IUFoST 2006
DOI: 10.1051/IUFoST:20060643

MOLECULAR METHODOLOGY IN FOOD MICROBIOLOGY DIAGNOSTICS: TRENDS AND CURRENT CHALLENGES

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ABSTRACT

Foodborne diseases are among the most serious public health concerns worldwide. Consequently, the application of microbiological controls within the quality assessment programs in the food industry is a premise to minimize the risk of infection for the consumer. Classical microbiological methods involve, in general, the use of appropriate pre-enrichment and enrichment, isolation on selective media, and subsequent confirmation using morphological, biochemical and/or serological tests. They are laborious, time consuming and not always reliable. A number of alternative, rapid and sensitive molecular methods for the detection, identification and quantification of foodborne pathogens have been developed to overcome these drawbacks. However, until now, they have shown low practical implementation for monitoring and microbiological control. Amplification techniques have a number of advantages including improved speed, excellent detection limit, specificity, sensitivity and potential for quantification as well as easy automation. Major drawbacks are the cost of the analysis and the qualified personnel needed to carry out the experiments, plus the lack of standardisation of protocols approved by normalisation bodies. Moreover, the cost of reagents is decreasing and more qualified people are available to perform this kind of analysis. Efforts on standardisation and normalisation of these techniques are a key priority.

The molecular methods, in conclusion, are a promising alternative that can substitute or complement the current reference methods in food microbiology diagnostics. More suitable and reliable results can be achieved in terms of speed and precision, and can detect and enumerate specifically viable microorganisms.

Foodborne diseases are among the most serious public health concerns all over the world and are a major cause of morbidity (Wallace *et al.*, 2000). Its incidence has been increased considerably during the last decades by the rapid globalisation of the food market, the increase of person and food transports, and the profound changes in the food consumption habbits(Käferstein *et al.*, 1997). More than 200 known diseases are transmitted trough food, with symptoms ranging from mild gastroenteritis to life-threatening syndromes, with the possibility of chronic complications or disability (Mead *et al.*, 1999). The causes of foodborne illness include pathogens, toxins and metals. More than 40 different foodborne pathogens are known to cause human illness (CAST, 1994), among which over 90 % of confirmed foodborne human illness cases and deaths caused by foodborne pathogens reported to the Center for Disease Control and Prevention (CDC) have been attributed to bacteria, being the rest due to fungi, parasites and viruses (Bean *et al.*, 1990). In consequence, microbiological quality control programs are being increasingly applied throughout the food production chain in order to minimize the risk of infection for the consumer. Surveillance systems that include quantification of reported foodborne illnesses and identification of emerging pathogens are needed (Blackburn and McClure, 2002).

The impact of foodborne pathogens in public health systems is very considerable. For example, in the USA, it has been estimated that foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalisations and 5,000 deaths each year, with known pathogens accounting for 14 million illnesses, 60,000 hospitalisations and 1,800 deaths per year (Mead *et al.*, 1999). Only three pathogens, *Salmonella*, *Listeria*, and *Toxoplasma*, are responsible for more than 1,500 deaths each year (Mead *et al.*, 1999). Foodborne illnesses account for around

DOI: 10.1051/IUFoST:20060643

1 % USA hospitalisation cases and 0.2 % deaths (Buzby *et al.*, 2001); with annual medical and productivity losses due to the five major foodborne pathogens estimated at around 6,500 million of dollars (Crutchfield and Roberts, 2000). In England and Wales, foodborne pathogens produce 1.3 million illnesses, 20,759 hospitalisations and 480 deaths each year (Adak *et al.*, 2002).

Classical microbiological methods involve, in general, the use of a non-selective pre-enrichment, selective enrichment, isolation on selective media, and subsequent confirmation using morphological, biochemical and/or serological test. Thus, they are laborious, time consuming and not always reliable (*e.g.* viable but non-culturable VBNC forms). A number of alternative, rapid and sensitive methods for the detection, identification and quantification of foodborne pathogens have been developed to overcome these drawbacks, including bioluminescence-based, immunological and molecular methods (Fung, 2002; Scheu *et al.*, 1998). Among the latter category, PCR has become the most popular microbiological diagnostic method, and recently, the introduction of a development of this technique, RTi-PCR, has produced a second revolution in the molecular diagnostic methodology in microbiology as stated by the increasing number of scientific publications and novel commercial kits available. RTi-PCR is highly sensitive and specific. Moreover, it allows accurate quantification of the bacterial target DNA. Main advantages of RTi-PCR for its application in diagnostic laboratories include quickness, simplicity, the closed-tube format that avoids risks of carryover contaminations and the possibility of high throughput and automation.

The efficiency of the molecular methods can be negatively affected by the presence of inhibitory substances generally existent in foods, and consequently they can produce the underestimation of the bacterial load as well as false negative results. Thus, the development of sample processing procedures is a priority for the implementation of molecular methods as diagnostic tools in food microbiology laboratories. In addition, other fundamental aspects rely on the adequate control of the amplification reaction efficiency. In this sense, the application of internal amplification controls allows the assessment and interpretation of the diagnostic results of the molecular techniques.

MOLECULAR AMPLIFICATION-BASED METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF FOODBORNE PATHOGENS

Pre-amplification processing of the samples

It is well known that components of food samples, growth media, and nucleic acids extraction reagents can reduce or even block amplification reactions. They are generally known as amplification inhibitors, and they may cause a dramatic decrease in sensitivity of these reactions compared to pure solution of nucleic acids (Rossen *et al.*, 1992). Consequently, sample preparation prior to the amplification reaction is crucial for the robustness and performance of amplification-based methods. Amplification inhibitors may interfere with the cell lysis, degrade or capture nucleic acids and/or inhibit the amplification reaction (Wilson, 1997). In PCR-based methods, the thermoestable DNA polymerase is probably the most important target site of PCR-inhibitory substances. A wide range of inhibitors has been reported; however the identities and modes of action of many of them remain unclear. Common inhibitors include various components of body fluids and reagents encountered in clinical samples (*e.g.*, haemoglobin, bile salts, complex polysaccharides, urea and heparin);

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DOI: 10.1051/IUFoST:20060643

food constituents (*e.g.*, organic and phenolic compounds, milk proteinases, glycogen, fats, and Ca^{2+}); environmental compounds (*e.g.*, phenolic compounds, humic acids, and heavy metals); constituents of bacterial cells; non-target nucleic acids; and laboratory contaminants such as glove powder, plasticware and cellulose. Consequently, the characterization and removal of inhibitory substances that may affect an amplification reaction represents an important step in the development of efficient sample preparation procedures to allow correct performance and robustness of the amplification-based assay.

Sample preparation

The purposes of sample preparation are to homogenise the sample for amplification, to increase the concentration of the target organism to the practical operating range of a given assay and to reduce or exclude amplification-inhibitory substances. Hence, pre-amplification treatment aims to convert biological samples into amplifiable samples. As food samples vary in homogeneity, consistency, composition, and accompanying microbiota, pre-amplification procedures should be adapted to each food matrix. A large range of pre-amplification procedures have been developed, many of them being laborious, expensive, and time-consuming (Jaffe *et al.*, 2001). They can either be biochemical; immunological; physical; or physiological procedures (Rådstöm *et al.*, 2003) (Tables 1 and 2); or a combination of them, *e.g.* a pre-step with a biochemical nucleic acid extraction protocol (Dahlenborg *et al.*, 2001; Chen *et al.*, 1997) or with a physical pre-amplification procedure (Lantz *et al.*, 1998).

Table 1. Sample preparation procedures used for different types of samples. Adapted from Rådström *et al.*, 2003.

Category	Subcategory	Sample preparation procedure	Sample
Biochemical	Adsorption	Lectin-based separation	Beef meat
		Protein adsorption	Blood
	Nucleic acids extraction	Nucleic acid purification procedures	Diverse matrixes
		Lytic procedures	Diverse matrixes
Immunological	Adsorption	Immunomagnetic capture	Diverse matrixes
Physical		Aqueous two-phase systems	Soft cheese
		Buoyant density centrifugation	Minced meat
		Centrifugation	Diverse matrixes
		Dilution	Diverse matrixes
		Filtration	Diverse matrixes
		Boiling	Diverse matrixes
		Other heat treatments	Diverse matrixes
Physiological		Enrichment	Diverse matrixes

Table 2. Comparison of the performance of different pre-PCR sample preparation procedures. Adapted from Rådström *et al.*, 2003.

Category	Product of sample preparation			Removal of inhibitors	Time required	Cost
	Type	Homogeneity	Concentration			
Biochemical: nucleic acid extraction	Nucleic acids	Good	Average	Yes	3-6 h	High
Immunological: Immunomagnetic capture	Cell/ nucleic acids	Average	Average	Average	2-4 h	High
Physical: Buoyant-density centrifugation	Cell	Average	Good	Average	30 min	Average
Physiological: Enrichment	Cell	Low	Good	Low	6-24 h	Low

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Amplification techniques

PCR

PCR is a simple, versatile, sensitive, specific and reproducible assay. PCR is an exponential amplification of a DNA fragment (Saiki *et al.*, 1988), 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.

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 ºC, 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 second phase, *i.e.* annealing of primers to ssDNA, takes place at temperatures closer to their T_m (usually 55-65 ºC). The oligonucleotides typically consist of relatively short sequences (15-25 nt) and 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 rehybridise 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 stabilise 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, resulting in a duplication of the starting target material. When the primers have been extended a few bases, they posses a stronger ionic attraction to the template, which reduces the probability of unbinding.

After each cycle, the newly synthesised DNA strands can serve as template in the next cycle. 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.

Real-time PCR

The development of real-time (RTi-) PCR represents a significant advance in many molecular techniques involving nucleic acids analysis. RTi-PCR 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, not only at the end of the reaction (as occurs in conventional PCR). RTi-PCR is used for many different purposes, particularly for quantifying nucleic acids and for genotyping.

The uniqueness of RTi-PCR is that the process of amplification is monitored in real time by using fluorescence (Heid *et al.*, 1996). The results of RTi-PCR consist of amplification curves (Figure 1), 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 RTi-PCR are the closed-tube format (that avoids risks of carryover 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 of the results compared to conventional PCR.

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DOI: 10.1051/IUFoST:20060643

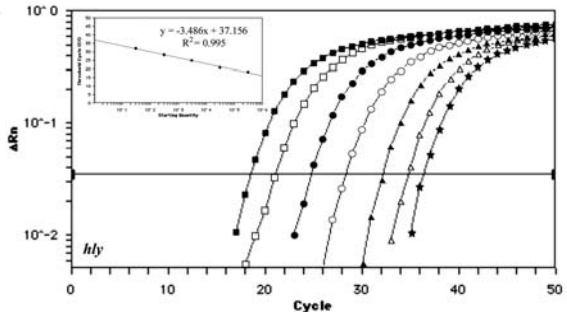

Figure 1 Amplification curves in semi-logarithmic view obtained from serial dilutions of a target DNA. Inset, regression curve obtained from C_T values.

Fluorescence signals that are proportional to the amount of PCR product can be generated by a **unspecific detection strategy** independent of the target sequence, *e.g.* through fluorescent dyes that have special fluorescent properties when bound to dsDNA (such as Syber Green), or by sequence-specific fluorescent oligonucleotide probes (such as TaqMan probes, Molecular beacons, Scorpion probes, Light-up probes, etc); *i.e.* a **sequence-specific strategy**.

Quantification analysis

The fluorescence is measured by the RTi-PCR device and can be visualized as an amplification plot (Figure 2). Typically, an amplification curve presents three different phases (Figure 3). 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.

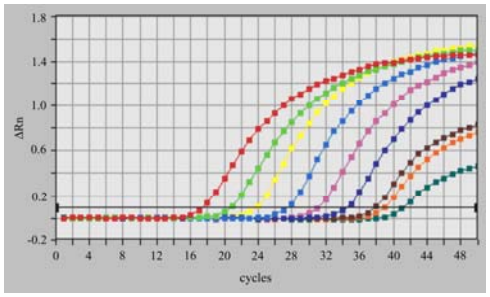

Figure 2. RTi-PCR amplification curves (linear representation). *x-axis:* RTi-PCR cycles; *y-axis*: normalised fluorescence. Amplification curves correspond to 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10, 4 and 1 copies, respectively. Black line: the threshold.

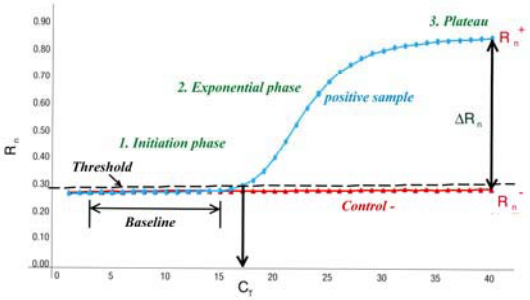

Figure 3. Phases of a PCR amplification curve. Blue: amplification curve of a positive sample. Red: negative control.

The **threshold cycle** or $\mathbf{C_T}$ value is the cycle at which fluorescence achieves a defined threshold. It corresponds to the cycle at which a statistically significant fluorescence increase is firstly detected. Therefore, the number of cycles needed for the amplification-associated fluorescence to reach a specific threshold level of detection (the C_T value) is inversely correlated to the amount of nucleic acid that was in the original sample (Walker, 2002). C_T 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 value in a linear **standard curve** of C_T values obtained from serially

diluted known-amount standards (Figure 4). This standard curve correlates the emitted fluorescence (C_T value) with the initial concentration of the standards used and the final result is achieved by interpolation of the produced fluorescence (C_T value) during the amplification of the sample in this standard curve. In practice, such curves are linear over more than five orders of magnitude.

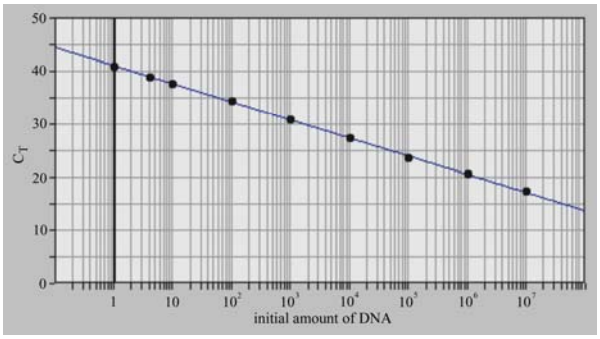

Figure 4. Standard curve built with DNA standards of known concentration (Figure 2). *x-axis*: initial amount of DNA (number of copies, ng, etc.); *y-axis*: C_T values. In this case, a dynamic range of 8 logarithmic units is shown. The slope of the curve is -3.41 and the regression coefficient or R^2 is 1.

Nucleic Acid Sequence based Amplification (NASBA)

The nucleic acid sequence-based amplification (NASBA) technique was first described by J. Compton in 1991, as a primer-dependent technology that can be used for the continuous amplification of nucleic acids, in a single mixture, at isothermal conditions (Compton, 1991). It is a sensitive transcription-based amplification (TAS) system specifically designed for the detection of RNA (Deiman *et al.*, 2002).

For the amplification process, NASBA employs three different enzymes: T7 RNA polymerase, RNase H and avian myeloblastosis virus (AMV) reverse transcriptase, which act in concert to amplify sequences from an original single-stranded RNA template (Rodríguez Lázaro et al., in press). The reaction also includes two oligonucleotide primers, complementary to the RNA region of interest, deoxyribunucleoside triphosphates for the activity of the AMV reverse transcriptase, and ribonucleoside triphosphates for the activity of the T7 RNA polymerase. One of the primers, primer 1, also contains a promoter sequence that is recognized by T7 RNA polymerase at the 5'-end. The reaction is performed at a single temperature, normally 41 ºC for 1 to 2 h in a self-sustained manner. At this temperature, the genomic DNA from the target remains double-stranded and does not become a substrate for amplification, and specificity values close to 100 % can be attained. The predominant product of the reaction is an RNA molecule of defined length limited by primer sequences complementary to the target.

Amplification process

Although NASBA is a continuous reaction, in order to simplify the description of the amplification process, it can be considered that the NASBA reaction is divided into two different phases (Rodríguez-Lázaro and Cook, 2003). In the first, so-called 'non-cyclic' phase, a double-stranded DNA product with a recognition sequence for T7 RNA polymerase is obtained. This product will be used as template in the second, 'cyclic' phase. In this second phase, RNA will be exponentially produced.

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DOI: 10.1051/IUFoST:20060643

In the 'non-cyclic' phase, the primer containing the T7 RNA polymerase promoter sequence (primer 1) anneals to the RNA target sequence. AMV reverse transcriptase uses the deoxynucleoside triphosphates to extend the 3' end of primer 1, thereby forming a cDNA copy of the template and resulting in a RNA:DNA hybrid. Subsequently, RNase H hydrolyses RNA from the RNA:DNA hybrids. Consequently, the original RNA is destroyed, leaving a single strand of DNA to which primer 2 anneals. AMV reverse transcriptase synthesizes the second DNA strand, rendering the promoter region double stranded.

The third enzyme, T7 RNA polymerase, transcribes RNA copies from the now transcriptionally active promoter, generating as many as 100 copies from each template molecule. Each new RNA molecule is now available as template for reverse transcriptase in the 'cyclic' phase of the NASBA process, but with the primer annealing steps reversed. In this phase, primer 2 binds to the template first and the action of AMV reverse transcriptase elongates the complementary DNA strand, thus generating a RNA:DNA hybrid as before. Then RNase H hydrolyses the RNA strand, so primer 1 is able to bind the resulting single-stranded DNA. AMV reverse transcriptase synthesizes DNA again, yielding the transcriptionally active promoter. Thus, the final product of a NASBA reaction is mainly single-stranded RNA.

Characteristics of NASBA

The NASBA reaction requires fewer 'cycles' than conventional PCR to produce a desired amplification of 1×10^9 molecules from each initial template (Chan and Fox, 1999). When using conventional PCR the number of molecules doubles in each step, so it requires approximately 20 cycles to produce an amplification of one million fold (Compton, 1991). However, with NASBA, 10-100 copies of RNA are generated in each transcription step, which means that only four to five 'cycles' are required to achieve a similar amplification (Chan and Fox, 1999). Errors that are inherent in some enzymatic activities (*e.g.* reverse transcriptase) are cumulative, so fewer 'cycles' should reduce the likelihood of these errors occurring. Compton (1991) evaluated the fidelity of the NASBA process by direct sequencing of the RNA product. From the sequences derived this way, 90 % were readable and 10 % were unreadable sequences which appeared to be due to either the inherent enzyme 'stops' in the sequence or strand degradation, which is to be expected in any amplification reaction. Furthermore, Chadwick *et al.* (1998) found that the NASBA error ratio by reverse transcription and sequencing of the NASBA product was 0.38 %.

As the NASBA is an exponentially amplification-based technique, theoretically a detection of 1 copy could be achieved. Experimental detection limits below 10 copies have been reported (reviewed in Cook, 2003; Rodríguez-Lázaro et al, in press), and several NASBA methods have been reported to be more sensitive than RT-PCR on equivalent targets (Cook, 2003).

NASBA is a potentially powerful technique for specific detection of viable cells. In contrast with RT-PCR, it does not required DNase treatment, since, as stated before, it runs at a single temperature in which dsDNA is not denatured and thus cannot became a substrate for NASBA.

The principal characteristics of NASBA are summarised in Table 3.

DOI: 10.1051/IUFoST:20060643

Table 3. Characteristics of NASBA. Modified from Deiman *et al.*, 2002.

- A single step isothermal amplification reaction at 41 ºC.
- Especially suited for RNA analytes because of the integration of RT into the amplification process.
- The single-stranded RNA product is an ideal target for detection by various methods including real-time detection using molecular beacons.
- The fidelity of NASBA is comparable to other amplification processes that use DNA polymerases lacking the 3' exonuclease activity.
- The use of a single temperature eliminates the need of special thermocycling equipment.
- Efficient ongoing process results in exponential kinetics caused by production of multiple RNA copies by transcription from a given cDNA product.
- Unlike amplification processes such as PCR, in which the initial primer level limits the maximum yield of product, the amount of RNA obtained in NASBA exceeds the level of primers by at least one order of magnitude.
- NASBA RNA product can be sequenced directly with a dideoxy method using RT and a labelled oligonucleotide primer.
- The intermediate cDNA product can be made double-stranded, ligated into plasmids, and cloned.
- Three enzymes are required to be active at the same reaction conditions.
- Low temperature can increase the non-specific interactions of the primers. However, these interactions are minimized by the inclusion of DMSO.
- A single melting step is required to allow the annealing of the primers to the target.
- The NASBA enzymes are not thermoestable and thus can only be added after the melting temperature.
- The length of the target sequence to be amplified efficiently is limited to approximately 100 to 250 nucleotides.

Analytical controls

The molecular-based detection techniques are instrumental techniques, and thus can produce false negative and false positive results (Knowk and Higuchi, 1989). Contamination remains an issue for diagnostic laboratories (Pellet *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). In order to correctly interpret the results of an amplification reaction , the following controls are recommended (Table 4).

Table 4. Analytical controls for molecular-based techniques. Adapted from Dieffenbach *et al.*, 1995; Hoorfar and Cook, 2003; and Stirling, 2003.

- **Processing Positive Control (PPC):** A negative sample spiked with sufficient amount of target (*e.g.* pathogen, species, etc), and processed throughout the entire protocol. A positive signal should be obtained indicating that the entire process (from nucleic acids extraction to amplification reaction) was correctly performed.
- **Processing Negative Control (PNC):** A negative sample spiked with sufficient amount of non-target or water, and processed throughout the entire protocol. A negative signal should be obtained indicating the lack of contamination along the entire process (from nucleic acids extraction to amplification reaction).
- **Premise Control or Environmental Control:** A tube containing the master mixture or water left open in the PCR setup room to detect possible contaminating nucleic acids in the environment.
- **Amplification Positive control:** A template known to contain the target sequence. A positive amplification indicates that amplification was performed correctly.
- **No Template Control (NTC) or Reagent Control (Blank):** Including all reagents used in the amplification except the template nucleic acids. Usually, water is added in stead of the template. A negative signal indicates the absence of contamination in the amplification assay.
- **Internal Amplification Control (IAC):** Chimerical non-target nucleic acid added to the master mixture in order to be co-amplified by the same primer set as the target nucleic acid but with an amplicon size visually distinguishable or different internal sequence region from the target amplicon. The amplification of IAC both in presence and absence of target indicates that the amplification conditions are adequate.

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DOI: 10.1051/IUFoST:20060643

Internal Amplification controls (IAC)

The efficiency of amplification-based techniques can be negatively influenced by several conditions including malfunction of equipment, incorrect reaction mixture, poor enzyme activity, or the presence of inhibitory substances in the original sample matrix (Rossen *et al.*, 1992). This can result in weak or negative signals and lead to underestimation of the amount of target nucleic acid in the sample. Many components of food products and culture media are amplification inhibitors. Their potential presence in the reaction is a serious problem that can compromise the applicability of the amplification-based techniques for analyses of these matrixes. Therefore, adequate control of the efficiency of the reaction is a fundamental aspect in such assays (Hoorfar and Cook, 2003, Hoorfar *et al.*, 2004). An internal amplification control or IAC is a non-target nucleic acid sequence which is co-amplified simultaneously with the target sequence (Cone *et al.*, 1992; Rodríguez-Lázaro et al., 2004; 2005). In a reaction without an IAC, a negative response (no signal) can mean that there was no target sequence present in the reaction. But, it could also mean that the reaction was inhibited. In a reaction with an IAC, a control signal will always be produced when there is no target sequence present. When no control signal is observed, this means that the reaction has failed, and the sample must be reanalysed.

In an amplification-based assay, an IAC should be based on flanking nucleic acid sequences (DNA for PCR assays, and RNA for RT-PCR and NASBA assays) with the same primer recognition sites as the target, with non-target internal sequences (Rodríguez-Lázaro et al., 2004; 2005). An optimal IAC should fulfil the criteria summarized in Table 5.

Table 5. Requirements of an optimal internal amplification control (IAC) for use in diagnostic assays. Modified from Hoorfar *et al.*, 2004.

- Target nucleic acid and IAC should share the same primer binding sites. This avoids two primer pairs to be present in a multiplex reaction.
- IAC amplicons should be easily distinguishable from the target nucleic acid amplicons. Depending on the detection method of the assay, an IAC should contain either different internal sequences to bind to IAC-specific probes, or produce an amplicon of a different size which is easily detected by gel electrophoresis.
- Only highly purified templates should be used.
- IAC concentration should be determined by titration of the IAC according the method described in Rosenstrauss *et al.* (1998) or by fluorescence methods.
- For dilution use polyallomer tubes and aerosol-resistant, sterile pipette tips. IAC nucleic acid can be stabilized, and storage should be at high concentrations of stabilized IAC nucleic acid (10^3 copies in aliquots at –20°C).
- The optimal amount of IAC in the assay shall be as low as possible, while still eliciting a signal through amplification. This will maximise the potential to identify false negatives by detecting amplification inhibition, and will ensure the reliable detection of low target concentrations.
- For PCR assays, the source of IAC should be plasmid DNA carrying the cloned IAC sequence, or purified PCR products. For RT-PCR and NASBA assays, the source of IAC should be an *in vitro*-synthesized chimerical single-stranded RNA.
- Add the IAC in the master mixture to ensure equal amount in each reaction tube.
- It is not strictly necessary that the amplification efficiency of target and IAC nucleic acid is identical. An experimental approach is needed to show the amplification efficiency of each nucleic acids during the amplification reaction.

There are two main strategies for use of an IAC in diagnostic assays: competitive IAC and non-competitive IAC. Both strategies are useful, although the first is recommended to avoid the risk of undesired interactions of multiple primers, and, especially, to get comparable amplification efficiency of the IAC and the target sequence.

In the competitive IAC strategy, the target and the IAC are co-amplified in a single reaction tube with the same primer set, and, in consequence, there is always some competition between target nucleic acid and IAC. Thus, the most critical parameter to consider is the optimal initial number of IAC copies in the diagnostic assay as it directly affects the target detection limit (Abdulmawjood *et al.*, 2002). If used at high concentration, the IAC might not allow detection of weak inhibition which could cause false-negative results if the target is present in low concentrations (Rosenstraus *et al.*, 1998). However, small amounts of initial IAC lead to substantial variations in IAC amplification, indicating poor reproducibility. The initial IAC copy number in the reaction must be determined in a compromise level that allows reproducible IAC detection and avoids inhibition of the target-specific reaction. In the non-competitive IAC strategy**,** the target and the IAC are amplified by two different primer sets. The IAC primer set can target a synthetic nucleic acid (DNA for PCR assays or RNA for RT-PCR or NASBA assays) or a gene different from the target.

Construction of an IAC

The simplest approach is to produce products which differ in size or internal region and hence can be easily distinguished from the target product in agarose gel electrophoresis, or with multiplex detection by colour using different fluorescent probes.

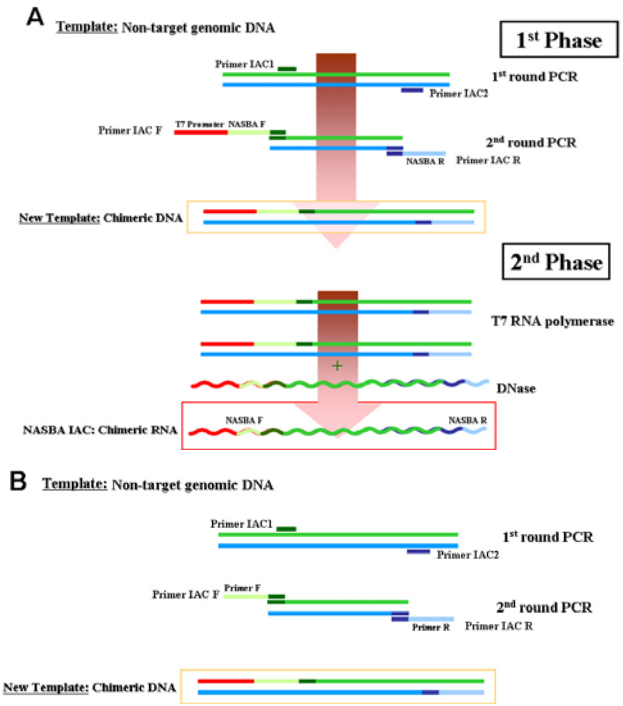

Figure 5. Illustration of the composite primer technique where the same primer set is used to amplify both the target and the non-target chimerical nucleic acid spiked in the reaction mixture. **A.** For diagnostic NASBA or RT-PCR assays. **B.** For diagnostic PCR assays.

For a size-dependent discrimination by gel electrophoresis of target and IAC nucleic acids, chimerical IACs may be produced by deleting, or inserting sequences between the

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DOI: 10.1051/IUFoST:20060643

recognition primer sites (Abdulmawjood *et al.*, 2002; Cubero *et al.*, 2002; Müller *et al.*, 1998; Sachadyn and Kur, 1998). A smaller size IAC can be constructed by deleting an internal sequence fragment. In this approach, the original amplicon is digested at 2 sites with restriction enzymes. After purification of the two outer fragments, they are re-ligated to create an amplicon which is smaller than the target gene. In order to generate an IAC larger than the target amplicon, it is cloned and digested at a unique site. Then, a new sequence of defined size is ligated in the linearized and dephosphorylated recombinant plasmid to create a new final recombinant plasmid containing the IAC (Cubero *et al.*, 2002). For sequence discrimination (*e.g.* multiplex by-colour) of target and IAC nucleic acids, modification of various nucleotides from the amplicon may be performed using mutagenesis technology (Courtney *et al.*, 1999). Alternatively, an IAC can be constructed by linking the two target specific primer sequences with a non-relevant sequence (Figure 5) *e.g.* from a fish virus (Hoorfar *et al.*, 2000) or a plant (Jofré *et al.*, 2005; Rodríguez-Lázaro *et al.*, 2004; 2005) for the detection of foodborne pathogens.

Finally, another IAC construction methodology allows the creation of an IAC with a completely designed nucleotide sequence (Rosenstraus *et al.* 1998). Several pairs of partially overlapping oligonucleotides that contain the entire IAC sequence are annealed and extended using an *E. coli* DNA polymerase. The product is finally reamplified with the terminal primers. DNA IACs may be used for PCR assays or as template for reverse transcription to produce IACs for RT-PCR or NASBA assays.

APPLICATION OF AMPLIFICATION TECHNIQUES FOR THE DETECTION OF FOODBORNE PATHOGENS

The promotion of a high level of Food Safety is a major policy priority worldwide. Food safety is compromised by foodborne diseases, which are a major problem in Public Health. The innocuousness of the foodstuffs is an inherent concept to food safety, and is related to many aspects of the agrarian production technologies as well as to manipulation and elaboration of foods. Thus, the application of exhaustive controls within Food Quality assurance programs is a premise to minimise the risk of diseases for the consumers. Therefore, the development and optimisation of novel alternatives for the monitoring, characterisation and enumeration of foodborne pathogens is one the key aspects in food microbiology (Stewart, 1997), and becomes increasingly important for the agricultural and food industry Malorny *et al.*, 2003). During the last decades, molecular approaches have significantly contributed to this field.

Classical microbiological methods for the presence of microorganisms in foods rely on the pre/enrichment and isolation of presumptive colonies of bacteria on solid media, and final confirmation by biochemical and/or serological identification. They are laborious and required a long process to obtain conclusive results. In addition, in certain circumstances they can not be reliable, *e.g.* some stressed viable but non-culturable VBNC *Campylobacter* spp. forms (Rollins and Colwell, 1986; Tholozan *et al.*, 1999). Thus, the adoption of amplification techniques in microbial diagnostics has been become in a promising alternative to overcome these disadvantages. In this scenario, the PCR has become in the most extensively used amplification method, but others have also been developed such as NASBA, although until now, they have had limited practical relevance for food monitoring and control. The inherent advantages of amplification techniques like short time to results, excellent detection limits, specificity and potential for automation can

foster its implementation in the food laboratories for routine testing of foodborne pathogens.

PCR-based methods

The general aspects of PCR as a food diagnostic tool have been reviewed amply elsewhere (e.g. Hill, 1996; Lantz *et al.*, 2000; Olsen *et al.*, 1995; Scheu *et al.*, 1998). PCR-based methods are predicted to be established as routine reference methods within the next ten years (Hoorfar and Cook, 2003), however further developments are needed for an effective implementation of this technique in food microbiology. The main issues that must be addressed for the effective adaptation of PCR in the Food Industry laboratories are the development of rational and easy-to-use strategies for pre-PCR treatment of the food samples, the lack of ability to unambiguously determination of viable forms, and distinguish from dead cells, the development of strategies for the direct use of quantitative RTi-PCR in food samples, and greater automation of the whole analytical process.

As the determination of the bacterial viability is a key issue for the application of food risk management, an approach to detect only viable bacterial cells by using PCR-based methodology is necessary, since PCR-based methods detect DNA, which survives cell death. For this purpose the use of mRNA as template for amplification can be a promising solution (Klein and Juneja, 1997). However, these require removing any trace of bacterial DNA in the reaction in order to avoid false-positive results in viability assays (Nogva *et al.*, 1999; Cook, 2003).

Enumeration of foodborne pathogens is a main aspect of molecular microbiological diagnostics, especially if it wants to be used for quantitative risk assessment. Although several methodological approaches have been described based on conventional PCR (Rijpens and Hermann, 2002; Scheu *et al.*, 1998), the most promising alternative is the application of adequate RTi-PCR assays.

NASBA-based methods

NASBA is a promising diagnostic tool for the analysis of viable microorganisms, since it is based on amplification of RNA rather than DNA. Critical reviews of the application of this technique to food and environmental microbiology have recently been published (Cook *et al.*, 2003; Rodríguez-Lázaro et al., in press). In those reviews, the authors state that NASBA for detection of foodborne pathogens is at around the same stage as PCR was a decade or so ago, with a few methods being sporadically published in the scientific press. Hence, considerable further development is required before NASBA can follow in PCR's footsteps to realize its potential for routine use. However, since NASBA can equal the rapidity and accuracy of PCR and has the additional potential advantage of unambiguous detection of viable pathogens, NASBA is a very promising diagnostic tool for food and clinical microbiology, and even can become a reference in future decades.

Finally, although RNA has been considered a good indicator of cell viability, not all RNA species (*i.e.* ribosomal RNA -rRNA-, transfer RNA -tRNA- and messenger RNA -mRNA-) are suitable for unambiguous detection of viable bacteria. Bacterial rRNA and tRNA have strong secondary structures and are much more stable than bacterial mRNA, which in general has a short half-life within viable bacterial cells, and is rapidly degraded by specific enzymes (RNases) (Rauhut and Klug, 1999). Thus, the detection of mRNA has

been proposed as an indicator of cell viability (del Mar Lleo, *et al.*, 2000), and NASBA could be a promising alternative tool for its study.

ACKNOWLEDGEMENTS

D.R-L is supported by a Marie Curie Intra-European fellowship (European Commision-funded program).

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