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REVIEW ARTICLE

# PCR inhibitors – occurrence, properties and removal

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## Summary

The polymerase chain reaction (PCR) is increasingly used as the standard method for detection and characterization of microorganisms and genetic markers in a variety of sample types. However, the method is prone to inhibiting substances, which may be present in the analysed sample and which may affect the sensitivity of the assay or even lead to false-negative results. The PCR inhibitors represent a diverse group of substances with different properties and mechanisms of action. Some of them are predominantly found in specific types of samples thus necessitating matrix-specific protocols for preparation of nucleic acids before PCR. A variety of protocols have been developed to remove the PCR inhibitors. This review focuses on the general properties of PCR inhibitors and their occurrence in specific matrices. Strategies for their removal from the sample and for quality control by assessing their influence on the individual PCR test are presented and discussed.

## Background

In the last few decades, the polymerase chain reaction (PCR) has become one of the most powerful molecular biological tools. PCR may be used for diagnosis of infectious or hereditary diseases and for genetic analyses in a large variety of sample types. The PCR is a very rapid and sensitive method, in which genomic DNA is exponentially amplified by a DNA polymerase using specific primer molecules. Variations of the PCR comprise the reverse transcription (RT-) PCR using RNA as template, which is first transcribed into DNA by a reverse transcriptase, or real-time PCR, which uses fluorescent probes for the detection of the PCR product providing quantitative information. The PCR is an enzymatic reaction and therefore sensitive to inhibitors. The occurrence of such so-called PCR inhibitors, which comprise all substances that have a negative effect on the PCR, is a major drawback of the PCR. PCR inhibitors can originate from the sample or may be introduced during sample processing or nucleic acid extraction. The major consequence of a partly or total inhibition of the PCR is a decreased sensitivity or false-negative results, respectively.

In the following sections, the different classes of PCR inhibitors and their mechanisms of action are presented. The incidence of PCR inhibitors in different types of matrices such as clinical, food or environmental specimens will be reviewed. Finally, different possibilities to remove PCR inhibitors as well as strategies for quality controls of the PCR will be presented and discussed.

## Substance classes of PCR inhibitors

PCR inhibitors are a very heterogeneous group of chemical substances. One certain matrix may contain many different inhibitory substances and the same inhibitors can be found in many different matrices. Organic as well as inorganic substances, which may be dissolved or solid, can appear as PCR inhibitors. Calcium ions are an example for inorganic substances with inhibitory effects on the PCR. However, most of the known inhibitors are organic compounds, for example, bile salts, urea, phenol, ethanol, polysaccharides, sodium dodecyl sulphate (SDS), humic acids, tannic acid, melanin as well as different proteins, such as collagen, myoglobin, haemoglobin, lactoferrin, immunoglobin G (IgG) and proteinases (Rossen *et al.*

1992; Rådström *et al.* 2004). Besides the substance class, the concentration of the compound is important for its inhibitory effect. For a detailed review on the relation of the concentration of inhibitors (e.g. sodium chloride, magnesium chloride, sucrose, phenol, SDS, ethanol or cetrimonium bromide) and their inhibitory effects, see the article of Rossen *et al.* (1992).

PCR inhibitors can be found in a variety of biological materials (organs, blood, body fluids etc.), environmental samples (water, soil, air etc.) and food (meat, milk, fruits, vegetables, seafood etc.). In addition, inhibitory substances may also be unintentionally added during transport, sample processing (e.g. pre-concentration procedures) or nucleic acid extraction. Table 1 shows some examples of matrices and their typical inhibitors.

## Mechanisms of action of PCR inhibitors

PCR inhibitors may interfere with different steps of a PCR analysis (Fig. 1). Generally, several PCR components, especially DNA, may adsorb to polymeric surfaces, for example, to the wall of vessels and reaction tubes, during sample processing, extraction or during PCR (Butot *et al.* 2007b; Fox *et al.* 2007; Gassilloud *et al.* 2007; Gonzalez *et al.* 2007). The efficacy of sample processing and nucleic acid extraction may be affected. Nucleases may degrade template RNA or DNA. Phenols may cross-link RNA under oxidizing conditions and thus hamper RNA isolation (Su and Gibor 1988; Wilkins and Smart 1996). In addition, the existence of polysaccharides may reduce the capacity to resuspend precipitated RNA (Wilkins and Smart 1996; Sipahioglu *et al.* 2006). Reverse transcription may be inhibited, for example, by direct interaction of the enzyme with melanin (Eckhart *et al.* 2000). The DNA used as template of the PCR can be modified or degraded by nucleases and other substances. Annealing of the primers to the DNA template may be disturbed by certain PCR inhibitors (Chandler *et al.* 1998; Abbaszadegan *et al.* 1999). As this effect is because of a competitive binding of the inhibitor to the template, appropriate primer design leading to higher melting points can overcome this problem (Huggett *et al.* 2008; Opel *et al.* 2010).

There are many PCR inhibitors that target the DNA polymerase directly or indirectly. Proteases or detergents present in the reaction can degrade this enzyme (Rossen *et al.* 1992; Powell *et al.* 1994). For example, urea (Saulnier and Andremont 1992; Wilson 1997) and phenol (Katcher and Schwartz 1994) are known to degrade DNA polymerases. Calcium, collagen, haematin and tannic acid may inhibit polymerase activity (Opel *et al.* 2010). Melanin forms a reversible complex with the DNA polymerase (Eckhart *et al.* 2000) and polysaccharides may disturb the enzymatic process by mimicking the structure of nucleic acid (Peist *et al.* 2001). Humic acids interact with the template DNA and the polymerase thus preventing the enzymatic reaction even at low concentrations (Sutlović *et al.* 2005, 2008). Other substances react with cofactors of the polymerase. High concentrations of calcium may lead to a competitive binding by the DNA polymerase instead of magnesium and complexing agents, for example, tannic acid, deplete magnesium. In both cases, magnesium is no longer available as cofactor for the polymerase and its activity is decreased (Opel *et al.* 2010). For real-time PCR assays, the interference with the fluorescent probes or increased background fluorescence represents additional mechanisms of action for PCR inhibitors decreasing sensitivity. Examples of PCR

**Table 1** Selected matrices and their identified polymerase chain reaction inhibitors

| Matrix | Contained inhibitors | References |
|---|---|---|
| Clinical specimens (e.g. blood; muscle tissues) | Antiviral substances (e.g. acyclovir), Haemoglobin, Heparin, Hormones, IgG, Lactoferrin, Myoglobin | Al-Soud and Rådström (2001); Burkardt (2000); Rådström *et al.* (2004); Yedidag *et al.* (1996) |
| Stool | Complex polysaccharides, Bile salts, Lipids, Urate | Kreader (1996); Monteiro *et al.* (1997); Rådström *et al.* (2004); Chaturvedi *et al.* (2008) |
| Seafood, bivalves, oysters | Algae, Glycogen, Polysaccharides | Atmar *et al.* (1993, 1995); Richards (1999) |
| Berries | Phenols, Polysaccharides | Seeram *et al.* (2006); Wei *et al.* (2008) |
| Plants | Pectin, Polyphenols, Polysaccharides, Xylan | Demeke and Adams (1992); Henson and French (1993); John (1992); Sipahioglu *et al.* (2006); Su and Gibor (1988); Wan and Wilkins (1994); Wei *et al.* (2008); Wilkins and Smart 1996 |
| Cheese, milk | Proteases (e.g. plasmin), Calcium ions, | Bickley *et al.* (1996); Powell *et al.* (1994); Rossen *et al.* (1992) |
| Water, environment | Debris, Fulmic acids, Humic acids, Humic material Metal ions, Polyphenol | Abbaszadegan *et al.* (1993); Ijzerman *et al.* (1997) |
| Palaeobiology, archaeology, forensic | Bone dust, Coprolite Peat extract, Clay-rich soil | Baar *et al.* (2011) |

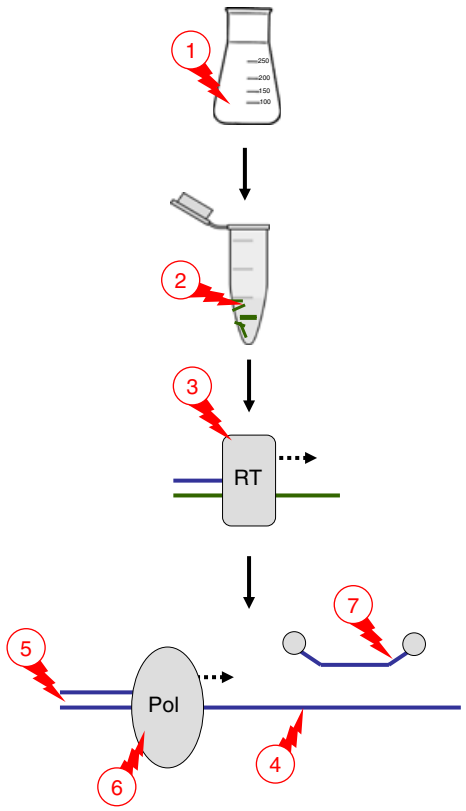


**Figure 1** Schematic presentation of the attack points of polymerase chain reaction (PCR) inhibitors during sample preparation and PCR. The nucleic acids may interfere with surfaces of the vessels (1) or substances may react with nucleic acids (2) during sample processing and extraction. Other substances inhibit reverse transcription (3) and degrade or modify the template DNA (4). Annealing of primers to the template can be hampered (5) or the DNA polymerase is degraded, inhibited or altered (6). Finally, substances may interfere with binding of probes or with their fluorophores (7).

inhibitors and their proposed mechanisms of action are presented in Table 2. However, it has to be mentioned that for many inhibitory substances, the distinct mechanism of action is not known so far.

## PCR inhibitors in clinical samples

In blood, serum or plasma samples, substances like IgG, haemoglobin and lactoferrin have been described as inhibitors of PCR (Al-Soud *et al.* 2000; Al-Soud and Rådström 2001). Anticoagulants, for example, heparin, may also inhibit the PCR (Costafreda *et al.* 2006). Many of these inhibitors are presumed to affect directly the RNA and not the enzymes of the reaction (Konet *et al.* 2000). In addition, hormones or antiviral substances like acyclovir can also affect amplification (Yedidag *et al.* 1996; Burkardt 2000).

The most critical component in urine samples is urea, which may lead to the degradation of the polymerase (Saulnier and Andremont 1992; Wilson 1997). However, the effect is dependant on the concentration of urea in the sample and initiates with a concentration of about 50 mmol $l^{-1}$ (Khan *et al.* 1991). As the urine excretion in humans is – dependent on diet and age – in the range between 340 and 580 mmol urea per day, inhibitory concentrations are not unusual.

Stool and faecal samples contain highly variable components dependant on nutrition, gut flora, lifestyle and environment of the patient (Oikarinen *et al.* 2009). Inhibitors may include polysaccharides or chlorophyll originating from herbs and vegetables, bile salts, urea, glycolipids, haemoglobin and heparin (Lantz *et al.* 1997; Monteiro *et al.* 1997; Pontiroli *et al.* 2011).

The most important inhibitory factors present in bile samples are the bile acids and their corresponding salts. However, efficient amplification can be achieved using a polymerase which is not sensitive to these substances (Al-Soud *et al.* 2005). The same applies to the analysis of eye fluids or muscle tissue containing myoglobin (Wiedbrauk *et al.* 1995; Bélec *et al.* 1998).

## PCR inhibitors in food and environmental samples

Many different PCR inhibitors have been identified in food. Fats, glycogen, polysaccharides, minerals as well as enzymes present in food may cause inhibition of the PCR (Powell *et al.* 1994; Richards 1999). In milk samples, PCR inhibition is mainly dependant on the concentration of calcium, whereas the fat content seems to have only minor influence on the amplification efficiency (Bickley *et al.* 1996). Additionally, Powell *et al.* (1994) identified plasmin, which degrades the *Taq* polymerase, as an inhibitor naturally occurring in milk.

In seafood, mainly polysaccharides seem to be responsible for PCR inhibition (Atmar *et al.* 1993, 1995). In addition, the glycogen content in the tissues of bivalve molluscs influences PCR efficiency (Enriquez *et al.* 1992; Richards 1999). Generally, the ability of bivalve molluscs to filter the water may lead to concentration of different inhibitory substances.

Plants carry many substances, such as polysaccharides, polyphenols, pectin and xylan, which may be co-extracted and thereafter hamper the PCR (Wei *et al.* 2008). Although for most of these polysaccharides (dextran, inulin, pectin or starch), no inhibitory effect was reported, some of them like dextran sulphate and ghatti gum affect the PCR efficiency. However, this effect was partly reversible by adding Tween 20, dimethyl sulphoxide or polyethylene glycol 400 (Demeke and Adams 1992). In another study, acidic polysaccharides (ghatti gum, xylan, dextran

**Table 2** Examples of PCR inhibitors and their mechanisms of action

| Inhibitor | Mechanism of action | References |
|---|---|---|
| Polyphenols<br>Polysaccharides | Co-precipitation with nucleic acid; reduction in the ability to resuspend precipitated RNA | John (1992), Sipahioglu *et al.* (2006), Su and Gibor (1988), Wan and Wilkins (1994) and Wilkins and Smart (1996) |
| Bacterial cells<br>Cell debris<br>Detergents<br>PCR additives<br>Proteins<br>Polysaccharides<br>Salts<br>Solvents | Degradation/sequestration of nucleic acids | Burkardt (2000), Katcher and Schwartz (1994), Peist *et al.* (2001), Rossen *et al.* (1992), Weyant *et al.* (1990) and Wilson (1997) |
| Polyphenols<br>Polysaccharides<br>Humic acids<br>Collagen<br>Melanin | Cross-linking with nucleic acids; change of chemical properties of nucleic acids | John (1992), Opel *et al.* (2010) and Wilkins and Smart (1996) |
| Humic acid<br>Humic matter | Binding/adsorption to nucleic acid and enzymes | Abbaszadegan *et al.* (1993) |
| Haematin<br>Indigo | Incomplete melting of DNA | Opel *et al.* (2010) |
| Metal ions | Reduction in specificity of primers | Abbaszadegan *et al.* (1993) |
| Detergents<br>Proteases<br>Urea | Degradation of polymerases | Powell *et al.* (1994), Rossen *et al.* (1992), Saulnier and Andremont (1992) and Wilson (1997) |
| Calcium<br>Collagen<br>Haematin<br>Herbal metabolites<br>IgG<br>Melanin<br>Myoglobin<br>Polysaccharides<br>Sodium<br>Tannic acid | Inhibition of DNA polymerase or reverse transcriptase activity | Al-Soud *et al.* (2000a), Al-Soud and Rådström (1998), Eckhart *et al.* (2000), Opel *et al.* (2010), Peist *et al.* (2001) and Wilkins and Smart (1996) |
| Polyphenols<br>Tannic acid | Chelation of metal ions | Abbaszadegan *et al.* (1993) and Opel *et al.* (2010) |
| EDTA | Chelation of metal ions including $Mg^{++}$ | Rossen *et al.* (1992) |
| Calcium ions | Competition with co-factors of the polymerase | Bickley *et al.* (1996), Opel *et al.* (2010) |
| Antiviral substances (e.g. acyclovir) | Competition with nucleotides, inhibition of DNA elongation | Yedidag *et al.* (1996) |
| Exogenic DNA | Competition with template | Tamariz *et al.* (2006) |

EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction.

sulphate), polyphenols as well as inulin and pectin found in tea extracts were proven to inhibit PCR amplification (Peist *et al.* 2001). Berries are generally rich in phenols (e.g. anthocyanin, flavonol, ellagitannin, proanthocyanidin and phenolic acids) and polysaccharides (Seeram *et al.* 2006; Wei *et al.* 2008). Substances present in berries and tomatoes seem to especially inhibit real-time PCR assays using TaqMan probes, whereas conventional PCR assays are less affected (Love *et al.* 2008).

Environmental samples can be very diverse and derived from different compartments including soil, water or air. This large variety leads to the presence of many different PCR inhibitors including those already described above. In addition, dead biomass and soil may contain humic and fulminic acids, which inhibit PCR even at low concentrations (Ijzerman *et al.* 1997). In sewage sludge, fats, proteins, polyphenols and heavy metals are found, and waste water contains polysaccharides, metal ions (e.g. iron and aluminium) and RNases – all of them are common PCR inhibitors of environmental samples (Shieh *et al.* 1995; Rock *et al.* 2010). Several different inhibitors have also been detected in animal feed (Löfström *et al.* 2004).

A large variety of water samples are frequently used for the detection of pathogens. To detect even low amounts of pathogens, large volumes of water are usually concentrated to very small volumes. However, this often results in concurrent concentration of the different inhibitors and increased interference with PCR (Abbaszadegan *et al.* 1993, 1999; Tsai *et al.* 1993; Jiang *et al.* 2005). Similar problems may occur during the collection of air samples, which is usually carried out by passing large air volumes through a variety of filters or other binding material. By this, inhibitory airborne components are often enriched leading to failure of PCR (Maher *et al.* 2001; Chen *et al.* 2010; Oppliger *et al.* 2011).

## PCR inhibitors introduced during sample preparation

Inhibitors may be added to the sample during sample processing or during nucleic acid extraction. This includes powder from gloves (Demeke and Jenkins 2010), different salts (e.g. sodium chloride or potassium chloride), detergents or organic molecules [ethylenediaminetetraacetic acid (EDTA), sarkosyl, ethanol, isopropyl alcohol or phenol] (Weyant *et al.* 1990; Katcher and Schwartz 1994; Burkardt 2000; Peist *et al.* 2001; Demeke and Jenkins 2010). These substances may be necessary for efficient cell lysis or for the preparation of pure nucleic acids, but they may also cause PCR inhibition at certain concentrations. Ionic detergents (e.g. sodium deoxycholate, sarkosyl and SDS) are highly inhibitory for the PCR, whereas non-ionic detergents (e.g. Nonidet P-40, Tween 20, Triton X-100 and N-octyl glucoside) cause PCR inhibition only at relatively high concentrations (Weyant *et al.* 1990). EDTA is found in several elution buffers of purification kits for preservation of DNA, but at certain concentrations, it may deplete magnesium ions and thus inhibit DNA polymerase activity. Additives of the PCR mixture, such as dithiothreitol, dimethyl sulphoxide or mercaptoethanol, may also be inhibitory at certain concentrations. For some of the polymerases commonly used in PCR, the inhibitory concentrations of certain substances have been determined and can be retrieved from the manufacturer.

Contact between polymeric surfaces and UV-irradiated plastic tubes with PCR chemicals is reported to decrease the sensitivity of the PCR (Butot *et al.* 2007b; Fox *et al.* 2007; Gassilloud *et al.* 2007; Gonzalez *et al.* 2007), although Tamariz *et al.* (2006) did not find any influence of UV irradiation. However, this discrepancy might be explained by different dosages of UV light used, which have been shown to correlate with the inhibitory effect (Burgess and Hall 1999). Generally, the material of swabs or the composition of transport media may also influence the sensitivity of the PCR (Wadowsky *et al.* 1994).

## General methods for removal of PCR inhibitors

Several methods for the removal of inhibitors or for the reduction of their effects have been proposed so far and some examples are shown in Table 3. Generally, the effects of the inhibitors may be reduced by selecting an appropriate method for sample processing and nucleic acid extraction, by the choice of a more robust DNA polymerase or by the use of specific PCR additives (Al-Soud and Rådström 2001). For example, guanidinium thiocyanate extraction may remove inhibitors from different sample matrices more efficient than other methods (Shieh *et al.* 1995; Hale *et al.* 1996). Other effective strategies include a phenol–chloroform extraction for the removal of inhibitory lipids or a treatment with activated carbon to eliminate inhibitory salts, such as urates (Wiedbrauk *et al.* 1995; Abolmaaty *et al.* 2007; Chaturvedi *et al.* 2008). These methods are reported to be more successful than gel filtration, treatment with proteinase K or heat treatment (Huppertz *et al.* 1993; Bergallo *et al.* 2006). Nevertheless, other reports show that the application of the phenol–chloroform extraction was not sufficient for complete removal of PCR inhibitors (Pachner and Delaney 1993).

Column chromatography using Sephacryl S-400, Sephadex G-200, Chelex, Sephadex or cetrimonium bromide can be used to remove salts and small proteins as well as polysaccharides from several sample matrices, for example, seminal fluid or stool (Da Silva *et al.* 1995; Schmidt *et al.* 1995; Hale *et al.* 1996; Croci *et al.* 2008). For example, cetrimonium bromide forms an insoluble complex with polysaccharides and denatured proteins and therefore efficiently removes them from the preparation (Alaeddini 2011). A repeated extraction using silica columns may also remove inhibitors (Kemp *et al.* 2006). In addition, cation exchange resins have been successfully used for the removal of PCR inhibitors (Jacobsen and Rasmussen 1992; Henson and French 1993). The use of magnetic silica beads for nucleic acid isolation has been repeatedly shown to efficiently remove a wide range of PCR inhibitors (Maher *et al.* 2001; Rutjes *et al.* 2005; Ngazoa *et al.* 2008; Sur *et al.* 2010).

Immunocapture methods are very efficient in removal of PCR inhibitors because the pathogen can be specifically separated from the sample matrix and the accordant inhibitors (Widjojoatmodjo *et al.* 1992; Croci *et al.* 2008; Schrader *et al.* 2011). For example, antigen-capture PCR has been successfully used for the sensitive detection of hepatitis A virus in seafood (Arnal *et al.* 1999). However, because of the use of specific antibodies, this method is not applicable to highly variable pathogens, such as noroviruses (Atmar *et al.* 1995). In this case, human histo-blood group antigens or pig gastric mucin, which

**Table 3** Strategies for the removal of PCR inhibitors from specific matrices

| Matrix | Strategy for removal of inhibitors | References |
|---|---|---|
| Stool | Additional extraction steps<br>Sedaphex G-200 chromatography<br>Heat treatment before the PCR<br>BSA or gp32<br>Selection of resistant polymerases<br>Chloroform extraction<br>Treatment with activated carbon<br>Dilution of the sample | Al-Soud and Rådström (1998), Chaturvedi *et al.* (2008), Hale *et al.* (1996), Kreader (1996), Monteiro *et al.* (1997), Scipioni *et al.* (2008a,b) and Wilde *et al.* (1990) |
| Seafood, bivalves, oysters | PEG precipitation<br>Cetyltrimethylammonium bromide treatment<br>Proteinase K treatment<br>Preparation of selected tissues (digestive gland)<br>Activated carbon treatment | Abolmaaty *et al.* (2007), Atmar *et al.* (1993, 1995) and Jothikumar *et al.* (2005) |
| Plants | RNA precipitation<br>Treatment with: Tween 20, DMSO, PEG 400, polyvinylpyrrolidone, ß-mercaptoethanol, dithiothreitol<br>dilution of nucleic acids<br>cation exchanger<br>Extraction with high concentration of borates<br>Precipitation of polysaccharides<br>proteinase K treatment<br>Desiccation (65°C for 2 days) | Demeke and Adams (1992), John (1992), Henson and French (1993), Sipahioglu *et al.* (2006), Su and Gibor (1988), Wan and Wilkins (1994) and Wilkins and Smart (1996) |
| Berries | Chloroform/butanol extraction<br>treatment with pectinases<br>Use of conventional PCR instead of real-time PCR | Butot *et al.* (2007a), Dubois *et al.* (2002) and Love *et al.* (2008) |
| Cheese, meat | Selection of resistant polymerase | Al-Soud and Rådström (1998) |
| Cheese | Inactivation of proteases by hot NaOH extraction | Rossen *et al.* (1992) |
| Milk | Addition of BSA, protease inhibitors, magnesium ions<br>Chelation of calcium ions | Bickley *et al.* (1996) and Powell *et al.* (1994) |
| Water, environmental samples | Combined treatment of Sedaphex G-100 and Chelex-100<br>Dialysis<br>Extraction with solvents<br>Ultrafiltration with positively charged membranes, UV irradiation<br>Antigen-capture PCR | Abbaszadegan *et al.* (1993), Cannon and Vinjé (2008), Ijzerman *et al.* (1997) and Tamariz *et al.* (2006) |
| Airborne and environmental samples | Magnetic bead DNA capture method | Maher *et al.* (2001) |

BSA, bovine serum albumin; PCR, polymerase chain reaction.

are proposed to be cellular receptors for noroviruses, have been successfully used for virus capturing and subsequent detection by PCR (Cannon and Vinjé 2008; Tian *et al.* 2008).

A more general and widely applied method is the dilution of the sample or the extracted nucleic acid, which will automatically result in a dilution of the PCR inhibitors (Widjojoatmodjo *et al.* 1992; Monteiro *et al.* 1997; Eckhart *et al.* 2000; Scipioni *et al.* 2008a,b). However, the dilution clearly is accompanied by a decrease in sensitivity. An opponent strategy is the addition of substances to the PCR mixture, which may include betaine, bovine serum albumin (BSA), dimethyl sulphoxide, formamide, glycerole, non-ionic detergents, polyethylene glycol, powdered milk, T4 bacteriophage gene 32 product (gp32) and proteinase inhibitors (Frackman *et al.* 1998; Al-Soud and Rådström 2000; Eckhart *et al.* 2000). Especially, BSA and gp32 are known to be effective against iron chloride, hemin, fulminic acid, humic acid, tannic acid, stool extracts and melanin (Al-Soud and Rådström 2000; Scipioni *et al.* 2008b; Opel *et al.* 2010). However, BSA is not effective against bile salts, bilirubin, EDTA, sodium chloride, SDS, Triton

X-100, calcium and collagen (Kreader 1996; Opel *et al.* 2010). Other described methods such as heating of the sample or DNase treatment were not always suitable for the removal of PCR inhibitors (Wilde *et al.* 1990).

The choice of the DNA polymerase and – if applicable – of the appropriate reverse transcription system is of great importance to prevent PCR inhibitory effects (Al-Soud and Rådström 1998; Löfström *et al.* 2004). Kermekchiev *et al.* (2009) showed that distinct mutations in the Taq DNA polymerase can overcome its inhibition by blood, plasma, haemoglobin, lactoferrin, serum IgG, soil extracts and humic acids. Baar *et al.* (2011) developed a chimeric polymerase consisting of parts from different polymerases of the genus *Thermus*, which shows a broad resistance against a variety of organic and inorganic inhibitors, as for example, humic acids, bone powder, fossilized excrements, coal tar or clay-rich earths. For real-time PCR using TaqMan probes, the 5′ exonuclease activity of the *Taq* polymerase is essential. In an experiment with 15 different *Taq* polymerases, considerable differences in the intensity of the fluorescence in different sample types were obvious (Kreuzer *et al.* 2000).

Manufacturer of commercially available kits for nucleic acid purification and PCR use a variety of the above-mentioned strategies for removal of PCR inhibitors and increasing robustness of PCR enzymes. Several studies investigated the performance of such kits (e.g. Ribao *et al.* 2004; Levesque-Sergerie *et al.* 2007; Demeke and Jenkins 2010); however, the efficiency is strongly dependent on the used matrix and no general recommendations can be retrieved from those studies.

## Methods for removal of specific PCR inhibitors

Several methods have been developed for removal of specific classes of inhibitors. Polysaccharides, which are mainly found in seafood or berries, may hamper the resuspension of precipitated nucleic acids (Atmar *et al.* 1993, 1995; Butot *et al.* 2007a). Either the precipitation of the polysaccharides before RNA isolation or application of an RNA isolation method without any polysaccharide contamination is used to avoid these negative effects (Fang *et al.* 1992; Wilkins and Smart 1996). This includes treatment with Tween 20, DMSO, polyethylene glycol or activated carbon (Demeke and Adams 1992; Abolmaaty *et al.* 2007). Pectinase treatment has been successfully used for the analysis of berries (Butot *et al.* 2007a).

Phenols can directly interact with RNA. Removal of phenols can be achieved by precipitation using polyvinylpyrrolidone (John 1992; Wilkins and Smart 1996). High concentrations of borates protect the RNA from interaction with polyphenols (Wan and Wilkins 1994; Wilkins and Smart 1996). Sipahioglu *et al.* (2006) removed these inhibitors from leaves by drying at 65°C for 2 days and conserving them at 4°C under hermetic conditions.

Humic and fulminic acids, which are often present in dead biomass, soil and water samples may be removed by dialysis, liquid–liquid extraction, flocculation using polyvalent cations, gel extraction, column-based methods and ultrafiltration, the latter one being most successful (Tsai and Olson 1992; Abbaszadegan *et al.* 1993; Tsai *et al.* 1993; Ijzerman *et al.* 1997; Braid *et al.* 2003). Queiroz *et al.* (2001) used electropositive filters for the analysis of sewage and water samples, which preferentially bind microorganisms and avoid co-purification of the inhibitory substances.

For the specific removal of other substances, only a few methods have been published. Urea present in urine samples may be effectively removed by dialysis or ultrafiltration (Khan *et al.* 1991). Proteases, which might occur in milk, may be eliminated by the addition of protease inhibitors or BSA (Powell *et al.* 1994). The inhibitory effects caused by calcium ions may be compensated by the addition of magnesium ions. Another possibility is the use of different chelating agents, which catch the calcium ions, resulting in increased amplification rates (Bickley *et al.* 1996).

## Assessing PCR inhibitions by control reactions

Despite extensive efforts to eliminate inhibitors from the sample, inhibitory substances which can affect PCR may still be present. Different analytical methods can be used to determine the presence of inorganic or organic compounds, for example, the amount of dissolved organic carbon indicative for humic acids (Chen *et al.* 2003; Rock *et al.* 2010). However, for most of the inhibitory substances, no practicable tools for their analysis are available.

To determine the inhibitory effect of all substances present in a nucleic acid preparation, it has been suggested to carry out PCR control reactions. This is especially necessary to exclude false-negative results, which may result from complete inhibition of the PCR, even in the presence of the target sequence (Parshionikar *et al.* 2004). Such controls generally consist of defined amounts of nucleic acids or microorganisms, which are added to the sample and analysed parallel to the target sequence. By comparison of the detected amount of the control with that originally added, the performance of the assay including the presence of PCR inhibitors can be assessed. Different types of controls can be distinguished according to the controlled steps (process control, amplification control) and the implementation of the control reaction (internal or external control).

A process control is added at the starting point of sample analysis, for example, before nucleic acid extraction, and passes therefore all preparation steps. In contrast, an amplification control is added to the nucleic acid extracted from the sample thus controlling only the performance of the PCR itself. An internal control is analysed in the same tube as the target, whereas the external control is analysed in a separate aliquot of the sample (Nolan *et al.* 2006).

The controls can be divided into competitive and non-competitive amplification controls. Competitive controls are amplified using the same primers and the same conditions but can be distinguished from the target by product length or sequence (Hoorfar *et al.* 2004; Villanova *et al.* 2007). One limiting factor of this application may be the concurrent use of primers, which may lead to a general decrease in PCR sensitivity (Hoorfar *et al.* 2004). In contrast, non-competitive controls composed of unrelated sequences are amplified by different primers as the target sequence and thus can also be used universally (Dingle *et al.* 2004; Hoorfar *et al.* 2004; Dreier *et al.* 2005; Villanova *et al.* 2007). A possible disadvantage of non-competitive controls is the use of separate primers, which may behave different than those used for the target sequence (Villanova *et al.* 2007). Generally, by using internal controls in multiplex PCR assays, the co-amplification of the control may inhibit the amplification of the target sequence, which might be solved by limiting the concentration of the control primers and control RNA (Hofmann 2003; Gall *et al.* 2007).

One special kind of non-competitive control is the endogenetic control, which uses housekeeping genes that directly originate from the sample (Hoffmann *et al.* 2005). Endogenetic controls act as process controls, which control the whole sample processing procedure. However, a high concentration of the endogenetic nucleic acids may hamper the amplification of the target sequence (Hoffmann *et al.* 2005). Externally added process controls are used mostly when endogenetic nucleic acids are not present due to the sample type (e.g. environmental samples) or sample preparation method (e.g. using virus purification). One possibility of such controls is the so-called armored RNA, which is packaged into phage proteins and thus resistant to RNases (Pasloske *et al.* 1998; Drosten *et al.* 2001; Hietala and Crossley 2006). Another strategy uses bacteriophages or animal viruses added to the sample. For example, bacteriophage MS2 and mengoviruses, feline calicivirus and enterovirus have been successfully used as process controls in food analysis (Dreier *et al.* 2005; Costafreda *et al.* 2006; Da Silva *et al.* 2007; Rolfe *et al.* 2007; Comelli *et al.* 2008; Lowther *et al.* 2008; Mattison *et al.* 2009; Blaise-Boisseau *et al.* 2010).

## Conclusions

In conclusion, PCR inhibitors are a heterogeneous class of substances that act at different steps of the diagnostic procedure. They are present in a large variety of sample types and may lead to decreased PCR sensitivity or even false-negative PCR results. Several strategies have been developed to remove PCR inhibitors during sample preparation. However, as at least in complex matrices, it cannot be guaranteed that the preparations are free of PCR inhibitors, all reactions should be analysed for the presence of inhibitory effects. The development of standardized controls has therefore been recommended for substantiated evaluation of diagnostic PCR results and for comparison of the efficiencies of different PCR protocols (Hoorfar *et al.* 2003, 2004; Mattison *et al.* 2009; Lees and CEN WG6 TAG4 2010). On the basis of the results of such efficiency-controlled assays, it might be necessary to modify the steps of sample analysis to remove specific PCR inhibitors or to use a PCR system, which is less sensitive to the inhibitory substances. This might be carried out by the application of general methods for removal of PCR inhibitors, for example, by changing the commercially available kits for nucleic acid preparation and PCR analysis. However, additional steps for removal of specific inhibitors may be necessary. The overview presented here might provide a collection of published methods, which can be selected and tested based on the sample type. In other cases, the different mechanisms of action of PCR inhibitors described here might be a starting point for the development of new methods for their removal or inactivation.

## Conflict of interest

There is no conflict of interests to declare.

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