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(54) Title: DISCRIMINATION OF BLOOD TYPE VARIANTS

	RHD			RHCE		RHD	Serology
	presence or absence of an RHD/RHCE hybrid exon 3 allele	exon 4 (position 602)	exon 7 (position 1048)	presence/absence of an RHCE* ^C allele	exon 7 (position 1006)	intron 3 at position 3100	
r's	presence	G	C	absence	T	G	C ^{rw}
DIIIa	presence	G	G	absence	G or T	A	C-
DIVa	presence	C	C	absence	G	A	C-
New variant	presence	G	G	absence	G	G	C-

Figure 3

(57) Abstract: The present invention provides a method for detecting the presence or absence of, or for discriminating between, blood type variants, including RHD*r 's, RHD*DIIIa and RHD*DIVa-2. The method comprises amplifying by PCR a sample obtained from a human subject at intron 3 of the *RHD* gene locus. The invention also provides products, in particular, probes, primers and kits for use in the method of the invention.

Discrimination of Blood Type Variants**Field of the Invention**

The invention relates to methods for genotyping and blood cell antigen determination, which in particular may discriminate the *RHD*r's* or *RHD*r's*-like blood type variants, which encode C^W antigen and no D antigen, from *RHD*DIIIa*, *RHD*DIVa-2* and other blood type variants. The invention also relates to products, in particular, probes, primers and kits for use in such methods.

Background to the Invention

The success of blood transfusion often depends on the degree of compatibility between donor and recipient. The degree of compatibility, in turn, is a function of the similarity in Red Blood Cell (RBC) antigen content between donor and recipient. Expression of many RBC antigens in an individual can be predicted from the analysis of their genomic DNA. Therefore, analysis of donor and/or recipient DNA can be used to facilitate blood matching and thus enable proper blood transfusion practice.

Hemolytic reactions are more common in multi-transfused than in singly transfused individuals, not only because of the increased probability of such an event as the number of transfused units increases, but also because of the accumulative nature of the immune response in the recipient. An example of a condition whose treatment includes repeated blood transfusions is Sickle Cell Disease (SCD). From the above follows that a high degree of compatibility with donor blood is often critical for the long-term success of transfusion in SCD patients.

While SCD is more prevalent among individuals of African ancestry, the blood donor population in the USA and other Western countries is largely Caucasian. As a consequence of this disparity, differences in RBC antigens between both

racial groups often become responsible for blood transfusion failures in SCD patients.

The genetic variant *RHD*DIIIa-CE(4-7)-D*, also known as *RHD-CE-D^S*, *RHD-CE(4-7)-D*, *(C)ce^S*, or *r'^S*, (*RHD*r'^S* henceforth) can be found in up to 5-10% of the African-American population, but is extremely rare in Caucasians. This variant poses a special challenge to blood transfusion because it encodes a rather complex antigen profile, which includes absence of D antigen, altered forms of C (C^{+W}) and e antigens, expression of low-frequency VS antigen, no expression of V antigen, and absence of the high-frequency hr^B antigen. Among them, D and C antigens are the clinically most relevant ones.

The antigenic complexity of *RHD*r'^S* correlates with its genetic complexity, which includes a substitution of part of *RHD* exon 3, *RHD* exons 4-7, and the intervening introns by their *RHCE* counterparts, a G>T substitution at position 186 (exon 2), a C>T substitution at position 410 (hybrid exon 3), a C>G substitution at position 733 (exon 5), and a G>T substitution at position 1006 (exon 7). In addition to the changes in the *RHD* gene, *RHD*r'^S* occurs in *cis* with *RHCE*ce^S1006T*, an *RHCE* gene that also encodes substitutions C>G at position 733 (exon 5) and G>T at position 1006 (exon 7).

To add to the antigenic and genetic complexity, knowledge about the molecular basis of *RHD*r'^S* is incomplete. For instance, the precise points of *RHCE/RHD* recombination have not been reported to date. Furthermore, two types of *RHD*r'^S* variant have been described and named Type 1 and Type 2, which differ not only in their genetic composition but also in their antigen profiles.

Several publications (Westhoff et al., Transfusion (2010), 50: 1303-1311, Pham et al., Transfusion (2009), 49: 495-504, and Tax et al., Transfusion (2002) 42: 6234-6644) have uncovered the genetic similarity between *RHD*r'^S* and other *RHD* variants,

in particular *RHD*DIIIa* and *RHD*DIVa/RHD*DIVa-2* (*RHD*DIVa-2* henceforth). A number of molecular methods for the specific detection of *RHD*r'^s* rely on the detection of polymorphisms located in D-CE hybrid exon 3 locus of RHD. These

5 polymorphisms are now known to be shared with variants *RHD*DIIIa* and *RHD*DIVa-2*. Consequently, to date, identification of *RHD*r'^s* in a sample by DNA analysis requires detection of hybrid exon 3 polymorphisms and discrimination from *RHD*DIIIa* and *RHD*DIVa-2*. This discrimination is
10 clinically relevant since the latter variants encode a different antigen profile, which includes expression of partial D and absence of C^{+W}.

EP1780217 describes the detection of RHD positive haplotypes
15 in D-negative individuals. Silvy et al., British Journal of Haematology, (2012 Dec 30) doi: 10.1111/bjh. 12179 [Epub ahead of print], describe identification of a polymorphism said to be restricted to the (C)ce^s type 1 haplotype. WO2012/171990 describes discrimination of blood type variants in a method
20 making use of polymorphisms in intron 7 of the RHD gene and/or intron 7 of the RHCE gene. EP2471949 describes a method for the identification by molecular techniques of genetic variants that encode no D antigen (D⁻) and altered C antigen (C^{+W}).

25 Antibody reagents commonly used to detect C antigen do not discriminate between C^{+W} and C⁺. Therefore, the phenotype is often reported as C⁺. In cases where the antibody reagent does discriminate between C^{+W} and C⁺ but the sample contains a normal *RHCE*C* allele in *trans* to a *RHD*r'^s* allele, C^{+W} is obscured by
30 C⁺, resulting in a C⁺ phenotype for the sample. Therefore, *RHCE*C* needs to be tested for and shown absent prior to assignment of a C^{+W} phenotype to a sample. Accordingly, there remains a need for further methods for distinguishing *RHD*r'^s* from *RHD*DIIIa* and *RHD*DIVa-2*. The present invention
35 addresses these and other objects.

Summary of the Invention

The present inventors have surprisingly found that methods and, in particular, PCR primers as described herein are able to amplify a portion of intron 3 of the RHD gene found in r'^s samples in a specific manner, and that this specificity is retained even under multiplex PCR conditions. Considerable disadvantages of previously-described primers and reaction methods are addressed by the methods and primers of the present invention, as demonstrated by the examples herein. The present invention mitigates false positive readings displayed by previously-described methods and primers, and is advantageously able to discriminate r'^s from closely-related alleles, including a newly-described variant of RHD*DIIIa.

Accordingly, the present invention provides, in a first aspect, an oligonucleotide polymerase chain reaction (PCR) primer, the nucleotide sequence of which is of the formula:

X-Y-Z

wherein:

X is X₁ or X₂, wherein:

X₁ is the final n nucleotides in the nucleotide sequence ATATGGAAATTTGATCATGT (SEQ ID NO: 1), wherein n is a number between 0 and 20, inclusive; and

X₂ is a variant of X₁ differing by no more than one nucleotide substitution;

Y is Y₁ or Y₂, wherein:

Y₁ is the nucleotide sequence **AS₁TAATS₂ATAC** (SEQ ID NO: 2), wherein S₁ and S₂ are independently selected from G and C; and

Y₂ is a variant of Y₁ differing from Y₁ by no more than one nucleotide substitution, provided that said nucleotide

substitution is not a substitution of the first **A** or final **C** of Y_1 ;

5 Z is the first m nucleotides in the nucleotide sequence TAAAG, wherein m is a number between 0 and 5, inclusive.

In some cases, the primer of this and other aspects of the invention is such that X_1 is the nucleotide sequence of AAATTTGATCATGT (SEQ ID NO: 3) or ATGT. That is to say, n may, 10 in some cases, be 14 or 4. In certain cases, n may be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20.

In some cases in accordance with this and other aspects of the present invention, Y_1 is selected from the group consisting of: 15 ACTAATCATAC (SEQ ID NO: 4); ACTAATGATAC (SEQ ID NO: 5); and AGTAATCATAC (SEQ ID NO: 6). Preferably, if S_1 is G, S_2 is C. Likewise, if S_1 is C, S_2 is preferably C. Thus, the selection of S_1 and S_2 may be chosen to keep the number of mismatches 20 with the intended target template sequence to not more than one nucleotide mismatch. However, it is specifically contemplated herein that the number of mismatches may be more than one. For example, in certain cases, both S_1 and S_2 may be G. Alternatively, the number of nucleotide mismatches may be 25 zero. In certain cases, both S_1 and S_2 may be C.

In some cases in accordance with this and other aspects of the present invention, m is 0. Therefore, the primer of the invention may have no Z, with the result that the last 30 nucleotide in Y (which is a C that corresponds to the C/A polymorphic nucleotide at position 3046 of RHD intron 3, as numbered in Figure 7) is the 3' nucleotide of the primer of the invention. Alternatively, m may be 1, 2, 3, 4 or 5.

35 In some cases in accordance with this and other aspects of the present invention, the primer is not more than 30, 29, 28, 27, 26, 25, 24, 23, 22, 21 or 20 nucleotides in length. In some

cases, the primer may be at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or at least 25 nucleotides in length. In certain cases the primer may be between 10 and 30 nucleotides in length, such as between 15 and 25 nucleotides in length. Particular cases include a primer in accordance with the first aspect of the invention which has a length of exactly 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides.

10 In some cases in accordance with this and other aspects of the present invention, the primer may be DNA or RNA. The primer is preferably DNA.

In some cases in accordance with this and other aspects of the present invention, the primer may include one or more, e.g. 1, 2, 3, 4 or 5 altered or non-natural bases and/or derivatised or modified nucleotide bases. In particular, one or more bases (e.g. a 5' or a 3' base) may be biotinylated or conjugated to a detectable label. Alternatively or additionally, one or more bases (e.g. 1, 2, 3, 4 or 5 nucleotide bases) may be locked nucleic acid (LNA) bases.

In some cases in accordance with this and other aspects of the present invention, the nucleotide sequence of the primer consists of a nucleotide sequence selected from the group consisting of:

- (i) AAATTTGATCATGTACTAATCATAC (SEQ ID NO: 7);
- (ii) ATGTACTAATCATAC (SEQ ID NO: 8);
- (iii) AAATTTGATCATGTACTAATGATAC (SEQ ID NO: 9); and
- 30 (iv) AAATTTGATCATGTAGTAATCATAC (SEQ ID NO: 10).

In some cases in accordance with this and other aspects of the present invention, the primer is suitable for use as a forward PCR primer in a PCR amplification of a portion of the r'^s allele of intron 3 of the RHD gene.

In a second aspect, the present invention provides a plurality of oligonucleotide primers comprising:

(i) an oligonucleotide primer of the first aspect of the invention; and

5 (ii) (a) a reverse primer that hybridises to a portion of intron 3 of r'^s , or its complement, which portion includes at least one position of single nucleotide polymorphism (SNP) that differs between r'^s and RHD; or

10 (b) one or more primers that hybridise to a region of the RHD gene and or the RHCE gene.

Preferably, said reverse primer (ii) (a) hybridises to the reverse complement of a portion of the r'^s intron 3 sequence that is shown in Figure 7 and which lies 3' of position 3050, 15 as numbered in Figure 7. In some cases in accordance with this and other aspects of the present invention said reverse primer hybridises to a portion of intron 3 of r'^s , or its complement, which portion includes the G/A polymorphism position 3189 of intron 3 of the *RHD* gene, said numbering 20 being as shown in Figure 7.

In some cases in accordance with the second aspect of the present invention, the plurality of primers comprises:

25 (i) an oligonucleotide primer of the first aspect of the invention; and

(ii) at least one primer selected from the group consisting of:

(a) an oligonucleotide primer of between 26 and 30 nucleotides in length comprising the nucleotide sequence 30 GGAAAAGGTTTGAGAGGAATTATAT~~T~~ (SEQ ID NO: 11) or a variant thereof differing by not more than 3 (e.g. not more than 2 or not more than 1) nucleotide substitutions from the nucleotide sequence of SEQ ID NO: 11, provided that said substitutions do not include substitution of the final T in the nucleotide 35 sequence of SEQ ID NO: 11;

(b) an oligonucleotide primer consisting of the nucleotide sequence GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11);

5 (c) the RHCE c forward primer consisting of the nucleotide sequence TGGGCTTCCTCACCTCAAA (SEQ ID NO: 12);

(d) the RHCE c reverse primer consisting of the nucleotide sequence TGATGACCACCTTCCCAGG (SEQ ID NO: 13);

(e) the RHCE C forward primer consisting of the nucleotide sequence GGCCACCACCATTTGAA (SEQ ID NO: 14);

10 (f) the RHCE C reverse primer consisting of the nucleotide sequence GGTAGCAGGCGTCTGTAAAAA (SEQ ID NO: 15);

(g) the RHCE exon 1 forward primer consisting of the nucleotide sequence CATAGACAGGCCAGCACAG (SEQ ID NO: 16);

15 (h) the RHCE exon 1 reverse primer consisting of the nucleotide sequence TGCCCCTGGAGAACCAT (SEQ ID NO: 17);

(i) the RHCE exon 5 forward primer consisting of the nucleotide sequence AAATTAAAATAAGCATTTGACCATC (SEQ ID NO: 18);

(j) the RHCE exon 5 reverse primer consisting of the nucleotide sequence CCTGAGATGGCTGTCACCAC (SEQ ID NO: 19);

20 (k) the RHCE exon 7 forward primer consisting of the nucleotide sequence ACATGCCATTGCCGTTC (SEQ ID NO: 20); and

(l) the RHCE exon 7 reverse primer consisting of the nucleotide sequence TCTCACCTGCCAATCTGCT (SEQ ID NO: 21).

25 In some cases the plurality of oligonucleotide primers comprise at least:

an oligonucleotide primer of the first aspect of the invention, and

30 an oligonucleotide primer of between 26 and 30 nucleotides in length comprising the nucleotide sequence GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11) or a variant thereof differing by not more than 3 (e.g. not more than 2 or not more than 1) nucleotide substitutions from the nucleotide sequence of SEQ ID NO: 11, provided that said substitutions do
35 not include substitution of the final T in the nucleotide sequence of SEQ ID NO: 11.

In some cases the plurality of oligonucleotide primers comprise at least the primer pair:

AAATTTGATCATGTACTAATCATAC (SEQ ID NO: 7); and
GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11).

5

In a third aspect the present invention provides a kit for assessing a subject's blood type, said kit comprising:

a plurality of primers of the second aspect of the invention;

10 optionally, one or more probes and/or primers that span one or more polymorphic positions in intron 3, exon 3, exon 4, intron 7 and/or exon 7 of the *RHD* gene locus; and/or

optionally, one or more probes and/or primers that span one or more polymorphic positions in exon 7 of the *RHCE* gene locus.
15

In a fourth aspect, the present invention provides a system for use in determining a subject's blood type, the system comprising:

20 a kit of the third aspect of the invention; and
at least one detector arranged to detect a signal from detectably labelled DNA obtained from said subject or a detectably labelled amplicon produced by PCR amplification carried out on DNA obtained from said subject;

25 at least one controller in communication with the at least one detector, the controller being programmed with computer-readable instructions to transform said signal into predicted blood type haplotypes, and optionally, to transform said predicted blood type haplotypes into a predicted blood
30 type phenotype.

In a fifth aspect the present invention provides a method for determining the presence or absence of, or for discriminating between, blood type alleles in a DNA-containing sample, which
35 method comprises amplification by polymerase chain reaction (PCR) of at least a portion of intron 3 of the *RHD* gene, wherein said PCR employs at least a forward primer and a

reverse primer each capable of hybridising to the r'^s sequence set forth in SEQ ID NO: 31 (Figure 7), or its complement, and wherein said forward primer is the primer of the first aspect of the invention.

5

In some cases in accordance with the method of this and other aspects of the invention, the blood type alleles are alleles that comprise an *RHD/RHCE* hybrid exon 3. In particular, the blood type alleles may be selected from the group consisting of: *RHD*r'^s*; *RHD*r'^s-like*; *RHD*r'^s Type 1*; *RHD*r'^s Type 2*; *RHD*DIIIa*; *RHD*DIIIa IVS3+3100G*; *RHD*DIII_FN*; *RHD*DIVa-2*; *RHD*DIVa*; *RHD*DIII-type4*; *RHD*DIII-type6*; *RHD*DIII-type7*; *RHD*DIII-type8*; *RHCE*ce^s*; *RHCE*ce^s1006T*; *RHCE*ce^s1006C*; *RHCE*ce733G*; *RHCE*ce48C,733G,1025T*; *RHCE*ce48C,697G,733G*; *RHCE*ce340T,733G*; and *RHCE*ce48C,733G,748A*.

In some cases in accordance with the method of this and other aspects of the invention the PCR amplifies r'^s , but does not amplify one or more of: *RHD*; *RHCE*ce*; *RHD*DIIIa*; *RHD*DIIIa IVS3+3100G*; and *RHD*DIVa*. In particular, said PCR may in certain cases amplify r'^s , but not any of *RHD*; *RHCE*ce*; *RHD*DIIIa*; *RHD*DIIIa IVS3+3100G*; and *RHD*DIVa*.

In some cases in accordance with the method of this and other aspects of the invention the method is for discriminating r'^s from *RHD*DIIIa IVS3+3100G*. The ability to distinguish r'^s from the newly-described variant, "*RHD*DIIIa IVS3+3100G*", which as far as the present inventors are aware has not previously been reported, is a major advance. The newly-described variant, "*RHD*DIIIa IVS3+3100G*" comprises the same *RHD* intron 3 position 3100 A to G polymorphism that has previously been reported to be specific for r'^s (see Silvy et al., 2012). Reliance on the presence of the *RHD* intron 3 position 3100 A to G polymorphism to uniquely identify r'^s would be expected to result in false positive results. The provision of a method that discriminates r'^s from *RHD*DIIIa IVS3+3100G* is expected to provide a significant clinical contribution.

The method in accordance with this and other aspects of the present invention utilises a forward primer of the first aspect of the invention. Particular examples include a primer
5 that consists of the nucleotide sequence
AAATTTGATCATGTACTAATCATAC (SEQ ID NO: 7). The method further utilises a suitable reverse primer, which may be any reverse primer capable of yielding a PCR product when used in combination with the forward primer of the first aspect of the
10 invention. In certain cases, the reverse primer is capable of hybridising to a portion of intron 3 of r'^s , or its complement, which portion includes at least one position of single nucleotide polymorphism (SNP) that differs between r'^s and *RHD*. In a certain cases, said position of SNP comprises the G/A
15 polymorphism at position 3189 of intron 3 of the *RHD* gene, said numbering being as shown in Figure 7 (see also SEQ ID NO: 31). In some cases, the reverse primer is of between 26 and 30 nucleotides in length and comprises the nucleotide sequence GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11). In certain cases,
20 the reverse primer consists of the nucleotide sequence
GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11).

In some cases in accordance with the method of this and other aspects of the invention the PCR is multiplex PCR. The
25 multiplex PCR may further comprise amplification of one or more *RHD* and/or *RHCE* gene segments other than *RHD* intron 3. In some cases, the one or more *RHD* and/or *RHCE* gene segments are selected from the group consisting of: *RHCE* c; *RHCE* C; *RHCE* exon 1; *RHCE* exon 5; and *RHCE* exon 7. Accordingly, the
30 method may employ additional PCR primers to be used in multiplex with the above-described PCR primers. In certain cases, the multiplex PCR further comprises employing 1, 2, 3, 4, 5, 6, 7, 8, 9 or all 10 of the following primers:

35 (i) the *RHCE* c forward primer consisting of the nucleotide sequence TGGGCTTCCTCACCTCAAA (SEQ ID NO: 12);

(ii) the RHCE c reverse primer consisting of the nucleotide sequence TGATGACCACCTTCCCAGG (SEQ ID NO: 13);

(iii) the RHCE C forward primer consisting of the nucleotide sequence GGCCACCACCATTGAA (SEQ ID NO: 14);

(iv) the RHCE C reverse primer consisting of the nucleotide sequence GGTAGCAGGCGTCTGTAAAAA (SEQ ID NO: 15);

(v) the RHCE exon 1 forward primer consisting of the nucleotide sequence CATAGACAGGCCAGCACAG (SEQ ID NO: 16);

(vi) the RHCE exon 1 reverse primer consisting of the nucleotide sequence TGCCCCTGGAGAACCAT (SEQ ID NO: 17);

(vii) the RHCE exon 5 forward primer consisting of the nucleotide sequence AAATTAAATAAGCATTTGACCATC (SEQ ID NO: 18);

(viii) the RHCE exon 5 reverse primer consisting of the nucleotide sequence CCTGAGATGGCTGTCACCAC (SEQ ID NO: 19);

(ix) the RHCE exon 7 forward primer consisting of the nucleotide sequence ACATGCCATTGCCGTTC (SEQ ID NO: 20); and

(x) the RHCE exon 7 reverse primer consisting of the nucleotide sequence TCTCACCTGCCAATCTGCT (SEQ ID NO: 21).

Preferably, the primers are employed in pairs, said primers being paired as follows: (i) with (ii); (iii) with (iv); (v) with (vi); (vii) with (viii); and (ix) with (x).

In some cases in accordance with the method of the present invention, the sample is a sample which has previously been determined to comprise an *RHD-RHCE* hybrid exon 3.

Alternatively, the sample may be suspected to comprise an *RHD-RHCE* hybrid exon 3 or be an unknown sample. The method may therefore further comprise a preceding step of determining whether the sample contains an *RHD-RHCE* hybrid exon 3.

In some cases in accordance with the method of the present invention, the method further comprises determining the presence or absence of an RHCE*C allele in the sample. In

some cases the method further comprises genotyping the sample at one or more positions of polymorphisms in the *RHD* and/or *RHCE* gene loci. In certain cases, the method comprises genotyping the sample at position 410 of the *RHD* exon 3. In certain cases, the method comprises genotyping the sample at position 602 of the *RHD* exon 4. In certain cases, method comprises genotyping the sample at position 1048 of the *RHD* exon 7. In certain cases, the method comprises genotyping the sample at position 1006 of the *RHCE* exon 7. In certain cases, the method comprises genotyping the sample at position 3100 of the *RHD* intron 3.

In some cases in accordance with the method of the present invention, the method comprises genotyping not more than 50, 40, 30, 25, 20, 15, or not more than 10, single nucleotide polymorphic positions in the *RHD* gene locus and/or the *RHCE* gene locus.

In some cases in accordance with the method of the present invention, the method further comprises predicting an *RHD* phenotype and/or an *RHCE* phenotype for the subject based on, at least, the degree of amplification by PCR of said at least one portion of intron 3 of the *RHD* gene. This may, for example, include a visual or computer-aided inspection of the intensity of a band on a gel where the intensity is related to the degree of amplification of the PCR product. The skilled person will of course be readily able to make use of suitable techniques to evaluate the degree of PCR amplification, e.g., for classification of a sample as containing r'^s . Such techniques include, without limitation, real time PCR (qPCR), Luminex bead-based detection and agarose gel-based evaluation. The method may comprise detecting a positive result for r'^s -specific PCR amplification and thereby classifying a sample as containing an r'^s allele. In some cases, the phenotype prediction is further based on:

- (i) the presence or absence of an *RHD*/*RHCE* hybrid exon 3;

(ii) the identity of one or both alleles present at position 602 of the RHD exon 4;

(iii) the identity of one or both alleles present at position 1048 of the RHD exon 7;

5 (iv) the presence or absence of an RHCE*C allele in the RHCE gene locus;

(v) the identity of one or both alleles present at position 1006 of the RHCE exon 7; and/or

10 (vi) the identity of one or both alleles present at position 3100 of the RHD intron 3.

In some cases the sample is found to contain r'^s and is therefore predicted to have C^{+w} serology.

15 In accordance with this and other aspects of the present invention, the sample is preferably obtained or has been previously obtained from a human subject. In some cases the subject is undergoing, or is a candidate for, blood transfusion or bone marrow transplantation. In some cases the
20 subject has sickle cell disease (SCD) or Thalassemia major. In some cases the subject has non-Caucasian ancestry. In some cases, the subject has African ancestry.

In accordance with this and other aspects of the present
25 invention, the sample may be any suitable biological sample from which it is possible to obtain nucleic acid, particularly genomic DNA, for use in a PCR reaction. Suitable samples include any material of bodily origin (liquid, solid or aspirate) such as blood, hair, cheek cells and skin cells.

30 In accordance with this and other aspects of the present invention, the sample may be subjected to one or more treatments to extract a nucleic acid prior to or as part of said amplification by PCR.

35

In accordance with this and other aspects of the present invention, the method may comprise Allele-Specific Polymerase Chain Reaction (ASP).

- 5 In accordance with this and other aspects of the present invention, the method may comprise labelling a nucleic acid obtained from the sample or labelling the amplicon produced by said PCR amplification.
- 10 In accordance with this and other aspects of the present invention, the method may further comprise carrying out serological analysis on a blood sample that has been obtained from the subject. This may be particularly useful to corroborate or clarify a phenotype prediction made. Combining
- 15 the genotype-based prediction of blood type with a serological-based prediction may be useful, e.g., to improve accuracy or to resolve ambiguous results. However, it is also specifically contemplated herein that the method of this and other aspects of the present invention may not comprise
- 20 carrying out serological analysis. Removing the need to carry out serological analysis provides considerable savings in terms of time, cost and/or resources.

In a sixth aspect the present invention provides a method of blood matching, the method comprising:

- 25 carrying out the method of the fifth aspect of the invention on a recipient sample from a recipient subject in need of donor blood and on a donor sample from a potential donor subject;
- 30 comparing the blood type alleles present in the recipient sample with those present in the donor subject and thereby determining the compatibility of the recipient subject to receive blood from the potential donor subject.
- 35 In accordance with the present invention, the subject may be undergoing, or may be a candidate for, blood transfusion. In some cases, the subject may have SCD, or any other disease

requiring repeated blood transfusions, such as Thalassemia major or certain blood cell malignancies.

5 In accordance with the present invention, the subject may be of non-Caucasian race. In particular, the subject may be of African ancestry (e.g. "Black persons"). In certain cases, the subject may have an ancestral origin in a Mediterranean country.

10 In accordance with the method of the invention, the sample may be any suitable biological sample from which it is possible to amplify at least a portion of intron 3 of the *RHD* gene locus and/or from which it is possible to determine the genotype of the subject at one or more polymorphic positions in the *RHD*
15 gene and/or the *RHCE* gene. In certain case, the sample may conveniently take the form of a blood sample.

In certain cases in accordance with the method of the invention, the method may comprise carrying out an Allele-Specific Polymerase Chain Reaction (ASP) and/or Allele-Specific Hybridization (ASH).
20

In certain cases in accordance with the method of this and other aspects of the invention, the method may comprise
25 labelling a nucleic acid obtained from the sample or labelling an amplicon derived from a nucleic acid obtained from the sample. The label is preferably a detectable label. In some cases, DNA derived from the sample, e.g. PCR product resulting from use of the DNA from the sample as template, may be
30 labelled using a fluorescent label or dye (e.g. by conjugating said fluorescent label or dye to the PCR product before or after fragmentation of the PCR product).

In some cases, the method in accordance with the sixth aspect
35 of the invention may be carried out for a plurality of recipient subjects and a plurality of potential donor subjects.

The invention will now be described in more detail, by way of example and not limitation, by reference to the accompanying drawings. Many equivalent modifications and variations will be apparent to those skilled in the art when given this disclosure. Accordingly, the exemplary embodiments of the invention set forth are considered to be illustrative and not limiting. Various changes to the described embodiments may be made without departing from the scope of the invention. All documents cited herein are expressly incorporated by reference.

Description of the Figures

Figure 1 shows a gel image depicting the PCR results of a reaction utilising primers for an amplification control (HgH) (upper band) and r'^S (lower band) for a variety of samples (including RhD⁺, DIIIIa, RHD*DIIIIa IVS3+3100G, r'^S , DIVa and a negative control;

Figure 2 shows Luminex intensity results for a variety of samples r'^S , RHD*DIIIIa IVS3+3100G and other non- r'^S samples following multiplex PCR using the r'^S -specific primers shown in Table 1 and one or more primer pairs shown in Tables 2 and 3;

Figure 3 shows a table of certain RHD and RHCE exon and intron features for r'^S , DIIIIa, DIVa and the new variant "RHD*DIIIIa IVS3+3100G";

Figure 4 shows a gel image depicting the PCR results of a reaction utilising primers for an amplification control (HgH) (upper band) and r'^S (lower band) for a variety of samples (including RhD⁺, DIIIIa, RhD⁻ and r'^S);

Figure 5 shows Luminex intensity results for a variety of samples r'^S , r'^S /RHD+ heterozygous, r'^S hemizygous, DIVa-2, DIIIIa, RHD- and RHD+ samples;

Figure 6 shows a gel image depicting the PCR results of a number of PCR reactions comparing different primer combinations. An amplification control (HgH) (upper band) and r'^s (lower band) are shown for a variety of samples (r'^s , RhD⁺, RhD⁻, DIIIIa and a negative control;

Figure 7 shows an alignment of a portion of intron 3 of the RHD gene numbered positions 3001 to 3250, numbered according to the RHD sequence (upper row). An identical nucleotide to that above is represented by a dot; a omitted/deleted nucleotide (i.e, a gap) is represented by a dash. The sequences shown are (from top to bottom): RHD, RHCE*ce, r'^s , DIVa, DIIIIa and the newly-described variant RHD*DIIIIa IVS3+3100G (shown as #425). The locations of the reference forward primer and reverse primer are indicated by shaded boxes.

Detailed Description of the Invention

The present invention finds use in the determination of the clinically relevant RHD- and RHCE- encoded antigen phenotypes of a blood sample. The invention provides a method for detecting the presence or absence of, or for discriminating between, blood type variants, which method comprises amplification by polymerase chain reaction (PCR) of at least a portion of intron 3 of the *RHD* gene, wherein said PCR employs at least a forward primer and a reverse primer each capable of hybridising to the r'^s sequence set forth in SEQ ID NO: , or its complement, and wherein said forward primer nucleotide sequence is of the formula:

X-Y-Z

wherein:

X is X_1 or X_2 , wherein:

X_1 is the final n nucleotides in the nucleotide sequence ATATGGAAATTTGATCATGT (SEQ ID NO: 1), wherein n is a number between 0 and 20, inclusive; and

X_2 is a variant of X_1 differing by no more than one
5 nucleotide substitution;

Y is Y_1 or Y_2 , wherein:

Y_1 is the nucleotide sequence **AS₁TAATS₂ATAC** (SEQ ID NO: 2), wherein S_1 and S_2 are independently selected from G and C;
10 and

Y_2 is a variant of Y_1 differing from Y_1 by no more than one nucleotide substitution, provided that said nucleotide substitution is not a substitution of the first **A** or final **C** of Y_1 ;
15

Z is the first m nucleotides in the nucleotide sequence TAAAG, wherein m is a number between 0 and 5, inclusive.

Advantageously, the method of the present invention may
20 further comprise determining the presence or absence of an *RHD*/*RHCE* hybrid exon 3 in said sample and/or genotyping a sample obtained from a human subject at one or more positions in intron 7 of the *RHD* gene and/or in intron 7 of the *RHCE* gene. Blood typing by making use of intron 7 polymorphisms is
25 described in WO2012/171990, the entire contents of which is expressly incorporated herein by reference. Blood typing by making use of a combination of polymorphisms in the *RHD* gene and/or the *RHCE* gene are described in US2012/0172239 and EP2471949, the entire contents of which are both expressly
30 incorporated herein by reference.

The Rh blood group D antigen is encoded by the *RHD* gene, which comprises 10 exons. The complete *RHD* gene sequence is available at NCBI Reference Sequence: NG_007494.1 No.
35 NG_007494.1, GI:171184448, (SEQ ID NO: 22), the entire contents of which is incorporated herein by reference.

The Rh blood group C antigen is encoded by the *RHCE* gene, which comprises 10 exons. The complete *RHCE* gene sequence is available at NCBI Reference Sequence: NG_009208.2, GI:301336136, (SEQ ID NO: 23), the entire contents of which is
5 incorporated herein by reference.

The term "sample" as used herein is intended to encompass any material (solid, liquid or aspirate) obtained directly or indirectly from a human subject and from which the identity of
10 one or more nucleotides in a relevant genomic locus (e.g. intron 7 or the RHD locus and/or intron 7 of the *RHCE* locus) can be determined. In particular, the term "sample" includes any biological fluid such as blood, plasma, urine, saliva, cerebrospinal fluid and interstitial fluid, any solid matter,
15 such as tissue, bone and hair, any cell or cell extract, any derived cell line, such as an immortalised tumour cell line and stem cell line, an extract of any of the preceding sample types, such as fixed or paraffin-embedded tissue. In certain preferred embodiments, the sample is an extract of human
20 genomic DNA, optionally amplified and/or purified.

As used herein, the term "genotyping" is intended to encompass any method for determining the identity of the nucleotide at a particular position such as a polymorphic position at a
25 specified locus. Thus, genotyping includes identifying one or both alleles of a particular gene. Genotyping may employ any of a variety of techniques, including but not limited to, allele-specific hybridisation, allele-specific PCR, sequencing of all or part of a gene.

30

Unless specified otherwise, all nucleic acid sequences, such as primer sequences, are set forth herein in the direct 5' to 3'. Thus, for example, the primer sequence
AAATTGATCATGTACTAATCATAC (SEQ ID NO: 7) may equally be
35 written as 5'-AAATTGATCATGTACTAATCATAC-3' (SEQ ID NO: 7).

As described herein in detail, certain blood type alleles are less common and are typically referred to as "variants" (e.g. *RHD*r'^s*). Variant blood type alleles are in some cases referred to herein simply as "blood type variants".

Broadly, the present invention provides methods and products for the identification by molecular techniques of genetic variants *RHD*r'^s* or *RHD*r'^s*-like, which encode no D antigen (*D⁻*), an altered form of D antigen (partial D), altered forms of C (*C^{+W}*) and e antigens, expression of low-frequency VS antigen, no expression of V antigen, and/or no *hr^B* antigen (*hr^{B-}*) in blood cells. The present inventors have found that amplification of a region of intron 3 of the *RHD* locus may be *r'^s*-specific when primers as described in detail herein are employed. This *r'^s*-specific amplification enables discrimination between variants *RHD*r'^s* or *RHD*r'^s*-like and other *RHD*/*RHCE* hybrid exon 3 variants, including but not limited to *RHD*DIIIa*, *RHD*DIIIa IVS3+3100G*, *RHD*DIII_FN* and *RHD*DIVa-2*. In certain embodiments, the method of the invention provides considerable efficiency savings in comparison with, for example, full DNA sequencing, or genotyping of a large number of polymorphisms, or determining the phenotype by serological methods. Nevertheless, it is specifically contemplated that the method of the invention may, in some cases, involve DNA sequencing in order to genotype the sample obtained from the subject.

A wide variety of techniques are suitable and may be used in accordance with the present invention. Allele-specific oligonucleotides, for example, used in a competitive or non-competitive PCR (ASP henceforth), can also be used to detect genetic variants.

In accordance with any aspect of the present invention, functional segments or their portions may be amplified, for example by PCR, using as a template genomic DNA. Amplified functional segments or their portions can be labelled (e.g. with a fluorescent label) to allow for their detection, and optionally fragmented to facilitate their pairing with oligonucleotide probes.

In accordance with any aspect of the present invention, labelled and fragmented functional segments or their portions may be incubated under conditions that maximize the sensitivity and specificity of pairing with probes attached to the solid support. The presence of probe-paired functional segments or their portions may be determined indirectly from the measurement of label, usually a fluorochrome, attached to the solid support. This measurement is referred to herein as signal intensity. By way of example, the fluorescence emitted by the fluorochrome may be collected by means of a fluorescence detection device, such as a confocal scanner.

20

Examples

Discrimination among genetic variants that share a RHD/RHCE hybrid exon 3 but encode different forms of D Ag (Partial D Ag vs. No D Ag) and RhC Ag (Normal C Ag vs. Altered/Weakened C Ag, sometimes abbreviated as C^W)

25

The following example relates to a method of discriminating among RHD/RHCE hybrid exon 3 variants RHD*r's, RHD*DIIIa and RHD*DIVa-2 and a newly-discovered variant designated herein as "RHD*DIIIa IVS3+3100G".

30

Genomic DNA was extracted from nucleated cells in a blood sample by cell lysis. Extracted DNA was purified on an affinity column. Both, cell lysis and DNA purification are

performed with a QIAamp Blood kit (Qiagen, Germany) by following manufacturer protocols and recommendations. Purity of DNA was determined by spectrophotometry on a Nanodrop instrument (Nanodrop, DE). Only DNA solutions with an OD₂₆₀/OD₂₈₀ 1.8±0.2 proceeded to subsequent analysis.

r^s-specific PCR amplification

Purified DNA was used as a template for multiplexed Polymerase Chain Reaction (PCR) amplification of the gene segments of interest in a GeneAmp 9700 thermal cycler (Perkin-Elmer, CA). Primer sequences were as follows (5'-3'):

Table 1			
r ^s	Forward	AAATTTGATCATGTACTAATCATAAC	SEQ ID NO: 7
	Reverse	GGAAAAGGTTTGAGAGGAATTATATT	SEQ ID NO: 11

Cycling conditions consist of a denaturation/polymerase activation step at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 80 sec, and a final extension step at 72°C for 7 min.

Amplified DNA was separated by electrophoresis on a 2% agarose gel, stained with SYBR Safe dye (Invitrogen, OR), and photographed under UV illumination. Amplification vs. no amplification of a segment was determined visually by a trained laboratory technician.

25

The following samples of known identity were subjected to allele-specific PCR using the above r^s-specific primer pair: RhD⁺ (one sample), DIIIIa (17 samples), the newly-discovered variant "RHD*DIIIIa IVS3+3100G" (one sample), r^s (one sample), DIVa (3 samples) and a water negative control (one sample). As a positive control for PCR amplification, primers for

amplification of HgH were also employed. The PCR reaction products were run on an agarose gel (see Figure 1).

As shown in Figure 1, the upper band which indicates the presence of the HgH positive control PCR product is visible for all samples with the exception of the water negative control. The r'^s -specific band (lower band) is visible only on the r'^s sample (lower panel; lane 4). These results clearly demonstrate that r'^s -specific PCR is able to discriminate between r'^s and other *RHD/RHCE* hybrid exon 3 variants such as DIIIIa, DIVa and the newly-discovered variant "RHD*DIIIIa IVS3+3100G".

Multiplex PCR and probe-based genotyping

The following primers (5'-3') were used in multiplex with the r'^s primers described above:

Table 2			
RHCE c	Forward	TGGGCTTCCTCACCTCAAA	SEQ ID NO: 12
	Reverse	TGATGACCACCTTCCCAGG	SEQ ID NO: 13
RHCE C	Forward	GGCCACCACCATTTGAA	SEQ ID NO: 14
	Reverse	GGTAGCAGGCGTCTGTAAAAA	SEQ ID NO: 15

Table 3			
RHCE Exon 1	Forward	CATAGACAGGCCAGCACAG	SEQ ID NO: 16
	Reverse	TGCCCCTGGAGAACCAT	SEQ ID NO: 17
RHCE Exon 5	Forward	AAATTAAATAAGCATTTGACCATC	SEQ ID NO: 18
	Reverse	CCTGAGATGGCTGTCACCAC	SEQ ID NO: 19
RHCE Exon 7	Forward	ACATGCCATTGCCGTTTC	SEQ ID NO: 20
	Reverse	TCTCACCTGCCAATCTGCT	SEQ ID NO: 21

The following probe sequence was used to determine the presence of absence of the intron 3 amplicon:

CAAAAGCTGATATGTCATGTTTAGTTA (SEQ ID NO: 24)

A single probe may be used to determine presence or absence of the intron 3 amplicon because the PCR is allele-specific (due to the design of the forward primer). In other words, it is not necessary for the detection probe itself to interrogate a sequence that is specific to r'^s.

As a non-limiting example, the procedure for Luminex® xMAP® - based detection employed herein, was as follows:

DNA SAMPLE PREPARATION

- Human genomic DNA was extracted from whole blood with EDTA as anti-coagulant. Genomic DNA extraction was carried out in the pre-PCR area.
- Genomic DNA input was 100 ng. Genomic DNA purity (OD₂₆₀/OD₂₈₀ ratio) was in the 1.63-1.95 range.
- Genomic DNA samples were stored frozen at -15 to -25 °C for up to one month. Multiple freeze/thaw cycles were avoided.

The following procedure was applied:

PROCEDURE

- The ID-CORE XT™ protocol consists of 4 steps:



- Each batch of samples was processed with a negative control (molecular biology grade water that is known to be free of any DNA contamination).

I. AMPLIFICATION

- Work in the pre-PCR area and use aerosol-barrier tips. Use a new tip for each DNA sample.
- Briefly vortex and spin tubes before use.
- It is not necessary to set up the PCR reaction on ice.

- Turn on the thermocycler.
- Take the following reagents out of the refrigerator and freezer: ID-CORE XT™ PCR Master Mix and HotStarTaq® DNA polymerase, respectively.
Note: HotStarTaq® DNA polymerase must be removed from the freezer immediately before use and returned to the freezer immediately after use. Alternatively use ice or a microtube cooler. The stock enzyme should be mixed by gently flicking the tube.
- Set up the ID-CORE XT™ PCR reaction mix as shown in the following table (all volumes in µL):

Number of samples	1	8	24	48
ID-CORE XT™ PCR Master Mix	22.5	180	540	1080
HotStarTaq® DNA Polymerase (5 U/µL)	0.5	4	12	24

NOTE: the stated volumes already include an excess to account for pipetting error.

4. Vortex and spin the ID-CORE XT™ PCR reaction mix.
5. Immediately dispense 20 µL per sample into the wells of a 96-well PCR plate.
6. Add 5 µL of sample DNA, positive control DNA and negative control to the appropriate wells in this order.
7. Seal the plate with the adhesive film.
- Work in the post-PCR area.
8. Spin down the PCR plate to collect the liquid at the bottom of the wells.
9. Verify that all wells are properly sealed and that the lid of the thermocycler has reached the pre-set temperature.
10. Place the plate in the thermocycler. Place the compression pad over the plate.
11. Close the thermocycler lid and start the **ID XT PCR** amplification program.

	Temperature	Time	Cycles
Polymerase Activation	95°C	15 min	1
Denaturation	95°C	30 s	40
Annealing	60°C	30 s	40
Extension	72°C	80 s	40
Final extension	72°C	7 min	1
Hold	4°C	∞	1
Amplification reaction volume: 25 µL			

II. HYBRIDIZATION

- Work in the post-PCR area. Use a new tip for each sample.
 - Whenever necessary during dispensing steps, dry the surface of the Costar plate using a low-lint wipe.
 - **Handling and storage of the ID-CORE XT™ Beads Master Mix:**
 - a. Beads settle and aggregate with time. Before pipetting, bring them to a homogeneous suspension by vigorous vortexing for 30 seconds.
 - b. During ID-CORE XT™ Beads Master Mix dispensing, keep beads in suspension by vortexing frequently (every 8 samples).
 - c. Do not centrifuge the plate once the ID-CORE XT™ Beads Master Mix has been dispensed to avoid bead aggregation.
 - d. Beads contain an internal fluorescent dye. To avoid photobleaching, protect them from light during storage and usage.
 - **Luminex® system preparation:**
 - a. Turn on the Luminex® 100/200™ system between 30 minutes and 4 hours before assaying the samples.
 - b. Set the XYP instrument heater temperature at 52 °C and verify that the heater block is on the plate holder.
 - c. Perform Luminex® 100/200™ daily startup maintenance.
1. Turn on the thermocycler.
 2. Take the ID-CORE XT™ Beads Master Mix out of the refrigerator and let equilibrate to room temperature.
 3. Vortex the ID-CORE XT™ Beads Master Mix for 30 seconds.
 4. Dispense 46 µL of the ID-CORE XT™ Beads Master Mix into a Costar plate. Avoid bubble formation during dispensing.
 5. Spin down the PCR plate to collect the liquid at the bottom of the wells.
 6. Add 4 µL of PCR product into each well of the Costar plate.
 7. Mix gently by pipetting up and down several times. Avoid bubble formation during dispensing and pipetting.
 8. Seal the plate with a BioRad sealing film.

9. Verify that all wells are properly sealed and that the lid of the thermocycler has reached the pre-set temperature.
10. Place the plate and the corresponding silicone compression mats on the thermocycler block.
- 5 11. Close the thermocycler lid and start the **ID XT HYB** hybridization program.

	Temperature	Time
Denaturation	95°C	2 min
Hybridization	52°C	30 min
Hold	52°C	∞

12. During the hybridization step prepare the labeling mix (see Labeling section) and perform the "Create a New Batch" step in the Luminex[®] software (see Data Acquisition section).

Note: PCR products must be labeled immediately after hybridization. The process cannot be stopped after the hybridization step.

15 III. LABELING

- Work in the post-PCR area.
1. Bring the following reagents to room temperature: SAPE and SAPE Dilution Buffer.
 2. Prepare the labeling mix as shown in the following table (all volumes in μL):

Number of samples	1	8	24	48
SAPE Dilution Buffer	87	696	2088	4176
SAPE	4.6	36.8	110	221

NOTE: the stated volumes already include an excess to account for pipetting error.

3. Vortex the labeling mix and keep it protected from light at room temperature.
4. At the 52 °C hold step, open the thermocycler lid and remove the compression pads and the sealing film with care, while keeping the plate on the thermocycler for the labeling step.
5. Dispense 80 μL of the labeling mix into each well of the hybridization plate and mix gently by pipetting up and down once.
6. Analyze the samples using the Luminex[®] system immediately after labeling.

30 IV. DATA ACQUISITION AND ANALYSIS

Luminex[®] system preparation:

- Refer to Luminex[®] User's Manual (Luminex[®] 100™ IS 2.3. User Manual, Luminex[®] 200™ User Manual, or xPONENT[®] 3.1 Software Manual) for instrument preparation and operation, including daily startup, calibration, maintenance, and shutdown procedures.
1. Select **Create a New Batch from an existing Protocol** in the HOME page (for Luminex[®] IS 2.3 software) or Batches tab option (for Luminex[®] xPONENT[®] 3.1 software) and select the corresponding protocol.
 2. Enter the sample IDs.
 3. Follow the stepwise instructions that appear on the screen for creating batches. For further instructions on creating batches and multibatches, refer to the corresponding Luminex[®] User's Manual.
 4. Select the **Eject icon** to eject the plate holder. Place the hybridization plate in the Luminex[®] XYP instrument heater block present on the plate holder.
 5. Select the **Retract icon**. The samples are now ready to be analyzed.
 6. Start the analysis process by clicking the **Start icon** (for Luminex[®] IS 2.3 software) or the **Run icon** (for Luminex[®] xPONENT[®] 3.1 software).
 7. After a batch is complete, the data are exported as a Comma Separated Values (csv) file. This file is saved in a folder with the Batch Name entered into the Luminex[®] software.
 8. Perform Luminex[®] 100/200™ daily shutdown maintenance.
 9. The system can be turned off at this point if it is not going to be used for the remainder of the day.

Proprietary software (Progenika Biopharma S.A.) is used to transform fluorescence intensity values for the particular allelic variants detected, singly or in combination, into
5 blood group genotypes, and from genotypes into predicted blood group phenotypes.

Figure 2 shows the output and graphical representation (vertical axis is "normalised intensity") of a Luminex assay
10 for r'^s samples, the newly-discovered variant "RHD*DIIIa IVS3+3100G", and other non- r'^s samples following multiplex PCR using the r'^s -specific primers shown in Table 1 and one or more primer pairs shown in Tables 2 and 3. The data shown in Figure 2 confirm that the r'^s samples are successfully
15 amplified by PCR *in multiplex* and that the Luminex normalised intensity signal is markedly higher for the r'^s samples than for non- r'^s samples, including the newly-discovered variant "RHD*DIIIa IVS3+3100G". Indeed, the separation of normalised signal (greater than 6-fold higher for r'^s samples) provides
20 clear unambiguous discrimination between r'^s samples and closely related non- r'^s samples, despite the relatively less stringent PCR conditions utilised for multiplex PCR. These results therefore underscore the surprising adaptability of the primers of the present invention, in particular the
25 forward primer as set forth in Table 1, for use in multiplex allele-specific PCR for identification of r'^s samples.

As shown in Figure 3, the ability of the r'^s -specific primers of the present invention to work in multiplex with primers for
30 amplification of other segments of the *RHD* gene and/or *RHCE* gene loci allows for efficient use of sample, e.g., to interrogate other polymorphisms relevant to the classification of blood type variants. In particular, segments including all or part of one or more *RHD* and/or *RHCE* exons and/or introns
35 may be amplified and the amplification products interrogated by allele-specific probes in order to detect the presence or

absence of, and/or identity of: an RHCE c; an RHCE C; RHCE exon 1; RHCE exon 5; RHCE exon 7; an RHD/RHCE hybrid exon 3 allele; a G/C polymorphism at position 602 of RHD exon 4; a C/G polymorphism at position 1048 of RHD exon 7; presence or
 5 absence of an RHCE*C allele; a G/T polymorphism at position 1006 of RHCE exon 7; and/or a G/A polymorphism at position 3100 of RHD intron 3.

Comparison with a previously-described primer

10

Silvy et al., British Journal of Haematology, (2012 Dec 30) doi: 10.1111/bjh. 12179 [Epub ahead of print], describe PCR amplification of a portion of intron 3 of the RHD gene. The amplification is said to be selective for r'^s (referred to
 15 therein as *(C)ce^s type 1 haplotype*. The primer pair employed by Silvy et al. included (see Fig. 1 and Supporting information Appendix S1 of Silvy et al.):

a forward primer ("RHD-for") that is specific for RHD-containing sequence having the sequence (5' to 3'):

GCAAATATGGAAATTTGATCATGTA (SEQ ID NO: 25);

a reverse primer ("RHCE-rev") that is specific for RHCE-containing sequence having the sequence (5' to 3'):

25 CTTAATCACAAGATTATTTTCAGAATCTAAC (SEQ ID NO: 26); and

a control reverse primer ("RHDCE-rev") that hybridised to both RHD-containing and RHCE-containing sequence having the sequence (5' to 3'):

30 GGGCCTTGGACAAGTTGTTA (SEQ ID NO: 27).

The method taught by Silvy et al. relies on the amplification of a 240bp amplicon by the primer pair RHD-for and RHCE-rev being specific for r'^s , as compared with, e.g., RHD*DIVa2 and
 35 RHD*DIIIa (see Silvy et al., Fig. 1(B)). Although Silvy et al. report amplification of 52 *(C)ce^s type 1* haplotype samples

and negative amplification of all other samples in a 118 sample set, regardless of the *RHD* and *RHCE* alleles present, this method suffers from a number of important drawbacks. In particular, the specificity is achieved by means of the
5 intersection of the two specific primers, *RHD*-for and *RHCE*-rev, rather than either primer itself being specific for r'^s . This means that, for example, the *RHD*-for primer also hybridises to other *RHD* sequence-containing template, including *RHD***DIIIIa* and *RHD***DIVa2*.

10

As described below, the present inventors have found that the method and primers of Silvy et al. result in poor discrimination of r'^s from, e.g., *RHD***DIIIIa* and *RHD***DIVa2* under the less stringent PCR conditions typically employed in
15 *multiplex* PCR.

20

Moreover, Silvy et al. teach that the IVS3+3100a>g SNP is specific to *(C)ce^s type 1* haplotype (i.e. r'^s). However, the present inventors have now discovered that this is not the
case. In particular, a variant provisionally termed "RHD**DIIIIa* IVS3+3100G" has been identified herein, which is not r'^s , but actually a variant of *RHD***DIIIIa* having the same intron 3 position 3100 A to G polymorphism. Therefore,
25 reliance on the presence of the intron 3 position 3100 A to G polymorphism in order to identify r'^s can and will lead to false positive results where a "RHD**DIIIIa* IVS3+3100G" sample would be incorrectly assigned as an r'^s sample. This is clinically relevant because "RHD**DIIIIa* IVS3+3100G" is C-serologically, rather than C^W. Thus, reliance on the presence
30 of intron 3 position 3100 A to G polymorphism in order to identify r'^s is undesirable.

35

As shown in the alignment of Figure 7, the forward primer of Silvy et al. (*RHD*-for) will anneal to all of the following:
RHD, r'^s , *DIVa*, *DIIIIa* and the newly-described "RHD**DIIIIa* IVS3+3100G" sample. Although the intersection of sequences that match the *RHD*-for and *RHCE*-rev primers of Silvy et al.

can be expected to be confined to r'^s , the amplification will only be specific under stringent conditions. Under less stringent conditions, the forward primer of Silvy *et al.* results in a number of non-specific amplifications. In contrast, the forward primer of the present invention is specific only to r'^s .

In the process of designing the forward primers of the present invention, the inventors sought to mitigate the drawbacks associated with the forward primer disclosed in Silvy *et al.*

Figure 4 shows the results of using the RHD-for primer of Silvy *et al.* having the sequence GCAAATATGGAAATTTGATCATGTA (SEQ ID NO: 25) together with the RHCE-specific reverse primer having the sequence GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11) under PCR conditions suitable for multiplex PCR. The upper band shows the amplification control (HgH), while the lower band is, theoretically, an amplicon which is specific for r'^s . Lane 1 shows that the sample "BGG-10-656", which is a known r'^s sample is indeed amplified by the above primer combination. However, lane 2, which is the sample "BGG-10-628", being a known DIIIIa sample, and lane 3, which is "L22", a known RhD+ sample, both exhibit positive amplification of the supposedly r'^s -specific amplicon. These results demonstrate that the forward primer RHD-for of Silvy *et al.* results in false positive results under the conditions tested. These results may be contrasted with the results obtained when using a forward primer of the present invention, in which case no such false positive results were found.

The results shown in Figure 5 likewise demonstrate that the forward primer of Silvy *et al.* results in non-specific amplification and therefore false positive results. In particular, samples which are known DIVa, DIIIIa and RhD+ all exhibited Luminex mean fluorescence intensity signal above the threshold line to be classified as r'^s (as did the samples known to be r'^s). Samples known to be RhD- did not exhibit

positive results in this experiment. These data can be contrasted with the r'^S -specific results shown in Figure 2, wherein a forward primer of the present invention was employed.

5

Variant primers of the invention

The present inventors sought to provide primers that contain sequence modifications compared with the "reference" forward primer having the sequence AAATTTGATCATGTACTAATCATAC (SEQ ID NO: 7). By way of example, the following changes were made to the "reference" forward primer sequence, while retaining r'^S -specificity.

In a first modification, the reference primer was shortened by deleting 10 nucleotides at the 5' end, in order to decrease annealing temperature for the PCR. This modification results in the forward primer of the invention having the following nucleotide sequence: ATGTACTAATCATAC (SEQ ID NO: 8).

In a second modification, the reference primer was altered by introducing one nucleotide substitution (replacing a C with a G) to increase specificity. This modification results in the forward primer of the invention having the following nucleotide sequence (the substitution position being underlined):

AAATTTGATCATGTACTAATGATAC (SEQ ID NO: 9).

In a third modification, the reference primer was altered by introducing one nucleotide substitution that differs from the substitution described above in relation to the second modification. This substitution was also a replacement of a C with a G to increase specificity, but was at a different position. This modification results in the forward primer of the invention having the following nucleotide sequence (the substitution position being underlined): AAATTTGATCATGTAGTAATCATAC (SEQ ID NO: 10).

35

Figure 6 shows PCR results obtained using: the reference forward primer of the invention AAATTTGATCATGTACTAATCATAC (SEQ ID NO: 7); the "inspecific" forward primer of Silvy et al. GCAAATATGGAAATTTGATCATGTA (SEQ ID NO: 25); the variant primer

of the invention having modification 1 ATGTACTAATCATAC (SEQ ID NO: 8); the variant primer of the invention having modification 2 AAATTTGATCATGTACTAATGATAC (SEQ ID NO: 9); the variant primer of the invention having modification 3
 5 AAATTTGATCATGTAGTAATCATAC (SEQ ID NO: 10); and a modified version of the "inspecific" forward primer of Silvy *et al.* having the sequence GGCAAATATGGAAATTTGATCATGTA (SEQ ID NO: 28). In all cases the reverse primer used was GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11).

10

Figure 6 shows the PCR amplification results of the above-described primers. The top band is a control and the lower band is intended to be an r'^S -specific amplification product, to the extent that the primers used in fact amplify in an r'^S -
 15 specific manner under the conditions employed. As shown in Figure 6, the reference forward primer (upper panel) exhibits r'^S -specific amplification (positive for three known r' s samples (lanes 1, 2 and 6) and negative for non- r'^S (RhD+ in lane 3, RhD- in lane 4 and DIIIIa in lane 5). The negative
 20 control of lane 7 shows no amplification.

The inspecific forward primer in box 1 (second panel down; left hand side) exhibits positive r'^S band presence of not only r'^S samples, but also RhD+ and DIIIIa (see lanes 3 and 5), thus
 25 confirming false positive results.

The primer modification 1 results are shown in box 2 (spanning the second panel, right hand side and third panel, left hand side). The r'^S band, although somewhat less bright than the
 30 upper control band, is clearly visible in all r'^S samples (lanes 1, 2 and 6), while all other lanes (RhD+, RhD-, DIIIIa and negative control) are without any r'^S band. These results confirm that the modification to the reference forward primer (shortening it by deleting the 5' 10 nucleotides) nevertheless
 35 retains r'^S specificity.

The primer modification 2 results are shown in box 3 (third panel, centre). The r'^s band is clearly visible in all r'^s samples (lanes 1, 2 and 6), while all other lanes (RhD+, RhD-, DIIa and negative control) are without any r'^s band. These results confirm that the modification to the reference forward primer (C to G substitution to generate a single base mismatch) nevertheless retains or even increases r'^s specificity.

The primer modification 3 results are shown in box 4 (spanning the third panel, right hand side and fourth panel, left hand side). The r'^s band is clearly visible in all r'^s samples (lanes 1, 2 and 6), while all other lanes (RhD+, RhD-, DIIa and negative control) are without any r'^s band. These results confirm that the modification to the reference forward primer (C to G substitution to generate a single base mismatch) nevertheless retains or even increases r'^s specificity.

The inspecific forward primer in box 5 (fourth panel right hand side) exhibits positive r'^s band presence of not only r'^s samples, but also RhD+ and DIIa (see lanes 3 and 5), thus confirming false positive results. There is even a faint r'^s band visible in the RhD- sample (lane 4).

Taken together, the present results demonstrate that the reference forward primer of the present invention exhibits superior performance compared with that of the Silvy *et al.* RHD-for primer and that the forward primer of the present invention is tolerant to a number of sequence modifications, including a 5' truncation of 10 nucleotides (i.e. reducing the value of n in the formula X-Y-Z as defined herein) and nucleotide substitutions that result in mismatches with the target sequence (i.e. selection of S_1 , S_2 and/or changes reflected in the substitution possibilities of X_2 and/or Y_2 of the formula X-Y-Z as defined herein. The present invention therefore provides, *inter alia*, a genus of related forward PCR primer designs that address the need for r'^s -specific

amplification and mitigate the drawbacks associated with a previously-described primer.

Equivalents

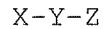
5

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as
10 a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing
15 description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

All references, including patent documents, disclosed herein
20 are incorporated by reference in their entirety for all purposes, particularly for the disclosure referenced herein.

Claims

1. An oligonucleotide polymerase chain reaction (PCR) primer, the nucleotide sequence of which is of the formula:



wherein:

X is X_1 or X_2 , wherein:

X_1 is the final n nucleotides of the nucleotide sequence ATATGGAAATTTGATCATGT (SEQ ID NO: 1), wherein n is a number between 0 and 20, inclusive; and

X_2 is a variant of X_1 differing by no more than one nucleotide substitution;

Y is Y_1 or Y_2 , wherein:

Y_1 is the nucleotide sequence **A** S_1 TAATS S_2 AT**A**C (SEQ ID NO: 2), wherein S_1 and S_2 are independently selected from G and C; and

Y_2 is a variant of Y_1 differing from Y_1 by no more than one nucleotide substitution, provided that said nucleotide substitution is not a substitution of the first **A** or final **C** of Y_1 ;

Z is the first m nucleotides in the nucleotide sequence TAAAG, wherein m is a number between 0 and 5, inclusive.

2. A primer according to claim 1, wherein the nucleotide sequence of said primer consists of a nucleotide sequence selected from the group consisting of:

- (i) AAATTTGATCATGTAGTAATCATAC (SEQ ID NO: 10);
- (ii) AAATTTGATCATGTACTAATCATAC (SEQ ID NO: 7);
- (iii) ATGTACTAATCATAC (SEQ ID NO: 8); and
- (iv) AAATTTGATCATGTACTAATGATAC (SEQ ID NO: 9).

3. A primer according to claim 1, wherein X_1 is the nucleotide sequence of AAATTTGATCATGT (SEQ ID NO: 3) or ATGT.

4. A primer according to claim 1 or claim 3, wherein Y_1 is selected from the group consisting of: ACTAATCATAC (SEQ ID NO: 4); ACTAATGATAC (SEQ ID NO: 5); and AGTAATCATAC (SEQ ID NO: 6).

5. A primer according to any one of claims 1 and 3 to 4, wherein m is 0.

6. A primer according to any one of claims 1 and 3 to 5, wherein the primer is between 15 and 25 nucleotides in length.

7. A primer according to any one of the preceding claims, wherein said primer is suitable for use as a forward PCR primer in a PCR amplification of a portion of the r'^s allele of intron 3 of the RHD gene.

8. A plurality of oligonucleotide primers comprising:

(i) an oligonucleotide primer as defined in any one of claims 1 to 7; and

(ii) at least one primer selected from the group consisting of:

(a) an oligonucleotide primer of between 26 and 30 nucleotides in length comprising the nucleotide sequence GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11) or a variant thereof differing by not more than 3 nucleotide substitutions from the nucleotide sequence of SEQ ID NO: 11, provided that said substitutions do not include substitution of the final T of the nucleotide sequence of SEQ ID NO: 11;

(b) an oligonucleotide primer consisting of the nucleotide sequence GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11);

(c) the RHCE c forward primer consisting of the nucleotide sequence TGGGCTTCCTCACCTCAAA (SEQ ID NO: 12);

- (d) the RHCE c reverse primer consisting of the nucleotide sequence TGATGACCACCTTCCCAGG (SEQ ID NO: 13);
- (e) the RHCE C forward primer consisting of the nucleotide sequence GGCCACCACCATTTGAA (SEQ ID NO: 14);
- (f) the RHCE C reverse primer consisting of the nucleotide sequence GGTAGCAGGCGTCTGTAAAAA (SEQ ID NO: 15);
- (g) the RHCE exon 1 forward primer consisting of the nucleotide sequence CATAGACAGGCCAGCACAG (SEQ ID NO: 16);
- (h) the RHCE exon 1 reverse primer consisting of the nucleotide sequence TGCCCCTGGAGAACCAT (SEQ ID NO: 17);
- (i) the RHCE exon 5 forward primer consisting of the nucleotide sequence AAATTAAAATAAGCATTTGACCATC (SEQ ID NO: 18);
- (j) the RHCE exon 5 reverse primer consisting of the nucleotide sequence CCTGAGATGGCTGTCACCAC (SEQ ID NO: 19);
- (k) the RHCE exon 7 forward primer consisting of the nucleotide sequence ACATGCCATTGCCGTTT (SEQ ID NO: 20); and
- (l) the RHCE exon 7 reverse primer consisting of the nucleotide sequence TCTCACCTGCCAATCTGCT (SEQ ID NO: 21).

9. A plurality of oligonucleotide primers according to claim 8, wherein said primers comprise at least (i) and (ii)(a).

10. A plurality of oligonucleotide primers according to claim 9, wherein said primers comprise the primer pair:

AAATTTGATCATGTACTAATCATAC (SEQ ID NO: 7); and
GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11).

11. A kit for assessing a subject's blood type, said kit comprising:

a plurality of primers as defined in any one claims 8 to 10;

optionally, one or more probes and/or primers that span one or more polymorphic positions in intron 3, exon 3, exon 4, intron 7 and/or exon 7 of the *RHD* gene locus; and

optionally, one or more probes and/or primers that span one or more polymorphic positions in exon 7 of the *RHCE* gene locus.

12. A system for use in determining a subject's blood type, the system comprising:

a kit as defined in claim 11; and

at least one detector arranged to detect a signal from detectably labelled DNA obtained from said subject or a detectably labelled amplicon produced by PCR amplification carried out on DNA obtained from said subject;

at least one controller in communication with the at least one detector, the controller being programmed with computer-readable instructions to transform said signal into predicted blood type haplotypes, and optionally, to transform said predicted blood type haplotypes into a predicted blood type phenotype.

13. A method for determining the presence or absence of, or for discriminating between, blood type alleles in a DNA-containing sample, which method comprises amplification by polymerase chain reaction (PCR) of at least a portion of intron 3 of the *RHD* gene, wherein said PCR employs at least a forward primer and a reverse primer each capable of hybridising to the portion of r'^S intron 3 sequence set forth in SEQ ID NO: 31, or its complement, and wherein said forward primer is as defined in any one of claims 1 to 7.

14. A method according to claim 13, wherein said blood type alleles are alleles that comprise an *RHD/RHCE* hybrid exon 3.

15. A method according to claim 13 or claim 14, wherein said blood type alleles are selected from the group consisting of:

*RHD** r'^S ; *RHD** r'^S -like; *RHD** r'^S Type 1; *RHD** r'^S Type 2;
*RHD**DIIIIa; *RHD**DIIIIa IVS3+3100G; *RHD**DIII_FN; *RHD**DIVa-2;
*RHD**DIVa; *RHD**DIII-type4; *RHD**DIII-type6; *RHD**DIII-type7;
*RHD**DIII-type8; *RHCE** ce^S ; *RHCE** ce^S1006T ; *RHCE** ce^S1006C ;
*RHCE** $ce733G$; *RHCE** $ce48C,733G,1025T$; *RHCE** $ce48C,697G,733G$;
*RHCE** $ce340T,733G$; and *RHCE** $ce48C,733G,748A$.

16. A method according to any one of claims 13 to 15, wherein said PCR amplifies r'^S , but does not amplify one or more of: RHD; RHCE*ce; RHD*DIIIIa; RHD*DIIIIa IVS3+3100G; and RHD*DIVa.

17. A method according to claim 16, wherein said PCR amplifies r'^S , but does not amplify any of RHD; RHCE*ce; RHD*DIIIIa; RHD*DIIIIa IVS3+3100G; and RHD*DIVa.

18. A method according to any one of claims 13 to 17, wherein the method is for discriminating r'^S from RHD*DIIIIa IVS3+3100G.

19. A method according to any one of claims 13 to 18, wherein said forward primer consists of the nucleotide sequence AAATTTGATCATGTAGTAATCATAC (SEQ ID NO: 10) or AAATTTGATCATGTACTAATCATAC (SEQ ID NO: 7).

20. A method according to any one of claims 13 to 19, wherein said reverse primer hybridises to a portion of intron 3 of r'^S , or its complement, which portion includes at least one position of single nucleotide polymorphism (SNP) that differs between r'^S and RHD.

21. A method according to claim 20, wherein said position of SNP comprises the G/A polymorphism at position 3189 of intron 3 of the *RHD* gene, said numbering being as shown in Figure 7.

22. A method according to any one of claims 13 to 21, wherein said reverse primer is of between 26 and 30 nucleotides in length and comprises the nucleotide sequence GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11).

23. A method according to claim 22, wherein said reverse primer consists of the nucleotide sequence GGAAAAGGTTTGAGAGGAATTATATT (SEQ ID NO: 11).

24. A method according to any one of claims 13 to 23, wherein said PCR is multiplex PCR, which further comprises

amplification of one or more other *RHD* and/or *RHCE* gene segments.

25. A method according to claim 24, wherein said one or more other *RHD* and/or *RHCE* gene segments are selected from the group consisting of: *RHCE* c; *RHCE* C; *RHCE* exon 1; *RHCE* exon 5; and *RHCE* exon 7.

26. A method according to claim 24 or claim 25, wherein said multiplex PCR further comprises employing 1, 2, 3, 4, 5, 6, 7, 8, 9 or all 10 of the following primers:

(i) the *RHCE* c forward primer consisting of the nucleotide sequence TGGGCTTCCTCACCTCAAA (SEQ ID NO: 12);

(ii) the *RHCE* c reverse primer consisting of the nucleotide sequence TGATGACCACCTTCCCAGG (SEQ ID NO: 13);

(iii) the *RHCE* C forward primer consisting of the nucleotide sequence GGCCACCACCATTTGAA (SEQ ID NO: 14);

(iv) the *RHCE* C reverse primer consisting of the nucleotide sequence GGTAGCAGGCGTCTGTAAAAA (SEQ ID NO: 15);

(v) the *RHCE* exon 1 forward primer consisting of the nucleotide sequence CATAGACAGGCCAGCACAG (SEQ ID NO: 16);

(vi) the *RHCE* exon 1 reverse primer consisting of the nucleotide sequence TGCCCCTGGAGAACCAT (SEQ ID NO: 17);

(vii) the *RHCE* exon 5 forward primer consisting of the nucleotide sequence AAATTAAAATAAGCATTTGACCATC (SEQ ID NO: 18);

(viii) the *RHCE* exon 5 reverse primer consisting of the nucleotide sequence CCTGAGATGGCTGTCACCAC (SEQ ID NO: 19);

(ix) the *RHCE* exon 7 forward primer consisting of the nucleotide sequence ACATGCCATTGCCGTTC (SEQ ID NO: 20); and

(x) the *RHCE* exon 7 reverse primer consisting of the nucleotide sequence TCTCACCTGCCAATCTGCT (SEQ ID NO: 21).

27. A method according to claim 26, wherein said multiplex PCR further comprises employing one or more pairs of primers as set forth in claim 26, said primers being paired as follows: (i) with (ii); (iii) with (iv); (v) with (vi); (vii) with (viii); and (ix) with (x).
28. A method according to any one of claims 13 to 27, wherein the sample is a sample which has previously been determined to comprise an *RHD*-*RHCE* hybrid exon 3.
29. A method according to any one of claims 13 to 27, wherein the method further comprises a preceding step of determining whether the sample contains an *RHD*-*RHCE* hybrid exon 3.
30. A method according to any one of claims 13 to 29, wherein the method further comprises determining the presence or absence of an *RHCE**C allele in the sample.
31. A method according to any one of claims 13 to 30, wherein the method further comprises genotyping the sample at one or more positions of single nucleotide polymorphism (SNP) in the *RHD* and/or *RHCE* gene loci.
32. A method according to claim 31, wherein the method comprises genotyping the sample at position 410 of the *RHD* exon 3.
33. A method according to claim 31 or claim 32, wherein the method comprises genotyping the sample at position 602 of the *RHD* exon 4.
34. A method according to any one of claims 31 to 33, wherein the method comprises genotyping the sample at position 1048 of the *RHD* exon 7.

35. A method according to any one of claims 31 to 34, wherein the method comprises genotyping the sample at position 1006 of the *RHCE* exon 7.

36. A method according to any one of claims 31 to 35, wherein the method comprises genotyping the sample at position 3100 of the *RHD* intron 3.

37. A method according to any one of claims 13 to 36, wherein the method comprises genotyping not more than 50, 40, 30, 25, 20, 15, or not more than 10, single nucleotide polymorphic positions in the *RHD* gene locus and/or the *RHCE* gene locus.

38. A method according to any one of claims 13 to 37, wherein the method further comprises predicting an *RHD* phenotype and/or an *RHCE* phenotype for the subject based on, at least, the degree of amplification by PCR of said at least one portion of intron 3 of the *RHD* gene.

39. A method according to claim 38, wherein the phenotype prediction is further based on:

- (i) the presence or absence of an *RHD*/*RHCE* hybrid exon 3;
- (ii) the identity of one or both alleles present at position 602 of the *RHD* exon 4;
- (iii) the identity of one or both alleles present at position 1048 of the *RHD* exon 7;
- (iv) the presence or absence of an *RHCE**C allele in the *RHCE* gene locus;
- (v) the identity of one or both alleles present at position 1006 of the *RHCE* exon 7; and/or
- (vi) the identity of one or both alleles present at position 3100 of the *RHD* intron 3.

40. A method according to claim 38 or claim 39, wherein the sample is predicted to have C^{+w} serology.

41. A method according to any one of claims 13 to 40, wherein the subject is undergoing, or is a candidate for, blood transfusion or bone marrow transplantation.

42. A method according to any one of claims 13 to 41, wherein the subject has sickle cell disease (SCD) or Thalassemia major.

43. A method according to any one of claims 13 to 42, wherein the subject has non-Caucasian ancestry.

44. A method according to claim 43, wherein the subject has African ancestry.

45. A method according to any one of claims 13 to 44, wherein the sample comprises a blood sample.

46. A method according to any one of claims 13 to 45, wherein the sample is subjected to one or more treatments to extract a nucleic acid prior to or as part of said amplification by PCR.

47. A method according to any one of claims 13 to 46, wherein the method comprises Allele-Specific Polymerase Chain Reaction (ASP).

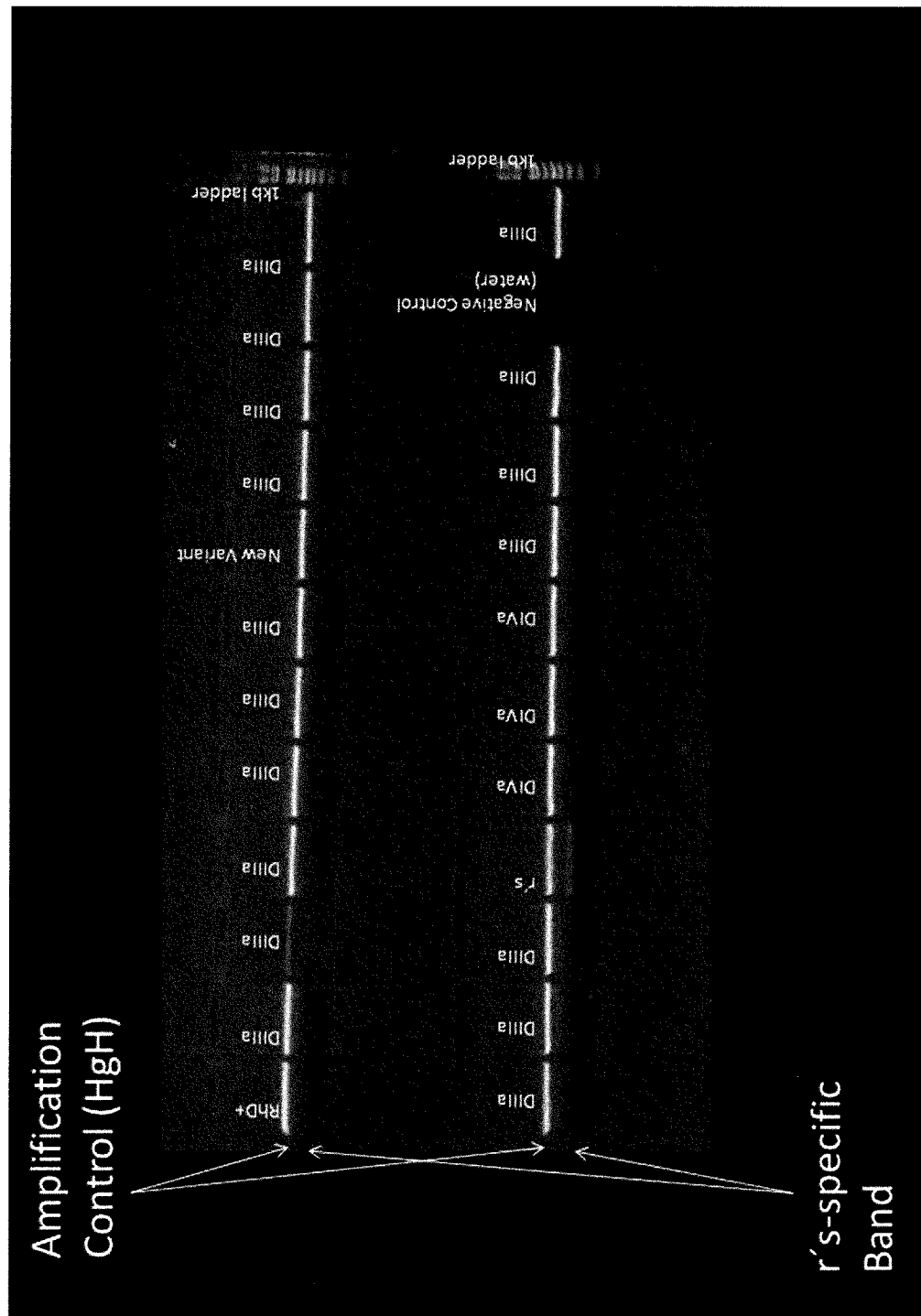
48. A method according to any one of claims 13 to 47, wherein the method comprises labelling a nucleic acid obtained from the sample or labelling the amplicon produced by said PCR amplification.

49. A method according to any one of claims 13 to 48, wherein the method further comprises carrying out serological analysis on a blood sample that has been obtained from the subject.

50. A method of blood matching, the method comprising:
carrying out the method according to any one of claims 13 to 49 on a recipient sample from a recipient subject in need

of donor blood and on a donor sample from a potential donor subject;

comparing the blood type alleles present in the recipient sample with those present in the donor subject and thereby determining the compatibility of the recipient subject to receive blood from the potential donor subject.



INTRON 03

Sample	CSV	MFI 1	MFI 2	Dist 13	Dist 2	Ratio	Cluster	Window	QCratio	EEGMFI	EE12MFI	PS Comment
118/140BA_BCGEN693	C0_207_2_150113_ID4663 CSV	5591	5685	0	0	9.258	P	5	1	0	0	
118/140BA_BGG-10-1643	C0_207_2_150113_ID4663 CSV	7952	8187	0	0	8.621	P	5	1	0	0	
118/140BA_BGGEN612-2	C0_207_2_150113_ID4663 CSV	1092	1076	0	0	0.869	A	5.2	1	0	0	
118/140BA_BC0106	C0_207_2_150113_ID4663 CSV	600	676	0	0	0.529	A	2.2	0	0	0	
118/140BA_NA22641	C0_207_2_150113_ID4663 CSV	758	948	0	0	0.656	A	3.2	0	0	0	
118/140BA_BGG-10-425	C0_207_2_150113_ID4663 CSV	1158	1213	0.04	0.086	0.977	AP		1	0	0	Indeter- -minate genotype

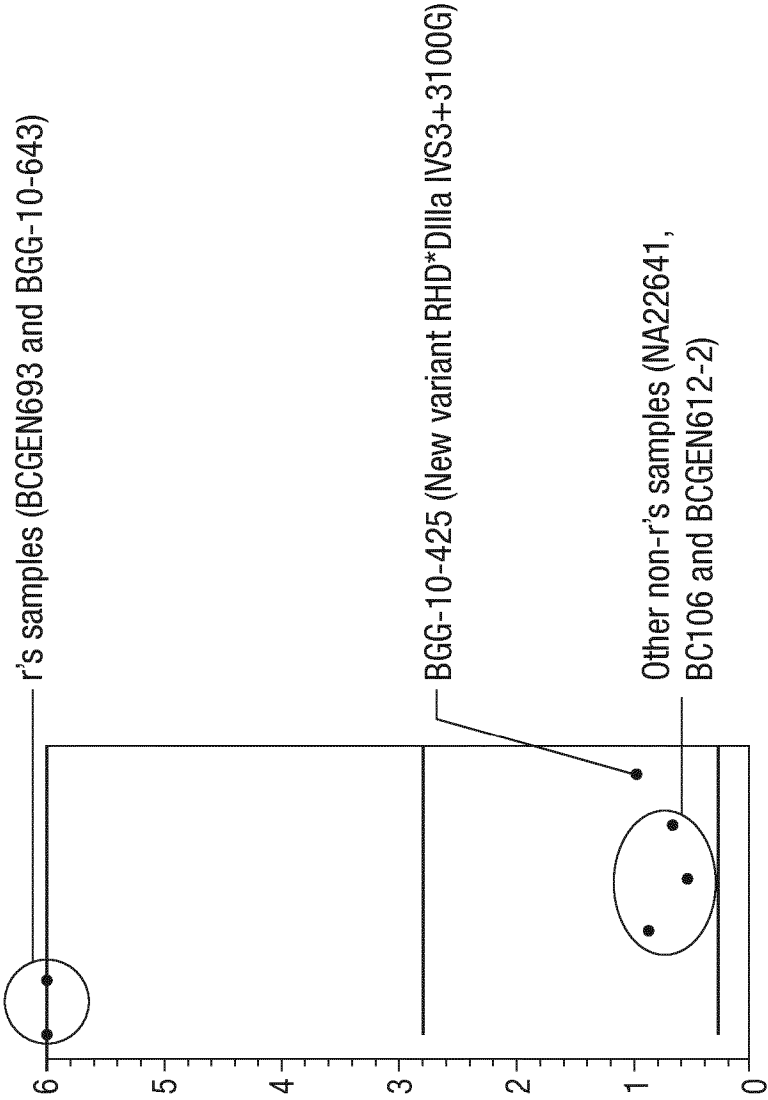


FIG. 2

	RHD			RHCE		RHD	Serology
	presence or absence of an RHD/RHCE hybrid exon 3 allele	exon 4 (position 602)	exon 7 (position 1048)	presence/absence of an RHCE*C allele	exon 7 (position 1006)		
r's	presence	G	C	absence	T	G	C ^{+w}
DIlla	presence	G	G	absence	G or T	A	C-
DIVa	presence	C	C	absence	G	A	C-
New variant	presence	G	G	absence	G	G	C-

Figure 3

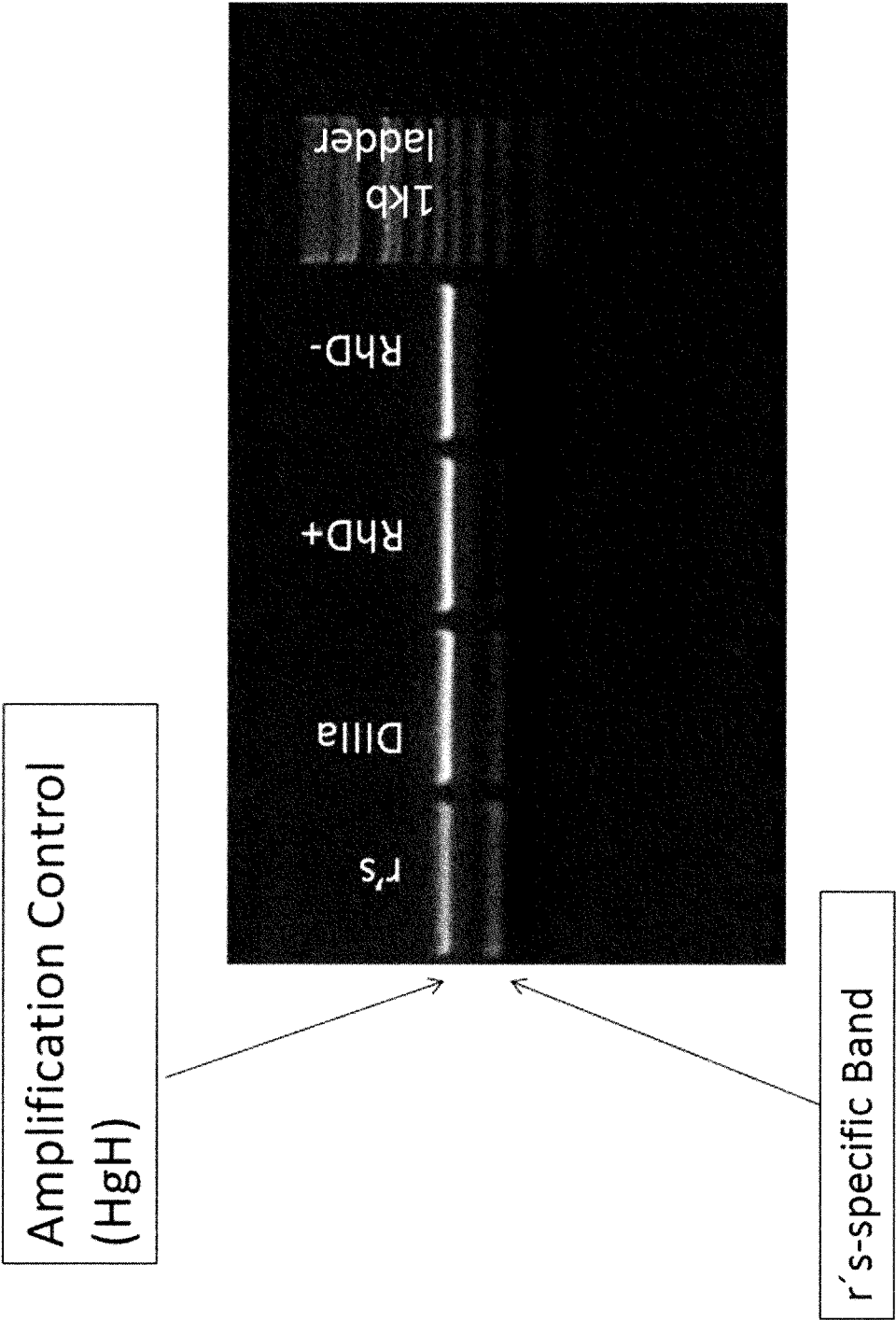
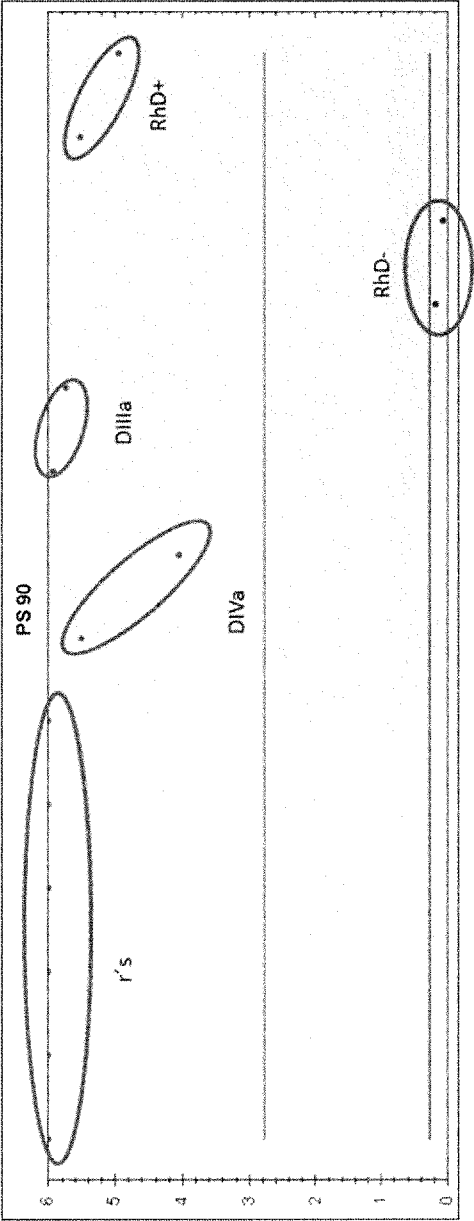


Figure 4



Only r's samples should be positive (above green line).
All others (DIVa, DIIa, RhD+ and RhD-) should be negative (below red line).

F_IDT_1/4_BCGEN693.1	r's
F_IDT_1/4_BCGEN693.2	r's
F_IDT_1/4_BGG-10-1423.1	r's,RHD-
F_IDT_1/4_BGG-10-1423.2	r's,RHD+
F_IDT_1/4_BGG-10-2335.1	r'shemizygous
F_IDT_1/4_BGG-10-2335.2	r'shemizygous
IDT_1/4_BCO105.1	DIVa-2
IDT_1/4_BCO105.2	DIVa-2
IDT_1/4_BCGEN612-2.1	DIIa
IDT_1/4_BCGEN612-2.2	DIIa
IDT_1/4_NA14706.1	RHD-
IDT_1/4_NA14706.2	RHD-
IDT_1/4_NA3264.1	RHD+
IDT_1/4_NA3264.2	RHD+

Figure 5

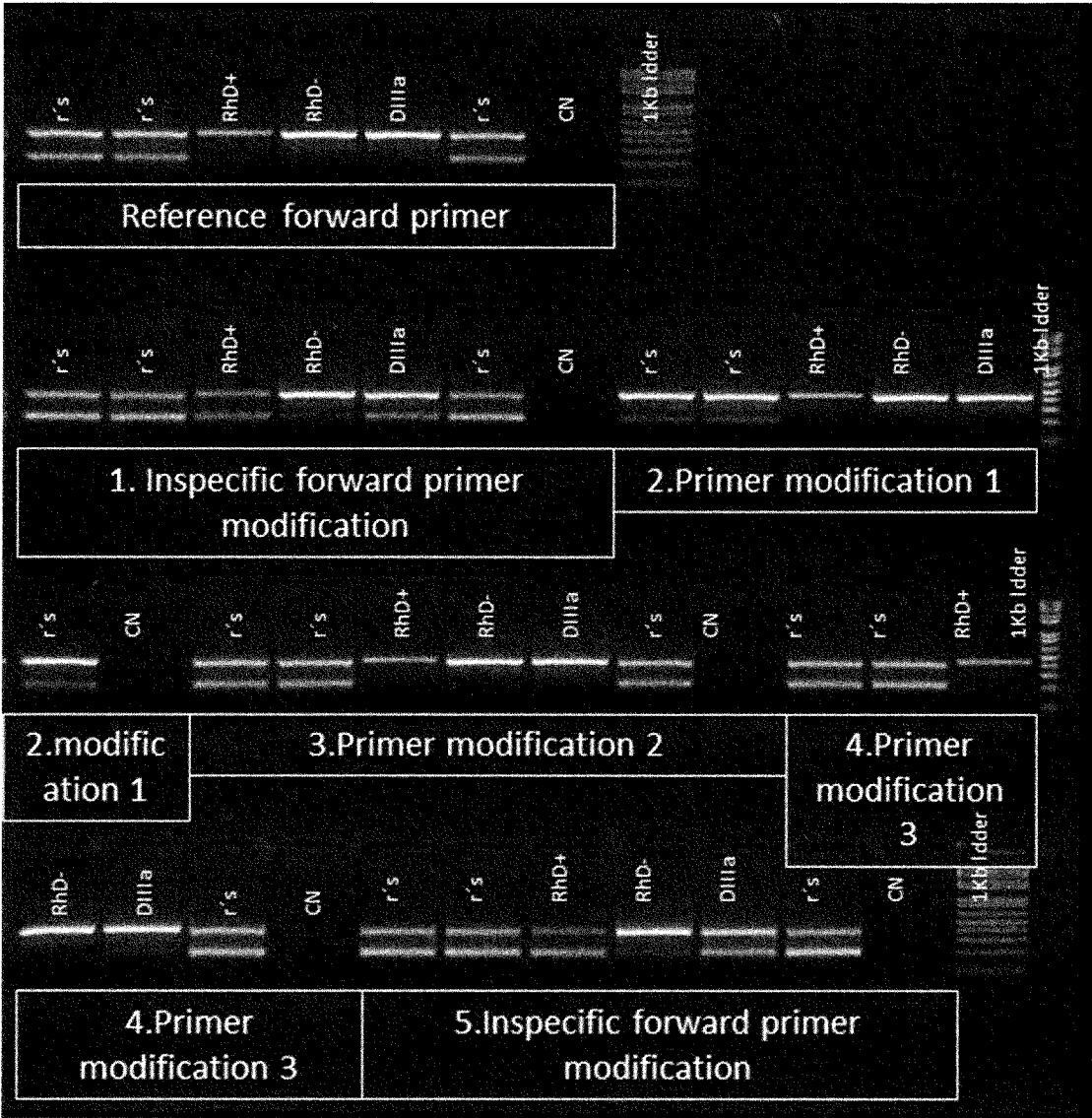


Figure 6

	3010	3020	3030	3040	3050
RHD	TTATTCCCAA	GGCAAATATG	GAAATTTGAT	CATGTACTAA	TCATAATAAA
RHCE*ce			...G.....	...A.G....	C....
r's			...A.....	...G.A....	C....
DIVa			...A.....	...G.A....	A....
DIIIa			...A.....	...G.A....	A....
#425			...A.....	...G.A....	A....
	3060	3070	3080	3090	3100
RHD	GCTGGATTCT	CTTTAAGAGA	TTGAGAAATT	AAAAGGCAAA	AGCTGATATA
RHCE*ce					A
r's					C
DIVa					A
DIIIa					A
#425					C
	3110	3120	3130	3140	3150
RHD	TCATGTTTAG	TTATATTGTG	AGTCTTATAA	GAAGCTGGGA	GGCAACCCCA
RHCE*ce		C....			
r's		T....			
DIVa		T....			
DIIIa		T....			
#425		T....			
	3160	3170	3180	3190	3200
RHD	TTAACTCACC	AGAATACAGA	ACTCAGTCTC	ACAACTTAGA	TATAATTCCT
RHCE*ce				A.	
r's				A.	
DIVa				G.	
DIIIa				G.	
#425				G.	
	3210	3220	3230	3240	3250
RHD	CTCAAACCTT	TTCCTCAAAG	ATTAAATTCT	GAAAATAATC	TTGTGATTAA
RHCE*ce			-.....		
r's			-.....		
DIVa			A.....		
DIIIa			A.....		
#425			A.....		
RHD	(SEQ ID NO: 29)				
RHCE*ce	(SEQ ID NO: 30)				
r's	(SEQ ID NO: 31)				
DIVa	(SEQ ID NO: 29)				
DIIIa	(SEQ ID NO: 29)				
#425	(SEQ ID NO: 32)				

Figure 7

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference CSCFP6960132	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/EP2014/052411	International filing date (<i>day/month/year</i>) 7 February 2014 (07-02-2014)	(Earliest) Priority Date (<i>day/month/year</i>) 8 March 2013 (08-03-2013)
Applicant PROGENIKA BIOPHARMA S.A.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 6 sheets.

☒

It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the **language**, the international search was carried out on the basis of:

☒

the international application in the language in which it was filed

☐

a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. ☐

This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6**bis**(a)).

c. ☒

With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐

Certain claims were found unsearchable (See Box No. II)

3. ☐

Unity of invention is lacking (see Box No III)

4. With regard to the **title**,

☒

the text is approved as submitted by the applicant

☐

the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒

the text is approved as submitted by the applicant

☐

the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. 3

☐

as suggested by the applicant

☒

as selected by this Authority, because the applicant failed to suggest a figure

☐

as selected by this Authority, because this figure better characterizes the invention

b. ☐

none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2014/052411

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
- a. (means)
- ☐ on paper
- ☒ in electronic form
- b. (time)
- ☒ in the international application as filed
- ☐ together with the international application in electronic form
- ☐ subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/052411

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

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)
C12Q

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)

EPO-Internal, WPI Data, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MONIQUE SILVY ET AL: "Identification of novel polymorphism restricted to the (C)ce s type 1 haplotype avoids risk of transfusion deadlock in SCD patients", BRITISH JOURNAL OF HAEMATOLOGY, vol. 160, no. 6, 1 March 2013 (2013-03-01), pages 863-867, XP055109233, ISSN: 0007-1048, DOI: 10.1111/bjh.12179 cited in the application page 864, column 2, paragraph 2; figure 1; table 1 Supporting information S1 ----- -/--	1-50



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 of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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

2 April 2014

Date of mailing of the international search report

10/04/2014

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/052411

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 2 471 949 A1 (PROGENIKA BIOPHARMA SA [ES]) 4 July 2012 (2012-07-04) cited in the application abstract paragraph [0011] - paragraph [0022] paragraph [0023] - paragraph [0042] -----	1-50
A	WO 2012/171990 A1 (PROGENIKA BIOPHARMA SA [ES]; OCHOA JORGE [ES]; LOPEZ MONICA [ES]; ESCO) 20 December 2012 (2012-12-20) cited in the application abstract page 9, line 28 - page 15, line 9; claims 39-47 page 3, line 3 - page 9, line 27; claims 1-38 -----	1-50
A	WO 01/77384 A2 (EPIGENOMICS AG [DE]; OLEK ALEXANDER [DE]; PIEPENBROCK CHRISTIAN [DE];) 18 October 2001 (2001-10-18) sequence 214802 -----	1-12
A	WO 02/38594 A1 (AGRICULTURE VICTORIA SERV PTY [AU]; AUSTRALIAN PORK LTD [AU]; PFIZER P) 16 May 2002 (2002-05-16) sequence 38 page 72 - page 73 -----	1-12
A	SALA N ET AL: "Analysis of the protein s gene in protein s deficiency", METHODS IN MOLECULAR MEDICINE, HUMANA PRESS, TOTOWA, NJ, US, vol. 31, 1 January 1999 (1999-01-01), pages 249-268, XP009177035, ISSN: 1543-1894 page 253; table 1; sequences PS-7.2 -----	1-12
A	BACH-NGA PHAM ET AL: "Heterogeneous molecular background of the weak C, VS+, hr(B)-, Hr(B)- phenotype in black persons", TRANSFUSION, AMERICAN ASSOCIATION OF BLOOD BANKS, BETHESDA, MD, US, vol. 49, no. 3, 1 March 2009 (2009-03-01), pages 495-504, XP002633278, ISSN: 0041-1132, DOI: 10.1111/J.1537-2995.2008.02005.X [retrieved on 2008-11-27] cited in the application the whole document ----- -/--	1-50

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/052411

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TAX MARTINE G H M ET AL: "RHC and RHc genotyping in different ethnic groups", TRANSFUSION, AMERICAN ASSOCIATION OF BLOOD BANKS, BETHESDA, MD, US, vol. 42, 1 May 2002 (2002-05-01), pages 634-644, XP003026240, ISSN: 0041-1132 cited in the application the whole document -----	1-50

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/052411

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			JP 2004512851 A	30-04-2004
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			NZ 520600 A	27-08-2004
			WO 0238594 A1	16-05-2002
