Post-PCR sterilization: a method to control carryover contamination for the polymerase chain reaction

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ABSTRACT
We describe a photochemical procedure for the sterilization of polynucleotides that are created by the Polymerase Chain Reaction (PCR). The procedure is based upon the blockage of Taq DNA polymerase when it encounters a photochemically modified base in a polynucleotide strand. We have discovered reagents that can be added to a PCR reaction mixture prior to amplification and tolerate the thermal cycles of PCR, are photoactivated after amplification, and damage a PCR strand in a manner that, should the damaged strand be carried over into a new reaction vessel, prevent it from functioning as a template for the PCR. These reagents, which are isosorosalen derivatives that form cyclobutane adducts with pyrimidine bases, are shown to stop Taq polymerase under conditions appropriate for the PCR process. We show that effective sterilization of PCR products requires the use of these reagents at concentrations that are tailored to the length and sequence of the PCR product and the level of amplification of the PCR protocol.

INTRODUCTION
Nucleic acid probe technology has been an essential tool for molecular biology since its inception 30 years ago. During the last decade, quantum developments in this field resulted from the introduction of automated DNA synthesizers. Short, synthetic oligonucleotides provided new tools for the advancement of cloning techniques, the creation of allele-specific hybridization protocols and, most importantly, template-dependent polymerase extension reactions were developed into nucleic acid amplification protocols by using the oligonucleotides as specific primers (1–4). The first of these procedures, the Polymerase Chain Reaction (PCR) (5,6), is now an established technique worldwide. One hundred to several thousand bases of a few molecules from a single target sequence in the presence of complex genomic nucleic acid sequences are routinely amplified to as many as $10^{12} - 10^{13}$ molecules in just a few hours. This technique has revolutionized the way molecular biologists conduct experiments, and has found application in a variety of different fields.

Contamination by ‘Amplicon’ Carry-Over
The widespread use of the PCR has come about because of its exquisite specificity and sensitivity. These features, however, have one drawback: the synthesis of specific PCR products that arise through the amplification of previously amplified sequences (‘amplicons’) which are inadvertently carried over into a subsequent PCR reaction (7–9). These carryover amplicons can contaminate reagents or sample specimens, causing systematic errors. Alternatively, individual reaction vessels can be contaminated during the preparative procedures and result in sporadic false positive PCR signals. The carryover problem is most acute in environments where the same amplification is run time and time again (e.g. a clinical diagnostic lab). Extreme care is essential when handling amplified samples so that carryover is minimized. The recommended sample handling conditions are similar to those used by microbiologists for the handling and growth of microorganisms(10,11). In fact, the problem is numerically analogous since similar amplifications occur when bacteria or viruses are grown in large volumes. Biological organisms are, however, effectively sterilized by numerous techniques that either thermally or chemically denature macromolecules. Simple denaturation is not adequate for sterilization of PCR products since a single strand of a nucleic acid is readily amplified and is also chemically resistant to the temperatures encountered in laboratory sterilizers. Effective sterilization of amplicons requires chemical, photochemical or enzymatic modification of the amplicons that prevent them from acting as templates for polymerase extension reactions.

General Considerations for the Sterilization of Amplicons Created by the PCR Procedure
From a temporal point of view, there are two places in a PCR-based diagnostic assay where sterilization can be implemented. When performed just before amplification (‘pre-PCR Sterilization’), carryover molecules present in the reaction mix will be rendered sterilized. When performed after amplification (‘post-PCR Sterilization’), all nucleic acids, including PCR products, will be sterilized. These two sterilization modes are distinguishable in that they impose specific and different

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requirements upon the nature of a chemical modification process and upon the reagents present during the modification procedure.

With the pre-PCR sterilization mode, PCR reagents susceptible to the chemical modification process must be left out and added back to the reaction mix before amplification can take place. These reagents most likely include Taq polymerase, the true target nucleic acid, and the primer oligonucleotides. Sporadic PCR errors due to amplicon carryover still exist with this mode since carryover molecules can be introduced during the addition of the other reaction components. Recently, a direct, shortwave UV irradiation procedure has been described as a pre-amplification sterilization process (12). This technique can sterilize some PCR reagents, but the process is inadequate for the entire complexity of carryover problems (13). An enzymatic approach that distinguishes amplicon from true target and primer oligonucleotides would be advantageous as a pre-PCR sterilization technique if the enzyme is also compatible with the PCR.

With post-PCR sterilization, all of the PCR reagents can be present since polymerase activity, primer integrity, and true target integrity are of concern following amplification. Sterilization in this mode can potentially obviate both the systematic and sporadic errors associated with the carryover problem if all of the amplicons are sterilized before the reaction tube is opened and exposed to the environment. With this mode, however, the chemically modified PCR strands must still be detectable. This usually implies that either a) the modified PCR amplicons be capable of specific and efficient hybridization to an oligonucleotide probe which is complementary to an internal sequence within one of the PCR strands, or b) that the modified amplicons be detectable as an oligonucleotide product of a specific length when analyzed by gel electrophoresis.

A post-PCR sterilization process that employs a photochemical reagent should have additional properties to be used conveniently with the PCR process. First, the non-activated reagent must not interfere with primer annealing or Taq polymerase activity, and must be thermally stable to the temperatures encountered with the PCR technique. This permits the reagent to be added prior to amplification. Second, the ideal reagent would be activated by wavelengths between 300 and 400 nm since the polypropylene tubes used for PCR reactions are transmissible to these wavelengths, direct UV damage to the amplicons is minimized, and PCR samples can be handled on the bench with normal fluorescent or incandescent lighting. Finally, the photochemical modification of the amplicons must prevent the modified amplicon from acting as a template for the PCR.

This last criteria assumes that an amplicon with a single modification is prohibited from acting as a PCR template molecule. Even with this assumption, the effectiveness of any nucleic acid sterilization process will also be dictated by the magnitude of the carryover problem, the extent of the amplification, and the average degree of modification of the amplicons. The population of carryover amplicons must be modified to a sufficient average extent per molecule so that statistically there is a small likelihood that some molecules are without any modifications, and therefore non-sterilized. Some of these non-sterilized carryover molecules, however, can be tolerated if their total number is less than the minimum detection limit of the PCR procedure.

Table I illustrates the relationship between detection sensitivity and PCR amplification at different numbers of initial target molecules. The amplification factor that is observed with the PCR technique is related to the number (n) of cycles of the PCR that have occurred and the efficiency of replication at each cycle (E), which in turn is a function of the priming and extension efficiencies during each cycle. Amplification follows the form \((1 + E^n)\), until high concentrations (approx. \(10^{-8} M\) or greater) of PCR product are made. PCR products eventually reach a plateau concentration. Also shown in Table I is a signal (CPM) for a hybridization assay that is used to detect the presence of PCR product. Using 100 CPM as a detection limit, the data of Table I show that detection of a single copy gene from 1 µg of genomic DNA requires only 20 cycles of amplification. Under these conditions, several thousand carryover molecules can escape detection. By contrast, the identification of 10 target molecules requires 30 cycles of amplification. Under these conditions, just a few molecules of carryover will generate a false positive PCR signal.

We describe here a photochemical process that destroys the template activity of PCR amplicons yet permits the modified amplicon to be probed in hybridization reactions. The process can be designed to ensure that carryover as high as \(10^{12}\) molecules are incapable of being amplified by the PCR procedure.

**MATERIALS AND METHODS**

**Materials**

Materials and light sources were obtained as specified in the accompanying manuscript (14). 5-MIP (5-methylisopсорalen) and \(^3\)H-5-MIP were obtained from HR1 Associates, Inc. (Berkeley, CA).

**PCR Amplifications**

An amplicon 115 bases in length was generated by using primers SK38 and SK39 according to published procedures (15) with either 1 µg of genomic DNA from an HIV-1 positive patient as the initial target or known copy numbers of the 115mer amplicon generated in a previous PCR reaction. A 500 base pair amplicon was also generated for comparative purposes according to the protocol described in the GeneAmp DNA amplification Reagent Kit (#N801-0055, Perkin-Elmer/Cetus) by utilizing primers PCR1/PCR02 with the control bacteriophage λ template or with PCR products generated in a previous PCR reaction with this primer/target system.

**Photoaddition Reactions**

Irradiations. All irradiations were performed with either a device (PTI) described previously (16) or the HRI-100 device. The PTI device exposes one sample at a time and has no emission below 320 nm. By contrast, the HRI-100 device is designed for the simultaneous irradiation of multiple Eppendorf tubes that are routinely used for PCR amplifications. This device consists of six fluorescent lamps (Black Light type FBTSBL) arranged parallel to each other. From a side view, the lamps form a U-shaped trough. A double-walled, UV-transparent plastic holder rests in the trough which supports up to 48 Eppendorf tubes (0.5 ml). With this arrangement, the sample tubes are exposed to approximately 20 mW/cm² of 300–400 nm light. Standard Eppendorf tubes have an average of 10% transmission for this wavelength region. During irradiations, samples were maintained at room temperature by passing water through the double-walled sample holder. We have observed that some amplicons are more efficiently sterilized when exposed to light at lower temperatures (4°C, data not shown).

Photoaddition to Genomic DNA. \(^3\)H-4′-AMDMIP (2.2×10⁵ CPM/µg), \(^3\)H-AMIP (3.1×10⁵ CPM/µg) or \(^3\)H-5-MIP...
### Table I: PCR amplification as a function of initial target number and cycle number.

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* 1 μg genomic DNA

Each block in this table contains three sets of data: (1) The total number of PCR product molecules present after the initial number of target strands are amplified for the number of cycles shown. These numbers of amplicons were calculated using an amplification factor of (1.85)^a. We have experimentally confirmed the value of this factor for the 115mer amplicon generated with the primers SK38 and SK39. (2) The concentration of PCR product when the amplification takes place in a 100 μl volume. The gray area indicates conditions that give rise to PCR products that are in excess of 10^10 M. In these regions, the PCR plateau effect will be acting on PCR product synthesis. And (3), a hybridization signal that is used to detect the presence of PCR product. This signal is calculated on the basis of having a 10% hybridization efficiency of a 32P labelled probe (3000 Ci/mM) to a 20 μl aliquot of the 100 μl PCR reaction mix.

(2.1 x 10^5 CPM/μg) at the concentrations described in the text were mixed with 100 μg of calf thymus DNA (Sigma Chemical Co.) in 200 μl of 1× TE buffer, pH = 7.0. 30 μl aliquots of these samples were irradiated at room temperature with the HRI-100 device for the time periods indicated. Photobinding levels were determined as described in the accompanying manuscript (14).

**Photoaddition to Synthetic 115mer.** Photobinding of 3H-4'-AMDMIP to the double stranded 115-mer (HRI-46/HRI-47) was determined as described in the accompanying manuscript (14).

**Sterilization Assays**

**115mer Sterilization.** 1 μg of genomic DNA from an HIV-1 positive patient was sufficiently amplified with primers SK38/SK39 to produce a plateau PCR reaction. Identical aliquots of this PCR mixture containing approximately 10^6 copies of the 115mer amplicon in 0.5 ml Eppendorf tubes were adjusted to 100 μg/ml 4'-AMDMIP in a total volume of 10 μl containing 1× Taq buffer (50 mM KCl, 2.5 mM MgCl2, 10 mM Tris, pH 8.5, 200 μg/ml gelatin). Half of these samples were irradiated for 15 minutes at room temperature with the PTI device. The irradiated samples, as well as control samples, were adjusted to 20 μl by the addition of new PCR reagents. To determine the template activity of these amplicons, the PCR reaction mixtures containing 1× Taq buffer, 200 μM of each dNTP, 5 μCi of α-32P-CTP (3000 Ci/mM, New England Nuclear), 0.5 unit of Taq DNA polymerase (Cetus/Perkin Elmer) and 0.5 μM of each SK38 and SK39 primers were then reamplified. After adding 5 μl of a dye solution, 10 μl of each of the reamplified mixtures was run on a 12% polyacrylamide/8M urea gel, and analyzed by autoradiography. By comparing with known copy numbers of a synthetic 115mer oligonucleotide, the alpha incorporation assay will detect at least 100 copies of template 115mer after 30 cycles of amplification.

**115mer and 500mer Comparison.** To compare the sterilization efficiency of these two different amplicons, equivalent copy
numbers of each amplicon were initially prepared in the following manner: A 30 cycle PCR reaction was first carried out for each system with the appropriate primers and starting template molecules. Aliquots (approximately 10^2-10^4 target copies) of each of these reactions were transferred to a second set of PCR reactions. The second sets of PCR amplifications were carried out in the presence of α-32P-dCTP (low specific activity), again for 30 cycles. Aliquots of these reactions were run on a 3% NuSilve/1% agarose gel, and then cut and counted by liquid scintillation counting in order to determine the exact concentrations of each of the two amplicon products in the second set of PCR reaction tubes. The concentrations of both the 115mer and the 500mer were then adjusted to 1×10^{-3} M by the addition of additional Taq buffer. These solutions were made 100 μg/ml in 4'-AMDMIP. Both samples were subsequently divided into two portions, one part being irradiated for 15 minutes at room temperature with the HRI-100 device and the other part kept in room lights. Serial dilutions of the irradiated and the unirradiated amplicons were carried over into new reaction tubes (20 μl total volume) containing new PCR reagents and high specific activity α-32P-dCTP, and then reamplified for another 30 cycles of PCR. 10 μl aliquots of these samples were analyzed on 12% polyacrylamide/8 M urea gels.

Template Dependent Extension Experiments with Taq DNA Polymerase

Synthesis of 71mer-MA Templates. Three template oligonucleotides (71mer-MA's) containing site-specific isopsoralen monoadducts (MA) were constructed. The 71mer sequence (5'-ATC CTG GGA TTA AAT AAA GTA AGA ATG TAT AGC CCT ACC AGC ATT CTG GAC ATA AGA CAA GGA CCA AA-3') is a subsequence of the 115mer amplicon. SK39 is complementary to the last 28 bases of the sequence above.

Preparation of 71mers which contain site-specific monoadducts involved 1) preparation of different 15mer monoadducted oligonucleotides from the same unmodified 15mer and 2) ligation of the different 15mer monoadducted oligonucleotides to the same 56mer extender oligonucleotide using a 25mer oligonucleotide as a split. Isopsoralen monoadducted 15mers (15mer-MA's) were made from reaction mixtures containing 100 μg of the 15mer (5'-ATC CTG GGA TTA AAT AAA GTA AGA ATG TAT AGC CCT ACC AGC ATT CTG GAC ATA AGA CAA GGA CCA AA-3') and 65 μg of a complementary 10mer (5'-ATT AAT AAT ACC C-3') in 185 μl of TE buffer (10 mM Tris, pH = 7.5, 1.0 mM EDTA) and 0.1 M NaCl. An isopsoralen derivative (either 4'-AMDMIP, AMIP, or 3H-5-MIP) was added to separate reaction mixtures in Eppendorf tubes at 20-30 μg/ml. These 3 solutions were irradiated with the PTI device for 15 minutes at 15°C. The mixtures were then extracted with CHCl3 (4×), and the oligonucleotides were recovered by ethanol precipitation (2×). Unmodified 15mer was separated from 15mer-MA's by fractionation on HPLC (C18 reverse phase) using a 0.1M NH4OAc/acetoniitrite gradient. Fractions containing 15mer-MA's were identified by 5' end labelling aliquots of the HPLC fractions and analyzing these 32P labelled oligonucleotides by autoradiography after separation on a sequencing gel. Fractions with 15mer isopsoralen monoadducts were further purified by ethanol precipitation. 71mer-MA's were constructed with a standard ligation reaction mixture (50 μl total volume) containing one of the 15mer-MA's (4×10^{-4} M), a 5' phosphorylated 56mer (5'-AAAA ATA GTA AGA ATG TAT AGC CCT ACC AGC ATT CTG GAC ATA AGA CAA GGA CCA AA-3'; 5×10^{-4} M) and a 26mer complementary oligonucleotide (5'-CAT TCT TAC TAT ATT TAA TCC C-3'; 5×10^{-4} M). After 2 hours of ligation, the reaction mixtures were applied to a 12% polyacrylamide/8 M urea gel. The 71mer-MA bands on the gel were identified, cut, eluted, and finally purified by ethanol precipitation.

Taq Extension Assay. For the extension experiments, SK39 was 5'-end labelled with 32P by a kinase reaction and purified on a sequencing gel (17). 32P-SK39 (1×10^{-8} M) was hybridized to each of the 71mers (1×10^{-9} M) in a 20 μl reaction mix consisting of 1×Taq buffer and 200 μM each of NTP's. The samples were initially heated to 95°C for 5 minutes, then incubated at 55°C for 3 minutes. The extension reactions were initiated by the addition of Taq polymerase (0.05 units/μl) and terminated by the addition of 0.1 μl of 0.5 M EDTA. Extension products (10 μl) were analyzed on a 12% polyacrylamide, 8 M urea gel.

RESULTS AND DISCUSSION

Photochemical Sterilization with Furocoumarins

Furocoumarins are a class of planar, tricyclic compounds that are known to intercalate between base pairs of nucleic acids (18). These compounds contain two reactive double bonds which, when excited by 320 nm-400 nm light, react with the 5,6 double bond of pyrimidines to form cyclobutane rings. Both monoadducts and crosslinks are formed with psoralens (linear furocoumarins) when they react with DNA. Psoralen crosslinks are known to block polymerase extension reactions presumably by preventing strand separation of complementary strands of DNA (19). Monoadducts of certain psoralen derivatives have also been reported to block extension reactions with some polymerases, but blockage by monoadducts is not observed in all cases (19-22). This may be due to the fact that, although psoralen crosslinks induce a deformation of a DNA helix at the adduct site (23), psoralen monoadducts do not produce similar alterations of DNA helices (24). Thermodynamic studies agree with the structural studies and show that the presence of a psoralen monoadduct on an oligonucleotide does not substantially effect the hybridization stability of the oligonucleotide (25). Isopsoralens (angular furocoumarins) have been presumed to be similar to psoralens in their reactivity with nucleic acids except that they form only monoadducts because of the relative orientation of the reactive bonds of these molecules. Oligonucleotides modified by some isopsoralen derivatives are known to retain hybridization capabilities (26).

We have evaluated derivatives of both psoralens and isopsoralens as photochemical reagents in a post amplification mode for PCR sterilization. While derivatives of both classes of compounds are capable of sterilizing PCR amplicons, the isopsoralen modified PCR product oligonucleotides can be probed by hybridization and are therefore favored. Modification requirements for effective sterilization of PCR products with isopsoralens are described below. Similar requirements apply to psoralen sterilization.

4'-AMDMIP (27) was evaluated for its ability to act as a photochemical sterilization agent for PCR amplicons. The sterilization protocol is outlined in Figure 1a. An amplicon 115 bases in length was generated by performing PCR on genomic DNA under conditions that yield amplicon at plateau concentrations. Several identical samples, each containing 10^6 copies of the amplicon, were aliquoted from the plateau amplification mix. 4'-AMDMIP was added to each sample. Half
amplification yield. Level 10^6 molecules of carryover without 4'-AMDMIP (lanes 2, 6, and 10) generate reamplification products. Direct irradiation of similar samples without 4'-AMDMIP present has no effect on reamplification (data not shown). The control reactions that contain carryover with 4'-AMDMIP but without light exposure generate reamplification products that are equivalent in magnitude as those reamplified in the absence of 4'-AMDMIP. 4'-AMDMIP, as a post PCR sterilization reagent, did not affect PCR amplification efficiency. And finally, the reactions that received carryover, 4'-AMDMIP and irradiation show isopsoralen sterilization of PCR amplicons to a degree that is cycle dependent. With 20 cycles, sterilization appears to be complete. With 25 cycles, reamplification product is visible (lane 8), albeit reduced relative to controls (lanes 6 and 7). And with 30 cycles, no significant sterilization is observed; PCR reamplification product (lane 12) is approximately the same relative to controls (lanes 10 and 11).

This data illustrates the interplay between sterilization sensitivity and the amplification factor. At 20 cycles of PCR amplification, sterilization appears to be completely effective. If 100 CPM is taken to be the threshold for seeing a band on the autoradiograph, then Table 1 shows that the sterilization protocol of this example with 4'-AMDMIP left less than 10^4 amplicons that were capable of being replicated by the PCR procedure. At 25 cycles of PCR, a very measurable band is observed, suggesting that at least 10^3 amplicons retained replicating capabilities. At 30 cycles of PCR it is difficult to distinguish the control signal from the sample obtained with the sterilized sample. This is consistent with both the control sample and the 4'-AMDMIP treated sample reaching the plateau region of the PCR amplification process.

There are at least two factors that would make the sterilization protocol just described inadequate for treating the 10^6 copies of carryover amplicons. First, the sterilization process will be incomplete if Taq polymerase is capable of polymerizing through an isopsoralen adduct on an amplicon. To evaluate the extent that Taq polymerase reads through isopsoralen monoadducts, we constructed three modified oligonucleotides, each of which contained a different isopsoralen at specific positions near the 5' ends of the oligonucleotides. These oligonucleotides were then hybridized to a labeled primer (SK39 in Figure 2a). This primer was extended with Taq polymerase and the length of the polymerase products were determined. The isopsoralen modified oligonucleotide templates were constructed by assuming that isopsoralens, like psoralens, would have as a preferentially reactive site a 5' TpA-3' sequence in duplex DNA (28,29). We began with two short complementary oligonucleotides that contain a single TpA site within their complementary sequence region. These oligonucleotides were reacted with either 5-MIP, 4'-AMDMIP or AMIP under conditions where the two oligonucleotides would be hybridized. 15mer monoadducted oligonucleotides were purified from non-modified oligonucleotides and then ligated at their 3' ends to another oligonucleotide to generate the monoadducted template oligonucleotides (71mer-MA's).

Extension products generated with the 71mer templates are shown in Figure 2b. The 71mer-MA template that was made with

![Diagram](image_url)

**Figure 1.** (A) Protocol for evaluation of 4'-AMDMIP as a photochemical sterilization reagent for PCR amplicons. (B) Autoradiograph of the reamplification of 10^6 copies of 4'-AMDMIP sterilized 115mer amplicon. The samples in lanes 1-4, 5-8, and 9-12 were reamplified for 20, 25, and 30 cycles of the PCR, respectively. Lanes 1, 5, and 9 are negative controls. Lanes 2, 6, and 10 indicate the level of reamplification of unsterilized amplicon. Lanes 3, 7 and 10 are control reactions which show that 4'-AMDMIP in a PCR reaction mix does not affect amplification yield. Lanes 4, 8 and 12 show the reamplification products obtained with sterilized 115mer templates.
5-MIP produced a single truncated extension product when the extension reaction was run for 1 minute at 55°C. Similar results were obtained when the extension protocol included a second 1 minute extension at 72°C. When the extension protocol was cycled 10 times to mimic PCR, evidence of a small amount of a full length extension product was visible. The 71mer-MA target containing 4'-AMDMIP monoadducts yielded similar results for the 3 extension conditions. However, multiple stops were
observed with this isopsoralen and the predominant stop was a position entirely different from that produced with the 5-MIP containing template molecule. The 71mer-MA target containing AMIP monoaducts produced 3 truncated products upon extension with Taq polymerase. Again, the 10 cycle extension product produce some full length product.

These extension experiments show that all 3 isopsoralen adducts are effective blocks for Taq polymerase. While some full length product did occur with each of the isopsoralen template molecules, our data is insufficient to conclude that true readthrough exists. We have quantitated the readthrough with another set of experiments using a 5-MIP modified template. For these experiments, the HPLC purified 15mer-MA oligonucleotide was further purified by PAGE prior to the ligation reaction to form the 71mer-MA. With this template molecule, the full length extension product accumulates at 0.2% per cycle relative to the truncated product band. This data implies that our 71mer-MA was either 99.8% pure or that readthrough occurs at a 0.2% rate. This issue is not resolved at present because we cannot determine that the initial 15mer-MA was greater than 99.8% pure.

The extension experiments also suggest that the three different isopsoralens have different preferential binding sites on DNA molecules. The stop with the 5-MIP template appears to be at about the position of the TpA sequence in the 15mer that was used to create the monoaduct. The longest extension products with the AMIP and ADMIP templates indicate that these isopsoralens probably reacted with the initial 15mer outside the region of the 10mer/15mer hybrid interaction. The sequence of the 15mer does not indicate any obvious secondary structure in this oligonucleotide, suggesting that the two positively charged photochemical reagents, 4'-AMDMIP and AMIP, may be interacting with single-stranded regions of DNA. This is a desirable feature for a PCR sterilization reagent and has been confirmed elsewhere (14).

The extension experiments performed with 4'-AMDMIP monoadducted template molecules show that it is unlikely that the data of Figure 1b arose from bypass synthesis by Taq polymerase during reamplification. Furthermore, while 5-MIP adducts are effective stops for Taq polymerase, this compound is inadequate as a photochemical sterilization reagent (see below; data not shown). A more plausible explanation for the inadequate sterilization is that the protocol used to generate the data in Figure 1b provided incomplete modification: some of the amplicon molecules were not modified and therefore, not sterilized.

Photochemical sterilization with isopsoralens is fundamentally a statistical process. This process is characterized by measuring the average number (a) of adducts per amplicon strand. If the addition reaction is governed by Poisson statistics, the fraction of molecules with no modifications in a large population of amplicons that have an average of a modifications is given by f_a(0) = e^{-a}. As in the example of Figure 1, if the carryover event consists of 10^6 amplicons, 2.5×10^3 non-sterilized template molecules will be present when the modification protocol generates an average of only 6 effective adducts per strand of PCR amplicon. Effective adducts are defined as adducts which occur in the segment of a target molecule bounded by the primer sequences. For the 115mer of Figure 1, this level of effective adducts occurs when there is an average strand modification density of 1 adduct per 9.3 bases. The data of Figure 1b can be explained if the sterilization protocol yielded a similar modification density. We have measured the binding of triitated 4'-AMDMIP to synthetic strands of the 115mer amplicon product. Under identical irradiation conditions (with the PTI device) as those for the sterilization experiment, a modification density of 1 adduct per 8.4 bases was measured. This data is consistent with sterilization efficiency being determined by the statistical nature of the photochemical modification procedure.

Optimization of Nucleic Acid Sterilization with Isopsoralens

Isopsoralens undergo photochemical sterilization concomitant with photochemical addition to polynucleotides. The ratio of degradation to binding is a function of the isopsoralen derivative and the irradiation wavelengths. These competing reactions result in a plateau level of addition of these compounds to nucleic acids that is dependent on the initial concentration of the photochemical reagent during the activation process and on the wavelengths used to excite the molecules. When the synthetic 115mer was irradiated for 15 minutes from a UV source that enhanced binding (HRI-100), a modification density of 1 adduct per 5 bases was obtained with 4'-AMDMIP at 100 μg/ml. This high level of modification is unique to this short amplicon which contains 70% AT in the sequence region bounded by the primers. Amplicons of this short length display sequence dependent modification densities. Figure 3 illustrates the modification densities that are achieved with 4'-AMDMIP when it is reacted with long, complex genomic DNA. Modification densities ranging from 1 adduct per 100 bases (at 9.6 μg/ml 4'-AMDMIP) to 1 adduct per 11 bases (at 359 μg/ml) can easily be attained. This data indicates the range that can be achieved when 4'-AMDMIP is reacted with complex DNA and shows that the modification density can be controlled by the concentration of this photo reagent. At approximately 100 μg/ml of 4'-AMDMIP, a modification density of 1 adduct per 15 bases was observed. With AMP at a similar concentration, a modification density of 1 adduct per 33 bases was obtained. With 5-MIP, which has a solubility limit of 10 μg/ml, the highest modification density observed was 1 adduct per 88 bases. Although 5-MIP adducts are effective stops for Taq polymerase, this compound is inadequate as a sterilization reagent because its solubility limit prevents high modification densities.

The important parameter to optimize sterilization is the average number of adducts per amplicon strand. A robust post-PCR sterilization protocol would ensure that a major spill of a PCR reaction mixture would not lead to a carryover problem. To ensure that all the strands in a plateau PCR reaction mix (approx. 6×10^11 amplicons/100 μl) contain at least one adduct, Poisson statistics require a minimum average of 28 effective adducts per amplicon strand. For the 115mer, this requirement is satisfied.
Table II: Expected number of non-sterilized amplicon molecules as a function of PCR product length.

<table>
<thead>
<tr>
<th>CASE A: (1 adduct per 5 bases)</th>
<th>Average Effective Adducts/Strand</th>
<th>Non-Sterilized Molecules per 6 x 10^11 Starting Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of PCR Product</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>2.7 x 10^7</td>
</tr>
<tr>
<td>150</td>
<td>20</td>
<td>1.2 x 10^3</td>
</tr>
<tr>
<td>200</td>
<td>30</td>
<td>&lt;1</td>
</tr>
<tr>
<td>250</td>
<td>40</td>
<td>&lt;1</td>
</tr>
<tr>
<td>300</td>
<td>50</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

CASE B: (1 adduct per 15 bases)

<table>
<thead>
<tr>
<th></th>
<th>Average Effective Adducts/Strand</th>
<th>Non-Sterilized Molecules per 6 x 10^11 Starting Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of PCR Product</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>13.3</td>
<td>5.7 x 10^5</td>
</tr>
<tr>
<td>300</td>
<td>16.6</td>
<td>3.4 x 10^4</td>
</tr>
<tr>
<td>350</td>
<td>20</td>
<td>1.2 x 10^3</td>
</tr>
<tr>
<td>400</td>
<td>23.3</td>
<td>4.4</td>
</tr>
<tr>
<td>450</td>
<td>26.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Assumes that to be effective, the adducts must be in the segment of the PCR product that is bounded by the primers. For calculation purposes, the primer lengths were taken to be 25 bases each.

A) 115-mer Reamplification

<table>
<thead>
<tr>
<th>Copies of Carryover</th>
<th>3 x 10^4</th>
<th>3 x 10^5</th>
<th>3 x 10^6</th>
<th>3 x 10^7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

B) 500-mer Reamplification

<table>
<thead>
<tr>
<th>Copies of Carryover</th>
<th>6 x 10^4</th>
<th>6 x 10^5</th>
<th>6 x 10^6</th>
<th>6 x 10^7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 4. Comparison of 4'-AMDMIP sterilization of a 115mer amplicon to sterilization of a 500mer amplicon. The reamplification of sterilized and non-sterilized amplicons are shown in the autoradiograph. The upper panel corresponds to reamplification of 115mer amplicon while the lower panel corresponds to reamplification of the 500mer amplicon. Lanes 1, 3, 5, and 7 are reamplifications of the non-irradiated controls. Lanes 2, 4, 6, and 8 are reamplifications from sterilized amplicons.

at an average modification density of 1 adduct per 2 bases. Although the modification density is controlled by the isopсорalen concentration, it is not possible to achieve such high modification densities. At concentrations exceeding 200 μg/ml of 4'-AMDMIP, PCR amplification of the 115mer, especially at low copy number, begins to be inhibited (14). We have determined that the inhibition is sequence dependent and that the concentration of isopсорalen that causes the inhibition varies with each amplicon system and each isopсорalen derivative (G.D. Cimino, unpublished observations). While this inhibition can be alleviated by the addition of a co-solvent such as DMSO or glycerol(14), the hybridization efficiency of amplicons modified by 4'-AMDMIP also diminishes with increasing 4'-AMDMIP concentration (G.D. Cimino, unpublished observations). When 4'-AMDMIP is used in excess of 200 μg/ml, a significant drop in hybridization efficiency of the modified 115mer amplicon occurs. If the detection protocol does not require the modified amplicon to hybridize to another probe molecule, this latter effect is not an issue and higher concentrations of isopсорalen (or psoralen) can be used.

For a fixed modification density there is an alternative method for improving amplicon sterilization with photochemically active reagents. By choosing PCR primers judiciously, the length of the PCR products can be varied, and therefore, the average number of adducts per strand can be varied. Table II illustrates this effect for two different modification densities. In Case A, a modification density of 1 adduct per 5 bases is evaluated. Under these conditions a 200 base long PCR product should have approximately 30 effective adducts per strand. At this level of modification, it is statistically unlikely that a single amplicon will have escaped modification; all of the potential carryover molecules should be sterilized. As pointed out earlier, we have experimentally determined that this level of modification density can be achieved with concentrations of 4'-AMDMIP that are compatible with hybridization detection formats and with amplicons that have a very high AT content. Case B in Table
II considers the more general situation with most amplicons where the modification density is taken, conservatively, to be 1 adduct per 15 bases. Under these conditions, the same level of sterilization will require an amplicon that is in excess of 450 bases in length.

We have experimentally evaluated the length dependency for 4'-AMDMIP sterilization of PCR amplicons. Figure 4 compares the sterilization level of the 115mer amplicon to the sterilization level of a longer 500mer amplicon. The 115mer reamplification series demonstrates that some, but not all, of the amplicons were rendered incapable of acting as carryover templates. By contrast, reamplification of the irradiated 500mer series did not result in any detectable PCR product for the highest copy number examined (6×10^7 copies). This experiment used a relatively low, non-optimal concentration of 4'-AMDMIP to illustrate the length dependence phenomena. However, as described in the accompanying manuscript, the use of optimal concentrations of 4'-AMDMIP provides highly effective sterilization of the 115-mer amplicon.

SUMMARY

Several monoadducts of isopsoralen derivatives were shown to stop polymerization reactions with Taq DNA polymerase. The use of isopsoralens as photochemical post-PCR sterilization reagents requires that the statistical nature of the modification process be matched with the level of amplification and the length and sequence of the amplicon. We have shown that effective sterilization is an operationally defined term. It implies that carryover cannot be detected under a precisely defined set of amplification and detection conditions. For optimum results it is therefore necessary to design and evaluate a post-PCR sterilization protocol for each amplicon system.

The design procedure should begin with an assessment of the tolerable level of carryover so that the length and sequence of an amplicon can be matched with a practical level of isopsoralen concentration. Figure 5 correlates different volumes of PCR carryover with the number of effective adducts necessary to be statistically confident that the carryover event does not contain a non-sterilized amplicon. 15 average adducts per PCR strand are sufficient to deal with carryover originating from 10^-4 M or less. If the carryover event results from larger volumes, more effective adducts per PCR strand are necessary. A practical sterilization procedure should be effective at least to a level equal to the routine contamination. In our experience, the magnitude of the carryover problem is much less than 10^5 molecules per event (typically 10 to 10^5 molecules). An additional consideration is a second type of false positive PCR signal which exists when carryover exceeds 10^2 amplicons. These amplicons are detected directly by hybridization to a 32P probe. Therefor, while it is desirable to design a sterilization protocol capable of sterilizing all amplicons in a PCR reaction tube, it is not necessary to achieve levels of sterilization that exceed direct detection of the carryover molecules themselves.

The above considerations will determine a range of isopsoralen concentrations that need to be appraised for their sterilization ability and for their effect on detection sensitivity. PCR amplification efficiency and, where appropriate, hybridization efficiency contribute to detection sensitivity. Amplification efficiency in the presence of isopsoralen should be evaluated under conditions that are far away from PCR plateau so that inhibitory effects can be seen. Using tolerable concentrations of isopsoralen, the hybridization efficiency of the modified amplicons should then be determined. Finally, the number of PCR cycles should be adjusted to bring the detection level back to the level that exists in the absence of the sterilization process. By following these steps, an effective and practical post-PCR sterilization protocol will emerge that eliminates the carryover problem associated with the PCR. The accompanying paper illustrates the design and refinement process of a post-PCR- amplification sterilization protocol for the detection of low copy numbers of a retroviral sequence in clinical samples (14).

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REFERENCES