Pre-PCR Processing

Strategies to Generate PCR-Compatible Samples

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Abstract

Polymerase chain reaction (PCR) is recognized as a rapid, sensitive, and specific molecular diagnostic tool for the analysis of nucleic acids. However, the sensitivity and kinetics of diagnostic PCR may be dramatically reduced when applied directly to biological samples, such as blood and feces, owing to PCR-inhibitory components. As a result, pre-PCR processing procedures have been developed to remove or reduce the effects of PCR inhibitors. Pre-PCR processing comprises all steps prior to the detection of PCR products, that is, sampling, sample preparation, and deoxyribonucleic acid (DNA) amplification. The aim of pre-PCR processing is to convert a complex biological sample with its target nucleic acids/cells into PCR-amplifiable samples by combining sample preparation and amplification conditions. Several different pre-PCR processing strategies are used: (1) optimization of the DNA amplification conditions by the use of alternative DNA polymerases and/or amplification facilitators, (2) optimization of the sample preparation method, (3) optimization of the sampling method, and (4) combinations of the different strategies. This review describes different pre-PCR processing strategies to circumvent PCR inhibition to allow accurate and precise DNA amplification.

Index Entries: Amplification facilitators; PCR-amplifiable samples; PCR-compatible samples; PCR facilitators; PCR inhibitors; PCR sample; pre-PCR processing; sample preparation; thermostable DNA polymerase.

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: (1) sampling, (2) sample preparation, (3) nucleic acid amplification, and (4) detection of PCR products (**Fig. 1**). Pre-PCR processing comprises all steps prior to the detection of PCR products. Thus, pre-PCR pro-

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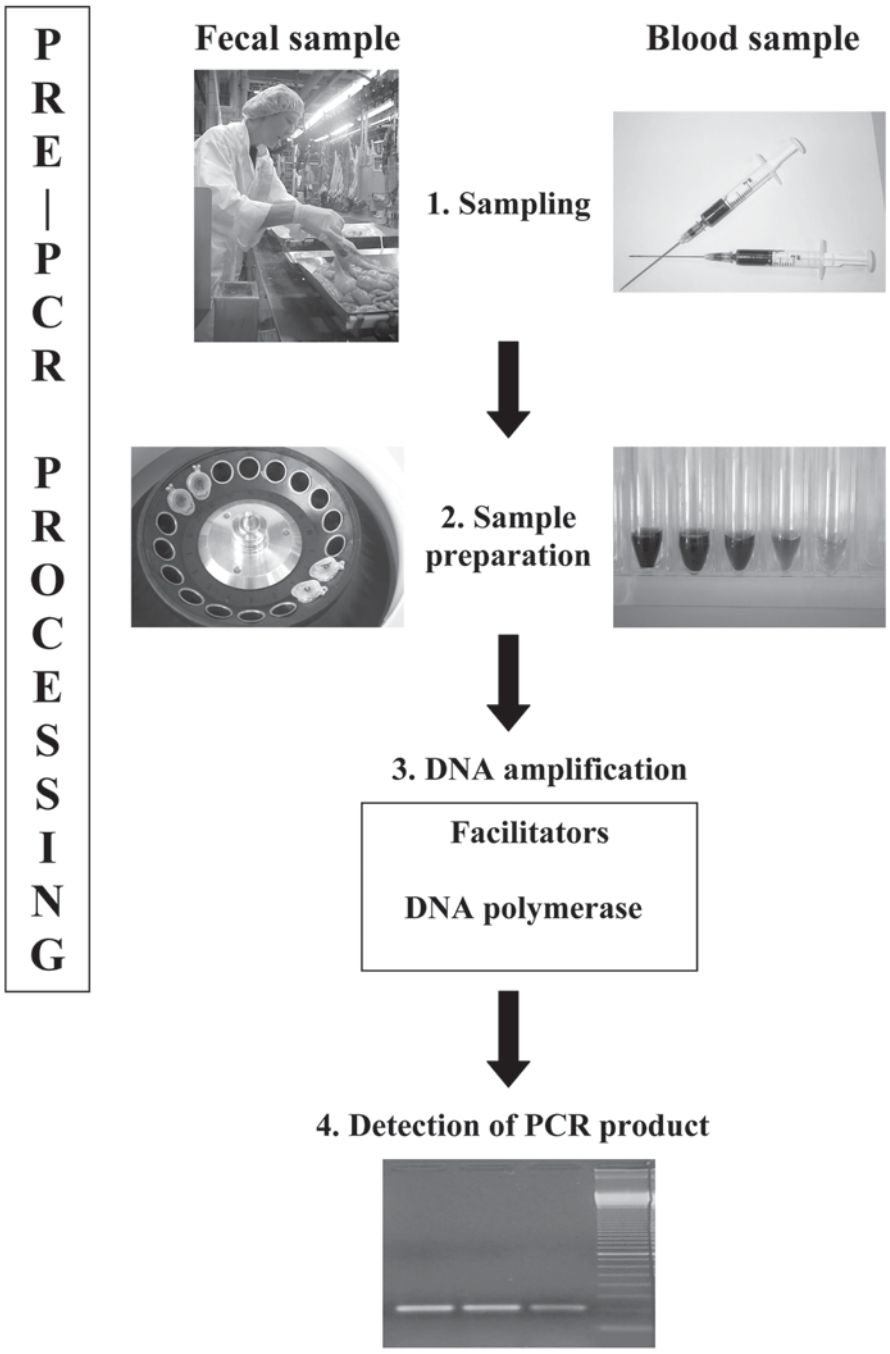

Fig. 1. Illustration of pre-PCR processing. The figure shows the different steps in diagnostic PCR. Pre-PCR processing refers to sampling, sample preparation, and DNA amplification with the addition of PCR facilitators and the use of an appropriate DNA polymerase.

cessing includes the composition of the reaction mixture of PCR and, in particular, the choice of DNA polymerase and amplification facilitators to be used.

This article focuses on sample preparation and the use of appropriate DNA polymerases and PCR facilitators for the development of efficient pre-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 from 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: (1) inactivation of the thermostable DNA polymerase, (2) degradation or capture of the nucleic acids, and (3) 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 either by increasing the concentration of purified template DNA, by 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 compare the effect of different inhibitors; perform a kinetic analysis of the DNA polymerase in the presence and absence of inhibitors; or 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)*, melanin and myoglobin in tissue *(11,12)*, polysaccharides in plants *(13)*, proteinases and calcium ions in milk *(14,15)*, and urea in urine *(16)*. 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 *(5,17)*. 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 protocols.

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-inhibiting. It has been suggested that heme regulates DNA polymerase activity and coordinates the synthesis of components in hemoglobin in erythroid cells by feedback inhibition *(18)*. 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 to exclude PCR-inhibitory substances that may reduce the amplification capacity of DNA and the efficiency of amplification, to increase the concentration of the target organism to the practical operating range of a given PCR assay, and to reduce the amount of the heterogeneous bulk sample and produce a homogeneous sample for amplification to ensure 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 ***(19)***. Because 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: (1) biochemical, (2) immunological, (3) physical, and (4) 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 (Qualicon, Wilmington, DE) ***(20)***, PrepMan (Applied Biosystems, Foster City, CA) ***(21)***, Purugene (Gentra Systems, Minneapolis, MN) ***(22)***, QIAmp® (Qiagen, Valencia, CA) ***(23)***, and XTRAX (Gull Laboratories, Salt Lake City, UT) ***(24)***. Consequently, several studies have compared and evaluated the quality of the extracted DNA ***(22,25,26)***, 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 because 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 ***(27)***. Because antibodies are used, the specificity will be influenced, 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 and the specificity of the antibodies. In general, after immunocapture, the sample requires lysis or washing ***(28)***, and agents such as viruses can then be detected directly ***(29)***. 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. Because part of the specificity depends on the antibodies themselves, false-positive results can be obtained from crossreactions. This methodology is quite expensive and, mostly, very laborious and time consuming.

3.3. Physical Methods

Many different physical methods have been used, such as aqueous two-phase systems ***(30)***, buoyant density centrifugation ***(31)***, centrifugation ***(32)***, filtration ***(33)***, and dilution ***(34)***. 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 ***(35)***. Density media, such as Percoll (Pharmacia, Uppsala, Sweden) ***(31)*** and BactXtractor (Quintessence Research AB, Bålsta, Sweden) ***(36)***, were used to

Table 1
Sample Preparation Methods Used for Different Types of Samples[a]

Category of Sample Preparation Method	Subcategory	Sample Preparation Method	Sample	Ref.
Biochemical	Adsorption	Lectin-based separation	Beef meat	*(82)*
		Protein adsorption	Blood	*(9)*
	DNA extraction	DNA purification method	Hemolytic serum	*(83)*
		Lytic methods	Blood anticoagulant	*(84)*
Immunological	Adsorption	Immunomagnetic capture	Blood	*(85)*
Physical		Aqueous two-phase systems	Soft cheese	*(86)*
		Buoyant density centrifugation	Minced meat	*(35)*
		Centrifugation	Urine	*(32)*
		Dilution	Blood	*(34)*
		Filtration	Milk	*(33)*
Physiological		Enrichment	Meat	*(37)*

[a]Modified from **ref. *1***.

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, that is, 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 *(37)*. 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 simplicity and low cost. The method provides viable cells to be used in PCR without further lysis steps *(38)*. However, it must be kept in mind that cells contain high concentrations of macromolecules that might influence and shift the equilibrium in many biochemical reactions *(39)* for instance, the DNA polymerase and its DNA template–primer binding properties *(40)*. 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 *Escherichia coli* DNA polymerase I, which needed to be replenished for every cycle *(41)*. The use of the thermostable DNA polymerase from *Thermus aquaticus* has greatly simplified PCR and enhanced the specificity *(42)*. 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

Table 2
Comparison of the Performance of Different Pre-PCR Sample Preparation Methods

Category of sample preparation method	Product of sample preparation	Homogeneity of product	Concentration of product	Removal of PCR inhibitors	Time required	Cost	Availability
Biochemical: DNA extraction	DNA	Good	Average	Yes	3–6 h	High	Complex
Immunological: Immunomagnetic capture	Cell/DNA	Average	Average	Average	2–4 h	High	Limited
Physical: Buoyant density centrifugation	Cell	Average	Good	Average	30 min	Average	Limited
Physiological: Enrichment	Cell	Low	Good	Low	6–24 h	Low	Good

degraded, denatured, or have its enzymatic activity reduced by a wide variety of compounds present in biological samples ***(3,5,9,43)***.

A number of DNA polymerases from other organisms are now commercially available. Examples of commonly used DNA polymerases include r*Tth* and *Tth*, isolated from *Thermus thermophilus,* DyNAzyme isolated from *Thermus brockianus*, as well as Ampli*Taq*® Gold and Platinum *Taq* with built-in hot start, both isolated from *T. aquaticus*. These polymerases exhibit very different properties regarding 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) ***(44)*** and random-amplified polymorphic DNA (RAPD) ***(45)***, multiplex PCR assays ***(46)***, differential display reverse transcription PCR (RT-PCR) ***(47)***, and autosticky PCR ***(48)***. 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 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 ***(49)***. The source of *Taq* DNA polymerase in the PCR step was also found to affect the banding patterns produced in differential display ***(47)***. Variations in the performance of DNA polymerases in coamplification PCR were also found to be salt dependent ***(50)***. The polymerase *Tth* maintains both DNA- and ribonucleic acid (RNA)-dependent DNA polymerase activities in the presence of 5% (v/v) phenol, whereas a trace amount of phenol was found to be

inhibitory to *Taq* DNA polymerase ***(43)***. 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 ***(51)***. *Tth* DNA polymerase was also shown to be less affected by inhibitors present in nasopharyngeal swab samples compared to *Taq* in an assay detecting influenza A virus ***(52)***. The use of hot-start enzymes, such as Ampli*Taq* Gold and Platinum *Taq*, increases the specificity, which was demonstrated for Ampli*Taq* Gold in a multiplex PCR assay detecting middle ear pathogens ***(46)***. Amplification of highly degraded DNA from paraffin-embedded tissue using Ampli*Taq* 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 Ampli*Taq* Gold compared to Platinum *Taq* when a time-release PCR protocol was applied ***(53)***.

4.2. Blood

When the inhibitory effect of blood on nine thermostable DNA polymerases was studied, Ampli*Taq* Gold and *Taq* DNA polymerases were totally inhibited in the presence of 0.004% (v/v) blood in the PCR mixture, whereas Hot*Tub*, *Pwo,* r*Tth* 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, whereas a target sequence in the presence of up to 4% (v/v) blood was amplified using *Tth* DNA polymerase ***(54)***. 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 Ampli*Taq* Gold DNA polymerase instead of Ampli*Taq* DNA polymerase in multiplex detection of DNA in blood was also reported ***(55,56)***.

4.3. Feces

In a comparison of the amplification efficiency of *Tth* DNA polymerase and *Taq* DNA polymerase in detecting *Helicobacter hepaticus* in mice feces, a 100-fold increase in sensitivity with *Tth* DNA polymerase over *Taq* DNA polymerase was observed ***(57)***. Furthermore, it has been reported that *Pwo* and r*Tth* 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 r*Tth* and *Taq* DNA polymerase was observed when detecting *Clostridium botulinum* ***(21)***. The results showed a decrease in sensitivity by 1 log unit when using *Taq* DNA polymerase instead of r*Tth*.

4.4. Cell Material

The DNA polymerases from *T. aquaticus* and *Thermus flavus* were found to bind to short double-stranded DNA fragments without sequence specificity ***(58)***. Furthermore, it was reported that the accumulation of amplification products during later PCR cycles also exerts an inhibitory effect on the DNA polymerases ***(59)***. It was indicated that the main factor contributing to the plateau phase in PCR was the binding of DNA polymerase to its amplification products. *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 ***(60)***.

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 $MgCl_2$ has been extended with numerous compounds to enhance the efficiency of amplification. Such compounds are called amplification enhancers or amplification facilitators ***(61)***. They can affect amplification at different stages and under different conditions by increasing or decreasing the

thermal stability of the DNA template, affecting the error rate of the DNA polymerase, affecting the specificity of the system, and 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 to the accompanying buffers (**Table 3**). A subdivision of facilitators into five groups was proposed ***(62)***: (1) proteins, (2) organic solvents, (3) nonionic detergents, (4) biologically compatible solutes, and (5) polymers. These groups are discussed in more detail in **Subheadings 5.1.–5.5.**, 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 ***(63–66)*** and the single-stranded DNA-binding protein gp32, which is a protein encoded by gene 32 of bacteriophage T4 ***(63,65,67,68)***. The addition of BSA to the amplification mixture was shown to relieve inhibition of amplification by several substances, such as blood, meat, feces ***(63)***, 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 ***(65)***. 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 ***(15)***. 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 ***(69)***. The protein gp32 may facilitate amplification in the same fashion as BSA. However, gp32 can bind single-stranded DNA, protecting it from nuclease digestion ***(70)***, 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 ***(54)***.

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 ***(61)***, thereby increasing the specificity of amplification ***(71)***. The effect on thermal stability seems to be caused by the general capability of organic solvents to destabilize DNA in solution ***(72,73)***.

5.3. Nonionic Detergents

The main nonionic 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 ***(74)***. 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 mixes was found to enhance specificity ***(71,75)*** and to reduce the formation of secondary structures caused by GC-rich regions ***(76)***. Also, glycerol may facilitate amplification by enhancing the hydrophobic interactions between protein domains and raising the thermal transition temperature of proteins ***(77)***. Glycerol can also lower the strand separation temperature of DNA, thus facilitating amplification ***(78)***.

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 ***(61)***. Also, PEG was reported to relieve the inhibition caused by feces ***(63)*** and dextran sulfate, a plant polysaccharide ***(13)***. Furthermore, PEG is known to possess enzyme-sta-

Table 3
Composition of Commercial Buffer Systems for DNA Polymerases

	Buffer Components							
DNA polymerase	Tris-HCl (m*M*)	KCl (m*M*)	$MgCl_2$ (m*M*)	Triton-X (vol%)	Tween-20 (vol%)	BSA (μg/mL)	EGTA (m*M*)	Glycerol (vol%)
DyNAzyme	100	500	15	1%	—	—	—	—
Plat. *Taq*	200	500	—	—	—	—	—	—
r*Tth*	100	1000	—	—	0.5%	—	7.5	5%
Taq	100	500	15	—	—	—	—	—
Tth	100	1000	15	—	0.5%	500	—	—

Table 4
Concentration of Facilitators Used in Different Applications

Facilitator	Concentration	Application	Reference
BSA	4 g/L	Relief of inhibition by meat, blood, and feces	***(63)***
BSA	0.4 g/L	Relief of inhibition by bilirubin and humics	***(65)***
BSA	0.6 g/L	Relief of inhibition by melanin in RT-PCR	***(87)***
gp32	0.1 g/L	Relief of inhibition by meat, blood, and feces	***(63)***
gp32	0.15 g/L	Relief of inhibition by bilirubin and humics	***(65)***
DMSO	5%	Rescue of failed amplification	***(61)***
DMSO	2%–10%	Facilitation of RT-PCR	***(88)***
Tween-20	2.5 g/L	Relief of inhibition by feces	***(63)***
Tween-20	0.5%	Relief of inhibition by plant polysaccharides	***(13)***
Betaine	117 g/L	Relief of inhibition by blood and meat	***(63)***
Betaine	2.5 *M*	PCR of GC-rich sequences	***(76)***
Glycerol	10–15%	Rescue of failed amplification	***(61)***
PEG 400	5%	Relief of inhibition by plant polysaccharides	***(13)***
PEG 400	10–15%	Rescue of failed amplification	***(61)***
PEG 400	2 g/L	Relief of inhibition by blood	***(63)***

Abbr: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; PEG, polyethylene glycol; RT-PCR, reverse transcription PCR.

bilizing properties comparable to BSA, which serve to maintain enzymatic activity ***(69)***. 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 ensure optimal conditions: (1) absence or low concentration of PCR-inhibitory components in the sample, and (2) sufficient concentration of target DNA.

Pre-PCR treatment aims to convert a complex biological sample containing the target microorganisms into PCR-amplifiable samples. Because 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 ***(79)***.

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 heterogeneous. Consequently, the conditions for DNA amplification can be optimized through efficient pre-PCR processing. Several different pre-PCR processing strategies can be used: optimization of the sample preparation method, optimization of the DNA amplification conditions by the use of alternative DNA polymerases and/or amplification facilitators, and 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 ***(21,80)*** or with a physical sample preparation method ***(24)***. The enrichment step is usually included to concentrate the target cells to PCR-detectable concentrations ***(37)***. The complexity of the various methods must be considered in light of the aim of the PCR analysis, that is, 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 because they do not affect the specificity of the PCR protocol as may the immunological and physiological methods. Because of the inhibitory components present in the enrichment broth, the simplest method is to take the PCR sample directly from the enrichment broth and dilute the sample ***(24)***. Recently, a PCR-compatible enrichment medium was developed for detection of *Yersinia enterocolitica*, thus making further pre-PCR processing of swab samples unnecessary ***(81)***. 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 r*Tth* 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 *C. botulinum* spores in porcine fecal samples based on inclusion of a sample preparation method and the use of a more robust DNA polymerase ***(21)***. 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 r*Tth* DNA polymerase.

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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