# Post-PCR sterilization: development and application to an HIV-1 diagnostic assay

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## ABSTRACT

**We have developed an effective post-PCR sterilization process and have applied the procedure to a diagnostic assay for HIV-1. The method, which is based on isopsoralen photochemistry, satisfies both the inactivation and hybridization requirements of a practical sterilization process. The key feature of the technique is the use of isopsoralen compounds which form covalent photochemical adducts with DNA. These covalent adducts prevent subsequent extension of previously amplified sequences (amplicons) by Taq polymerase. Isopsoralens have minimal inhibitory effect on the PCR, are activated by long wavelength ultraviolet light, provide sufficient numbers of covalent adducts to impart effective sterilization, modify the amplified sequence such that it remains single-stranded, and have little effect on subsequent hybridization. The sterilization procedure can be applied to a closed system and is suitable for use with commonly used detection formats. The photochemical sterilization protocol we have devised is an effective and pragmatic method for eliminating the amplicon carryover problem associated with the PCR. While the work described here is limited to HIV-1, proper use of the technique will relieve the concern associated with carryover for a wide variety of amplicons, especially in the clinical setting.**

## INTRODUCTION

The advent of nucleic acid hybridization methodology has revolutionized both the detection of infectious disease pathogens and the identification of genetic variation associated with human disease. By providing sufficient target molecules for analysis by these methods, the polymerase chain reaction (PCR) has rapidly become a key component in the development of hybridization based clinical diagnostic tests (1–6). The PCR exponentially increases the number of target sequences available for detection and simultaneously reduces the complexity of the nucleic acid to be analyzed. The repetitive denaturing, annealing and polymerase extension procedures, repeated 25 or more times, greatly reduces the problem of extraneous signal for the detection step. Finally, unlike antibody based diagnostics, PCR provides direct detection of the nucleic acid for the genotype or its transcribed products.

While the advantages of the PCR are well accepted, this remarkable technique has not attained its rightful place as a routine diagnostic procedure in major clinical laboratories primarily because it is too sensitive. Without a complementary technique to control the billions of amplifiable target sequences ('amplicons') which are produced by the reaction, testing laboratories which repetitively amplify the same sequence consistently become contaminated with these same amplicons from the PCR. Uncontrolled release of these amplicons can lead to erroneous positive results with subsequent clinical specimens which are in fact negative (7–10). Although the contamination considered here is not due to a viable organism, its control demands procedures which rival, and in some respects surpass, the requirements of microbiological sterility. Since the PCR can detect a single molecule of DNA, the 'carryover' of a single amplicon from a previous PCR reaction can in fact produce a false-positive signal.

Following the PCR, a major source of contamination is the creation of aerosols which contain amplicons from the reaction. Once a sample has been prepared for the PCR and amplified, the reaction vessel should never be opened to the environment without prior application of a sterilization procedure. There are basically two periods during the PCR process when sterilization may be applied. *Pre-PCR* sterilization precedes the PCR and must inactivate any carryover amplicons which are present. The fact that amplification follows sterilization requires that the sterilization process be compatible with the template activity of the target DNA and the other components of the PCR. One procedure has been described which utilizes short wavelength ultraviolet irradiation of the reaction mix prior to amplification (11). While this method renders long amplicons unamplifiable (12), it requires both the target sequence and the Taq polymerase to be absent during the treatment. Since these components are not sterilized and are added to the PCR reaction following the irradiation step, they remain a potential source of contamination. The alternative is the use of a *post-PCR* approach, which modifies

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the amplicon such that it may no longer serve as an effective template for DNA polymerase. The modification process is performed following amplification, prior to opening the PCR container, and before the detection step. The limitation of this strategy is that most protocols for detection of the amplicon involve a DNA hybridization step. Since most modifications which block polymerase template activity also interfere with hybridization, post-PCR sterilization procedures using such modifications are impractical.

We have developed and refined an effective procedure for post-PCR sterilization which satisfies both the inactivation and the hybridization requirements of a practical sterilization process. The key feature of the technique is the use of photochemical reagents which 1) have minimal inhibitory effects on the PCR; 2) are activated by long-wave ultraviolet light; 3) provide sufficient numbers of covalent adducts which are effective polymerase blocks; 4) modify the amplicon such that it remains single-stranded; and 5) have little effect on the base pairing of the modified amplicon to its complement. We have found that particular isopsoralen compounds have the properties which satisfy these requirements. The isopsoralens described in this report can be present during the PCR under appropriate conditions, yet they remain highly photoreactive molecules following amplification. Upon irradiation, the isopsoralens form covalent adducts which effectively block the progression of DNA polymerases, and provide amplicons with an average 'modification density' sufficient for sterilization by a factor of 100 million or more. These compounds address the limitations associated with the Poisson distribution of modified amplicons, which we have addressed in a separate publication (13). Unlike the related but linearly annulated psoralens, which readily crosslink double-stranded nucleic acid, the isopsoralens form only monoadducts with DNA, thereby leaving the modified amplicons single-stranded and available for hybridization.

## MATERIALS AND METHODS

### Materials

Unlabelled and tritium labelled 4'-aminomethyl-4,5'-dimethyl-isopsoralen (4'-AMDMIP), 5-aminomethylisopsoralen (AMIP) and 6-aminomethyl-4,5'-dimethylisopsoralen (6-AMDMIP) were prepared as described elsewhere (14–16). $\alpha$-$^{32}$P-dCTP was obtained from DuPont-NEN (3000 or 6000 Ci/mmol), T4 DNA ligase was obtained from Bethesda Research Labs, and DNA kinase was obtained from New England BioLabs. Taq polymerase was obtained from Perkin-Elmer/Cetus (Norwalk, CT). Plasmid pBKBH10S was provided by Cetus Corporation (Emeryville, CA). Clinical HIV samples were provided by Roche Diagnostic Research (Alameda, CA) and Cetus Corporation. The Cetus sample was derived from a Multiple AIDS Cohort Study (MACS). The HRI-100 irradiation device was obtained from HRI Associates, Inc. (Berkeley, CA). The PTI irradiation device was constructed from components obtained from Photon Technology International (Princeton, NJ).

### Synthetic Oligonucleotides

Oligonucleotides were synthesized by the phosphoramidite method on a Biosearch 8750 DNA synthesizer then further purified by polyacrylamide gel electrophoresis and HPLC. Names and sequences (5'–3') of the synthetic oligonucleotides used in this report. *SK-38*: ATAATCCACCTATCCCAGTAGGAG-AAAT; *SK-39*: TTTGGTCCTTGTCTTATG TCCAGAATGC; *SK-19*: ATCCTGGG ATTAAATAAAATAGTAAGAATGTA-TAGC CCTAC; *SK-19-MA*: ATCCTGGGATT*AAATAAAA-TAGTAAGAATGTATAGCCC TAC (T* = position of mono-adduct); *HRI-42*: ATCCTGGGATTAAAT; *HRI-43*: ATTT-AATCCC; *HRI-44*: AAAATAGTAAGAATGTATA GCCC-TAC; *HRI-45*: CATTCTTACTATTTTATTTAATCCC; *HRI-46*: ATAATCCACCTATCCCAGTAGGAGAAATTT-ATAAAAGATGGATAATCCTGGGATTAAATAAAATAG-TAAGAATGTATAGCCCTACCAGCATTCTGGACATAA-GACAAGGACCAAA; *HRI-47*: TTTGGTCCTTGTCTTAT-GTCCAGAATGCTGGTAGGGCTATACATTCTTACTATT-TTATTTAATCCCAGGATTATCCATCTTTTATAAATTT-CTCCTACTGGGATAGGGGATTAT.

### Synthetic Crosslinkable Oligonucleotide

The preparation of SK-19-MA was performed as described (13) using a 10-mer/15-mer pair (HRI-42/HRI-43) and the psoralen derivative 4'-hydroxymethyl-4,5',8-trimethylpsoralen (17) to provide monoadducted 15-mer (HRI-42-MA). HRI-42-MA was then ligated to a 26-mer (HRI-44) with DNA ligase to provide SK-19-MA. A complementary 25-mer (HRI-45) was used as a template for the ligation.

### Irradiation Conditions

Unless specified otherwise, all irradiations were performed for 15 min with the HRI-100 device. Samples were placed in the central positions of the sample rack for irradiation. The inner trough of the device was filled with distilled water at ambient temperature. The sole use of the PTI device was for the crosslinking step in the COP analysis. The PTI source provided 320–400 nm light, obtained by using a 9 cm water filter, a 0.6 cm pyrex filter, and a 9 cm aqueous 1.7% $Co(NO_3)_2$/2% NaCl filter. The COP irradiations were performed for 15 min at ambient temperature.

### PCR Amplification

Unless specified otherwise, the conditions used for amplification of the 115-mer sequence were as follows. In addition to the appropriate target sequence and/or isopsoralen compound, each PCR reaction mixture contained 10 pmol primer pair SK-38/39, 200 $\mu$M each of the four deoxynucleoside triphosphates, 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 2.5 mM $MgCl_2$, 200 $\mu$g/ml gelatin and 1 unit Taq polymerase in a total PCR volume of 20 $\mu$l. For labelled experiments, 20 $\mu$Ci of $\alpha$-$^{32}$P-dCTP was included. The amplification profile used was 93°C (60 sec.), 55°C (30 sec.), 72°C (60 sec.), then finally 72°C (7 min.).

### Dark Effect of 4'-AMDMIP and AMIP on the PCR

For the high copy number experiments, $10^7$ copies of double-stranded synthetic 115-mer (based on optical density measurement) were amplified 30 cycles in the presence of either 4'-AMDMIP (100, 200, 300 & 400 $\mu$g/ml) or AMIP (100, 200, 300 & 400 $\mu$g/ml). The PCR reaction mix contained 20 $\mu$Ci of $\alpha$-$^{32}$P-dCTP. Following amplification, an aliquot of each reaction mix was removed and analyzed by denaturing polyacrylamide gel electrophoresis using a 12%/8M urea gel (PAGE). For experiments addressing PCR efficiency as a function of target copy number and 4'-AMDMIP concentration, different starting copy numbers of the double-stranded 115-mer ($10^2$, $10^3$, $10^4$, $10^5$ or $10^6$ copies) were amplified 30 cycles in the presence of different concentrations of 4'-AMDMIP (100, 200, 300 or 400 $\mu$g/ml). PCR product was quantified by PAGE

followed by autoradiography, band excision and scintillation counting.

### Sterilization of 115-mer Amplified from Synthetic Template with either 4'-AMDMIP or AMIP

$10^7$ copies of double-stranded synthetic 115-mer were amplified 30 cycles in the presence of either 4'-AMDMIP (400 μg/ml) or AMIP (400 μg/ml). Following amplification, the samples were divided into two equal portions then one portion irradiated. Aliquots were removed from both the irradiated and unirradiated portions of each sample containing $10^8$, $10^6$, $10^4$ or $10^2$ copies (based on a post-PCR amplicon concentration of $1\times10^{-8}$ M; see below), then reamplified 30 cycles in the presence of 20 μCi of α-$^{32}$P-dCTP. Following reamplification, the samples were analyzed by denaturing PAGE.

Verification that $10^7$ copies of the 115-mer amplified 30 cycles provides a plateau reaction mix was confirmed in separate experiments. Amplification of $10^7$ copies was performed in the presence of α-$^{32}$P-dCTP followed by denaturing PAGE. Excision of amplicon bands followed by scintillation counting verified a final amplicon concentration between $1\times10^{-8}$ M and $3\times10^{-8}$ M. Agarose gel comparison of the labelled reaction product with an identical PCR reaction without the α-$^{32}$P-dCTP showed that the unlabelled reaction provided slightly more amplicon. The unlabelled amplicon concentration was therefore at least $1\times10^{-8}$ M.

### Sterilization of Double-Stranded and Single-Stranded 115-mer with 6-AMDMIP

Each of the synthetic single-strands of the double-stranded 115-mer sequence (HRI-46 and HRI-47) were used separately or as a 1:1 mix to investigate sterilization of single-stranded amplicons. $10^{11}$, $10^{10}$ and $10^8$ copies of HRI-46, HRI-47, and HRI-46 plus HRI-47 were prepared in amplification buffer (100 ml) containing 6-AMDMIP (200 μg/ml). The samples were divided into two equal portions then one portion irradiated. A 1:100 dilution of the unirradiated (control) and irradiated (sterilized) samples were then placed into new tubes providing $10^9$, $10^8$ and $10^6$ challenge copies of either sterilized or unsterilized 115-mer. Fresh PCR reagents were added along with 20 μCi of α-$^{32}$P-dCTP, the mixtures reamplified 30 cycles, then analyzed by denaturing PAGE followed by autoradiography.

### Sterilization of an Amplified Clinical Sample with 4'-AMDMIP and 6-AMDMIP

Peripheral blood mononuclear cells from an antibody positive HIV-1 patient were collected and processed as previously described (18,19). 10 μl of cell lysate (approximately $10^5$ cells) was used for each PCR. Each sample was amplified 30 cycles in the presence of either 4'-AMDMIP (200 μg/ml) or 6-AMDMIP (200 μg/ml) using the standard profile with high temperature Taq addition (Taq was added to each tube after heating both the sample and the Taq to 80°C). Following amplification, the samples were divided into two equal portions then one portion irradiated. Aliquots were removed from both the irradiated and unirradiated portions of each sample containing $10^9$, $10^8$ or $10^6$ copies, then reamplified 30 cycles in the presence of 20 μCi of α-$^{32}$P-dCTP. Following reamplification, the samples were analyzed by PAGE followed by autoradiography.

### Oligomer Hybridization (OH) and Crosslinkable Oligonucleotide Probe Analysis (COP)

Targets (either $10^6$ copies of synthetic 115-mer or 1 μg clinical lysate) were prepared for PCR either with or without 4'-AMDMIP (200 μg/ml). The reaction components were mixed, then the samples amplified for either 20 or 30 cycles. Following PCR, the samples containing 4'-AMDMIP were irradiated. Following the irradiation, OH and COP assays were performed as follows. 10 μl of each PCR reaction mixture was added to 3.3 μl of 'probe mix' [5' labelled SK-19 or SK-19-MA (normal for OH; monoadducted for COP) at $10^{-8}$ M containing EDTA and an appropriate salt mixture (20)], overlaid with 30−40 μl of light mineral oil, then heated to 95−100°C for 5 minutes. For OH reactions, the hybridization mixture was incubated at 56°C for 30 minutes, loading dyes added, and aliquots loaded directly onto a native PAGE gel followed by electrophoresis under native conditions. For COP reactions, the hybridization mixture was placed in the PTI light source then irradiated for 15 min at 56°C. Following this, loading dyes (containing urea or formamide) were added, the sample heated to 95−100°C for 5 minutes, quick chilled on wet ice, then loaded on a denaturing PAGE gel followed by electrophoresis under denaturing conditions.

### Isopsoralen Binding to DNA

Experiments to evaluate the binding of 4'AMDMIP and AMIP to the double-stranded synthetic 115-mer sequence were done as follows. $^3$H-AMDMIP (100 μg/ml; $2.2\times10^5$ CPM/μg) or $^3$H-AMIP (100 μg/ml; $3.1\times10^5$ CPM/μg) was added to a mixture of HRI-46 (5 μg) and HRI-47 (5 μg) in a total volume of 100 μl 1× Taq buffer (50 mM KCl, 10 mM Tris, pH 8.5, 2.5 mM $MgCl_2$ and 200 μg/ml gelatin). The solutions were irradiated then processed by chloroform extraction (equal vol ×4) and ethanol precipitation (×2; 0.2 M NaCl, 2.5 vol ethanol). The resulting pellets were dissolved in 0.5 ml distilled water and covalently bound 4'-AMDMIP and AMIP determined by optical density and scintillation counting. To evaluate the effect of DMSO on the photochemical binding of 4'-AMDMIP to DNA, samples were prepared containing calf thymus DNA (10 μg), tritium labelled 4'-AMDMIP (50 μg/ml), and DMSO (0, 1, 3, 5, 10 & 20%) in 10 mM Tris/1 mM EDTA, pH 7. The samples were irradiated then processed by chloroform extraction (equal volume ×4) and ethanol precipitation (×2). The resulting pellet was dissolved in 0.5 ml distilled water and covalently bound 4'-AMDMIP determined by optical density and scintillation counting.

## RESULTS AND DISCUSSION

### Sterilization of the HIV-1 115-mer Amplicon Provided by SK-38/SK-39

The 115-mer amplicon provided by primer pair SK-38/SK-39 is located in the viral nucleocapsid gene (*gag*) of the HIV-1 genome. This region is highly conserved among the sequenced isolates (21). The sterilization procedure was applied to this particular amplicon for several reasons. First, this amplicon is widely used for the detection of HIV-1 (22,23), and therefore an optimized sterilization protocol for this sequence will be immediately useful. Second, the sequence is relatively short, which provided a challenging test for the sterilization technique since sterilization becomes increasingly difficult with decreasing

amplicon length (13). Third, the short length permitted the chemical synthesis of the sequence which provided a standard for quantitative experiments. Important parameters evaluated during the development of the sterilization methodology included the effect of the sterilization reagent on the PCR amplification, the efficiency of the sterilization process using the HIV-1 115-mer amplicon from both synthetic and clinical sources, and the detectability of the sterilized amplicon by different hybridization techniques.

### PCR Amplification in the Presence of Isopsoralens

With clinical samples, the HIV-1 infectivity level can be 1 in 10,000 T4 cells or lower (24). Such low copy number samples will require effective amplification of only a few initial template molecules. A robust sterilization protocol must anticipate the full range of starting templates which may be encountered, and the presence of isopsoralen during the PCR must not interfere with efficient amplification. On the other hand, an important element of the sterilization protocol is addition of the isopsoralen to the PCR reaction mixture prior to the PCR. If addition follows amplification and the reaction vessel is opened, contamination of the laboratory may occur prior to sterilization. Thus including isopsoralen as a component of the PCR reaction, which is necessary for sterilization, requires either isopsoralens which do not inhibit the PCR, or alternatively, PCR conditions which minimize any such inhibition.

To examine these concerns, experiments were performed to determine what effect the isopsoralens have on the PCR and to then optimize isopsoralen concentration for a wide range of starting copy numbers. The first set of experiments was designed to quantify amplification efficiency at high starting copy number using different concentrations of 4′-AMDMIP and AMIP. When $10^7$ copies of the 115-mer were cycled 30 times, the presence of 100 to 400 μg/ml of either compound did not depress the PCR yield (relative to the zero isopsoralen control; data not shown). The second set of experiments was carried out to evaluate the effect of 4′-AMDMIP on amplification as the starting copy number was decreased (to as few as 100 starting copies). The experiments determined amplification efficiency at different 4′-AMDMIP concentrations with $10^2$ to $10^6$ starting copies. The results showed that amplification efficiency dropped with both increasing 4′-AMDMIP concentration and decreasing starting template copies. As shown in Figure 1, no difference in the PCR product yield was observed up to 400 μg/ml 4′-AMDMIP when the starting copy number was $10^5$ or higher. However, when the starting copies were reduced to $10^4$, the yield dropped at 400 μg/ml 4′-AMDMIP, while further reduction of the starting copy number to $10^3$ or $10^2$ resulted in lower yields between 200 and 300 μg/ml of 4′-AMDMIP (data not shown).

Recent work with a different HIV-1 amplicon (142-mer) has shown the inhibitory effect due to isopsoralen (i.e. low amplification efficiency) can be more pronounced when less than 100 starting copies are present (25). Interestingly, the inhibitory effect of the isopsoralens can be overcome by the use of co-solvents during the PCR. The presence of either DMSO (3−5%) or glycerol (10−20%) in the PCR mix alleviates the observed inhibition, restoring amplification efficiency to the level which occurs in the absence of isopsoralen. The co-solvent effect is quite broad, and will reestablish amplification of a low copy sample (e.g. 10 copies) in the presence of a high concentration of isopsoralen. This technique allows the use of an isopsoralen concentration which provides a high level of sterilization under conditions which do not compromise the PCR.

While the precise mechanism is not clear, these results may be understood by considering that the presence of positively charged isopsoralens act to stabilize the double-stranded amplicon and/or kinetically inhibit polymerization, and either of these effects will be more pronounced as the length of the amplicon increases. When a high copy number is amplified ($10^5$/$10^6$ starting copies), the inhibitory effect is concealed because the reaction still achieves plateau after 30 cycles. When this occurs, the amplification efficiency appears to be the same. As the starting copy number decreases, plateau is no longer reached and differences in PCR efficiency become apparent. By addition of a polar organic compound to the solution mix, the interaction between the isopsoralen and the DNA prior to photoreaction is weakened, presumably reducing the enhanced stability of the amplicon duplex, and also reducing the inhibition of the propagation of the Taq polymerase during polymerization.

While a concomitant reduction in isopsoralen photoaddition to DNA in the presence of effective concentrations of the co-solvent might be expected (e.g. 5% DMSO), the observed reduction in photoreactivity was not significant. To evaluate the effect of DMSO on the photochemical binding of isopsoralens to DNA, samples were prepared containing calf thymus DNA, tritium labelled 4′-AMDMIP, and differing amounts of DMSO (0, 1, 3, 5, 10 & 20%). Covalently bound 4′-AMDMIP was determined as a function of DMSO concentration and found to be 119 covalent 4′-AMDMIP adducts/$10^3$ base pairs (BP) with 0% DMSO; 108 adducts/$10^3$ BP with 3%; 101 adducts/$10^3$ BP with 5%; 88 adducts/$10^3$ BP with 10%; and 60 adducts/$10^3$ BP with 20%.

These experiments demonstrate that careful selection of the isopsoralen concentration is important. If the initial copy number is high (e.g. genomic DNA), a relatively high concentration of isopsoralen is preferred. If the copy number is low or unknown, then either a lower isopsoralen concentration or a co-solvent approach is advised to assure that all samples will amplify. In the work discussed below with the HIV-1 115-mer amplicon, non-inhibitory concentrations of 4′-AMDMIP or 6-AMDMIP were used (200 μg/ml for most experiments).

### Sterilization of the 115-mer Amplicon

Two sources of the HIV-1 115-mer amplicon were used for the sterilization experiments: synthetic 115-mer, prepared by chemical synthesis, and patient samples, provided by HIV-1 positive individuals. The methodology used to determine sterilization efficiency consisted of 1) amplifying a known or unknown number of target molecules a given number of cycles in the presence of a specific concentration of an isopsoralen compound, 2) dividing the fully amplified PCR mixture into two portions followed by the irradiation of one, 3) preparing a dilution series from both the sterilized and unsterilized halves of the sample, and 4) reamplifying each dilution point to determine if and how much reamplification occurred. Detection was accomplished by the use of $\alpha$-$^{32}$P-dCTP in the second PCR, during which every reamplified molecule was radiolabelled.

Two important points about this methodology are the following. First, the use of an internal label for the analysis assures that only the *reamplified* molecules are detected. The sensitivity limit of this procedure is about 100 CPM, which corresponds to approximately $10^8$ amplicon copies at the specific activity used for these experiments. While analysis by hybridization methods such as oligomer hybridization (OH) or crosslinkable oligonucleotide probe analysis (COP, 26) are more sensitive than the internal labelling technique, they also detect the copies of

the amplicon ('challenge copies') used to seed the reamplification experiment (whether sterilized or not). Second, the protocol irradiates a fully amplified PCR reaction mixture (instead of a dilution), and then prepares the dilution series from the irradiated solution. This procedure simulates real amplified samples which will contain up to $10^{12}$ amplicon copies during the irradiation.

A representative sterilization experiment using the synthetic 115-mer target is shown in Figure 2. The PCR mixtures used in the experiment initially contained $10^7$ starting copies of the 115-mer sequence which had been amplified 30 cycles in the presence of 400 μg/ml 4'-AMDMIP or 400 μg/ml AMIP. The amplified reaction mixtures were divided into two equal portions then one portion was irradiated. The sterilization efficiency was then evaluated as described in the Methods section. Inspection of Figure 2 shows that with 4'-AMDMIP, sterilization was essentially complete at all challenge copy numbers (compare lanes 1/2, 3/4, 5/6, 7/8), while with AMIP, the sterilization efficiency was much lower (compare lanes 9/10, 11/12, 13/14, 15/16). While all of the carryover samples irradiated with AMIP showed some reduction in signal relative to the unirradiated control, sterilization was significant in only the lowest case ($10^2$ challenge copies). These disparate results are consistent with the relative modification densities provided by the two compounds.

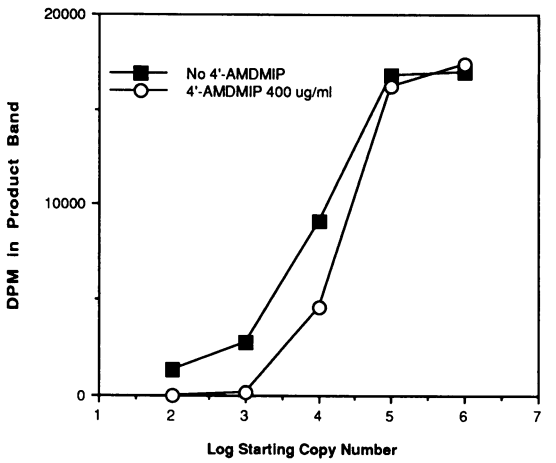


**Figure 1.** Dark effect of 4'-AMDMIP on the PCR as a function of target copy number and 4'-AMDMIP concentration. DPM in the product bands are plotted against the log of the initial copy numbers of 115-mer template.

In separate experiments, synthetic 115-mer was reacted with tritiated 4'-AMDMIP and AMIP under similar conditions and found to provide an average modification density of 1:5 and 1:15.2 adducts per base, respectively. This corresponds to 17.4 and 5.8 effective adducts per 115-mer single strand (adducts in the priming region are not counted). The fraction of molecules that contain zero modifications in a large population of molecules that have an average of $a$ modifications is given by $f_a(0) = e^{-a}$. Therefore, if there are $10^8$ carryover molecules where the average molecule contains 17.4 modifications, the number of molecules with zero modifications (isopsoralen adducts) will be $e^{-17.4} \times 10^8 = 2.8$ (see reference 13). These calculations reveal that the expected number of non-sterilized molecules for $10^8$, $10^6$, $10^4$ and $10^2$ carryover 115-mer molecules with an average modification density of 17.4 effective adducts per strand are 2.8, .028, .00028, and .0000028, respectively. The corresponding number of unsterilized molecules for 115-mer with an average modification density of 5.8 effective adducts per strand are

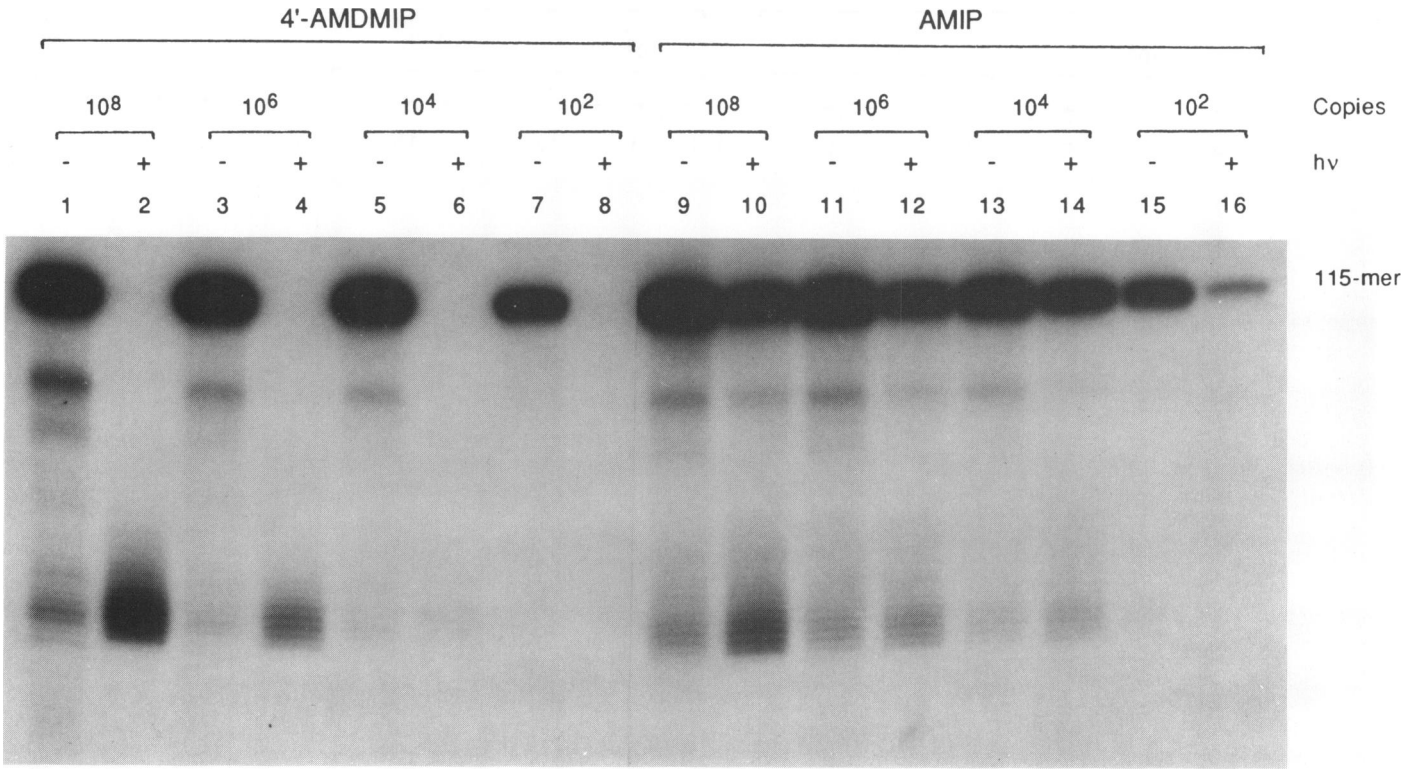


**Figure 2.** Sterilization of the 115-mer amplicon amplified from the synthetic 115-mer template with either 4'-AMDMIP (400 μg/ml) or AMIP (400 μg/ml). Unirradiated (control) amplicons (4'-AMDMIP/AMIP): $10^8$ copies (lanes 1/9), $10^6$ copies (lanes 3/11), $10^4$ copies (lanes 5/13), $10^2$ copies (lanes 7/15); irradiated (sterilized) amplicons (4'-AMDMIP/AMIP): $10^8$ copies (lanes 2/10), $10^6$ copies (lanes 4/12), $10^4$ copies (lanes 6/14), $10^2$ copies (lanes 8/16).

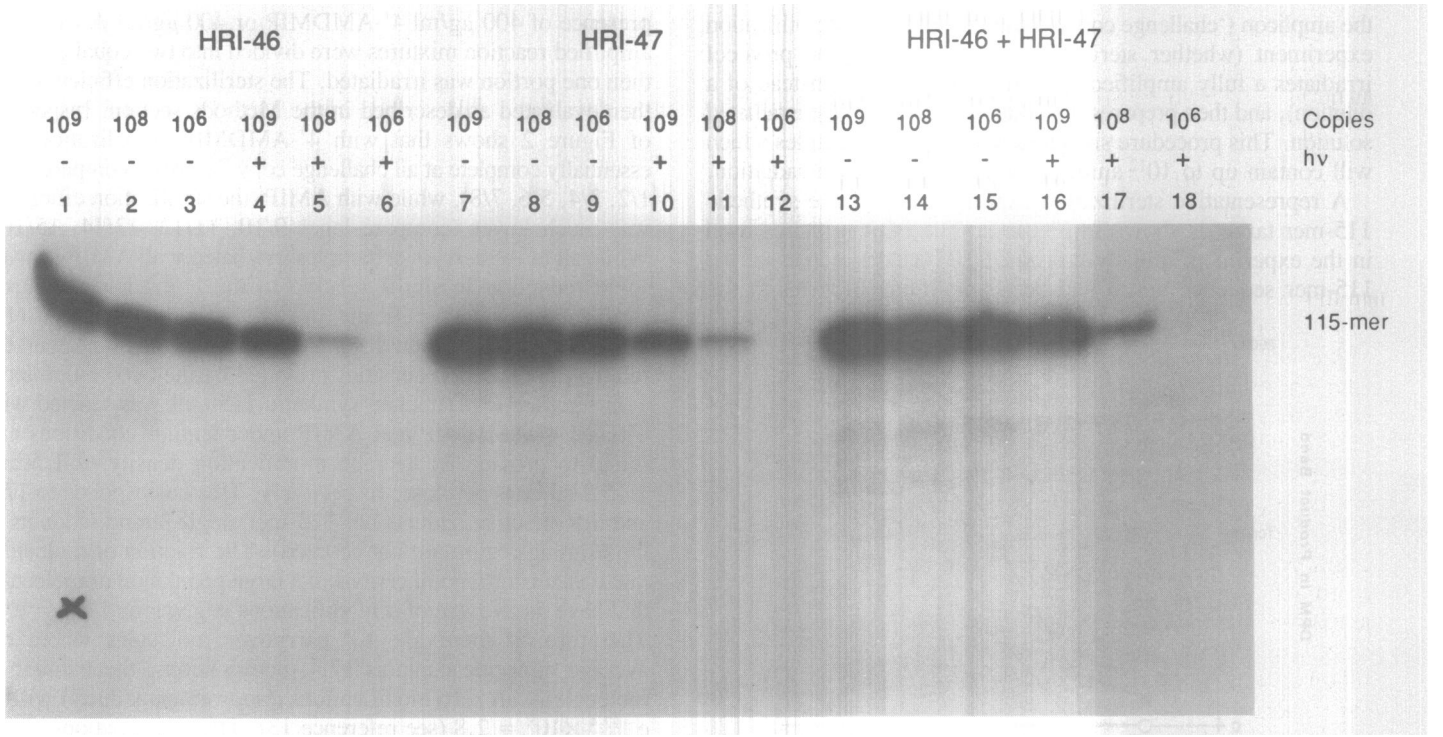


**Figure 3.** Sterilization of double-stranded and single-stranded 115-mer with 6-AMDMIP. Each of the synthetic single strands of the double-stranded 115-mer sequence (HRI-46 and HRI-47) were used separately or as a 1:1 mix to determine sterilization with each of the single-stranded amplicons. HRI-46 unirradiated (control) target: $10^9$, $10^8$ and $10^6$ copies (lanes 1–3); HRI-46 irradiated (sterilized) target: $10^9$, $10^8$ and $10^6$ copies (lanes 4–6); HRI-47 unirradiated (control) target: $10^9$, $10^8$ and $10^6$ copies (lanes 7–9); HRI-47 irradiated (sterilized) target (lanes 10–12); HRI-46 + HRI-47 unirradiated (control) target: $10^9$, $10^8$ and $10^6$ copies (lanes 13–15); HRI-46 + HRI-47 irradiated (sterilized) target: (lanes 16–18).

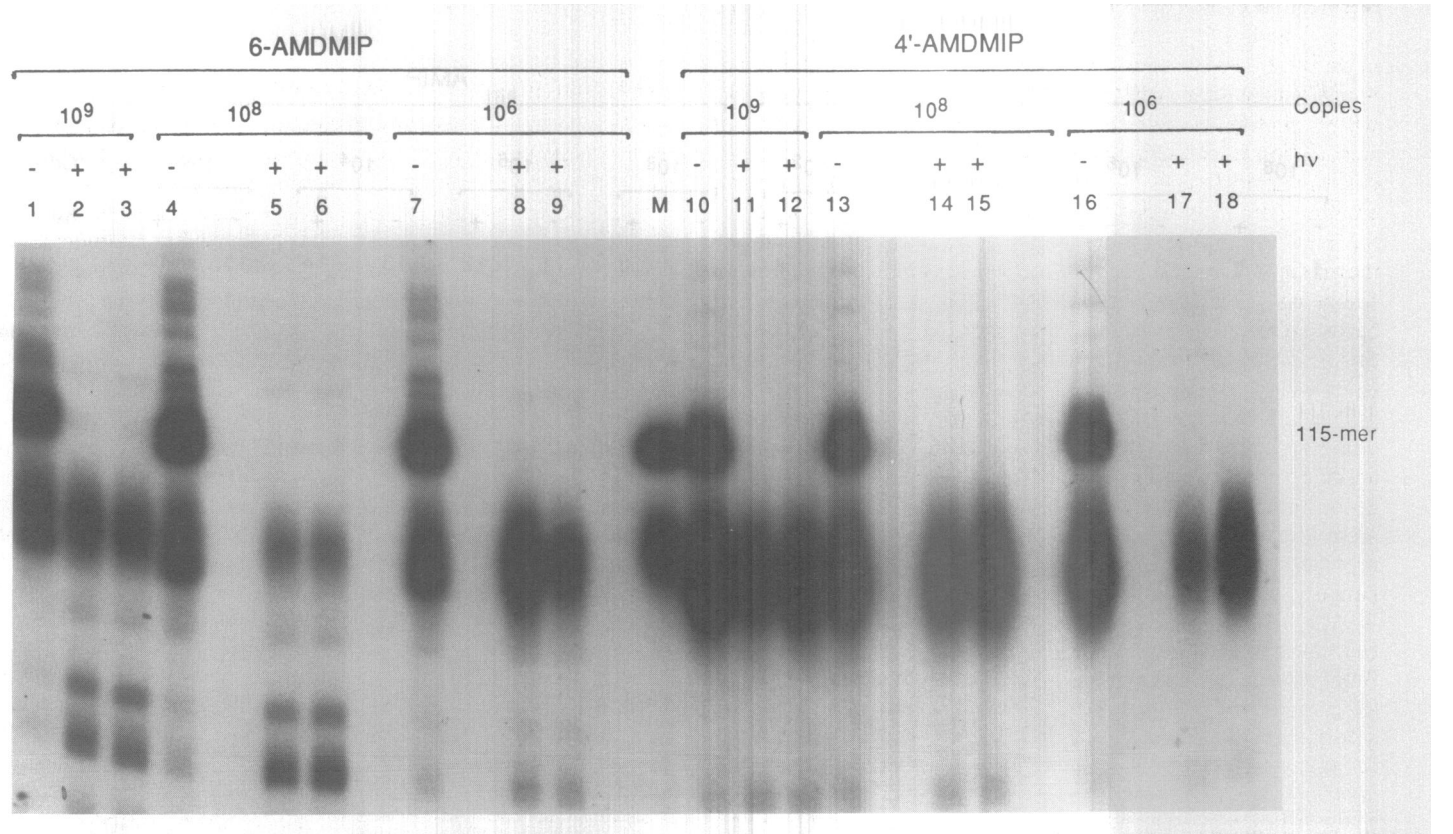


**Figure 4.** Sterilization of an amplified clinical sample with 4'-AMDMIP and 6-AMDMIP. The autoradiogram compares sterilization with 6-AMDMIP (lanes 1–9) and 4'-AMDMIP (lanes 10–18). Unirradiated (control) amplicons (6-AMDMIP/4'-AMDMIP): $10^9$ copies (lanes 1/10); $10^8$ copies (lanes 4/13); $10^6$ copies (lanes 7/16); irradiated (sterilized) amplicons (6-AMDMIP/4'-AMDMIP): $10^9$ copies (lanes 2, 3/11,12); $10^8$ copies (lanes 5, 6/14,15); $10^6$ copies (lanes 8, 9/17,18). Marker ('M') is $10^6$ copies of a control plasmid (pBKBH10S) amplified under the same conditions with SK-38/39.

300000, 3000, 30, and .3, respectively. Thus while only a weak potential signal is expected for the $10^8$ copy sample of the 4′-AMDMIP treated sample following a 30 cycle reamplification, it is reasonable to expect a detectable signal to appear in all but the $10^2$ copy number case after sterilization with AMIP. The model 115-mer experiments with tritiated 4′-AMDMIP and AMIP were done at an isopsoralen concentration of 100 μg/ml, while the sterilization experiment used these compounds at 400 μg/ml. Use of the higher concentration will increase the modification density by 20−30% and decrease the number of unsterilized molecules in each of the dilutions. The magnitude of the decrease, however, would not be expected to significantly alter the observed levels of sterilization provided by the two compounds.

A potential concern for the isopsoralen sterilization technique is the ability of the isopsoralens to react with single-stranded amplicons. For some PCR applications, it is desirable to perform a 'biased' amplification, whereby an excess of one of the two amplicon strands is purposely made (27). Moreover, there may be an unintended yet considerable population of single-stranded amplicons following normal PCR, depending on the particular amplicon sequence and details of how the samples are handled following amplification. To address this concern, a sterilization experiment was performed using each of the synthetic single-strands of the double-stranded 115-mer sequence (HRI-46 and HRI-47). Samples of each single strand or a 1:1 mixture of the two were irradiated in the presence of 6-AMDMIP. Following irradiation, a dilution series was prepared from each sample (along with the corresponding unirradiated controls) followed by reamplification and PAGE analysis. As shown in Figure 3, sterilization of the individual strands was essentially the same as sterilization of the two strands combined. A slight increase in sterilization was noted with the pyrimidine-rich strand HRI-47, which is consistent with the fact that it is primarily pyrimidines which photoreact with the isopsoralens. The equivalence of sterilization with single and double strands may result from 1) extensive intramolecular secondary structure of the single-strands, creating double-stranded reaction sites for the isopsoralens, 2) the existence of one or more reactive 'hot spots' within each of the single-strands, and/or 3) reaction of single-strands directly, following stacking of the isopsoralen between single-stranded bases. Whatever the actual mechanism, the ultimate result is effective sterilization of single-stranded amplicons with the isopsoralen technique.

The final set of sterilization experiments was performed with clinical samples. Peripheral blood mononuclear cells from antibody positive HIV-1 patients were collected and processed according to the Methods section. The sterilization experiments were identical in design to those with the synthetic 115-mer. A representative result is shown in Figure 4. In this experiment, patient samples were amplified then sterilized with either 4′-AMDMIP or 6-AMDMIP. A dilution series was then prepared (nominally $10^9$, $10^8$ or $10^6$ copies), reamplified and evaluated. Inspection of Figure 4 demonstrates successful sterilization at all three levels with both isopsoralen derivatives. The difference in sterilization results with 6-AMDMIP in this experiment relative to the experiment shown in Figure 3 is probably due to the clinical sample actually containing less than the indicated copy numbers at the dilution points. While the synthetic 115-mer provided a 'hard' copy number, since it was measured spectrophotometrically, the clinical sample copy number was based upon the assumption that the PCR mix was in fact $10^{-8}$ M following amplification.

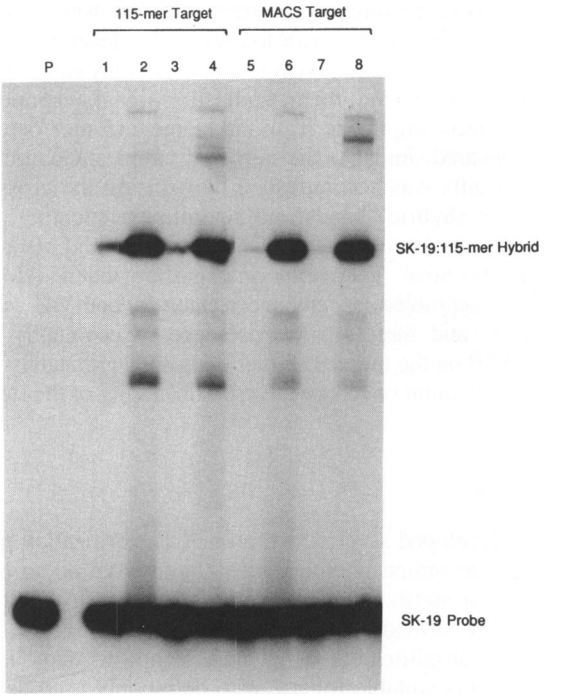


**Figure 5.** OH detection of the sterilized 115-mer amplicon. Samples were analyzed by OH as described in the Methods section. Samples 1−4 contain $10^6$ copies of 115-mer amplified either 20 (lanes 1, 3) or 30 (lanes 2, 4) cycles. Lanes 1 and 2 are the no 4′-AMDMIP (unsterilized) controls; lanes 3 and 4 are the plus 4′-AMDMIP (sterilized) samples. Lanes 5−8 are the analogous samples generated from the clinical DNA (MACS) sample.

## Detection of the Sterilized 115-mer Amplicon

Although isopsoralens are bifunctional reagents, their angular shape prevents crosslink formation with nucleic acid. The absence of interstrand crosslinks and the minor influence of isopsoralen monoadducts on base pairing provides single-stranded, sterilized amplicons, which remain detectable by hybridization procedures. The following two experiments demonstrate the compatibility of isopsoralens with two different hybridization formats: oligomer hybridization with the 41-mer probe SK-19, and crosslinkable oligonucleotide probe analysis with the crosslinkable analogue of this same probe, SK-19-MA.

The experimental design and details are provided in the Methods section. Synthetic 115-mer ($10^6$ copies) or genomic DNA (1 μg) from an individual known to be infected with HIV-1 were used as starting templates. PCR samples were prepared for each target both with and without 4′-AMDMIP then amplified for 20 or 30 cycles. Following PCR, the samples which contained 4′-AMDMIP were irradiated. Both control and irradiated samples were then analyzed by the OH and COP procedures. The results of the OH assay are shown in Figure 5. Samples 1−4 contain $10^6$ copies of 115-mer reamplified either 20 (lanes 1, 3) or 30 (lanes 2, 4) cycles (only the 30 cycle reamplifications provided significant product). Lanes 1 and 2 are the no 4′-AMDMIP (unsterilized) controls, while lanes 3 and 4 are the plus 4′-AMDMIP (sterilized) samples. Lanes 5−8 are the analogous samples generated from the clinical DNA (MACS) sample. Visual inspection of Figure 5 shows the band intensities of the unsterilized and sterilized samples are similar (compare lanes 1/3, 2 /4, 6 /8). The same set of samples were used for the COP experiment and the results were essentially the same. With COP,

the bands corresponding to 115-mer amplicon were covalently crosslinked to SK-19-MA prior to analysis by denaturing PAGE. The trends of the 20 and 30 cycle amplifications were the same as the OH assay. Again, the hybridization signal generated with the unsterilized amplicons from either the 115-mer or clinical sample appeared similar to the sterilized samples. Quantification of these results was accomplished by excising the crosslinked probe-target (hybrid) bands and scintillation counting, which showed the sterilized signal to be between 20 and 50% of the unsterilized control. These experiments show that 4'-AMDMIP sterilized target molecules remain detectable by both OH and COP procedures, and therefore the presence of covalently bound 4'-AMDMIP on the target 115-mer does not significantly inhibit probe hybridization or reduce the crosslinkability of the sterilized target molecules.

## SUMMARY

We have developed an effective post-PCR sterilization process based on isopsoralen photochemistry, and have optimized the procedure for sterilization of a important retroviral amplicon. The method provides a high level of sterilization while maintaining amplification efficiency with low copy number samples, and is suitable for use with commonly used detection formats which utilize hybridization. In short, the photochemical sterilization protocol we have described is an effective and pragmatic method for eliminating the amplicon carryover problem which has been associated with the PCR. While the work described here is limited to HIV-1, proper use of the technique will go far towards relieving the concern associated with the carryover problem with a wide variety of amplicons, especially in the clinical setting.

The example which has been used for this study, the 115-mer HIV-1 amplicon, is a particularly stringent test of the sterilization protocol because of its short length. We have predicted and observed that sterilization becomes easier to achieve as the amplicon becomes longer (13). Due to the statistical nature of isopsoralen-based sterilization, the success of the method depends on obtaining the appropriate modification density of the amplicon being sterilized, and the primary factors controlling modification are the reactivity of the isopsoralen derivative and the length and particular sequence of the amplicon. Since each amplicon has its own base sequence and length, optimal sterilization conditions for each amplicon must currently be evaluated on a test by test basis.

For the majority of diagnostically important sequences, amplicon length, and to some degree, sequence, can be selected by the judicious choice of primers. This versatility will allow isopsoralen sterilization to be successfully applied to a wide variety of important diagnostic targets. Moreover, while the sterilization compounds of choice are currently 4'-AMDMIP and 6-AMDMIP, we are developing new reagents which bind more effectively to DNA at lower concentrations. Such reagents will reduce the concern of system-dependent PCR inhibition, minimize the need to optimize sterilization on a test by test basis, and will allow the ultimate goal of a generic sterilization procedure to be attained. At the present time, it is prudent to include 10% glycerol in the PCR reaction to insure the successful amplification of low copy number samples. This is especially important when applying the procedure to a wide range of samples where copy number is unknown.

A final point concerns the sterilization efficiency provided by the isopsoralen procedure. While absolute sterilization (i.e. modification of essentially every amplicon) can be realized with long, pyrimidine-rich sequences, the current procedure with the 115-mer amplicon provides adequate sterilization for the routine level of carryover which occurs. Since most false positives observed in both research and diagnostic testing facilities seem to arise from carryover of between 10 and 10,000 copies, a procedure which provides sterilization of $10^8$ copies is effective. Furthermore, since carryover of $10^8$ copies will provide a hybridization signal independent of sterilization, it is not necessary to provide a sterilization level that exceeds the direct detection level of the carryover molecules themselves. Thus, at the level of contamination observed in most laboratory situations, the probability of a false positive test following the application of the post-PCR sterilization protocol we have developed is very low.

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