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A Biochemical Perspective of the Polymerase Chain Reaction

Will Bloch
Cetus Corporation, Emeryville, California 94608
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In the five years since the first publication (Saiki et al., 1985) on PCR,[1] this method of nucleic acid replication and modification in vitro has grown to rival in popularity traditional microbiological procedures for cloning and related manipulations. The year 1989 saw the publication of over 800 research articles, three review volumes (Erlich, 1989; Erlich et al. 1989; Innis et al., 1989), at least three major review articles (Guatelli et al., 1989; Vosberg, 1989; White et al., 1989), and one set of symposium abstracts [(1989) *J. Cell. Biochem., Suppl. B 13*, 269–313] dealing with PCR, as well as a PCR chapter in the standard molecular biological methods manual (Sambrook et al., 1989). The review volumes and methods manual all present useful strategic and tactical summaries, sufficient to introduce any biochemist to the whys and hows of the technique. How could the world possibly benefit from still another PCR review? To date the PCR literature has emphasized five areas of application: genetic mapping, genetic polymorphism, detection of mutations, molecular virology (especially retrovirology), and transcriptional splicing and regulation (including molecular immunology). The overwhelming focus of concern has been on human pathology and microbiology: infectious disease, genetic disease and disease predisposition, cancer, and the human cellular and humoral responses to disease. The following discussion avoids this well-traveled ground as much as possible. Instead it surveys the PCR literature from the viewpoint of a student of biochemical structure and function who lacks extensive molecular biological experience and needs to choose the optimal methods to manipulate or analyze nucleic acids. For this purpose it divides the biochemist's activities into four major classes: discovery (e.g., of new genes or genotypes), analysis (e.g., of nucleic acid sequence, of allelic or mutational variation, and of quantity of specific sequences), modification (random and directed mutagenesis), and synthesis (e.g., of proteins or of nucleic acids derived from PCR-manipulated nucleic acid). Readers who find this treatment too advanced might want first to study an excellent recent introduction to PCR by Gibbs (1990).

Summary of the Method

In simplest form, PCR is a chemical, as opposed to biological, method of greatly increasing the concentration of a specific nucleic acid sequence relative to that of other nucleic acid in the reaction mixture. Its success depends on intelligent or lucky choice of the following elements: (1) a target sequence in genomic DNA or cDNA, optimally in the 100–1000 nt size range but possibly as long as 10^4 nt, known in sufficient detail to design primers that will anneal to the ends; (2) at least two synthetic oligonucleotide primers significantly complementary to the ends of target sequence on opposite strands, oriented so that their 3′-OH ends point toward one another along the intervening sequence; (3) a DNA polymerase catalyzing 5′ → 3′ extension of primer under the direction of a template DNA strand that has been annealed to primer; (4) concentrations of dNTP's and of a divalent metal ion (Mg^{2+} much preferred) chosen empirically to satisfy poorly understood enzymological requirements; (5) a thermal cycle normally of several minutes duration, repeated 10–50 times, which provides a 40–75 °C temperature for primer-template annealing and enzyme-catalyzed primer extension and a 90–99 °C temperature so that the dsDNA product of one cycle can strand separate to provide the ssDNA template to direct primer extension in the next cycle; and (6) a sealed reaction tube, chemically and physically compatible with the cycling temperature-controlled environment, normally containing only 20–200 μL of buffered reaction mixture so that the heat capacity of the vessel and mixture does not greatly retard attainment of the intended cycle temperatures.

The sequence of events in an amplification is the following: (1) mixing of the buffered PCR reagents and a test sample that might contain target DNA, shortly before thermal cycling is to begin; (2) DNA strand separation at 90–99 °C for up

[1] Abbreviations: PCR, polymerase chain reaction; cDNA, complementary DNA (used here primarily to indicate in vitro reverse transcript); dNTP, deoxyribonucleoside triphosphate; dsDNA, double-stranded DNA; dU, deoxyuridine; nt, nucleotide; NTP, ribonucleoside triphosphate; pol I, DNA polymerase I (or repair polymerase); ssDNA, single-stranded DNA; *Taq*, *Thermus aquaticus*.

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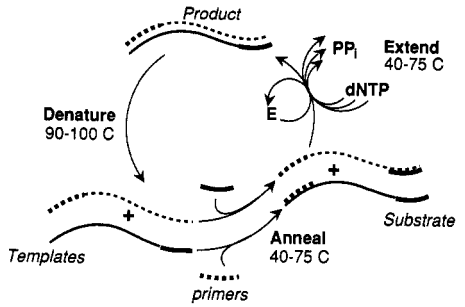

FIGURE 1: Microscopic model of a single PCR cycle. Because each cycle generates up to two molecules of product (completely extended substrate) from each product molecule input from the preceding cycle, exponential accumulation of product over a series of cycles is possible.

Table I: PCR Reactants

component	typical size	typical concn (M)
Taq pol I	94 kDa	10^{-9}
Mg^{2+}		10^{-3}–10^{-2}
dNTP		10^{-5}–10^{-3} (each)
primer	15–30 nt	10^{-7}–10^{-6} (each)
target DNA		
genomic DNA	10^{5}–10^{7} nt	10^{-20}–10^{-15} (1–10^{5} copies/100 μL)
cDNA	10^{2}–10^{4} nt	
intermediate strand	10^{2}–10^{4} nt	10^{-19}–10^{-14} (final)
short strand	10^{2}–10^{3} nt	10^{-10}–10^{-7} (final)

to several minutes; (3) primer annealing and extension for several minutes at 40–75 °C to create a double-stranded product of indefinite length (only one end of each primer extension is specified by a primer-complementary sequence); (4) strand separation of the extended-primer-template duplex at 90–99 °C, usually for no more than a minute; (5) primer annealing and extension for several minutes at 40–75 °C, this time with at least half of the template being supplied by extended primer from the previous cycle ("intermediate template") and therefore directing primer extension to exactly the length defined by spacing of the two primers along the template ("short template"); and (6) repeat of steps 4 and 5 for many cycles, resulting in a linear accumulation of intermediate template and an exponential accumulation of short template. The latter rapidly outnumbers the former to the degree that it forms the only product molecules normally detectable when amplification is stopped.

Table I summarizes the key chemical parameters of an amplification. Figure 1 diagrams the reaction steps without showing the maturation of target structure over the first two cycles. Figure 2 illustrates the evolution of intermediate and short templates from the target DNA in the test sample. Although primer extension commonly is done at a higher temperature than primer-template annealing, there is no theoretical justification for this practice; annealing and extension at a single temperature work just as well (Kim & Smithies, 1988).

Exponential accumulation of the short template usually drops to linear accumulation at a product concentration near 10^{-8} M, and accumulation usually ceases altogether near 10^{-7} M. Typical gain per cycle during the exponential accumulation phase is 1.8–1.95, slightly less than the theoretical maximum of 2.0; therefore 10, 20, and 30 cycles can experience total gains of up to 800, 6.4×10^{5}, and 5.0×10^{8}, respectively, unless limited by onset of the linear accumulation phase.

The preferred enzyme has been a single-subunit bacterial DNA repair polymerase (polymerase I, or pol I), originally the Klenow fragment of *Escherichia coli* pol I (Saiki et al., 1985) but now overwhelmingly the thermoresistant pol I of *Thermus aquaticus* (Saiki et al., 1988; Lawyer et al., 1989). However, another thermoresistant bacterial DNA polymerase (Elie et al., 1988) and thermolabile viral DNA polymerases (Keohavong et al., 1988a,b) also have been made to catalyze the PCR. Use of a thermoresistant enzyme allows a complete amplification reaction to be performed without opening the reaction tube, thereby greatly increasing reliability, precision, convenience, and productivity with respect to most laboratories' limiting resource, labor. Productivity has been enhanced farther by the design of automated, rapidly cycling, reasonably precise, thermostated blocks, ovens, and baths; the various commercially available thermal cyclers allow completely unattended amplification of 25–100 reactions at a time. The great versatility of the method follows from the fact that primer of practically any length and composition and template of any degree of single strandedness are recognized by repair DNA polymerases, unlike most RNA polymerases and some other DNA polymerases. Analytical, synthetic, or modification applications of PCR entail complete or amost complete knowledge of the target sequence; but in discovery PCR, the target sequence is incompletely known, often nothing more than a hypothesis based on genetic homology arguments. Targets at least as long as 3000 nt have been amplified sufficiently for visual detection on an ethidium-stained electrophoresis gel (Grady & Campbell, 1989; Schwarz et al., 1990); a 10 200 nt amplified target has been detected by an isotopically labeled hybridization probe following Southern blotting (Jeffreys et al., 1988). The practical upper bound on PCR product length is unclear; Jeffreys et al. (1988) and Kim and Smithies (1988) showed that longer targets require longer thermal cycle intervals at 40–75 °C. It should not depend strongly on polymerase processivity; *Taq* pol I now appears

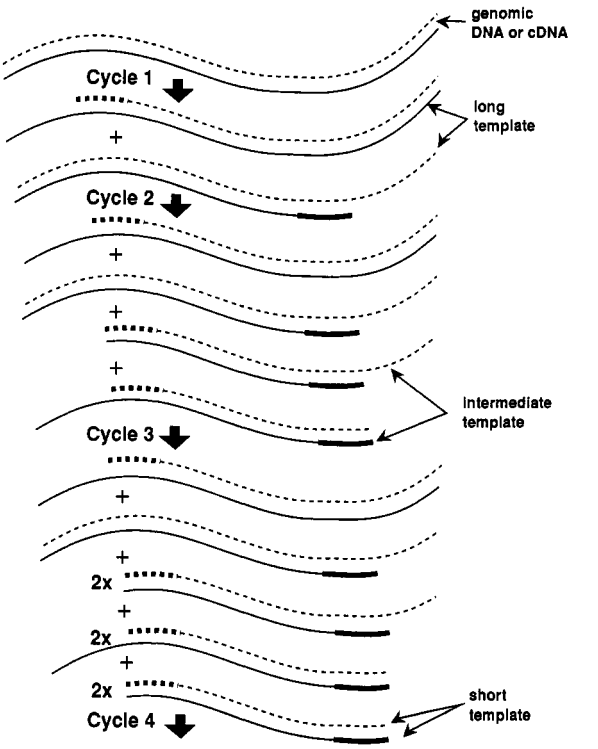

FIGURE 2: Evolution of PCR template size over the first few cycles of an amplification. The ends of the DNA molecules presenting the target sequence in a PCR test sample usually extend beyond the target sequence, defined by the two primers. The initial products of target replication still extend beyond the target sequence in one direction. Only in the third cycle can there begin to accumulate the relatively short duplexes generally described as PCR product.

(Olsen & Eckstein, 1989) not to be nearly as processive as was originally thought (Innis et al., 1988), yet it amplifies well sequences longer than 10^3 nt. Simultaneous use of multiple primer pairs allows coamplification of multiple products (Chamberlain et al., 1988).

PCR is highly tolerant of impurities in the DNA sample being amplified. The degree of nucleic acid purification needed before PCR depends on the complexity and chemistry of the sample matrix as well as the concentration of the target sequence in the sample. A biological sample normally should be deproteinized before introduction into the reaction mixture, if only to assure removal of proteases, nucleases, and phosphatases that might destroy reactants. Phenol–chloroform extraction followed by ethanol precipitation, the standard molecular biological solution to this problem, is PCR-compatible. However, a rich assortment of faster and simpler methods has been validated for particular test samples, including simple incubation of human cells at 100 °C in hypotonic medium (Kogan et al., 1987; Kumar & Barbacid, 1988; Saiki et al., 1986), proteinase K digestion followed by heating at 85–95 °C to kill the proteinase (Li et al., 1988), a combination of thermal lysis and proteinase K treatment (Kim & Smithies, 1988), alkalinization and subsequent neutralization of serum containing viral targets (Kaneko et al., 1989), exposure of bacteria to low osmolarity (Weier & Rosette, 1990), antibody capture of virus followed by detergent lysis (Brown & Robertson, 1990), and chemisorption of base-denatured DNA on a cationic nylon membrane (Kadokami & Lewis, 1990). Nuclei can be isolated from complex, protein-rich, matrices such as blood by osmotic lysis; if proteinase K digestion occurs in a solvent containing 6 M guanidinium chloride as well as detergent, thermal inactivation of the proteinase appears to be unnecessary, having been preempted by autolysis (Jeanpierre, 1987). The simpler methods have been validated for test samples containing low cell numbers, whereas proteinase K digestion or phenol–chloroform extraction is more likely to be needed when the total protein concentration is high.

PCR commonly shows great specificity, in that amplification of a single target sequence from a complex genome, initially present in very low copy number, often results in a single product of exactly the expected size and sequence, despite the myriad opportunities for primer annealing to nontarget sequences. This specificity derives from two important features of the current PCR art: the requirement for annealing of two primers, correctly oriented, within a span of less than about 10^4 nucleotides and the use of a thermoresistant enzyme that allows the annealing and extension temperature(s) to be raised to the highest value at which the lower melting primer-template duplex is stable. If necessary, specificity can be increased by "nested priming"; after an initial amplification, primers are replaced by a primer pair defining a target that lies within the sequence specified by the original primer pair (Haqqi et al., 1988; Kaneko et al., 1989; Kemp et al, 1989; Schowalter & Sommer, 1989). This process, which radically reduces the probability of successful priming of nontargeted amplification, practically assures that only a single PCR product will be obtained.

Discovery PCR

Protein and Gene Discovery. Most discovery efforts focus on protein structural genes that are expressed in a target tissue, often in just a few cells or in low yield. Rappolee et al. (1989) coupled RNA microisolation, reverse transcription, and PCR to recover specific cDNA's from less from 100 copies of a mRNA. Belyavsky et al. (1989) demonstrated a PCR method of developing a cDNA library from low-abundance mRNA. PCR has revealed trace expression of genes in cells not normally expected to transcribe them (Chelly et al., 1989; Sarkar & Sommer, 1989); the Belyavsky method might be able to fish the entire expression repertoire of an organism from the mRNA of a single tissue. Reverse transcriptase often copies rare transcripts incompletely; in other cases, sequence information is too incomplete to specify primers bracketing the entire target sequence. Frohman et al. (1988) and Loh et al. (1989) showed how PCR with a single sequence-specific primer can capture the ends of a reverse transcript to identify entire coding regions and flanking regulatory elements. For examples of how well this strategy works, see Casella et al. (1989) and Delort et al. (1989). The latter authors showed that intermediate in vitro transcription, DNase digestion, and reverse transcription can increase the specificity of the Frohman/Loh procedures, which have reduced ability to discriminate against nontarget sequences because they use only one target-specific primer.

Discovery PCR often exploits homology within gene families to identify and sequence new genes and the proteins for which they code. Protein sequence comparison among a few family members usually identifies evolutionarily conserved regions suitable for designing primers that might bracket the intervening sequence of a new but related gene. "Mixed oligonucleotide", or "degenerate", priming (Lee et al., 1988a; Girgis et al., 1988) is used to seek a PCR product of the length predicted from the known sequences. In degenerate priming, reverse translation yields the complete collection of nucleotide sequences, any one of which the genetic code predicts might encode a conserved amino acid sequence. Automated solid-phase nucleotide synthesis easily introduces base multiplicity at the degenerate positions in the coding sequence so that a single oligonucleotide preparation can supply all possible coding sequences, only one of which might match perfectly the corresponding region of the target genome or cDNA. Primer degeneracy also can be created with deoxyinosine residues (Knoth et al., 1988), which base pair promiscuously. Although base multiplicity is the most common method of creating primer degeneracy, deoxyinosine substitution alone has served this function (Patil & Dekker, 1990). Both sources of degeneracy have been included at different positions in a single degenerate primer pool, deoxyinosine being used where the degeneracy exceeds 2 and pairs of nucleotides serving at 2-fold-degenerate positions (Moremen, 1989).

Primer degeneracy favors nonspecific amplification by increasing the probability of pairwise combination of primer sequences that anneal to the test sample DNA outside of the target region but with the appropriate spacing and orientation to permit amplification. In at least one instance (Bernasconi et al., 1989), control amplifications using each degenerate primer pool without the other have served to identify and exclude nonspecific PCR products resulting from fortuitous priming by oligonucleotides within a single pool. During the design of degenerate pools, degeneracy normally is minimized, especially at the 3′ end of each primer, by strategic placement of the primer sequence along the target sequence. In addition, amplification conditions (reagent concentrations, temperatures, times, cycle number) are sought which yield just one or a few PCR products in the expected size range, reducing the sequencing effort needed to verify that a new member of the gene family has been found. Such discovery PCR has successfully identified new examples of the following proteins: *Drosophila* and human forms of a sequence-specific transcriptional factor (Peterson et al., 1990), an archaebacterial H^+-ATPase regu-

latory subunit (Bernasconi et al., 1989), murine G-protein α subunits (Strathmann et al., 1989), a bovine brain G-protein α subunit (Gautam et al., 1989), the 27-kDa succinate dehydrogenase subunit from several species (Gould et al., 1989), feline herpes virus thymidine kinase (Nunberg et al., 1988), murine protein tyrosine kinases (Wilks, 1989; Wilks et al., 1989), murine immunoglobulin heavy- and light-chain variable regions (Orlandi et al., 1989; Sastry et al., 1989), nematode tyrosine kinases and human K^+ channels (Kamb et al., 1989), human thyroid G-protein-coupled receptors (Libert et al., 1989), rat liver Golgi mannosidase II (Moremen, 1989), nematode and trypanosome serine and cysteine proteases (Sakanari et al., 1989), a putative *Plasmodium* drug-resistance glycoprotein (Wilson et al., 1989), multiple reverse transcriptase coding sequences embedded in the human genome (Shih et al., 1989), and a human integrin β subunit (Suzuki & Naitoh, 1990).

Of course, discovery methods can target genetic material that does not necessarily code for protein sequence, such as 5′-terminal transcriptional regulatory regions (Delort et al., 1989; Brunk & Sadler, 1990), rRNA structural genes (Medlin et al., 1988; Edwards et al., 1989), regulatory cytosine methylation sites and genomic sequences recognized by DNA binding proteins (Saluz & Jost, 1989; Pfeifer et al., 1989; Mueller & Wold, 1989; Steigerwald et al., 1990; Kinzler & Vogelstein, 1989, 1990), proviral integration sites in host cellular DNA (Silver & Keerikatte, 1989), genomic sequences flanking transposable elements (Earp et al., 1990) and telomere-associated tandem repeats (Weber et al., 1990), and initiation sites of DNA replication (Vasilev & Johnson, 1989). In fact, PCR is suited to the characterization of any sequence flanking a region for which primers can be designed (Ochman et al., 1988; Triglia et al., 1988), and often a sequence-specific primer is needed on only one side of a target sequence (Mueller & Wold, 1989). A particularly elegant discovery method for DNA sequences recognized by specific DNA binding proteins screens random oligomers bracketed by a PCR primer sequence and a primer-complementary sequence that can direct the amplification of trace amounts of oligonucleotide recovered from DNA–protein complexes (Thiesen & Bach, 1990). Tuerk and Gold (1990) developed an analogous selection procedure for RNA ligands to specific binding proteins; these authors used several cycles of sequence selection to explore the affinity hierarchy among closely related sequences.

A logically different form of molecular biological discovery seeks nucleic acid sequences present in one test sample and not in another sample closely related to the first one. This "subtractive" strategy entails annealing of the two nucleic acid populations and subsequent separation of single-stranded and double-stranded nucleic acids under conditions enriching for single-stranded nucleic acid which reports on the greater endowment of the first population. Several sequential annealing and separation cycles help to compensate for relatively low enrichment efficiency. However, low recovery from such enrichment, coupled with the fact that the differentially represented sequence usually is a very small fraction of the total nucleic acid, jeopardizes detection of the subtractive "signal". PCR amplification of trace enriched nucleic acid sequences has been used to report on differential gene expression in related cell lines (Timblin et al., 1990) and to model the discovery of genomic differences such as might arise from a large deletion mutation or a host–parasite interaction (Straus & Ausubel, 1990; Wieland et al., 1990).

Discovery of Mutations and Polymorphism. By virtue of the large yield of pure or easily purified amplified target sequence, PCR provides ideal material for the standard methods of discovering mismatches in heteroduplexes between two nucleic acid populations ostensibly containing the same sequence: denaturing gradient gel electrophoresis, hydroxylamine/osmium tetraoxide chemical cleavage, and RNase A cleavage. PCR actually improves the first procedure by simplifying the addition of an "artifical" GC-rich sequence to the 5′ end of one primer to heighten sensitivity to single base-pair mismatches (Sheffield et al., 1989). A recent comparison of the three methods in combination with PCR found such GC-clamped denaturing gradient gel electrophoresis to be the most sensitive (Theophilus et al., 1989); other workers report satisfaction with chemical cleavage (Grompe et al., 1989; Montandon et al., 1989). PCR also interfaces effectively with a new method of mutation screening that does not rely on heteroduplex formation, instead exploiting an electrophoretic mobility shift induced by incorporation of biotinylated mononucleotides into single-stranded DNA (Kornher & Livak, 1989). Furthermore, it is claimed that single-base substitutions are indicated reliably by mobility shifts when denatured PCR product is polyacrylamide gel electrophoresed under nondenaturing conditions, simply as a result of conformation changes in ssDNA (Orita et al., 1989). This phenomenon provides a second way to screen for mutations without forming heteroduplexes. The major limitation of PCR with respect to screening for genetic variation lies in the effects of infidelity, discussed below under *Sequencing*; PCR-generated mutational "background" gets worse as the target size or total cycle number is increased (Reiss et al., 1990).

Analysis

Sequencing. PCR discovery requires validation by sequencing. PCR also is a natural bridge between classical molecular biological discovery and sequencing, thanks to the variety of ingenious methods developed for direct sequencing of the PCR product. The linkage between PCR and sequencing was reviewed recently by Gyllensten (1989). PCR product can be sequenced after subcloning into standard sequencing plasmids. This process is facilitated by the ease with which PCR product is 5′-phosphorylated and blunt-end ligated. However, blunt-end ligation is unnecessary because restriction sites can be included in the 5′-terminal regions of PCR primers (along with sequencing-primer-complementary sites, if desired). Because subcloning is vulnerable to errors introduced during PCR or prior reverse transcription, subclone sequencing created a natural concern about polymerase infidelity, or error generation, during amplification.

Efforts to characterize *Taq* pol I fidelity fall into three classes: those simply reporting the sequence error rate in 5–10 replicate clones after standard PCR (Fucharoen et al., 1989; Newton et al., 1988; Collins et al., 1988; Dunning et al., 1988; Saiki et al., 1988), one isolating approximately 10 mutant/native target heteroduplexes from a single amplification by denaturing gradient gel electrophoresis and sequenced reamplified mutant species (Keohavong & Thilly, 1989), and one applying an M13 bacteriophage model system in a nonamplification context where sequencing is optional and where it is possible to collect reasonable error frequency statistics (Tindall & Kunkel, 1988; Eckert & Kunkel, 1990). These studies, reporting mutant frequencies ranging from zero per five clones for a 3000-nt target to three per five clones for a 220-nt target, provide no predictive consensus on *Taq* pol I and PCR fidelity for several reasons.

(1) Two major solvents are used: one recommended by Cetus Corp., which contains no thiol, adjusts ionic strength with KCl, and buffers at pH 8.3–8.5 (room temperature) with

10 mM Tris-HCl; and one suggested by New England Biolabs, which contains 10 mM mercaptoethanol, adjusts ionic strength in part with ammonium sulfate, and buffers at pH 8.8 with 67 mM Tris-HCl. The latter solvent appears to give a higher error rate than the former. Eckert and Kunkel (1990) reported (without comment) a severalfold enhancement of mutation rate upon shifting from 20 mM MES to 20 mM Tris buffer. If this is truly a specific ion effect, 67 mM Tris should be proportionately more mutagenic, and replacement of Tris with a zwitterionic or anionic buffer might be generally beneficial.

(2) Experimental values of dNTP concentration range from 200 μM to 1.5 mM (for each dNTP), although there are sound indications from a second model system, not yet applied to *Taq* pol I, that the error rate should vary directly with dNTP concentration and that reducing dNTP concentration to near 10 μM might increase fidelity without reducing enzyme activity (Petruska et al., 1988; Mendelman et al., 1990). *Taq* pol I results of Kwok et al. (1990) also favor much lower dNTP concentrations.

(3) Some of the papers that report sequenced PCR product subclones are short on experimental detail.

(4) There is no uniform language or conceptual structure for describing polymerase fidelity. Mutant frequencies originally expressed per clone sequenced may be divided by the length of the PCR product sequenced to obtain an error rate per nucleotide; but the calculations of Tindall and Kunkel (1988) obtained a somewhat larger number, dividing the mutant frequency (per plaque counted) by the number of sites in the target sequence where sequencing has located mutations. These loci represented less than half of the total number of residues in the particular target sequence chosen. This model system normalization is unavailable to the average PCR user, whose sample size is too small to saturate the polymerase-induced mutational hot spots unique to each target sequence. PCR users also should correct the error rate per nucleotide for the fact that the final observed mutation frequency reflects mutational events occurring at any cycle of the amplification, with mutations in earlier cycles being more heavily weighted in the final distribution (Saiki et al., 1988). What ultimately limits the predictive value of most error-rate estimates is the expense of subclone sequencing, which subjects any experiment to the statistics of small numbers. Precision estimates normally do not accompany experimental error-rate values.

Given these manifold uncertainties, the absence of an experimentally tested model for the template sequence dependence of *Taq* pol I infidelity, and the absence of a sufficiently comprehensive experimental survey of the independent variables controlling fidelity to teach us how to minimize the error rate confidently, the advisable remedies for the fact of polymerase infidelity are (1) if subcloning, to sequence several subclones derived from any amplification, (2) to avoid subclone sequencing of long ($>10^3$ nt) PCR products, which have a significant probability of containing no error-free sequences (Reiss et al., 1990), (3) preferably to sequence PCR product directly without subcloning, so that widely distributed but infrequent errors will be masked by the population-average sequence, and (4) to direct-sequence or subclone-sequence the PCR product only from large ($>10^5$) target copy numbers in order to minimize the number of cycles and the statistical weight of any early-cycle error (Krawczak et al., 1989). Eckert and Kunkel (1990) suggested that fidelity can be increased an order of magnitude by working at low pH and strictly limiting the Mg^{2+} concentration, but the practicality of such conditions for PCR is unclear. The sequence data from all three kinds of fidelity studies suffice to reject one, nonenzymatic, mechanistic hypothesis for PCR infidelity; by far the majority of recorded *Taq* pol I induced mutations alter A or T residues, not G or C, so that cytidine deamination to uracil at the high temperatures of thermal cycling cannot be a major mutational pathway. Single-base deletions are much rarer than substitutions, and insertions are much rarer than deletions (Tindall & Kunkel, 1988). The growing body of literature analyzing DNA polymerase fidelity in terms of the catalytic mechanism (Petruska et al., 1988; Mendelman et al., 1989, 1990; Lai & Beattie, 1988; El-Deiry et al., 1988; Kuchta et al., 1988) provides the intellectual framework for understanding *Taq* pol I fidelity; but the substantive conclusions, many of which are based on enzymes possessing a $3' \rightarrow 5'$ exonuclease "proofreading" activity absent in *Taq* pol I (Tindall & Kunkel, 1988), are not easily generalizable to PCR. Given the natural movement from subclone sequencing to direct sequencing of PCR product, the most serious practical consequence of *Taq* pol I infidelity is not in the sequencing realm but rather in the mutational background that it imposes on heteroduplex-based mutation detection (Reiss et al., 1990) and in the upper bound it sets on the size of the PCR product that can be cloned accurately. However, fidelity control also influences three practical applications of PCR discussed below, allele-specific amplification, the detection of target sequences from genetically plastic or highly polymorphic sources, and random mutagenesis by PCR.

Most direct sequencing of double-stranded PCR products has relied on procedures optimized for covalently closed circular plasmids (Edwards et al., 1989; Newton et al., 1988; Higuchi et al., 1988a; Wrischnik et al., 1987). Such methods may be suboptimal for relatively short, linear PCR products, which shows accelerated kinetics for complementary strand reannealing; this reaction competes with sequencing primer annealing and extension. Casanova et al. (1990) reported the systematic optimization of reaction conditions to minimize the seriousness of this competition. Several strategies have been reported for recovering from PCR amplifications relatively pure single strands that can be sequenced with high efficiency; asymmetric PCR (Gyllensten & Erlich, 1988; Innis et al., 1988), exonuclease digestion of one strand extended from a 5'-phosphorylated primer (Higuchi & Ochman, 1989), avidinylated solid-phase capture of PCR product containing only one biotinylated primer, followed by DNA denaturation and removal of the unbiotinylated strand under conditions that do not destroy biotin–avidin binding (Hultman et al., 1989; Syvänen et al., 1989; Mitchell & Merril, 1989), and sequencing of RNA transcripts of PCR product strands extended from primers containing a bacteriophage promoter sequence recognized by a phage RNA polymerase (Sarkar & Sommer, 1988; Stoflet et al., 1988). A different approach to efficient sequencing of PCR product single strands employs phosphorothioate incorporation during amplification with primers, only one of which is ^{32}P-labeled, followed by random iodoethanol or 2,3-epoxy-1-propanol alkylation, unimolecular cleavage of the resulting phosphotriester, and gel electrophoresis of the cleavage fragments (Nakamaye et al., 1988). An analogous chemical sequencing method that employs different cleavage chemistries is even simpler because it requires only a single PCR rather than a separate reaction for each α-thio-dNTP; it has been validated for nonisotopic detection with fluorescent primers (Voss et al., 1989). A variant of phosphorothioate-directed PCR product fragmentation for sequencing exploits the facts that *Taq* polymerase is not very processive and that snake venom $3' \rightarrow 5'$ exonuclease is effectively stopped by a 3'-terminal phosphorothioate (Olsen & Eckstein, 1989) to

create all possible phosphorothioate-terminated exonuclease digestion fragments. Except for asymmetric PCR, each of these methods of avoiding complementary-strand interference chooses the strand to be sequenced by 5′-terminal tagging one primer. The procedure selected determines whether the tag consists of a nonradioactive or radioactive phosphoryl group, biotin, a phage promoter sequence, or a fluorophore. Asymmetric PCR accomplishes strand selection simply by controlling the mole ratio of the two amplification primers.

Tangential to the evolution of PCR technology has been the use of thermoresistant DNA polymerases not only for amplification but also for reverse transcription of rRNA (Jones & Foulkes, 1989) and mRNA (Tse & Forget, 1990) and for sequencing, regardless of the source of the sequencing template (Innis et al., 1988; Bechtereva et al., 1989; Mardis & Roe, 1989). These developments permit cDNA generation and sequencing at elevated temperatures, where template secondary structure is less likely to interfere with primer extension. A further antidote to template conformational interference with sequencing and PCR is the incorporation of 7-deaza-2′-deoxyguanosine into the PCR product in complete or partial replacement of G (Barr et al., 1986; McConlogue et al., 1988). This base analogue weakens the stacking interactions that stabilize nucleic acid secondary structure, without interfering with base-pair specificity.

Detection of Mutation and Polymorphism. The methods for detecting genetic variation reviewed in this section differ from the discovery procedures described previously in that they require prior knowledge of the variable genetic locus and usually of the exact sequence differences expected. Most PCR analyses of previously described genetic variants have used standard molecular biological detection methods based on restriction fragment length polymorphism (Kogan et al., 1987), variable number tandem repeats (Weber & May, 1989), or DNA probing under stringent conditions (Saiki et al., 1986, 1989). The major benefits that PCR brings to such studies are a greatly reduced detection limit and such abundance of amplified target sequence that nonisotopic signal generation is practical, though amplification of variable-number tandem repeats may experience yield limitation as a consequence of out-of-register product strand reannealing; this side reaction results in artifactual product species that obscure the expected band pattern (Jeffreys et al., 1988). However, the special features of PCR amplification offer opportunities to simplify the analysis of genetic variation. Most straightforward is the detection of insertion or deletion by change in the electrophoretically determined size of PCR product. Almost as simple is the identification of chromosomal translocations, such as the bcr/abl mutation associated with chronic myelogenous leukemia and the 14:18 translocation common in B-cell lymphomas; if a PCR primer is chosen from each side of the translocation, specific product should be seen only when the mutation has occurred (Lee et al., 1988b; Kawasaki et al., 1988; Crescenzi et al., 1988). Detection of base substitutions benefits from a greater range of technical options. A "mutation" can be introduced into a primer sequence covering a polymorphic site so that one genetic variant will generate a new restriction site in the amplified target sequence (Haliassos et al., 1989). Provided that primer length is kept below about 20 nt and PCR annealing and extension are performed with sufficient stringency, single interallelic base differences within the primer sequence suffice to discriminate thermodynamically among primer-template perfect matches and mismatches when alternate primers compete for the same target (Gibbs et al., 1989). Most of this competition must occur during the first two cycles of an amplification, because once a mismatched primer has been extended and its extension product has served as a template, the "error" will be faithfully replicated in subsequent cycles. Positioning a primer so that its 3′ end lies at a polymorphic site strengthens discrimination against mismatched primer extension for reasons rooted in polymerase mechanism rather than annealing thermodynamics. DNA polymerases perfer to add a residue to the 3′-OH of a perfectly matched primer 3′ end. This bias depends strongly on the exact identities of the mismatched bases and is reinforced by multiple mismatches near the 3′ end (Kwok et al., 1990). There are theoretical reasons for expecting discrimination against 3′-terminal mismatch extension to be sharpened at low dNTP concentrations (Mendelman et al., 1990), but experience in three different systems, using dNTP concentrations ranging from 2 μM to 1.5 mM, suggests that low dNTP concentrations are unnecessary for such allele-specific amplification (Newton et al., 1989; Wu et al., 1989; Ehlen & Dubeau, 1989). Kwok et al. (1990) showed that dNTP concentrations must be reduced below 50 μM before mismatch extension specificity increases and that 6 μM dNTP strongly disfavors extension of any mismatch except T·G. Ehlen and Dubeau (1989) found considerable C·T mismatch extension specificity at 20 μM dNTP and complete C·T, C·A, and C·C mismatch discrimination at 2 μM dNTP.

Qualitative Analysis of the PCR Product. PCR product detection and identification can be accomplished by size-dependent or sequence-dependent means. Ethidium-stained gel electrophoresis is sufficiently sensitive to provide size-dependent detection for most amplifications, as efficient PCR easily yields the approximately 1 ng of specific product per 10 μL of reaction mixture needed to give visible ethidium staining. Acrylamide gels give greater resolution than agarose gels, often revealing minor PCR product polydispersity that is attributed to the low processivity of *Taq* pol I (Olsen & Eckstein, 1989; Voss et al., 1989). Acrylamide gel electrophoresis of denatured PCR product under nondenaturing conditions adds some resolving power because each strand gives a band, the mobility of which also is sequence-dependent (Orita et al., 1989). Acrylamide gel sensitivity can be increased by silver staining, and use of a discontinuous buffer system increases resolution (Allen et al., 1989). If α-thio-dNTP's have been used in the amplification, one can fluorescently label PCR product with bimanes before and after carrying out size-dependent fractionation (Hodges et al., 1989), though the labeling reaction is a bit slow to be competitive with ethidium or silver staining. A recent report (Glazer et al., 1990) shows that ethidium dimer is about 2 orders of magnitude more sensitive than ethidium in gel staining and describes an automated scanner for recording fluorescent gel patterns. Other instrumentally oriented detection methods, such as HPLC (Katz & Dong, 1990) and capillary electrophoresis, offer resolution approaching and sensitivity exceeding ethidium-stained gels with obvious opportunities for speed, automation, quantitation, and (in the case of HPLC) easy product recovery that conventional gel electrophoresis cannot match. HPLC shows sequence-dependent departures from strict size-dependent sorting that may give enhanced detection specificity, especially if they turn out to be HPLC solvent-dependent. The logic of size-dependent product identification is simply (1) that PCR primer design normally requires enough understanding of the target sequence that the length of the expected product is predictable to within a few nucleotides and (2) that the probability of primer annealing to nontarget sequences in the test sample with identical orientation and spacing is very low. This rea-

soning is supported by the fact that a large fraction of target/primer systems yield only the predicted product when primer annealing is sufficiently stringent; i.e., there are no easily detectable nonspecific products of any size, except possibly for some primer-oligomer species, which would still arise in the absence of added test sample DNA.

Increasingly, it has become clear that a major source of PCR nonspecificity is primer-template annealing and primer extension under the permissive conditions that exist when all reactants are mixed at room temperature or below and are allowed to stand for a poorly controlled interval between mixing and the first amplification cycle. Once primer sequence has been extended, even in just a single cycle or during a single preamplification incubation and even if initially mismatched to the native template directing extension, its extension product can template a primer extension reaction in the next cycle, which creates a DNA strand that will match the original primer perfectly in all subsequent cycles. Many test samples contain significant amounts of single-stranded DNA even before they have been heated deliberately to 95–100 °C to induce first-cycle strand separation; nontarget single-stranded DNA and 15–30-nt primers can anneal promiscuously at room temperature in PCR solvent, and *Taq* pol I is sufficiently active at room temperature to extend the annealed primers. Partial test-sample DNA denaturation would be expected in some sample preparation methods, such as proteinase K digestion followed by heating to inactivate the proteinase (Li et al., 1988; Kim & Smithies, 1988). The drying of ethanol-precipitated DNA, conventionally considered to be completely innocuous, also induces strand separation (Svaren et al., 1987). A simple way to reduce PCR nonspecificity resulting from preamplification misprimed primer extension (F. Faloona, personal communication; Faloona and Mullis, manuscript in preparation) is to withhold enzyme from the reaction until the rest of the reagents have been heated to at least 60 °C; this procedure originally was used by some workers for other reasons (Frohman et al., 1988; Newton et al., 1989; Ward et al., 1989).

In many cases, PCR users require more evidence of amplification specificity than can be assured by product size. The standard remedy is to look for annealing of an oligonucleotide probe to a product sequence between those of the two primers, effectively requiring target complementarity to defined sequences in three different regions. Practically every imaginable format variant for sequence-dependent detection has been published: Southern blot (revealing size specificity as well) (Saiki et al., 1988; Kim & Smithies, 1988), gel electrophoresis following solution-phase probe–product annealing (influenced by product size) (Kumar & Barbacid, 1988; Kumar et al., 1989), direct dot blot (Farr et al., 1988; Higuchi et al., 1988b; Larzul et al., 1989), reverse dot blot (Saiki et al., 1989), restriction fragment analysis after reconstitution of a restriction site by probe–product annealing (Saiki et al., 1985; Kwok et al., 1987), and sandwich capture modes that exploit the ease with which binding moieties such as biotin are added to primer 5′ ends (Syvänen et al., 1988; Saiki et al., 1989; Kemp et al., 1989). Most intriguing is the recent report of amplification and post-PCR-probed detection in fixed cells, despite the poor control in such samples of the concentration conditions that result in efficient PCR (Haase et al., 1990); soon PCR targets may be identified on a cell-by-cell basis in mounted specimens without treating each cell as a separate test sample. Because probe annealing, like sequencing-primer annealing, can suffer reduced efficiency from the competing renaturation of separated product strands, sequence-specific detection should benefit from the product strand selection methods described above under *Sequencing*. Although radioactive label of PCR primers or probes generates signal simply and sensitively, the high yield of PCR product renders nonisotopic detection practical (Higuchi et al., 1988b; Kemp et al., 1989; Saiki et al., 1989; Keller et al., 1989; Larzul et al., 1989). DNA probe methods commonly have required some type of separation step to resolve the analyte-dependent signal from the background signal of excess probe. Fluorescence energy transfer between tags on probes annealed to adjacent regions of a target sequence (Cardullo et al., 1988) or annealed to one another in a reaction that competes with annealing to complementary target strands (Morrison et al., 1989) combines the benefits of a nonisotopic signal with elimination of the need for probe–hybrid separation. However, sequence-specific detection by fluorescence energy transfer shows a much higher ratio of background to target-specific signal than is routinely obtained by separation-dependent probe hybridization.

Isotopic and sequence-dependent detection often is recommended when PCR is attempted near the theoretical detection-limit minimum of one target molecule per reaction volume (Li et al., 1988; Kumar & Barbacid, 1988), especially because a low target copy number generally favors the formation of primer oligomers that may obscure specific product in non-sequence-dependent separations. However, single-copy detection via ethidium-stained gel electrophoresis of the PCR product is practical provided enough amplification cycles are used and misprimed nonspecificity is sufficiently controlled (Kim & Smithies, 1988). Given the uncertainties of measuring and delivering such low quantities of DNA or of cells, claims of single-copy detection either should be based on microscopically monitored cell micromanipulation or should be accompanied by enough replication to demonstrate the binomially predicted frequency of target-free samples in a homogeneous nucleic acid solution or cell suspension (Saiki et al., 1988). The most serious difficulty with detection near the single-copy limit is vulnerability to sample contamination with target-bearing DNA from the environment, especially if bacterial or human sequences are targeted. Contamination sources include DNA-carrying dust or other debris, samples being prepared or amplified simultaneously, and PCR product leaked, most likely in aerosols, from previous amplifications of the same target. Experienced PCR users, especially those amplifying the same target repeatedly over an extended period, adopt multiple common sense laboratory precautions to minimize reagent, equipment, and reaction mixture contamination (Kwok & Higuchi, 1989; Kitchin et al., 1990; Sarkar & Sommer, 1990).

PCR product detection has been proposed that is neither size-dependent nor sequence-dependent, instead relying on the PCR primers to incorporate into amplified double-stranded DNA binding moieties such as biotin and tags such as fluorophores and radioisotopes (Chehab & Kan, 1989; Sauvaigo et al., 1990). Molecules containing both primers will bear both a binding moiety and a tag and therefore can be resolved from the background signal of excess tagged primer. Although particular primer/target systems may always be amplified so specifically that this strategy will be reliable, it is generally vulnerable to high background and false positives caused by nonspecific amplification, including primer oligomerization.

Quantitative PCR. The current analytical frontier in PCR lies at methods that relate the magnitude of an analytical signal to the initial concentration of target sequence with the precision, dynamic range, detection limit, and resolution required for quantitative analysis. Such methods need two things: (1) amplification with adequate precision and dynamic range and

(2) optimized quantitation of PCR product. Less work has been done on the first problem than on the second, probably because a reliable quantitative assay of PCR product is needed in order to monitor amplification optimization. Consideration of amplification dynamic range focuses on the observation that PCR product accumulates exponentially up to about 10^{-8} M, rapidly declining to linear accumulation between 10^{-8} and about 10^{-7} M product. As product concentration remains proportional to starting target concentration only as long as product accumulates exponentially, the 10^{-8} M limit sets an upper bound on the preferred concentration of PCR product to be analyzed. Combined with this limit, the target sequence dynamic range in test samples then sets a lower bound on the concentration of PCR product that must be detectable, thereby specifying the detection limit of the PCR product assay. Of course, calibration curves do not have to be linear, so that the 10^{-8} M upper bound on PCR product is not an absolute requirement for quantitative PCR. By compression of the dynamic range of the analytical signal, the postexponential phase of PCR product accumulation effectively extends the analyzed dynamic range of target sequence in the test sample at some cost in resolution.

Isotopic size-dependent quantitation of PCR product can be as simple as gel electrophoresis of PCR product prepared with ^{32}P-labeled primers or [α-^{32}P]dNTP's, followed by scintillation counting of excised bands (Wang et al., 1989; Gilliland et al., 1990; Choi et al., 1989) or by densitometry of gel autoradiographs (Neubauer et al., 1990). The two most obvious nonisotopic size-dependent modes of PCR product detection that might also quantitate DNA are HPLC (Katz & Dong, 1990) and densitometry of fluorescently stained electrophoretic gels either directly (Glazer et al., 1990) or indirectly via an intermediate photographic image (Ribeiro et al., 1989). Small-diameter nonporous anion-exchange packings give fast HPLC separations with high recoveries. State-of-the-art UV absorbance HPLC detectors give a detection limit near 0.1 ng of DNA with a dynamic range of about 10^4. The dynamic range of PCR product concentration is extended another factor of 10 by the practical range of injection volumes; 0.1 ng corresponds to 100 μL of 1.5×10^{-11} M 100-nt PCR product or 1.5×10^{-12} M 1000-nt PCR product. Fluorescent gel densitometry is unlikely to match the reliability of spectrophotometrically detected HPLC, minimally because of the needs for tight kinetic control over staining and destaining with reversibly binding dyes and for a frequently repeated concentration-standard calibration curve. The ethidium dimer staining method of Glazer et al. (1990) must be modified to achieve a practical calibration curve. In its current form, DNA saturation with dye precipitates DNA, thereby interfering with electrophoretic separation and frustrating the requirement that an analytical reagent be in stoichiometric excess over an analyte if the signal is to show a useful analyte concentration dependence; the utility of such ethidium dimer staining depends on prior knowledge of the DNA concentration. Ethidium staining (Ribeiro et al., 1989) avoids this difficulty but is less sensitive because of a lower dye affinity for DNA. Video densitometry of gels (Freeman et al., 1990; Boniszewski et al., 1990) is much faster and just as accurate as traditional two-dimensional mechanical scanning, but remains dependent on staining precision and user sophistication in applying image analysis software to compensate for common irregularities in band shape.

Most development of quantitative PCR has relied on sequence-dependent detection to add assurance that the signal will not be biased high or show false positives as a result of nonspecific amplification. One sequence-dependent detection mode that is unlikely to be practical for quantitation relies on the competition between two solution-phase annealing reactions of sequence-complementary probes carrying fluorescent tags that can undergo energy transfer (Morrison et al., 1989); the dynamic range is too limited and the PCR product concentration range must be known in advance in order to apply probe concentrations giving useful analytical sensitivity. Traditional probing with stoichiometrically excess single-stranded nucleic acid, followed by a separation step to remove unreacted probe, has a practical dynamic range limited at the top end by PCR product reannealing kinetics and at the bottom end by probe specific activity. Published efforts at quantitative probed detection have used both isotopic (Larzul et al., 1988; Syvänen et al., 1988; Singer-Sam et al., 1990) and nonisotopic, enzyme-generated (Coutlée et al., 1989; Keller et al., 1989) signals. PCR product has been captured (to facilitate removal of excess detection probe) by dot blotting (Larzul et al., 1988; Singer-Sam et al., 1990), avidin-coated beads (Syvänen et al., 1988), an anti-biotin-coated microtiter plate (Coutlée et al., 1989), and a DNA-probe-coated microtiter plate (Keller et al., 1989).

A major concern in quantitative PCR has been the inclusion of internal amplification controls to correct the yield of specific product for reaction-to-reaction variation in amplification efficiency, although Singer-Sam et al. (1990) claim to obtain satisfactory uncorrected precision. Three different strategies have been applied to this end: coamplification with the same primers of an added synthetic internal standard of different length from the target (Wang et al., 1989), coamplification with the same primers of an added synthetic internal standard identical with the target except for a base substitution introducing a restriction site (Gilliland et al., 1990), and coamplification with different primers of endogenous sequence expected to be quantitatively invariant in the experimental system but funtionally related to the target (Choi et al., 1989; Neubauer et al., 1990). The major uses of quantitative PCR to date have been to monitor gene dosage, gene expression, and viral infection. The efforts to incorporate internal standards have been limited so far to gene dosage and expression studies, where the molar ratio of target to internal standard observes a relatively limited dynamic range and is easy to predict or control. These systems also are more amenable to size-dependent detection because background nucleic acid is sufficiently limited in quantity that misprimed nonspecificity is rarely a problem. It is much harder to design convenient and relevant internal standards for monitoring infectious disease, because the background genomic material (e.g., host DNA) is likely to have many orders of magnitude higher copy number than the microbial target. The challenge is to prevent amplification of a host-originated control sequence from quantitatively overpowering target amplification. The vast excess of host genome also increases the probability of misprimed nonspecificity that will confound purely size-dependent detection, thereby motivating the use of sequence-dependent detection. Simultaneous detection of target and control sequences is harder to incorporate into sequence-dependent formats than into size-dependent modes. A final concern in internal standard design is that target and standard be presented in similar structures that will experience similar strand-separation, primer-annealing, and primer-extension kinetics during the critical first two amplification cycles, before short PCR product begins to accumulate. cDNA consists of relatively short, monodisperse, single-stranded material. Free viruses also have predictable structures that can be modeled.

Genomic eukaryotic or genomically integrated viral targets exist in variably cleaved structures, which are much harder to predict, model, or match with control sequences.

A major limitation of quantitative PCR efforts to date is the paucity of precision and accuracy determinations and comparison with competing detection modes. Until quantitative PCR meets the validation standards routinely applied in other areas of analytical biochemistry, such as immunoassay and receptor binding, it remains a goal rather than a reality.

Modification by PCR

Random Mutagenesis. The infidelity of PCR, which limits its utility for subcloning-dependent applications and for error-free amplification of very long targets, becomes a virtue when random mutagenesis is the goal. Understanding how a variety of independent variables influenced fidelity, one would simply amplify at the extreme conditions maximizing the error rate. The main problem currently is the absence of well-controlled efforts to maximize or minimize fidelity. Leung et al. (1989) reported an increase in substitution rate and a shift from transitions to transversions, induced by limiting the concentration of one dNTP and by partially replacing Mg^{2+} with Mn^{2+}. However, the number of nucleotides sequenced for each test condition was low, and the error rate varied only 5-fold over all of the conditions tested. The number of mutations observed for each conditions was not stated explicitly but appears to have ranged from about 8 to about 40. The maximum reported mutation frequency was 2% of sequenced nucleotides. These limitations raise the concern that some or all of the reported increases in error rate lie within the statistical expectations for random sampling from a single population. The denaturing gradient gel electrophoresis method of Keohavong and Thilly (1989) and the native gel analysis of denatured PCR product described by Orita et al. (1989) allow the researcher to isolate a population of PCR product enriched for mutant sequences, not only streamlining the collection of reliable statistics on error rate but also reducing the need to increase the amplification error rate in order to get a practical mutation yield. Enriching for relatively rare mutants serves to reduce the likelihood of isolating multiple mutants.

Of course, PCR-induced random mutagenesis is not really random. As discussed above under *Sequencing*, *Taq* pol I preferentially substitutes A,T with G,C, and target sequences contain hot spots and cold spots (Tindall & Kunkel, 1988). The experimental design of Leung et al. (1989) would bias further against truly random mutagenesis by limiting the concentration of only one of the four dNTP's. Truly random mutagenesis cassettes for short sequences can be inserted into a longer PCR product by combining the oligonucleotide template constructs of Thiesen and Bach (1990), which bracket random oligomers with sequences representing a PCR primer and a primer complement, with the directed-mutagenesis approach described below.

Site-Directed Mutagenesis. In its simplest form, site-directed mutagenesis is performed by designing a mismatch to target sequence in one primer; by the third PCR cycle, this error is securely encoded in the short template that will direct the exponential accumulation of PCR product. The mismatch can be internal to a genomic sequence recognized by the primer (Hoffman & Hundt, 1988) or can represent a 5′-terminal primer extension that bears no resemblance to the native sequence; such an extension usually adds a useful function such as a restriction site (Scharf et al., 1986) or a flanking transcriptional or translational regulatory site (Skoglund et al., 1990). However, site-directed mutagenesis by PCR approached its full potential when it became clear that mutation could be introduced anywhere in a gene, not just at the ends, by amplifying the gene in two sections with the break close to the mutagenesis target. Provided the primers covering the junction contain 5′-terminal extensions into one another's sequence, the complete gene can be reconstructed after one section has been mutated, simply by 3′-terminal overlap annealing and *Taq* pol I extension of each 3′ end templated by the complementary fragment (Higuchi et al., 1988b; Ho et al., 1989). This basic strategy can be modified to make large deletions (Kahn et al., 1990) or gene fusions (Horton et al., 1989; Yon & Fried, 1989). A further modification streamlines the mutagenesis process and assures that essentially all amplified DNA will contain the mutation (Nelson & Long, 1989). A more radical use of PCR primers to join two sequences is especially suited to rearranging large regions of genetic material, allowing the insertion, deletion, or replacement of entire introns, exons, or, after transcription and translation, protein domains (Clackson & Winter, 1989). It combines (1) amplification of the new sequence, (2) annealing of the primer-defined ends of the PCR product to the old sequence while the latter lies in a single-stranded vector prepared under conditions that replaced T with dU, (3) extension and ligation of the annealed (T-containing) PCR product strand to create a double-stranded vector comprising one dU-containing and one T-containing strand, and (4) transfection of a host containing an enzyme that destroys the dU-containing strand bearing the native sequence, leaving behind only the T-containing mutated strand.

Synthetic PCR

As PCR is itself a method of synthesizing DNA, "synthetic PCR" is a literal redundancy. The distinction intended here is between amplifications where the target sequence is the focus of concern and those in which PCR product is a tool or intermediate in a larger scale process. The most common examples of the latter situation concern (1) the use of PCR product as a probe, e.g., in traditional cloning and screening, (2) the use of PCR in the creation of expression vectors for polypeptide production, and (3) PCR mutagenesis to create a chimeric virus. PCR also has been used to amplify full-length product selectively from a mixture containing abundant failure sequences generated by solid-phase polynucleotide synthesis, simplifying and extending the size range of total gene synthesis (Barnett & Erfle, 1990).

PCR product is ideal probe material. It lies in the right size range to show high specificity, as well as to tolerate the levels of mismatch expected in gene families and with polymorphic hybridization targets, without carrying superfluous (e.g., vector) sequences, which can generate background from nonspecific interactions. It is much more homogeneous than a nick-translation product. It can be labeled isotopically or nonisotopically, internally via dNTP's or only at the 5′ end via primer, to almost any desired specific activity. It can be recovered in double-stranded or, as often is preferred, in single-stranded form. PCR can generate abundant and essentially pure probe material with unmatched convenience, productivity, and speed. This enumeration of the probing advantages of PCR product has the effect of extracting virtue from necessity. PCR product is often too short to encompass a complete gene or mRNA sequence, though perfectly suited to identify longer clones from a library.

Schowalter and Sommer (1989) described the PCR preparation of both dsDNA and (with little detail) single-stranded RNA probes in which incorporation of a [^{32}P]dNTP or [^{32}P]NTP was used to obtain high specific activity; nested

priming assured probe specificity. For other examples of isotopically tagged probed with PCR product, see Suzuki and Naitoh (1990), Shih et al. (1989), and Oskenberg et al. (1989). Weier and Rosette (1988) showed how to get a single-stranded RNA probe, nonisotopically tagged via biotin-11-UTP incorporation during the in vitro transcription of the PCR product. Lo et al. (1988) achieved nonisotopic labeling of a dsDNA probe with biotin-11-dUTP. Nonisotopic tagging also should be attainable by post-PCR alkylation of amplified DNA prepared with phosphorothioate mononucleotides (Hodges et al., 1989). In principle, any of the methods described under *Sequencing* for isolating single-stranded PCR product should serve to generate a ssDNA probe.

Olsnes et al. (1989) showed how synthetic PCR could be combined with in vitro transcription and in vitro translation to prepare a functional protein without resort to any biological processing. In this case, the target was a bacterial toxin judged too dangerous to express fermentatively. Mackow et al. (1990) used this strategy to map epitopes with nested polypeptides. Skoglund et al. (1990) employed PCR to create a bacterial system for high-level heterologous expression of an endonuclease by combining the structural gene with optimal flanking regions in an *E. coli* plasmid, also engineering a codon switch intended to disrupt mRNA secondary structure. A cassette construction for overproduction of heterologous proteins in *E. coli* is claimed reliably to require less than 2 weeks from primer design to polypeptide recovery (MacFerrin et al., 1990). Less intricate PCR-assisted engineering of an expression vector achieved high-level homologous expression in *E. coli* of a tRNA methyltransferase (Gu & Santi, 1990). A synthetic PCR effort which should revolutionize academic and industrial immunology has involved the PCR cloning of individual mouse immunoglobulin heavy-chain and light-chain variable regions (Orlandi et al., 1989) or of complete splenic variable-region repertoires (Sastry et al., 1989; Ward et al., 1989) and expression of these clones in *E. coli* or mouse myeloma cells. Expression in mouse myeloma cells occurred after fusion to a human heavy-chain or light-chain constant region (Orlandi et al., 1989); this procedure leads directly to mouse/human hybrid antibodies of whatever subclass is desired. Chaudhary et al. (1990) adapted PCR cloning of immunoglobulin variable regions to express an active single-chain immunotoxin in *E. coli*. For an example of PCR synthesis of a chimeric virus, see Wychowski et al. (1990).

Conclusion

The last five years have seen an explosive growth of our ability to exploit the strengths of PCR; ways to minimize the method's limitations evolve almost as rapidly as problems are discovered. The near future should see the introduction of methodological modifications and new reagents (e.g., enzymes) that strengthen the technology even farther. Less predictable is whether study of the physical–chemical foundations of in vitro nucleic acid amplification will introduce an element of conceptual maturity into the enterprise or whether the impressive empirical vigor of PCR practitioners will suffice to meet their needs. Even today, PCR renders macromolecular alchemy available to every biochemist with a minimum of intellectual or physical retooling.

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