Boost Your Real-Time Results

Refresh your knowledge of the fundamentals of real-time PCR

Sample & Assay Technologies

1 Introduction

Real-time PCR and RT-PCR are highly sensitive techniques that enable you to amplify, quantify, and detect a specific nucleic acid sequence in a sample. The name comes from the product being detected during the reaction after each round of amplification (in real time) rather than afterward (at the end point). Real-time detection means getting results faster. It also involves less interaction with the PCR product, thus decreasing contamination results and increasing process safety.

Quantifying the DNA, cDNA, or RNA targets is easily done during the first cycle when the PCR product is detected. This is in contrast with end-point detection in conventional PCR, which doesn't enable accurate quantification of nucleic acids. Real-time PCR is also called quantitative PCR (qPCR) because of the ease of quantification.

Highly suited for a wide range of applications, real-time PCR is regularly used in gene expression analysis, genotyping, viral load determination, and library quantification, and is of high importance in areas such as cancer and pathogen research, food quality and safety control, and human identity testing.

2 Detecting PCR products in real-time

Real-time PCR and RT-PCR enable accurate quantification of the starting amounts of your DNA, cDNA, and RNA targets. The reaction product is measured as it is produced using a fluorescent reporter. Fluorescence measurements take place during each qPCR cycle. The fluorescence signal is proportional to the amount of qPCR product, and the linear correlation between the qPCR product and fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction. Fluorescence reporters used for qPCR include fluorescent dyes that bind to double-stranded DNA or fluorescently labeled, sequence-specific probes that hybridize with the qPCR product during amplification.

2.1 SYBR® Green dye

The fluorescent dye SYBR Green I binds to the minor groove of any double-stranded DNA molecule, emitting a fluorescent signal of a defined wavelength on binding (Figure 1). The excitation and emission maxima of SYBR Green I are respectively 494 nm and 521 nm, meaning the dye is suitable for use with any real-time cycler. Detection takes place during the extension step of real-time PCR. The signal intensity increases with increasing cycle number due to the accumulation of PCR product.

Using SYBR Green enables the analysis of many different targets without the need to synthesize target-specific labeled probes. However, nonspecific PCR products and primer–dimers will also contribute to the fluorescent signal, so high PCR specificity is essential when using SYBR Green.

2.2 EvaGreen dye

EvaGreen is a third-generation, saturating fluorescent dye which selectively binds to double-stranded DNA. In contrast to conventional SYBR Green I, EvaGreen can be used at higher concentrations without PCR inhibition and shows equal binding affinity for GC-rich and AT-rich regions with no apparent sequence preference. This makes EvaGreen highly suitable for HRM analysis of all types of PCR products. It enables the generation of distinct melting curves due to visualization of lower fluorescent differences, thereby ensuring standardized results.

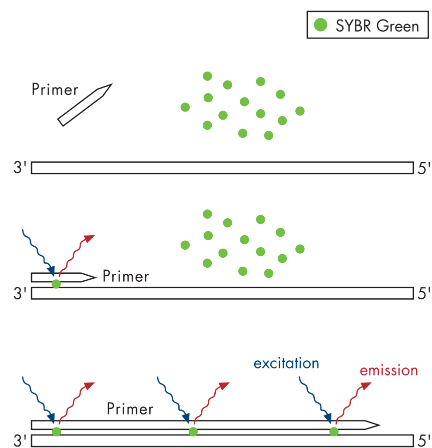

Figure 1. SYBR Green principle. Principle of SYBR Greenbased detection of PCR products in real-time PCR.

2.3 Fluorescently labeled, sequence-specific probes

Fluorescently labeled probes provide a highly sensitive and specific method of detection: 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 can also be produced, resulting 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.

TaqMan® probes are sequence-specific oligonucleotide probes that carry 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. While the probe is intact, the reporter signal from the fluorophore is suppressed or quenched. 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.

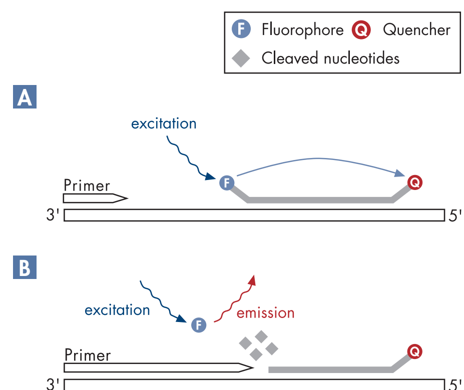

Figure 2. TaqMan probe principle. **A** Both the TaqMan probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the quencher to the fluorophore strongly reduces the fluorescence emitted by the fluorophore. **B** During the PCR extension step, *Taq* DNA 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.

2.2.3 Dyes used for fluorogenic probes in real-time PCR

Various fluorescent dyes are available for real-time PCR with sequence-specific probes. Each has its own excitation and emission maxima (Table 1). The wide variety of dyes makes it possible to detect two or more different specific nucleic acid sequences in the same reaction (multiplex real-time PCR). There are two conditions: the dyes must all be

compatible with the excitation and detection capabilities of the real-time cycler used, and their emission spectra have to be sufficiently distinct from one another. When carrying out multiplex PCR, it's best to use dyes with the widest possible channel separation to avoid any potential signal crosstalk.

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.

3 Methods in real-time PCR

3.1 DNA analysis

You can directly use genomic DNA or plasmid DNA as your starting template for real-time PCR. The technique lets you quantify genomic DNA or copy numbers, and allows qualitative analysis, such as single nucleotide polymorphism (SNP) detection.

3.1.1 Allelic discrimination using TaqMan Probes

An allelic discrimination assay is a multiplex end-point assay that detects variants of a single nucleic acid sequence. The presence of 2 primer/probe pairs in each reaction allows genotyping of the 2 possible variants at the single-nucleic polymorphism (SNP) site in a target template sequence. During PCR, the primers anneal to the target sequence during the annealing step. Two TaqMan probes including a unique pair of fluorescent dyes, each specific for one of the available alleles (e.g., C or T) are present in the reaction, specifically annealing to the target region between the 2 primers. One fluorescent dye detector is a perfect match to the wild type (allele 1) and the other fluorescent dye detector is a perfect match to the mutation (allele 2). The allelic discrimination assay measures the change in fluorescence of the dyes associated with the probes.

3.1.2 Copy number quantification

Copy number variances (CNVs) are known to affect approximately 10–20% of the human genome and have been associated with many types of cancer and various complex diseases, such as autism and autoimmune disorders. Quantitative real-time PCR is an excellent method for CNV validation and association studies. First, a reference gene is defined, the copy number of which is presumed to be constant in all the samples. The copy number changes of the genes of interest are then calculated based on the C_T difference between the genes of interest and reference gene in the various samples, using the $\Delta\Delta C_T$ method for relative quantification (see chapter 4 for more information).

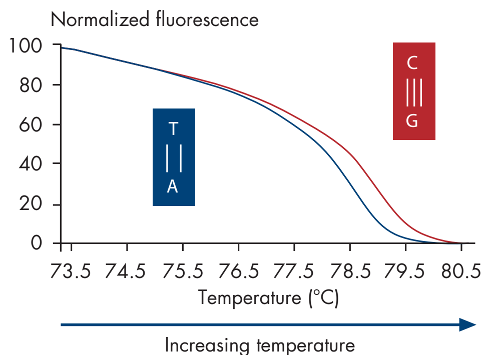

Figure 3. Principle of HRM technology. HRM analysis is based on the dissociation behavior of dsDNA due to increasing temperature. Melting of dsDNA depends on GC-content. AT-rich regions melt faster.

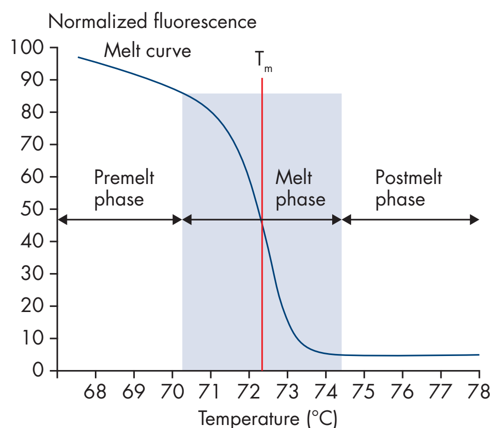

Figure 4. A typical HRM plot. The melt curve plots the transition from the high fluorescence of the initial pre-melt phase, through to the decrease in fluorescence of the melt phase, to the basal level of fluorescence at the post-melt phase. Fluorescence decreases as the DNA intercalating dye is released from dsDNA as it melts into single strands. The midpoint of the melt phase, at which the rate of change in fluorescence is greatest, defines the melting Temperature Tm of the DNA under analysis.

3.1.3 High-resolution melting technology

HRM analysis is based on the dissociative behavior of double-stranded DNA in increasing temperature. The melting of double-stranded DNA depends on its GC content and the overall distribution of the bases. AT-rich regions melt faster than GC-rich regions (Figure 3). In HRM analysis, a PCR product is generated through amplification and then subjected to a gradual temperature increase. This is done in the presence of a dye that fluoresces when bound to double-stranded DNA.

To perform HRM, the temperature is increased from low to high. The fluorescence of third-generation intercalating dyes (e.g., EvaGreen) is measured continuously and plotted against increasing temperature. Increased fluorescence is only measured as long as the dye is bound to double-stranded DNA. At lower temperatures, fluorescence is high because the DNA is entirely double-stranded. As the temperature increases, the DNA starts to disassociate into two single strands, resulting in DNA melting. At this point, the dye is released and the fluorescence decreases. The melting temperature (T_m) of the DNA sample occurs at the point of the melt phase where the rate of change in fluorescence is greatest (Figure 4). When the DNA is completely melted, only background fluorescence is detectable.

The DNA strands of a PCR product are bound together by hydrogen bonds and additional interactions, such as base stacking forces. The strength of these interactions depends on certain parameters, such as the length of the amplicon, the overall base composition, and the local GC content within the PCR amplicon. All of these parameters affect the melting behavior of your PCR product, meaning each product delivers a characteristic melting pattern. HRM even enables the detection of very subtle differences between two DNA sequences, allowing you to accurately detect variations in the DNA sequence down to the single nucleotide level, such as in SNP detection.

3.2 RNA analysis

RNA templates are used for the analysis of gene expression levels or to examine viral load of RNA viruses. Real-time RT-PCR comprises two reactions and is the method for RNA work. In the first reaction, the RNA is transcribed into cDNA using a reverse transcriptase, which is an enzyme that is generally derived from an RNA-containing retrovirus. In the second reaction, the cDNA is amplified and quantified

during the qPCR reaction. Real-time RT-PCR can be either a two-step or a one-step procedure (Figure 5 and Table 2).

3.2.1 Two-step and one-step RT-PCR

In **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 (RT) reaction is then added to the real-time PCR. You have a choice of RT primers, depending on your experimental needs. Using oligo-dT primers or random oligomers for reverse transcription means that several different transcripts can be analyzed from a single RT reaction. In addition, you can immediately transcribe labile RNA samples 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. This is possible due to specialized reaction chemistries and cycling protocols. One-step RT-PCR 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.

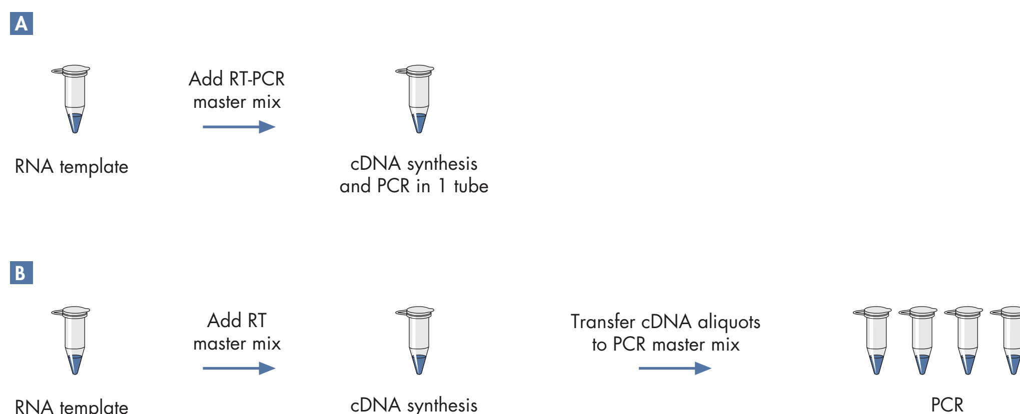

Figure 5. Comparison of two-step and one-step RT-PCR. A In one-step RT-PCR, reverse transcription and PCR take place sequentially in the same tube. B In two-step RT-PCR, cDNA is synthesized in 1 tube, and aliquots of the cDNA are transferred to other tubes for PCR.

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

3.3 Multiplex real-time PCR and RT-PCR

In multiplex real-time PCR, you can quantify several genomic DNA targets simultaneously in one reaction. Multiplex real-time RT-PCR is similar, 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.

Multiplex real-time PCR and RT-PCR have many advantages for applications such as gene expression analysis, viral load monitoring, and genotyping. The target gene(s) and internal control are coamplified in the same reaction, eliminating the well-to-well variability that would occur with separate amplification reactions. The internal control can be either an endogenous gene that does not vary in expression between different samples (e.g., a reference gene) or an exogenous nucleic acid.

For viral load monitoring, the use of an exogenous nucleic acid as an internal control allows you to check the following parameters: 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 real-time PCR and RT-PCR are possible thanks to 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. 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).

3.4 Whole genome amplification and whole transcriptome amplification

Deep genome and transcriptome analysis is often limited due to the small amount of sample available (6 pg genomic DNA and 0.5 pg messenger RNA/human cell). A thorough analysis requires a few hundred nanograms up to micrograms of RNA or DNA. To generate these amounts, whole genome amplification (WGA) and whole transcriptome amplification (WTA) can be performed from samples as small as a single cell. The methods include a new variation of the enzyme Phi29 polymerase, which has high processivity, proofreading activity, and high affinity for a low template amount. It is a phage-derived DNA polymerase with 3'→5' prime exonuclease activity (proofreading activity) that delivers up to 1000-fold higher fidelity than *Taq* DNA polymerase. Phi29 polymerase easily solves secondary structures such as hairpin loops, thereby preventing slipping, stoppage, and dissociation of the polymerase during amplification. This enables the generation of DNA fragments up to 100 kb without sequence bias.

WTA allows amplification of entire transcriptomes from very small amounts of RNA. WTA of limited RNA samples or single cells can be achieved by reverse transcription and cDNA ligation prior to multiple displacement amplification (MDA). The cDNA library is prepared covering all of the transcript sequences, including both the 5' and 3' regions (Figure 6). 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. This is critical for accurate gene expression analysis.

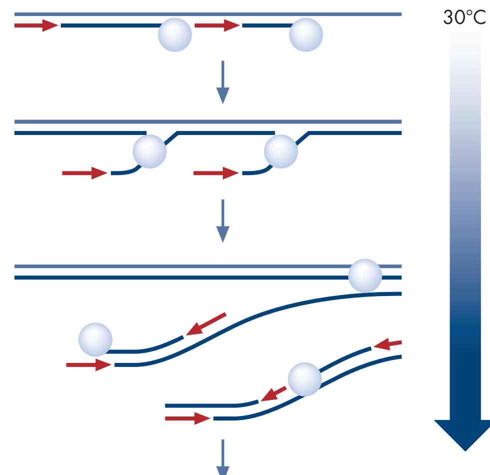

Figure 6. Multiple Displacement Amplification (MDA). Primers (arrows) anneal to the template DNA and are extended at 30°C by Phi 29 polymerase, which moves along the DNA template strand, displacing the complementary strand while becoming a template itself for replication. In contrast to PCR amplification, MDA does not require different temperatures and ends in very long fragments with low mutation rates.

4 Terminology used in real-time PCR

Before you can quantify the levels of your nucleic acid target using real-time PCR, you have to analyze the raw data and set baseline and threshold values. 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), you must adjust the baseline and threshold settings 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.

Some of the 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. Amplification plots are also called qPCR curves.

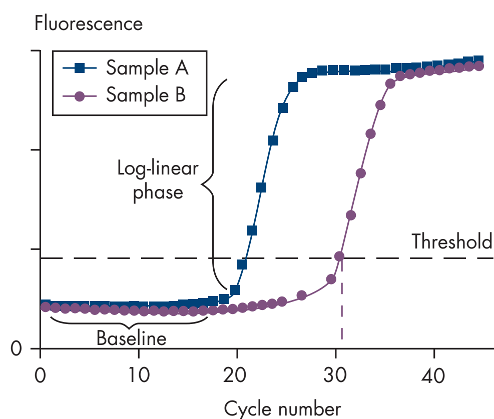

Figure 7. Typical amplification plot. Amplification plots showing increases in fluorescence from 2 samples (A and B). Sample A contains a higher amount of starting template than sample B. The Y-axis is on a linear scale.

Baseline: The baseline represents 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 (Figure 7). The number of cycles used to calculate the baseline can be changed. Reduce it if high template amounts are used or if the expression level of the target gene is high. 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 8). 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 the amplification products. Recent versions of software for various real-time cyclers allow automatic, optimized baseline settings for individual samples.

Background: Nonspecific fluorescence in the reaction is the background. Examples of background causes are inefficient quenching of the fluorophore or the presence of large amounts of double-stranded DNA template when using SYBR Green. The software algorithm of the real-time cycler mathematically removes the background component of the signal.

Reporter signal: The reporter signal is the fluorescent signal that is generated during real-time PCR by either SYBR Green or a fluorescently labeled sequence-specific probe.

Normalized reporter signal (Rn): The emission intensity of the reporter dye divided by the emission intensity of the passive reference dye measured in each cycle gives you the normalized reporter signal.

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 it has a completely different emission spectrum from the fluorescent dyes commonly used for probes.

SNP classification: Single nucleotide polymorphisms are typically divided into 4 different classes, depending on the type of base exchange. A simple exchange of an A to a T will have only a very subtle influence on the T_m, but will still affect the melting behavior of the amplicon.

Table 3. The four SNP classes

SNP class	Base change	Typical Tm shift	Abundance (in humans)*
I	C/T and G/A	Large: >0.5°C	64%
II	C/A and G/T		20%
III	C/G		9%
IV	A/T	Very small: <0.2°C	7%

* Abundance in humans according to Venter et al. 2001.†

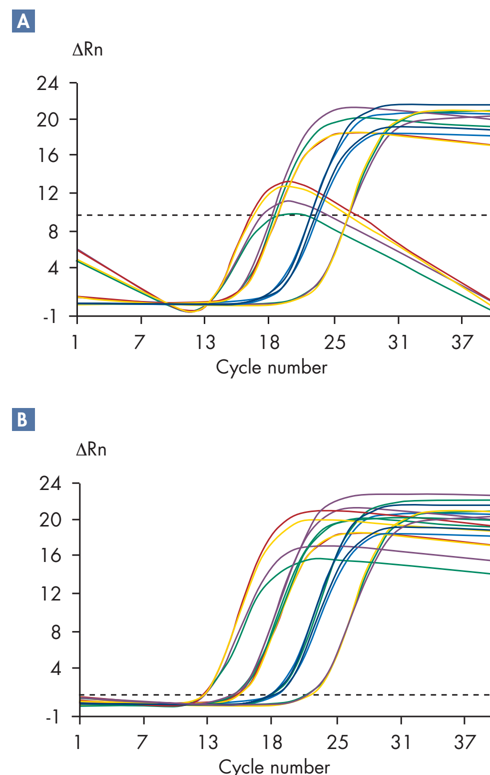

Figure 8. Setting baseline and threshold values. Using the correct baseline and threshold values is important for accurate quantification. **A** Amplification product becomes detectable within the baseline setting of cycles 6 to 15 and generates a wavy curve with the highest template amount. **B** Setting 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.

Threshold: The threshold is adjusted to a value above the background and significantly below the plateau of the amplification plot (Figure 7). It must be placed within the linear region of the amplification curve, which represents the detectable log-linear range of the PCR. Set the threshold value within the logarithmic amplification plot view to enable easy identification of the log-linear phase of the PCR (Figure 8). When using several targets in the real-time experiment, you must set the threshold for each target.

Threshold cycle (C_T) or quantification cycle (C_q): 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. It allows the calculation of the starting template amount.

T_m: The melting temperature of the PCR product is indicated by the highest rate of change in fluorescence. Melting of dsDNA depends on its GC content and the overall distribution of bases.

† Venter, J.C. et al. (2001) The sequence of the human genome. Science 291, 1304

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. It is used to normalize for the amount of template according to the formula:

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

$\Delta\Delta C_T$ 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 are normalized to this when performing relative quantification, according to the formula:

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

Reference gene or housekeeping gene: The reference gene's expression level should not differ between samples. 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 the presence of inhibitors in the RNA sample, and for variation in RNA content, reverse-transcription efficiency, nucleic acid recovery, and sample handling.

Internal control: An internal control is crucial for avoiding false negative results. It is a control sequence that is amplified in the same reaction as the target sequence and detected with a different probe. An internal control is often used to rule out failure of amplification in cases where the target sequence is not detected. If the internal control is also undetected, then amplification has failed. If the internal control is detected and the target sequence is not, then the target sequence was not present.

Calibrator sample: A reference sample that is used for comparison with all the other samples in relative quantification (e.g., RNA purified from a cell line or tissue) is called a calibrator sample. It is used to determine the relative expression level of a gene and it 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: The positive control 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): 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.

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

Standard: A sample of known concentration or copy number used to construct a standard curve is called a standard.

Standard curve: To generate a standard curve, the C_T values 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 that 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) indicates a PCR efficiency less than 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 that 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.

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

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

Dynamic range: The range over which an increase in starting material concentration results in a corresponding increase in the real-time PCR product is called the dynamic range.

Absolute quantification: Absolute quantification describes a quantification method in which samples of known quantity are serially diluted and then amplified to generate a standard curve. The amount of target in the unknown sample is determined by comparison with the standard curve.

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.

QIAGEN PCR products

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QuantiNova SYBR Green PCR Kit

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QuantiFast SYBR Green RT-PCR Kit

Designed for fast, one-step qRT-PCR using SYBR Green I dye for gene expression analysis, this kit enables shorter run times on fast and on standard real-time cyclers. Learn more at www.qiagen.com/QuantiFast-SYBR-Green

QuantiFast Probe RT-PCR Kit

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Type-it CNV Probe PCR +qC Kit

This kit is designed for quantification of gene copy number using TaqMan probe-based multiplex real-time PCR. It includes a universal control reference assay for reliable CNV validation. Learn more at www.qiagen.com/Type-it-CNV-Prob

Type-it HRM PCR Kit

This kit is designed for quantification of gene copy number using TaqMan probe-based multiplex real-time PCR. It includes a universal control reference assay for reliable CNV validation. Learn more at www.qiagen.com/Type-it-HRM

Type-it Fast SNP Probe PCR Kit

This kit is designed for accurate and reliable SNP genotyping using TaqMan or TaqMan MGB probes, even with difficult SNP loci (e.g., GC rich) or low amounts of starting template. Learn more at www.qiagen.com/Type-it-Fast-SNP

REPLI-g® Mini Kit

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REPLI-g Single Cell Kit

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REPLI-g WTA Single Cell Kit

Designed for whole transcriptome amplificationf total RNA or mRNA from single cells, this kit has an innovative lysis buffer that effectively stabilizes cellular RNA, ensuring the resulting RNA accurately reflects the in vivo gene expression profile. Learn more at www.qiagen.com/REPLI-g-WTA-Single-Cell

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