Enzymatic Control of Carryover Contamination in PCR

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INTRODUCTION

PCR produces an abundance of amplified DNA product from traces of input DNA. It was apparent early that due to its exquisite sensitivity, PCR is especially susceptible to contamination. In some applications (e.g., cloning a gene, preparing a probe), contamination is 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 has 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.

Early users of PCR noted that carryover contamination could be a significant problem because of the abundance of DNA generated by PCR and the ease with which such DNA can be reamplified (Gibbs and Chamberlain 1989; Kwok and Higuchi 1989). 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 probably needs to cease until a thorough clean-up can be effected. This most likely means 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.

This chapter focuses on the enzymatic elimination of PCR product carryover. This approach modifies the PCR so that the products of previous PCR amplifications are discriminated against. 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 sub-

sequent PCR. Because PCR uses DNA primers to detect a DNA target, this process must either act on PCR product DNA before primers and target DNA are added, or it must discriminate in favor of the true target and against possible PCR-derived DNA. Various ways of achieving this discrimination have been proposed. Physical methods are discussed in Dieffenbach et al. and in Cone and Fairfax, both this volume.

Several enzymatic ways of eliminating carryover contamination have been demonstrated. Pretreatment of PCR products with nucleases is based on the principle 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 (DeFilippes 1991). This was, in fact, observed, and different restriction enzymes have provided different degrees of decontamination. Surprisingly, DNase I could also be used successfully (Furrer et al. 1990). Target DNA must be added after inactivation of the nucleases.

In a second class of methods, the PCR primers are modified. These methods 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 (Longo et al. 1990), reamplification of PCR products may be inhibited with the enzyme uracil DNA glycosylase (UDG; also called uracil *N*-glycosylase or UNG). If primers contain a 3′ ribonucleotide, treatment of PCR products with ribonuclease or alkali releases primer sequences and inhibits reamplification (Rys and Persing 1993; Walder et al. 1993).

The most widely used decontamination method for diagnostic PCR is based on substituting PCR product DNA with deoxyuracil bases in place of thymines (Longo et al. 1990). A schematic of this method 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 [Bodnar et al. 1983; Wang et al. 1992] and hybridizes to probes [Wang et al. 1992]), except that it contains tens or hundreds of deoxyuridines. Preincubation of all amplification reactions with the enzyme UDG results in removal of dU from carryover DNA (but does not affect 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 contain uracil and are 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.

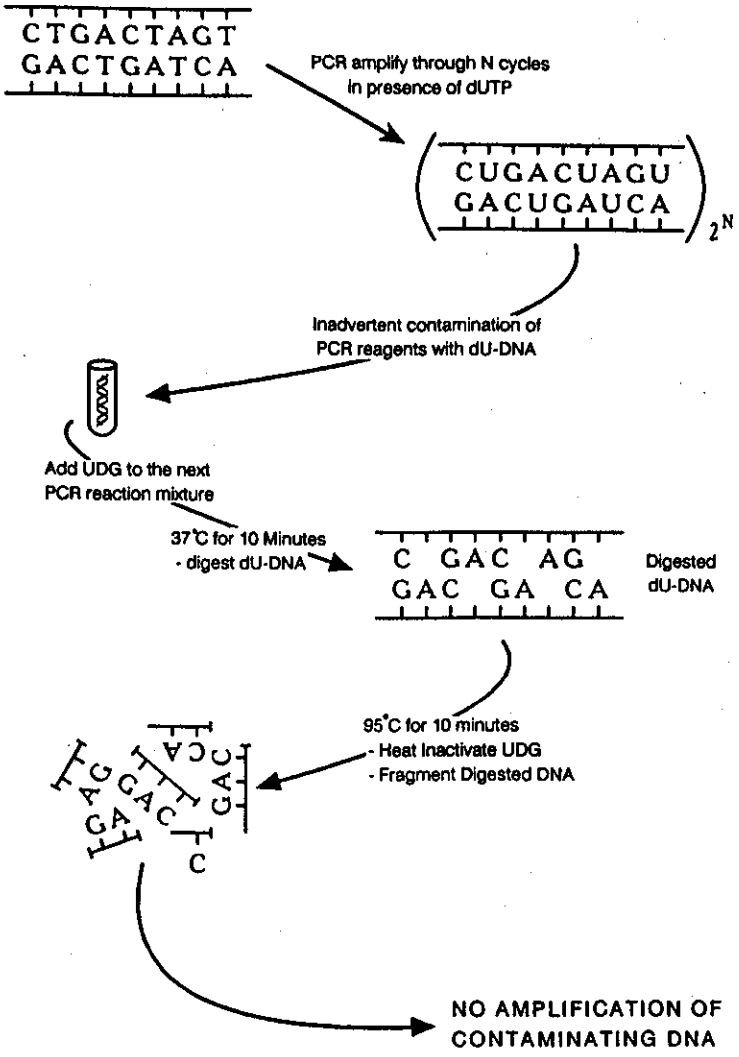

Figure 1 Schematic representation of UDG method for prevention of carryover contamination in PCR.

It is important to note that longer PCR products make UDG decontamination more efficient (but see discussion below for application to "long PCR"). In one study, contamination of product shorter than 100 bp could not be completely eliminated with UDG (Espy et al. 1993). Smaller DNAs may contain too few uracil bases to guarantee complete destruction by UDG.

REAGENTS

dUTP nucleotide mix: dATP, dCTP, dGTP, and dUTP mix at 10 mM each (see below for higher dUTP concentration if amplification yield is low)

Uracil DNA glycosylase, 1 unit/μl (Life Technologies, cat. no. 18054-015)

10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl)

25 mM $MgCl_2$ solution

Taq DNA polymerase, 5 units/μl (Life Technologies, cat. no. 18038-018)

PROTOCOL

To a 0.5-ml microcentrifuge tube on ice add the following:

10x PCR buffer	5 μl
10 mM dUTP mix	1 μl
PCR primers (10 μM each)	1 μl
Uracil DNA glycosylase, 1 unit/μl	1 unit
Taq DNA polymerase, 5 units/μl	0.5 μl
25 mM $MgCl_2$	3 μl (see below)
Sterile distilled water	38.5 μl

Notes

- *Magnesium concentration.* For most PCR primer pairs, $MgCl_2$ at a concentration of 1.5 mM is satisfactory. If amplification is not adequate, adjustments to Mg concentration may be helpful. If nucleotide (e.g., dUTP) concentrations are increased, the Mg concentration must also be raised, since nucleotides chelate Mg ions.
- *dUTP concentration.* The fundamental requirement for UDG control of carryover amplification is that all PCR products contain uracil bases replacing thymine. This is accomplished by replacing dTTP with dUTP in reaction mixtures. For some amplifications this direct substitution (usually 0.2 mM) has no effect on amplification potential (Rys and Persing 1993; Kox et al. 1994); for some target/primer combinations, however, the concentration of dUTP must be raised to 0.6 mM or even 1 mM to reach comparable sensitivities (Wang et al. 1992; Hohlfeld et al. 1994). It is important that magnesium concentrations also are raised in such situations (see above). Recommendation: Start with dUTP equimolar with other dNTPs, test higher concentrations if necessary.
- *Decontamination.* To decontaminate PCR procedures, UDG is added to all assays and they are incubated prior to temperature cycling. Because the UDG of commerce is derived from *Escherichia coli*, this step was originally done at 37°C (Longo et al. 1990). Studies have verified decontamination at room temperature (Wang et al. 1992; De Wit et al. 1993). Subsequently, a short time at 50°C has been found to be effective (Hohlfeld et al. 1994; Kox et al. 1994). In addition, it has been claimed that this step increases PCR specificity (Mulder et al. 1994). The amount of UDG required ap-

pears to be quite variable: As little as 0.01 unit has been used (Kox et al. 1994). The balance is between cost of enzyme and completeness of decontamination. Recommendation: If UDG cost is an issue, test different amounts with different levels of expected contamination. Otherwise, one unit per assay is effective for at least 10^9 contaminant molecules (Longo et al. 1990).

- *Temperature cycling, gel electrophoresis, and hybridization.* Normal procedures are followed. Many clinical studies have tested the specificity of amplification by a variety of hybridization tests (see, e.g., Rys and Persing 1993; Mulder et al. 1994). Recommendation: Use normal protocols.
- *Residual UDG activity.* UDG from *E. coli* that has been through many PCR cycles recovers a small fraction of its catalytic activity when it is returned to lower temperatures (Thornton et al. 1992). Although this activity is easily detectable with appropriate assays, PCR products often undergo subsequent treatment (gel electrophoresis, denaturation, hybridization, etc.) that eliminates residual UDG activity. However, it is only prudent to store reactions with dU-containing DNA and residual UDG activity at –20°C when not in use. One study added an equal volume of chloroform to each reaction after cycling was complete to keep the UDG inactive (Hohlfeld et al. 1994). Recommendation: Store PCR products at –20°C.

DISCUSSION

The efficacy of the use of UDG for decontamination of PCR has been demonstrated by several groups. Longo et al. (1990) 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-minute UDG incubation. In the clinical laboratory setting, the UDG decontamination procedure has been used in the development of several diagnostic assays based on PCR, such as the detection of *Mycobacterium leprae* (De Wit et al. 1993), *Mycobacterium tuberculosis* (Nolte et al. 1993), human immunodeficiency virus (HIV) (Butcher and Spadoro 1992), and Lyme disease (Dodge et al. 1992). Commercial versions of these tests incorporate the UDG decontamination technology under the trademark AmpErase (Roche Diagnostic Systems).

In "long PCR," DNAs longer than 30 kb have been amplified using mixtures of DNA polymerases, one with and one without a 3′ exonuclease (proofreading) activity (Barnes 1994; Cheng et al. 1994). Addition of dUTP to these reactions has uniformly inhibited amplification of long products (>4 kb; D. Shuster and A. Rashtchian, unpubl.). This phenomenon appears to be related to inhibition of the tested proofreading polymerases (*Pfu*, Vent, and DeepVent) by dU-

containing DNA. Therefore, UDG-based decontamination of long PCR may not be possible with currently available enzymes.

Carryover contamination is a significant source of false-positive results when primer pairs are used repeatedly in DNA amplification. Routine procedures for detecting carryover contamination are essential, but they must be complemented by prevention. Enzymatic control measures such as those described here carry a cost; e.g., modification of the standard PCR or special procedures and facilities. It is necessary to balance the costs of these measures with the benefit derived from controlling contamination and obtaining reliable results.

REFERENCES

Barnes, W.M. 1994. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. *Proc. Natl. Acad. Sci.* **91:** 2216–2220.

Bodnar, J.W., W. Zempsky, D. Warder, C. Bergson, and D.C. Ward. 1983. Effect of nucleotide analogs on the cleavage of DNA by the restriction enzymes *Alu*I, *Dde*I, *Hin*fI, *Rsa*I, and *Taq*I. *J. Biol. Chem.* **258:** 15206–15213.

Butcher, A. and J. Spadoro. 1992. Using PCR for detection of HIV-1 infection. *Clin. Immunol. News* **12:** 73–76.

Cheng, S., C. Fockler, W.M. Barnes, and R. Higuchi. 1994. Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci.* **91:** 5695–5699.

DeFillipes, F.M. 1991. Decontaminating the polymerase chain reaction. *BioTechniques* **10:** 26–29.

De Wit, M.Y.L., J.T. Douglas, J. McFadden, and P.R. Klatser. 1993. Polymerase chain reaction for detection of *Mycobacterium leprae* in nasal swab specimens. *J. Clin. Microbiol.* **31:** 502–506.

Dodge, D.E., R. Nersesian, and R. Sun. 1992. Diagnosis of the Lyme disease spirochete *Borrelia burgdorferi*. *Clin. Immunol. News* **12:** 69–73.

Espy, M.J., T.F. Smith, and D.H. Persing. 1993. Dependence of polymerase chain reaction product inactivation protocols on amplicon length and sequence composition. *J. Clin. Microbiol.* **31:** 2361–2365.

Furrer, B., U. Candrian, P. Wieland, and J. Luthy. 1990. Improving PCR efficiency. *Nature* **346:** 324.

Gibbs, R.A. and J.S. Chamberlain. 1989. The polymerase chain reaction: A meeting report. *Genes Dev.* **3:** 1095–1098.

Hohlfeld, P., F. Daffos, J.-M. Costa, P. Thulliez, F. Forestier, and M. Vidaud. 1994. Prenatal diagnosis of congenital toxoplasmosis with a polymerase-chain-reaction test on amniotic fluid. *New Engl. J. Med.* **331:** 695–699.

Kox, L.F.F., D. Rhienthong, A. Miranda, N. Udomsantisuk, K. Ellis, J. van Leeuwen, S. van Heusden, S. Kuijper, and A.H.J. Kolk. 1994. A more reliable PCR for detection of *Mycobacterium tuberculosis* in clinical samples. *J. Clin. Microbiol.* **32:** 672–678.

Kwok, S. and R. Higuchi. 1989. Avoiding false positives with PCR. *Nature* **339:** 237–238.

Longo, M.C., M.S. Berninger, and J.L. Hartley. 1990. Use of uracil DNA glycosylase to control carryover contamination in polymerase chain reactions. *Gene* **93:** 125–128.

Mulder, J., N. McKinney, C. Christopherson, J. Sninsky, L. Greenfield, and S. Kwok. 1994. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: Application to acute retroviral infection. *J. Clin. Microbiol.* **32:** 292–300.

Nolte, F.S., B. Metchock, J.E. McGowan, A. Edwards, O. Okwumabua, C. Thurmond, P.S. Mitchell, B. Plikaytis, and T. Shinnick. 1993. Direct detection of *Mycobacterium tuberculosis* in sputum by polymerase chain reaction and DNA hybridization. *J. Clin. Microbiol.* **31:** 1777–1782.

Rys, R.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.

Thornton, C.G., J.L. Hartley, and A. Rashtchian. 1992. Utilizing uracil DNA glycosylase to control carryover contamination in PCR: Characterization of residual UDG activity following thermal cycling. *BioTechniques* **13:** 180–183.

Walder, R.Y., J.R. Hayes, and J.A. Walder. 1993. Use of

PCR primers containing a 3′-terminal ribose residue to prevent cross-contamination of amplified sequences. *Nucleic Acids Res.* **21:** 4339–4343.

Wang, X., T. Chen, D. Kim, and S. Piomelli. 1992. Prevention of carryover contamination in the detection of β^s and β^c genes by polymerase chain reaction. *Am. J. Hematol.* **40:** 146–148.

Ultraviolet Irradiation of Surfaces to Reduce PCR Contamination

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INTRODUCTION

False-positive PCRs arise from contamination with exogenous genomes, plasmids, or PCR products (Kwok and Higuchi 1989). Contaminated laboratory surfaces represent one of the many potential sources of exogenous DNA.

UV irradiation of dry DNA provides just one tool in the arsenal necessary to prevent PCR contamination. Although this type of decontamination was recommended previously as a way to "quickly damage any DNA left on exposed surfaces" (Kwok and Higuchi 1989), further work has revealed a slow time course and sequence dependence (Fairfax et al. 1991; Fox et al. 1991; Sarkar and Sommer 1991). UV irradiation has also been proposed for decontaminating DNA in reagent solutions (Isaacs et al. 1991; Meier et al. 1993; Sarkar and Sommer 1993), a procedure that has met with mixed reviews (Fox et al. 1991; Dwyer and Saksena 1992; Frothingham et al. 1992).

Most UV-induced DNA damage occurs via the formation of cyclobutane rings between neighboring pyrimidine bases, thymidine or cytidine. The cyclobutane rings form intrastrand pyrimidine dimers that inhibit polymerase-mediated chain elongation. Dimer formation is reversible, establishing a steady-state equilibrium that favors monomers over dimers. As such, <10% of the possible pyrimidine dimers actually exist in irradiated DNA at one time (Gordon and Haseltine 1982).

UV irradiation of laboratory surfaces has some important limitations. First, the surface must be perpendicular to the light source to achieve optimal light intensity. Skewed surfaces dilute the intensity,

and three-dimensional objects, such as pipettors, cannot be effectively decontaminated by UV light because only a fraction of the surface actually faces the light source. This drawback is compounded by the fact that almost all laboratory surfaces, such as pipettors, centrifuges, door handles, and test tube racks, present potential sources of contamination (Cone et al. 1990). Second, other materials dried with the target DNA, such as irrelevant DNA and nucleotides, can shield the target, making inactivation less efficient (Frothingham et al. 1992). Third, very short PCR products may not contain adequate numbers of neighboring pyrimidines to make them susceptible targets. The UV sensitivity of an amplified region can be estimated by counting the number of dimerizable sites (neighboring pyrimidines: CT, TT, TC, CC) in each single strand of the sequence. Based on theoretical considerations (Gordon and Haseltine 1989) and limited experimental data (Fairfax et al. 1991; Meier et al. 1993), sequences with <10 dimerizable sites will be relatively UV-resistant.

This procedure describes a method for reducing DNA contamination on laboratory surfaces by using UV light to inactivate dried DNA (Fairfax et al. 1991). Different procedures have been proposed for UV inactivation of contaminating DNA in solutions (Isaacs et al. 1991; Meier et al. 1993; Sarkar and Sommer 1993). Although UV irradiation can be helpful, meticulous technique remains the most important method for preventing contamination. In particular, UV irradiation is not an effective replacement for the physical separation of sample preparation in a pre-PCR laboratory from PCR product analysis in a post-PCR laboratory. UV irradiation can, however, provide an additional margin of safety for keeping the PCR laboratory contamination-free.

SUPPLIES AND REAGENTS

UV light ballast UF-36-2 (American Ultraviolet)
Two UV lamps, model G36T6L (American Ultraviolet)
Markline timer switch (M.H. Rhodes)
Shortwave UV radiometer J-225 (American Ultraviolet)
Purified template DNA
35 x 10-mm tissue culture dishes (Corning)
10 mM Tris-HCl (pH 8.0)

PROTOCOL

UV Irradiation of Surfaces

Caution: UV irradiation is mutagenic and can cause visual loss or blindness. Wear UV-protective glasses and cover exposed skin when working with UV light.

INSTALLATION

1. Mount the ballast and two lamps approximately 1 meter over the work surface. The UV light source can be located at any distance from the surface, but as the distance increases, stronger lights will be necessary to achieve the same light intensity at the work surface. Installation of an in-line timer switch for automatic lamp shutoff can help to conserve the limited UV lamp life.

2. Document the UV light (254 nm) intensity at the work surface by measuring it with a UV meter. This measure of UV intensity establishes the baseline performance of the UV lamp installation. Lamp performance can then be checked by comparing future light intensity measurements with this one. We achieved effective decontamination with an intensity of 400 $\mu W/cm^2$ at the work surface using the above equipment.

Measuring DNA Inactivation

STANDARDS

1. Obtain a concentrated solution of purified template DNA, such as genomic DNA, plasmid DNA, or PCR products.

2. Establish the minimum amplifiable concentration by making duplicate tenfold dilutions of the DNA in 10 mM Tris-HCl (pH 8.0) and then amplifying an aliquot of each dilution.

3. Determine the most dilute specimen that was PCR positive in duplicate and call the DNA concentration in that dilution the minimum amplifiable concentration.

4. Prepare a concentrated DNA standard from the original DNA solution that is 10^6–10^8 times more concentrated than the minimum amplifiable concentration.

5. Prepare 12 test targets, each composed of 100 µl of the concentrated DNA standard spread in the center of a plastic petri dish and dried at room temperature.

EXPERIMENTAL PROTOCOL

1. Place all 12 uncovered petri dishes with dry DNA in the area to be decontaminated. When ready to begin this 8-hour experiment, remove 3 dishes from the area and cover them. Turn on the UV lights.

2. After each UV irradiation time point (2, 4, and 8 hours), remove and cover 3 more dishes. Resuspend the DNA by adding 100 µl of 10 mM Tris-HCl (pH 8.0) to each dish. Agitate thoroughly by pipetting repeatedly and swirling the dish for several minutes, and remove the liquid to a labeled tube.

DETERMINING UV SENSITIVITY

1. Quantitate the amount of amplifiable DNA in each sample by amplifying serial tenfold dilutions as described above.

2. Plot the results with time on the *x*-axis (0, 2, 4, and 8 hours) and the number of tenfold dilutions to achieve the minimum amplifiable concentration on the *y*-axis (Fairfax et al. 1991).

The data should reveal a time-dependent decrease in DNA concentration. For instance, if the minimum amplifiable concentration of the 0-hour time point was reached at a 10^{-7} dilution and the minimum amplifiable concentration of the 4-hour time point was reached at a 10^{-4} dilution, then 4 hours of irradiation would have resulted in a 1,000-fold reduction. Although inactivation of even tenfold could be considered useful, susceptible targets can routinely be inactivated by 10,000-fold or more.

Decontamination Procedure

Decontaminate the work space after use by turning on the UV lights. Turn off the UV lights before resuming work. The minimum duration of UV illumination required for effective DNA inactivation can be determined from the measurement of DNA inactivation procedure described above. Alternatively, the UV lights can remain on at all times when the work area is not in use.

TROUBLESHOOTING

If contamination persists, look for shadowed work space areas and nonperpendicular surfaces that escape effective irradiation, and seek other contamination sources, such as reagents, equipment, or surfaces outside of the immediate work area that could contact the operator during setup.

If contamination recurs after it was eliminated by UV irradiation, remeasure the UV intensity at the work surface as described above and compare it with the original intensity. Replace the UV lamps as necessary. UV lamps will still look blue even though their UV output has decreased.

Recurrent contamination may also indicate that separation of contaminating DNA from the PCR setup area is not adequate. It is essential to maintain strict isolation of specimens to prevent contamination

from PCR products, plasmids containing target DNA, and very high levels of concentrated target DNA, such as purified viral or bacterial DNA. Therefore, UV irradiation is an adjunct to proper technique.

REFERENCES

Cone, R.W., A.C. Hobson, M.W. Huang, and M.R. Fairfax. 1990. Polymerase chain reaction decontamination: The wipe test. *Lancet* **336:** 686–687.

Dwyer, D.E. and N. Saksena. 1992. Failure of ultraviolet irradiation and autoclaving to eliminate PCR contamination [letter]. *Mol. Cell. Probes* **6:** 87–88.

Fairfax, M., M. Metcalf, L. Corey, and R.W. Cone. 1991. Slow inactivation of dry PCR templates by UV light. *PCR Methods Appl.* **1:** 142–143.

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.

Frothingham, R., R.B. Blitchington, D.H. Lee, R.C. Greene, and K.H. Wilson. 1992. UV absorption complicates PCR decontamination. *BioTechniques* **13:** 208–210.

Gordon, L.K. and W.A. Haseltine. 1982. Quantitation of cyclobutane pyrimidine dimer formation in double- and single-stranded DNA fragments of defined sequence. *Radiat. Res.* **89:** 99–112.

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.

Kwok, S. and R. Higuchi. 1989. Avoiding false positives with PCR. *Nature* **339:** 237–238.

Meier, A., D.H. Persing, M. Finken, and E.C. Bottger. 1993. Elimination of contaminating DNA within polymerase chain reaction reagents: Implications for a general approach to detection of uncultured pathogens. *J. Clin. Microbiol.* **31:** 646–652.

Sarkar, G. and S.S. Sommer. 1991. Parameters affecting the susceptibility of PCR contamination to UV inactivation. *BioTechniques* **10:** 589–594.

——. 1993. Removal of DNA contamination in polymerase chain reaction reagents by ultraviolet irradiation. *Methods Enzymol.* **218:** 381–388.

Specificity, Efficiency, and Fidelity of PCR

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INTRODUCTION

The efficacy of PCR is measured by its specificity, efficiency (i.e., yield), and fidelity. A highly specific PCR generates one and only one amplification product that is the intended target sequence. More efficient amplification generates more products with fewer cycles. A highly accurate (i.e., high-fidelity) PCR contains a negligible amount of DNA polymerase-induced errors in its product. An ideal PCR would have high specificity, yield, and fidelity. Studies indicate that each of these three parameters is influenced by numerous components of PCR, including the buffer conditions, the PCR cycling regime (i.e., temperature and duration of each step), and DNA polymerases. Unfortunately, adjusting conditions for maximum specificity may not be compatible with high yield; likewise, optimizing for the fidelity of PCR may result in reduced efficiency. Thus, when setting up a PCR, one should know which of the three parameters is the most important for its intended application, and optimize the PCR accordingly. For instance, for direct sequencing analysis of a homogeneous population of cells (either by sequencing or by restriction fragment length polymorphism [RFLP]), the yield and specificity of PCR is more important than the fidelity. On the other hand, for studies of individual DNA molecules or rare mutants in a heterogeneous population, fidelity of PCR is vital. This chapter discusses essential components of PCR and how each influences the specificity, efficiency, and fidelity of PCR.

SETTING UP PCR

Template

Virtually all forms of DNA and RNA are suitable substrates for PCR. These include genomic, plasmid, and phage DNA, previously amplified DNA, cDNA, and mRNA. Samples prepared via standard molecular methodologies (Sambrook et al. 1989) are sufficiently pure for PCR, and usually no extra purification steps are required. Shearing of genomic DNA during DNA extraction does not affect the efficiency of PCR (at least for the fragments that are less than about 2 kb). In general, the efficiency of PCR is greater for smaller-size template DNA (i.e., previously amplified fragment, plasmid, or phage DNA) than for high-molecular-weight (i.e., undigested eukaryotic genomic) DNA. Thus, mechanical shearing and/or rare restriction enzyme digestion of genomic DNA prior to PCR are suggested for increasing the yield (Coen 1991).

Typically, 0.1–1 μg of mammalian genomic DNA is utilized per PCR (Saiki et al. 1985; Scharf et al. 1986; Mullis and Faloona 1987; Keohavong et al. 1988b; Sambrook et al. 1989). For reproducible PCR, less than 10 μg of DNA is recommended. Assuming that a haploid mammalian genome (3×10^9 bp) weighs about 3.4×10^{-12} g, 1 μg of genomic DNA corresponds to approximately 3×10^5 copies of autosomal genes. For bacterial genomic DNA or a plasmid DNA, which represent a much less complex genome, as little as picogram (10^{-12} g) to nanogram (10^{-9} g) quantities are used per reaction (Sambrook et al. 1989; Coen 1991). Previously amplified DNA fragments have also been utilized as PCR templates. Purification of the amplified product is highly recommended if the initial PCR generated a number of unspecific bands, or if a different set of primers (i.e., internal primers) are to be utilized for the subsequent PCR. On the other hand, if the amplification reaction contains only the intended target product, and the purpose of the subsequent PCR is simply to increase the overall yield utilizing the same set of primers, no further purification is required. One could simply take out a small aliquot of the original PCR mixture and subject it to a second round of PCR. In addition to the purified form of DNA, PCR from cells has also been demonstrated. In this laboratory, direct amplification of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) exon 3 fragment from 1×10^5 human cells (following proteinase treatment to open up the cells) has been routinely carried out (P. Keohavong, unpubl.).

Primer Design

For many applications of PCR, primers are designed to be exactly complementary to the template. However, for other applications, such as allele-specific PCR, the engineering of mutations or new restriction endonuclease sites into a specific region of the genome, and cloning of

homologous genes where sequence information is lacking, base pair mismatches are introduced either intentionally or unavoidably (Coen 1991). In either case, an ideal set of primers should hybridize efficiently to the target sequence with negligible hybridization to other related sequences that are present in the sample. Primers are typically 15–30 bases long. Assuming that the nucleotide sequences of the genome are randomly distributed, the probability of finding a match using a set of 20-base-long primers is $(1/4)^{(20+20)} = 9 \times 10^{-26}$. Because there are 3×10^9 bp per haploid mammalian genome, it is highly unlikely that this set of primers will find another perfectly matched template in the genome. However, amplification of unspecific products in PCR using a set of 20-base-long primers is not uncommon. This is likely due to the fact that primers containing a number of mismatches still are amplified under most PCR conditions, and that the nucleotide sequences of the genome are, in fact, not randomly distributed. Researchers have been successful in eliminating unspecific PCR products by adding the final ingredient (usually the polymerase) when the reaction mixture is hot (hot start PCR; D'Aquila et al. 1991) or by using nested primers (Mullis and Faloona 1987). To optimize the specificity of the genes suspected to be duplicated in the genome, primer sequences should be selected from intronic regions of the gene, because they are divergent even in members of tandemly repeated gene families.

Reaction Mixture

The "standard" buffer for *Taq* polymerase-mediated PCR contains 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at room temperature), and 1.5 mM $MgCl_2$ (Coen 1991). The "standard" buffers for other DNA polymerases including modified T7 or Sequenase (Keohavong et al. 1988b), T4 (Keohavong et al. 1988a), Klenow (Mullis and Faloona 1987), Vent (New England Biolabs 1990, 1991), and *Pfu* (see Stratagene catalog) are also available. Although the standard buffer works well for a wide range of templates and oligonucleotide primers, the "optimal" buffer for a particular PCR varies depending on the target and the primer sequences, and the concentrations of other components in the reaction (i.e., dNTP and primers). Therefore, these so-called "standard" conditions should be regarded as a point of departure to explore modifications and potential improvements. In particular, the concentration of Mg^{++} should be optimized whenever a new combination of target and primers is first used or when the concentration of dNTPs or primers is altered. dNTPs are the major source of phosphate groups in the reaction, and any change in their concentration affects the concentration of available Mg^{++}. The presence of divalent cations is critical, and it has been shown that magnesium ions are superior to manganese, and that calcium ions are ineffective (Chien et al. 1976). In addition to the

standard components of the PCR buffer mentioned above, some researchers routinely use additional components such as gelatin, Triton X-100, or bovine serum albumin for stabilizing enzymes, and glycerol (Cha et al. 1992; Cheng et al. 1994; Varadaraj and Skinner 1994), dimethylsulfoxide (DMSO; Mullis and Faloona 1987; Cheng et al. 1994; Varadaraj and Skinner 1994), or formamide (Sarkar et al. 1990; Cheng et al. 1994; Varadaraj and Skinner 1994) for enhancing specificity. It has been proposed that these reagents enhance the specificity of PCR by lowering melting and strand separation temperatures (Cheng et al. 1994). This in turn facilitates denaturation of the template and increases the specificity of primer annealing.

Primers and dNTP

To maximize the efficiency of PCR, one must ensure that the reaction mixture contains nonlimiting amounts of primers and dNTPs. Typically, in a 100-μl reaction mixture, between 0.3 μM (1.8×10^{13} molecules) and 3 μM (1.8×10^{14} molecules) of each primer and between 37 μM (2.2×10^{15} molecules) and 1.5 mM (9×10^{16} molecules) of each dNTP are utilized. For a genomic DNA PCR containing 1 μg of template DNA (3×10^5 copies of autosomal genes), the molar ratio between the primers and the genomic target sequence is at least 10^8 to 1. Having such a large excess of primers ensures that once template DNA becomes denatured, it will anneal to primers rather than to itself. Because the maximum copy number of amplified target sequence is about 10^{12} copies (see Fig. 2 and Exponential Phase of PCR, below), each primer is always in at least 10-fold excess of the target sequence (assuming that primers are not consumed by generating unspecific amplification products). The ratio between the primer and template is also important with regard to the specificity of PCR. If the ratio is too high, PCR is more prone to generate unspecific amplification products, and primer-dimers are also formed. However, if the ratio is too low (i.e., <0.1% of the standard condition for the genomic DNA PCR), the efficiency of PCR is greatly compromised.

For primers, the fraction of free (i.e., unincorporated) primers is strictly dependent on how many target sequences are generated. The fraction of free dNTPs, however, depends not only on the number of target sequences generated, but also on the size of the target sequence. For example, generating 10^{12} copies of a 100-bp target sequence consumes $10^{12} \times 100 = 10^{14}$ dNTP molecules. On the other hand, generating 10^{12} copies of a 2-kb fragment consumes 2×10^{15} dNTP molecules and effectively decreases the concentration of free dNTP. This, in turn, has a deteriorative effect on the overall efficiency of PCR. Thus, for amplifying a large target sequence, a higher concentration of dNTP is recommended (Keohavong et al. 1988b).

PCR Cycle

A typical PCR cycle consists of three steps: (1) a denaturation step (1–2-minute incubation at ≥94°C); (2) a primer annealing (or hybridization) step (1–2-minute incubation at 50–55°C); and (3) an extension step (1–2-minute incubation at 72°C). It has been hypothesized that each of the three steps in the cycle requires a minimal amount of time to be effective, whereas too much time at each step can be both wasteful (time wise) and deleterious to the DNA polymerase (Coen 1991). On the other hand, at least for relatively short DNA fragments (i.e., 100–200 bp long), PCR consisting of two steps (e.g., a denaturation step: 94°C incubation step for 1 minute followed by a primer hybridization/extension step at 50–57°C for 1 minute) can generate as much product as a three-step PCR (Cha et al. 1992). This has been the case for at least four different sets of primers tested on two different genes (Cha et al. 1992). It is possible that due to the high processivity of *Taq*, primers that anneal to the template become fully extended during the short time period during which the reaction mixture reaches the optimal temperature for *Taq* polymerase (70°C–75°C) between the 50°C to 94°C transition.

This notion is also consistent with the results of "rapid PCR" (Wittwer and Garling 1991). In an attempt to increase the speed of temperature cycling (i.e., reduce ramp times), researchers have used capillary tubes as containers and air as the heat-transfer medium for PCR. Standard protocols for a 30-cycle amplification using microfuge tubes are usually 2–6 hours in length. Using a rapid cycler, the authors completed 35 cycles of three-step PCR in 15 minutes. In this rapid PCR, each cycle consisted of a ≤1-second denaturation step at 94°C, a ≤1-second annealing step at 45°C, and a 10-second elongation step at 72°C. In addition to improving cycle times, the rapid cycle PCR amplification was more specific than three-step PCR using a conventional thermal cycler. One possible limitation of the currently available rapid PCR technique is its small reaction volume (10 μl). Because of these volume constraints, only 50 ng of DNA was used as a PCR template. Since 50 ng of mammalian DNA represents about 1.5×10^4 copies, it would not be useful for detecting rare mutations in mammalian cells. Nevertheless, rapid PCR could be used effectively for the analysis of less complex genomes (i.e., bacteria, plasmid, or phage) and/or homogeneous populations. It should also be pointed out that rapid PCR generates as much product as conventional heat-block PCR (i.e., $1–5 \times 10^{12}$ copies) (P. Andre and W. Thilly, unpubl.).

Finally, as in the case of the "standard" PCR buffer, the "standard" three-step PCR regime should also be viewed as a point of departure from which further improvement can be made. In general, higher annealing temperature and shorter time allowed for annealing and extension steps improve the specificity of PCR. Also, in amplifying large

fragments (i.e., >1 kb), it is necessary to increase the duration of each step to get efficient amplification (Kwok et al. 1990; Coen 1991).

EXPONENTIAL PHASE OF PCR

To set up an informative and analytical PCR, one must understand the kinetics of specific product accumulation during PCR. A schematic representation of different products accumulating as a function of cycle is depicted in Figure 1. The desired blunt-ended duplex fragments appear for the first time during the third cycle of the PCR, and from this point on, this product accumulates exponentially according to the formula, $N_f = N_o (1 + Y)^n$, where N_f is the final copy number of the double-stranded target sequence, N_o is the initial copy number, Y is the efficiency of primer extension per cycle, and n is the number of PCR cycles under conditions of exponential amplification (Keohavong et al. 1988b). As depicted in Figure 2, in most cases, once the final copy number of the desired fragment (N_f) reaches about 10^{12}, its efficiency per cycle (Y) drops dramatically, and the product stops accumulating exponentially. This drop in efficiency likely reflects that enzymes become limiting in the reaction. Because products are accumulating exponentially, adding twice as much enzyme at this stage will only support one additional cycle of PCR. The exponential phase of a PCR refers to the early cycle period during which the products accumulate in a manner that is consistent with the equation above. Continuing PCR beyond this point often results in amplification of unspecific bands, the appearance of small deletion mutant bands, and, in certain instances, the disappearance of the specific product (G. Hu, unpubl.). One can overcome these undesired effects of "overamplification" and achieve additional amplification by taking a small aliquot of the reaction mixture that has already undergone 10^6–10^7 doublings and placing it in a fresh reaction mixture.

For many applications of PCR, especially the ones that are quantitative in nature, it is critical that amplification is carried out in the exponential phase of PCR (Fig. 2). Numerous laboratories have studied the efficiencies of different DNA polymerases that are utilized in PCR. As a result, we now have a fairly good idea regarding how efficient different DNA polymerases are in a typical PCR (Keohavong and Thilly 1988; Ling et al. 1991). By using the equation above, a knowledge of the initial copy number permits one to estimate how many cycles will be required for the final copy number to reach about 10^{12}. For example, for *Taq* PCR (assuming efficiency per cycle of 70%) starting with 1 μg of genomic DNA (e.g., 3×10^5 copies of mammalian genome), the equation becomes $10^{12} = 3 \times 10^5 (1 + 0.7)^n$. Solving for n gives 28.6, indicating that in this hypothetical case, the desired product will accumulate exponentially up to about cycle number 29. Thus, an analysis that is quantitative in nature must be carried out on the samples that are taken out at or before the 29th cycle. In fact, since

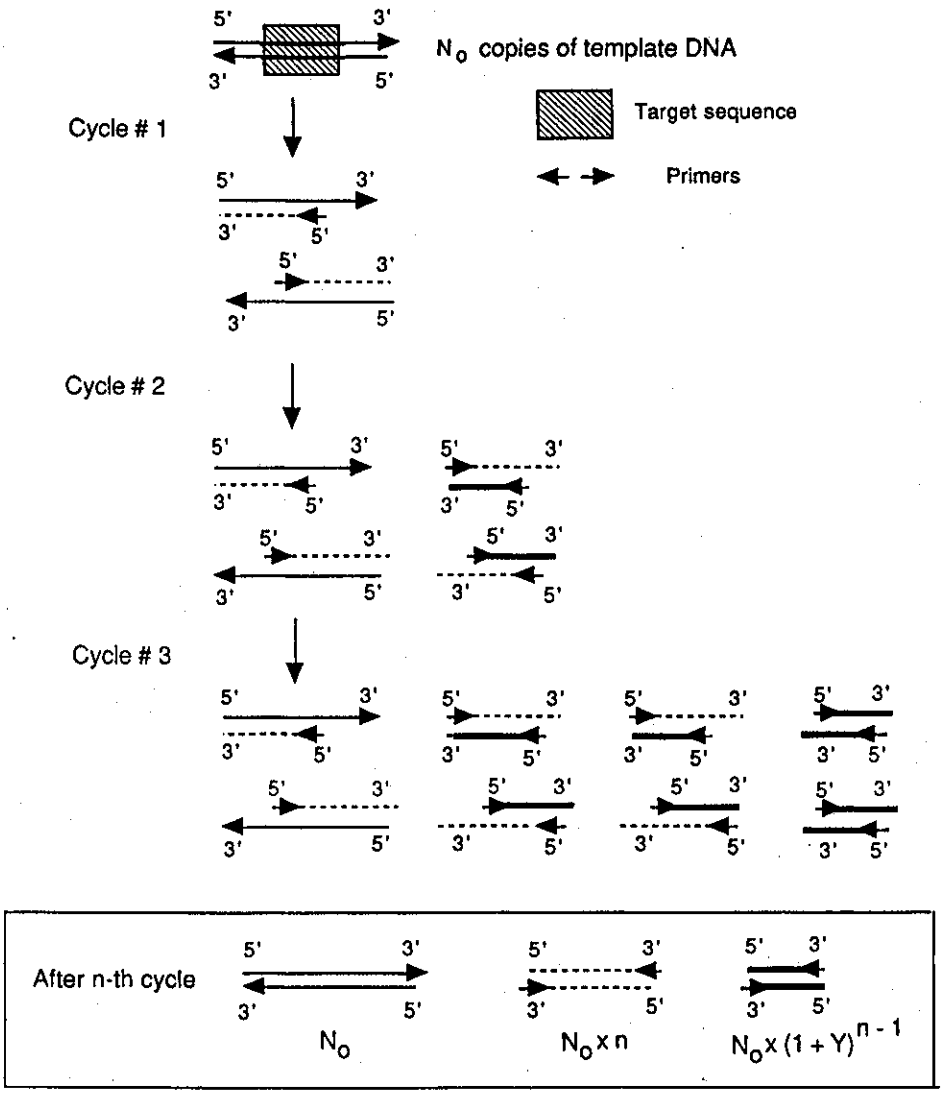

Figure 1 Schematic representation of PCR. N_0 copies of duplex template DNA are subjected to n cycles of PCR. During each cycle, duplex DNA is denatured by heating, which then allows primers (arrows) to anneal to the target sequence (hatched square). In the presence of DNA polymerase and dNTPs, primer extension takes place. The desired blunt-ended duplex product (thick bars with arrows) appears during the third cycle and accumulates exponentially during subsequent cycles. Following n cycles of exponential PCR, there will be $N_0 (1 + Y)^{n-1}$ copies of the duplex target sequence.

10^{12} copies of a particular sequence are sufficient for most applications in molecular biology, there is no apparent reason to carry out additional cycles.

The efficiency of the same polymerase can vary significantly, depending on the nature of the target sequence, the primer sequences, and the reaction conditions (Eckert and Kunkel 1991; Ling et al.

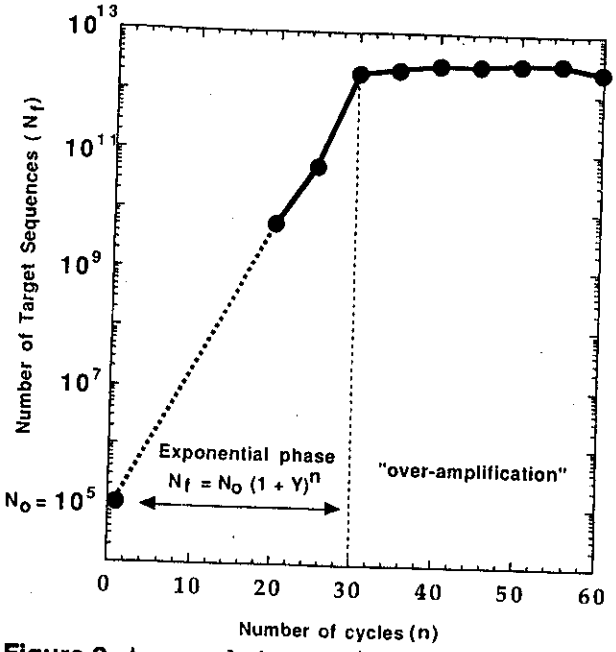

Figure 2 Accumulation of target sequence during PCR as a function of number of cycles. Approximately 10^5 (N_0) copies of rat Ha-*ras* gene exon 1 are subjected to 60 cycles of PCR under a standard *Taq* PCR condition (Chien et al. 1976). A 2.5-μl aliquot is taken at 20, 25, 30, 35, 40, 45, 50, 55, and 60 cycles (n) and analyzed on a polyacrylamide gel. The number of target sequences generated at each stage (N_f) is estimated based on the intensity of the band following ethidium bromide staining. *Taq* (2.5 units) is added following 30 cycles of PCR.

1991). Therefore, the efficiencies listed in Table 1 may not reflect the efficiency of a different PCR carried out under different conditions. The reported values can be used to make a reasonable estimate. Nevertheless, because each specific PCR amplification has a different efficiency, to carry out an accurate quantitative analysis, one needs to determine the efficiency of the particular PCR (see Fig. 2).

DNA POLYMERASES AND PCR

In vitro DNA replication has been accomplished by DNA polymerases from many different sources (Saiki et al. 1985, 1988; Mullis and Faloona 1987; Keohavong et al. 1988a; New England Biolabs 1990, 1991; see also Stratagene catalog). The initial PCR procedure described by Saiki et al. (1985) used the Klenow fragment of *Escherichia coli* DNA polymerase I. This enzyme was heat labile, and, as a result, fresh enzyme had to be added during each cycle following the denaturation and primer hybridization steps. Introduction of the thermostable *Taq* polymerase in PCR (Saiki et al. 1988) subsequently alleviated this tedium and made possible automation of the thermal cycling portion of the procedure. For PCR, thermostable DNA

Table 1 Summary of PCR Conditions

Enzyme	dNTP (mM)	pH	Mg (mM)	Efficiency per cycle (%)	Error rate (error/bp incorporated)	PCR-induced mutant fraction[a] (%)	No. of cycles required[b]	References
Pfu	0.1	8.4	1.5	60	7×10^{-7}	0.3	30	P. Andre (unpubl.)
T4	2.15	8.0	5	56	3×10^{-6}	2	32	Keohavong and Thilly (1989)
T7	3.5	8.0	2.5	90	4.4×10^{-5}	13	22	Keohavong and Thilly (1989)
Vent	0.5–1.5	8.5	7.5	70	4.5×10^{-5}	16	26	Ling et al. (1991)
Taq	0.5–1.5	8.0	5	36	7.2×10^{-5}	25	45	Ling et al. (1991)
Taq	16.6	8.8	10	88	2×10^{-4}	56	22	Dunning et al. (1988); Keohavong and Thilly (1989)
Klenow	1.5	7.9	10	80	1.3×10^{-4}	41	24	Mullis and Faloona (1987); Keohavong and Thilly (1989)

[a]Fraction of PCR-induced noise following 10^6-fold amplification of 200-bp target sequence given the error rate.
[b]Number of cycles required to obtain 10^6-fold amplification given the efficiency per cycle.

polymerases (e.g., *Taq*, Vent, and *Pfu*) are preferred over heat-labile polymerases (e.g., T4, T7, and Klenow) simply because they are much easier to handle and, most importantly, they are amenable to automation.

Studies have shown that different DNA polymerases have distinct characteristics that affect the efficacy of PCR. For example, *Taq* polymerase does not have the 3′→5′ exonuclease proofreading function, and, as a result, it has a relatively high error rate in PCR (Table 1). On the other hand, its inability to edit mispaired 3′ ends has been an asset for researchers who developed the allele-specific PCR based on the concept that primers containing mismatches at the 3′ end were not extended as efficiently as the perfectly matched primers (Newton et al. 1989; Wu et al. 1989; Kwok et al. 1990; Cha et al. 1992; Bottema and Sommer 1993). This concept would not have worked for enzymes with exonuclease activities, because once the 3′ mismatch was recognized by the polymerase, it would first be repaired and would then be extended, thus abolishing the specificity conferred by the 3′ mismatches. As applications of PCR become increasingly sophisticated and specific, distinctive properties of polymerases should be utilized to meet specific needs.

Fidelity of in vitro DNA polymerization is perhaps one of the most intensively studied subjects in PCR. For many applications of PCR, where a relatively homogeneous DNA population is analyzed (i.e.,

direct sequencing or restriction endonuclease digestion), the polymerase-induced mutations during PCR are of little concern. In general, polymerase-induced mutations are randomly distributed over the amplified fragment, and an accurate consensus sequence is usually obtained. However, PCR is also used for studies of rare molecules in heterogeneous populations. Examples include the study of allelic polymorphisms in individual mRNA transcripts (Frohman et al. 1988; Lacy et al. 1989), the characterization of the allelic stages of single sperm cells (Li et al. 1990) or single DNA molecules (Jeffreys et al. 1990; Ruano et al. 1990), and the characterization of rare mutations in a tissue (Cha et al. 1992) or a population of cells in culture. For these applications, it is vital that the polymerase-induced mutant sequences do not mask the rare DNA sequences. Each polymerase-induced error, once introduced, is amplified exponentially along with the original wild-type sequences during subsequent cycles. This results in an overall increase in the fraction of polymerase-induced mutant sequences as a function of the number of amplification cycles. Analyses that use small amounts of template DNA are especially prone to PCR-induced artifacts. For example, if one were to carry out PCR with 10 copies of template DNA, any polymerase-induced mutation during the first few cycles would appear as a major mutant population in the final PCR products. Because the number of copies of template DNA is low and the error rate of *Taq* polymerase is about 10^{-4}, the probability of this event occurring is low (i.e., 10^{-3}). However, if such an event should occur, the particular mutation induced by the polymerase would comprise as much as 10% of the final PCR products. One can prevent this "jackpot" artifact by starting with a large amount of template DNA (i.e., $\geq 10^5$ copies). In this case, about 10 mutations are introduced on the average during the first cycle of the PCR; however, all of these mutations constitute only about $1/10^5$ of the final products.

Under low-fidelity conditions (i.e., *Taq* or Klenow PCR), this mutant fraction can become significant. For example, following one million-fold amplification by a DNA polymerase with an error rate of 10^{-4}, the PCR-induced error constitutes as much as 33% of the 200-bp-long amplified products.[1] Assuming that polymerase errors are uniformly distributed, the error frequency per base, on average, is 1.7×10^{-3} ($0.33 \times 200 = 0.0017$). This level of PCR-induced noise will certainly hinder attempts to characterize rare mutations in tissue cul-

[1]The fraction of PCR-induced mutants is calculated according to a formula $F(>1) = 1 - e^{-bfd}$, where b is the length of the target sequence, f is the error rate, and d is the number of doublings (Newton et al. 1989; Wu et al. 1989). Thus, following a 10^6-fold amplification (e.g., 20 doublings) of a 200-bp fragment at an error rate of 10^{-4}/bp incorporated will lead to an estimated PCR-induced mutant fraction of 33% ($1 - e^{-(200)(10^{-4})(20)} = 0.33$).

ture or in animals and humans, where the expected mutant frequency of a particular mutation could be as low as 10^{-7} or 10^{-8}.

The fidelity of PCR varies depending on reaction conditions and the nature of the target sequences. In the past, several groups have found conditions that permitted more accurate PCR by modifying reaction buffer conditions. For instance, Ling et al. (1991) were able to reduce the error rate of *Taq* PCR by a factor of 2.8 (from 2×10^{-4} to 7.2×10^{-5}) by modifying reaction conditions. One may assess the significance of this 2.8-fold improvement on *Taq* PCR fidelity by comparing the fractions of PCR-induced noise before and after the improvement. According to the formula, $F(>1) = 1 - e^{-bfd}$ (see footnote 1) (Eckert and Kunkel 1991), 56% of the PCR product amplified under the low-fidelity condition is *Taq* polymerase-induced noise (Table 1). On the other hand, only 25% of the PCR product generated under the high-fidelity condition is polymerase-induced noise. In this case, a 2.8-fold reduction in the *Taq* polymerase error rate reduced the overall PCR-induced mutant fraction by more than half (Table 1). Thus, it is indeed possible to improve the overall fidelity of PCR substantially by adjusting reaction conditions. Nevertheless, it must be pointed out that despite much effort to optimize the fidelity of *Taq*, T7, and Vent PCR by altering reaction conditions, their improved fidelity has never reached the level of *Pfu* or T4 polymerase (Keohavong and Thilly 1989; Ling et al. 1991), suggesting that some intrinsic properties of the polymerase also contribute to its overall error rate. Regarding the error rates of exo+ polymerases, one should realize that the measured error rate reflects the average value of a heterogeneous population of DNA polymerases; this heterogeneity presumably arises as a result of errors during transcription of the gene. It is possible that some of the transcription errors are introduced in the region of the gene that is critical for fidelity of the polymerase (i.e., the proofreading function), and thus increase the average error rate. If this is the case, one may be able to enhance the fidelity of exo+ polymerase PCR by devising a means to physically separate or biologically inactivate these rare exo– mutant polymerases (W. Thilly, unpubl.).

In addition to the error rate during PCR, the kinds of mutations that are introduced during PCR are also dependent on DNA polymerases. Whereas GC to AT transitions are the predominant mutations for T4 and T7 polymerases, AT to GC transitions are most frequently observed with *Taq* polymerase (Keohavong and Thilly 1989). *Taq* polymerase is also highly prone to generating deletion mutations if the template DNA has the potential to form secondary structures (Cariello et al. 1991). The Klenow fragment induces possible transitions and deletions of 2 and 4 bp. These observations again suggest that each polymerase has distinctive modes of operation regarding fidelity in in vitro replication.

The findings that different polymerases induce different types mutations in PCR also have a very practical value in designing PC based experiments. For example, if one were to look for a rare alle that had undergone a GC to AT transition, it would be best to use *Ta* polymerase for PCR. Because *Taq* predominantly induces AT to G transitions (Keohavong and Thilly 1989), using *Taq* will minimiz false-positive cases that may arise as a result of a *Taq* polymeras induced artifact. In another hypothetical case, assume that *Taq* PC followed by sequencing analysis, either by cloning and sequencing o by denaturant gradient gel electrophoresis (DGGE)-type analysis fo lowed by sequencing, reveals that in the population of cells analyzed a rare AT to GC mutant allele exists at a frequency of 10^{-3}. However this mutation is the type of mutation expected from *Taq* amplification thus one is not sure if this is a true variant in the original sample or PCR artifact. To distinguish between these two possibilities, the sam analysis can be carried out again using a T7 or T4 polymerase, to se whether the AT to GC mutations appear again. If this AT to GC muta tion appears following PCR mediated by two different enzymes with different mutational specificities, then it is fair to say that the mutatio existed in the original sample.

Because of its thermostability, reliability, and durability, *Taq* DN polymerase has been most widely used in PCR. However, as summarized in Table 1, the fidelity of *Taq* (2×10^{-4} error/bp per duplication) is the lowest among DNA polymerases whose fidelity has bee measured. This, in turn, effectively prevents using *Taq* polymerase i a PCR where the fidelity is of concern. Recently, a number of addi tional thermostable enzymes have been isolated. Unlike *Taq*, which does not have the $3' \rightarrow 5'$ exonuclease proofreading function, these newly isolated enzymes (e.g., Vent and *Pfu*) do have the editing function, and, as expected, they are more accurate than *Taq* polymerase. Error rates for *Pfu* and Vent have been estimated to be 7×10^{-7} and 4.5×10^{-5} errors/bp per duplication, respectively (Table 1). The fraction of PCR-induced noise in a 200-bp target sequence following a 10^6-fold amplification in these cases would be 0.3% for *Pfu* and 16% for Vent polymerases (56% for *Taq* PCR; see Table 1).

ANALYZING FIDELITY OF PCR

For many applications of PCR where rare variants are involved, the fidelity of PCR is an important concern. A number of laboratories have studied the fidelity of PCR, and the error rates of commonly utilized DNA polymerases are known (Table 1). However, because the fidelity of a polymerase varies significantly depending on the reaction conditions and the nature of the target sequences, it needs be determined on a sequence-by-sequence and/or a reaction condition-by-reaction condition basis. There are at least three independent methods of measuring the fidelity of PCR: (1) the forward mutation assay, (2) the reversion mutation assay, and (3) the DGGE-type analysis.

Forward Mutation Assay

The forward mutation assay consists of cloning individual DNA molecules from the amplified population and determining the number of DNA sequence changes according to what fraction of the cloned population displays a particular phenotype (Loeb and Kunkel 1982; Eckert and Kunkel 1990, 1991). For example, one can assess the error rate during synthesis of the *lacZ* gene by the frequency of light blue and colorless (mutant) plaques among the total plaques scored. The nature of mutations can also be determined by DNA sequence analysis of a collection of the mutants.

Reversion Mutation Assay

The second method is a reversion mutation assay using a phage template DNA that contains specific mutations resulting in a measurable phenotype (i.e., *lacZ* $^-$, or colorless phenotype). In these assays, polymerase-induced errors are scored as DNA sequence changes that revert the mutant to a wild-type or pseudo-wild-type phenotype. This approach is especially useful for highly accurate polymerases (Eckert and Kunkel 1991).

Reversion assays are focused on a limited subset of errors occurring at only a few sites. As mentioned above, in general, polymerase-induced mutations are randomly distributed throughout a target sequence. However, a number of locations in the target sequence are more prone to polymerase-induced errors (Keohavong and Thilly 1989). Thus, error rates measured by the reversion assay may vary significantly depending on the nature of the initial mutations placed in the phage template.

DGGE-type Analysis

DGGE is a system that separates DNA fragments harboring small changes (i.e., single-base substitutions, small additions, or deletions) based on their sequences. In this case, DGGE is used to separate polymerase-induced mutant sequences from the correctly amplified sequences. By measuring the fraction of signals coming from the portion of the gel corresponding to the polymerase-induced mutant sequences (heteroduplex fraction), one can calculate the fidelity of the enzyme according to a formula, $f = HeF/(b \times d)$, where f is the error rate (errors per bp incorporated per duplication), HeF is the heteroduplex fraction, b is the length of the single-strand low-melting domain in which mutants can be detected, and d is the number of DNA duplications (Keohavong and Thilly 1989; Ling et al. 1991). Unlike the other two assays in which only the changes that result in phenotypic changes are scored as PCR-induced mutations, DGGE al-

lows the visualization and detection of all the mutations introduced in the target sequence. This feature makes DGGE the most comprehensive and sensitive means of measuring PCR fidelity among the currently available techniques.

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REFERENCES

Bottema, C.D.K. and S.S. Sommer. 1993. PCR amplification of specific alleles: Rapid detection of known mutation and polymorphisms. *Mutat. Res.* **288:** 93–102.

Cariello, N.F., W.G. Thilly, J.A. Swenberg, and T.R. Skopek. 1991. Deletion mutagenesis during polymerase chain reaction: Dependence on DNA polymerase. *Gene* **99:** 105–108.

Cha, R.S., H. Zarbl, P. Keohavong, and W.G. Thilly. 1992. Mismatch amplification mutation assay (MAMA): Application to the c-H-*ras* gene. *PCR Methods Appl.* **2:** 14–20.

Cheng, S., C. Fockler, W.M. Barnes, and R. Higuchi. 1994. Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci.* **91:** 5695–5699.

Chien, A., D.B. Edgar, and J.M. Trela. 1976. Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus. J. Bacteriol.* **127:** 1550.

Coen, D.M. 1991. The polymerase chain reaction. In *Current protocols* (ed. F.M. Ausubel et al.), pp. 15.01–15.40. Wiley, New York.

D'Aquila, R.T., L.J. Bechtel, J.A. Videler, J.J. Eron, P. Gorczyaca, and J.C. Kaplan. 1991. Maximizing sensitivity and specificity of PCR by preamplification heating. *Nucleic Acids Res.* **19:** 3749.

Dunning, A.M., P. Talmud, and S.E. Humphries. 1988. Errors in the polymerase chain reaction. *Nucleic Acids Res.* **16:** 10393.

Eckert, K.A. and T.A. Kunkel. 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Nucleic Acids Res.* **18:** 3739–3744.

——. 1991. DNA polymerase fidelity and the polymerase chain reaction. *PCR Methods Appl.* **1:** 17–24.

Frohman, M.A., M.K. Dush, and G.R. Martin. 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene specific oligonucleotide primer. *Proc. Natl. Acad. Sci.* **85:** 8998–9002.

Jeffreys, A.J., R. Neumann, and V. Wilson. 1990. Repeat unit sequence variation in minisatellites: A novel source of DNA polymorphism for studying variation and mutation by single molecule analysis. *Cell* **60:** 473–485.

Keohavong, P. and W.G. Thilly. 1989. Fidelity of DNA polymerases in DNA amplification. *Proc. Natl. Acad. Sci.* **86:** 9253–9257.

Keohavong, P., A.G. Kat, N.F. Cariello, and W.G. Thilly. 1988a. Laboratory methods: DNA amplification in vitro using T4 DNA polymerase. *DNA* **7:** 63–70.

Keohavong, P., C.C. Wang, R.S. Cha, and W.G. Thilly. 1988b. Enzymatic amplification and characterization of large DNA fragments from genomic DNA. *Gene* **71:** 211–216.

Kwok, S., D.E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, and J.J. Sninsky. 1990. Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. *Nucleic Acids Res.* **18:** 999–1005.

Lacy, M.J., L.K. McNeil, M.E. Roth, and D.M. Kranz. 1989. T-cell receptor δ-chain diversity in peripheral lymphocytes. *Proc. Natl. Acad. Sci.* **86:** 1023–1026.

Li, H., X. Cui, and N. Arnheim. 1990. Direct electrophoretic detection of the allelic state of a single DNA molecule in human sperm by using the polymerase chain reaction. *Proc. Natl. Acad. Sci.* **87:** 4580–4584.

Ling, L.L., P. Keohavong, C. Dias, and W.G. Thilly. 1991. Optimization of the polymerase chain reaction with regard to fidelity: Modified T7, *Taq*, and Vent DNA polymerases. *PCR Methods Appl.* **1:** 63–69.

Loeb, L.A. and T.A. Kunkel. 1982. Fidelity of DNA synthesis. *Annu. Rev. Biochem.* **52:** 429–457.

Mullis, K.B. and F.A. Faloona. 1987. Specific synthesis

of DNA in vitro via a polymerase-catalysed chain reaction. *Methods Enzymol.* **155:** 335–350.

New England Biolabs. 1990, 1991. Vent™ DNA polymerase technical bulletin. Beverly, Massachusetts.

Newton, C.R., A. Graham, L.E. Heptinstall, S.J. Powell, C. Summers, N. Kalsheker, J.C. Smith, and A.F. Markham. 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system. *Nucleic Acids Res.* **17:** 2503–3516.

Ruano, G., K.K. Kidd, and J.C. Stephens. 1990. Haplotype of multiple polymorphisms resolved by enzymatic amplification of single DNA molecules. *Proc. Natl. Acad. Sci.* **87:** 6296–6300.

Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230:** 1350–1354.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science* **239:** 487–491.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual,* 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sarkar, G., S. Kapelner, and S.S. Sommer. 1990. Formamide can dramatically improve the specificity of PCR. *Nucleic Acids Res.* **18:** 7465.

Scharf, S.J., G.T. Horn, and H.A. Erlich. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science* **233:** 1076–1078.

Varadaraj, K. and D.M. Skinner. 1994. Denaturants or cosolvents improve the specificity of PCR amplification of a G+C rich DNA using genetically engineered DNA polymerases. *Gene* **140:** 1–5.

Wittwer, C.T. and D.J. Garling. 1991. Rapid cycle DNA amplification: Time and temperature optimization. *BioTechniques* **10:** 76–83.

Wu, D.Y., L. Ugozzoli, B.J. Pal, and R.B. Wallace. 1989. Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia. *Proc. Natl. Acad. Sci.* **86:** 2757–2760.

Optimization and Troubleshooting in PCR

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INTRODUCTION

The use of PCR to generate large amounts of a desired product can be a double-edged sword. Failure to amplify at optimum conditions can lead to the generation of multiple undefined and unwanted products, even to the exclusion of the desired product. At the other extreme, no product may be amplified. A typical response at this point is to vary one or more of the many parameters that are known to contribute to primer-template fidelity and primer extension. High on the list of optimization variables are Mg^{++} concentrations, buffer pH, and cycling conditions. With regard to the last variable, the annealing temperature is most important. The situation is further complicated by the fact that some of the variables are quite interdependent. For example, because dNTPs directly chelate a proportional number of Mg^{++} ions, an increase in the concentration of dNTPs decreases the concentration of free Mg^{++} available to influence polymerase function.

Touchdown PCR

Touchdown (TD) PCR represents a fundamentally different approach to PCR optimization (Don et al. 1991). Rather than using multiple reaction tubes, each with different reagent concentration and/or cycling parameters, a single tube or a small set of tubes is run under cycling conditions that inherently favor amplification of the desired amplicon, often to the exclusion of artifactual amplicons and primer-dimers. Multiple cycles are programmed so that the annealing segments in sequential cycles are run at incrementally lower temperatures (see below). As cycling progresses, the annealing-segment temperature, which was selected to be initially above the suspected T_m, gradually declines to, and falls below, this level. This strategy

helps ensure that the first primer-template hybridization events involve only those reactants with the greatest complementarity, i.e., those yielding the target amplicon. Even though the annealing temperature may eventually drop down to the T_m of nonspecific hybridizations, the target amplicon will have already begun its geometric amplification and is thus in a position to outcompete any lagging (nonspecific) PCR products during the remaining cycles. Because the aim is to avoid low-T_m priming during the earlier cycles, it is imperative that the hot start modification (D'Aquila et al. 1991; Erlich et al. 1991; Mullis 1991) (see below) be used with TD PCR. TD PCR should be viewed not as a method of determining the optimum cycling conditions for a specific PCR, but as a potential one-step method for approaching optimal amplification. We have found that a variety of otherwise satisfactory single-amplicon-yielding reactions are rendered more robust (i.e., yield more product) when subjected to TD PCR (K.H. Roux and K.H. Hecker, unpubl.).

TD PCR is of particular value when the degree of identity between the primer and template is unknown (Roux 1994). This situation often arises when primers are designed on the basis of amino acid sequences, members of a multigene family are amplified, or evolutionary PCR is attempted; i.e., amplification of DNA from one species using primers with identity to a homologous segment of another species. In such cases, the mismatches between the primers and template may have lowered the T_m of the target amplicon enough to approach those of the spurious priming sites. Degenerate primers with multiple base variation or inosine residues are often used in such situations (Knoth et al. 1988; Lee et al. 1988; Patil and Dekker 1990; Batzer et al. 1991; Peterson et al. 1991), but the greater variety of sequences in the former case and the relaxed stringency in the latter case might tend to increase the chances of nonspecific priming. Moreover, in some cases the locations of potential base mismatches are unknown. Although TD PCR can be used with degenerate primers (Batzer et al. 1991), we have shown that nondegenerate primers displaying a significant degree of template-sequence mismatch can yield single-target amplicons of single-copy genes from genomic DNA under standard buffer conditions (Roux 1994). Even mismatches clustered near the 3′ end of the primer are tolerated.

PROTOCOL

Programming the Thermal Cycler for TD PCR

The goal in programming for TD PCR is to produce a series of cycles with progressively lower annealing temperatures. The annealing temperature range should span about 15°C and extend from at least a few degrees above to 10 or so degrees below the estimated T_m. For example, for a calculated primer-template T_m of 62°C with no degen-

eracy, program the thermal cycler to decrease the annealing temperature 1°C every second cycle (i.e., run 2 cycles per degree) from 65°C to 50°C, followed by 15 additional cycles at 50°C.

Some thermal cyclers (e.g., Perkin-Elmer model 9600 and MJ Research model PTC-100) readily accommodate TD PCR and are easily programmed to decrease the temperature of a segment automatically by a fixed amount per cycle (e.g., 0.5°C/cycle). For others, a long series of files must be linked or extensive strings of commands entered. In these latter cases, it may be more convenient to create a "generic" TD PCR program covering a broader temperature range (~20°C) than to reprogram every time the range needs to be modified by a few degrees. Another alternative to programming restrictions and inconvenience is to use fewer but more abrupt steps (e.g., seven 2°C steps or five 3°C steps); however, doing so may decrease the chances for discriminating between products with two closely spaced T_m values.

The continued presence of spurious bands following TD PCR indicates that the initial annealing temperature was too low, that there is a relatively small gap between the T_m values of the target and unwanted amplicons, and/or that the unwanted amplicons are being more efficiently amplified. Raising the number of cycles per 1°C descending step to 3 or 4 will give the target amplicon added competitive advantage before the initiation of the spurious amplification. A proportional number of cycles should be removed from the end of the program to prevent excess cycling and the concomitant degradation of the amplicon and generation of high-molecular-weight smears (Bell and DeMarini 1991).

Modifications of TD PCR for use with degenerate and mismatched primers include lowering the annealing temperature range (e.g., 50°C declining to 35°C) and running the last 15 cycles at 50°C.

Optimization Strategy

The example given is for TD PCR, but the same principles apply to conventional PCR.

1. Design optimal primer pairs that are closely matched in T_m. For additional discussion of primer design, see Dieffenbach et al. in Section 3 (this volume).

2. Calculate or estimate approximate T_m. Program the thermal cycler for TD PCR as described above.

3. Set up several standard hot start PCR mixes incorporating a range of Mg^{++} concentrations and including appropriate positive and negative controls. Use 10^4–10^5 copies of the template.

4. Amplify as above and analyze products.
   a. If weak or no product is detected:
      - Subject reaction tubes to 10 additional cycles at constant annealing temperature (i.e., 55°C) and recheck.
      - Reamplify 10-fold dilutions (1:10 to 1:1000) of initial TD PCR at fixed annealing temperature for 30 cycles.
      - Use more template and check for inhibitor in template preparation by spiking original PCR mix with dilutions of known positive (demonstrably amplifiable) template.
      - Add, extend, or increase the temperature of the initial template denaturation step prior to cycling (5 minutes at 95°C is standard).
      - Vary concentrations of buffer components (pH, *Taq* DNA polymerase, dNTPs, primers).
      - Add enhancers to PCR mix (see below).
      - Reamplify dilutions (1:10 to 1:1000) of the first reaction using nested primers.
      - Abandon this primer set, design new primers, and begin again. Depending on one's degree of impatience and tolerance for frustration, this step might supersede any of the above.
   b. If multiple products or a high-molecular-weight smear is observed:
      - Raise the maximum and minimum annealing temperatures (i.e., move the range upward) in the TD PCR program.
      - Remove some cycles from the bottom of the range and/or from the terminal constant temperature cycles.
      - Increase the number of cycles per degree annealing temperature by 1 cycle, i.e., to 3 cycles per degree. Doing so may necessitate removing some lower end and/or terminal cycles to prevent smearing due to excess cycling.
      - Vary concentrations of buffer components (pH, *Taq* DNA polymerase, dNTPs, primers).
      - Attempt band purification followed by reamplification. Target bands can be cut from gels and allowed to diffuse out or be liberated by freeze/thaw cycles or enzymatic gel digestion. Alternatively, a small plug of gel can be removed with a micropipette tip or, most simply, by stabbing the band directly in the gel with an autoclaved toothpick and inoculating a fresh reaction tube.

- Reamplify $1:10^4$ and $1:10^5$ dilutions of first reaction using nested primers.
- If all else fails, abandon primer set, design new primers, and begin again.

OTHER OPTIMIZATION STRATEGIES

Several other optimization strategies have been developed for standard PCR, although most are applicable to TD PCR as well. Each is discussed briefly below. Variables that affect PCR product specificity and yield are listed in Table 1.

Enhancing Agents

Various additives such as DMSO (1–10%), PEG-6000 (5–15%), glycerol (5–20%), nonionic detergents, formamide (1.25–10%), and bovine serum albumin (10–100 μg/ml) can also be incorporated into the reaction to increase specificity and yield (Pomp and Medrano 1991; Newton and Graham 1994). In fact, some reactions may amplify only in the presence of such additives (Pomp and Medrano 1991). Several optimization kits incorporating these and other enhancing agents and a variety of buffers are currently marketed (e.g, by Continental Laboratory Products, Invitrogen, Perkin-Elmer, and Stratagene). Additional discussion of PCR optimization and contamination-avoidance strategies can be found in Newton and Graham (1994).

Table 1 Conditions Favoring Enhanced Specificity

	Use hot start
	Use TD PCR (favors enhanced specificity and yield)
	Optimize primer design
↓	Mg^{++}
↓	dNTP (also favors higher fidelity)
	Optimize pH
↓	*Taq* DNA polymerase
↓	Cycle segment lengths
↓	Number of cycles
↑	Annealing temperature
↓	Inhibitors
↑	Ramp speed
↑	Chance that target temperature is achieved in each tube
	Add and optimize enhancer(s)
↓	Primer concentration
↓	Primer degeneracy
↑	Template denaturation efficiency

Adjusting conditions in the direction opposite that listed above usually favors increased sensitivity (i.e., more product) and the concomitant risk of nonspecific amplification. The aim is to strike a balance between these two opposing tendencies. ↑ and ↓ signify increase and decrease, respectively.

Matrix Analyses

The basic challenge is to devise an optimization protocol that is efficient in both time and cost. A full matrix analysis in which several values for each of the variables are tested in combination with each of the other variables can quickly become overwhelmingly cumbersome and costly. The size of the matrix can be significantly pared down by applying the Taguchi method (Taguchi 1986), in which several key variables are simultaneously altered (Cobb and Clarkson 1994). A more typical strategy is to run a simple matrix analysis focused on those parameters most likely to have the greatest impact on PCR primer hybridization and enzyme fidelity, e.g., Mg^{++} concentration and annealing temperature.

Mg^{++} Concentration

Mg^{++} concentration is the easiest parameter to manipulate because all concentration variations can be run simultaneously in separate tubes. Suppliers of *Taq* polymerase now provide the $MgCl_2$ solution separate from the standard reaction buffer to simplify its adjustment. A typical two-step optimization series might first include Mg^{++} at 0.5-mM increments from 0.5 to 5.0 mM and, after the range is narrowed, a second round covered by several 0.2- or 0.3-mM increments.

Annealing Temperature

Optimization of annealing temperature begins with calculation of the T_m values of the primer-template pairs by one of several methods, the simplest being $T_m = 4(G + C) + 2(A + T)$. A single-base mismatch lowers the T_m by about 5°C. More complex formulas can also be used (Sambrook et al. 1989; Sharrocks 1994), but in practice, because the T_m is variously affected by the individual buffer components and even the primer and template concentrations, any calculated T_m value should be regarded as an approximation. Several reactions run at temperature increments (2–5°C) straddling a point 5°C below the calculated T_m give a first approximation of the optimum annealing temperature for a given set of reaction conditions. It should be noted that some primers, for reasons that are not entirely apparent, are refractory to optimization (He et al. 1994). One possible explanation may be that unique characteristics of the target amplicon give a T_m above the temperature of the denaturation cycle segment (Sharrocks 1994). If permissible, it may be more time- and cost-efficient simply to design a second set of primers that hybridize to neighboring DNA.

Cycle Number, Reamplification, and Product Smearing

Increasing the number of cycles may enhance an anemic reaction, but this modification can also lead to the generation of spurious bands and to smears composed of high-molecular-weight products rich in single-stranded DNA (Bell and DeMarini 1991). Similar smearing can occur under normal conditions if the quantity of starting template is too great, as often occurs in attempts to reamplify from a previous PCR. A general rule of thumb is to use 1 μl of a $1:10^4$ to $1:10^5$ dilution of a PCR product if a gel band is detectable.

Nested PCR

Nested and semi-nested PCRs are often quite successful in reducing or eliminating unwanted products while at the same time dramatically increasing sensitivity (Mullis and Faloona 1987; Gibbs 1990; Mullis 1991; Zhang and Ehrlich 1994; Zimmerman et al. 1994). An initial set of primers straddling the DNA segment of interest is first amplified under standard conditions. Spurious products are frequently primed with one or both primers and contain irrelevant sequences internally. An aliquot of the reaction-product mix is then subjected to an additional round of amplification using primers complementary to the sequences internal to the first set of primers. Only the legitimate product should be amplified in this second round. This approach is often successful even if the desired product is initially below the level of detection by ethidium bromide staining and in the presence of visible spurious bands. Semi-nested PCR, in which a second primer is internal to only one end of the target segment, can be equally effective (Zhang and Ehrlich 1994). This variation is often required for gene walking or attempts at 5′ or 3′ RACE in which the template DNA sequence internal to only one of the primers is known.

A second form of artifact, known as jumping PCR, may not be eliminated by nested PCR. Incompletely extended products can occasionally rehybridize to an adjacent segment of DNA, perhaps to a similar gene element, to prime an unintended product (Huang and Jeang 1994). In such instances, the sequence internal to one or both primers is still present, but the amplicon size differs.

If nested PCR methods are employed, better results may be obtained if the first and second rounds of amplification are terminated after 20 or so cycles rather than the usual 30–35. This modification minimizes the chances of generating unwanted high-molecular-weight bands and smears (Bell and DeMarini 1991; Zhang and Ehrlich 1994). Such artifacts often contain considerable single-stranded DNA and appear to be the result of mispriming by DNA products amplified in earlier cycles. Nested PCR is extremely sensitive; as little as a single copy of a viral gene has been detected in a background of 10^6 genomes (Zimmerman et al. 1994).

Hot Start PCR

Even brief incubations of a PCR mix at temperatures significantly below the T_m can result in primer-dimer and nonspecific priming. Hot start PCR methods (D'Aquila et al. 1991; Erlich et al. 1991; Mullis 1991) can dramatically reduce these problems. The aim is to withhold at least one of the critical components from participating in the reaction until the temperature in the first cycle rises above the T_m of the reactants. For example, in smaller assays incorporating an oil overlay, one of the components common to all tubes (e.g., *Taq* DNA polymerase) can be initially withheld and added only after the temperature rises above 80°C during the first denaturing stage. Alternatively, a wax bead can be melted over the bulk of the reaction mix in each tube and allowed to solidify, and the withheld component can be pipetted on top of the wax cap. These beads can be made in the laboratory (Bassam and Caetano-Anolles 1993; Wainwright and Seifert 1993) or purchased (Ampliwax PCR Gems, Perkin-Elmer). During the temperature ramp into the first denaturation segment, the wax melts and the final component becomes incorporated and mixed by convection in each tube, a great convenience when dealing with large numbers of tubes. A recent hot start variation involves adding specific anti-*Taq* DNA polymerase antibody (TaqStart Antibody, CLONTECH) to the PCR tubes prior to the addition of *Taq* DNA polymerase. The antibody prevents polymerase activity from beginning until the temperature rises to dissociate and denature the blocking antibody. This modification is compatible with newer thermal cyclers and techniques that seek to avoid the extra handling and purification steps accompanying oil and wax addition and sample recovery.

TROUBLESHOOTING

- *Little or no detectable product.* You have adjusted the Mg^{++} concentration, buffer pH, and cycling parameters; added more cycles; and tried lower annealing temperatures and TD PCR. You still see no product on ethidium bromide-stained gels (acrylamide gels are considerably more sensitive than agarose gels), yet your positive controls indicate no reagent problems. What should be the next step? Lengthening the initial denaturation step and/or increasing the temperature increases the likelihood that the template DNA is fully denatured to provide the maximal number of priming sites. Standard conditions for this optional step are 5 minutes at 95°C. An in-tube thermocouple can be used to predetermine that the indicated temperature corresponds to the actual sample temperature. Amplification may have occurred, but it could have been inefficient. If so, the amplicons can be revealed by a probe of the dried gel or a blot. A secondary amplification using the same primers or,

preferably, nested primers may be all that is needed to generate a specific product. Serial 10-fold dilutions ranging from 1:100 to 1:10,000 should be used.

Little or no product may indicate the presence of inhibitors in the DNA sample. Numerous inhibitors of PCR have been described. These include ionic detergents (e.g., SDS and Sarkosyl) (Weyant et al. 1990), phenol, heparin (Beutler et al. 1990), xylene cyanol, and bromphenol blue (Hoppe et al. 1992). Test for inhibitor in the template preparation by spiking the original PCR mix with dilutions of known positive (demonstrably amplifiable) template. Reextraction, ethanol precipitation, and/or centrifugal ultrafiltration may resolve the problem. Proteinase K carryover can serve to digest the *Taq* DNA polymerase, but it is readily denatured by a 5-minute incubation at 95°C.

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REFERENCES

Bassam, B.J. and G. Caetano-Anolles. 1993. Automated "hot start" PCR using mineral oil and paraffin wax. *BioTechniques* **14:** 30–34.

Batzer, M.A., J.E. Carlton, and P.L. Deininger. 1991. Enhanced evolutionary PCR using oligonucleotides with inosine at the 3′-terminus. *Nucleic Acids Res.* **19:** 5081.

Bell, D.A. and D. DeMarini. 1991. Excessive cycling converts PCR products to random-length higher molecular weight fragments. *Nucleic Acids Res.* **19:** 5079.

Beutler, E., T. Gelbart, and W. Kuhl. 1990. Interference of heparin with the polymerase chain reaction. *BioTechniques* **9:** 166.

Cobb, B.D. and J.M. Clarkson. 1994. A simple procedure for optimizing the polymerase chain reaction (PCR) using modified Taguchi methods. *Nucleic Acids Res.* **22:** 3801–3805.

D'Aquila, R.T., L.J. Bechtel, J.A. Viteler, J.J. Eron, P. Gorczyca, and J.C. Kaplin. 1991. Maximizing sensitivity and specificity of PCR by preamplification heating. *Nucleic Acids Res.* **19:** 3749.

Don, R.H., P.T. Cox, B.J. Wainwright, K. Baker, and J.S. Mattick. 1991. "Touchdown" PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* **19:** 4008.

Erlich, H.A., D. Gelfand, and J.J. Sninsky. 1991. Recent advances in the polymerase chain reaction. *Science* **252:** 1643–1651.

Gibbs, R.A. 1990. DNA amplification by the polymerase chain reaction. *Anal. Chem.* **62:** 1202–1214.

He, Q., M. Marjamaki, H. Soini, J. Mertsola, and M.K. Viljanen. 1994. Primers are decisive for sensitivity of PCR. *BioTechniques* **17:** 82–87.

Hoppe, B.L., B.M. Conti-Tronconi, and R.M. Horton. 1992. Gel-loading dyes compatible with PCR. *BioTechniques* **12:** 679–680.

Huang, L.-M. and K.-T. Jeang. 1994. Long-range jumping of incompletely extended polymerase chain fragments generates unexpected products. *BioTechniques* **16:** 242–246.

Knoth, K., S. Roberds, C. Poteet, and M. Tamkun. 1988. Highly degenerate inosine-containing primers specifically amplify rare cDNA using the polymerase chain reaction. *Nucleic Acids Res.* **16:** 10932.

Lee, C., X. Wu, R.A. Gibbs, R.G. Cook, D.M. Muzny, and C.T. Caskey. 1988. Generation of cDNA probes directed by amino acid sequence: Cloning of urate oxidase. *Science* **239:** 1288–1291.

Mullis, K.B. 1991. The polymerase chain reaction in an anemic mode: How to avoid cold oligodeoxyribonuclear fusion. *PCR Methods Appl.* **1:** 1–4.

Mullis, K. and F.A. Faloona. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155:** 335–350.

Newton, C.R. and A. Graham. 1994. *PCR.* Bios Scientific, Oxford.

Patil, R.V. and E.E. Dekker. 1990. PCR amplification of an *Escherichia coli* gene using mixed primers containing deoxyinosine at ambiguous positions in degenerate amino acid codons. *Nucleic Acids Res.* **18:** 3080.

Peterson, M.G., J. Inostroza, M.E. Maxon, O. Flores, A. Adomon, D. Reinberg, and R. Tjian. 1991. Structure and functional properties of human general transcription factor IIE. *Nature* **354:** 369–373.

Pomp, D. and J.F. Medrano. 1991. Organic solvents as facilitators of polymerase chain reaction. *BioTechniques* **10:** 58–59.

Roux, K.H. 1994. Using mismatched primer-template pairs in touchdown PCR. *BioTechniques* **16:** 812–814.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sharrocks, A.D. 1994. The design of primers for PCR. In *PCR technology: Current innovations* (ed. H Griffin and A.M. Griffin), pp. 5–11. CRC Pre Boca Raton, Florida.

Taguchi, G. 1986. *Introduction to quality engineeri* Asian Productivity Organisation, UNIPUB, N York.

Wainwright, L.A. and H.S. Seifert. 1993. Paraffin bead can replace mineral oil as an evaporation barri in PCR. *BioTechniques* **14:** 34–36.

Weyant, R.S., P. Edmonds, and B. Swaminathan. 199 Effect of ionic and nonionic detergents on the *Ta* polymerase. *BioTechniques* **9:** 308–309.

Zhang, X-Y. and M. Ehrlich. 1994. Detection and quantitation of low numbers of chromosomes con taining *bcl-2* oncogene translocations using semi- nested PCR. *BioTechniques* **16:** 502–507.

Zimmermann, K., K. Pischinger, and J.W. Mannhalte 1994. Nested primer PCR detection limits of HIV- in a background of increasing numbers of lysed cells. *BioTechniques* **17:** 18–20.