higher yields of cDNA from any transcript region, allowing high sensitivity in real-time two-step RT-PCR.

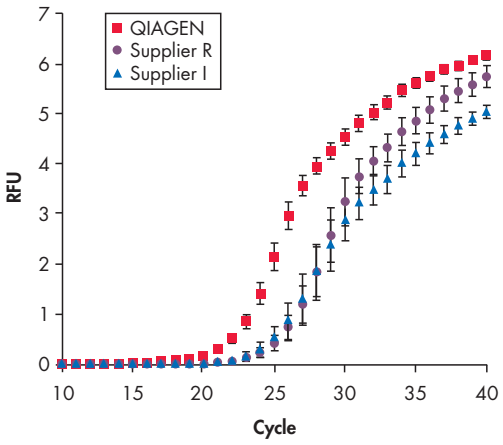


Figure 24. Sensitive detection of a target at the 5' region of a 12.5-kb transcript. Total RNA from mouse testis was reverse transcribed using the QuantiTect Reverse Transcription Kit or reverse transcriptases from Supplier I and Supplier R. Identical volumes of triplicate RT reactions were used in real-time PCR on the LightCycler system to analyze a target located at the 5' region of the dystrophin gene (about 12.5 kb upstream of the poly-A site). The error bars show the standard deviation for each set of triplicates. Compared with the other 2 kits, the QuantiTect Reverse Transcription Kit generated much higher amounts of cDNA (indicated by the lower C_T values) and provided greater reproducibility in real-time RT-PCR (indicated by the smaller error bars). (Data kindly provided by Dr. Andrej-Nikolai Spiess, Department of Molecular Andrology, University Hospital Hamburg, Germany.)

8.2 Effect of RT volume added to two-step RT-PCR

In two-step RT-PCR, the addition of the completed reverse-transcription reaction to the subsequent amplification reaction transfers not only cDNA template, but also salts, dNTPs, and RT enzyme. The RT reaction buffer, which has a different salt composition to that of the real-time PCR buffer, can adversely affect real-time PCR performance. However, if the RT reaction forms 10% or less of the final real-time PCR volume, performance will not be significantly affected (Figure 25). Use of 3 µl of RT reaction in a 20 µl PCR (i.e., 15% of the final volume) can lead to significant inhibition of real-time PCR (Figure 25). 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 of the RT reaction mix that might affect accurate transcript quantification.

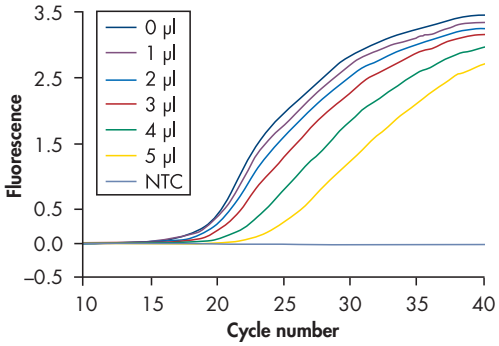


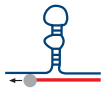
Figure 25. Inhibition of real-time PCR by addition of RT reaction. Real-time PCR (20 µl volume) was carried out using plasmid DNA as template. The volumes of RT reaction (without template RNA) indicated above were added to the PCR to determine their effect on amplification.

8.3 Reverse-transcription conditions for two-step RT-PCR

The QuantiTect Reverse Transcription Kit provides efficient and sensitive reverse transcription of any template with Quantiscript® Reverse Transcriptase, which is a unique mix consisting of Omniscript® and Sensiscript Reverse Transcriptases. Omniscript Reverse Transriptase is designed for reverse transcription of RNA amounts greater than 50 ng, and Sensiscript Reverse Transcriptase is optimized for use with very small amounts of RNA (<50 ng). This enzyme combination is compatible with a wide range of RNA template amounts, from as little as 10 pg of RNA up to 1 µg of RNA (Figure 26).

RNA secondary structure can affect RT-PCR in several ways. Regions of RNA with complex secondary structure can cause the reverse transcriptase to stop or dissociate from the RNA template (Table 11A).

Table 11. 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

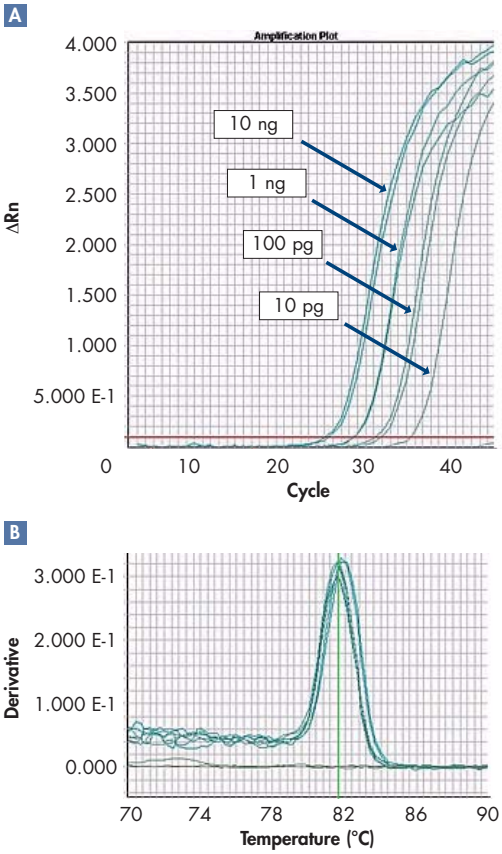


Figure 26. High specificity in real-time two-step RT-PCR. A cDNA was synthesized from HeLa cell RNA using the QuantiTect Reverse Transcription Kit. Tenfold serial dilutions of cDNA (10 ng to 10 pg) were then analyzed on the ABI PRISM® 7900 using the QuantiTect SYBR Green PCR Kit and a QuantiTect Primer Assay for BCL2 (B-cell CLL/lymphoma 2). B Melting curve analysis showed a single peak, indicating high specificity.

The truncated cDNAs, missing the downstream primer-binding site, are then not amplified during PCR. Alternatively, the reverse transcriptase can skip over looped-out regions of RNA, which are then excluded from the synthesized cDNA. In the PCR step, these cDNAs with internal deletions are amplified and appear as shortened PCR products (Table 11B). Ideally, the reverse transcriptase should not be affected by RNA secondary structure (Table 11C) and should be capable of reverse transcribing any template, without the need for reaction optimization. Quantiscript Reverse Transcriptase's unique combination of Omniscript and Sensiscript Reverse Transcriptases has a particularly high affinity for RNA, which 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 (Figure 24).

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 12A). RNase H, an enzyme activity which is intrinsic to the Omniscript and Sensiscript Reverse Transcriptases of Quantiscript Reverse Transcriptase, removes RNA in RNA:DNA hybrids to allow primer binding and second-strand DNA synthesis (Table 12B). RNase H digestion 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).

Table 12. 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

In addition to Quantiscript Reverse Transcriptase, the QuantiTect Reverse Transcription Kit also contains unique Quantiscript RT Buffer, which is specifically designed to be compatible with downstream real-time PCR analysis, in contrast to common RT buffer formulations developed for other applications such as the preparation of cDNA libraries. Quantiscript RT Buffer, together with Quantiscript Reverse Transcriptase, ensures accurate and sensitive results in real-time two-step RT-PCR (Figure 27), providing high yields of cDNA and helping to keep nonspecific products to a minimum. When the QuantiTect Reverse Transcription Kit was used in real-time two-step RT-PCR analysis of BCL2 transcript, subsequent melting curve analysis showed a distinct peak in the melting curve for the specific product (Figure 26).

References

1. Omniscript and Sensiscript RT Kits for highly efficient reverse transcription. QIAGEN News **1999**, No. 2, 1. (www.qiagen.com/literature/qiagennews)
2. Tacke, E. et al (1995) Transposon tagging of the maize Glossy2 locus with the transposable element En/Spm. Plant J. **8**, 907.

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, a component of Quantiscript Reverse Transcriptase, consistently gives higher sensitivity and a linear response (Figure 28).

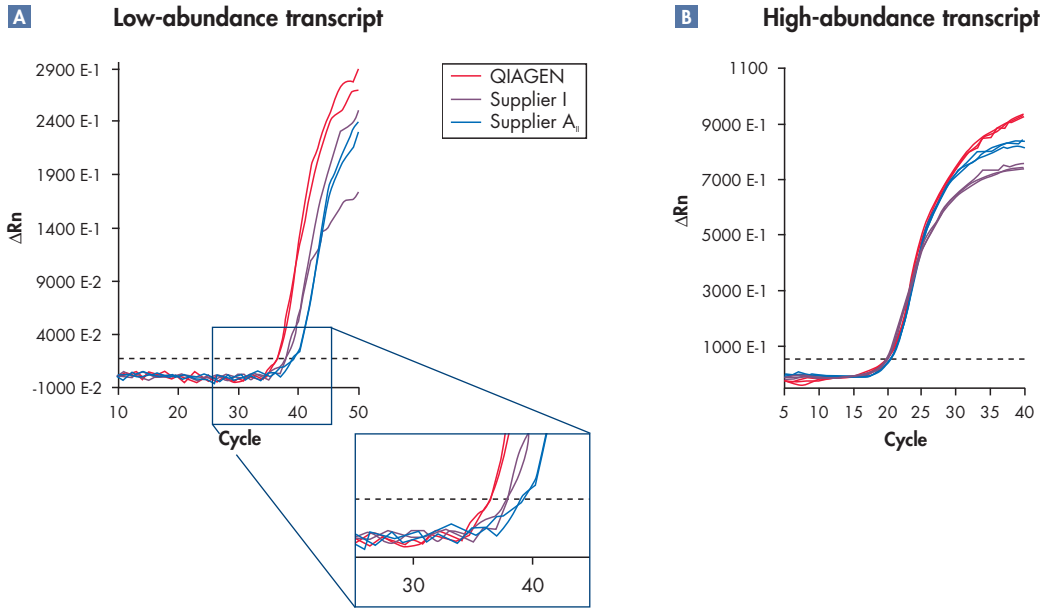


Figure 27. Higher sensitivity in real-time two-step RT-PCR. Real-time two-step RT-PCR analysis of A TGFB2 (low expression) and B IL8 (higher expression) was carried out. Total RNA was purified from human whole blood using the PAXgene Blood RNA system. cDNA was then synthesized from 1 µg RNA using the QuantiTect Reverse Transcription Kit, a kit from Supplier A$_{II}$, or a kit from Supplier I. Real-time PCR was performed in duplicate on the ABI PRISM 7900 using the QuantiTect Probe PCR Kit and a gene expression assay for TGFB2 or IL8. The C_T values for TGFB2 were lowest with the QuantiTect Reverse Transcription Kit, demonstrating that even low-abundance genes can be efficiently reverse transcribed and sensitively detected in real-time PCR.

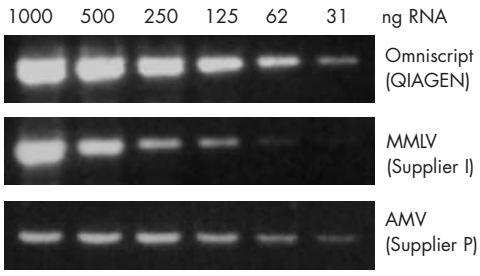


Figure 28. Superior sensitivity and dynamic range. Reverse transcription was carried out with different reverse transcriptases according to suppliers' specifications, using the indicated amounts of total RNA from HeLa cells. 1/20 of the reverse-transcription reaction was used in a 25-cycle PCR amplification with QIAGEN *Taq* DNA Polymerase. A 1.7-kb β-actin fragment was amplified.

8.4 Reverse-transcription conditions for one-step RT-PCR

The ideal reverse transcriptase for one-step RT-PCR should also exhibit the same properties as those described above for reverse transcriptases for two-step RT-PCR. However, one of the main problems in one-step RT-PCR is the inhibitory effect of the reverse transcriptase on the PCR step, which can lead to increased C_T values and thus reduced sensitivity and specificity when compared with two-step RT-PCR. QuantiTect and QuantiFast Kits for one-step RT-PCR have a patented additive in the reaction buffer to prevent this problem of PCR inhibition. In addition, other buffer components allow reverse transcription at high temperatures (50–60°C). As a result, the optimized ratio of reverse transcriptases to HotStarTaq® DNA Polymerase or HotStarTaq *Plus* DNA Polymerase enables highly sensitive and efficient one-step RT-PCR that is comparable to two-step RT PCR (Figure 29).

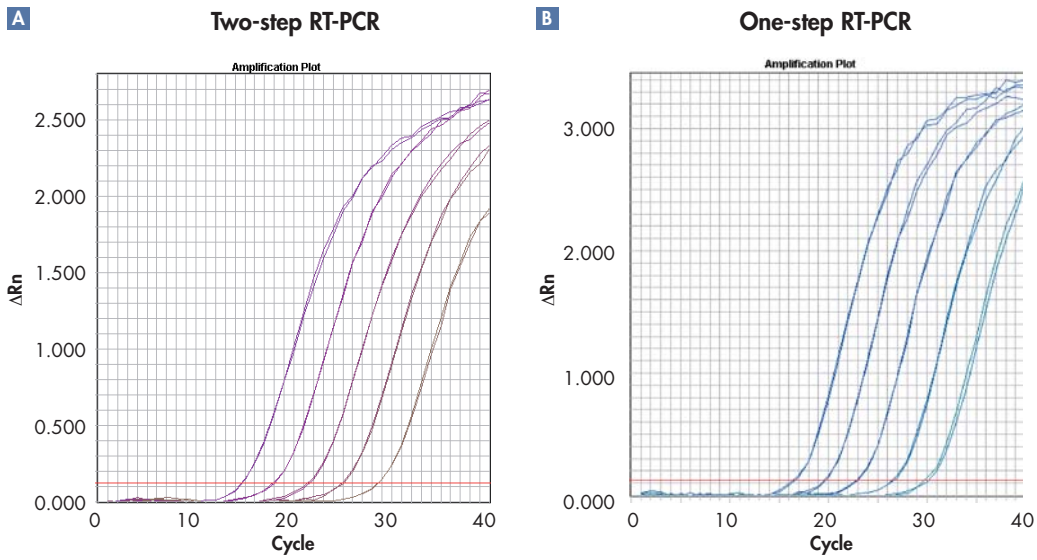


Figure 29. Comparable dynamic range and high sensitivity in two-step and one-step RT-PCR. Ubiquitin expression in human leukocytes was analyzed using a Primer Express® designed TaqMan assay. Reactions were run in duplicate on the ABI PRISM 7900. A 10-fold cDNA dilutions (100 ng to 10 pg) were analyzed using the QuantiFast Probe PCR Kit. B 10-fold RNA dilutions (100 ng to 10 pg) were analyzed using the QuantiFast Probe RT-PCR Kit.

8.5 Removal of genomic DNA contamination

Contamination of RNA samples with trace amounts of genomic DNA can interfere with real-time RT-PCR quantification if the PCR primers used are also able to amplify genomic DNA sequences. To avoid the negative effects of genomic DNA contamination, careful primer design is required (see section 9.7, page 52). If this is not possible, RNA samples should be treated with DNase I to digest contaminating DNA. A faster alternative to DNase digestion is RNeasy Plus Kits, which purify DNA-free RNA using gDNA Eliminator columns. For two-step RT-PCR applications, the QuantiTect Reverse Transcription Kit provides cDNA synthesis with integrated genomic DNA removal.

9. Guidelines for successful real-time PCR

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 noncomplementary 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 for detection. As SYBR Green 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.

9.1 Primer design

Prerequisites for successful real-time PCR and RT-PCR include design of optimal primer pairs for each reaction, use of appropriate primer concentration, and correct storage of primer and probe solutions (see section 6.2, page 27). For efficient amplification in real-time PCR and 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 C_T values, and sensitivity of the reaction drop significantly with increasing amplicon size (Figure 30).

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 (Table 13). However, even when primers and probes 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 a low level. These problems can be minimized by using an appropriate reaction chemistry (see section 9.2, page 42).

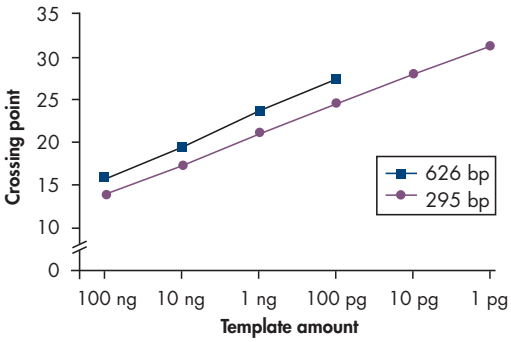


Figure 30. Improved PCR efficiency and sensitivity with shorter amplicons. Quantitative, real-time one-step RT-PCR of a 295-bp fragment and a 625-bp fragment of the β-actin gene was carried out using the QuantiTect SYBR Green RT-PCR Kit on the LightCycler system. Reactions were performed using varying amounts of template RNA, as indicated. Crossing-point (Cp or C_T) values are shown for each reaction.

Table 13. 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 x (A+T) + 4°C x (C+G)

9.1.1 Assays for SYBR Green detection

Primer design can be circumvented by using bioinformatically validated primer sets, such as QuantiTect Primer Assays, which are ready to use in SYBR Green-based real-time RT-PCR on any real-time cycler. Each assay, comprising 2 gene-specific primers as a lyophilized 10x mix, provides high PCR efficiency and accurate quantification of as few as 10 copies of template when used in combination with QuantiTect, QuantiFast, or FastLane® Kits for SYBR Green-based real-time RT-PCR. Assays are available for any gene from human, mouse, rat, and many other species, and can be easily ordered online at www.qiagen.com/GeneGlobe (Figure 31). The assays have been optimized and validated to provide maximum sensitivity and a wide dynamic range (Figures 32–34; for more data, visit www.qiagen.com/SYBRGreen).

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 51, page 53). This prevents coamplification of genomic DNA, which can compromise assay sensitivity and efficiency by competition between the desired PCR product and the product derived from genomic DNA.

A GeneGlobe search page

B Assay details page

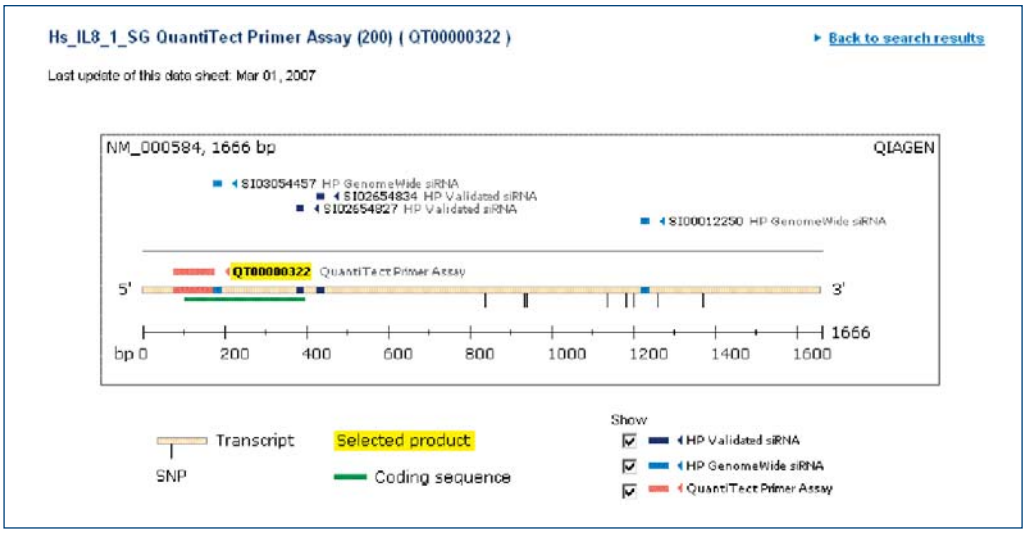


Figure 31. Easy ordering of assays at GeneGlobe®. A Visit the GeneGlobe Web portal (www.qiagen.com/GeneGlobe), enter the gene ID and species, and start the search. B After GeneGlobe finds the relevant assay, you can click on a link to view details such as the position of the assay primers and amplicon size. (GeneGlobe can also search for assays for multiple genes. Simply enter search terms into a single field or upload a list of search terms, and start the search.)

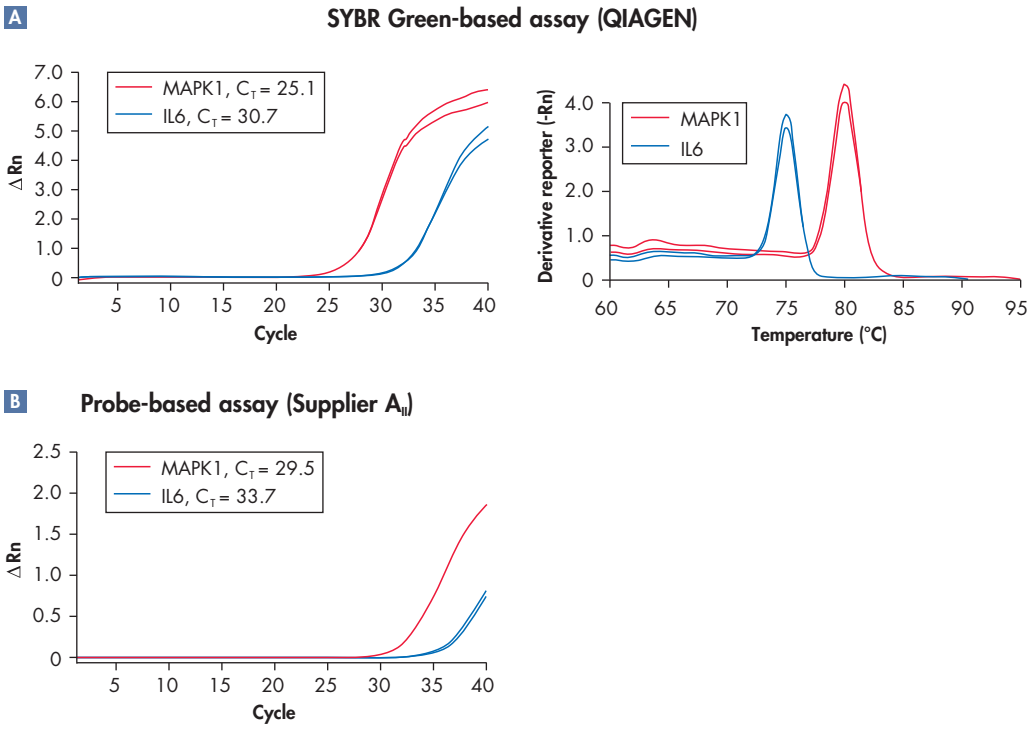


Figure 32. Superior sensitivity in real-time RT-PCR on the StepOnePlus™. MAPK1 (a protein kinase) and IL6 (a cytokine) in human leukocyte cDNA (1 ng) were quantified in duplicate using A QuantiTect Primer Assays and the QuantiTect SYBR Green PCR Kit, or B probe-based assays and a real-time PCR kit from Supplier A$_{II}$. QuantiTect Primer Assays provided greater sensitivity than the probe-based assays, as demonstrated by the lower C_T values, as well as high specificity, as indicated by the single peaks in melting curve analysis.

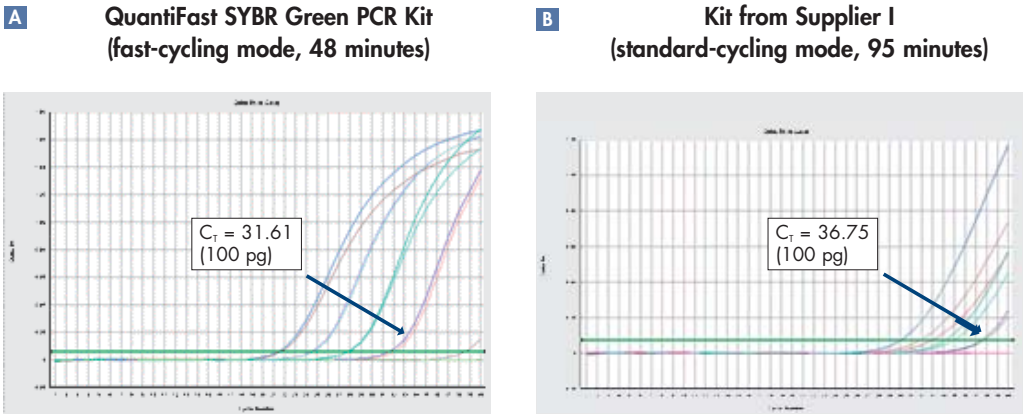


Figure 33. Sensitive detection of low copy numbers in fast real-time RT-PCR. Tenfold serial dilutions of human leukocyte cDNA (100 ng to 100 pg) were analyzed in duplicate on the Applied Biosystems 7500 Fast System using the QuantiTect Primer Assay for BCL2 and the indicated kits. A The QuantiFast SYBR Green PCR Kit provided significantly more sensitive detection of low copy numbers in half the time than B the kit from Supplier I.

Application data

High PCR efficiency for reliable relative quantification

QuantiTect Primer Assays provide highly efficient amplification in SYBR Green-based real-time RT-PCR. This enabled reliable relative quantification of Nos2 and Il1b expression in cultured rat aorta cells by the $\Delta\Delta C_T$ method.

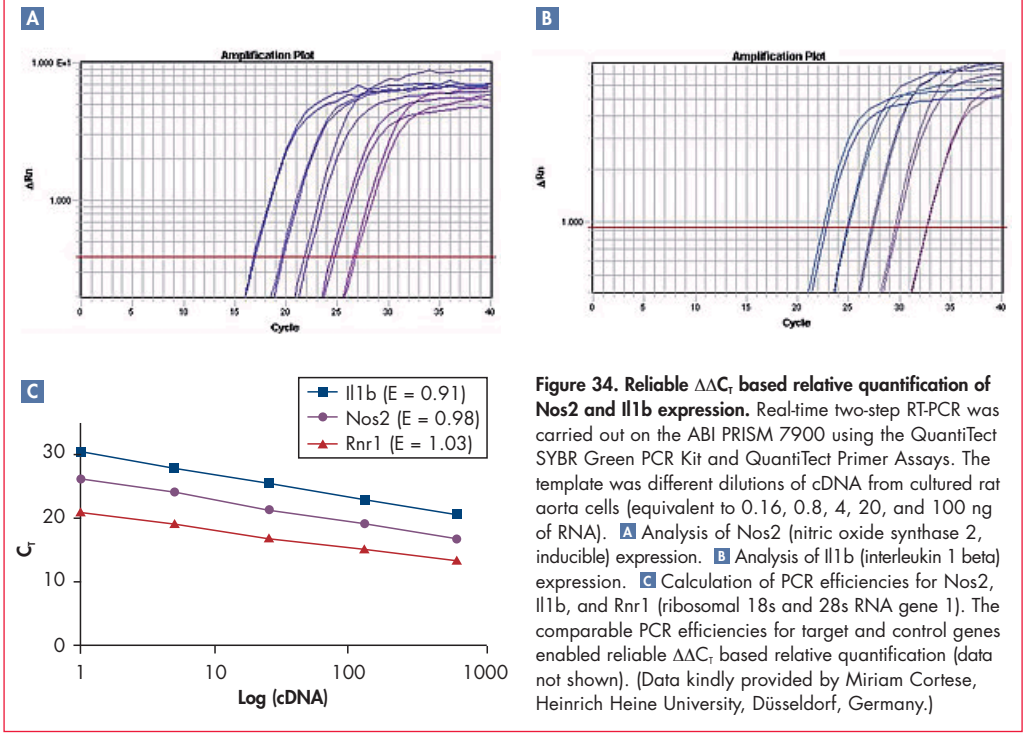


Figure 34. Reliable $\Delta\Delta C_T$ based relative quantification of Nos2 and Il1b expression. Real-time two-step RT-PCR was carried out on the ABI PRISM 7900 using the QuantiTect SYBR Green PCR Kit and QuantiTect Primer Assays. The template was different dilutions of cDNA from cultured rat aorta cells (equivalent to 0.16, 0.8, 4, 20, and 100 ng of RNA). A Analysis of Nos2 (nitric oxide synthase 2, inducible) expression. B Analysis of Il1b (interleukin 1 beta) expression. C Calculation of PCR efficiencies for Nos2, Il1b, and Rnr1 (ribosomal 18s and 28s RNA gene 1). The comparable PCR efficiencies for target and control genes enabled reliable $\Delta\Delta C_T$ based relative quantification (data not shown). (Data kindly provided by Miriam Cortese, Heinrich Heine University, Düsseldorf, Germany.)

? Can I use QuantiTect Primer Assays for validation of siRNA experiments?

Yes. We have seen that 24 hours or later after transfection of cells with siRNA from QIAGEN, the reduction in transcript level could be reliably detected with QuantiTect Primer Assays, both with assays encompassing the cleavage site as well as with assays located up- and downstream of it. For more information, read *QIAGEN News* weekly article **e14**, September 2006 at www.qiagen.com/literature/qiagennews.

? Can I use QuantiTect Primer Assays and assays using TaqMan probes in the same run?

Yes, this may be possible when using QuantiFast Kits, as the kits allow a combined annealing/extension step for both types of assay. Please follow the cycling recommendations in the handbook supplied with the QuantiFast SYBR Green Kit you are using.

However, if using QuantiTect Kits, QuantiTect Primer Assays and TaqMan assays should be run separately. With QuantiTect Kits, QuantiTect Primer Assays are used in a 3-step cycling protocol with data acquisition during the extension step, whereas TaqMan probes require a 2-step cycling protocol with data acquisition during the combined annealing/extension step.

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

The master mix supplied with QuantiTect or QuantiFast SYBR Green Kits can be mixed with primers and stored for more than 4 weeks at –20°C or 4°C. If carrying out real-time one-step RT-PCR, we do not recommend storing the primers, master mix, and RT mix as a single solution since reverse transcriptases are sensitive to freezing in an aqueous environment.

Can I use QuantiTect Primer Assays to detect DNA?

No. The assays are designed to detect RNA where possible.

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

Make sure that primers and amplification reagents are stored correctly. Avoid multiple freeze–thaw cycles for primers. 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.

Can I use QuantiTect Primer Assays in fast real-time PCR?

Yes. QuantiTect Primer Assays are fully compatible with QuantiFast SYBR Green Kits, which enable fast cycling in SYBR Green-based real-time PCR and RT-PCR. The fast two-step cycling (i.e., denaturation followed by combined annealing/extension) with QuantiFast SYBR Green Kits provides the same sensitivity and specificity in real-time analysis as three-step cycling (i.e., denaturation, annealing, and extension) with QuantiTect SYBR Green Kits. The differences in annealing temperature between QuantiFast Kits and QuantiTect Kits are due to differences in buffer composition.

9.2 Reaction chemistry

9.2.1 Effect of cations 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 35). 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 $(NH_4)_2SO_4$ or NH_4Cl in the buffer promotes specific primer annealing without any need for optimization of Mg^{2+} concentration, maximizing yields of specific PCR product (Figures 35 and 36).

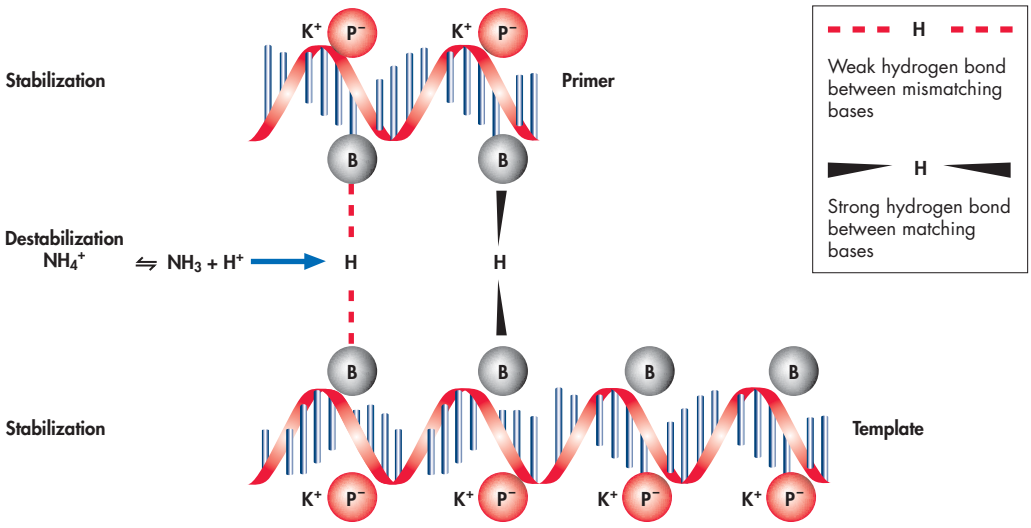


Figure 35. NH_4^+ and K^+ cations in QIAGEN PCR Buffer increase specific primer annealing. K^+ binds to the phosphate groups (**P**) on the DNA backbone, stabilizing the annealing of the primers to the template. NH_4^+, which exists both as the ammonium ion and as ammonia under thermal-cycling conditions, can interact with the hydrogen bonds between the bases (**B**), destabilizing principally the weak hydrogen bonds at mismatched bases. The combined effect of the 2 cations maintains the high ratio of specific to nonspecific primer–template binding over a wide temperature range.

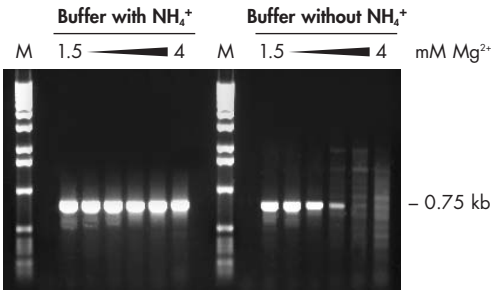


Figure 36. Specific primer annealing over a range of Mg^{2+} concentrations. PCR amplification of the single-copy human prion protein gene was carried out at increasing Mg^{2+} concentrations from 1.5 mM to 4 mM. PCR was performed using either QIAGEN PCR Buffer and QIAGEN *Taq* DNA Polymerase (**Buffer with NH_4^+**), or PCR buffer and *Taq* DNA polymerase from Supplier P_{II} (**Buffer without NH_4^+**). **M**: markers.

9.2.2 Effect of a hot start on real-time PCR specificity

Real-time PCR specificity can also be increased by using a hot start, where an inactive DNA polymerase is activated at the start of PCR by incubation at a high temperature. A hot start increases PCR specificity because it prevents the formation of primer–dimers and nonspecific products during reaction setup and the initial heating step. After a hot start, these PCR artifacts are also absent in every PCR cycle. HotStarTaq DNA Polymerase and HotStarTaq *Plus* DNA Polymerase, which are modified forms of QIAGEN *Taq* DNA Polymerase, provide a stringent hot start. This hot start, in combination with unique QIAGEN PCR Buffer, enables high specificity in PCR (Figure 37). A hot start also allows reverse transcription and PCR to be performed sequentially in one-step RT-PCR, as the *Taq* DNA polymerase remains inactive during the RT step until activated at the start of PCR.

QIAGEN offers a wide range of reaction chemistries for real-time PCR, two-step RT-PCR, and one-step RT-PCR with SYBR Green or probe detection: QuantiTect Kits, which include chemistries for multiplex analysis, and QuantiFast Kits, which provide fast cycling on any cycler (to see the full range of kits, refer to the selection guide on page 62). All QuantiTect Kits contain an application-specific buffer based on QIAGEN PCR Buffer and HotStarTaq DNA Polymerase. All QuantiFast Kits contain an adapted QIAGEN PCR Buffer for fast cycling as well as HotStarTaq *Plus* DNA Polymerase for a fast hot start.

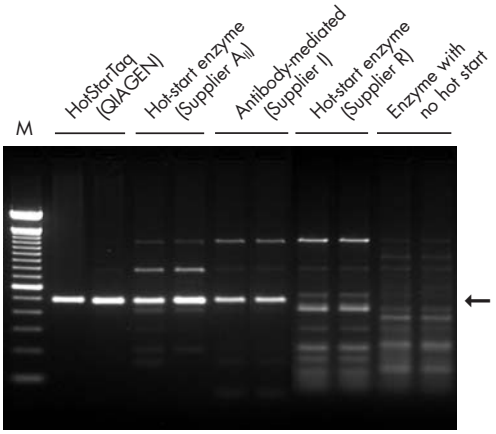


Figure 37. Different hot-start methods. A 497-bp fragment was amplified from 50 copies of an HIV-pol-gene construct which had been added to 1 µg human genomic DNA. Different hot-start enzymes were employed: HotStarTaq DNA Polymerase from QIAGEN; hot-start enzymes from Suppliers A_{II} and R; *Taq*–antibody 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.

9.3 SYBR Green-based detection in real-time PCR

Reduced specificity is often a major problem when using SYBR Green for quantitative, real-time PCR and RT-PCR. In comparison with kits from other suppliers, QuantiTect and QuantiFast Kits for SYBR Green detection show improved PCR specificity when using SYBR Green (Figure 38). ▶

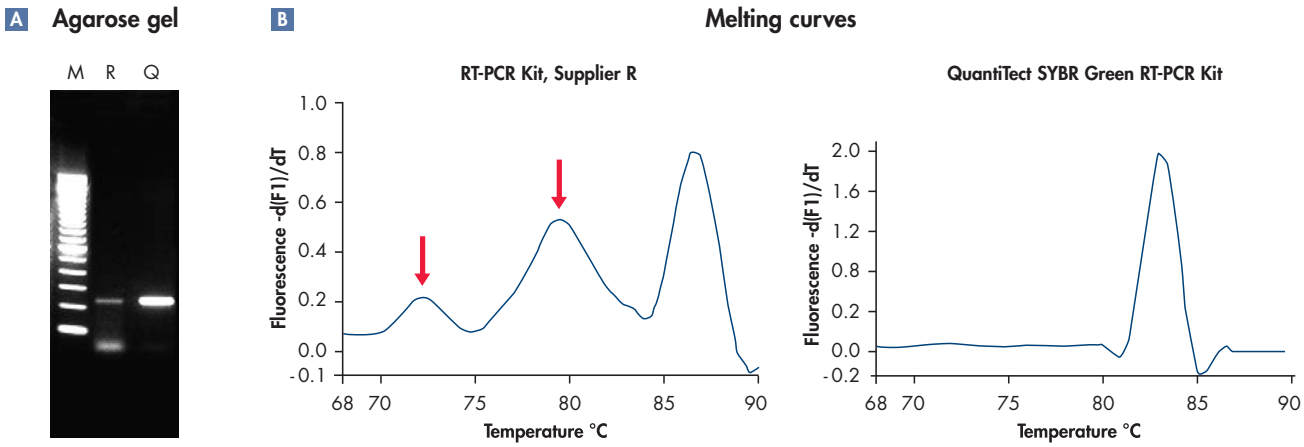


Figure 38. Highly specific real-time RT-PCR. RT-PCR products were amplified from the β-actin transcript in 1 ng HeLa cell RNA on the LightCycler using an RT-PCR kit from Supplier R (**R**) or the QuantiTect SYBR Green RT-PCR Kit (**Q**). **A** Agarose gel electrophoresis and **B** melting curve analysis of the reactions showed a specific band and a single peak with the QuantiTect Kit, indicating high specificity. In contrast, the kit from Supplier R gave a faint specific band and additional nonspecific bands as well as several peaks, demonstrating low specificity. **M**: markers.

Low sensitivity can be a problem when using SYBR Green 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 templates is therefore often unreliable. QuantiTect and QuantiFast SYBR Green Kits provide highly specific amplification, allowing accurate quantification of just a few copies of template (Figure 39). Specific and sensitive quantification is achieved with QuantiTect and QuantiFast SYBR Green Kits, whatever the cycler used.

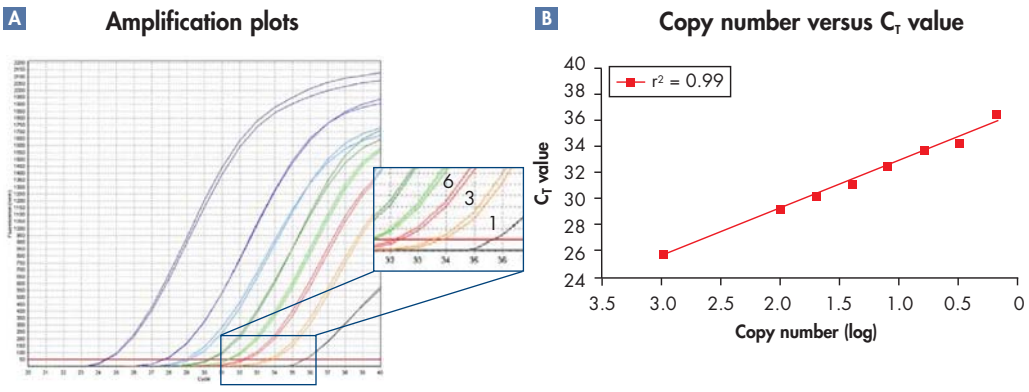


Figure 39. Resolution of small differences in copy number. The QuantiFast SYBR Green PCR Kit and the Mastercycler® ep *realplex* were used to detect the Y-chromosome-specific single-copy gene SRY in genomic DNA from a male donor. **A** Curves for 1000 down to 1 copy can be clearly distinguished from each other. **B** A plot of copy number (log) versus C_T value demonstrates high linearity.

Visit www.qiagen.com/SYBRGreen to view application data for QuantiTect and QuantiFast SYBR Green Kits.

9.4 Probe-based detection in real-time PCR

Sequence-specific probes provide a highly sensitive method of detection in real-time PCR and RT-PCR. In contrast to detection with SYBR Green, primer–dimers and other nonspecific PCR products do not contribute to the fluorescent signal when probes are used for detection. 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 a low level may not be detected. The amplification efficiency (see section 5.3.1, page 18) is lower, and is often indicated by nonuniform C_T distances between dilutions of the template.

Improved PCR specificity leads to higher PCR efficiency, sensitivity, and dynamic range (Figures 40 and 41). When expression levels of the human cytokine gene IL1RN were analyzed, the QuantiFast Probe PCR Kit enabled sensitive detection from just 10 pg of cDNA template (Figure 40A). In contrast, the kit from Supplier A_{II} allowed detection from 100 pg of cDNA template (Figure 40B). The increased sensitivity of the QuantiFast Probe PCR Kit is also reflected by its lower C_T values. At the highest template amount (100 ng), human IL1RN could be detected 2 cycles earlier with the QuantiFast Probe PCR Kit, increasing the dynamic range of the reaction. The C_T values obtained with the QuantiFast Probe PCR Kit were evenly spaced over the whole 10-fold dilution series.

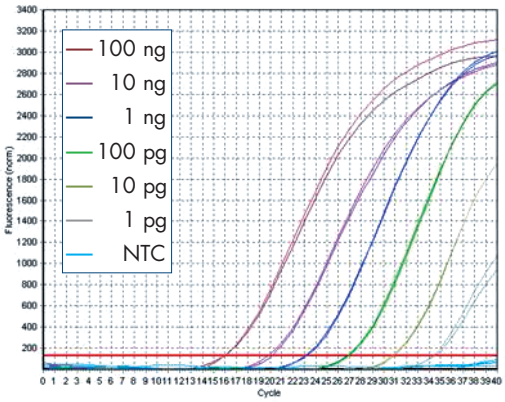
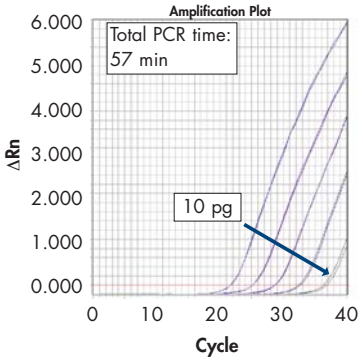


Figure 41. Wide dynamic range and high sensitivity in two-step RT-PCR. Duplicate reactions were performed on the Mastercycler ep *realplex* using 10-fold dilutions of human leukocyte cDNA and a Primer Express designed TaqMan assay for ubiquitin (a regulatory protein). The QuantiFast Probe PCR +ROX Vial Kit provided accurate gene expression analysis from low to high template amounts with a PCR efficiency of 93%. **NTC**: no template control.

A **QuantiFast Probe PCR Kit (IL1RN transcript)**



B **Kit from Supplier A_{II} (IL1RN transcript)**

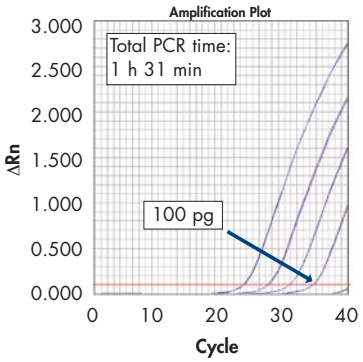


Figure 40. Greater sensitivity in two-step RT-PCR. Reactions were run in duplicate on the ABI PRISM 7900 using 10-fold dilutions of human leukocyte cDNA (100 ng to 0.01 ng) and a TaqMan gene expression assay for IL1RN (a cytokine). The QuantiFast Kit showed greater sensitivity than the standard-cycling kit from Supplier A_{II} (which was used according to the standard-cycling protocol), providing much lower C_T values and transcript quantification from down to 10 pg cDNA.

Visit www.qiagen.com/fastPCR to view more application data for QuantiFast Probe Kits.

9.5 Real-time PCR with fast cycling

Fast real-time PCR can be achieved by optimizing the reaction and cycling conditions of existing assays. Such optimization includes reducing the DNA polymerase activation time, shortening the duration of each PCR cycle, combining the annealing and extension steps, and shortening the RT step if carrying out one-step RT-PCR. However, changes to these parameters often reduce PCR sensitivity and increase the variability of quantification data.

QuantiFast Kits are supplied with a specially developed PCR buffer that allows a rapid hot start as well as a significant reduction of PCR cycling times. HotStarTaq *Plus* DNA Polymerase, which is inactive at ambient temperature, requires only a brief 3- or 5-minute incubation at 95°C for activation. The enzyme helps to increase PCR specificity by ensuring that no nonspecific products are formed during reaction setup and the initial denaturation step. Reduced denaturation, annealing, and extension times are enabled by the patent-pending additive Q-Bond® and other components of the PCR buffer (Figure 42). High annealing specificity is maintained by a balanced combination of KCl and NH_4Cl in the buffer (see section 9.2.1, page 42). For one-step RT-PCR, an optimized mix of reverse transcriptases enables cDNA synthesis in just 10 minutes. Fast real-time PCR is achieved without compromising specificity and sensitivity (Figures 43–46 and Table 14; for more data, visit www.qiagen.com/fastPCR).

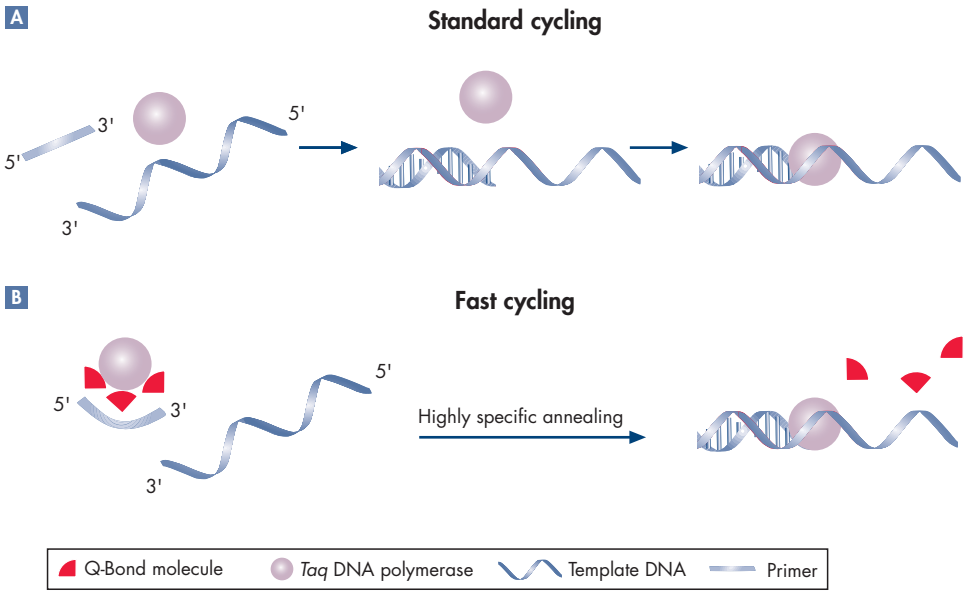


Figure 42. Fast primer annealing. Q-Bond dramatically increases the binding affinity of DNA polymerase to single-stranded DNA, enabling faster annealing and extension. A Under standard-cycling conditions, denaturation, annealing, and extension usually occur as a 3-step process where the template is first denatured, followed by primer annealing to form a binary complex and then polymerase binding to form a tertiary complex. B With Q-Bond in the reaction, this 3-step process becomes a faster 2-step process, where the template is first denatured and the tertiary complex is then formed by the simultaneous binding of primer and polymerase, allowing extension to start within seconds. Other components of the buffer support the melting of double-stranded DNA, allowing reduced denaturation and extension times.

A QuantiFast SYBR Green PCR Kit (fast-cycling mode)

B QuantiTect SYBR Green PCR Kit (standard-cycling mode)

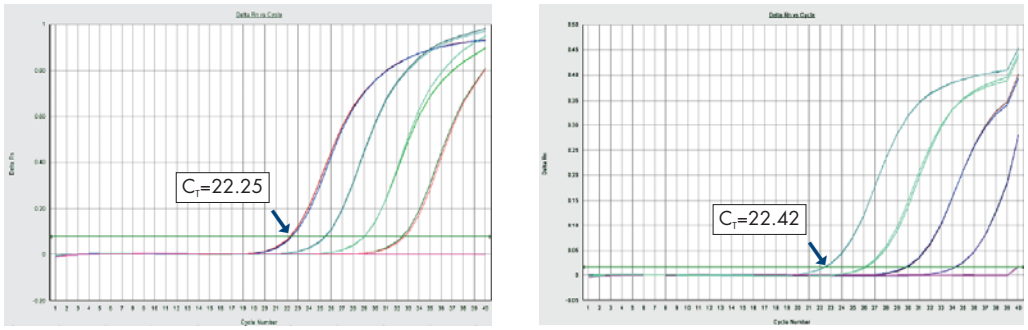


Figure 43. Faster results without compromising sensitivity. Expression of MYC (a proto-oncogene) in human leukocytes was analyzed by real-time two-step RT-PCR. Duplicate reactions were run on the Applied Biosystems 7500 Fast System using10-fold cDNA dilutions (10 ng to 10 pg). **A** Fast-cycling mode with the QuantiFast Kit gave similar C_T values to **B** standard-cycling mode with the QuantiTect Kit.

Application data

High sensitivity in fast real-time RT-PCR

Performing real-time RT-PCR under fast-cycling conditions can reduce the sensitivity of transcript detection. The QuantiFast Probe PCR Kit enabled fast real-time RT-PCR with much higher sensitivity than a kit from another supplier.

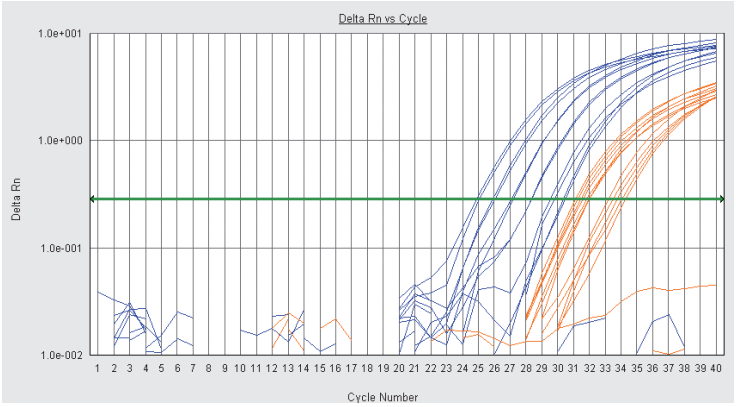


Figure 44. Fast cycling using TaqMan assays without compromising performance. Real-time PCR was carried out on the ABI PRISM 7000 under fast-cycling conditions using the QuantiFast Probe PCR Kit (blue curves) or a kit from Supplier E_{II} (orange curves). The template was twofold serial dilutions of human skeletal muscle cDNA (100 ng to 3.13 ng). Reactions were run in duplicate using a TaqMan assay for human NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 [p105]). (Data kindly provided by Despina Constantin and Tim Constantin, University of Nottingham Medical School, Nottingham, United Kingdom.)

Application data

Sensitive parasite detection

The formation of nonspecific products in SYBR Green-based real-time PCR reduces PCR specificity and sensitivity. The QuantiFast SYBR Green PCR Kit overcomes this problem with a unique PCR buffer. Two parasite DNA targets were amplified with high PCR efficiency and were sensitively detected.

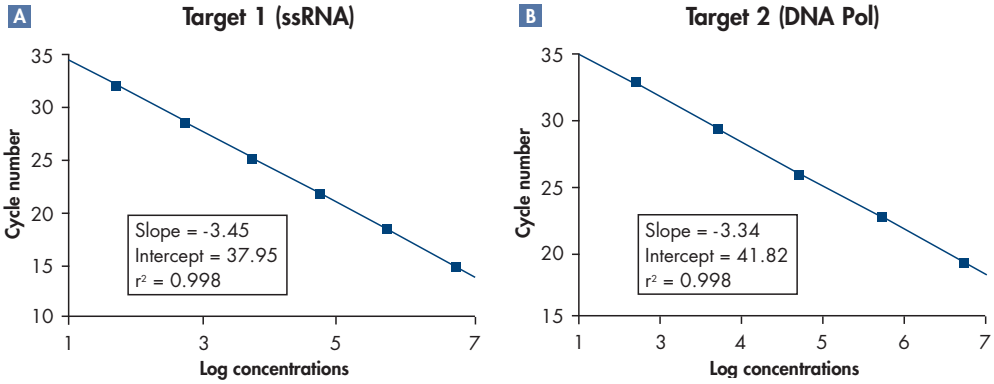


Figure 45. High PCR efficiences in parasite detection. Fast real-time PCR with SYBR Green detection was carried out on the LightCycler 1.5 using the QuantiFast SYBR Green PCR Kit. The template was 6 or 5 dilutions of parasite genomic DNA from 5 ng to 0.5 pg or 0.05 pg. Gene-specific primers for ssrRNA (5S ribosomal RNA) and DNA Pol (DNA polymerase) were used. The standard curves show that the QuantiFast SYBR Green PCR Kit enabled high PCR efficiencies of 95% (for ssrRNA) and 99% (for DNA Pol). (Data kindly provided by Eric Prina, Department of Parasitology and Mycology, Institut Pasteur, Paris, France.)

Application data

Fast, efficient real-time PCR on any cycler

Fast real-time PCR with SYBR Green detection was carried out on various cyclers using the QuantiFast SYBR Green PCR Kit and primers specific for RNase P. A high PCR efficiency of around 100% was achieved with all cyclers tested.

Table 14. Fast real-time PCR with high efficiency on various different cyclers

	Copy number							
Cycler	14,300	1430	143	14.3	1.43	Slope	Efficiency	R^2
Applied Biosystems 7500 Fast	22.66	25.46	28.81	32.4	35.75	–3.31	101%	1.00
iQ5	20.98	23.98	27.31	30.02	34.05	–3.219	104%	0.99
LightCycler 1.2	–	21.54	24.52	27.97	31.36	–3.349	99%	0.99
Mx3000®	20.31	23.28	26.78	30.23	33.41	–3.314	100%	0.99

The template was five 10-fold serial dilutions of human genomic DNA, each analyzed in triplicate (Data kindly provided by Karen McCaustland, Centers for Disease Control and Prevention [CDC], Atlanta, GA, USA. Presentation of these data does not imply endorsement by the CDC or any CDC employee.)

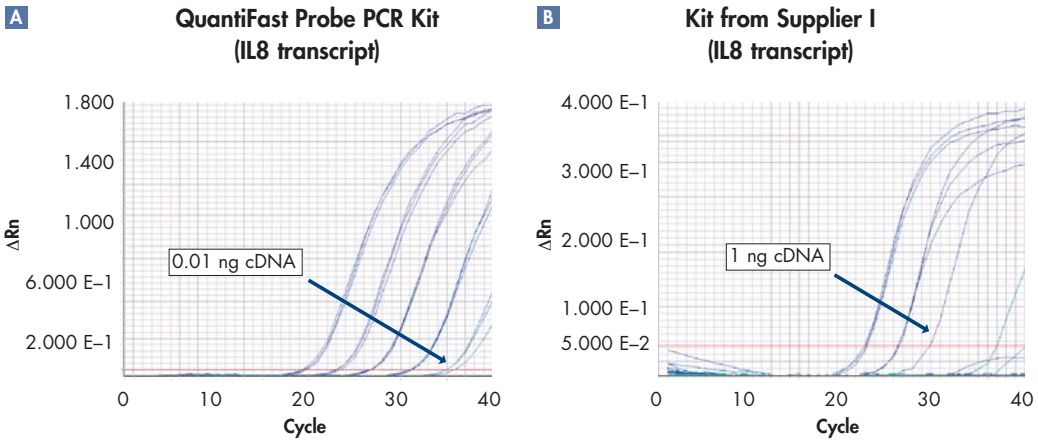


Figure 46. Sensitive two-step RT-PCR. Reactions were run in triplicate on the ABI PRISM 7900 using 10-fold dilutions of human leukocyte cDNA (100 ng to 0.01 ng) and a Primer Express designed TaqMan assay for IL8 (a chemokine). The QuantiFast Kit showed greater sensitivity than the kit from Supplier I (which was used according to the standard-cycling protocol), providing lower C_T values and transcript quantification from down to 0.01 ng cDNA.

9.6 Real-time RT-PCR direct from cell lysates

The ability to use cell lysates directly as template in real-time RT-PCR significantly streamlines the gene expression analysis workflow, especially for high-throughput analyses. However, several important factors need to be considered. First of all, RNA in the cell lysate must be protected from degradation, and the upregulation and downregulation of mRNA transcripts immediately after cell lysis must be prevented. Secondly, the use of cell lysate as template in real-time two-step or one-step RT-PCR must not compromise PCR specificity and sensitivity.

FastLane Kits are supplied with a lysis buffer that provides cell lysis with RNA stabilization. Transcript representation is preserved to ensure accurate results in gene expression analysis. The cell lysates also undergo a short reaction to remove genomic DNA. This eliminates genomic DNA contamination in subsequent real-time RT-PCR and avoids the need to specially design primers or probes to prevent coamplification of genomic DNA sequences. The cell lysates are prepared in only 12 minutes, and can be used directly in real-time two-step or one-step RT-PCR using QuantiTect or QuantiFast technology without compromising performance (Figures 47 and 48).

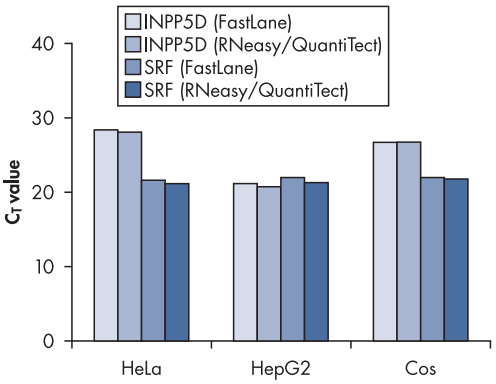


Figure 47. Sensitive detection without RNA purification. Two low-abundance transcripts, INPP5D (a phosphatase) and SRF (serum response factor), were quantified in 3 different cell lines by real-time one-step RT-PCR. The FastLane Cell SYBR Green Kit provided C_T values comparable to those achieved with the RNeasy Mini Kit and QuantiTect SYBR Green RT-PCR Kit.

Application data

High-throughput screening of gene expression

Analysis of gene expression in multiple cell samples can be cumbersome due to the need to purify RNA from many samples. The FastLane Cell Multiplex Kit allows multiplex, real-time one-step RT-PCR using cell lysates, and provided similar results as multiplex, real-time two-step RT-PCR in the analysis of forskolin inhibition of SOST expression in UMR-106 cells.

A

Assay method	Forskolin inhibition (IC_{50})
Traditional method	557 ± 205 nM (CV = 0.37)*
FastLane Cell Multiplex Kit	564 ± 217 nM (CV = 0.38)*

* From 2 independent experiments, each performed in duplicate.

B

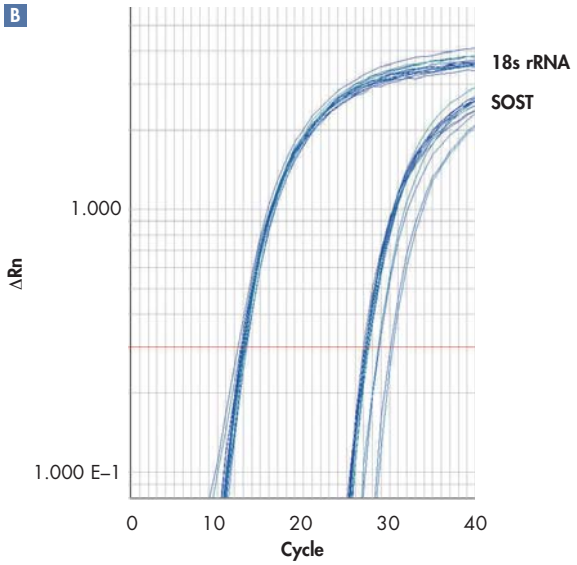


Figure 48. Streamlined high-throughput screening without compromising performance. UMR-106 cells were treated with four 10-fold dilutions of forskolin (10 µM to 0.01 µM). Relative quantification of SOST expression was then carried out on the ABI PRISM 7900 using TaqMan assays for SOST and 18S rRNA (18S rRNA was used to normalize SOST expression). IC_{50} values for forskolin inhibition were determined for assays done using either the traditional method (RNA purification followed by cDNA synthesis and duplex, real-time PCR) or the FastLane Cell Multiplex Kit (duplex, real-time one-step RT-PCR using cell lysates). Amplification plots from multiplex reactions with the FastLane Cell Multiplex Kit are shown here for 1 experiment. (Data kindly provided by Angela Furrer and Hansjoerg Keller, Bone & Cartilage Unit, Musculoskeletal Disease Area, Novartis Institutes for BioMedical Research, Basel, Switzerland.)

? How many copies of template can I detect?

This depends on the amplification reagents used and on successful primer design. With QuantiTect and QuantiFast Kits, <10 copies of template can be detected when the kits are used in combination with optimal primer–probe sets or primer sets, such as QuantiTect Primer Assays.

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

We recommend using a hot start to PCR to prevent primer–dimer formation. In addition, use a PCR buffer that promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. All QuantiTect and QuantiFast Kits provide a hot start and contain a PCR buffer specifically adapted for quantitative, real-time PCR and RT-PCR. 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 experiment (e.g., see Figure 38, page 44). 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 2 different targets with identical primer binding sites (e.g., 2 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 96-well plates) or 20 µl (for 384-well plates and LightCycler capillaries). However, volumes may be reduced to 20 µl and 10 µl, respectively. QuantiFast Kits for real-time PCR and RT-PCR have been optimized for a final reaction volume of 25 µl (for 96-well plates), 20 µl (for LightCycler capillaries), or 10 µl (for 384-well plates). We strongly recommend using the primer and probe concentrations, reaction volumes, and amount of starting template given in the QuantiTect and QuantiFast handbooks.

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

With QuantiTect and QuantiFast Kits, reaction plates can be stored for several hours at 4°C. To avoid bleaching of probes or SYBR Green, plates should be stored protected from light. Reactions set up using QuantiTect and QuantiFast PCR Kits can be stored overnight at –20°C. The effects 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 and QuantiFast 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 in quantitative PCR, the cycling program may be optionally followed by melting curve analysis according to the instructions supplied with the real-time cycler. 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 1 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 detection?

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

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

This phenomenon is sometimes observed when using nonhydrolyzable probes such as FRET probes. During the late phase of PCR, where many PCR products have been generated, there is strong competition between hybridization of the probe to the target strand and reassociation ▶

of the 2 complementary product strands. For some primer–probe combinations, reassociation occurs more quickly than probe hybridization toward the end of the PCR. Therefore, the yield of PCR product seems to decrease (Figure 49). 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.

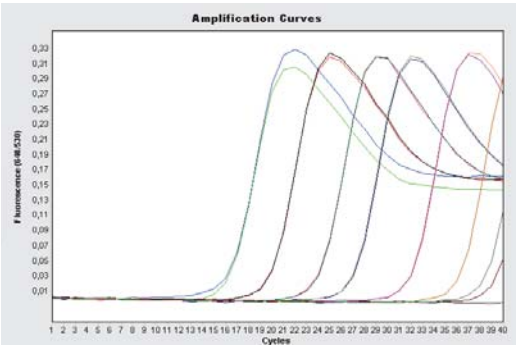


Figure 49. Hook-shaped amplification plot. Amplification plot showing apparent decrease in yield of PCR product toward the end of PCR. This phenomenon is typical for LightCycler instruments.

The concentration of ROX dye in QuantiTect Probe Kits is too high for "Auto C_T" analysis on my Applied Biosystems 7500. What should I do?

We recommend setting the threshold manually. For further details, refer to the user manual supplied with your instrument.

My Bio-Rad cycler (iCycler iQ®, iQ5, or MyiQ) requires use of fluorescein calibration dye. Can I still use QuantiTect and QuantiFast Kits?

Yes, we recommend collecting external well factors with an external well factor plate containing fluorescein solution before starting your real-time PCR run. Our studies show that collecting external factors provides more reliable data with QuantiTect and QuantiFast Kits than collecting dynamic well factors (which is achieved by spiking fluorescein into the PCR mix). In addition, collecting well factors is more convenient as the external well factor plate can be stored long-term and reused many times. Details on collecting well factors can be found in the instructions supplied with your Bio-Rad cycler, and also in our Technical Information (www.qiagen.com/goto/WellFactors).

9.7 Genomic DNA in real-time RT-PCR

Contaminating genomic DNA in RNA samples may also be detected in real-time RT-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 primer or 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.

9.7.1 Eliminating genomic DNA contamination

As an alternative to DNase I digestion, RNA can be purified using RNeasy Plus Kits, which include gDNA Eliminator columns, or cDNA can be synthesized using the QuantiTect Reverse Transcription Kit, which includes integrated removal of genomic DNA (Figure 50). 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 51).

9.7.2 Designing primers to avoid detection of genomic DNA

The primer or probe can be designed so that one half hybridizes to the 3' end of one exon and the other half to the 5' end of the adjacent exon. The primer or probe will therefore anneal to cDNA synthesized from spliced mRNAs, but not to genomic DNA, eliminating detection of contaminating DNA. Alternatively, RT-PCR primers can be designed to flank a region that contains at least 1 intron. Products amplified from cDNA (no introns) will be smaller than those amplified from genomic DNA (containing introns). If possible, a target with very long introns should be selected: the RNA target may then be preferentially amplified because of the higher PCR efficiency of this shorter PCR product without introns. If genomic DNA is detected (i.e., presence of amplification product in "No RT" control), the template RNA should be treated with RNase-free DNase. Alternatively, the primers should be redesigned to avoid amplification of genomic DNA.

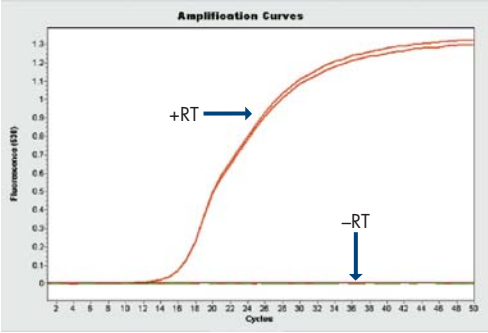


Figure 50. Effective genomic DNA removal for accurate real-time RT-PCR. Real-time two-step RT-PCR using the QuantiTect Reverse Transcription Kit in combination with the QuantiTect SYBR Green PCR Kit was carried out with (**+RT**) or without (**–RT**) reverse transcriptase. β-actin-specific primers designed to detect both mRNA and genomic DNA sequences were used. Reactions were run in duplicate on the LightCycler 2.0. Control reactions with no template were also performed (green). The red, flat **–RT** plot indicates efficient removal of residual genomic DNA.

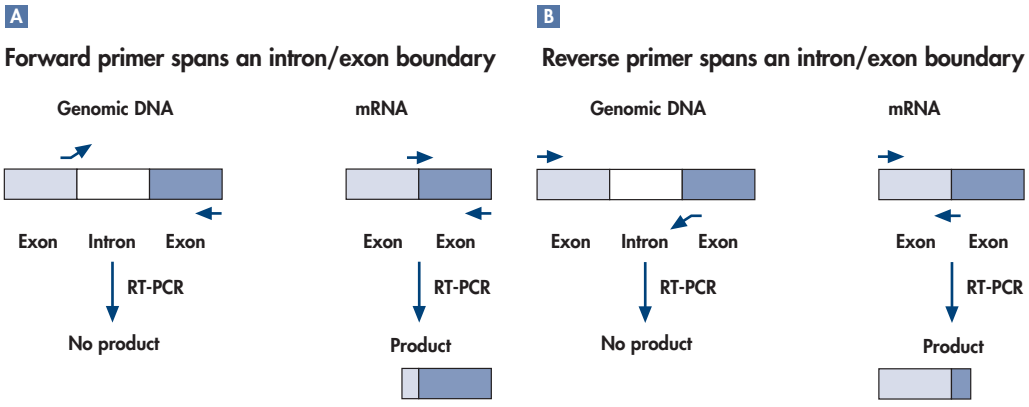


Figure 51. No coamplification of genomic DNA. Primer design to eliminate signals from contaminating genomic DNA. A Forward primer crosses an intron/exon boundary. B Reverse primer crosses an intron/exon boundary.

10. Guidelines for successful multiplex real-time PCR

Optimizing the conditions for multiplex, real-time PCR, two-step RT-PCR, and one-step RT-PCR can be tedious and time-consuming (Figure 52). Several factors need to be considered, including the concentrations of the primers, Mg^{2+}, *Taq* DNA polymerase, and dNTPs as well as the composition of the PCR buffer. In addition, care must be taken to design optimal primers and probes and to choose appropriate reporter dyes and quenchers. If multiplex, real-time PCR under fast-cycling conditions is desired, shortening cycling times while maintaining reliable amplification of multiple targets is also a challenge.

10.1 Guidelines for designing primers and probes

- Try to keep the size of the amplicons as small as possible, ideally 60–150 bp.
- Follow the guidelines for good assay design:
  - Use specialized design software to design primers and probes.
  - All assays should be designed using the same settings to ensure that they will work optimally under the same cycling conditions.
- Check the specificity of your primers by performing a BLAST® search (www.ncbi.nlm.nih.gov/blast). Ensure that primer sequences are unique for your template sequence.
- For two-step and one-step RT-PCR assays, in which detection of genomic DNA must be avoided, design primers or probes so that one half anneals to the 3' end of one exon and the other half to the 5' end of the adjacent exon (see Figure 51, page 53).

10.2 Guidelines for selecting appropriate reporter dyes and quenchers for the probes

- For accurate detection of the different targets in a real-time, multiplex PCR assay, it is essential that the sequence-specific probes are labeled with reporter dyes whose fluorescence spectra are well separated or exhibit only minimal overlap. See Table 1 (page 7) for reporter dyes commonly used in real-time, multiplex PCR.
- Choose suitable combinations of reporter dyes and quenchers that are compatible with multiplex analysis using the detection optics of your real-time cycler (for details, refer to the instrument user manual; for examples, see Figures 54 and 55). For details on reporter dyes tested and recommended by QIAGEN, see Table 15.
- Use nonfluorescent quenchers (e.g., use BHQ instead of TAMRA). Triplex and 4-plex analysis may only be possible using nonfluorescent quenchers.

Table 15. Combinations of reporter dyes for multiplex assays using QuantiFast and QuantiTect Multiplex Kits

Cycler	Reference dye	Dye 1†	Dye 2†	Dye 3†	Dye 4†
ABI PRISM 7700	ROX	FAM	HEX, JOE, VIC	—	—
ABI PRISM 7000 and 7900, Applied Biosystems 7300, StepOnePlus	ROX	FAM	HEX, JOE, VIC	Bodipy TMR, NED	—
Applied Biosystems 7500	ROX	FAM	HEX, JOE, VIC	Bodipy TMR, NED	Alexa Fluor 647, Cy5
iCycler iQ and iQ5	Not required	FAM	HEX, JOE, TET, VIC	Texas Red, ROX	Cy5
LightCycler 2.0	Not required	FAM	HEX, JOE, VIC	Texas Red, ROX	Alexa Fluor 660, Bodipy 630/650, Pulsar® 650
Mx3000P®, Mx3005P®	Not required	FAM	HEX, JOE, VIC	Texas Red, ROX	Cy5
Rotor-Gene 6000	Not required	FAM	HEX, VIC	ROX	Quasar® 705

* Visit www.qiagen.com/multiplex to view dye combinations for other cyclers, including dye combinations for 5-plex PCR.

† Preferably, select Dye 1 for the least abundant target, Dye 2 for the second least abundant target, and Dyes 3–4 for the most abundant targets.

10.3 Choice of PCR buffer

The amplification of multiple targets in the same reaction presents the challenge of ensuring that all targets are amplified with the same high efficiency. If the reaction contains targets that vary significantly in abundance, the more abundant targets may be amplified more efficiently than the less abundant targets, resulting in inaccurate quantification data. A typical strategy to overcome this problem is to determine the limiting primer concentration for the more abundant targets. However, this may require numerous optimization steps until the multiplex assay works satisfactorily (Figure 52).

QuantiTect Multiplex Kits provide a ready-to-run solution for multiplex, real-time PCR and RT-PCR. The supplied master mix, which contains novel QuantiTect Multiplex PCR Buffer and HotStarTaq DNA Polymerase, eliminates the need for optimization of reaction and cycling conditions, enabling successful results at the first attempt. All PCR products in a multiplex reaction are amplified with the same efficiency as the PCR products in the corresponding single-amplification reactions, resulting in comparable sensitivities. The master mix also provides high sensitivity and specificity, with detection of as little as 10 copies of each target.

QuantiFast Multiplex Kits combine fast-cycling, real-time PCR and RT-PCR with the same advantages as QuantiTect Multiplex Kits. A ready-to-use master mix containing QuantiFast Multiplex PCR Buffer and HotStarTaq *Plus* DNA Polymerase provides time savings of up to 50% in multiplex analysis on any real-time cycler, and allows reliable quantification of up to 4 targets per reaction without the need for PCR optimization. Reduced PCR run times are achieved through the use of HotStarTaq *Plus* DNA Polymerase and the PCR additive Q-Bond (see section 9.5, page 46). ▶

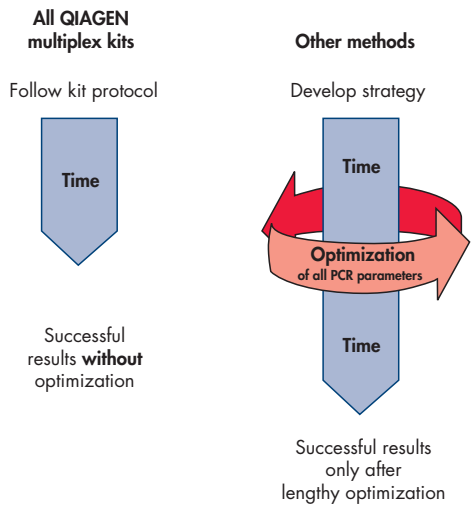


Figure 52. Prerequisites for success in real-time, multiplex PCR. Developing multiplex assays often requires lengthy optimization steps. With QuantiTect Multiplex Kits, these steps are avoided.

In addition to various salts and additives, QuantiFast and QuantiTect Multiplex PCR Buffers contain a specially optimized combination of K^+ and NH_4^+ ions, which promotes a high ratio of specific to nonspecific binding of primers and probes during each PCR annealing step (see Figure 35, page 42). With these buffers, primer and probe annealing is only marginally influenced by $MgCl_2$ concentration, so optimization by titration of Mg^{2+} is usually not required. The buffers also contain synthetic Factor MP, which facilitates multiplex PCR by increasing the local concentration of primers and probes at the template and stabilizing specifically bound primers and probes (Figure 53).

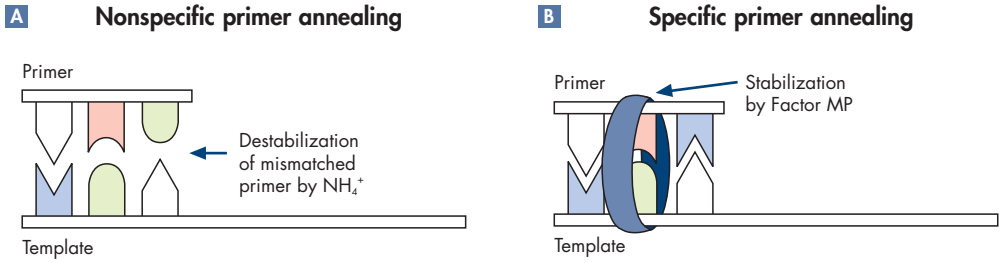


Figure 53. Unique buffer promotes stable and efficient annealing. A NH_4^+ ions prevent nonspecific primers and probes from annealing to the template. B Synthetic Factor MP, an innovative PCR additive, increases the local concentration of primers and probes at the template. Together with K^+ and other cations, synthetic Factor MP stabilizes specifically bound primers and probes, allowing efficient primer extension by HotStarTaq DNA Polymerase or HotStarTaq *Plus* DNA Polymerase.

HotStarTaq DNA Polymerase and HotStarTaq *Plus* DNA Polymerase are inactive at room temperature and become active only upon incubation at 95°C. This stringent hot start prevents the formation of misprimed products and primer–dimers during reaction setup and the first denaturation step. Competition for reactants by these PCR artifacts is therefore avoided (see section 9.2.2, page 43).

To suit the requirements of different real-time cyclers, QuantiTect Multiplex Kits are available with or without ROX dye (see selection guide, page 62). Kits supplied with a master mix containing ROX dye are intended for cyclers that require ROX dye for fluorescence normalization (e.g., instruments from Applied Biosystems). Kits supplied with a master mix free of ROX dye are recommended for all other cyclers, as they allow greater multiplexing through the use of probes labeled with ROX, Texas Red, or other equivalent dye. These include cyclers from Bio-Rad/MJ Research, Cepheid, Corbett/QIAGEN, Eppendorf, Roche, and Stratagene.

QuantiFast Multiplex Kits are also available in 2 formats (see selection guide, page 62). Kits supplied with a master mix containing ROX dye are optimized for use with all instruments from Applied Biosystems except the Applied Biosystems 7500. Kits supplied with a ROX-free master mix and a separate solution of ROX dye are intended for use with the Applied Biosystems 7500 and cyclers from Bio-Rad/MJ Research, Cepheid, Corbett/QIAGEN, Eppendorf, Roche, and Stratagene.

The protocols provided with QuantiFast and QuantiTect Multiplex Kits have been developed on a wide range of real-time cyclers by QIAGEN. Since all reaction and cycling parameters are already established, there is no need to perform optimization steps, such as determining limiting primer concentrations or adjusting cycling conditions. Data demonstrating the performance of QuantiFast and QuantiTect Multiplex Kits in duplex, triplex, and 4-plex PCR can be viewed at www.qiagen.com/multiplex.

10.4 Guidelines for evaluating the performance of a multiplex assay

- Check the functionality of each set of primers and probe in individual PCR assays before combining the different sets in a multiplex PCR assay.
- Compare the performance of the multiplex PCR assay with the corresponding singleplex PCR assays. Assay performance can be tested by, for example, assaying serial dilutions of a sample containing the target nucleic acids (e.g., see Figure 54). In addition, the dynamic range of the multiplex assay can be tested by, for example, making several dilutions of one target nucleic acid and keeping the concentration of the other target nucleic acid constant; as template, target nucleic acids cloned in a plasmid or prepared as a PCR product can be used.
- Check the multiplex PCR assay for linearity by performing reactions with 10-fold dilutions of template, and check whether the C_T values obtained are similar to those obtained with the corresponding singleplex PCR assays. A standard curve can be used to evaluate the linear range and the PCR efficiency of the assay (e.g., see Figures 55 and 56).

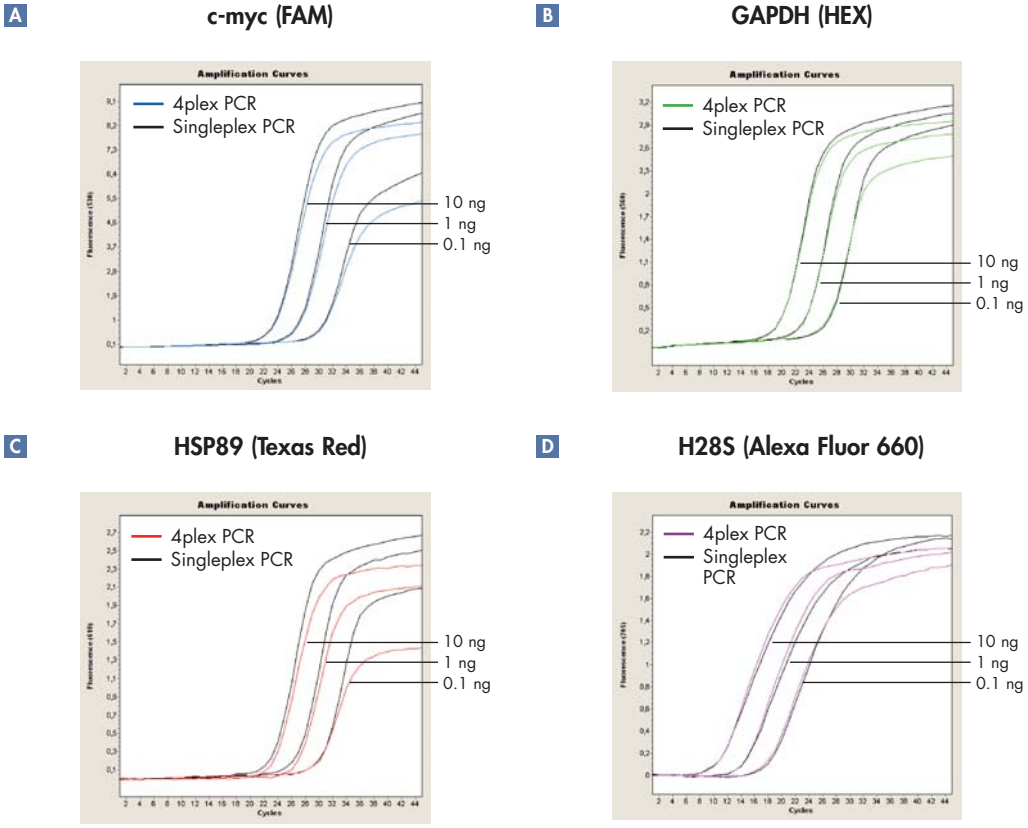


Figure 54. Comparable amplification in 4-plex PCR and singleplex PCRs. 4-plex, real-time one-step RT-PCR was performed on the LightCycler 2.0 using the QuantiTect Multiplex RT-PCR NR Kit and TaqMan probes. The template was 10, 1, or 0.1 ng of total RNA purified from K562 cells. A c-myc (a proto-oncogene) was detected using a FAM labeled probe. B GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was detected using a HEX labeled probe. C HSP89 (a heat shock protein) was detected using a Texas Red labeled probe. D H28S (28S rRNA) was detected using an Alexa Fluor 660 labeled probe. For comparison, the targets were also quantified by singleplex, real-time one-step RT-PCR (black curves). Curves for 4-plex and singleplex PCRs overlap, showing comparable amplification (i.e., equivalent C_T values).

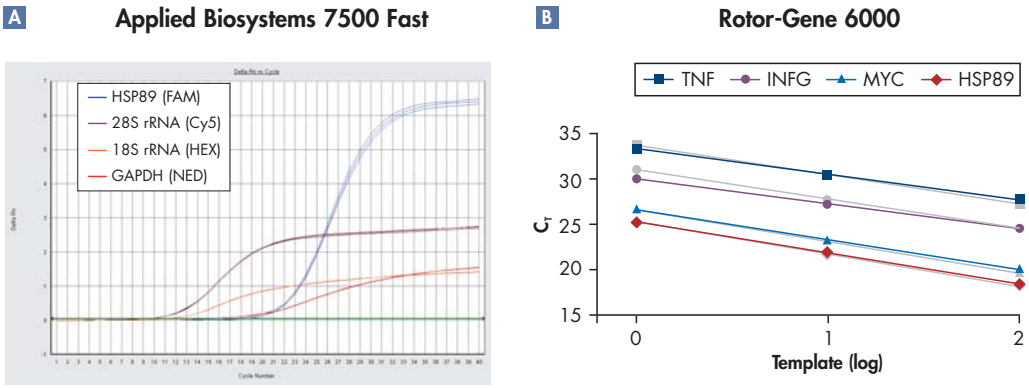


Figure 55. Uncompromised sensitivity on any cycler. 4-plex, real-time PCR was carried out using the QuantiFast Multiplex PCR +R Kit and Primer Express designed TaqMan assays. A Triplicate reactions run using 10 ng Ramos cell line cDNA B Duplicate reactions run using leukocyte cDNA (100, 10, or 1 ng). The graph of C_T values against cDNA amount shows that targets of greatly differing abundance were amplified with similar efficiency. 4-plex data (colored lines) were comparable to singleplex data (gray lines).

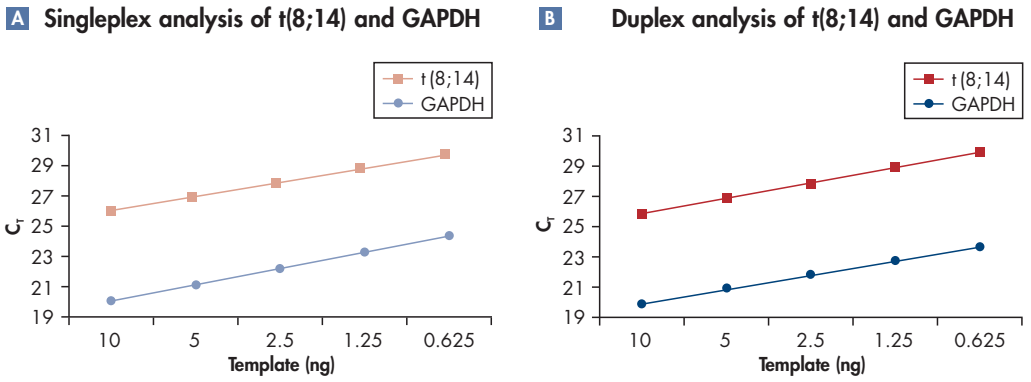


Figure 56. Linear C_T values over twofold decreases in template. Duplex and singleplex PCR were carried out on the Applied Biosystems 7500 Fast System using the QuantiFast Multiplex PCR +R Kit and assays for the t(8;14) chromosomal translocation and for GAPDH. Quadruplicate reactions were run using genomic DNA from the Ramos cell line as template (twofold dilutions from 10 ng to 0.625 ng). When analyzing template amounts that differ by twofold (e.g., 10 ng and 5 ng), the expected difference in C_T value would be 1. The data show that C_T values increased linearly by 1 C_T value with decrease in template dilution for both the A singleplex and B duplex reactions, demonstrating the ability of the kit to precisely discriminate between small differences in template amount.

10.5 Guidelines for programming the real-time cycler

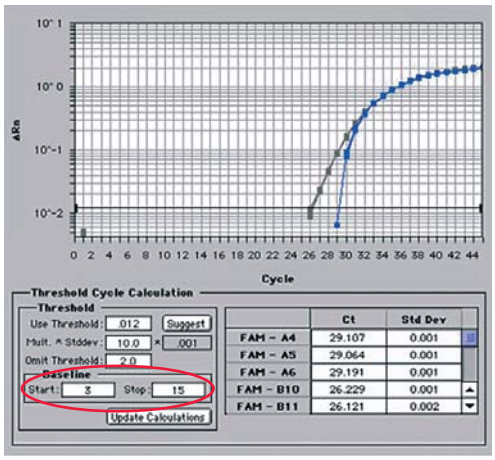
- Be sure to activate the filters or detectors for the reporter dyes used in the multiplex PCR assay. For details on setting up your real-time cycler for multiplex PCR analysis, refer to the instrument user manual.
- Follow the optimized protocols in the handbooks supplied with QuantiFast or QuantiTect Multiplex Kits. It is important to follow the specified cycling conditions, even for assays where the cycling conditions have already been established using a different kit or reagent.

10.6 Guidelines for analyzing data from a multiplex assay

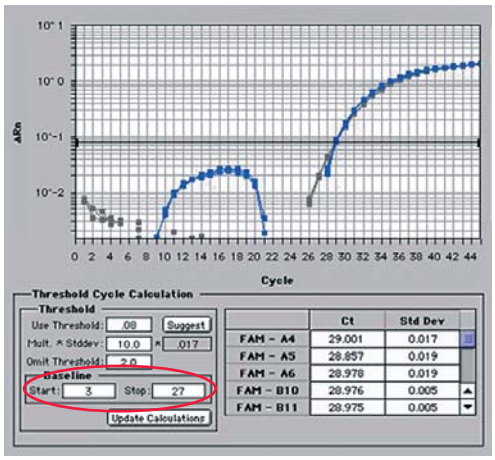
- Optimal analysis settings (i.e., baseline settings and threshold values) for each reporter dye are a prerequisite for accurate quantification data:
  - Adjust the analysis settings for every reporter dye channel in every run (Figure 57). It is important to analyze the data for each channel separately.

- Note that the default analysis settings provided by the instrument software may not provide accurate results and may need to be adjusted.
- Save the multiplex reactions after amplification so that the PCR products can be checked on a gel if required.

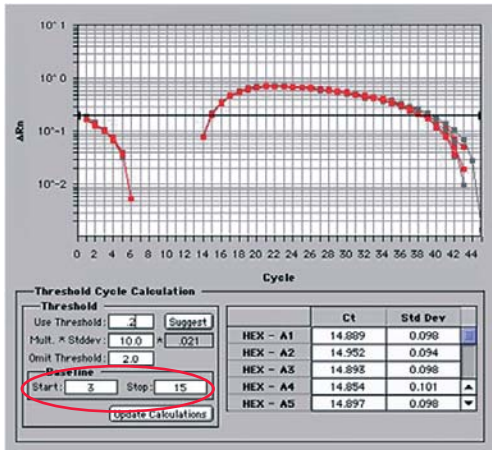
A **FAM channel (default baseline settings, cycle 3–15)**



B **FAM channel (adjusted baseline settings, cycle 3–27)**



C **HEX channel (default baseline settings, cycle 3–15)**



D **HEX channel (adjusted baseline settings, cycle 3–10)**

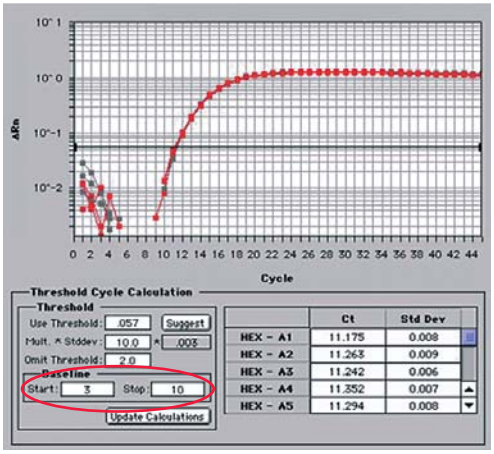


Figure 57. Importance of optimal baseline settings on data analysis. Two targets were analyzed by real-time, singleplex PCR (gray) or real-time, duplex PCR (blue or red). **A** The singleplex PCR and duplex PCR data for the FAM detected target are shown on the same graph. The standard baseline settings (cycles 3–15) have been applied. However, the singleplex PCR and duplex PCR data have different C_T values. Note that the differences in C_T values may not be very obvious when inspecting the amplification plots in linear view (not shown). **B** The baseline settings for the data shown in **A** have been adjusted (cycles 3–27; the upper limit is set just before an increase in the fluorescent signal becomes detectable). The singleplex PCR and duplex PCR data now show identical C_T values. **C** The singleplex PCR and duplex PCR data for the HEX detected target are shown on the same graph. The standard baseline settings (cycles 3–15) have been applied. Since high template amounts were used, detectable amplification appears already within the baseline cycles. Due to software algorithms, wavy curves are displayed. **D** The baseline settings for the data shown in **C** have been adjusted (cycles 3–10) so that no detectable amplification appears within the baseline cycles. The wavy curves are now eliminated.

I quantify my target gene and control gene in separate reactions and have already optimized the primer–probe concentrations. Can I use these concentrations when analyzing both genes in a multiplex assay?

Yes. If using QuantiFast or QuantiTect Multiplex Kits, you can continue to use the same primer–probe concentrations. However, be sure to follow the cycling conditions specified in the handbook supplied with the kits.

I have already designed primer–probe sets for my target gene and control gene. Do I need to redesign them if I want to analyze both genes in a multiplex assay?

First of all, check whether the primers and probes are complementary to each other. If they are not, redesign of the primer–probe sets is not necessary if combining them for use with QuantiFast or QuantiTect Multiplex Kits. However, it may be necessary to label the probes with new fluorophores. Each probe should be labeled with a distinct fluorophore and detected via a particular channel of your real-time cycler. For details on choosing an appropriate combination of fluorophores for multiplex assays, see section 10.2, page 54 or refer to the handbook supplied with the QuantiFast or QuantiTect Multiplex Kit.

How many targets can I detect in 1 reaction vessel by multiplex PCR?

Up to 5 targets can be simultaneously detected in the same reaction vessel, depending on the real-time cycler used. For details, refer to Table 15, page 55, or visit www.qiagen.com/multiplex.

11. Guidelines for successful virus load quantitation

When applying real-time PCR and RT-PCR to detection of viral nucleic acids (DNA and/or RNA), the inclusion of an internal, positive control is often desired to rule out the possibility of false negatives. In other words, a multiplex reaction is carried out to quantify both the target viral nucleic acids as well as control nucleic acid. Carrying out multiplex PCR and RT-PCR also provides the advantage of detecting several viruses from the same sample simultaneously, which saves times and conserves sample.

QuantiTect Virus Kits, which are specially designed for sensitive detection of viral DNA and/or RNA, use similar multiplex PCR technology to QuantiTect Multiplex Kits (Figure 58), allowing detection of up to 4 targets in a single reaction. As little as 1 copy of viral DNA or RNA target can be detected either in multiplex reactions with an internal control or in single-target amplification reactions. Multiplex assays without any optimization steps are achieved through QuantiTect Virus PCR Buffer, which contains an optimized combination of KCl and NH_4Cl as well as synthetic factor MP for effective primer annealing, and HotStarTaq *Plus* DNA Polymerase, which provides a stringent hot start with a short activation time.

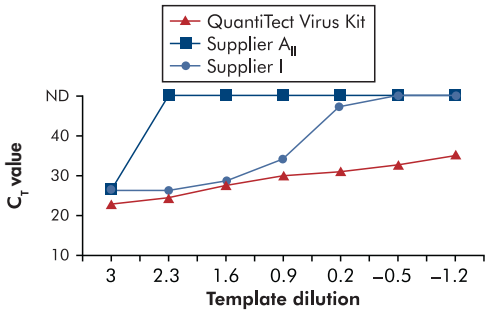


Figure 58. Improved detection of low amounts of viral RNA compared with other real-time kits. Viral RNA was diluted in serial fivefold dilutions and amplified in duplex with an internal control using the QuantiTect Virus Kit or kits from Suppliers A and I. The QuantiTect Virus Kit provided much higher sensitivity than the other reagents tested, enabling reliable analysis of unknown samples. **ND**: Not detected after 50 PCR cycles.

? How can I safely dilute RNA and DNA standards to very low concentrations?

We recommend using QuantiTect Nucleic Acid Dilution Buffer (supplied with QuantiTect Virus Kits) to dilute nucleic acids (RNA or DNA) used to generate standard curves or used as positive controls in real-time PCR or RT-PCR. The buffer stabilizes RNA and DNA standards during dilution and reaction setup and prevents loss of nucleic acids on plastic surfaces, such as tubes or pipet tips. The buffer is ready to use and is free of RNases and DNases. Proper use of the buffer enables safe and accurate dilution of the small amounts of nucleic acids typically used as standards for analysis of viral nucleic acids. Aliquots of diluted standards can be stored in QuantiTect Nucleic Acid Dilution Buffer at –20°C for up to 6 months. Repeated freezing and thawing should be avoided.

? My virus sample is very dilute. Is real-time PCR still possible?

QuantiTect Virus Kits are supplied with a highly concentrated 5x master mix. This means that only one-fifth of the final PCR volume is taken up by the PCR reagent, allowing use of larger volumes of dilute viral template in the reaction.

QIAGEN products for real-time PCR and real-time RT-PCR

Technology	Detection	Procedure	ROX dye	Kit
Fast cycling* (UNG treatment not possible)	SYBR Green	PCR and two-step RT-PCR	In master mix	QuantiFast SYBR Green PCR Kit†‡
		One-step RT-PCR	In master mix	QuantiFast SYBR Green RT-PCR Kit†
	Probe	PCR and two-step RT-PCR	In master mix	QuantiFast Probe PCR Kit‡
			In separate tube	QuantiFast Probe PCR +ROX Vial Kit‡
		One-step RT-PCR	In master mix	QuantiFast Probe RT-PCR Kit
			In separate tube	QuantiFast Probe RT-PCR +ROX Vial Kit
	Multiplex	PCR and two-step RT-PCR	In master mix	QuantiFast Multiplex PCR Kit‡
			In separate tube	QuantiFast Multiplex PCR +R Kit‡
		One-step RT-PCR	In master mix	QuantiFast Multiplex RT-PCR Kit
			In separate tube	QuantiFast Multiplex RT-PCR +R Kit
Standard cycling (UNG treatment possible)	SYBR Green	PCR and two-step RT-PCR	In master mix	QuantiTect SYBR Green PCR Kit†‡
		One-step RT-PCR	In master mix	QuantiTect SYBR Green RT-PCR Kit†
	Probe	PCR§ and two-step RT-PCR	In master mix	QuantiTect Probe PCR Kit‡
		One-step RT-PCR§	In master mix	QuantiTect Probe RT-PCR Kit
	Multiplex	PCR§ and two-step RT-PCR	In master mix	QuantiTect Multiplex PCR Kit‡
			Not included	QuantiTect Multiplex PCR NoROX Kit‡
		One-step RT-PCR§	In master mix	QuantiTect Multiplex RT-PCR Kit
			Not included	QuantiTect Multiplex RT-PCR NR Kit
Real-time one-step RT-PCR without RNA purification¶	SYBR Green	One-step RT-PCR	In master mix	FastLane Cell SYBR Green Kit†
	Probe	One-step RT-PCR	In master mix	FastLane Cell Probe Kit
	Multiplex	One-step RT-PCR	In master mix	FastLane Cell Multiplex PCR Kit
			Not included	FastLane Cell Multiplex PCR NR Kit

*For ultrafast cycling, use Rotor-Gene Kits and the Rotor-Gene Q cycler.

† Can be used with QuantiTect Primer Assays (predesigned, genomewide primer sets).

‡ For fast cDNA synthesis with gDNA removal, use the QuantiTect Reverse Transcription Kit. To prepare a cDNA archive from very limited RNA amounts, use the QuantiTect Whole Transcriptome Kit.

§ For highly sensitive detection of viral RNA and/or DNA in singleplex or multiplex reactions, use QuantiTect Virus Kits.

¶ For real-time two-step RT-PCR without RNA purification, use the FastLane Cell cDNA Kit with a QuantiFast, QuantiTect, or Rotor-Gene PCR Kit.

Useful links

- RNA sample technologies: www.qiagen.com/RNA
- PCR technologies: www.qiagen.com/PCR
- RNAi solutions: www.qiagen.com/siRNA
- GeneGlobe (assays and siRNAs): www.qiagen.com/GeneGlobe
- ProductFinder: www.qiagen.com/ProductFinder
- Gene expression analysis: www.qiagen.com/geneXpression
- SYBR Green-based real-time PCR: www.qiagen.com/SYBRGreen
- Multiple, real-time PCR: www.qiagen.com/multiplex
- Fast real-time PCR: www.qiagen.com/fastPCR

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

www.qiagen.com

Australia ▪ Orders 03-9840-9800 ▪ Fax 03-9840-9888 ▪ Technical 1-800-243-066

Austria ▪ Orders 0800/28-10-10 ▪ Fax 0800/28-10-19 ▪ Technical 0800/28-10-11

Belgium ▪ Orders 0800-79612 ▪ Fax 0800-79611 ▪ Technical 0800-79556

Brazil ▪ Orders 0800-557779 ▪ Fax 55-11-5079-4001 ▪ Technical 0800-557779

Canada ▪ Orders 800-572-9613 ▪ Fax 800-713-5951 ▪ Technical 800-DNA-PREP (800-362-7737)

China ▪ Orders 0086-21-3865-3865 ▪ Fax 0086-21-3865-3965 ▪ Technical 800-988-0325

Denmark ▪ Orders 80-885945 ▪ Fax 80-885944 ▪ Technical 80-885942

Finland ▪ Orders 0800-914416 ▪ Fax 0800-914415 ▪ Technical 0800-914413

France ▪ Orders 01-60-920-920 ▪ Fax 01-60-920-925 ▪ Technical 01-60-920-930

Germany ▪ Orders 02103-29-12000 ▪ Fax 02103-29-22000 ▪ Technical 02103-29-12400

Hong Kong ▪ Orders 800 933 965 ▪ Fax 800 930 439 ▪ Technical 800 930 425

Ireland ▪ Orders 1800 555 049 ▪ Fax 1800 555 048 ▪ Technical 1800 555 061

Italy ▪ Orders 02-33430-420 ▪ Fax 02-33430-426 ▪ Technical 800-787980

Japan ▪ Telephone: 03-6890-7300 ▪ Fax 03-5547-0818 ▪ Technical 03-6890-7300

Korea (South) ▪ Orders 1544 7145 ▪ Fax 1544 7146 ▪ Technical 1544 7145

Luxembourg ▪ Orders 8002-2076 ▪ Fax 8002-2073 ▪ Technical 8002-2067

Mexico ▪ Orders 01-800-7742-639 ▪ Fax 01-800-1122-330 ▪ Technical 01-800-7742-639

The Netherlands ▪ Orders 0800-0229592 ▪ Fax 0800-0229593 ▪ Technical 0800-0229602

Norway ▪ Orders 800-18859 ▪ Fax 800-18817 ▪ Technical 800-18712

Singapore ▪ Orders 65-67775366 ▪ Fax 65-67785177 ▪ Technical 65-67775366

Spain ▪ Orders 91-630-7050 ▪ Fax 91-630-5145 ▪ Technical 91-630-7050

Sweden ▪ Orders 020-790282 ▪ Fax 020-790582 ▪ Technical 020-798328

Switzerland ▪ Orders 055-254-22-11 ▪ Fax 055-254-22-13 ▪ Technical 055-254-22-12

UK ▪ Orders 01293-422-911 ▪ Fax 01293-422-922 ▪ Technical 01293-422-999

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