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This invention relates to a series of PCR primers that will allow the simultaneous amplification of regions of the clinically significant ABO and RHD genes.

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(54) Title: RHD AND ABO GENOTYPING BY MULTIPLEX PCR

(57) Abstract: This invention relates to a series of PCR primers that will allow the simultaneous amplification of regions of the clinically significant *ABO* and *RHD* genes.

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Gene Analysis

This invention relates to the field of gene analysis. More particularly, the invention relates to the study of the genotype of a subject in order to perform blood group analysis.

Background

Blood group definition is currently performed using serological techniques for a relatively limited number of clinically significant blood groups. Recent advances have included the determination of blood groups using molecular genetic techniques, but these have only been used in circumscribed situations, for example: the prenatal determination where the isolation of foetal blood for serological investigation would be dangerous or the determination of blood type in multiply transfused patients where serology is difficult because of the admix of patient/donor blood.

Currently large-scale blood group genotyping is not performed due to limitations of molecular-genetic based technologies and the relatively low cost of the current serological testing methodology. However, blood group serology has significant drawbacks. For example, the number of reagents available for testing some blood group antigen specificities is limited or such reagents may not exist. As a consequence, not all blood group antigens are tested for routinely. This can lead to primary alloimmunisation events where the recipients of blood become immunised to the antigens carried on the donated red blood cells. Blood group genotyping of all blood donors would result in more comprehensive blood testing and may result in a reduction in the incidence of alloimmunisations and subsequent transfusion reactions.

The ABO blood group is the most significant of all human blood groups and can cause immediate transfusion reactions, possibly leading to death, when ABO-incompatible blood is transfused. This is because blood group A, B and O individuals have preformed anti-A and/or anti-B in their serum (made to bacterial carbohydrate antigens) that will cross react with red cell A and/or B antigens not found on their own red cells. ABO compatibility is a major cause of transfusion associated morbidity and mortality and every blood donor and patient receiving blood, blood products or solid organ transplants must have their ABO status defined.

Red cell serology is used routinely for defining the ABO status of human red cells utilized in transfusion therapy. Despite this widespread and cheap application of serological techniques, ABO genotyping has some applications in routine Transfusion Medicine. Rare A and B alleles have depressed expression of both sets of antigens (e.g. A3, B3, A_{el}, B_{el}, A_x and B_x). These rare variants can be missed by routine automated ABO typing, with some of these potentially being typed as blood group O. Many of these alleles are caused by hybrid ABO genes and can only be classified using molecular genetic techniques. If blood grouping by molecular genetic techniques becomes a frontline replacement to red cell serology, then robust tests for ABO genotype will need to be developed and utilized (Olsson (2001) Blood **98** 1584-1593).

The A and B antigens of the ABO histo-blood group system are synthesized by glycosyltransferases encoded by the *ABO* locus on chromosome 9. The gene encoding the A glycosyltransferase was the first to be isolated, cloned and sequenced (Clausen *et al* (1990) J. Biol. Chem. **265** 1139-1145 ; Yamamoto *et al* (1990) Nature **345** 229-233). Sequence analysis revealed a coding region of 1062bp that corresponds to a 41 kDa protein. This coding region was shown subsequently to be distributed over 7 exons (Yamamoto *et al* (1995) Glycobiology **5** 51-58; Bennett *et al* (1995) Biochem. Biophys. Res. Commun. **211** 347) and the gene spans a region of ~20 kb on 9q34. The consensus coding sequence is the *A101* allele and all polymorphisms that affect the specificity and efficacy of the glycosyltransferase are considered mutations of this allele.

Most of the mutations that affect the specificity and/or efficacy of the encoded glycosyltransferase occur in exons 6 and 7. However there are a few important mutations in the earlier exons (Chester & Olsson (2001) Trans. Med. Rev. **11** 295-313). Mutations that encode the major alleles are shown in Table 1.

Nucleotide	261	297	467	526	703	796	802	803	1060
A1 (A101)	G	A	C	C	G	C	G	G	C
A2 (A201)	-	-	**T**	-	-	-	-	-	Deletion
B (B101)	-	G	-	**G**	**A**	**A**	-	**C**	-
O1 (O01)	Deletion	-	-	-	-	-	-	-	-
O1^v (O02)	Deletion	G	-	-	-	-	-	-	-
O2 (O03)	-	G	-	**G**	-	-	**A**	-	-

Table 1. Selected nucleotide polymorphisms between the major alleles of the *ABO* gene located in exons 6 and 7. No change is indicated by "-". Nucleotides that generate a change in the amino acid coded are shown in bold font. Alternative allele names are shown in parentheses (http://www.bioc.aecom.yu.edu/bgmut.index.htm).

The Rh system is the most polymorphic blood group system and is of significant importance in transfusion medicine. The Rh system is involved in haemolytic transfusion reactions, neonatal haemolytic disease and autoimmune haemolytic anaemia. There are two different, but highly homologous, genes in the Rh system. One gene (*RHD*) encodes the D polypeptide and the other (*RHCE*) the CcEe polypeptide. *RHD* carries the D antigen as the most potent blood group immunogen. This antigen is absent from a relatively large segment (15-17%) of the population (i.e. the Rh-negative phenotype), as a result of *RHD* gene deletion or other gene alterations. *RHCE* exists in four allelic forms and each allele determines the expression of two antigens in Ce, ce, cE or CE combination (*RHCE* is the collective name of the four alleles).

Multiplex (MPX) Polymerase Chain Reaction (PCR) is a variation on the well-known PCR technique, and employs different primer pairs in the same amplification reaction. It has been used in the analysis of blood groups. MPX PCR primers for amplification of Rh D sequences have been previously produced. Avent N D *et al.,* Blood, 1997, **89** 2568-77 discloses a multiplex *RHD* genotyping assay based on amplification of *RHD* intron 4 and the 3' non-coding region. Subsequently, six further *RHD* gene primer sequences have been produced for use in MPX PCR (Maaskant-van Wijk P A *et al* Transfusion **38**, November/December 1998, 1015-1021). In this disclosure, primers were designed to amplify various exons of the *RHD* gene. It was also indicated that *RHD* assays should not be dependent on non coding regions of the *RHD* gene (i.e.

introns) and that the technique might be of great value in prenatal *RH* genotyping. Wagner *et al.*, 1999, Blood, **93**, 385-393 disclosed a normal PCR based method involving primers to amplify relatively large PCR products. Due to the size of the products amplified, the PCR primers could not be used in a multiplex PCR method.

The inventors have prepared primers that can be used in multiplex PCR for use in blood group genotyping analysis, in particular, *RHD* and *ABO* genotyping analysis. The primers have been identified and selected to amplify fragments of an appropriate size for MPX PCR (in this case they are smaller than 1315bp) and have also been selected for functionality, that is to say, the selected primers provide good amplification of the desired fragments and are specific to the desired fragments.

Summary of the invention

According to a first aspect of the present invention there is provided a method of *RHD* genotyping analysis, by multiplex PCR, the method comprising contacting *RHD* gene nucleic acids from a subject with one or more of the following primer pairs 1,2;3,4 or 4A;5,6;7,8 or 8A;9 or 9A or 10 or 10A or 10B,11 or 11A;12,13;14 or 14A,15 or 15A;16,17;18,19; and 30,31 from the following table (table 2), wherein the primer pairs may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
1	101F	gccgcgaattcactagtgCCATAGAGAGGCCAGCACAA
2	198R	ggccgcgggaattcgattTGCCCCTGGAGAACCAC
3	int1F	gccgcgaattcactagtgTGACGAGTGAAACTCTATCTCGAT
4	297R	ggccgcgggaattcgattCCACCATCCCAATACCTGAAC
4A	296R	ggccgcgggaattcgattAGAAGTGATCCAGCCACCAT
5	303F	gccgcgaattcactagtgTCCTGGCTCTCCCTCTCT
6	397R	ggccgcgggaattcgattGTTGTCTTTATTTTTCAAAACCCT
7	403F	gccgcgaattcactagtgGCTCTGAACTTTCTCCAAGGACT
8	499R	ggccgcgggaattcgattCAAACTGGGTATCGTTGCTG
8A	498R	ggccgcgggaattcgattATTCTGCTCAGCCCAAGTAG
9	502F	gccgcgaattcactagtgCTTTGAATTAAGCACTTCACAGA
9A	503F	gccgcgaattcactagtgTTGAATTAAGCACTTCACAGAGCA
10	5Aluint4F (RoHar)	gccgcgaattcactagtgAAGGACTATCAGGCCACG
10A	RoHar4	gccgcgaattcactagtgCTGAAAGGAGGGAAACGGAC
10B	RoHar8	gccgcgaattcactagtgGGGCAGTGAGCTTGATAGTAGG
11	599R	ggccgcgggaattcgattCACCTTGCTGATCTTCCC
11A	598R	ggccgcgggaattcgattTGTGACCACCCAGCATTCTA

12	601F	gccgcgaattcactagtgAGTAGTGAGCTGGCCCATCA
13	697R	ggccgcgggaattcgattCTTCAGCCAAAGCAGAGGAG
14	702F	gccgcgaattcactagtgCTGGGACCTTGTTAGAAATGCTG
14A	701F	gccgcgaattcactagtgACAAACTCCCCGATGATGTGAGTG
15	799R	ggccgcgggaattcgattCAAGGTAGGGGCTGGACAG
15A	798R	ggccgcgggaattcgattGAGGCTGAGAAAGGTTAAGCCA
16	801F	gccgcgaattcactagtgCTGGAGGCTCTGAGAGGTTGAG
17	899R	ggccgcgggaattcgattGGCAATGGTGGAAGAAAGG
18	901F	gccgcgaattcactagtgACTGTCGTTTTGACACACAAT
19	998R	ggccgcgggaattcgattTGTCACCCGCATGTCAG
30	1001F	gccgcgaattcactagtgCAAGAGATCAAGCCAAAATCAGT
31	1097R	ggccgcgggaattcgattGTGGTACATGGCTGTATTTTATTG
32	MAPH-rev	gccgcgaattcactagtg
33	MAPH-forw	ggccgcgggaattcgatt

Table 2

and amplifying the *RHD* gene nucleic acids. Each of the primers indicated in the Table comprises a 5' MAPH tag (the first 18 nucleotides of the primer sequences shown in lower case) and a gene-specific sequence (shown in upper case). The MAPH tag is used to assist in the amplification of the nucleic acids. Specifically, once the RHD gene nucleic acids have been PCR amplified using the primers, primers to the MAPH tags (32 and 33) are used to further amplify the sequences. Preferably, both amplification steps are performed simultaneously. As will be appreciated by those skilled in the art, primers without the 5' MAPH tag (primer sequences represented by the sequence in uppercase only) can be used in the method of the invention in order to amplify the *RHD* gene nucleic acids. Alternatively, the primer sequences can comprise different tag sequences to the MAPH tags indicated in the table.

The method of the invention is advantageous because it allows the simultaneous amplification of ten regions, exons 1 to 10 of the highly clinically significant *RHD* gene. This includes most known *RHD* alleles, including the clinically significant partial and weak D variants. In particular, it includes exon 10, in which there is a mutation that results in the Del phenotype recently described in Gassner C, Doescher A, Drnovsek TD, Rozman P, Eicher NI, Legler TJ, Lukin S, Garritsen H, Kleinrath T, Egger B, Ehling R, Kormoczi GF, Kilga-Nogler S, Schoenitzer D, Petershofen EK. (2005) Transfusion 45(4) 527-538 Presence of *RHD* in serologically D-, C/E+ individuals: a European multicenter study. The method permits even more comprehensive blood testing and should result in a reduction in the incidence of alloimmunisations and subsequent transfusion reactions.

The method is also advantageous in that it can distinguish some common partial D phenotypes that are caused by hybrid *RHD-RHCE* genes including the DV and DVI phenotypes. These phenotypes will lack predicted fragments following amplification. DVI phenotypes are relatively common, occurring once in every 4000 individuals of Western European descent There are at least eight different genetic bases associated with the DV phenotype and at least four different genetic bases associated with the DVI phenotype. All known DV phenotypes can be differentiated following subsequent further analysis of the MPX products. DVI phenotype individuals lack a large number of D epitopes and can become alloimmunised to the *RHD* antigen by transfusion or pregnancy. In the UK DVI mothers are deliberately typed as D-negative, so they receive anti-D to avoid alloimmunisation. However, if blood donors of DVI phenotype are typed as D-negative, this blood may be transfused to "true" D-negative individuals and alloimmunisation may result. Genotyping using the assay of the invention would identify DVI persons and they can be excluded from the donor population for transfusion to D-negative individuals.

The method is further advantageous in that it can be used for analysis of adult donor subjects. This is important in connection with subjects who receive frequent transfusions, for example, those with sickle cell anaemia.

The DHAR phenotype is associated with a hybrid *RHCE-RHD* gene where exon 5 of *RHCE* is replaced by *RHD* (Beckers E A *et al.*, Br J Haematol. 1996 Mar;92(3):751-7.). DHAR red cells express a small but significant number of D epitopes. Using conventional serological techniques these individuals may type as Rh D-negative and their blood could potentially be transfused into D-negative individuals. These individuals may become immunised. DHAR is a very rare blood group. The assay of the invention permits the detection of the DHAR phenotype.

At least one of the primers used in the method is preferably labelled to allow detection of the amplified product. Suitable labels are well known to those skilled in the art. For example, it may be desirable to label one of the primers with 6-FAM.

The nucleic acids used in this and subsequent aspects of the invention may be derived from any appropriate source, such as, but not limited to blood, a buccal smear, urine, amniotic fluid. The nucleic acids are preferably derived from blood.

The blood may be utilized in any known manner, for example, *ex vivo*. In particular, the method of the invention may be performed on blood directly removed from an individual, for example, a patient requiring a blood transfusion or may be performed on a sample of blood to be delivered to an individual, for example, blood from a blood donation.

The nucleic acid is preferably DNA, most preferably genomic DNA.

The annealing temperature may be from 54-63°C. Preferably the annealing temperature is about 60°C. Most preferably the annealing temperature is 60°C.

The method of the invention may be combined with other MPX PCR methods to genotype other blood group genes. For example the method of the invention may be combined with MPX PCRs for the ABO/MNS/P1/RHCE/LU (Lutheran)/KE(Kell)/LE(Lewis)/FY(Duffy)/JK(Kidd)/DI(Diego)/YT(Cartwright)/XG/SC(Scianna)/DO(Dombrock)/CO(Colton)/LW/CH/RG(Chido/Rodgers)/Hh/XK/GE(Gerbich)/CROM(Cromer)/KN(Knops)/IN(Indian)/OK/RAPH/JMH(JohnMiltonHagen)/IGNT/P and/or GIL systems and/or any other blood group system that is known or becomes known.

Nucleic acids amplified by the method of the invention may be detected using any suitable method. For example, the amplified nucleic acid may be hybridised with a suitable nucleic acid probe specific for the sequence to be detected. Suitable nucleic acid probes can be provided in a format such as a gene chip. Preferably, the gene chip includes nucleic acid probes which hybridise to nucleic acids specific for other blood group genotypes.

In a preferred method of the invention the *RHD* gene nucleic acids are contacted with one or more of the following primer pairs: 1,2;3,4;5,6;7,8;9 or 10,11;12,13;14,15;and 18,19, preferably all of those primer pairs.

In an alternative preferred embodiment, the *RHD* gene nucleic acids are contacted with one or more of the following primer pairs 1,2;3,4A;5,6;7,8A;9A or 10A or 10B,11A;12,13;14A,15A; 16,17; 18,19; and 30,31, preferably all of those primer pairs.

Most preferably the *RHD* gene nucleic acids are contacted with all the following primer pairs 1,2;3,4 or 4A;5,6;7,8 or 8A;9 or 9A or 10 or 10A or 10B,11 or 11A;12,13;14 or 14A,15 or 15A;16,17;18,19; and 30,31.

According to a second aspect of the invention there is provided a method of *RHD* genotyping analysis, by multiplex PCR, the method comprising contacting *RHD* gene nucleic acids derived from blood from a subject with at least one primer selected from the following table (table 2A) wherein the primer may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
1	101F	gccgcgaattcactagtgCCATAGAGAGGCCAGCACAA
4	297R	ggccgcgggaattcgattCCACCATCCCAATACCTGAAC
4A	296R	ggccgcgggaattcgattAGAAGTGATCCAGCCACCAT
5	303F	gccgcgaattcactagtgTCCTGGCTCTCCCTCTCT
6	397R	ggccgcgggaattcgattGTTGTCTTTATTTTTCAAAACCCT
7	403F	gccgcgaattcactagtgGCTCTGAACTTTCTCCAAGGACT
8A	498R	ggccgcgggaattcgattATTCTGCTCAGCCCAAGTAG
9	502F	gccgcgaattcactagtgCTTTGAATTAAGCACTTCACAGA
9A	503F	gccgcgaattcactagtgTTGAATTAAGCACTTCACAGAGCA
10	5Aluint4F (RoHar)	gccgcgaattcactagtgAAGGACTATCAGGCCACG
10A	RoHar4	gccgcgaattcactagtgCTGAAAGGAGGGAAACGGAC
10B	RoHar8	gccgcgaattcactagtgGGGCAGTGAGCTTGATAGTAGG
11	599R	ggccgcgggaattcgattCACCTTGCTGATCTTCCC
11A	598R	ggccgcgggaattcgattTGTGACCACCCAGCATTCTA
12	601F	gccgcgaattcactagtgAGTAGTGAGCTGGCCCATCA
13	697R	ggccgcgggaattcgattCTTCAGCCAAAGCAGAGGAG
14	702F	gccgcgaattcactagtgCTGGGACCTTGTTAGAAATGCTG
14A	701F	gccgcgaattcactagtgACAAACTCCCCGATGATGTGAGTG
15	799R	ggccgcgggaattcgattCAAGGTAGGGGCTGGACAG
15A	798R	ggccgcgggaattcgattGAGGCTGAGAAAGGTTAAGCCA
17	899R	ggccgcgggaattcgattGGCAATGGTGGAAGAAAGG
18	901F	gccgcgaattcactagtgACTGTCGTTTTGACACACAAT
19	998R	ggccgcgggaattcgattTGTCACCCGCATGTCAG
31	1097R	ggccgcgggaattcgattGTGGTACATGGCTGTATTTTATTG

Table 2A

and amplifying the *RHD* gene nucleic acids. As will be appreciated by those skilled in the art, a pair of primers needs to be used to obtain amplification. Both primers may be selected from table 2A or one of the primers can be selected from table 2A and used with any suitable second primer, for example, a primer from table 2 or any other suitable primer. The pair of primers may be used alone or with any other primers. Preferably, the method comprises contacting the *RHD* gene nucleic acids with one or more of the following primer pairs: 1,2;3,4 or 4A;5,6;7,8 or 8A;9 or 9A or 10 or 10A or 10B, 11 or 11A;12,13;14 or 14A,15 or 15A;16,17;18,19; and 30,31.

In an alternative preferred embodiment, the method comprises contacting the *RHD* gene nucleic acids with one or more of the following primer pairs: 1 and 2; 5 and 6; 10 and 11; 12 and 13; 14 and 15 and 18 and 19.

In a further alternative preferred embodiment, the method comprises contacting the *RHD* gene nucleic acids with one or more of the following primer pairs: 1,2;3, 4A;5,6;7,8A; 9A or 10A or 10B,11A;12,13;14A, 15A;16,17;18,19; and 30,31.

In this and subsequent methods of the invention, primer pairs may be used individually or in combination to amplify, for example, one, several or all exons of interest.

As indicated above for the first aspect of the present invention, each of the primers indicated in table 2 comprises a 5' MAPH tag (the first 18 nucleotides of the primer sequences shown in lower case) and a gene-specific sequence. As will be appreciated by those skilled in the art, primers without the 5' MAPH tag (primer sequences represented by the sequence in uppercase only) can be used in the method of the invention in order to amplify the *RHD* gene nucleic acids. Alternatively, the primer sequences can comprise different tag sequences to the MAPH tags indicated in the table.

According to a third aspect of the invention there is provided a method of *ABO* genotyping analysis, by multiplex PCR, the method comprising contacting *ABO* gene nucleic acids from a subject with one or more of the following primer pairs 20,21; 22,23; 24 or 24A,25; 26,27 and 28,29 from the following table (table 3), wherein the

primer pairs may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
20	int1-49f	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG
21	int2+62r	ggccgcgggaattcgattATTGGCTGCTGTGGTCA
22	int3-33f	gccgcgaattcactagtgcCTGCTCCTAGACTAAACTTC
23	int4+52r	ggccgcgggaattcgattAAGGGAGGCACTGACATTA
24	int5-44f	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC
24A	int5-367f	gccgcgaattcactagtgGATTTGCCCGGTTGGAGTC
25	int6+31r	ggccgcgggaattcgattAGTCACTCGCCACTGCC
26	ABO432f	gccgcgaattcactagtgcCACCGTGTCCACTACTATG
27	ABO766r	ggccgcgggaattcgattTGTAGGCCTGGGACTGG
28	ABO723f	gccgcgaattcactagtgGGAGGCCTTCACCTACG
29	ABO1147r	ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC

Table 3

and amplifying the *ABO* gene nucleic acids. Preferably the *ABO* gene nucleic acids are contacted with all of the primer pairs mentioned.

Each of the primers indicated in table 3 comprises a 5' MAPH tag (the first 18 nucleotides of the primer sequences shown in lower case) and a gene-specific sequence (shown in upper case). The MAPH tag is used to assist in the amplification of the nucleic acids. Specifically, once the ABO gene nucleic acids have been PCR amplified using the primers, primers to the MAPH tags are used to further amplify the sequences. Preferably, both amplification steps are performed simultaneously. As will be appreciated by those skilled in the art, primers without the 5' MAPH tag (primer sequences represented by the sequence in uppercase only) can be used in the method of the invention in order to amplify the ABO gene nucleic acids. Alternatively, the primer sequences can comprise different tag sequences to the MAPH tags indicated in the table.

These primers amplify *ABO* exons 2, 4, 6, and 7 in a gene-specific manner such that allele specificity is determined by the use of oligonucleotide probes specific for a given allele. The primer sequences have been selected to deliberately exclude any known *ABO* nucleotide polymorphism, so as to be gene but not allele specific. Amplification of the *ABO* gene by this primer set permits the identification by sequence-specific oligonucleotide probes of all known ABO variants.

The blood may be utilized in any known manner, for example, *ex vivo*. In particular, the method of the invention may be performed on blood directly removed from an individual, for example, a patient requiring a blood transfusion or may be performed on a sample of blood to be delivered to an individual, for example, blood from a blood donation.

The nucleic acid is preferably DNA, more preferably genomic DNA.

The annealing temperature may be from 54-63°C. Preferably the annealing temperature is about 57°C. Most preferably the annealing temperature is 57°C.

The method of the third aspect of the invention may be combined with other MPX PCR methods to genotype other blood group genes. For example the method of the invention may be combined with MPX PCRs for the RHD//MNS/P1/RHCE/LU (Lutheran)/KE(Kell)/LE(Lewis)/FY(Duffy)/JK(Kidd)/DI(Diego)/YT(Cartwright)/XG/ SC(Scianna)/DO(Dombrock)/CO(Colton)/LW/CH/RG(Chido/Rodgers)/Hh/XK/GE(G erbich)/CROM(Cromer)/KN(Knops)/IN(Indian)/OK/RAPH/JMH(JohnMiltonHagen)/ IGNT/P and/or GIL systems and/or any other blood group system that is known or becomes known.

Nucleic acids amplified by the method of the third aspect of the invention may be detected as indicated above.

Preferably, the method comprises contacting *ABO* gene nucleic acids derived from blood from a subject with one or more, preferably all, of the following primer pairs 20,21; 22,23; 24,25; 26,27 and 28,29.

Alternatively, the method comprises contacting *ABO* gene nucleic acids derived from blood from a subject with one or more, preferably all, of the following primer pairs 20,21; 22,23; 24A,25; 26,27 and 28,29.

According to a fourth aspect of the present invention, there is provided a method of ABO genotyping analysis, by multiplex PCR, the method comprising contacting ABO gene nucleic acids derived from blood from a subject with at least one primer selected

from the following table (table 3), wherein the primer may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
20	int1-49f	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG
21	int2+62r	ggccgcgggaattcgattATTGGCTGCTGTGGTCA
22	int3-33f	gccgcgaattcactagtgcCTGCTCCTAGACTAAACTTC
23	int4+52r	ggccgcgggaattcgattAAGGGAGGCACTGACATTA
24	int5-44f	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC
24A	int5-367f	gccgcgaattcactagtgGATTTGCCCGGTTGGAGTC
25	int6+31r	ggccgcgggaattcgattAGTCACTCGCCACTGCC
26	ABO432f	gccgcgaattcactagtgcCACCGTGTCCACTACTATG
27	ABO766r	ggccgcgggaattcgattTGTAGGCCTGGGACTGG
28	ABO723f	gccgcgaattcactagtgGGAGGCCTTCACCTACG
29	ABO1147r	ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC

Table 3

and amplifying the ABO gene nucleic acids. As will be appreciated by those skilled in the art, a pair of primers needs to be used to obtain amplification. Both primers may be selected from table 3 or one of the primers can be selected from table 3 and used with any suitable second primer. The pair of primers may be used alone or with any other primers. Preferably, the method comprises contacting the ABO gene nucleic acids with one or more of the following primer pairs: 20 and 21; 22 and 23; 24 or 24A and 25; 26 and 27; 28 and 29.

In a preferred embodiment, the method comprises contacting the ABO gene nucleic acids with one or more of the following primer pairs: 20 and 21; 22 and 23; 24 and 25; 26 and 27; 28 and 29.

In an alternative embodiment, the method comprises contacting the ABO gene nucleic acids with one or more of the following primer pairs: 20 and 21; 22 and 23; 24A and 25; 26 and 27; 28 and 29.

According to a fifth aspect of the invention, there is provided a method of *ABO* and *RHD* genotyping analysis, by multiplex PCR, the method comprising contacting *ABO* gene and *RHD* gene nucleic acids derived from blood from a subject with one or more of the following primer pairs 1,2; 3,4 or 4A; 5,6; 7,8 or 8A; 9 or 9A or 10 or 10A or 10B,11 or 11A; 12,13; 14 or 14A,15 15A; 16,17; 18,19; 20,21; 22,23; 24 or 24A,25; 26,27; 28,29; and 30,31 from the following table (table 4), wherein the primer pairs

may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
1	101F	gccgcgaattcactagtgCCATAGAGAGGCCAGCACAA
2	198R	ggccgcgggaattcgattTGCCCCTGGAGAACCAC
3	int1F	gccgcgaattcactagtgTGACGAGTGAAACTCTATCTCGAT
4	297R	ggccgcgggaattcgattCCACCATCCCAATACCTGAAC
4A	296R	ggccgcgggaattcgattAGAAGTGATCCAGCCACCAT
5	303F	gccgcgaattcactagtgTCCTGGCTCTCCCTCTCT
6	397R	ggccgcgggaattcgattGTTGTCTTTATTTTTCAAAACCCT
7	403F	gccgcgaattcactagtgGCTCTGAACTTTCTCCAAGGACT
8	499R	ggccgcgggaattcgattCAAACTGGGTATCGTTGCTG
8A	498R	ggccgcgggaattcgattATTCTGCTCAGCCCAAGTAG
9	502F	gccgcgaattcactagtgCTTTGAATTAAGCACTTCACAGA
9A	503F	gccgcgaattcactagtgTTGAATTAAGCACTTCACAGAGCA
10	5Aluint4F (RoHar)	gccgcgaattcactagtgAAGGACTATCAGGCCACG
10A	RoHar4	gccgcgaattcactagtgCTGAAAGGAGGGAAACGGAC
10B	RoHar8	gccgcgaattcactagtgGGGCAGTGAGCTTGATAGTAGG
11	599R	ggccgcgggaattcgattCACCTTGCTGATCTTCCC
11A	598R	ggccgcgggaattcgattTGTGACCACCCAGCATTCTA
12	601F	gccgcgaattcactagtgAGTAGTGAGCTGGCCCATCA
13	697R	ggccgcgggaattcgattCTTCAGCCAAAGCAGAGGAG
14	702F	gccgcgaattcactagtgCTGGGACCTTGTTAGAAATGCTG
14A	701F	gccgcgaattcactagtgACAAACTCCCCGATGATGTGAGTG
15	799R	ggccgcgggaattcgattCAAGGTAGGGGCTGGACAG
15A	798R	ggccgcgggaattcgattGAGGCTGAGAAAGGTTAAGCCA
16	801F	gccgcgaattcactagtgCTGGAGGCTCTGAGAGGTTGAG
17	899R	ggccgcgggaattcgattGGCAATGGTGGAAGAAAGG
18	901F	gccgcgaattcactagtgACTGTCGTTTTGACACACAAT
19	998R	ggccgcgggaattcgattTGTCACCCGCATGTCAG
30	1001F	gccgcgaattcactagtgCAAGAGATCAAGCCAAAATCAGT
31	1097R	ggccgcgggaattcgattGTGGTACATGGCTGTATTTTATTG
20	int1-49f	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG
21	int2+62r	ggccgcgggaattcgattATTGGCTGCTGTGGTCA
22	int3-33f	gccgcgaattcactagtgcCTGCTCCTAGACTAAACTTC
23	int4+52r	ggccgcgggaattcgattAAGGGAGGCACTGACATTA
24	int5-44f	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC
24A	int5-367f	gccgcgaattcactagtgGATTTGCCCGGTTGGAGTC
25	int6+31r	ggccgcgggaattcgattAGTCACTCGCCACTGCC
26	ABO432f	gccgcgaattcactagtgcCACCGTGTCCACTACTATG
27	ABO766r	ggccgcgggaattcgattTGTAGGCCTGGGACTGG
28	ABO723f	gccgcgaattcactagtgGGAGGCCTTCACCTACG
29	ABO1147r	ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC

Table 4

and amplifying the RHD and ABO gene nucleic acids. Preferably the nucleic acids are contacted with all the pairs mentioned above.

As indicated above for the previous aspects of the present invention, each of the primers indicated in table 4 comprises a 5' MAPH tag (the first 18 nucleotides of the primer sequences shown in lower case) and a gene-specific sequence (shown in upper case). As will be appreciated by those skilled in the art, primers without the 5' MAPH tag (primer sequences represented by the sequence in uppercase only) can be used in the method of the invention in order to amplify the *RHD* and ABO gene nucleic acids. Alternatively, the primer sequences can comprise different tag sequences to the MAPH tags indicated in table 4.

Preferably, the method comprises contacting the *ABO* gene and *RHD* gene nucleic acids with one or more, preferably all, of the following primer pairs: 1,2; 3,4; 5,6; 7,8; 9 or 10,11; 12,13; 14,15; 18,19; 20,21; 22,23; 24,25; 26,27 and 28,29.

Alternatively, the method comprises contacting the *ABO* gene and *RHD* gene nucleic acids with one or more, preferably all, of the following primer pairs: 1,2; 3,4; 5,6; 7,8A; 9A or 10A or 10B,11A; 12,13; 14A,15A; 16, 17;18,19; 20,21; 22,23; 24A,25; 26,27; 28,29; and 30,31.

The blood may be utilized in any known manner, for example, *ex vivo*. In particular, the method of the invention may be performed on blood directly removed from an individual, for example, a patient requiring a blood transfusion or may be performed on a sample of blood to be delivered to an individual, for example, blood from a blood donation.

The nucleic acid is preferably DNA, more preferably genomic DNA.

The annealing temperature may be from 54-63°C. Preferably the annealing temperature is about 60°C or about 57°C. Most preferably the annealing temperature is 60°C.

The method of the fifth aspect of the invention may be combined with other MPX PCR methods to genotype other blood group genes. For example the method of the invention may be combined with MPX PCRs for the MNS/P1/RHCE/LU (Lutheran)/KE(Kell)/LE(Lewis)/FY(Duffy)/JK(Kidd)/DI(Diego)/YT(Cartwright)/XG/

SC(Scianna)/DO(Dombrock)/CO(Colton)/LW/CH/RG(Chido/Rodgers)/Hh/XK/GE(Gerbich)/CROM(Cromer)/KN(Knops)/IN(Indian)/OK/RAPH/JMH(JohnMiltonHagen)/IGNT/P and/or GIL systems and/or any other blood group system that is known or becomes known.

Nucleic acids amplified by the method of the fifth aspect of the invention may be detected as indicated above.

According to a sixth aspect of the invention, there is provided a method of *ABO* and *RHD* genotyping analysis, by multiplex PCR, the method comprising contacting *ABO* gene and *RHD* gene nucleic acids derived from blood from a subject with one or more primer from the following table (table 4A), wherein the primer may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
1	101F	gccgcgaattcactagtgCCATAGAGAGGCCAGCACAA
4	297R	ggccgcgggaattcgattCCACCATCCCAATACCTGAAC
4A	296R	ggccgcgggaattcgattAGAAGTGATCCAGCCACCAT
5	303F	gccgcgaattcactagtgTCCTGGCTCTCCCTCTCT
6	397R	ggccgcgggaattcgattGTTGTCTTTATTTTTCAAAACCCT
7	403F	gccgcgaattcactagtgGCTCTGAACTTTCTCCAAGGACT
8A	498R	ggccgcgggaattcgattATTCTGCTCAGCCCAAGTAG
9	502F	gccgcgaattcactagtgCTTTGAATTAAGCACTTCACAGA
9A	503F	gccgcgaattcactagtgTTGAATTAAGCACTTCACAGAGCA
10	5Aluint4F (RoHar)	gccgcgaattcactagtgAAGGACTATCAGGCCACG
10A	RoHar4	gccgcgaattcactagtgCTGAAAGGAGGGAAACGGAC
10B	RoHar8	gccgcgaattcactagtgGGGCAGTGAGCTTGATAGTAGG
11	599R	ggccgcgggaattcgattCACCTTGCTGATCTTCCC
11A	598R	ggccgcgggaattcgattTGTGACCACCCAGCATTCTA
12	601F	gccgcgaattcactagtgAGTAGTGAGCTGGCCCATCA
13	697R	ggccgcgggaattcgattCTTCAGCCAAAGCAGAGGAG
14	702F	gccgcgaattcactagtgCTGGGACCTTGTTAGAAATGCTG
14A	701F	gccgcgaattcactagtgACAAACTCCCCGATGATGTGAGTG
15	799R	ggccgcgggaattcgattCAAGGTAGGGGCTGGACAG
15A	798R	ggccgcgggaattcgattGAGGCTGAGAAAGGTTAAGCCA
17	899R	ggccgcgggaattcgattGGCAATGGTGGAAGAAAGG
18	901F	gccgcgaattcactagtgACTGTCGTTTTGACACACAAT
19	998R	ggccgcgggaattcgattTGTCACCCGCATGTCAG
31	1097R	ggccgcgggaattcgattGTGGTACATGGCTGTATTTTATTG
20	int1-49f	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG
21	int2+62r	ggccgcgggaattcgattATTGGCTGCTGTGGTCA
22	int3-33f	gccgcgaattcactagtgcCTGCTCCTAGACTAAACTTC
23	int4+52r	ggccgcgggaattcgattAAGGGAGGCACTGACATTA
24	int5-44f	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC
24A	int5-367f	gccgcgaattcactagtgGATTTGCCCGGTTGGAGTC

25	int6+31r	ggccgcgggaattcgattAGTCACTCGCCACTGCC
26	ABO432f	gccgcgaattcactagtgcCACCGTGTCCACTACTATG
27	ABO766r	ggccgcgggaattcgattTGTAGGCCTGGGACTGG
28	ABO723f	gccgcgaattcactagtgGGAGGCCTTCACCTACG
29	ABO1147r	ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC

Table 4A

and amplifying the RHD and ABO gene nucleic acids. As will be appreciated by those skilled in the art, a pair of primers needs to be used to obtain amplification. Both primers may be selected from table 4A or one of the primers can be selected from table 4A and used with any suitable second primer, for example a primer from table 4 or any other suitable primer. The pair of primers may be used alone or with any other primers.

Preferably the method comprises contacting *ABO* gene and *RHD* gene nucleic acids with one or more of the following primer pairs: 1,2; 3,4; 5,6; 7,8; 9 or 10,11; 12,13; 14,15; 18,19; 20,21; 22,23; 24,25; 26,27 and 28,29.

Alternatively, the method comprises contacting *ABO* gene and *RHD* gene nucleic acids with one or more of the following primer pairs: 1,2; 3,4; 5,6; 7,8A; 9A or 10A or 10B,11A; 12,13; 14A,15A; 18,19; 20,21; 22,23; 24A,25; 26,27; 28,29; and 30,31.

According to a seventh aspect of the invention there are provided one or more of the following PCR primers, wherein the primers may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
1	101F	gccgcgaattcactagtgCCATAGAGAGGCCAGCACAA
4	297R	ggccgcgggaattcgattCCACCATCCCAATACCTGAAC
4A	296R	ggccgcgggaattcgattAGAAGTGATCCAGCCACCAT
5	303F	gccgcgaattcactagtgTCCTGGCTCTCCCTCTCT
6	397R	ggccgcgggaattcgattGTTGTCTTTATTTTTCAAAACCCT
7	403F	gccgcgaattcactagtgGCTCTGAACTTTCTCCAAGGACT
8A	498R	ggccgcgggaattcgattATTCTGCTCAGCCCAAGTAG
9	502F	gccgcgaattcactagtgCTTTGAATTAAGCACTTCACAGA
9A	503F	gccgcgaattcactagtgTTGAATTAAGCACTTCACAGAGCA
10	5Aluint4F (RoHar)	gccgcgaattcactagtgAAGGACTATCAGGCCACG
10A	RoHar4	gccgcgaattcactagtgCTGAAAGGAGGGAAACGGAC
10B	RoHar8	gccgcgaattcactagtgGGGCAGTGAGCTTGATAGTAGG
11	599R	ggccgcgggaattcgattCACCTTGCTGATCTTCCC
11A	598R	ggccgcgggaattcgattTGTGACCACCCAGCATTCTA
12	601F	gccgcgaattcactagtgAGTAGTGAGCTGGCCCATCA
13	697R	ggccgcgggaattcgattCTTCAGCCAAAGCAGAGGAG

14	702F	gccgcgaattcactagtgCTGGGACCTTGTTAGAAATGCTG
14A	701F	gccgcgaattcactagtgACAAACTCCCCGATGATGTGAGTG
15	799R	ggccgcgggaattcgattCAAGGTAGGGGCTGGACAG
15A	798R	ggccgcgggaattcgattGAGGCTGAGAAAGGTTAAGCCA
17	899R	ggccgcgggaattcgattGGCAATGGTGGAAGAAAGG
18	901F	gccgcgaattcactagtgACTGTCGTTTTGACACACAAT
19	998R	ggccgcgggaattcgatttGTCACCCGCATGTCAG
31	1097R	ggccgcgggaattcgattGTGGTACATGGCTGTATTTTATTG
20	int1-49f	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG
21	int2+62r	ggccgcgggaattcgattATTGGCTGCTGTGGTCA
22	int3-33f	gccgcgaattcactagtgcCTGCTCCTAGACTAAACTTC
23	int4+52r	ggccgcgggaattcgattAAGGGAGGCACTGACATTA
24	int5-44f	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC
24A	int5-367f	gccgcgaattcactagtgGATTTGCCCGGTTGGAGTC
25	int6+31r	ggccgcgggaattcgattAGTCACTCGCCACTGCC
26	ABO432f	gccgcgaattcactagtgcCACCGTGTCCACTACTATG
27	ABO766r	ggccgcgggaattcgatttGTAGGCCTGGGACTGG
28	ABO723f	gccgcgaattcactagtgGGAGGCCTTCACCTACG
29	ABO1147r	ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC

Table 4A

As indicated above, each of the primers indicated in table 4A comprises a 5' MAPH tag (the first 18 nucleotides of the primer sequences shown in lower case) and a gene-specific sequence (shown in upper case). The present invention also provides one or more of the primers indicated in table 4A above without the 5' MAPH tag (primer sequences represented by the sequence in uppercase only). Such primers can be used to amplify the *RHD* and ABO gene nucleic acids. As will be appreciated by those skilled in the art the primer sequences indicated in uppercase in table 4A can be modified by the addition of additional sequences, such as different tag sequences.

Primers according to the invention may be used with or without the MAPH tags shown above. Without the tags, the primers have the following sequences:

Primer no.	Primer name	Sequence (5'-3')
1	101F	CCATAGAGAGGCCAGCACAA
2	198R	TGCCCCTGGAGAACCAC
3	int1F	TGACGAGTGAAACTCTATCTCGAT
4	297R	CCACCATCCCAATACCTGAAC
4A	296R	AGAAGTGATCCAGCCACCAT
5	303F	TCCTGGCTCTCCCTCTCT
6	397R	GTTGTCTTTATTTTTCAAAACCCT
7	403F	GCTCTGAACTTTCTCCAAGGACT
8	499R	CAAACTGGGTATCGTTGCTG
8A	498R	ATTCTGCTCAGCCCAAGTAG

9	502F	CTTTGAATTAAGCACTTCACAGA
9A	503F	TTGAATTAAGCACTTCACAGAGCA
10	5Aluint4F (RoHar)	AAGGACTATCAGGCCACG
10A	RoHar4	CTGAAAGGAGGGAAACGGAC
10B	RoHar8	GGGCAGTGAGCTTGATAGTAGG
11	599R	CACCTTGCTGATCTTCCC
11A	598R	TGTGACCACCCAGCATTCTA
12	601F	AGTAGTGAGCTGGCCCATCA
13	697R	CTTCAGCCAAAGCAGAGGAG
14	702F	CTGGGACCTTGTTAGAAATGCTG
14A	701F	ACAAACTCCCCGATGATGTGAGTG
15	799R	CAAGGTAGGGGCTGGACAG
15A	798R	GAGGCTGAGAAAGGTTAAGCCA
16	801F	CTGGAGGCTCTGAGAGGTTGAG
17	899R	GGCAATGGTGGAAGAAAGG
18	901F	ACTGTCGTTTTGACACACAAT
19	998R	TGTCACCCGCATGTCAG
30	1001F	CAAGAGATCAAGCCAAAATCAGT
31	1097R	GTGGTACATGGCTGTATTTTATTG
20	int1-49f	GTGAGAGAAGGAGGGTGAG
21	int2+62r	ATTGGCTGCTGTGGTCA
22	int3-33f	CTGCTCCTAGACTAAACTTC
23	int4+52r	AAGGGAGGCACTGACATTA
24	int5-44f	CTGCCAGCTCCATGTGAC
24A	int5-367f	GATTTGCCCGGTTGGAGTC
25	int6+31r	AGTCACTCGCCACTGCC
26	ABO432f	CACCGTGTCCACTACTATG
27	ABO766r	TGTAGGCCTGGGACTGG
28	ABO723f	GGAGGCCTTCACCTACG
29	ABO1147r	CAGAGTTTACCCGTTCTGC

The primers of the present invention can be used in any method. In particular, the primer sequences may be used as probes or as primers. Preferably the primers are used in genotyping analysis, particularly blood group analysis, especially methods of *RHD* and/or *ABO* genotyping analysis.

In use, the primers are used in pairs, as indicated in the methods of the invention. The preferred pairs are as follows:

1,2;

3,4 or 4A;

5,6;

7,8 or 8A;

9 or 9A or 10 or 10A or 10B,11 or 11A;

12,13;

14 or 14A,15 or 15A;

16,17;

18,19;

20,21;

22,23;

24 or 24A,25;

26,27;

28,29; and

30,31

The primers may be labelled to allow easy detection.

The primers of the invention and those used in methods of the invention may be varied by the skilled addressee. For example, the lengths of the primers may be varied. This would lead to a change in T_m for the primers. This could then affect the annealing temperature of the PCR reaction. The length of the primers may be chosen so that the T_m value for a primer is under 70°C.

Substitution of bases could be made at the 5' end of the primers without affecting the *RHD* specificity of the PCR reaction.

It is preferred that the ΔG value for primer-duplexing is less than -10 kcal/mole.

The primers according the seventh aspect of the invention and the primers used in the earlier aspects of the invention may be modified by shortening or extending the primers to include further parts of the sequence to be recognised, or by moving the primer sequence along the sequence to be recognised. Equally the primers may be modified slightly by changing one or more, preferably no more than five, more preferably no more than three, even more preferably no more than two nucleotides. Resultant primers are known as functional variants, namely variants of the original primers that are specific to the same sequences and form part of the invention.

According to an eighth aspect of the invention, there is provided a gene chip having a plurality of attached probe sequences enabling the identification of one or more of the PCR products produced by the methods indicated above. Preferably the gene chip comprises sufficient probe sequences to enable the detection of all possible PCR products produced by using the methods indicated above.

As will be appreciated by those skilled in the art the methods of the present invention may be performed in combination with any other genotyping methods. For example, the methods of genotyping the RHD and ABO genes may be combined with methods of genotyping other blood genes or any other genes. Preferably all the genotyping methods are performed using multiplex PCR. It is particularly preferred that a series of primers are used to amplify specific nucleotides sequences to be genotyped. The primers used preferably all have the same 5' tag sequences enabling subsequent amplification of all the nucleotide sequences using primers specific to the tag sequences.

Methods and primers in accordance with the invention will now be described, by way of example only, with reference to Figures 1 to 11 in which:

Fig. 1 illustrates the location design of the *RHD* primers;
Fig. 2 illustrates *RHD* primers for amplification of exon 1 (Fig 2A), exon 2 (Fig 2B), exon 3 (Fig 2C), exon 4 (Fig 2D), exon 5 (fig 2E), exon 6 (Fig 2F), exon 7 (Fig 2G), exon 7 alternative primers (Fig 2H), exon 8 (Fig 2I) exon 9 (Fig 2J) and exon 10 (Fig 2K) in the *RHD* MPX PCR method of the invention;
Fig. 3 shows *RHD* primer sequences in accordance with the invention;
Fig. 4 shows a *RHD* primer mix used in a method in accordance with the invention;
Fig. 5A shows *ABO* primer sequences in accordance with the invention, and Fig. 5B shows the primer location in the ABO gene sequence, wherein shaded letters denote the gene-specific primer sequences, lower case letters denote intron sequence, upper case letters denote exon sequence, bold font letters denote important allele-discriminating nucleotides. The numbers indicate the nucleotide number in the *ABO* gene coding sequence. The A^1 allele sequence is the consensus sequence and is shown in this figure;

Fig. 6 illustrates the results of the gel electrophoresis of *RHD* gene amplification products from a *RHD* MPX PCR reaction in accordance with the invention including a primer pair for exon 8;

Fig. 7 illustrates the results of the gel electrophoresis of *ABO* gene amplification products from an *ABO* MPX PCR reaction in accordance with the invention;

Fig. 8 illustrates the results of the gel electrophoresis of *RHD* and ABO gene amplification products from a *RHD* and ABO MPX PCR reaction in accordance with the invention including a primer pair for exon 8.

Fig. 9A shows alternative *ABO* primer sequences in accordance with the invention, and Fig. 9B shows the primer location in the ABO gene sequence, wherein shaded letters denote the gene-specific primer sequences, lower case letters denote intron sequence, upper case letters denote exon sequence, bold font letters denote important allele-discriminating nucleotides. The numbers indicate the nucleotide number in the *ABO* gene coding sequence. The A^1 allele sequence is the consensus sequence and is shown in this figure;

Fig. 10 illustrates the results of the gel electrophoresis of *ABO* gene amplification products from an *ABO* MPX PCR reaction in accordance with the invention; and

Fig.11 illustrates the results of the gel electrophoresis of *RHD* gene amplification products from a *RHD* MPX PCR reaction in accordance with the invention including a primer pair for exon 8;

Fig. 12 shows primers according to the invention.

EXAMPLES

RHD Primer Design

The primers were designed or selected to ensure that the exon sequence for exons 1 to 10 inclusive of *RHD* is amplified by the *RHD* MPX PCR of the invention. The location design of the *RHD* primers is illustrated in Figure 1. *RHD* primers are shown in Figure 3.

The design of primers was performed using Oligo v6.0 primer design software (Molecular Biology Insights, Inc.). The Oligo v6.0 software allows a collection of primer sequences to be electronically multiplexed – this enables detection of any conflicts between the primers and checking for possible primer-dimer formations. Primers were redesigned if they were found to self-dimerize or if they were found to

be incompatible with a large majority of the other primers in the multiplex. The primer sequences of a pair were chosen so that they were compatible i.e. ensuring that primer-dimer formation was limited. The lengths of the primers were chosen so that the T_m value for a primer was under 70°C.

Primers were also assessed using NetPrimer (PREMIER Biosoft International), a web-based program that gives each primer a rating up to 100% and also checks for primer-dimer formation. Primers were chosen for the multiplex using a combination of choosing the highest rating primers from NetPrimer results and ones which were compatible with the highest number of other primers from the Oligo v6.0 MPX results.

Primers were designed to ensure that the region amplified included the known single nucleotide polymorphisms (SNPs) to be detected for the *RHD* gene. This generally meant that the primer positions were located in the intron sequence surrounding the exon in question. The SNP positions for the *RHD* gene were mapped onto the sequence data for this gene, with the *RHD* sequence data (introns and exons) having been aligned with the sequence data for the closely related gene *RHCE*. Variant *RHD* alleles will be detected by the MPX PCR in combination with a gene chip.

An example is illustrated in Figure 2 for *RHD* exons 1 to 10 primers. Primers for the *RHD* MPX were checked against the *RHCE* sequence to ensure specificity for the *RHD* gene.

Figure 2A shows an alignment of *RHD* and *RHCE* sequences for exon 1 (shown in italics). The differences between the two genes in the exon are underlined. The positions of three SNPs are shown (double underlined):

SNP	*allele*
C8G	weak D type 3
G48A	*RHD* W16X (*RHD* negative allele)
C121T	*RHD* Q41X (*RHD* negative allele)

The primer sequence positions (101F, 198R) are shown in bold (without the MAPH tags).

Similarly, figures 2B-2K show the *RHD* and *RHCE* sequences, and SNPs and primers for exons 2 to 10.

The initial exon 2 forward primer was found to amplify from *RHC* as well as *RHD* so the primer sequence was changed to the one disclosed in Legler, T J *et al.*, Transfusion Medicine 2001 **11**, 383-388). A total of 10 different primers were tried for exon 2 in order to achieve *RHD* specificity. Six primers were tried for exon 2 where base changes have been introduced into the sequence. These were tested because they would have amplified a smaller product for exon 2 but the sequence changes did not result in *RHD* specificity.

The majority of the primers have 3' *RHD* specific ends but two of the primers are complementary to *RHD* and *RHCE* sequence (exon 2 reverse and exon 8 reverse). Exon 5 forward primer spans a region of sequence where there is an insert in *RHCE* but not in *RHD*.

For exons 4 and 5, previously published reverse primer sequences could be used (Maaskant-van Wijk *et al.*, Transfusion, 38, 1015-1021,1998).

ABO Primer Design

ABO primers are shown in Figure 5A. Primers were designed to amplify exons 2, 4, 6 and 7 of the *ABO* gene. In one design, for optimal amplification in a multiplex reaction, PCR products of 400 bp or less were desired and consequently, primers were selected to amplify exon 7 in two parts: 7A and 7B. Fragment 7B is 461 base pairs long but is readily amplified under the conditions described and is required to incorporate all known allele variants within this DNA sequence. The primer pairs were designed to be inclusive of all known mutations in the exon and were placed in non-variable regions of the introns. Allele-determining mutations are denoted in bold font in figure 5B and their position in the coding sequence of the gene denoted by the nucleotide number given in superscript. Subsequent to the initial design, an intron 5 polymorphism was found in primer int5-44F. Other intron 5 gene specific primers were identified and int5-367F was substituted into the assay (see figs 9A and 9B). The

intent of the microarray is that allele-specificity is determined by specific oligonucleotide probes that will bind to gene-specific PCR products, and that was our goal for *ABO*-specific, exon-specific primer selection.

Primer sequences were designed *de novo*. All primer pairs were checked using the Oligo v6.0 primer design software to evaluate melting temperatures, possible primer-dimer formation and hairpin formation. The length of the primers was selected to give a melting temperature of ~60°C. The sequences of the primers are shown in the following table:

nr.	MAPH PCR primer	ABO exon	Sequence (5'-3')
20	int1-49f	2	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG
21	int2+62r		ggccgcgggaattcgattATTGGCTGCTGTGGTCA
22	int3-33f	4	gccgcgaattcactagtgCCTGCTCCTAGACTAAACTTC
23	int4+52r		ggccgcgggaattcgattAAGGGAGGCACTGACATTA
24	int5-44f	6	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC
25	int6+31r		ggccgcgggaattcgattAGTCACTCGCCACTGCC
26	ABO432f	7A	gccgcgaattcactagtgCCACCGTGTCCACTACTATG
27	ABO766r		ggccgcgggaattcgattTGTAGGCCTGGGACTGG
28	ABO723f	7B	gccgcgaattcactagtgGGAGGCCTTCACCTACG
29	ABO1147r		ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC

Multiplex primer details for *ABO*-specific amplification. Lower case letters denote the MAPH tag sequence. Upper case letters denote the gene-specific sequence.

Multiplex PCR blood *RHD* gene analysis

Genomic DNA was isolated from adult peripheral blood using the QIAamp DNA Blood Mini kit (Qiagen Ltd.). The amount of genomic DNA in each sample was quantitated by measuring the absorbance at 260nm. Standard genomic DNA samples were used to assess the reliability of the multiplex PCR:

R1R1 = CDe/CDe

R2R2 = cDE/cDE

rr = cde/cde

r'r = Cde/cde

r''r = cdE/cde

R0r = cDe/cde

A 25µl PCR mix consisted of:

per 25 µl MPX reaction	
12.5 µl	2x Mastermix #
0.06 µl	*RHD* primer mix
0.8 µl	100 µM MAPH forward
0.8 µl	100 µM MAPH reverse
0.25 µl	Mg^{2+} (50mM, Bioline)
9.59 µl	H_2O
1 µl	100 ng/µl DNA
25 µl	**Total**

2x Mastermix = Qiagen multiplex PCR buffer which comprises all the necessary components for performing the PCR reaction, including HotStarTaq DNA Polymerase, Mg^{2+} and necessary dNTPs.

Primers were supplied by Operon Biotechnologies (formerly Qiagen). A suitable primer mix is shown in Fig. 4. The primer mix shown in Fig. 4 is a guide and variations may be made to the primer mix to change the ratio of the various primer pairs used.

Multiplex amplification and probe hybridization (MAPH) -tagged PCR primers are used to multiplex amplify gene fragments by producing "hybrid" PCR primers that have a 5' end MAPH tag and a 3' gene specific fragment. In the initial stages of the PCR the gene fragments will be amplified by these hybrid primers. Included in the PCR mix are MAPH forward and reverse primers that will amplify every PCR product amplified by the hybrid primers. This provides the multiplex reaction with uniformity and up to 20 gene fragments can be amplified in this manner. A modification of MAPH is disclosed by White *et al* (White, S *et al* Am. J. Hum. Genet. 2002 Aug;**71**(2):365-74) including the flanking sequences, which are referred to as "MAPH forward" and "MAPH reverse"(Fig. 3). The flanking sequences were supplied by Sanquin.

The amplification protocol was:

Multiplex PCR programme	
15 min	95°C
45 sec	94°C
90 sec	60°C } 38 cycles
90 sec	72°C
10 min	72°C

This was an adaptation of the protocol detailed by Qiagen for the Multiplex PCR buffer kit. The denaturation time has been extended, the annealing temperature chosen is in the middle of the range given (57 - 63°C) and the number of cycles is in the middle of the range given (30 – 45 cycles).

DHAR genomic DNA samples will have intron 4 of *RHCE* rather than intron 4 of *RHD*. Due to the location of the forward primer for exon 5, no exon 5 product would be amplified for DHAR samples with the original set of MPX primers. Therefore we have designed a forward primer 5' of the *Alu* sequence in intron 4 in a region that is *RHCE* specific. This primer is compatible with the reverse primer for exon 5 (*RHD*-specific).

Multiplex PCR blood *ABO* gene analysis

Genomic DNA was isolated from adult peripheral blood by either the QIAamp DNA Blood Mini Kit (Qiagen Ltd.) or by a modified salting-out procedure (Miller *et al* (1988) Nuc. Ac. Res. **16** 1215). DNA concentration was determined spectrophotometrically at 260nm, and diluted to 100ng/μL. Samples of different common ABO blood groups were selected for amplification.

per 25 µl MPX reaction	
12.5 µl	2x Mastermix #

0.25 µl	*ABO* primer mix (0.5 µM)
0.5 µl	50 µM MAPH forward
0.5 µl	50 µM MAPH reverse
10.25 µl	H_2O
1 µl	100 ng/µl DNA
25 µl	**Total**

2x Mastermix = Qiagen multiplex PCR buffer which comprises all the necessary components for performing the PCR reaction, including HotStarTaq DNA Polymerase, Mg^{2+} and necessary dNTPs.

The ABO primer mix comprises:

ABO Primer	Volume 10µM stock (µl)
int1-49f	2
int2+62r	2
int3-33f	2
int4+52r	2
int5-44f	2
int6+31r	2
ABO432f	2
ABO766r	2
ABO723f	2
ABO1147r	2
10mM Tris pH8	20
Total	40

Amplification was performed in 0.2 mL PCR tubes in either a PE 9700 or a PE 2700 thermal cycler (Perkin Elmer/Cetus, Norwalk, CT) under the following conditions:

Multiplex PCR programme

15 min	95°C	
30 sec	94°C	45 cycles
90 sec	57°C	
90 sec	72°C	

10 min 72°C

Amplified products were assessed by running 10 µL of each reaction on either a 3% agarose gel (prepared in house) or a 5-20% polyacrylamide gel (Novex Gels, Invitrogen, Inc.). A representative gel is shown in Figure 7 and shows the robust nature of the amplification reaction. Faint bands of 700 bp and higher indicate the low levels of amplification of larger gene-specific fragments as predicted.

In an alternative example, the following mixes were used:

per 25ul MPX reaction	
12.5uL	2x Mastermix
0.25uL	ABO primer mix
0.4uL	50uM MAPH forward
0.4uL	50uM MAPH reverse
10.45uL	H_2O
1uL	100ng/uL DNA
25uL	Total

Stock ABO primer mix used in the reaction above was prepared as follows:

ABO primer	Volume 10uM stock (uL)
int1-49f	2.5
int2+62r	2.5
int3-33f	2.5
int4+52r	2.5
int5+367f	2.5
int6+31r	5
ABO432f	5
ABO1147r	5
10mM Tris pH8	72,5
Total	100

The primers used in this example, the regions amplified and the resulting gel are shown in figures 9A, 9B and 10.

Multiplex PCR blood *RHD* and *ABO* gene analysis

Genomic DNA was isolated and quantified as before. The primer mixes used were as indicated for the individual RHD MPX PCR and the ABO MPX PCR. However, final concentrations of primers in the reaction were different to those detailed above due to the reaction mix setup below.

A 25µl PCR mix consisted of:

per 25 µl MPX reaction	
12.5 µl	2x Mastermix #
0.085 µl	*RHD* primer mix
0.2 µl	*ABO* primer mix
1.3 µl	100 µM MAPH forward
1.3 µl	100 µM MAPH reverse
0.6 µl	Mg^{2+} (50mM, Bioline)
8.015 µl	H_2O
1 µl	100 ng/µl DNA
25 µl	**Total**

2x Mastermix = Qiagen multiplex PCR buffer which comprises all the necessary components for performing the PCR reaction, including HotStarTaq DNA Polymerase, Mg^{2+} and necessary dNTPs.

The PCR amplification reactions were performed as indicated above, except that the following programme was used:

Multiplex PCR programme		
15 min	95°C	
60 sec	94°C	40 cycles
2 min	60°C	
2 min	72°C	
10 min	72°C	

Amplified products were assessed as indicated above. A representative gel is shown in Figure 8 and shows the robust nature of the amplification reaction.

In an alternative example, the following mixes were used:

ABO and RHD primer mix

ABO Primer	Volume 10uM stock (ul)
int1-49f	1.25
int2+62r	1.25
int3-33f	1.25
int4+52r	1.25
int5-44f	1.25
int6+31r	1.25
ABO432f	5
ABO766r	5
ABO723f	5
ABO1147r	5
RHD Primer	Volume 20uM stock (ul)
101F	1.25
198R	1.25
int1F	12.5
296R	12.5
303F	1.25
397R	1.25
403F	2.5
498R	2.5
503F	2.5
598R	2.5

5Aluint4F	2.5
601F	1.25
697R	1.25
701F	1.25
798R	1.25
801F	1.5
899R	1.5
901F	1.1
998R	1.1
1001F	1.75
1097R	1.75
10mM Tris pH8	16.3
Total	100

per 25 ul mpx reaction		
12.5 ul	2x Mastermix #	
1.5 ul	ABO/RHD primer mix	
0.625 ul	100 mM MAPH forw	
0.625 ul	100 uM MAPH rev	
8.75 ul	H_2O	
1 ul	100 ng/ul DNA	
25 ul	**Total**	
# 2x Mastermix = Qiagen multiplex PCR buffer		
NOTE: MAPH volumes will vary according to stock concentration and depending on whether the primers are added from one combined MAPH mix		NOTE: DNA has been used at 100ng/ul but volumes could be adjusted to use at 40ng/ul

Multiplex PCR programme		
15 min	95°C	
30 sec	95°C	
90 sec	57°C	45 cycles
90 sec	72°C	
10 min	72°C	

Multiplex PCR Results

The MPX PCR amplifies all the products required. These products are visible by gel electrophoresis as shown in Figure 6 (*RHD* gene amplification products) and Figure 7 (*ABO* gene amplification products). Alternatively, the products are visible by GeneScan® analysis software (Applied Biosystems) using a capillary microsequencer (Applied Biosystems). The products have also been sequenced to ensure that the correct amplicons are being amplified.

The size of each amplicon and the *RHD* exon from which it is derived are indicated on the left of Fig. 6. In Fig. 6 "gDNA" means genomic DNA. A product specific for exon 5 of the *RHD* R_0^{Har} gene variant (DHAR) is also highlighted. This product is not obtained from normal D-positive and D-negative samples. Primer pairs were also tested individually to ensure *RHD* specificity.

In Figure 7, the *ABO* exon from which each amplicon is derived is indicated on the right of the figure. Exon 4 is 151 bp; exon 2 is 217 bp; exon 6 is 263 bp; exon 7A is 371 bp; and exon 7B is 461 bp. The numbers on the left of the figure indicate the size of the DNA marker bands.

In Figure 8, the *RHD* and *ABO* exon from which each amplicon is derived is indicated on the right of the figure. The numbers on the left of the figure indicate the size of the DNA marker bands.

The amplified nucleic acids may then be hybridized to further sequences in an array such as gene chip.

Although conditions for MPX PCR are described herein, those skilled in the art will be aware that any appropriate MPX PCR conditions may be used.

Claims

1. A method of *RHD* genotyping analysis, by multiplex PCR, the method comprising contacting *RHD* gene nucleic acids from a subject with the one or more of the following primer pairs 1,2;3,4 or 4A;5,6;7,8 or 8A;9 or 9A or 10 or 10A or 10B, 11 or 11A;12,13;14 or 14A,15 or 15A;16,17;18,19; and 30,31 from the following table, wherein the primer pairs may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
1	101F	gccgcgaattcactagtgCCATAGAGAGGCCAGCACAA
2	198R	ggccgcgggaattcgattTGCCCCTGGAGAACCAC
3	int1F	gccgcgaattcactagtgTGACGAGTGAAACTCTATCTCGAT
4	297R	ggccgcgggaattcgattCCACCATCCCAATACCTGAAC
4A	296R	ggccgcgggaattcgattAGAAGTGATCCAGCCACCAT
5	303F	gccgcgaattcactagtgTCCTGGCTCTCCCTCTCT
6	397R	ggccgcgggaattcgattGTTGTCTTTATTTTTCAAAACCCT
7	403F	gccgcgaattcactagtgGCTCTGAACTTTCTCCAAGGACT
8	499R	ggccgcgggaattcgattCAAACTGGGTATCGTTGCTG
8A	498R	ggccgcgggaattcgattATTCTGCTCAGCCCAAGTAG
9	502F	gccgcgaattcactagtgCTTTGAATTAAGCACTTCACAGA
9A	503F	gccgcgaattcactagtgTTGAATTAAGCACTTCACAGAGCA
10	5Aluint4F (RoHar)	gccgcgaattcactagtgAAGGACTATCAGGCCACG
10A	RoHar4	gccgcgaattcactagtgCTGAAAGGAGGGAAACGGAC
10B	RoHar8	gccgcgaattcactagtgGGGCAGTGAGCTTGATAGTAGG
11	599R	ggccgcgggaattcgattCACCTTGCTGATCTTCCC
11A	598R	ggccgcgggaattcgattTGTGACCACCCAGCATTCTA
12	601F	gccgcgaattcactagtgAGTAGTGAGCTGGCCCATCA
13	697R	ggccgcgggaattcgattCTTCAGCCAAAGCAGAGGAG
14	702F	gccgcgaattcactagtgCTGGGACCTTGTTAGAAATGCTG
14A	701F	gccgcgaattcactagtgACAAACTCCCCGATGATGTGAGTG
15	799R	ggccgcgggaattcgattCAAGGTAGGGGCTGGACAG
15A	798R	ggccgcgggaattcgattGAGGCTGAGAAAGGTTAAGCCA
16	801F	gccgcgaattcactagtgCTGGAGGCTCTGAGAGGTTGAG
17	899R	ggccgcgggaattcgattGGCAATGGTGGAAGAAAGG
18	901F	gccgcgaattcactagtgACTGTCGTTTTGACACACAAT
19	998R	ggccgcgggaattcgattTGTCACCCGCATGTCAG
30	1001F	gccgcgaattcactagtgCAAGAGATCAAGCCAAAATCAGT
31	1097R	ggccgcgggaattcgattGTGGTACATGGCTGTATTTTATTG
32	MAPH-rev	gccgcgaattcactagtg
33	MAPH-forw	ggccgcgggaattcgatt

and amplifying the *RHD* gene nucleic acids.

2. A method according to claim 1, in which *RHD* gene nucleic acids are contacted with one or more of the following primer pairs 1,2; 3,4; 5,6; 7,8; 9 or 10,11; 12,13; 14,15; and 18,19.

3. A method according to claim 2, in which *RHD* gene nucleic acids are contacted with the following primer pairs 1,2; 3,4; 5,6; 7,8; 9 or 10,11; 12,13; 14,15; and 18,19.

4. A method according to claim 1, in which *RHD* gene nucleic acids are contacted with one or more of the following primer pairs 1,2; 3,4A; 5,6; 7,8A; 9A or 10A or 10B,11A; 12,13; 14A,15A; 16,17; and 18,19.

5. A method according to claim 4, in which *RHD* gene nucleic acids are contacted with the following primer pairs 1,2; 3,4A; 5,6; 7,8A; 9A or 10A or 10B,11A; 12,13; 14A,15A; 16,17; and 18,19.

6. A method according to claim 1, in which *RHD* gene nucleic acids are contacted with the following primer pairs 1,2;3,4 or 4A;5,6;7,8 or 8A;9 or 9A or 10 or 10A or 10B, 11 or 11A;12,13;14 or 14A,15 or 15A;16,17;18,19; and 30,31.

7. A method according to claim 2 or 3 in which exons 1 to 7 and 9 of the *RHD* gene are amplified.

8. A method according to claim 4, 5 or 6 in which exons 1 to 10 of the *RHD* gene are amplified.

9. A method according to any preceding claim in which partial and weak *RHD* variants are amplified.

10. A method of *RHD* genotyping analysis, by Multiplex PCR, the method comprising contacting *RHD* gene nucleic acids from a subject with at least one primer selected from the following table, wherein the primer may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer	Primer	Sequence (5'-3')

no.	name	
1	101F	gccgcgaattcactagtgCCATAGAGAGGCCAGCACAA
4	297R	ggccgcgggaattcgattCCACCATCCCAATACCTGAAC
4A	296R	ggccgcgggaattcgattAGAAGTGATCCAGCCACCAT
5	303F	gccgcgaattcactagtgTCCTGGCTCTCCCTCTCT
6	397R	ggccgcgggaattcgattGTTGTCTTTATTTTTCAAAACCCT
7	403F	gccgcgaattcactagtgGCTCTGAACTTTCTCCAAGGACT
8A	498R	ggccgcgggaattcgattATTCTGCTCAGCCCAAGTAG
9	502F	gccgcgaattcactagtgCTTTGAATTAAGCACTTCACAGA
9A	503F	gccgcgaattcactagtgTTGAATTAAGCACTTCACAGAGCA
10	5Aluint4F (RoHar)	gccgcgaattcactagtgAAGGACTATCAGGCCACG
10A	RoHar4	gccgcgaattcactagtgCTGAAAGGAGGGAAACGGAC
10B	RoHar8	gccgcgaattcactagtgGGGCAGTGAGCTTGATAGTAGG
11	599R	ggccgcgggaattcgattCACCTTGCTGATCTTCCC
11A	598R	ggccgcgggaattcgattTGTGACCACCCAGCATTCTA
12	601F	gccgcgaattcactagtgAGTAGTGAGCTGGCCCATCA
13	697R	ggccgcgggaattcgattCTTCAGCCAAAGCAGAGGAG
14	702F	gccgcgaattcactagtgCTGGGACCTTGTTAGAAATGCTG
14A	701F	gccgcgaattcactagtgACAAACTCCCCGATGATGTGAGTG
15	799R	ggccgcgggaattcgattCAAGGTAGGGGCTGGACAG
15A	798R	ggccgcgggaattcgattGAGGCTGAGAAAGGTTAAGCCA
17	899R	ggccgcgggaattcgattGGCAATGGTGGAAGAAAGG
18	901F	gccgcgaattcactagtgACTGTCGTTTTGACACACAAT
19	998R	ggccgcgggaattcgattTGTCACCCGCATGTCAG
31	1097R	ggccgcgggaattcgattGTGGTACATGGCTGTATTTTATTG

and amplifying the *RHD* gene nucleic acids.

11. A method according to claim 10, comprising contacting the *RHD* gene nucleic acids with one or more of the following primer pairs: 1,2;3,4 or 4A;5,6;7,8 or 8A;9 or 9A or 10 or 10A or 10B, 11 or 11A;12,13;14 or 14A,15 or 15A;16,17;18,19; and 30,31.

12. A method according to claim 10, comprising contacting the *RHD* gene nucleic acids with one or more of the following primer pairs: 1,2;3,4;5,6;7,8;9 or 10, 11;12,13;14,15;18,19.

13. A method according to claim 10, comprising contacting the *RHD* gene nucleic acids with one or more of the following primer pairs: 1,2;3,4A;5,6;7, 8A;9A or 10A or 10B,11A;12,13;14A,15A;16,17;18,19; and 30,31.

14. A method of *ABO* genotyping analysis, by multiplex PCR, the method comprising contacting *ABO* gene nucleic acids from a subject with one or more of the following primer pairs 20,21; 22,23; 24 or 24A,25; 26,27 and 28,29 from the following table, wherein the primer pairs may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
20	int1-49f	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG
21	int2+62r	ggccgcgggaattcgattATTGGCTGCTGTGGTCA
22	int3-33f	gccgcgaattcactagtgcCTGCTCCTAGACTAAACTTC
23	int4+52r	ggccgcgggaattcgattAAGGGAGGCACTGACATTA
24	int5-44f	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC
24A	int5-367f	gccgcgaattcactagtgGATTTGCCCGGTTGGAGTC
25	int6+31r	ggccgcgggaattcgattAGTCACTCGCCACTGCC
26	ABO432f	gccgcgaattcactagtgcCACCGTGTCCACTACTATG
27	ABO766r	ggccgcgggaattcgatttGTAGGCCTGGGACTGG
28	ABO723f	gccgcgaattcactagtgGGAGGCCTTCACCTACG
29	ABO1147r	ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC

and amplifying the *ABO* gene nucleic acids.

15. A method according to claim 14, wherein the *ABO* gene nucleic acids are contacted with one or more of the following primer pairs: 20,21; 22,23; 24,25; 26,27 and 28,29.

16. A method according to claim 15, wherein the *ABO* gene nucleic acids are contacted with the following primer pairs: 20,21; 22,23; 24,25; 26,27 and 28,29

17. A method according to claim 16, wherein the *ABO* gene nucleic acids are contacted with one or more of the following primer pairs: 20,21; 22,23; 24A,25; 26,27 and 28,29.

18. A method according to claim 17, wherein the *ABO* gene nucleic acids are contacted with the following primer pairs: 20,21; 22,23; 24A,25; 26,27 and 28,29.

19. A method of ABO genotyping analysis, by multiplex PCR, the method comprising contacting ABO nucleic acid from a subject with at least one primer

selected from the following table, wherein the primer may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
20	int1-49f	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG
21	int2+62r	ggccgcgggaattcgattATTGGCTGCTGTGGTCA
22	int3-33f	gccgcgaattcactagtgcCTGCTCCTAGACTAAACTTC
23	int4+52r	ggccgcgggaattcgattAAGGGAGGCACTGACATTA
24	int5-44f	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC
24A	int5-367f	gccgcgaattcactagtgGATTTGCCCGGTTGGAGTC
25	int6+31r	ggccgcgggaattcgattAGTCACTCGCCACTGCC
26	ABO432f	gccgcgaattcactagtgcCACCGTGTCCACTACTATG
27	ABO766r	ggccgcgggaattcgattTGTAGGCCTGGGACTGG
28	ABO723f	gccgcgaattcactagtgGGAGGCCTTCACCTACG
29	ABO1147r	ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC

and amplifying the ABO gene nucleic acids.

20. A method according to any of claims 14 to 18, in which exons 2, 4, 6 and 7 of the *ABO* gene are amplified.

21. A method according to any of claims 14 to 18 in which rare *ABO* variants are amplified.

22. A method of *ABO* and *RHD* genotyping analysis, by multiplex PCR, the method comprising contacting *ABO* gene and *RHD* gene nucleic acids from a subject with one or more of the following primer pairs 1,2; 3,4 or 4A; 5,6; 7,8 or 8A; 9 or 9A or 10 or 10A or 10B,11 or 11A; 12,13; 14 or 14A,15 15A; 18,19; 20,21; 22,23; 24 or 24A,25; 26,27; 28,29; and 30,31 from the following table, wherein the primer pairs may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
1	101F	gccgcgaattcactagtgCCATAGAGAGGCCAGCACAA
2	198R	ggccgcgggaattcgattTGCCCCTGGAGAACCAC
3	int1F	gccgcgaattcactagtgTGACGAGTGAAACTCTATCTCGAT
4	297R	ggccgcgggaattcgattCCACCATCCCAATACCTGAAC
4A	296R	ggccgcgggaattcgattAGAAGTGATCCAGCCACCAT
5	303F	gccgcgaattcactagtgTCCTGGCTCTCCCTCTCT
6	397R	ggccgcgggaattcgattGTTGTCTTTATTTTTCAAAACCCT
7	403F	gccgcgaattcactagtgGCTCTGAACTTTCTCCAAGGACT
8	499R	ggccgcgggaattcgattCAAACTGGGTATCGTTGCTG
8A	498R	ggccgcgggaattcgattATTCTGCTCAGCCCAAGTAG
9	502F	gccgcgaattcactagtgCTTTGAATTAAGCACTTCACAGA

9A	503F	gccgcgaattcactagtgTTGAATTAAGCACTTCACAGAGCA
10	5Aluint4F (RoHar)	gccgcgaattcactagtgAAGGACTATCAGGCCACG
10A	RoHar4	gccgcgaattcactagtgCTGAAAGGAGGGAAACGGAC
10B	RoHar8	gccgcgaattcactagtgGGGCAGTGAGCTTGATAGTAGG
11	599R	ggccgcgggaattcgattCACCTTGCTGATCTTCCC
11A	598R	ggccgcgggaattcgattTGTGACCACCCAGCATTCTA
12	601F	gccgcgaattcactagtgAGTAGTGAGCTGGCCCATCA
13	697R	ggccgcgggaattcgattCTTCAGCCAAAGCAGAGGAG
14	702F	gccgcgaattcactagtgCTGGGACCTTGTTAGAAATGCTG
14A	701F	gccgcgaattcactagtgACAAACTCCCCGATGATGTGAGTG
15	799R	ggccgcgggaattcgattCAAGGTAGGGGCTGGACAG
15A	798R	ggccgcgggaattcgattGAGGCTGAGAAAGGTTAAGCCA
16	801F	gccgcgaattcactagtgCTGGAGGCTCTGAGAGGTTGAG
17	899R	ggccgcgggaattcgattGGCAATGGTGGAAGAAAGG
18	901F	gccgcgaattcactagtgACTGTCGTTTTGACACACAAT
19	998R	ggccgcgggaattcgattTGTCACCCGCATGTCAG
30	1001F	gccgcgaattcactagtgCAAGAGATCAAGCCAAAATCAGT
31	1097R	ggccgcgggaattcgattGTGGTACATGGCTGTATTTTATTG
20	int1-49f	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG
21	int2+62r	ggccgcgggaattcgattATTGGCTGCTGTGGTCA
22	int3-33f	gccgcgaattcactagtgcCTGCTCCTAGACTAAACTTC
23	int4+52r	ggccgcgggaattcgattAAGGGAGGCACTGACATTA
24	int5-44f	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC
24A	int5-367f	gccgcgaattcactagtgGATTTGCCCGGTTGGAGTC
25	int6+31r	ggccgcgggaattcgattAGTCACTCGCCACTGCC
26	ABO432f	gccgcgaattcactagtgcCACCGTGTCCACTACTATG
27	ABO766r	ggccgcgggaattcgattTGTAGGCCTGGGACTGG
28	ABO723f	gccgcgaattcactagtgGGAGGCCTTCACCTACG
29	ABO1147r	ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC

and amplifying the RHD and ABO gene nucleic acids.

23. A method according to claim 22, wherein the *ABO* gene and *RHD* gene nucleic acids are contacted with one or more of the following primer pairs 1,2; 3,4; 5,6; 7,8; 9 or 10,11; 12,13; 14,15; 18,19; 20,21; 22,23; 24,25; 26,27; 28,29; and 30,31.

24. A method according to claim 23, wherein the *ABO* gene and *RHD* gene nucleic acids are contacted with the following primer pairs 1,2; 3,4; 5,6; 7,8; 9 or 10,11; 12,13; 14,15; 18,19; 20,21; 22,23; 24,25; 26,27; 28,29; and 30,31.

25. A method according to claim 22, wherein the *ABO* gene and *RHD* gene nucleic acids are contacted with the following primer pairs 1,2; 3,4A; 5,6; 7,8A;9A or 10 or 10A or 10B,11A; 12,13; 14A,15A; 16,17;18,19; 20,21; 22,23; 24A,25; 26,27; 28,29; and 30,31.

26. A method according to claim 25, wherein the *ABO* gene and *RHD* gene nucleic acids are contacted with one or more of the following primer pairs 1,2; 3,4A; 5,6; 7,8A;9A or 10 or 10A or 10B,11A; 12,13; 14A,15A; 16,17;18,19; 20,21; 22,23; 24A,25; 26,27; 28,29; and 30,31.

27. A method of *ABO* and *RHD* genotyping analysis, by multiplex PCR, the method comprising contacting *ABO* gene and *RHD* gene nucleic acids from a subject with one or more primer from the following table wherein the primer may comprise the entire sequence shown in the table or the sequence shown in uppercase:

Primer no.	Primer name	Sequence (5'-3')
1	101F	gccgcgaattcactagtgCCATAGAGAGGCCAGCACAA
4	297R	ggccgcgggaattcgattCCACCATCCCAATACCTGAAC
4A	296R	ggccgcgggaattcgattAGAAGTGATCCAGCCACCAT
5	303F	gccgcgaattcactagtgTCCTGGCTCTCCCTCTCT
6	397R	ggccgcgggaattcgattGTTGTCTTTATTTTTCAAAACCCT
7	403F	gccgcgaattcactagtgGCTCTGAACTTTCTCCAAGGACT
8A	498R	ggccgcgggaattcgattATTCTGCTCAGCCCAAGTAG
9	502F	gccgcgaattcactagtgCTTTGAATTAAGCACTTCACAGA
9A	503F	gccgcgaattcactagtgTTGAATTAAGCACTTCACAGAGCA
10	5Aluint4F (RoHar)	gccgcgaattcactagtgAAGGACTATCAGGCCACG
10A	RoHar4	gccgcgaattcactagtgCTGAAAGGAGGGAAACGGAC
10B	RoHar8	gccgcgaattcactagtgGGGCAGTGAGCTTGATAGTAGG
11	599R	ggccgcgggaattcgattCACCTTGCTGATCTTCCC
11A	598R	ggccgcgggaattcgattTGTGACCACCCAGCATTCTA
12	601F	gccgcgaattcactagtgAGTAGTGAGCTGGCCCATCA
13	697R	ggccgcgggaattcgattCTTCAGCCAAAGCAGAGGAG
14	702F	gccgcgaattcactagtgCTGGGACCTTGTTAGAAATGCTG
14A	701F	gccgcgaattcactagtgACAAACTCCCCGATGATGTGAGTG
15	799R	ggccgcgggaattcgattCAAGGTAGGGGCTGGACAG
15A	798R	ggccgcgggaattcgattGAGGCTGAGAAAGGTTAAGCCA
17	899R	ggccgcgggaattcgattGGCAATGGTGGAAGAAAGG
18	901F	gccgcgaattcactagtgACTGTCGTTTTGACACACAAT
19	998R	ggccgcgggaattcgattTGTCACCCGCATGTCAG
31	1097R	ggccgcgggaattcgattGTGGTACATGGCTGTATTTTATTG
20	int1-49f	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG
21	int2+62r	ggccgcgggaattcgattATTGGCTGCTGTGGTCA
22	int3-33f	gccgcgaattcactagtgcCTGCTCCTAGACTAAACTTC
23	int4+52r	ggccgcgggaattcgattAAGGGAGGCACTGACATTA
24	int5-44f	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC
24A	int5-367f	gccgcgaattcactagtgGATTTGCCCGGTTGGAGTC
25	int6+31r	ggccgcgggaattcgattAGTCACTCGCCACTGCC
26	ABO432f	gccgcgaattcactagtgcCACCGTGTCCACTACTATG
27	ABO766r	ggccgcgggaattcgattTGTAGGCCTGGGACTGG
28	ABO723f	gccgcgaattcactagtgGGAGGCCTTCACCTACG

29	ABO1147r	ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC

and amplifying the RHD and ABO gene nucleic acids.

28. A method according to any preceding claim in which a Multiplex PCR reaction in respect of other blood genes is also performed simultaneously, sequentially or separately.

29. A method according claim 28 in which the MPX PCR reaction are in relation to the ABO/MNS/P1/RH/LU(Lutheran)/KE(Kell)/LE(Lewis)/FY(Duffy)/JK (Kidd)/DI(Diego)/YT(Cartwright)/XG/SC(Scianna)/DO(Dombrock)/CO(Colton)/ LW/CH/RG(Chido/Rodgers)/Hh/XK/GE(Gerbich)/CROM(Cromer)/KN(Knops)/I N(Indian)/OK/RAPH/JMH(JohnMiltonHagen)/IGNT/P and/or GIL systems and/or any other blood group system that is known or becomes known.

30. A method according to any preceding claim in which the blood is *ex vivo*.

31. A method according to any preceding claim in which the nucleic acid is genomic DNA.

32. A method according to any preceding claim in which the annealing temperature is from 54 to 63°C.

33. A method according to claim 32 in which the annealing temperature is about 57°C.

34. A method according to claim 32 in which the annealing temperature is about 60°C.

35. A method according to any preceding claim in which the amplified gene nucleic acids are then hybridised to nucleic acid probes specific for the sequences to be detected.

36. A method according to claim 35 in which the nucleic acid probes are arranged in an array.

37. A method according to claim 35 or 36 in which the method is arranged to be performed on a solid substrate.

38. A method according to claim 37 wherein the nucleic acid probes are attached to a gene chip.

39. A method according to any preceding claim, wherein at least one primer is replaced with a functional variant.

40. A PCR primer shown in the following table, wherein the primer may comprise the entire sequence shown in the table or the sequence shown in uppercase, or a functional variant thereof:

Primer no.	Primer name	Sequence (5'-3')
1	101F	gccgcgaattcactagtgCCATAGAGAGGCCAGCACAA
4	297R	ggccgcgggaattcgattCCACCATCCCAATACCTGAAC
4A	296R	ggccgcgggaattcgattAGAAGTGATCCAGCCACCAT
5	303F	gccgcgaattcactagtgTCCTGGCTCTCCCTCTCT
6	397R	ggccgcgggaattcgattGTTGTCTTTATTTTTCAAAACCCT
7	403F	gccgcgaattcactagtgGCTCTGAACTTTCTCCAAGGACT
8A	498R	ggccgcgggaattcgattATTCTGCTCAGCCCAAGTAG
9	502F	gccgcgaattcactagtgCTTTGAATTAAGCACTTCACAGA
9A	503F	gccgcgaattcactagtgTTGAATTAAGCACTTCACAGAGCA
10	5Aluint4F (RoHar)	gccgcgaattcactagtgAAGGACTATCAGGCCACG
10A	RoHar4	gccgcgaattcactagtgCTGAAAGGAGGGAAACGGAC
10B	RoHar8	gccgcgaattcactagtgGGGCAGTGAGCTTGATAGTAGG
11	599R	ggccgcgggaattcgattCACCTTGCTGATCTTCCC
11A	598R	ggccgcgggaattcgatttGTGACCACCCAGCATTCTA
12	601F	gccgcgaattcactagtgAGTAGTGAGCTGGCCCATCA
13	697R	ggccgcgggaattcgattCTTCAGCCAAAGCAGAGGAG
14	702F	gccgcgaattcactagtgCTGGGACCTTGTTAGAAATGCTG
14A	701F	gccgcgaattcactagtgACAAACTCCCCGATGATGTGAGTG
15	799R	ggccgcgggaattcgattCAAGGTAGGGGCTGGACAG
15A	798R	ggccgcgggaattcgattGAGGCTGAGAAAGGTTAAGCCA
17	899R	ggccgcgggaattcgattGGCAATGGTGGAAGAAAGG
18	901F	gccgcgaattcactagtgACTGTCGTTTTGACACACAAT
19	998R	ggccgcgggaattcgatttGTCACCCGCATGTCAG
31	1097R	ggccgcgggaattcgattGTGGTACATGGCTGTATTTTATTG
20	int1-49f	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG
21	int2+62r	ggccgcgggaattcgattATTGGCTGCTGTGGTCA
22	int3-33f	gccgcgaattcactagtgcCTGCTCCTAGACTAAACTTC
23	int4+52r	ggccgcgggaattcgattAAGGGAGGCACTGACATTA
24	int5-44f	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC

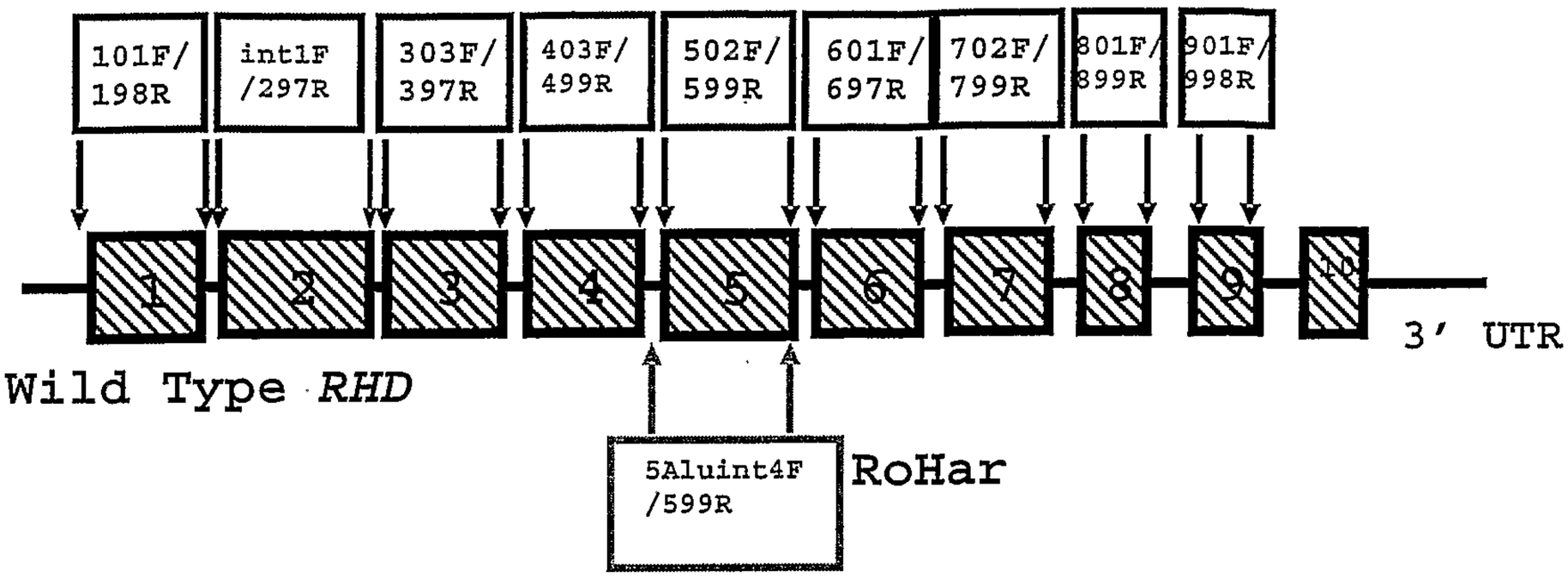
24A	int5-367f	gccgcgaattcactagtgGATTTGCCCGGTTGGAGTC
25	int6+31r	ggccgcgggaattcgattAGTCACTCGCCACTGCC
26	ABO432f	gccgcgaattcactagtgcCACCGTGTCCACTACTATG
27	ABO766r	ggccgcgggaattcgattTGTAGGCCTGGGACTGG
28	ABO723f	gccgcgaattcactagtgGGAGGCCTTCACCTACG
29	ABO1147r	ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC

41. Use of a PCR primer according to claim 40 in a PCR reaction.

42. Use of a PCR primer according to claim 40 in a method of genotyping analysis.

43. A gene chip having a plurality of attached probe sequences enabling the identification of one or more of the PCR products produced by the method of any one of claims 1 to 39.

Figure 1



Key:
Shaded boxes = exons; numbers refer to exon number
Each box has forward primer/reverse primer detailed
Reverse primers for exons 2, 4 and 5 span at least some of the 3' end of the exon in question; all other primers are in the intron sequence
RHD Exon 5 will only be amplified from *RHD* R_0^{Har} genomic DNA samples by using an *RHCE*-specific intron 4 forward primer pairing with the *RHD*-specific reverse primer (599R)

Fig. 2A

Exon 1

RHCE : CTTCCGTGTTAACTCCATAGACAGGCCAGCACAGCCAGCCTTGCAGCCTGAGATAAGGCCTTTGG
RHD : CTTCCGTGTTAACT**CCATAGAGAGGCCAGCACAA**CCAGCCTTGCAGCCTGAGATAAGGCCTTTGG
CTTCCGTGTTAACTCCATAGA AGGCCAGCACA CCAGCCTTGCAGCCTGAGATAAGGCCTTTGG

RHCE : CGGGTGTCTCCCCTATCGCTCCCTCAAGCCCTCAAGTAGGTGTTGGAGAGAGGGGTGATGCCTGG
RHD : CGGGTGTCTCCCCTATCGCTCCCTCAAGCCCTCAAGTAGGTGTTGGAGAGAGGGGTGATGCCTGG
CGGGTGTCTCCCCTATCGCTCCCTCAAGCCCTCAAGTAGGTGTTGGAGAGAGGGGTGATGCCTGG

RHCE : *TGCTGGTGGAACCCCTGCACAGAGACGGACACAGGATGAGCTCTAAGTACCCGCGGTCTGTCCGG*
RHD : *TGCTGGTGGAACCCCTGCACAGAGACGGACACAGGATGAGCTCTAAGTACCCGCGGTCTGTCCGG*
TGCTGGTGGAACCCCTGCACAGAGACGGACACAGGATGAGCTCTAAGTACCCGCGGTCTGTCCGG

RHCE : *CGCTGCCTGCCCCTCTGCGCCCTAACACTGGAAGCAGCTCTCATTCTCCTCTTCTATTTTTTTAC*
RHD : *CGCTGCCTGCCCCTCTGGGCCCTAACACTGGAAGCAGCTCTCATTCTCCTCTTCTATTTTTTTAC*
CGCTGCCTGCCCCTCTG GCCCTAACACTGGAAGCAGCTCTCATTCTCCTCTTCTATTTTTTTAC

RHCE : *CCACTATGACGCTTCCTTAGAGGATCAAAAGGGGCTCGTGGCATCCTATCAAGGTGAGAGTTCAT*
RHD : *CCACTATGACGCTTCCTTAGAGGATCAAAAGGGGCTCGTGGCATCCTATCAAGGTGAGAGTTCAT*
CCACTATGACGCTTCCTTAGAGGATCAAAAGGGGCTCGTGGCATCCTATCAAGGTGAGAGTTCAT

RHCE : *TGGAACAGTGGTCACAGGAGCAAATAGCAGGGGCAGGGGCGGGGGAGGCCTATGGTTCTCCAGGG*
RHD : *TGGAAAAGTGGTCACAGGAGCAAATAGCAGGGGCAGGGGCGGGGGAGGCCT***GTGGTTCTCCAGGG**
TGGAA AGTGGTCACAGGAGCAAATAGCAGGGGCAGGGGCGGGGGAGGCCT TGGTTCTCCAGGG

RHCE : *GCACAGATGTTCCTTTCTACAAAATCCCGAGGAAAA-GATTCCCCCATCTTCTTCCGTAGATTGC*
RHD : ***GCA***CAGATGTTCCTTTCTACAAAATCCCAAGGAAAAAGATTCCCCCATCTTCTTCCGTAGATTGC
GCACAGATGTTCCTTTCTACAAAATCCC AGGAAAA GATTCCCCCATCTTCTTCCGTAGATTGC

Key:

exon : *italics*
primers : **bold**
differences between *RHD* and *RHCE* in exon : underlined
SNPs for variant *RHD* alleles : double underlined

Consensus sequence is shown underneath each set of *RHCE/RHD* sequence.

Fig 2B

Exon 2

```
RHCE : TTGAACCCAGGAGGCAGAGGTTGCAGTGAGCCAAGATCTCGCCACTGTACTCCAGCCTGGGTGAC
RHD  : TTGAACCCAGGAGGCAGAGGTTGCAGTGAGCCAAGATCTTGCCACTGTACTCCAGCCTGGGTGAC
       TTGAACCCAGGAGGCAGAGGTTGCAGTGAGCCAAGATCT GCCACTGTACTCCAGCCTGGGTGAC

RHCE : AAGAGTGAAACTCTATCTCAAAATTAAAAAAAAAAAATCTTAGCTCTACCCACCGGGGCAAGTTA
RHD  : --GAGTGAAACTCTATCTCGATATTAAAAAAAAAAA-TCTTAGCTCTACCCACCGGGGCAAGTTA
         GAGTGAAACTCTATCTC A ATTAAAAAAAAAAA TCTTAGCTCTACCCACCGGGGCAAGTTA

RHCE : CATAACGCCTCTGTGCCTTGGTTTTCATATCTGTAAAATGGTGACAGTAACAGCACCCATGTCAA
RHD  : CGTAACGCCTCTGTGCCTTGGTTTTCATATCTGTAAAATGGTGACAGTAACAGCACCCACGTCAA
       C TAACGCCTCTGTGCCTTGGTTTTCATATCTGTAAAATGGTGACAGTAACAGCACCCA GTCAA

RHCE : AGTGTGGTTGTGAGAACGAAACAAGATAGTCTATGTAAAGTGATTAAAACAGCGTAGGCACATGG
RHD  : AGTGTGGTTGTGAGAACGAAACAAGATAGTCTATGTAAAGTGATTAAAACAGCGTAGGCACATGG
       AGTGTGGTTGTGAGAACGAAACAAGATAGTCTATGTAAAGTGATTAAAACAGCGTAGGCACATGG

RHCE : TAAACGCTTAGGAAATGTAGGCTGTTATAAAGCTCAGAGATGTTAAGTAACTAGATCAAGACCAC
RHD  : TAAACGCTTAGGAAATGTAGGCTGTTATAAAGCTCAGAGATGTTAAGTAACTAGATCAAGATCAC
       TAAACGCTTAGGAAATGTAGGCTGTTATAAAGCTCAGAGATGTTAAGTAACTAGATCAAGA CAC

RHCE : ACAGTTAGAGGGTGCCACAGTCTTGATTTGAACCCAAATTTGTCTCGTTCTGGAGCTCAAGCTGC
RHD  : ACAGTTAGAGGGTGCCAGAGTCCTGATTTGAACCCAAGTTTGTCTCGTTCTGGAGCTCAAGCTGC
       ACAGTTAGAGGGTGCCA AGTC TGATTTGAACCCAA TTTGTCTCGTTCTGGAGCTCAAGCTGC

RHCE : TAACCCTTTTTCAAAACTGGAATTAAACCAAAGTGCTCACCCTCCGCTTTGCTGGGCCCCTCCCT
RHD  : TAACCCTTTTTCAAAACTGGAATTAAACCAAAGTGCTCACCCTCCGCTTTGCTGGGCCCCTCCCT
       TAACCCTTTTTCAAAACTGGAATTAAACCAAAGTGCTCACCCTCCGCTTTGCTGGGCCCCTCCCT

RHCE : GCCCTCAGGTGCATCTCTTCCACTCACCTGCCACAGCAGCCTCTGCTCAGGGTCTGAGACTGGGA
RHD  : GCCCTCAGGTGCGTCTCTTCCACTCACCTGCCACAGCAGCCTCTGCTCAGGGTCTGAGACCGGGA
       GCCCTCAGGTGC TCTCTTCCACTCACCTGCCACAGCAGCCTCTGCTCAGGGTCTGAGAC GGGA

RHCE : AAGGTGAGGGCTACCCAGGTGGCCCTGATGTTTTCTGCCAGCCAGCTCACCAGGTCCCTCGCAGC
RHD  : AAGGTGAGGGCTACCCAGGTGGCCCTGATGTTTTCTGCCAGCCAGCTCACCAGGTCCCTCGCAGC
       AAGGTGAGGGCTACCCAGGTGGCCCTGATGTTTTCTGCCAGCCAGCTCACCAGGTCCCTCGCAGC

RHCE : AGGCGGCAAAGGGAGGGAGGTTTGCTGTGAAGATTATGTGGTTCCCAACAACAAGAGCACTGGGC
RHD  : AGGCGGCAAAGGGAGGGAGGTTTGCTGTGAAGATTATGTGGTTCCCAACAACAAGAGCGCTGGGC
       AGGCGGCAAAGGGAGGGAGGTTTGCTGTGAAGATTATGTGGTTCCCAACAACAAGAGC CTGGGC

RHCE : CTATCTCTGCCCTCTCTTTTCTGTGTGTCCTGGGACAAGTCACTTGGCTTCTGTGGCTTTATTTT
RHD  : CTATCTCTGCCCTCTCTTTTCTGTGTGTCCTGGGACAAGTCACTTGGCTTCTGTGGCTTCATTTT
       CTATCTCTGCCCTCTCTTTTCTGTGTGTCCTGGGACAAGTCACTTGGCTTCTGTGGCTT ATTTT

RHCE : CTCATGTGCCCAGCCAGGGGGTTGGCCCTCATATGCAATAACAGCAGCAATGACCTTTACTGAGT
RHD  : CTCATGTGCCCAGCCAGGGGGTTGGCCCTCATATGCAATAACAGCAGCAATGACCTTTACTGAGT
       CTCATGTGCCCAGCCAGGGGGTTGGCCCTCATATGCAATAACAGCAGCAATGACCTTTACTGAGT

RHCE : GTCCATGTGCATCAAGCACGTGTACTTTACACTTGTTCTTATTATTAGGTTTAATAATAGAATAA
RHD  : GTCCATGTGCGTCAAGCACGTGTGCTTTACACTTGTTCTTATTATTAGGTTTAATAATAGAATAA
       GTCCATGTGC TCAAGCACGTGT CTTTACACTTGTTCTTATTATTAGGTTTAATAATAGAATAA

RHCE : TTGCCACATTTACTGAGCACTCATTATGGGCCAGGCCCTGCCCTAAGTGCTTAATTAGCTTTAGC
RHD  : TTGCCACATTTACTGAGCACTCATTATGGGCCAGGCCCTGCCCTAAGTGCTTAATTAGCTTTAGC
       TTGCCACATTTACTGAGCACTCATTATGGGCCAGGCCCTGCCCTAAGTGCTTAATTAGCTTTAGC

RHCE : TCCTCTAATCCTTACCTTATCCCCACACGGCATGTTATGTTATCCCCATTATTCAGTTGAGAACA
RHD  : TCCTCTAATCCTTATCTTATCCCCACACGGCATGTTATGTTATCCCCATTATTCAGTTGAGAACA
       TCCTCTAATCCTTA CTTATCCCCACACGGCATGTTATGTTATCCCCATTATTCAGTTGAGAACA

RHCE : TTGAGGCTCAAAGAGGCAAAGTAACTTGACCAAATACTTGTAAACGATCTTGCATGCCCCTTCCA
RHD  : TTGAGGCTCAAAGAGGCAAAGTAACTTGACCAAATACTTGTAAACGATCTTGCATGCCCCTTCCA
       TTGAGGCTCAAAGAGGCAAAGTAACTTGACCAAATACTTGTAAACGATCTTGCATGCCCCTTCCA
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RHCE : GCTGCCATTTAGTAAGACTCTAATTTCATACCACCCTAAATCTCGTCTGCTTCCCCCTCCTCCTT
RHD  : GCTGCCATTTAGTAAGACTCTAATTTCATACCACCCTAAATCTCGTCTGCTTCCCCCTCGTCCTT
       GCTGCCATTTAGTAAGACTCTAATTTCATACCACCCTAAATCTCGTCTGCTTCCCCCTC TCCTT

RHCE : CTCACCATCTCCCCACCGAGCAGTCGGCCAAGATCTGACCGTGATGGCGGCCCTTGGCTTGGGCT
RHD  : CTCGCCATCTCCCCACCGAGCAGTTGGCCAAGATCTGACCGTGATGGCGGCCATTGGCTTGGGCT
       CTC CCATCTCCCCACCGAGCAGT GGCCAAGATCTGACCGTGATGGCGGCC TTGGCTTGGGCT

RHCE : TCCTCACCTCAAATTTCCGGAGACACAGCTGGAGCAGTGTGGCCTTCAACCTCTTCATGCTGGCG
RHD  : TCCTCACCTCGAGTTTCCGGAGACACAGCTGGAGCAGTGTGGCCTTCAACCTCTTCATGCTGGCG
       TCCTCACCTC A TTTCCGGAGACACAGCTGGAGCAGTGTGGCCTTCAACCTCTTCATGCTGGCG

RHCE : CTTGGTGTGCAGTGGGCAATCCTGCTGGACGGCTTCCTGAGCCAGTTCCCTCCTGGGAAGGTGGT
RHD  : CTTGGTGTGCAGTGGGCAATCCTGCTGGACGGCTTCCTGAGCCAGTTCCCTTCTGGGAAGGTGGT
       CTTGGTGTGCAGTGGGCAATCCTGCTGGACGGCTTCCTGAGCCAGTTCCCT CTGGGAAGGTGGT

RHCE : CATCACACTGTTCAGGTATTGGGATGGTGGCTGGATCACTTCTGGGTCATAGAGGGAATGGACCC
RHD  : CATCACACTGTTCAGGTATTGGGATGGTGGCTGGATCACTTCTGGGTCATAGAGGGAATGGACCC
       CATCACACTGTTCAGGTATTGGGATGGTGGCTGGATCACTTCTGGGTCATAGAGGGAATGGACCC
```

Key:

exon : *italics*
primers : **bold**
differences between *RHD* and *RHCE (little c)* in exon : underlined
SNPs for variant *RHD* alleles : double underlined
first and last nucleotide of exon : shaded

Consensus sequence is shown underneath each set of *RHCE/RHD* sequence.
RH little c sequence is shown; RH big C sequence is highly homologous to RHD sequence in this region

Exon 3

RHCE : CCTTCTCAGTCATCCTGGCTCTCCTTCTCACCCCCAG*TATTCGGCTGGCCACCATGAGTGCTATG*
RHD : CCTTCTCAGTCGTCCTGGCTCTCCCTCTCTCCCCCAG*TATTCGGCTGGCCACCATGAGTGCTTTG*
CCTTCTCAGTC TCCTGGCTCTCC TCTC CCCCCAGTATTCGGCTGGCCACCATGAGTGCT TG

RHCE : *TCGGTGCTGATCTCAGCGGGTGCTGTCTTGGGGAAGGTCAACTTGGCGCAGTTGGTGGTGATGGT*
RHD : *TCGGTGCTGATCTCAGTGGATGCTGTCTTGGGGAAGGTCAACTTGGCGCAGTTGGTGGTGATGGT*
TCGGTGCTGATCTCAG GG TGCTGTCTTGGGGAAGGTCAACTTGGCGCAGTTGGTGGTGATGGT

RHCE : *GCTGGTGGAGGTGACAGCTTTAGGCACCCTGAGGATGGTCATCAGTAATATCTTCAACGTGAGTC*
RHD : *GCTGGTGGAGGTGACAGCTTTAGGCAACCTGAGGATGGTCATCAGTAATATCTTCAACGTGAGTC*
GCTGGTGGAGGTGACAGCTTTAGGCA CCTGAGGATGGTCATCAGTAATATCTTCAACGTGAGTC

RHCE : ATGGTGCTGGGAGGAGGGACCTGGGAGAAAAGGGCCAAAAGCTCCATTTGGTGGGGCTTCCGGGG
RHD : ATGGTGCTGGGAGGAGGGACCTGGGAGAAAAGGGCCAAAAGCTCCATTTGGTGGGGTTTCC**AGGG**
ATGGTGCTGGGAGGAGGGACCTGGGAGAAAAGGGCCAAAAGCTCCATTTGGTGGGG TTCC GGG

RHCE : TTTTGAAAAATAAAGACAACCTGTAATCCCAGCTACTTGGGAGGTTGAGGAGGGAAGATCACTTG
RHD : **TTTTGAAAAATAAAGACAAC**CTGTAATCCCAGCTACTTGGGAGGTTGAGGAGGGAAGATCACTTG
TTTTGAAAAATAAAGACAACCTGTAATCCCAGCTACTTGGGAGGTTGAGGAGGGAAGATCACTTG

Key:

exon : *italics*
primers : **bold**
differences between *RHD* and *RHCE* in exon : underlined
SNPs for variant *RHD* alleles : double underlined

Consensus sequence is shown underneath each set of *RHCE/RHD* sequence.

Fig 2D

Exon 4

RHCE : TGGGTTGGGCTGGGTAAGCTCTGAACACCAGTCTCGTGGCTTCAAGTCACACCTCCTAAGTGAAG
RHD : TGGGTTGGGCTGGGTAAGCTCTGAACACCAGTCTCATGGCTTCAAGTCACACCTCCTAAGTGAAG
TGGGTTGGGCTGGGTAAGCTCTGAACACCAGTCTC TGGCTTCAAGTCACACCTCCTAAGTGAAG

RHCE : CTCTGAACTTTCTCCAAGGACCATCAGGGCTTTCCCCTGGGCAGAGGATGCCGACACTCACTGCT
RHD : **CTCTGAACTTTCTCCAAGGACT**ATCAGGGCTTGCCCC-GGGCAGAGGATGCCGACACTCACTGCT
CTCTGAACTTTCTCCAAGGAC ATCAGGGCTT CCCC GGGCAGAGGATGCCGACACTCACTGCT

RHCE : *TATTTTGGGCTGACTGTGGCCTGGTGCCTGCCAAAGCCTCTACCCAAGGGAACGGAGGATAATGA*
RHD : *TATTTTGGGCTGTCTGTGGCCTGGTGCCTGCCAAAGCCTCTACCCGAGGGAACGGAGGATAAAGA*
TATTTTGGGCTG CTGTGGCCTGGTGCCTGCCAAAGCCTCTACCC AGGGAACGGAGGATAA GA

RHCE : *TCAGAGAGCAACGATACCCAGTTTGTCTGCCATGCTGGG*TAAGGACAAGGTGGGGTGAGTGGTCT
RHD : *TCAGACAGCAACGATACCCAGTTTGTCTGCCATGCTGGG*TAAGGACAAGGTGGGGTGAGTGGTCT
TCAGA AGCAACGATACCCAGTTTGTCTGCCATGCTGGGTAAGGACAAGGTGGGGTGAGTGGTCT

RHCE : CATACTTGGGCTGAGCAGAATGGCTCAGAAAAGGCTCTGGCTGAAAAAATCTCCCTCCTTTACCA
RHD : C**CTACTTGGGCTGAGCAGAAT**GGCTCAGAAAAGGCTCTGGCTGAAAAAATCTCCCTCCTTTACCA
C TACTTGGGCTGAGCAGAATGGCTCAGAAAAGGCTCTGGCTGAAAAAATCTCCCTCCTTTACCA

Key:

exon : *italics*
primers : **bold**
differences between *RHD* and *RHCE* in exon : underlined
SNPs for variant *RHD* alleles : double underlined
first and last nucleotide of exon are shaded

Consensus sequence is shown underneath each set of *RHCE/RHD* sequence.

Fig 2E

Exon 5

```
rhce : CATACCTTTGAATTAAGCACTTCCTTTTAGGGACCTCTCTTCATTAATATCCACTAGAAAGGAGA
rhd  : CATACCTTTGAATTAAGCACTTC----------------------------------------
       CATACCTTTGAATTAAGCACTTC

rhce : GACTCATTATGTGTGAGTTTCAATAAGTTTATCCAATCCCTTTGTTTTCAACTGAAAGGAGGGAA
rhd  : ----------------------------------------------------------------

rhce : ACGGACAAGTGAAGAAGGTAGGGCCCAGGAGTGAAGGAACAAGGGTGGGAATAGTAATAATGTTG
rhd  : ----------------------------------------------------------------

rhce : TACTTTGAAAATCTACTGGGAAAATGATGAACTTAGACTGCTGGGAGAGGCTAATAGAAAATCGG
rhd  : ----------------------------------------------------------------

rhce : GCAGTGAGCTTGATAGTAGGCAAAGGACTATCAGGCCACGGGGTCAAGTTAAAGCAGCACATTCA
rhd  : ----------------------------------------------------------------

rhce : TTAAAAAAAAAATAAATAAGCGTTTGGGCCAGGCGTGGTGGCTCAAGCCTGTAATCCCAGCACTT
rhd  : ----------------------------------------------------------------

rhce : TGGGAGGCCAAGGTGGGTGGATCACCTGAGGTCAGGAGTTCGAGACCAGCCTGGCCAACAGGGCG
rhd  : ----------------------------------------------------------------

rhce : AAACCCCATCTCTACTAAAAATACAAACAAATTAGCTGGGCATGGTGGTGCACGCCTGTAATCCC
rhd  : ----------------------------------------------------------------

rhce : AGCTACTTGGGAGGCTGAGGCAGGAGAATCTTTTGAATCCAGGTGGTGGAGGTTGCAGTGAGCCA
rhd  : ----------------------------------------------------------------

rhce : AGATCGCGCCACTGCACTCCAGCCTGGGCAACAGAGCAAGAGTCCATCTCAATTAAAAAGAAAAA
rhd  : ----------------------------------------------------------------

rhce : AAAATTAAAATAAGCATTTGACCATCACAGAGCAGGTTCAGGAGGCCTGGGGTATGCAGATTTCA
rhd  : ----------------------------ACAGAGCAGGTTCAGGAGGCCTGGGGTATGCAGATTTCA
                                    ACAGAGCAGGTTCAGGAGGCCTGGGGTATGCAGATTTCA

rhce : ACCCTCTTGGCCTTTGTTTCCTTGTCTGTAAAATGTGGTTAGCTGGTATCAGCTTGAGAGCTCGG
rhd  : ACCCTCTTGGCCTTTGTTTCCTTGTCTGTAAAATGTGGTTAGCTGGTATCAGCTTGAGAGCTCGG
       ACCCTCTTGGCCTTTGTTTCCTTGTCTGTAAAATGTGGTTAGCTGGTATCAGCTTGAGAGCTCGG

rhce : AGGGGAGACGTGACTTCCCCATCTAACTCTAAGTGACAAGGCTGAGACTCTCCAGCCCTAGGATT
rhd  : AGGGGAGACGTGACTTCCCCATCTAACTCTAAGTGACAAGGCTGAGACTCTCCAGCCCTAGGATT
       AGGGGAGACGTGACTTCCCCATCTAACTCTAAGTGACAAGGCTGAGACTCTCCAGCCCTAGGATT

rhce : CTCATCCAAAACCCCTCGAGGCTCAGACCTTTGGAGCAGGAGTGTGATTCTGGCCAACCACCCTC
rhd  : CTCATCCAAAACCCCTCGAGGCTCAGACCTTTGGAGCAGGAGTGTGATTCTGGCCAACCACCCTC
       CTCATCCAAAACCCCTCGAGGCTCAGACCTTTGGAGCAGGAGTGTGATTCTGGCCAACCACCCTC

rhce : TCTGGCCCCCAGGCGCCCTCTTCTTGTGGATGTTCTGGCCAAGTGTCAACTCTGCTCTGCTGAGA
rhd  : TCTGGCCCCCAGGCGCCCTCTTCTTGTGGATGTTCTGGCCAAGTTTCAACTCTGCTCTGCTGAGA
       TCTGGCCCCCAGGCGCCCTCTTCTTGTGGATGTTCTGGCCAAGT TCAACTCTGCTCTGCTGAGA

rhce : AGTCCAATCCAAAGGAAGAATGCCATGTTCAACACCTACTATGCTCTAGCAGTCAGTGTGGTGAC
rhd  : AGTCCAATCGAAAGGAAGAATGCCGTGTTCAACACCTACTATGCTGTAGCAGTCAGCGTGGTGAC
       AGTCCAATC AAAGGAAGAATGCC TGTTCAACACCTACTATGCT TAGCAGTCAG GTGGTGAC
```

rhce : *AGCCATCTCAGGGTCATCCTTGGCTCACCCCCAAAGGAAGATCAGCATGGTGA*GCAGGGCGCTGC
rhd : *AGCCATCTCAGGGTCATCCTTGGCTCACCCCCAAGGGAAGATCAGCAAGGTGA*GCAGGGCGCTGC
AGCCATCTCAGGGTCATCCTTGGCTCACCCCCAA GGAAGATCAGCA GGTGAGCAGGGCGCTGC
rhce : CCTTGGGCAGCACTTGGGTCTAACAGGACTAGCACACATATTTATGCCCCTCCCCACCCCAGGGC
rhd : CCTTGGGCAGCACTTGGGTCTAACAGGACTAGCACACATATTTATGCCCCTCCCCACCCCAGGGC
CCTTGGGCAGCACTTGGGTCTAACAGGACTAGCACACATATTTATGCCCCTCCCCACCCCAGGGC

rhce : CAGCGTGGGTTGGGAGAGGACATGCCGGGTGGTGGAGCTGTGCCTGCCTCTACAGTGGAGCTCTA
rhd : CAGCGTGGGTTGGGAGAGGGCATGCCGGGTGGTGGAGCTGTGCCTGCCTCTACAGTGGAGCTCTA
CAGCGTGGGTTGGGAGAGG CATGCCGGGTGGTGGAGCTGTGCCTGCCTCTACAGTGGAGCTCTA

rhce : GGAAGAATGCTGGGTGGTCACAGGGGGCCTGGGACTCAGGAGACTGTCCAGTGATCAAAGGCTTT
rhd : GG**TAGAATGCTGGGTGGTCACA**GTGGGCCTGGGACTCAGGAGACTGTCCAGTGATCAAAGGCTTT
GG AGAATGCTGGGTGGTCACAG GGGCCTGGGACTCAGGAGACTGTCCAGTGATCAAAGGCTTT

Key:

exon : *italics*
primers : **bold**
differences between *RHD* and *RHCE* in exon : underlined
SNPs for variant *RHD* alleles : double underlined
first and last nucleotide of exon are shaded

Consensus sequence is shown underneath each set of *RHCE/RHD* sequence.

Fig. 2F

Exon 6

RHCE : CCTAAGAGGCAGTAGTGAGCTGGCCCACCGTGTCCACTGATGAAGGACACGTAGCCCCAACACAG
RHD : CCTAAGAGGC**AGTAGTGAGCTGGCCCATCA**TGTCCACTGATGAAGGACACGTAGCCCCAACACAG
CCTAAGAGGCAGTAGTGAGCTGGCCCA C TGTCCACTGATGAAGGACACGTAGCCCCAACACAG

RHCE : GGGAGAGGTGGTTTCAGGATCAGCAAAGCAGGGAGGATGTTACAGGGTTGCCTTGTTCCCAGCGT
RHD : GGGAGAAGTGGTTTCAGGATCAGCAAAGCAGGGAGGATGTTACAGGGTTGCCTTGTTCCCAGCGT
GGGAGA GTGGTTTCAGGATCAGCAAAGCAGGGAGGATGTTACAGGGTTGCCTTGTTCCCAGCGT

RHCE : GCTGGTCACTTGCAGCAAGATGGTGTTCTCTCTCTACCTTGCTTCCTTTACCCACACGCTATTTC
RHD : GCTGGTCACTTGCAGCAAGATGGTGTTCTCTCTCTACCTTGCTTCCTTTACCCACACGCTATTTC
GCTGGTCACTTGCAGCAAGATGGTGTTCTCTCTCTACCTTGCTTCCTTTACCCACACGCTATTTC

RHCE : TTTGCAG*ACTTATGTGCACAGTGCGGTGTTGGCAGGAGGCGTGGCTGTGGGTACCTCGTGTCACC*
RHD : TTTGCAG*ACTTATGTGCACAGTGCGGTGTTGGCAGGAGGCGTGGCTGTGGGTACCTCGTGTCACC*
TTTGCAGACTTATGTGCACAGTGCGGTGTTGGCAGGAGGCGTGGCTGTGGGTACCTCGTGTCACC

RHCE : *TGATCCCTTCTCCGTGGCTTGCCATGGTGCTGGGTCTTGTGGCTGGGCTGATCTCCATCGGGGGA*
RHD : *TGATCCCTTCTCCGTGGCTTGCCATGGTGCTGGGTCTTGTGGCTGGGCTGATCTCCGTCGGGGGA*
TGATCCCTTCTCCGTGGCTTGCCATGGTGCTGGGTCTTGTGGCTGGGCTGATCTCC TCGGGGGA

RHCE : *GCCAAGTGCCTGCCG*GTAAGAAACTAGACAACTAATGCTCTCTGCTTTGGCTGAAGGCCAGCAGG
RHD : *GCCAAGTACCTGCCG*GTAAGAAACTAGACAACTAAC**CTCCTCTGCTTTGGCTGAAG**GCCAGCAGG
GCCAAGT CCTGCCGGTAAGAAACTAGACAACTAA CTCTGCTTTGGCTGAAGGCCAGCAGG

Key:

exon : *italics*
primers : **bold**
differences between *RHD* and *RHCE* in exon : underlined
SNPs for variant *RHD* alleles : double underlined

Consensus sequence is shown underneath each set of *RHCE/RHD* sequence.

Fig. 2G

Exon 7

RHCE : GTGCCTACACTAGACCCTTGCTACTCATAGTGTGGTCCGTAGATGAGCAGCATTGGCATCACCTG
RHD : GTGTCTACACTAGACCCTTGCTACTCATAGTGTGGTCCGTAGACCAGCAGCATTGGCATCACCTG
GTG CTACACTAGACCCTTGCTACTCATAGTGTGGTCCGTAGA AGCAGCATTGGCATCACCTG

RHCE : GGACCTTGTTAGAAATGCTCTTAGACCCCACCCCACATCCACTAAAGCCAGCTCTTCATTTCAAC
RHD : **GGACCTTGTTAGAAATGCTG**TTAGACCCCACCCCACATCCACTAAAGCCAGCTCTTCATTTCAAC
GGACCTTGTTAGAAATGCT TTAGACCCCACCCCACATCCACTAAAGCCAGCTCTTCATTTCAAC

RHCE : AAACTCCCCATTGATGTGAGTACACATTCAAGTCTGAGAAGGGCTTCTTTGAGGTGAGCCTTAGT
RHD : AAACTCCCCGATGATGTGAGTGCACATTCAAGTCTGAGAAGGGCTTCTTTGAGGTGAGCCTTAGT
AAACTCCCC TGATGTGAGT CACATTCAAGTCTGAGAAGGGCTTCTTTGAGGTGAGCCTTAGT

RHCE : GCCCATCCCCATTTGGTGGCGCCGGATACCAAGGGTGTGTGAAAGGGGTGGGTAGGGAATATGGG
RHD : GCCCATCCCCCTTTGGTGGCCCCGGATACCAAGGGTGTGTGAAAGGGGTGGGTAGGGAATATGGG
GCCCATCCCC TTTGGTGGC CCGGATACCAAGGGTGTGTGAAAGGGGTGGGTAGGGAATATGGG

RHCE : TCTCACCTGCCAATCTGCTTATAATAACACTTGTCCACAG*GTGTGTTGTAACCGAGTGCTGGGGA*
RHD : TCTCACCTGCCAATCTGCTTATAATAACACTTGTCCACAG*GGGTGTTGTAACCGAGTGCTGGGGA*
TCTCACCTGCCAATCTGCTTATAATAACACTTGTCCACAGG GTGTTGTAACCGAGTGCTGGGGA

RHCE : *TTCACCACATCTCCGTCATGCACTCCATCTTCAGCTTGCTGGGTCTGCTTGGAGAGATCACCTAC*
RHD : *TTCCCCACAGCTCCATCATGGGCTACAACTTCAGCTTGCTGGGTCTGCTTGGAGAGATCATCTAC*
TTC CCACA CTCC TCATG CT CA CTTCAGCTTGCTGGGTCTGCTTGGAGAGATCA CTAC

RHCE : *ATTGTGCTGCTGGTGCTTCATACTGTCTGGAACGGCAATGGCATGTGGGTCACTGGGCTTACCCC*
RHD : *ATTGTGCTGCTGGTGCTTGATACCGTCGGAGCCGGCAATGGCATGTGGGTCACTGGGCTTACCCC*
ATTGTGCTGCTGGTGCTT ATAC GTC G CGGCAATGGCATGTGGGTCACTGGGCTTACCCC

RHCE : CCATCCCCTTAACACTCCCCTCCAACTCAGGAAGAAATGTGTGCAGAGTCCTTAGCTGGGGCGTG
RHD : CCATCCCCTTAACACTCCCCTCCAACTCAGGAAGAAATGTGTGCAGAGTCCTTAGCTGGGGCGTG
CCATCCCCTTAACACTCCCCTCCAACTCAGGAAGAAATGTGTGCAGAGTCCTTAGCTGGGGCGTG

RHCE : TGCACTCGGGGCCAGGTGCTCAGTAGGCTTCGGTGAATATTTGTTGGCTGATTTATTCAGAAATT
RHD : TGCACTCGGGGCCAGGTGCTCAGTAGGCTTCGGTGAATATTTGTTGGCTGATTTATTCAGAAATT
TGCACTCGGGGCCAGGTGCTCAGTAGGCTTCGGTGAATATTTGTTGGCTGATTTATTCAGAAATT

RHCE : ATGTCCAGCCCCTACCTTGGATGGATTTATCACCTCTCCAGGCCACCTCTTCTTTCCAAATAGGA
RHD : **CTGTCCAGCCCCTACCTTG**GATGGATTTATCACCTCTCCAGGCCACCTCTTCTTTCCAAATAGGG
TGTCCAGCCCCTACCTTGGATGGATTTATCACCTCTCCAGGCCACCTCTTCTTTCCAAATAGG

Key:

exon : *italics*
primers : **bold**
differences between *RHD* and *RHCE* in exon : <u>underlined</u>
SNPs for variant *RHD* alleles : <u>double underlined</u>

Consensus sequence is shown underneath each set of *RHCE/RHD* sequence.

Fig 2H

Exon 7

RHCE : GGACCTTGTTAGAAATGCTCTTAGACCCCACCCCACATCCACTAAAGCCAGCTCTTCATTTCAAC
RHD : GGACCTTGTTAGAAATGCTGTTAGACCCCACCCCACATCCACTAAAGCCAGCTCTTCATTTCAAC
GGACCTTGTTAGAAATGCT TTAGACCCCACCCCACATCCACTAAAGCCAGCTCTTCATTTCAAC

RHCE : AAACTCCCCATTGATGTGAGTACACATTCAAGTCTGAGAAGGGCTTCTTTGAGGTGAGCCTTAGT
RHD : **AAACTCCCCGATGATGTGAGTG**CACATTCAAGTCTGAGAAGGGCTTCTTTGAGGTGAGCCTTAGT
AAACTCCCC TGATGTGAGT CACATTCAAGTCTGAGAAGGGCTTCTTTGAGGTGAGCCTTAGT

RHCE : GCCCATCCCCATTTGGTGGCGCCGGATACCAAGGGTGTGTGAAAGGGGTGGGTAGGGAATATGGG
RHD : GCCCATCCCCCTTTGGTGGCCCCGGATACCAAGGGTGTGTGAAAGGGGTGGGTAGGGAATATGGG
GCCCATCCCC TTTGGTGGC CCGGATACCAAGGGTGTGTGAAAGGGGTGGGTAGGGAATATGGG

RHCE : TCTCACCTGCCAATCTGCTTATAATAACACTTGTCCACAG*GTGTGTTGTAACCGAGTGCTGGGGA*
RHD : TCTCACCTGCCAATCTGCTTATAATAACACTTGTCCACAG*GGGTGTTGTAACCGAGTGCTGGGGA*
TCTCACCTGCCAATCTGCTTATAATAACACTTGTCCACAGG GTGTTGTAACCGAGTGCTGGGGA

RHCE : *TTCACCACATCTCCGTCATGCACTCCATCTTCAGCTTGCTGGGTCTGCTTGGAGAGATCACCTAC*
RHD : *TTCCCCACAGCTCCATCATGGGCTACAACTTCAGCTTGCTGGGTCTGCTTGGAGAGATCATCTAC*
TTC CCACA CTCC TCATG CT CA CTTCAGCTTGCTGGGTCTGCTTGGAGAGATCA CTAC

RHCE : *ATTGTGCTGCTGGTGCTTCATACTGTCTGGAACGGCAATGGCATG*TGGGTCACTGGGCTTACCCC
RHD : *ATTGTGCTGCTGGTGCTTGATACCGTCGGAGCCGGCAATGGCA*TGTGGGTCACTGGGCTTACCCC
ATTGTGCTGCTGGTGCTT ATAC GTC G CGGCAATGGCATGTGGGTCACTGGGCTTACCCC

RHCE : CCATCCCCTTAACACTCCCCTCCAACTCAGGAAGAAATGTGTGCAGAGTCCTTAGCTGGGGCGTG
RHD : CCATCCCCTTAACACTCCCCTCCAACTCAGGAAGAAATGTGTGCAGAGTCCTTAGCTGGGGCGTG
CCATCCCCTTAACACTCCCCTCCAACTCAGGAAGAAATGTGTGCAGAGTCCTTAGCTGGGGCGTG

RHCE : TGCACTCGGGGCCAGGTGCTCAGTAGGCTTCGGTGAATATTTGTTGGCTGATTTATTCAGAAATT
RHD : TGCACTCGGGGCCAGGTGCTCAGTAGGCTTCGGTGAATATTTGTTGGCTGATTTATTCAGAAATT
TGCACTCGGGGCCAGGTGCTCAGTAGGCTTCGGTGAATATTTGTTGGCTGATTTATTCAGAAATT

RHCE : ATGTCCAGCCCCTACCTTGGATGGATTTATCACCTCTCCAGGCCACCTCTTCTTTCCAAATAGGA
RHD : CTGTCCAGCCCCTACCTTGGATGGATTTATCACCTCTCCAGGCCACCTCTTCTTTCCAAATAGGG
TGTCCAGCCCCTACCTTGGATGGATTTATCACCTCTCCAGGCCACCTCTTCTTTCCAAATAGG

RHCE : CCACCTAGGTATAGACCAAAGACACGAAATCTTCTGTGACCCCACAAACACAGAGCAGGTCAAAT
RHD : CCACCTAGGTATAGACCAAAGACACGAAATCTTTTGTGATCCCACAAACACAGAGCAGGTCAAAT
CCACCTAGGTATAGACCAAAGACACGAAATCTT TGTGA CCCACAAACACAGAGCAGGTCAAAT

RHCE : AGGCCCAAGCCAATTGAGACTGTGGTTCAGGTCGTGATGCAGAGCTTTGCTGTGGACGTGCTCCC
RHD : AGGCCCAAGCCAATTGAGACTGTGGTTCAGGTCGTGATGCAGAGCTTTGCTGTGGACGTGCTCCC
AGGCCCAAGCCAATTGAGACTGTGGTTCAGGTCGTGATGCAGAGCTTTGCTGTGGACGTGCTCCC

RHCE : ACTGCGTACTAGCTGGGCATGCGGCTTAACCTTTCTCAGCCTCAGTCGCCCCCTTGTAAATGGAG
RHD : ACTGCGTACTAGCTGGGCATG**TGGCTTAACCTTTCTCAGCCTC**AGTCGCCCCATTGTAAATGGAG
ACTGCGTACTAGCTGGGCATG GGCTTAACCTTTCTCAGCCTCAGTCGCCCC TTGTAAATGGAG

Key:

exon : *italics*
primers : **bold**
differences between *RHD* and *RHCE* in exon : underlined
SNPs for variant *RHD* alleles : double underlined
first and last nucleotide of exon are shaded

Consensus sequence is shown underneath each set of *RHCE/RHD* sequence.

Fig 2I

Exon 8

RHCE : CAGAAAAAAAAAAAAAAAAAGAGAGAGAGAGAAAACTGGAGGCTCTGAGAGGTTAAAGGACTTG
RHD : CAGAAAAAAAAAAAAAAAAA-GAGAGAGAGAGAAAA**CTGGAGGCTCTGAGAGGTTGAG**GGACTTG
CAGAAAAAAAAAAAAAAAAA GAGAGAGAGAGAAAACTGGAGGCTCTGAGAGGTT A GGACTTG

RHCE : CCCAGGGTCTTGCAGCTAGTAAGTGACAGAGCTGGGACTTGAGCTTGGGTTTTCTGACTCCTGGT
RHD : CCCAGGGTCTTGCAGCTAGTAAGTGACAGAGCTGGGACTTGAGCTTGGGTTTTCTGACTCCTGGT
CCCAGGGTCTTGCAGCTAGTAAGTGACAGAGCTGGGACTTGAGCTTGGGTTTTCTGACTCCTGGT

RHCE : CTGGTTCATTATCCATGAGGTGCTGGGAACTAAAATAAGCCACAATCTTGGAATCTCCGTCGCCT
RHD : CTGGTTCATTATCCATGAGGTGCTGGGAACTAAAATAAGCCACAATCTTGGAATCTCCGTCGCCT
CTGGTTCATTATCCATGAGGTGCTGGGAACTAAAATAAGCCACAATCTTGGAATCTCCGTCGCCT

RHCE : CCCTCCCTCCCACATGTCTGCGTGGCTTTTTGGGAAAATGCCAGGGGAATGTACCAGCCAGGGAG
RHD : CCCTCCCTCCCACATGTCTGCGTGGCTTTTTGGGAAAATGCCAGGGGAATGTACCAGCCAGGGAG
CCCTCCCTCCCACATGTCTGCGTGGCTTTTTGGGAAAATGCCAGGGGAATGTACCAGCCAGGGAG

RHCE : AGGACCCTTGTTTTCCTCATGGCCCTTCCTGGCAATGGCACTACTGACACCGACAGTCCTTTTTG
RHD : AGGACCCTTGTTTTCCTCATGGCCCTTCCTGGCAATGGCACTACTGACACCGACAGTCCTTTTTG
AGGACCCTTGTTTTCCTCATGGCCCTTCCTGGCAATGGCACTACTGACACCGACAGTCCTTTTTG

RHCE : TCCCTGATGACCTCTGCTGCCTGATGCCCAAGTGACCACCTCTGCTTTGTCATTTCTAG*GATTGG*
RHD : TCCCTGATGACCTCTGCTGCCTGATGCCCAAGTGACCACCTCTGCTTTGTCATTTCTAG*GATTGG*
TCCCTGATGACCTCTGCTGCCTGATGCCCAAGTGACCACCTCTGCTTTGTCATTTCTAGGATTGG

RHCE : *CTTCCAGGTCCTCCTCAGCATTGGGGAACTCAGCTTGGCCATCGTGATAGCTCTCATGTCTGGTC*
RHD : *CTTCCAGGTCCTCCTCAGCATTGGGGAACTCAGCTTGGCCATCGTGATAGCTCTCACGTCTGGTC*
CTTCCAGGTCCTCCTCAGCATTGGGGAACTCAGCTTGGCCATCGTGATAGCTCTCA GTCTGGTC

RHCE : *TCCTGACAG*GTCAGTGTGAGGCCA**CCTTTCTTCCACCATTGCC**AGGACACAGCACCCACGTCCAG
RHD : *TCCTGACAG*GTCAGTGTGAGGCCACCTTTCTTCCACCATTGCCAGGACACAGCACCCACGTCCAG
TCCTGACAGGTCAGTGTGAGGCCACCTTTCTTCCACCATTGCCAGGACACAGCACCCACGTCCAG

Key:

exon : *italics*
primers : **bold**
differences between *RHD* and *RHCE* in exon : underlined
SNPs for variant *RHD* alleles : double underlined
first and last nucleotide of exon are shaded

Consensus sequence is shown underneath each set of *RHCE/RHD* sequence.

Forward primer has 2 RHD specific nucleotides including one at the 3' end.
Reverse primer has universal sequence.

Fig. 2J

Exon 9

RHCE : GAAAAAGGATTTCTGTTGAGACACTGTCGTTTTGACACACACAATATTTTGATTAATCTTGAGAT
RHD : GAAAAAGGATTTCTGTTGAGAT**ACTGTCGTTTTGACACACA**--**AT**ATTTCGATTAATCTTGAGAT
GAAAAAGGATTTCTGTTGAGA ACTGTCGTTTTGACACACA ATATTT GATTAATCTTGAGAT

RHCE : TAAAAATCCTGTGCTCCAAATCTTTTAACATTAAATTATGCATTTAAACAG*GTTTGCTCCTAAAT*
RHD : TAAAAATCCTGTGCTCCAAATCTTTTAACATTAAATTATGCATTTAAACAG*GTTTGCTCCTAAAT*
TAAAAATCCTGTGCTCCAAATCTTTTAACATTAAATTATGCATTTAAACAGGTTTGCTCCTAAAT

RHCE : *CTCAAAATATGGAAAGCACCTCATGTGGCTAAATATTTTGATGACCAAGTTTTCTGGAAGGTAAG*
RHD : *CTTAAAATATGGAAAGCACCTCATGAGGCTAAATATTTTGATGACCAAGTTTTCTGGAAGGTAAG*
CT AAAATATGGAAAGCACCTCATG GGCTAAATATTTTGATGACCAAGTTTTCTGGAAGGTAAG

RHCE : ATTTTTCACCTATTAACGTGATAGATTTTGAGTGCATGAACTTAAAAACATACCTGGGTATATAT
RHD : ATTTTTCACCTATTAACGTGATAGATTTTGAGTGCATGAACTTAAAAACATACCTGAGTATATAT
ATTTTTCACCTATTAACGTGATAGATTTTGAGTGCATGAACTTAAAAACATACCTG GTATATAT

RHCE : GTTGACTTGCTGTTTATGAGTAAAACAAAAACAAAAATGGAGTAAGGAGCATTGCAGGAGGAACT
RHD : GTTGACTTGCTGTTTATGAGTAAAACAAAAACAAAAATGGAGTAAGGAGCATTGCAGGAGGAACT
GTTGACTTGCTGTTTATGAGTAAAACAAAAACAAAAATGGAGTAAGGAGCATTGCAGGAGGAACT

RHCE : AGAGGAGAAACAAATCCATGATATGCATGTGTGTGGGGGAGGGTGGCGGGGAGGTGGTAAAGGTC
RHD : AGAGGAGAAACAAATCCATGATATGCATGTGTGTGGGGGAGGGTGGCGGGGAGGTGGTAAAGGTC
AGAGGAGAAACAAATCCATGATATGCATGTGTGTGGGGGAGGGTGGCGGGGAGGTGGTAAAGGTC

RHCE : ACCATTTCCCTGATACCTCAAATTCATTCAGAGTCAGGGATGAGACAGCTTTCACTGGCCACACT
RHD : ACCATTTCCCTGATACCTCAAATTCATTCAGAGTCAGGGATGAGACAGCTTTCACTGGCCACACT
ACCATTTCCCTGATACCTCAAATTCATTCAGAGTCAGGGATGAGACAGCTTTCACTGGCCACACT

RHCE : TCCCCTCCCGCTATCTGCAGTCCTCAGCGTAGCCAAATAGTTTGACATGCGGGTGACAGAACCCC
RHD : TCCCCTCCCCCTATCTGCAGTCCTCAGCGTAGCCAAATAGT**CTGACATGCGGGTGACA**GAACCCC
TCCCCTCCC CTATCTGCAGTCCTCAGCGTAGCCAAATAGT TGACATGCGGGTGACAGAACCCC

Key:

exon : *italics*
primers : **bold**
differences between *RHD* and *RHCE* in exon : underlined
SNPs for variant *RHD* alleles : double underlined

Consensus sequence is shown underneath each set of *RHCE/RHD* sequence.

Fig 2K

Exon 10

```
RHCE     : CTGTTTCAAGAGATCAAGCCAAAATCAGTATGTGGGTTCATCTGCAATAAAAATGTTTGTTTTG
RHD      : CTGTTTCAAGAGATCAAGCCAAAATCAGTATGTGGGTTCATCTGCAATAAAAATGTTTGTTTTG
           CTGTTTCAAGAGATCAAGCCAAAATCAGTATGTGGGTTCATCTGCAATAAAAATGTTTGTTTTG

RHCE     : CTTTTACAGTTTCCTCATTTGGCTGTTGGATTTTAAGCAAAAGCATCCAAGAAAAACAAGGCCT
RHD      : CTTTTACAGTTTCCTCATTTGGCTGTTGGATTTTAAGCAAAAGCATCCAAGAAAAACAAGGCCT
           CTTTTACAGTTTCCTCATTTGGCTGTTGGATTTTAAGCAAAAGCATCCAAGAAAAACAAGGCCT

RHCE     : GTTCAAAAACAAGACAACTTCCTCTCACTGTTGCCTGCATTTGTACGTGAGAAACGCTCATGAC
RHD      : GTTCAAAAACAAGACAACTTCCTCTCACTGTTGCCTGCATTTGTACGTGAGAAACGCTCATGAC
           GTTCAAAAACAAGACAACTTCCTCTCACTGTTGCCTGCATTTGTACGTGAGAAACGCTCATGAC

RHCE     : AGCAAAGTCTCCT-TATGTATAATGAAACAAG-GTCAGAGACAGATTTGATATTAAAAAATTAA
RHD      : AGCAAAGTCTCCAATGTTCGCGCAGGCACTGGAGTCAGAGAAAA---TGGAGTTGAATCCTTTC
           AGCAAAGTCTCC  T T       G  AC  G GTCAGAGA A    TG   TT AA   TT

RHCE     : AGACTAAAAACTTAGTTTAAGAGTCA--ATTTAATAAG---TTTAAAATA-AATGTTTAGTTTC
RHD      : TCTGCCACTCTTTGAGGAGAATCTCACCATTTATTATGCACTGTAGAATACAACAATAAAATAC
                 A    TT      A   TCA  ATTTA TA G   T TA AATA AA   T A  T C

RHCE     : ATTAGGATGATGC-TATCAATATTTTCTTGGTTA-CAGAC-ACATTATTAAAGTTTTGGGTTAA
RHD      : AGCCATGTACCACATAACAACATCTTGGTAAACAACAGACTGCATATATGATGGTGGTCATCCA
           A      T    C TA CAA AT TT  T    A CAGAC  CAT   T A G T     T  A
```

Key:

exon : *italics*
primers : **bold**
first and last nucleotide of exon are shaded
Consensus sequence is shown underneath each set of *RHCE/RHD* sequence.

Figure 3

Primer No.	MAPH PCR primer	RHD exon	Sequence (5'-3')	Tm (°C)	Product size (bp)	[primer] in mpx (nM)
1	101F		gccgcgaattcactagtgCCATAGAGAGGCCAGCACAA	69.8		1.2
2	198R	1	ggccgcgggaattcgattTGCCCCTGGAGAACCAC	69.2	415	1.2
3	int1F		gccgcgaattcactagtgTGACGAGTGAAACTCTATCTCGAT	67.8		3.36
4	297R	2	ggccgcgggaattcgattCCACCATCCCAATACCTGAAC	69.8	1302	3.36
5	303F		gccgcgaattcactagtgTCCTGGCTCTCCCTCTCT	66		1.2
6	397R	3	ggccgcgggaattcgattGTTGTCTTTATTTTTCAAAACCCT	65.3	304	1.2
7	403F		gccgcgaattcactagtgGCTCTGAACTTTCTCCAAGGACT	68.8		1.2
8	499R	4	ggccgcgggaattcgattCAAACTGGGTATCGTTGCTG	67.7	256	1.2
9	502F	5	gccgcgaattcactagtgCTTTGAATTAAGCACTTCACAGA	64.8	470	1.44
10	5Aluint4F (RoHar)	5 (RoHar)	gccgcgaattcactagtgAAGGACTATCAGGCCACG	64.7	838	1.44
11	599R		ggccgcgggaattcgattCACCTTGCTGATCTTCCC	65.1		1.44
12	601F		gccgcgaattcactagtgAGTAGTGAGCTGGCCCATCA	69.3		1.2
13	697R	6	ggccgcgggaattcgattCTTCAGCCAAAGCAGAGGAG	68.7	407	1.2
14	702F		gccgcgaattcactagtgCTGGGACCTTGTTAGAAATGCTG	70.5		1.2
15	799R	7	ggccgcgggaattcgattCAAGGTAGGGGCTGGACAG	69.1	578	1.2
18	901F		gccgcgaattcactagtgACTGTCGTTTTGACACACAAT	64.1		1.056
19	998R	9	ggccgcgggaattcgattTGTCACCCGCATGTCAG	67.3	525	1.056
20	MAPH-rev		gccgcgaattcactagtg	57.94		3200
21	MAPH-forw		ggccgcgggaattcgatt	67.55		3200

Figure 4

RHD Primer	Volume 20μM stock (μl)
101F	1.25
198R	1.25
int1F	3.5
297R	3.5
303F	1.25
397R	1.25
403F	1.25
499R	1.25
502F	1.5
5Aluint4F	1.5
599R	1.5
601F	1.25
697R	1.25
702F	1.25
799R	1.25
801F	1.5
899R	1.5
901F	1.1
998R	1.1
10mM Tris pH8	20.8
Total	50

Figure 5A

nr.	MAPH PCR primer	ABO exon	Sequence (5'-3')	Tm (°C)	Product size (bp)	[primer] in mpx (nM)
20	int1-49f	2	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG	57.9	217	5
21	int2+62r		ggccgcgggaattcgattATTGGCTGCTGTGGTCA	59.0		5
22	int3-33f	4	gccgcgaattcactagtgCCTGCTCCTAGACTAAACTTC	58.0	151	5
23	int4+52r		ggccgcgggaattcgattAAGGGAGGCACTGACATTA	59.6		5
24	int5-44f	6	gccgcgaattcactagtgCTGCCAGCTCCATGTGAC	61.2	263	5
25	int6+31r		ggccgcgggaattcgattAGTCACTCGCCACTGCC	60.6		5
26	ABO432f	7A	gccgcgaattcactagtgCCACCGTGTCCACTACTATG	60.4	371	5
27	ABO766r		ggccgcgggaattcgattTGTAGGCCTGGGACTGG	60.7		5
28	ABO723f	7B	gccgcgaattcactagtgGGAGGCCTTCACCTACG	59.8	461	5
29	ABO1147r		ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC	60.0		5
30	MAPH-rev		gccgcgaattcactagtg	57.94		1000
31	MAPH-forw		ggccgcgggaattcgatt	67.55		1000

Figure 5B

Exon 2 (nt29-98)

To detect 87_88insG

gtgagagaaggagggtgagtgatgtgatttttctactcctgttttccagGAAAACCAAAATGCC
AC[46]GCACTTC[53]GACCTATGATCCTTTTCCTAATAATGCTTGTCTT[87]GGTCTTGTTTGGgtaag
acacatttgaccatcgaggctggcctggtttggggagaagtgaccacagcagccaat

Exon 4 (nt156-203)

To detect 188G>A; 189C>T

cctgctcctagactaaacttcatctcctgtgttctcattctgcagCATGGCTGTTAGGGAACCTGACCA
TCTGCAGC[188]G[189]CGTCTCGTTGCCAA[203]Ggtataatgtcagtgcctcccctt

Exon 6 (nt240-374)

To detect 261delG; 297A>G; 322C>T

ctgccagctccatgtgaccgcacgcctctctccatgtgcagTAGGAAGGATGTCCTCGTGGT[261]
GACCCCTTGGCTGGCTCCCATTGTCTGGGAGGGCAC[297]ATTCAACATCGACATCCTCAACGAG[32]
[2]CAGTTCAGGCTCCAGAACACCACCATTGGGTTAACTGTGTTTGCCATCAAGAAgtaagtcagt
gaggtggccgagggtagagacccaggcagtggcgagtgact

Exon 7 (nt375-1062)

7A

CCACCGTGTCCACTACTATGTCTTCACCGACCAGC[467]CGGCCGCGGTGCCCCGCGTGACGCTGGGGACC
GGTCGGCAGCTGTCAGTGCTGGAGGTGCGCGCCTACAAGCGCT[542]GGCAGGACGTGTCCATGCGCCGCA
TGGAGATGATCAGTGACTTCTGCGAGCGGCGCTTCCTCAGCGAGGTGGATTACCTGGTGTGCGTGGACG
TGGACATGGAG[646]TTCCGCGACCACGTGGGCGTGGAGATCCTGACTCCGCTGTTCGGCACCCTGCAC[700]
CCC[703]GGCTTCTACGGAAGCAGC[721]CGGGAGGCCTTCACCTACGAGCGCCGGCCCCAGTCCCAGGCCTA
CA

7B

GGAGGCCTTCACCTACGAGCGCCGGCCCCAGTCCCAGGCCTACAT[768]CCCCAAGGACGAGGGCGATTTC
TACTAC[796]CT[798]GGGG[802]G[803]GGTTCTTCGGGGGGTCGGTGCAAGAGGTGCAGCGGCTCACCAGGGCCT
GCCACCAGGCCATGATGGTCGACCAGGCCAACGGCATCGAGG[893]CCGTGTGGCACGACGAGAGCCACCT
GAACAAGTA[927]CCTGCTGCGCCACAAACCCACCAAGGTGCTCTCCCCCGAGTACTTGTGGGACCAGCAG
CTGCTGGGCTGGCCCGCCGTCCTGAGGAAGCTGAGGTTCACTGCGGTGCCCAAGAACCACCAGGCGGTC
CGGAAC[1060]CCGTGAGCGGCTGCCAGGGGCTCTGGGAGGGCTGCC[1096]GGCAGCCCCGTCCCCCTCCCGC
CCTTGGTTTTAGCAGAACGGGTAAACTCTG

Figure 6

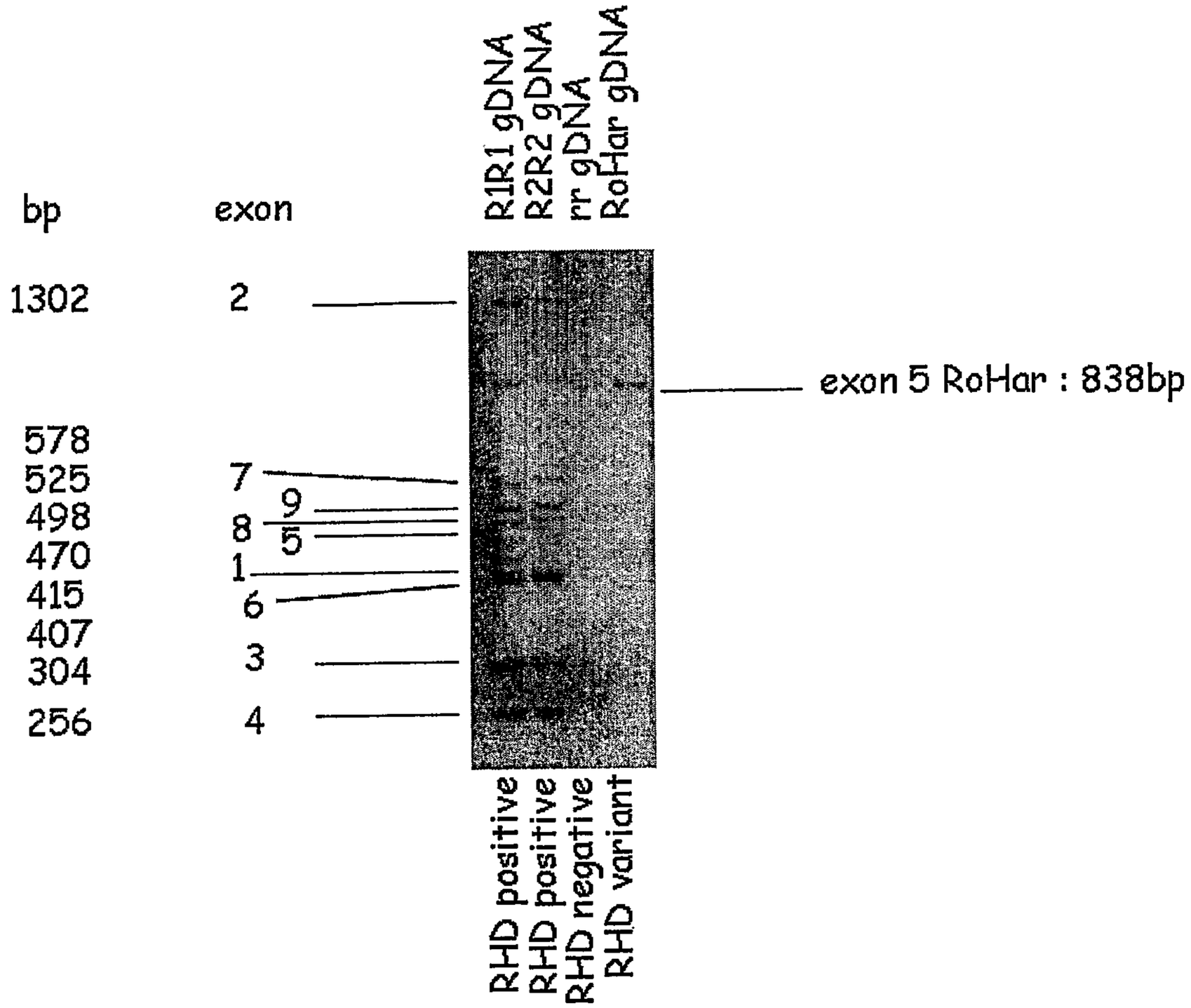

bp
exon
1302
578
525
498
470
415
407
304
256
2
7
9
8
5
1
6
3
4
R1R1 gDNA
R2R2 gDNA
rr gDNA
RoHar gDNA
exon 5 RoHar : 838bp
RHD positive
RHD positive
RHD negative
RHD variant

Figure 7

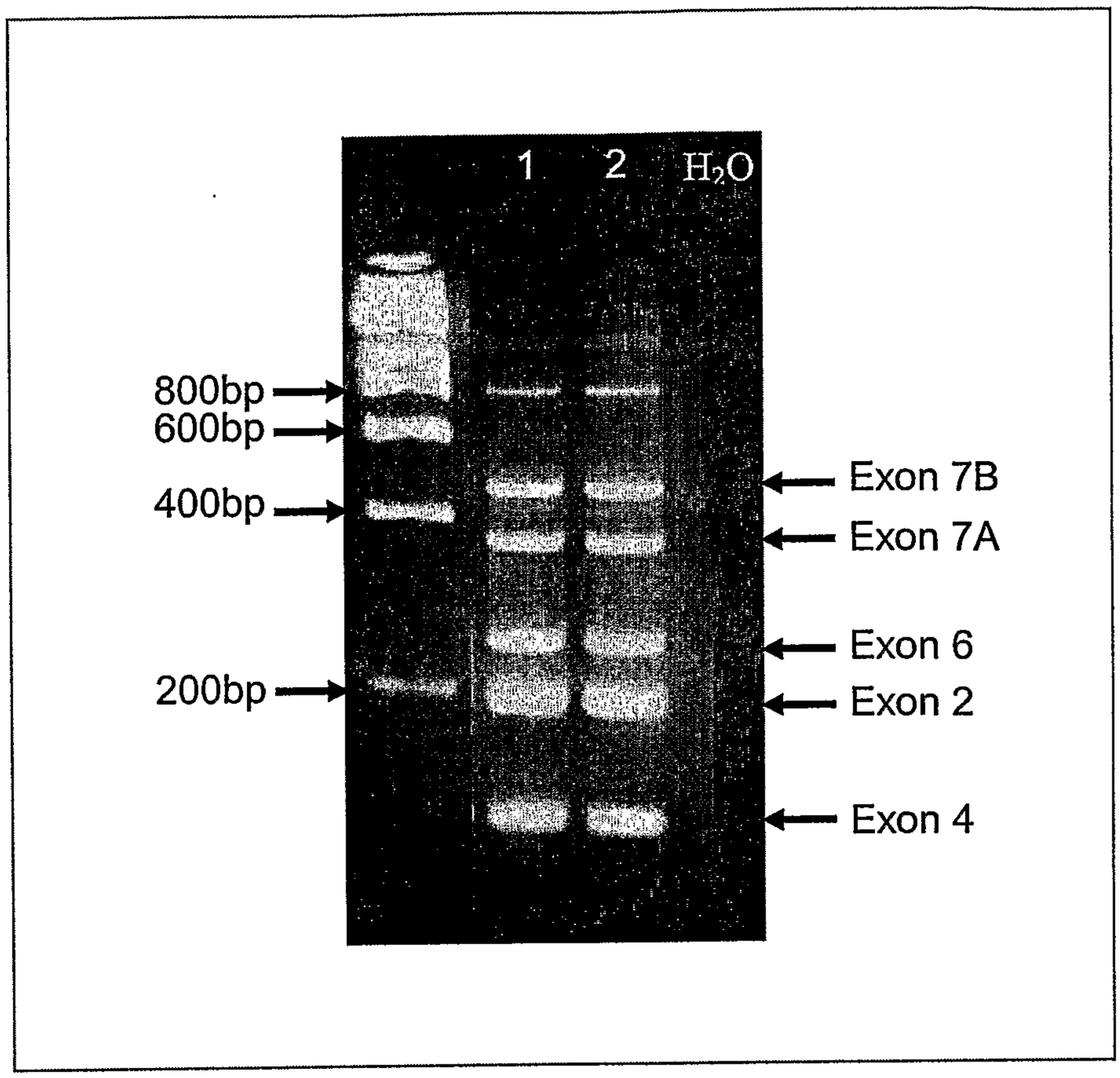
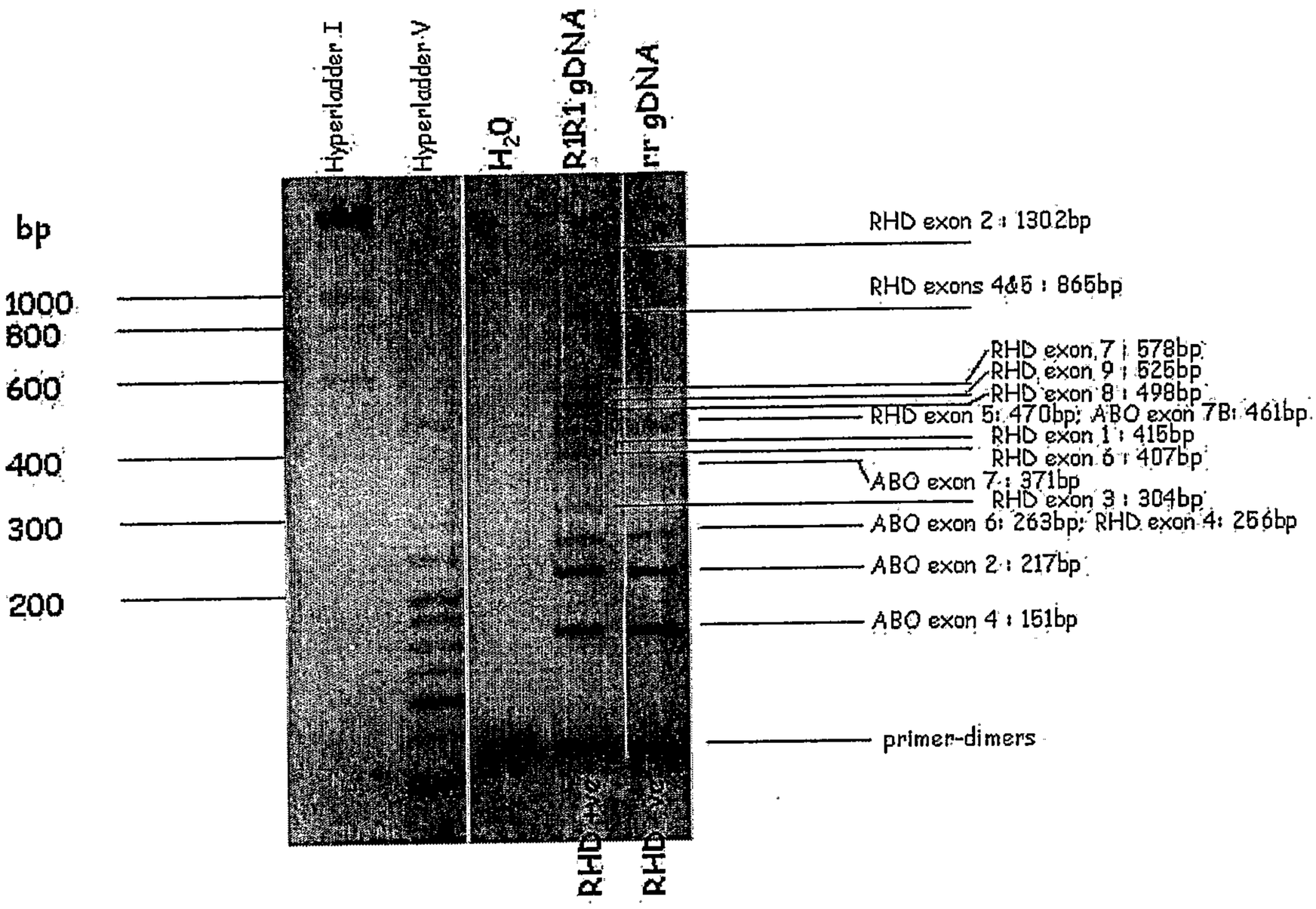

1
2
H_2O
800bp
600bp
400bp
200bp
Exon 7B
Exon 7A
Exon 6
Exon 2
Exon 4

Figure 8


Hyperladder I
Hyperladder V
H_2O
R1R1 gDNA
rr gDNA
bp
1000
800
600
400
300
200
RHD exon 2 : 1302bp
RHD exons 4&5 : 865bp
RHD exon 7 : 578bp
RHD exon 9 : 525bp
RHD exon 8 : 498bp
RHD exon 5: 470bp; ABO exon 7B: 461bp
RHD exon 1 : 415bp
RHD exon 6 : 407bp
ABO exon 7 : 371bp
RHD exon 3 : 304bp
ABO exon 6: 263bp; RHD exon 4: 256bp
ABO exon 2 : 217bp
ABO exon 4 : 151bp
primer-dimers
RHD
RHD

WO 2006/032897 PCT/GB2005/003659

CA 02581240 2007-03-21

Figure 9A

Nr.	MAPH PCR primer	ABO exon	Sequence 5′ to 3′	Tm (°C)	Product size (bp)	[primer] in mpx (nM)
	int1-49f	2	gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG	57.9	217	5
	int2+62r		ggccgtgggaattcgattATTGGCTGCTGTGGTCA	59.0		5
	int3-33f	4	gccgcgaattcactagtgCCTGCTCCTAGACTAAACTTC	58.0	151	5
	int4+52f		ggccgtgggaattcgattAAGGGAGGCACTGACATTA	59.6		5
	int5+367f	6	gccgcgaattcactagtgGATTTGCCCGGTTGGAGTC	60.0	410	5
	int6+31r		ggccgtgggaattcgattAGTCACTCGCCACTGCC	60.6		10
	ABO732f	7	gccgcgaattcactagtgCCACCGTGTCCACTACTATG	60.4	716	10
	ABO1147r		ggccgtgggaattcgattCAGAGTTTACCCGTTCTGC	60.0		10
	MAPH-rev		gccgcgaattcactagtg	57.94		1000
	MAPH-for		ggccgtgggaattcgatt	67.55		1000

Figure 9B

Exon 2 (nt29-98)

To detect 87_88insG

gtgagagaaggagggtgagtgatgtgatttttctactcctgttttccagGAAAACCAAAATGCC
AC[46]*G*CACTTC[53]*G*ACCTATGATCCTTTTCCTAATAATGCTTGTCTT[87]*G*GTCTTGTTTGGgtaag
acacatttgaccatcgaggctggcctggtttggggagaagtgaccacagcagccaat

Exon 4 (nt156-203)

To detect 188G>A; 189C>T

cctgctcctagactaaacttcatctcctgtgttctcattctgcagCATGGCTGTTAGGGAACCTGACCA
TCTGCAGC[188]***G***[189]***C***GTCTCGTTGCCAA[203]Ggtataatgtcagtgcctcccctt

Exon 6 (nt240-374)

To detect 261delG; 297A>G; 322C>T

gatttgcccggttggagtcgcatttgcctctggttggtttcccggggaagggcggctgcctctggaagg
gtggtcagaggaggcagaagctgagtggagtttccaggtgggggcggccgtgtgccagaggcgcatgtg
ggtggcaccctgccagctccatgtgaccgcacgcctctctccatgtgcagTAGGAAGGATGTCCTCGTG
GT[261]***G***ACCCCTTGGCTGGCTCCCATTGTCTGGGAGGGCAC[297]***A***TTCAACATCGACATCCTCAACGAG[322]
CAGTTCAGGCTCCAGAACACCACCATTGGGTTAACTGTGTTTGCCATCAAGAAgtaagtcagtgaggtg
gccgagggtagagacccaggcagtggcgagtgact

Exon 7 (nt433-1248)

To detect 467C>T; 542G>A, 646T>A; 703G>A; 796C>A; 802G>A; 803G>C;
798_804ins G, 893C>T; 927C>A; 1060delC.

[433]CCACCGTGTCCACTACTATGTCTTCACCGACCAGC[467]***C***GGCCGCGGTGCCCCGCGTGACGCTGGGGA
CCGGTCGGCAGCTGTCAGTGCTGGAGGTGCGCGCCTACAAGCGCT[542]***G***GCAGGACGTGTCCATGCGCCG
CATGGAGATGATCAGTGACTTCTGCGAGCGGCGCTTCCTCAGCGAGGTGGATTACCTGGTGTGCGTGGA
CGTGGACATGGAG[646]***T***TCCGCGACCACGTGGGCGTGGAGATCCTGACTCCGCTGTTCGGCACCCTGCAC
[700]CCC[703]***G***GCTTCTACGGAAGCAGC[721]CGGGAGGCCTTCACCTACGAGCGCCGGCCCCAGTCCCAGGCC
TACAT[768]CCCCAAGGACGAGGGCGATTTCTACTAC[796]***C***T[798]***GGGG***[802]***G***[803]***G***GTTCTTCGGGGGGTCGGT
GCAAGAGGTGCAGCGGCTCACCAGGGCCTGCCACCAGGCCATGATGGTCGACCAGGCCAACGGCATCGA
GG[893]***C***CGTGTGGCACGACGAGAGCCACCTGAACAAGTA[927]***C***CTGCTGCGCCACAAACCCACCAAGGTGC
TCTCCCCCGAGTACTTGTGGGACCAGCAGCTGCTGGGCTGGCCCGCCGTCCTGAGGAAGCTGAGGTTCA
CTGCGGTGCCCAAGAACCACCAGGCGGTCCGGAAC[1060]***C***CGTGAGCGGCTGCCAGGGGCTCTGGGAGGG
CTGCC[1096]GGCAGCCCCGTCCCCCTCCCGCCCTTGGTTTTAGCAGAACGGGTAAACTCTG

Notes:

The nucleotides in underlined italics are the SNPs that will detected by the microarray technology. The nucleotides in italics are known polymorphisms of the *ABO* gene that encode rare variants and which we may wish detect on subsequent versions of the microarray. The normal stop codon in exon 7 is underlined; the stop codon that results from 1060delC is double-underlined. Bold font in intron sequences indicates allelic polymorphism.

Figure 10

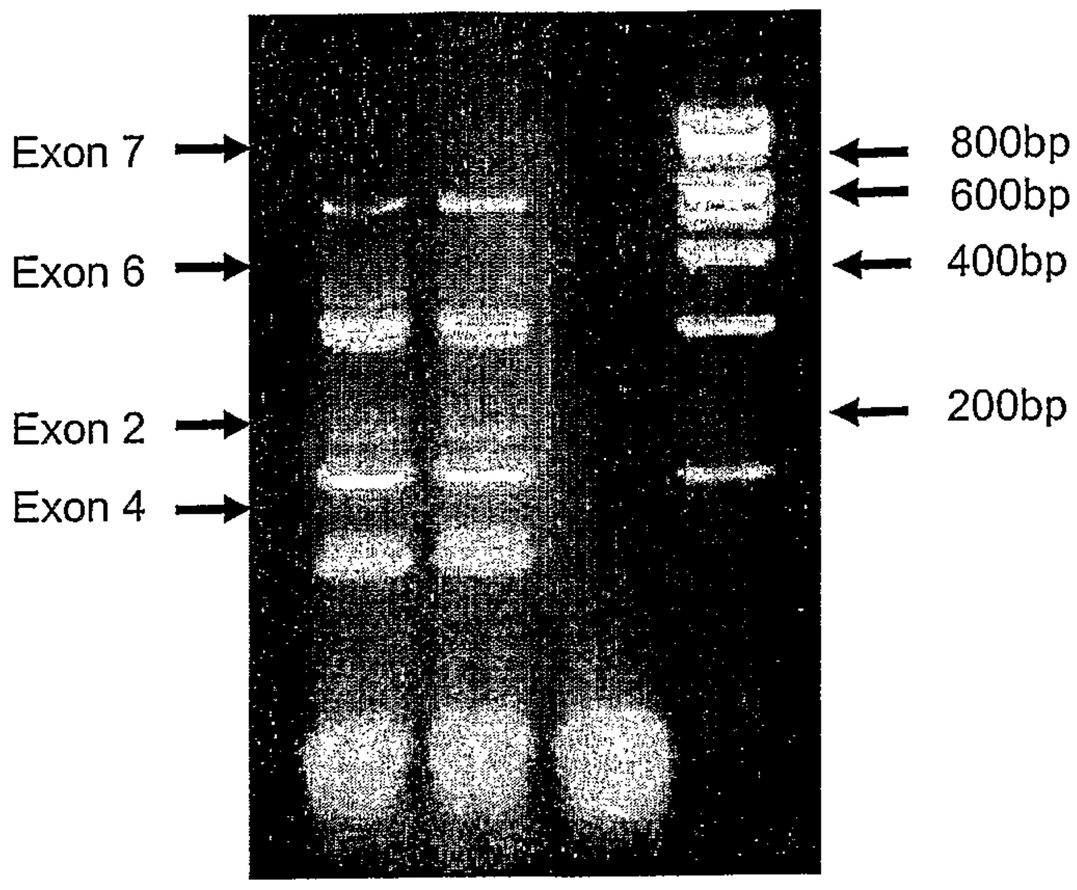


Figure 11

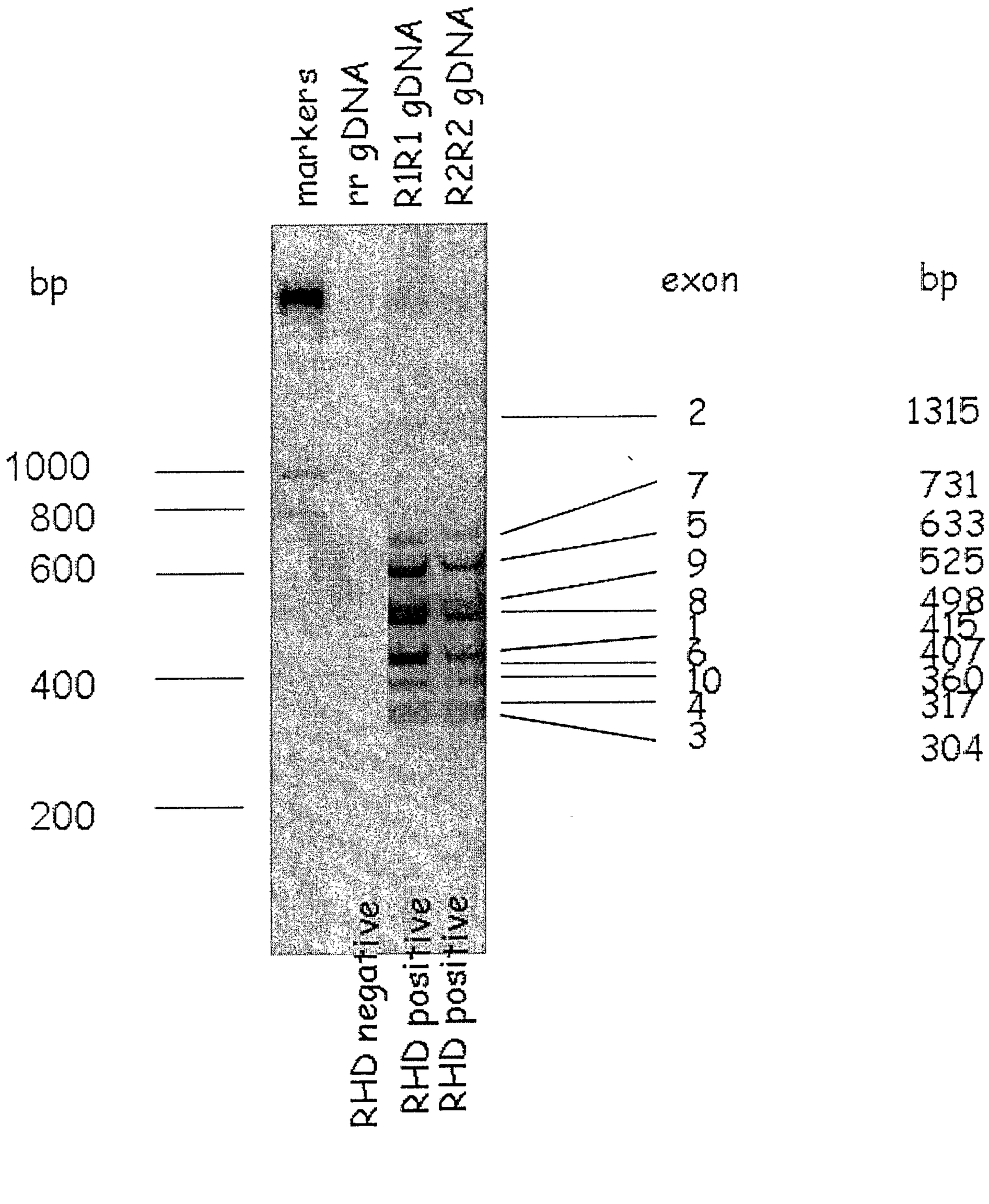

bp
1000
800
600
400
200
RHD negative
RHD positive
RHD positive
exon
2
7
5
9
8
6
10
4
3
bp
1315
731
633
525
498
415
407
369
317
304

Figure 12

nr.	MAPH PCR primer	ABO exon	RHD exon	Sequence (5'-3')	Tm °C	Product size (bp)	[primer] in mpx (nM)
1	int1-49f			gccgcgaattcactagtgGTGAGAGAAGGAGGGTGAG	57.9	217	7.5
2	int2+62r	2	n/a	ggccgcgggaattcgattATTGGCTGCTGTGGTCA	59.0		7.5
3	int3-33f			gccgcgaattcactagtgCCTGCTCCTAGACTAAACTTC	58.0	151	7.5
4	int4+52r	4	n/a	ggccgcgggaattcgattAAGGGAGGCACTGACATTA	59.6		7.5
5	int5-44f			gccgcgaattcactagtgCTGCCAGCTCCATGTGAC	61.2	263	7.5
6	int6+31r	6	n/a	ggccgcgggaattcgattAGTCACTCGCCACTGCC	60.6		7.5
7	ABO432f			gccgcgaattcactagtgCCACCGTGTCCACTACTATG	60.4	371	30
8	ABO766r	7	n/a	ggccgcgggaattcgatttGTAGGCCTGGGACTGG	60.7		30
9	ABO723f			gccgcgaattcactagtgGGAGGCCTTCACCTACG	59.8	461	30
10	ABO1147r	7B	n/a	ggccgcgggaattcgattCAGAGTTTACCCGTTCTGC	60.0		30
11	101F			gccgcgaattcactagtgccatagagaggccagcacaa	69.8		15
12	198R	n/a	1	ggccgcgggaattcgatttgcccctggagaaccac	69.2	415	15
13	int1F			gccgcgaattcactagtgtgacgagtgaaactctatctcgat	67.8		150
14	296R	n/a	2	ggccgcgggaattcgattagaagtgatccagccaccat	68	1315	150
15	303F			gccgcgaattcactagtgtcctggctctccctctct	66		15
16	397R	n/a	3	ggccgcgggaattcgattgttgtctttatttttcaaaaccct	65.3	304	15
17	403F			gccgcgaattcactagtggctctgaactttctccaaggact	68.8		30
18	498R	n/a	4	ggccgcgggaattcgattattctgctcagcccaagtag	65.6	317	30
19	503F	n/a	5	gccgcgaattcactagtgttgaattaagcacttcacagagca	69.6	633	30
20	5Aluint4F (RoHar)	n/a	5 (RoHar)	gccgcgaattcactagtgaaggactatcaggccacg	64.7	1010	30
21	598R			ggccgcgggaattcgatttgtgaccacccagcattcta	68.6		30
22	601F			gccgcgaattcactagtgagtagtgagctggcccatca	69.3		15
23	697R	n/a	6	ggccgcgggaattcgattcttcagccaaagcagaggag	68.7	407	15
24	701F			gccgcgaattcactagtgacaaactccccgatgatgtgagtg	74.9		15
25	798R	n/a	7	ggccgcgggaattcgattgaggctgagaaaggttaagcca	70.4	731	15
26	801F			gccgcgaattcactagtgctggaggctctgagaggttgag	71.3		18
27	899R	n/a	8	ggccgcgggaattcgattggcaatggtggaagaaagg	68.9	498	18
28	901F			gccgcgaattcactagtgactgtcgttttgacacacaat	64.1		13.2
29	998R	n/a	9	ggccgcgggaattcgatttgtcacccgcatgtcag	67.3	525	13.2
30	1001F			gccgcgaattcactagtgcaagagatcaagccaaaatcagt	68.1		21
31	1097R	n/a	10	ggccgcgggaattcgattgtggtacatggctgtattttattg	65.9	360	21
32	MAPH-rev			gccgcgaattcactagtg	57.94		2500
33	MAPH-forw			ggccgcgggaattcgatt	67.55		2500