Brief Review: Preventing PCR Amplification Carryover Contamination in a Clinical Laboratory

Jaber Aslanzadeh
Department of Pathology and Laboratory Medicine, Hartford Hospital, Hartford, Connecticut

Abstract. During the past two decades PCR and several other DNA/RNA amplification techniques have become important diagnostic tools in clinical laboratories. Amplification products contamination has been the main impediment to using these techniques routinely in diagnostic laboratories. Over the years, several creative pre- and post-amplification methods have been developed that prevent amplicon carryover contamination. These procedures, coupled with automated systems that employ real-time amplification and simultaneous detection in a closed system, have substantially reduced the possibility of false positive results due to amplification products carryover contamination. (received 28 July 2004; accepted 15 September 2004)

Keywords: polymerase chain reaction, isopsoralen, uracil N-glycosylase, amplicon carryover contamination

Introduction

The ability to amplify specific regions of microbial DNA or RNA by various amplification techniques has provided rapid and sensitive detection of infectious pathogens [1]. Amplification-based techniques are now used routinely in clinical laboratories to detect organisms that do not grow in conventional culture media. The techniques facilitate the study of epidemics and the detection of mutations associated with drug resistance in viral, bacterial, and fungal agents [2]. Unfortunately, the exquisite sensitivity of these techniques makes them vulnerable to contamination [3-8]. At least 2 documented cases of Lyme disease, one with fatal outcome, have been attributed to false-positive PCR findings [9,10]. Similarly, formal retraction of published manuscripts has occurred as a result of false-positive PCR reactions [6].

Potential sources of contamination include (a) the large numbers of target organisms in clinical specimens that may result in cross-contamination, (b) plasmid clones derived from organisms that have been previously analyzed and that may be present in large numbers in the laboratory environment [11], and (c) most importantly, repeated amplification of the same target sequence, which leads to accumulation of amplification products in the laboratory environment [2,6]. A typical PCR generates as many as $10^9$ copies of target sequence and if aerosolized, even the smallest aerosol will contain as many as $10^6$ amplification products [6]. If uncontrolled, within a relatively short time the buildup of aerosolized amplification products will contaminate laboratory reagents, equipment, and ventilation systems [6].

Several pre- and post-amplification amplicon sterilization techniques have been proposed in order to prevent contamination from impeding the routine use of these techniques in clinical laboratories [2,12-19]. The objectives of these measures are, first, to prevent amplicon carryover contamination of reaction tubes with previously generated amplicons and, second, to sterilize all the generated amplicons so that even if they contaminate a reaction tube they are either ineligible targets for amplification or are destroyed prior to the next amplification reaction.

The following protocols outline reliable control measures for PCR-based assays and for other DNA-
based amplification techniques such as ligase chain reaction (LCR) or strand displacement amplification (SDA) tests. RNA-based amplification assays, such as self-sustaining sequence replication (3SR, also known as NASBA), transcription mediated amplification (TMA), and \(Q\) b replicase, are less prone to contamination, due to the nature of their amplification products (ie, RNA) and their inability to resist RNase in the environment.

**Barriers to Amplification Product Carryover**

Mechanical and chemical barriers both help to prevent amplification products carryover contamination. These barriers must be in place prior to the initiation of any amplification studies. While they are inadequate, per se, to control amplification carryover contamination, their judicious use complements additional pre- or post-amplification products sterilization protocols.

**Mechanical barriers.** Mechanical barriers to prevent amplification products carryover include the strict separation of areas of the laboratory where samples and reagents are prepared from the areas where amplification is performed and amplification products are analyzed. All traffic must be unidirectional from the reagent preparation area to the sample preparation area, to the amplification area, and to the detection area. These sites optimally are physically separated and are preferably at a substantial distance from each other. Each area must be equipped with the necessary instruments, disposable devices, laboratory coats, gloves, aerosol-free pipettes, and ventilation systems. All reagents and disposables used in each area must be delivered directly to that area. The technologists must be alert to the possibility of transferring amplification products on their hair, glasses, jewelry, and clothing from contaminated rooms to clean rooms.

**Chemical barriers.** Work stations should all be cleaned with 10% sodium hypochlorite solution (bleach), followed by removal of the bleach with ethanol. Bleach causes oxidative damage to nucleic acid and prevents it from being reamplified in subsequent PCR reactions [20]. Because bleach does not discriminate between the extracted target DNA and PCR amplicons, specimens treated with bleach are not proper targets for amplification studies. In the rare occasions that an item, such as an amplification tray, must be transferred from a contaminated area to a clean area, the item should be placed in 2% to 10% bleach solution overnight and extensively washed before the transfer.

**Sterilization of Amplification Products**

Several creative methods to prevent amplification products carryover contamination have been developed [2,12-19]. While most of these techniques are optimized for use with PCR, they may also be useful for sterilizing the products of other amplification techniques. Generally, most PCR-based diagnostic assays consist of 3 steps: sample processing, PCR amplification, and amplification products detection.

Two criteria must be met by amplification products control procedures to ensure that subsequent diagnostic assays are not compromised by a carryover event [2,6,21]. The first is that the reaction products are not exposed to the environment until they are modified in a specific manner that distinguishes them from true sequences in subsequent amplification. This requires that the distinguishing modification occur before the reaction tube is opened for the detection phase of the diagnostic assay. Second, the modification(s) must not interfere with the detection steps.

Both pre- and post-amplification sterilization should be used. Pre-amplification sterilization techniques assume that it is possible for amplification products from previous amplifications to contaminate a new PCR mix, but provide an effective remedy to eliminate the contaminants prior to the start of the next round of amplification [2,6,21]. Post-PCR sterilization relies on modification of the amplified products prior to exposing them to the laboratory environment. Since the modified amplification products are unsuitable as PCR targets in subsequent amplification reactions, they do not cause false-positive reactions.
Sterilization Prior to Polymerase Chain Reaction

_Ultra-violet light irradiation._ UV light was the first sterilization technique used to eliminate amplification products carryover contamination [7,8]. The technique is based on the property of UV light to induce thymidine dimers and other covalent modifications of DNA that render the contaminating nucleic acid inactive as a template for further amplification. Briefly, a reaction tube containing all the amplification reagents except the target is exposed to UV light (combination of 254 and 300 nm) for 5 to 20 min, followed by the addition of target DNA [22]. The UV irradiation of the reaction tube sterilizes the potential contaminants in the reaction tube and prevents their subsequent amplification. The technique has been reported to yield varying degrees of success in several publications [23-25].

While the technique is simple, inexpensive, and does not require modification of existing protocols, it exhibits sub-optimal efficacy in sterilizing short (<300 nucleotides) and G+C-rich templates [2]. In addition, the efficacy of UV irradiation depends on the distance of the nucleic acids from the light source [2]. Nucleotides present in the PCR reaction mix may protect contaminating amplification products from the UV irradiation [26]. UV irradiation may have deleterious effects on the enzyme Taq polymerase and oligonucleotide primers [21,27].

Despite such limitations, UV irradiation should be an integral feature of any PCR laboratory. After the packages have been opened, all pipettes and other disposable devices should be stored in a UV light box. Preparation of the amplification master mix and specimen processing should also be carried out in a UV light box.

_Engzymatic inactivation with uracil-N-glycosylase._ Uracil-N-glycosylase (UNG) is a DNA repair enzyme that is found in a wide variety of bacterial species. The main function of this enzyme is to recognize and remove uracil residues that are generated by spontaneous deamination of cytosine residues in double-stranded DNA as a component of excision-repair processes [28,29]. In 1990, Longo et al [16] reported the utility of UNG to sterilize the 284 bp PCR amplification products of HPV type 16. The technique was subsequently evaluated by several investigators [30-32] and UNG is now the most widely used contamination control technique. UNG is incorporated in all PCR kits currently manufactured by Roche Diagnostics Corp. The technique relies on substituting uracil (dUTP) for thymine (dTTP) during PCR to generate amplification products with distinguishing characteristics relative to the native DNA template.

Because the newly synthesized amplicons contain dUTP, they are susceptible to hydrolysis by the bacterial enzyme UNG. The addition of this enzyme to the amplification mix allows selective hydrolysis and removal of the contaminating amplification products from the PCR mix. The enzyme is most active at room temperature. Therefore, following inoculation of the reaction tube with target specimens, amplification tubes are incubated at room temperature for 10 min. During this time, the UNG hydrolyzes and removes any contaminating amplification products that may be present in the PCR reaction tube. In addition, any product generated by Taq polymerase by non-specific binding of the primers to the target DNA at this reduced temperature is hydrolyzed. This unique property of UNG potentially eliminates the need for hot start PCR. The subsequent incubation of the reaction tubes at 95°C effectively inactivates the UNG and allows the PCR reaction to proceed and generate new amplification products [21].

As expected, UNG works best with thymine-rich amplification products and has reduced activity with G+C-rich targets. Under certain circumstances, UNG may not be completely inactivated and the residual enzymatic activity may be enough to degrade amplification products generated during the early amplification cycles [33]. All PCR products should be frozen at -20°C or held at 72°C until they are analyzed [33]. To achieve optimum sterilization activity, UNG and dUTP concentrations must be optimized individually for each amplification reaction. Depending on the amplification target, it may be necessary to include dTTP as well as dUTP in the reaction mix. Further disadvantages of the UNG procedure are that U-containing DNA may not hybridize on Southern blots as well as native DNA.
DNA [34] and many restriction endonucleases cleave U-DNA with low efficiency or not at all [35].

**Sterilization After Polymerase Chain Reaction**

*Inactivation of nucleic acids with furocoumarins.* Furocoumarin compounds such as psoralen and isopsoralen represent a class of planar tricyclic reagent that is known to intercalate between base pairs of nucleic acids [36]. These compounds contain 2 reactive double bonds that, when excited by UV irradiation (300-400 nm), covalently bind to nucleic acid polymers by forming cyclobutane adducts with pyrimidine bases [36]. The mechanism of the amplification products sterilization protocol is based on the blockage of Taq polymerase-mediated primer extension reaction when the enzyme encounters a photochemically modified base in a target sequence [6,37,38]. Therefore, psoralen modified amplification products, if carried over to new reaction tubes, cannot serve as targets in subsequent amplification reactions.

Although psoralen and isopsoralen compounds are both capable of sterilizing amplification products, the psoralen compounds form cross-links between the complementary strands of DNA and therefore the amplification products may not be detected using common hybridization protocols [39]. In contrast, isopsoralen does not cross-link the complementary strands and the modified DNA is easily detected using common amplification products detection protocols. At least 3 isopsoralen compounds have been used to modify amplification products: 6-aminomethyl-4,5’-dimethylisopsoralen (6-AMDMIP), 4’-aminomethyl-4,5’-dimethylisopsoralen (4’-AMDMIP), and IP-10 [34]. In 1991 Cimino et al [37] explored the possibility of using the unique chemical property of these compounds to control amplification products carryover contamination. They added these compounds to the PCR reaction mix that amplified either a 115-mer sequence unique to HIV-1 or a 500-mer sequence unique to bacteriophage lambda [37,40]. Following amplification, isopsoralen was activated by irradiating the reaction tubes with UV light for 15 min. Then a known concentration of the isopsoralen modified amplification products was reamplified in subsequent PCR reactions. Isopsoralen did not appear to have an inhibitory effect on PCR and successfully sterilized up to $10^4$ of 115-mer HIV specific amplification products and $10^8$ of 500-mer bacteriophage-specific amplification products. They concluded that the level of sterilization depended on the length and sequence of the amplification products [37,40]. Long and A+T-rich amplification products were reactive with isopsoralen and were thus easier to sterilize than short and G+C-rich amplification products.

The sterilization efficiency improved with increasing isopsoralen concentration [41]. Isopsoralen concentrations 25 mg/ml were generally inadequate to sterilize most PCR products and did not give much protection from PCR carry-over contamination [42]. Isopsoralen concentrations of 100 mg/ml may have an inhibitory effect on PCR [33,42]. Generally, the inhibitory effects of psoralen are reduced by adding 10% glycerol or 1% BSA as cosolvents [2]. The inhibitory effects of psoralen may persist in some PCR reactions, even in the presence of glycerol. It is recommended that the psoralen concentrations be optimized for each primer and PCR assay. In one study, an inhibitory concentration of IP-10 was successfully utilized to sterilize amplification products by addition of this compound to the reaction tubes after completion of PCR reactions [42]. PCR was performed without IP-10; the compound was added to the tube after completion of PCR and activated to sterilize all the amplification products. Amplitube beads (Roche Diagnostics, Indianapolis, IN) were included in the PCR reaction tubes to prevent amplification products aerosolization when the PCR tubes were opened to add IP-10. The tubes were placed at room temperature for a few min to allow the Amplitube to solidify. The solidified wax formed a barrier between the PCR products and the laboratory environment. The tubes were then opened without aerosolizing the amplification products and the desired concentration of IP-10 was layered onto the wax. The tube was reheated for a few min to melt the wax and mix the IP-10 with PCR products. Finally, the tube was exposed to UV light for 15 min to sterilize the amplification products. While this modification requires post-amplification
addition of IP-10 to the reaction mix, the modification virtually eliminates the inhibitory effects of IP-10 on PCR. As in the case of UV irradiation and the UNG protocol, isopsoralen is ineffective for sterilizing G+C-rich and short amplification products and requires added equipment. These compounds are carcinogenic and must be used with care [36].

**Primer hydrolysis.** The primer hydrolysis method of amplification products sterilization relies on uniquely synthesized chimeric primers that contain one or more ribose linkages at the 3’ end [32]. The generated amplification products containing ribose residue(s) are susceptible to alkaline hydrolysis at the site of the ribose molecule(s). PCR products are usually exposed to 1M NaOH and incubated for 30 min to hydrolyze the amplification products at the site(s) of ribose. The addition of NaOH to the final PCR products cleaves the amplification products and removes the primers from the rest of the PCR products. Once the amplification products have been hydrolyzed, the truncated amplicons lack the primer binding sites. Even if they are aerosolized and introduced into the subsequent amplification reaction mix, they will not be recognized as targets during the next amplification cycles. Opening of the tubes at the time that NaOH is added, however, may provide an opportunity for contamination by the aerosolized amplification products. Primer hydrolysis protocols have varied efficiency, depending on the number and position of 3’-ribose residues incorporated in the primers. The sterilization efficiency of this protocol may range from $10^4$ to $10^9$ amplification products [32].

**Hydroxylamine.** Addition of hydroxylamine hydrochloride to PCR reaction tubes after amplification provides an alternative sterilization technique, especially for short (<100 bp) and G+C-rich amplification products. The compound preferentially reacts with the oxygen atoms of the cytosine

Table 1. Comparison of amplification products sterilization techniques to control PCR carryover contamination.

<table>
<thead>
<tr>
<th>Method</th>
<th>Mode of action</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV light</td>
<td>thymidine dimer</td>
<td>inexpensive, requires no change in PCR protocol</td>
<td>ineffective against G+C-rich and short (&gt;300 bp) amplification products</td>
</tr>
<tr>
<td>UNG*</td>
<td>enzymatic hydrolysis of the aerosolized amplicons</td>
<td>easy to incorporate, most active against T-rich amplicons</td>
<td>expensive, may reduce amplification efficiency</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>chemically modifies C and prevents C+G pairing</td>
<td>inexpensive, effective on short and G+C-rich amplicons</td>
<td>carcinogenic, may interfere with amplicon analysis</td>
</tr>
<tr>
<td>Isoporalen (IP)</td>
<td>modifies target by cyclobutane adduct</td>
<td>relatively inexpensive, requires minor modification of the PCR protocol</td>
<td>carcinogenic, inhibitory effect on PCR not very effective for controlling G+C-rich and short amplicons, requires added equipment</td>
</tr>
<tr>
<td>Psoralen</td>
<td>same as IP</td>
<td>same as IP</td>
<td>may interfere with amplicon analysis</td>
</tr>
<tr>
<td>Primer hydrolysis</td>
<td>post PCR hydrolysis of RNA residues of the amplicons by NaOH</td>
<td>equally effective on G+C-rich amplicons</td>
<td>variable efficacy, may generate aerosol during NaOH addition</td>
</tr>
</tbody>
</table>

*Uracil-N-glycosylase
residue [43,44] and creates covalent adducts that prevent base-pairing with guanine residues in subsequent reactions. Thus, modified amplification products are not recognized as amplification targets in subsequent PCR reactions. Hydroxylamine has been shown to inactivate a 156 bp PCR product of the ospA gene of *B. burgdorferi* and a 92-bp amplification product of the DNA polymerase gene of herpes simplex virus [13]. Hydroxylamine binds to these products in a dose-dependent manner as determined by their migration in agarose gel. Because hydroxylamine binds and modifies cytosine, the technique is highly effective in sterilizing short and G+C-rich amplification products. The compound, must be added to the reaction tubes following the amplification, which may result in amplification products aerosolization.

The techniques described above and presented in Table 1 all have advantages and disadvantages. Therefore, the implementation of one or more of these methods does not replace good laboratory practice. In fact, the sterilizing limit of these techniques may be saturated with few uncontrolled PCR assays. Espy et al [30] compared the efficacy of UNG and IP-10 to sterilize amplification products generated from human herpes simplex virus, cytomegalovirus, Epstein-Barre virus and human papilomavirus-16. Both techniques effectively controlled amplification products that were 240 bp in length. Neither assay effectively controlled short (92 bp) and G+C-rich amplification products unique to HSV.

Rys and colleagues [32] compared UNG, isopsoralen, and primer hydrolysis to sterilize 156 bp amplification products unique to the *B. burgdorferi* ospA gene. The 3 protocols were equally reliable in controlling amplification carry-over contamination. IP-10 and UNG consistently sterilized as many as 10⁹ amplification products. The primer hydrolysis protocol, on the other hand, gave varying efficiency depending on the number and position of 3′-ribose residue(s) incorporated in the primers. The sterilization efficiency of this technique ranged from 10⁴ to 10⁹ amplification products. The authors recommend the use of a combined approach to control PCR carry-over contamination. Their protocol includes isopsoralen and dUTP (instead of dTTP) in PCR reactions. This approach allows them to use isopsoralen as the first-line control measure. Because the amplification products contain dUTP, the UNG is reserved and used only if they encounter contamination in the presence of isopsoralen and is continued until the source of contamination is identified [32].

**Conclusions**

During the past decade there have been significant improvements in virtually every area of amplification technology. Initially, the traditional agarose gel electrophoresis followed by Southern blot hybridization was replaced with the micro-well plate assays that employed enzymatic detection of amplification products (MPA). More recently, automated amplification and detection systems such as COBAS Amplicor (Roche Diagnostics) have been introduced. The latter uses a robotic arm with a syringe and needle for removal of PCR products in a relatively enclosed area that limits amplification products aerosolization.

A new generation of amplification techniques, such as 5-exonuclease (Taqman), light cyclers, and other real-time amplification and detection techniques, simultaneously amplify and detect microbial DNA without exposing the amplification products to the laboratory environment [45,46]. In addition, the introduction of creative and new amplification techniques, such as linked linear amplification, that are inherently immune from carry-over contamination [47] and use of automated nucleic acid extraction systems will reduce hands-on manipulation of clinical samples and potentially reduce the chance of contamination. It should be emphasized that despite these improvements, PCR and other amplification techniques remain susceptible to carry-over contamination. False-positive findings have been reported with all the commercially-available automated systems [48].

**References**

2. Tang Y, Persi DH. Molecular detection and identification of microorganisms, In: Manual of Clinical Microbiology,
PCR contamination control


44. Freese E, Strack HB. Induction of mutations in transforming DNA by hydroxylamine. PNAS USA 1962;48:1796-1803.