JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1993, p. 2361–2365
0095-1137/93/092361-05$02.00/0

Vol. 31, No. 9

Dependence of Polymerase Chain Reaction Product Inactivation Protocols on Amplicon Length and Sequence Composition

MARK J. ESPY, THOMAS F. SMITH,* AND DAVID H. PERSING

Division of Clinical Microbiology, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905

Received 17 March 1993/Returned for modification 29 April 1993/Accepted 11 June 1993

Specific diagnostic test results generated by polymerase chain reaction (PCR) depend upon control of amplicon contamination in the clinical laboratory. We compared photochemical (isopsoralen [IP]) and enzymatic (uracil *N*-glycosylase [UNG]) methods for their ability to prevent carryover of amplicons generated from genomic targets of five viruses. PCR products (amplicons) (herpes simplex virus, 342 bp; cytomegalovirus, 250 bp; Epstein-Barr virus, 240 bp) exposed to UV light in the presence of various concentrations of IP compound 10 (IP-10) resulted in apparent increased molecular sizes of the products, as indicated by migration patterns after gel electrophoresis, and were predictive of inactivation by the agent. For amplicons of ≤100 bp, IP-10-induced electrophoretic shifts were related to the guanidine-cytidine (G+C) content of the PCR product; no apparent shift and no inactivation were observed for a 92-bp herpes simplex virus amplicon (G+C content, 65%), whereas the 100-bp human papillomavirus product (G+C content, 42%) showed a concentration-dependent shift (25 to 100 μg/ml) in electrophoretic migration and was partially inactivated. UNG effectively controlled amplicon carryover for target DNA of ≥240 bp; however, this treatment did not inactivate the two amplicons of ≤100 bp, regardless of the G+C content of the product. Larger products were inactivated efficiently by both methods, regardless of their G+C contents. We concluded that both IP and UNG effectively inactivate PCR amplicons but not short amplicons of ≤100 bp. We recommend that with the adoption of PCR technology in clinical laboratories, primers should be designed to produce amplicons of at least 240 to 350 bp (depending on G+C content) and that at least one effective method of controlling carryover contamination should be incorporated into each PCR protocol.

The polymerase chain reaction (PCR) is capable of producing up to 10^{12} copies of a target nucleic acid sequence in a few hours' time (12). Accordingly, this technique generally provides test results that have high sensitivity compared with those of conventional culture or antigen detection assays (12, 13). However, the widespread use of PCR has commonly led to false-positive results because of contamination by amplified nucleic acids carried over from a previous reaction rather than the specific amplification of target sequences in clinical samples (5, 9, 10). In recognition of this problem, guidelines regarding physical laboratory requirements and technical procedures for the control of inadvertent PCR product (amplicon) contamination have been published (7, 14). These recommendations include physical separation of areas for reagent preparation, sample addition, target amplification, and analysis of products. In addition, the use of positive displacement pipettes and air-flow-controlled laboratories and the judicious selection of controls have been recommended. In practice, however, implementation of these procedures reduces but does not completely eliminate false-positive problems because of carryover contamination.

The transfer of PCR technology from experimental applications to the diagnostic laboratory for routine testing of clinical samples will demand new methods that improve the specificity of PCR by eliminating carryover contamination or its effects. Inactivation procedures for control of amplicon carryover, one enzymatic (uracil *N*-glycosylase [UNG]) and one photochemical (isopsoralen [IP]), have been described (4, 8). However, for both the UNG and IP methods, the efficiency of inactivating amplified DNA may vary widely depending on amplicon length and G+C content. Our objective in the study described here was to assess the efficiency of these two methods for eliminating amplicon contamination in a clinical setting of PCR use.

MATERIALS AND METHODS

Cells. Human diploid lung fibroblast cells (MRC-5; ViroMed Laboratories, Minneapolis, Minn.) and human squamous cervical carcinoma SiHa cells (HTB35; American Type Culture Collection, Rockville, Md.) were grown in Eagle minimal essential medium containing final concentrations of 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (10 μg/ml). Human Burkitt lymphoma cells (Daudi) (CCL213; American Type Culture Collection) were grown in RPMI 1640 medium containing 10% fetal bovine serum, 200 mM L-glutamine, penicillin, streptomycin, and gentamicin.

Sample preparation. Herpes simplex virus (HSV) and cytomegalovirus (CMV) were inoculated in culture tubes seeded with MRC-5 cells. SiHa and Daudi cells, which contain a portion of the human papillomavirus type 16 (HPV-16) and Epstein-Barr virus (EBV) genomes, respectively, were grown in culture flasks. When 3+ to 4+ cytopathic effects were observed in culture tubes, HSV-infected cells were scraped into the fluid medium and the cell suspension was used without further preparation. CMV-infected cells, SiHa cells, and Daudi cells were lysed with a solution containing TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]), 0.5% sodium dodecyl sulfate (SDS), and 20 mg of

* Corresponding author.

TABLE 1. Characteristics and nucleotide base sequence of primers and probes used to detect target nucleic acids

Organism	Target gene	GenBank accession no.	Position	Size (bp)	Sequence (5'–3')
HSV	DNA polymerase	M16321	3060–3151	92	CATCACCGACCCGGAGAGGGAC (primer)
					GGGCCAGGCGCTTGTTGGTGTA (primer)
			3109–3128		GTCCTCACCGCCGAACTGAG (probe)
HSV	Glycoprotein B	K03541	1792–2133	342	CTGGTCAGCTTTCGGTACGA (primer)
					CAGGTCGTGCAGCTGGTTGC (primer)
			1982–2009		AGGTCGATGAAGGTGCTGACGGTGGTGA (probe)
CMV	Immediate early	K01090	1234–1483	250	CCTAGTGTGGATGACCTACGGGCCA (primer)
					CAGACACAGTGTCCTCCCGCTCCTC (primer)
			1348–1367		CCAGAGTCCCCTGTACCCGC (probe)
EBV	Glycoprotein 220	V01555	2381–2620	240	GGCTGGTGTCACCTGTGTTA (primer)
		V01509			CCTTAGGAGGAACAAGTCCC (primer)
		V01554	2466–2501		GGTGGAGGGGCTGAGTGTCTCTGGGTTTGAACTGGG (probe)
HPV-16	E6 region	K02718	50–149	100	ACCGAAACCGGTTAGTATAAAAGC (primer)
					ATAACTGTGGTAACTTTCTGGGTC (primer)
			99–125		CTGCAATGTTTCAGGACCCACAGGAGC (probe)

proteinase K per ml. The nucleic acids were extracted twice with phenol-chloroform (70 and 40%, respectively; Applied Biosystems, Foster City, Calif.) and once with chloroform-isoamyl alcohol (24:1) and were then precipitated with absolute ethanol.

Oligonucleotide primers and probes. Oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems) at the Mayo Clinic Molecular core facility, Rochester, Minn. Information regarding the primer and probe sets is given in Table 1.

IP. IP compound 10 (IP-10) was purchased from HRI Associates (Concord, Calif.).

UNG. UNG (N808-0096) was purchased from Perkin-Elmer Cetus (Norwalk, Conn.).

PCR amplification. The PCR reaction mixture contained the following: 200 μM (each) deoxyribonucleoside triphosphates, 10× buffer (500 mM KCl, 100 mM Tris-Cl [pH 8.3], 15 mM $MgCl_2$, 2.5 mg of bovine serum albumin per ml), 100 pmol each of appropriate primer, 25 μg of IP-10 per ml or 1 μl of UNG per 100 μl, 10% glycerol, and 1.25 U of *Taq* polymerase. Each reaction tube received 45 μl of the reaction mixture, 2 drops of mineral oil, and 5 μl of target DNA. A no-target control reaction tube received 50 μl of reaction mixture only. Reactions were amplified in a DNA thermal cycler (model 480; Perkin-Elmer Cetus) by using a two-step protocol: 3 min of denaturation at 94°C and then 1 min of annealing at 60°C for one cycle and 30 s of denaturation at 94°C and then 30 s of annealing at 60°C for 60 cycles. When IP-10 was added to the reaction mixture, the tubes were placed in an UV transilluminator (HRI-100; HRI Associates) for 15 min at 4°C after amplification.

Identification of amplicon sequences. PCR products were visualized with UV light as a single band by staining with ethidium bromide (10 mg/ml) after agarose gel electrophoresis (3%: 1.5% NuSieve [FMC, Rockland, Maine], 1.5% electrophoresis grade agarose [Bethesda Research Laboratories, Gaithersburg, Md.]). The products were then transferred to a nylon membrane (Magnagraph; Fisher) by Southern blotting. Membranes were prehybridized for 1 h at 42°C with a solution containing 5× SSPE (20× SSPE is 3.6 M NaCl, 0.2 M $NaPO_4$, and 0.02 M EDTA [pH 7.4]), 0.2% SDS, and 100 μg of *Escherichia coli* tRNA (Calbiochem-Behring, San Diego, Calif.) per ml. The ^{32}P-end-labeled probes (100 pmol) were added and were allowed to hybridize to the membranes for an additional hour. The membranes were washed twice with 100 ml of wash buffer (0.1× SSPE, 0.1% SDS) at 42°C for 10 min and were then exposed to X-ray film for 24 h. The film was then developed by a Kodak X-Omat X-ray film processor.

Evaluation of PCR inactivation. After initial amplification, serial 10-fold dilutions of each IP-10-treated (25, 50, or 100 μg/ml) amplicon plus a IP-10-free control were made in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). To determine the effectiveness of inactivation, an aliquot (5 μl) of each dilution was then reamplified in a PCR without IP-10. The products of the reactions were examined by agarose gel electrophoresis and Southern blotting as described previously (1, 2).

To study the effect of the UNG protocol, deoxyuridine was first substituted for thymidine in the nucleotide mixture, which was then used to amplify serial 10-fold dilutions of the six target sequences in a PCR. After amplification, aliquots (5 μl) of each amplicon were reamplified in the presence of UNG (10 min at 37°C and then 10 min at 95°C for 1 cycle and 30 s of denaturation at 94°C and then 30 s of annealing at 60°C for 60 cycles). The products were examined by agarose gel electrophoresis and Southern blotting as described previously (1, 2).

RESULTS

Photochemical inactivation. For most of our PCR products, treatment with increasing concentrations of IP (25, 50, or 100 μg/ml) resulted in an increase in the apparent molecular size of the product, as indicated by electrophoretic migration. Such an effect, indicated by the reduced distance of migration after gel electrophoresis, was most apparent with the largest PCR products. In contrast, a significant electrophoretic shift in band migration was not observed with the 92-bp amplicon of HSV (G+C content 65%).

For the two amplicons of less than or equal to 100 bp, the IP-induced band migrations were related to the G+C content of the PCR product. No apparent electrophoretic band shifts were observed for the smaller HSV amplicon (92 bp; G+C

TABLE 2. Inactivation of PCR amplicons after reaction with IP and UNG

Target	Amplicon		Log_{10} remaining product after treatment					
			Isopsoralen (μg/ml)					
	Size (bp)	G+C content (%)	None	25	50	100	None	UNG
HSV	92	65	10^{12}	10^{12}	10^{12}	10^{12}	10^5	10^5
	342	65	10^{11}	0	0	0	10^5	0
CMV	250	56	10^{10}	10^1	10^1	10^1	10^5	0
EBV	240	49	10^{10}	10^0	10^0	10^0	10^5	0
HPV-16	180	42	10^{12}	10^{10}	10^8	10^7	10^5	10^5

content, 65%), whereas HPV-16 (100 bp; G+C content, 42%) products showed a concentration-dependent shift in electrophoretic migration after reaction with IP. All of the IP-treated PCR products of 240 bp or larger that were assayed by gel electrophoresis uniformly produced the expected band migration shifts.

To determine whether the IP-induced band shifts observed in agarose gels were predictive for inactivation by IP, 10-fold dilutions of the IP-treated products were reamplified in a reaction mixture that did not include the agent. PCR products were analyzed by agarose gel electrophoresis, transferred to a nylon membrane by Southern blotting, and hybridized with an internal radiolabeled probe. All of the products that demonstrated an electrophoretic band shift in agarose gels were inactivated by IP; levels of amplifiable products were reduced by ranges of 5 log_{10} units for the 100-bp HPV-16 product to 11 log_{10} units for the 342-bp product of HSV (Table 2). EBV, CMV, and HSV (342 bp) products required only 25 μg of IP per ml for maximal inactivation. Low-molecular-mass amplicons (HPV-16) were inactivated less effectively and required higher concentrations of IP (100 μg/ml) compared with PCR products of 200 to 300 bp. The 92-bp, G+C-rich amplicon (HSV) was not inactivated by any concentration of IP tested (Fig. 1A and B).

Enzymatic inactivation. UNG was also studied for inactivation of carryover PCR amplicons. The UNG protocol is based on the ability of the enzyme UNG to excise deoxyuracil residues from DNA products containing this nucleotide. Heating of the abasic PCR products under the slightly alkaline conditions of PCR results in strand scission, thus rendering them refractory to PCR amplification. We therefore substituted deoxyuridine for thymidine in our nucleotide mixture prior to amplification.

As a control for UNG specificity, the original thymidine-containing targets were added to the dUTP-substituted reaction mixture and were amplified in the presence of UNG (Fig. 2A and B, bottom, lanes 8 to 13). Products were analyzed by agarose gel electrophoresis (Fig. 2A), transferred to a nylon membrane by Southern blotting, and detected with a radiolabeled probe internal to the amplicon (Fig. 2B).

While incorporation of dUTP did not seem to adversely affect PCR amplification (Fig. 2A and B; top, lanes 1 to 6; bottom, lanes 8 to 13), nonspecific electrophoretic bands (primer-dimer) were present in these amplicon mixtures, which may affect sensitivity. No amplification of dUTP-containing amplicons occurred after treatment of the products with UNG (Fig. 2A and B, bottom, lanes 1 to 6).

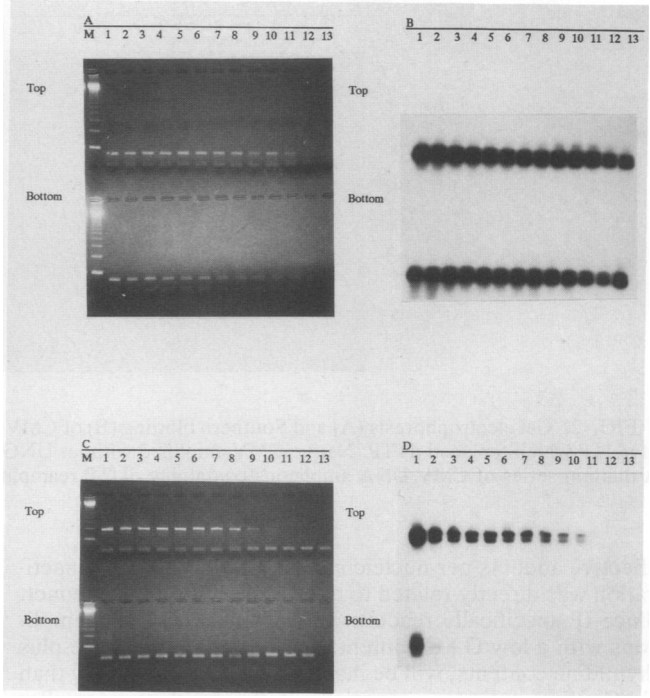

FIG. 1. Gel electrophoresis (A and C) and Southern blotting (B and D) of HSV (A and B) and CMV (C and D) amplicons without (top) and with (bottom) IP (25 μg/ml) treatment. Lane designations correlate with dilution (e.g., lane 1, 10^{-1} dilution; lane 12, 10^{-12} dilution). M, molecular size markers.

Complete inactivation of CMV (Fig. 2A and B, bottom, lanes 1 to 6), EBV, and HSV (342 bp) occurred; however, HSV (92 bp) and HPV-16 were refractory to inactivation by UNG (Table 2).

DISCUSSION

Control of carryover amplicon contamination can be achieved by photochemical (IP), enzymatic (UNG), or other (primer hydrolysis; see accompanying paper [9a]) methods; inactivation occurs before UNG or after IP amplification (4, 8). These procedures must be initiated prospectively; contamination caused by previous amplicon buildup in the laboratory is not prevented by either of these methods.

Photochemical inactivation is implemented after thermal cycling has been completed. IP, in the presence of long-wavelength (320 to 400 nm) UV light, forms covalent adducts with thymidine residues in the PCR product. The geometries of IPs are such that once a monoadduct has been formed, subsequent reaction of the remaining double bond with a pyrimidine on the complementary nucleic acid strand does not occur because of a misalignment of the two reactive double bonds (3, 6). Thus, although the amplicons cannot be used as PCR templates, they can be detected by hybridization. Most important, this inactivation can be performed in a closed system, i.e., prior to opening the PCR vessels (6).

As demonstrated by Cimino et al. (4), the size of an amplicon (in base pairs) was the most important factor in determining the effectiveness of IP as an inactivating agent. The monoadducts formed by IP and nucleic acid polymers block extension reactions with *Taq* polymerase in some, but not all, instances. They demonstrated that the average

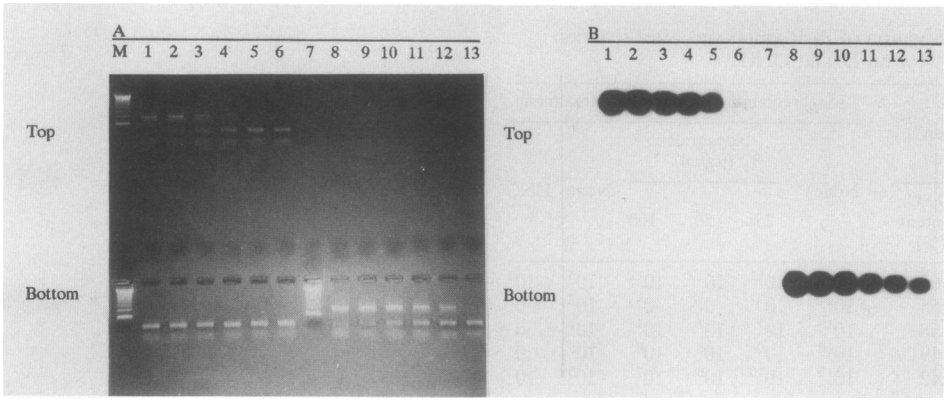

FIG. 2. Gel electrophoresis (A) and Southern blotting (B) of CMV DNA amplification products. CMV DNA was amplified in the presence of dUTP which replaced dTTP. Native CMV amplified without UNG (lanes 1 to 6; top) and with UNG (lanes 8 to 13, bottom) pretreatment. A dilution series of CMV DNA amplicons containing dUTP reamplified after UNG pretreatment is also shown (lanes 1 to 6, bottom).

effective adducts per nucleic acid strand for effective inactivation was directly related to the length of the PCR product. Since IP specifically reacts with thymidine residues, amplicons with a low G+C content, and hence high adenine plus thymidine contents, will be inactivated more effectively than G+C-rich amplicons, especially if the product is smaller than 240 bp. Consistent with their results, IP did not inactivate the 92-bp amplicon (G+C content, 65%) specific for HSV, whereas complete inactivation was obtained with the larger PCR products, even though the G+C contents of these products were high (CMV, EBV) (Fig. 1; Table 2).

Since the 92-bp HSV amplicon was not inactivated with IP, we attempted to modify the primers by attaching tails rich in adenine plus thymidine residues to the 5′ ends of the HSV primer sequence to serve as an IP target (data not shown). Fifteen adenine or thymidine residues were incorporated into the tails of the sense (GGG ATA AAT GCT TAT TTA AT) and antisense (GGG ATA AAT GCT TAT TTA AT) primers. Five guanidine or cytidine bases were added to this polynucleotide tail to add stability to the resultant amplified 132-bp product. However, the additional adenine or thymidine bases and the increased length of the DNA product did not provide for inactivation by IP. Similarly, reaction of 8-methoxypsoralen, which has a linear ring structure similar to that of the parent psoralen compound, which would therefore span the double-stranded DNA molecule to form covalent adducts (and thus produce double-stranded cross-links in the A+T-rich regions of the amplicon), did not inactivate the amplicon in additional amplification reactions. Presumably, despite the cross-linking of PCR products at the adenine plus thymidine ends, enough priming can occur internally to serve as an adequate target to initiate PCR.

Evidence of intercalation of IP between base pairs of nucleic acids can be detected by a shift in electrophoretic mobility. The degree of the observed shift depended on the number of AT residues in the amplicons and the concentration of IP used in the reaction. For most amplification reactions, a final IP concentration of 25 μg/ml rendered amplicons refractory to amplification in additional reactions. Nevertheless, the migration pattern of the IP cross-linked products was not always predictive of optimal inactivation. For example, a stepwise increase in molecular mass occurred with the 100-bp amplicon of HPV-16, but inactivation was reduced by only a factor of 5 $\log_{10}$ units (100 μg/ml; Table 2), compared with 9 to 10 $\log_{10}$ units for other PCR products (CMV, EBV) after treatment with IP.

We initially expected the UNG system to perform better than the IP protocol for inactivation of the short G+C-rich products of HSV (92 bp) and HPV-16 (100 bp) because of the presumed high efficiency of enzymatic cleavage of deoxyribonucleotide residues from the DNA backbone. However, the results that we observed were the opposite; UNG did not inactivate these products effectively. In fact, the IP protocol was more effective than expected for the HPV-16 product. Additional experience with other targets will be required to determine the lower threshold for effective UNG and IP inactivation; effective inactivation of a 156-bp *Borrelia burgdorferi* amplification product by both methods is described in the accompanying manuscript (9a).

Interestingly, the presence of UNG in the same PCR mixture had no adverse effect on the activity of IP; similarly, IP did not inhibit the activity of UNG in similar experiments (data not shown). On the basis of this observation, we suggest that an optimal and highly cost-effective strategy for amplicon inactivation for the clinical laboratory might include elements of both protocols. Inactivation of PCR amplicons can be inexpensively and efficiently achieved with IP, particularly for AT-rich products and long (≥240-bp) G+C-rich products. Since replacement of thymidine with deoxyuracil residues had little effect on the efficiency of PCR amplification in our systems, an efficient strategy for the clinical laboratory may be to use the IP method (18¢ per reaction) as a frontline method for controlling amplicon carryover, but to routinely substitute deoxyuridine for thymidine in PCR products. UNG would then be added only when amplicon contamination is suspected (63¢ per reaction). This may allow continued use of an assay, despite a low-level contamination problem; in the interim, the source of contamination can be identified and the problem can be rectified.

Of the total viruses recovered in cell cultures in our laboratory during 1 year, herpesviruses accounted for 83% (CMV, 43%; HSV, 37%; varicella-zoster virus, 3%) (11). Because of the prevalence of these viruses in our tertiary-care practice, our goal was to implement PCR technology for the diagnosis of these infections, especially from cerebrospinal fluid and tissue specimens, which generally do not yield viruses by conventional tube or shell vial assays. In this regard, the primers specific for a sequence of the DNA

polymerase gene of HSV have been capable of amplifying a 92-bp sequence which was at least sixfold more sensitive than the detection of fluorescent foci in shell vial cell cultures (2). Furthermore, this PCR assay has had demonstrated utility for the detection of HSV in culture-negative cerebrospinal fluid specimens and in paraffin-embedded tissue specimens (1, 2).

For the diagnosis of herpesvirus infections, accurate PCR results are critical for patient management. For example, a false-positive report of HSV DNA disease would likely commit a patient to a costly 14-day hospitalization period for administration of acyclovir by the intravenous route. Perhaps more importantly, clinical and laboratory evaluations of the patient for the actual etiology of the central nervous system disease may not be pursued. Because of the extreme sensitivity of PCR technology, prevention and control of amplicon carryover must be maintained to ensure the confidence of the clinical laboratory and clinician of accurate results.

ACKNOWLEDGMENT

This work was supported in part by NIH grants AI30548, AI32403, and AR41497.

REFERENCES

1. **Aslanzadeh, J., K. F. Helm, M. J. Espy, S. A. Muller, and T. F. Smith.** 1992. Detection of HSV-specific DNA in biopsy tissue of patients with erythema multiforme by polymerase chain reaction. Br. J. Dermatol. **126**:19–23.
2. **Aslanzadeh, J., D. R. Osmon, M. P. Wilhelm, M. J. Espy, and T. F. Smith.** 1992. A prospective study of the polymerase chain reaction for detection of herpes simplex virus in cerebrospinal fluid submitted to the clinical virology laboratory. Mol. Cell. Probes **6**:367–373.
3. **Cimino, G. D., H. B. Gamper, S. T. Isaacs, and J. E. Hearst.** 1985. Psoralens as photoactive probes of nucleic acid structure and function: organic chemistry, photochemistry, and biochemistry. Annu. Rev. Biochem. **54**:1151–1193.
4. **Cimino, G. D., K. C. Metchette, J. W. Tessman, J. E. Hearst, and S. T. Isaacs.** 1991. Post-PCR sterilization: a method to control carryover contamination for the polymerase chain reaction. Nucleic Acids Res. **19**:99–107.
5. **Fox, J. C., M. Ait-Khaled, A. Webster, and V. C. Emery.** 1991. Eliminating PCR contamination: is UV irradiation the answer? J. Virol. Methods **33**:375–382.
6. **Isaacs, S. T., J. W. Tessman, K. C. Metchette, J. E. Hearst, and G. D. Cimino.** 1991. Post-PCR sterilization: development and application to an HIV-1 diagnostic assay. Nucleic Acids Res. **19**:109–116.
7. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339**:237–238.
8. **Longo, M. C., M. S. Berninger, and J. L. Holley.** 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene **93**:125–128.
9. **Persing, D. H.** 1991. Polymerase chain reaction: trenches to benches. J. Clin. Microbiol. **29**:1281–1285.

9a. **Rys, P. N., and D. H. Persing.** 1993. Preventing false positives: quantitative evaluation of three protocols for inactivation of polymerase chain reaction amplification products. J. Clin. Microbiol. **31**:2356–2360.

10. **Sarkar, G., and S. S. Sommer.** 1990. Shedding light on PCR contamination. Nature (London) **343**:27.
11. **Smith, T. F., and A. D. Wold.** 1991. Changing trends of diagnostic virology in a tertiary care medical center, p. 1–20. *In* L. M. de la Maza and E. M. Peterson (ed.), Medical virology 10. Plenum Press, New York.
12. **Syvänen, A.-C.** 1992. From one to millions—the polymerase chain reaction in diagnosis. Am. Med. **24**:179–182.
13. **Tompkins, L. S.** 1992. The use of molecular methods in infectious diseases. N. Engl. J. Med. **327**:1290–1297.
14. **Williams, S. D., and S. Kwok.** 1992. Polymerase chain reaction: applications for viral detection, p. 147–173. *In* E. H. Lennette (ed.), Laboratory diagnosis of viral infections. Marcel Dekker, Inc., New York.