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Amplification of Nucleic Acids by Polymerase Chain Reaction (PCR) and Other Methods and their Applications

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ABSTRACT: The *in vitro* replication of DNA, principally using the polymerase chain reaction (PCR), permits the amplification of defined sequences of DNA. By exponentially amplifying a target sequence, PCR significantly enhances the probability of detecting target gene sequences in complex mixtures of DNA. It also facilitates the cloning and sequencing of genes. Amplification of DNA by PCR and other newly developed methods has been applied in many areas of biological research, including molecular biology, biotechnology, and medicine, permitting studies that were not possible before. Nucleic acid amplification has added a new and revolutionary dimension to molecular biology. This review examines PCR and other *in vitro* nucleic acid amplification methodologies — examining the critical parameters and variations and their widespread applications — giving the strengths and limitations of these methodologies.

KEY WORDS: polymerase chain reaction (PCR), nucleic acid amplifaction, DNA amplification.

Less than a decade ago, a novel method — the polymerase chain reaction (PCR) — was discovered that permits the *in vitro* replication of DNA. The ability to replicate specific segments of DNA *in vitro* allows the detection and cloning of specific genes. Whereas previously only minute amounts of a specific gene could be obtained from a cell, now even a single gene copy can be detected following nucleic acid amplification. Amplification of DNA by PCR and other newly developed methods have already been applied in many areas of biological research, including molecular biology, biotechnology, and medicine, permitting studies that were not possible before. Nucleic acid amplification has added a new and revolutionary dimension to molecular biology. This review examines PCR and other *in vitro* nucleic acid amplification methodologies and their widespread applications.

I. THE POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is an *in vitro* method for amplifying selected nucleic acids (DNA or RNA) sequences.[1] The method

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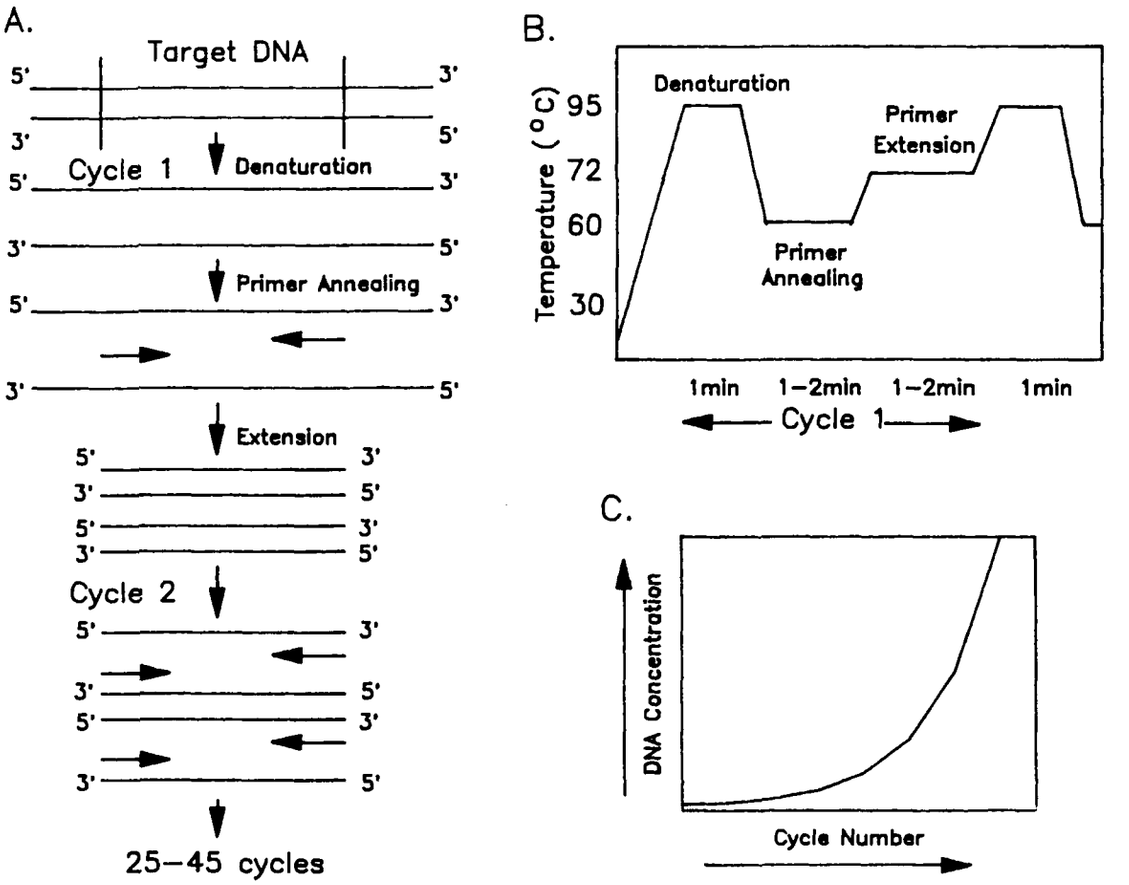

FIGURE 1. (A) schematic diagram of PCR amplification. Each cycle consists of denaturation of target DNA, primer annealing, and primer extension. (B) A graphic representation of a typical PCR cycle, which consists of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 to 2 min, and primer extension at 72°C for 1 to 2 min. (C) Quantitation of amplified DNA product. The amount of amplified DNA increases exponentially as the cycle number increases.

consists of repetitive cycles of DNA denaturation, primer annealing, and extension by DNA polymerase[2,3] (Figure 1A). Two oligonucleotide primers flank the DNA segment to be amplified and are repeatedly heat denatured, hybridized to their complementary sequences, and extended with DNA polymerase. The two primers hybridize to opposite strands of the target sequence, such that synthesis proceeds across the region between the primers, replicating that DNA segment. The product of each PCR cycle is complementary to and capable of binding primers, and so the amount of DNA synthesized is doubled in each successive cycle[4] (Figure 1C). The original template DNA can be in a pure form and as a discrete molecule or it can be a very small part of a complex mixture of biological substances. It can be a tissue specimen, a human hair, dried blood, mummified brain tissue, or tissue from a 40,000-year-old woolly mammoth frozen in a glacier.[3]

A. Amplification of Genomic DNA

The amplification of a specific DNA fragment from complex genomic samples is one of the most common applications of this technique. The human β-globin gene was one of the first DNA sequences to be amplified by PCR.[5] There are several common elements for the amplification of genomic DNA target sequences. These include the use of a thermostable DNA polymerase, primers that flank the region being am-

plified, a suitable buffer solution for the reaction to occur, and the repetitive cycling between temperatures that permit melting of the DNA, permit primer annealing to the target DNA, and permit the addition of nucleotides to the primers by the action of the DNA polymerase.

Due to the tremendous amplification power of the PCR, great care is taken to guard against accidental contamination of solutions and samples with exogenous DNA. Negative controls are constantly run to monitor the purity of solutions used in the procedure. Laboratory space should be allocated for pre-PCR work and post-PCR work to prevent cross-contamination with amplified sequences via aerosols. Sample preparation for PCR is carried out in the pre-PCR area, and detection of PCR products is done in the post-PCR area. Separate micropipettes and positive displacement micropipettes are recommended to be used in the two areas.[6] It has also been suggested that reagents be made in batches and stored in a −20°C freezer after running appropriate tests on them.[6]

Because of its amplification power, one must also be concerned with the fidelity of DNA replication by PCR and the potential for producing artifacts. The error rate during PCR amplification has been reported by Dunning et al.,[7] based on the amplification of a 798-bp fragment of human apolipoprotein B (ApoB) gene. The amplified product of this gene was sequenced from 10 individuals (8000 bases), and 22 differences were found that were detected as artifacts generated by PCR. The most common changes found were A to G and T to C. 77% of the changes noted were associated with run-off bases of the same sequence. This type of artifact generated by PCR may have a profound effect on the nature of the amplified cloned DNAs.

1. Temperature Cycling Parameters

In a typical PCR cycle, the DNA is denatured by heating to 94°C for 1 min, primers are annealed by cooling to 40 to 60°C for 1 min, followed by primer extension at 72°C for 1 min (Figure 1B). The first cycle is preceded by an initial denaturation step at 94 to 95°C for 3 to 5 min. After the last cycle, the sample is allowed to heat to 70 to 72°C for 3 to 5 min to ensure that the amplified DNA is double stranded. The most critical temperatures are the denaturation and reannealing temperatures. Inadequate heating leads to failure of DNA melting and no DNA amplification. The reannealing step determines the specificity of PCR. Using too low a temperature results in mispriming and amplification of nontarget sequences. Too high a temperature causes a lack of primer annealing and hence no DNA amplification. In the case of amplification of a short target sequence (≤100 to 300 bases), a rapid and convenient two-step PCR amplification can be performed. In two-step PCR, the primer extension step is set at the same temperature as the reannealing temperature.[8] For amplification of a greater than 1-kb target DNA, the primer extension step can be between 1 and 7 min, depending on the length of the target DNA to be amplified.[9] In combination with longer primer extension time, agents such as gelatin or bovine serum albumin are required in the PCR reaction buffer for longer activity and stability of the *Taq* DNA polymerase.[9] Use of 15 to 20% glycerol as a cosolvent in PCR reaction helps to amplify larger DNA fragments of about 2.5 kb.[10] At the end of the amplification cycles, the sample is cooled and held at 4°C until retrieved. This automated program in the DNA thermal cycler has made the amplification method easy and rapid.

As the temperatures are critical, it is important that thermal cyclers that permit the automated cycling of temperatures according to a temperature program have accurate temperature regulation. Since the emergence of the first DNA thermal cycler by Perkin-Elmer Cetus Corporation, there have been about a dozen companies who manufacture this machine under different trade names and engineering set-ups. It is not easy to determine the performance and quality of the thermal cycler without performing tests on it. Recently, Hoelzel[11] ran performance tests on nine different thermal cyclers. In his study, out of all nine thermal cyclers tested, only one gave uniform heating and cooling, consistently gave the same results in all wells, and produced a profile in the sample tube that was essentially the same as the cycle that had been programmed. According to this study, a thermal cycler with a metal block heated by a heating element pad or a Peltier pump and cooled by a Peltier pump seems to be consistent.

2. Taq DNA Polymerase

The *Thermus aquaticus* (*Taq*)[4] DNA polymerase has replaced the Klenow fragment of *Escherichia coli* DNA polymerase I in PCR. Due to the thermolability of the Kelnow fragment,[1,5] fresh enzyme was required to be added during each cycle. *Taq* polymerase does not need to be replenished at each cycle and also improves the specificity, yield, and sensitivity of the reaction.[4,12-14] Thermostable DNA polymerase was first isolated from a thermophilic eubacterial microorganism, *Thermus aquaticus* YTI,[15] which is capable of growing at 70 to 75°C. The DNA polymerase isolated from *T. aquaticus* has a molecular weight close to 93.910 kDa, with a specific activity of 200,000 U/mg.[13,16-18] This 94-kDa *Taq* DNA polymerase has a temperature optimum (T_{opt}) of 75 to 80°C, with a nucleotide incorporation rate (K_{cat}) of 150 nucleotides/s/enzyme molecule.[16]

The rate of DNA strand extension was found to be >60 nucleotide/s at 70°C with *Taq* DNA polymerase for a GC-rich 30-mer primer on M13 DNA and 24 nucleotide/s at 55°C.[16] Although very little DNA synthesis activity was observed at higher temperature (>90°C), possibly due to the instability of the primer-template duplex, the DNA polymerization activity of the *Taq* DNA polymerase is retained about 50% after 130, 40, and 5 to 6 min at 92.5, 95, and 97.5°C, respectively.[16] A PCR reaction of 50 cycles, with an upper limit of 95°C for 20 s in each cycle, can retain 65% polymerization activity of this enzyme.[16] A genetically engineered *E. coli* strain into which the *taq* DNA polymerase gene has been cloned is now used to produce *Taq* DNA polymerase (Ampli*Taq*, Perkin-Elmer Cetus).

3. Other Reaction Components

In addition to target DNA, primers, and *Taq* DNA polymerase, the standard PCR buffer contains 50 m*M* KCl, 10 m*M* Tris·HCl (pH 8.4), 1.5 m*M* $MgCl_2$, and 100 μg/ml of gelatin (GenAmp Amplification kit, Perkin-Elmer Cetus). $MgCl_2$ plays an important role in PCR amplification.[19] Varying concentrations of $MgCl_2$, usually in the range of 1.5 to 4 m*M* can be used for a specific and higher yield of the amplified products.[19] The presence of EDTA or other chelating agents in the reaction may interfere with the Mg^{2+} concentration. Deoxynucleotide triphosphates are added at a concentration of 200 μ*M* each. It is important to keep the four dNTP concentrations above the K_m of each dNTP (10 to 15 μ*M*) and balanced for best base incorporation fidelity. Greater that 50 m*M* (final concentration) of dNTP in the PCR reaction inhibits *Taq* DNA polymerase activity.[14] Changing the buffering capacity of the PCR reaction sometimes increases the yield of the amplified DNA products, for example, by increasing the concentrations of the Tris·Cl up to 50 m*M* (pH 8.9).[8]

During PCR amplification it is recommended to overlay 80 to 100 μl light mineral oil on top of the reaction mix, since evaporation of the liquid, particularly at the denaturation step, can greatly affect the PCR product yield. A study performed by Mezei[20] showed that the use of light mineral oil increased the yield of the amplified product approximately five times. The reason for this may be the maintenance of heat stability and salt concentrations throughout the reaction mixture. Similar results were observed by us when PCR amplifications were performed in 100 to 150 μl reaction without the addition of any mineral oil (A. Bej, unpublished observation).

Since some template DNA for PCR amplification may not denature completely due to the presence of a high G + C content and primers may not reanneal at the secondary structure regions of the template DNA, cosolvents in the PCR reaction, such as 1 to 10% dimethyl sulfoxide (DMSO)[10,21,22] or 5 to 20% glycerol,[10] have been shown to greatly increase the amount of PCR amplified DNA. Also, Dermer and Johnson[23] showed that use of 20% glycerol increases the reproducibility of duplicate PCR reactions. Since in some cases glycerol seems to inhibit PCR amplification, it is advised to evaluate the use of this cosolvent for each case.[20]

4. Target DNA

The amount of DNA typically used for PCR of single-copy genomic targets is 0.05 to 1.0 μg. There should be at least one intact DNA strand.

Impurities should be eliminated or diluted so as not to inhibit polymerization. A sample may be prepared by lysing the cells, by boiling in a hypotonic solution, or by freeze-thaw cycling. DNA may be extracted and purified, but extensive purification often is not required for successful DNA amplification.[24]

A serious problem in PCR amplification is contamination with previously amplified DNA[25-27] or other exogenous DNAs, which can serve as target for specific primers. Enzymes (e.g., Ampli*Taq* or *Taq* DNA polymerase; *E. coli* DNA polymerase, etc.) themselves are often contaminated with DNA. This problem becomes more serious when a single molecule detection is necessary or quantitative evaluation needs to be performed. Several methods, such as UV treatment,[28] restriction enzyme digestion,[29] or use of psoralen,[30] have been described to overcome this problem. UV treatment of the PCR reaction prior to PCR amplification involves exposure of the target DNA[28] to a combination of 254- and 300-nm UV lights for 5 to 20 min. An alternative protocol described by Furrer et al.[29] involved treatment of the PCR reaction either with restriction endonuclease or with DNAse I before adding target DNA and *Taq* DNA polymerase. In their study, they showed that pretreatment of the PCR reaction with restriction endonuclease reduced DNA contamination by a factor of 5 to 10 without decreasing the efficiency of the PCR amplification, whereas using DNAse I reduced contamination by a factor of 1000. More recently, psoralen, which intercalates into double-stranded DNA and forms a covalent interstrand cross-link after UV treatment (320 to 400 nm), has been used successfully to remove the contamination with exogenous double-stranded DNA/RNA. Treatment with psoralen requires two steps: first incubation of the PCR sample containing contaminated DNA with 8-MOP (8-methoxypsoralen) in the dark for 30 min to overnight; second, treatment with UV (long wave, 365 nm) for 1 h. By following this method, 99.9% of the contaminated DNA could be removed. Of these three procedures, the simplest method is treatment with UV light. Using a Fotodyne Foto/PrepI UV transilluminator (Fotodyne Inc., New Berlin, WI), it was shown that after 1-min treatment with UV light 95% of the DNA contamination was removed. After 2-min treatment, no evidence of amplified contaminating DNA was seen. Jinno et al.[30] found that UV (254 to 300 nm) treatment alone did not eliminate the problem of contamination. However, the use of psoralen and UV treatment would be more time consuming than UV treatment alone. The use of DNAse or restriction enzyme requires complete inactivation or removal of these enzymes after treatment. Also, the additional cost of these enzymes makes this method unpopular. Moreover, restriction endonuclease or DNAse I themselves may be contaminated with DNA or RNA.

Porphyrin-derived compounds present in DNA from human blood samples were found to inhibit the *Taq* DNA polymerase. Removal of this inhibitor was achieved when the DNA sample was boiled for 5 min and immediately centrifuged through a 1-ml Sephadex® G50 column, preequilibrated in 1 m*M* Tris · Cl pH 8.0 and 0.1 m*M* EDTA. Neither boiling the DNA nor passage through the column alone abolishes the inhibitor. Humic acids from soils and high concentrations of clay also interfere with PCR (A. Bej, unpublished).

5. Primers

Pairs of primers are selected that flank the DNA region to be amplified. Primers are oligonucleotides that are single-stranded fragments of DNA, complementary to the 5′ ends of the target DNA to be amplified. For primer annealing to occur specifically at the sites flanking the DNA region to be amplified, there must be nearly complete homology between the target DNA and the primer nucleotide sequence. Typically the primers are 15 to 30 nucleotides long, with no more than 2 bp complementary overlap at their 3′ ends.[24] DNA polymerase adds nucleotides to the 3′ end. It is important that the 5 to 6 bases at the 3′ ends of the primers exhibit precise base pairing with the target DNA. The terminal base match at the 3′ end is critical, and an exact match is generally required for effective amplification; a T on the primer that is a mismatch at the 3′ terminal sometimes still allows amplification, and degenerate primers (see discussion below) can overcome the stringency of complete matching. While the

primer should match the target, complementarity between the paired primers at the 3′ ends should be avoided, because they may produce an unwanted product, called a *primer dimer*.[24,31] A primer-dimer is a double-stranded fragment of DNA that is formed when one primer is extended by the polymerase over the other primer and has a length close to the sum of the two primers. It is an amplification artifact that can become the predominant product in a reaction. All primers for a target amplification should have the same melting temperature, or more specifically, the temperature of annealing (T_a) to the template, with an average G + C content of 40 to 60%, with no stretches of polypurines or polypyrimidines. Significant secondary structures (measured by the negative value of ΔG) internal to the primers or immediately downstream (for left-hand-side primer) or immediately upstream (for right-hand-side primer) of the template should be avoided.[24,31] Usually a range of 0.1 to 1 μM of each primer is used for symmetric PCR amplifications. For optimum results, primers should be purified after synthesis from ''run-off'' products and other impurities by HPLC or gel purification.[24-32] For applications such as cloning, a 5′ overhang containing a restriction site or for detection purposes 5′ biotin-labeled primers can be used successfully for PCR amplification. The primers should be stored at −20°C when not in use; the shelf life of oligonucleotide primers is at least 6 months when stored in liquid and 12 to 24 months when stored after lyophilization. The primers can also be stored at 4°C in 20% acetonitrile solution after HPLC purification, which prevents microbial growth.

Defined DNA templates from the *hunchback* gene of *Drosophila melanogaster* and *D. virilis* were used to determine the minimum homology required for PCR primers.[35] In this experiment one primer had perfect complementarity, whereas the other primer had partial homology, with the template strand for amplification of 200 to 1000 bases. It was found that a primer length between 17 and 20 bases with 3-base homologies at its 3′-OH end was necessary for successful amplification. Primers with 20 to 24 bases are preferable for optimal amplification and three bases should match completely at the 3′-OH end for best results. To ensure this, it has been suggested that for primers based on amino acid sequence the primer be ended with the amino-acid codon either for Met or Trp residue or by synthesizing appropriate redundant oligonucleotides for these positions.

The annealing temperature (T_a) of the primers should be kept between 60 and 65°C for specific priming and removal of unnecessary ''ghost'' amplified DNA bands when genomic DNA or mRNA is used for PCR amplification.[8,24] The T_a can be calculated either from the melting temperature (T_m) of the primers using the equation: $T_a = T_m - 5°C = 2(A + T) + 4(G + C) - 5°C$[36] or by using a computer-aided program such as ''oligo''.[37]

a. Degenerate Primers for DNA Amplification and cDNA Cloning

The exact nucleic acid sequence of a segment of DNA cannot be determined from amino acid sequence, since degeneracy is inherent to the genetic triplet code.[38] However, for PCR amplification ''degenerate'' primers can be designed so that every possible combination of nucleic acid sequence that could code for a given amino acid sequence can be generated and used for PCR amplification.[39,40] When only a limited portion of a protein sequence is known for a gene of interest, or when searching for uncharacterized sequences related to a known gene family, it may be necessary to use ''degenerate'' primers, a mixture of oligonucleotides varying in nucleotide sequence but having the same number of nucleotides. The urate-oxidase gene,[41] the diabetes-associated peptide,[40] and the mammalian and avian hepadnaviruses[42] have been cloned using degenerate primers. When designing these primers, amino acids with minimal degeneracy are selected and up to 516-fold degeneracy is recommended. Degeneracy at the 3′ end of the primer should be avoided.[43]

Mixed primers representing all codon choices using the partial amino acid sequence of the protein have been used for PCR amplification of the unknown target DNA followed by cloning and sequence analysis.[41,44] PCR amplification using primers containing deoxyinosine was used to solve the problem of having numerous degenerate co-

dons for proteins.[44,45] The specificity of such deoxyinosine-containing primers varied depending upon the cDNA concentration.[45]

b. PCR Amplification Using "Nested" Primers

In this method, PCR is performed for 15 to 30 cycles with 1 primer set, followed by an additional 15 to 30 cycles using a second primer set internal to the amplified DNA.[46] This generates the sequence of interest with high yield and minimal amplification of secondary sites. The first primer set is removed before continuing with the second primer set, by centrifugation through Centricon 30 (Amicon) molecular filters, or by using limiting amounts of the initial primers. This approach has been used for the molecular analysis of mutations in Chinese hamster ovary (AS52) cells.[47] The Chinese hamster ovary, AS52 cell line contains a single copy of the bacterial guanine phosphoribosyltransferase (*gpt*) gene, which is analogous to the mammalian hypoxanthine-guanine. The problem of recovering mutant gene sequences form the mammalian genome has been overcome by directly amplifying mutant *gpt* gene sequences by PCR. The PCR-amplified product was sequenced without cloning, thus generating point mutational spectra derived in mammalian cells. PCR by nested primers provides an additional level of specificity and increases amplification efficiency by minimizing nonspecific primer annealing. This method is very effective for detecting organisms in environmental samples where the presence of unknown target DNA is likely and for amplification of single-copy gene targets.

c. Multiplex PCR

It is possible to amplify several DNA segments simultaneously using multiple pairs of primers. This procedure, called multiplex PCR, was developed by Chamberlain et al. to detect human genes.[22,48] Bej et al. modified the approach of simultaneous PCR amplification to detect gene sequences associated with different groups of bacteria in environmental samples.[49] Multiplex PCR amplification of two different *Legionella* genes, one specific for *L. pneumophila* (*mip*) and the other for the genus *Legionella* (5S rRNA), was achieved by the "staggered" addition of primers. The *mip* primers were first used for amplification in 7 PCR cycles followed by the addition of the 5S rRNA primers to the reaction for 38 PCR cycles. Multiplex PCR amplification using differing amounts of primers specific for *lac*Z and *lam*B genes permitted the detection of coliform bacteria and those associated with human fecal contamination, including the indicator bacterial species *E. coli* and enteric pathogens *Salmonella* and *Shigella*.[8]

For multiplex PCR amplification, it is necessary to design the primers in such a way that they all have very close T_a values. A difference of $\pm$ 10°C T_a value between the two sets of primers may lead to differential amounts of amplified products and often no visible amplification for one or the other target. Also the lengths of the target DNAs should be close. Large differences in the lengths of target DNAs will favor the amplification of the shorter target over the longer one. As a result, differential yield of amplified products will be seen. In cases of differential T_a of two sets of primers and/or variable target DNA lengths, "staggered" DNA amplification and variable amounts of primers can be used to achieve equal amounts of amplified products.[49]

d. Inverse PCR

Inverse PCR permits the amplification of DNA flanking a region of known sequence.[50] In this reaction the DNA is synthesized outward from the primer pairs, rather than inward between the two primers (Figure 2). The source DNA is cleaved with restriction enzyme and circularized with DNA ligase before amplification. The primers used for inverse PCR are synthesized in the opposite orientations to those employed in normal PCR. Upstream and/or downstream segments are thus amplified. Hybridization probes have been produced by this technique for aligning large DNA fragments cloned as yeast artificial chromosomes.[50] Such probes can be used for chromosome walking in any gene library with overlapping DNA fragments and are useful for

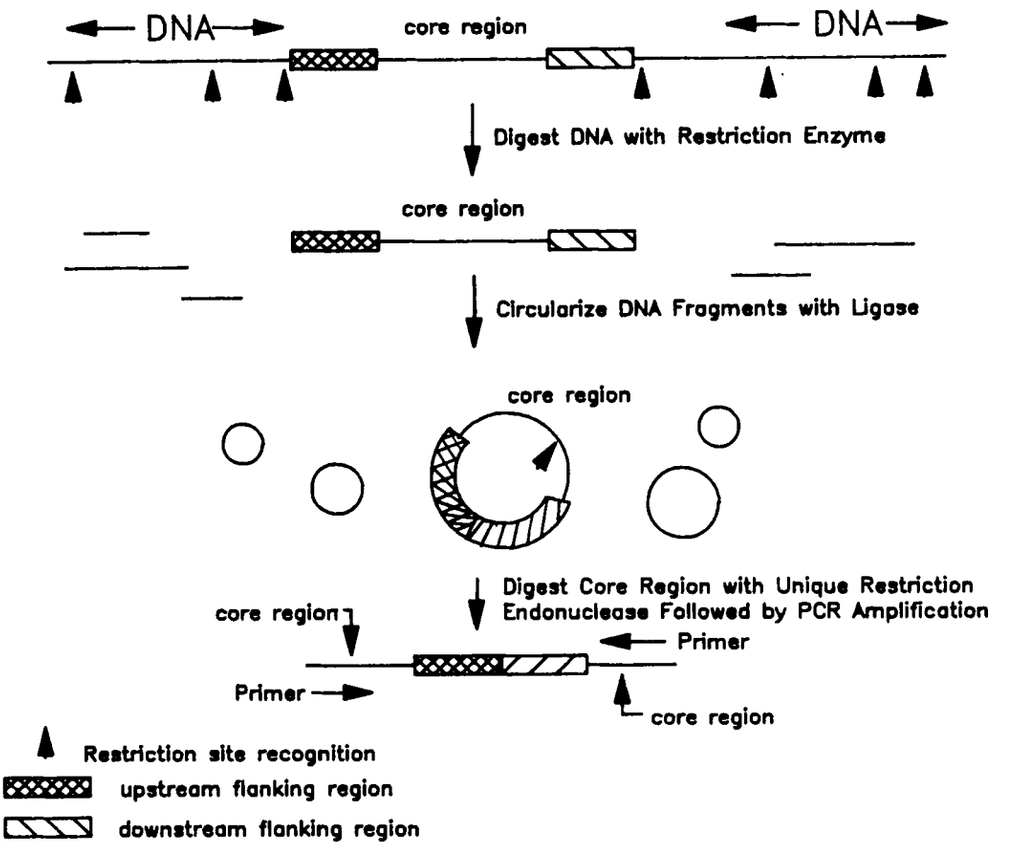

FIGURE 2. Amplification of flanking regions by inverse PCR.

determining the insertion sequences of transposons. Helmsley et al. have utilized inverse PCR for site-directed mutagenesis.[51] One of the primers is designed to contain the desired mutation. The 5′ ends of the primers hybridize to adjacent nucleotides on opposite stands of a circular double-stranded molecule containing the region of interest. The 3′ ends of the primers prime synthesis in opposite directions around the circular template. PCR is performed followed by phosphorylation with T_4 polynucleotide kinase, which provides a 5′ phosphate for the primers, ligation, and transformation into a host.

Some of the limitations of inverse PCR have been described by Ochman et al.[50] The first limitation is the unknown nature of the flanking sequence, since selection of the restriction enzyme requires pilot experiments that need many enzymes or selection of an enzyme that gives the proper size. Selection of restriction enzyme(s) should not cut the vector DNA at unsuitable sites. Another limitation is that most eukaryotic genomes contain significant amounts of moderately or highly repetitive DNA, and unknown junction sequences in YACs or cosmids will sometimes include these sequences. Thus probes obtained by the inverse PCR method could potentially hybridize with many genome sequences.

IPCR has been used to amplify and clone a genomic sequence flanking transposable element, *Ac* (activator) in tobacco.[52] The amplified DNA was cloned by blunt-end cloning and transformed into *E. coli*. Amplification was established in a model transgenic tobacco plant carrying an *Ac* element and applied to the cloning of a *Spm* element from a maize line carrying multiple *Spm* hybridizing sequences. The method may be used to facilitate the isolation of wild-type genes. Rich and Willis have reported the use of IPCR for amplifying genomic DNA sequences flanking a Tn5 insertion in the chromosome of a *Pseudomonas syringae* strain.[53] The

2.5-kb amplified product was used as a hybridization probe to isolate the homologous fragment from a cosmid library of wild-type *P. syringae* genomic DNA. The method may be used to isolate DNA sequences adjacent to both ends of a chromosomal Tn5 insertion.

e. PCR Amplification with a Single Specific Primer

Sometimes it is desirable to amplify DNA fragments that contain only a single known sequence that is long enough to enable synthesis of a functional primer in PCR reactions. Such a method has been described by Kalman et al.[55] The first step in this method consists of restriction endonuclease digestion of chromosomal DNA to generate 5′ overhanging cohesive ends with 5′ phosphorylated termini. A double-stranded linker with one flush end and the other end complementary to the overhang was generated by the restriction enzyme. The linker-primer DNA contained no phosphomonoesters. As a result, ligation of linker DNA to chromosomal DNA with T4 DNA ligase resulted in covalent attachment of only one of the strands of the linker DNA to 5′ termini of restricted chromosomal DNA. Following this, a specific primer was synthesized complementary to the single known sequence, and the PCR reaction was performed in the presence of this specific primer, as well as additional linker-primer.

Although this method described by Kalman et al.[55] is similar in many ways with the "single-sided PCR" described by Mueller and Wold,[56] there are differences, such as (1) the generation of ends suitable for ligation by linear amplification, rather than by restriction enzymes; (2) the use of flush-ended linker primers vs. more readily ligatable cohesive ends; and (3) the use of the method for DNA footprinting vs. cloning.

B. "Booster PCR"

One of the problems in PCR amplification is the formation of "primer dimer" and other spurious products when fewer than 1000 copies of the target DNA are amplified. Primers and enzyme are thus consumed with a consequent reduction in yield of target. To overcome this problem "booster PCR" has been described by Ruano et al.[57] During stage I, primers are diluted to obtain an initial 10^7-fold molar excess of primer over template. At the beginning of stage II, primer concentration is brought up to 0.1 μM, instead of the starting primer concentration of 0.1 to 1.0 μM in the standard PCR method. The "booster PCR" amplifies more target, since it reduces primer-dimer formation. It seems that the possibility of missing the single target altogether can be eliminated by using the "booster PCR" method.

C. Anchored PCR

Anchored PCR (A-PCR) is used in the analysis of sequences that have variable termini. Loh et al. have used A-PCR to analyze the diversity of the T-cell receptor α-chain mRNAs from human peripheral blood lymphocytes.[58] cDNA was first synthesized and a poly (dG) tail was annexed to the 3′ end using terminal deoxynucleotidyl transferase (Figure 3). PCR was performed using a 3′ primer and another primer, called the *anchor*, which contained a poly (dC) tail attached to a sequence with convenient restriction sites. A-PCR has also been used to obtain the 5′-untranslated regions of two nonallelic preproinsulin genes.[59]

Furthermore, A-PCR can be applied to clone a segment of a gene or a complete gene from the genome when the amino acid sequence of either the NH_2- or the $COOH-$ terminal end is known. Using a single specific primer, the upstream or the downstream region of the gene can be amplified. The complementary strand can be amplified by A-PCR. A-PCR precludes the DNA sequencing of the synthesized first strand to obtain the upstream primer sequence information.

D. Membrane-Bound PCR

This method is useful when there are limited amounts of template DNA or when the DNA is contaminated. The DNA can be purified by electrophoresis and then blotted, or it can be blotted

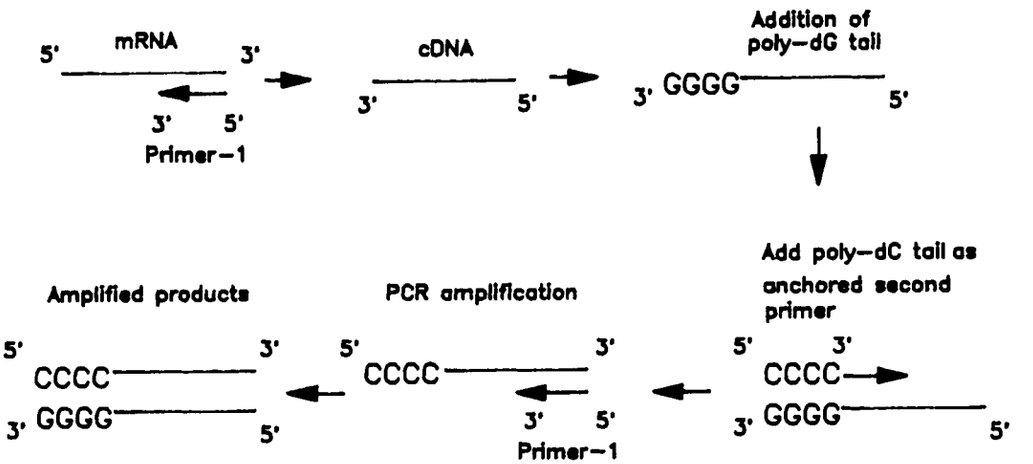

FIGURE 3. Amplification of sequences with variable termini using anchored PCR.

then washed prior to PCR. The efficiency is somewhat lower than solution PCR; membrane-bound PCR requires about 40 cycles of PCR amplification. This method has been used to amplify anchored cDNA from a spider abdomen (*Nephila clavipes*).[60]

E. Expression Cassette PCR

A human CD4 protein-overproducing strain of *E. coli* was constructed by MacFerrin et al. using expression-cassette polymerase chain reaction (ECPCR).[61] In ECPCR any contiguous coding sequence is inserted between sequences that direct high-level protein biosynthesis in *E. coli*. The gene expression cassettes obtained by ECPCR are inserted in a regulated overexpression plasmid, which is then transferred into competent *E. coli* cells by transformation. Also, ECPCR permits the facile generation of mutant proteins with N- and/or C-terminal truncations by modifying the 5′- or 3′-end of a coding sequence. The ECPCR method has permitted the dissection of a multidomain protein into its component domains.

F. Ligation Mediated PCR

In mammals and other vertebrates, DNA methylation plays an important role in the transcription-silencing system. Conventionally DNA methylation at specific sites is studied by use of methylation-sensitive restriction enzymes, followed by Southern blotting or by genomic sequencing. The sensitivity of these conventional methods is poor. For Southern blot assay about 10^5 cells (1 μg DNA) per lane is required, and for genomic sequence assay 50 μg DNA is required, both of which give poor quality data. The sensitivity of this method has been increased several-hundred-fold by using ligation-mediated-PCR (LM-PCR) following enzyme treatment.[62] In this method, after cleaving two portions of the same DNA sample simultaneously with two restriction enzymes, one sensitive and the other insensitive to methylation, a gene-specific oligonucleotide primer is used for primer extension, followed by linker ligation and then conventional PCR amplification. Using this method it is possible to analyze DNA methylation quantitatively from 100 cells (approximately 0.6 ng), which is 1000 times more sensitive than Southern blotting.

G. Amplification of RNA

Methods commonly used for RNA analysis include *in situ* hybridization, Northern blots, S-1 nuclease assays, and RNase A protection studies. The level of detection is about 10^5 to 10^6 target sequence molecules, except for *in situ* hybridization, which can detect 10 to 100 molecules

in a single cell.[63] The PCR technique can be modified to detect target RNA. If target RNA sequence is to be amplified by PCR, a DNA copy of it (cDNA) must be synthesized by using reverse transcriptase before the PCR is begun. The nucleotide sequence of point mutations at the mouse HPRT locus has been determined using *in vitro* amplification of HPRT mRNA sequences.[64] Starting with 1 μg each of poly(A)$^+$ RNA of two mutants, a 740-bp fragment containing the entire HPRT coding region was amplified. First a cDNA copy was synthesized using avian myeloblastosis virus (AMV) reverse transcriptase and a HPRT-specific oligonucleotide primer, which can anneal 3′ of the stop codon of the HPRT coding sequence. Then a second primer was added that can anneal to the newly synthesized DNA strand just upstream of the AUG start codon. After 30 PCR cycles the amplified HPRT DNA segment was sequenced and the sequences compared with the published sequence of wild-type mouse HPRT cDNA. To ensure that a base-pair change is not caused through misincorporation by the reverse transcriptase or the DNA polymerase, it is necessary to sequence a second clone from an independent amplification or to pool >10 M13 isolates when sequencing. Polymerase-induced mutations at a base in a given isolate will be "diluted out" by the wild-type sequence in the other isolates. The biological mutation, however, will be present in all isolates, since it was present in all of the original cDNA copies and will therefore be detected. This method can be used to sequence incorrectly spliced mRNAs and can therefore be applied to the study of factors that play a role in the choice of splice acceptor sites. Mahbubani et al.[65] have reported a method for the detection of bacterial mRNAs that involves brief inhibition of protein synthesis with chloramphenicol, followed by reverse transcription, PCR amplification of cDNA, and Southern blot hybridization. Detection of mRNAs by this method was several orders of magnitude more sensitive than Northern blot hybridization but less sensitive than direct DNA target amplification by PCR.

1. RACE: Rapid Amplification of cDNA Ends

The synthesis of full-length cDNA copies of mRNA transcripts by reverse transcription can be hard to achieve. The RACE protocol generates cDNAs by PCR amplification of the region between a single point in the transcript and the 3′ or 5′ end.[66] For the 3′ end, a "hybrid" primer containing 17 oligo(dT) residues linked to a unique 17-mer primer (adapter) is used for reverse transcription. The adapter primer, together with another unique primer, is used for subsequent PCR amplification. For the 5′ end, cDNA is synthesized using a gene-specific primer. The first strand reaction product is tailed with a monopolymer. Then PCR amplification is achieved using the hybrid primer and a gene-specific primer. One of the problems encountered with this method is nonspecific amplification. This can be minimized by raising the annealing temperature and choosing primers with similar melting temperatures. The specific product can be traced and isolated from agarose gel after Southern blot analysis.

2. Quantitation of mRNA Using PCR

Quantitation of mRNA can be measured by using "competitive PCR". From a small number of MLA-144 cells (200 cells), the expression of two cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3), were PCR amplified by "competitive PCR" methods following cDNA synthesis.[67] In their method, instead of analyzing a different reporter gene product,[68] they added a competitor DNA fragment that differed from the cDNA of interest by having altered restriction enzyme sites or a small intron. Thus, the same primers coamplified the unknown template and the competitor. After PCR amplification the competitor-amplified DNA can be identified by restriction digestion. The ratio of the products remained constant throughout the amplification. The relative amounts of the amplified DNAs are measured by

direct scanning of ethidium bromide-stained gels or by radionucleotide incorporation. The ratio of the two amplified DNAs is used to calculate the concentration of the target cDNA. Following this method an accurate measurement of mRNA species in low abundance or from a low number of cells was determined. cDNA prepared from as few as 10 cells can be quantitated. Random hexamer primers should be used to prime the reverse transcriptase reaction to obtain an internal mRNA control, as the efficiency of this reaction may not be 100%.

Wang and Mark have reported a technique using synthetic AW106 cRNA as an internal standard.[69] The synthetic gene has upstream and downstream primers of 12 target genes, connected in sequence. The AW106 cRNA is reverse transcribed and amplified with target mRNA in the same tube. The PCR products are distinguished by gel electrophoresis on the basis of size. The amount of target mRNA is calculated from the standard curve of the AW106 cRNA.

A PCR-aided transcript titration assay (PATTY) has been described by Becker-Andre and Hahlbrock for quantification of absolute mRNA.[70] A site-directed mutated cDNA, containing a new restriction site and derived from the mRNA to be analyzed, serves as an internal standard. Equal amounts of total RNA are "spiked" with increasing known amounts of internal standard RNA. After cDNA synthesis and PCR, the amplified DNA is cleaved with the appropriate restriction enzyme. The samples contain progressively more mutated DNA fragments and less endogenous target DNA. One sample contains equal amounts of both types of DNA. The known amount of mutated RNA added is equal to the mRNA to be analyzed. The detection level achieved is 100 target molecules.

Quantitation of RNA is conventionally done by Northern blot hybridization and RNase protection assays.[71,72] Moderately abundant total RNA from cells is sufficient to carry out these assays. However, there are several limitations, such as quantity of RNA required, efficiency of binding of RNA to hybridization membranes, and time-consuming manipulation of RNA by the conventional methods. Moreover, various rare transcripts may not be identified and quantified by these methods.

PCR has been used to amplify specific mRNA transcripts into many copies following cDNA synthesis. Also, relative expression of a specific RNA and quantitation of rare messages can be determined by PCR. Expression of the multidrug resistance gene (*mdr1*) in human breast tumors was detected at a low level of total cellular RNA (1 ng).[73] The entire method was simplified and was performed in a single tube.

Using PCR, cloning of a full-length cDNA of low-abundance mRNA from a single protein was developed by Cooper and Isola.[39] In their method, the first strand cDNA of the mRNA of serum p80 protein was synthesized by a poly dT_{17}GGCC "universal" primer that ultimately introduces an *Xcy*I site into one terminus of the cDNA using T4 DNA polymerase that has 3′ exonucleolytic strand degradation. The second strand synthesis was primed by annealing a specific degenerate deoxyoligonucleotide sequence (DOS) containing all possible codon combinations of the N-terminal amino acid sequence. The 5′ end of the specific primers were modified by the addition of a synthetic *Eco*RI linker sequence. Following PCR amplification they were able to clone the amplified products in the M13 vector. Although this method produced a considerable number of nonspecific amplified DNA bands in an agarose gel because of usage of "universal" and degenerate primers, hybridization analysis showed that about 90 to 95% of the clones were the target mRNA.

The amplification and quantitation of mRNA has been further simplified utilizing the reverse transcriptase activity of *Taq* DNA polymerase. Reverse transcriptase (RT) activity of *E. coli* DNA polymerase was first described by Loeb et al.[74] Recently, it has been shown that similar *in vitro* RT activity persists for *Taq* DNA polymerase at 68°C with a 2 to 3 m*M* Mg^{2+} concentration.[75] The RT activity of *Taq* polymerase was combined with PCR amplification and a one-step, one-enzyme analysis was performed by Shaffer et al. for a spliced interleukin-2 (IL-2) mRNA from gibbon T cells (MLA144).[76] Use of a thermostable *Taq* RT permits the reaction to proceed at a higher temperature, eliminating the RNA secondary structure, increasing primer stringency, and speeding up reaction time. Most RT-PCR protocols described require up to an hour of re-

action time in the presence of viral RT,[63,67,77-81] whereas the protocol described by Shaffer et al.[76] and Singer-Sam et al.[82] using *Taq* RT takes only minutes. Although the reverse transcription using *Taq* polymerase enzyme was performed using different concentrations of Mg^{2+}, some researchers found that a complete and efficient synthesis of the first strand may require Mn^{2+} (D. Gelfand, personal communication). They found that without Mn^{2+}, the RT activity of *Taq* DNA polymerase is very slow and that it fails to extend more than 150 to 250 bases.

The mini-exon-donor RNA (med RNA) of an insect trypanosomatid *Heptomonas seymouri* from 4×10^6 live cells was immersed in a boiling water bath for 5 min and cooled quickly on ice. Reverse transcription and PCR amplification was performed from the supernatent of the sample without any further purification within 8 h.[83,84] Since the amount of medRNA per cell was not known, they could not calculate the efficiency of this simple method. However, using defined amounts of different RNA synthesized *in vitro*, the reverse transcription and PCR steps by using this procedure yielded greater than 10^7-fold amplification of a 70-bp product.

A RT-PCR assay was used to measure quantitatively the accumulated levels of RNA transcripts in total mouse RNAs derived from male germ cells at various stages of spermatogenesis.[82] Using this method it was determined that RNA levels for two X-linked enzymes, phosphoglycerate kinase (PGK-1), and hypoxanthine phosphoribosyl transferase (HGPRT), decreased during spermatogenesis. In contrast to this, the Y-linked ZFY (zinc finger protein) was elevated in all spermatogenic cell fractions tested.

The accurate quantitation of PCR-amplified DNA or RNA is greatly affected by plateau effects, uneven priming, or variable cycle efficiencies. A combination of PCR amplification of the target DNA or RNA and temperature gradient gel electrophoresis (TGGE), described by Hencod and Heibey,[85] could overcome such problems. In this method an internal standard template is used. In PCR amplification, the ratio between the template and standard remains constant during PCR amplification. Subsequently a small amount of labeled standard (approximately 1 ng per 0.1 to 1 μg of amplified DNA) is added, which after denaturation and reannealing form a homoduplex with amplified standard and a heteroduplex with the amplified template. Since the standard duplex has more thermal stability than the template DNA, in TGGE the heteroduplex template will migrate a shorter distance than the homoduplex standard. From this the initial template copy number is calculated as follows:

Template copy number
= (intensity of heteroduplex/intensity of homoduplex) × number of initial standard copies.

The quantitation by this method based on the heteroduplexes is found to be more accurate than other methods.

II. APPLICATIONS OF PCR

A. Cloning with PCR

PCR-amplified DNA can be directly cloned into a plasmid or M13 vector. A restriction site can be created in the PCR primers, and the amplified DNA can be digested and cloned. This method is simpler and faster than the construction of phage or cosmid libraries, screening of recombinant clones, and restriction mapping and subcloning.[86,87] A restriction site is created at the 5′ end of a primer by adding bases. A "GG" or "CTC" clamp is also added to prevent "breathing" of the DNA during digestion. An "internal" restriction site is created by modifying the existing sequence near the 5′ end of the primer. Any base changes in the primer are made only at the 5′ end, so as not to interfere with 3′ extension by *Taq* polymerase. PCR amplification is performed for 26 to 28 cycles to produce sufficient DNA for cloning. More PCR cycles may produce nonspecific products.[88] For blunt-end cloning the amplified product may be cloned after repairing the 3′ termini with the Klenow fragment.[89] Since PCR allows the incorporation of any restriction site or promoter sequence, it obviates the necessity of using site-directed mutagensis to modify a sequence. Auch and Reth have described a PCR-based method for the rapid de-

tection and cloning of exons from genomic DNA fragments.[90] An exon trap vector has been constructed that contains the LTR of Rous sarcoma virus (RSV) as a strong promoter in front of a truncated 213-bp sequence of the phosphatase gene followed by the 3′ part of the rat preproinsulin gene. Genomic fragments are cloned in the vector and transfected into trypsinized COS cells and RNA is analyzed by RNA-PCR amplification. The method is useful for the identification of unknown genes and for the determination of exon-intron structures.

B. DNA Sequencing

PCR products can either be cloned prior to sequencing or can be directly sequenced. Cloning and sequencing allows the use of standard sequencing protocols but is sensitive to the error rate of *Taq* polymerase. *Taq* polymerase incorporates one incorrect nucleotide per $1/10^4$ to $1/(5 \times 10^4)$ base additions. Therefore, several independent isolates have to be analyzed to determine the correct sequence.

Direct sequencing methods include genomic amplification with transcript sequencing (GAWTS)[91] and RNA amplification with transcript sequencing (RAWTS).[92,93] The two methods are similar, except that GAWTS uses genomic DNA and RAWTS involves cDNA synthesis. Both methods require the attachment of a phage promoter onto at least one of the PCR primers. A transcription step further increases the signal and provides an abundance of single-stranded template for reverse transcriptase-mediated dideoxy sequencing. An end-labeled reverse transcriptase primer complementary to the desired sequence generates the additional specificity required to generate unambiguous sequence data. The four steps involved in the procedures are (1) cDNA synthesis for RAWTS, genomic DNA is used in GAWTS; (2) PCR in which either or both primers contain a phage promoter; (3) transcription with a phage polymerase; and (4) dideoxy sequencing using reverse transcriptase. Koeberl et al.[94] have reported the use of GAWTS for measuring the rate of polymorphism in regions of functional significance in the factor IX gene.[94] RAWTS has been used to determine that a low level of expression of "tissue-specific" mRNAs occur in many tissues.[93]

Direct sequence analysis of the three-allelic polymorphism of the apolipoprotein E (apoE) gene of humans was performed by using PCR.[95] In this method the PCR amplification was performed using one of the primers containing biotin at the 5′ end. The synthesized biotinylated fragments were then captured on an avidin matrix and rendered single stranded, whereafter the nucleotide sequence of the immobilized strand is determined by the chain termination method. A similar method was also described to separate the biotinylated amplified strand on streptavidin-coated magnetic beads followed by chain-termination sequencing of gibbon interleukin-2 gene.[76] This simplified method of PCR amplification, followed by rapid automated sequence analysis, of human genes can be used for routing diagnostic purposes.

A semi-automated method of DNA sequence analysis using PCR amplification described from glycerol preserved bacterial stock cultures in a microtiter plate and robotic work station has been described.[96] Using differential amounts of primers, it is possible to generate single-stranded DNA by PCR. This method is called "asymmetric" PCR, which has revolutionized the DNA sequencing method by saving time, accuracy, and effort.[97,98] Asymmetric PCR is less efficient than conventional PCR, and more cycles need to be run to achieve maximum yield. Generation of a single-stranded DNA template using asymmetric PCR amplification of the target DNA, coupled with fluorescent-labeled chain termination, automated DNA sequence analysis has been described by Wilson et al.[99]

C. Molecular Analysis of Mutations

Germ-line and somatic single-base substitutions are responsible for some inherited and acquired diseases.[100] PCR can be used for the direct detection of point mutations. Three techniques that are used following PCR in the diagnosis of point mutations are dot-blot hybridization,[101] restriction analysis,[102,103] and direct sequencing.[4,104,105] The RNase-A mismatch cleavage method is a powerful tool for the detection and

characterization of single-base substitutions in eukaryotic genes. The method is based on the ability of bovine pancreatic RNase to recognize and cleave a large percentage of single-base mismatches in RNA:RNA[106] or DNA:RNA[107-109] duplexes. A labeled RNA probe is hybridized to cellular RNA or DNA, and the hybrids are digested with RNase A. The products of digestion are analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. Point mutations are detected by mismatch-specific subbands. PCR greatly increases the scope of this method by providing an increased concentration of target sequences. Also, PCR is possible on formalin-fixed, paraffin-embedded tissue, which further widens the scope of this approach. This method has been used for the diagnostic detection of mutant *ras* genes in human tumors.[110] While not all base mismatches are cleaved by RNase A, chemical cleavage of the heteroduplex using hydroxylamine and osmium tetroxide will identify all mismatched thymidine and cytosine residues.[111]

Radiolabeled DNA probes can be produced by asymmetric PCR, and this also facilitates heteroduplex mapping. A normal gene segment is amplified and single-labeled DNA strands are generated. These labeled strands are then hybridized to DNA amplified with the same primers from a suspected mutant. The heteroduplex is analyzed by chemical cleavage.[111]

DNA polymerase in PCR-amplified products can be identified by denaturing gradient gel electrophoresis (DGGE). This method allows the separation of DNA molecules differing by single base changes.[107-109,112,113] DNA is electrophoresed through acrylamide gels, containing a gradient of formamide and urea. As the fragments migrate into a region where partial denaturation begins, the electrophoretic movement stops. The temperature at which a fragment begins to melt is altered by single DNA base substitutions. Hence, the position in the gel is determined by the DNA sequence. DGGE can detect about 50% of the single base substitutions in DNA fragments from 100 to 1000 bases in length.

While the RNase A cleavage method cannot detect all mutations because its efficiency depends on the mismatch and its context,[107-109] DGGE loses its resolution when strand dissociation occurs.[114,115] One approach to solving this problem is the inclusion of GC-rich high-temperature melting "clamps" at the 5′ terminus of one of the oligonucleotide primers.[116] The solution melting method[117,118] provides another approach to detecting mutations in the high-melting domains of PCR products that is based on melting heteroduplexes in solution, followed by polyacrylamide gel electrophoresis to monitor for strand dissociation. As the concentration of the denaturant increases, the melting of double-stranded nucleic acid proceeds step-wise through a series of discrete domains. The sequence of the high-melting domain, and not the length or composition of the other domains present, determines the conditions under which strand dissociation occurs. Radioactively labeled heteroduplexes are heated at various concentrations of formamide, cooled, electrophoresed, and autoradiographed. The radiolabeled probe is identified in either a double-stranded (fast migrating) or single-stranded (slow migrating) form. The method is sensitive enough to detect destabilization of a high-melting domain in a RNA-DNA heteroduplex by a single-base mismatch, as revealed by the earlier disappearance of the relevant double-stranded species by autoradiography.[118] *Taq* polymerase introduces approximately 1 mutation per 400 nucleotides, based on the assumption of 30 PCR cycles and a 2×10 E-4 error rate.[4] Therefore, the analysis of PCR fragments containing high melting domains approximately 130 bp and shorter should be unaffected by the introduction of mutations during amplification. Because of this length limitation, this method is not suitable for screening large fragments of DNA for polymorphisms, but is useful for screening multiple exons for mutations.

D. Gene Fusion by PCR

Yon and Freid have reported an elegant method for constructing hybrid fusion genes using PCR.[119] PCR is performed with the two DNA fragments carrying the sequences to be fused and three primers. While the "outer oligos" anneal to different fragments of DNA, the "linking oligos", present at a much lower concentration (0.1 μM), hybridizes with both DNA fragments around

the joint. Thus the desired fusion product is produced.

PCR has been used to recombine DNA molecules of two different mouse class-I major histocompatibility genes at their precise junctions, irrespective of the nucleotide sequences at the recombination site, thus, engineering hybrid genes without using restriction endonucleases or ligase.[120] In this method fragments from the genes that are to be recombined are generated in separate polymerase chain reactions. Then a set of primers is designed so that the ends of the products contain complementary sequences. When these PCR products are mixed, denatured, and reannealed, the strands having the matching sequences at their 3′ ends overlap and act as primers for each other. Thus, extension of this overlap by DNA polymerase produces a molecule in which the original sequences were "spliced" together.

E. Identification of DNA Sequences that Bind to Regulatory Proteins by Whole Genome PCR

The total genomic DNA is cleaved and DNA fragments are ligated to "catch linkers" consisting of a 20-bp DNA fragment. Each linker has one half of the *Xho*I site. Catch-linkers ligated to themselves are cleaved with *Xho*I. Using catch oligomers as primers, the ligated DNA is amplified by PCR. Amplified DNA is selected by protein binding, eluted, and reamplified. This approach has been used for the identification and cloning of human DNA sequences that bind to the *Xenopus laevis* transcription factor IIIA.[121]

F. Mapping of Transposon Insertion Sites

Barnes has described a method for mapping transposon insertion sites.[54] Primers are designed to prime DNA synthesis from the short inverted sequences at the ends of transposons. Whenever the primer hybridizes with a complementary genomic sequence, a product, called *echo* is formed, which is detected on an agarose gel. The amplified DNA can be cloned and sequenced to determine the nature of the fragment.

G. PCR Amplification Following Chromosome Microdissection

Using the PCR method a simple rapid procedure has been described to isolate clones carrying sequences from specific regions of the polytene chromosome of *Drosophila melanogaster*.[122] In this procedure a specific region of the polytene chromosome *D. melanogaster* was dissected out and used as a template for PCR amplification using nonspecific primers. The amplified DNA was used as a probe to screen a standard *D. melanogaster* library. The positive plaques are those clones carrying sequences homologous to the region from which the DNA was dissected. The isolated segments can be sequenced and characterized. This procedure overcomes the difficulties and limitations of the conventional procedures described for the purpose[123] and is simpler than the one developed by Ludecke et al.[124] to clone the defined regions of the human genome.

H. Diagnosis

1. Bacteria

PCR is being used for the rapid detection of pathogens, especially those whose *in vitro* cultivation is difficult, lengthy, or unavailable. *Borrelia burgdorferi*, the etiologic agent for Lyme disease, has an intermediate vector, the deer tick, *Ixodes dammini*. The mid-gut contents of the live tick are screened for the spirochaete using fluorescent antibodies and culture. PCR extends the range of specimens that can be analyzed for the presence of the organism.[125] The diagnosis of syphilis is made difficult by the fact that the pathogen *Treponema pallidum* cannot be cultivated on artificial medium. Dark-field microscopy, currently used for the detection of syphilis, is insensitive.[126] Burstain et al.[127] have described a sensitive PCR-based assay for *T. pallidum* that amplifies the gene encoding the pathogen-specific and highly conserved 47-kDa membrane immunogen. Detection of *T. pallidum* by amplification of a part of the *tmpA* gene has been reported by Hay et al.[128] PCR-based detection of *Mycoplasma genitalium* is being used to investigate

the pathogenicity of the organism and to elucidate its main tissue tropism.[129] As few as four organisms can be detected by amplification of a segment of the 140-kDa adhesin gene. The amplified DNAs of different *M. genitalium* strains were cleaved with restriction enzymes to detect point mutations and geographic variation. Conventional means of identification of *Mycobacterium tuberculosis* takes up to 6 weeks, owing to the long generation time. More recently, Wit et al.[130] have developed a PCR-based *M. tuberculosis* detection assay using repetitive DNA sequences present in the genome. The detection limit is 10 organisms. Different mycobacterial species were identified by PCR using a 65-kDa mycobacterial antigen,[131] MPB 64 protein,[132] and a repeated DNA sequence.[133] Using a protein antigen b sequence as target, Sjobring et al.[134] were able to detect mycobacteria from the *M. tuberculosis* complex. *M. leprae* was also detected by PCR with low sensitivity.[135] The use of PCR has been reported for the identification of pathogenic rickettsiae.[136] PCR provides several advantages over ELISA and DFA in the identification of *Rickettsia typhi*. Unlike ELISA, DFA, and plaque assays, PCR requires no fresh or properly frozen specimens. Since PCR can be applied to fixed tissues (frozen or formalin fixed),[137] it makes it very advantageous in field studies by reducing the potential dangers involved in the transportation of infected vectors. Besides, PCR detection of *R. typhi* is significantly more sensitive than ELISA, DFA, or plaquing techniques.[136]

Specific primer sets have been designed for the detection of the whooping cough pathogen *Bordetella pertussis*.[138] These primers are based on the sequence of the pertussis toxin, discriminate between the pathogen and related species, and can detect down to six bacteria. A multiple gene amplification system has been prepared for the simultaneous detection of enterotoxigenic *Escherichia coli* (ETEC) and *Shigella*.[139] Three primer sets are used in PCR to amplify the heat-stable and the heat-labile enterotoxins of ETEC and the invasion-associated loci of the large *Shigella* virulence plasmid. Both pathogens cause diarrheal illnesses, claiming four to five million infant lives each year in developing countries.[140] The enteroinvasive *S. flexneri* was detected from food (inoculated with 10^4 cells per gram of lettuce) within 1 d by PCR amplification of a 0.760-kb fragment of the 220-kb invasive plasmid.[141] As few as 1000 toxigenic *E. coli* were detected colorimetrically by PCR using the heat-labile toxin gene.[142] Single *E. coli* cells from stool samples were detected colorimetrically by PCR using the LT gene as the target DNA.[143]

Verotoxin producing *E. coli* strains were reliably detected by PCR amplification coupled with gene probe methods with a sensitivity of 100 pg to 1 ng of genomic DNA.[144] Victor et al.[145] have described a PCR-based procedure for the diagnosis of toxigenic *E. coli*. Serological typing and tissue culture techniques currently used for the detection of enterotoxigenic *E. coli* lack the specificity and sensitivity required for routine diagnosis.[146-148] A highly conserved region of the A subunit of the heat-labile enterotoxin gene is amplified. Bacteria are preselected on plates from stool samples, lysed by boiling, and amplified. The sensitivity achieved was 1 bacterium in 10 μl. Specific detection of aerolysin producing *Aeromonas hydrophila* was demonstrated by PCR amplification of the *aer* gene[149] with a sensitivity of 1 ng of genomic DNA.[150]

Toxigenic *Clostridium difficile* have been differentiated from nontoxigenic strains by PCR amplification of the *toxinA* gene.[151] No amplification was evidenced with the serologically cross-reacting *C. sordellii*. Wren et al. have also described a PCR assay for *C. difficile* using the *toxinA* target.[152] Their primers, however, showed cross-reactivity with *C. sordellii*. The toxigenicity of *C. difficile* is conventionally determined by a time-consuming and cumbersome cell structure assay.

PCR amplification of the MOMP gene has been used for the detection and the distinction of *Chlamydia trachomatis, C. psittaci,* and *C. pneumoniae* in tissue culture and for the detection of *C. trachomatis* in direct patient specimens.[153]

PCR-based detection of eubacterial species has been developed based on the conserved regions of the 16S rRNA/rDNA sequence of *E. coli* by several groups.[99,154,155] The small subunit of rRNA contains segments that are conserved at the species, genus, or kingdom level. Another set of *E. coli* 16S rRNA-based primers was used for PCR amplification of the rDNA of more than 100 different bacterial species, and the amplified

DNAs were detected colorimetrically (Bej et al., in preparation). PCR amplification using such rRNA/rDNA-based universal eubacterial primers can potentially be applied for monitoring bacterial DNA or bacterial cells in environmental, clinical, food, and pharmaceutical samples. The use of a PCR amplification system based on the 16S rDNA sequence has simplified the study of molecular systematics.[155]

2. Viruses

The retroviruses are etiologic agents for malignancies such as lymphomas, leukemias, sarcomas, and carcinomas, autoimmune diseases such as arthritis and lupus, and cytopathic diseases leading to anemias and immunodeficiency states. HTLV-1 causes adult T-cell lymphoma/leukemia, myelopathy, and immunodeficiency. HTLV-II causes T-cell and hairy-cell leukemias. HIV-1 and HIV-2 cause AIDS. Most humans infected with a retrovirus do not manifest symptoms for many years. Serological assays do not detect seronegative infected stages. Also, for detection of infection in newborns, treatment efficacy, and for analyzing genetic variation of different virus isolates, it is important to be able to detect the retroviruses themselves. The low level of circulating free virus may hinder direct detection of virus in patient samples without *in vitro* propagation. Virus culturing is time consuming, expensive, and tedious. Often the results are inconclusive. Tests performed on cultured cells, microscopy, immunofluorescence, and Southern blot analysis on extracted DNA, do not have the required sensitivity.[156] The use of PCR has greatly facilitated the detection of retroviruses. PCR has been used in epidemiological studies and in the diagnosis of HTLV-I and -II.[157-159] The identification of HIV by PCR has aided diagnostics and research.[160,161]

Hepatitis B virus infection is diagnosed by the presence of HBsAg, anti-HBsAg, anti-HBcAg in the serum using radioimmunoassays or ELISA. The finding of HBV DNA and DNA polymerase in serum indicates active virus replication. In some cases of chronic hepatitis, the HBV DNA cannot be detected in serum due to the low level of virus. PCR-gene probe detection has been found to be 10-fold more sensitive than direct detection of HBV DNA in serum by slot-blot analysis.[42]

The genital human papillomaviruses (HPVS) consist of over 20 virus types associated with a number of diseases and cancers. The HPVs share interspersed regions of DNA sequence homology. PCR primers have been designed that will amplify distinct regions from over 25 HPVs.[162] The PCR method has been applied to develop the detection of 11 different HPVs using only two general primer sets.[163] With these primer sets it is possible to identify novel HPV genotypes in dysplasias and squamous cell carcinomas suspected of having a HPV etiology.

The enteroviruses (EV) comprises more than 60 serotypes and are responsible for the most common causes of childhood infection. Current diagnostic methods are slow, lack sensitivity, and are complicated by the serotypic diversity of the EVs and by low viral titers in clinical specimens. PCR provides an accurate and rapid diagnostic test.[164]

The cytomegalovirus (CMV) is the most common congenital viral infection in humans, leading to mental retardation and nonhereditary sensorineural deafness. A reliable test for CMV is needed. PCR gene probe detection of CMV has been shown to have a sensitivity and a specificity of 100%.[165] The human cytomegalovirus (HCMV) strains are differentiated by the presence of *a*-sequence, which is located between the long and short unique sequences of the virus. Using PCR followed by restriction enzyme polymorphism of this hypervariable region of HCMV, Zaia et al. demonstrated differences among 38 of 40 isolates of HCMV.[166] This study showed that PCR amplification, coupled with restriction analysis of the amplified DNA, can be used for the rapid diagnosis of differences among different strains of HCMV.

Using the PCR amplification method, a fast, sensitive, and reliable detection of gastroenteritis causing adenoviruses, including Ad40 and Ad41, directly from diluted stool samples has been described by Allard et al.[167] PCR has been utilized to amplify and detect Epstein-Barr virus (EBV) DNA from blood and tissue biopsies of a patient with Sjogren's syndrome,[168] Hodgkin's lymphoma,[169] and in immunocompromised patients.[170,171] Telenti et al. described the use of the

PCR method to demonstrate very close association between EBV and lymphoproliferative disorders occurring in the presence of immunosuppression.[172]

In the majority of cases, diarrhea in children is caused by a double-stranded RNA virus known as rotavirus. Although detection of rotavirus is routine for diagnostic procedure, serotyping for different strains remains difficult. PCR amplification using serotype-specific primers of gene 9 of rotavirus[173] allows the detection and typing of this virus directly from stool specimens. Other double-stranded RNA viruses, such as infectious pancreatic necrosis virus from salmonid fish, was detected with higher sensitivity than the conventional probe detection method using PCR amplification followed by colorimetric detection.[174]

The human parvovirus B19, which is the causative agent for several syndromes, such as chronic arthritis, fetal death, chronic anemia, and neutropenia in immunocompromised patients, has been detected by PCR-gene probe methods.[175] The detection limit after PCR amplification was 10^4 times more sensitive than dot-blot hybridization with a radiolabled cloned portion of the B19 genome as a probe. Also, PCR was 10^7 times more sensitive than dot-blot hybridization when an internal radiolabeled probe was used.[175] Several thousand deaths occur in West and Central Africa every year due to an outbreak of Lassa fever, caused by Lassa virus. It has been predicted that early detection of this virus can prevent such morbidity and mortality. PCR has been utilized successfully for rapid and reliable detection of this virus from serum and urine specimens of many suspected patients.[176] The PCR method has been used successfully for specific detection of herpes simplex virus (HSV) from the lung tissue of a patient with unsuspected HSV pneumonia.[177]

3. Human Genetic Diseases

PCR has achieved widespread use in the analysis of genetic diseases. The target region is usually amplified from genomic DNA and cDNA, and examined for mutations or polymorphisms by sequencing, hybridization with allele-specific oligonucleotides, restriction analysis, or enzymatic or chemical cleavage.[158] Deletions in genomic DNA can be identified by determining if a segment of DNA can be amplified.[178]

Lesch-Nyhan syndrome is an X-linked genetically lethal disease that results from severe deficiency of the purine salvage enzyme hypoxanthine-guanine phosphoribosyl transferase (HPRT).[179] Prior to the invention of PCR, the identification of altered DNA sequences in a single patient was too big a task for routine diagnosis. PCR and automated DNA sequencing have made it possible to define the nucleotide mutation causing the disease in the majority of cases.[180] Mutations identified in affected males are used as a basis for testing the carrier status or early diagnosis of other family members.

Monogenic diseases such as β-thalassemia[104] and hemophilia A[181] are being more accurately and rapidly diagnosed by PCR. Diagnosis of a large gene, such as Duchenne muscular dystrophy, necessitates multiplex PCR using up to nine sets of primer pairs.[48] Many altered sequences have been characterized from patients with HPRT deficiency[64] and ornithine δ-amino transferase deficiency.[182] Prenatal diagnosis for cystic fibrosis is carried out on samples of fetal chorion obtained in the first trimester. While Southern blot analysis takes 2 weeks, PCR results are made available in 1 d, permitting a clinically safer termination of pregnancy or more time to think.[183] PCR has made possible the detection of extremely small numbers of cells carrying chromosomal translocations. Reciprocal chromosomal translocations are observed in hematopoietic neoplasia, such as follicular lymphoma and chronic myleogenous leukemia.[184,185] The hybrid DNA sequence or the unique mRNA sequences specific for the translocation is amplified by PCR.[186-189] HLA typing is useful in tissue typing for transplantation to minimize graft rejection by selecting "HLA-matched" donor and recipient pairs. HLA typing is important in the analysis of genetic susceptibility to autoimmune diseases. It can also be used in forensic analysis and paternity determination. The evolution of the HLA class II polymorphism and its relation to disease susceptibility has been greatly facilitated by PCR-based sequencing studies.[97,190] Recently, it has been discovered that Duchenne and Becker muscular dystrophy (DMD and BMD) in humans are

due to the defect in the X-linked dystrophin gene.[191] Using a dystrophin transcript for PCR amplification, the deletion in the message was detected for both DMD and BMD types of abnormalities from muscle biopsy samples.[192] Using PCR amplification of exon 6, a variant of antithrombin III was identified from a female patient with a history of recurrent thromboses.[193] Using this method they reported that this is due to the change of a proline for alanine as a result of the point mutation in the gene.

In cases where the mutation is unknown, classic linkage analysis can be used, such as in the case of prenatal diagnosis of hemophilia A.[103] PCR amplification of nuclear as well as mitochondrial DNAs in such cases using gene-specific primers, followed by restriction length polymorphism (RFLP), has been achieved successfully.[194] Molecular lesions, which do not alter restriction sites in the gene, have been detected by PCR amplification followed by RNase A mismatch and gel electrophoresis analyses.[110]

Recently a chromosomal assignment method for acidic fibroblast growth factor gene located on human chromosome 5, based on the PCR amplification of the target DNAs from somatic cell hybrids, has been described.[195] This approach is faster, less labor intensive, and much more sensitive than the method of assignment, conventionally done by Southern blot hybridization of somatic cell hybrid DNAs.

An X-linked recessive disorder, hemophilia B (Christmas disease) in humans, affects 1 in 30,000 males and is difficult to diagnose accurately by conventional methods. These problems were overcome by utilizing PCR amplification for the clinical diagnosis of hemophilia B.[196]

The long-term applications of the PCR method in transfusion medicine are enormous. Chromosomal translocations have been detected by PCR in residual follicular lymphoma cells from patients in remission who failed to show any abnormality by morphologic examination and conventional Southern Blot analysis.[186,188,197-200] One lymphoma cell was specifically detected by PCR method in 1 million normal cells by Crescenzi et al.[188] They also described the use of PCR to purge malignant cells from bone marrow and rapid DNA sequencing of chromosomal breakpoints without molecular cloning. Preliminary work using PCR for ABO blood typing has been described.[201]

I. Detection of Targeted Gene Modifications by PCR

When DNA is introduced into a cell such that the exogenous DNA contains sequences that match the target gene, homologous recombination occurs, leading either to the insertion of the incoming DNA into the target gene or to replacement of sequences in the target. PCR has been applied for the detection and isolation of cells containing the correctly modified target loci.[202] Primers are chosen so that only the required modified target is amplified. The PCR product of the recombinant fragment is seen in an ethidium-bromide-stained agarose electrophoresis gel. The method can be used for human gene therapy, i.e., to study the possibility of replacing a defective human gene with its normal counterpart.

J. Forensic Analysis and the Determination of Familial Relationships

DNA sequence analysis is today the most powerful tool in forensic analysis. The analysis of the sample depends on the quality and the quantity of the DNA in the sample. PCR allows amplification from very small amounts of starting DNA and is also possible on degraded DNA. The analysis is also rapid and technically simple to perform. The HLA-DQα system is a well-characterized typing system,[12] which can distinguish two individuals about 91% of the time.[203] DNA typing has been performed on single hairs[204] by amplifying segments of the HLA DQα gene and the mitochondrial D-loop DNA. Identification of hairs is of considerable forensic importance. The D-loop mitochondrial DNA appears to be suitable for ascertaining genetic individuality.[204-206] Since mitochondrial DNA is maternally inherited, it is suitable for testing whether individuals are maternally related.

K. DNA Fingerprint Analysis

DNA fingerpinting is a common method used in many laboratories for paternity check or sample mixup, and has a number of different medical applications.[208-211] For DNA fingerprint analysis, sometimes copious amounts of DNA may be re-

quired. PCR amplification of the target DNA has made DNA fingerprint analysis easy and time efficient. It also enables the production of large amounts of specific probes for the hybridization process.[12,27,212,213] Contaminated DNA from fungal or bacterial sources sometimes interferes with RFLP analysis. This problem can be eliminated by PCR amplification when specific primers for human DNA sequences are used. PCR amplification and sensitive colorimetric (ASO) probe detection of the human HLA-DQα (six alleles) has been studied extensively and can conveniently be used for human DNA typing.[4] It has been found that the PCR-labeled probes give 10 times more hybridization signal for RFLP analysis than the conventional radiolabeled M13 probe.[9] PCR fingerprint has been applied in a phytopathogenic fungus, *Phoma tracheiphila*. A complete fingerprint for this fungus was achieved within a 4-h time period.[214] A study by Fujimura has revealed an important pitfall in the use of PCR in molecular diagnosis.[215] A patient who appeared to be homozygous for the absence of a restriction enzyme according to PCR was found to be heterozygous by Southern blot analysis. Such an error may be detected by performing RFLP analysis on DNA amplified with several independent primer sets for each polymorphism.

L. PCR in the Human Genome Project

The application of PCR methods in human genome mapping and sequencing is enormous, as is evident from discussions at several scientific meetings.[216] The first goal is to determine the location of the estimated 100,000 human genes, followed by the most important and ultimate goal, which is to sequence the entire human genome and to develop an understanding of how the human genome functions. Much of the emphasis using PCR genome mapping and sequencing is focused on the "sequence-tagged sites" (STS) proposal described by Olson et al.[217] Since the human genome size is so enormous, the STS method will help to organize the fragments generated to construct genetic and physical maps. Each fragment will be identified by a 100- to 1000-bp site, and will be sequenced with the primers generated to perform PCR amplification of each site.

The role of PCR in human genome organization and the generation of region-specific probes for mapping was focused on the determination of variation in the *Alu* sequence; LINE sequences [long interspersed repeated sequence, such as *Kpn*, or (CA)n] for obtaining unique signatures of clones for identification, and for generating new STSs. *Alu*-base PCR was originally described by Nelson et al.[218] and further developed by his group in isolating, mapping, fingerprinting, and sequencing the human X-chromosome. *Alu*-PCR appears to be a commonly used technique for the generation of region-specific probes, such as a probe for the fragile X region, and GTG banded chromosome to obtain a specific clone by "coincidence cloning". Microdissection of a specific region of a chromosome described by Wesley et al.[122] can be used to clone various regions of the human chromosomes.

Current nucleotide sequence methods are neither rapid not accurate. The PCR method and *Taq* DNA polymerase seem to play a great role in human genome sequencing. Development of various new and existing DNA sequencing methods are well described by Rose.[216] The role of future versions of the DNA thermal cycler and modifications of the basic PCR method and the *Taq* DNA polymerase will enhance and provide accurate human DNA sequence analysis.

M. Organismal and Population Biology

1. Phyllogenetic Studies

PCR amplification enables comparative DNA sequence study among species. PCR amplification and sequencing of a mitochondrial ribosomal RNA gene has revealed a close relationship between species of false truffles and mushrooms, which had previously been placed in a different fungal family, the *Boletaceae* family.[219] Besides studies on the relationships of contemporary species, PCR also allows studies on extinct species, since it can be performed on materials in museum collections.[213,220] Evolutionary studies on two extinct species, the marsupial wolf[221] and the quagga,[207] have been based on the PCR amplification of museum specimens. PCR studies have revealed that the marsupial wolf may have under-

gone convergent evolution with both true wolves and South American carnivorous marsupials.

2. Evolutionary Studies

PCR-amplified DNA sequence analysis of mitochondrial DNA from various human populations has confirmed the African origin for human mitochondrial DNA.[222] DNA amplified from a 7000-year-old brain[207] revealed the presence of mitochondrial DNA not found among living Native Americans. Primers based on conserved regions of mitochondrial DNA have been used to amplify DNA from more than 100 animal species, including mammals, birds, amphibians, fishes, and invertebrates.[223] DNA sequencing of PCR-amplified DNA revealed that transitions are more common than transversions in birds and fishes. However, the thymine to cytosine transition is less common in birds, since bird DNA is deficient in thymine. This study also revealed that amino acid replacement in a cytochrome b gene segment is faster in birds and mammals than in fishes. Variation in gene frequencies among different populations can be studied by designing synthetic DNA probes based on sequence data from a group of individuals and can be used to identify specific alleles. A wide range of evolutionary issues can be addressed by these studies.

3. Conservation

The population size of geographic ranges of rare animals can be estimated by identifying these animals by PCR amplification of traces left by them, such as fragments of skin or hair, urine, scent marks, and feces.[224] Identification of processed wildlife parts, such as worked ivory, by PCR will allow inforcement of international conservation laws.

4. Ecology

The species and strains in symbiotic relationships can be successfully identified using specific DNA primers and probes. Symbiotic strains present in the fungus *Laccaria* have been identified from a mycorrhizae by amplifying the spacer region of the nuclear ribosomal RNA gene repeat.[224] Zehr and McReynold[225] used degenerate oligonucleotide primers to amplify nitrogen fixation (*nif*) genes from the marine cyanobacterium *Trichodesmium thiebautii*, an organism that has never been maintained in pure culture. *Trichodesmium* bundles were isolated from the west Caribbean Sea by using plankton tows and were then washed in buffer and frozen for later analysis. DNA was isolated from the bundles by using a phenol/chloroform extraction protocol and then subjected to PCR amplification with a degenerate primer mixture of 126 and 96 oligomers for the up- and downstream primers, respectively. The resulting amplified product was resolved by electrophoresis, cloned into a M13 cloning/sequencing vector, and later subjected to sequence analysis.

N. Environmental

The PCR method was first applied to monitor genetically engineered microorganisms by Steffen and Atlas.[226] They used the polymerase chain reaction (PCR) to amplify a 1.3-kb probe-specific region of DNA from the herbicide-degrading bacterium *Pseudomonas cepacia* AC1100 in order to increase the sensitivity of detecting genetically engineered microbes in soil. PCR performed after bacterial DNA was isolated from sediment samples permitted the detection of as few as 100 cells of *P. cepacia* AC1100 per 100 g of sediment sample against a background of 10^{11} diverse nontarget organisms, that is, *P. cepacia* AC1100 was positively detected at a concentration of 1 cell per gram of sediment. This represented at 10^3-fold increase in sensitivity compared with nonamplified samples.

Chaudhry et al.[227] also used PCR for detecting genetically engineered microorganisms (GEMs). They cloned 0.3-kb napier grass (*Pennisetum purpureum*) genomic DNA into a derivative of a 2,4-dichlorophenoxyacetic acid-degradative plasmid, pRC10, and transferred the construct into *Escherichia coli*. The PCR method amplified and detected the DNA marker of the GEM, even after 10 to 14 d of incubation. They concluded that the method may be useful for monitoring GEMs in complex environments, where discrimination between GEMs and indig-

enous microorganisms is either difficult or requires time-consuming tests.

PCR is useful for the identification of pathogens and indicator species for environmental surveillance.[228] Bej et al.[33] used PCR amplification and gene probe detection of regions of two genes, *lacZ* and *lamB*, to detect coliform bacteria in environmental waters. They demonstrated the potential use of PCR amplification of *lacZ* and *lamB* as a method to detect indicators of fecal contamination of water. They showed that amplification of *lamB*, in particular, permits the detection of *E. coli* and enteric pathogens (*Salmonella* and *Shigella* spp.) with the necessary specificity and sensitivity for monitoring the bacterialogical quality of water so as to ensure the safety of water supplies. Bej et al.[8] and Cleuziat and Robert-Baudouy[229] developed a method for the detection of the fecal coliform bacterium *E. coli* using PCR and gene probes based upon amplifying regions of the *uid* gene that code for β-glucuronidase — the expression of which forms the basis for fecal coliform detection by the commercially available Colilert method.

Starnbach et al.[230] reported the detection of *Legionella pneumophila* by amplification of a fragment of DNA of unknown function from *Legionella* using PCR. Their sensitivity of detection was equivalent to 35 colony forming units detected by viable plating. Mahbubani et al.[34] developed a method for the detection of *Legionella* in environmental water sources, based upon PCR and gene probes. All species of *Legionella*, including all 15 serogroups of *L. pneumophila* tested, were detected by PCR amplification of a 118-bp DNA sequence that codes for a region of 5S rRNA, followed by radiolabeled oligoprobe hybridization to an internal region of the amplified DNA. Strains of *L. pneumophila* (all serogroups) were specifically detected based upon amplification of a portion of the coding region of the macrophage infectivity potentiator (*mip*) gene. *Pseudomonas* spp. that exhibit antigenic cross-reactivity in serological detection methods did not produce positive signals in the PCR-gene probe method using Southern blot analysis. Single cell, single gene *Legionella* detection was achieved with the PCR-gene probe methods. Both viable culturable and viable nonculturable cells of *L. pneumophila*, formed during exposure to hypochlorite, showed positive PCR amplification, whereas nonviable cells did not. Viable cells of *L. pneumophila* were also specifically detected by using *mip* mRNA as the target, reverse transcription to form DNA, and PCR to amplify the signal. When cells were killed by elevated temperature, only viable culturable cells were detected, and detection if viable culturable cells corresponded precisely with positive PCR amplification.[231] Specific primers and a probe have been developed for the protozoan pathogen, *Giardia lamblia* using the *giardin* gene as a target for the detection of this organism in water (Mahbubani et al., submitted). A single cyst of *G. lamblia* was isolated by using a micromanipulator, and detection of the single cyst was achieved using the PCR method and RNA-PCR. Another set of primers and a probe were developed for the detection of both *G. lamblia* and *G. muris* from water by Mahbubani et al. (submitted). The sensitive detection of *G. lamblia* in drinking water supplies by PCR will prevent outbreaks of water-borne diarrhea caused by this pathogen.

III. OTHER NUCLEIC ACID AMPLIFICATION METHODS

A. Transcription Based Amplification System (TAS)

In this method of nucleic acid amplification, the target RNA (or denatured DNA) is hybridized to a primer that contains a RNA polymerase (T7, T3, or SP6) binding sequence and a target complementary sequence. Reverse transcriptase is used to elongate the primer to yield a DNA strand complementary to the target RNA. the RNA-DNA duplex is heat denatured, and a second primer is annealed to the cDNA containing the RNA polymerase binding site. Now reverse transcriptase is added to produce double-stranded cDNA and a new DNA-RNA heteroduplex. Next the double-stranded cDNA is incubated with RNA polymerase to produce multiple RNA transcripts from the polymerase binding site containing a double-stranded DNA template. Some of the RNA transcripts are reverse transcribed using the second

primer and reverse transcriptase in the mixture to form RNA-DNA heteroduplex. A second cycle of cDNA synthesis and RNA transcription can then begin. Relatively few cycles are required to achieve 10^5- to 10^6-fold amplification.[232]

The method has been applied for the amplification of the *vif* region of HIV-1.[232] The specificity of the system has been enhanced by using a bead-based sandwich hybridization system (BBSHS). Sephacryl beads containing an oligonucleotide probe are used to capture the amplified sequence hybridized to another ^{32}P-labeled oligonucleotide probe. The beads are centrifuged, the hybridization solution is removed, and the beads are washed. Cerenkov counts of the hybridization solution, washes, and beads are measured. The sensitivity level of the TAS-BBSHS method is the HIV-1 sequences present in one quarter of a sample derived from one infected cell in a population of 10^6 uninfected cells. Although the basic goals of the PCR and the TAS methods are to amplify target nucleic acids *in vitro*, there are several advantages, as well as disadvantages, of the TAS method over the PCR method. Because of the inherent property of the RNA polymerase to produce 10 to 10^3 copies of RNA per copy of DNA template, the TAS method requires fewer cycles to achieve a large number of copies (10^6 copies in six cycles) with a few thermal denaturation steps. Like the conventional PCR method, the TAS method also needs 3.25 to 4.0 h to achieve a million-fold amplified product.

B. Amplification of RNA Using Qβ Replicase

Lizardi et al.[233] have described a nucleic acid amplification procedure using Qβ replicase that exponentially amplifies probe sequences, rather than target sequences, as in PCR. This Qβ RNA amplification system is an alternate to PCR. A recombinant RNA molecule has been synthesized containing a probe for *Plasmodium falciparum* embedded within the MDV-1 RNA, which serves as a template for Qβ replicase. After replication, both the template and product are released from the replication complex and serve as templates in the next cycle of synthesis. Replication initiated with 1000 RNA molecules produces 129 ng of recombinant RNA in 30 min. The Qβ assay is performed in four steps: (1) several probes are added to an RNA sample or to single-stranded DNA, (2) Qβ-labeled probes are added, (3) nonspecifically bound MDV-1 probes are removed by washing, and (4) Qβ replicase is added, which recognizes the MDV-1 variant molecules specifically attached to the hybrid complex and amplifies them up to 10^6 to 10^9 fold in 15 min at 37°C.[234] In PCR the target sequence is first amplified and labeled, and then is isolated. The Qβ assay first isolates, then labels and amplifies, only the probe sequences. The advantage of Qβ is the uniformity of the procedure for the preparation of samples from different sources. The disadvantage is that unhybridized probes may give a nonspecific signal. Also the sensitivity of this method is not as high as that achieved by the PCR method. This method is not as versatile as the PCR method and hence finds limited use in the area of diagnostics. Although the Qβ replicase system amplifies the target nucleic acids faster than PCR, it has limited scope and application in the various areas of biological research.

C. Ligase Chain Reaction (LCR)

Two single-stranded DNA probes 10 to 20 bases long and complementary to either one or both DNA strands start the reaction. First DNA is denatured. Then the probes are allowed to anneal to target DNA, lining up end to end. The probes are joined by ligase and can serve as target in the next cycle. The process is repeated 20 to 50 times. Although this is an alternative method of target nucleic acid amplification, the sensitivity, specificity, and overall efficiency of this method have yet to be demonstrated. This method has been used to detect 200 target molecules and to discriminate between normal βA- and sickle β^5-globin genotypes from 10-μl blood samples.[235]

D. Cycling Probe Reaction

A probe amplifier system based on the cycling probe reaction has been described by Duck et al.[236] In the cycling probe reaction, a chimeric

DNA-RNA-DNA probe, designated as the cycling probe, is used to hybridize with a target DNA molecule at a specific constant temperature, which is dependent upon the sequence and length of the probe. Ribonuclease H is used to cleave the cycling probe, and the resulting fragment dissociates from the target. As a result, the target molecule is available to pair with another cycling probe molecule and the cycle is repeated. As the reaction proceeds the probe fragments accumulate, serving as the basis for detection of the target. This system functions by allowing a single target molecule to act as a catalyst in converting a large number of probe molecules to a unique detectable form. This probe amplifier system is useful for large-scale automated clinical diagnostic systems. It is fast, sensitive, and performed at a constant temperature. Using a thermostable enzyme, this reaction can be performed at an elevated temperature, which increases its specificity. With the further development of this method with proven specificity and sensitivity in the amplification of target nucleic acids, this method has the potential of making use of the DNA thermal cycler optional.

IV. CONCLUDING REMARKS

The application of *in vitro* DNA amplification — in particular, PCR technology — in research and diagnostics has proceeded at an unprecedented rate. Because of its extreme sensitivity and versatility, the long-term implications of this technology in all areas of biological research and medicine are potentially tremendous. The use of PCR technology has accelerated the discoveries of the molecular basis of many infectious, malignant, and genetic diseases. The application of this method has simplified and enhanced research in the areas of forensic science, evolutionary biology, and developmental biology. Although conceptually the PCR method is simple and is based on the long-understood DNA replication process in life forms, in reality the power and applicability of this method are phenomenal. It has transformed our approaches to both fundamental and applied biological problems. PCR has assumed a prominent place in genetics, molecular biology, biotechnology, and medical research. The long-term implications of nucleic acid amplification technology will rapidly provide us more information about complex biological and medical problems. These methods will change our practice in many fields of medical and basic research.

REFERENCES

1. **Mullis, K. B. and Faloona, F. A.,** Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction, *Meth. Enzymol.*, 155, 335, 1987.
2. **Mullis, K., Faloona, F., Scharf, S., Horn, G., and Erlich, H.,** Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction, *Cold Spring Harbor Symp. Quant. Biol.*, 51, 263, 1986.
3. **Mullis, K. B.,** The unusual origin of the polymerase chain reaction, *Sci. Am.*, 262, 56, 1990.
4. **Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A.,** Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase, *Science*, 239, 487, 1988.
5. **Saiki, R., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N.,** Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, *Science*, 230, 1350, 1985.
6. **Orrego, C.,** Organizing a laboratory for PCR work, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds., Academic Press, San Diego, 1990, 447.
7. **Dunning, A. M., Talmud, P., and Humphries, S. E.,** Errors in the polymerase chain reaction, *Nucleic Acids Res.*, 16, 10393, 1988.
8. **Bej, A. K., DiCesare, J. L., Haff, L., and Atlas, R. M.,** Detection of *E. coli* and *Shigella* spp. in water by using polymerase chain reaction (PCR) and gene probes for *uid*, *Appl. Environ. Microbiol.*, submitted.
9. **Jeffreys, A. J., Wilson, V., Neumann, R., and Keyte, J.,** Amplification of human minisatellites by the polymerase chain reaction: toward DNA fingerprinting of single cells, *Nucleic Acids Res.*, 16, 10953, 1988.
10. **Smith, K. T., Long, C. M., Bowman, B., and Manos, M. M.,** Using cosolvents to enhance PCR amplification, *Amplifications*, 5, 16, 1990.
11. **Hoelzel, R.,** The trouble with "PCR" machines, *Trends Genet.*, 6, 237, 1990.
12. **Saiki, R. K., Bugawan, T. L., Horn, G. T., Mullis, K. B., and Erlich, H. A.,** Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele-specific oligonucleotide probes, *Nature*, 324, 163, 1986.

13. **Lawyer, F. C., Stoffel, S., Saiki, R. K., Myambo, K., Drummond, R., and Gelfand, D. H.,** Isolation, characterization, and expression in *Escherichia coli* of the DNA polymerase gene from *Thermus aquaticus*, *J. Biol. Chem.*, 264, 6427, 1989.
14. **Innis, M. A., Myambo, K. B., Gelfand, D. H., and Brow, M. A. D.,** DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA, *Proc. Natl. Acad. Sci. U.S.A.*, 85, 9436, 1988.
15. **Brock, T. D. and Freeze, H.,** *Thermus aquaticus* gen. n. and sp. n., a non-sporulating extreme thermophile, *J. Bacteriol.*, 98, 289-297, 1969.
16. **Gelfand, D. H. and White, T. J.,** Thermostable DNA polymerases, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds., Academic Press, San Diego, 1990, 129.
17. **Chien, A., Edgar, D. B., and Trela, J. M.,** Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*, *J. Bacteriol.*, 127, 1550, 1976.
18. **Kaledin, A. S., Slyusarenko, A. G., and Gorodetskii, S. I.,** Isolation and properties of DNA polymerase from extremely thermophilic bacterium, *Thermus aquaticus*, *Biokhimiya*, 45, 644, 1980.
19. **Oste, C.,** Optimization of magnesium concentration in the PCR reaction, *Amplifications — A Forum for PCR Users*, 1, 10, 1989.
20. **Mezei, L. M.,** Effect of oil overlay on PCR amplification, *Amplifications — A Forum for PCR Users*, 4, 11, 1990.
21. **Winship, P. R.,** An improved method for directly sequencing PCR amplified material using dimethyl solfoxide, *Nucleic Acids Res.*, 17, 1266, 1989.
22. **Chamberlain, J. S., Gibbs, R. A., Ranier, J., and Caskey, C. T.,** Multiplex PCR for the diagnosis of Duchenne muscular dystrophy, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds., Academic Press, San Diego, 1990, 272.
23. **Dermer, S. J. and Johnson, E. M.,** Rapid DNA analysis of α-antitrypsin deficiency: application of an improved method for amplifying mutated gene sequences, *Lab. Invest.*, 59, 403, 1988.
24. **Saiki, R. K.,** The design and optimization of the PCR, in *PCR Technology: Principles and Applications for DNA Amplification*, Erlich, H. A., Ed., Stockton Press, New York, 1989, 7.
25. **Kwok, S. and Higuchi, R. G.,** Avoiding false positives with PCR, *Nature*, 339, 237, 1989.
26. **Williams, J. F.,** Optimization strategies for the polymerase chain reaction, *BioTechniques*, 7, 762, 1989.
27. **Li, H. H., Gyllensten, U. B., Cui, X. F., Saiki, R. K., and Erlich, H. A.,** Amplification and analysis of DNA sequences in single human sperm and diploid cells, *Nature*, 335, 414, 1988.
28. **Sarkar, G. and Sommer, S. S.,** Shedding light on PCR contamination, *Nature*, 343, 27, 1990.
29. **Furrer, B., Candrian, U., Wieland, and Luthy, J.,** Improving PCR efficiency, *Nature*, 346, 324, 1990.
30. **Jinno, Y., Yoshiura, K., and Niikawa, N.,** Use of psoralen as extinguisher of contaminated DNA in PCR, *Nucleic Acids Res.*, 18, 6739, 1990.
31. **Saiki, R.,** Amplification of genomic DNA, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M. A., Gelfand, D., Sninsky, J. J., and White, T. J., Eds., Academic Press, San Diego, 1990, 13.
32. **Ausubel, F. M., Brent, R., Kingston, R. F., and Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds.,** *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley-Interscience, New York.
33. **Bej, A. K., Steffan, R. J., DiCesare, J., Haff, L., and Atlas, R. M.,** Detection of coliform bacteria in water by polymerase chain reaction and gene probes, *Appl. Environ. Microbiol.*, 56, 307, 1990.
34. **Mahbubani, M. H., Bej, A. K., Miller, R., Haff, L., DiCesare, J., and Atlas, R. M.,** Detection of *Legionella* with polymerase chain reaction and gene probe methods, *Mol. Cell. Probes*, 4, 175, 1990.
35. **Sommer, R. and Tautz, D.,** Minimal homology requirements for PCR primers, *Nucleic Acids Res.*, 17, 6749, 1989.
36. **Suggs, S. V., Hirose, T., Miyake, T., Kawashima, E. H., Johnson, M. J., Itakura, K., and Wallace, R. B.,** Use of synthetic oligodeoxyribonucleotides for the isolation of cloned DNA sequences, in *Developmental Biology Using Purified Genes*, Brown, D. D. and Fox, C. F., Eds., Academic Press, New York, 1981, 683.
37. **Rychlik, W. and Rhods, R. E.,** A computer program for choosing optimal oligonucleotides for filter hybridization sequencing and *in vitro* amplification of DNA, *Nucleic Acids Res.*, 17, 8543, 1989.
38. **Crick, F. H. C.,** The origin of the genetic code, *J. Mol. Biol.*, 38, 377, 1965.
39. **Cooper, D. L. and Isola, N.,** Full-length cDNA cloning utilizing the polymerase chain reaction, a degenerate oligonuceotide sequence and a universal mRNA primer, *BioTechniques*, 9, 60, 1990.
40. **Girgis, S. I., Alevizaki, M., Denny, P., Ferrier, G. J. M., and Legon, S.,** Generation of DNA probes for peptides with highly degenerate codons using mixed primer PCR, *Nucleic Acids Res.*, 16, 10371, 1988.
41. **Lee, C. C., Wu, X., Gibbs, R. A., Cook, R. G., Muzny, D. N., and Caskey, C. T.,** Generation of cDNA probes directed by amino acid sequence: cloning of urate oxidase, *Science*, 239, 1288, 1988.
42. **Mack, D. H. and Sninsky, J. J.,** A sensitive method for the identification of uncharacterized viruses related to known virus groups: hepadnavirus model system, *Proc. Natl. Acad. Sci. U.S.A.*, 85, 6977, 1988.
43. **Compton, T.,** Degenerate primers for DNA amplification, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds., Academic Press, San Diego, 1990, 39.

44. **Patil, R. V. and Dekker, E. E.,** PCR amplification of an *Escherichia coli* gene using mixed primers containing deoxyinosine at ambiguous positions in degenerate amino acids codons, *Nucleic Acids Res.*, 18, 3080, 1990.
45. **Knoth, K., Roberds, S., Poteet, C., and Tamkun, M.,** Highly degenerate, inosine-containing primers specifically amplify rare cDNA using the polymerase chain reaction, *Nucleic Acids Res.*, 16, 10932, 1988.
46. **Haqqi, T. M., Sarkar, G., David, C. S., and Sommer, S. S.,** Specific amplification with PCR of a refractory segment of genomic DNA, *Nucleic Acids Res.*, 16, 11844, 1988.
47. **Tindall, K. R. and Stankowski, L. F.,** Molecular analysis of spontaneous mutations at the gpt locus in Chinese hamster ovary (AS52) cells, *Mutat. Res.*, 220, 241, 1989.
48. **Chamberlain, J. S., Gibbs, R. A., Ranier, J., Nguyen, P. N., and Caskey, C. T.,** Deletions screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification, *Nucleic Acids Res.*, 16, 11141, 1988.
49. **Bej, A. K., Mahbubani, M. H., Miller, R., DiCesare, J. L., Haff, L., and Atlas, R. M.,** Multiplex PCR amplification and immobilized capture probes for detection of bacterial pathogens and indicators in water, *Mol. Cell. Probes*, 4, 353, 1990.
50. **Ochman, H., Ajioka, J. W., Garza, D., and Hartl, D. L.,** Inverse polymerase chain reaction, *Biotechnology*, 8, 759, 1990.
51. **Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G., and Galas, D. J.,** A simple method for site-directed mutagenesis using the polymerase chain reaction, *Nucleic Acids Res.*, 17, 6545, 1989.
52. **Earp, D. J., Lowe, B., and Baker, B.,** Amplification of genomic sequences flanking transposable elements in host and heterologous plants: a tool for transposon tagging and genomic characterization, *Nucleic Acids Res.*, 18, 3271, 1990.
53. **Rich, J. J. and Willis, D. K.,** A single oligonucleotide can be used to rapidly isolate DNA sequences flanking a tansposon Tn5 insertion by the polymerase chain reaction, *Nucleic Acids Res.*, 18, 6673, 1990.
54. **Barnes, T. M.,** Echolocation: a PCR-based strategy for reliably and rapidly mapping transposon insertion sites, *Nucleic Acids Res.*, 18, 6741, 1990.
55. **Kalman, M., Kalman, E. T., and Cashel, M.,** Polymerase chain reaction (PCR) amplification with a single specific primer, *Biochem. Biophys. Res. Comm.*, 167, 504, 1990.
56. **Meuller, P. R. and Wold, B.,** *In vivo* footprinting of a muscle specific enhancer by ligation mediated PCR, *Science*, 246, 780, 1989.
57. **Ruano, G., Fenton, W., and Kidd, K. K.,** Biphasic amplification of very dilute DNA samples via "booster" PCR, *Nucleic Acids Res.*, 17, 5407, 1989.
58. **Loh, E. Y., Elliott, J. F., Cwirla, S., Lanier, L. L., and Davis, M. M.,** Polymerase chain reaction with single-sided specificity: analysis of T cell receptor delta chain, *Science*, 243, 217, 1989.
59. **Shuldiner, A. R., Nirula, A., and Roth, J.,** Hybrid DNA artifact from PCR of closely related target sequences, *Nucleic Acids Res.*, 17, 4409, 1989.
60. **Kadokami, Y. and Lewis, R. V.,** Membrane bound PCR, *Nucleic Acids Res.*, 18, 3982, 1990.
61. **MacFerrin, K. D., Terranova, M. P., Schreiber, S. L., and Verdine, G. L.,** Overproduction and dissection of proteins by the expression-cassette polymerase chain reaction, *Biochemistry*, 87, 1937, 1990.
62. **Steigerwald, S. D., Pfeifer, G. P., and Riggs, A. D.,** Ligation-mediated PCR improves the sensitivity of methylation analysis by restriction enzymes and detection of specific DNA strand breaks, *Nucleic Acids Res.*, 18, 1435, 1990.
63. **Kawasaki, E. S.,** Sample preparation from blood, cells, and other fluids, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds., Academic Press, San Diego, 1990, 146.
64. **Vrieling, H., Simons, J. W. I. M., and van Zeeland, A. A.,** Nucleotide sequence determination of point mutations at the mouse HPRT locus using *in vitro* amplification of HPRT mRNA sequences, *Mutat. Res.*, 198, 107, 1988.
65. **Mahbubani, M., Bej, A., DiCesare, J., Miller, R., Haff, L., and Atlas, R. M.,** Detection of bacterial mRNA using PCR, *BioTechniques*, 10, 48, 1991.
66. **Frohman, M. A., Dush, M. K., and Martin, G. R.,** Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer, *Proc. Natl. Acad. Sci. U.S.A.*, 85, 8998, 1988.
67. **Gilliland, G., Perrin, S., Blanchard, K., and Bunn, H. F.,** Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction, *Proc. Natl. Acad. Sci. U.S.A.*, 87, 2725, 1990.
68. **Chelly, J., Kaplan, J. C., Maire, Gautron, S., and Kahn, A.,** Transcription of the dystrophin gene in human muscle and non-muscle tissue, *Nature*, 333, 858, 1988.
69. **Wang, A. M. and Mark, D. F.,** Qualitative PCR, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds., Academic Press, San Diego, 1990, 70.
70. **Becker-Andre, M. and Hahlbrock, K.,** Absolute mRNA quantification using the polymerase chain reaction: a novel approach by a PCR-aided transcript titration assay (PATTY), *Nucleic Acids Res.*, 17, 9437, 1989.
71. **Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R.,** Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage PS 6 promoter, *Nucleic Acids Res.*, 12, 7035, 1984.
72. **Thomas, P. S.,** Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose,

Proc. Natl. Acad. Sci. U.S.A., 77, 5201, 1980.
73. **Fuqua, S. A. W., Fitzgerald, S. D., and McGuire, W. L.,** A simple polymerase chain reaction method for detection and cloning of low-abundance transcripts, *BioTechniques*, 9, 206, 1990.
74. **Loeb, L. A., Tartof, K. D., and Tavaglini, E. C.,** Copying natural RNAs with *E. Coli* DNA polymerase I, *Nature New Biol.*, 242, 66, 1973.
75. **Jones, M. D. and Foulkes, N. S.,** Reverse transcription of mRNA by *Thermus aquaticus* DNA polymerase, *Nucleic Acids Res.*, 17, 8387, 1989.
76. **Shaffer, A. L., Wojnar, W., and Nelson, W.,** Amplification, detection, and automated sequencing of gibbon interleukin-2 mRNA by *Thermus aquaticus* DNA polymerase reverse transcription and polymerase chain reaction, *Anal. Biochem.*, 190, 292, 1990.
77. **Brenner, C. A., Tam, A., Nelson, A. W., Engleman, P. A., Suzuki, N., Fry, K. E., and Larrick, J. W.,** Message amplification phenotyping (Mapping): a technique to simultaneously measure multiple mRNAs from small numbers of cells, *BioTechniques*, 7, 1096, 1989.
78. **Wang, A. M., Doyle, M. V., and Mark, D. F.,** Quantitation of mRNA by the polymerase chain reaction, *Proc. Natl. Acad. Sci. U.S.A.*, 86, 9717, 1989.
79. **Chang, H.-L., Zaroukian, M. H., and Essellman, W. J.,** T200 alternated exon use in murine lymphoid cells determined by reverse transcription-polymerase chain reaction, *J. Immunol.*, 143, 315, 1989.
80. **Lipson, K. E. and Baserga, R.,** Transcriptional activity of the human thymidine kinase gene determined by a method using the polymerase chain reaction and an intron-specific probe, *Proc. Natl. Acad. Sci. U.S.A.*, 86, 9447, 1989.
81. **Lee, M.-S., LeMaister, A., Kantarjian, H. M., Talpaz, M., Freireich, E. J., Trujillo, J. M., and Strass, S. A.,** Detection of two alternative bcr/abl mRNA junctions and minimal residual disease in Philadelphia chromosome positive chronic myelogenous leukemia by polymerase chain reaction, *Blood*, 73, 2165, 1989.
82. **Singer-Sam, J., Robinson, M. O., Bellve, A. R., Simon, M. I., and Riggs, A. D.,** Measurement by quantitative PCR of changes in HPRT, PGK-1, PGK-2, APRT, MTase, and Zfy gene transcripts during mouse spermatogenesis, *Nucleic Acids Res.*, 18, 1255, 1990.
83. **Byrne, B. C., Li, J. J., Sninsky, J., and Poiesz, B. J.,** Detection of HIV-1 RNA sequences by *in vitro* DNA amplification, *Nucleic Acids Res.*, 16, 4165, 1988.
84. **Bellofatto, V., Cooper, R., and Cross, G. A. M.,** Discontinuous transcription in *Leptomonas seymouri*: presence of intact and interrupted mini- exon gene families, *Nucleic Acids Res.*, 16, 7437, 1988.
85. **Henco, K. and Heibey, M.,** Quantitative PCR: the determination of template copy numbers by temperature gradient gel electrophoresis (TGGE), *Nucleic Acids Res.*, 18, 6733, 1990.
86. **Scharf, S. J., Horn, G. T., and Erlich, H. A.,** Direct cloning and sequence analysis of enzymatically amplified genomic sequences, *Science*, 233, 1076, 1986.
87. **Scharf, S. and Erlich, H. A.,** The polymerase chain reaction: *in vitro* enzymatic amplification of DNA, in *Current Protocols in Molecular Biology*, Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds., Greene Publishing Associates and Wiley-Interscience, New York, 1988, 1.
88. **Scharf, S. J.,** Cloning with PCR, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds., Academic Press, San Diego, 1990, 84.
89. **Tabor, S. and Struhl, K.,** Klenow fragment of *Escherichia coli* DNA polymerase I, in *Current Protocols in Molecular Biology*, Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds., Greene Publishing Associates and Wiley-Interscience, New York, 1988, 1.
90. **Auch, D. and Reth, M.,** Exon trap cloning: using PCR to rapidly detect and clone exons form genomic DNA fragments, *Nucleic Acids Res.*, 18, 6743, 1990.
91. **Stoflet, E. S., Koeberl, D. D., Sakar, G., and Sommer, S. S.,** Genomic amplification with transcript sequencing, *Science*, 239, 491, 1988.
92. **Sarkar, G. and Sommer, S. S.,** RNA amplification with transcript sequencing (RAWTS), *Nucleic Acids Res.*, 16, 5197, 1988.
93. **Sarkar, G. and Sommer, S. S.,** Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity, *Science*, 244, 331, 1989.
94. **Koeberl, D. D., Bottema, C. D., Buerstedde, J. M., and Sommer S. S.,** Functionally important regions of the factor IX gene have a low rate of polymorphism and a high rate of mutation in the dinucleotide CpG, *Am. J. Hum. Genet.*, 45, 448, 1989.
95. **Syvanen, A., Aalto-Setala, K., Kontula, K., and Soderlund, H.,** Direct sequencing of affinity-captured amplified human DNA application to the detection of apolipoprotein E polymorphism, *FEBS Lett.*, 258,71, 1989.
96. **Schofield, J. P., Vaudin, M., Kettle, S., and Jones, D. S. C.,** A rapid semi-automated microtiter plate method for analysis and sequencing by PCR from bacterial stocks, *Nucleic Acids Res.*, 17, 9498, 1989.
97. **Gyllensten, U. B. and Erlich, H. A.,** Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQα locus, *Proc. Natl. Acad. Sci. U.S.A.*, 85, 7652, 1988.
98. **McCable, P. C.,** Production of single-stranded DNA by asymmetric PCR, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T.J., Eds., Academic Press, San Diego, 1990, 76.
99. **Wilson, R. K., Chen, C.,and Hood, L.,** Optimi-

zation of asymmetric polymerase chain reaction for rapid fluorescent DNA sequencing, *BioTechniques*, 8, 184, 1990.

100. **Landegren, U., Kaiser, R., Sanders, J., and Hood, L.,** A ligase-mediated gene detection technique, *Science*, 241, 1077, 1988.
101. **Saiki, R. K., Chang, C. A., Levenson, C. H., Boehm, C. D., Kazazian, H. H., and Erlich, H. A.,** Rapid genetic analysis of enzymatically amplified DNA with non-radioactive allele-specific oligonucleotide probes, *Am. J. Hum. Genet.*, 41, 237, 1987.
102. **Chehab, F. F., Doherty, M., Cai, S., Kan, Y. W., Cooper, S., and Rubin, E. M.,** Detection of sickle cell anemia and thalassaemia, *Nature*, 329, 293, 1987.
103. **Kogan, S. C., Doherty, M., and Gitschier, J.,** An improved method for prenatal diagnosis of genetic disease by analysis of amplified DNA sequences, *N. Engl. J. Med.*, 317, 985, 1987.
104. **Wong, C., Dowling, C. E., Saiki, R. K., Higuchi, R. G., Erlich, H. A., and Kazazian, H. H.,** Characterization of β-thalassaemia mutations using direct genomic sequencing of amplified single copy DNA, *Nature*, 330, 384, 1987.
105. **Engelke, D. R., Hoerner, P. A., and Collins, F. S.,** Direct sequencing of enzymatically amplified human genomic DNA, *Proc. Natl. Acad. Sci. U.S.A.*, 85, 544, 1988.
106. **Winter, E., Yamamoto, F., Almoguera, C., and Perucho, M.,** A method to detect and characterize point mutations in transcribed genes: amplification and overexpression of the mutant c-Ki-*ris* allele in human tumor cells, *Proc. Natl. Acad. Sci. U.S.A.*, 82, 7575, 1985.
107. **Myers, R. M., Lumelsky, N., Lerman, L. S., and Maniatis, T.,** Detection of single base substitutions in total genomic DNA, *Nature*, 313, 495, 1985.
108. **Myers, R. M., Fischer, S. G., Maniatis, T., and Lerman, L. S.,** Modifications of the melting properties of duplex DNA by attachment of a GC-rich DNA sequence as determined by denaturing gradient gel electrophoresis, *Nucleic Acids Res.*, 13, 3111, 1985.
109. **Myers, R. M., Fishers, S. G., Lerman, L. S., and Maniatis, T.,** Nearly all single base subsitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis, *Nucleic Acids Res.*, 13, 3131, 1985.
110. **Almoguera, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N., and Perucho, M.,** Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes, *Cell*, 53, 549, 1988.
111. **Grompe, M., Muzny, D. M., and Caskey, C. T.,** Scanning detection of mutations in human ornithin tanscarbamoylase by chemical mismatch cleavage, *Proc. Natl. Acad. Sci. U.S.A.*, 86, 5888, 1989.
112. **Fischer, S. G. and Lerman, L. S.,** DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 1579, 1983.
113. **Myers, R. M. and Maniatis, T.,** Recent advances in the development of methods for detecting single-base substitutions associated with human genetic diseases, *Cold Spring Harbor Symp. Quant. Biol.*, 51, 275, 1986.
114. **Lerman, L. S., Silverstein, K., and Grinfeld, E.,** Searching for gene defects by denaturing gradient gel electrophoresis, *Cold Spring Harbor Symp. Quant. Biol.*, 51, 285, 1986.
115. **Lerman, L. S. and Silverstein, K.,** Computational simulation of DNA melting and its application to denaturing gradient gel electrophoresis, *Meth. Enzymol.*, 155, 482, 1987.
116. **Sheffield, V. C., Cox, D. R., Lerman, L. S., and Myers, R. M.,** Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes, *Proc. Natl. Acad. Sci. U.S.A.*, 86, 232, 1989.
117. **Smith, M.,** Molecular genetic studies on alcohol and aldehyde dehydrogenase: individual variation, gene mapping and analysis of regulation, *Biochem. Soc. Trans.*, 16, 227, 1988.
118. **Latham, T. and Smith, F. I.,** Detection of single-base mutations in DNA molecules using the solution melting method, *DNA*, 8, 223, 1989.
119. **Yon, J. and Fied, M.,** Precise gene fusion by PCR, *Nucleic Acids Res.*, 17, 4895, 1989.
120. **Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R.,** Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension, *Gene*, 77, 61, 1989.
121. **Kinzler, K. W. and Vogelstein, B.,** Whole genome PCR: amplification to the identification of sequences bound by gene regulatory proteins, *Nucleic Acids Res.*, 17, 3645, 1989.
122. **Wesley, C. S., Ben, M., Kretiman, M., Hagag, N., and Eanes, W. F.,** Cloning regions of the *Drosophila* genome by microdissection of polytene chromosome DNA and PCR with nonspecific primer, *Nucleic Acids Res.*, 18, 599, 1990.
123. **Pirotta, V.,** Cloning *Drosophila* genes, in *Drosophila: a Practical Approach*, Robert, D. B., Ed., IRL Press, Oxford, 1986, 83.
124. **Ludecke, H., Senger, G., Claussen, U., and Hrsthemke, B.,** Cloning defined regions of the human genome by microdissection of banded chromosomes and enzymatic amplification, *Nature*, 338, 348, 1989.
125. **Pershing, D. H., Telford, S. R., Speilman, A., and Barthold, S. W.,** Detection of *Borrelia burgdorferi* infection in *Ixodes dammini* ticks with the polymerase chain reaction, *J. Clin. Microbiol.*, 28, 566, 1990.
126. **Tramont, E. C.,** *Treponema pallidum* (syphilis), in *Principles and Practice of Infectious Diseases*, 3rd ed., Mandell, G. L., Douglas, R. G., and and Bennett, J. E., Eds., John Wiley & Sons, New York, 1990, 1974.
127. **Burstain, J. M., Grimprel, E., Lukehart, S. A.,**

Norgard, M. V., and Radolf, J. D., Sensitive detection of *Treponema pallidum* by using the polymerase chain reaction, *J. Clin. Microbiol.*, 29, 62, 1991.

128. **Hay, P. E., Clark, J. R., Strugnell, R. A., Taylor-Robinson, D., and Goldmeier, D.,** Use of the polymerase chain reaction to detect DNA sequences specific to pathogenic treponemes in cerebrospinal fluid, *FEMS Microbiol. Lett.*, 68, 233, 1990.
129. **Skov Jensen, J., Uldum, S. A., Sondergard-Andersen, J., Vuust, J., and Lind, K.,** Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples, *J. Clin. Microbiol.*, 29, 46, 1991.
130. **De Wit, D., Steyn, L., Shoemaker, S., and Sogin, M.,** Direct detection of *Mycobacterium tuberculosis* in clinical specimens by DNA amplification, *J. Clin. Microbiol.*, 28, 2437, 1990.
131. **Brisson-Noel, A., Lecossier, D., Nassif, X., Gecquel, B., Levy-Frebault, V., and Hance, A. J.,** Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples, *Lancet*, 22, 1069, 1989.
132. **Shanker, P., Manjunath, Lakshmi, R., Aditi, and Shriniwas, P. S.,** Identification of *Mycobacterium tuberculosis* by polymerase chain reaction, *Lancet*, 335, 423, 1989.
133. **Eisenbach, K. D., Crawford, J. T., and Bates, J. H.,** Repetitive DNA sequences as probes for *Mycobacterium tuberculosis*, *J. Clin. Microbiol.*, 26, 2240, 1989.
134. **Sjobring, U., Mecklenburg, M., Andersen, A. B., and Miorner, H.,** Polymerase chain reaction for detection of *Mycobacterium tuberculosis*, *J. Clin. Microbiol.*, 28, 2200, 1990.
135. **Hartskeeri, R. A., De Wit, M. Y. L., and Llatser, P. R.,** Polymerase chain reaction for the detection of *Mycobacterium leprae*, *J. Gen. Microbiol.*, 135, 2357, 1989.
136. **Webb, L., Carl, M., Malloy, D. C., Dasch, G. A., and Azad, A. F.,** Detection of marine typhus infection in fleas by using the polymerase chain reaction, *J. Clin. Microbiol.*, 28, 530, 1990.
137. **Shibata, D., Martin, W. J., Appleman, M. D., Causey, D. M., Leedom, J. M., and Arnheim, N.,** Detection of cytomegalovirus DNA in peripheral blood of patients infected with human immunodeficiency virus, *J. Infect. Dis.*, 158, 1185, 1988.
138. **Houard, S., Hackel, C., Herzog, A., and Bollen, A.,** Specific identification of *Bordetella pertussis* by the polymerase chain reaction, *Res. Microbiol.*, 140, 477, 1989.
139. **Frankel, G., Giron, J. A., Vallmassoi, and Schoolnik, G. K.,** Multi-gene amplification: simultaneous detection of three virulence genes in diarrhoeal stool, *Mol. Microbiol.*, 3, 1729, 1989.
140. **Snyder, J. D. and Merson, M. H.,** The magnitude of the global problem of acute diarrhoeal disease: a review of active surveillance data, *Bull WHO*, 60, 605, 1982.
141. **Lampel, K. A., Jagow, J. A., Trucksess, M., and Hill, W. E.,** Polymerase chain reaction for detection of invasive *Shigella flexneri* in food, *Appl. Environ. Microbiol.*, 56, 1536, 1990.
142. **Olive, D. M., Atta, A. I., and Setti, S. K.,** Detection of toxigenic *Escherichia coli* using biotin-labeled DNA probes following enzymatic amplification of the heat labile toxin gene, *Mol. Cell Probes*, 2, 47, 1988.
143. **Olive, D. M.,** Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase, *J. Clin. Microbiol.*, 27, 261, 1990.
144. **Pollard, D. R., Johnson, W. M., Lior, H., Tyler, D., and Rozee, K. R.,** Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction, *J. Clin. Microbiol.*, 28, 540, 1990.
145. **Victor, T., DuToit, R., VanZyl, J., Bester, A. J., and VanHelden, P. D.,** Improved method for the routine identification of toxigenic *Escherichia coli* by DNA amplification of a conserved region of the heat-labile toxin a subunit, *J. Clin. Microbiol.*, 29, 158, 1991.
146. **Konowalchuk, J. and Speirs, J. I.,** Response of various cell lines to *Escherichia coli* toxic products, *Can. J. Microbiol.*, 25, 335, 1979.
147. **Sack, D. A. and Sack, R. B.,** Test for enterotoxigenic *Escherichia coli* using Y1 adrenal cells in miniculture, *Infect. Immunol.*, 11, 334, 1975.
148. **Speirs, J. L., Stavri, S., and Konowalchuk, J.,** Assay of *Escherichia coli* heat-labile enterotoxin with Vero cells, *Infect. Immun.*, 16, 617, 1977.
149. **Howard, S. P., Garland, W. J., Green, M. J., and Buckley, J. T.,** Nucleotide sequence of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*, *J. Bact.*, 169, 2869, 1987.
150. **Pollard, D. R., Johnson, W. M., Lior, H., Tyler, D., and Rozee, K. R.,** Detection of the aerolysin gene in *Aeromonas hydrophila* by the polymerase chain reaction, *J. Clin. Microbiol.*, 28, 2477, 1990.
151. **Kato, N., Ou, C.-Y., Dato, H., Bartley, S., Brown, V. K., Dowell, V. R., and Ueno, K.,** Identification of toxigenic *Clostridium difficile* by the polymerase chain reaction, *J. Clin. Microbiol.*, 29, 33, 1991.
152. **Wren, B., Clayton, C., and Tabaqchali, S.,** Rapid identification of toxigenic *Clostridium difficile* by polymerase chain reactions, *Lancet*, 1, 423, 1990.
153. **Holland, S. M., Gaydos, C. A., and Quinn, T. C.,** Detection and differentiation of *Clyamydia trachomatis*, *Chlamydia psittaci*, and *Chlamydia pneumoniae* by DNA amplification, *J. Infect. Dis.*, 162, 984, 1990.
154. **Chen, K., Neimark, H., Rumore, P., and Steinman, C. R.,** Broad range DNA probes for detecting and amplifying eubacterial nucleic acids, *FEMS Microbiol. Lett.*, 57, 19, 1989.
155. **Wisotzkey, J. D., Jurtshuk, P., and Fox, G. E.,** PCR amplification of 16S yDNA from pypholized cell cultures facilitates studies in molecular system-

atics, *Curr. Microbiol.*, 21, 325, 1990.

156. **Erlich, G., Davey, F., Kirshner, J., Sninsky, J., Kwok, S., Slamon, D., Kalish, R., and Poiesz, B.,** A polyclonal CD4+ and CD8+ lymphocytosis in a patient doubly infected with HTLV-1 and HIV-1: a clinical and molecular analysis, *Am. J. Hematology*, 30, 128-139, 1989a.
157. **Erlich, H. A., Kazazian, H. H., Jr., and Gibbs, R. A.,** Introduction, in *Current Communications in Molecular Biology*, Erlich, H. A., Gibbs, R., and Kazazian, H. H., Jr., Eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, 1.
158. **Ehrlich, H. A., Ed.,** *PCR Technology: Principles and Applications for DNA Amplification*, Stockton Press, New York, 1989.
159. **Greenberg, S. J., Ehrlich, G. D., Abbott, M. A., Hurwitz, B. J., Waldman, T. A., and Poiesz, B. J.,** Detection of sequences of homologous to human retroviral DNA in multiple sclerosis by gene amplification, *Proc. Natl. Acad. Sci. U.S.A.*, 86, 2878, 1989.
160. **Ou, C. Y., Kwok, S., Mitchell, S. W., Mack, D. H., Sninsky, J. J., Krebs, J. W., Feorino, P., Warfield, D., and Schochetman, G.,** DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells, *Science*, 239, 295, 1988.
161. **Kwok, S., Mack, D. H., Sninsky, J. J., Ehrlich, G. D., Poiesz, B. J., Dock, N. L., Alter, H. J., Mildvan, D., and Grieco, M. H.,** Diagnosis of human immunodeficiency virus in seropositive individuals: enzymatic amplification of HIV viral sequences in peripheral blood mononuclear cells, in *HIV Detection by Genetic Engineering Methods*, Luciw, P. A. and Steimer, K. S., Eds., Marcel Dekker, New York, 1989, 243.
162. **Ting, Y. and Manos, M. M.,** Detection and typing of genital human papillomaviruses, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds., Academic Press, San Diego, 1990, 356.
163. **Snijders, P. J. F., van den Brule, A. J. C., Schrijnemakers, H. F. J., Snow, G., Meijer, C. J. L. M., and Balboomers, J. M. M.,** The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes, *J. Gen. Virol.*, 71, 173, 1990.
164. **Rotbart, H. A.,** PCR amplification of enteroviruses, in *PCR Protocols: A Guide to Methods and Applicatons*, Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds., Academic Press, San Diego, 1990, 372.
165. **Demmler, G. J., Buffone, G. J., Schimbor, C. M., and May, R. A.,** Detection of cytomegalovirus in urine from newborns by using polymerase chain reaction DNA amplification, *J. Infect. Dis.*, 158, 1177, 1988.
166. **Zaia, J. A., Gallez-Hawkins, G., Churchill, M. A., Morton-Blackshere, A., Pande, H., Adler, S. P., Schmidt, G. M., and Forman, S. J.,** Comparative analysis of human cytomegalovirus *a*-sequence in multiple clinical isolates by using polymerase chain reaction and restriction fragment length polymorphism assays, *J. Clin. Microbiol.*, 28, 2602, 1990.
167. **Allard, A., Birones, R., Juto, P., and Wadell, G.,** Polymerase chain reaction for detection of adenoviruses in stool samples, *J. Clin. Microbiol.*, 28, 2659, 1990.
168. **Saito, I., Servenius, B., Compton, T., and Fox, R. I.,** Detection of Epstein-Barr virus DNA by polymerase chain reaction in blood and tissue biopsies from patients with Sjogren's syndrome, *J. Exp. Med.*, 169, 2191, 1989.
169. **Bignon, Y., Bernard, D., Cure, H., Fonck, Y., Pauchard, J., Travade, P., Legros, M., Dastugue, B., and Plagne, R.,** Detection of Epstein-Barr viral genomes in lymph nodes of Hodgkin's disease patients, *Mol. Carcinog.*, 3, 9, 1990.
170. **Rogers, B. B., Alpert, L. C., Hine, E. A. S., and Buffone, G. J.,** Analysis of DNA in fresh and fixed tissue by the polymerase chain reaction, *Am. J. Pathol.*, 135, 541, 1990.
171. **Ferrand, J. P., Gozlan, J., Bendelac, A., Delauche-Cavallier, M. C., Brouet, J. C., and Morinet, F.,** Detection of Epstein-Barr virus in epidermal skin lesions of an immunocompromised patient, *Ann. Intern. Med.*, 112, 511, 1990.
172. **Telenti, A., Marshall, W. F., and Smith, T. F.,** Detection of Epstein-Barr virus by polymerase chain reaction, *J. Clin. Microbiol.*, 28, 2187, 1990.
173. **Gouvea, V., Glass, R. I., Woods, P., Taniguchi, K., Clark, H. F., Forrester, B., and Fank, Z.-H.,** Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens, *J. Clin. Microbiol.*, 28, 276, 1990.
174. **Rimstad, E., Hornes, E., Olsvik, O., and Hyllseth, B.,** Identification of a double-stranded RNA virus by using polymerase chain reaction and magnetic separation of the synthesized DNA segments, *J. Clin. Microbiol.*, 28, 2275, 1990.
175. **Koch, W. C. and Adler, S. P.,** Detection of human parvovirus B19 DNA by using the polymerase chain reaction, *J. Clin. Microbiol.*, 28, 65, 1990.
176. **Lunkenheimer, K., Hufert, F. T., and Schmitz, H.,** Detection of Lassa fever virus RNA in specimens from patients with Lassa fever using the polymerase chain reaction, *J. Clin. Microbiol.*, 28, 2689, 1990.
177. **Geradts, J., Warnock, M., and Yen, T. S. B.,** Use of the polymerase chain reaction in the diagnosis of unsuspected herpes simplex viral pneumonia: report of a case, *Human Pathol.*, 21, 118, 1990.
178. **Chamberlain, J. S., Ranier, J., Pearlman, J. A., Nguyen, P. N., Farwell, N. J., Gibbs, R., Muzny, D. M., and Caskey, T.,** Analysis of Duchenne muscular dystrophy gene mutations in mice and humans, *UCLA Symp. Cell. Mol. Biol. New Ser.*, 93, 951, 1989.

179. **Lesch, M. and Nyhan, W. L.,** A familial disorder of uric acid metabolism and central nervous system function, *Am. J. Med.*, 36, 561, 1964.
180. **Gibbs, R. A., Nguyen, P. N., McBridge, L. J., Keopf, S. M., and Caskey, C. T.,** Identification of mutations leading tothe Lesch-Nyhan syndrome by automated direct DNA sequencing of *in vitro* amplified cDNA, *Proc. Natl. Acad. Sci. U.S.A.*, 86, 1919, 1989.
181. **Kogan, S. C. and Gitschier, J.,** Genetic prediction of hemophilia A, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds., Academic Press, San Diego, 1990, 288.
182. **Valle, D., Mitchell, G. A., Brody, L. C., Martin, L. S., Steel, G., and Sipila, I.,** Mutation detection and structure-function studies at the ornithin δ-aminotransferase locus, in *Current Communications in Molecular Biology*, Erlich, H. A., Gibbs, R., and Kazazian, H. H., Jr., Eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, 63.
183. **Williamson, R.,** Application of polymerase chain reaction to cystic fibrosis: prenatal diagnosis, carrier testing, and community genetics, in *Current Communications in Molecular Biology*, Erlich, H. A., Gibbs, R., and Kazazian, H. H., Jr., Eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, 69.
184. **Rowley, J. D.,** A new consistent chromosmal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and Giemsa staining, *Nature*, 243, 290, 1973.
185. **Yunis, J. J., Oken, M. M., Kaplan, M. I., Ensrud, K. M., Howe, R. R., and Theologides, A.,** Distinctive chromosomal abnormalities in histologic subtypes of non-Hodgkin's lymphomas, *N. Engl. J. Med.*, 37, 1231, 1982.
186. **Lee, M.-S., Chang, K.-S., Cabanillas, F., Freireich, E., Trujillo, J. M., and Stass, S. A.,** Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification, *Science*, 237, 175, 1987.
187. **Lee, M.-S., Chang, K.-S., Kantarjian, H. M., Talpaz, M., Freireich, E., Trujillo, J. M., and Stass, S. A.,** Detection of minimal residual *bcr/abl* transcripts by a modified polymerase chain reaction, *Blood*, 72, 89, 1988.
188. **Crescenzi, M., Seto, M., Herzige, G. P., Weiss, P. D., Griffith, R. C., and Korsmeyer, S. J.,** Thermostable DNA polymerase chain amplification of t(14;18) chromosomal breakpoints and detection of minimal residual disease, *Proc. Natl. Acad. Sci. U.S.A.*, 85, 4869, 1988.
189. **Kawasaki, E. S., Clardk, S. S., Coyne, M. Y., Smith, S. D., Champlin, R., Witte, O. N., and McCormick, F. P.,** Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia-specific mRNA sequences amplified *in vitro*, *Proc. Natl. Acad. Sci. U.S.A.*, 85, 5698, 1988.
190. **Todd, J. A., Bell, J. I., and McDevitt, H. O.,** HLA-DQβ gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus, *Nature*, 329, 599, 1987.
191. **Emery, A. E. H.,** *Duchenne Muscular Dystrophy*, Oxford Monographs on Medical Genetics 15, Oxford University Press, Oxford, 1987.
192. **Muntoni, F. and Strong, P. N.,** Transcription of the dystrophin gene in Duchenne muscular dystrophy muscle, *FEBS Lett.*, 252, 95, 1989.
193. **Perry, D. J., Harper, P. L., Fairham, S., Daly, M., and Carrell, R. W.,** Antithrombin Cambridge, 384 Ala to Pro: a new variant identified using the polymerase chain reaction, *FEBS Lett.*, 254, 174, 1989.
194. **Wallace, D. C., Singh, G., Lott, M. T., Hodge, J. A., Schurr, T. G., Lezza, A. M. S., Elsas II, L. J., and Nikoskelainen, E. K.,** Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy, *Science*, 242, 1426, 1988.
195. **Dionne, C. A., Kapaln, R., Seuanez, H., O'Brien, S. J., and Jaye, M.,** Chromosome assignment by polymerase chain reaction techniques: assignment of the oncogene FGF-5 to human chromosome 4, *BioTechniques*, 8, 190, 1990.
196. **Reiss, J., Neufeldt, Wieland, K., and Zoll, B.,** Diagnosis of haemophilia B using the polymerase chain reaction, *Blut*, 60, 31, 1990.
197. **Jackson, J. B.,** The polymerase chain reaction in transfusion medicine, *Transfusion*, 30, 51, 1990.
198. **Stetlen-Stevenson, M., Raffled, M., Cohen, P., and Cossman, J.,** Detection of occult follicular lymphoma by specific DNA amplification, *Blood*, 72, 1822, 1988.
199. **Cunningham, D., Hickish, T., Rosin, R. D., Sauven, P., Baron, J. H., Farrell, P. J., and Isaacson, P.,** Polymerase chain reaction for detection of dissemination in gastric lymphoma, *Lancet*, 1, 695, 1989.
200. **Ngan, B. Y., Nourse, J., and Cleary, M. L.,** Detection of chromosomal translocation t(14;18) within the minor cluster region of bc1-2 by polymerase chain reaction and direct genomic sequencing of the enzymatically amplified AND in follicular lymphomas, *Blood*, 73, 1759, 1989.
201. **Yamamoto, F., Clausen, H., White, T., Marken, J., and Hakomari, S.,** Molecular genetic basis of the histo-blood group ABO system, *Nature*, 345, 229, 1990.
202. **Kim, H. S. and Smithies, O.,** Recombinant fragment assay for gene targeting based on the polymerase chain reaction, *Nucleic Acids Res.*, 16, 8887, 1988.
203. **Sensabaugh, F. G. and von Beroldingen, C.,** The application of polymerase chain reaction in forensic science, in *Current Communications in Molecular Biology*, Erlich, H. A., Gibbs, R., and Kazazian, H. H., Jr., Eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, 147.
204. **Higuchi, R. G., von Beroldingen, C. H.,**

Sensabaugh, G. F., and Erlich, H. A., DNA typing from single hairs, *Nature*, 332, 543, 1988.

205. **Cann, R. L., Stoneking, M., and Wilson, A. C.,** Mitochondrial DNA and human evolution, *Nature*, 325, 31, 1987.
206. **Orrego, C., Wilson, A. C., and King, M. C.,** Identification of maternally related individuals by amplification and direct sequencing of a highly polymorphic, noncoding region of mitochondrial DNA, *Am. J. Hum. Genet.*, 43, A219, 1988.
207. **Paabo, S., Gifford, J. A., and Wilson, A. C.,** Mitochondrial DNA sequences from a 7000-year-old brain, *Nucleic Acids Res.*, 16, 9775, 1988.
208. **Jeffreys, A. J., Wilson, V., and Thein, S. L.,** Hypervariable "minisatellite" regions in human DNA, *Nature*, 314, 67, 1985.
209. **Tyler, M. G., Kirby, L. T., Wood, S., and Ferris, J. A. J.,** Human blood stain identification and sex determination in dried blood stains using recombinant DNA techniques, *Forensic Sci. Int.*, 31, 267, 1986.
210. **Giusti, A., Baird, M., Pasquale, S., Balazs, I., and Glassberg, J.,** Application of deoxyribonucleic acid (DNA) polymorphisms to the analysis of DNA recovered from sperm, *J. Forensic Sci.*, 31, 409, 1986.
211. Federal Bureau of Investigation, *Crime in the United States*, FBI Law Endorsement Bulletin, August 1988, 6–9, 1987.
212. **Higuchi, R. G. and Ochman, H.,** Production of single-stranded DNA templates by exonuclease digestion following the polymerase chain reaction, *Nucleic Acids Res.*, 17, 5865, 1989.
213. **Paabo, S., Higuchi, R. G., and Wilson, A. C.,** Ancient DNA and the polymerase chain reaction: the emerging field of molecular archeology, *J. Biol. Chem.*, 264, 9709, 1989.
214. **Rollo, F., Salvi, R., Amici, A., and Anconetani, A.,** Polymerase chain reaction fingerprints, *Nucleic Acids Res.*, 15, 9094, 1987.
215. **Fujimura, F. K., Jorthrup, H., Beaudet, A. L., and O'Brien, W. E.,** Genotyping errors with the polymerase chain reaction, *N. Engl. J. Med.*, 322, 61, 1990.
216. **Rose, E.,** PCR and the Human Genome Project: the 1990 Cold Spring Harbor meeting on genome mapping and sequencing, *Amplifications*, 5, 1, 1990.
217. **Olson, M. V., Hood, L., Cantor, C., and Botstein, D.,** A common language for physical mapping of the human genome, *Science*, 245, 1434, 1989.
218. **Nelson, D. L., Ledbetter, S. A., Carbo, V. M. F., Ramirez-Solis, R., Webster, T. D., Ledbetter, D. H., and Caskey, C. T.,** *Alu* polymerase chain reaction: a method for rapid isolation of human-specific sequences from complex DNA sources, *Proc. Natl. Acad. Sci. U.S.A.*, 86, 6686, 1989.
219. **Bruns, T. D., Fogel, R., White, T. J., and Palmer, J. D.,** Accelerated evolution of a false-truffle from a mushroom ancestor, *Nature*, 399, 140, 1989.
220. **Paabo, S.,** Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification, *Proc. Natl. Acad. Sci. U.S.A.*, 86, 1939, 1989.
221. **Thomas, R. H., Schaffner, W., Wilson, A. C., and Paabo, S.,** DNA phylogeny of the extinct marsupial wolf, *Nature*, 340, 465, 1989.
222. **Vigilant, L., Pennington, R., Harpending, H., Kocher, T. D., and Wilson, A. C.,** Mitochondrial DNA sequences in single hairs from southern African population, *Proc. Natl. Acad. Sci. U.S.A.*, 86, 9350, 1989.
223. **White, T. J., Arnheim, N., and Erlich, H. A.,** The polymerase chain reaction, *Trends Genet.*, 5, 185, 1989.
224. **Arnheim, N., White, T., and Rainey, W. E.,** Application of PCR: organismal and population biology, *BioScience*, 40, 174, 1990.
225. **Zehr, J. P. and McReynolds, L. A.,** Use of degenerate oligonucleotides for the amplification of the nifH gene from the marine cyanobacterium *Trichodesmium thiebautii*, *Appl. Environ. Microbiol.*, 55, 2522, 1989.
226. **Steffan, R. J. and Atlas, R. M.,** DNA amplification to enhance the detection of genetically engineered bacteria in environmental samples, *Appl. Environ. Microbiol.*, 54, 2185, 1988.
227. **Chaudry, G. R., Toranzos, G. A., and Bhatti, A. R.,** Novel method for monitoring genetically engineered microorganisms in the environment, *Appl. Environ. Microbiol.*, 55, 1301, 1989.
228. **Atlas, R. M. and Bej, A. K.,** Detecting bacterial pathogens in environmental water samples by using PCR and gene probes, in *PCR Protocols: A Guide to Methods and Applications*, Innis, M., Gelfand, D., Sninsky, D., and White, T., Eds., Academic Press, New York, 1990, 399.
229. **Cleuziat, P. and Robert-Baudouy, J.,** Specific detection of *Escherichia coli* and *Shigella* species using fragments of genes coding for β-glucuronidase, *FEMS Microb. Lett.*, 72, 315.
230. **Starnbach, M. N., Flakow, S., and Tompkins, L. S.,** Species-specific detection of *Legionella pneumophila* in water by DNA amplification and hybridization, *J. Clin. Microbiol.*, 27, 1257, 1989.
231. **Bej, A. K., Mahbubani, M. H., and Atlas, R. M.,** Detection of viable *Legionella pneumophila* in water by polymerase chain reaction and gene probe methods, *Appl. Environ. Microbiol.*, 57, 1991, in press.
232. **Kwok, O. Y., Davis, G. R., Whitfield, K. M., Chappelle, H. L., DiMichele, L. J., and Gineras, T. R.,** Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format, *Proc. Natl. Acad. Sci. U.S.A.*, 86, 1173, 1989.
233. **Lizardi, P. M., Guerra, C. E., Lomeli, H., Tussie-Luna, I., and Kramer, F. R.,** Exponential amplification of recombinant-RNA hybridization probes, *Biotechnology*, 6, 1197, 1988.
234. **Knight, P.,** Amplifying probe assays with Q-beta

replicase, *Biotechnology*, 7, 609, 1989.
235. **Barany, F.,** Genetic disease detection and DNA amplification using cloned thermostable ligase, *Proc. Natl. Acad. Sci. U.S.A.*, 88, 189, 1991.
236. **Duck, P., Alvarado-Urbina, G., Burdick, B., and Collier, B.,** Probe amplifier system based on chimeric cyling oligonucleotides, *BioTechniques*, 9, 142, 1990.