Pre-PCR Processing of Samples

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1. Introduction

Diagnostic polymerase chain reaction (PCR) is an extremely powerful rapid method for diagnosis of microbial infections and genetic diseases, as well as for detecting microorganisms in environmental and food samples. However, the usefulness of diagnostic PCR is limited, in part, by the presence of inhibitory substances in complex biological samples, which reduce or even block the amplification capacity of PCR in comparison with pure solutions of nucleic acids (1). Thus, the presence of substances interfering with amplification will directly influence the performance of diagnostic PCR and, in particular, the assay’s sensitivity of detection. Some inhibitors may dramatically interfere with amplification, even at very small amounts. For example, PCR mixtures containing the widely used Taq DNA polymerase are totally inhibited in the presence of 0.004% (v/v) human blood (2). Consequently, sample processing prior to PCR is required to enable DNA amplification of the target nucleic acids in the presence of even traces of PCR-inhibitory substances. To improve diagnostic PCR for routine analysis purposes, the processing of the sample is crucial for the robustness and the overall performance of the method. In general, diagnostic PCR may be divided into four steps: (i) sampling; (ii) sample preparation; (iii) nucleic acid amplification; and (iv) detection of PCR products (Fig. 1). Pre-PCR processing comprises all steps prior to the detection of PCR products. Thus, pre-PCR processing includes the composition of the reaction mixture of PCR and, in particular, the choice of DNA polymerase and amplification facilitators to be used.

This chapter will focus on sample preparation and the use of appropriate DNA polymerases and PCR facilitators for the development of efficient pre-
Fig. 1. Illustration of pre-PCR processing. The figure shows the different steps in diagnostic PCR. Pre-PCR processing refers to sampling, sample preparation, and DNA amplification with the addition of PCR facilitators and the use of an appropriate DNA polymerase.
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PCR processing strategies for various categories of samples, as well as substances and mechanisms involved in inhibition.

2. PCR Inhibitors

PCR inhibitors originate either from the original sample or from sample preparation prior to PCR, or both (3). In a review by Wilson (4), a systematic list of PCR inhibitors was presented, and the mechanisms by which the inhibitors may act were divided into the following three categories: (i) inactivation of the thermostable DNA polymerase; (ii) degradation or capture of the nucleic acids, and (iii) interference with the cell lysis step. Although many biological samples were reported to inhibit PCR amplification, the identities and biochemical mechanisms of many inhibitors remain unclear.

2.1. Approaches to the Characterization of PCR Inhibitors

The effect of PCR inhibitors can be studied by either increasing the concentration of purified template DNA or adding different concentrations of the PCR-inhibitory samples or by both ways. Increasing the concentration of target DNA may be useful to overcome the effect of inhibitors (interfering with DNA and/or binding reversibly to the DNA-binding domain of the DNA polymerase), whereas adding different concentrations of the inhibitory sample is an alternative approach to evaluate the strength of the inhibitory samples on the amplification capacity of PCR. On the other hand, studying the effect of inhibitors on the polymerization activity of the DNA polymerase can be useful to (i) compare the effect of different inhibitors; (ii) perform a kinetic analysis of the DNA polymerase in the presence and absence of inhibitors; and (iii) evaluate the effect of adding substances that relieve the inhibition, such as bovine serum albumin (BSA). The recent introduction of thermal cyclers with real-time detection of PCR product accumulation offers the possibility to study the quantitative effects of inhibitors more efficiently. These instruments may be used to study the efficiency of the PCR performance and/or to study the DNA polymerase efficiency for the synthesis of DNA in the presence and absence of PCR inhibitors (5).

2.2. Identification of PCR Inhibitors

A limited number of components have been identified as PCR inhibitors, namely, bile salts and complex polysaccharides in feces (6,7), collagen in food samples (8), heme in blood (9), humic substances in soil (10), proteases in milk (11), and urea in urine (12). The thermostable DNA polymerase is probably the most important target site of PCR-inhibitory substances (2). In a recent study, using various chromatographic procedures, hemoglobin,
immunoglobulin G (IgG), and lactoferrin were identified as three major PCR inhibitors in human blood (5,13). The mechanism of PCR inhibition by IgG was found to be dependent on its ability to interact with single-stranded DNA. Furthermore, this interaction was enhanced when DNA was heated with IgG. By testing different specific clones of IgGs, blocking of amplification through the interaction of single-stranded target DNA was found to be a general effect of IgGs. Therefore, in the case of blood specimens, it is not advisable to use boiling as a sample preparation method or to use hot-start PCR protocol.

Hemoglobin and lactoferrin were found to be the major PCR inhibitors in erythrocytes and leukocytes, respectively (5), and both hemoglobin and lactoferrin contain iron. The mechanism of inhibition may be related to the ability of these proteins to release iron ions into the PCR mixture. When the inhibitory effect of iron was investigated, it was found to interfere with DNA synthesis. Furthermore, bilirubin, bile salts and hemin, which are derivatives of hemoglobin, were also found to be PCR inhibitory. It has been suggested that heme regulates DNA polymerase activity and coordinates the synthesis of components in hemoglobin in erythroid cells by feedback inhibition (14). In the same study, it was observed that hemin was a competitive inhibitor with the target DNA and a noncompetitive inhibitor with the nucleotides through direct action against the DNA polymerase. As a result, characterization of PCR inhibitors and detailed knowledge of inhibitory capacities and mechanisms are important prerequisites for the development of more efficient sample preparation methods, which will eliminate the need for extensive processing of biological samples prior to diagnostic PCR.

3. Sample Preparation

The objectives of sample preparation are (i) to exclude PCR-inhibitory substances that may reduce the amplification capacity of DNA and the efficiency of amplification (see Chapter 1); (ii) to increase the concentration of the target organism to the practical operating range of a given PCR assay; and (iii) to reduce the amount of the heterogeneous bulk sample and produce a homogeneous sample for amplification in order to insure reproducibility and repeatability of the test. All these factors affect the choice of sample preparation method. However, many sample preparation methods are laborious, expensive, and time-consuming or do not provide the desired template quality (15). Since sample preparation is a complex step in diagnostic PCR, a large variety of methods have been developed, and all these methods will affect the PCR analysis differently in terms of specificity and sensitivity (1). The most frequently used sample preparation methods may be divided into four different categories: (i) biochemical; (ii) immunological; (iii) physical; and (iv) physiological methods (Table 1).
3.1. Biochemical Methods

The most widely employed biochemical method is DNA extraction. Many different commercial kits are available, such as BAX (Quallcon Inc., Wilmington, DE) (16), PrepMan (Applied BioSystems, Foster City, CA, USA) (17), Purugene (Gentra Systems Inc., Minneapolis, MN, USA) (18), QIAamp® (Qiagen, Valencia, CA, USA) (19), and XTRAX (Gull Laboratories Inc., Salt Lake City, UT, USA) (20). Consequently, several studies have compared and evaluated the quality of the extracted DNA (18,21,22), and a kit that provides the highest yield, concentration, and purity of DNA can be recommended. The advantage of DNA extraction is that a homogeneous sample with high quality is provided for amplification. Most PCR inhibitors are removed, since the template is usually purified and stored in appropriate buffers, such as Tris-EDTA (TE) buffer. The drawback of DNA extraction methods is that the target microorganism usually has to be pre-enriched in medium or on an agar plate prior to extraction. In addition, most DNA extraction methods are laborious and costly. Batch-to-batch variation after DNA extraction may also exist with respect to purity and concentration of the template.

3.2. Immunological Methods

This category is mainly based on the use of magnetic beads coated with antibodies (23). Since antibodies are used, the specificity will be influenced,

Table 1
Sample Preparation Methods Used for Different Types of Samples

<table>
<thead>
<tr>
<th>Category of sample preparation method</th>
<th>Sample preparation method</th>
<th>Sample</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochemical</td>
<td>Lectin-based separation</td>
<td>Beef meat</td>
<td>(78)</td>
</tr>
<tr>
<td></td>
<td>Protein adsorption</td>
<td>Blood</td>
<td>(9)</td>
</tr>
<tr>
<td>DNA extraction</td>
<td>DNA purification method</td>
<td>Hemolytic serum</td>
<td>(79)</td>
</tr>
<tr>
<td></td>
<td>Lytic methods</td>
<td>Blood anticoagulant</td>
<td>(80)</td>
</tr>
<tr>
<td>Immunological</td>
<td>Immunomagnetic capture</td>
<td>Blood</td>
<td>(81)</td>
</tr>
<tr>
<td>Physical</td>
<td>Aqueous two-phase systems</td>
<td>Soft cheese</td>
<td>(82)</td>
</tr>
<tr>
<td></td>
<td>Buoyant density centrifugation</td>
<td>Minced meat</td>
<td>(31)</td>
</tr>
<tr>
<td></td>
<td>Centrifugation</td>
<td>Urine</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>Dilution</td>
<td>Blood</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td>Filtration</td>
<td>Milk</td>
<td>(29)</td>
</tr>
<tr>
<td>Physiological</td>
<td>Enrichment</td>
<td>Meat</td>
<td>(33)</td>
</tr>
</tbody>
</table>

* Modified with permission from Ref. 1.
and the captured cells will be those containing the corresponding antigen. The specificity of the PCR protocol will depend on both the PCR assay used, as well as the specificity of the antibodies. In general, after immunocapture, the sample requires lysis or washing (24), and viruses can then be used directly (25). In most cases, these methods increase the concentration of the target organism. The homogeneity of the PCR sample may differ depending on the processing steps that follow the capture, but usually the template is of appropriate quality after this treatment. Since part of the specificity depends on the antibodies themselves, false negative results can be obtained as a result of cross-reactions. This methodology is quite expensive and also very laborious and time-consuming.

3.3. Physical Methods

Many different physical methods have been used, such as aqueous two-phase systems (26), buoyant-density centrifugation (27), centrifugation (28), filtration (29) and dilution (30). These methods are dependent on the physical properties of the target cells, for example cell density and size. Aqueous two-phase systems provide a gentle way of partitioning PCR inhibitors and target cells between two immiscible phases. For instance, a polyethylene glycol (PEG) 4000 and dextran 40-based system was used in a PCR detection assay for *Helicobacter pylori* in human feces (6). Density centrifugation was shown to be a promising method if fast detection is of importance (31). Density media, such as Percoll (Pharmacia, Uppsala, Sweden) (27) and BactXtractor (Quintesserence Research AB, Bålsta, Sweden) (32), were used to concentrate the target organism and remove PCR-inhibitory substances of different density. After this treatment, whole cells were obtained, which could be used as a PCR sample. The homogeneity of the sample may differ depending on the kind of biological sample matrices. If components of the sample matrix have the same density as the cells these may inhibit DNA amplification. The advantage of density centrifugation is that the target organism is being concentrated, which allows rapid detection response. Furthermore, these methods are relatively user friendly.

3.4. Physiological Methods

These methods are based on bacterial growth and biosynthesis of cell components, i.e., genome, cytoplasm, and cell surface constituents. Culture can be carried out in enrichment broth or on agar plates. Again, the aim is to provide detectable concentrations of viable target cells prior to PCR (33). Selective or nonselective agar or enrichment medium can be used, and the specificity will depend partly on the characteristics of the medium. The template quality, as well as the homogeneity of the PCR sample, may differ with respect to the presence of cell components. The advantages of this methodology are its sim-
plicity and low cost. The method provides viable cells to be used in PCR without further lysis steps (34). However, it must be borne in mind that cells contain high concentrations of macromolecules, which might influence and shift the equilibrium in many biochemical reactions (35), for instance the DNA polymerase and its DNA template–primer binding properties (36) (see Chapter 1). Therefore, the DNA polymerase has a key function during DNA amplification in terms of DNA synthesis and resistance to PCR inhibitors.

A comparison of the performance of sample preparation methods described in this section is shown in Table 2.

4. DNA Polymerases

The first PCR experiments were carried out with the thermolabile Klenow fragment of *Eschericia coli* DNA polymerase I, which needed to be replenished for every cycle (37). The use of the thermostable DNA polymerase from *Thermus aquaticus* (Taq) has greatly simplified PCR and enhanced the specificity (38). With high specific activity, fidelity, and temperature range, Taq DNA polymerase and its derivatives became and still are the most widely used enzymes in PCR. Thermostable DNA polymerase is a key component in the amplification reaction, and any factor interfering with the enzymatic activity will affect the amplification capacity. The DNA polymerase can be degraded, denatured, or have its enzymatic activity reduced by a wide variety of compounds present in biological samples (3, 5, 9, 39).

A number of DNA polymerases from other organisms are now commercially available. Examples of commonly used DNA polymerases include rTth and Tth, isolated from *Thermus thermophilus*, DyNazyme isolated from *T. brockianus*, as well as AmpliTaq® Gold (Applied BioSystems, Foster City, CA, USA) and Platinum Taq with built-in hot start, both isolated from *T. aquaticus*. These polymerases exhibit very different properties with regard to resistance to various components in biological samples and performance in the presence of these components. The choice of DNA polymerase was shown to influence the performance of several PCR-based applications, such as genotyping using restriction fragment-length polymorphism (RFLP) (40) and random-amplified polymorphic DNA (RAPD) (41), multiplex PCR assays (42), differential display reverse transcription PCR (RT-PCR) (43), and autosticky PCR (44). Recent research indicated that different polymerases have different susceptibilities to PCR inhibitors (2). Therefore, the inhibition of PCR by components of biological samples can be reduced or eliminated by choosing an appropriate thermostable DNA polymerase without the need for extensive sample processing prior to PCR.

The choice of DNA polymerase is determined by several factors related to the application. The level of resistance of DNA polymerase to PCR inhibitors
<table>
<thead>
<tr>
<th>Category of sample preparation method</th>
<th>Product of sample preparation</th>
<th>Homogeneity of product</th>
<th>Concentration of product</th>
<th>Removal of PCR inhibitors</th>
<th>Time required</th>
<th>Cost</th>
<th>Availability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochemical: DNA extraction</td>
<td>DNA</td>
<td>Good</td>
<td>Average</td>
<td>Yes</td>
<td>3–6 h</td>
<td>High</td>
<td>Complex</td>
</tr>
<tr>
<td>Immunological: Immunomagnetic capture</td>
<td>Cell/DNA</td>
<td>Average</td>
<td>Average</td>
<td>Average</td>
<td>2–4 h</td>
<td>High</td>
<td>Limited</td>
</tr>
<tr>
<td>Physical: Buoyant density centrifugation</td>
<td>Cell</td>
<td>Average</td>
<td>Good</td>
<td>Average</td>
<td>30 min</td>
<td>Average</td>
<td>Limited</td>
</tr>
<tr>
<td>Physiological: Enrichment</td>
<td>Cell</td>
<td>Low</td>
<td>Good</td>
<td>Low</td>
<td>6–24 h</td>
<td>Low</td>
<td>Good</td>
</tr>
</tbody>
</table>

Table 2: Comparison of the Performance of Different Pre-PCR Sample Preparation Methods
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can be determined by intrinsic factors, such as enzyme purification techniques and reaction buffer composition, as well as its production from native or recombinant strains. Furthermore, the sample preparation protocol and the presence of trace levels of extraction reagents in the purified sample can affect the extraction efficiency and the sensitivity of PCR. Taq DNA polymerase from different commercial sources was reported to be inhibited to a different extent by humic substances in soil extracts (45). The source of Taq DNA polymerase in the PCR step was also found to affect the banding patterns produced in differential display (43). Variations in the performance of DNA polymerases in co-amplification PCR were also found to be salt-dependent (46). The polymerase Tth maintains both DNA- and RNA-dependent DNA polymerase activities in the presence of 5% (v/v) phenol, while a trace amount of phenol was found to be inhibitory to Taq DNA polymerase (39). Several studies evaluated the usefulness and characteristics of different DNA polymerases with respect to various PCR samples including clinical samples, blood, feces, and cell material.

4.1. Clinical Samples

It was noted that both Tfl and Tth DNA polymerases are more resistant to aqueous and vitreous fluids of the eye than the polymerases Taq, Tli, and the Stoffel fragment (47). Tth DNA polymerase was also shown to be less affected by inhibitors present in nasopharyngeal swab samples compared to Taq DNA polymerase in an assay detecting influenza A virus (48). The use of hot-start enzymes, such as AmpliTaq Gold and Platinum Taq, reduces the possibility of carryover contamination. Furthermore, increased specificity using AmpliTaq Gold was demonstrated for a multiplex PCR assay detecting middle ear pathogens (42). Amplification of highly degraded DNA from paraffin-embedded tissue using AmpliTaq Gold or Platinum Taq increased the yield by up to 20 times compared to Taq. Improved PCR amplification with less background was observed in the same study for AmpliTaq Gold compared to Platinum Taq when a time-release PCR protocol was applied (49).

4.2. Blood

When the inhibitory effect of blood on nine thermostable DNA polymerases was studied, AmpliTaq Gold and Taq DNA polymerases were totally inhibited in the presence of 0.004% (v/v) blood in the PCR mixture, while HotTub, Pwo, rTth, and Tfl DNA polymerases were able to amplify DNA in the presence of at least 20% (v/v) blood without reduced amplification sensitivity (2). Furthermore, it was found that the addition of 1% (v/v) blood was totally inhibitory to Taq DNA polymerase, while a target sequence in the presence of up to 4% (v/v) blood was amplified using Tth DNA polymerase (50). Different PCR conditions and target DNA concentrations may
explain these conflicting results regarding the effect of blood on Taq DNA polymerase. The enhancement of amplification yield and specificity using AmpliTaq Gold DNA polymerase instead of AmpliTaq DNA polymerase in multiplex detection of DNA in blood was also reported (51,52).

4.3. Feces

In a comparison of the amplification efficiency of Tth polymerase and Taq DNA polymerase in detecting Helicobacter hepaticus in mice feces, a 100-fold increase in sensitivity with Tth polymerase over Taq DNA polymerase was observed (53). Furthermore, it has been reported that Pwo and rTth DNA polymerases could amplify DNA in the presence of 0.4% (v/v) feces without reduced sensitivity (2). The inhibitory effect of the microbial flora in pig feces on the amplification capacity of rTth and Taq DNA polymerase was observed when detecting Clostridium botulinum (17). The results showed a decrease in sensitivity by one log unit when using Taq DNA polymerase instead of rTth.

4.4. Cell Material

The DNA polymerases from T. aquaticus and T. flavus were found to bind to short double-stranded DNA fragments without sequence specificity (54). Furthermore, it was reported that the accumulation of amplification products during later PCR cycles also exerts an inhibitory effect on the DNA polymerases (55). It was indicated that the main factor contributing to the plateau phase in PCR was the binding of DNA polymerase to its amplification products (see Chapter 1). Taq DNA polymerase was replaced with Tth DNA polymerase for more sensitive detection of Staphylococcus aureus DNA in bovine milk (8). Also, the detection of cells of the poultry pathogen Mycoplasma iowae was significantly improved by replacement of Taq DNA polymerase with Tth DNA polymerase (56).

5. Amplification Facilitators

In the course of the development of PCR methodology the basic master mixture containing DNA polymerase, primers, nucleotides, and a reaction buffer containing Tris-HCl, KCl, and MgCl₂, has been extended with numerous compounds to enhance the efficiency of amplification. Such compounds are called amplification enhancers or amplification facilitators (57). They can affect amplification at different stages and under different conditions by (i) increasing or decreasing the thermal stability of the DNA template; (ii) affecting the error rate of the DNA polymerase; (iii) affecting the specificity of the system; and (iv) relieving the inhibition of amplification caused by complex biological samples. With the introduction of new DNA polymerases, a number of suppliers have already added amplification facilitators into the
accompanying buffers (Table 3). A subdivision of facilitators into five groups was proposed (58): (i) proteins; (ii) organic solvents; (iii) non-ionic detergents; (iv) biologically compatible solutes; and (v) polymers. These groups will be discussed in more detail, including some of the commonly used compounds within the different groups. Specific amounts of facilitators used by different research groups are listed in Table 4.
5.1. Proteins

The two proteins most commonly used to facilitate amplification are BSA (59–61) and the single-stranded DNA-binding protein gp32, which is a protein encoded by gene 32 of bacteriophage T4 (59, 61–63). The addition of BSA to the amplification mixture was shown to relieve inhibition of amplification by several substances, such as blood, meat, feces (61) and heme-containing compounds (9). It has been suggested that BSA can help to overcome PCR inhibition by blood or heme-containing compounds by binding them. Furthermore, it was shown that BSA can bind phenolics and relieve PCR inhibition in this way (59). Inhibition of amplification by fecal samples can be caused by the degradation of DNA polymerase by proteinases. It has been suggested that proteins such as BSA and gp32 can relieve this inhibition effect by serving as a target for the proteinases (11). BSA is often used for the stabilization of proteins in solution, and thus, a possible way of facilitating amplification may consist in stabilization of the DNA polymerase (64). The protein gp32 may facilitate amplification in the same fashion as BSA. However, gp32 can bind single-stranded DNA, protecting it from nuclease digestion (65), and it has been suggested that, in blood, the protein can improve the accessibility of the DNA polymerase when large amounts of coagulated organic material are present in the PCR sample (50).

5.2. Organic Solvents

Examples of frequently used organic solvents as PCR facilitators include dimethyl sulfoxide (DMSO) and formamide. It has been suggested that both solvents affect the thermal stability of the primers and the thermal activity profile of the DNA polymerase (57), thereby increasing the specificity of amplification (66). The effect on thermal stability seems to be caused by the general capability of organic solvents to destabilize DNA in solution (67, 68).

5.3. Non-ionic Detergents

The main non-ionic detergents used as PCR facilitators are Tween 20 and Triton-X. It was shown that the addition of Tween 20 stimulates the activity of Taq DNA polymerase and reduces false terminations of the enzyme (69). The mechanisms behind these findings are still unclear.

5.4. Biologically Compatible Solutes

Betaine and glycerol are the most common facilitators in the group of biologically compatible solutes. The solutes are used by organisms and cellular systems to maintain biological activity under extreme conditions. For that reason, glycerol is used in the storage buffer of thermostable enzymes. The addition of both
betaine and glycerol to amplification reaction mixtures was found to enhance specificity \((66,70)\) and to reduce the formation of secondary structures caused by GC-rich regions \((71)\). Also, glycerol may facilitate amplification by enhancing the hydrophobic interactions between protein domains and raising the thermal transition temperature of proteins \((72)\). Glycerol can also lower the strand separation temperature of DNA, thus facilitating amplification \((73)\).

5.5. Polymers

PEG and dextran are polymers that can be used as amplification facilitators. It was shown that PEG can facilitate amplification in similar ways as organic solvents \((57)\). Also, PEG was reported to relieve the inhibition caused by feces \((61)\) and dextran sulfate, a plant polysaccharide \((74)\). Furthermore, PEG is known to possess enzyme stabilizing properties comparable to BSA, which serve to maintain enzymatic activity \((64)\). This action could enhance amplification by stabilizing the DNA polymerase.

6. Pre-PCR Processing Strategies

The treatment of complex biological samples prior to amplification is a crucial factor determining the performance of diagnostic PCR assays. The following requirements should be fulfilled to insure optimal conditions \((1)\): (i) absence or low concentration of PCR-inhibitory components in the sample; and (ii) sufficient concentration of target DNA.

Pre-PCR treatment aims to convert a complex biological sample containing the target microorganisms into PCR-amplifiable samples. Since complex biological samples often contain PCR inhibitors \((4)\), numerous pre-PCR processing protocols have been developed. The reason for the variety in PCR protocols and pre-PCR methods is that the most suitable approach depends on the nature of the sample and the purpose of the PCR analysis. For instance, various sample preparation methods were developed to remove or reduce the effects of PCR inhibitors without knowing the identity of the PCR inhibitors and/or understanding the mechanism of inhibition. Therefore, the characterization of PCR inhibitors represents an important step in the development of efficient sample preparation methods designed to overcome the effects of inhibitory factors. For example, the PCR-inhibitory effect of collagen was partially relieved by adjusting the magnesium ion concentration in the amplification mixture \((75)\).

Once the sample matrix has been characterized regarding PCR inhibitors and concentrations of target DNA, one can predict whether the sample is suitable for PCR analysis or not. Samples can be divided into heterogeneous and homogeneous samples, with most complex biological samples being heteroge-
neous. Consequently, the conditions for DNA amplification can be optimized through efficient pre-PCR processing. Several different pre-PCR processing strategies can be used: (i) optimization of the sample preparation method; (ii) optimization of the DNA amplification conditions by the use of alternative DNA polymerases, and/or amplification facilitators; and (iii) a combination of both strategies.

Selection and optimization of sample preparation methods is the most frequently used approach to circumvent PCR inhibition (1). Many PCR protocols combine sample preparation methods from different categories. A common strategy for diagnostic PCR consists in the combination of a pre-enrichment method with a biochemical DNA extraction method (17,76) or with a physical sample preparation method (20). The enrichment step is usually included to concentrate the target cells to PCR-detectable concentrations (33). The complexity of the various methods must be considered in light of the aim of the PCR analysis, i.e., if the results are to be used for risk assessments or for hazard analysis critical control point (HACCP) purposes.

A summary of the different sample preparation categories is presented in Table 2. In general, DNA extraction methods provide templates of high quality, but the method is usually complex. However, automated robust DNA extraction methods have been introduced. Physical methods are favorable, since they do not affect the specificity of the PCR protocol, as may the immunological and physiological methods. The simplest method is to take the PCR sample directly from the enrichment broth and dilute the sample, because of the inhibitory components present in the enrichment broth (20). Recently, a PCR-compatible enrichment medium was developed for detection of *Yersinia enterocolitica*, thus making pre-PCR processing of swab samples unnecessary (77). However, complex matrices present in the culture medium may have a detrimental effect on PCR performance.

The DNA amplification reaction mixture can be optimized by selection of a robust DNA polymerase and by the addition of amplification facilitators, to circumvent the PCR-inhibitory effects of sample components and to maintain the amplification efficiency. This strategy has been employed in the laboratory of the authors for blood samples, and by using the *rTth* DNA polymerase combined with BSA, it was possible to amplify DNA in the presence of at least 20% (v/v) blood without loss of sensitivity (2). Furthermore, a pre-PCR processing protocol was developed for detection of *Clostridium botulinum* spores in porcine fecal samples, based on inclusion of a sample preparation method and the use of a more robust DNA polymerase (17). After a heat shock (10 min at 70°C; and pre-enrichment for 18 h at 30°C, the feces homogenate was exposed to DNA extraction prior to PCR, and PCR was performed using the more robust *rTth* DNA polymerase.
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In the future development of diagnostic PCR assays, research on pre-PCR processing is most likely to expand in response to the growing demand for rapid, robust and user-friendly PCR protocols. A future challenge for pre-PCR processing strategies is the design of PCR procedures integrating both sampling and DNA amplification as automated operations.

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