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Dealing with Contamination: Enzymatic Control of Carryover Contamination in PCR

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PCR produces an abundance of product DNA from traces of input DNA. It was apparent early that because it is so sensitive, PCR is especially susceptible to contamination. In a research setting (e.g., cloning a gene, preparing a probe), contamination is generally not a concern. However, if a primer pair is used many times, if the PCR is designed to be very sensitive, or if the presence or the absence of amplification of a target sequence will have diagnostic implications, then possible contamination must be eliminated for the PCR results to be meaningful.

Contaminating DNA can originate from three sources: DNA from other test samples, DNA from experimental materials such as recombinant clones, or DNA generated by previous PCR amplification of the same target sequence. This last source of contamination, often called "carryover" contamination, has proven to be the most troublesome source.

Early users of PCR noted that carryover contamination could be a significant problem owing to the abundance of DNA generated by PCR and the ease with which such DNA can be reamplified.(1,2) Detecting carryover contamination, e.g., by including negative control reactions, is essential. Prevention is clearly preferred, however, because correcting the problem can be costly and testing of samples will probably need to cease until a thorough clean-up can be effected. This will most likely mean discarding all suspected reagents, and cleaning, or even replacing, equipment. A last resort, one not always possible, is to change to a different primer pair, so as to amplify a different region of the target DNA.

Two general approaches may be taken to control carryover contamination: physical isolation of PCR products from newly assembled reactions (see also Setting up a PCR Laboratory, pp. S2–S9) and design of the PCR so that products of a first PCR are not efficiently reamplifiable in a subsequent PCR. Physical measures to control carryover contamination are listed in approximate order of difficulty and cost:

1. Use dedicated pipetting devices. Use pipette tips that contain an aerosol barrier, or use a positive-displacement pipette with disposable tips and plungers.
2. Decontaminate surfaces. Aqueous sodium hypochlorite(3) has been suggested.
3. Premix reagents before aliquoting into individual reactions to reduce the number of pipetting steps and thus chances of contamination.(1)
4. Assemble new PCRs in a separate area, preferably where contamination control measures can be taken, e.g., laminar flow hood with UV lighting. This area should be isolated from the temperature cycler and the area where the amplification reactions are opened.
5. Amplify for the minimum number of cycles to make only as much product DNA as is needed to obtain the desired results.
6. Open amplification reactions cautiously to avoid aerosols. It has been suggested that reactions be frozen before opening.(2)
7. Wear disposable gloves. It is essential to change gloves after handling tubes containing amplification products. Wear hats and face masks, since laboratory workers have been found to carry PCR products.(4)

The second general approach is to modify the PCR so that the products of previous PCR amplifications are discriminated against in a new PCR. For this approach to work, a discriminating process of some kind must intervene after the last cycle of a first PCR or before the first cycle of a subsequent PCR. Because PCR uses DNA primers to detect DNA target, this process must either act on PCR product DNA before primers and target DNA are added, or must discriminate in favor of true target and against possible PCR-derived DNA. Various ways of achieving this discrimination have been proposed:

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1. Irradiation of samples with UV light(5–7) or gamma radiation(8) prior to amplification. The rationale is that nucleotides and primers are much more resistant to radiation than larger DNA. Target must be added after this pretreatment. The efficacy of this method has not been complete or uniform.(9–14)

2. Pretreatment of PCR reaction with nucleases. Here the principle is that oligonucleotide primers, being single stranded, are resistant to restriction endonucleases, but carryover contaminants with known (and preferably multiple) cleavage sites should be cut efficiently and made unamplifiable.(11,15) This was, in fact, observed, and different restriction enzymes provided different degrees of decontamination. Surprisingly, DNase I could also be used successfully.(15) Target DNA must be added after inactivation of the nucleases.

3. Inactivation of psoralen-containing PCR products with UV light immediately after completion of amplification. Psoralens, which covalently link the complementary strands of DNA upon ultraviolet irradiation, have been found to block amplification of double-stranded DNA effectively.(16,17) Isopsoralens can be included in PCR reactions under conditions chosen to minimize their effect on amplification.(18) Irradiation of reactions with a powerful UV source for 15 min yielded products that hybridized normally to specific probes, but that would not reamplify to detectable levels when 10^8 molecules were added to new reactions and cycled 30 times.(18)

4. Methods in which the oligonucleotide primers are modified. These are based on the fact that for PCR to proceed, the primer DNA, which after PCR is found at the 5′ end of each DNA strand, must itself be copied at each cycle. If primers contain uracil bases,(19) reamplification of PCR products may be inhibited with the enzyme uracil DNA glycosylase (UDG) (see also below). If primers contain a 3′ ribonucleotide, treatment of PCR products with base releases primers and inhibits reamplification (J. Walder, Integrated DNA Technologies, Coralville, IA).

The most widely used decontamination method for diagnostic PCR is based on substituting PCR product DNA with uracil bases in place of thymine.(19) A schematic representation of this methodology is shown in Figure 1. The DNA produced in such reactions is normal in most respects (e.g., it is cut by many restriction enzymes(20) and hybridizes to oligonucleotide probes,(21) except that it contains tens or hundreds of uracil bases. Preincubation of all amplification reactions with the enzyme UDG results in removal of uracil from carryover DNA (but not from sample DNA, dUTP, or RNA), creating tens or hundreds of abasic sites. DNA polymerases stall at these sites. Furthermore, such sites are heat labile and break during temperature cycling. Either type of damage prevents amplification. If dUTP is used routinely in all PCR amplifications, then all PCR products will contain uracil and be susceptible to UDG. The method is robust and, because it acts on complete reactions just prior to temperature cycling (i.e., all components including target DNA are present), no carryover PCR product, regardless of source, should escape destruction. In some cases, PCR does not proceed with quite the same efficiency when dTTP is completely replaced with dUTP.(19) This inefficiency appears to be sequence specific and is not necessarily related to the length of the fragment to be amplified (unpublished data). Higher concentrations of dUTP, with compensating magnesium concentrations (which is chelated by dNTPs), can increase product yield.(21)

The efficacy of the use of UDG for decontamination of PCR has been demonstrated by several groups. Longo et al.(19) showed that intentional contamination with $>10^{10}$ molecules of PCR product did not yield product detectable by ethidium bromide staining when reamplification was attempted following a 10-min UDG incubation. Kolk et al.(22) and Wang et al.(21) have demonstrated UDG decontamination in diagnostic tests. In the clinical lab-

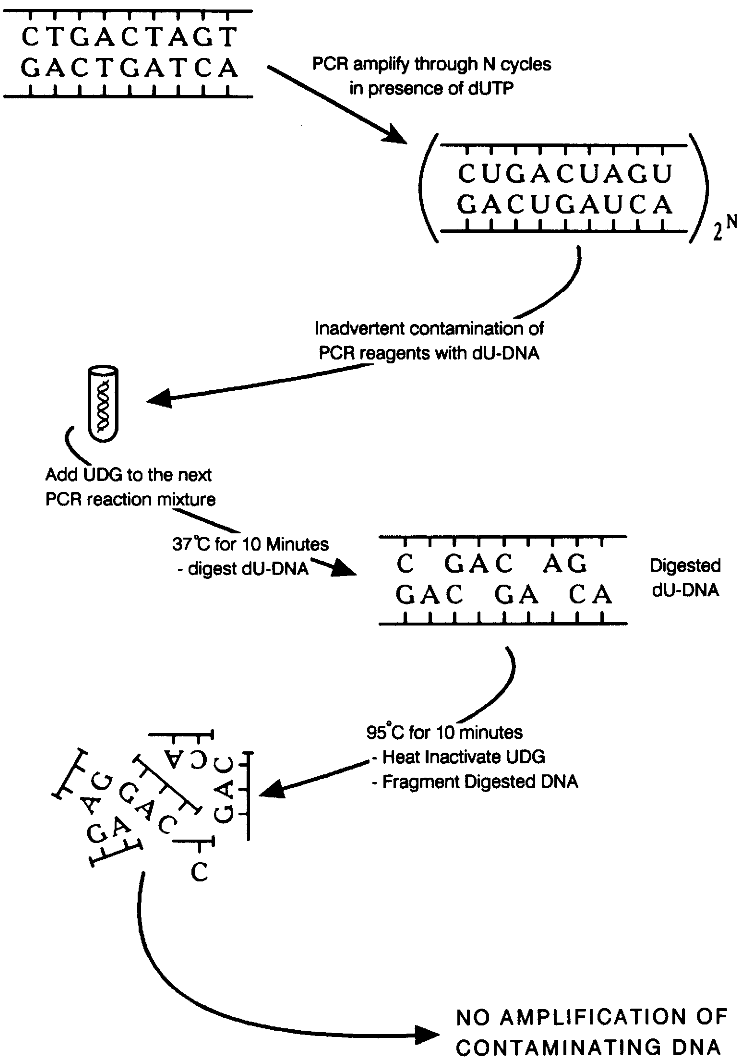

FIGURE 1 Schematic representation of UDG method for prevention of carryover contamination in PCR.

oratory setting, the UDG decontamination procedure has been used in development of several diagnostic assays based on PCR, such as detection of *Mycobacterium leprae,*(23) HIV,(24) Lyme disease,(25) and chlamydia.(26) Commercial versions of these tests incorporate the UDG decontamination technology under the trademark AmpErase (Roche Diagnostic Systems).

The stability and characteristics of dU-containing PCR products have been studied in detail. Although there are no significant differences in thermal stability of dU-containing DNA, these DNA fragments are extremely sensitive to UDG. Thornton et al.(27) have shown that the majority of the UDG is inactivated during PCR amplification. However, when the PCR reactions are stored at temperatures varying from 4°C to 42°C, some residual reactivation of UDG is observed that can degrade dU-containing DNA. Two solutions have been proposed to avoid this problem. (1) Following PCR amplification, PCR products should be kept in a soak file set at 72°C, instead of 4°C or 25°C soak files normally used for dT PCR products. (2) A specific protein that can inhibit UDG, UDG inhibitor protein, can be added to PCR products to inactivate the residual UDG activity.(28) Of course, a UDG that is irreversibly inactivated during PCR would completely eliminate this problem.

PCR products may be used for a variety of purposes. They may be cloned, digested with restriction enzymes, hybridized with probes, etc. These procedures are often essential for accurate diagnostic applications of PCR. Bebee et al.(29) have studied the characteristics of the dU-containing PCR products regarding digestion with restriction enzymes and cloning. Restriction endonuclease cleavage of dU-containing DNA is dependent on the specific endonuclease used as well as the sequences flanking the endonuclease recognition site. Wang et al.(21) showed that amplified hemoglobin gene DNA containing uracils can be digested with the restriction enzyme *Dde*I, but not with *Bsu*36I or *Cvn*I. Cloning of dU-containing PCR products is easily achieved using an *ung*$^-$ (UDG-deficient) strain of *E. coli* host.

Carryover contamination is a significant source of error when PCR is being used in a diagnostic context. Routine procedures for detecting carryover contamination are essential but must be complemented by prevention. Enzymatic or physical control measures such as those described above carry a cost, e.g., modification of the standard PCR or special procedures and facilities. Judgment is required to balance the costs these measures impose with the benefit derived from controlling contamination and obtaining reliable results.

PROTOCOL FOR UDG DECONTAMINATION

Note: The UDG method cannot work unless all of the PCR products that can cause contamination are substituted with uracil bases. UDG has no effect on thymine-containing DNA.

Reagents

1. dUTP nucleotide mix: dATP, dCTP, dGTP, and dUTP at equimolar concentrations as per normal PCR (but see above for higher dUTP concentrations if amplification yield is low).
2. Uracil DNA glycosylase, 1 U/μl (BRL/Life Technologies, Inc., Gaithersburg, MD).

Protocol

1. Add 1 unit of UDG to each PCR reaction (50–100 μl).
2. Change the thermal cycler program to include an initial 37°C incubation for 15 min followed by 10 min at 95°C. During this time, UDG cleaves uracil bases from any carryover contaminants, and the high temperature cleaves abasic sites and inactivates UDG.
3. Temperature cycle as usual; however, be aware that some UDG activity returns when reaction temperatures fall below 50°C. Keep reactions at 72°C or frozen until analysis of results.

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