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(54) Title: DIAGNOSIS OF β-THALASSEMIA USING A MULTIPLEX AMPLIFICATION REFRACTORY MUTATION SYSTEM

(57) Abstract

The present invention provides methods of diagnosing diseases such as β-thalassemia using a multiplex amplification re-fractory mutation system. In addition, kits useful for diagnosing diseases such as β-thalassemia are provided.

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DIAGNOSIS OF β-THALASSEMIA USING A MULTIPLEX
AMPLIFICATION REFRACTORY MUTATION SYSTEM

CROSS REFERENCE TO RELATED APPLICATIONS

This application is related to U.S. application
5 Serial No. 850,703 filed March 13, 1992 entitled "Diagnosis
of Cystic Fibrosis using Allele Specific Multiplex Polymerase
Chain Reactions", assigned to the assignee of the present
application and incorporated by reference in its entirety.
(Attorney Docket No. CH-0224)

10 ## FIELD OF THE INVENTION

This invention is directed to the diagnosis of β-
thalassemia using a novel multiplex amplification refractory
mutation system.

BACKGROUND OF THE INVENTION

15   Beta thalassemia is a heterogenous genetic disease
associated with defective expression of β chain human
hemoglobin (Hb). Weatherall, et al., *The Thalassemia
Syndromes* 3rd Ed., (Blackwell, Oxford 1981). Its distribution
encompasses the Mediterranean basin including North Africa
20 and the Middle East, as well as China, India and South-East
Asia. Kazazian, H.H., *Semin Hematol* 27: 209 (1990). More than
100 mutations have been defined which lead to aberrant
expression of the β-globin gene in humans.
Stamatoyannopoulos, G., et al., *The Molecular Basis of Blood
25 Disease* (W.B. Sauders Co., Philadelphia PA 1987). Of the
several different types, the IVS-1 nt 1, IVS-1 nt 6, codon
39, IVS-1 nt 110, IVS-2 nt 1, IVS-2 nt 745 changes are the

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most common mutations among Mediterraneans, and account for
approximately 92% of the β-thalassemia defects in this area.
Cao, A., et al. *Br. J. Haematol* 71: 309 (1989). A wide
variety of strategies and techniques are currently available
5   to detect mutations. Orkin, et al., *Nature* 296: 627 (1982);
Saiki, et al., *N. Engl. J. Med* 319: 537 (1988); Losekoot, M.,
et al., *Br. J. Haematol*. 76 : 269 (1990). However, none
appear ideal for rapid screening of large numbers of samples.
Foremost issues are detection of as many mutations as
10   possible, cost effectiveness, rapidity and accuracy in
obtaining the diagnosis, and eventually, the ability to
handle large numbers of samples by automated methods.
Methodology should also be simple enough to be utilized in
clinical laboratories.

15   Analyses of DNA frequently take the form of
restriction fragment length polymorphism (RFLP) using
Southern blotting techniques. While this technique has been
useful, it is relatively slow and only allows for the
detection of limited number of polymorphic base changes which
20   either create or destroy a restriction endonuclease
recognition site.

           In addition, β-thalassemia has been diagnosed by
using denaturing gradient gel electrophoresis and direct
sequencing of PCR amplified genomic DNA. This procedure is
25   limited by the labor intensive nature of the procedure.
Thereby increasing cost and decreasing the number of samples
which can be tested.

           The amplification refractory mutation system is
another method which has been used to diagnose β-thalassemia.
30   Varawalla, et al., *Brit. J. of Haematology* 78: 242-247
(1991). This technique involves PCR primers having allele
specific 3' nucleotides and corresponding "normal" primers
having normal 3' nucleotides. In addition, because in some
cases a single 3' allele specific base does not allow
35   amplification to proceed, additional mismatches near the 3'
end of appropriate primers are introduced. This procedure
was used to screen individually for a number of different β-

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thalassemia mutations including IVS1 nt110, IVS1 nt1, IVS1
nt6, codon 39, codon 6, and IVS2 nt 745. However,
simultaneous detection of multiple mutant alleles has not
been accomplished. The ability to detect multiple allelic
5   mutations simultaneously is of utmost importance for the
detection of as many mutations as possible and for cost
effectiveness, rapidity and accuracy in obtaining diagnosis.
Furthermore, the detection of multiple mutant alleles is
important for the eventual automation of a diagnostic test.
10   Multiplex PCR is another procedure which has been
used for the diagnosis of diseases characterized by mutant
alleles. Multiplex is useful for the simultaneous
amplification of multiple target sequences permitting
multiple mutant alleles to be scanned in a single lane of an
15 agarose gel. This strategy involves appropriate choice of
primer pairs so that PCR fragments (either normal or mutant)
are generated of different size which can be easily resolved
by comparison of samples run in parallel lanes. In males,
deletional forms of X-linked diseases such as Lesch-Nyhan
20 syndrome and Duchenne muscular dystrophy are immediately
obvious with this procedure, since missing exons are readily
apparent in the amplification pattern. (Gibbs, et al.,
Genomics, 7: 235-244 (1990); Chamberlain, et al., *Nucleic
Acid Research 16*: 11141-11156 (1988); Beggs, et al., *Human*
25 *Genet.* 86: 45-48 (1990). Such a diagnostic tool, effective
for detecting multiple allelic mutations, quickly and
accurately, is greatly desired for the detection of β-
thalassemia.
In particular, a cost-effective, simple, rapid test that
30 specifically detects the five prevalent mutations of the β-
globin gene associated with β-thalassemia would be of major
benefit, and could be applied to common mutations in other
population groups as well.

SUMMARY OF THE INVENTION

35   Methods of diagnosing β-thalassemia which are
effective for simultaneously detecting multiple allelic

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mutations quickly and accurately are provided by the present
invention. The methods of the present invention comprise the
steps of obtaining genomic DNA from a patient suspected of
carrying a genetic mutation characteristic of β-thalassemia

5 and selecting at least two primer sets for detecting at least
two mutations characteristic of β-thalassemia. Each primer
set is comprised of two primer pairs, a first primer pair
comprising a specific primer for a normal allele, and a
second primer pair comprising a specific primer for a mutant

10 allele. Each pair further comprises a common primer. A
polymerase chain reaction is performed in accordance with
methods of the present invention using said genomic DNA and
said at least two primer sets whereby primer pairs comprising
a specific primer for a normal allele are used simultaneously

15 and primer pairs comprising a specific primer for a mutant
allele are used simultaneously. Two or more polymerase chain
reaction products are detected whereby the detection of a
polymerase chain reaction product of a specific primer for a
mutant allele indicates the likelihood that said patient

20 carries a mutation characteristic of the phenotype β-
thalassemia. In some embodiments of the present invention,
each specific primer is differentially labeled. In still
other embodiments of the present invention the genomic DNA
and all differentially labeled primer sets are used

25 simultaneously to perform the polymerase chain reaction.
        In another embodiment of the present invention kits
are provided comprising four dNTPs and at least two primer
sets selected from the group consisting of

IVS-1 nt1-N	TTAAACCTGTCTTGTAACCTTGATACGAAC	(SEQ ID NO: 1),
30 IVS-1 nt1-M	TTAAACCTGTCTTGTAACCTTGATACGAAT	(SEQ ID NO: 2),
and β-CRP1	ACCTCACCCTGTGGAGCCAC	(SEQ ID NO: 3);
IVS-1 nt6-N	TCTCCTTAAACCTGTCTTGTAACCTTCATA	(SEQ ID NO: 4),
IVS-1 nt6-M	TCTCCTTAAACCTGTCTTGTAACCTTCATG	(SEQ ID NO: 5),
and β-CRP1	ACCTCACCCTGTGGAGCCAC	(SEQ ID NO: 3);
35 IVS-1 nt110-N	ACCAGCAGCCTAAGGGTGGGAAAATAGTCC	(SEQ ID NO: 6),
IVS-1 nt110-M	ACCAGCAGCCTAAGGGTGGGAAAATAGTCT	(SEQ ID NO: 7),
and β-CRP1	ACCTCACCCTGTGGAGCCAC	(SEQ ID NO: 3);
Codon 39-N	CAGATCCCCAAAGGACTCAAAGAACCTGTG	(SEQ ID NO: 8),

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Codon 39-M    CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),
and β-CRP1    ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3; and

IVS-2 nt 1-N  AAGAAAACATCAAGGGTCCCATAGACTGAC (SEQ ID NO: 10),
IVS-2 nt 1-M  AAGAAAACATCAAGGGTCCCATAGACTGAT (SEQ ID NO: 11),
5 and β-CRP1   ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3).
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It is therefore an object of the invention to
provide methods of diagnosing β-thalassemia. It is a further
object of the invention to provide kits useful for diagnosing
β-thalassemia. These and other objects will become apparent
10 by an examination of the detailed description and
accompanying claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the
strategy for multiplex amplification of the areas
15 encompassing the most common β-thalassemia mutations in
Mediterraneans. Approximate locations of the five most
common β-thalassemia mutations are indicated (▲) within the
β-globin gene. Multiplex amplification is accomplished using
a common upstream primer (β-CRP1) and a mixture selected from
20 five normal and/or five mutant primers in each polymerase
chain reaction. The size of expected PCR products in base
pairs is also shown.

Figure 2 shows PCR amplification of normal DNA
using normal primers (lanes 2, 4, 6, 8 and 10) or mutant
25 primers for the same regions (lanes 3, 5, 7, 9 and 11).
Multiplex amplification of four regions in normal DNA
encompassing IVS-2 nt 1, codon 39, IVS-1 nt 110 and IVS-1 nt
6 with normal primers (lane 12) or mutant primers (lane 13)
is also shown. Lanes 14 and 15 show the same pattern of
30 multiplex allele-specific PCR using the IVS-1 nt 6 normal and
mutant primers, respectively. PCR products are sized relative
to markers generated from a Hae III digest of φX174 Rf DNA
(lane 1). Arrows show location of each PCR product using
indicated primers.

35   Figure 3 shows the detection of common
Mediterranean β-thalassemia mutations by a multiplex
amplification refractory mutation system. All amplifications

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were done with either normal (N) or mutant (M) primer pairs
corresponding to the IVS-1 nt 1, IVS-2 nt 1, codon 39, IVS-1
nt 110 and IVS-1 nt 6 regions of the human β-globin gene.
Multiplex amplification was done using separate reactions
5 containing a mixture of either normal or corresponding mutant
primer pairs with normal genomic DNA (lanes 10, 11, and 12),
DNA from a heterozygote for the IVS-2 nt 1 mutation (lanes 2
and 3), DNA from a heterozygote for the IVS-1 nt 1 mutations
(lanes 4 and 5), DNA from a compound heterozygote for the
10 codon 39 and IVS-1 nt 1 mutations (lanes 6 and 7), DNA from a
compound heterozygote for -29 and codon 24 mutations (lanes 8
and 9), respectively. PCR products are sized relative to
markers generated from a Hae III digest of φX174 Rf DNA (lane
1).

15 **DETAILED DESCRIPTION OF THE INVENTION**

        Rapid, simple, non-radioactive methods for
detection of mutations causing a disease such as β-
thalassemia are provided by the present invention. In some
methods of the present invention, genomic DNA is obtained
20 from a patient suspected of carrying a genetic mutation
characteristic of a disease such as β-thalassemia. Genomic
DNA may be extracted by methods described by Poncz, et al.,
Hemoglobin 6: 27-33 (1982) or with an automated extractor
(Applied Biosystems, Inc., Foster City, CA). Other methods
25 for extraction of genomic DNA known to those skilled in the
art are also encompassed by the present invention.
        More than 100 mutations have been defined which
lead to aberrant expression of β-globin gene in humans.
Stamatoyannopoulos, G., et al., *The Molecular Basis of Blood*
30 *Disease* (W.B. Sauders Co., Philadelphia PA 1987). Some of
the most common, accounting for approximately 92% of the β-
thalassemia defects in the Mediterranean, are IVS-1 nt 1,
IVS-1 nt 6, IVS-1 nt 110, codon 39 and IVS-2 nt 1.
        A multiplex amplification refractory mutation
35 system can be used to detect mutations such as the mutations
described above by the appropriate choice of primers. In

some embodiments of the present invention a primer strategy
such as the strategy set forth in Figure 1 can be used to
detect at least two β-thalassemia mutations. Primers are
designed so that the size of the resulting PCR products
5 differ, thereby facilitating detection. Oligonucleotide
primers of the present invention can be synthesized by
procedures known to those skilled in the art such as by solid
state phosphoramidite synthesis.

In accordance with methods of the present invention
10 at least two primer sets for detecting at least two mutations
characteristic of a disease such as β-thalassemia are
selected. In some embodiments of the present invention four
primer sets are selected useful for diagnosing four mutations
characteristic of β-thalassemia. In still further preferred
15 embodiments of the present invention five primer sets are
selected which are useful for detecting five mutations
characteristic of β-thalassemia. Each primer set is comprised
of two primer pairs. A first primer pair is comprised of a
3' primer specific for a normal allele. A second primer pair
20 is comprised of a 3' primer specific for a mutant allele such
as an allele specific for β-thalassemia. A mismatched residue
is incorporated at the third nucleotide in from the 3'
nucleotide, Newton, et al., *Nucleic Acid Research* 17: 2503
(1989), in both normal and mutant 3' primers in order to
25 ensure selective amplification. The 3' primers differ from
each other only at their terminal 3' nucleotide. Thus, for
example, the 3' nucleotide of the 3' primer of the first
primer pair is substantially complementary to the nucleic
acid sequence of a normal allele. The 3' nucleotide of the 3'
30 primer of the second primer pair may be substantially
complementary to the nucleic acid sequence of a β-thalassemia
mutation. Each 3' primer has a mismatched incorporated at
the third nucleotide in from its 3' nucleotide. Each primer
pair further comprises a common primer. Thus, the nucleic
35 acid sequence of the common primer is the same for both
primer pairs comprising a primer set. Under proper annealing
temperatures and polymerase chain reaction conditions, these

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primers pairs only direct amplification of their
complementary allele. For example, normal genomic DNA will be
amplified by the first primer pair which is specific for a
normal allele. The second primer pair, specific for a mutant
5 allele will not amplify genomic DNA from a normal patient.
     In some preferred embodiments of the present
invention, each 3' specific primer is differentially labeled,
resulting in differentially labeled PCR products. Any label
known to those skilled in the art which can be easily
10 differentiated clinically are encompassed by the present
invention. For example, dyes known to those skilled in the
art may be useful to distinguish PCR products based upon
color differentiation. In preferred embodiments of the
present invention fluorescent dyes such as FAM™ (blue), JOE™
15 (green), TAMRA™ (yellow) and ROX™ (red) (Applied Biosystems,
Inc., Foster City, CA) may be used. Differential labels may
be linked to oligonucleotide primers of the present invention
by methods known to those skilled in the art, such as by
linker molecules. Linker molecules useful in the present
20 invention may be selected from any of a variety of linker
molecules available to those skilled in the art, such as a
reactive aminohexyl linker (Aminolink). In other embodiments
of the present invention differential label may be
incorporated during synthesis of the oligonucleotide primers.
25 In still other embodiments of the present invention, PCR
products are labeled by differential recognition by a labeled
probe or chemical moiety such as a rhodamine coupled
antibody. For example, the specific primer for the IVS-1 nt 1
mutant allele may be labeled with yellow dye and the IVS-1 nt
30 1 normal allele labeled with blue dye. By detecting a yellow
signal, one skilled in the art would be apprised that the
patient has a IVS-1 nt 1 mutant allele. A blue signal would
indicate a normal allele.
     Some primer sets useful in the present invention
35 are set forth in Table I.

TABLE I

OLIGONUCLEOTIDE PRIMER SETS USED FOR MUTATION DETECTION

Set No.	SEQ. ID NO:	Primer	Coordinates	Primer Type	Sequence	pM
1	1	IVS-1 nt 1-N	+143 to +172	normal	TTAAACCTGTCTTGTAACCTTGATACGAAC	90
	2	IVS-1 nt 1-M	+143 to +172	mutant	TTAAACCTGTCTTGTAACCTTGATACGAAT	90
	3	β-CRP1	-109 to -89	common	ACCTCACCCTGTGGAGCCAC	90
2	4	IVS-1 nt 6-N	+149 to +178	normal	TCTCCTTAAACCTGTCTTGTAACCTTCATA	10
	5	IVS-1 nt 6-M	+149 to +178	mutant	TCTCCTTAAACCTGTCTTGTAACCTTCATG	10
	3	β-CRP1	-109 to -89	common	ACCTCACCCTGTGGAGCCAC	90
3	6	IVS-1 nt 110-N	+253 to +282	normal	ACCAGCAGCCTAAGGGTGGGAAAATAGTCC	20
	7	IVS-1 nt 110-M	+253 to +282	mutant	ACCAGCAGCCTAAGGGTGGGAAAATAGTCT	20
	3	β-CRP1	-109 to -89	common	ACCTCACCCTGTGGAGCCAC	90
4	8	Codon 39-N	+298 to +327	normal	CAGATCCCCAAAGGACTCAAAGAACCTGTG	10
	9	Codon 39-M	+298 to +327	mutant	CAGATCCCCAAAGGACTCAAAGAACCTGTA	10
	3	β-CRP1	-109 to -89	common	ACCTCACCCTGTGGAGCCAC	90
5	10	IVS-2 nt 1-N	+496 to +525	normal	AAGAAAACATCAAGGGTCCCATAGACTGAC	90
	11	IVS-2 nt 1-M	+496 to +525	mutant	AAGAAAACATCAAGGGTCCCATAGACTGAT	90
	3	β-CRP1	-109 to -89	common	ACCTCACCCTGTGGAGCCAC	90

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Coordinates are given relative to the cap site (+1) of the β-globin gene, with "N" indicating normal and "M" mutant primers, respectively. β-CRP1 refers to the common primer having a sequence common to both primer pairs. Bold letters

5 identify 3' mutation and second base change at 3 nucleotides in from the 3' nucleotide. Picomoles (pM) of each primer used per primer pair is also indicated. Sequences are provided in the 5' to 3' direction.

Other primer sets useful to detect a particular

10 disease such as β-thalassemia can be identified using methods known to those skilled in the art. The first member in each set is the specific primer for a normal allele, the second member of each set is the specific primer for the corresponding mutant allele, and the third member of each set

15 is the common primer included in each primer pair.

At least two mutant alleles can be detected simultaneously by methods of the present invention. It is encompassed by some embodiments of the present invention to perform two polymerase chain reactions per diagnosis. In one

20 PCR reaction mixture, primer pairs for normal alleles from each primer set are used. In a second reaction mixture, all primer pairs for mutant alleles from each primer set are used. Thus each polymerase chain reaction in such a diagnostic test causes amplification of genomic DNA using

25 either primers specific for mutant or normal alleles, i.e. half of each primer set per reaction, an entire primer set per diagnosis. Resulting PCR products are run in parallel on gels to detect the presence or absence of bands. For example, diagnosis of β-thalassemia is accomplished in some

30 embodiments of the present invention, by comparison of normal and mutant polymerase chain reaction products. Figure 3, for example, shows four, two PCR diagnoses using five primer sets each. In each diagnosis, one PCR reaction was performed with 3' primers specific for normal alleles (lanes 2,4,6 and 8)

35 and one PCR reaction was performed with primers specific for mutant alleles (lanes 3,5,7 and 9). PCR products run in parallel on a gel confirmed the following genotypes, a

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heterozygote for the IVS-2 nt 1 mutation (comparison of lanes
2 and 3), heterozygote for the IVS-1 nt 1 mutation
(comparison of lanes 4 and 5), compound heterozygote for the
codon 39 and IVS-1 nt 1 mutations (comparison of lanes 6 and
5   7). Diagnosis, may be used in the context of the present
invention to encompass a procedure whereby two or more primer
sets are amplified and interpreted in order to determine the
presence or absence of selected normal and mutant alleles in
a particular genomic DNA sample. As has been exemplified
10 above, a diagnosis may encompass one or more polymerase chain
reactions.

In preferred embodiments of the present invention
both members of each primer pair of at least two primer sets
are used simultaneously in a single polymerase chain reaction
15 which is run on a single lane of a gel. Differential labels,
as described above, are useful herein for distinguishing
polymerase chain reaction products, especially those having
similar mobilities. Thus PCR products can be distinguished
by mobility and label. In addition, labels, such as
20 fluorescent labels may be particularly amenable to automated
methods.

In some preferred embodiments of the present
invention at least two of the primer sets provided in Table I
are selected. It is still more preferred in some embodiments
25 of the present invention to select all of the primer sets of
Table I. In some embodiments of the present invention the
primer sets 1 (IVS-1 nt 1 N and M), 3 (IVS-1 nt 110 N and M),
4 (Codon 39 N and M) and 5 (IVS-2 nt 1 N and M) are selected.
In still other embodiments of the present invention the
30 primer sets 1 (IVS-1 nt 1 N and M), 3 (IVS-1 nt 110 N and M)
and 4 (Codon 39 N and M) in accordance with some embodiments
of the present invention. In still other embodiments of the
present invention the primer sets 3 (IVS-1 nt 110 N and M)
and 4 (Codon 39 N and M) are selected.
35   Kits are also provided by the present invention
comprising four dNTPs and at least two primer sets selected
from the primer sets provided in Table I.

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The following examples are illustrative, but should
not be construed as limiting the present invention.

EXAMPLES

EXAMPLE 1

5 **DNA Samples**

DNA samples were obtained from normal controls and
patients either homozygous or heterozygous for the common β-
thalassemia Mediterranean mutations. Genomic DNA was
extracted using protocols previously described in Poncz,

10 M.D., et al., *Hemoglobin* 6: 27-33 (1982) or with an automated
extractor (ABI, Foster City, CA). Genotypes were confirmed
either by DNA sequence analysis using PCR based, cycle-
sequencing approach employing laser-activated fluorescence-
emission DNA sequencer; Tamary, et al., *Amer. J. Hemat.* in

15 press (1992); Trifillis, et al., *Blood* 78: 3298 (1991);
McBride, et al., *Clin Chem* 35: 2196 (1989); or by denaturing
gradient gel electrophoresis. Diazani, et al., *Genomics* (in
press), Losekoot, et al., *Br. J. Haematol* 76: 269 (1990).

EXAMPLE 2

20 **Primer Synthesis**

Unlabelled oligonucleotide primers were prepared on
a 380B DNA Synthesizer (ABI, Foster City, CA) by the
phosphoramidite method at 0.2 mmol scale with (2-O-cyano-
ethyl)-phosphoramidites; Caruthers, M.H., et al., *Methods in*

25 *Enzymol.* 154: 287-313 (1987); and were then purified.
Oligonucleotide primers were prepared for fluorescent
labeling following standard phosphoramidite chemistry
preparation by attachment of a reactive aminohexyl linker
group (Aminolink) to the 5' end of the primer. Draper, D. and

30 L.E. Gold, *Biochemistry* 19: 1774-1781 (1980). Following
cleavage from the solid support, and deprotection, each
modified primer was then reacted with an N-hydroxyl
succinimide ester derivative of a distinct dye. ABI 370 user
bulletin (1989). The dye labeled primer was then removed

35 from excess reactants via high performance liquid

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chromatography purification. Sequences of the
oligonucleotide primers are as provided in Table I.

EXAMPLE 3

Polymerase Chain Reaction

5   PCR was performed according to methods previously
described by Saiki, R.K., et al., *Science* 239: 487-489
(1988). Reaction mixtures (25μl) contained 100ng of genomic
DNA, 1.5 μM of each dNTP, the common primer and normal and/or
mutant primers in a buffer containing 6.7mM $MgCl_2$, 16.6 mM
10 $(NF_4)_2SO_4$, 5.0 μM βME, 6.8 mM EDTA, 67.0 mM Tris HCl pH 8.8,
10% (v/v) DMSO. The mixture was heated at 95°C for 5 minutes
to denature the DNA, and then quickly chilled on ice. Taq
DNA polymerase (1.5 U, Perkin Elmer, Norwalk, CT) was added
before overlaying the samples with 25 μl of mineral oil. The
15 samples were then subjected to 30 cycles on a DNA thermal
cycler (Perkin-Elmer Cetus, Norwalk, CT) with denaturation at
95°C for 1 minute, annealing at 60°C for 1 minute and
extension at 72°C for 1 minute. The last cycle had a 5
minute extension at 72°C. Approximately 15μl of the PCR
20 product were then analyzed following electrophoresis on a 3%
(w/v) agarose (NuSieve GTG) gel.

EXAMPLE 4

Selectivity of Oligonucleotide Primers

        Differently sized PCR products were produced based
25 upon the strategy illustrated in Figure 1. Unlabelled normal
and mutant PCR primers were synthesized so their terminal 3'
nucleotide corresponds to either a normal or mutant sequence.
An additional mismatched residue was also incorporated at 3
nucleotides in from the 3' nucleotide, Newton, et al.,
30 *Nucleic Acid Research* 17: 2503 (1989), in both normal and
mutant specific primers in order to ensure selective
amplifications. Primers were tested with normal and available
mutant DNA samples under stringent PCR conditions to ensure
that selective amplification occurred with either normal or
35 mutant primers. The strategy for multiplex generation of

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differently sized PCR products was accomplished using a
single common primer located at -109 base pairs upstream of
the β-globin gene. Sequence of this primer was identical to
the normal sequence. Poncz, et al., *J. Biol. Chem.* 258: 11599
5   (1982). Normal and mutant products were scored by the
presence or absence of correctly sized bands following
electrophoresis on agarose gel. PCR was performed in
accordance with Example 3.

        Separate amplifications using normal primers for
10 each of the five regions in normal DNA gives single bands of
expected size (Figure 2, lanes 2,4,6,8 and 10). No
amplification products were seen with the mutant primer set
(Figure 2, lanes 3,5,7,9 and 11). Multiplex amplification of
4 regions in normal DNA encompassing IVS-2 nt 1, codon 39,
15 IVS-1 nt 110 and IVS-1 nt 6 with normal primers (lane 12) or
mutant primers (lane 13) yields the expected combined
pattern. Lanes 14 and 15 show the same pattern of multiplex
allele-specific PCR using the IVS-1 nt 6 normal and mutant
primers respectively.

20 **EXAMPLE 5**
Diagnosis of β-Thalassemia

        Different known β-Thalassemia genotypes were used
to test the accuracy of the method. Unlabeled mutant and
normal PCR products IVS-1 nt 1 and IVS-1 nt 6 are too similar
25 in size to be resolved using this multiplex method. Thus, in
order to test the primers four separate PCR reactions were
performed in accordance with Example 3. Four mutations were
tested by doing two separate PCR reactions. Each reaction
contained the common primer and either four mutant or four
30 normal primers for the IVS-1 nt 1, IVS-1 nt 110, codon 39 and
IVS-2 nt 1 mutations. Screening for the IVS-1 nt 6 mutations
was done in two separate PCR reactions (with normal and
mutant primer). Data are shown in Figure 3 confirming the
following genotypes, a heterozygote for the IVS-2 nt 1
35 mutation (comparison of lanes 2 and 3), heterozygote for the
IVS-1 nt 1 mutation (comparison of lanes 4 and 5), compound

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heterozygote for the codon 39 and IVS-1 nt 1 mutations
(comparison of lanes 6 and 7).

EXAMPLE 7

Diagnosis of Five Common β-Thalassemia Mutations using
5 **Fluorescently Labeled Primers**

        Diagnosis is carried out as provided in Example 5,
except that both normal and mutant allele primers are
combined in a single PCR reaction mixture. Each reaction
contains the common primer and five mutant and five normal
10 primers for the IVS-1 nt 1, IVS-2 nt 1, IVS-1 nt 6, codon 39
and IVS-1 nt 110 regions of the human β-globin gene. Primers
are labeled as follows: IVS-1 nt 1-N (blue), IVS-1 nt 1-M
(green), IVS-1 nt 6-N (yellow), IVS-1 nt 6-M (red), IVS-1 nt
110-N (blue), IVS-1 nt 110-M (green) Codon 39-N (yellow),
15 Codon 39-M (red), IVS-2 nt 1-N (blue), IVS-2 nt 1-M (green).
A fluorescently labeled marker lane in run in a separate lane
to facilitate sizing PCR products. The PCR product is then
analyzed following electrophoresis on a 3% (w/v) agarose
(NuSieve GTG) gel using a multi-line argon ion laser such as
20 a GENE SCANNER™ (Applied Biosystems, Inc. Foster City, CA) to
detect the fluorescently labeled PCR products. Multicolored
bands indicate heterozygosity for an allele, while single
colored bands indicate homozygosity for an allele.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Fortina, Paolo
              Surrey, Saul

    (ii) TITLE OF INVENTION: DIAGNOSIS OF B-THALASSEMIA USING A
         MULTIPLEX AMPLIFICATION REFRACTORY MUTATION SYSTEM

    (iii) NUMBER OF SEQUENCES: 11

    (iv) CORRESPONDENCE ADDRESS:
         (A) ADDRESSEE: Woodcock Washburn Kurtz Mackiewicz and Norris
         (B) STREET: One Liberty Place - 46th Floor
         (C) CITY: Philadelphia
         (D) STATE: PA
         (E) COUNTRY: U.S.A.
         (F) ZIP: 19103

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:
         (A) APPLICATION NUMBER:
         (B) FILING DATE:
         (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
           (A) NAME: Johnson, Philip S.
           (B) REGISTRATION NUMBER: 27,200
           (C) REFERENCE/DOCKET NUMBER: CH-0225

(ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 215-569-3100
        (B) TELEFAX: 215-568-3439

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTAAACCTGT CTTGTAACCT TGATACGAAC   30

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTAAACCTGT CTTGTAACCT TGATACGAAT   30

- 18 -

(2) INFORMATION FOR SEQ ID NO:3:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 20 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACCTCACCCT GTGGAGCCAC   20

(2) INFORMATION FOR SEQ ID NO:4:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 30 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCTCCTTAAA CCTGTCTTGT AACCTTCATA   30

(2) INFORMATION FOR SEQ ID NO:5:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 30 base pairs
          (B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCTCCTTAAA CCTGTCTTGT AACCTTCATG   30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACCAGCAGCC TAAGGGTGGG AAAATAGTCC   30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACCAGCAGCC TAAGGGTGGG AAAATAGTCT   30

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAGATCCCCA AAGGACTCAA AGAACCTGTG   30

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CAGATCCCCA AAGGACTCAA AGAACCTGTA   30

(2) INFORMATION FOR SEQ ID NO:10:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 30 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: DNA (genomic)

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGAAAACAT CAAGGGTCCC ATAGACTGAC   30

(2) INFORMATION FOR SEQ ID NO:11:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 30 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: DNA (genomic)

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AAGAAAACAT CAAGGGTCCC ATAGACTGAT   30

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What is claimed is:

        1.   A method of diagnosing β-thalassemia
comprising the steps of:
        obtaining genomic DNA from a patient suspected of
5   carrying a genetic mutation characteristic of the phenotype
β-thalassemia;
        selecting at least two primer sets for detecting at
least two mutations characteristic of β-thalassemia, each set
being comprised of two primer pairs, a first primer pair
10   comprising a specific primer for a normal allele, and a
second primer pair comprising a specific primer for a mutant
allele, each pair further comprising a common primer;
        detecting two or more polymerase chain reaction
products whereby the detection of a polymerase chain reaction
15   product of a specific primer for a mutant allele indicates
the likelihood that said patient carries a mutation
characteristic of the phenotype β-thalassemia.

        2.   The method of claim 1 wherein at least four
primer sets are selected.

20   3.   The method of claim 1 wherein at least five
primer sets are selected.

        4.   The method of claim 1 wherein the step of
selecting at least two primer sets further comprises
selecting primer sets in which each specific primer is
25   differentially labeled.

        5.   The method of claim 4 wherein the step of
performing a polymerase chain reaction further comprises
using said genomic DNA and all differentially labeled primer
sets simultaneously.

30   6.   The method of claim 1 wherein the step of
selecting further comprises selecting primer sets comprising

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a first, second and third member, the first member being a
specific primer for a normal allele, the second member being
a specific primer for a mutant allele, and the third member
being a common allele from the group consisting of:

5   IVS-1 nt1-N   TTAAACCTGTCTTGTAACCTTGATACGAAC (SEQ ID NO: 1),
     IVS-1 nt1-M   TTAAACCTGTCTTGTAACCTTGATACGAAT (SEQ ID NO: 2),
     and β-CRP1   ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);

     IVS-1 nt6-N   TCTCCTTAAACCTGTCTTGTAACCTTCATA (SEQ ID NO: 4),
     IVS-1 nt6-M   TCTCCTTAAACCTGTCTTGTAACCTTCATG (SEQ ID NO: 5),
10   and β-CRP1   ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);

     IVS-1 nt110-N ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),
     IVS-1 nt110-M ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),
     and β-CRP1   ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);

     Codon 39-N   CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),
15   Codon 39-M   CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),
     and β-CRP1   ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3); and

     IVS-2 nt 1-N   AAGAAAACATCAAGGGTCCCATAGACTGAC (SEQ ID NO: 10),
     IVS-2 nt 1-M   AAGAAAACATCAAGGGTCCCATAGACTGAT (SEQ ID NO: 11),
     and β-CRP1   ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3).

20   7.   The method of claim 1 wherein the polymerase
chain reaction is performed by using said genomic DNA and the
primer sets:

     IVS-1 nt1-N   TTAAACCTGTCTTGTAACCTTGATACGAAC (SEQ ID NO: 1),
     IVS-1 nt1-M   TTAAACCTGTCTTGTAACCTTGATACGAAT (SEQ ID NO: 2),
25   and β-CRP1   ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);

     IVS-1 nt6-N   TCTCCTTAAACCTGTCTTGTAACCTTCATA (SEQ ID NO: 4),
     IVS-1 nt6-M   TCTCCTTAAACCTGTCTTGTAACCTTCATG (SEQ ID NO: 5),
     and β-CRP1   ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);

     IVS-1 nt110-N ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),
30   IVS-1 nt110-M ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),
     and β-CRP1   ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);

     Codon 39-N   CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),
     Codon 39-M   CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),
     and β-CRP1   ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3); and

35   IVS-2 nt 1-N   AAGAAAACATCAAGGGTCCCATAGACTGAC (SEQ ID NO: 10),
     IVS-2 nt 1-M   AAGAAAACATCAAGGGTCCCATAGACTGAT (SEQ ID NO: 11),
     and β-CRP1   ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3).

each of said primer sets being comprised of a first, second
and third member, the first member being a specific primer
40   for a normal allele, the second member being a specific

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primer for a mutant allele, and the third member being a
common allele.

8.   The method of claim 7 wherein the polymerase
chain reaction is performed by using said genomic DNA and
5   said primer sets, the said first and second members of each
primer set further being differentially labelled.

9.   The method of claim 8 wherein the polymerase
chain reaction is performed by using said genomic DNA and all
differentially labeled primer sets simultaneously.

10   10.   The method of claim 1 wherein the polymerase
chain reaction is performed by using said genomic DNA and the
primer sets:

IVS-1 nt1-N	TTAAACCTGTCTTGTAACCTTGATACGAAC (SEQ ID NO: 1),
IVS-1 nt1-M	TTAAACCTGTCTTGTAACCTTGATACGAAT (SEQ ID NO: 2),
15 and β-CRP1	ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);
IVS-1 nt110-N	ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),
IVS-1 nt110-M	ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),
and β-CRP1	ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);
Codon 39-N	CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),
20 Codon 39-M	CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),
and β-CRP1	ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3); and
IVS-2 nt 1-N	AAGAAAACATCAAGGGTCCCATAGACTGAC (SEQ ID NO: 10),
IVS-2 nt 1-M	AAGAAAACATCAAGGGTCCCATAGACTGAT (SEQ ID NO: 11),
25 and β-CRP1	ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3).

each of said primer sets being comprised of a first, second
and third member, the first member being a specific primer
for a normal allele, the second member being a specific
primer for a mutant allele, and the third member being a
30   common allele.

11.   The method of claim 10 wherein the polymerase
chain reaction is performed by using said genomic DNA and
said primer sets, the said first and second members of each
primer set further being differentially labelled.

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12. The method of claim 11 wherein the polymerase
chain reaction is performed by using said genomic DNA and all
differentially labeled primer sets simultaneously.

13. The method of claim 1 wherein the polymerase
chain reaction is performed by using said genomic DNA and the
primer sets:

```
IVS-1 nt1-N    TTAAACCTGTCTTGTAACCTTGATACGAAC (SEQ ID NO: 1),
IVS-1 nt1-M    TTAAACCTGTCTTGTAACCTTGATACGAAT (SEQ ID NO: 2),
and β-CRP1     ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);

IVS-1 nt110-N ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),
IVS-1 nt110-M ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),
and β-CRP1     ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3); and

Codon 39-N     CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),
Codon 39-M     CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),
and β-CRP1     ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3).
```

each of said primer sets being comprised of a first, second
and third member, the first member being a specific primer
for a normal allele, the second member being a specific
primer for a mutant allele, and the third member being a
common allele.

14. The method of claim 13 wherein the polymerase
chain reaction is performed by using said genomic DNA and
said primer sets, the said first and second members of each
primer set further being differentially labelled.

15. The method of claim 14 wherein the polymerase
chain reaction is performed by using said genomic DNA and all
differentially labeled primer sets simultaneously.

16. The method of claim 1 wherein the polymerase
chain reaction is performed by using said genomic DNA and the
primer sets:

```
IVS-1 nt110-N ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),
IVS-1 nt110-M ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),
and β-CRP1     ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3); and

Codon 39-N     CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),
```

```
Codon 39-M      CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),
and β-CRP1      ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3).
```

each of said primer sets being comprised of a first, second
and third member, the first member being a specific primer
5 for a normal allele, the second member being a specific
primer for a mutant allele, and the third member being a
common allele.

        17. The method of claim 16 wherein the polymerase
chain reaction is performed by using said genomic DNA and
10 said primer sets, the said first and second members of each
primer set further being differentially labelled.

        18. The method of claim 17 wherein the polymerase
chain reaction is performed by using said genomic DNA and all
differentially labeled primer sets simultaneously.

15   19. A kit comprising four dNTPs and at least two
primer sets selected from the group consisting of:
```
IVS-1 nt1-N     TTAAACCTGTCTTGTAACCTTGATACGAAC (SEQ ID NO: 1),
IVS-1 nt1-M     TTAAACCTGTCTTGTAACCTTGATACGAAT (SEQ ID NO: 2),
and β-CRP1      ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);
```

```
20 IVS-1 nt6-N     TCTCCTTAAACCTGTCTTGTAACCTTCATA (SEQ ID NO: 4),
   IVS-1 nt6-M     TCTCCTTAAACCTGTCTTGTAACCTTCATG (SEQ ID NO: 5),
   and β-CRP1      ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);
```

```
   IVS-1 nt110-N   ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),
   IVS-1 nt110-M   ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),
25 and β-CRP1      ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);
```

```
   Codon 39-N      CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),
   Codon 39-M      CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),
   and β-CRP1      ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3); and
```

```
   IVS-2 nt 1-N    AAGAAAACATCAAGGGTCCCATAGACTGAC (SEQ ID NO: 10),
30 IVS-2 nt 1-M    AAGAAAACATCAAGGGTCCCATAGACTGAT (SEQ ID NO: 11),
   and β-CRP1      ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3).
```

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20. A method of diagnosing a disease characterized
by multiple allelic mutations comprising the steps of:
                obtaining genomic DNA from a patient suspected of
carrying a genetic mutation characteristic of the disease;
5   selecting at least two primer sets for detecting at
least two mutations characteristic of the disease, each set
being comprised of a first primer pair comprising a specific
primer for a normal allele, a second primer pair comprising a
specific primer for a mutant allele, each specific primer
10 being differentially labeled, each pair further comprising a
common primer;
                performing a polymerase chain reaction by using
said genomic DNA and said at least two primer sets whereby
primer pairs comprising a specific primer for a normal allele
15 are used simultaneously and primer pairs comprising a
specific primer for a mutant allele are used simultaneously;
and
                detecting one or more polymerase chain reaction
products wherein detection of a polymerase chain reaction
20 product of a specific primer for a mutant allele indicates
the likelihood that said patient carries a mutation
characteristic of the disease.

                21. The method of claim 20 wherein at least 4
primer sets are selected.

25   22. The method of claim 20 wherein at least 5
primer sets are selected.

                23. The method of claim 20 wherein the step of
selecting at least two primer sets further comprises
selecting primer sets in which each specific primer is
30 differentially labelled.

                24. The method of claim 23 wherein the step of
performing a polymerase chain reaction further comprises
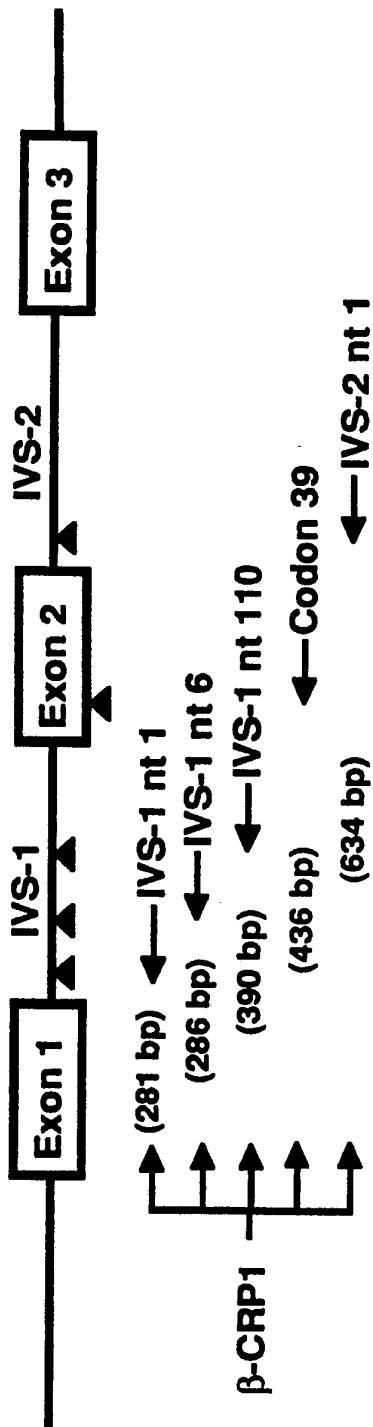using said primer sets simultaneously.
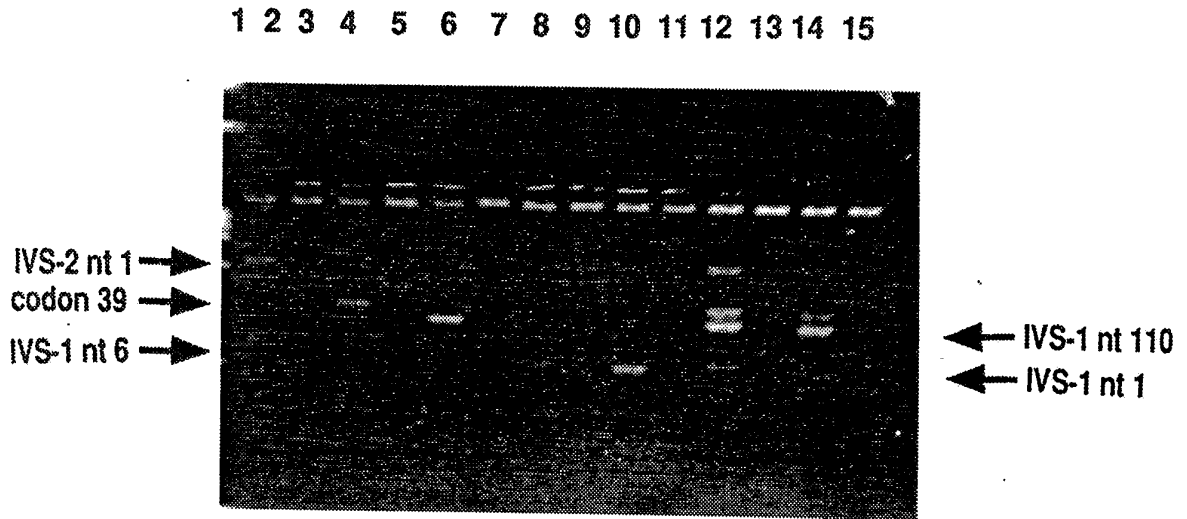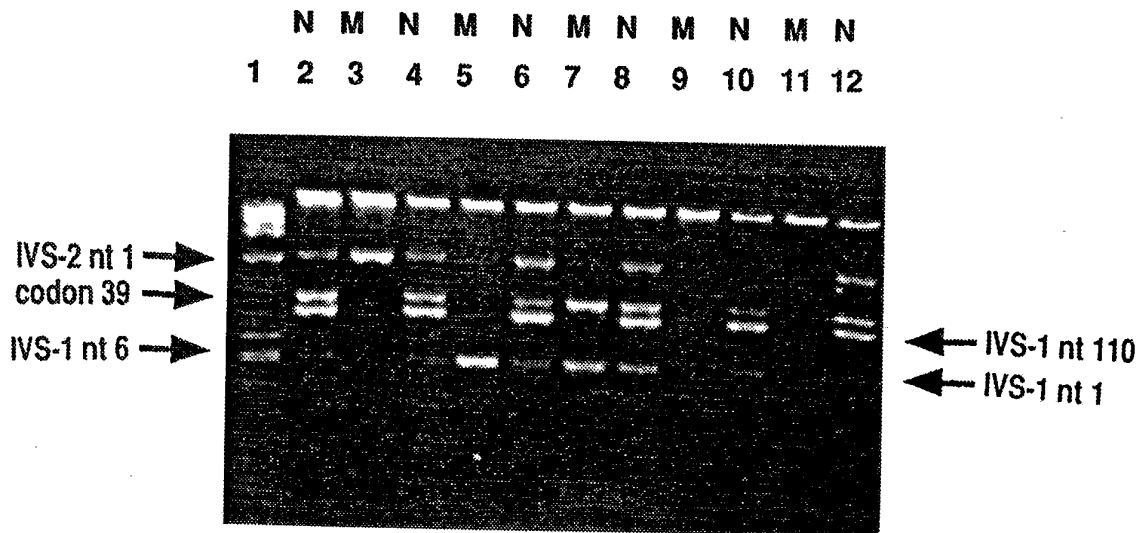
1 / 2

FIG.1

FIG.2

FIG.3

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12P 19/34; CO7H 21/04
US CL :435/91; 536/24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91; 536/24.3, 24.33; 935/76, 77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci., Volume 86, issued April 1989, (U.S.A), D.Y. Wu et al., "Allele-Specific Enzymatic Amplification of Beta-globin Genomic DNA for Diagnosis of Sickle Cell Anemia", pages 2757-2760, see page 2758 and Figure 1.	1-18, 20-24
Y	Genomics, Volume 7, issued 1990, R.A. Gibbs et al., "Multiplex DNA Deletion Detection and Exon Sequencing of the Hypoxanthine Phosphoribosyltransferase Gene in Lesch-Nyhan Families", pages 235-244, see page 236.	1-18, 20-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 April 1993	1 3 MAY 1993

| Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	British Journal of Haemotology, Volume 78, issued 1991, N.Y. Varawalla et al., "The Spectrum of Beta-thalassaemia Mutations on the Indian Subcontinent: the Basis for Prenatal Diagnosis", pages 242-247, see page 243 and Table 1.	1-24
Y	Genomics, Volume 2, issued 1988, M.H. Skolnick et al., "Simultaneous Analysis of Multiple Polymorphic Loci Using Amplified Sequence Polymorphisms (ASPs)", pages 273-279, see entire document.	1-18, 20-24