Chapter 2

Overcoming Inhibition in Real-Time Diagnostic PCR

Johannes Hedman and Peter Rådström

Abstract

PCR is an important and powerful tool in several fields, including clinical diagnostics, food analysis, and forensic analysis. In theory, PCR enables the detection of one single cell or DNA molecule. However, the presence of PCR inhibitors in the sample affects the amplification efficiency of PCR, thus lowering the detection limit, as well as the precision of sequence-specific nucleic acid quantification in real-time PCR. In order to overcome the problems caused by PCR inhibitors, all the steps leading up to DNA amplification must be optimized for the sample type in question. Sampling and sample treatment are key steps, but most of the methods currently in use were developed for conventional diagnostic methods and not for PCR. Therefore, there is a need for fast, simple, and robust sample preparation methods that take advantage of the accuracy of PCR. In addition, the thermostable DNA polymerases and buffer systems used in PCR are affected differently by inhibitors. During recent years, real-time PCR has developed considerably and is now widely used as a diagnostic tool. This technique has greatly improved the degree of automation and reduced the analysis time, but has also introduced a new set of PCR inhibitors, namely those affecting the fluorescence signal. The purpose of this chapter is to view the complexity of PCR inhibition from different angles, presenting both molecular explanations and practical ways of dealing with the problem. Although diagnostic PCR brings together scientists from different diagnostic fields, end-users have not fully exploited the potential of learning from each other. Here, we have collected knowledge from archeological analysis, clinical diagnostics, environmental analysis, food analysis, and forensic analysis. The concept of integrating sampling, sample treatment, and the chemistry of PCR, i.e., pre-PCR processing, will be addressed as a general approach to overcoming real-time PCR inhibition and producing samples optimal for PCR analysis.

Key words: Amplification facilitators, Clinical diagnostics, Diagnostic PCR, DNA extraction, Forensic analysis, Internal amplification control, Microbial analysis, PCR inhibitors, Pre-PCR processing, Sample treatment, Real-time PCR, Swab techniques, Thermostable DNA polymerase

1. Introduction

The polymerase chain reaction (PCR) (1) is a powerful analytical tool in molecular diagnostics. Only one or a few nucleic acid molecules are required for analysis and denatured and/or partly

degraded DNA can be analyzed, as well as high-molecular-weight DNA. PCR provides fast analysis in a matter of hours, compared with the days required in culture-based microbial methods. In real-time PCR the growth of the amplification product is monitored continuously, through the detection of fluorescently labeled probes or dyes intercalating with DNA. This removes the need for gel electrophoresis, further shortening the analysis time, and allowing PCR to be used for nucleic acid quantification. Table 1 provides examples of applications of real-time PCR.

Thermostable DNA polymerases are a vital component of PCR. The polymerase from *Thermus aquaticus* (*Taq*) and its commercial derivatives, i.e. mutants or chemically enhanced variants, are most widely used because of their tolerance to high temperatures and good processivity. Polymerases from a range of other organisms are also readily available, such as *Thermus thermophilus* (*Tth*), *Thermus flavus* (*Tfl*), and *Pyrococcus furiosus* (*Pfu*). Some manufacturers have made use of the different properties of polymerases by providing mixtures of enzymes from different organisms.

PCR is extremely sensitive and reproducible when pure DNA samples are analyzed. However, the amplification efficiency (AE) and detection limit are sometimes changed by molecules interfering with the DNA polymerase or affecting the nucleotides and nucleic acids (2). Several substances have been identified as PCR inhibitors and a few have been partially characterized regarding their molecular PCR-inhibitory mechanism(s) (Table 2). PCR

Table 1
Different applications of diagnostic real-time PCR

Discipline	Target cell type[a]	Concentration/ number of target molecules[b]	Sample homogeneity	Degree of PCR inhibition	Reference
Archeological analysis	Human cells, animal cells	Low	Medium	High	(12, 30)
Clinical diagnostics	Human cells, microorganisms	High/medium	Medium	Medium	(31–33)
Environmental analysis	Micro- and macro-organisms	Low/medium	Low	High	(34–37)
Food analysis	Micro- and macro-organisms	Low	Low	High	(38–41)
Forensic science	Human cells, animal cells	Low	Low	High	(42–46)

Assays for the detection and/or quantification of nucleic acids

[a]The target cells in clinical and food analysis are often pathogenic organisms, and in archeological, environmental, and forensic analysis partly degraded nucleic acids from macroorganisms

[b]Concentration of target nucleic acids in pathogens or relevant cells in the sample

Table 2
Overview of ions and molecules inhibiting PCR

Type of inhibitor	Molecule or ion	Source	Mechanism/s[a]	Reference
Polymerase inhibitors	Al^{3+}	Sampling using aluminum-shafted swabs	Alters ion composition	(27)
	Alginate	Sampling with calcium alginate swabs	Adsorption of Mg^{2+} or entrapment of polymerase	(27)
	Bile salts (cholic and deoxycholic acid)	Feces	Direct effect on polymerase	(15, 61)
	Calcium ions	Milk	Competition with the polymerase cofactor Mg^{2+}	(24)
	Collagen	Bone	Alteration of ion composition by binding cations	(62)
	EDTA	Anticoagulant	Chelation of Mg^{2+}	(63)
	$FeCl_3$		Release of iron ions	(51)
	Free radicals	UV treatment of PCR tubes	Reaction with polymerase	(64–66)
	Fulvic acid	Soil	Binding to polymerase	(51)
	Heme	Blood	Release of iron ions and competition with template	(18, 48)
	$KAc/K_2Cr_2O_7$	DNA extraction/preservative	Alteration of ion composition	(63, 67)
	Lactoferrin	Blood	Release of iron ions	(18)
	LiCl	Growth medium	Alteration of ion composition	(63)
	Melanin	Skin, hair	Binding to polymerase	(11)
	$MgCl_2$	Growth medium	Alteration of level of Mg^{2+} in PCR	(63)
	Myoglobin	Muscle tissue	Release of iron ions	(22)
	NaCl		Alteration of ion composition	(51, 63)
	NaOH	DNA extraction	Degradation of DNA, pH-mediated denaturation of polymerase	(63)
	NH_4Ac	DNA extraction	Alteration of ion composition	(63)
	Phenol	Soil, DNA purification	Denaturation of polymerase or binding to the polymerase via hydrogen bonds	(21)
	Phytic acid	Feces	Chelation of Mg^{2+} or change in ion content if present as salt	(68)

(continued)

**Table 2
(continued)**

Type of inhibitor	Molecule or ion	Source	Mechanism/s[a]	Reference
	Polysaccharides	Feces	Binding to polymerase	(69)
	Proteinases (plasmin)	Milk	Degradation of polymerase	(23)
	Reverse transcriptase	RT-PCR	Competition with DNA polymerase and/or formation of complex with ssDNA	(49, 70)
	Tannic acid	Soil	Binding to polymerase	(51)
	Urea	Urine	Prevents non-covalent bonding, acting directly on polymerase or hindering primer annealing	(54, 71)
Nucleic acid inhibitors	Bilirubin	Feces	Competition with template	(18, 51)
	Cellulose, nitrocellulose	Sampling filters	Binding to DNA	(72)
	Ethanol	DNA extraction	Precipitation of DNA	(63)
	Ethidium bromide	DNA extraction	Binding to DNA	(63)
	Formaldehyde	Preservative	Interference with DNA and DNA polymerase	(67)
	Heparin	Anticoagulant	Binding to DNA, competition with template and direct interaction with polymerase	(18, 73)
	Immunoglobulin G	Blood	Binding to ssDNA	(16)
	Isopropanol	DNA extraction	Precipitation of DNA	(63)
	PEG	DNA extraction	Precipitation of DNA	(63)
	SYBR Green I	Detection dye	Binding to dsDNA with high affinity	(57)
	SYTOX Orange	Detection dye	Binding to dsDNA with high affinity	(57)
	TO-PRO-3	Detection dye	Binding to dsDNA with high affinity	(57)
Fluorescence inhibitors	Humic compounds	Soil	Fluorescence quenching, direct interaction with polymerase and primer annealing	(19, 20, 51, 52, 59)
	Polymeric surfaces	Miniaturized real-time PCR instruments	Binding of detection dye, e.g., SYBR Green	(60)

[a]Confirmed or probable mechanism. Inhibitors affecting the ion composition of the reaction could act as polymerase inhibitors, nucleotide/nucleic acid inhibitors, and/or fluorescence inhibitors

inhibitors are especially prominent in food and forensic analysis, where the amount of target cells/nucleic acids is often small and sample matrices are diverse and complicated. Therefore, the issue of PCR inhibitors has been discussed mainly in these areas (3–7). However, inhibition must be considered in all kinds of PCR-based diagnostic analysis, in particular when quantifying nucleic acids. The effect of inhibitors is complex. Different DNA polymerases and their buffer systems have different abilities to maintain functionality in the presence of PCR-inhibitory molecules (8, 9); samples with low levels of target nucleic acids are more severely affected than those with more DNA (10), and amplification of longer amplicons is more easily inhibited than that of shorter ones (11). The dNTP sequences of the target amplicon and primers affect tolerance of PCR to inhibition (12), and there is some evidence that amplicons with a lower GC content and primers with lower Tm are more affected by inhibitors than amplicons with a higher GC content and primers with higher Tm (13). Inhibitors may be thermolabile, or present at low amounts, both retarding the reaction (12). This results in a delay in amplification, but the PCR efficiency is not affected, making it difficult to detect the presence of inhibitors. Understanding PCR inhibition is made even more difficult by the fact that other factors can cause similar problems. DNA degradation can also lead to lower amplification yields than expected, and DNA lesions, such as *cis–syn* thymidine dimers, have been shown to lower the sensitivity of real-time PCR (14) or completely block amplification.

Real-time PCR inhibitors can be categorized into three groups, depending on their PCR-inhibitory mechanism(s), namely (1) DNA polymerase inhibitors, (2) nucleotide/nucleic acid inhibitors, and (3) fluorescence inhibitors. DNA polymerase inhibitors can either affect the enzyme directly, e.g., by degrading or denaturing it, or indirectly, e.g., by chelating the essential Mg^{2+} ions. Nucleotide or nucleic acid inhibitors can block amplification by binding to single- or double-stranded DNA or by destroying it. Fluorescence inhibitors affect the detection of amplicons in real-time PCR. They may form precipitates blocking fluorescence or interact with the fluorophore. Cell lysis inhibitors have been proposed as a fourth group of PCR inhibitors (5). These supposedly come into play when whole cells are used in the PCR reaction, i.e. no cell lysis or DNA extraction is performed prior to PCR. However, to our knowledge no one has been able to distinguish between inhibitors affecting cell lysis and those affecting the DNA polymerase and/or the nucleic acids.

PCR-inhibiting molecules originate from one or more of the following steps in sample processing: the sample matrix itself, the methods and materials used for sampling, or the sample treatment process. Inhibitors present in the sample matrices themselves include bile salts and polysaccharides in feces (15), heme,

immunoglobulin G and lactoferrin in blood (16–18), humic acids in soil (19–21), melanin in skin and hair (11), myoglobin in muscle tissue (22), plasmin and Ca^{2+} ions in milk (23, 24), particulate matter in indoor air (25), and indigo dyes in denim fabric (26). Inhibitors arising from the sampling process may originate from materials on which the sample is found or from the equipment used for sampling. The alginate of calcium-alginate swabs used, for example, for the collection of nasopharyngeal samples, is a potent PCR inhibitor (27). Possible inhibitors arising from sample treatment are phenol from organic DNA purification (21), proteases used for DNA extraction (28), and growth media used for pre-enrichment of microorganisms (29).

For good performance of PCR all the steps in sample processing must be optimized bearing in mind the type of sample and the DNA target in question. The concept of pre-PCR processing implies combining sampling, sample treatment and PCR chemistry optimally in each particular case (7). Different applications place different demands on pre-PCR processing. In clinical analysis, PCR samples are often fairly homogeneous, for example, blood, cerebrospinal fluid, and urine. Sampling is performed directly on the patient and the sampling medium best suited for the analysis can be used, minimizing PCR-inhibiting substances. In contrast, in forensic DNA analysis, crime scene sampling involves a range of different cell types and sample matrices that may inhibit PCR. Not only are more PCR inhibitors present, but also sample treatment may be more complex, costly, and time consuming.

2. The Nature of PCR Inhibitors

2.1. DNA Polymerase Inhibitors

Interactions between proteins and DNA polymerases are a major cause of inhibition. For example, human blood contains an excess of proteins compared with the amount of DNA. In one study, 1 μl of blood was found to contain about 35 ng of DNA and about 150 μg of proteins (47). Many of these proteins interfere with the polymerization process in PCR.

Heme, part of hemoglobin, has been identified as a key PCR inhibitor in human blood (17, 18). It is thought to affect PCR by releasing iron ions, affecting the ion balance, and thereby disturbing polymerase activity as well as primer and probe annealing. Heme can therefore be regarded as a universal PCR inhibitor. This compound is also found in proteins such as myoglobin, cytochrome *b*, and catalase and could therefore cause inhibition in tissues other than blood. Inhibition due to heme has been found to be most notable in dried blood stains, while fresh blood shows comparably low degrees of inhibition. This suggests that the inhibition resulting from heme arises from a degenerated heme complex. Hemoglobin that had been digested with proteinase K was shown to inhibit PCR, whereas non-digested hemoglobin did not (48),

further strengthening the notion that the form of heme is important regarding its PCR-inhibiting activity. The iron-releasing molecule lactoferrin is another key inhibitor in blood, affecting PCR in a manner similar to heme (18).

Different polymerases show considerable differences in their resistance to inhibitors in blood. In the first study systematically comparing different polymerases with respect to inhibitor tolerance, *Taq* and Ampli*Taq* Gold were found to be inhibited by 0.004% (v/v) blood, whereas r*Tth* and *Tfl* functioned in 20% (v/v) blood (8). r*Tth* is also considerably less susceptible to inhibition by the heme-containing myoglobin in muscle samples than *Taq* (22). Even different batches of the same brand of *Taq* polymerase have been shown to give different detection limits with the same amounts of blood (18).

The protein melanin, present in skin and hair, is known to completely inhibit *Taq* (11). Melanin binds reversibly to the *Taq* polymerase, thereby hindering its activity. When performing reverse transcription PCR (RT-PCR), the reverse transcriptase enzyme may inhibit PCR, possibly by competing with the DNA polymerase (49). Since *Tth* has reverse transcription activity, it can be used for both steps of RT-PCR to circumvent PCR inhibition. A nucleotide analogue, acyclovir triphosphate, is used to treat patients who have immunodeficiency symptoms or prior to transplantation. This molecule inhibits viral DNA polymerase by premature chain termination and has also been found to inhibit PCR (50).

Humic, fulvic, and tannic acids, together with other polyphenolic compounds, are potent PCR inhibitors present in soil (19, 20, 51). Humics probably affect PCR both by binding to the polymerase via hydrogen bonds and by changing the melting temperature of dsDNA, preventing primer annealing (52). A method of dealing with inhibition by humic substances in soil by simply increasing the concentration of *Taq* polymerase has been proposed (53). However, increasing the amount of polymerase is costly and not advisable as a standard method. Replacing the polymerase may be a better approach, since *Tth* has been found to withstand phenol considerably better than *Taq* (21).

Polyvalent as well as monovalent metal ions are important in PCR. Mg^{2+} is a cofactor for the DNA polymerase, and the overall ion content should create a favorable ion environment for denaturation and primer and probe annealing, as well as for the polymerase. Ca^{2+} ions in milk compete with Mg^{2+} for the binding sites on the polymerase, thereby inhibiting PCR (24). K^+ ions are often used in the PCR buffer to ensure a suitable ion content. Changing the amount of K^+ ions or introducing other ions, e.g. Na^+, can cause inhibition (8). Different polymerases can cope with different amounts of ions. r*Tth* retains its activity at almost twice the K^+ ion content which causes inactivation of *Taq* (8). Salt crystals altering the ion content of the PCR reaction are probable inhibitors in urine (54).

2.2. Nucleotide/Nucleic Acid Inhibitors

Nucleic acid inhibitors are molecules that affect the melting temperature or conformation of DNA in such a way that primer annealing or extension is inhibited, either completely or partially. Cations are potent nucleic acid inhibitors (see above). Nontarget DNA may inhibit amplification by sterically preventing the primers from annealing or by providing nonspecific binding sites for the primers (55). Immunoglobulin G in blood plasma is believed to form a complex with ssDNA, thereby inhibiting PCR (16). The effect was found to be greater when immunoglobulin G was heated to 95°C together with the DNA template. Similar complexes can be formed with ssDNA and other proteins during heating and denaturation, which could explain the inhibition seen with reverse transcriptase (16). This should be kept in mind when using boiling as a step in DNA extraction, as in Chelex® extraction (56). The enzyme r*Tth* can amplify DNA in the presence of undiluted immunoglobulin G, which is not possible with *Taq*, Ampli*Taq* Gold, *Pwo* or *Tfl* (16). The DNA-intercalating dye SYBR Green I, used for real-time PCR target detection, has been shown to partly inhibit PCR when used at the recommended concentration (57). This is explained by the high affinity of SYBR Green to dsDNA. Dyes with lower affinity, such as SYTO-13 and SYTO-82, showed no PCR-inhibiting properties in the same study.

2.3. Fluorescence Inhibitors

The advent of real-time PCR allowed the introduction of a new set of PCR-inhibiting molecules, i.e., those that directly affect the detection of fluorescence. The choice of detection chemistry and probe technology influences fluorescence inhibition. Hydrolysis probes (*Taq*Man) emit fluorescence when hydrolyzed by the DNA polymerase. Changes in the ion content of the reaction could affect probe hybridization, thus causing inhibition. Molecules inhibiting the polymerization of DNA polymerases could also lower their 5′–3′ exonuclease activity, thereby inhibiting the hydrolysis of *Taq*Man probes (58), leading to a double inhibition effect. Different fluorochromes have different biophysical properties, which probably affects their susceptibility to quenching inhibitors in the sample matrix. However, this has not yet been extensively studied. Humic acids quench the fluorescence of SYBR Green, probably by binding to the fluorophore or by collisional quenching (59). Surface-bound SYBR Green fluoresces most, and humics may prevent surface binding. Humic acids sequester ethidium bromide, thereby reducing the amount available for DNA interaction. Humics may thus affect the analysis using classic PCR as well as real-time PCR. SYBR Green has been shown to adsorb onto polymeric tubes, which could be used to create miniature PCR systems (60), resulting in a lower signal. The effect increases with greater tube length and lower volume and would lead to fluorescence inhibition in a miniaturized real-time PCR assay.

3. Quantifying the Level of PCR Inhibition

In diagnostic PCR it is vital to know if a sample is affected by PCR inhibitors. The risk of false-negative results is one of the major drawbacks of diagnostic PCR (74). A low level of inhibition could affect the efficiency of amplification and the detection limit, leading to underestimation of DNA concentrations. In forensic analysis it is important to distinguish between samples with little DNA and those with reasonable amounts of DNA and also containing PCR inhibitors, in order to perform the appropriate analysis. Samples with too little DNA should be concentrated, while those with inhibitors must be purified. Methods of measuring the quantity and quality of DNA based on optical density (OD) are not ideal for estimating the level of PCR inhibitors, since there is no direct correlation between OD and successful PCR amplification (10, 75). OD ratios at different wavelengths such as OD_{260}/OD_{280}, mainly predict the presence of proteins, while other substances not detected may interfere significantly with PCR. For example, RNA samples that were found to be pure when analyzed with UV spectrophotometry and different types of RNA chips, showed inhibition using real-time PCR (76). This indicates that the classical techniques are less sensitive to inhibitors and should be complemented with PCR assays. Also, one-third of the scientists using RT-PCR do not check the quality of the RNA before cDNA synthesis (77), further stressing the importance of real-time PCR quality control to avoid false-negative results.

The end-point fluorescence of real-time PCR is usually lowered by fairly small amounts of inhibitors (78). However, lowering of the end-point fluorescence does not necessarily affect the detection limit of the assay (79), and it should therefore not be used to estimate inhibition. Quantification cycle (C_q) shifts are better means of measuring the effects of inhibitors. C_q is derived from the amplification curve, generally using the threshold method or the second derivative maximum method. There are three methods of calculating PCR inhibition (1) using an assay with an internal amplification control (IAC), (2) calculating the amplification efficiency, or (3) modeling the amplification curve.

3.1. Internal Amplification Control

The fastest and most straightforward way of monitoring PCR inhibition in routine analysis is by using an IAC, which involves adding a known amount of a DNA fragment, which is amplified simultaneously with the target (80–82). The presence of inhibitors results in either complete failure to amplify the IAC, or slower amplification than expected, evidenced by a higher C_q value than that of a pure sample. The use of IACs is strongly recommended in

order to avoid false-negative results (83) and is a legal requirement in the detection of pathogens in food in Europe (EN ISO 22174). The notation used differs, and IC (internal control) (84), IPC (internal PCR control) (82), and internal standard DNA (25) are also used. Note that the abbreviation IPC is also used for internal process control (see below).

IACs can either have primer sites equivalent to the target, so that only one primer pair is required to amplify the two fragments (85) or different primer sites, creating a need for separate primer pairs (86). In the first case there is competition for the primers. Incorporating a second primer pair makes the assay more complex. Several systems employing specific IACs have been developed during the past few years for a range of purposes (81, 82). A universal IAC for hydrolysis probe assays, with specific primers, has been proposed (86). It uses a fabricated DNA fragment and has been applied in assays to detect agents posing a biological threat, such as *Bacillus anthracis* and *Clostridium botulinum*. An IAC with primer sites for five different human virus detection primers has been developed (85). It can be used in multiplex assays amplifying any of the five targets and the IAC with the same primers.

If the concentration of the IAC is too high, the amplification of the target may be inhibited by IAC DNA. The appropriate number of IAC molecules depends on the assay and must be titrated during assay optimization. Low numbers of IAC molecules, such as 20 (84), 58 (85), or 100 molecules (41) per reaction, have been successfully applied.

In order to detect all levels of inhibition, the amplification of the IAC must be at least as sensitive to PCR inhibitors as the target. The size of the IAC is important in this respect, since shorter fragments are usually more readily amplified in the presence of inhibitors than longer ones (11). An IAC amplicon longer than the target is therefore recommended (85).

Since the amplification of target DNA competes with IAC amplification when using one set of primers, the value of C_q of the IAC is elevated if the amount of target DNA is high. Therefore, the direct use of C_q shifts is not an ideal measure of inhibition. Hudlow et al. (46) proposed an equation incorporating the effect of target DNA concentration on IAC C_q. The equation can be used to predict the possibility of successful short tandem repeat (STR) analysis, e.g., for a forensic DNA sample. The difference between the C_q of the IAC in the sample and the C_q of the IAC in a pure reaction is calculated. This value, called "delta C_q," is divided by the amount of DNA in ng, giving a normalized inhibition factor (NIF). NIF values have been shown to be well correlated to the possibility of obtaining complete STR profiles; values over 1 indicate unsuccessful STR typing (46).

If a real-time quantitative PCR assay is used to optimize a subsequent PCR reaction, such as a multiplex human identity PCR for

forensic or parental investigations, the relative amounts of sample in both assays must be considered. The quantification kit Quantifiler™ Human (Applied Biosystems, Foster City, CA) is more sensitive to hematin inhibition than the Identifiler® STR analysis kit (kits inhibited by 16 and 20 µM hematin, respectively) (82), suggesting that all the inhibitors affecting the Identifiler analysis should also be seen in the results using the Quantifiler kit. However, in the Quantifiler kit the sample is only 2 of the 25 µl reaction volume, compared with 10 of 25 µl in the Identifiler kit. The level of PCR inhibitors in a real sample is therefore up to five times larger in an analysis using the Identifiler kit than in one using Quantifiler. A sample that appears to be uninhibited according to the IAC using Quantifiler can give a completely inhibited blank profile when using Identifiler or comparable systems, such as SGM Plus® (Applied Biosystems) (unpublished data, Swedish National Laboratory of Forensic Science). The sample-to-reaction-volume ratio should preferably be the same for all PCR-based analyses that are performed on the same sample.

When using an assay without an IAC, negative samples can be confirmed by spiking the reaction with an alien plasmid (26) or target DNA molecules, e.g., 50 copies (40). Roussel et al. (10) spiked DNA extracts from mouse stomachs with *Helicobacter pylori* DNA. The resulting C_q values were compared with those from the same amount of DNA molecules in water, giving an inhibition ratio.

Alien DNA, or cells, can be introduced before the sample is collected or before DNA extraction. An internal process control monitors not only the PCR but also the analysis steps prior to PCR, such as sampling and sample treatment. Murphy et al. (87) used a genetically modified *Escherichia coli* strain as an internal process control, which was added to pathogen-containing food samples prior to sampling. Lenticule discs were used to encapsulate *E. coli* to enable the addition of equal amounts to all samples. *E. coli* is detected using specific primers and can therefore be used universally in process control. Juen and Traugott (88) developed a multiplex with primers for both prey and predator for the detection of *Amphimallon solstitiale* in the gut content of soil-living invertebrates. A negative result from prey DNA amplification is a sign of inhibition, or failure in the DNA extraction.

3.2. Amplification Efficiency (AE)

The AE may be calculated from the slope of a standard curve obtained from the analysis of a dilution series of DNA, using the formula $AE = 10^{(-1/slope)} - 1$. A slope of –3.32 indicates the ideal efficiency of 1.0 (exponential amplification). The deviation of the calculated value of AE from 1.0 provides a measure of the level of PCR inhibition in the sample. The most systematic way of measuring inhibition using the AE is to spike the sample with different amounts of pure DNA from a source other than the target and generate a standard curve based on this alien DNA (34). In this

way, the level of PCR inhibitors is kept constant for all DNA dilutions and an inhibited sample would give an AE of less than 1.0. This method can be used to compare different DNA extraction methods with regard to their ability to produce inhibitor-free extracts (38). The use of alien DNA removes the effect of the DNA yield, which would make the results biased. Zebra fish DNA (78) and potato DNA (76) have been as used, and these are suitable for testing extracts from all organisms and plants, except for zebra fish and potato, respectively.

Another way of measuring inhibition through the AE is to dilute the sample directly and create a standard curve based on the target DNA. However, the inhibitors are also diluted, which leads to a larger inhibition effect for high concentration dilutions than for lower ones. This gives a steeper standard curve slope, and the AE for a sample containing inhibitors could deviate from the expected low value, and instead exceed 1.0. If the objective is to thoroughly investigate the inhibition effect that a certain sample matrix has on PCR, it is better to use alien DNA than a dilution series of the sample itself.

Pure DNA is often used to obtain standard curves for absolute quantification. For the quantification to be correct, the AE of the sample must be the same as for the standard DNA. This is not the case for partially inhibited samples. A new data analysis method, taking the slope of the amplification curve, which is affected by inhibitors, into account has been proposed as a way of dealing with this problem (89). There, C_q values are defined as the intersection of the x-axis and the tangent to the inflection point of the amplification curve. This alternative method was found to give better consistency between inhibited and pure samples than both the threshold method and the second derivative maximum method.

3.3. Modeling

The PCR inhibition of a sample can be investigated by mathematical interpretation of the amplification curve using computer modeling (29, 90). The shape of a curve that is affected by inhibitors differs from that given by a pure sample (43) due to differences in the kinetics of the reaction. An inhibited sample generally gives a flatter, "less exponential" curve. The end-point fluorescence value can also be lowered by inhibitors. Tissue-specific inhibition in bovine RNA analysis has been confirmed using mathematical modeling of the amplification curve (91).

In absolute quantification the DNA concentration will be underestimated if the AE of the sample is lower than that of the DNA standards. Using a mathematical model, it has been shown that differences in AE greater than 0.2 caused more than 30% underestimation of the DNA in samples from genetically modified organisms (38). Accurate, absolute quantification of DNA using real-time PCR therefore requires that the AE of the external standard and samples are the same or at least that the differences in

efficiency are known. This can be achieved through computer modeling. A model can be fitted to experimental data to analyze a part of the amplification curve, and a sample-specific AE can be calculated, without the laborious work of spiking the sample with alien DNA and obtaining a standard curve as described above. The resulting AE is compared with the AE of the pure standard DNA. Samples with efficiencies outside a predetermined interval are considered outliers and cannot be confidently quantified using the standard curve (92).

4. Amplification Facilitators and PCR Buffer Systems

The difference in AE between different polymerases can to some extent be explained by differences in the buffers, the presence of PCR facilitators, the pH, and salt concentration (29). The resistance to inhibitors of a given polymerase can therefore be altered by using different buffers (9, 93). Both the AE and the detection window can be improved by changing the buffer (9). Several compounds have been shown to facilitate PCR (Table 3). Facilitators were first used to increase the specificity and fidelity of PCR, but some have also shown the capacity to relieve PCR inhibition (94). Recently, manufacturers have started adding PCR facilitators to buffers for commercial DNA polymerases. The *Tth* buffer (Roche Diagnostics, Mannheim, Germany) and the Klen*taq* buffer (DNA Polymerase Technologies, St. Louis, MO) both contain the detergent Tween 20, the *Tth* buffer also contains BSA. CertAmp buffer (Biotools, Madrid, Spain) contains glycerol. The effect of PCR facilitators is dependent on the facilitator concentration, and overloading will result in the inhibition of amplification, as has been shown for BSA, Tween 20, Triton X-100 (63), formamide, and glycerol (95). Synergistic effects between different types of facilitators should not be expected (94), on the contrary combining facilitators can cause inhibition (95). Categorization of PCR facilitators into five groups has been proposed (7): (1) proteins, (2) nonionic detergents, (3) organic solvents, (4) biologically compatible solutes, and (5) polymers.

4.1. Proteins

Bovine serum albumen (BSA) is the most commonly used PCR facilitator. BSA is a transport protein that binds fatty acids (lipids) and organic molecules. Its excellent binding capacity makes it suitable for reducing various types of inhibition in *in vitro* amplification. BSA binds the inhibitors heme and melanin (11), in the latter case preventing it from binding to the polymerase. BSA may also act as a competitive target for proteases, thereby sheltering the polymerase from these. When BSA is present, phenols preferentially bind to these molecules instead of to the polymerase. A range of different BSA concentrations has been used to deal with inhibition

Table 3
PCR facilitators used to alleviate inhibition

Type of facilitator	Facilitator	Concentration	Observed effect	Reference
Protein	BSA	0.1–1.28 g/l	Relieves inhibition due to bile salts, feces, $FeCl_3$, fulvic acid, hemoglobin, humic acid, immunoglobulin G, lactoferrin, meat, melanin, tannic acid, waste water sludge, 50-year-old saliva stains	(11, 16–18, 45, 51, 61, 88, 94, 96)
Protein	Gp32	0.1–0.15 g/l	Relieves inhibition due to feces, $FeCl_3$, fulvic acid, hemoglobin, humic acid, lactoferrin, meat, tannic acids	(16, 51, 94)
Protein	A-macroglobulin	0.04 g/l	Relieves inhibition due to proteases in milk	(23)
Protein	Casein	0.01% (w/v)	Relieves inhibition caused by bile salts	(61)
Protein	Lima bean trypsin inhibitor	0.02 g/l	Relieves inhibition caused by blood	(8)
Protein	Phytase	50 U/ml	Relieves inhibition due to phytic acids in feces	(68)
Protein	Proteinase inhibitor	1×	Relieves inhibition in samples of feces	(94)
Protein	Soybean trypsin inhibitor	0.04 g/l	Relieves inhibition due to proteases in milk	(23)
Nonionic detergent	Tween 20	0.5–2.5%	Relieves inhibition in samples of feces, phenolic compounds, and plant polysaccharides	(94, 99, 100)
Organic solvent	DMSO	2–10%	Rescue of failed amplification	(103, 104)
Organic solvent	Formamide	0.01% (w/v)	Relieves inhibition due to bile salts	(61)
Biologically compatible solute	Betaine	11.7% (w/v)	Relieves inhibition due to hemoglobin	(16, 94)
Polymer	PEG 400	5–15%	Relieves inhibition due to blood and plant polysaccharides	(94, 99, 103)

in different assays (Table 3). In the *Taq*-mediated detection of hydrogenase A associated with *Clostridia* in sludge waste water and manure, 100 ng BSA/µl gave more efficient and specific amplification, and a better detection limit, than both lower and higher concentrations (96). When comparing different PCR facilitators for blood, feces, and meat samples, with the enzymes *Taq* and r*Tth*, BSA was found to have the best performance and reduced inhibition in all these types of sample (94). Tween 20, glycerol, polyethylene glycol (PEG), dextran, formamide, and dimethyl sulphoxide (DMSO) had no effect on inhibition in this study. The protein Gp32 shows similar inhibition-alleviation properties to BSA (51, 94). However, the use of Gp32 instead of BSA is not recommended since it is far more costly. Specific enzymes have been successfully used to reduce inhibition from some compounds, such as phytase for phytic acid (68) and heparinase for the anticoagulant heparin (73). Skim-milk alleviates inhibition of *Taq* amplification of plant samples containing polyphenolic compounds (97) and humic material containing environmental samples (98) and also increases the specificity of amplification (98). The active substance is probably the protein casein, and it is believed to function in the same manner as BSA, preferentially binding compounds that would otherwise bind to the polymerase lowering its efficiency.

4.2. Nonionic Detergents

The detergents Tween 20 and Triton X-100 are frequently used as amplification facilitators, to reduce inhibition in samples from feces (94), plant polysaccharides (99), and phenolic compounds (100), although the mechanism is unclear. However, Triton X-100 at a concentration of 2% v/v was found to decrease the specificity of a *Taq* polymerase assay, indicating that care should be taken not to overload the reaction. Tween 20 did not show this effect (101).

4.3. Organic Solvents

DMSO and formamide are commonly used for PCR facilitation. Formamide denatures DNA and can facilitate amplification for assays with insufficient thermal denaturation (102), or if a high GC content elevates the melting temperature, making denaturation more difficult. Formamide also has some inhibitor-alleviation capabilities, as has been shown for bile salts (61). The effect of organic solvent facilitators is based on their ability to destabilize DNA.

4.4. Biologically Compatible Solutes

Betaine and glycerol are frequently used facilitators. Betaine increases the thermostability of proteins and has been reported to reduce inhibition in blood and meat samples (94).

4.5. Polymers

PEG and dextran are the most common polymer PCR facilitators. PEG stabilizes the DNA polymerase and has been used to reduce inhibition in samples of blood, feces (94), and plant polysaccharides (99).

5. Sampling

Sampling is a key step in diagnostic PCR analysis. Good sampling should provide a sample that is representative of the material to be tested, maximize sample uptake and minimize PCR inhibitor uptake. In clinical diagnostics, the size of the sample is usually not a limiting factor, and sampling is straightforward and reproducible, e.g., the sampling of venous blood. In microbial detection for environmental or food analysis, as well as forensic DNA analysis, sampling is more complicated. The amount of target material can be very low, and the background may be any type of surface or liquid, such as animal carcasses or waste water. In practice, one cannot be certain that amplifiable DNA is actually present in the sample until it has been subjected to PCR. Great care is needed during sampling in order to minimize the risk of false-negative results.

5.1. Standardization

Standardization of sampling is vital to obtain reliable and reproducible PCR results. However, standardized methods are lacking in several scientific fields. In microbial food and feedstuff testing, there are specific international standards governing the sampling of different substrates, e.g., carcasses (105) and horizontal surfaces (106). The standards define where samples should be taken, the size of the sampling surface, approved sampling methods and how each method is to be applied, including how to hold the swab, how many times the area should be covered, and which buffer to use to moisten the sampling swab or material. These standards also govern the transportation and handling of samples before analysis. Prior to the terrorist actions involving *B. anthracis* spores in the USA in 2001, there were no standardized sampling methods for biocontaminants on different surfaces (107). The events clearly illustrated the need for standardization, and significant work has since been carried out to develop and evaluate sampling methods for bacterial spores on different surfaces, both for PCR (108, 109) and classical microbial analysis (107–111).

There are no sampling standards in forensic science; partly because of the wide variety of materials and tissues investigated and also for historical reasons. Efforts have been focused on developing sensitive and highly discriminatory PCR-based identification systems, while little has been done to develop standardized sampling methods.

Several sampling techniques used for diagnostic PCR, e.g. swabbing and excision, are based on protocols for classical, non-PCR analysis methods such as cultivation-based microbial detection (112) and immunoassays (113). This can be problematic since the biochemical and physical process of PCR is very different from that of classical methods. The material of the sampling swab itself may inhibit PCR, as is the case with calcium-alginate swabs and Al^{3+}-releasing

aluminum swab shafts (27). Cotton has been shown to inhibit PCR (101), but the pure cotton used in dedicated PCR swabs supplied by several manufacturers should not cause inhibition.

5.2. Direct Sampling

In direct sampling, the material carrying the target cells is placed directly into a tube for sample treatment, e.g., excision of meat and soil sampling. This gives a relatively high amount of target cells, but a high level of PCR inhibitors may also be released from the background material. Excision of beef carcasses for pathogen detection gives higher microbial yields than surface swabbing using cotton swabs for cultivation methods (114), but maceration releases a great deal of tissue debris into the extract, inhibiting PCR. Excision can only be performed on a rather small area, it is time consuming, requires special skills, and destroys the sample. Thus, excision is not a suitable sampling method for online diagnostic PCR analysis. Direct sampling of soil for microbial diagnostics also shows substantial levels of inhibition (19, 20), and extensive sample treatment is needed. In forensics, clothing such as suede and denim introduce PCR inhibitors when used directly for analysis (115).

In clinical analysis, e.g., the screening of blood from newborns, filter paper provides a stable sampling matrix without the need for cooling. Filter paper has been used for many years in various enzyme and immunoassays, as well as being a good medium for PCR-based analysis (113). FTA® cards (GE Healthcare, Little Chalfont, UK) are chemically treated filter paper, on which the cells are lysed and the proteins degraded directly on contact, and DNA is immobilized within the paper structure. By shielding DNA from oxidation and nucleases FTA cards allow for long-term storage at room temperature (116).

5.3. Swabbing Techniques

Surface swabbing can be used instead of direct sampling in order to lower the amount of PCR inhibitors released from the substrate. Polyurethane sponge swabbing has been shown to give yields comparable to those from excision for beef, pork, and lamb carcasses (117). Cotton swabbing of stains on suede has been shown to reduce the amount of PCR inhibitors in the sample, compared with direct sampling (unpublished data, Swedish National Laboratory of Forensic Science).

Swabbing using a moistened cotton swab has been used for microbial cultivation methods for many years (112). However, cotton is not an ideal material for sensitive analysis because of its high cell absorption; furthermore there are difficulties in standardizing sampling methods and the reproducibility is low (112). Despite these drawbacks, it is still probably the single most popular method of diagnostic PCR sampling. Various liquids have been used to moisten swabs before sampling. Physiological saline was found to improve cell recovery for several kinds of crime scene stains compared with water (unpublished data, Swedish National

Laboratory of Forensic Science), and ethanol is sometimes used for sampling of contact traces (118). Moistening swabs with extraction buffer was shown to increase DNA yield in the sampling of saliva from skin (119). Surfactants improve the collection of spores from nonporous surfaces (120), further showing that both the collection material and the buffer must be appropriate for the sample in question.

The double-swab technique (114), or wet and dry swab method (105), is a slight modification of cotton swab sampling. The surface is first swabbed with a moistened cotton swab, and then with a dry one to soak up excess fluid. As for the single-swab method, the double-swab technique was first used for microbial cultivation methods (114) and then employed in PCR analysis. The method is commonly used in forensics, e.g., for collecting saliva from bite marks (121) and contact stains from a range of different surfaces (122).

Nylon flocked swabs are a newer sampling medium, developed especially for PCR analysis. Flocked swab sampling give RNA yields comparable to nasal secretions for clinical virus detection and also allow for a more standardized sampling procedure (123). Thanks to their design, with thousands of short nylon fibers extending from the swab head enabling sampling of only the outermost cells, less PCR inhibitors are probably introduced than in the case of nasal secretions and other swab materials. Also, the target cells remain on the surface of the fibers and are easily released.

For the detection of biocontaminants on surfaces, large areas must be sampled to avoid false-negative results. Neither ordinary cotton swabs nor swabs made of other materials are suitable for collecting samples from large surfaces. A commercially available biological sampling kit (BiSKit) containing a thick foam material enables both wet and dry sampling of a surface area of 1 m² for subsequent PCR analysis (109). BiSKit foam has been shown to perform better than cotton and foam swabs, which only enable swabbing of around 100 cm² of both metal and wood laminate surfaces. Gauze can also be used for swabbing of large surfaces, e.g., for carcass sampling (105).

Swabbing involves the use of water or another liquid to release cells, but soluble inhibitors are released at the same time. Dry sampling, e.g., tape lifting, is a way of avoiding the collection of inhibitors with the sample. A piece of tape, e.g., hydrophilic adhesive tape (HAT) (124), is pressed against the substrate a number of times, usually until it no longer adheres. Cells are transferred to the tape, which is placed in a tube for DNA extraction. Tape lifting is ideal for contact DNA on nonporous surfaces. In the investigation of a bank robbery in Sweden, one of the perpetrators had put his hand on the shoulder of one of the bank employees. A complete DNA profile of the suspect was obtained using tape lifting and he was apprehended (125). Tape lifting has been shown

to give a higher DNA yield and visibly purer extracts than the double-swab technique and excision in forensic DNA analysis of shoe insoles (126).

6. Sample Treatment

The objectives of sample treatment are to prepare the sample for PCR amplification by (1) concentrating target nucleic acids, (2) removing/neutralizing PCR inhibitors, and (3) making the sample more homogeneous to ensure better repeatability in the amplification. Sample treatment may involve cell separation, cell lysis, and DNA purification. Direct cell lysis followed by DNA purification is the most common approach; several DNA extraction methods combine these two steps (Table 4). To enable sensitive microbial detection using PCR, a pre-enrichment step may also be necessary before nucleic acid purification. Generally, PCR is regarded as extremely sensitive, but in the context of finding one *Salmonella* bacterium in 25 g of minced meat, which is the legislative requirement for food safety in Europe, PCR is in fact rather insensitive compared with culture-based methods (74).

Table 4
Sample treatment methods

Analysis step	Method	Reference
Cell separation	Aqueous two-phase system (solubility separation)	(15, 154)
Cell separation	Flotation (buoyant density separation)	(129–131)
Cell separation	Flow cytometry	(67)
Cell separation	Immuno-magnetic affinity separation	(67, 128)
Cell separation	Laser capture microdissection	(132–135)
DNA extraction	Agarose gel diffusion of inhibitors (size separation)	(155)
DNA extraction	Chelex DNA extraction	(56, 156)
DNA extraction	Detergent treatment (non-lysis DNA release)	(101)
DNA extraction	Phenol chloroform purification (solubility separation)	(136, 137)
DNA extraction	Protease treatment (cell lysis, protein degradation)	(28, 152)
DNA extraction	Silica beads (DNA binding)	(145, 146, 148, 149)
DNA extraction	Solid-phase extraction (protein adsorption)	(26, 47, 98, 142, 143)
DNA purification	Coated activated charcoal (protein adsorption)	(140)
DNA purification	Dilution	(19, 20, 54)
DNA purification	Filter purification	(139)
DNA purification	NaOH treatment	(115)

6.1. Cell Separation

The separation of target cells from the surrounding matrix is often complicated and time consuming. However, since nucleic acid inhibitors are removed before they can interfere with the released DNA molecules, it may be beneficial for certain types of samples and complex sample matrices. Whole cells also provide a more stable environment than free DNA. Affinity bead separation is a classical way of separating a certain type of cell from other cells and the sample matrix. The beads are either coated with generic proteins or specific antibodies, depending on the level of specificity required. Dynabeads® coated with lectin have been shown to provide successful purification of gram-positive bacteria from meat samples (127). Antibody separation is very specific and has been successfully applied to cell separation in oocyst samples (67), as well as in the isolation of *Listeria* in milk (128). However, the specificity can be a drawback, as PCR simply confirms the antibody separation and the capacity of PCR to distinguish the target from a DNA background is not used.

Flow cytometry is another very specific cell separation method that has been used with success for PCR analysis (67). Both antibody separation and flow cytometry are quite expensive and perform poorly in the presence of complex sample matrices. Flotation is an efficient separation system that exploits the difference in the buoyant densities of cells and particles to separate whole cells from PCR-inhibitory substances and free nucleic acids (129). It is robust and specific, and also laborious in its present form. Flotation isolates target cells in a thin band using centrifugation in a medium such as Percoll® (130). The method has been shown to effectively remove PCR inhibitors from various food samples (131). Laser capture microdissection (LCM) utilizes an infrared laser beam to lift cells from a surface, preferably a microscope slide (132). LCM has been used in forensic DNA analysis to separate sperm cells from epithelial cells (133) and to separate leukocytes from buccal cells, in order to avoid mixed DNA profiles (134). The technique has also enabled DNA typing of crime scene samples mixed with soil, by separating cells from the matrix (135). LCM requires expensive equipment and is time consuming, but can be valuable in solving serious crimes.

6.2. DNA Extraction and Purification

There are many rather expensive commercial DNA extraction kits whose compositions have not been disclosed. Most less costly methods are based on either Chelex 100 resin or phenol chloroform. Chelex is a chelating styrene divinylbenzene copolymer that binds polyvalent metal ions that may otherwise catalyze DNA degradation at high temperatures and at low ion contents (56). Water or an aqueous buffer is added to the sample, and the cells are removed from the substrate by vortexing. The cells are then pelleted using centrifugation, and water-soluble inhibitors can be discarded with the supernatant. However, disrupted cells and free

DNA are lost in the process, lowering the yield. The sample is heated to 56°C to achieve lysis and boiled to degrade the proteins, both in one tube without sample transfer. The Chelex method is described as "quick and dirty," i.e., it is a rapid, cheap method that can be applied to most types of sample, but it is not a powerful inhibitor remover. Care should be taken not to include Chelex beads in the PCR as they would chelate the necessary Mg^{2+} ions. Since centrifugation is a vital step in the Chelex method, it is difficult to automate the procedure.

Phenol chloroform purification, or organic purification as it is also called, is a powerful method for obtaining inhibitor-free extracts (136, 137). It has long been the preferred method of extracting DNA from "dirty" samples, such as bone and soil. One drawback is that phenol is toxic, and working with it thus constitutes a health hazard. Phenol chloroform effectively removes inhibitors that are soluble in the alcohol phase, e.g. many types of proteins. For inhibitors with solubilities similar to DNA, such as humic substances, polysaccharides, hemoglobin, and urea, another purification strategy is needed. For example, alkaline and acid hematin have been successfully removed from DNA extracts from blood using phenol chloroform, but complexes of ferric heme and serum proteins were simultaneously purified with DNA in the water phase and inhibited PCR (48). Washing with water, exclusion by size or binding of proteins to a solid phase such as silica beads, are possible ways of removing water-soluble inhibitors. Phenol inhibits PCR (21) and must be completely removed from the DNA template after purification. Protease treatment, usually employing proteinase K, is a common step in phenol chloroform purification and is applied in many commercial DNA extraction kits. The proteases mediate cell lysis and degrade cell proteins and proteins bound to DNA, which could otherwise interfere with the PCR (28). Collagenase has been suggested as an alternative to proteinase K in phenol purification of human bones, as human collagen was identified as a key inhibitor of such samples (62). Ethanol and isopropanol are commonly used for the precipitation of DNA after treatment with phenol chloroform, e.g., when purifying environmental soil samples (138). A combination of PEG and NaCl could provide an alternative to the alcohols, since this has been shown to produce extracts from soil with less humic substances and more DNA than when using isopropanol (98).

If the initial DNA extraction using phenol chloroform or Chelex fails to remove inhibitors, the template can be further purified. Filtration and dilution are two quick and simple DNA purification methods. In filtration, the sample, together with an aqueous buffer, is washed through a filter with pores that allow the smaller inhibitor molecules to pass through, while retaining the larger DNA molecules. Microcon filter tubes (Millipore, Billerica, MA) have been used to purify forensic DNA extracts from cigarette

butts (139). Dilution, i.e., simply adding water or a buffer to the extracts, has been successfully used to circumvent inhibition from humic substances (19, 20, 54), urine (54) and reverse transcriptase used in cDNA formation (49). Other DNA purification methods of varying complexity are also available, depending on the sample in question. Sodium hydroxide (NaOH) treatment alleviates inhibition in blood samples from various substrates, including soil and wood (115). NaOH denatures the DNA strands, releasing PCR inhibitors that bind to double-stranded DNA, and alkaline conditions are believed to inactivate protein inhibitors. Because of the risk of DNA degradation, NaOH treatment is not recommended for samples with minute amounts of DNA. Activated charcoal coated with *Pseudomonas fluorescens* has been found to adsorb water-soluble PCR inhibitors (140). Using uncoated charcoal resulted in complete adsorption of all DNA. Chemical flocculation, developed as a method of removing suspended organic solids from waste water, has been successfully applied as a DNA purification step for soil samples (141).

An alternative to phenol chloroform purification is solid-phase extraction, a method in which particulate matter, such as silica beads, is used to adsorb aqueous proteins (142). Small size and large surface area make adsorption a rapid process. The negatively charged bead surface binds positively charged proteins with high affinity, whereas the negatively charged DNA is repelled. Solid-phase extraction can be performed in two ways: (1) by adding the beads to the template tube, which is then agitated allowing the proteins to become adsorbed, and then removing the proteins by filtration or centrifugation or (2) by passing the sample through a column packed with beads upon which the proteins are adsorbed. Sepharose 6B affinity beads have been used to purify extracts from indigo dyes in denim (26), bone samples and saliva on envelopes (143), by adding the resin to the samples to allow inhibitor adsorption, and then removing them by centrifugation. A great deal of DNA is lost in this process, and caution should therefore be exercised when applying the method (143). The column approach is better suited for small volumes and samples with high protein concentrations. Sepharose 4B beads are used in spin column purification, having both size-exclusion and adsorption capabilities. Polyvinyl polypyrrolidone (PVPP) is another frequently used spin column medium, which forms a complex together with PCR-inhibiting phenolic compounds via hydrogen bonds (144). The complex is then precipitated and removed. Arbeli and Fuentes (98) showed that a combined Sepharose-PVPP spin column gave more efficient soil sample purification than separate Sepharose and PVPP columns. The Sepharose first separates humic compounds from the DNA by size exclusion, and the PVPP then absorbs most of the remaining humics. Others have found PVPP methods to be unreliable in removing PCR-inhibiting compounds (141). PVPP treatment has also been shown to lower the DNA yield (138).

Solid-phase extraction is quick and safe, in contrast to phenol chloroform, and the risk of phenol impurities in the extract is removed. Today, several column-based solid-phase extraction kits are commercially available. In a recent survey, 71% of real-time PCR users stated that they used spin-column-based methods for RNA extraction, while only 8% used home-made reagents and protocols such as phenol chloroform (77). The future for solid-phase extraction could lie in automated miniaturized systems. These could make DNA purification quicker, by reducing the running time, and cheaper, since smaller amounts of sample and chemicals are needed. Analysis could also be performed in the field. Silica and silica-coated beads are promising media for use in miniaturized systems. Wen et al. (47) developed a dual-phase microchip. As the lysed blood sample flows through the chip, proteins, such as hemoglobin and lactoferrin are bound to the silica beads. The DNA is bound to a monolithic column and subsequently released.

In the presence of a chaotropic salt, such as guanidinium thiocyanate, DNA binds to silica particles. The chaotropic salt also lyses cells and inactivates proteins. These properties can be used for efficient DNA purification. When DNA binds and forms a complex with the silica, unbound material such as cell debris and proteins can be washed away in a series of washing steps using washing buffers and/or ethanol (145). Incubating the silica–DNA complex in a low-ionic-strength buffer releases the DNA into solution. The method has been improved by introducing magnetic silica-coated beads. The magnetic force can be used either to move the beads from tube to tube during washing or to immobilize the beads in one tube while changing the washing liquid, simplifying sample handling. The technique is suitable for automation, since no centrifugation is needed. Several commercially available kits can be used either manually or with pipetting robots. Promising miniaturized systems with DNA-binding silica beads have also been developed (146).

6.3. Automation

Automation of DNA extraction is important for increasing sample throughput and ensuring the quality of laboratory analysis. Automating the extraction of DNA from heterogeneous, single-source samples in simple sample matrices, such as fresh venous blood in clinical analysis, is quite straightforward. A cheap, fast, and simple two-step method of automating the analysis of blood samples on filter paper punches in 96-well plates has been developed by Lin et al. (113). Leukocytes are released from the filter paper using methanol and lysed by heating in Tris buffer. When lysed in water a large amount of heme was released, but using Tris buffer pure extracts were produced. DNA binding FTA filter paper punches can be used directly in PCR analysis, after a simple washing procedure using, as in an automated system for forensic DNA reference samples (147).

Automation of DNA extraction from complicated and hetero-geneous samples and sample matrices, such as forensic crime scene samples, is more complicated. The methods available today are mainly commercial kits relying on DNA-binding magnetic beads, which require extensive pretreatment, e.g. lysis and removal of the substrate in order to prevent the pipettes from becoming clogged. Biorobot M-48 (Qiagen, Hilden, Germany) utilizes a closed system of magnetic silica-coated beads and a DNA purification chemical in pre-filled cartridges. In a study by Nagy et al. (148) the Biorobot M-48 gave higher DNA yields than phenol chloroform extraction, and produced inhibitor-free extracts from a range of sample and substrate types. However, Kishore et al. (149) found it necessary to modify the system using carrier RNA to give results comparable to those obtained using the manual phenol chloroform method. Biorobot EZ1 (Qiagen) and Maxwell® 16 (Promega, Madison, WI) are automated, small-scale, desktop plug-and-play systems for low- to medium-throughput analysis. The EZ1 has been extensively evaluated and has been shown to produce results comparable to those obtained with phenol chloroform and Chelex extraction (118, 149, 150). Open robotic systems, such as the Hamilton Star (Hamilton, Reno, NV), Freedom EVO® (Tecan, Männedorf, Switzerland), and versions of BioMek (Beckman Coulter, Fullerton, CA) have been used together with automatable magnetic bead sys-tems such as DNA IQ™ (Promega) and ChargeSwitch® (Invitrogen, Carlsbad, CA). In a forensic DNA laboratory, the Freedom EVO and DNA IQ were found to generally perform slightly worse than phenol chloroform extraction for crime scene samples, but pro-duced acceptable DNA yields and low amounts of PCR inhibitors in most samples (151).

Apart from the standard magnetic bead procedures, there are a number of promising new methods that can improve DNA purification in samples. A simple, automatable closed-tube method using a novel protease (EA1) has been developed by Moss et al. (152). A buffer containing protease is added to the sample, which is heated to 75°C for 15 min to achieve cell lysis and protein break-down, and then to 94°C for 15 min to break down protease remains. The method gave better results than the Chelex system for crime scene blood stains on different materials. Although the extracts were visibly red, the inhibitor heme had been broken down and inactivated. In samples that had been subjected to different kinds of stress the method also produced higher DNA yields than Chelex, where free DNA is washed away. The EA1 method under-standably failed in the analysis of samples from denim and cigarette butts, since these substrates have an abundance of non-protein PCR inhibitors.

Synchronous coefficient of drag alteration (SCODA) is a novel and possibly automatable technique for DNA purification in complex samples, e.g., in forensics (153). A rotating electric

field separates the DNA molecules from the cell lysate and other particulate matter on an agarose gel, using the long-charged property of DNA as the basis for separation. Direct release of DNA mediated by a detergent is a possible alternative to maceration for analysis of plants. An automatable 96-well DNA extraction system suitable for different types of plant samples has been developed, using Tween 20 as detergent (101).

7. Pre-PCR Processing

Knowledge concerning PCR-inhibitory mechanisms at the molecular level is vital for the development of accurate and efficient real-time PCR-based systems for rapid diagnostics. To achieve the full potential of diagnostic PCR, the issue of inhibitors must be thoroughly addressed. Since inhibiting molecules can be introduced at any stage of the process, coming from the sample itself, the sampling equipment or the procedure used for sample preparation, all steps must be verified and controlled for each type of sample. Pre-PCR processing includes all the steps prior to the detection/quantification of the PCR product, i.e., sampling, sample treatment, and PCR. Choosing the appropriate thermostable DNA polymerase and using PCR facilitators are central components of this integrated concept (7). PCR inhibitors are defined by the PCR chemistry, in other words, the performance of PCR in the presence of inhibiting molecules depends on the choice of thermostable DNA polymerase, buffer, and facilitators (8, 9, 94). Therefore, the first step of pre-PCR processing must be the identification of the PCR chemistry best suited for the sample and assay to be used. Minimizing the effect of inhibitors in this fashion does not affect the sample itself, in contrast to sample treatment. Extensive DNA extraction and purification should be avoided if possible, since this may lower the amount of target DNA. If the inhibitory effects are not eliminated, sampling and sample treatment must be improved. In the scientific community, sampling is often performed in "the usual way." The development of sampling methods has not followed the development of PCR instruments and PCR-based analysis methods. Most of the techniques in use today, e.g., cotton swabbing and direct sampling, were developed for classical analysis methods (112). As the result of the analysis depends greatly on the sample and the way in which it is collected, more effort should be devoted to the development of sampling strategies.

The idea of pre-PCR processing is to generate PCR-compatible samples allowing high analytical precision. Simon et al. (100) were able to amplify inhibited *Listeria monocytogenes* samples in two separate ways: by employing silica column separation and by adding the PCR facilitator Tween 20. Juen and Traugott (88) reported

successful amplification of 98% of their severely inhibited soil samples when adding BSA as a facilitator, and applying DNA purification using silica beads only gave a success rate of 56%, while, at the same time, over 90% of the DNA was lost. In some cases, the combination of improved sample treatment and PCR chemistry is required to produce acceptable results. Abu Al-Soud et al. (61) alleviated the inhibition caused by bile salts in *Helicobacter* detection by combining dilution and heating with the addition of the PCR facilitators casein and formamide. The combined effect was greater than when using only dilution and heating or facilitator addition. The concept of pre-PCR processing can be the basis for fast and efficient analysis systems, such as a newly developed 12-h method for *Salmonella* detection, combining pre-enrichment, sample treatment, and an optimized quantitative real-time PCR assay (39). Reducing the amount of manual handling in the pre-PCR processing and integrating this into an automated system are future challenges.

Acknowledgments

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