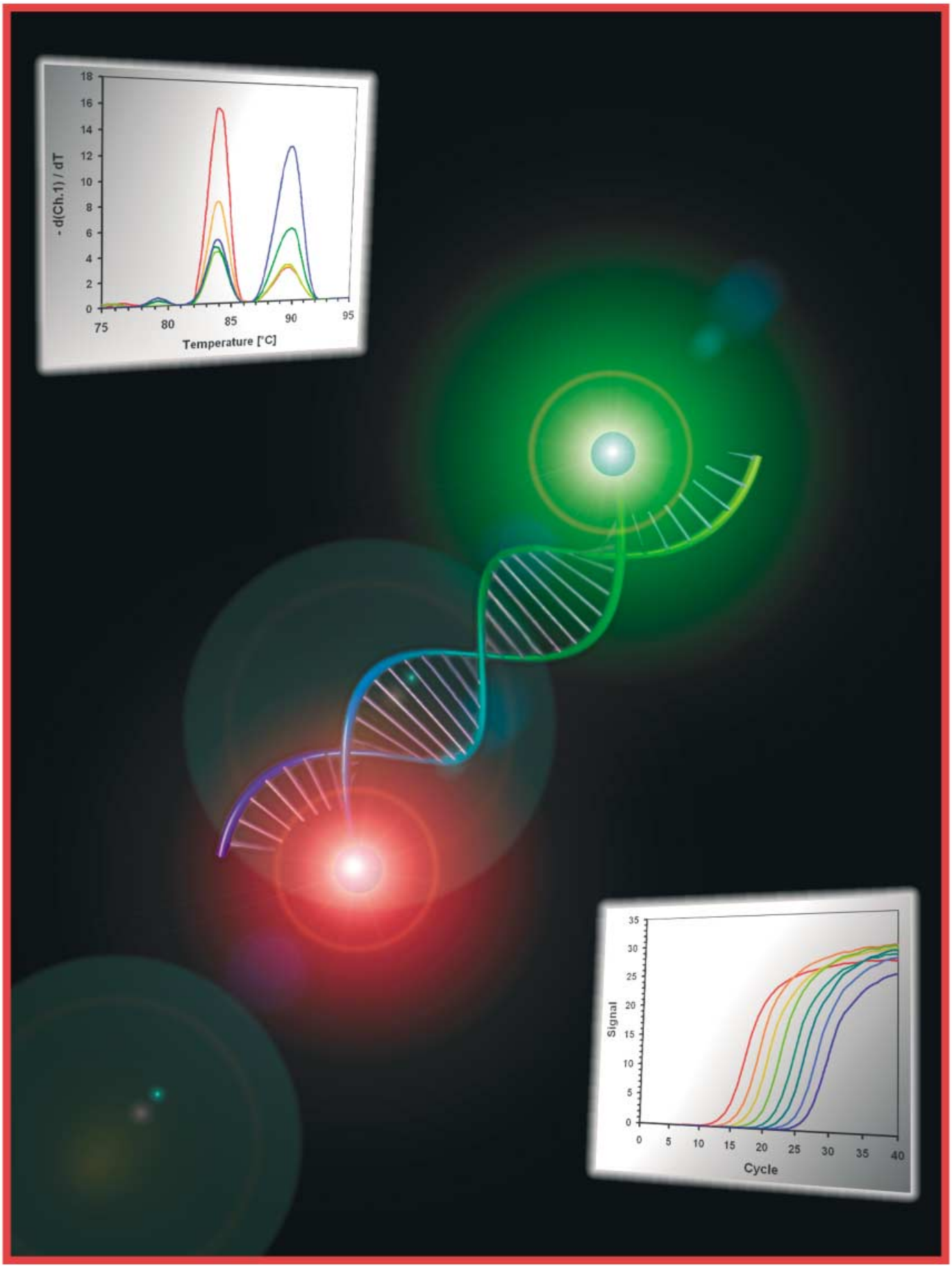

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Real-Time Polymerase Chain Reaction

Jochen Wilhelm*[a, b] and Alfred Pingoud[b]

Real-time PCR is the state-of-the-art technique to quantify nucleic acids for mutation detection, genotyping and chimerism analysis. Since its development in the 1990s, many different assay formats have been developed and the number of real-time PCR machines of different design is continuously increasing. This review provides a survey of the instruments and assay formats available and discusses the pros and cons of each. The principles of quantitative real-time PCR and melting curve analysis are explained. The quantification algorithms with internal and external standardization are derived mathematically, and potential pitfalls for the data analysis are discussed. Finally, examples of applications of this extremely versatile technique are given that demonstrate the enormous impact of real-time PCR on life sciences and molecular medicine.

Real-Time PCR

Real-time PCR is used for many different purposes, particularly for quantifying nucleic acids and for genotyping. Since its invention in 1996, the number of publications dealing with real-time PCR has increased nearly exponentially (Figure 1).

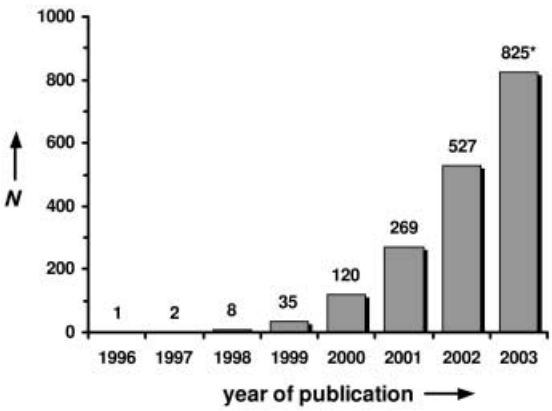

Figure 1. *Result of a* PubMed *search using the keywords "real-time PCR", sorted by year of publication. The value for 2003 (*) is estimated by a linear extrapolation from the number of publications cited in* PubMed *between January and March 2003.* N = *number of* PubMed *entries.*

The peculiarity of real-time PCR is that the process of amplification is monitored in real time by using fluorescence techniques.[1–3] The information obtained, that is the amplification curves (Figure 2), can be used to quantify the initial amounts of template molecules with high precision over a wide range of concentrations. In melting curve analyses performed subsequently (Figure 3), the amplified sequences can be characterized with respect to their apparent melting temperature (T_m), which is a function of product length and base composition.[4, 5] This closed-tube analysis is fast, easy to perform and avoids carry-over contaminations.

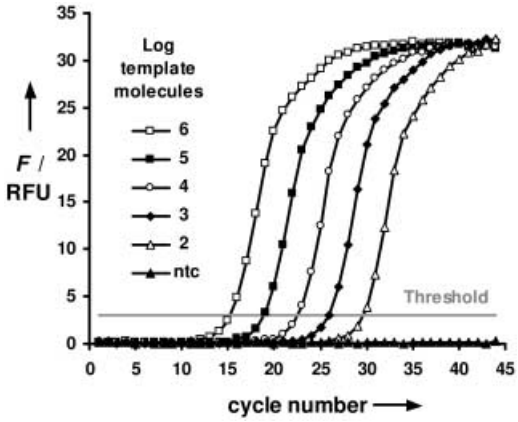

Figure 2. *Amplification curves of a dilution series containing 100–1 000 000 template copies. During the first cycles, no signal increase is detectable. The observable exponential phases are shifted to higher cycle numbers for samples containing fewer target molecules; ntc = no template control.*

The major advantages of real-rime PCR compared with other methods for the quantification of nucleic acids are the extremely wide dynamic range (more than eight orders of magnitude)[1] and the significantly higher reliability of the results compared with conventional PCR, because with real-time PCR, the whole amplification profile is known. Individual reactions deviating in their amplification efficiency (e.g. owing to the presence of polymerase inhibitors) can be identified easily. Quantitative real-time PCR is more precise than end-point determinations.[6]

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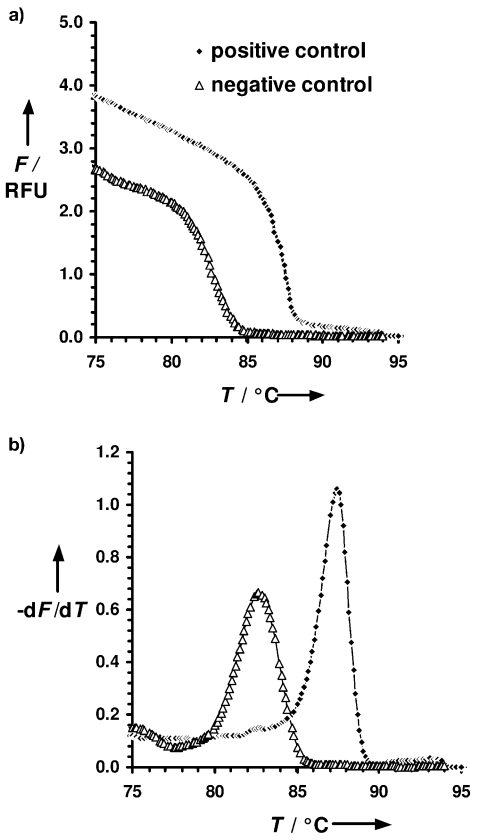

Figure 3. Result of a melting curve analysis of a positive and a negative control. The positive control contains the specific PCR product with an apparent T_m of 87.5 °C, whereas the negative control contains primer dimers with a lower apparent T_m of 83 °C. a) Melting curve data. The initial slight decrease is due to the temperature-dependent quench. The following steep decrease depicts the melting process of the product. b) Melting peak representation of the data shown in (a).

Instrumentation

The first real-time thermocyclers, produced by Applied Biosystems, became commercially available in 1997. Today, the ABI 7700 is the best-selling real-time PCR instrument. It is based on a conventional 96-well blockcycler with an additional fluorimeter device. Only a little later, Roche Diagnostics started distributing the LightCycler based on an entirely different instrument design, which was originally developed by Idaho Technologies.[7–9] The LightCycler uses small glass capillaries as reaction tubes. They are placed in a sample carrousel, within an air-thermostated chamber of the instrument. The combination of a small sample volume, the cylindrical shape of the capillary tubes and temperature adjustment with air allows very steep temperature gradients and short cycle times to be used, which increases the specificity of the reaction. A whole PCR run with 40 cycles can be performed within only 15 – 20 min. Therefore, this technique is sometimes termed rapid-cycle PCR. As a result of the short times required per PCR, the smaller number of samples per run (32 compared to 96 in the ABI 7700) is more than compensated with respect to sample throughput. For high-throughput analyses, Roche now also offers a LightCycler with a sample carrousel with 64 capillary positions. Additionally, in contrast to all other instruments available, the LightCycler instrument uses a robust blue light-emitting diode for excitation instead of a delicate laser.

With the iCycler, RioRad offers a cost-effective fluorimeter device, which can be combined with their conventional PCR machines. Real-time PCR systems are now available from many companies, such as MJ Research (DNA Engine Opticon Continuous Fluorescence Detection System), Stratagene (Mx400), Thermo Hybaid (Chimaera Quantitative Detection System), Corbett Research (Rotor-Gene 3000) and Cepheid (Smartcycler). Many more are likely to follow. Eppendorff, for example, is currently developing its own real-time PCR system, which will soon be ready to be introduced onto the market. All these instruments differ considerably in price, flexibility and user-friendliness of the software.

There are as yet no publications comparing the accuracies and precisions of the available real-time PCR machines. These performance parameters are largely affected by the signal-to-noise ratio (depending on the detection format (see below) and the quality of the optics). Another crucial point is the temperature homogeneity within the instrument. Small local deviations can result in substantial errors for quantifications.[10, 11] Roche assures temperature homogeneity in the LightCycler by using a continuously rotating sample carousel. The Rotor-Gene 3000 contains a 36- or 72-well rotor for 0.2-mL propylene PCR tubes that spin at 500 rpm. Corbett Research claims that this guarantees sample-to-sample temperature variations of less than 0.01 K.

The investments of so many companies in real-time PCR systems are absolutely profitable. The Frost & Sullivan report B114 of 2002 states that the market has good potential for growth in the coming years: in 2002, sales were worth about 310 million US dollars (120 million US dollars in Europe), with Applied Biosystems, Roche and BioRad sharing 85 %. Sales are predicted to grow to more than 350 million US dollars in 2006 in Europe alone. The most important future fields for real-time PCR systems will be clinical diagnostics and food control.

Dr. Jochen Wilhelm was born in 1972. He is currently conducting research on data analysis for the evaluation of DNA microarray experiments for expression profiling at the Department of Pathology, Justus Liebig University of Giessen (Germany). He obtained his Diploma in Biology in 1997 from the same university, where he also earned a PhD in 2003 for research on real-time PCR conducted under the guidance of Prof. A. Pingoud in the Institute of Biochemistry and sponsored by the Deutsche Forschungsgemeinschaft.

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Detection Formats

Fluorescence signals that are proportional to the amount of PCR product can be generated by fluorescent dyes that are specific for double-stranded DNA (dsDNA) or by sequence-specific fluorescent oligonucleotide probes. The first dye used for this purpose was ethidium bromide.[2, 8, 12] Other intercalating dyes such as YO-PRO-1 have since been used.[13, 14]

SYBR Green I

SYBR Green I is the most frequently used dsDNA-specific dye in real-time PCR today (Figure 4 a). It is an asymmetric cyanine dye, structurally related to the dsDNA-specific dyes YOYO-1 and TOTO-1.[15, 16] In contrast to ethidium bromide, intercalation of cyanine dyes is negligible under the assay conditions of real-time PCR experiments. Instead, SYBR Green I largely binds sequence independently to the minor groove of dsDNA. The binding affinity is more than 100 times higher than that of ethidium bromide. The fluorescence of the bound dye is more than 1000-fold higher than that of the free dye and, therefore, is well suited for monitoring the product accumulation during PCR.[8, 17] SYBR Green I can be excited with blue light with a wavelength of 480 nm. Its emission spectrum is comparable to that of fluorescein with a maximum at 520 nm and a quantum yield of 0.8.[15] The reported self-quenching of SYBR Green I bound to dsDNA[18] is not an error source in quantitative real-time PCR assays because in such experiments the signal of the exponential phase is used for which the dye-to-base-pair ratio is larger than 2.[19] Thus, the PCR products are saturated with bound dye, and the self-quenching is proportional to the amount of PCR product. It is often stated that the nonspecific detection of any dsDNA is a disadvantage that does not occur when sequence-specific detection formats are used. This is not absolutely true. Amplified nonspecific products alter the amplification efficiency for the specific products. Therefore, the amplification of non-specific products, such as primer dimers, will result in a systematic error for the quantification regardless of whether the nonspecific products are detected or not. Therefore, for quantitative analysis, the assay must be optimised anyway, so that nonspecific products do not occur.

Provided that the assay is optimised, SYBR Green I detection is sensitive enough to identify a single target molecule in the reaction mixture. If primer dimers accumulate in the late cycles, they mask the signal resulting from the target amplification. The sensitivity can be recovered by using a higher measurement temperature at which the primer dimers are molten and hence do not contribute to the signal measured. However, in this case, accurate quantification may be impaired.

Recently, a new minor groove binding asymmetric cyanine reporter dye (BEBO) was introduced. BEBO and SYBR Green I are reportedly similar in all important aspects of their behaviour, such as specificity, PCR inhibition and quantum efficiency.[20]

For sequence-specific detection, fluorophor-labelled oligonucleotide probes are used.[21–23] The fluorescence signal intensity can be related to the amount of PCR product by a product-dependent decrease of the quench of a reporter fluorophor or by an increase of the fluorescence resonance energy transfer (FRET) from a donor to an acceptor fluorophor. FRET, also called Förster transfer, is the radiationless transfer of excitation energy by dipole – dipole interaction between fluorophors with overlapping emission and excitation spectra. The FRET and the quench efficiency are strongly dependent on the distance between the fluorophors.[24, 25] Therefore, the PCR-product-dependent change in the distance between the fluorophors is used to generate the sequence-specific signals. There are several different formats used. In principle, all of them could function by a decrease of quench or an increase of FRET; in practice, most formats are based on a decrease of quench. The most commonly used quenchers are TAMRA, DABCYL and the recently developed Black Hole Quencher (BHQ; Biosearch Technologies, Novato, CA, USA). This new class of quencher is able to quench the most commonly used fluorescent dyes more efficiently than other quenchers and does not fluoresce itself. For quantifications, SYBR Green I was reported to yield results more precise than,[6] or of at least similar precision to those from fluorogenic probes.[26] Sequence-specific probes, in contrast, allow multiplexing[27–29] and easy identification of point mutations.[30–32] A common drawback of probe systems that use the decrease-of-quench mechanism is unwanted generation of a signal due to probe

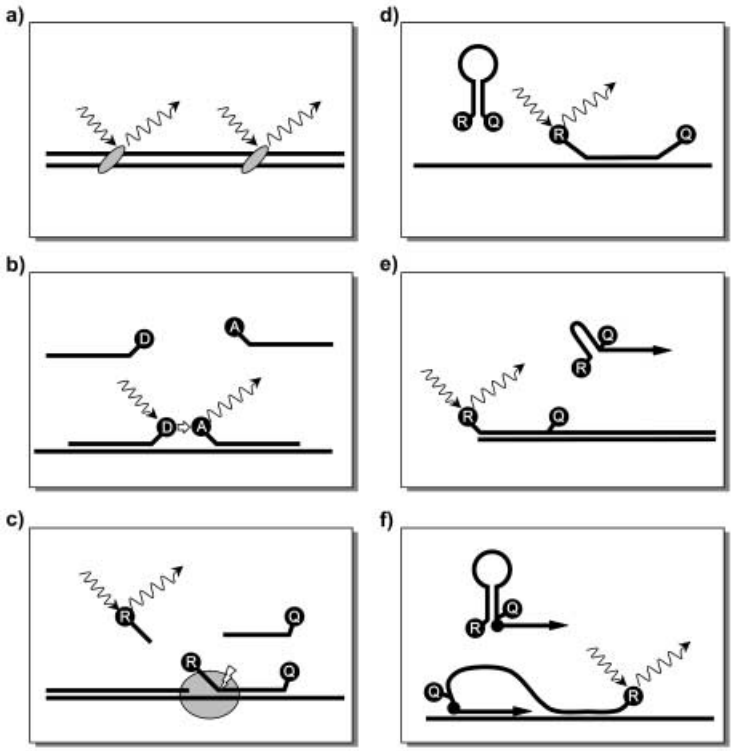

Figure 4. Detection formats commonly used for real-time PCR. For explanations, see the text; D, donor; A, acceptor; R, reporter; Q, quencher. a) SYBR Green I; b) hybridisation probes; c) TaqMan *probes. The grey circle indicates the* Taq *polymerase hydrolysing the* TaqMan *probe; d) molecular beacon; e) Sunrise primer; f) Scorpion primer.*

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destruction (e.g. by unintentional hydrolysis of the probes by the *Taq* DNA polymerase) or by formation of secondary structures of the probes that lead to a decrease in quench.

Hybridisation probes

Hybridisation probes (Figure 4b) are used in the only format in which FRET is measured directly. The donor probes are 3′-terminally labelled with a reporter fluorophor (usually 6-carboxy-fluorescein, FAM) and the acceptor probes are 5′-terminally labelled with an acceptor fluorophor (cyanine dyes Cy3, Cy5; 6-carboxy-4,7,2′,7′-tetrachlorofluorescein, TET; 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine, TAMRA; 6-carboxyrhodamine X, ROX). Only the donor fluorophor is excited, so that no acceptor fluorescence is observed from probes free in solution. During the primer-annealing phase, the probes hybridise adjacently on the single-stranded DNA and the excitation energy is transferred from the donor to the acceptor. When *Taq* DNA polymerase is used, the probes can in part be hydrolysed by the inherent endonucleolytic activity of the polymerase (see below). This leads to a decrease of the effective probe concentration during the PCR and finally yields suboptimal signal-to-noise ratios. This problem can be overcome by using polymerases lacking endonucleolytic activities.[33] The hybridisation probe format is used mostly for LightCycler experiments. The LightCycler has special filters for detection and Roche has developed fluorophors (RED-640 and RED-705) with corresponding emission characteristics.[9] Nevertheless, fluorophores with similar spectroscopic properties like Cy5 have been used.[34]

Hydrolysis probes

A *Taq*Man probe (Figure 4c) is an oligonucleotide 5′-terminally labelled with a reporter fluorophor like fluorescein and labelled internally or 3′-terminally with a quencher. Intact probes do not fluoresce because they are quenched. During the extension phase of the primers, the probe, which is complementary to the amplicon sequence, is bound to the single-stranded PCR product like the primers. When the *Taq* DNA polymerase reaches the probe, it is sheered and endonucleolytically cut. The quencher is hence released from the fluorophor, which now fluoresces after excitation.[1, 35, 36] In contrast to all other formats, in this case the hydrolysis of the probes by the *Taq* DNA polymerase is not only intended but is actually required for signal generation. Sheering without cleavage is detrimental for the precision of quantifications as a result of decreased signal-to-noise ratios. The proportion of hydrolysed probes is dependent on the probe position and sequence, which has to be considered for probe design.[37]

Molecular beacons

Molecular beacons (Figure 4d) are labelled on both ends. One end is attached to a reporter fluorophor, and the other end is attached to a quencher. Only the middle part of the probe is complementary to the amplicon sequence, whereas the terminal 10–15 nucleotides are self-complementary. The free probe forms a stem-loop structure in which the reporter is kept close to the quencher. During the annealing phase of the PCR, the loop can bind to the PCR product. In this conformation, the stem is opened and the quenching is relieved.[38–40] The signal yield is very sensitive to the hybridisation conditions, which are difficult to optimise.

Sunrise primers

Sunrise primers (Figure 4e) are primers with a 5′-terminal hairpin structure, labelled with a reporter fluorophor and a quencher. The hairpin structure keeps the reporter and quencher together. In the first step, the sunrise primer, as the forward primer, is extended. This extended product serves as template for the reverse primer in the second step. In the end, the polymerase opens the hairpin structure and a double-stranded PCR product is formed in which reporter and quencher are separated.[3] Because the stem is also opened in primer dimers and other unspecific products, the signals obtained are not really specific for the PCR product.

Scorpion primers

Scorpion primers (Figure 4f) are structurally and functionally related to molecular beacons, but serve as primers in the PCR reaction. Scorpion primers have self-complementary sequences that form a 5′-terminal stem-loop structure, with the loop sequence complementary to the amplicon sequence, which follows the primer sequence. The 3′-end serves as the primer. The stem region is labelled with a reporter fluorophor and a quencher. In the first step, the primer is extended, yielding a single-stranded template for the reverse primer in the second step. The stem then opens and the loop binds to the product, separating reporter and quencher. In contrast to the sunrise primers, the reverse extension is blocked by a hexethylene glycol group. This ensures that the reporter of the scorpion primer remains quenched in unspecific products like primer dimers.[41–42]

Light-up probes

Light-up probes are peptide nucleic acids (PNAs) that use thiazole orange as the fluorophor. Upon hybridisation with DNA, duplex or triplex structures are formed with increased fluorescence intensity of the fluorophor. A quencher is not required. This technique is limited by unspecific fluorescence, which increases during PCR and therefore restricts the achievable sensitivity.[43–45]

Some other formats use the increasing quench as indicator for product accumulation.[46–47] In this case, the fluorescence is quenched by a guanine residue of the PCR product. These probes are comparatively inexpensive and easy to construct; however, measurement of the decrease of a signal is problematic, especially during the early exponential phase in which only very few probes are quenched.

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Quantitative Analyses

For quantitative analysis, the amplification curves are evaluated. The amplification process is monitored either through the fluorescence of dsDNA-specific dyes (like SYBR Green I) or of sequence-specific probes. Each curve consists of at least three distinct phases: 1) an initial lag phase in which no product accumulation can be measured, 2) an exponential phase, and 3) a plateau phase. The exponential phase in principle could be extrapolated to the start of the reaction (Cycle 0) to calculate the template copy number, but the error would be too high. The template copy number can be estimated with greater precision from the number of cycles needed for the signal to reach an arbitrary threshold (Figure 2). The threshold must intersect the signal curve in its exponential phase, in which the signal increase correlates with product accumulation. The intersection point is the so-called threshold value (C_T) or crossing point (C_P). This point may be between two successive cycles (i.e. it may be a fractional number). For exact quantifications, the efficiency of the amplification reaction must be known. It is crucial that the amplification efficiencies of standards and unknowns are identical. The efficiency can be estimated from the C_T values of samples with known template concentrations ('standards') as described below.

During the exponential phase, the signal S can be described by Equation 1:

$$S = pN_0\varepsilon^c \quad (1)$$

where p is a proportionality factor to relate PCR product concentration and signal intensity, N_0 is the amount of template, ε is the amplification efficiency ($1 \leq \varepsilon \leq 2$; $\varepsilon = 2$ means 100% efficiency) and c is the cycle number.

Solving for c results in Equation 2:

$$c = -(\log\varepsilon)^{-1}(\log N_0 + \log p - \log S) \quad (2)$$

With $m = -(\log\varepsilon)^{-1}$ and $b = -(\log\varepsilon)^{-1}(\log p - \log S)$, Equation 2 simplifies to Equation 3:

$$c = m\log N_0 + b \quad (3)$$

This equation describes the linear relationship between the C_T values determined and the log of the template concentration (N_0). The parameters m and b can be determined by a regression analysis of the C_T values of the standards. When solved for N_0, this equation serves as a calibration curve for the calculation of the unknowns according to Equation 4:

$$N_0 = 10^{(C_T - b)/m} \quad (4)$$

The efficiency can be calculated from the parameter m by using Equation 5:

$$\varepsilon = 10^{-1/m} \quad (5)$$

By inserting ε back into Equation 4, one obtains Equation 6:

$$N_0 = \varepsilon^{(b - C_T)} \quad (6)$$

The maximum value for ε is 2.0 (i.e. the amount of product is doubled in each cycle). The experimental value for ε usually varies between 1.5 and 1.9. Lower efficiencies limit the sensitivity of the assay but allow quantifications with higher precisions. Therefore, reactions should be optimised for high efficiency. The effect of the efficiency on the precision, however, is not pronounced.

With more than six orders of magnitude, the dynamic range of this procedure is extraordinarily high.[48–50] The accuracy of this technique is limited by the precision of the determination of the C_T values. The error of the C_T values results from the signal noise and the C_T calculation method. In highly optimised assays, standard errors of less than ± 0.2 cycles can be achieved. By assuming an amplification efficiency of 2 (i.e. 100%), this implies that the minimum relative error for the quantification is about 10–20%. The effects of different analysis and calculation methods and the effects of amplification-independent signal trends on the accuracy and precision of quantifications by real-time PCR are described in detail in papers by Lui et al.[51] and Wilhelm et al.[52]

Quantification is relative to the standard used. Only when the absolute concentration of the template molecules in the standard sample is known can the results be absolute. However, in most cases, determination of absolute concentrations is not required. That real-time PCR allows absolute quantification is demonstrated in principle by the reported determination of genome sizes.[26]

All quantifications by PCR are relative—either to a standard or to a reference gene. Interestingly, Equation 6 nicely illustrates the relative character of the quantifications using a dilution series of a standard; the meaning of the parameter b is the expected C_T value of a sample with 'one' copy (or any other unit as defined by the operator). The difference of this value minus the C_T value determined for the unknown sample ($\Delta C_T = b - C_T$) is a direct measure for the relative difference in template concentrations of the unknown and standard.

To analyse relative changes in transcript levels, the chosen standard is usually a reference transcript, for example from a housekeeping gene, itself with unknown template concentration. The calculation of ΔC_T values between reference and sample transcript in a reference and a test sample then provides a simple tool to estimate relative changes. The derivation, assumptions and applications of the so-called $2^{\Delta\Delta C_T}$ method are described elsewhere by Livak et al.[53] The results of this method are only semiquantitative because the efficiency ε is assumed to be 2.0 in all experiments and for all templates, which is at best an optimistic estimate. More precise results are obtained with a procedure introduced by Pfaffl et al.,[54] which includes a measured value for ε.

In general, care must also be taken for accurate quantifications with external standardization, especially with respect to polymerase inhibitors, which may be present in different concentrations in the unknowns and standards. This problem is circumvented by internal standardization. Here, an analytically distinguishable standard template ('competitor') is added to the

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sample and co-amplified in the same reaction.[55, 56] The direct and simultaneous quantitative analysis of both products in real-time PCR also poses problems. These difficulties are mostly due to the fact that different fluorophors have to be used to distinguish the sequences of competitor and sample. As a result of different FRET and quantum efficiencies, the C_T values obtained for competitor and sample are not directly comparable. The problem of where to set the threshold makes relative quantifications difficult if not impossible. However, a simple trick can be used to combine the advantages of both methods:[36] the reaction mixtures are prepared in duplicate. To one of these mixtures, the probe specific for the competitor sequence is added, whereas the probe specific for the sample sequence is added to the other mixture. This process is carried out for a series of reactions with different amounts of competitor added. With this procedure, two calibration lines are obtained and the intersection of the two lines is the equivalence point.

Melting Curve Analyses

Melting curves represent the temperature dependence of the fluorescence (Figure 3). They are recorded subsequent to the amplification of the target sequence by PCR. The detection can be performed either with dsDNA-specific dyes like SYBR Green I or with sequence-specific probes such as the molecular beacons and the hybridisation probes (scorpion and sunrise primers cannot be used for melting curve analysis because they are integrated into the PCR products; *Taq*Man probes cannot be used for melting curve analyses either, since their signal generation depends on the hydrolysis of the probe). Melting curves of sequence-specific probes are used for genotyping, resolving single base mismatches between target sequence and probe,[30, 42] whereas SYBR Green I is used most frequently for product characterization.[4] It has been reported that melting curves measured with SYBR Green I can also be utilized for genotyping of insertion/deletion polymorphisms[57] and of single nucleotide polymorphisms (SNPs).[58]

In melting curves, the signal decreases gradually as a result of a temperature-dependent quench and more abruptly at a certain temperature because of the melting of the products (dsDNA or ssDNA/probe hybrid; Figure 3 A). The melting temperature (T_m) of a product is defined as the temperature at which the steepest decrease of signal occurs. This can be identified conveniently as the peak value(s) (global or local maxima) in the negative derivative of the melting curve (Figure 3 b). Additionally, the area under the curve (AUC) of the peaks is proportional to the amount of product. Therefore, melting curve analysis may be used for quantifications with internal standardization when the T_m values of sample and competitor products are significantly different.[59] However, well-performed normalization is required to reduce the systematic error due to the temperature-dependent quench. This quench also limits the sensitivity of melting curve analyses. At present, there is only one software package available that can remove the quench effects from the data.[52]

With SYBR Green I, the amplification of the correct target sequence can be confirmed. In most cases, nonspecific products have different lengths and therefore deviating melting temperatures.[4] Hybridisation probes, molecular beacons and *Taq*Man probes are used for mutation detection,[28, 30, 60] genotyping[42, 61–63] and SNP screening.[64, 65]

Applications

Real-time PCR is used for absolute and relative quantifications of DNA and RNA template molecules and for genotyping in a variety of applications.

Quantitative real-time PCR is used to determine viral loads,[66] gene expression,[56, 67] titers of germs and contaminations (in food, blood, other body fluids and tissues),[68–71] allele imbalances[72] and the degrees of amplification and deletion of genes.[73, 74]

Real-time PCR is also becoming increasingly important in the diagnosis of tumours, such as for the detection and monitoring of minimal residual diseases,[50, 75–78] the identification of micrometastases in colorectal cancer,[79] neuroblastoma[80] and prostate cancer.[81] It has been used to quantify amplifications of oncogenes[82–85] as well as deletions of tumour suppressor genes[86] in tumour samples. Also, the response of human cancer to drugs has been studied.[87–89] Other clinically relevant applications are cytokine mRNA profiling in immune responses[90, 91] and tissue-specific gene expression analysis.[92–94] Also, the results of DNA chip experiments are validated by real-time PCR quantifications.[95–97]

Chimerism analysis is possible when sequence-specific probes are utilized to differentiate and quantify alleles. High dynamic ranges can be achieved with allele-specific real-time PCR.[98] Robust chimerism analyses with extremely large dynamic ranges based on insertion/deletion polymorphisms and on SNPs are also possible.[99, 100] Genetic chimerisms have been monitored by Y-chromosome-specific real-time PCR for sex-mismatched transplantations[101–103] and by allele-specific real-time PCR.[98, 100] This combination of allele-specific amplification with real-time PCR has been shown to reveal detection limits of down to 0.01 % for SNPs.[100] Real-time PCR is increasingly used in forensic analyses,[104–106] but also to monitor disease- or age-related accumulation of deletions in the mitochondrial genome.[107, 108]

Melting curve analyses are used for real-time competitive PCR,[59, 85] gene dosage tests[72] and genotyping and SNP detection.[63, 109, 110] These applications will have a particularly strong impact on pharmacogenetics.[111] Profiling of DNA methylation is also possible by melting curve analysis,[112, 113] which simplifies the analysis of epigenetic variations of the genome and developmental processes.

Summary and Outlook

Real-time PCR techniques have had an will continue to have a substantial impact on life sciences for several reasons: the robust assays are fast and easy to perform; the risk of carry-over contamination is minimal because of the closed-tube formats of the analyses; post-PCR processing is not required; and the results obtained have a high precision, provided that the evaluation is done correctly. The evaluation of the data is still a

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limiting factor for reliability and precision of the results. However, as a result of the increasing market competition, more robust and user-friendly software will be provided. Additionally, the instrumentation and the chemistry involved will become more economical.

Quantitative analyses with precisions of less than 15% within a dynamic range of more than six orders of magnitude make this technique a valuable tool in nearly all investigations in which the amounts or concentrations of known nucleic acid target sequences in biological samples have to be determined. Combination with sequence-specific detection allows rapid and quantitative genotyping. By using allele-specific primers for real-time PCR, quantitative chimerism analyses are possible with detection limits of 0.01%. Melting curve analyses, performed directly after the PCR, allow product characterization, genotyping, mutation detection, competitive analyses and gene-dosage analyses.

The advantages of real-time PCR are exploited in clinical diagnosis and the monitoring of infectious diseases and tumours. The technique is applied for the analysis of age-dependent diseases, cytokine and tissue-specific expression, forensic samples, epigenetic factors like DNA methylation and for food monitoring. The field of applications is still growing rapidly, which suggests that real-time PCR will become one of the most important techniques in molecular life sciences and medicine.

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Keywords: FRET (fluorescence resonance energy transfer) · melting curve analysis · nucleic acids · polymerase chain reaction · *Taq*Man

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