Specific Enzymatic Amplification of DNA In Vitro: The Polymerase Chain Reaction

K. MULLIS, F. FALOONA, S. SCHARF, R. SAIKI, G. HORN, AND H. ERLICH
Cetus Corporation, Department of Human Genetics, Emeryville, California 94608

The discovery of specific restriction endonucleases (Smith and Wilcox 1970) made possible the isolation of discrete molecular fragments of naturally occurring DNA for the first time. This capability was crucial to the development of molecular cloning (Cohen et al. 1973); and the combination of molecular cloning and endonuclease restriction allowed the synthesis and isolation of any naturally occurring DNA sequence that could be cloned into a useful vector and, on the basis of flanking restriction sites, excised from it. The availability of a large variety of restriction enzymes (Roberts 1985) has significantly extended the utility of these methods.

The de novo organic synthesis of oligonucleotides and the development of methods for their assembly into long double-stranded DNA molecules (Davies and Gassen 1983) have removed, at least theoretically, the minor limitations imposed by the availability of natural sequences with fortuitously unique flanking restriction sites. However, de novo synthesis, even with automated equipment, is not easy; it is often fraught with peril due to the inevitable indelicacy of chemical reagents (Urdea et al. 1985; Watt et al. 1985; Mullenbach et al. 1986), and it is not capable of producing, intentionally, a sequence that is not yet fully known.

We have been exploring an alternative method for the synthesis of specific DNA sequences (Fig. 1). It involves the reciprocal interaction of two oligonucleotides and the DNA polymerase extension products whose synthesis they prime, when they are hybridized to different strands of a DNA template in a relative orientation such that their extension products overlap. The method consists of repetitive cycles of denaturation, hybridization, and polymerase extension and seems not a little boring until the realization occurs that this procedure is catalyzing a doubling with each cycle in the amount of the fragment defined by the positions of the 5′ ends of the two primers on the template DNA, that this fragment is therefore increasing in concentration exponentially, and that the process can be continued for many cycles and is inherently very specific.

The original template DNA molecule could have been a relatively small amount of the sequence to be synthesized (in a pure form and as a discrete molecule) or it could have been the same sequence embedded in a much larger molecule in a complex mixture as in the case of a fragment of a single-copy gene in whole human DNA. It could also have been a single-stranded DNA molecule or, with a minor modification in the technique, it could have been an RNA molecule. In any case, the product of the reaction will be a discrete double-stranded DNA molecule with termini corresponding to the 5′ ends of the oligonucleotides employed.

We have called this process polymerase chain reaction or (inevitably) PCR. Several embodiments have been devised that enable one not only to extract a specific sequence from a complex template and amplify it, but also to increase the inherent specificity of this process by using nested primer sets, or to append sequence information to one or both ends of the sequence as it is being amplified, or to construct a sequence entirely from synthetic fragments.

MATERIALS AND METHODS

PCR amplification from genomic DNA. Human DNA (1 μg) was dissolved in 100 μl of a polymerase buffer containing 50 mM NaCl, 10 mM Tris-Cl (pH 7.6), and 10 mM $MgCl_2$. The reaction mixture was adjusted to 1.5 mM in each of the four deoxynucleoside triphosphates and 1 μM in each of two oligonucleotide primers. A single cycle of the polymerase chain reaction was performed by heating the reaction to 95°C for 2 minutes, cooling to 30°C for 2 minutes, and adding 1 unit of the Klenow fragment of *Escherichia coli* DNA polymerase I in 2 μl of the buffer described above containing about 0.1 μl of glycerol (Klenow was obtained from U.S. Biochemicals in a 50% glycerol solution containing 5 U/μl). The extension reaction was allowed to proceed for 2 minutes at 30°C. The cycle was terminated and a new cycle was initiated by returning the reaction to 95°C for 2 minutes. In the amplifications of human DNA reported here, the number of cycles performed ranged from 20 to 27.

Genotype analysis of PCR-amplified genomic DNA using ASO probes. DNA (1 μg) from various cell lines was subjected to 25 cycles of PCR amplification. Aliquots representing one thirtieth of the amplification mixture (33 ng of initial DNA) were made 0.4 N in NaOH, 25 mM in EDTA in a volume of 200 μl and applied to a Genatran-45 nylon filter with a Bio-Dot spotting apparatus. Three replicate filters were prepared. ASO probes (Table 1) were 5′-phosphorylated with [λ-^{32}P]ATP and polynucleotide kinase and purified by spin dialysis. The specific activities of the probes were between 3.5 and 4.5 μCi/pmole. Each filter

THE UNIVERSITY OF MICHIGAN LIBRARIES

Tecan Ex. 2040, Page 1 of 11
QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00026

was prehybridized individually in 8 ml of 5× SSPE, 5× DET, and 0.5% SDS for 30 minutes at 55°C. The probe (1 pmole) was then added, and hybridization was continued at 55°C for 1 hour. The filters were rinsed twice in 2× SSPE and 0.1% SDS at room temperature, followed by a high stringency wash in 5× SSPE and 0.1% SDS for 10 minutes at 55°C (for 19C) or 60°C (for 19A and 19S) and autoradiographed for 2.5 hours at −80°C with a single intensification screen.

Cloning from PCR-amplified genomic DNA using linker primers. An entire PCR reaction was digested at 37°C with *Pst*I (20 units) and *Hin*dIII (20 units) for 90 minutes (for β-globin) or *Bam*HI (24 units) and *Pst*I (20 units) for 60 minutes (for HLA-DQα). After phenol extraction, the DNA was dialyzed to remove low-molecular-weight inhibitors of ligation (presumably the dNTPs used in PCR) and concentrated by ethanol precipitation. All (β-globin) or one tenth (DQα) of the material was ligated to 0.5 μg of the cut M13 vector under standard conditions and transformed into approximately 6×10^9 freshly prepared competent JM103 cells in a total volume of 200 μl. These cells (10–30 μl) were mixed with 150 μl of JM103 culture, plated on IPTG/X-Gal agar plates, and incubated overnight. The plates were scored for blue (parental) plaques and lifted onto BioDyne A filters. These filters were hybridized either with one of the labeled PCR oligonucleotide primers to visualize all of the clones containing PCR-amplified DNA (primer plaques) or with a β-globin oligonucleotide probe (RS06) or an HLA-DQα cDNA probe to visualize specifically the clones containing target sequences. Ten β-globin clones from this latter category were sequenced by using the dideoxy extension method. Nine were identical to the expected β-globin target sequence, and one was identical to the homologous region of the human δ-globin gene.

PCR construction of a 374-bp DNA fragment from synthetic oligodeoxyribonucleotides. 100 pmoles of TN10, d(CCTCGTCTACTCCCAGGTCCTCTTCAAGGGCCAAGGCTGCCCCGACTATGTGCTCCTCACCCACACCGTCAGCC), and TN11, d(GGCAGGGGCTCTTGACGGCAGAGAGGAGGTTGACCTTCTCCTGGTAGGAGATGGCGAAGCGGCTGACGGTGTGG), designed so as to overlap by 14 complementary bases on their 3′ ends, were dissolved in 100 μl of buffer containing 30 mM Tris-acetate (pH 7.9), 60 mM sodium acetate, 10 mM magnesium acetate, 2.5 mM dithiothreitol, and 2 mM each dNTP. The solution was heated to 100°C for 1 minute and cooled in air at about 23°C for 1 minute; 1 μl containing 5 units Klenow fragment of *E. coli* DNA polymerase I was added, and the polymerization reaction was allowed to proceed for 2 minutes. Gel electrophoresis on 4% NuSieve agarose in the presence of 0.5 μg/ml ethidium bromide indicated that eight repetitions of this procedure were required before the mutual extension of the two primers on each other was complete (Fig. 5, lane I). A 2-μl aliquot of this reaction without purification was added to 100 μl of a second-stage reaction mixture identical to the one above except that 300 pmoles each of oligonucleotides LL09, d(CCTGGCCAATGGCATGGATCTGAAAGATAACCAGCTGGTGGTGCCAGCAGATGGCCTGTACCTCGTCTACTCCC), and LL12, d(CTCCCTGATAGATGGGCTCATACCAGGGCTTGAGCTCAGCCCCCTCTGGGGTGTCCTTCGGGCAGGGGCTCTTG), were substituted for TN10 and TN11. LL09 and LL12 were designed so that their 3′ ends would overlap with 14 complementary bases on the 3′ ends of the single-stranded fragments released when the 135-bp product of the previous reaction was denatured (see Fig. 4, no. 2). The cycle of heating, cooling, and adding Klenow fragment was repeated 15 times in order to produce the 254-bp fragment in lane II of Figure 5; 2 μl of this reaction mixture without purification was diluted into a third-stage reaction mixture as above but containing 300 pmoles of TN09, d(TGTAGCAAACCATCAAGTTGAGGAGCAGCTCGAGTGGCTGAGCCAGCGGGCCAATGCCCTCCTGGCCAATGGCA), and TN13, d(GATACTTGGGCAGATTGACCTCAGCGCTGAGTTGGTCACCCTTCTCCAGCTGGAAGACCCCTCCCTGATAGATG). After 15 cycles of PCR, the 374-bp product in lane III of Figure 5 was evident on gel electrophoresis. After gel purification, restriction analysis of the 374-bp fragment with several enzymes resulted in the expected fragments (data not shown).

PCR amplification with oligonucleotide linker primers. DNA was amplified by mixing 1 μg of genomic DNA in the buffer described above with 100 pmoles of each primer. Samples were subjected to 20 cycles of PCR, each consisting of 2 minutes of denaturation at 95°C, 2 minutes of cooling to 37°C, and 2 minutes of polymerization with 1 unit of Klenow DNA polymerase. After amplification, the DNA was concentrated by enthanol precipitation, and half of the total reaction was electrophoresed on a gel of 4% NuSieve agarose in TBE buffer. The ethidium-bromide-stained gel was photographed (see Fig. 6A), and the DNA was transferred to Genatran nylon membrane and hybridized to a labeled probe (RSO6) specific for the target sequence (Fig. 6B). The blot was then washed and autoradiographed. For the amplification of β-globin, the starting DNA was either from the Molt-4 cell line or from the globin deletion mutant GM2064 For lane 5, the starting material was 11 pg of the β-globin recombinant plasmid pBR328::β^A, the molar equivalent of 5 μg of genomic DNA. For lane 6, the reaction was performed as in lane 1 except that no enzyme was added. To increase the efficiency of amplification of the longer HLA-DQα segment, DMSO was added to 10% (v/v), and the polymerization was carried out at 37°C for 27 cycles. The starting DNA for the amplification of DQα was either from the consanguineous HLA typing cell line LG-2 or from the HLA class II deletion mutant LCL721.180.

Tecan Ex. 2040, Page 2 of 11
QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00026

DISCUSSION

Extraction and Amplification

Figure 1 describes the basic PCR process that results in the extraction and amplification of a nucleic acid sequence. "Extraction" is used here in the sense that the sequence, although contained within a larger molecule that may in fact be a heterogeneous population of larger molecules, as in the case of a chromosomal DNA preparation, will be amplified as a single discrete molecular entity, and thus extracted from its source. This feature of the chain reaction can be, in some cases, as important as the amplification itself. The source DNA is denatured and allowed to hybridize to an excess of primers that correspond to the extremities of the fragment to be amplified (Fig. 1A). The oligonucleotide primers are employed in micromolar concentrations, and thus the hybridization is rapid and complete but not particularly stringent. A DNA polymerase is added to the reaction, which already contains the four deoxynucleoside triphosphates, and the primers are extended (Fig. 1B,C). After a short time, the reaction is stopped, and the DNA is denatured again by heating. On cooling, the excess of primers again hybridizes rapidly, and this time there are twice as many sites for hybridization on sequences representing the target. Sequences not representing the target, which may have been copied in the first polymerization reaction by virtue of an adventitious interaction with one of the primers, will only very rarely have generated an additional site for either of the primers. The unique property of the targeted sequence for regenerating new primer sites with each cycle is intrinsic to the support of a chain reaction, and the improbability of its happening by chance accounts for the observed specificity of the overall amplification.

In Figure 1D, the discrete nature of the final product becomes evident. Whereas initial extension of the primers on templates with indefinite termini results in products with definite 5′ ends but indefinite 3′ ends (Fig. 1C), their extension on a template that is itself an extension product from a previous cycle results in a DNA strand that has both ends defined (Fig. 1D). It is the number of these DNA fragments, with both ends defined by primers, that increases exponentially during subsequent cycles. All other products of the reaction increase in a linear fashion. Thus, the reaction effectively amplifies only that DNA sequence that has been targeted by the primers.

Nested Primer Sets

Despite its intrinsic specificity, the amplification of fragments of single-copy genes from whole human DNA sometimes produces a molecule exclusively representative of the intended target, and sometimes it does not. Given the complexity of the human genome and the tendency for many genes to have some sequences in common with other related genes, this lack of complete specificity is not surprising. In many applications, extreme specificity is not required of the amplification process because a second level of specificity is invoked by the process of detecting the amplified products, e.g., the use of a labeled hybridization probe to detect a PCR-amplified fragment in a Southern blot. However, in other applications, such as attempting to visualize an amplified fragment of the β-globin gene by the ethidium bromide staining of an agarose gel, further specificity than that obtained by a simple amplification protocol was required. We achieved this by doing the amplification in two stages (Fig. 2). The first stage amplified a 110-bp fragment; the second stage employed two oligomers that primed within the sequence of this fragment to produce a subfragment of 58 bp (Table 1). By thus employing the specificity inherent in the requirement of four independent but coordinated priming events, we were able to amplify exclusively a fragment of the human β-globin gene approximately 2,000,000-fold (Mullis and Faloona 1986).

Addition and Amplification

Figure 3 depicts a PCR in which one of the oligonucleotides employed, primer B, has a 5′ sequence that is not homologous to the target sequence. During cycle 1 of the amplification, priming occurs on the basis of the homologous 3′ end of the oligomer, but the nonhomologous bases become a part of the extension product. In later cycles, when this extension product is copied by virtue of the fact that it contains a site for primer A, the entire complement to primer B is incorporated in this copy; thereafter, no further primer-template interactions involving nonhomologous bases are required, and the product that increases exponentially in the amplification contains sequence information on one end that was not present in the original target.

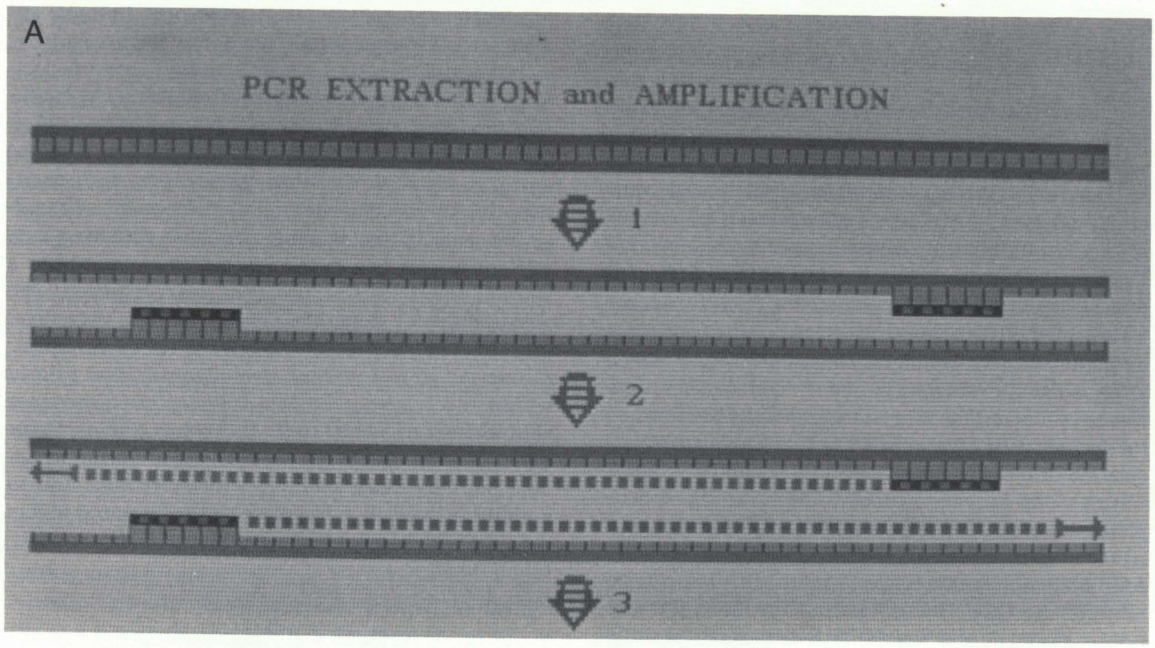
A similar situation results if both primers carry an additional 5′ sequence into the reaction, except that now both ends of the product will have been appended in the course of amplification. We have employed this embodiment of the PCR to insert restriction site linkers onto amplified fragments of human genomic sequences to facilitate their cloning; it also underlies the strategy described below for construction of DNA from oligonucleotides alone.

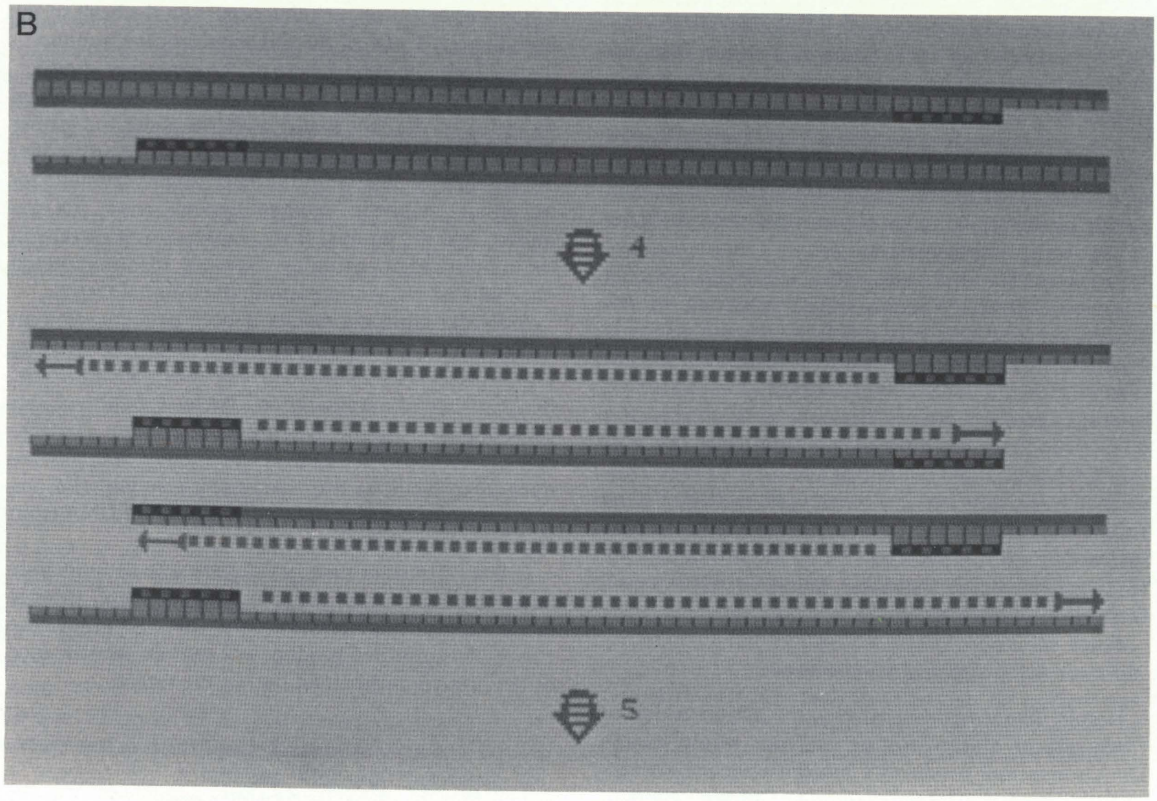
If one or both primers are designed so as to include an internal mismatch with the target sequence, specific in vitro mutations can be accomplished. As in the case above, the amplified product will contain the sequence of the primers rather than that of the target.

Construction and Amplification

The process whereby extrinsic sequences are appended to a fragment during the PCR amplification can be utilized in the stepwise construction of a totally synthetic sequence from oligonucleotides. The advan-

THE UNIVERSITY OF MICHIGAN LIBRARIES





Tecan Ex. 2040, Page 4 of 11
QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00026

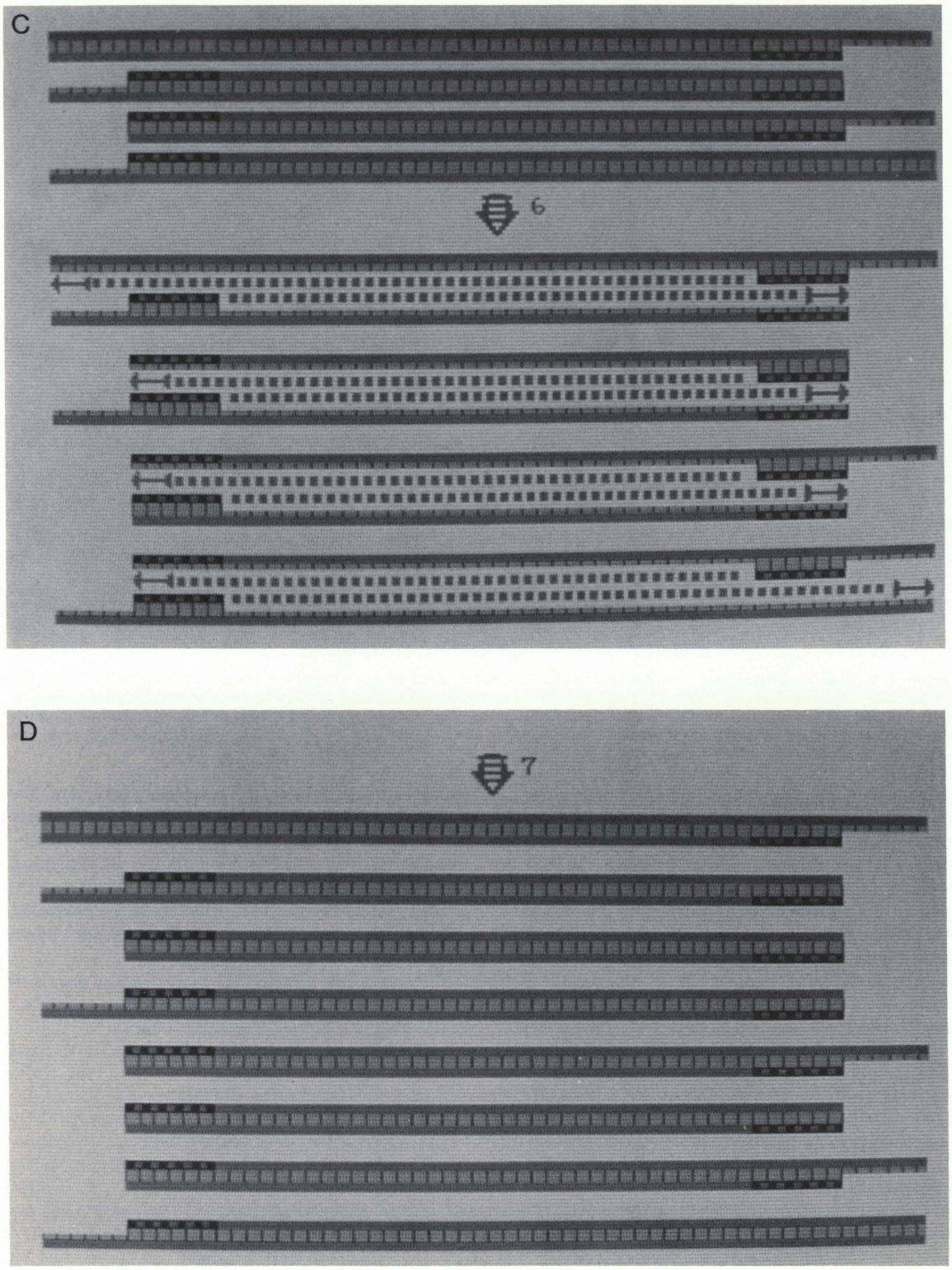


Figure 1. Three complete cycles of the polymerase chain reaction resulting in the eightfold amplification of a template sequence defined by the 5′ ends of two primers hybridized to different strands of the template are depicted above. The first cycle is shown in detail as reactions 1, 2, and 3. The second cycle is depicted in less detail as reactions 4 and 5, and the third cycle, as reactions 6 and 7. By cycle 3, a double-stranded DNA fragment is produced that has discrete termini, and thus the targeted sequence has been extracted from its source as well as amplified.

THE UNIVERSITY OF MICHIGAN LIBRARIES

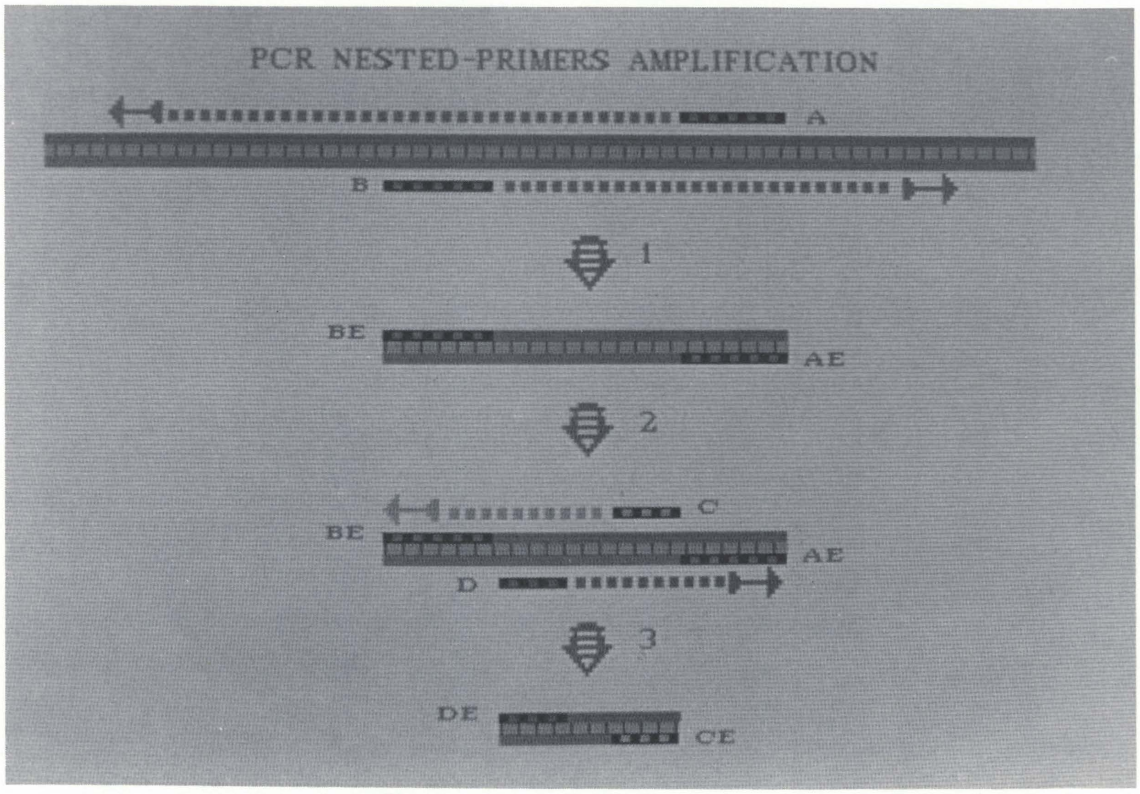


Figure 2. A PCR amplification employing oligonucleotides A and B results after a number of cycles in the fragment depicted as AE:BE. A subfragment, CE:DE, of this product can be extracted and further amplified by use of oligonucleotides C and D. This overall process requires four independent and coordinated primer-template interactions and is thereby potentially more specific than a single-stage PCR amplification.

tages of this approach (Figs. 4 and 5) over methods currently in use are that the PCR method does not require phosphorylation or ligation of the oligonucleotides. Like the method employed by Rossi et al. (1982), which involves the mutual extension of pairs of oligonucleotides on each other by polymerase (Fig. 4, no. 1), the PCR method does not require organic synthesis of both strands of the final product. Unlike this method, however, the PCR method is completely general in that no particular restriction enzyme recognition sequences need be built into the product for purposes of accomplishing the synthesis. Furthermore, the PCR method offers the convenience of enabling the final product, or any of the several intermediates, to be amplified during the synthesis or afterwards to produce whatever amounts of these molecules are required.

Table 1. Oligodeoxyribonucleotides

	Sequence	Use
PC03	ACACAACTGTGTTCACTAGC	produce a 110-bp fragment
PC04	CAACTTCATCCACGTTCACC	from β-globin 1st exon
PC07	CAGACACCATGGTGCACCTGACTCCTG	produce a 58-bp subfragment
PC08	CCCCACAGGGCAGTAACGGCAGACTTCTCC	of the 110-bp fragment above
GH18	CTTCTGCAGCAACTGTGTTCACTAGC	as PC03 and PC04, but with *Pst*I
GH19	CACAAGCTTCATCCACGTTCACC	and *Hin*dIII linkers added
GH26	GTGCTGCAGGTGTAAACTTGTACCAG	242-bp from HLA-DQα
GH27	CACGGATCCGGTAGCAGCGGTAGAGTTG	with *Pst*I and *Bam*HI linkers
RS06	CTGACTCCTGAGGAGAAGTCTGCCGTT ACTGCCCTGTGGG	probe to central region of 110-bp fragment produced by PC03 and PC04
19A	CTCCTGAGGAGAAGTCTGC	ASO probe to β^A-globin
19S	CTCCTGTGGAGAAGTCTGC	ASO probe to β^S-globin
19C	CTCCTAAGGAGAAGTCTGC	ASO probe to β^C-globin

Tecan Ex. 2040, Page 6 of 11
QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00026

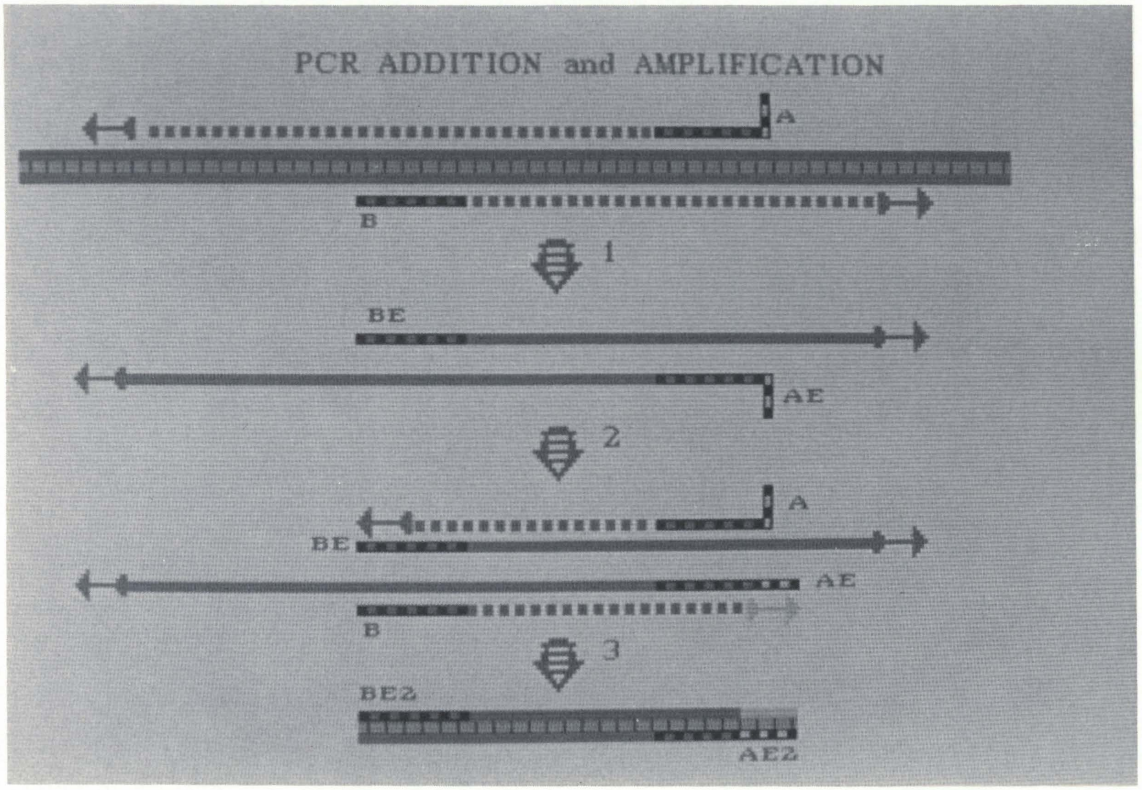


Figure 3. Addition of the nonhomologous sequence on the 5′ end of oligonucleotide A results, after the first PCR cycle, in the incorporation of this nonhomologous sequence into extension product AE, which, in a later cycle as template for B, directs the synthesis of BE2 that together with AE2 comprises the double-stranded DNA fragment AE2:BE2. This fragment will accumulate exponentially with further cycles.

Genetic Analysis

In addition to its use as an in vitro method for enzymatically synthesizing a specific DNA fragment, the PCR, followed by hybridization with specific probes, can serve as a powerful tool in the analysis of genomic sequence variation. Understanding the molecular basis of genetic disease or of complex genetic polymorphisms such as those in the HLA region requires detailed nucleotide sequence information from a variety of individuals to localize relevant variations. Currently, the analysis of each allelic variant requires a substantial effort in library construction, screening, mapping, subcloning, and sequencing. Here, using PCR primers modified near their 5′ ends to produce restriction sites, we describe a method for amplification of specific segments of genomic DNA and their direct cloning into M13 vectors for sequence analysis. Moreover, the cloning and sequencing of PCR-amplified DNA represents a powerful analytical tool for the study of the specificity and fidelity of this newly developed technique, as well as a rapid method of genomic sequencing. In addition, allelic variation has been analyzed using PCR amplification prior to hybridization with allele-specific oligonucleotide probes in a dot-blot format. This is a simple, general, and rapid method for genetic analysis and recently has been demonstrated in crude cell lysates, eliminating the need for DNA purification.

PCR Cloning and Direct Sequence Analysis

To develop a rapid method for genomic sequence determination and to analyze the individual products of PCR amplification, we chose the oligonucleotide primers and probes previously described for the diagnosis of sickle cell anemia (Saiki et al. 1985). These primers amplify a 110-bp segment of the human β-hemoglobin gene containing the Hb-S mutation and were modified near their 5′ ends (as described above in Fig. 3) to produce convenient restriction sites (linkers) for cloning directly into the M13mp10 sequencing vector. These modifications did not affect the efficiency of PCR amplification of the specific β-globin segment. After amplification, the PCR products were cleaved with the appropriate restriction enzymes, ligated into the M13 vector, and transformed into the JM103 host, and the resulting plaques were screened by hybridization with a labeled oligonucleotide probe to detect the β-globin clones. The plaques were also screened with the labeled PCR oligonucleotide primers to identify all of the clones containing amplified DNA (Table 2). In-

THE UNIVERSITY OF MICHIGAN LIBRARY

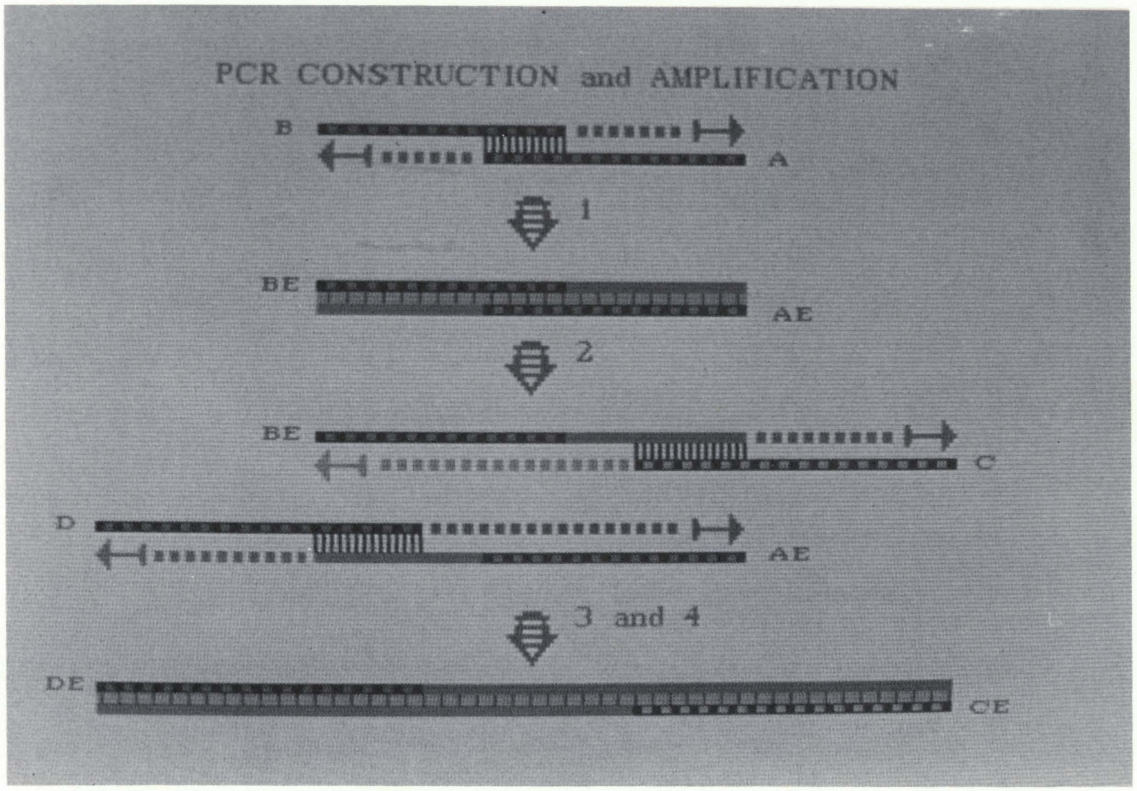


Figure 4. PCR construction and amplification. Oligonucleotides with complementary overlapping 3′ ends are extended on each other using DNA polymerase. The double-stranded DNA product of this reaction is extended on both ends by a second stage of several PCR cycles depicted by reactions 2, 3, and 4.

dividual clones were then sequenced directly by using the dideoxy primer-extension method.

Over 80% of the clones contained DNA inserts with the PCR primer sequences, but only about 1% of the clones hybridized to the internal β-globin probe. These nonglobin fragments presumably represent amplifications of other segments of the genome. This observation is consistent with the gel and Southern blot analysis of the PCR-amplified DNA from a β-globin deletion mutant and a normal cell line (Fig. 6). The similarity of the observed gel profiles reveals that most of the amplified genomic DNA fragments arise from nonglobin templates. Sequence analysis of two of these nontarget clones showed that the segments between the PCR primer sequences were unrelated to the β-globin gene and contained an abundance of dinucleotide repeats, similar to some genomic intergenic spacer sequences.

When ten of the clones that hybridized to the β-globin probe were sequenced, nine proved to be identical to the β-globin gene and one contained five nucleotide differences but was identical to the δ-globin gene. Each β-globin PCR primer has two mismatches with the δ-globin sequence. Each of these ten sequenced clones contains a segment of 70 bp originally synthesized from the genomic DNA template during the PCR amplification process. Since no sequence alterations were seen in these clones, the frequency of nucleotide misincorporation during 20 cycles of PCR amplification is less than 1 in 700.

To analyze the molecular basis of genetic polymorphism and disease susceptibility in the HLA class II loci, this approach has been extended to the amplification and cloning of a 242-bp fragment from the second exon, which exhibits localized allelic variability, of the HLA-DQα locus. In this case, the primer sequences (based on conserved regions of this exon) contain 5′-terminal restriction sites that have no homology with the DQα sequence. The specificity of amplification achieved by using these primers is greater than that achieved with the β-globin primers, since gel electrophoresis of the PCR products reveals a discrete band at 240 bp, which is absent from an HLA deletion mutant (Fig. 6). In addition, hybridization screening of the M13 clones from this amplification indicates that about 20% are homologous to the DQα cDNA probe (data not shown), an increase of 20-fold over the β-globin amplification. At this time, the basis for the difference in the specificity of amplification, defined here as the ratio of target to nontarget clones, is not clear, but may reflect the primer sequences and their genomic distribution. As described above, in some cases, the specificity of the PCR amplification can be significantly enhanced by using nested sets of PCR primers (Fig. 2).

Three HLA-DQα PCR clones derived from the

Tecan Ex. 2040, Page 8 of 11
QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00026

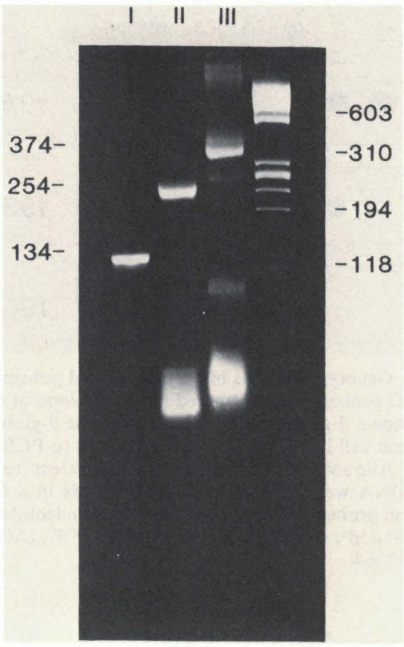


Figure 5. PCR construction of a 374-bp DNA fragment from synthetic oligodeoxynucleotides. (Lane I) 134-bp mutual extension product of TN10 and TN11; (lane II) 254-bp fragment produced by polymerase chain reaction of LL09 and LL12 with product in lane I; (lane III) 374-bp fragment produced by polymerase chain reaction of TN08 and TN13 with product in lane II; extreme right-hand lane is molecular-weight markers.

homozygous typing cell LG2 were subjected to sequence analysis. Two clones were identical to an HLA-DQα cDNA clone from the same cell line. One differed by a single nucleotide, indicating an error rate of approximately 1/600, assuming the substitution occurred during the 27 cycles of amplification. We are also currently using this procedure to analyze sequences from polymorphic regions of the HLA-DQα and DRβ loci. These preliminary studies suggest that the error rate over many cycles of amplification appears to be sufficiently low so that reliable genomic sequences can be determined directly from PCR amplification and cloning. This approach greatly reduces the number of cloned DNA fragments to be screened, circumvents the need for full genomic libraries, and allows cloning from nanogram quantities of genomic DNA.

Table 2. Cloning β-Globin Sequences from PCR-amplified DNA

Category	Number	Frequency (%)
Total plaques	1496	100
White plaques	1338	89
Primer plaques	1206	81
Globin plaques	15	1

Analysis of PCR-amplified DNA with Allele-specific Oligonucleotide Probes

Allelic sequence variation has been analyzed by oligonucleotide hybridization probes capable of detecting single-base substitutions in human genomic DNA that has been digested by restriction enzymes and resolved by gel electrophoresis (Conner et al. 1983). The basis of this specificity is that, under appropriate hybridization conditions, an allele-specific oligonucleotide (ASO) will anneal only to those sequences to which it is perfectly matched, a single-base-pair mismatch being

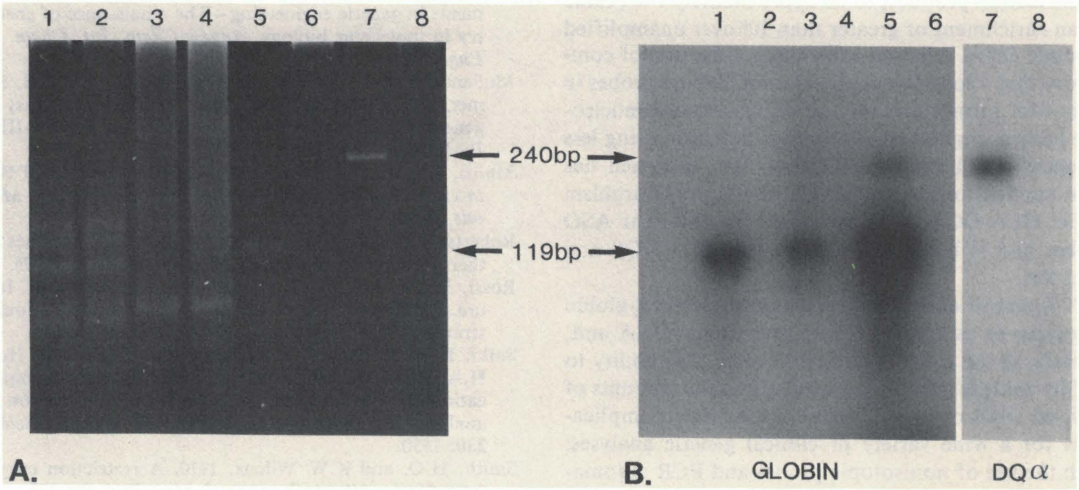


Figure 6. PCR amplification with oligonucleotide linker primers. (*A*) Ethidium-bromide-stained gel showing total amplified products. (*1*) Primers PC03 and PC04 on Molt-4 DNA; (*2*) PC03 and PC04 on GM2064 DNA; (*3*) GH18 and GH19 on Molt-4; (*4*) GH18 and GH19 on GM2064; (*5*) GH18 and GH19 on pBR328::β^A DNA; (*6*) PC03 and PC04 on Molt-4, no enzyme; (*7*) primers GH26 and GH27 on LG-2 DNA; (*8*) GH26 and GH27 on LCL721.180 DNA. (*B*) Southern blots showing specific amplified products. Lanes are numbered as in *A*. Lanes *1* through *6* were hybridized to the labeled RS06 oligonucleotide probe. Lanes *7* and *8* were hybridized to a cloned DBα cDNA probe labeled by nick translation.

THE UNIVERSITY OF MICHIGAN LIBRARY

sufficiently destabilizing to prevent hybridization. To improve the sensitivity, specificity, and simplicity of this approach, we have used the PCR procedure to amplify enzymatically a specific β-globin sequence in human genomic DNA prior to hybridization with ASOs. The PCR amplification, which produces a greater than 10^5-fold increase in the amount of target sequence, permits the analysis of allelic variation with as little as 1 ng of genomic DNA and the use of a simple "dot-blot" for probe hybridization. As a further simplification, PCR amplification has been performed directly on crude cell lysates, eliminating the need for DNA purification.

To develop a simple and sensitive method, we have chosen the sickle cell anemia and hemoglobin C mutations in the sixth codon of the β-globin gene as a model system for genetic diagnosis. We have used ASOs specific for the normal (β^A), sickle cell (β^S), and hemoglobin C (β^C) sequences as probes to detect these alleles in PCR-amplified genomic samples. The sequences of the 19-base ASO probes used here are identical to those described previously (Studenski et al. 1985).

DNA was extracted from six blood samples from individuals whose β-globin genotypes comprise each possible diploid combination of the β^A, β^S, and β^C alleles and from the cell line GM2064, which has a homozygous deletion of the β-globin gene. Aliquots (1 μg) of each sample were subjected to 25 cycles of PCR amplification, and one thirtieth of the reaction product (33 ng) was applied to a nylon filter as a dot-blot. Three replicate filters were prepared and each was hybridized with one of the three ^{32}P-labeled ASOs under stringent conditions. The resulting autoradiogram (Fig. 7) clearly indicates that each ASO annealed only to those DNA samples containing at least one copy of the β-globin allele to which the probe was perfectly matched and not at all to the GM2064 deletion mutant. The frequency of the specific β-globin target to PCR-amplified DNA has been estimated by the analysis of cloned amplification products to be approximately 1% (Table 2), an enrichment of greater than 10^5 over unamplified genomic DNA. It is this substantial reduction of complexity that allows the application of 19-base probes in a dot-blot format and the use of shorter oligonucleotide probes, capable of allelic discrimination using less stringent conditions. In addition, this approach has been applied to the analysis of genetic polymorphism in the HLA-DQα locus by using four different ASO probes and is being extended to the HLA-DQβ and DRβ loci.

PCR amplification has been used to detect β-globin genotypes in as little as 0.5 ng of genomic DNA and, recently, in the crude lysate of 75 cells. This ability to rapidly analyze genetic variation of minute amounts of purified DNA or in cell lysates has important implications for a wide variety of clinical genetic analyses. With the use of nonisotopic probes and PCR automation, this procedure combining in vitro target amplification and ASO probes in a dot-blot format promises to be a general and simple method for the detection of allelic variation.

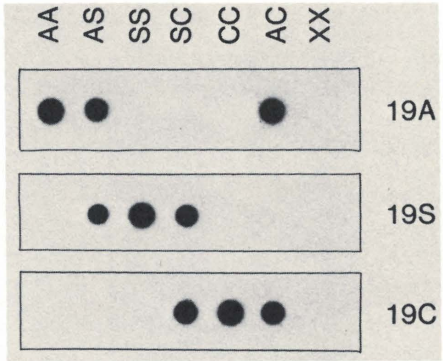


Figure 7. Genotype analysis of PCR-amplified genomic DNA using ASO probes. DNA extracted from the blood of individuals of known β-globin genotype and from the β-globin deletion mutant cell line GM2064 were subjected to PCR amplification. Aliquots of PCR reactions equivalent to 33 ng starting DNA were applied to replicate filters in a dot-blot format and probed with allele-specific oligonucleotides. (AA) $\beta^A\beta^A$; (AS) $\beta^A\beta^S$; (SS) $\beta^S\beta^S$; (SC) $\beta^S\beta^C$; (CC) $\beta^C\beta^C$; (AC) $\beta^A\beta^C$; (XX) GM2064.

ACKNOWLEDGMENTS

We thank Corey Levenson, Lauri Goda, and Dragan Spasic for the synthesis of oligonucleotides, Eric Ladner for graphic support, and Kathy Levenson for preparation of the manuscript.

REFERENCES

Cohen, S., A. Chang, H. Boyer, and R. Helling. 1973. Construction of biologically functional bacterial plasmids *in vitro*. *Proc. Natl. Acad. Sci.* **70:** 3240.

Conner, B.J., A. Reyes, C. Morin, K. Itakura, R. Teplitz, and R.B. Wallace. 1983. Detection of sickle cell β-S-globin allele by hybridization with synthetic oligonucleotides. *Proc. Natl. Acad. Sci.* **80:** 272.

Davies, J.E. and H.G. Gaessen. 1983. Synthetic gene fragments in genetic engineering—The renaissance of chemistry in molecular biology. *Angew Chem. Int. Chem. Ed. Engl.* **22:** 13.

Mullenbach, G.T., A. Tabrizi, R.W. Blacher, and K.S. Steimer. 1986. Chemical synthesis and expression in yeast of a gene encoding connective tissue activating peptide-III. *J. Biol. Chem.* **261:** 719.

Mullis, K.B. and F. Faloona. 1986. Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. *Methods Enzymol.* (in press).

Roberts, R.J. 1985. Restriction and modification enzymes and their recognition sequences. *Nucleic Acids Res.* **9:** 75.

Rossi, J.J., R. Kierzek, T. Huang, P.A. Walker, and K. Itakura. 1982. An alternate method for synthesis of double-stranded DNA segments. *J. Biol. Chem.* **257:** 9226.

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

Smith, H.O. and K.W. Wilcox. 1970. A restriction enzyme from *Hemophilus influenzae*. *J. Mol. Biol.* **51:** 379.

Studencki, A.B., B. Conner, C. Imprain, R. Teplitz, and R.B. Wallace. 1985. Discrimination among the human β-a, β-s, and β-c-globin genes using allele-specific oligonucleotide hybridization probes. *Am. J. Hum. Genet.* **37:** 42.

Urdea, M.S., L. Ku, T. Horn, Y.G. Gee, and B.D. Warner. 1985. Base modification and cloning efficiency of oligodeoxynucleotides synthesized by phosphoramidite method; methyl versus cyanoethyl phosphorous protection. *Nucleic Acids Res.* **16:** 257.

Watt, V.M., C.J. Ingles, M.S. Urdea, and W.J. Rutter. 1985. Homology requirements for recombination in *Escherichia coli*. *Proc. Natl. Acad. Sci.* **82:** 4768.

THE UNIVERSITY OF MICHIGAN
BIOMEDICAL LIBRARIES