-continued

3' 5'
. . .zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .
original template strand −

5' 3'
AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
newly synthesized long product 3

If these four strands are allowed to rehybridize with Primers 1 and 2 in the next cycle, agent for polymerization will catalyze the following reactions:

Primer 2 5' AAAAAAAAAA ——————→ extends to here
3' . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5'
newly synthesized long product 1

extends ←—————— GGGGGGGGGG 5' Primer 1
5' . . .zzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzz . . . 3'
original template strand +

Primer 2 5' AAAAAAAAAA ——————→ extends
3' . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzz . . . 5'
original template strand −

extends to here ←—————— GGGGGGGGGG 5' Primer 1
5' AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzz . . . 3'
newly synthesized long product 2

If the strands of the above four duplexes are separated, the following strands are found:

5' AAAAAAAAAAXXXXXXXXXXCCCCCCCCCC 3'
newly synthesized [S+]

3' . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5'
first cycle synthesized long product 1

3' . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5'
newly synthesized long product 1

5' . . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzz . . . 3'
original template strand +

5' AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzz . . . 3'
newly synthesized long product 2

3' . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzz . . . 5'
original template strand −

3' TTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5'
newly synthesized [S−]

5' AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzz . . . 3'
first cycle synthesized long product 2

It is seen that each strand which terminates with the oligonucleotide sequence of one primer and the complementary sequence of the other is the specific nucleic acid sequence [S] that is desired to be produced.

The steps of this process can be repeated indefinitely, being limited only by the amount of Primers 1 and 2, the agent for polymerization and nucleotides present. For detection, the number of cycles used is that required to produce a detectable signal, an amount which will depend, e.g., on the nature of the sample. For example, if the sample is pure or diluted, fewer cycles may be required than if it is a complex mixture. If the sample is human genomic DNA, preferably the number of cycles is from about 10–30.

The amount of original nucleic acid remains constant in the entire process, because it it not replicated. The amount of the long products increases linearly because they are produced only from the original nucleic acid. The amount of the specific sequence increases exponentially. Thus, the specific sequence will become the predominant species. This is illustrated in the following table, which indicates the relative amounts of the species theoretically present after n cycles, assuming 100% efficiency at each cycle:

	Number of Double Strands After 0 to n Cycles		
Cycle Number	Template	Long Products	Specific Sequence [S]
0	1	—	—

-continued

Cycle Number	Number of Double Strands After 0 to n Cycles: Template	Long Products	Specific Sequence [S]
1	1	1	0
2	1	2	1
3	1	3	4
5	1	5	26
10	1	10	1013
15	1	15	32,752
20	1	20	1,048,555
n	1	n	$(2^n - n - 1)$

When a single-stranded nucleic acid is utilized as the template, only one long product is formed per cycle.

The method herein may be utilized to clone a particular nucleic acid sequence for insertion into a suitable expression vector. The vector may then be used to transform an appropriate host organism to produce the gene product of the sequence by standard method of recombinant DNA technology.

Normally, such cloning would either involve direct ligation into a vector or the addition of oligonucleotide linkers followed by restriction enzyme cleavage. Both of these methods involve, however, the inefficient blunt-end ligation reaction. Also, neither technique would control for the orientation or multiplicity of insertion of the amplified product into the cloning vector.

The amplification process herein may yield a mixture of nucleic acids, resulting from the original template nucleic acid, the expected target amplified products, and various background non-target products. The amplified product can also be a mixture if the original template DNA contains multiple target sequences, such as in a heterozygous diploid genome or when there is a family of related genes.

The primers herein may be modified to assist the rapid and specific cloning of the mixture of DNAs produced by the amplification reaction. In such modification the same or different restriction sites are incorporated at the 5′ ends of the primers to result in restriction sites at the two ends of the amplified product. When cut with the appropriate enzymes, the amplified product can then be easily inserted into plasmid or viral vectors and cloned. This cloning allows the analysis or expression of individual amplified products, not a mixture.

Although the same restriction site can be used for both primers, the use of different sites allows the insertion of the product into the vector with a specific orientation and suppresses multiple insertions as well as insertions arising from amplifications based on only one of the two primers. The specific orientation is useful when cloning into single-strand sequencing vectors, when single-strand hybridization probes are used, or when the cloned product is being expressed.

One method to prepare the primers is to choose a primer sequence which differs minimally from the target sequence. Regions in which each of the primers is to be located are screened for homology to restriction sites appropriate to the desired vector. For example, the target sequence "CAGTATCCGA . . . " differs by only one base from one containing a BamHI site. A primer sequence is chosen to match the target exactly at its 3′ end, and to contain the altered sequence and restriction site near its 5′ end (for example, "CAGgATCCGA . . . ", where the lower case letter symbolizes a mismatch with the target sequence). This minimally altered sequence will not interfere with the ability of the primer to hybridize to the original target sequence and to initiate polymerization. After the first amplification cycle the primer is copied, becomes the target, and matches exactly with new primers. After the amplification process, the products are cleaved with the appropriate restriction enzymes, optionally separated from inhibitors of ligation such as the nucleotide triphosphates and salts by passing over a desalting column or molecular weight chromatography column, and inserted by ligation into a cloning vector such as bacteriophage M13. The gene may then be sequenced and/or expressed using well known techniques.

The second method for preparing the primers involves taking the 3′ end of the primers from the target sequence and adding the desired restriction site(s) to the 5′ end of the primer. For the above example, a HindIII site could be added to make the sequence "cgaagctt-CAGTATCCGA . . . ", where lower case letters are as described above. The added bases would not contribute to the hybridization in the first cycle of amplification, but would match in subsequent cycles. The final amplified products are then cut with restriction enzyme(s) and cloned and expressed as described above. The gene being amplified may be, for example, human beta-hemoglobin or the human HLA DQ, DR or DP-α and -β genes.

In addition, the process herein can be used for in vitro mutagenesis. The oligodeoxyribonucleotide primers need not be exactly complementary to the DNA sequence which is being amplified. It is only necessary that they be able to hybridize to the sequence sufficiently well to be extended by the polymerase enzyme or by whatever other inducing agent is employed. The product of a polymerase chain reaction wherein the primers employed are not exactly complementary to the original template will contain the sequence of the primer rather than the template, thereby introducing as in vitro mutation. In further cycles this mutation will be amplified with an undiminished efficiency because no further mispaired primings are required. The mutant thus produced may be inserted into an appropriate vector by standard molecular biological techniques and might confer mutant properties on this vector such as the potential for production of an altered protein.

The process of making an altered DNA sequence as described above could be repeated on the altered DNA using different primers so as to induce further sequence changes. In this way a series of mutated sequences could gradually be produced wherein each new addition to the series could differ from the last in a minor way, but from the original DNA source sequence in an increasingly major way. In this manner changes could be made ultimately which were not feasible in a single step due to the inability of a very seriously mismatched primer to function.

In addition, the primer can contain as part of its sequence a non-complementary sequence provided that a sufficient amount of the primer contains a sequence which is complementary to the strand to be amplified. For example, a nucleotide sequence which is not complementary to the template sequence (such as, e.g., a promoter, linker, coding sequence, etc.) may be attached at the 5′ end of one or both of the primers, and thereby appended to the product of the amplification process. After the extension primer is added, sufficient cycles are run to achieve the desired amount of new

template containing the non-complementary nucleotide insert. This allows production of large quantities of the combined fragments in a relatively short period of time (e.g., two hours or less) using a simple technique.

Moreover, the process herein may be used to synthesize a nucleic acid fragment from an existing nucleic acid fragment which is shorter than its product (called the core segment) using certain primers the 3′ ends of which are complementary to or substantially complementary to the 3′ ends of the single strands produced by separating the strands of the original shorter nucleic acid fragments, and the 5′ ends of which primers contain sequence information to be appended to the core segment. This process comprises:

(a) treating the strands of said existing fragment with two oligonucleotide primers under conditions such that an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primers are selected so as to be substantially complementary to the 3′ end of each strand of said existing fragment such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each primer contains, at its 5′ end, a sequence of nucleotides which are not complementary to said existing fragment and which correspond to the two ends of the nucleic acid fragment being synthesized;

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating tthe single-stranded molecules generated from step (b) with the primers of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template so as to produce two intermediate double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ end of one of the oligonucleotide primers, and two full-length double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ ends of both of the oligonucleotide primers;

(d) repeating steps (b) and (c) for a sufficient number of times to produce the full-length double-stranded molecule in an effective amount;

(e) treating the strands of the product of step (d) with two primers so as to lengthen the product of step (d) on both ends; and

(f) repeating steps (a)–(d) using the product of step (d) as the core fragment and two oligonucleotide primers which are complementary or substantially complementary to the 3′ ends of the single strands produced by separating the strands of the product of step (d).

Steps (b) and (c) are repeated as often as necessary, usually at least 5 times, to produce the required amount of the full-length double-stranded product to synthesize the final product (i.e., the effective amount). In addition, the core segment may be obtained as the product of a previous amplification cycle. The product produced in step (d) may be purified before a new cycle of extension and amplification, or used directly by employing the reaction mixture containing the product.

If the 3′ ends of the primers are not exactly complementary to the 3′ ends of the single strands of the original shorter nucleic acid, the core fragment of the product will not be exactly the same as the sequence information resident in the original shorter nucleic acid. Therefore, mutants of the original nucleic acid may be made by using primers which are substantially complementary at their 3′ ends to the 3′ ends of the single strands of the original shorter nucleic acid.

If restriction site linkers are incorporated into the primers, then the amplified double-stranded products can be digested with the appropriate restriction enzymes and ligated directly into an M13 vector for rapid cloning and sequencing. The M13 plaques containing the specific amplified target sequences can be identified by hybridizing plaque lift filters with a probe specific for the target sequence.

The method herein may also be used to enable detection and/or characterization of specific nucleic acid sequences associated with infectious diseases, genetic disorders or cellular disorders such as cancer, e.g., oncogenes. Amplification is useful when the amount of nucleic acid available for analysis is very small, as, for example, in the prenatal diagnosis of sickle cell anemia using DNA obtained from fetal cells. Amplification is particularly useful if such an analysis is to be done on a small sample using non-radioactive detection techniques which may be inherently insensitive, or where radioactive techniques are being employed but where rapid detection is desirble.

For purposes of this invention genetic diseases may include specific deletions and/or mutations in genomic DNA from any organism, such as, e.g., sickle cell anemia, cystic fibrosis, α-thalassemia, β-thalassemia, and the like. Sickle cell anemia can be readily detected via oligomer restriction analysis or a RFLP-like analysis following amplification of the appropriate DNA sequence by the present method. α-Thalassemia can be detected by the absence of a sequence, and β-thalassemia can be detected by the presence of a polymorphic restriction site closely linked to a mutation which causes the disease.

All of these genetic diseases may be detected by amplifying the appropriate sequence and analyzing it by Southern blots without using radioactive probes. In such a process, for example, a small sample of DNA from, e.g., amniotic fluid containing a very low level of the desired sequence is amplified, cut with a restriction enzyme, and analyzed via a Southern blotting technique. The use of non-radioactive probes is facilitated by the high level of the amplified signal.

In another embodiment a small sample of DNA may be amplified to a convenient level and then a further cycle of extension reactions performed wherein nucleotide derivatives which are readily detectable (such as ^{32}P-labeled or biotin labeled nucleoside triphosphates) are incorporated directly into the final DNA product, which may be analyzed by restriction and electrophoretic separation or any other appropriate method. An example of this technique in a model system is demonstrated in FIG. 5.

In a further embodiment, demonstrated in a model system in FIG. 3, the nucleic acid may be exposed to a particular restriction endonuclease prior to amplification. Since a sequence which has been cut cannot be amplified, the appearance of an amplified fragment, despite prior restriction of the DNA sample, implies the absence of a site for the endonuclease within the amplified sequence. The presence or absence of an amplified sequence can be detected by an appropriate method.

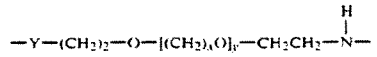
A practical application of this technique can be illustrated by its use in facilitating the detection of sickle cell anemia via the oligomer restriction technique described

herein and in copending U.S. application Ser. No. 716,982 filed Mar. 27, 1985. Sickle cell anemia is a hemoglobin disease which is caused by a single base pair change in the sixth codon of the β-globin gene. FIG. 6 illustrates the sequences of normal and sickle cell β-globin genes in the region of their polymorphism, where the single bars mark the location of a DdeI site present only in the normal gene and where the double bars mark the location of a HinfI site which is non-polymorphic and thus present in both the normal and sickle cell alleles. FIG. 7 illustrates the process of oligomer restriction of normal β-globin DNA using a probe spanning both restriction sites and labeled where the asterisk appears. (The probe is preferably labeled at the end which is fewer base pairs from the restriction site than the other end of the probe.) The DNA, amplified as provided herein, is denatured and annealed to the labeled probe. The amplification may be carried out at elevated temperatures (35°–40° C.) in the presence of dimethyl sulfoxide to minimize formation of secondary structure. The enzyme DdeI cleaves the DNA at the reformed DdeI site and generates a labeled octamer. Under the conditions used in the test the octamer is short enough to dissociate from the duplex. The subsequent addition of the enzyme HinfI has no effect on the now single-stranded octamer. FIG. 8 illustrates the same process applied to the sickle cell allele of β-globin DNA. The enzyme DdeI cannot cleave the duplex formed by the amplified DNA and the labeled probe because of the A-A base pair mismatch. The enzyme HinfI, however, does restrict the hybrid and a labeled trimer is produced. In practice the method can diagnose the DNA of an individual as being either homozygous for the wild type, homozygous for the sickle type or a heterozygous carrier of the sickle cell trait, since a specific signal is associated with the presence of eithr allele. Use of this above-described method to amplify the pertinent sequence allows for a rapid analysis of a single copy gene using a probe with only a single ^{32}P label.

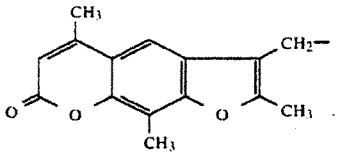
Various infectious diseases can be diagnosed by the presence in clinical samples of specific DNA sequences characteristic of the causative microorganism. These include bacteria, such as Salmonella, Chlamydia, and Neisseria; viruses, such as the hepatitis viruses; and parasites, such as the Plasmodium responsible for malaria. U.S. Pat. No. 4,358,535 issued to Falkow describes the use of specific DNA hybridization probes for the diagnosis of infectious diseases. A problem inherent in the Falkow procedure is that a relatively small number of pathogenic organisms may be present in a clinical sample from an infected patient and the DNA extracted from these may constitute only a very small fraction of the total DNA in the sample. Specific amplification of suspected sequences prior to immobilization and hybridization detection of the DNA samples could greatly improve the sensitivity and specificity of these procedures.

Routine clinical use of DNA probes for the diagnosis of infectious diseases would be simplified considerably if non-radioactively labeled probes could be employed as described in EP No. 63,879 to Ward. In this procedure biotin-containing DNA probes are detected by chromogenic enzymes linked to avidin or biotin-specific antibodies. This type of detection is convenient, but relatively insensitive. The combination of specific DNA amplification by the present method and the use of stably labeled probes could provide the convenience and sensitivity required to make the Falkow and Ward procedures useful in a routine clinical setting.

In addition, the probe may be a biotinylated probe in which the biotin is attached to a spacer arm of the formula:



where Y is O, NH or N—CHO, x is a number from 1 to 4, and y is a number from 2 to 4. The spacer arm is in turn attached to a psoralen moiety of the formula:



The psoralen moiety intercalates into and crosslinks a "gapped circle" probe as described by Courage-Tebbe et al., *Biochim. Biophys. Acta*, 697 (1982) 1–5, wherein the single-stranded hybridization region of the gapped circle spans the region contained in the primers. The details of this biotinylation and dot blot procedure are described more fully in commonly assigned copending U.S. application Ser. Nos. 683,263 filed Dec. 18, 1984 and 791,332 filed Oct. 25, 1985, the disclosures of which are incorporated herein by reference.

The amplification process can also be utilized to produce sufficient quantities of DNA from a single copy human gene such that detection by a simple non-specific DNA stain such as ethidium bromide can be employed so as to make a DNA diagnosis directly.

In addition to detecting infectious diseases and pathological abnormalities in the genome of organisms, the process herein can also be used to detect DNA polymorphism which may not be associated with any pathological state.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner. In these examples all percentages are by weight if for solids and by volume if for liquids, and all temperatures are in degrees Celsius unless otherwise noted.

EXAMPLE 1

A 25 base pair sequence having the nucleotide sequence

5' CCTCGGCACCGTCACCCTGGATGCT 3'

3' GGAGCCGTGGCAGTGGGACCTACGA 5'

contained on a 47 base pair FokI restriction fragment of pBR322 obtainable from ATCC was prepared as follows. A FokI digest of pBR322 containing the 47-bp fragment was produced by digesting pBR322 with FokI in accordance with the conditions suggested by the supplier, New England Biolabs Inc. The primers which were utilized were 5' d(CCTCGGCACCG) 3' and 5' d(AGCATCCAGGGTG) 3', and were prepared using conventional techniques. The following ingredients were added to 33 μl of buffer which consisted of 25 mM potassium phosphate, 10 mM magnesium chloride and 100 mM sodium chloride at pH 7.5: 2433 pmoles of each of the primers described above, 2.4 pmoles of the FokI digest of pBR322, 12 nmoles of dATP, 22 nmoles of dCTP, 19 nmoles of dGTP and 10 nmoles of TTP.

The mixture was heated to 85° C. for five minutes and allowed to cool to ambient temperature. Five units of the Klenow fragment of *E. coli* DNA polymerase I were added and the temperature was maintained for 15 minutes. After that time, the mixture was again heated to 85° C. for five minutes and allowed to cool. Five units of the Klenow fragment were again added and the reaction was carried out for 15 minutes. The heating, cooling and synthesis steps were repeated eleven more times.

After the final repetition, a 5 μl aliquot was removed from the reaction mixture. This was heated to 85° C. for three minutes and allowed to cool to ambient temperature. 12.5 pmoles of α-P^{32}-deoxycytidine triphosphate and 5 units of Klenow fragment were added and the reaction was allowed to proceed for 15 minutes. The labeled products were examined by polyacrylamide gel electrophoresis. The FokI digest was labeled in a similar fashion and served as a control and molecular weight markers. The only heavily labeled band visible after the 13 cycles was the intended 25 base pair sequence.

EXAMPLE 2

The desired sequence to be amplified was a 94 base pair sequence contained within the human beta-globin gene and spanning the MstII site involved in sickle cell anemia. The sequence has the nucleotide sequence shown in FIG. 1.

I. Synthesis of Primers

The following two oligodeoxyribonucleotide primers were prepared by the method described below:

5′ CACAGGGCAGTAACG 3′ Primer A

and

5′ TTTGCTTCTGACACA 3′ Primer B

Automated Synthesis Procedures: The diethylphosphoramidites, synthesized according to Beaucage and Caruthers (*Tetrahedron Letters* (1981) 22:1859–1862), were sequentially condensed to a nucleotide derivatized controlled pore glass support using a Biosearch SAM-1. The procedure included detritylation with trichloroacetic acid in dichlormethane, condensation using benzotriazole as activating proton donor, and capping with acetic anhydride and dimethylaminopyridine in tetrahydrofuran and pyridine. Cycle time was approximately 30 minutes. Yields at each step were essentially quantitative and were determined by collection and spectroscopic examination of the dimethoxytrityl alcohol released during detritylation.

Oligodeoxyribonucleotide Deprotection and Purification Procedures: The solid support was removed from the column and exposed to 1 ml concentrated ammonium hydroxide at room temperature for four hours in a closed tube. The support was then removed by filtration and the solution containing the partially protected oligodeoxyribonucleotide was brought to 55° C. for five hours. Ammonia was removed and the residue was applied to a preparative polyacrylamide gel. Electrophoresis was carried out at 30 volts/cm for 90 minutes after which the band containing the product was identified by UV shadowing of a fluorescent plate. The band was excised and eluted with 1 ml distilled water overnight at 4° C. This solution was applied to an Altech RP18 column and eluted with a 7–13% gradient of acetonitrile in 1% ammonium acetate buffer at pH 6.0. The elution was monitored by UV absorbance at 260 nm and the appropriate fraction collected, quantitated by UV absorbance in a fixed volume and evaporated to dryness at room temperature in a vacuum centrifuge.

Characterization of Oligodeoxyribonucleotides: Test aliquots of the purified oligonucleotides were ^{32}P labeled with polynucleotide kinase and γ-^{32}P-ATP. The labeled compounds were examined by autoradiography of 14–20% polyacrylamide gels after electrophoresis for 45 minutes at 50 volts/cm. This procedure verifies the molecular weight. Base composition was determined by digestion of the oligodeoxyribonucleotide to nucleosides by use of venom diesterase and bacterial alkaline phosphatase and subsequent separation and quantitation of the derived nucleosides using a reverse phase HPLC column and a 10% acetonitrile, 1% ammonium acetate mobile phase.

II. Source of DNA

A. Extraction of Whole Human Wild-Type DNA

Human geonomic DNA homozygous for normal β-globin was extracted from the cell line Molt4 (obtained from Human Genetic Mutant Cell Repository and identified as GM2219c) using the technique described by Stetler et al., *Proc. Nat. Acad. Sci.* USA (1982), 79:5966–5970.

B. Construction of Cloned Globin Genes

A 1.9 kb BamHI fragment of the normal β-globin gene was isolated from the cosmid pFC11 and inserted into the BamHI site of pBR328 (Soberon, et al., Gene (1980) 9:287–305). This fragment, which encompasses the region that hybridizes to the synthetic 40-mer probe, includes the first and second exons, first intron, and 5′ flanking sequences of the gene (Lawn et al., *Cell* (1978), 15:1157–1174). This clone was designated pBR328:HbA and deposited under ATCC No. 39,698 on May 25, 1984.

The corresponding 1.9 kb BamHI fragment of the sickle cell allele of β-globin was isolated from the cosmid pFC12 and cloned as described above. This clone was designated pBR328:HbS and deposited under ATCC No. 39,699 on May 25, 1984.

Each recombinant plasmid was transformed into and propagated in *E. coli* MM294 (ATCC No. 39,607).

C. Digestion of Cloned Globin Genes with MstII

A total of 100 μg each of pBR328:HbA and pBR328:HbS were individually digested with 20 units of MstII (New England Biolabs) for 16 hours at 37° C. in 200 μl of 150 mM NaCl, 12 mM Tris HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 100 μg/ml bovine serum albumin (BSA). The products are designated pBR328:HbA/MstII and pBR328:HbS/MstII, respectively.

III. Polymerase Chain Reaction

To 100 μl of buffer consisting of 60 mM sodium acetate, 30 mM Tris acetate and 10 mM magnesium acetate at pH 8.0 was added 2 μl of a solution containing 100 picomoles of Primer A (of the sequence d(CACAGGGCACTAACG)), 100 picomoles of Primer B (of the sequence d(TTTGCTTCTGACACA)) and 1000 picomoles each of dATP, dCTP, dGTP and TTP. In addition, one of the following sources of DNA described above was added:

10 μg whole human wild-type DNA (Reaction I)

0.1 picomole pBR328:HbA (Reaction II)

0.1 picomole pBR328:HbS (Reaction III)

0.1 picomole pBR328:HbA/MstII (Reaction IV)
0.1 picomole pBR328:HbS/MstII (Reaction V)
No target DNA (Reaction VI)

Each resulting solution was heated to 100° C. for four minutes and allowed to cool to room temperature for two minutes, whereupon 1 μl containing four units of Klenow fragment of *E. coli* DNA polymerase was added. Each reaction was allowed to proceed for 10 minutes, after which the cycle of adding the primers, nucleotides and DNA, heating, cooling, adding polymerase, and reacting was repeated nineteen times for Reaction I and four times for Reactions II–VI.

Four microliter aliquots of Reactions I and II removed before the first cycle and after the last cycle of each reaction were applied to a 12% polyacrylamide gel 0.089M in Tris-borate buffer at pH 8.3 and 2.5 mM in EDTA. The gel was electrophoresed at 25 volts/cm for four hours, transferred to a nylon membrane serving as solid phase support and probed with a 5'-^{32}P-labeled 40 pb synthetic fragment, prepared by standared techniques, of the sequence

5'd(TCCTGAGGAGAAGTCTGCCGT-
TACTGCCCTGTGGGGCAAG)3'

in 30% formamide, 3×SSPE, 5×Denhardt's, 5% sodium dodecyl sulfate at pH 7.4. FIG. 2 is an autoradiograph of the probed nylon membrane for Reactions I and II. Lane 1 is 0.1 picomole of a 58-bp synthetic fragment control one strand of which is complementary to the above probe. Lane 2 is 4 μl of Reaction I prior to the first amplification cycle. Lane 3 is 4 μl of Reaction I after the 20th amplification cycle. Lane 4 is 4 μl of Reaction II after five amplification cycles. Lane 5 is a molecular weight standard consisting of a FokI (New England Biolabs) digest of pBR322 (New England Biolabs) labeled with alpha-^{32}P-dNTPs and polymerase. Lane 3 shows that after twenty cycles the reaction mixture I contained a large amount of the specific sequence of the proper molecular weight and no other detectable products. Reaction mixture II after five cycles also contained this product, as well as the starting nucleic acid and other products, as shown by Lane 4.

To 5.0 μl aliquots of Reactions II–VI after the fourth cycle were added 5 pmoles of each primer described above. The solutions were heated to 100° C. for four minutes and allowed to equilibrate to room temperature. Three pmoles each of alpha-^{32}P-dATP, alpha-^{32}P-dCTP, alpha-^{32}P-dGTP and alpha-^{32}P-TTP and four units of Klenow fragment were added. The reaction, in a final volume of 10 μl and at the salt concentrations given above, was allowed to proceed for 10 minutes. The polymerase activity was terminated by heating for 20 minutes at 60° C. Four μl aliquots of Reactions II–VI were loaded onto a 12% polyacrylamide gel 0.089M in Tris-borate buffer at pH 8.3 and 2.5 mM in EDTA. The gel was electrophoresed at 25 volts/cm for four hours after which autoradiography was performed.

FIG. 3 is an autoradiograph of the electrophoresis. Lane 1 is a molecular weight standard, Lane 2 is Reaction II, Lane 3 is Reaction III, Lane 4 is Reaction IV and Lane 5 is Reaction V. Another lane for Reaction VI with no DNA as control had no images in any of the lanes. It can be seen from the figure that the 94-bp fragment predicted from the target DNA was present only where intact β-globin DNA sequences were available for amplification, i.e., pBR328:HbA (Lane 2), pBR328:HbS (Lane 3) and pBR328:HbS(MstII (Lane 5). MstII digestion cuts pBR328:HbA in the 94-mer sequence rendering it incapable of being amplified, and the 94-mer band does not appear in Lane 4. In contrast, the 94-mer sequence in pBR328:HbS does not cut when the plasmid is digested with MstII and thus is available for amplification as shown in Lane 5.

FIG. 4 illustrates the chain reaction for three cycles in amplifying the 94-bp sequence. PC01 and PC02 are Primers A and B. The numbers on the right indicate the cycles, whereas the numbers on the left indicate the cycle number in which a particular molecule was produced.

EXAMPLE 3

This example illustrates amplification of a 110 bp sequence spanning the allelic MstII site in the human hemoglobin gene.

A total of 1.0 microgram whole human DNA, 100 picomoles d(ACACAACTGTGTTCACTAGC) and 100 picomoles d(CAACTTCATCCACGTTCACC), the primers having been prepared by the technique of Example 2, were dissolved in 100 μl of a solution which was:

1.5 mM in each of the four deoxyribonucleoside triphosphates
30 mM in Tris acetate buffer at pH 7.9
60 mM in sodium acetate
10 mM in magnesium acetate
0.25 mM in dithiothreitol

The solution was heated to 100° C. for one minute and brought rapidly to 25° C. for one minute, after which was added 2.5 units Klenow fragment of DNA polymerase. The polymerase reaction was allowed to proceed for two minutes at 25° C., after which the cycle of heating, cooling, adding Klenow, and reacting was repeated as often as desired.

With 70% efficiency at each cycle, 15 cycles resulted in the synthesis of 1.4 femtomoles of the desired 110 bp fragment of the β-globin gene.

EXAMPLE 4

This example illustrates amplification of a 240 bp sequence spanning the allelic MstII site in the human hemoglobin gene. This sequence contains NcoI, HinfI and MstII restriction sites.

To 100 μl of a mixture of 60 mM sodium acetate, 30 mM Tris acetate and 10 mM magnesium acetate at pH 8.0 containing 0.1 pmole pBR328:HbA was added 2 μl of Solution A containing:

100 pmoles d(GGTTGGCCAATCTACTC-CCAGG) primer
100 pmoles d(TAACCTTGATAC-CAACCTGCCC) primer
1000 pmoles each of dATP, dCTP, dGTP and TTP

The two primers were prepared by the technique described in Example 2. The solution was heated to 100° C. for four minutes and allowed to cool in ambient air for two minutes, after which was added 1 μl containing four units Klenow fragment of *E. coli* DNA polymerase. The reaction was allowed to proceed for 10 minutes after which the cycle of solution A addition, heating, cooling, adding polymerase, and reacting was repeated three times. To a 5.0 μl aliquot of the reactions was added 5 picomoles of each oligonucleotide primer described above. The solution was heated to 100° C. for four minutes and allowed to come to ambient temperature, after which 3 picomoles each of the alpha-^{32}P-labeled deoxyribonucleoside triphosphates and 4 units Klenow fragment were added. The reaction, in a final volume of 10 μl and at the salt concentrations given

above, was allowed to proceed for 10 minutes. The polymerase activity was terminated by heating for 20 minutes at 60° C. Two μl aliquots were digested with NcoI, MstII, or HinfI and loaded onto a 12% polyacrylamide gel 0.089M in Tris-borate at pH 8.3 and 2.5 mM in EDTA. The gel was electrophoresed at 25 volts/cm for four hours and autoradiography was performed. FIG. 5 illustrates the autoradiograph of the electrophoresis, where Lane 1 is the molecular weight standard, Lane 2 is without digestion with enzyme (240 bp intact), Lane 3 is digestion with NcoI (131 and 109 bp), Lane 4 is digestion with MstII (149 and 91 bp), and Lane 5 is digestion with HinfI (144 and 96 bp). The autoradiograph is consistent with the amplification of the 240 bp sequence.

EXAMPLE 5

This example illustrates use of the process herein to detect sickle cell anemia by sequential digestion.

Synthesis and Phosphorylation of Oligodeoxyribonucleotides

A labeled DNA probe, RS06, of the sequence:

5′ *CTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGG 3′

where * indicates the label, and an unlabeled blocking oligomer, RS10, of the sequence:

3′ GACAGAGGTCACCTCTTCAGACGGCAATGACGGGACACCC 5′

which has three base pair mismatches with RS06 were synthesized according to the procedures provided in Example 2(I). The probe RS06 was labeled by contacting five pmole thereof with 4 units of T4 polynucleotide kinase (New England Biolabs) and 50 pmole γ-^{32}P-ATP (New England Nuclear, about 7200 Ci/mmole) in a 40 μl reaction volume containing 70 mM Tris buffer (pH 7.6), 10 mM $MgCl_2$, 1.5 mM spermine, and 2.5 mM dithiothreitol for 90 minutes at 37° C. The total volume was then adjusted to 100 μl with 25 mM EDTA and purified according to the procedure of Maniatis et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory, 1982), pp. 464–465 over a 1 ml Bio Gel P-4 spin dialysis column from Bio-Rad equilibrated with Tris-EDTA (TE) buffer (10 mM Tris buffer, 0.1 mM EDTA, pH 8.0). The labeled probe was further purified by electrophoresis on a 18% polyacrylamide gel (19:1 acrylamide:BIS, Bio-Rad) in Tris-boric acid-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) for 500 vhr. After localization by autoradiography, the portion of the gel containing the labeled probe was excised, crushed and eluted into 0.2 ml TE buffer overnight at 4° C. TCA precipitation of the reaction product indicated that the specific activity was 4.9 Ci/mmole and the final concentration was 20 pmole/ml.

The labeled RS10 blocking oligomer was used at a concentration of 200 pmole/ml.

Isolation of Human Genomic DNA from Cell Lines

High molecular weight genomic DNA was isolated from the lymphoid cell lines Molt4, SC-1 and GM2064 using essentially the method of Stetler et al., *Proc. Natl. Acad. Sci.* USA (1982), 79, 5966–5970 (for Molt4) and Maniatis et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory, 1982), pp. 280–281.

Molt4 (Human Mutant Cell Repository, GM2219C) is a T cell line homozygous for normal β-globin, and SC-1, deposited with ATCC on Mar. 19, 1985, is an EBV-transformed B cell line homozygous for the sickle cell allele. GM2064 (Human Mutant Cell Repository, GM2064) was originally isolated from an individual homozygous for hereditary persistence of fetal hemoglobin (HPFH) and contains no beta- or delta-globin gene sequences. All cell lines were maintained in RPMI-1640 with 10% fetal calf serum.

Isolation of Human Genomic DNA from Clinical Blood Samples

A clinical blood sample designated CH12 from a known sickle cell carrier (AS) was obtained from Dr. Bertram Lubin of Children's Hospital in Oakland, Calif. Genomic DNA was prepared from the buffy coat fraction, which is composed primarily of peripheral blood lymphocytes, using a modification of the procedure described by Nunberg et al., *Proc. Nat. Acad. Sci. USA*, 75, 5553–5556 (1978).

The cells were resuspended in 5 ml Tris-EDTA-NaCl (TEN) buffer (10 mM Tris buffer pH 8, 1 mM EDTA, 10 mM NaCl) and adjusted to 0.2 mg/ml proteinase K, 0.5% SDS, and incubated overnight at 37° C. Sodium perchlorate was then added to 0.7M and the lysate gently shaken for 1–2 hours at room temperature. The lysate was extracted with 30 ml phenol/chloroform (1:1), then with 30 ml chloroform, and followed by ethanol precipitation of the nucleic acids. The pellet was resuspended in 2 ml of TE buffer and RNase A added to 0.005 mg/ml. After digestion for one hour at 37° C., the DNA was extracted once each with equal volumes of phenol, phenol/chloroform, and chloroform, and ethanol precipitated. The DNA was resuspended in 0.5 nm.

Polymerase Chain Reaction to Amplify Selectively β-Globin Sequences

Two micrograms of genomic DNA was amplified in an initial 100 μl reaction volume containing 10 mM Tris buffer (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 150 pmole of Primer A of the sequence d(CACAGGGCACTAACG), and 150 pmole of Primer B of the sequence d(CTTTGCTTCTGACACA) and overlayed with about 100 μl mineral oil to prevent evaporation.

Each DNA sample underwent 15 cycles of amplification where one cycle is composed of three steps:

(1) Denature in a heat block set at 95° C. for two minutes.

(2) Transfer immediately to a heat block set at 30° C. for two minutes to allow primers and genomic DNA to anneal.

(3) Add 2 μl of a solution containing 5 units of the Klenow frgment of *E. coli* DNA polymerase I (New England Biolabs), 1 nmole each of dATP, dCTP, dGTP and TTP, in a buffer composed of 10 mM Tris (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, and 4 mM dithiothreitol. This extension reaction was allowed to proceed for 10 minutes at 30° C.

After the final cycle, the reaction was terminated by heating at 95° C. for two minutes. The mineral oil was extracted with 0.2 ml of chloroform and discarded. The final reaction volume was 130 μl.

Hybridization/Digestion of Amplified Genomic DNA with Probes and DdeI/HinfI

Forty-five microliters of the amplified genomic DNA was ethanol precipitated and resuspended in an equal volume of TE buffer. Ten microliters (containing the

pre-amplification equivalent of 154 ng of genomic DNA) was dispensed into a 1.5 ml Microfuge tube and 20 μl of TE buffer to a final volume of 30 μl. The sample was overlayed with mineral oil and denatured at 95° C. for 10 minutes. Ten microliters of 0.6M NaCl containing 0.02 pmole of labeled RS06 probe was added to the tube, mixed gently, and immediately transferred to a 56° C. heat block for one hour. Four microliters of unlabeled RS10 blocking oligomer (0.8 pmole) was added and the hybridization continued for an additional 10 minutes at the same temperature. Five microliters of 60 mM $MgCl_2$/0.1% BSA and 1 μl of DdeI (10 units, New England Biolabs) were added and the reannealed DNA was digested for 30 minutes at 56° C. One microliter of HinfI (10 units, New England Biolabs) was then added and incubated for another 30 minutes. The reaction was stopped by the addition of 4 μl 75 mM EDTA and 6 μl tracking dye to a final volume of 61 μl.

The mineral oil was extracted with 0.2 ml chloroform, and 18 μl of the reaction mixture (45 ng genomic DNA) was loaded onto a 30% polyacrylamide mini-gel (19:1, Bio-Rad) in a Hoeffer SE200 apparatus. The gel was electrophoresed at approximately 300 volts for one hour until the bromophenol blue dye front migrated to 3.0 cm off-origin. The top 1.5 cm of the gel was removed and the remaining gel was exposed for four days with one intensification screen at −70° C.

Discussion of Photograph (FIG. 9)

Each lane contains 45 ng of amplified genomic DNA. Lane A contains Molt4 DNA; Lane B, CH12; Lane C, SC-1; and Lane D, GM2064. Molt4 represents the genotype of a normal individual with two copies of the β^A gene per cell (AA), CH12 is a clinical sample from a sickle cell carrier with one β^A and one β^S gene per cell (AS), and SC-1 represents the genotype of a sickle cell individual with two copies of the β^S gene per cell (SS), GM2064, which contains no beta- or delta-globin sequences, is present as a negative control.

As seen in the photograph, the DdeI-cleaved, β^A-specific octamer is present only in those DNA's containing the β^A gene (Lanes A and B), and the HinfI-cleaved, β^S-specific trimer is present only in those DNA's containing the β^S gene (Lanes B and C). The presence of both trimer and octamer (Lane B) is diagnostic for a sickle cell carrier and is distinguishable from a normal individual (Lane A) with only octamer and a sickle cell afflicted individual (Lane C) with only trimer.

As a comparison, repeating the experiment described above using non-amplified genomic, DNA revealed that the amplification increased the sensitivity of detection by at least 1000 fold.

EXAMPLE 6

This example illustrates direct detection of a totally unpurified single copy gene in which human DNA on gels without the need for a labeled probe.

Using the technique described in Example 3, a 110-bp fragment from a sequence in the first exon of the beta-globin gene was amplified from 10 micrograms of whole human DNA after 20 cycles. This 110-bp fragment produced after 20 cycles was easily visualized on gels stained with ethidium bromide.

The sequence was not amplified when it was first cut with the restriction enzyme DdeI unless, as in the beta-globin S allele, the sequence does not contain the restriction site recognized by the enzyme.

EXAMPLE 7

A. A total of 100 fmoles pBR328 containing a 1.9 kb insert from the human beta-globin A allele, 50 nmoles each alpha-32P-dNTP at 500 Ci/mole, and 1 nmole of each of the primers used in Example 3 were dissolved in a solution containing 100 μl 30 mM Tris-acetate at pH 7.9, 60 mM sodium acetate, 100 mM dithiothreitol, and 10 mM magnesium acetate. This solution was brought to 100° C. for two minutes and cooled to 25° C. for one minute. A total of 1 μl containing 4.5 units Klenow fregment of *E. coli* DNA polymerase I and 0.09 units inorganic pyrophosphatase was added to prevent the possible build-up of pyrophosphate in the reaction mixture, and the reaction was allowed to proceed for two minutes at 25° C., after which the cycle of heating, cooling, adding enzyme, and reacting was repeated nine times. Ten-μl aliquots were removed and added to 1 μl 600 mM EDTA after each synthesis cycle. Each was analyzed on a 14% polyacrylamide gel in 90 mM Tris-borate and 2.5 mM EDTA at pH 8.3 and 24 volts/cm for 2.5 hours. The completed gel was soaked for 20 minutes in the same buffer with the addition of 0.5 μg/ml ethidium bromide, washed with the original buffer, and photographed in UV light using a red filter.

The 110-bp fragment produced was excised from the gel under ultraviolet light and the incorporated ^{32}P counted by Cerenkov radiation. An attempt to fit the data to an equation of the form: pmoles/10 μl=0.01 $[(1+y)^N-yN-1]$, where N represents the number of cycles and y the fractional yield per cycle, was optimal with y=0.619. This indicates that a significant amplification is occurring.

B. The above experiment was repeated except that 100 nmoles of each dNTP was added to a 100 μl reaction, no radiolabel was employed, and aliquots were not removed at each cycle. After 10 cycles the reaction was terminated by boiling for two minutes and rehybridization was performed at 57° C. for one hour. The sequence of the 110-bp product was confirmed by subjecting 8 μl aliquots to restriction analysis by addition of 1 μl bovine serum albumin (25 mg/ml) and 1 μl of the appropriate restriction enzyme (HinfI, MnlI, MstII, NcoI) and by reaction at 37° C. for 15 hours. PAGE was performed as described above.

EXAMPLE 8

This example illustrates the use of different primers to amplify various fragments of pBR328 and 322.

A. The experiment described in Example 7A was repeated except using the following primers: d(TTTGCTTCTGACACAACTGTGTTCACTAGC) and d(GCCTCACCACCAACTTCATCCACGTTCACC) to produce a 130-bp fragment of pBR328.

B. The experiment described in Example 7A was repeated except using the following primers: d(GGTTGGCCAATCTACTCCCAGG) and d(TGGTCTCCTTAAACCTGTCTTG) to produce a 262-bp fragment of pBR328. The reaction time was 20 minutes per cycle.

The experiment described in Example 8B was repeated except that 100 fmoles of an MstII digest of pBR328 containing a 1.9 kb insert from the human beta-globin S allele was used as initial template. This plasmid was cleaved several times by MstII but not inside the sequence to be amplified. In addition, the primers employed were as follows:

d(GGTTGGCCAATCTACTCCCAGG) and
d(TAACCTTGATACCAACCTGCCC)
to produce a 240-bp fragment.

D. The experiment described in Example 7B was repeated except that 100 fmoles of an NruI digest of pBR322 was used as template, 200 nmoles of each dNTP were used in the 100 μl reaction, and the primers were:
d(TAGGCGTATCACGAGGCCCT) and
d(CTTCCCCATCGGTGATGTCG)
to produce a 500-bp fragment from pBR322. Reaction times were 20 minutes per cycle at 37° C. Final rehybridization was 15 hours at 57° C. Electrophoresis was on a 4% agarose gel.

EXAMPLE 9

This example illustrates the invention process wherein an in vitro mutation is introduced into the amplified segment.

A. A total of 100 fmoles of pBR322 linearized with NruI, 1 nmole each of the primers:
d(CGCATTAAAGCTTATCGATG) and
d(TAGGCGTATCACGAGGCCCT)
designed to produce a 75-bp fragment, 100 nmole each dNTP, in 100 μl 40 mM Tris at pH 8, 20 mM in $MgCl_2$, 5 mM in dithiothreitol, and 5 mg/ml bovine serum albumin were combined. The mixture was brought to 100° C. for one minute, cooled for 0.5 minutes in a water bath at 23° C., whereupon 4.5 units Klenow fragment and 0.09 units inorganic pyrophosphatase were added, and a reaction was allowed to proceed for three minutes. The cycle of heating, cooling, adding enzymes, and reacting was repeated nine times. The tenth reaction cycle was terminated by freezing and an 8-μl aliquot of the reaction mixture was applied to a 4% agarose gel visualized with ethidium bromide.

B. The experiment described in Example 9A was repeated except that the oligonucleotide primers employed were:
d(CGCATTAAAGCTTATCGATG) and
d(AATTAATACGACTCACTATAGG-
GAGATAGGCGTATCACGAGGCCCT).
Thse primers are designed to produce a 101-bp fragment, 26 nucleotides of which (in the second listed primer) are not present in pBR322. These nucleotides represent the sequence of the T7 promoter, which was appended to the 75-bp sequence from pBR322 by using the primer with 20 complementary bases and a 26-base 5′ extension. The procedure required less than two hours and produced two picomoles of the relatively pure 101-bp fragment from 100 fmoles of pBR322.

The T7 promoter can be used to initiate RNA transcription. T7 polymerase may be added to the 101-bp fragment to produce single-stranded RNA.

C. The experiment described in Example 8D was repeated except that the oligonucleotide primers employed were as follows:
d(TAGGCGTATCACGAGGCCCT) and
d(CCAGCAAGACGTAGCCCAGC)
to produce a 1000-bp fragment from pBR322.

D. The experiment described in Example 9C was repeated except that the oligonucleotide primers employed were as follows:
d(TAGGCGTATCACGAGGCCCT) and
d(AATTAATACGACTCACTATAGG-
GAGATAGGCGTATCACGAGGCCCT)
so as to produce a 1026-bp fragment, 26 nucleotides of which (in the second listed primer) are not present in pBR322 and represent the T7 promoter described above. The promoter has been inserted adjacent to a 1000-bp fragment from pBR322.

The results indicate that a primer which is not a perfect match to the template sequence but which is nonetheless able to hybridize sufficiently to be enzymatically extended produces a long product whwich contains the sequence of the primer rather than the corresponding sequence of the original template. The long product serves as a template for the second primer to introduce an in vitro mutation. In further cycles this mutation is amplified with an undiminished efficiency, because no further mispaired primings are required. In this case, a primer which carries a non-complementary extension on its 5′ end was used to insert a new sequence in the product adjacent to the template sequence being copied.

EXAMPLE 10

This example illustrates employing nested sets of primers to decrease the background in the amplification of single copy genes.

Whole human DNA homozygous for the wild-type β-globin allele was subjected to twenty cycles of amplification as follows: A total of 10 μg DNA, 200 picomoles each of the primers:
d(ACACAACTGTGTTCACTAGC) and
d(CAACTTCATCCACGTTCACC)
and 100 nanomoles each dNTP in 100 μl of 30 mM Tris-acetate pH 7.9, 60 mm sodium acetate, 10 mM dithiothreitol, and 10 mM magnesium acetate were heated to 100° C. for one minute, cooled to 25° C. for one minute, and treated with 2 units Klenow fragment for two minutes. The cycle of heating, cooling and adding Klenow as repeated 19 times. A ten-μl aliquot was removed from the reaction mixture and subjected to a further ten cycles of amplification using each of the primers:
d(CAGACACCATGGTGCACCTGACTCCTG)
and
d(CCCCACAGGGCAGTAACG-
GCAGACTTCTCC),
which amplify a 58-bp fragment contained within the 110-bp fragment produced above. This final ten cycles of amplification was accomplished by diluting the 10-μl aliquot into 90 μl of the fresh Tris-acetate buffer described above containing 100 nanomoles each dNTP and 200 pmoles of each primer. Reaction conditions were as above. After ten cycles a 10-μl aliquot (corresponding to 100 nanograms of the original DNA) was applied to a 6% NuSieve (FMC Corp.) agarose gel and visualized using ethidium bromide.

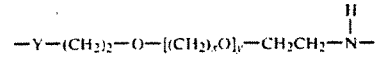
FIG. 10 illustrates this gel illuminated with UV light and photographed through a red filter as is known in the art. Lane 1 is molecular weight markers. Lane 2 is an aliquot of the reaction described above. Lane 3 is an aliquot of a reaction identical to that described above, except that the original wild-type DNA was cleaved with DdeI prior to amplification. Lane 4 is an aliquot of a reaction identical to that described above, except that human DNA homozygous for the sickle betaglobin allele was treated with DdeI prior to amplification (the sickle allele does not contain a DdeI site in the fragment being amplified here). Lane 5 is an aliquot of a reaction identical to that described above, except that salmon sperm DNA was substituted for human DNA. Lane 6 is an aliquot of a reaction identical to that described above, except that the aliquot was treated with DdeI

after amplification (DdeI should convert the 58-bp wild-type product into 27- and 31-bp fragments). Lane 7 is an aliquot of the Lane 4 material treated with DdeI after amplification (the 58-bp sickle product contains no DdeI site).

Detection of a 58-bp fragment representative of a single-copy gene from one microgram of human DNA using only ethidium bromide staining of an agarose gel requires an amplification of about 500,000-fold. This was accomplished by using the two nested sets of oligonucleotide primers herein. The first set amplifies the 110-bp fragment and the inner nested set amplifies a sub-fragment of this product up to the level of convenient detection shown in FIG. 10. This procedure of using primers amplifying a smaller sequence contained within the sequence being amplified in the previous amplification process and contained in the extension products of the other primers allows one to distinguish the wild-type from the sickle allele at the betaglobin locus without resorting to either radioisotropic or non-radioisotopic probe hybridization methodology such as that of Conner et al., *Proc. Natl. Acad. Sci.* USA, 80:278 (1983) and Leary et al., *Proc. Natl. Acad. Sci.* USA, 80:4045 (1983).

EXAMPLE 11

The present process is expected to be useful in detecting, in a patient DNA sample, a specific sequence associated with an infectious disease such as, e.g., Chlamydia using a biotinylated hybridization probe spanning the desired amplified sequence and using the process described in U.S. Pat. No. 4,358,535, supra. The biotinylated hybridization probe may be prepared by intercalation and irradiation of a partially double-stranded DNA with a 4'-methylene substituted 4,5'-8-trimethylpsoralen attached to biotin via a spacer arm of the formula:



where Y is O, NH or N—CHO, x is a number from 1 to 4, and y is a number from 2 to 4, as described in U.S. Pat. Nos. 4,582,789 issued Apr. 15, 1986 and 4,617,261 issued Oct. 14, 1986, the disclosures of which are incorporated herein by reference. Detection of the biotinyl groups on the probe may be accomplished using a streptavidin-acid phosphatase complex commercially obtainable from Enzo Biochemical using the detection procedures suggested by the manufacturer in its brochure. The hybridized probe is seen as a spot of precipitated stain due to the binding of the detection complex, and the subsequent reaction catalyzed by acid phosphatase, which produces a precipitable dye.

EXAMPLE 12

In this example, the process of Example 7 was basically used to amplify a 119 base pair fragment on the human β-hemoglobin gene using the primers:

5'-CTTCTGcagCAACTGTGTTCACTAGC-3' (GH18)

5'-CACaAgCTTCATCCACGTTCACC-3' (GH19)

where lower case letters denote mismatches from wild-type sequence to create restriction enzyme sites. The full scheme is shown in Table I. Table I illustrates a diagram of the primers GH18 and GH19 which are used for cloning and sequencing a 119-base pair fragment of the human β-globin gene and which are designed to contain internal restriction sites. The start codon ATG is underlined. GH18 is a 26-base oligonucleotide complementary to the negative strand and contains an internal PstI site. GH19 is a 23-base oligonucleotide complementary to the plus strand and contains an internal HindIII recognition sequence. Arrows indicate the direction of extension by DNA polymerase I. The boxed sequences indicate the restriction enzyme recognition sequences of each primer. These primers were selected by first screening the regions of the gene for homology to the PstI and HindIII restriction sites of bacteriophage M13. The primers were then prepared as described in previous examples.

TABLE I

DdeI

GH19

←CCACTTGCACCTAC[TTCgAa]CAC

CTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTG(+)

GAAGACTGTGTTGACACAAGTGATCGTTGGAGTTTGTCTGTGGTACCACGTGGACTGAGGACTCCTCTTCAGACGGCAATGACGGGACACCCCGTTCCACTTGCACCTACTTCAACCAC(−)

CTTCTG[cagCAA]CTGTGTTCACTAGC→

GH18

Pst I

5' CTTCTG[cagCAA]CTGTGTTCACTAGC 3' GH18 left linker primer

5' CAC[aAgCTT]CATCCACGTTCACC 3' GH19 roght linker primer

Hind III

Amplification and Cloning

After twenty cycles of amplification of 1 microgram of human genomic DNA isolated from the cell line Molt 4 as described in Example 2, 1/14th of the reaction product was hybridized to the labeled β-globin specific oligonucleotide probe, RS06, of the sequence 5'-CTGACTCCTGAGGAGAAGTCTGCCGT-TACTGCCCTGTGGG-3' using the methods described above for oligomer restriction. Following solution hybridization, the reaction mixture was treated with DdeI under restriction digestion conditions as described above, to produce an 8-base pair oligonucleotide. The amount of this 8-base pair product is proportional to the amount of amplified product produced. The digestion products were resolved on a 30% polyacrylamide gel and visualized by autoradiography.

Analysis of the autoradiogram revealed that the amplification was comparable in efficiency to that of amplification with primers PC03 (5'-ACACAACTGTGTTCACTAGC-3') and PC04 (5'-CCACTTGCACCTACTTCAAC-3'), which are complementary to the negative and positive strands, respectively, of the wild-type β-globin.

The amplified product was ethanol precipitated to desalt and concentrate the sample, redissolved in a restriction buffer of 10 mM Tris pH 8, 10 mM $MgCl_2$, 1 mM DTT, 100 mM $NaCl_2$, and simultaneously digested with PstI and HindIII. After digestion the sample was desalted with a Centricon 10 concentrator and ligated overnight at 12° C. with 0.3 micrograms of the PstI/-HindIII digested vector M13mp10w, which is publicly available from Boehringer-Mannheim.

The entire ligation mixture was transformed into *E. coli* strain JM103, which is publicly available from BRL in Bethesda, MD. The procedure followed by preparing the transformed strain is described in Messing, J. (1981) *Third Cleveland Symposium on Macromolecules:Recombinant DNA*, ed. A. Walton, Elsevier, Amsterdam, 143–153.

The transformation mixture was plated onto x-gal media for screening via plaque hybridization with nylon filters. The filters were probed with a β-globin-specific oligonucleotide probe RS24 of the sequence 5'-CCCACAGGGCAGTAACGGCAGACTTCTCCT-CAGGAGTCAG-3' to determine the number of β-globin inserts. The filters were then reprobed with the primer PC04 to determine the total number of inserts.

Plating and Screening

Table II summarizes the plating and plaque hybridization data. The filters were probed with the primer PC04 to determine the percentage of inserts resulting from amplification and cloning; 1206 clear plaques (90% of total number of clear plaques) hybridized to the primer. Fifteen plaques hybridized to the β-globin specific probe RS24. The percentage of β-globin positive plaques among the amplified primer-positive plaques is approximately 1%.

TABLE II

Plate No.	Blue Plaques	No Inserts*	Inserts**	β-Globin Inserts
1	28	25	246	1
2	29	18	222	2
3	11	26	180	0
4	24	20	192	5
5	22	27	185	5
6	39	21	181	3
TOTAL	158	132	1206	15

% of plaques containing amplified sequences which contain β-globin insert 15/1206 × 100 = 1.24%

% of total plaques which contain β-globin insert 15/1496 × 100 = ca.1%

% of total plaques which contain amplified sequences 1206/1496 × 100 = 0.8%

*Clear plaques which do not hybridize to primer PC04

**Clear plaques which hybridize to primer PC04

Restriction Enzyme and Southern Blot Analysis

DNAs from phage DNA minipreparation of three β-globin positive and two β-globin negative (but PC04 primer positive) plaques were analyzed by restriction enzyme analysis. MstII digestion of DNA from M13 clones containing the amplified β-globin fragment should generate a characteristic 283 base-pair fragment. Following MstII digestion, the three β-globin positive clones all produced the predicted 283 base pair fragment, while the two clones which were positive only with the primer produced larger fragments.

The gel from this analysis was transferred to a MSI nylon filter and hybridized with a radiolabeled nick-translated β-globin probe prepared by standard nick translation methods as described by Rigby et al., *J. Mol. Biol.* (1977), 113:237–51. The only bands which hybridized to the β-globin probe were the three β-globin positive clones. The two other clones had inserts which did not hybridize to the β-globin probe.

Sequence Analysis

Ten β-globin positive clones which were shown by restriction enzyme analysis to contain the β-globin insert were sequenced using the M13-dideoxy sequencing method. Of the ten clones, nine were identical to the β-globin wild-type sequence. The other clone was identical to the ∂-globin gene which had been shown to be amplified to only a small degree by the β-globin primers.

In conclusion, the modified linker primers were nearly as efficient as the unmodified primers in amplifying the β-globin sequence. The primers were able to facilitate insertion of amplified DNA into cloning vectors. Due to the amplification of other segments of the genome, only 1% of the clones contained hemoglobin sequences.

Nine of the ten clones were found to be identical to the published β-globin sequence, showing that the technique amplifies genomic DNA with high fidelity. One clone was found to be identical with the published ∂-globin sequence, confirming that the primers are specific for the β-globin gene despite their having significant sequence homology with δ-globin.

When cloning was carried out with a 267 base pair fragment of the β-globin gene, cloning was effective only when dimethylsulfoxide was present (10% by volume at 37° C.) in the amplification procedure.

Restriction site-modified primers were also used to amplify and clone and partially sequence the human N-ras oncogene and to clone 240-base pair segments of the HLA DQ-α and DQ-β genes. All of these amplifications were carried out in the presence of 10% by volume dimethylsulfoxide at 37° C. The primers for amplifying HLA DQ-α and DQ-β genes were much more specific for their intended targets than were the β-globin and DR-β primers, which, rather than giving a

discrete band on an ethidium bromide stained agarose gel, produced only a smear. In addition, the HLA DQ-α primers produced up to 20% of clones, with amplified inserts which contained the desired HLA target fragment, whereas 1% of the β-globin clones contained the target sequence. The HLA DQ-α and DQ-β gene cloning was only effective when the DMSO was present and the temperature was elevated.

EXAMPLE 13

This example illustrates the use of the process herein to prepare the TNF gene of 494 base pairs starting from two oligonucleotides of 74 base pairs each.

PRIMERS

The primers employed were prepared by the method described in Example 2 and are identified below, each being 74 mers.

(TN10) 5'-CCTCGTCTACTCCCAGGTCCTCTTCAAGGGCCAAGGCTGCCCCGACTATGTGCTCCTCACCCACACCGTCAGCC-3'

(TN11) 5'-GGCAGGGGCTCTTGACGGCAGAGAGGAGGTTCACCTTCTCCTGGTAGGAGATGGCGAAGCGGCTGACGGTGTGG-3'

(LL09) 5'-CCTGGCCAATGGCATGGATCTGAAAGATAACCAGCTGGTGGTGCCAGCAGATGGCCTGTACCTCGTCTACTCCC-3'

(LL12) 5'-CTCCCTGATAGATGGGCTCATACCAGGGCTTGAGCTCAGCCCCCTCTGGGGTGTCCTTCGGGCAGGGGCTCTTG-3'

(TN08) 5'-TGTAGCAAACCATCAAGTTGAGGAGCAGCTCGAGTGGCTGAGCCAGCGGGCCAATGCCCTCCTGGCCAATGGCA-3'

(TN13) 5'-GATACTTGGGCAGATTGACCTCAGCGCTGAGTTGGTCACCCTTCTCCAGCTGGAAGACCCCTCCCTGATAGATG-3'

(LL07) 5'-CCTTAAGCTTATGCTCAGATCATCTTCTCAAAACTCGAGTGACAAGCCTGTAGCCCATGTTGTAGCAAACCATC-3'

(TN14) 5'-GCTCGGATCCTTACAGGGCAATGACTCCAAAGTAGACCTGCCCAGACTCGGCAAAGTCGAGATACTTGGGCAGA-3'

OVERALL PROCEDURE

I. Ten cycles of the protocol indicated below were carried out using primers TN10 and TN11, which interact as shown in the diagram below, step (a).

II. A total of 2 μl of the reaction mixture from Part I above was added to the primers LL09 and LL12. The protocol described below was carried out for 15 cycles, so that the primers would interact with the product of Part I as shown in the diagram below, step (b).

III. A total of 2 μl of the reaction mixture from Part II above was added to the primers TN08 and TN13. The protocol described below was carried out for 15 cycles, so that the primers would interact with the product of Part II as shown in the diagram below, step (c).

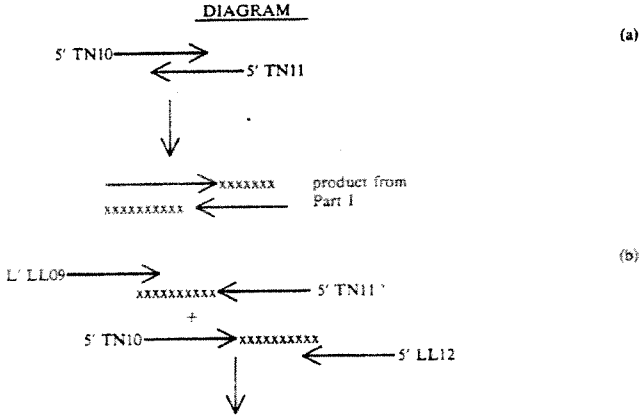
IV. A total of 2 μl of the reaction mixture from Part III above was added to the primers LL07 and LL14. The protocol described below was carried out for 15 cycles, so that the primers would interact with the product of Part III as shown in the diagram below, step (d).

PROTOCOL

Each reaction contained 100 μl of:
- 2 mM of each of dATP, dCTP, dGTP and TTP
- 3 μM of each of the primers used at that step
- 1×polymerase buffer, (30 mM Tris-acetate, 60 mM Na-acetate, 10 mM Mg-acetate, 2.5 mM dithiothreitol)

Each cycle constituted:
- (1) 1 min. in boiling water
- (2) 1 min. cooling at room temperature
- (3) add 1 μl (5 units) of the Klenow fragment of DNA polymerase
- (4) allow the polymerization reaction to proceed for 2 min. For the next cycle start again at step 1.

DIAGRAM



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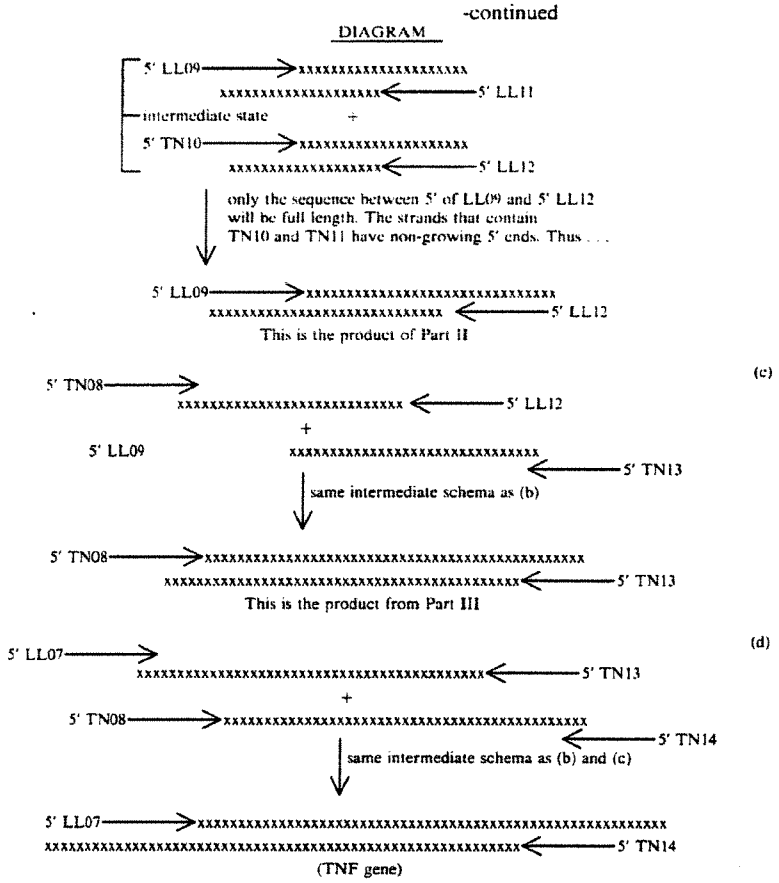

Deposit of Materials

The cell line SC-1 (CTCC #0082) was deposited on Mar. 19, 1985 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852 USA, with ATCC Accession No. CRL#8756. The deposit of SC-1 was made pursuant to a contract between the ATCC and the assignee of this patent application, Cetus Corporation. The contract with ATCC provides for permanent availability of the progeny of this cell line to the public on the issuance of the U.S. patent describing and identifying the deposit or the publications or upon the laying open to the public of any U.S. or foreign patent application, whichever comes first, and for availability of the progeny of this cell line to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 CFR §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the cell line on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable culture of the same cell line.

In summary, the present invention is seen to provide a process for detecting sequences in nucleic acids by first amplifying one or more specific nucleic acid sequences using a chain reaction in which primer extension products are produced which can subsequently act as templates for further primer extension reactions. The process is especially useful in detecting nucleic acid sequences which are initially present in only very small amounts. Also, the amplification process can be used for molecular cloning.

Other modifications of the above described embodiments of the invention which are obvious to those of skill in the area of molecular biology and related disciplines are intended to be within the scope of the following claims.

What is claimed is:

1. A process for detecting the presence or absence of at least one specific nuclei acid sequence in a sample containing a nucleic acid or mixture of nucleic acids, or distinguishing between two different sequences in said sample, wherein the sample is suspected of containing said sequence or sequences, which process comprises:

(a) treating the sample with one oligonucleotide primer for each strand of each different specific sequence, under hybridizing conditions such that for each strand of each different sequence to which an oligonucleotide primer is hybridized an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primer or primers are selected so as to be sufficiently complementary to each strand of each specific sequence to hybridize therewith such

that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

(b) treating the sample under denaturing conditions to separate the primer extension products from their templates if the sequence or sequences to be detected are present;

(c) treating the sample with oligonucleotide primers such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the specific nucleic acid sequence or sequences if present;

(d) adding to the product of step (c) a labeled oligonucleotide probe for each sequence being detected capable of hybridizing to said sequence or a mutation thereof; and

(e) determining whether said hybridization has occurred.

2. The process of claim **1**, wherein steps (b) and (c) are repeated at least once.

3. The process of claim **1**, wherein steps (a) and (c) are accomplished by treatment with four different nucleoside triphosphates and an agent for polymerization, which are added together with or separately from the primer(s).

4. The process of claim **1**, wherein said nucleic acid is double stranded and its strands are separated by denaturing before or during step (a).

5. The process of claim **1**, wherein said nucleic acid is single stranded.

6. The process of claim **4**, wherein said nucleic acid is DNA and said primers are oligodeoxyribonucleotides.

7. The process of claim **4**, wherein said nucleic acid is RNA and said primers are oligodeoxyribonucleotides.

8. The process of claim **5**, wherein said nucleic acid is DNA and said primers are oligodeoxyribonucleotides.

9. The process of claim **5**, wherein said nucleic acid is RNA and said primers are oligodeoxyribonucleotides.

10. The process of claim **1**, wherein each primer employed contains a restriction site on its 5′ end which is the same as or different from a restriction site on another primer, and after step (c) and before step (d) the product of step (c) is cleaved with a restriction enzyme specific for each of said restriction sites and the cleaved products are separated from the uncleaved products and used in step (d).

11. The process of claim **1**, wherein the specific nucleic acid sequence contains at least one specific deletion or mutation that causes a genetic disease.

12. The process of claim **11**, wherein the genetic disease is sickle cell anemia.

13. The process of claim **11**, wherein after step (c) and before step (d) the treated sample is cut with a restriction enzyme and electrophoresed and step (e) is accomplished by Southern blot analysis.

14. The process of claim **1**, wherein the specific nucleic acid sequence is contained in a pathogenic organism or is contained in an oncogene.

15. The process of claim **1**, wherein steps (a) and (c) are accomplished using an enzyme selected from the group consisting of *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, reverse transcriptase wherein the template is RNA or DNA and the extension product is DNA, and an enzyme that after being exposed to a temperature of about 65°–90° C. forms said extension products at the temperature of reaction during steps (a) and (c).

16. A process for detecting the presence or absence of a nucleic acid sequence containing a polymorphic restriction site specific for sickle cell anemia which sequence is suspected of being contained in a sample, which process comprises:

(a) treating the sample, together or separately, with an oligodeoxyribonucleotide primer for each strand, four different nucleoside triphosphates, and an agent for polymerization under hybridizing conditions, such that for each strand of the nucleic acid sequence an extension product of each primer is synthesized which is sufficiently complementary to each strand of the nucleic acid sequence being detected to hybridize therewith and contains the region of the β-globin gene known potentially to contain the mutation that causes sickle cell anemia, wherein said primers are selected such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

(b) treating the sample under denaturing conditions to separate the primer extension products from the templates on which they are synthesized if the sequence to be detected is present;

(c) treating the product of step (b) with oligodeoxyribonucleotide primers, four different nucleoside triphosphates, and an agent polymerization such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the sequence to be detected if present;

(d) hybridizing said primer extension products of step (c) with a labeled oligodeoxyribonucleotide probe complementary to a normal β-globin gene;

(e) digesting the hybridized mixture from step (d) with a restriction enzyme for the restriction site specific for sickle cell anemia; and

(f) detecting whether the digest contains a restriction fragment correlated with the presence of sickle cell anemia.

17. The process of claim **16**, wherein in step (d) the probe spans DdeI and HinfI restriction sites, in step (e) the restriction enzyme is DdeI, and after step (e) and before step (f) the mixture is digested with restriction enzyme HinfI.

18. The process of claim **16**, wherein in steps (d)–(f) are present a positive control which contains a nucleic acid with the polymorphic restriction site specific for sickle cell anemia and a negative control which does not contain such nucleic acid.

19. A process for synthesizing a nucleic acid fragment from an existing nucleic acid fragment having fewer nucleotides than the fragment being synthesized and two oligonucleotide primers, wherein the nucleic acid being synthesized is comprised of a left segment, a core segment and a right segment, and wherein the core segment is sufficiently complementary to the nucleotide sequence of said existing nucleic acid fragment to hybridize therewith, and the right and left segments represent the nucleotide sequence present in the 5′ ends of the two primers, the 3′ ends of which are complementary to, or sufficiently complementary to hybridize with, the 3′ ends of the single strands produced by separating the strands of said existing nucleic acid fragment, which process comprises:

(a) treating the strands of said existing fragment with two oligonucleotide primers under conditions such

that an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primers are selected so as to be sufficiently complementary to the 3' end of each strand of said existing fragment to hybridize therewith, such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each primer contains, at its 5' end, a sequence of nucleotides which are not complementary to said existing fragment and which correspond to the two ends of the nucleic acid fragment being synthesized;

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating the single-stranded molecules generared from step (b) with the primers of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template so as to produce two intermediate double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5' end of one of the oligonucleotide primers, and two full-length double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5' ends of both of the oligonucleotide primers;

(d) repeating steps (b) and (c) for a sufficient number of times to produce the full-length double-stranded molecule in an effective amount;

(e) treating the strands of the product of step (d) with two primers so as to lengthen the product of step (d) on both ends; and

(f) repeating steps (a)–(d) using the product of step (d) as the core fragment and two oligonucleotide primers which are complementary to, or sufficiently complementary to hybridize with, the 3' ends of the single strands produced by separating the strands of the product of step (d).

20. The process of claim **19**, wherein steps (b) and (c) are repeated at least five times.

21. The process of claim **20**, wherein the core segment used is the product of step (f).

22. The process of claim **19**, wherein the core fragment used is obtained by the steps comprising:

(a) reacting two oligonucleotides, each of which contain at their 3' ends a nucleotide sequence which is complementary to the other oligonucleotide at its 3' end, and which are non-complementary to each other at their 5' ends, with an agent for polymerization and four nucleoside triphosphates under conditions such that an extension product of each oligonucleotide is synthesized which is complementary to each nucleic acid strand;

(b) separating the extension products from the templates on which they were synthesized to produce single-stranded molecules; and

(c) treating the single-stranded molecules generated from step (b) with the oligonucleotides of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the core fragment.

23. The process of claim **19**, wherein the product of step (d) is purified before step (e).

24. The process of claim **19**, wherein the product of step (d) is not purified before step (e).

25. The process of claim **19**, wherein steps (a) and (c) are accomplished by treatment with four different nucleoside triphosphates and an agent for polymerization, which are added together with or separately from the primers.

26. A process for cloning into bacteriophage M13 a polymorphic genetic sequence on the human HLA DQ, DR or DP Class Ii α and β genes, which process comprises:

(a) treating a genetic sequence of human HLA DQ, DR, or DP Class II α and β genes with one oligonucleotide primer for each strand of said sequence, under conditions such that for each strand an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primers are selected so as to be sufficiently complementary to each strand to hybridize therewith such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each of said primers contains a restriction site on its 5' end which is different from the restriction site on the other primer;

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating the single-stranded molecules generated from step (b) with oligonucleotide primers such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, wherein steps (a) and (c) are carried out in the presence of an effective amount of dimethylsulfoxide to amplify sufficiently the amount of sequence produced and at a temperature of 35°–40° C.;

(d) adding to the product of step (c) a restriction enzyme for each of said restriction sites to obtain cleaved products in a restriction digest; and

(e) ligating the cleaved products into said bacteriophage M13 with a specific orientation.

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