United States Patent [19]
Mullis et al.

[11] **Patent Number: 4,683,195**
[45] **Date of Patent: * Jul. 28, 1987**

[54] **PROCESS FOR AMPLIFYING, DETECTING, AND/OR-CLONING NUCLEIC ACID SEQUENCES**

[75] Inventors: **Kary B. Mullis**, Kensington; **Henry A. Erlich**, Oakland; **Norman Arnheim**, Woodland Hills; **Glenn T. Horn**, Emeryville; **Randall K. Saiki**, Richmond; **Stephen J. Scharf**, Berkeley, all of Calif.

[73] Assignee: **Cetus Corporation**, Emeryville, Calif.

[*] Notice: The portion of the term of this patent subsequent to Jul. 28, 2004 has been disclaimed.

[21] Appl. No.: **828,144**

[22] Filed: **Feb. 7, 1986**

Related U.S. Application Data

[60] Continuation-in-part of Ser. No. 824,044, Jan. 30, 1986, abandoned, which is a division of Ser. No. 791,308, Oct. 25, 1985, which is a continuation-in-part of Ser. No. 716,975, Mar. 28, 1985, abandoned.

[51] **Int. Cl.**[4] **C12Q 1/68**; C12P 19/34; C12N 1/00; C12N 15/00; G01N 33/48; G01N 33/00; G01N 33/566; G01N 33/564; C07H 21/02; C07H 21/04

[52] **U.S. Cl.** **435/6**; 435/91; 435/172.3; 435/317; 436/63; 436/94; 436/501; 436/508; 536/27; 536/28; 536/29; 935/17; 935/18; 935/76; 935/77; 935/78

[58] **Field of Search** 435/91, 172.3, 317, 435/6; 536/27, 28, 29; 935/17, 18, 78, 77, 76; 436/63, 94, 501, 508

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,395,486 7/1983 Wilson et al. 435/6

OTHER PUBLICATIONS

Gorski et al, "Molecular organization of the HLA-SB Region of the Human Major Histocompatibility Complex and Evidence for Two SB Beta-chain Genes", *Proc. Natl. Acad. Sci.* USA 81: 3934 (1984).

Boss et al, "Cloning and Sequence Analysis of the Human Major Histocompatibility Complex Gene DC-3beta", Proc. Nat. Acad. Sci. USA 81: 5199 (1984).

Okada et al, "Gene Organization of DC and DX Subregions of the Human Major Histocompatibility Complex", Proc. Natl. Acad. Sci. USA 82: 3410 (1985).

Salser, "Cloning cDNA sequences: A general Technique for Propagating Eukaryotic Gene Sequences in Bacterial Cells", in Genetic Engineering, 1978, Charrabarty (ed.), CRC Press, Inc. Boca Raton, Fla., pp. 53–81.

Gaubatz et al, "Strategies for Constructing Complementary DNA for Cloning", J. Theor. Biol. 95: 679 (1982).

Rossi et al., *J. Biol. Chem.*, 257, 9226–9229 (1982).

Primary Examiner—James Martinell
Attorney, Agent, or Firm—Janet E. Hasak; Albert P. Halluin

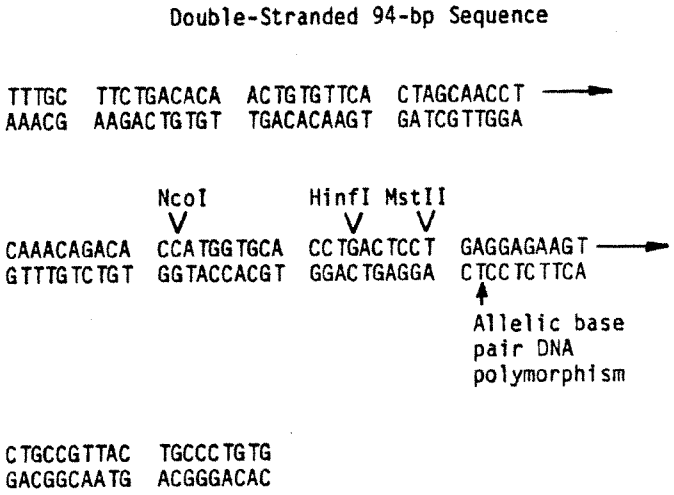
[57] **ABSTRACT**

The present invention is directed to a process for amplifying and detecting any target nucleic acid sequence contained in a nucleic acid or mixture thereof. The process comprises treating separate complementary strands of the nucleic acid with a molar excess of two oligonucleotide primers, extending the primers to form complementary primer extension products which act as templates for synthesizing the desired nucleic acid sequence, and detecting the sequence so amplified. The steps of the reaction may be carried out stepwise or simultaneously and can be repeated as often as desired.

In addition, a specific nucleic acid sequence may be cloned into a vector by using primers to amplify the sequence, which contain restriction sites on their non-complementary ends, and a nucleic acid fragment may be prepared from an existing shorter fragment using the amplification process.

26 Claims, 12 Drawing Figures

FIG. 1


Double-Stranded 94-bp Sequence
TTTGC TTCTGACACA ACTGTGTTCA CTAGCAACCT
AAACG AAGACTGTGT TGACACAAGT GATCGTTGGA
NcoI
HinfI MstII
CAAACAGACA CCATGGTGCA CCTGACTCCT GAGGAGAAGT
GTTTGTCTGT GGTACCACGT GGACTGAGGA CTCCTCTTCA
Allelic base pair DNA polymorphism
CTGCCGTTAC TGCCCTGTG
GACGGCAATG ACGGGACAC

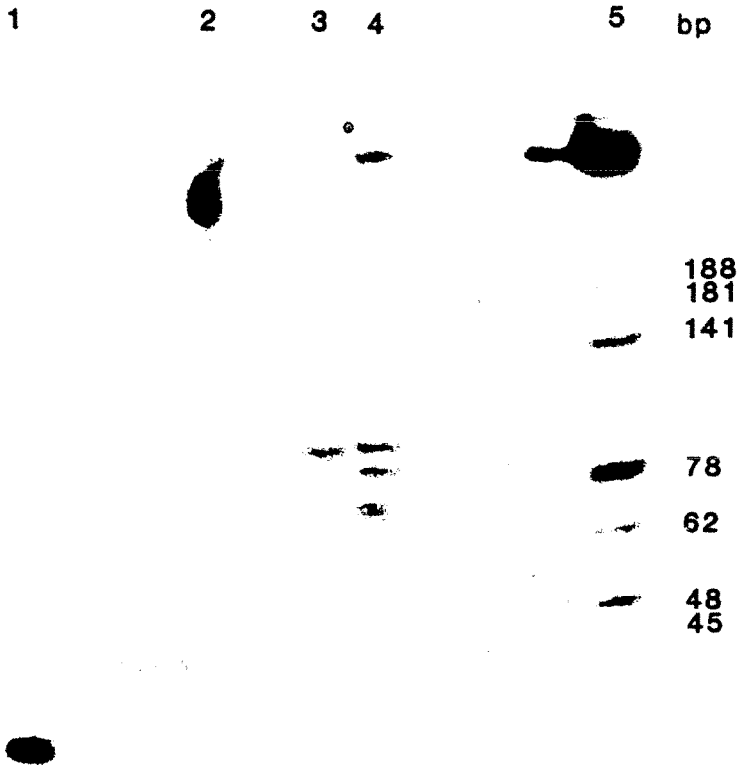


FIG.2

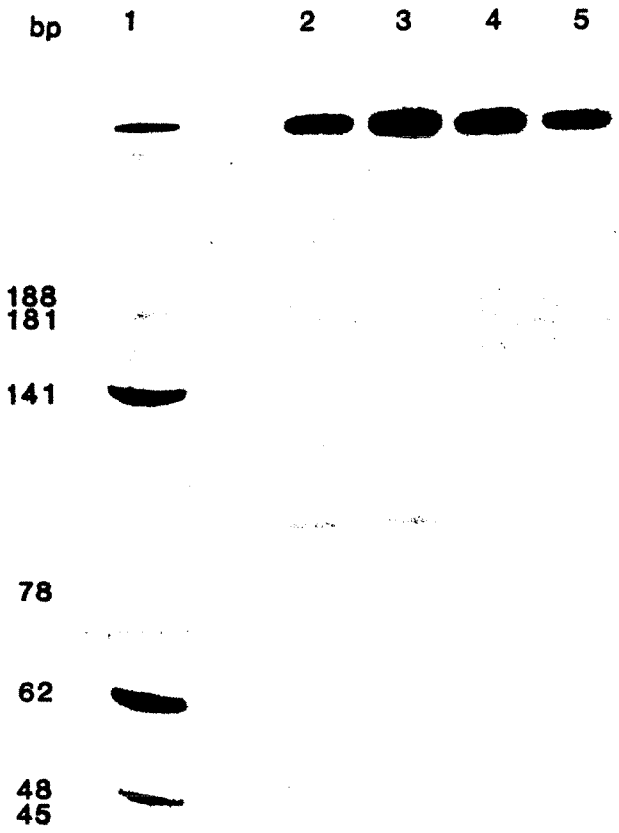


FIG.3

FIG.4-1

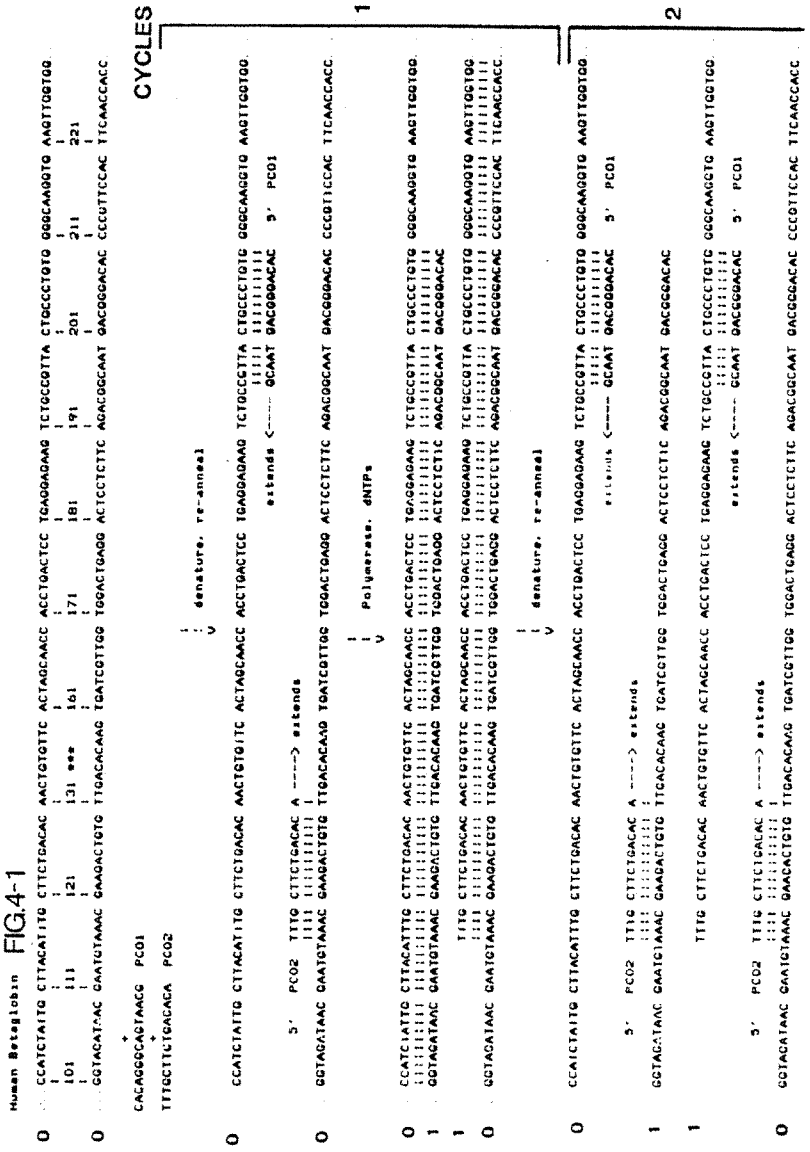

Human Beta-globin
CYCLES
PCO1
PCO2
denature, re-anneal
extends
Polymerase, dNTPs

FIG.4-2

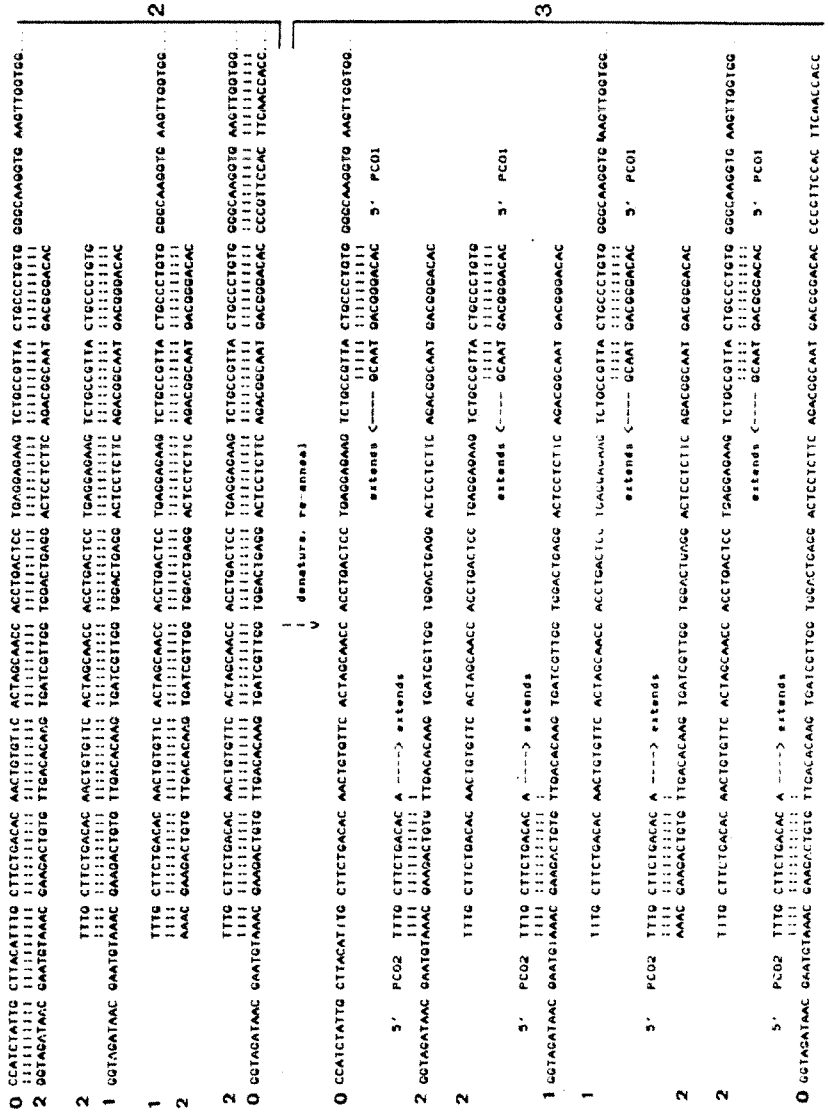
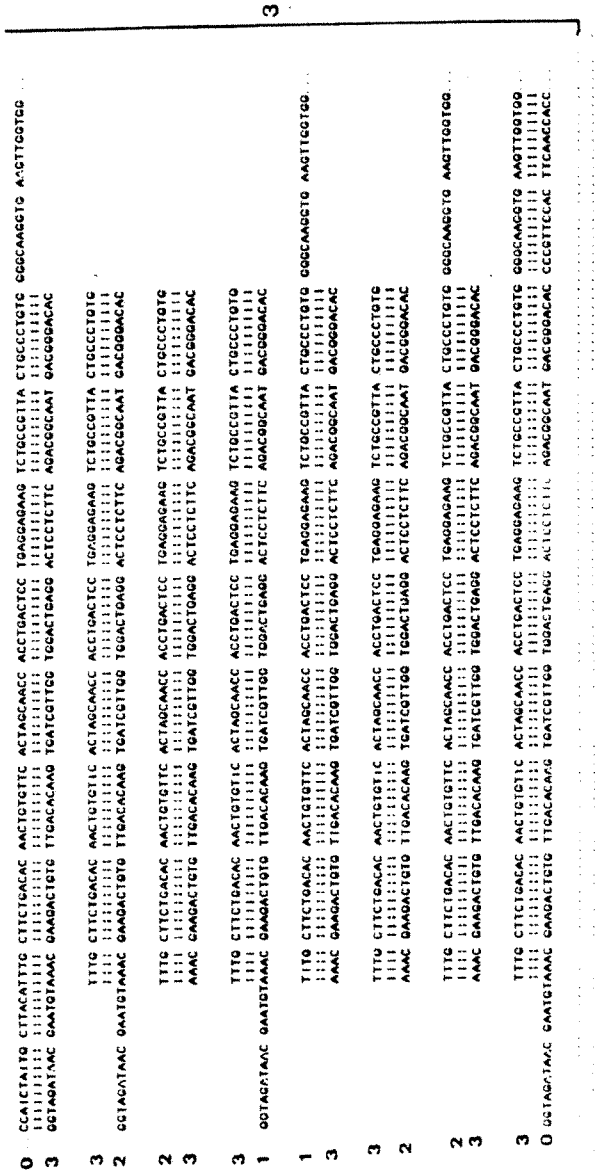

Polymerase, dNTPs
denature, re-anneal
extends
PCO1
PCO2
5'
3'
2
3

FIG.4-3


Polymerase. dNTPs
COPIES OF DNA SEQUENCE AFTER N CYCLES
template
long product
short product
=2(exp N)-N-1
1,048,555

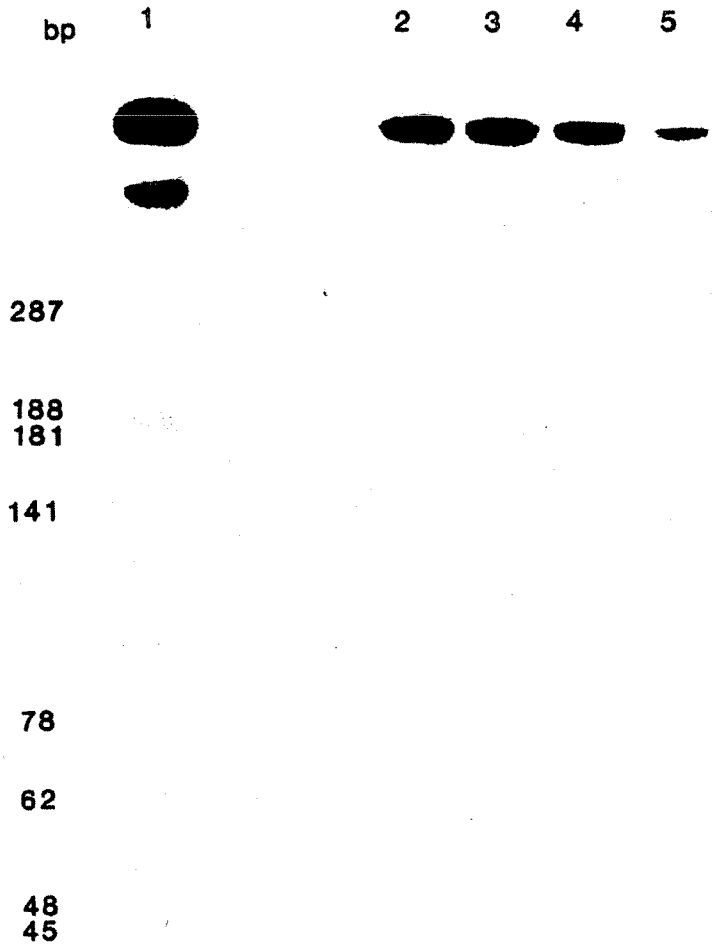
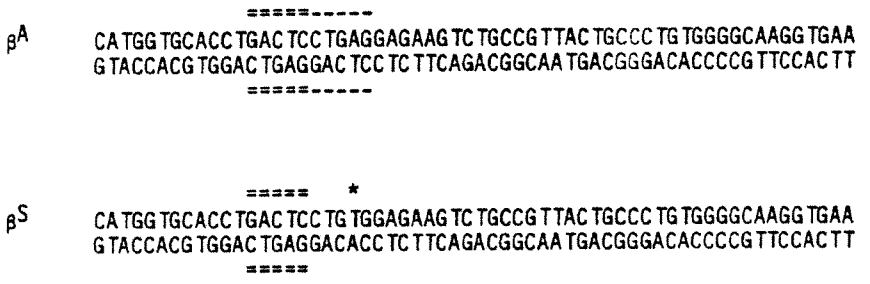
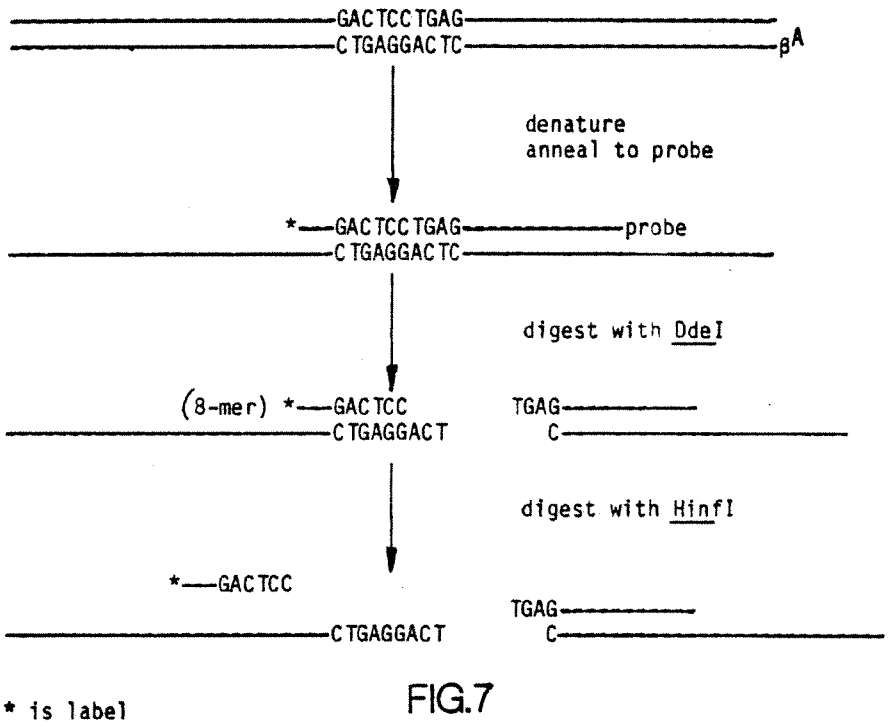


FIG.5

FIG.6



* Marks the mutation (A to T) in the sickle cell gene which disrupts the DdeI site



* is label

FIG.7

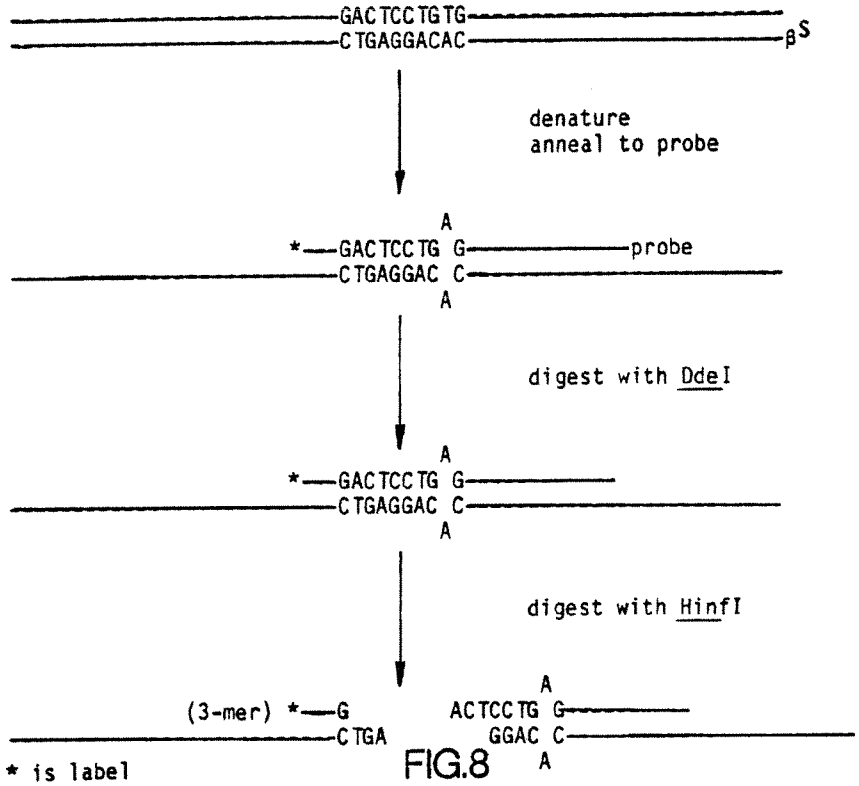

GACTCCTGTG
CTGAGGACAC
βS
denature
anneal to probe
A
*—GACTCCTG G
probe
CTGAGGAC C
A
digest with DdeI
A
*—GACTCCTG G
CTGAGGAC C
A
digest with HinfI
A
(3-mer) *—G
ACTCCTG G
CTGA
GGAC C
A
* is label

FIG.8

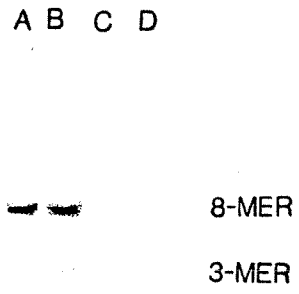
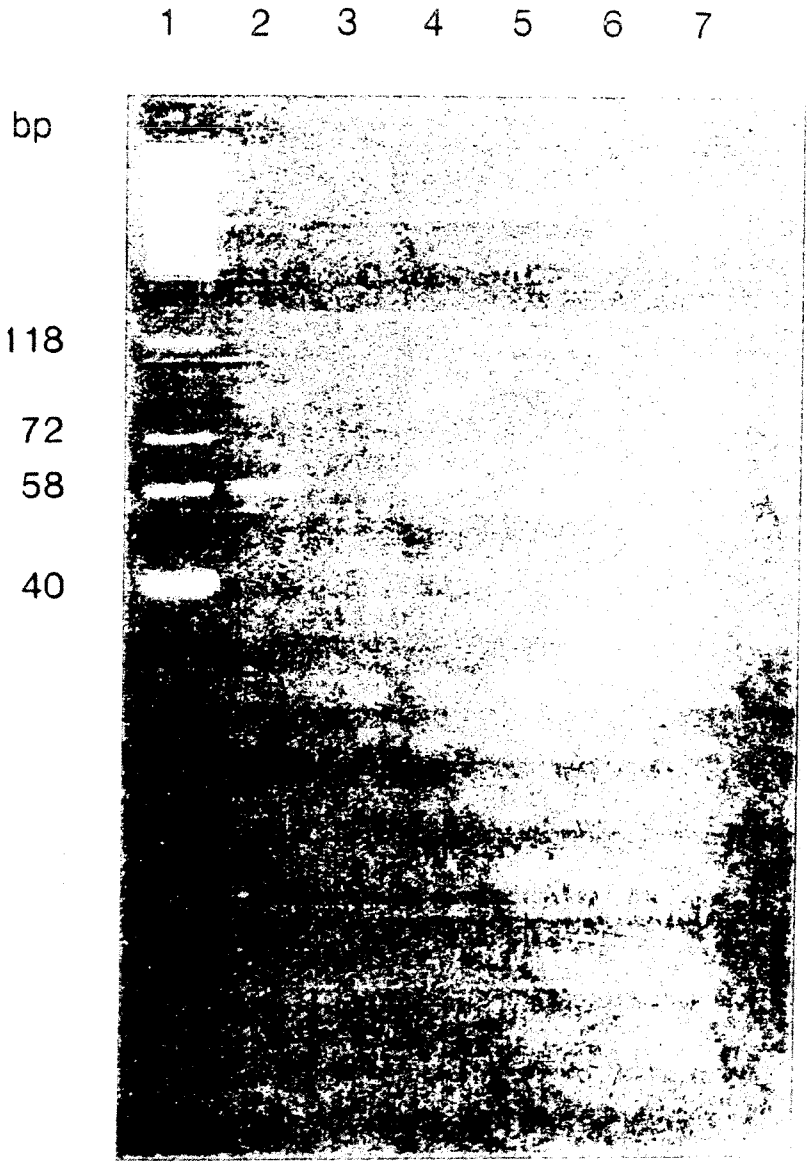


FIG.9

FIG. 10



PROCESS FOR AMPLIFYING, DETECTING, AND/OR-CLONING NUCLEIC ACID SEQUENCES

BACKGROUND OF THE INVENTION

This application is a continuation-in-part application of copending U.S. Ser. No. 824,044 filed Jan. 30, 1986, now abandoned, which is a divisional application of copending U.S. Ser. No. 791,308 filed Oct. 25, 1985, which is a continuation-in-part application of copending U.S. application Ser. No. 716,975 filed Mar. 28, 1985, now abandoned.

FIELD OF THE INVENTION

The present invention relates to a process for amplifying existing nucleic acid sequences if they are present in a test sample and detecting them if present by using a probe. More specifically, it relates to a process for producing any particular nucleic acid sequence from a given sequence of DNA or RNA in amounts which are large compared to the amount initially present so as to facilitate detection of the sequences. The DNA or RNA may be single- or double-stranded, and may be a relatively pure species or a component of a mixture of nucleic acids. The process of the invention utilizes a repetitive reaction to accomplish the amplification of the desired nucleic acid sequence.

DESCRIPTION OF RELATED DISCLOSURES

For diagnostic applications in particular, the target nucleic acid sequence may be only a small portion of the DNA or RNA in question, so that it may be difficult to detect its presence using nonisotopically labeled or end-labeled oligonucleotide probes. Much effort is being expended in increasing the sensitivity of the probe detection systems, but little research has been conducted on amplifying the target sequence so that it is present in quantities sufficient to be readily detectable using currently available methods.

Several methods have been described in the literature for the synthesis of nucleic acids de novo or from an existing sequence. These methods are capable of producing large amounts of a given nucleic acid of completely specified sequence.

One known method for synthesizing nucleic acids de novo involves the organic synthesis of a nucleic acid from nucleoside derivatives. This synthesis may be performed in solution or on a solid support. One type of organic synthesis is the phosphotriester method, which has been utilized to prepare gene fragments or short genes. In the phosphotriester method, oligonucleotides are prepared which can then be joined together to form longer nucleic acids. For a description of this method, see Narang, S. A., et al., *Meth. Enzymol.*, 68, 90 (1979) and U.S. Pat. No. 4,356,270. The patent describes the synthesis and cloning of the somatostatin gene.

A second type of organic synthesis is the phosphodiester method, which has been utilized to prepare a tRNA gene. See Brown, E. L., et al., *Meth. Enzymol.*, 68, 109 (1979) for a description of this method. As in the phosphotriester method, the phosphodiester method involves synthesis of oligonucleotides which are subsequently joined together to form the desired nucleic acid.

Although the above processes for de novo synthesis may be utilized to synthesize long strands of nucleic acid, they are not very practical to use for the synthesis of large amounts of a nucleic acid. Both processes are

laborious and time-consuming, require expensive equipment and reagents, and have a low overall efficiency. The low overall efficiency may be caused by the inefficiencies of the synthesis of the oligonucleotides and of the joining reactions. In the synthesis of a long nucleic acid, or even in the synthesis of a large amount of a shorter nucleic acid, many oligonucleotides would need to be synthesized and many joining reactions would be required. Consequently, these methods would not be practical for synthesizing large amounts of any desired nucleic acid.

Methods also exist for producing nucleic acids in large amounts from small amounts of the initial existing nucleic acid. These methods involve the cloning of a nucleic acid in the appropriate host system, where the desired nucleic acid is inserted into an appropriate vector which is used to transform the host. When the host is cultured the vector is replicated, and hence more copies of the desired nucleic acid are produced. For a brief description of subcloning nucleic acid fragments, see Maniatis, T., et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, pp. 390–401 (1982). See also the techniques described in U.S. Pat. Nos. 4,416,988 and 4,403,036.

A third method for synthesizing nucleic acids, described in U.S. Pat. No. 4,293,652, is a hybrid of the above-described organic synthesis and molecular cloning methods. In this process, the appropriate number of oligonucleotides to make up the desired nucleic acid sequence is organically synthesized and inserted sequentially into a vector which is amplified by growth prior to each succeeding insertion.

The present invention bears some similarity to the molecular cloning method; however, it does not involve the propagation of any organism and thereby avoids the possible hazards or inconvenience which this entails. The present invention also does not require synthesis of nucleic acid sequences unrelated to the desired sequence, and thereby the present invention obviates the need for extensive purification of the product from a complicated biological mixture.

SUMMARY OF THE INVENTION

The present invention resides in a process for amplifying one or more specific nucleic acid sequences present in a nucleic acid or mixture thereof using primers and agents for polymerization and then detecting the amplified sequence. The extension product of one primer when hybridized to the other becomes a template for the production of the desired specific nucleic acid sequence, and vice versa, and the process is repeated as often as is necessary to produce the desired amount of the sequence. This method is expected to be more efficient than the methods described above for producing large amounts of nucleic acid from a target sequence and to produce such nucleic acid in a comparatively short period of time. The present method is especially useful for amplifying rare species of nucleic acid present in a mixture of nucleic acids for effective detection of such species.

More specifically, the present invention provides a process for detecting the presence or absence of at least one specific nucleic acid sequence in a sample containing a nucleic acid or mixture of nucleic acids, or distinguishing between two different forms of sequences in said sample, wherein the sample is suspected of contain-

ing said sequence or sequences, which process comprises:

(a) treating the sample with one oligonucleotide primer for each strand of each different specific sequence suspected of being present in the sample, under hybridizing conditions such that for each strand of each different sequence to be detected 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 substantially complementary to each strand of each specific sequence 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 oligonucleotides 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 probe capable of hybridizing to said sequence being detected or a mutation thereof; and

(e) determining whether said hybridization has occurred.

The steps (a)–(c) may be conducted sequentially or simultaneously. In addition, steps (b) and (c) may be repeated until the desired level of sequence amplification is obtained.

In other embodiments the invention relates to diagnostic kits for the detection of at least one specific nucleic acid sequence in a sample containing one or more nucleic acids at least one of which nucleic acid is suspected of containing said sequence, which kit comprises, in packaged form, a multicontainer unit having

(a) one container for each oligonucleotide primer for each strand of each different sequence to be detected, which primer or primers are substantially complementary to each strand of each specific nucleic acid sequence such that an extension product synthesized from one primer, when it is separated from its complement, can serve as a template for the synthesis of the extension product of the other primer;

(b) a container containing an agent for polymerization;

(c) a container for each of four different nucleoside triphosphates;

(d) a container containing a probe capable of detecting the presence of said sequence in said sample; and

(e) a container containing means for detecting hybrids of said probe and said sequence.

In yet another embodiment, the invention relates to a process for cloning into a vector a specific nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, which process comprises:

(a) treating the nucleic acid(s) with one oligonucleotide primer for each strand of each different specific sequence being amplified, under conditions such that for each strand of each different sequence being amplified 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 substantially complementary to each strand of each specific sequence 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 said primer or primers each contain a restriction site on its 5' end which is the same as or different from the restriction site(s) on the other primer(s);

(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 depending on the particular sequence being amplified, steps (a) and (c) are carried out in the presence of from 0 up to an effective amount of dimethylsulfoxide or at a temperature of up to about 45° 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 product(s) into one or more cloning vectors.

In yet another embodiment, the invention herein relates to 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 represents at least substantially the nucleotide sequence of said existing nucleic acid fragment, and the right and left segments represent the sequence nucleotide present in the 5' ends of the two primers, the 3' ends of which are complementary or substantially complementary to 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 condition 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 the 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).

The core fragment may be 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.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** illustrates a 94 base pair length sequence of human β-globin desired to be amplified. The single base pair change which is associated with sickle cell anemia is depicted beneath the 94-mer.

FIG. **2** illustrates a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating amplification of the 94-mer contained in human wild-type DNA and in a plasmid containing a 1.9 kb BamHI fragment of the normal β-globin gene (pBR328:HbA).

FIG. **3** illustrates a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating amplification of any of the specific target 94-mer sequence present in pBR328:HbA, a plasmid containing a 1.9 kb BamHI fragment of the sickle cell allele of β-globin (pBR328:HbS), pBR328:HbA where the sequence to be amplified is cleaved with MstII, and pBR328:HbS where the sequence to be amplified has been treated but not cleaved with MstII.

FIG. **4** illustrates in detail the steps and products of the polymerase chain reaction for amplification of the desired 94-mer sequence of human β-globin for three cycles using two oligonucleotides primers.

FIG. **5** represents a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating amplification after four cycles of a 240-mer sequence in pBR328:HbA, where the aliquots are digested with NcoI (Lane 3), MstII (Lane 4) or HinfI (Lane 5). Lane 1 is the molecular weight standard and Lane 2 contains the intact 240-bp product.

FIG. **6** illustrates the sequence of the normal (β^A) and sickle cell (β^S) β-globin genes in the region of the DdeI and HinfI restriction sites, where the single lines for β^A mark the position of the DdeI site (CTGAG) and the double bars for β^A and β^S mark the position of the HinfI site (GACTC).

FIG. **7** illustrates the results of sequential digestion of normal β-globin using a 40-mer probe and DdeI followed by HinfI restriction enzymes.

FIG. **8** illustrates the results of sequential digestion of sickle β-globin using the same 40-mer probe as in FIG. **7** and DdeI followed by HinfI restriction enzymes.

FIG. **9** illustrates a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating the use of the same 40-mer probe as in FIG. **7** to specifically characterize the beta-globin alleles present in samples of whole human DNA which have been subjected to amplification, hybridization with the probe, and sequential digestion with DdeI and HinfI.

FIG. **10** illustrates a photograph of a 6% NuSieve agarose gel visualized using ethidium bromide and UV light. This photograph demonstrates amplification of a sub-fragment of a 110-bp amplification product which sub-fragment is an inner nested set within the 110-bp fragment.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "oligonucleotide" as used herein in referring to primers, probes, oligomer fragments to be detected, oligomer controls and unlabeled blocking oligomers is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase and at a suitable temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and source of primer. For example, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15–25 or more nucleotides, although it may contain fewer nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with template.

The primers herein are selected to be "substantially" complementary to the different strands of each specific sequence to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to be am-

plified to hybridize therewith and thereby form a template for synthesis of the extension product of the other primer.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "DNA polymorphism" refers to the condition in which two or more different nucleotide sequences coexist in the same interbreeding population in a DNA sequence.

The term "restriction fragment length polymorphism" ("RFLP") refers to the differences in DNA nucleotide sequences that are randomly distributed throughout the entire human genome and that produce different restriction endonuclease patterns.

The present invention is directed to a process for amplifying any one or more desired specific nucleic acid sequences suspected of being in a nucleic acid. Because large amounts of a specific sequence may be produced by this process, the present invention may be used for improving the efficiency of cloning DNA or messenger RNA and for amplifying a target sequence to facilitate detection thereof.

In general, the present process involves a chain reaction for producing, in exponential quantities relative to the number of reaction steps involved, at least one specific nucleic acid sequence given (a) that the ends of the required sequence are known in sufficient detail that oligonucleotides can be synthesized which will hybridize to them, and (b) that a small amount of the sequence is available to initiate the chain reaction. The product of the chain reaction will be a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

Any source of nucleic acid, in purified or nonpurified form, can be utilized as the starting nucleic acid or acids, provided it is suspected of containing the specific nucleic acid sequence desired. Thus, the process may employ, for example, DNA or RNA, including messenger RNA, which DNA or RNA may be single stranded or double stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of any of these nucleic acids may also be employed, or the nucleic acids produced from a previous amplification reaction herein using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified may be only a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as a portion of the β-globin gene contained in whole human DNA or a portion of nucleic acid sequence due to a particular microorganism which organism might constitute only a very minor fraction of a particular biological sample. The starting nucleic acid may contain more than one desired specific nucleic acid sequence which may be the same or different. Therefore, the present process is useful not only for producing large amounts of one specific nucleic acid sequence, but also for amplifying simultaneously more than one different specific nucleic acid sequence located on the same or different nucleic acid molecules.

The nucleic acid or acids may be obtained from any source, for example, from plasmids such as pBR322, from cloned DNA or RNA, or from natural DNA or RNA from any source, including bacteria, yeast, viruses, and higher organisms such as plants or animals. DNA or RNA may be extracted from blood, tissue material such as chorionic villi or amniotic cells by a variety of techniques such as that described by Maniatis et al., *Molecular Cloning: A Laboratory Manual*, (New York: Cold Spring Harbor Laboratory, 1982), pp 280–281.

Any specific nucleic acid sequence can be produced by the present process. It is only necessary that a sufficient number of bases at both ends of the sequence be known in sufficient detail so that two oligonucleotide primers can be prepared which will hybridize to different strands of the desired sequence and at relative positions along the sequence such that an extension product synthesized from one primer, when it is separated from its template (complement), can serve as a template for extension of the other primer into a nucleic acid of defined length. The greater the knowledge about the bases at both ends of the sequence, the greater can be the specificity of the primers for the target nucleic acid sequence, and thus the greater the efficiency of the process. It will be understood that the word primer as used hereinafter may refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding the terminal sequence(s) of the fragment to be amplified. For instance, in the case where a nucleic acid sequence is inferred from protein sequence information a collection of primers containing sequences representing all possible codon variations based on degeneracy of the genetic code will be used for each strand. One primer from this collection will be homologous with the end of the desired sequence to be amplified.

The oligonucleotide primers may be prepared using any suitable method, such as, for example, the phosphotriester and phosphodiester methods described above, or automated embodiments thereof. In one such automated embodiment diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage et al., *Tetrahedron Letters* (1981), 22:1859–1862. One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066. It is also possible to use a primer which has been isolated from a biological source (such as a restriction endonuclease digest).

The specific nucleic acid sequence is produced by using the nucleic acid containing that sequence as a template. If the nucleic acid contains two strands, it is necessary to separate the strands of the nucleic acid before it can be used as the template, either as a separate step or simultaneously with the synthesis of the primer extension products. This strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One physical method of separating the strands of the nucleic acid involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation may involve temperature ranging from about 80° to 105° C. for times ranging from about 1 to 10 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or the enzyme RecA, which has helicase activity and in the presence of riboATP is known to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are described by *Cold Spring Harbor Symposia on Quantitative Biology*, Vol. XLIII "DNA: Replication and Recombination" (New York:

Cold Spring Harbor Laboratory, 1978), B. Kuhn et al., "DNA Helicases", pp. 63–67, and techniques for using RecA are reviewed in C. Radding, *Ann. Rev. Genetics.* 16:405–37 (1982).

If the original nucleic acid containing the sequence to be amplified is single stranded, its complement is synthesized by adding one or two oligonucleotide primers thereto. If an appropriate single primer is added, a primer extension product is synthesized in the presence of the primer, an agent for polymerization and the four nucleotides described below. The product will be partially complementary to the single-stranded nucleic acid and will hybridize with the nucleic acid strand to form a duplex of unequal length strands that may then be separated into single strands as described above to produce two single separated complementary strands. Alternatively, two appropriate primers may be added to the single-stranded nucleic acid and the reaction carried out.

If the original nucleic acid constitutes the sequence to be amplified, the primer extension product(s) produced will be completely complementary to the strands of the original nucleic acid and will hybridize therewith to form a duplex of equal length strands to be separated into single-stranded molecules.

When the complementary strands of the nucleic acid or acids are separated, whether the nucleic acid was originally double or single stranded, the strands are ready to be used as a template for the synthesis of additional nucleic acid strands. This synthesis can be performed using any suitable method. Generally it occurs in a buffered aqueous solution, preferably at a pH of 7–9, most preferably about 8. Preferably, a molar excess (for cloned nucleic acid, usually about 1000:1 primer:template, and for genomic nucleic acid, usually about 10^6:1 primer:template) of the two oligonucleotide primers is added to the buffer containing the separated template strands. It is understood, however, that the amount of complementary strand may not be known if the process herein is used for diagnostic applications, so that the amount of primer relative to the amount of complementary strand cannot be determined with certainty. As a practical matter, however, the amount of primer added will generally be in molar excess over the amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred to improve the efficiency of the process.

The deoxyribonucleoside triphosphates dATP, dCTP, dGTP and TTP are also added to the synthesis mixture in adequate amounts and the resulting solution is heated to about 90°–100° C. for from about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period the solution is allowed to cool to from 20°–40° C., which is preferable for the primer hybridization. To the cooled mixture is added an agent for polymerization, and the reaction is allowed to occur under conditions known in the art. This synthesis reaction may occur at from room temperature up to a temperature above which the agent for polymerization no longer functions efficiently. Thus, for example, if DNA polymerase is used as the agent for polymerization, the temperature is generally no greater than about 45° C. Preferably an amount of dimethylsulfoxide (DMSO) is present which is effective in detection of the signal or the temperature is 35°–40° C. Most preferably, 5–10% by volume DMSO is present and the temperature is 35°–40° C. For certain applications, where the sequences to be amplified are over 110 base pair fragments, such as the HLA DQ-α or -β genes, an effective amount (e.g., 10% by volume) of DMSO is added to the amplification mixture, and the reaction is carried at 35°–40° C., to obtain detectable results or to enable cloning.

The agent for polymerization may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase, and other enzymes, including heatstable enzymes, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be agents, however, which initiate synthesis at the 5' end and proceed in the other direction, using the same process as described above.

The newly synthesized strand and its complementary nucleic acid strand form a double-stranded molecule which is used in the succeeding steps of the process. In the next step, the strands of the double-stranded molecule are separated using any of the procedures described above to provide single-stranded molecules.

New nucleic acid is synthesized on the single-stranded molecules. Additional inducing agent, nucleotides and primers may be added if necessary for the reaction to proceed under the conditions prescribed above. Again, the synthesis will be initiated at one end of the oligonucleotide primers and will proceed along the single strands of the template to produce additional nucleic acid. After this step, half of the extension product will consist of the specific nucleic acid sequence bounded by the two primers.

The steps of strand separation and extension product synthesis can be repeated as often as needed to produce the desired quantity of the specific nucleic acid sequence. As will be described in further detail below, the amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion.

When it is desired to produce more than one specific nucleic acid sequence from the first nucleic acid or mixture of nucleic acids, the appropriate number of different oligonucleotide primers are utilized. For example, if two different specific nucleic acid sequences are to be produced, four primers are utilized. Two of the primers are specific for one of the specific nucleic acid sequences and the other two primers are specific for the second specific nucleic acid sequence. In this manner, each of the two different specific sequences can be produced exponentially by the present process.

The present invention can be peformed in a step-wise fashion where after each step new reagents are added, or simultaneously, where all reagents are added at the initial step, or partially step-wise and partially simultaneous, where fresh reagent is added after a given number of steps. If a method of strand separation, such as heat, is employed which will inactivate the agent for polymerization, as in the case of a heat-labile enzyme, then it is necessary to replenish the agent for polymerization after every strand separation step. The simultaneous method may be utilized when a number of puri-

fied components, including an enzymatic means such as helicase, is used for the strand separation step. In the simultaneous procedure, the reaction mixture may contain, in addition to the nucleic acid strand(s) containing the desired sequence, the strand-separating enzyme (e.g., helicase), an appropriate energy source for the strand-separating enzyme, such as rATP, the four nucleotides, the oligonucleotide primers in molar excess, and the inducing agent, e.g., Klenow fragment of *E. coli* DNA polymerase I. If heat is used for denaturation in a simultaneous process, a heat-stable inducing agent such as a thermostable polymerase may be employed which will operate at an elevated temperature, preferably 65°–90° C. depending on the inducing agent, at which temperature the nucleic acid will consist of single and double strands in equilibrium. For smaller lengths of nucleic acid, lower temperatures of about 50° C. may be employed. The upper temperature will depend on the temperature at which the enzyme will degrade or the temperature above which an insufficient level of primer hybridization will occur. Such a heat-stable enzyme is described, e.g., by A. S. Kaledin et al., *Biokhimiya*, 45, 644–651 (1980). Each step of the process will occur sequentially notwithstanding the initial presence of all the reagents. Additional materials may be added as necessary. After the appropriate length of time has passed to produce the desired amount of the specific nucleic acid sequence, the reaction may be halted by inactivating the enzymes in any known manner or separating the components of the reaction.

The process of the present invention may be conducted continuously. In one embodiment of an automated process, the reaction may be cycled through a denaturing region, a reagent addition region, and a reaction region. In another embodiment, the enzyme used for the synthesis of primer extension products can be immobilized in a column. The other reaction components can be continuously circulated by a pump through the column and a heating coil in series; thus the nucleic acids produced can be repeatedly denatured without inactivating the enzyme.

The present invention is demonstrated diagrammatically below where double-stranded DNA containing the desired sequence [S] comprised of complementary strands [S+] and [S−] is utilized as the nucleic acid. During the first and each subsequent reaction cycle extension of each oligonucleotide primer on the original template will produce one new ssDNA molecule product of indefinite length which terminates with only one of the primers. These products, hereafter referred to as "long products," will accumulate in a linear fashion; that is, the amount present after any number of cycles will be proportional to the number of cycles.

The long products thus produced will act as templates for one or the other of the oligonucleotide primers during subsequent cycles and will produce molecules of the desired sequence [S+] or [S−]. These molecules will also function as templates for one or the other of the oligonucleotide primers, producing further [S+] and [S−], and thus a chain reaction can be sustained which will result in the accumulation of [S] at an exponential rate relative to the number of cycles.

By-products formed by oligonucleotide hybridizations other than those intended are not self-catalytic (except in rare instances) and thus accumulate at a linear rate.

The specific sequence to be amplified, [S], can be depicted diagrammatically as:

[S+] 5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCC 3′
[S−] 5′ TTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′

The appropriate oligonucleotide primers would be: .

Primer 1: GGGGGGGGGG
Primer 2: AAAAAAAAAA

so that if DNA containing [S]

. . . zzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzz . . .
. . . zzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzz . . .

is separated into single strands and its single strands are hybridized to Primers 1 and 2, the following extension reactions can be catalyzed by DNA polymerase in the presence of the four deoxyribonucleoside triphosphates:

3′ 5′
extends ← GGGGGGGGGG Primer 1
. . . zzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzz . . .
original template strand+

original template strand−
. . . zzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzz . . .
Primer 2 AAAAAAAAAA → extends
5′ 3′

On denaturation of the two duplexes formed, the products are:

3′ 5′
. . . zzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG
newly synthesized long product 1

5′ 3′
. . . zzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzz . . .
original template strand+