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Patent	Patentes
Health Permit	Registros Sanitarios
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Design	Diseños

Señor
JEFE DIVISIÓN DE NUEVAS CREACION
SUPERINTENDENCIA DE INDUSTRIA Y
E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 04-088188-00006-0000

Fecha: 2008-01-14 16:11:04 Dep. 2020 NUECREACIONE
No. 2 PATENTES Eje. 1 REGDEPOSITO
Por 11 RESPUESTAREQUE Fojos: 3

REF: EXPEDIENTE: 04-88188
TITULO: "PROCESO Y OBTENCIÓN DE MARCADORES
MOLECULARES PARA DETERMINAR..."
ACTUACIÓN: RESPUESTA A EXAMEN DE PATENTABILIDAD

Estimados señores:

CARLOS ORLANDO MAYA-RODRÍGUEZ, mayor de edad, vecino de esta ciudad, identificado con la Cédula de Ciudadanía No. 17.117.818 expedida en Bogotá, Abogado titulado en ejercicio y portador de la Tarjeta Profesional No. 17.402 del Consejo Superior de la Judicatura, obrando en calidad de apoderado de la UNIVERSIDAD TECNOLÓGICA DE PEREIRA, según poder debidamente conferido y legalizado, adjunto a las diligencias administrativas de la referencia.

Encontrándome dentro del término legal comparezco para responder el oficio de Requerimiento No. 14072 del Examen de Patentabilidad emanado de esa Oficina, y con fundamento en el Artículo 45 de la Decisión 486 de 2.000 de C.A.N, damos respuesta en los siguientes términos.

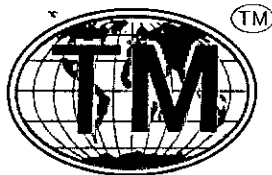
1. REIVINDICACIONES

Para atender el requerimiento y sugerencias del señor examinador, respecto a la claridad de la solicitud y según el Art. 30 de la Decisión 486, se presenta:

1.1.- Presentamos un nuevo Título

**"JUEGO DE PRIMERS Y PROCEDIMIENTO PARA DETECTAR UN
MARCADOR DE SEXO MASCULINO EN PLANTAS DE BOROJO"**

1.2.- Nuevo pliego reivindicatorio que se compone de dos reivindicaciones, el que se ajusta correctamente para reclamar el Nivel Inventivo, sobre los primers diseñados por el solicitante y el procedimiento para detección de un marcador de sexo en plantas de borojó.



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Las anteriores con un contenido que se ajusta a los límites impuestos por la memoria descriptiva original, por lo que no exceden el alcance de la invención.

Por lo anteriormente expuesto, ruego a usted dar por subsanado dicho requerimiento, sin perjuicio de lo cual desde ya nos acogemos a lo dispuesto por el inciso segundo del artículo 45 de la decisión 486 de la Comunidad Andina de Naciones que a la letra dice: "(...) Cuando la oficina nacional competente estimare que ello es necesario para los fines del examen de patentabilidad, podrá notificar al solicitante dos o más veces (...)".

NOTIFICACIONES

TM

Mi poderdante y yo recibiremos notificaciones en la Secretaría de su Despacho o en mi Oficina de Abogado ubicada en la Carrera 13A No. 28-38 Ofc. 246 MZ. 2 Parque Central Bavaria de la ciudad de Bogotá D.C.

Del señor Jefe,


CARLOS ORLANDO MAYA RODRIGUEZ
No. 17.117.818 expedida en Bogotá
T.P. No. 17.402 del C.S.J.

Patentes

REIVINDICACIONES

1. Un juego de primers para detección de un marcador de sexo masculino en plantas de borojó, caracterizado porque el juego de primers para detección de un marcador de sexo masculino en plantas de borojó tienen las secuencias:

BM1a: 5'- TGTTGATGATTGTTGTCATTCC- 3'

BM1b: 5'- AAATTGCAAAGAACTGATGG - 3'

2. Procedimiento de detección del marcador de sexo masculino en plantas de borojó que emplea los primers de la reivindicación 1 caracterizado porque se realiza según los siguientes pasos:
 - a) Preparación de mezcla de reacción para la PCR con 7.53 µl de H₂O, 1.5 µl de buffer 10X, 0.9 µl de MgCl₂ 25 mM, 0.3 µl de una mezcla de dNTPs 2.5 mM, 0.88 µl del iniciador BM1a, 0.79 µl de iniciador BM1b, 0.1 µl de Taq DNA polimerasa y 3 µl de ADN.
 - b) Ciclos de temperatura para la PCR de 94°C un minuto, 35 ciclos de 94°C 30 segundos, 55°C 30 segundos y 72°C 30 segundos
 - c) Determinación del producto de PCR en geles de agarosa al 2% con 10ul del producto de la reacción de PCR mezclado con 3 ul de buffer de carga (30% de glicerol en agua, 0.25% de azul de bromofenol y 0.25% de cilencianol), se visualiza en gel de agarosa al 2% teñido con 1ul de bromuro de etidio (10mg/ml).

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 Com-

plex 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).

Salsler, "Cloning cDNA sequences: A general Technique for Propagating Eukaryotic Gene Sequences in Bacterial Cells", in *Genetic Engineering*, 1978, Charabarty (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

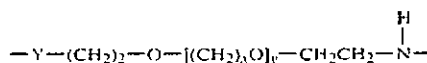
herein and in copending U.S. application Ser. No. 716,982 filed Mar. 27, 1985. Sickle cell anemia is a hemoglobin disease which is caused by a single base pair change in the sixth codon of the β -globin gene. FIG. 6 illustrates the sequences of normal and sickle cell β -globin genes in the region of 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 either 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.

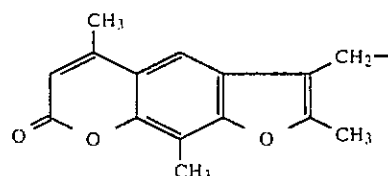
Routine clinical use of DNA probes for the diagnosis of infectious diseases would be simplified considerably if non-radioactively labeled probes could be employed as described in EP No. 63,879 to Ward. In this procedure biotin-containing DNA probes are detected by chromogenic enzymes linked to avidin 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' CCTCGGCACCGTCACCTGGATGCT 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-P³²-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 dichloromethane, 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.

rated to dryness at room temperature in a vacuum centrifuge.

Characterization of Oligodeoxyribonucleotides: Test aliquots of the purified oligonucleotides were ³²P labeled with polynucleotide kinase and γ-³²P-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 genomic 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 MgCl₂, 1 mM dithiothreitol (DTT), and 100 µg/ml bovine serum albumin (BSA). The products are designated pBR328:HbA/MstII and pBR328:HbS/MstII, respectively.

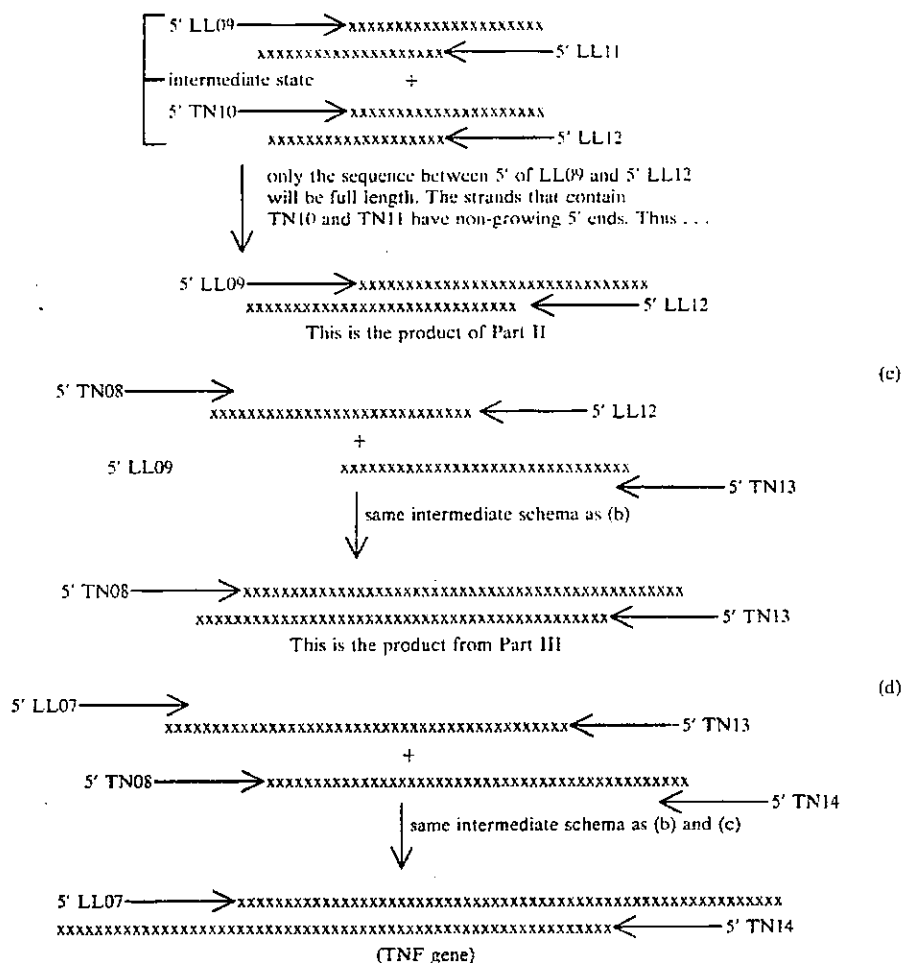
III. Polymerase Chain Reaction

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(CACAGG-GCACTAACG)). 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)

-continued

DIAGRAM



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 exten-

sion 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 nucleic 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



US006180345B1

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(12) **United States Patent**
Ranjekar et al.

(10) **Patent No.:** **US 6,180,345 B1**
(45) **Date of Patent:** **Jan. 30, 2001**

(54) **PROCESS FOR SIMULTANEOUS
PREPARATION OF SEX SPECIFIC AND
GENDER-NEUTRAL SEMISYNTHETIC
AMPLICONS USEFUL FOR SEX
DETERMINATION**

(75) **Inventors:** **Prabhakar K. Ranjekar; Anjali S.
Parasnis; Vidya S. Gupta, all of Pune
(IN)**

(73) **Assignee:** **Counsel of Scientific & Industrial
Research, New Delhi (IN)**

(*) **Notice:** Under 35 U.S.C. 154(b), the term of this
patent shall be extended for 0 days.

(21) **Appl. No.:** **09/259,473**

(22) **Filed:** **Feb. 26, 1999**

Related U.S. Application Data

(63) Continuation-in-part of application No. 09/052,505, filed on
Mar. 31, 1998, now Pat. No. 6,037,128.

(30) **Foreign Application Priority Data**

Aug. 13, 1998 (IN) 2377/DEL/98

(51) **Int. Cl.⁷** **C12Q 1/68; C12P 19/34;
C07H 21/02; C07H 21/04**

(52) **U.S. Cl.** **435/6; 435/91.2; 435/91.5;
536/23.1; 536/23.6; 536/24.3; 536/24.33**

(58) **Field of Search** **435/6, 91.2, 91.5;
536/23.1, 23.6, 24.3, 24.33**

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,747,257 5/1998 Jensen 435/6

OTHER PUBLICATIONS

Jones et al., "Refined Papain", *Process Biochemistry* vol. 9,
No. 6 pp 21-24 (1974).

Madrigal et al., "The Dependence of Crude Papain Yields on
Different Collection ('Tapping') Procedures for Papaya
Latex", *J. Sci. Food Agric.* 31, 279-285 (1980).

Polley et al., "Identification of sex in hop (*Humulus Lupu-
lus*) using molecular markers", *Genome*, 40: 357-361
(1997).

Sondur et al., "A genetic linkage map of papaya based on
randomly amplified polymorphic DNA markers", *Theor
Appl Genet*, 93: 547-553 (1996).

Taberlet et al., "Universal primers for amplification of three
non-coding regions of chloroplast DNA", *Plant Molecular
Biology* 17:1105-1109 (1991).

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Assistant Examiner—Diana Johannsen

(74) *Attorney, Agent, or Firm*—Fish & Richardson P.C.

(57) **ABSTRACT**

An improved process for simultaneous preparation of sex
specific and gender-neutral semisynthetic amplicons
obtained by amplifying sequences of synthetic oligonucle-
otide primers identified as SEQ ID NOS:4 to 7
(5'GGATCCCTATAGTG 3'; 5'GGATCCCITTTGCACTC
3'; 5CGAAATCGGTAGACGATACG3' and 5'GGGGATA-
GAGGGACTTGAAC 3') useful for sex determination, said
process comprising isolating nucleic acids from any part of
a papaya plant by conventional methods, amplifying the said
nucleic acids in a conventional Random Amplification of
polymorphic DNA Polymerase Chain Reaction (RAPD-
PCR), resolving the amplified products by conventional
electrophoresis method, eluting the sex specific, double
stranded amplified product from the gel piece by known
methods, cloning the said product in a known vector by
conventional methods, sequencing the said cloned product
by known methods, synthesizing the single stranded chains
of synthetic oligonucleotides by known method based on the
said sequence data, amplifying the said nucleic acid in a
Conventional Sequence Tagged Site Polymerase Chain
Reaction by using synthetic oligonucleotides as primers to
get sex specific and gender-neutral semisynthetic amplicons
simultaneously.

12 Claims, No Drawings

-continued

<210> SEQ ID NO 6
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Papaya cultivar pusa-giant

<400> SEQUENCE: 5
cgaaatcggt agacgatacg

20

<210> SEQ ID NO 7
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Papaya cultivar pusa-giant

<400> SEQUENCE: 7
ggggatagag ggacttgaac

20

What is claimed is:

1. A method for sex determination of a dioecious papaya plant by the simultaneous preparation of a male sex-specific semisynthetic amplicon and a gender-neutral semisynthetic amplicon comprising amplifying nucleic acids isolated from any part of a papaya plant in a polymerase chain reaction using (i) a pair of male sex-specific synthetic oligonucleotides that specifically anneal to and specifically amplify the male sex-specific sequence of SEQ ID NO:1 to produce a male sex-specific semisynthetic amplicon, and (ii) a pair of gender-neutral synthetic oligonucleotides that specifically anneal to and specifically amplify a gender-neutral sequence to produce a gender-neutral semisynthetic amplicon, wherein following amplification the presence of the male sex-specific semisynthetic amplicon and the gender-neutral semisynthetic amplicon indicates that the papaya plant is male, and the presence of only the gender-neutral amplicon indicates that the papaya plant is female.
2. The method of claim 1, wherein the pair of male sex-specific synthetic oligonucleotides is SEQ ID NO:2 and 3, or SEQ ID NO: 4 and 5.
3. The method of claim 2, wherein the male sex-specific semisynthetic amplicon is 831 bp in size.
4. The method claim 1, wherein the gender-neutral synthetic oligonucleotides are SEQ ID NOS:6 and 7.

5. The method of claim 1, wherein the gender-neutral semisynthetic amplicon is 600 bp in size.

6. The method of claim 1, wherein the male sex-specific semisynthetic amplicon and the gender-neutral semisynthetic amplicon differ in size to allow for differentiation between the amplicons.

7. The method of claim 6, wherein the amplicons are differentiated by resolving on agarose gel, polyacrylamide gel, or a mixture of polyacrylamide and agarose.

8. The method of claim 1, wherein the nucleic acids are from a papaya plant at a juvenile stage.

9. The method of claim 1, wherein any or all of the oligonucleotides are labeled.

10. The method of claim 9, wherein the label with which any or all of the oligonucleotides are labeled is radioactive or non-radioactive.

11. The method of claim 10, wherein the label is a radioactive label and wherein the radioactive label is ³²P, ³H, ¹⁴C, or ¹²⁵I.

12. The method of claim 10, wherein the label is a non-radioactive label and wherein the non-radioactive label is an alkaline phosphate, a biotin bromodeoxyuridine, a fluorescent molecule, or a chromogenic molecule.

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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES**

2020

Bogotá, D.C., 28 de febrero de 2008

Abogado

Carlos Orlando Maya Rodríguez

ASUNTO	Radicación	04-88188
	Trámite	002
	Evento	001
	Actuación	430
	Oficio	3896
	Folios	010

Patente de Invención



Patente de Modelo de Utilidad ☐

Vía Nacional



Como resultado del examen definitivo, realizado con fundamento en el artículo 48 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados

- 1.1. Descripción: Folios: 8 a 17 como se radicó inicialmente.
- 1.2. Reivindicaciones: Folios: 47 según modificación de 14/01/2008..
- 1.3. Rta. del solicitante: Folios: 45 a 46.

2. Objeto de la invención

Título de la solicitud: "Juego de Primers y Procedimiento para detectar un marcador de sexo masculino en plantas de Borojó" (Folio 45).

El objeto de la presente solicitud de patente de invención consiste en un juego de primers y el Procedimiento que los aplica para detectar un marcador de sexo masculino en plantas de Borojó.

Reivindicación 1: Juego de primers para detección de un marcador de sexo masculino en plantas de Borojó.

Reivindicación 2: Procedimiento de 3 etapas detección del marcador de sexo masculino en plantas de Borojó que emplea los primers de la cláusula 1 caracterizado por los pasos de una PCR convencional.

El problema planteado en esta solicitud es la necesidad de desarrollar estrategias de identificación sexual temprana en plantas dioicas, particularmente en plantas de Borojó.

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Para solucionar este problema técnico la solicitud en estudio proporciona un juego de primers y un procedimiento de identificación de sexo masculino en plantas de Borojó que los emplea.

El objeto de la solicitud definida anteriormente se clasificó en la IPC C12N 15/00, C12N 15/09, A61K 39/02.

3. Determinación del Estado de la Técnica

La fecha que se ha tenido en cuenta para determinar el Estado de la Técnica es:

Fecha de presentación de la solicitud nacional - 07/09/2004.

Teniendo en cuenta la fecha indicada se realizó la búsqueda en el estado de la técnica de documentos relacionados con la solicitud encontrándose los siguientes documentos más relevantes:

Nº	Documento	Título	Fecha de publicación
D1	US4683195	"Process for amplifying, detecting, and/or-cloning nucleic acid sequences"	28/07/1987
D2	US6180345	"Process for simultaneous preparation of sex specific and gender-neutral semisynthetic amplicons useful for sex determination"	30/01/2001

4. Análisis de patentabilidad (según requisito) (artículo 14 D486)

4.1. Nivel inventivo (artículo 18 D486)

La solicitud en estudio reclama un juego de primers para detección de un marcador de sexo masculino en plantas de Borojó (reivindicación 1) y un procedimiento de detección del marcador de sexo masculino en plantas de Borojó que emplea los primers de la cláusula 1 caracterizado porque comprende las etapas de: i) Preparación de la mezcla de reacción para la PCR; ii) Ciclos de amplificación; y iii) Determinación del producto de PCR en geles de agarosa. (Estas etapas corresponden a las características de una PCR convencional).

Considerando: 1) que el documento de US4683195 (D1) divulga el proceso de PCR como inicialmente fue descrito, detallando las mismas etapas del proceso reclamado en la solicitud en estudio (ver *Todo el Documento: p.e. Ejemplos, Reivindicaciones*); 2) que el D1 también enseña como debe realizarse el diseño de los cebadores según la secuencia blanco (ver *Ejemplo 2*); 3) que tanto la técnica de PCR (1986) como el diseño de primers se constituyen como técnicas de rutina y actividades habituales en un laboratorio de biología molecular, inclusive los primers pueden diseñarse desde hace más de una década por medio de un software (como es el caso de los reclamados) o en línea en el internet; 4) que la única diferencia que se presenta con D1 se refiere a la aplicación específica de la PCR y el diseño de primers para determinar el sexo en plantas dioicas, específicamente en Borojó, a partir de una secuencia blanco de las plantas masculinas; 5) y que D2 enseña precisamente la aplicación de técnicas biología molecular, entre las que se encuentra el PCR, el diseño de los primers y la determinación de la secuencia blanco, para determinación del sexo en papaya (ver

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Resumen y Reivindicaciones), que es también una planta dioica, el desarrollo reclamado solo puede reconocerse como la aplicación de una metodología conocida a un problema técnico específico, que por demás arroja los resultados esperados.

En definitiva, D2 sugiere enteramente la aplicación de PCR, antecedida de la obtención de una secuencia blanco, para determinación molecular del sexo en plantas; y adicionalmente, además de que como ya se expresó el diseño de primers es una actividad cotidiana para un técnico medio, de ninguna manera una estructura diseñada a partir de un software puede reconocerse como proveniente del aporte del investigador y por ende carece de actividad inventiva. De igual manera, y como ya igualmente se indicó, para la aplicación de un procedimiento conocido, reconocido además como principal en biología molecular y que para el momento de la presente solicitud se encontraba ya totalmente automatizado y realizado por un aparato.

Por todo lo anterior, los desarrollos reclamados no cumplen con el requisito de nivel inventivo establecido en el artículo 18 de la Decisión 486.

5. Conclusión:

Los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

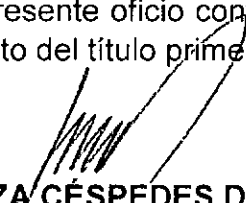
Nº	Documento Nº	Reivindicaciones afectadas	Requisito que afecta
D1	US4683195	1-2	Nivel Inventivo
D2	US6180345	1-2	Nivel Inventivo

6. Respuesta a los argumentos del solicitante:

En reconsideración de sus conceptos anteriores, a la luz del estado de la técnica, este despacho se acoge a lo anteriormente expuesto.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de 60 días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única Nº 10 de 2001.


ALIX CARMENZA CÉSPEDES DE VERGEL
Jefe de División de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista, de fecha
13 MAR. 2008

CONCEPTO TÉCNICO No. 714	EXAMINADOR TECNICO Código No.202081
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