Photochemical Sterilization of 3SR Reactions

John Versailles, Kelly Berckhan, Soumitra S. Ghosh, and Eoin Fahy

Life Sciences Research Laboratory, Baxter Diagnostics, Inc., San Diego, California 92121 USA

The self-sustained sequence replication (3SR) reaction is an extremely efficient method for amplifying target DNA and RNA sequences that may be present in minute quantities. A serious problem often encountered in its practice is carryover contamination from products of previous 3SR reactions. A postamplification treatment of 3SR reaction products with the photoactive agent 4'-aminomethyl-4,5-dimethylisopsoralen (IP-10) was investigated as an approach for preventing carryover contamination by 3SR amplicons. Initially, inhibition of the amplification reaction by high concentrations of the reagent was observed. This problem was circumvented by developing a gel-based delivery of IP-10, and the method was found to provide highly efficient sterilization (~10⁶-fold) of 3SR amplicons. Evaluation of this strategy on a number of 3SR targets has indicated that the degree of sterilization is dependent on the length of the amplified region and on the concentration of IP-10. It appears that the sterilization effect is caused by covalent modification of the pyrimidine bases of RNA and DNA, which renders them unusable as templates for the 3SR reaction. Modification of a purified RNA transcript with IP-10 was shown to prevent effectively reverse transcription by avian myeloblastosis virus reverse transcriptase (AMV RT). Similarly, treatment of a T7 RNA polymerase promoter-containing DNA template with IP-10 eliminated full-length transcription by T7 RNA polymerase. This isopsoralen method may be used to sterilize multiple 3SR reactions in a clinical assay with a convenient UV irradiation step.

The advent of in vitro nucleic acid amplification techniques, such as PCR,⁽¹⁾ ligase chain reaction,⁽²⁾ and the self-sustained sequence replication (3SR) reaction⁽³⁾ has facilitated the development of new methods for the clinical diagnosis of infectious diseases⁽⁴⁾ and genetic disorders.⁽⁵⁾ Although these techniques are capable of rapid synthesis of picomole amounts of specific RNA and DNA sequences, they suffer from the drawback that minute quantities of amplified sequences (amplicons) inadvertently may be carried over into a subsequent reaction. These sequences can be amplified very efficiently, thereby giving rise to a false-positive signal. This problem is particularly acute in situations where the same target region is amplified repeatedly, such as those carried out in clinical diagnostic laboratories.

A number of pre- and postamplification techniques have been proposed for PCR sterilization⁽⁶⁻⁸⁾ and one approach that has shown promise is a contamination-control strategy based on isopsoralens.^(9,10) Isopsoralens are angular aromatic molecules that form cyclobutane adducts with the pyrimidine bases of DNA (or RNA) on irradiation with long-wavelength UV light. Once an isopsoralen mono-adduct is formed, the covalently modified DNA strands cannot serve as substrates for subsequent amplification by *Taq* polymerase.⁽⁹⁾ Moreover, the angular shape of the isopsoralen molecule precludes the cross-linking of the complementary DNA strands, thus providing sterilized amplicons that can be detected by hybridization procedures. Whereas the PCR method generates double-stranded DNA (dsDNA) products, the 3SR reaction gives rise to a mixture of single-stranded RNA (ssRNA) and dsDNA products in a ratio of ~100:1. To use this strategy for sterilization of 3SR reactions,

it is essential that the RNA products are also modified efficiently, because both RNA and DNA amplicons are sources of carryover contamination. It is also critical that amplification and subsequent product hybridization are not adversely affected by the presence of the sterilizing agent and that the process can occur in a closed system to prevent contamination through aerosol transfer.

This report describes the use of 4'-aminomethyl-4,5-dimethylisopsoralen (IP-10) as a sterilization agent in a format that permits efficient 3SR amplification and detection of the ssRNA reaction products from a variety of targets. Furthermore, it is demonstrated that the sterilization effect is the result of inhibition of strand synthesis by T7 RNA polymerase and avian myeloblastosis virus reverse transcriptase (AMV RT) resulting from modification of their dsDNA and ssRNA templates, respectively, with IP-10.

MATERIALS AND METHODS

Reagents

The HRI-100 UV illuminator and an aqueous solution of IP-10 (4.6 mg/ml) were obtained from HRI Associates, Inc., (Concord, CA). NuSieve agarose (genetic technology grade) was purchased from FMC (Rockland, ME). Geneamp (0.5 ml) reaction tubes were obtained from Perkin-Elmer Cetus, (Norwalk, CT). *Escherichia coli* RNase H and DNase were purchased from Bethesda Research Laboratories. RNase was obtained from Sigma Chemical Corp., and AMV RT was obtained from Life Sciences (St. Petersburg, FL). T7 RNA polymerase was purchased from Stratagene (La Jolla, CA). Oligonucleotides were synthesized on an Applied Biosystems 380A automated

DNA synthesizer and were purified by reverse-phase HPLC using a 0.1 M triethylammonium acetate (pH 6.8)/acetonitrile gradient. Oligonucleotides were checked for purity by electrophoresis on a 20% polyacrylamide gel. The sequences of the oligonucleotides used in these experiments are as follows: [Oligonucleotides containing a T7 promoter sequence are denoted with an asterisk (*)] 88-211(*), 5'-AATTTAATACGACTCACTATAGGGATCTATTGTGCCCCGGCTGGTTTTGCGATTCTA-3'; 88-347(*), 5'-AATTTAATACGACTCACTATAGGGA-TGTACTATTATGGTTTTAGCATTGTCTGTGA-3'; 90-422, 5'-AATTAGGCCAGTAGTATCAACTCAACTGCT-3'; 86-273, 5'-AGTCTAGCAGAAGAAGAGGTAGTAA-TTAGA-3'; 89-255, 5'-TTATTGTGCCCCGGCTGGTTTTGCGATTCTA-3'; 89-263(*), 5'-AATTTAATACGACTCACTATAGGGA-TTTTAAAGTGTT ATTCCATTTTGCTCTACT-3'; 92-135(*), 5'-AATTTAATACGACTCACTATA GGGAAAGCCATAATCTCATCAGGG-3'; 90-635, 5'-GATGGGAGGATGTTTGCGAATGCCTT-3'; 92-132, 5'-GCTAAAAGGATGAAGTCTAGGAG-3'; 92-155, 5'-GATGGCTTGTTAAGGAGCTGCATGATGT-3'; 90-626, 5'-GATACACTTGATGTACTGCCAAGTGGG-3'; 90-621(*), 5'-AATTTAATACGACTCATATAGGGATTATATCCCATATATGGAGTTCCGCG-3'; 92-212, 5'-CTATGGGAACATACGTCATTATTG-3'; 92-214, 5'-ATGGGCGGGGGTCTGTTGGGCGGTC-3'. Underlined bases in the oligonucleotide sequences denote the presence of a T7 promoter at the 5' ends. Transcription initiation sequences are in boldface type.

Target Preparation

Plasmid pARV7A/2 was constructed by inserting a cDNA copy of the human immunodeficiency virus-1 (HIV-1) genome into the *EcoRI* site of pUC19.⁽¹¹⁾ HIV-1 RNA was extracted from CEM cells and quantified by comparative hybridization to predetermined concentrations of pARV7A/2.⁽¹²⁾

Plasmid pACYC-HCMV-*EcoRI*-J, based on pACYC184⁽¹³⁾ and containing an *EcoRI* fragment from the genome of the AD 169 strain of cytomegalovirus (CMV), was obtained from Dr. Deborah Spector (University of California, San Diego). The 10,026-bp fragment includes the sequences spanning the immediate/early promoter-enhancer (IEP) and major immediate/early protein (MIE) regions of CMV. The *EcoRI* fragment was

used as a DNA target in 3SR amplifications of the IEP region. A 1066-bp *Bgl*III-*Pvu*II fragment from pACYC-HCMV-*EcoRI*-J was cloned into the transcription vector pSP6/T7 α 19 and transcribed by T7 RNA polymerase to yield a sense RNA product spanning the MIE region of CMV. This transcript was used as an RNA target for 3SR amplifications of the MIE region.

3SR Amplification and Detection

RNA targets were amplified in a volume of 50 μ l in the presence of 10% DMSO/15% sorbitol (HIV-1), or 5% DMSO/10% sorbitol (CMV) as described previously.⁽¹⁴⁾ DNA targets were heated to 100°C for 3 min in 3SR solution, equilibrated at 42°C, and 10 units of AMV RT was added. After 10 min, the reaction tube was heated to 100°C for 1 min, equilibrated at 42°C, and the 3SR enzymes were added to initiate the amplification reaction. The RNA products from 3SR reactions were detected by bead-based sandwich hybridization (BBSH)^(15,16) using ³²P-labeled oligonucleotide probes and capture sequences covalently attached to a Trisacryl support.⁽¹⁶⁾ The detection probes were labeled at their 5' ends using T4 kinase and [γ -³²P]ATP.⁽¹⁷⁾

Endpoint Titration of 3SR Product

The product used for the assay was obtained from an amplification of the HIV-1 *env* region using primer pair 88-211* and 88-347*. The concentration of RNA in the stock solution of the 3SR product was estimated by BBSH to be 2824 fmole/ μ l. Before the reamplification reaction, the 3SR product stock solution was serially diluted in 10 mM Tris-HCl and 1 mM EDTA (pH 8.1) (TE buffer) to deliver target amounts ranging from 10⁻⁶ to 10⁻¹² μ l of the original stock solution. These solutions were then reamplified using the primers 88-211* and 88-347*.

Reamplification of 3SR Reactions after Degradation of the RNA Component of the 3SR Product

RNase (10 mg) was dissolved in 1 ml of 10 mM Tris-HCl and 15 mM NaCl (pH 7.5) and heated at 100°C for 15 min to inactivate contaminating DNase. The solution was then cooled slowly to room

temperature and stored at -20°C for later use.

For digestion of the RNA with RNase, 3.54 μ l (1 $\times$ 10⁻¹¹ mole) of the 3SR pooled product was incubated with 1 unit of RNase in 50 μ l of 50 mM sodium acetate (pH 5.00), and the solution was kept at room temperature for 1 hr. Alkaline hydrolysis of the RNA was achieved by treating 3.54 μ l (1 $\times$ 10⁻¹¹ mole) of the 3SR product with 50 μ l of a solution of 1 mM EDTA and 300 mM NaOH at 60°C for 30 min. The solution was then cooled to room temperature and neutralized with 5 μ l of 3 N HCl. The residual cDNA in the reaction mixture was reannealed before the endpoint titration analysis by heating at 100°C for 2 min, then at 60°C for 3 hr, followed by overnight incubation at 42°C. The RNase and alkali-treated products were then diluted to give input target amounts ranging from 10⁻⁶ μ l to 10⁻¹² μ l of the original stock solution and then reamplified using primers 88-211* and 88-347*.

Reamplification of 3SR Reactions after Degradation of the 3SR DNA Products

The 3SR pooled product (3.54 μ l, 1 $\times$ 10⁻¹¹ mole) was treated with a solution of 0.5 mg of RNase-free DNase (7.75 units) in 50 mM sodium acetate, 10 mM MgCl₂, and 2 mM CaCl₂ (pH 6.5), in a final volume of 50 μ l. The enzymatic hydrolysis was allowed to proceed for 30 min at 37°C. The DNase-treated product was then diluted to deliver target amounts ranging from 10⁻⁶ to 10⁻¹² μ l of the original stock solution and then reamplified using primers 88-211* and 88-347*.

Sterilization of 3SR Reactions with IP-10

Aliquots of 0.5% NuSieve agarose containing 0, 600, 1200, and 1800 μ g/ml of IP-10 were prepared by mixing equal volumes of 1% NuSieve agarose and aqueous IP-10 solutions. Gel volumes of 10 μ l were added to the caps of reaction tubes and allowed to solidify. A control reaction containing no gel was also prepared in each experiment. 3SR amplifications were carried out in a volume of 50 μ l as described previously,⁽¹⁵⁾ and all reactions were performed in duplicate. All reaction tubes were heated to 80°C for 1 min immediately after the 1-hr incubation.

tion, vortexed, and centrifuged briefly. Reaction tubes containing IP-10 were UV irradiated at 4°C for 20 min before freezing. Irradiation of 3SR products was performed with the HRI-100 illuminator according to the manufacturer's instructions. The resulting 3SR products were quantitated by BBSH.

Reamplification of 3SR Reactions

The 3SR products from the IP-10-containing amplification experiments described above were diluted in TE buffer to concentrations ranging from 10 fmole/5 μ l to 0.0001 amole/5 μ l. These diluted 3SR products were used as targets in a subsequent 3SR amplification with the same primers but in the absence of IP-10. The resulting 3SR products were quantitated by BBSH.

Modification of dsDNA with IP-10

A 406-bp region of plasmid pARV7A/2 containing the HIV-1 sequence was PCR amplified using primers 89-255 and 89-263. This product, containing a T7 RNA polymerase promoter sequence, was purified by agarose gel electrophoresis and quantitated by absorbance at 260 nm. The PCR product (4 pmoles) was added to a 3SR reaction solution (50 μ l) and irradiated in the presence of final concentrations of 0, 100, 200, and 300 μ g/ml of IP-10 as described above. A reaction tube containing the PCR product and 300 μ g/ml of IP-10, was not UV irradiated and was used as a control. A portion of the modified PCR product (400 fmoles) from each reaction was used as a template in a transcription reaction with 40 units of T7 RNA polymerase in 20 μ l of a solution containing 50 mM Tris-HCl (pH 7.5); 6 mM MgCl₂; 2 mM spermidine; 10 mM NaCl; 10 mM DTT; 0.5 mM each of GTP, UTP, and ATP; 100 μ M CTP; 50 μ Ci [³²P]CTP; and 1 μ l of Inhibit-ACE (5 Prime-3 Prime, Inc.). After incubation at 37°C for 60 min, the nucleic acids were isolated by phenol/chloroform extraction and ethanol precipitation. The ³²P-labeled RNA products were then analyzed on a 10% denaturing polyacrylamide gel.

Modification of ssRNA with IP-10

The 406-bp PCR product (12 pmoles) described above was used as a template in a cold transcription reaction (200 μ l) with T7 RNA polymerase. The transcript was

applied to a Nick G-50 spin column (Pharmacia) to remove unincorporated nucleotides and analyzed on a denaturing 8% polyacrylamide gel (Novex) for the presence of full-length product (381 bases). The RNA (25 pmoles) was treated with IP-10 in 3SR solution as described for the PCR product above. The modified transcripts (2.5 pmoles) were used as templates in 20- μ l reverse transcription reactions with 24 units of AMV RT in 50 mM Tris-HCl (pH 8.5), 8 mM MgCl₂, 30 mM KCl, 1 mM DTT, 0.75 mM dNTPs, and 0.2 μ M ³²P-labeled primer 89-255. After incubation for 60 min at 42°C, the ³²P-labeled cDNA products were analyzed on a 10% denaturing polyacrylamide gel.

RESULTS

Endpoint Titration of 3SR Product Carryover

In the 3SR reaction, RNA transcripts are synthesized from cDNA templates and are the predominant products. Whereas these RNA product molecules are probably the principal source of carryover contamination, the cDNA products may also have a significant contribution. This premise is derived from a previous analysis of the 3SR reaction products by filter-based hybridization assays,⁽³⁾ which indicated that there is an ~100:1 ratio of RNA/cDNA products. It was important to estimate the ratio of RNA to cDNA by an independent analysis and to ascertain their respective roles in carryover contamination. This was accomplished by degradation of the RNA and cDNA components of a 3SR reaction in separate experiments, followed by serial dilution

and reamplification of the digestion products. A 212-base region of the *env* gene of HIV-1 RNA was amplified by 3SR using primers 88-211* and 88-347*, and the RNA products were quantitated by BBSH. A sample of the reaction mixture was digested with DNase-free RNase or alkali to destroy the RNA component. Similarly, another portion of the reaction was treated with RNase-free DNase to remove the cDNA product. The digestion products, as well as an unmodified control sample, were serially diluted in TE buffer, and amounts varying from 1×10^{-18} to 1×10^{-24} mole (based on the original BBSH quantitation of the 3SR product) were used as targets in a further 3SR amplification using the same primer pair. The results of the endpoint titration analysis, presented in Table 1, shows that both RNA transcript and cDNA function as effective templates in a 3SR reaction. The ~100:1 RNA/cDNA product ratio predicted by the endpoint titration analysis is in good agreement with the conclusions from earlier filter-based hybridization assays.⁽³⁾ Thus, an effective contamination control strategy for the 3SR system requires the efficient removal of both nucleic acids. Importantly, the endpoint titration analysis clearly illustrates the problem of carryover contamination because as little as 10^{-12} μ l of the original 3SR product mixture was sufficient to give a signal upon reamplification.

Sterilization of 3SR Reactions with RNA Targets

A postamplification photochemical approach using IP-10 was chosen for 3SR

TABLE 1 Endpoint Titration of 3SR Product Subjected to Enzymatic or Chemical Degradation

Target volume (μ l)	Amount of product detected by BBSH (fmole/ μ l) ^a			
	untreated ^b	RNase ^b treatment	alkali ^b hydrolysis	DNase ^b treatment
10^{-6}	325	100	130	256
10^{-7}	195	20	21	201
10^{-8}	173	2	3	48
10^{-9}	27	0	0	4
10^{-10}	2.1	0	0	0.6
10^{-11}	0.28	0	0	0
10^{-12}	0	0	0	0

^aThe values are an average of amplification duplicates. A no-target amplification control was used as background and its value was subtracted from all data points.

^bBefore serial dilution and amplification, the target (consisting of the product of a previous 3SR amplification of HIV-1 *env* RNA) was either untreated or treated with RNase, NaOH, or DNase.

sterilization, on the basis of its successful use in PCR sterilization studies^(9,10) and also on the ability of linear psoralens to modify RNA effectively.⁽¹⁸⁾ Initial experiments determined that irradiation of 3SR products in the presence of IP-10 led to significant levels of sterilization but suffered from the drawback that IP-10 itself inhibited 3SR amplification when concentrations of ≥ 40 $\mu\text{g/ml}$ were used. The degree of inhibition was found to vary with target sequence and was more acute (10- to 100-fold reduction in amplification yield) when the amplification efficiency of the target was low ($\leq 10^6$ -fold, data not shown). This inhibition problem was circumvented by sequestering the isopsoralen in a pellet of low-melting-point agarose gel in the cap of the reaction tubes. The 3SR reaction components were then introduced into the tubes, and amplifications were performed under standard conditions. On completion of the reaction, the tubes were heated at 80°C to melt the gel and the mixture was vortexed to give a homogeneous solution that remained liquid at room temperature. The tubes were finally UV irradiated to effect the sterilization process.

Sterilization experiments were performed on four targets of current interest in this laboratory, with amplicon lengths ranging from 212 to 382 bases. The amounts of IP-10 used varied from 0 to 1800 $\mu\text{g/ml}$ of gel, corresponding to final concentrations of 0–300 $\mu\text{g/ml}$ in the 3SR solution after the gel-melting step. The effect of increasing amounts of IP-10 on 3SR amplification with primer pair 88-211*/88-347* and the detection of a 212-base region of the *env* gene of HIV-1 are shown in Figure 1. The spatial separation of IP-10 from the 3SR reaction components resulted in highly efficient amplification. It appears that the slight decrease in product detected with increasing IP-10 concentrations is primarily the result of a decrease in hybridization efficiency of the modified 3SR products rather than a decrease in amplification efficiency. This is supported by the observation that omission of the UV irradiation step resulted in an equivalent amplification efficiency for the 300- $\mu\text{g/ml}$ IP-10 reaction compared with that of a control reaction containing no IP-10 (see Fig. 1). No significant IP-10-dependent decrease in amplification was observed for 3SR reactions of a 382-base region of the *env* gene of HIV-1 with

FIGURE 1 Effect of 0.5% NuSieve agarose containing IP-10 on 3SR amplification reactions. Aliquots (10 μl) of melted agarose gel containing various amounts of IP-10 were added to the interior of the caps and allowed to solidify. 3SR reactions were performed in a volume of 50 μl using HIV-1 *env* RNA as target and priming oligonucleotides 88-211*/88-347*. The control reaction contained no agarose. After completion of the 3SR amplification reaction, all agarose-containing tubes were heated to 80°C for 1 min, vortexed, and centrifuged to give final IP-10 concentrations ranging from 0 to 300 $\mu\text{g/ml}$. All tubes except the 300- $\mu\text{g/ml}$ (UV-) tube were then UV irradiated for 20 min at 4°C . Antisense reaction products were detected by BBSH with Trisacryl OligoBeads, 86-273, and ^{32}P -labeled probe 90-422.

FIGURE 2 Relationship between input target and detected product in reamplification of IP-10-treated 3SR products generated with CMV-MIE RNA as target and primer pair 90-635/92-135*. Irradiated products from 3SR reactions containing 0 ($\times$), 100 ($\blacksquare$), 200 ($\blacktriangle$), and 300 ($\bullet$) μg of IP-10/ml were diluted in TE buffer and amplified with primer pair 90-635/92-135* in the absence of IP-10 and NuSieve agarose gel. Amplified products were detected by BBSH with Trisacryl OligoBeads, 92-155, and ^{32}P -labeled probe 92-132.

primer pair 89-255/89-263*, a 343-base region of the *pol* gene of HIV-1 (primer pair 90-047/89-391*), or a 377-base region of the MIE gene of CMV (primer pair 90-635/92-135*) (data not shown).

The 3SR products from reactions containing 0, 100, 200, and 300 $\mu\text{g/ml}$ of IP-10 in the experiments described above were serially diluted in TE buffer. The control reaction containing no IP-10 was diluted to provide concentrations of 1, 0.1, 0.01, and 0.001 amole of 3SR product/5 μl , whereas the IP-10-containing reactions were diluted to give 10, 1, 0.1, and 0.01 fmole of 3SR product/5 μl . These 5- μl aliquots of the diluted 3SR products were used as targets in a subsequent round of 3SR amplifications with the same primer pair employed in the initial amplifications. In these reamplifications no IP-10 or agarose was included in the reaction tubes. Figure 2 illustrates the effect of IP-10 on reamplification of the targets using primer pair 90-635/92-135*, where the amount of product in the second amplification is plotted against the amount of input target. Reamplification of the 3SR product that had contained no IP-10 was very efficient ($>2 \times 10^8$ -fold). In contrast, amplification was severely inhibited for all the targets that had been photochemically modified with IP-10. Figure 2 indicates that the amount of 3SR product generated from 10 fmoles of isoprosalen-modified target is less than the amount of 3SR product resulting from amplification of the unmodified target, corresponding to a sterilization efficiency of at least 10^7 -fold.

The results of a reamplification of IP-10-modified targets with primer pair 88-211*/88-347*, shown in Figure 3, suggests a relationship between the level of sterilization and the concentration of IP-10. A significant amount of 3SR product was obtained from the reamplification of the 212-base-long target, which had been treated originally with 100 $\mu\text{g/ml}$ of IP-10. The reamplification of the longer HIV-1 *pol* and HIV-1 *env* amplicons that had been modified with isoprosalen produced results similar to those observed for the long CMV region (Table 2), with the levels of 3SR product detected being close to background for all IP-10 concentrations tested.

Sterilization of 3SR Reactions with DNA Targets

The isoprosalen sterilization strategy was

FIGURE 3 Relationship between input target and detected product in reamplification of IP-10-treated 3SR products generated with HIV-1 *env* RNA as target and primer pair 88-211*/88-347*. Irradiated products from 3SR reactions containing 0 (x), 100 (■), 200 (▲), and 300 (●) μg IP-10/ml were diluted in TE buffer and amplified with primer pair 88-211*/88-347* in the absence of IP-10 and NuSieve agarose gel. Amplified products were detected by BBSH with Trisacryl OligoBeads, 86-273, and ^{32}P -labeled probe 90-422.

TABLE 2 Total 3SR Product Detected in Reamplification Reactions of Isoprosalen-Modified Targets

	Input target (amole)	<i>env</i> ^a 89-255 89-263	<i>env</i> ^a 88-211 88-347	<i>pol</i> ^a 90-047 89-391	CMV ^a 90-630 92-135	CMV ^a 92-626 92-621
Reamp time (min)		40	40	60	40	60
No IP-10	1.0	14,580	100,050	5,128	12,521	73,772
	0.1	9,159	110,893	3,888	12,589	56,986
	0.01	2,198	93,851	2,768	10,506	14,642
	0.001	7,411	87,945	674	9,602	1,056
100 $\mu\text{g/ml}$ IP-10	10,000	169	52,215	658	176	45,372
	1,000	42	58,061	639	29	45,839
	100	11	52,966	419	310	43,228
	10	0	57,014	586	396	7,117
200 $\mu\text{g/ml}$ IP-10	10,000	30	6,433	165	29	10,355
	1,000	11	8,234	191	0	6,853
	100	8	8,577	116	176	6,157
	10	0	6,921	78	98	448
300 $\mu\text{g/ml}$ IP-10	10,000	0	4,213	87	0	3,922
	1,000	0	7,876	41	156	2,777
	100	7	2,834	28	72	579
	10	80	5,907	6	0	591

Comparison of total 3SR products (fmole/50 μl) detected after reamplification of various amounts of 3SR products that had been treated with 0, 100, 200, and 300 $\mu\text{g/ml}$ of IP-10 in the initial amplification step.

^aThis refers to the target sequences and primer pairs used in 3SR amplification (see text).

applied to a 3SR reaction containing a DNA target, by amplifying a 197-bp region from the IEP gene of CMV (primer pair 90-626/90-621*). An initial reverse transcription step was performed, followed by addition of the IP-10-containing gel to the cap of the reaction tube. After addition of the 3SR enzymes and incubation for 1 hr, the reactions were sterilized as described for the RNA targets. Reamplification of the IP-10-treated 3SR products indicated a dependence of the degree of sterilization on isopsoralen concentration (Fig. 4).

Transcription of IP-10-modified RNA and DNA Templates by 3SR Enzymes

The high degree of 3SR sterilization achieved with IP-10 strongly suggested that this molecule modified both the cDNA and RNA reaction products, thereby preventing further strand synthesis by T7 RNA polymerase and AMV RT. This hypothesis was tested by photochemically modifying purified dsDNA and ssRNA targets with IP-10, and performing T7 RNA polymerase and AMV RT transcription of the modified DNA and RNA templates, respectively. Thus, the 382-bp region of the *env* gene of HIV-1 RNA was amplified by PCR with primers 89-255 and 89-263* to generate a T7 promoter-containing DNA product that is essentially identical to the cDNA intermediate in a corresponding 3SR reaction. After UV modification by IP-10 (0–300 $\mu\text{g}/\text{ml}$) in 3SR solution, the DNA was used as a template in a T7 RNA polymerase assay in which [α - ^{32}P]CTP was incorporated into the transcription products. Gel electrophoretic analysis of the reaction products (Fig. 5A) shows that UV irradiation of the template in the presence of IP-10 (100–300 $\mu\text{g}/\text{ml}$) prevented subsequent transcription. In contrast, the DNA sample containing 300 $\mu\text{g}/\text{ml}$ of IP-10, which had not been UV treated, and the control DNA irradiated in the absence of IP-10, both produced full-length transcripts.

Isopsoralen-modified RNA templates were prepared in two steps. The PCR product described above was used as a template in a cold transcription reaction with T7 RNA polymerase, and the purified transcript (analogous to the ssRNA product from a 3SR reaction) were photochemically treated with various concentrations of IP-10. The modified tran-

FIGURE 4 Relationship between input target and detected product in reamplification of IP-10-treated 3SR products generated with primer pair 90-621/90-626* and a dsDNA target. Irradiated products from 3SR reactions containing 0 ($\times$), 100 ($\blacksquare$), 200 ($\blacktriangle$), and 300 ($\bullet$) μg IP-10/ml were diluted in TE buffer and amplified with primer pair 90-621/90-626* in the absence of IP-10 and NuSieve agarose gel. Amplified products were detected by BBSH with Trisacryl OligoBeads, 92-212, and ^{32}P -labeled probe 92-214.

scripts were then used as templates in reverse transcription reactions containing the ^{32}P -labeled primer 89-255. Gel analysis of the labeled cDNA products

(Fig. 5B) shows that AMV RT was unable to reverse transcribe the templates that had been irradiated in the presence of IP-10.

FIGURE 5 Transcription of IP-10-modified RNA and DNA templates by AMV RT and T7 RNA polymerase, respectively. (A) An autoradiograph of a 10% denaturing polyacrylamide gel shows the ^{32}P -labeled products from a T7 RNA polymerase reaction using a DNA template that had been irradiated in the presence of 0, 100, 200, and 300 $\mu\text{g}/\text{ml}$ of IP-10 (lanes 2–5) or that had not been irradiated in the presence of 300 $\mu\text{g}/\text{ml}$ of IP-10 (lane 6). (Lane 1) *Msp*I digest of pBR322 DNA that had been treated with phosphatase and ^{32}P -labeled T4 kinase. (B) An autoradiograph of a 10% denaturing polyacrylamide gel shows the ^{32}P -labeled products from a reaction with AMV RT using an RNA template that had been irradiated in the presence of 0, 100, 200, and 300 $\mu\text{g}/\text{ml}$ of IP-10 (lanes 2–5), or that had not been irradiated in the presence of 300 $\mu\text{g}/\text{ml}$ of IP-10 (lane 6). (Lane 1) *Msp*I digest of pBR322 DNA that had been treated with phosphatase and ^{32}P -labeled T4 kinase.

FIGURE 6 Relationship among target copy-number, 3SR amplification efficiency, and amount of product. Values in the shaded area represent amounts of 3SR product that are below the detection limit of BBSH using a ^{32}P -labeled probe. (Bottom, right) Values (i.e., 10^{13} , 10^{14} copies) represent amounts of product that exceed the dynamic range ($\sim 1.5 \times 10^{12}$ copies per 50- μl reaction) of the 3SR reaction.

DISCUSSION

The efficacy of any sterilization method depends on the degree of carryover contamination and the efficiency of the subsequent amplification reaction. The relationship among efficiency of amplification (fold amplification), the amount of target, and the amount of 3SR-amplified product is presented in Figure 6. The underlying parameter defining the relationship among the three variables is set by the limit of detection of the hybridization detection method; in the case of our BBSH method, the limit of sensitivity is 6×10^8 amplicons per total 3SR reaction volume of 50 μl . Thus, if 10^4 amplicons are carried over and the subsequent 3SR amplification proceeds with 10^7 -fold efficiency, 10^{11} amplicons would be produced. To be successful, a sterilization method would then require reducing the number of viable target amplicons from 10^4 to 10 molecules to be below the limit of BBSH detection. If the extent of the contamination is $>10^4$ molecules, or if the amplification efficiency is $>10^7$ -fold, a greater degree of sterilization would be necessary to prevent the appearance of false-positive signals. This study presents data indicating that for 3SR amplicons of ≥ 200 bases, IP-10 can prevent false-positive signals that may arise from carry

over of as much as 10 fmoles of product, even in cases where amplification efficiency is $>10^8$ -fold. Because amplicon aerosols appear to be the primary source of carryover contamination and the amount carried over is believed to be <1 amole in most cases, the sterilization protocol described in this study should provide a wide margin of error for controlling contamination in situations where the same amplicon is repeatedly amplified by the 3SR reaction.

A key feature of the present method is the mode of delivery of IP-10. Because IP-10 strongly inhibits the 3SR reaction at the high concentrations required to ensure efficient sterilization, it was necessary to sequester the reagent in low-melting point agarose in the cap of the reaction tube during 3SR amplification.

The 0.5% NuSieve gel, impregnated with IP-10, remains solid during the 65°C denaturation and incubation steps required for 3SR amplification. The gel liquefies at $75\text{--}80^\circ\text{C}$, thus enabling a postamplification delivery of IP-10 to the 3SR solution following a thermal melt step. The gel-based isoprosoralen method also is effective in sterilizing 3SR amplification reactions of DNA targets. The requirement for a prior reverse transcription step, which involves target denaturation at 100°C , necessitates the addition of IP-10 in agarose after this procedure. The 3SR enzymes are then added to initiate the amplification reaction.

The mechanism of action of IP-10 in the sterilization process was investigated using isoprosoralen-modified RNA and DNA as templates for AMV RT and T7 RNA polymerase, respectively. Full-length strand synthesis was not observed for both transcription reactions, confirming that inhibition of the reamplification reactions occurs because RNA and cDNA molecules modified with IP-10 cannot function as effective templates. Although modification by IP-10 impairs the nucleic acids as templates, they can still function as efficient targets in hybridization-detection reactions.

In agreement with previous studies,^(9,10) the results show a correlation between amplicon length and the concentration of the sterilization reagent. Thus, IP-10 concentrations of 200 and 300 $\mu\text{g}/\text{ml}$ were highly efficient in sterilizing the four target regions that are routinely amplified in this laboratory (Table 2). However, while 100 $\mu\text{g}/\text{ml}$ of IP-10 was adequate for long targets, it failed to provide complete sterilization with the short, 212-bp-long HIV-1 *env* region defined by the primer pair 88-211*/88-347*. Table 3 shows the lengths of amplicons used in the study and the total

TABLE 3 Characteristics of Amplified Regions

Region	<i>env</i> 89-255 89-263	<i>env</i> 88-211 88-347	<i>pol</i> 90-047 89-391	CMV 90-635 92-135	CMV 90-626 90-621
Length (bases)	382	212	352	382	197
T's (U's) in sense strand	89	55	79	106	51
T's (U's) in antisense strand	149	74	133	94	50

Comparison of regions used for IP-10 sterilization studies. Shown are the length of the region defined by amplification primers and the number of T (U) residues on each strand.

number of thymine and uracil residues present in the sense and antisense strands of their sequences. The most likely explanation for the inverse relationship between amplicon length and IP-10 concentration therefore appears to be the number of thymine/uracil residues that are available for cross-linking to the isopsoralen molecule.

IP-10 appears to be quite stable when stored in agarose gel at room temperature. Samples that have been stored in the dark for at least 6 months show no noticeable decline in sterilization efficiency. The gel-based technique is amenable to various amplification reaction formats and has been performed successfully in standard 1.5-ml Eppendorf tubes as well as the smaller 0.5-ml PCR tubes.

The ability of this strategy to modify efficiently the thymine and uracil residues of polynucleotides implies a general utility as a sterilization agent for amplification protocols that generate DNA (PCR and ligase chain reaction) or RNA (Q-beta replication)⁽¹⁹⁾ reaction products, or a combination of both nucleic acids. Importantly, the physical separation of the sterilization agent from the reaction mixture during amplification should preclude inhibition of polymerase activity and consequent decrease in product yields.

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