

Real-time PCR in Food Science: Introduction

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Abstract

Food safety and quality control programmes are increasingly applied throughout the production food chain in order to guarantee added value products as well as to minimize the risk of infection for the consumer. The development of real-time PCR has represented one of the most significant advances in food diagnostics as it provides rapid, reliable and quantitative results. These aspects become increasingly important for the agricultural and food industry. Different strategies for real-time PCR diagnostics have been developed including unspecific detection independent of the target sequence using fluorescent dyes such as SYBR Green, or by sequence-specific fluorescent oligonucleotide probes such as TaqMan probes or molecular beacons.

Introduction

Kleppe *et al.* described the principles of polymerase chain reaction (PCR) for first time in 1971 (Kleppe *et al.*, 1971). But it was in 1985, with the introduction of a thermostable DNA polymerase, that Saiki and collaborators working in Cetus Corporation published the first experimental data using this technique (Saiki *et al.*, 1985, 1988; Mullis *et al.*, 1986). The discovery of PCR is attributed to a collaborator of Saiki, Kary B. Mullis, winner of the Nobel Prize in Chemistry in 1993. Since then, PCR has been used in more than 300,000 scientific publications (search 'PCR' in pubmed.com). This technique has been applied in different areas due to its versatility, specificity and sensitivity. Accordingly, PCR has been successfully used for microorganism identification (Rodríguez-Lázaro *et al.*, 2007; Rodríguez-Lázaro, 2013), for the detection of ingredients of food products (Hernandez *et al.*, 2005) and different animal and fish species (Chisholm *et al.*, 2005; Rodríguez-Lázaro, 2013).

The principle of PCR

PCR is a simple, versatile, sensitive, specific and reproducible assay (Saiki *et al.*, 1988; Cha *et al.*, 1993). It consists of an exponential amplification of a DNA fragment, and its principle is based on the mechanism of DNA replication *in vivo*: dsDNA is denatured to ssDNA, duplicated, and this process is repeated along the reaction according to the following formula (Higuchi *et al.*, 1993; Innis *et al.*, 1999).

$$C = C_0(1 + E)^n$$

where C is the final amount of DNA, C_0 is the initial amount of DNA, E is efficiency ($E = 10^{-1/s} - 1$;

if $E = 1$ then $s = -3.3219$), n is the number of cycles and s is the slope of the exponential phase.

During the denaturation step, the dsDNA melts opening up to ssDNA, and all enzymatic reactions stop (i.e. the extension from a previous cycle). To DNA denaturation, the temperature is usually raised to 93–96°C, breaking the H-bonds and thus increasing the number of non-paired bases. The temperature at which half of the dsDNA is single-stranded is known as the melting temperature, T_m . The type of solvent, the salt concentration and the pH influence the denaturation process. The concentration of G/C and T/A can also affect the T_m value. G/C-rich DNA sequences have higher T_m values compared with those T/A rich. The second phase, i.e. annealing of primers to ssDNA, takes place at temperatures closer to their T_m (usually 55–65°C) and is named as temperature of annealing (T_a). The oligonucleotides used as primers typically consist of relatively short sequences (15–25 nt) complementary to recognition sites, flanking the segment of target DNA to be amplified. Once the temperature is reduced, the two complementary ssDNA chains tend to rehybridize into a dsDNA molecule. In this phase, ionic bonds are constantly formed and broken between the single-stranded primer and the single-stranded template. If primers adequately anneal to the template, the ionic bond is strong enough between the template and the primer to stabilize the nascent double stranded structure and allow the polymerase to attach and begin copying the template. The extension phase is carried out across the target sequence by using a heat-stable DNA polymerase in the presence of dNTPs and $MgCl_2$, resulting in a duplication of the starting target material. This enzyme has 5'→3' DNA polymerase activity, i.e. it adds dNTPs from 5' to 3', reading the template from 3' to 5'. When the primers have been extended a few bases, they possess a stronger ionic attraction to the template, which reduces the probability of unbinding. The duration of the extension step can be increased if the region of DNA to be amplified is long (> 1000 bp); however, for the majority of PCR experiments 1 min is sufficient to get a complete extension.

After each cycle, the newly synthesized DNA strands can serve as template in the next cycle. As shown in Figure 1, the major product of this exponential reaction is a segment of ds-DNA whose termini are defined by the 5' termini of the 2 primers and whose length is defined by the distance between the primers. The products of a successful first round of amplification are heterogeneously sized DNA molecules, whose lengths may exceed the distance between the binding sites of the two primers. In the second round, these molecules generate DNA strands of defined length that will accumulate in an exponential fashion in later rounds of amplification and will form the dominant products of the reaction.

Thus, amplification, as a final number of copies of the target sequence, is expressed by the following equation:

$$(2^n - 2n)x$$

where n is the number of cycles, $2n$ is the first product

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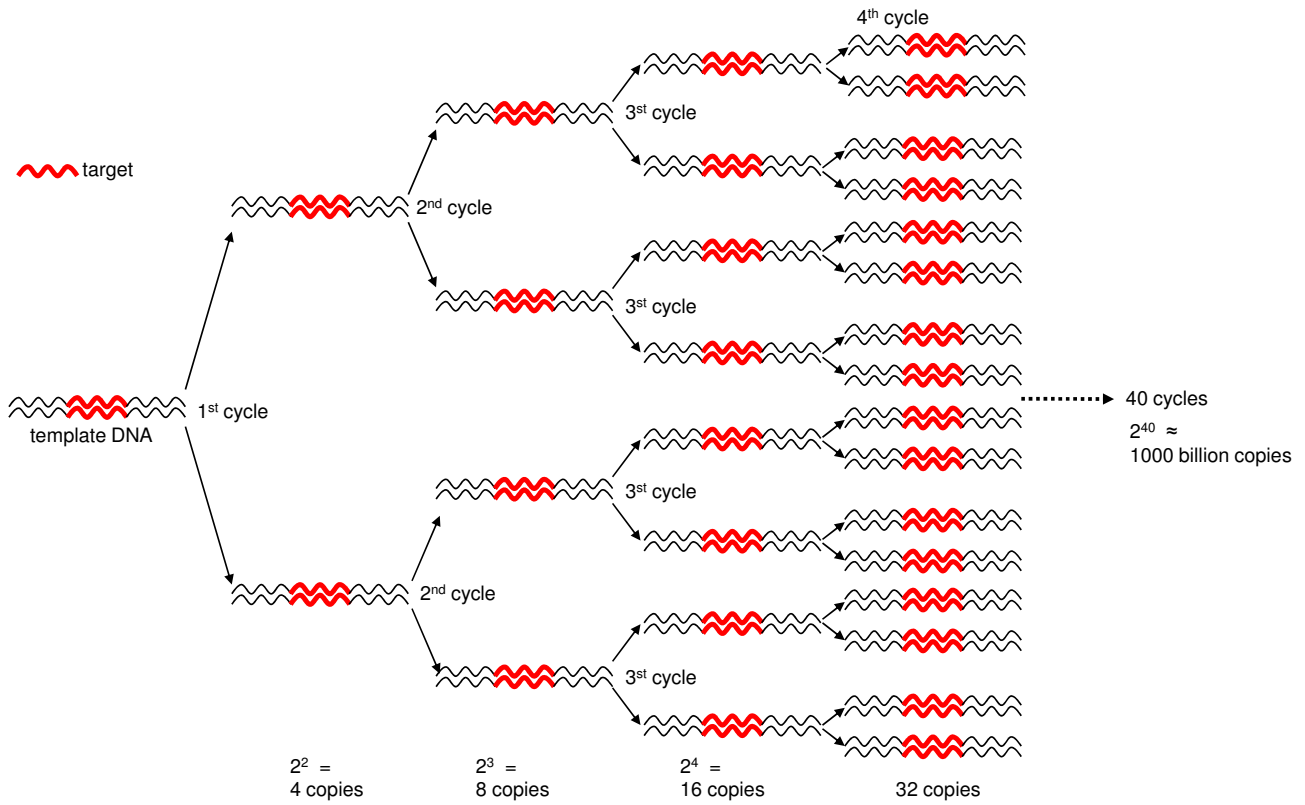


Figure 1. The exponential amplification of DNA in PCR.

obtained after the first cycle and second products obtained after the second cycle with undefined length and x is the number of copies of the original template.

Potentially, after 30 PCR cycles there will be around 2^{30} -fold amplification, assuming 100% efficiency during each cycle. The efficiency of a PCR will vary from template

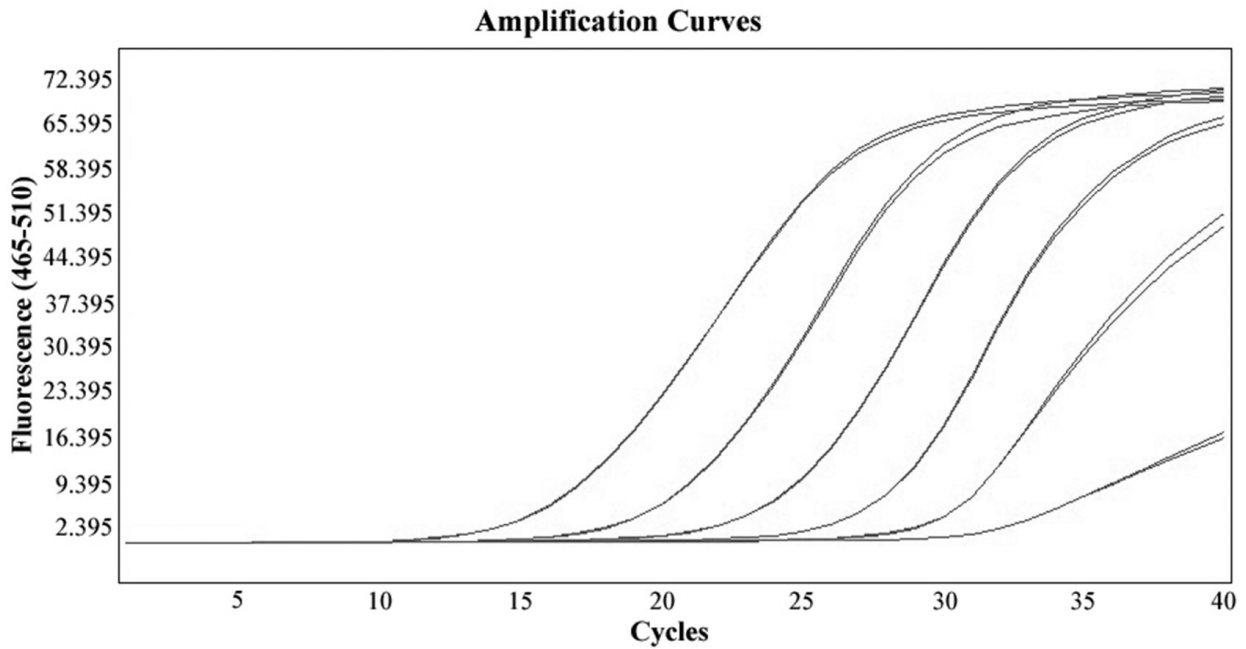


Figure 2. Amplification curves in semilogarithmic view obtained from serial dilutions of a target DNA.

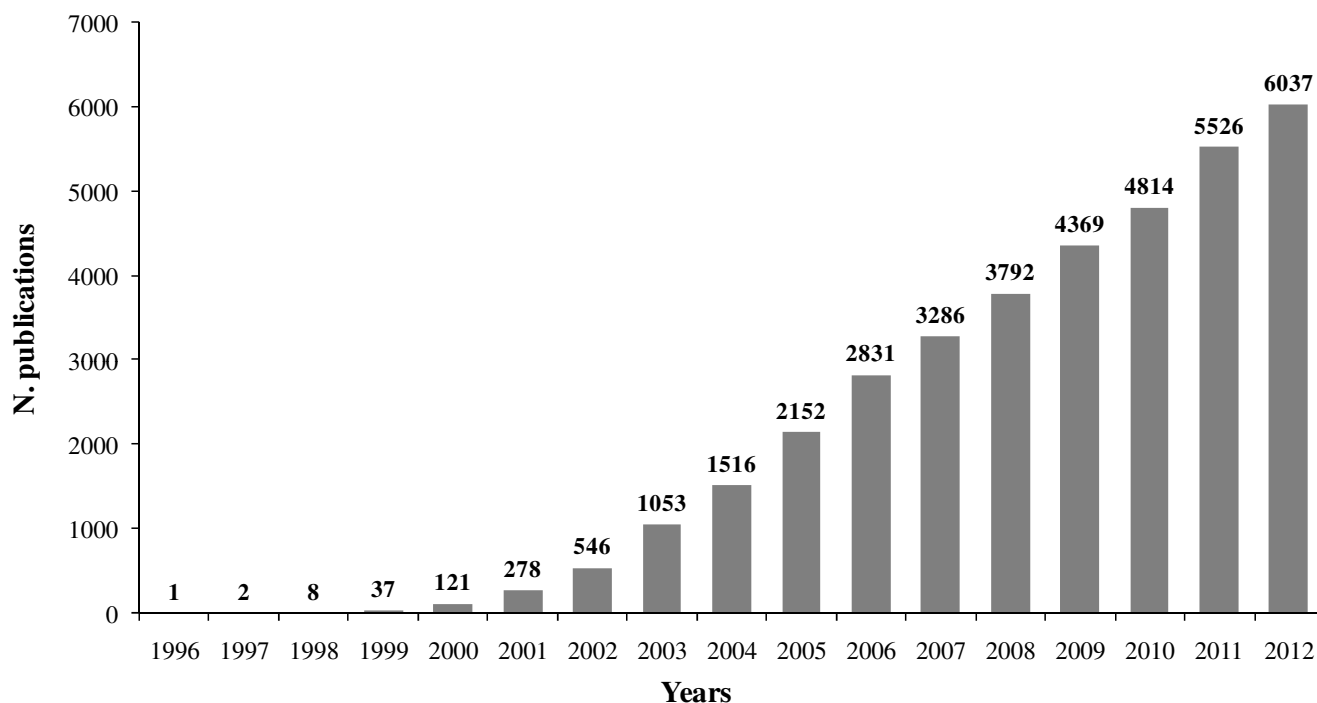


Figure 3. Result of a PubMed search using 'real-time PCR', sorted by year of publication.

to template and according to the degree of optimization that has been carried out.

Multiplex PCR

Whereas standard PCR usually uses one pair of primers to amplify a specific sequence, multiplex PCR allows the simultaneous amplification of more than one target sequence in a single reaction by using a set of primers (Elnifro *et al.*, 2000; Higuchi *et al.*, 1992; Wittwer *et al.*, 2001). This saves considerable time and effort, and decreases the number of reactions that need to be performed to detect the desired targets in the sample. The presence of many PCR primers in a single tube can cause some problems, such as the increased formation of misprimed PCR products, 'primer dimers', and the amplification discrimination of longer DNA fragments (Atlas and Bej, 1994; Higuchi *et al.*, 1992). For multiplex PCR, primers should be chosen with similar annealing temperatures. The lengths of amplified products should be similar; as large differences in the lengths of the target DNAs may favour the amplification of shorter targets over the longer ones, which results in differential yields of amplified products.

Real-time PCR

The development of real-time (q-) PCR represents a significant advance in many molecular techniques involving nucleic acids analysis. qPCR allows monitoring of the synthesis of new amplicon molecules during the PCR (i.e., in real time). Data is therefore collected throughout the PCR process, i.e. monitoring of the process of amplification in real time by using fluorescence, and not only at the end of the reaction (as occurs in conventional PCR) (Heid *et al.*, 1996; Higuchi *et al.*, 1993; Nazarenko *et al.*, 1997). The qPCR results consist of amplification curves (Figure 2),

that can be used to quantify the initial amounts of template DNA molecules with high precision over a wide range of concentrations (Schmittgen *et al.*, 2000). Major advantages of qPCR are the closed-tube format (that avoids risks of carry-over contamination), fast and easy to perform analysis, the extremely wide dynamic range of quantification (more than eight orders of magnitude) (Heid *et al.*, 1996) and the significantly higher reliability and sensitivity of the results compared to conventional PCR. qPCR can be used for many different purposes like conventional PCR, particularly for quantifying nucleic acids and for genotyping. Since the first scientific work published in 1996 (Heid *et al.*, 1996), the number of publications where qPCR is used has increased nearly exponentially (Figure 3).

Chemistries or detection formats

The fluorescence that is monitored along all the qPCR process can be detected by a unspecific detection strategy independent of the target sequence, e.g. through fluorescent dyes that have special fluorescent properties when bound to dsDNA, or by sequence-specific fluorescent oligonucleotide probes, i.e. a sequence-specific strategy.

Unspecific detection formats

Ethidium bromide was the first dye used for this purpose (Higuchi *et al.*, 1993; Le Pecq and Paoletti, 1966; Wittwer *et al.*, 1997). Other intercalating dyes such as YO-PRO-1, BEBO or SYBR Green I have since been used (Bengtsson *et al.*, 2003; Ishiguro *et al.*, 1995; Tseng *et al.*, 1997). SYBR Green I is the most frequently used dsDNA-specific dye in qPCR. It is an asymmetrical cyanine dye, structurally related to the dsDNA-specific dyes YOYO-1 and TOTO-1 (Jin *et al.*, 1994; Singer *et al.*, 1994). Its binding affinity is more than 100 times higher than that of ethidium bromide. SYBR Green

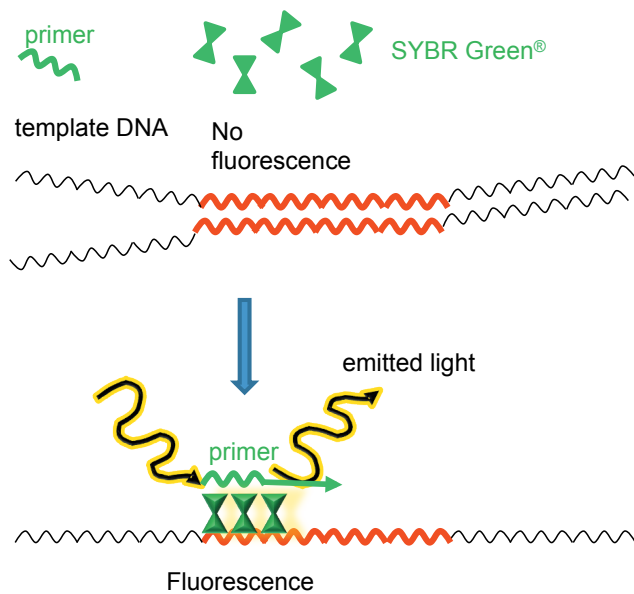


Figure 4. Principle of detection using Sybr Green.

It largely binds sequence independently to the minor groove of ds-DNA. It can be excited with blue light with a wavelength of 480 nm, and its emission spectrum is comparable to that of fluorescein with a maximum at 520 nm and a quantum yield of 0.8 (Jin *et al.*, 1994). The fluorescence of the bound dye is above 1000-fold higher than that of the free dye and, therefore, is well suited for monitoring the product accumulation during PCR (Wittwer *et al.*, 1997; Morrison *et al.*, 1998). When monitored in real time, this results in an increase in the fluorescence signal that can be observed during the polymerization step, and that falls off when the DNA is denatured (Figure 4). Consequently, fluorescence measurements have to be performed at the end of the elongation step of every PCR cycle. This method obviates the need for target-specific fluorescent probes, and hence it can be used with any pair of primers for any target, making its use less expensive (Giulietti *et al.*, 2001). However, its major disadvantage is that specificity is determined entirely by the primers and thus the risk of amplifying non-specific PCR products has to be considered during optimization (Simpson *et al.*, 2000). However, PCR product verification can be achieved at the end-point by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon (Ririe *et al.*, 1997).

The *AmpliFluor* system is an unspecific detection system developed by Intergen co. AmpliFluor technology uses a universal energy-transfer hairpin primer (UniPrimer) which emits a fluorescent signal when unfolded during its incorporation into an amplification product. The UniPrimer contains a 18-nucleotide sequence (Z sequence: 5'-act gaa cct gac cgt aca-3') at its 3' end, that is also present at the 5' end of one of the target-specific primers so that it anneals to the PCR product and acts as universal PCR primer. In the first step, 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 (Nazarenko *et al.*, 1997) (Figure 5).

Sequence-specific fluorescent oligonucleotide probes

There are different types of specific-sequence fluorescent probes, and they can be classified into two major groups, *hydrolysis probes* and *hybridization probes*, both types being homologous to the internal region amplified by the two primers. The fluorescence signal intensity can be related to the amount of PCR product (i) by a product-dependent decrease of the quench of a reporter fluorophore or (ii) by an increase of the fluorescence resonance energy transfer (FRET) from a donor to an acceptor fluorophore. FRET, also called Förster transfer, is the radiationless transfer of excitation energy by dipole-dipole interaction between fluorophores with overlapping emission and excitation spectra. The FRET and the quench efficiency are strongly dependent on the distance between the fluorophores (Förster, 1948; Clegg, 1992). Therefore, the PCR-product-dependent change in the distance between the fluorophores is used to generate the sequence-specific signals. Several different formats can be 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 fluorescent reporter dyes are FAM, TET (tetrachloro-6-carboxyfluorescein), JOE (2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein)

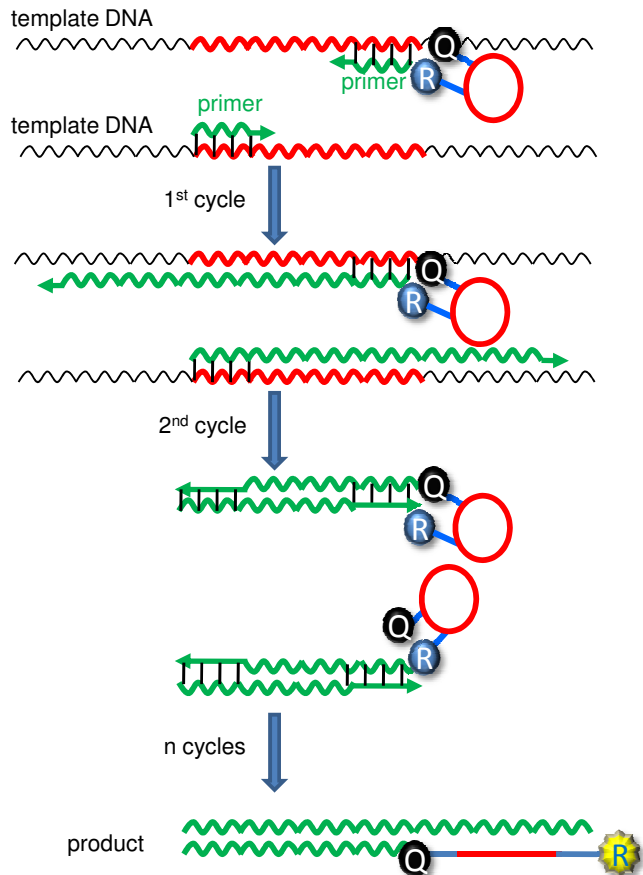


Figure 5. Principle of detection using AmpliFluor system. Adapted from Whitcombe *et al.* (1999).

Table 1. Excitation and emission spectre of different fluorophores

Fluorophores	Excitation (nm)	Emission (nm)
DABCYL	453	–
6-FAM	492	515
SYBR Green I	497	520
TET	521	536
JOE	527	548
HEX	535	556
TAMRA	555	580
ROX	575	602
VIC	538	554

or HEX (hexachloro-6-carboxyfluorescein), and the most frequently used quenchers are TAMRA, DABCYL and Black Hole Quencher (BHQ) (Table 1). Many commercial fluorophores have been developed by biotech companies such as VIC, Alexa Fluor, or Yakina Yelow.

Sequence-specific probes allow multiplexing (Bernard *et al.*, 1998; Dupont *et al.*, 2002; Wittwer *et al.*, 2001) and easy identification of point mutations (Giesendorf *et al.*, 1998; Lay and Wittwer, 1997; Lyon, 2001). A common drawback of probe systems that use the decrease-of-quench mechanism is unwanted generation of a signal due to probe 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 (Wilhem and Pingoud, 2003).

Hydrolysis probes

The hydrolysis probes are cleaved when hybridized by the 5'–3' exonuclease activity of particular DNA polymerases (Holland *et al.*, 1991) during the elongation phase of primers, yielding a real time measurable fluorescence emission directly proportional to the concentration of the target sequence (Heid *et al.*, 1996). It usually utilizes either *Taq* or *Tth* polymerase, but any enzyme with equivalent

5'–3' exonuclease activity properties (e.g. *Tth*) can be used (Gut *et al.* 1999). The best known hydrolysis probes are TaqMan® probes and TaqMan® MGB (minor groove binder) probes, both developed by Applied Biosystems.

A *TaqMan probe* is an oligonucleotide double-labelled with a reporter fluorophore at the 5' end (reporter dye) and with a quencher internally or at the 3' end (quencher dye). In addition, the probes must be blocked at their 3'- end to prevent the extension during the annealing step. The TaqMan assay (Figure 6) uses three oligonucleotides. Two conventional primers allow amplification of the product, to which the TaqMan probe will anneal. The quencher dye absorbs the fluorescence of the reporter dye due to its proximity, which permits FRET. When the correct amplicon is amplified, the probe can hybridize to the target after the denaturation step. It remains hybridized while the polymerase extends the primers until it reaches the probe. Then, it displaces its 5' end to hold it in a forked structure. The enzyme continues to move from the now free end to the bifurcation of the duplex, where cleavage takes place (Lyamichev *et al.* 1993). The quencher is hence released from the fluorophore, which now fluoresces after excitation (Heid *et al.*, 1996; Holland *et al.*, 1991; Gibson *et al.*, 1996). As the polymerase will cleave the probe only while it remains hybridized to its complementary strand, the temperature conditions of the polymerization phase of the PCR must be adjusted to ensure probe binding. Most probes have a T_m of around 70°C; therefore, the TaqMan system uses a combined annealing and polymerization step at 60–63°C. This ensures that the probe remains bound to its target during the primer extension step. It also ensures maximum 5'–3' exonuclease activity of the *Taq* and *Tth* DNA polymerases (Tomblin *et al.* 1996).

The *TaqMan MGB probes* are similar to TaqMan probes. They contain a non-fluorescent quencher (NFQ) and an oligopeptide at the 3' end. This oligopeptide is a DNA minor groove binder (MGB), with very high affinity for the minor groove of AT-rich double-stranded DNA (Afonina *et al.*, 1997). Addition of the MGB ligand significantly enhances duplex stability. The shorter the probe, the greater the MGB contribution to the overall duplex stability: with 12- to 18-bp oligonucleotides, it raises the T_m from 44–56°C up to 66–70°C (Kutyavin *et al.*, 2000). This allows designing suitable

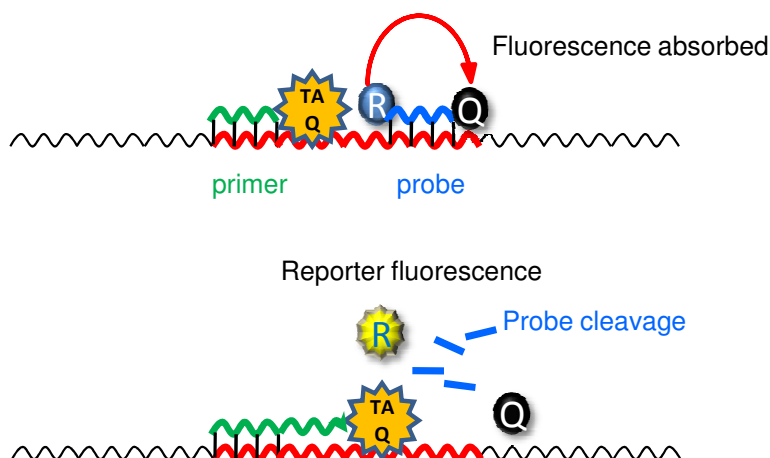


Figure 6. Principle of detection using TaqMan probes. 5'–3' polymerase and exonuclease activity of the Taq DNA polymerase (TAQ). R, reporter; Q, quencher.

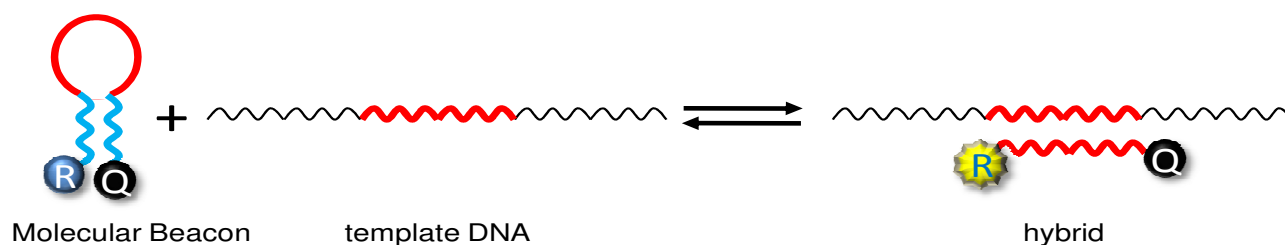


Figure 7. Principle of detection using Molecular Beacons. During hybridization PCR step, the fluorophore and quencher components of molecular beacons become spatially separated and the fluorescence is generated. R: reporter; Q: quencher.

probes in sequences such as those rich in AT, in which conventional TaqMan probes require an excessive length.

Hybridization probes

In contrast to hydrolysis probes, hybridization probes are not hydrolysed during PCR. The fluorescence is generated by a change in its secondary structure during the hybridization phase, which results in an increase of the distance separating the reporter and the quencher dyes. The most relevant hybridization probes are those containing hairpins (Molecular Beacons, Scorpion primers, etc.), and FRET hybridization probes.

Molecular beacons form a stem-and-loop structure through complementary sequences on the 5' and 3' ends of the probe (Tyagi and Kramer 1996). The loop portion is complementary to the target nucleic acid (Tan *et al.*, 2000). A reporter and a quencher fluorophore are attached one at the end of each arm. The quencher is a non-fluorescent chromophore that dissipates the energy it receives from the fluorophore as heat. The fluorescence is quenched when the probe is in a hairpin-like structure (stem-and-loop structure) due to the proximity between quencher and fluorophore allowing FRET (Cardullo *et al.*, 1998; Stryer, 1978). In the presence of a complementary sequence, designed internal to the primer binding sites, the probe undergoes a conformational transition that forces the stem apart and

results in the formation of a probe/target hybrid that is more stable than the former stem (Bonnet *et al.*, 1999). This conformational change separates the fluorophore from the quencher and consequently FRET no longer occurs, thus increasing reporter fluorescence emission (Kostrikis *et al.*, 1998; Tyagi *et al.*, 1998) (Figure 7). Molecular beacons have been reported to be significantly more specific than conventional oligonucleotide probes of equivalent length, due to the presence of a stem structure (Bonnet *et al.*, 1999; Kaboev *et al.*, 2000; Tyagi *et al.*, 1998). The main drawback of molecular beacons is associated with its design as the fluorescence yield is very sensitive to the hybridization conditions.

FRET probes or *hybridization probes* use four oligonucleotides, i.e. two primers and two sequence-specific probes (Wittwer *et al.*, 1997). Each probe has a single label: either a donor fluorophore at the 3'-end or an acceptor fluorophore at the 5'-end. The emission spectrum of the donor fluorophore overlaps the excitation spectrum of the acceptor fluorophore. The FRET probes must be blocked at their 3'-end to prevent the extension during the annealing step. The two probes hybridize to the target sequences in a head-to-tail arrangement, thus bringing the two dyes close (typically 1–5 nucleotides distant), allowing FRET. During PCR, only the donor fluorophore is excited. In solution, only background fluorescence is emitted by

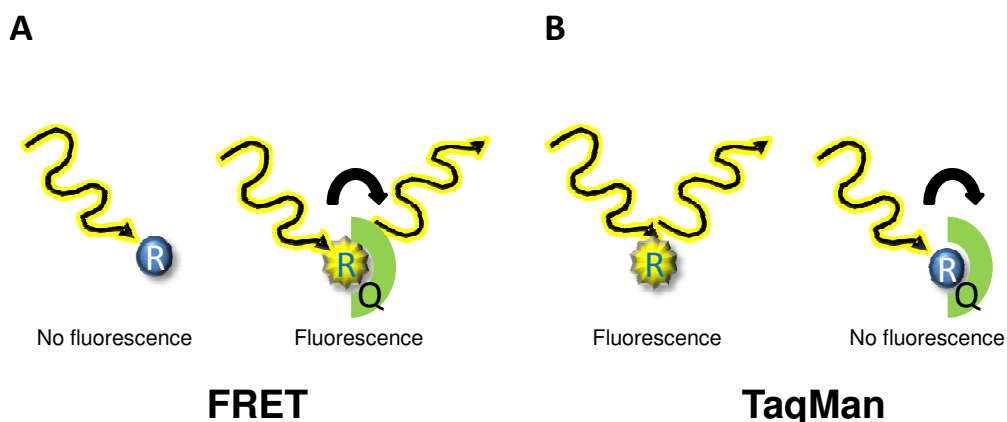


Figure 8. Differences between detection using FRET probes and TaqMan probes. A. Energy transfer in FRET probes (the e^- acceptor emits fluorescence when placed close to the e^- donor). B. Energy transfer in hydrolysis probes (TaqMan) (the e^- donor emits fluorescence when the distance to the acceptor or quencher is higher than 10 nm. R: reporter).

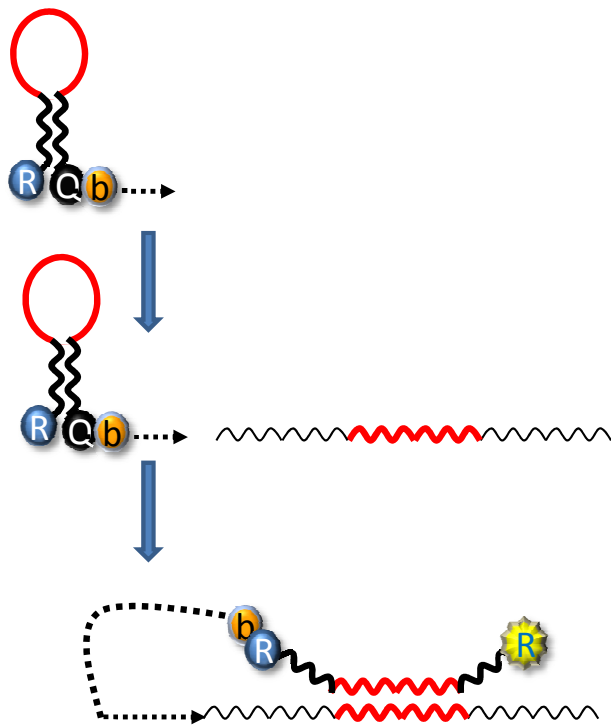


Figure 9. Principle of detection using Scorpion primers. R: reporter; Q: quencher; b: blocker. Adapted from Whitcombe *et al.* (1999).

the donor. During annealing, the two probes hybridize adjacently to their target sequence and thus the excitation energy is transferred by FRET from the donor dye in one of the probes to the acceptor dye in the other probe (Figure 8), allowing the acceptor dye to dissipate fluorescence at a different wavelength (Bernard and Wittwer, 2000). The use of two independent probes results in high specificity and flexibility for probe design. Furthermore, as the probes are not hydrolysed, fluorescence is reversible and allows the generation of melting curves.

Scorpion primers are structurally and functionally related to molecular beacons, but serve as primers in the PCR. They consist of a probe sequence linked to the 5' end of a primer via a non-amplifiable stopper moiety (Whitcombe *et al.*, 1999). The probe presents a fluorophore linked at the 5'-end and a quencher at the 3'-end, and is held in a hairpin loop structure by complementary sequences on its 5'- and 3'-ends. This configuration brings the fluorophore in close proximity with the quencher and avoids fluorescence similarly to Molecular Beacons. In addition, the probe sequence is complementary to an internal region of the sequence extended by the adjacent primer. In the first step, the primer is extended, yielding a single-stranded template for the reverse primer in the second step. Upon hybridization, the hairpin is opened, producing a physical separation of the fluorophore and quencher such that increases in signal are observed (Figure 9). 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 (Whitcombe *et al.*, 1998, 1999).

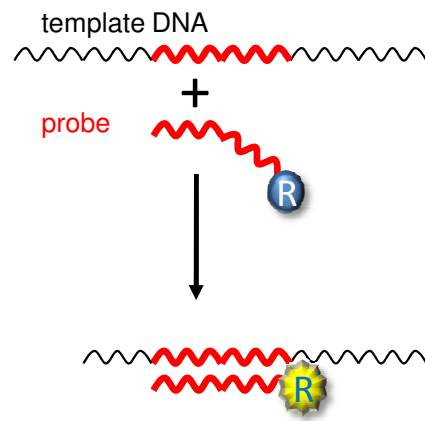


Figure 10. Principle of detection using light-up probes. R: reporter.

The *light-up technology* utilizes a nucleic acid analogue instead of natural DNA as sequence recognizing element (Isacsson *et al.*, 2000). Light-up probes are peptide nucleic acids (PNAs) (Nielsen *et al.*, 1991) that use thiazole orange, a derivative of cyanine, as reporter fluorophore (Svanvik *et al.*, 2000a). It forms sequence-specific complexes with DNA and RNA which are more stable than double-stranded natural nucleic acids (Iyer *et al.*, 1995; Egholm *et al.*, 1993). These features are attributed mainly to the charge-neutral nature of PNA, which eliminates the electrostatic repulsion between the hybridizing strands. The probe has low fluorescence when free in solution, however they show increased fluorescence intensity upon hybridization with DNA (Figure 10).

Real-time PCR design

There are already defined prerequisites to design TaqMan oligonucleotides, and most of them can be also applied to other probes. They are summarized in Table 2.

When a qPCR assay is designed, the most important parameters are the amplicon length and the T_m of the primers and probe. The optimal amplicon length should be less than 150 bp, but it is advisable to reduce the length below 80 bp. However, amplicons up to 300 bp amplify efficiently. Shorter amplicons amplify more efficiently than longer ones and are more tolerant to suboptimal reaction conditions. This is because they are more likely to be denatured during the 92–95°C PCR step, allowing the probes and primers to compete more effectively for binding to their complementary targets. As the extension rate of *Taq* polymerase is between 30 and 70 bases per second (Jeffreys *et al.*, 1988), polymerization times as short as 5 s are sufficient to replicate such amplicon, making amplification of artefacts less likely and reducing the time of the assay. Primers are generally used in the 50–300 nM range. Higher concentrations may promote mispriming and accumulation of non-specific products, and lower concentrations may lead to primer exhaustion, although target copy numbers will have been calculated well before. Non-specific priming can be minimized by selecting primers that have only one or two G/Cs within the 3' last five nucleotides. A relative instability at the 3' ends

Table 2. Requisites for the design of oligonucleotides for RTi-PCR assays

Parameter	Primers	Probes
GC content (%)	30–80%	30–80%
Calculated T_m	50–60°C, always >55°C as UNG works at 50°C T_m of the primers should not differ >2°C	68–70°C (5–10°C above primer T_m)
Runs of identical nucleotides	Maximum 3 (no Gs!!)	Maximum 3 (no Gs!!)
Sequence length	Minimum 15 bp (15–30 bp)	Maximum. 30 bp
Amplicon length	The shorter the better. With TaqMan probes, 50–150 bp	
Distance forward primer to probe	Maximum 50 bp	
Primer dimers, hairpin loops	Avoid	Avoid
3'- instability (3'- rule)	Primers only. Maximum two Gs/Cs in the last 5 bp	
Autoquenching		No G at 5'-end
G–C ratio		Select the strand with C>G

makes primers less likely to hybridize transiently causing non-specific extension.

The T_a of the probe is also a critical parameter. Since amplification primers are extended as soon as they bind to their targets, the hybridization target sequence is rapidly masked with newly synthesized DNA. Therefore, the T_a of the probes must be significantly greater (approximately 10°C) than that of the primers. The presence of G at the 5' end of the probe is to be avoided, because it slightly quenches the reporter signal, even after probe cleavage. Furthermore, the probe should contain more Cs than Gs; if this is not

the case, the antisense probe should be used. The probe should never overlap with, or be complementary to either of the primers. The optimum concentration of fluorogenic probes will vary with the type of probe, as it depends on background fluorescence: quenching of hydrolysis probes is often below 100%, and thus they produce background fluorescence levels higher than molecular beacons and FRET probes.

An additional requirement of the FRET system is that the target sequence should be located towards the 3' end of the amplicon, close to the reverse primer-binding site on

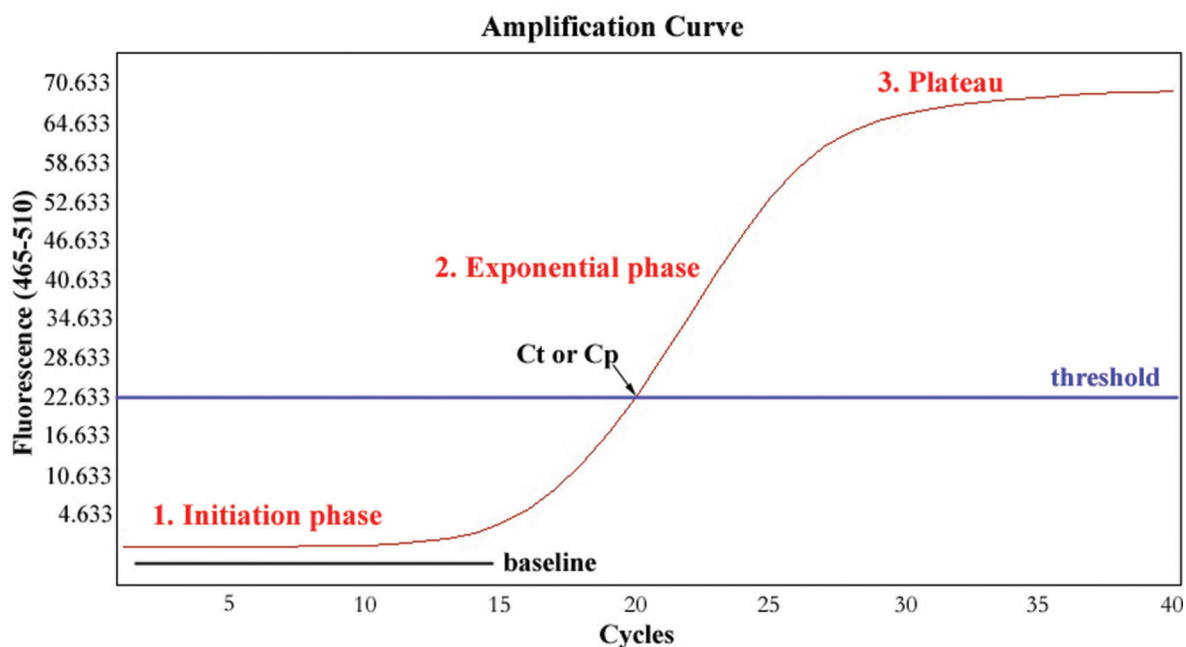


Figure 11. Phases of a PCR amplification curve. Red: amplification curve of a positive sample. Blue: threshold. Black: baseline cycles.

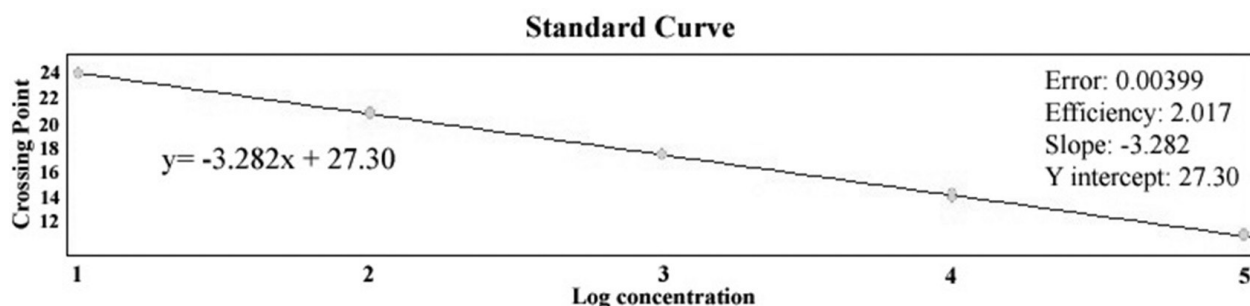


Figure 12. Standard curve built with DNA standards of known concentration (Figure 2). x-axis, initial amount of DNA (number of copies, ng, etc.); y-axis, C_T values.

the opposite strand. As fluorescence is measured during the annealing phase, this arrangement allows the qPCR device to measure fluorescence before the polymerase displaces the probes. Paired probes used for quantification should have equal T_a , whereas for mutational analysis the T_m of the sensor probe should be lower than that of the anchor probe, thus ensuring that the sensor probe controls the generation of the fluorescent signal.

Quantification analysis

As indicated above, the fluorescence emission during qPCR is proportional to the synthesized DNA, and can be visualized as an amplification plot (Figure 2). Typically, an amplification curve presents three different phases (Figure 11). The first is called the *initiation phase*, it occurs during the first PCR cycles where the emitted fluorescence can not be distinguished from the baseline. During the *exponential* or *log phase* there is an exponential increase in fluorescence, before the plateau phase is reached. In this last phase, the reagents are exhausted, and no increase in fluorescence is observed. Only in the exponential phase, quantification is possible.

A fluorophore (so-called passive reference) may be present in the PCR buffer in order to well-to-well normalize the fluorescence (Table 1). This helps to overcome the effects of pipetting errors. This normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the passive reference to obtain a ratio, i.e. R_n (normalized reporter) for any given reaction. At any given cycle, R_n^+ is the R_n value of a reaction containing all components including the template and R_n^- is the R_n value of an unreacted sample obtained from the early cycles of the PCR, i.e. those prior to a detectable increase in fluorescence, or from a reaction not containing template. The ΔR_n is the difference between the R_n at the end point (R_n^+) and at the starting point (R_n^-). It reliably indicates the magnitude of the signal generated by the given set of PCR conditions, and is proportional to the DNA amount during the exponential phase. The fluorescence emitted in the first cycles is used to calculate the baseline. A threshold is established at the fluorescence value of the average standard deviation of R_n for the baseline cycles, multiplied by an adjustable factor (usually 10 times). Alternatively, it can be established by the operator in order to compare different qPCR experiments. The C_P (crossing point, PCR cycle) or C_T (threshold cycle) value is the cycle at which fluorescence achieves a defined threshold. It corresponds

to the cycle at which a statistically significant increase in fluorescence (ΔR_n or non-normalized) is first detected. This concept is the basics for accurate and reproducible quantification using qPCR; the number of cycles needed for the amplification-associated fluorescence to reach a specific threshold level of detection (the C_T or C_P value) is inversely correlated to the amount of nucleic acid that was in the original sample (Walker, 2002). This value always is in the exponential phase of amplification, when amplification is most efficient, and therefore quantification is least affected by reaction-limiting conditions.

The quantity of DNA at the start of the PCR can then be determined by interpolation of the resulting C_T or C_P value in a linear standard curve of values obtained from serially diluted known-amount standards (Figure 12). This standard curve correlates the emitted fluorescence (C_T or C_P value) with the initial concentration of the standards used and the final result is achieved by interpolation of the produced fluorescence (C_T or C_P value) during the amplification of the sample in this standard curve. In practice, such curves are linear over more than five orders of magnitude.

Diagnostic real-time PCR parameters

An international standard guideline (ISO 16140:2003) was prepared by CEN/TC275 in collaboration with the Technical Committee ISO/TC34 to establish the general principle and technical procedure for the validation of alternative methods in the field of microbiological analysis of food, animal feeding stuffs, and environmental and veterinary samples. Many of the performance criteria are well recognized and accepted by numerous internationally recognized organizations for standardization (ISO, EMEA, Codex Alimentarius, etc.). There are a series of concepts that define an analytical method and that are applied to diagnostic qPCR. The main concepts are: linearity, accuracy, precision, sensitivity and specificity.

Linearity can be defined as the ability of the method, when used with a given matrix, to give results that are in proportion to the amount of analyte present in the sample; that is, an increase in analyte corresponds to a proportional increase in the result (Anonymous, 2003). In the same manner, linearity has been defined by Paoletti and Weighardt (2002) as the proportionality of the signal to the amount of reference material, demonstrated by the calculation of a regression line with an adequate statistical method. The European Commission, in the particular case of genetically modified organisms (GMO), has established that

the linearity within the range of applicability of the method should be not less than 98% (Paoletti and Weighardt, 2002).

The accuracy describes the veracity of the test results (Skog and Leary, 1992), and can be defined as closeness of agreement between a test result and the accepted reference value (Anonymous, 1993; Paoletti and Weighardt, 2002; Hoorfar and Cook, 2003). The precision describes the reproducibility of the test results (Skog and Leary, 1992), and can be defined as the closeness of agreement between independent test results obtained under stipulated conditions of repeatability and reproducibility (Anonymous, 1993; Thompson *et al.*, 2002).

Sensitivity is the ability of the alternative method to distinguish two different amounts of analyte (as measured by the reference method) within a given matrix, at a specified average value, or over the whole measurement range; that is, the minimal quantity variation which gives a significant variation of the measurement signal (Anonymous, 2003). The sensitivity differs from detection limit because it is calculated for each value of the measurement range. From a qualitative approach (i.e. for qualitative methods), the sensitivity can also be defined as the ability of the alternative method to detect the analyte when it is detected by the reference method (Anonymous, 2003; Hoorfar and Cook, 2003). The limit of detection is the smallest critical level detected with a probability $(1-\beta)$, which has to be well over 50%, for example 95% (Anonymous, 2003) or 99% (Hoorfar and Cook, 2003; Knutsson *et al.*, 2002). The limit of detection has been also described as the smallest amount or concentration of analyte in the test that can be distinguished from zero (Anonymous, 1997; IUPAC, 1995; Thompson *et al.*, 2002); the smallest number of culturable target microorganisms necessary to create a positive test response (Hoorfar and Cook, 2003), or the minimum level at which the analyte can reliably be detected with a probability of 95% (Paoletti and Weighardt, 2002). The limit of quantification is the smallest amount of analyte which can be measured and quantified with defined precision and accuracy by the method under validation (Anonymous, 2003). The Association of Analytical Communities (AOAC) defines the quantification limit for quantitative methods as: $LOQ = 10SD_0$ (Coleman *et al.*, 1997).

The specificity is the degree to which a method is affected by the other components present in a multi-component sample (Anonymous, 2003; Malorny *et al.*, 2003b). In other words, the ability of a method to measure a given analyte within the sample without interference from non-target components or background noise (Anonymous, 2003). For food microbiology diagnostics other three related concepts have been defined: selectivity, inclusivity and exclusivity. Selectivity is defined as a measure of the degree of non-interference in the presence of non-target analytes (Anonymous, 2003). A method is selective if it can be used to detect the analyte and that a guarantee can be provided that the detected signal can only be a product by the specific analyte. Inclusivity is defined as the ability of an alternative method to detect the target analyte from a wide range of strains, and exclusivity as the lack of interference from a relevant range of non-target strains of the alternative method.

Quality control of the real-time PCR

The qPCR is an instrumental technique, and thus can produce false negative and false positive results. Contamination remains an issue for diagnostic laboratories (Pellet *et al.*, 1999; Scherczinger *et al.*, 1999). The main causes of production of false positive results are the accidental contamination of the samples or the reagents with positive samples (cross-contamination) or with amplification products and plasmid clones (carry-over contamination). To minimize these risks, several practices should be applied (Dieffenbach *et al.*, 1995; Hernández, 2003; Mifflin, 2003). For example, it is advisable to establish space and time separation of pre- and post-amplification activities (Heinrich, 1991; Yap *et al.*, 1994), in particular to separate pre-PCR activities from recombinant DNA activities, as well as to define two different pre-PCR areas: for the preparation of the master mix and for the addition the nucleic acid template to the reaction mixture. Each working area must have its own separate set of equipment, including pipettes, reagents, pipette tips, racks, centrifuges, vortex, lab coats, etc., and lab coats and gloves should be worn at all times, and gloves should be changed frequently. In addition, all reagents should be prepared in large volumes and aliquoted into single-use volumes for storage and free from contaminating nucleic acids and/or nucleases. Other actions to prevent or reduce the risk of PCR contaminations are the use of UV light before carrying out the experiments in the sample preparation lab and in the cabinet where the amplification mixture is prepared, and samples should be manipulated with specialized barrier or positive-displacement pipettes, which prevent the carry-over of aerosols created during pipetting. Finally, the use of uracil-DNA-glycosylase (UDG; also called uracil-*N*-glycosylase or UNG) is recommended, as it can eliminate the contamination arising from PCR products (Longo *et al.*, 1990; Thornton *et al.*, 1992). During amplification, dTTP is substituted with dUTP, so the DNA product will contain dU instead of dT. Subsequent PCR runs are programmed to include a 50°C incubation step with UDG, which will mediate cleavage of any contaminating DNA strands containing dU.

In addition to the prevention measures for avoiding contamination, a suite of controls must be included in order to correctly interpret the results of a qPCR.

Application of real-time PCR for the detection of food-borne pathogens

Bacterial detection and control are two key aspects in food microbiology (Stewart, 1997). Microbiological quality control programmes are increasingly applied throughout food chain production in order to minimize the risk of infection for the consumer. Thus, the availability of reliable, rapid and accepted test systems to detect the presence or absence, or even the degree of contamination of pathogens, becomes increasingly important for the agricultural and food industry. Such systems would also find a place within the framework of legislative control measures. During the last 15 years, PCR has significantly contributed to this field (Rodríguez-Lázaro, 2013).

Traditional and standardized analysis of food for the presence of microorganisms relies on the enrichment and isolation of presumptive colonies of bacteria on agar media, using approved diagnostic artificial media. This is generally followed by biochemical and/or serological identification.

Traditional methods of detection, although reliable and efficient, require several days to weeks before results are produced. Furthermore, phenotypic properties by which the bacteria are identified may not be always expressed; and when expressed, they may be difficult to interpret and classify. Another disadvantage of traditional methods is that cells which are viable but otherwise non-culturable cannot be detected, e.g. some stressed *Campylobacter* spp. (Rollins and Colwell, 1986; Tholozan *et al.*, 1999). Thus, introduction of amplification techniques in microbial diagnostics has been established in research laboratories as a valuable alternative to traditional detection methods. The most extensively used amplification method is PCR, but other methods have also been developed such as NASBA, the Q-beta replicase amplification system and the ligase chain reaction (LCR), although until now, they have had limited practical relevance for food monitoring and control. Speed, excellent detection limit, selectivity, specificity, sensitivity and potential for automation are among the most important advantages of qPCR. These advantages compared to traditional detection methods might well encourage end-users to adopt amplification techniques in routine testing for food-borne pathogens.

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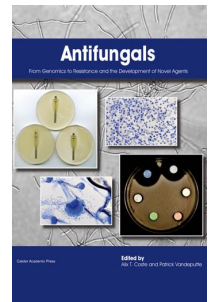
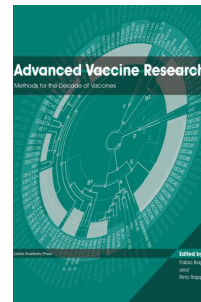
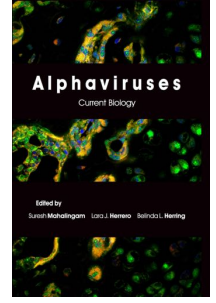
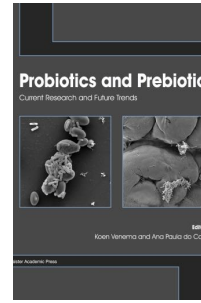
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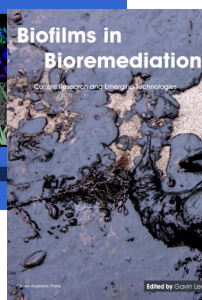
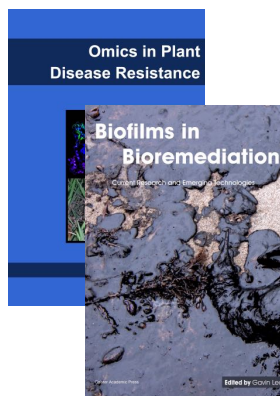
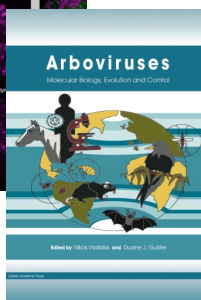
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