Molecular background of serological D negative phenotype in the Omani population

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ABSTRACT

Many *RHD* allele molecular bases studies on serological D negative phenotype have been conducted in Caucasian and African populations. Study of *RHD* alleles was lacking in Arabs from Gulf (Omanis) which could be very helpful to come up with a molecular screening protocol for managing donors and patients carrying an *RHD* molecular variant. In the present study, we analyse the molecular background of serological D negative Omani population.

A total of 203 dry blood samples on Whatman's FTA card were collected from Omani cohort from different regions of the country. The samples were first analysed serologically followed by *RHD* genotyping using allele specific real-time PCR with melting curve analysis, a commercial allele specific PCR (BAGene kits), digital PCR and Sanger sequencing. Among the 203 serological D negative samples, 189 (93.1%) were phenotyped as true D negative. The true serological D negative samples indicated two patterns that were presence of 37 bp insertion in exon 4 that is unique for *RHDΨ* (*n* = 8) and presence of *RHDΨ* in cis to partial D without *RHCE* hybrid (*n* = 2). Total of 9 samples (4.43%) were reclassified as D positive (serological false D negative) indicated patterns that are; *RHD-CE-D*^{s}/*RHD-CE(4)-D* (*n* = 1), *RHD-CE(5)-D* in cis to *weak D DAR2.00* (*n* = 1), partial D without *RHCE* hybrid *(DIIIb)* in cis to *weak D type 45* (*n* = 1), *RHD-CE(4)-D* in cis to *weak D DAR2.00* (*n* = 1), *weak D type 4.2* in cis to *weak D type 41* (*n* = 1), *RHD-CE(4)-D* in cis to *RHD-CE(8-9)-D (n* = 1), *RHD-CE(4)-D* in cis to *weak D type 4.2* (*n* = 2,) and *DVI.2 (RHD-CE(4-6)-D* in cis or trans to *DEL(IVS8-31T>C)* (*n* = 1). Total of 5 samples were unclassified. Altogether, these studies showed serological limitation existence in 4.4% of the samples that reinforce *RHD* molecular typing and lay the foundation for the development of PCR based *RHD* genotyping screens in Oman.

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ASP-PCR	Allele specific primer-polymerase chain reaction
BCSH	British Committee for Standards in Haematology
BLAST	Basic Local Alignment Search Tool
CBB	Central Blood Bank
bp	Base pair
cdPCR	Chip-based Digital PCR
cffDNA	cell-free fetal DNA
C_t	Cycle threshold
DAT	Direct antihuman globulin test
ddPCR	Droplet Digital PCR
DHTR	Delayed haemolytic transfusion reaction
dNTPs	Deoxyribonucleotide triphosphates.
EDTA	Ethylene Diamine Tetra Acetic acid
HDFN	Hemolytic disease of the foetus & newborn
HGH	Human Growth Hormone
HRM	High Resolution Melt
HTR	Haemolytic transfusion reaction
IAT	Indirect anti human test
LR-PCR	Long-range PCR
ISBT	International Society of Blood Transfusion
MLPA	Multiplex Ligation-Dependent Probe Amplification
MOH	Ministry of Health

mRNA	messenger RNA
NCBI	National Centre for Biotechnology Information
NGS	Next Generation Sequencing
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PCR-SSP	Polymerase chain reaction with sequence-specific primers
qPCR	Quantitative real-time PCR
RBC	Red blood cell
RHDΨ	*RHD* pseudogene
SCD	Sickle cell disease
SMM	Single missense mutation
SNP	Single Nucleotide Polymorphisms
T_m	Melting Temperature
UTR	Un-Translated Region

1 CHAPTER ONE

1.1 Introduction

Effective and safe blood transfusion requires donor and recipient to have exact blood types in most of the cases. Therefore, compatibility testing is essential for both donor and recipient before any transfusion can take place. Serological and molecular techniques can help to identify different blood types. Performing ABO and RhD (Rh blood group, D antigen) compatibility test is necessary in blood transfusion as incompatibility may cause severe haemolysis that possibly lead to death (ABO blood group incompatibility). Based on RhD typing, blood donors and blood transfusion recipients have been categorized as either D positive or D negative (Sandler, Chen and Flegel, 2017). This categorization is very important during blood transfusions as the D antigen is clinically significant and the correct D typing helps to reduce the risk of alloimmunisation in the transfusion recipient and avoid haemolytic transfusion reaction (HTR). Correct RhD typing is also important for childbearing D negative women for Rh immunoprophylaxis with Rh immune globulin in case if the foetus is D positive and thus avoiding haemolytic disease of foetus and newborn (HDFN).

Serological methods which is a common method worldwide for RhD typing may result in false D negative that are in fact *RHD* variants (Sassi et al., 2014). These variants can be quantitative and reduce D antigen number on red blood cells (RBCs) such as weak D and DEL phenotypes or qualitative with or without weakening of D antigen such as partial D and *RHD* hybrid (Avent and Reid, 2000). In serologic D phenotyping it is necessary to detect these variants, which result from the amino acid substitutions (Sandler, Chen and Flegel, 2017). Serological methods are less effective in identifying various types of antigens, while RH genotyping can detect more complex Rh specificities, and hence, molecular testing is preferred.

1.2 Rh blood group history

In 1939, blood transfusions were based on ABO compatibility alone. It first came in to the spotlight in 1939 (Levine and Stetson, 1939) in New York when a woman had a severe acute haemolytic transfusion reaction (AHTR) after having had a matching ABO blood transfusion from her husband following the delivery of her stillborn infant. Her serum showed antibody that reacted at 25^{o}C and 37^{o}C with husband's RBCs. It was then postulated that father and infant had a factor mother lacked and this factor was introduced by the infant during pregnancy which produced antibody that reacted with father´s transfused RBCs in-vivo and 80% of other ABO compatible blood in-vitro. This antibody was named human anti-Rh in 1940. Landsteiner and Wiener injected Rhesus monkey RBCs into rabbit and isolated antibodies that agglutinated Rhesus monkey RBCs and 85% of human RBCs, hence the name Rh (Rhesus) was given (Landsteiner and Wiener, 1940). By 1962, it was determined that anti-Rh antibody differed to human derived anti-D and was renamed anti-LW in honour of Landsteiner and Wiener, while a human antibody as anti-D of the Rh (not rhesus) was retained.

Continuous research and investigations showed Rh as a common cause of AHTR and HDFN. From 1943 onwards, the Rh started to be more complex and appreciated to compose of numerous alleles as many other Rh factors were defined associated with original agglutinin (Race et al., 1944; Mourant, 1945; Stratton, 1946). Fisher and Race had 4 different Rh-specificities anti-sera, anti-C, anti-E, anti-c and anti-e that determined seven alleles and they postulated three loci producing D/d, E/e and C/c (Race et al., 1944), whereas Wiener defined 8 alleles and suggested only one Rh locus (Wiener, 1943). In 1986, Tippett postulated an alternative theory of two loci; one encoding D or no d and the other encoding C or c and E or e (Tippett, 1986). It was validated by genetic studies and it has been accepted theory till today.

After the determination of the Rh factor in 1939, blood recipients and donors Rh RBCs had been categorized as D positive or D negative. However, the first D variant was defined in 1946, when red cells from a donor at Manchester Royal infirmary was tested with a panel 32 different human anti-D sera. Agglutination was only seen with 12 of these anti-sera (Stratton, 1946). Such D variants were named D^u. Another class of D variant was initially defined as blood factors as Rh positive individual's has anti-Rh which failed to react with own cells and cells of some other rare Rh positive (Unger, Wiener and Katz, 1959). It was then defined mosaics (Wiener and Unger, 1962) and eventually renamed as partial D by Salmon *et al*, in 1984 (Issitt and Telen, 1996). DEL phenotype a third class of D variant detected by adsorption and elution of anti-D was first reported in 1984 (Okubo et al., 1984). Many cases later were reported some D^u / D-positive who have had D positive blood transfusion or exposed to D positive foetus RBCs during pregnancy developed anti-D (Argall, Ball and Trentelman, 1953; Simmons and Krieger, 1960; Ostgard, Fevang and Kornstad, 1986).

1.3 Rh terminology

Number of Rh alleles increases as methods for molecular blood group determination are widely used. Many developed countries adopted strategies for transfusion support from genotyping data. As reporting of genotypes is becoming a routine, and the results obtained could develop to a major task for transfusion scientists. Therefore, knowledge of nomenclatures on alleles is essential (Wagner, 2019).

Four systems have been developed to explain the relationships and inheritance of the 5 original Rh antigens. Two systems proposed by Fisher & Race and Weiner were based on serological tests and inheritance studies. Additional two theories were developed by Rosenfield & International Society of Blood Transfusion (ISBT) to symbolise the terminology to computer system (Harmening, 2012). Fisher and Race investigations

postulated that the Rh antigens were inherited as haplotype from each parent and follow Mendelian inheritance (Race, 1948). Each haplotype comes from three separate loci and 3 alleles (locus one = *D* or *d* allele, locus two = *C* or *c* allele and locus three = *E* or *e* allele). However, it was later considered *d* as amorphic allele and now it is very well known that there is no d antigen. Loss of D antigen expression can be due to entire *RHD* gene deletion (Caucasian) or inactive or silent gene due to *RHDΨ* or *RHD-CE-D*^{s} hybrid gene (African) (Moussa et al., 2012). For example, individual with the genotype DCe/DcE would have D, c, e, C and E antigens whereas dCe/dcE would have all the antigens except D.

Weiner proposed shorthand designation for Rh common antigens haplotype and phenotype based on symbols. He believed that Rh gene responsible for the production of phenotype called agglutinogen (allele) that has at least 3 three factors (Wiener, 1943). These agglutinogens and blood factors are shown in Table 1.1. This theory has been proven to be inaccurate; however, the terminology has been in use and developed further.

As more Rh antigens discovered, the system became more complex and difficult to give names to the new antigens. In 1962, Rosenfield with colleagues numbered each Rh antigen based on its discovery order (Rosenfield et al., 1962). The proposed system, determines only the presence or absence of Rh antigen. The absence of Rh antigen is preceded by negative sign. For the Rh major antigens D, C, E , c and e numbers 1, 2, 3 , 4 and 5 respectively were assigned. For example, RBCs that type positive for D, c & e antigens and negative for C and E would be designated as Rh: 1, -2. -3, 4 , 5. If the same sample was not tested for E antigen the designation would be Rh: 1, -2, 4 , 5 (3 which represents E antigen is not stated).

Further development of the blood groups system was necessary as the blood transfusion world became to share data. In 1980, the ISBT working party formed a committee that aimed to establish a universal language for the RBC antigens nomenclature that can be

read by eye and machine keeping with the blood groups genetic bases. The monograph was published in 1990 describing 242 RBCs antigen (The ISBT Working Party on Terminology for Red Cell Surface Antigens, 1990), 254 antigens with 23 blood group systems in 1995 (Daniels et al., 1995), 284 antigens with 29 blood group systems (Daniels et al., 2004) and >300 antigens with 36 blood group systems in 2016 (Storry et al., 2016).

Table 1.1 Wiener proposed Terminology for Rh common antigens, haplotype and phenotype

Gene*	Agglutinogen	Factors	Shorthand designation	Fisher & Race
Rh^0	Rh_0	$Rh_0hr'hr''$	R_0	Dce
Rh^1	Rh_1	$Rh_0rh'hr''$	R_1	DCe
Rh^2	Rh_2	$Rh_0hr'rh''$	R_2	DcE
Rh^z	Rh_z	$Rh_0rh'rh''$	R_z	DCE
rh	rh	hr'hr″	r	ce
rh'	rh'	rh'hr″	r'	Ce
Rh″	Rh″	hr'rh″	r″	cE
rh^y	rh_y	rh'rh″	r_y	CE

Agglutinogen represents a single haplotype capable of expressing 3 different antigens. The uppercase R indicates D antigen presence whereas the lowercase r denotes D antigen absence. The Uppercase C is denoted by a single prime (') or a 1. Lowercase c is implicit when there is no ' or 1 denoted and the third antigen (e) presence is assumed. For example, R1 denotes for the presence of D, C and e antigens whereas R0 indicates D, c & e antigens presence. Number 2 or double prime (″) indicates the presence of uppercase E antigen whereas lowercase e is implied when there is no double prime (″) or 2. To illustrate, R2 indicates the presence of D, c & E antigens, r″ for c & E antigens and r for c and e antigens (where the presence of c antigen is assumed). When both uppercase C & E are present, Letter Z or y is denoted with presence or absence of D antigen respectively - That is R_z indicates D,C & E antigens whereas r_y indicates C & E antigens. The presence of Rh factors (antigens) D, C, c, E and e in agglutination were denoted Rh_0, rh', hr', rh″ and hr″ respectively as rh was given to C with (') or E with (″), hr was given to c with (') or e with (″) and Rh_0 was given to D factors. *(Harmening, 2012)

1.3.1 International Society of Blood Transfusion (ISBT) RH nomenclature

Each antigen belonging to a blood group system was given a six-digit number for each by ISBT. The first three numbers represent the system and the remaining three the antigenic specificity (Harmening, 2012). Number 004 was assigned to the Rh blood group system, and then each antigen assigned to the Rh system was given a unique number to complete the six-digit computer number. Table A1.1 in Appendix 1 provides a listing of these numbers. When referring to individual antigens, an alphanumeric designation similar to the Rosenfield nomenclature may be used. The alphabetic names formerly used were left unchanged but were converted to all uppercase letters (e.g., Rh, Kell became RH, KELL). Therefore, D is RH1, C is RH2, E is RH3, c is RH4 and e is RH5 and so on (Table A1.1 in Appendix 1).

For *RH* alleles a special ISBT nomenclature rules were adopted (Storry et al., 2011). The ISBT *RH* allele names consist of the gene symbol (*RHCE* or *RHD*), followed by a number or name indicating the main antigenic group, and as many groups of numbers following to indicate the allele (Figure 1.1). Non-expressed alleles are characterized by addition of an ‘N’ to the initial number, weak D alleles by an additional ‘W’, DEL alleles by an additional ‘EL’.

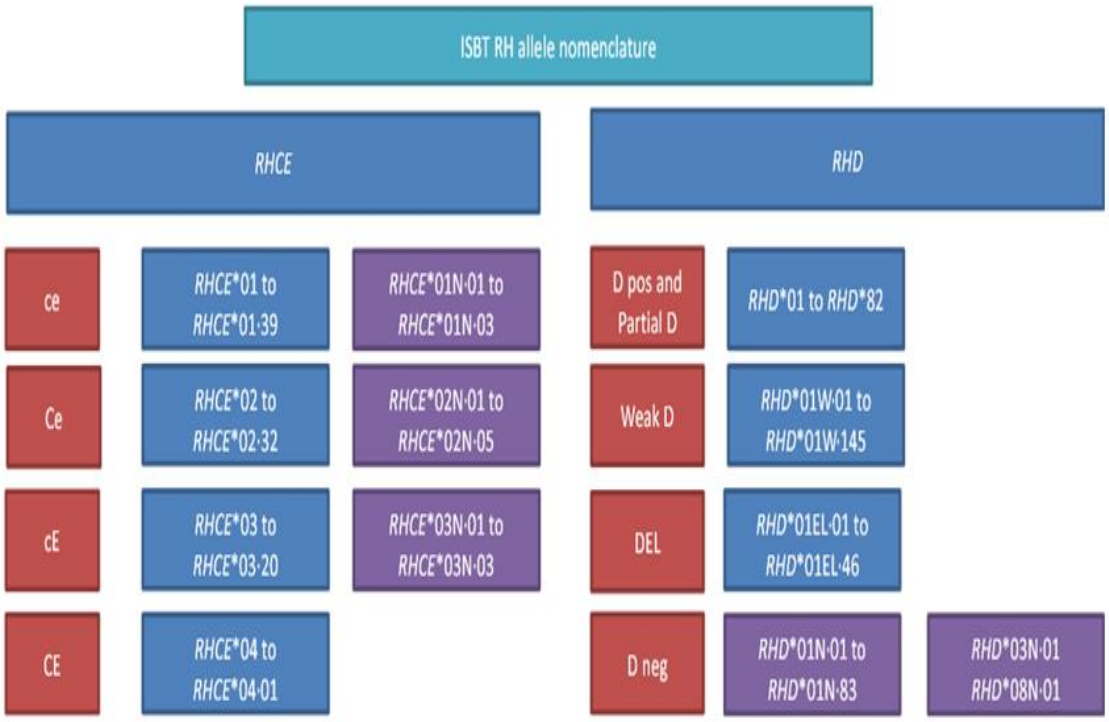


Figure 1.1 Overview of the ISBT *RH* allele nomenclature

For *RHCE*, the 'standard'' *ce* allele is *RHCE**01, *Ce RHCE*02*, *cE RHCE*03* and *CE RHCE*04*. Variant alleles are derived from the respective 'standard allele' indicated by numbers following a dot, possibly with subgrouping for related alleles. *RHD* alleles with relevant partial D (or without weakening) are grouped according to their phenotype up to *RHD*62*. Non-expressed alleles are characterized by addition of an 'N' to the initial number, weak D alleles by an additional 'W', DEL alleles by an additional 'EL'. The blue and purple boxes indicate respective allele number range up to 2018 (Wagner, 2019).

1.4 Rh proteins

The Rh blood group system is highly polymorphic and complex system composed of RBCs specific protein antigens such as D and others over 58 different proteins as per the last report in 2015 (Harmening, 2012; EL Wafi et al., 2016). Scientists spent several years trying to uncover the complexity of the Rh blood group system from serology, biochemical structure of the Rh proteins, mode of inherence and the genetic control. These efforts revealed the importance of some Rh blood group proteins such as (D) in clinical practice, as they can be associated with HDFN and HTR.

The Rh proteins can be divided into Rh blood group erythrocyte proteins such as D, C, c, E and e, Rh associated glycoproteins on erythrocytes such as RhAG and non-erythroid proteins such as RhBG and RhCG found in other tissues (Westhoff, 2007). The complex of the Rh protein family is estimated by density ultracentrifugation to be 170 000 daltons (Hartel-Schenk and Agre, 1992). The complex structure as tetramer was originally proposed in 2000 but since 2005, a trimeric structure has been favoured by several authors. RhCE and RhD associate as Rh core complex that comprises one RhD/CcEe protein and most likely two Rh-associated glycoproteins (RhAG) as a trimer (Avent et al., 2006). Conroy and colleagues used *E.coli* ammonium channel (AmtB) as a template for the generation of Rh proteins models (Conroy et al., 2005). The tetramer structure that was widely quoted contrasted with trimeric structure of *E.coli* AmtB. By sequence alignment between *E.coli* AmtB, RhD and RhAG and comparing homology model of these, they suggested trimeric structure of Rh complex on erythrocytes. Merrick and colleagues study used the structure of *E. coli* AmtB as a template, together with secondary structure predictions for Rh polypeptides and the extensive available biochemical data on Rh proteins, constructed homology models for human RhAG and RhD and concluded the possibility for the Rh proteins to adopt a trimeric structure (Merrick et al., 2006).

1.4.1 Biochemical composition of RhD and RhCE proteins

Blood group antigen can be carbohydrates, proteins, glycolipids or glycoproteins attached to a component on the surface of the RBCs membrane (Dean, 2005; Alimba, Adekoya and Oboh, 2010). Unlike ABO blood group sugar antigens, the biochemical appearance of Rh antigens is hydrophobic non-glycosylated protein which is physically supported by lipid in the RBCs membrane and exist as integral cell membrane (Hillyer, 2007; Whitlock, 2010). Among the current 62 Rh antigens recognized by the ISBT, proteins D, C, c, E and e are the most immunogenic and are the most common antigens in Rh blood group system (Flegel, 2007; Flores-Bello et al., 2018). The immunogenicity of Rh antigens differs, with D being the most immunogenic followed by c, E, C and e antigens (Harmening, 2012).

The RhD protein expresses the D antigen, while the RhCcEe protein carries either C or c antigens (involving the second extracellular loop) together with E or e antigens (involving the fourth extracellular loop) on the same protein (Mouro et al., 1993; Blunt, Daniels and Carritt, 1994; Avent et al., 1996b; Smythe et al., 1996). Rh antigens appear early during erythropoietic differentiation. RhCcEe protein is detectable on CD34 progenitors isolated from cord blood after 5 to 7 days, while RhD appears after 9 to 11 days of culture (Southcott, Tanner and Anstee, 1999).

The RhD and RhCE proteins are composed of 417 amino acids that transverse the RBC membrane 12 times and display short loops of amino acids on the exterior (Cartron, 1994) with N and C termini located in the cytoplasm of the RBC (Scott, 2004)- (**Figure 1.2**). The N terminal methionine, which represents the mRNA translation-initiation signal, is cleaved from the mature proteins (Flegel, 2007).

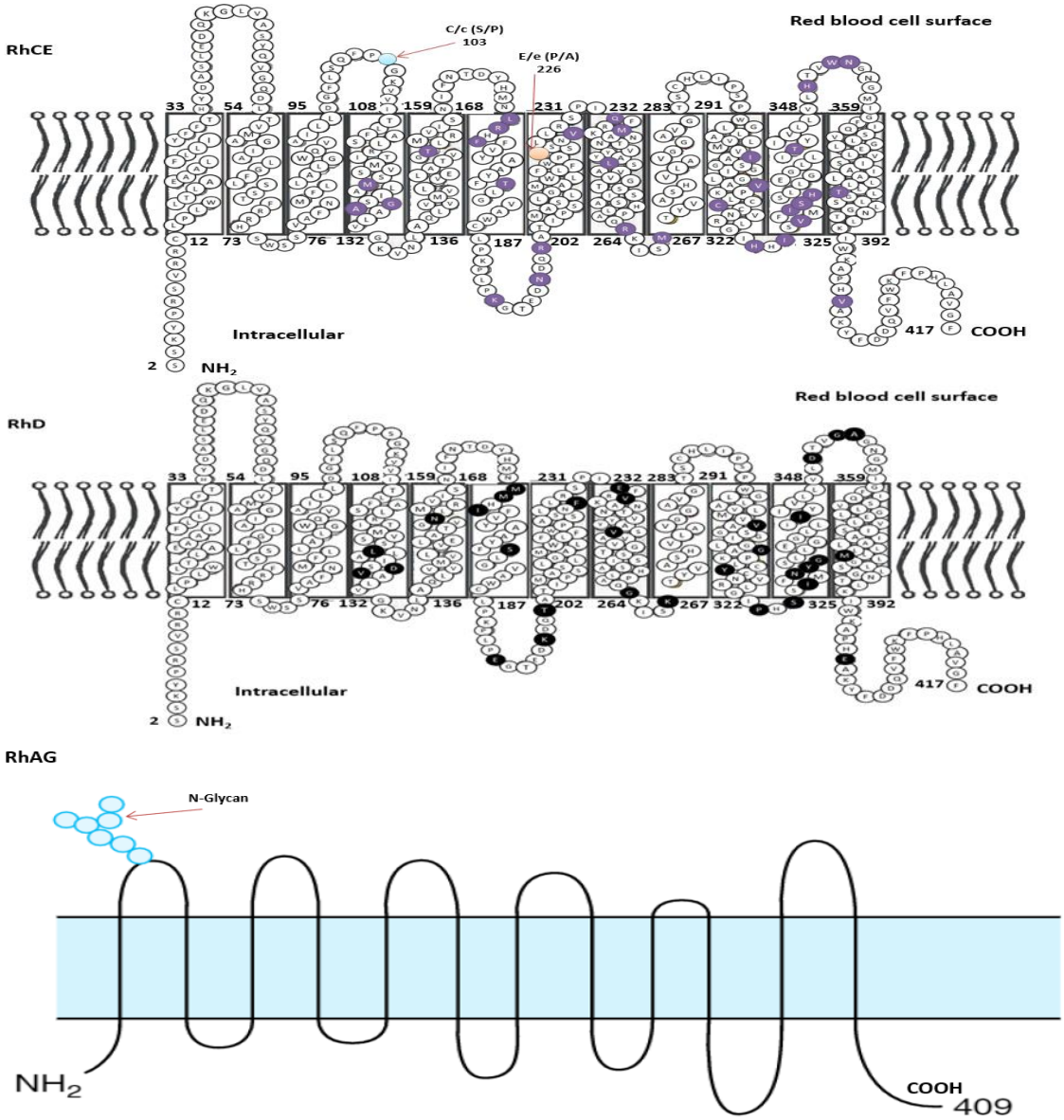


Figure 1.2 RhD , RhCE and RhAG model protein.

The Rh proteins RhD, RhCE and RhAG transverse the membrane 12 times and display short loops of amino acids on the exterior with N and C termini located in the cytoplasm of the RBC. The amino acids sequence from amino acid 2 (first amino acid methionine is cleaved) to 417 with their names for RhD and RhCE protein (417 amino acids) are shown. The difference in amino acid sequence between RhD and RhCE indicated as black and violet circles respectively. Positions 103 for (C/c) [Ser103Pro] and 226 for (E/e) [Pro226Ala] in RhCE are indicated as blue and orange circles respectively. The RhAG protein consist of 409 amino acids with N-Glycan at the first extracellular loop as branched structure. RhD and RhCE structure was adapted from (Flegel, 2007) and modified by the author. RhAG structure was adapted from (Vege and Westhoff, 2019)

Although the *RHD* and *RHCE* cDNA open reading frames encode 417 amino acid polypeptides, the N terminal methionine, which represents the mRNA translation-initiation signal, is cleaved from the mature proteins (Merrick et al., 2006). Both RhD and RhCE proteins have 6 extracellular loops (Flegel and Wagner, 2002) with unknown function, but RBCs lacking all Rh antigens have structural abnormalities such as stomatocytes (Vege and Westhoff, 2019). The difference in amino acids sequence in the antigen is the residue number 103 for Cc (C: Serine & c: Proline) and 226 for Ee (E: Proline & e: Alanine) [Figure 1.2]. In RhCE, the protein of C differs from c by four amino acids and E protein differs from e by one amino acid (Daniels, 2013b). On the other hand, there is a difference of 32 to 35 amino acids for the RhCE and RhD proteins (Hillyer, 2007). The small loops at exterior part on the surface of the RBCs provide the different serological antigenic shape called epitopes for different Rh blood types. Nonetheless, the relation between the RhD epitopes and the polymorphisms of proteins of the Rh system, are not completely understood (Howe and Stack, 2017).

RhD and RhCE proteins are also called Rh30 (Table 1.2) proteins as they migrate in sodium dodecyl sulphate polyacrylamide gel electrophoresis gels (SDS-PAGE) with about molecular weight ratio of 30,000 (Hillyer, 2007). The Rh complex at its core is a hetero-oligomeric trimer consisting of Rh polypeptides (RhD and RhCE) and Rh-associated glycoproteins (RhAG) (Burton and Daniels, 2011). The expression of RhD and RhCE proteins on erythrocytes is mediated by co-expression of RhAG protein (Raud, Ferec and Fichou, 2017).

Table 1.2 RhD, RhCE and RhAG proteins synonyms

Rh protein	Synonyms
RhD	Rh30, Rh30B, Rh30D, D30, Rh30 polypeptide [30 kd], RhXIII, Rh13
RhCE	Rh30, Rh30A, Rh30C [RhCE], Rh30 polypeptide [32 kd], RhIXb cDNA, [RhcE], Rh21 cDNA [RhcE], R6A32, Rhce, RhCe, RhcE, RhCE, CcEe
RhAG	Rh50, Rh glycoprotein Rh50A, D50, MB-2D10 protein, R6A45, GP50, GP50A

1.4.2 RhAG protein

As stated, the Rh proteins carry Rh antigens but are only expressed on the erythrocyte surface if glycoprotein RhAG is also present. RhAG protein is detectable on CD34 progenitors isolated from cord blood, after culture for 3 to 5 days (Southcott, Tanner and Anstee, 1999). The amino acid sequence homology (approximately 40%) of the Rh and RhAG proteins indicates an ancestral relationship, and collectively they are referred to as the ``Rh protein family." Hydrophobicity profiles, immunochemical analyses, and data obtained through site-directed mutagenesis imply that RhAG proteins are similar to Rh proteins and have 12 transmembrane spans with both the N-terminus and C-terminus oriented to the cytoplasm like Rh proteins (Figure 1.2) (Westhoff, 2004). One of the 2 potential N-glycan sites is glycosylated. A third site is predicted to be cytoplasmic and, therefore, not accessible for glycosylation (Ridgwell et al., 1992; Ridgwell et al., 1994). Based on the amino acid sequence, RhAG shares 39.2% and 38.5% amino acid sequence identity with, respectively, the Rhce and RhD proteins (Avent et al., 1990; Cherif-Zahar et al., 1990; Le van Kim et al., 1992; Ridgwell et al., 1992; Arce et al., 1993).

The RhAG protein is controlled by approximately 32 kb *RHAG* gene located on chromosome 6p12.3 with 10 exons. *RHAG* gene exons 2–9 are 47% homologous to *RHCE* and *RHD* (Daniels, 2013a). RhAG is 409 amino acids with a molecular weight between 40,000 and 100,000 protein is associated with the expression of the Rh system and is part of a membrane channel, which is responsible for transporting ammonium and possibly carbon dioxide through the membrane of RBCs (Huang, 1998). RhAg antigen does not carry Rh antigens but is required for the correct assembly of the Rh proteins (RhD & RhCE) in the red cell membrane for expression. Mutations in the *RHAG* gene can lead to regulator type of Rh_{null} phenotype (Hou, Yan and Tian, 2017). In 2010, RHAG blood group system was established based on the recognition of three RBCs antigens on RhAG protein encoded by *RHAG* gene (Tilley et al., 2010). The RHAG blood group system consists of two high frequency antigens and two low frequency antigens (Table 1.3).

Table 1.3 Antigens of the RHAG blood group system

Antigen			
No	**Name**	**Frequency**	**Amino acids***
RHAG1	Duclos	High	Gln106Glu
RHAG2	O1[a]	Low	Ser227Leu
RHAG3	DSLK	High	Lys164Gln
RHAG4		Low	Val270Ile, Ala280Ala

*Amino acid substitution for negative phenotype. Adapted from (Vege and Westhoff, 2019)

1.5 RHD and RHCE genes

The human *RH* locus is responsible for the expression of the Rh blood group antigens. The Rh blood group system has two main genes (*RHD* and *RHCE*) that encode for the Rh protein. Both genes (loci) are located on chromosome 1p36.11 and are in very close proximity to each other (Westhoff, 2004) but are in opposite orientation 5'-*RHD*-3'-3'-*RHCE*-5' with 10 exons each (Wagner and Flegel, 2000). *RHD* is flanked by two 9000 base regions of 98.6% homology, named the Rhesus boxes (Daniels. G., 2008; Khosroshahi et al., 2019). *RHCE* & *RHD* composed of 57831 and 57295 bp respectively (Okuda et al., 2000) separated by small membrane protein 1 *SMP1* gene (Wagner and Flegel, 2000) which has been renamed *TMEM50A* (Figure 1.3).

Bioinformatics using online Ensembl browser showed, messenger RNA (mRNA) transcript known as complementary DNA (cDNA) of both *RHD* and *RHCE* genes composed of 2814 bp and 1660 bp respectively (Figure A1.1, Appendix 1). However, the coding sequence that is translated into 417 amino acids polypeptide is composed of 1254 bp for both *RHD* and *RHCE* genes.

RHD and *RHCE* genes carry different antigens, one carries the D antigen and the other carries CE antigens which can be ce, Ce, cE or CE (Arce et al., 1993). The RhD protein is encoded by *RHD* (synonyms: *RH30, RH30B, RH30D, RHXIII, RH13*); the RhCcEe protein is encoded by *RHCE* (synonyms: *RH30, RH30A, RH30C (RHCE), RHIXB, RH21*) (Figure 1.3); and the RhAG glycoprotein is encoded by *RHAG* (synonyms: *RH50, RH50A*).

Previous studies have found the *RHD* and *RHCE* genes to be 93.8% identical to each other (Westhoff, 2004; Granier et al., 2013). The homology even higher between *RHD* and *RHCE* exons. For example, both *RHD* and *RHCE* exon 8 are 100% identical. There are no differences or single or very few polymorphism(s) that can differentiate *RHD* and *RHCE* (*ce*, *Ce*, *cE*) exons (Figure 1.4). These observations suggest that they are derived from a relatively

recent duplication event. Previously a study of nonhuman primate *RH*-like genes demonstrated that ancestral *RH* gene duplication occurred in the common ancestor of man, gorillas and chimpanzees. The two genes differences is responsible for the amino acids differences along the entire protein dependant on the *RHCE* allele (Avent and Reid, 2000; Flegel, 2006; Daniels, 2013b), exons 1–7 encoding 50–60 amino acids each and exons 8–10 encoding the last 58 residues (Table 1.4 and Figure 1.5) (Cherif-Zahar et al., 1994; Cherif-Zahar, Raynal and Cartron, 1997). The most frequently occurring forms of *RHCE* and *RHD* encode 8 haplotypes: *Dce*, *dce*, *DCe*, *dCe*, *DcE*, *dcE*, *DCE*, and *dCE*, known in short, respectively, as R_0, r, R_1, r'', R_z and r_y.

The intronic region of the gene has a notable difference in intron 4, as *RHD* gene has a 600 bp deletion compared to *RHCE* gene (Arce et al., 1993; Avent et al., 1997c; Okuda et al., 1997; Okuda et al., 2000). Other intronic region over 100 bp deletions or insertions were detected (Okuda et al., 2000).

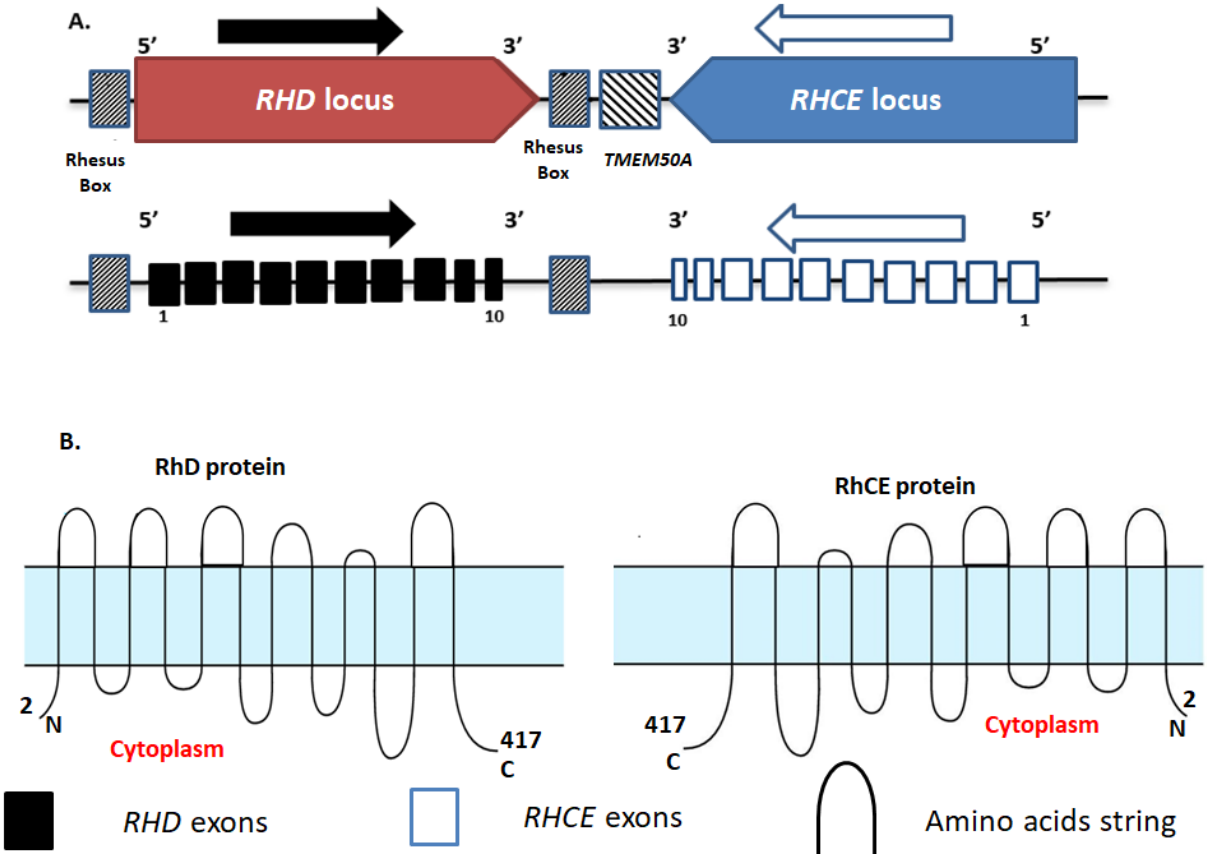


Figure 1.3 *RHD* and *RHCE* loci and their product protein.

Both genes are in apposite orientation with 10 exons each and separated by *TMEM50A* gene, *RHD* exons represented as black box and *RHCE* exons as white box (A). Structure of RhD & RhCE protein in the RBCs membrane as products of *RHD* & *RHCE* genes respectively, N & C termini located in the cytoplasm of the RBC (B). The amino acids arrangment from amino acid 2 to 417 for RhD and RhCE protein (B). (Daniels, 2013).

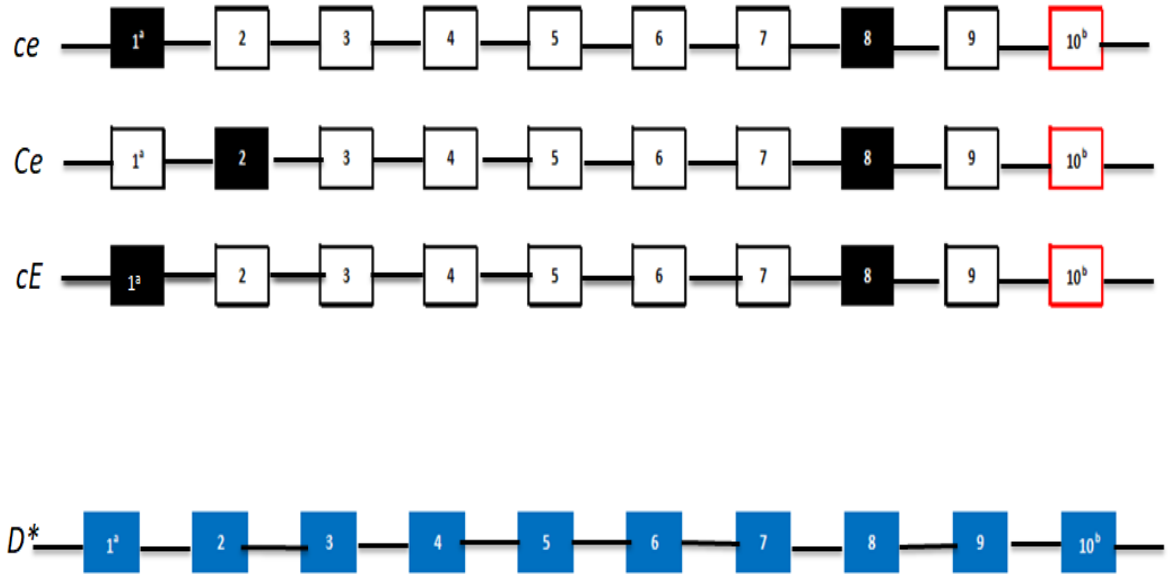


Figure 1.4 Schematic representation of the coding sequence (transcript) of wild type *RHD* gene (*D) compared with alleles of *RHCE* gene (*ce, Ce* and *cE*).**

The 10 exons of the *RHD* & *RHCE* genes are symbolized by squares and numbered 1 to 10. Letters [a] and [b] denote the coding sequence transcript of exon 1 with 5´UTR and exon 10 with 3´UTR respectively. The blue boxes denote the coding sequence exons for wild type *RHD* gene. The black boxes denote 100% homology between wild type *RHD* and *RHCE* allele exons. The white boxes with black borders denote presence of polymorphism(s) within the exon distinguishing *RHD* and *RHCE* allele. The white boxes with red borders denote polymorphisms distinguishing *RHD* and *RHCE* alleles at 3´UTR, with sequence share in exon 10 between *RHD* & *RHCE*. *RHce* and *RHcE* alleles share sequence with wild type *RHD* gene; whereas *RHCe* allele show polymorphism. Wild type *RHD* & all three *RHCE* alleles shown; share sequence in exon 8, but are different in exon 2 through 7 and exons 9 and 10 with exception of *RHCe* allele that share also sequence in exon 2.

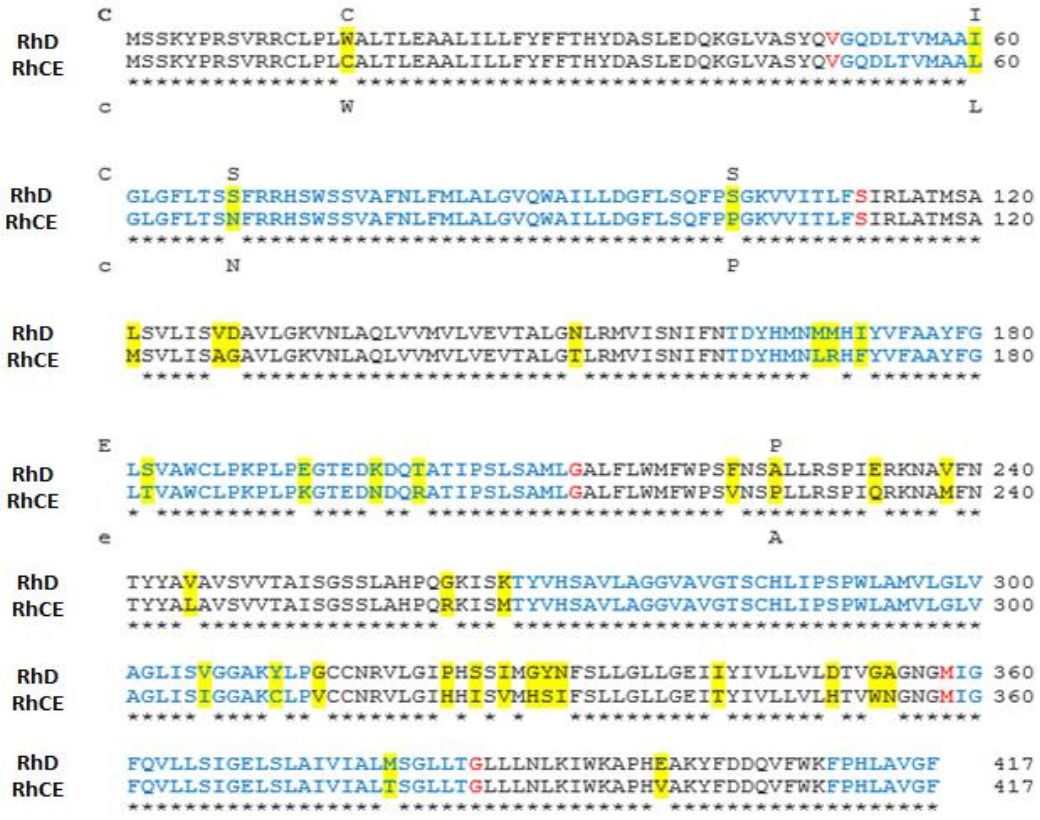


Figure 1.5 Amino acids difference between RhD and RhCE protein.

Sequence alignment of RhD and RhCE proteins as displayed in online software CLUSTAL O (1.2.4) multiple sequence alignment. The amino acid sequence data were deduced from cDNA nucleotide sequences from Ensembl genome browser. Black letters indicate amino acids translated from exon and blue letters indicate the same from another exon. Red letters indicate residue overlaps splice site. Yellow highlights the amino acid difference between RhD and RhCE polypeptides C, c, E and e. Four amino acid changes (Cys16Trp; Ile60Leu; Ser68Asn; Ser103Pro) are associated with the C to c polymorphism, whereas one amino acid change (Pro226Ala) is associated with E to e polymorphism.

Table 1.4 Organizations of *RHD* & *RHCE* codons in each exon of *RHD* & *RHCE* genes

RHD/RHCE **Exon***	***RHD/RHCE*** **codons**
1	1 – 49
2	50 – 112
3	113 – 162
4	163 – 211
5	212 – 267
6	268 – 313
7	314 – 358
8	359 – 384
9	385 – 409
10	410 – 417

*(Daniels. G., 2008)

1.6 Molecular basis of D

Since the first descriptions of Rh cDNAs (Avent et al., 1990; Cherif-Zahar et al., 1990; Le van Kim et al., 1992; Arce et al., 1993), much effort has been made in differentiating the molecular bases underlying the antigens of the Rh system. The different genetic mechanisms that change the Rh protein include gene deletion (D-negative phenotype); gene conversion (C/c polymorphism); antithetical missense mutations (E/e); gene hybrid (*RHD-CE-D*), single-nucleotide polymorphisms (DEL variant *K409K*) and other missense mutations such as *RHD-CE-D*^{s} and *RHD*Ψ*. The *RH* genes appear to be a source of massive diversity, and combinations of these different genetic rearrangements abound among all racial groups.

Most individuals are either D positive or D negative. However, there is a grey area in the form of D variants that affects qualitative and/or quantitative appearance of D antigen. The D negative phenotype has different genetic backgrounds with variable distribution in different populations (Figure 1.6). The D negative phenotype can arise either due to complete absence of the *RHD* gene which is common in Caucasians or few D variants that are common in African. These D variants may give inactive or silent *RHD* gene such as *RHD* pseudogene (*RHD*Ψ*) or *RHD-CE-D*^{s} hybrid gene that does not express D but altered C (Moussa et al., 2012). List of other alleles that cause D negative can be viewed in Appendix 1 (Table A1.2).

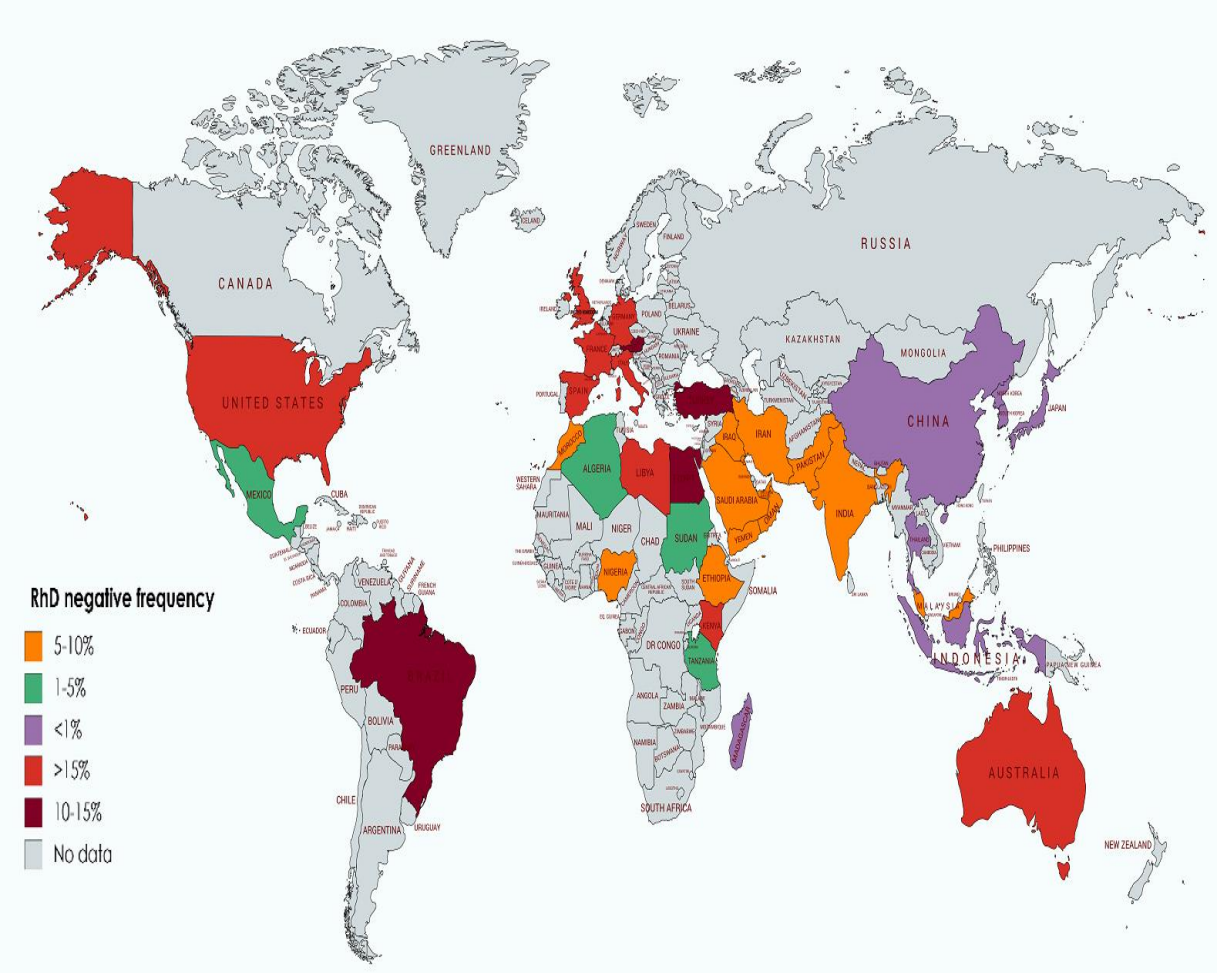


Figure 1.6 Frequency of D negative among different world populations.

The map was created using mapchart.net online software. The frequency of D negative decreases as we go far East from America continental and Europe towards Middle East and rest of Asian countries with exception of Australia. Some American continental countries such as Mexico and Brazil show different frequencies of 1-5% (*green*) and between 10-15% (*dark brown*) respectively. Other Americans, Europe and Australian population have the highest D negative frequency (>15% - Red). African have mixed frequency whereas all Middle East and Gulf countries have frequency between 5 and 10% (*orange*). The lowest frequency of D negative is found in East Asia countries such as China, Thailand and Indonesia with <1% frequency (*violet*).

There are two types molecular bases of D variants: (1) one or more nucleotide substitution in *RHD* gene that changes the amino acid in RhD protein, (2) genetic recombination which causes replacement of one or more *RHD* exons by *RHCE* (Daniels, 2013b). These variants can be categorized as partial D, weak D and DEL phenotype.

1.6.1 Complete *RHD* gene deletion

The D negative phenotype usually results from a complete absence of the RhD protein that results from complete deletion of *RHD* gene, explaining the high immunogenicity of D. As the D negative phenotype is associated with the absence of the RhD protein, there is no antigen negative D is found. Typically, the symbol d indicates that there is no D. Among Caucasians the D negative phenotype is associated with homozygosity for the complete *RHD* deletion, with percentage between 15% and 17% (Westhoff, 2007). Homozygous deletion of *RHD*, appears to have occurred between a 1463-bp region of identity in each of the Rh boxes (Figure 1.7) (Wagner and Flegel, 2000). In a very recent study, it has been observed that Basques presents the highest frequency (47.2%) of the *RHD* deletion (Flores-Bello et al., 2018).

Few studies have been conducted on D negative phenotype on Asian and African populations. Khosroshahi and colleagues detected Hybrid Rhesus box was detected in all tested samples, and Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) confirmed that 99% were homozygous for *RHD* gene deletion (Khosroshahi et al., 2019). Moussa and colleagues observed *RHD* gene deletion is the most prevalent cause of D-negative phenotype (98.6%) among Tunisian population (Moussa et al., 2012). Both studies showed that D negativity cause at molecular level is similar to Caucasian.

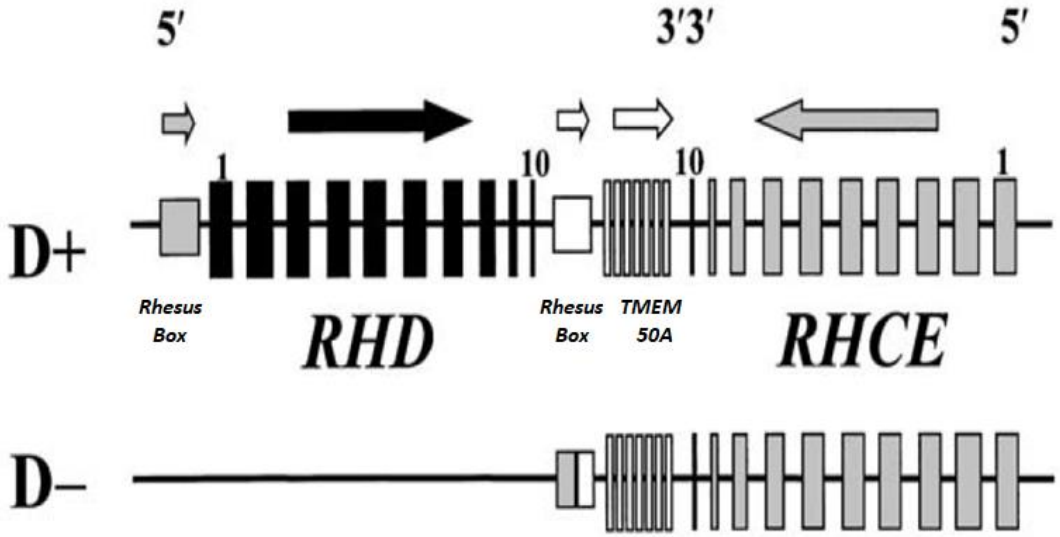


Figure 1.7 Genomic organization of the *Rh* genes in typical D positive (D+) and D negative (D-) haplotypes.

The *RH* gene shows *RHD* gene with exons 1-10 (black boxes), the two homologous Rhesus boxes, and *TMEM50A* in 5´ to 3´ orientation, and *RHCE* gene with exons 1-10 (grey boxes) in 3´ to 5´ orientation. In the D negative haplotype, there is a deletion of part of each Rhesus box and of *RHD* whole gene (Daniels. G., 2008).

1.6.2 RHD pseudogene (RHD*Ψ)-RHD*8N.01

RHD pseudogene is characterized by inactivation of D gene by insertion of 37 bp at the intron 3/exon 4 boundary of *RHD* gene that introduces a frame shift and translation termination (Figure 1.8). In addition, a nonsense (Tyr>stop) mutation in exon 6 that causes premature termination of translated protein (Singleton et al., 2000). *RHD**Ψ* associated nucleotides and amino acids changes related to wild type *RHD* gene can be viewed in Table 1.5 and Figure 1.9.

RHD gene deletion is a common cause of D negative in African, however around 67% are at least heterozygous to *RHD**Ψ* (Daniels, 2013b). Molecular analysis of *RHD* alleles in native Congolese revealed that 20.5% of D negative samples have *RHD**Ψ* (Touinssi et al., 2009). Moussa and colleagues found that Tunisian population has very low frequency of *RHD**Ψ* (0.5%) compared with that described in the African population (Moussa et al., 2012).

Table 1.5 *RHDΨ* associated nucleotides and amino acids changes related to wild type RHD gene**

RHD intron/exon*	Nucleotide changes relative to "standard *RHD* gene	Amino acid changes relative to standard protein	Remarks
Intron 3/Exon 4	IVS3-19 dupl 37	(D209N) reading frame or (D216N) new reading frame	Stop codon at 210
Exon 4	609G>A	-	No change
Exon 5	654G>C 667T>G 674C>T	(M218I) (F223V) (S225F)	
Exon 6	807T>G	(Y269X)	Stop codon at 269

*Information deduced from rhesusbase.info website and (Singleton et al., 2000)

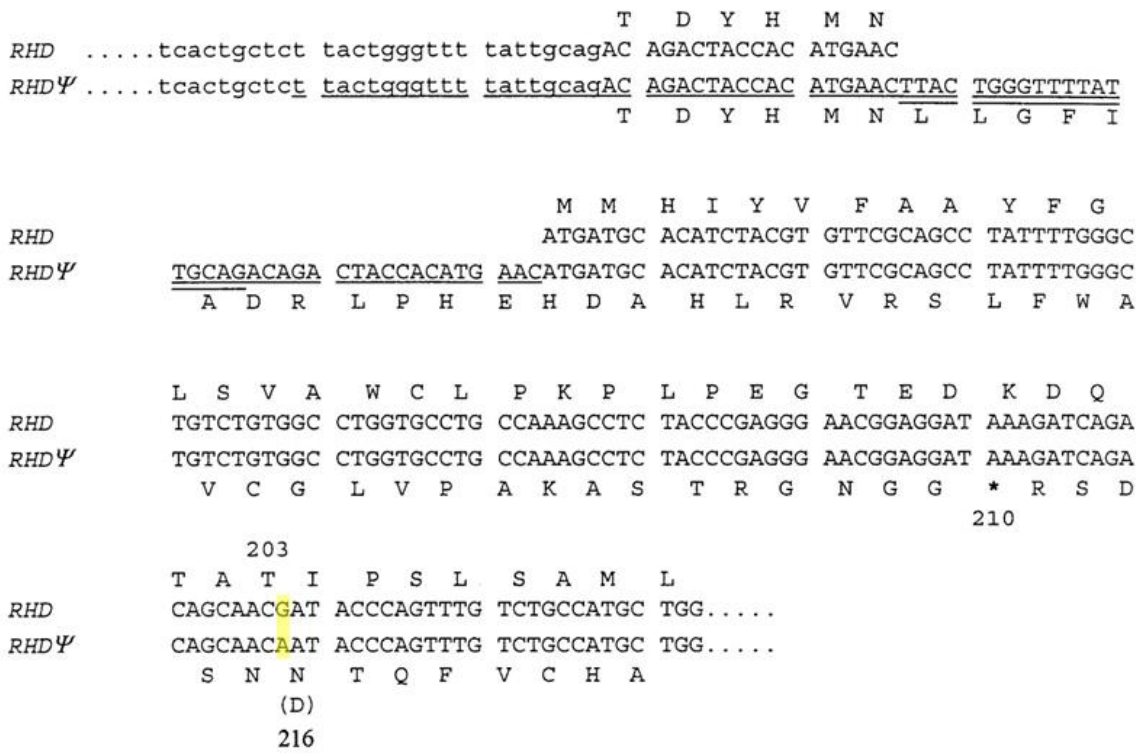


Figure 1.8 The presence of *RHD*Ψ* in Africans with the Rh D-negative blood group phenotype.

RHD intron 3 sequence shown in small letters whereas exon 4 sequence shown in capital letters. The region of the 37 bp duplication is underlined (single and double). The putative exon sequence derived from intron 3 is double-underlined. This insert introduce a reading frameshift and a potential translation-termination codon at codon 210 (indicated as *). The 37 bp insertion and subsequent frameshift gives also rise to an aspartic acid to asparagine substitution at codon 203 (original reading frame) or codon 216 (new reading frame) due to 609G>A point mutation (highlighted in yellow) (Singleton et al., 2000).

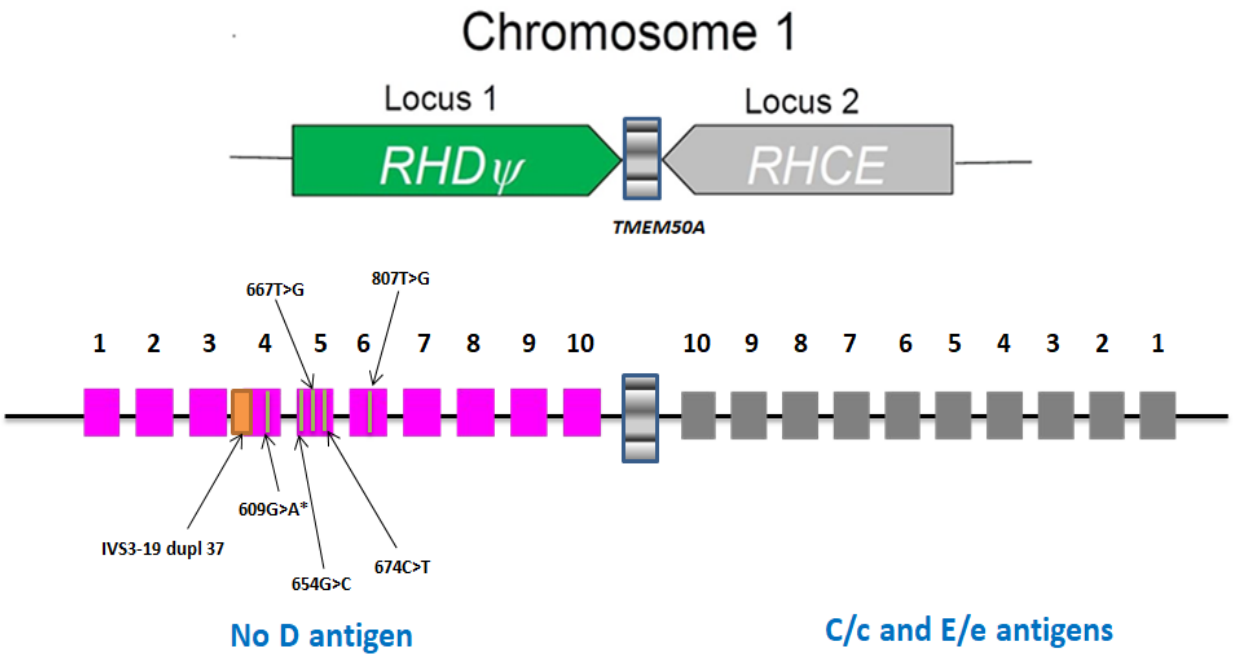


Figure 1.9 Schematic diagram on molecular background of *RHDΨ* gene that gives D negative phenotype.

The numbers indicate exon number on the *RHD* and *RHCE* genes. Pink box represent *RHD* exons and grey box represents *RHCE* exons. The orange box within pink box of *RHD* exon 4 represents 37 bp insert which is a duplication of a sequence spanning the intron 3 (at -19 nucleotide sequence)–exon 4 boundary (IVS-19 dup 37). Green lines represents point mutations associated with *RHDΨ* gene at *RHD* exon 4 (609G>A), *RHD* exon 5 (654G>C, 667T>G and 674C>T) and *RHD* exon 6 (807T>G). * indicates a point mutation that does not result in an amino acid change. *RHDΨ* gene has no effect on *RHCE* gene exons.

1.6.3 Partial D

The D antigen is a collection of conformation-dependent epitopes along the entire RhD protein. These epitopes are the binding sites of antibody. Changes in the amino acids and one part of the protein may affect the expression of the epitope sequence or result in new epitopes. As such, the antigens of the Rh D blood group may exhibit variations of their expression in the erythrocyte membrane of the RBCs. In partial D, RhD protein is altered from wild type protein and results in absence of one or more epitopes. The altered protein have amino acids changes in the extracellular loop (Daniels, 2013b). People whose RBCs have an altered form of RhD protein (partial D) may make allo-anti-D against altered or missing epitope. Such RBCs, depending on which D epitopes are altered, are agglutinated by a proportion of monoclonal anti-D reagents. Three types of molecular bases of partial D have been found: *RHD-CE* hybrid alleles, missense mutations in the extracellular protein segments, and dispersed missense mutations (Flegel and Wagner, 2002). According to RhesusBase website, more than 50 partial D alleles have been reported, the latest was in 2018 a weak partial D due to single missense mutation *RHD*(L214F)-*CE*(7)-*D* and a hybrid allele *RHCE*(5:697-5:744)-*RHD* (Fichou et al., 2018).

RHD/CE hybrid alleles results from rearrangements and genetic recombination between *RHD* and *RHCE* due to gene conversion events. This may result in part of an exon, a whole exon, or several exons of *RHCE* to combine with *RHD* gene (Figure 1.10) (Daniels, 2013b). As a result, *RHD* encode hybrid proteins that have RhCE-specific amino acids. This change affects a long string of amino acids, which is always located on the erythrocyte surface and the new hybrid can generate antigens; for example, DIV & DVI RBCs carry the Rh antigens called Evans (RH37) & BARC (RH52) respectively (Flegel and Wagner, 2002; Flegel, 2007; Quinley, 2011).

DIV has been divided serologically into DIV^{a} which is Go^{a} positive and DIV^{b} which is Go^{a} negative using polyclonal anti-Go^{a}, an antibody defining the low-prevalence D antigen RH30 (Go^{a}) (Tippett and Sanger, 1977). Several molecular subtypes have been reported for DIV^{b} while DIV^{a} is less diverse at the molecular level. A low frequency antigen called Evans carried by DIV^{b} is due to hybrid *RHCE* (7-9) seen in $CD^{IVb}e$ and $cD^{IVb}E$ haplotypes (Daniels, 2002).

DVI occurs in CDe, cDE, and cDe haplotypes (Jones, Scott and Voak, 1995). The $CD^{VI}e$ haplotype is due to hybrid *RHCE* (4-6) (Mouro et al., 1994) and $cD^{VI}E$ haplotype is due to hybrid *RHCE(4-5)* (Avent et al., 1997b). Most $CD^{VI}e$ haplotypes carry the low frequency antigen BARC (Flegel and Wagner, 1996). According to Rhesus base website, DVI type 2 and DVI type 3 carry BARC antigen with hybrid *RHCE* (4-6) and hybrid *RHCE* (3-6) respectively.

There are many other low frequency antigens that are carried by partial D caused by hybrid *RHCE*. For instance, D^{W} (RH23) is an antigenic marker for category DV^{a} partial D that is raised from hybrid *RHCE* (5) and can be seen in $CD^{Va}e$ and $cD^{Va}e$ haplotypes. RH32 is another low frequency antigen associated with $CD^{BT-2}e$ haplotype that is due to hybrid *RHCE* (5-9) that is carried by DBT-2 partial D where it shows weak expression of both C & e antigens (Daniels, 2002).

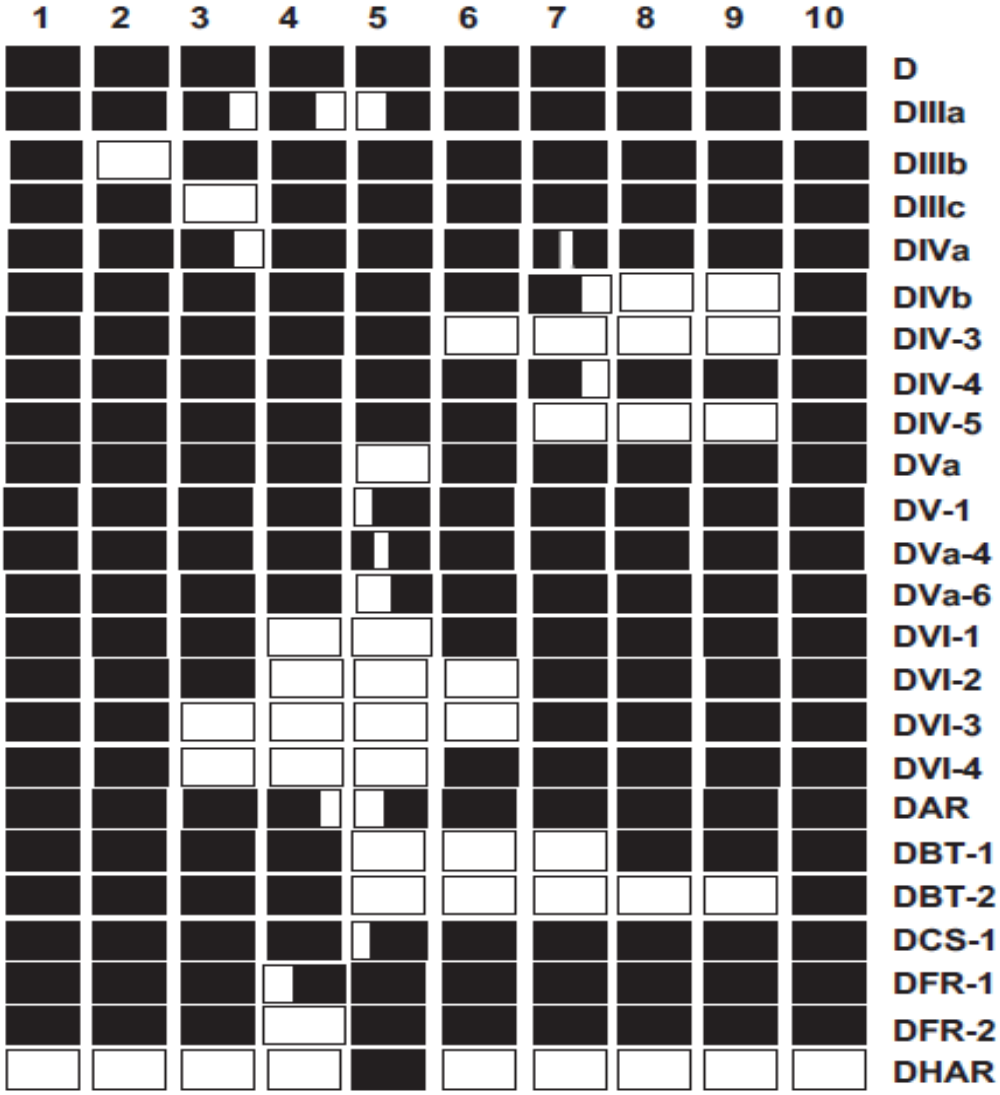


Figure 1.10 Partial D hybrid alleles compared to normal *RHD* gene.

The numbers indicate exon number. Black box represents *RHD* exon, whereas white box represents *RHCE* exon. The first row represents wild type *RHD* gene, whereas second row onwards represents different partial D types caused by *RHCE* hybrid. Many partial D arise from part of *RHCE* exon hybrids with *RHD* exon (e.g DV-1, DVa-4, DVa-6 and DCS-1 result from part of *RHCE* exon 5 hybrids with *RHD* exon 5). Other types of partial D result from full *RHCE* exon hybrid (e.g DIIIb type has exon 1 and 3 through 10 of *RHD* and exon 2 of *RHCE*, DIIIC with exons 1, 2 and 4 through 10 of *RHD* and exon 3 of *RHCE*. Two or more full exons of *RHCE* hybrid can also result in partial D (e.g DBT-2 results from *RHCE* hybrid of exon 5 through 9, DHAR with *RHCE* hybrid of exon 1 through 4 and 6 through 10, DVI-3 from *RHCE* hybrid of exon 3 through 6) (Daniels, 2013b).

According to the updated data from the RhesusBase website (http://www.rhesusbase.info/), 27 partial D are caused by single missense mutations (SMM), the latest reported mutation is *RHD*52* which is 668 T>C (F223S) predicted to cause damaging effect on RhD protein (Filosa et al., 2016). The phenotypes of SMM causing partial D are much more diverse than the phenotypes caused by gene conversion and determined by the localization and type of substitution (Figure 1.11). In addition, fewer epitopes are affected in partial D caused by SMM compared to partial D that results from gene conversions (Flegel and Wagner, 2002).

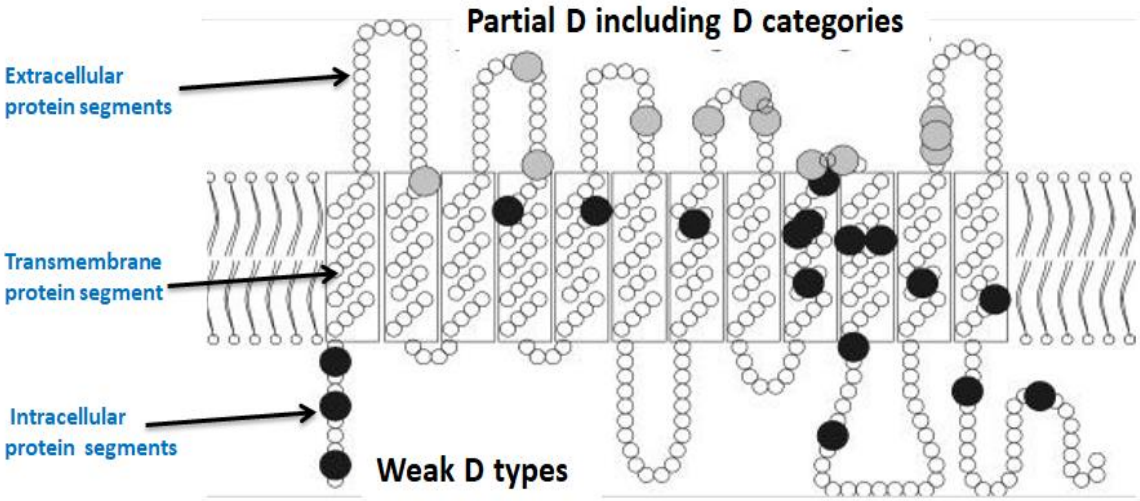


Figure 1.11 Aberrant *RHD* alleles with single missense mutations.

Amino acid positions affected in weak D types are shown in black; those affected in partial D, including D categories, are shown in grey. Amino acid substitutions in weak D are located in the intracellular or transmembraneous protein segments, those found in partial D phenotypes in the extracellular protein segments (Flegel and Wagner, 2002).

Multiple missense mutation results in multiple amino acid substitutions which are dispersed in the RhD protein may also cause partial D variant. D category, DII, DIII type 4, DIII type 5 and DIV type 1.0 are few examples of dispersed amino acid substitutions. More than 30 multiple missense mutation causing partial D have been reported as per the data from RhesusBase website. ISBT *RHD*DAR6* with multiple mutations *RHD* (150T>C,178A>C,201G>A,203G>A,602C>G,667T>G,957G>A,1025T>C) is the latest type that has been noted in African ancestry.

Few aberrant *RHD* alleles can easily cause clinical problems in transfusion recipients and pregnant women. The carrier of these alleles may easily be anti-D immunised (Flegel and Wagner, 2002). For example, carriers of partial D type DVI can be anti-D immunised if transfused with D positive RBCs. Therefore, most transfusion services in the hospital use type of monoclonal anti-D that does not react with DVI RBCs and thus deliberately mistyping transfusion recipients and pregnant women as D negative. This is to avoid exposure of D positive RBCs for the missing epitope(s) and prevent allo-anti-D immunisation and select the pregnant candidates for anti-D immunoglobulin.

Cases of anti-D immunisation in women with partial D causing mild to severe HDFN have been reported and involve mothers with DNB (Lukacevic Krstic et al., 2016; Quantock et al., 2017), DIIIa (Beckers et al., 1996), DIVb (Okubo et al., 1991), DVa (Lukacevic Krstic et al., 2016), DVI (Lacey et al., 1983) and DBT (Wallace et al., 1997). Very few cases of HTR due to anti-D in D positive recipient have been reported and involve HTR in sickle cell disease (SCD) patient with homozygous partial D for *RHD*DAU4* (Ipe et al., 2015). Therefore, partial D patients with anti-D should be transfused D negative blood (Daniels, 2013b). Child bearing women with partial D should be treated as D negative to get anti-D immunoglobulin prophylaxis and avoid anti-D immunisation to protect the foetus from HDFN.

Donors RBCs that can be used to immunise D negative recipients for anti-D should be labelled D positive. For new donors it is essential to test the RBCs for partial D types DIV, DV and DVI and for known repeated donors a blended anti-D reagent of DIV, DV and DVI and weak D (Daniels, 2013b).

1.6.4 Weak D

Wagner and colleagues proposed that only amino acid substitutions located within the membrane spanning or cytosolic domains of the D protein are responsible for weak D (Figure 1.11) (Wagner et al., 1999). Through *RHD* sequencing, they identified many different types of weak D, numbered weak D type 1, 2, 3. This has now been expanded to many weak D types, with several subtypes (e.g. weak D type 4·1) Most serological weak D phenotypes (>95% in Northern Europeans) are the expression of weak D types 1, 2, 3 or 4·0/4·1 (Flegel, 2011).

To date, 159 weak D types have been detected out of which 154 are listed on the Rhesus database (http://www.rhesusbase. info/). These weak D types cannot be distinguished serologically, though they are associated with different degrees of weakness and some can. Among white German and Austrian blood donors with weak D red cells, 70%, 18%, and 5% were weak D types 1, 2, and 3 respectively (Wagner et al, 1999), whereas among French patients the equivalent frequencies were 38%, 27%, and 8% (Noizat-Pirenne et al., 2007). Novel weak D (not yet given ISBT nomenclature) types 150, 151 and 152 have been reported recently (Fichou et al., 2018).

The estimated number of D antigen on RBCs has been noticed to vary between different D phenotypes (Table 1.6). Fluorescence flow cytometry using mono and polyclonal anti-D shown the following decrease in number of D antigens on the phenotypes: D-- > R_2R_2 > R_1R_2 > R_1R_1 > R_2r > R_0r > R_1r (Nicholson et al., 1991; Harmening, 2012). The number of D antigen sites per red cell in the ‘weak D types’ varies from about 60 to 3800 (Table 1.7).

This is in contrast with 13,000–24,000 for 'normal' D+ red cells and 3000 on DVI type 2 and 33,000 on DIII type 4 red cells (Wagner et al, 2000).

Table 1.6 Estimated number of D antigens on different Rh phenotypes

Rh Phenotype	Number of D antigen sites*
R_1r	9,900 – 14,600
R_0r	12,000 – 20,000
R_2r	14,000 – 16,600
R_1R_1	14,500 – 19,300
R_1R_2	23,000 – 31,000
R_2R_2	15,800 – 33,300
D--	110,000 – 202,000

The highest number of D antigen can be found in D-- phenotype, whereas the lowest number could be seen in R_1r, R_0r R_2r phenotypes. *(Harmening, 2012)

Table 1.7 Few weak D types with number of D antigens on RBCs and their ability to enhance anti-D allo-immunisation

Weak D type	Anti-D	Number of D antigen	References
DOL	Yes	4,700	(Avent, Poole and Singleton, 1999)
1		1285	(Wagner et al., 1999)
2		489	(Wagner et al., 1999)
3		1932	(Wagner et al., 1999)
4		2288	(Wagner et al., 1999)
4.1		3811	(Wagner et al., 1999)
15	Yes	297	(Wagner et al., 1999)

The lowest number of D antigen on RBCs can be seen in weak D types 2 and 15. Individuals with weak D type DOL and 15 are capable of producing anti-D.

Serological weak D phenotype is negative or less than +2 reactivity of RBCs with an anti-D reagent in initial testing, followed by moderate to strong agglutination with anti-human globulin (Sandler et al., 2015). In many countries, longstanding laboratory practice is not to test for weak D any patient or child bearing woman who shows D negative in initial serological anti-D test. Thus, typing them as D negative and protect them against anti-D immunisation. However, this practice results in unnecessary transfusion of D negative RBCs and unnecessary administration of anti-D immunoglobulin prophylaxis. The practice used to test the donor for weak D if direct agglutination test is negative.

Current protocol requires a weak D test for the donor, thus protecting D negative recipients from immunogenic RBCs with a serological weak D phenotype (Ooley, 2015). A study by Wagner and colleagues in central Europe showed that transfusion recipients with a weak D

type 1, 2 or 3 in the homozygous or hemizygous state are not at risk for forming alloanti-D when exposed to D positive RBCs. As a current protocol, transfusion recipient with weak D should be tested for weak D type 1, 2 and 3, if positive transfusion of D positive RBCs blood should be safe, if negative transfusion of D negative RBCs should be offered (Figure 1.12). Likewise, homozygous and hemizygous weak D types 1, 2 and 3 pregnant and women with childbearing age typed as D positive. They do not require anti-D immunoglobulin prophylaxis, as they are not at risk of anti-D immunisation if foetus is D positive (Figure 1.12) (Sandler et al., 2015).

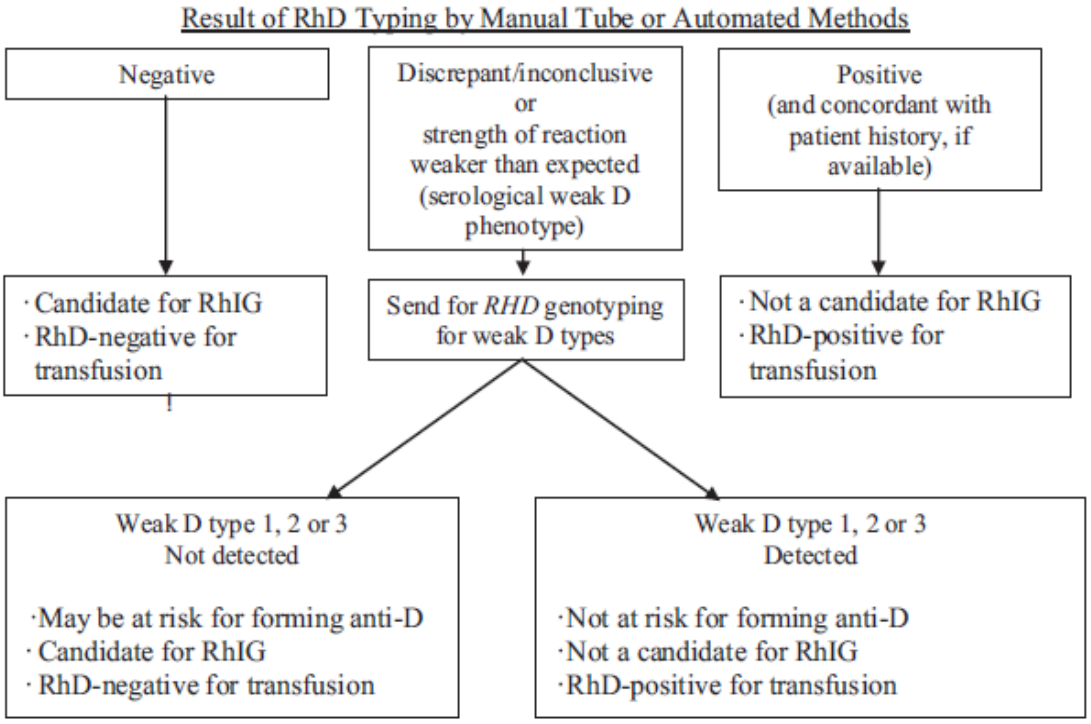


Figure 1.12 Management of serological weak D phenotype.

If the result of serological RhD typing is D negative, the individual should be managed as D negative and offered D negative unit for transfusion / candidate for RhIG; and if D positive, the individual should be managed as D positive and offered D poitive unit for transfusion / not a candidate for RhIG. If the result of RhD typing is a serological weak D phenotype, the laboratory should retest the blood sample and do *RHD* genotyping for Weak D type 1, 2 and 3. If a weak D type 1, 2 or 3 is not detected, the individual should be managed as D negative. If a weak D type 1, 2 or 3 is detected, the individual can be managed safely as D poitive (Sandler, Chen and Flegel, 2017).

1.6.5 DEL phenotypes

DEL is a very weak form of D that cannot be detected by conventional serological tests but by adsorption and elution tests (Kulkarni et al., 2018). The antigen site density per red cell was estimated to be less than 22 (Kormoczi et al., 2005). However, density below 200 may also be involved in DEL phenotype. DEL phenotype is linked with RhCE*Ce or RhCE.cE as noted previously (Flegel and Wagner, 2002; Shao et al., 2002).

DEL phenotype is usually caused by single nucleotide polymorphism (SNP) that causes change in the transmembrane or cytosolic domains of the RhD protein (Nuchnoi et al., 2014). They can also be associated with certain partial or hybrid DEL alleles (Sandler, Chen and Flegel, 2017). According to RhesusBase website, more than 40 DEL alleles are currently listed. The commonest DEL alleles are found in Eastern Asia population (1227G>A, K409K) and Caucasian (M295I) and (IVS3+1 G>A).

In Eastern Asia, DEL (also called Asian-type DEL) was found to be between 10% and 33% of D negative RBCs tested by conventional serological (Daniels, 2013b). This DEL associate allele has SNP (1227G>A, K409K) in a splice site on exon 9 that leads to reduced D antigenic density (Luettringhaus et al., 2006). A study conducted by (Shao, 2010) showed that Asian-type DEL is not usually associated with alloanti-D production. Another retrospective study by Wang and colleagues on 227 D negative transfusion recipients in China who received transfusion, some of which were D positive. None of the transfusion recipients with anti-D had a DEL phenotype associated with the (K409K) allele (Wang et al., 2014).

Caucasian DEL allele (IVS3+1G>A) has the splice site mutation *RHD* at first nucleotide of intron 3 (Wagner, Frohmajer and Flegel, 2001). This type of DEL allele has been associated with alloanti-D production. Four individuals with *RHD* (IVS3+1G>A) had anti-D, with one of the antibodies causing mild HDFN (Gardener et al., 2012). Pregnant

women with a complete DEL phenotype (K409K) who deliver an D positive newborn are not at risk for forming anti-D, but pregnant women with certain partial or hybrid DEL alleles are at risk for forming anti-D (Sandler, Chen and Flegel, 2017).

Donors must be tested for DEL phenotype as evidences have shown that alloanti-D immunisation occurred on individuals who were exposed to DEL RBCs. Despite low number of D antigens on RBCs of DEL phenotype, *RHD* (K409K) and *RHD* (IVS2-2A>G) have shown to cause primary & secondary immunisation when transfused to D negative patient (Wagner et al., 2005).

1.7 Unusual Rh phenotypes

There are many less frequently encountered Rh antigens that can be found in different ethnic groups. Few antigens were already described in section 1.6.3.

1.7.1 C^w (RH8)

The first example of anti-C^w was found in the serum of a DCe/DCe patient who had been transfused with DCe/DCe C^w+ red cells (Callender and Race, 1946). The prevalence of this antigen in Northern Eurpean and white American is 2.6% (Daniels, 2002). C^w+ red cells are almost always C+, but the C antigen associated with C^w is weaker than normal C, though recognition of this weakness depends on the anti-C used. Although C^w is usually produced by a DCe haplotype, C^w associated with dCe (Callender and Race, 1946) and dCE (Dunsford and Aspinall, 1951) have also been found. C^w is usually produced by a *Ce* allele of *RHCE* encoding Arg41. C^w associated with c is produced by a *ce* allele encoding Arg41 and Cys16 (Mouro et al., 1995). CC^we and cC^we alleles therefore have exons 1 of identical sequence and cC^we could have arisen by recombination between CC^we (exon 1) and ce (exons 2–10) alleles of *RHCE*.

1.7.2 VS (RH20) and V (RH10) antigens

VS and V define antigens that are common on the red cells of Africans with a frequency of up to 50%, but are very rare in other populations. VS is associated with unusual weak e expression called e^s. When VS is produced by *RHCE* that encodes for c and e^s but not C, another antigen called V is also expressed (Denatale et al., 1955). VS is produced by the haplotypes *Dce*^{s}, *dce*^{s}, and *d(C)ce*^{s}, whereas V is produced by *Dce*^{s} and *dce*^{s}, but not *d(C)ce*^{s}. The haplotype *d(C)ce*^{s} produces partial c, e^s, ce (f), a weak partial C, G, and VS; it does not produce D, Ce, V, hr^B (RH31) and Bastian antigen Hr^B (RH34) (Daniels, 2002; Pham et al., 2009a; Pham et al., 2009b). Some individuals with d(C)ce^s phenotype have produced alloanti-C, confirming that the weak C is altered C antigen.

At molecular and protein level, the presence of V and VS occur with a nucleotide polymorphism 733C>G in exon 5 of *RHCE* gene that leads to amino acid substitution (Leu245Val) predicted to be within a transmembrane domain (Daniels GL, et al, 1998). The V antigen (in the presence of VS) is not expressed when another transmembrane amino acid substitution at residue Gly336Cys is present due to a nucleotide polymorphism 1006G>T in exon 7 of *RHCE* (Faas et al., 1997; Daniels et al., 1998). The membrane location of residues 245 and 336 suggests Rh antigen expression is affected significantly by non-exofacial amino acids and the prediction of some Rh epitope expression cannot be based solely on externalized residues.

The *(C)ce*^{s} haplotype is composed of hybrid *RHD-CE-D* allele linked to *ce*^{s} allele of the *RHCE* gene. There are two types of *(C)ce*^{s} haplotype . *(C)ce*^{s} type 1 haplotype when the hybrid *RHD-CE-D* allele consists of exons 1 and 2, part of exon 3, and exons 8 through 10 from *RHD* and the remainder of exon 3 and exons 4 through 7 from *RHCE*. *(C)ce*^{s} type 2 haplotype exists with hybrid *RHD-CE-D* allele consists of exons 1 and 2, complete exon 3, and exons 8 through 10 from *RHD*, and exons 4 through 7 from *RHCE*. Both types harbor

733C>G (Leu245Val) and 1006G>T (Gly336Cys) nucleotide polymorphisms (Fasano et al., 2010).

1.8 RHD detection in molecular Immunohaematology laboratory

Serological tests usually precede molecular tests and inform the choice of molecular biology technique for subsequent testing (Olsson et al., 2005). Hemagglutination is the original method to test for blood group antigens using commercially available antibodies. However, it does not reliably predict a foetus at risk of HDFN, it is difficult to phenotype RBCs from a recently transfused patient or when RBCs are coated with IgG, and has poor ability to predict zygosity in Rh-positive individuals (Reid and Denomme, 2011).

The blood of serologically negative donors with weak D antigen expression may be immunogenic and induce the formation of immunogenic alloantibodies in the recipient (Flegel and Wagner, 2002). In addition, D detecting serological methods can mistype few D positive individuals (e.g DEL phenotype) as D negative and increase risk of HTR. Therefore, a proper DNA testing for *RH* gene could help a lot to get rid of these problems.

Since 1990s, molecular biology methods have gradually been introduced into the diagnostic process. Methods are based on the polymerase chain reaction (PCR). The earliest developed PCR based assays rely on the amplification using sequence specific primers (PCR-SSP) and pattern analysis of the PCR products in agarose gel or on the electrophoretic analysis of PCR-RFLP. The conventional PCR is based on in-house primers design and optimization of PCR or on standardized and optimized in-vitro diagnostics (IVD) commercial tests such as BAGene kit. Most of the common *RHD* alleles involved in serological D negative in Caucasians and African were determined using conventional end point PCR (Singleton et al., 2000; Wagner, Frohmajer and Flegel, 2001). In a study to determine the polymorphisms associated with antigen expression for *RHD* and *RHCE* in 39 multi-transfused patients including thalassemia and SCD; BAGene RH-Type kit was used and

detected discrepancies between genotyping and phenotyping results in many alleles (Bakanay et al., 2013). Additional tests such as Sanger sequencing might be required if serological testing yields equivocal results. Many studies used PCR-RFLP for the detection of *RHD* deletion and D zygosity (Wagner and Flegel, 2000; Kacem et al., 2015; Khosroshahi et al., 2019). A more recent modification of this technique is PCR based on real-time PCR with allele specific TaqMan or HybProbe probes or with SYBR Green DNA intercalating dyes. Real-time PCR technique has also been useful to determine different *RHD* alleles in serological D negative phenotype (Moussa et al., 2012; Szulman et al., 2012; Khosroshahi et al., 2019).

The blood transfusion service in some countries makes use of a technique based on Multiplex Ligation-dependent Probe Amplification (MLPA) (Haer-Wigman et al., 2013). Kim and colleagues investigated the molecular basis of serologically D negative phenotypes, including the DEL type using MLPA and found that 17.9% of the Korean cohort had DEL *RHD* (1227G>A) allele (Kim et al., 2018). A growing number of tests started to use technologies designed for larger-scale research, including technologies based on digital PCR (dPCR). Recent studies used dPCR and were successfully able to do fetal *RHD* genotyping from cell free fetal DNA (Sillence et al., 2015; Svobodova et al., 2015; Lucie et al., 2016).

Following 1000 genome project, there was a need of sequencing with a high throughput, the development of tools for reading DNA sequences and for collecting and analyzing the data obtained, and as a result, the development of the high-throughput solution known as Next Generation Sequencing (NGS). The various levels of sequencing may be achieved by NGS, these are either the whole genome (WGS), exomes or only selected genes or regions of interest. It has been successfully implemented in transplantation medicine for testing donors genotypes of HLA antigens in high-throughput mode (Schöfl et al., 2017). Complete *RHD* gene sequencing has been possible using NGS by amplifying the whole gene

(Tounsi, Madgett and Avent, 2018). Another study used NGS to genotype *RHD* and *RHCE* using gene specific primers that successfully identified 10 and 25 *RHD* and *RHCE* alleles respectively (Dezan et al., 2017).

Efforts were also made in prenatal diagnosis of fetal D status for the management of D negative women with partners heterozygous for the *RHD* gene. Due to the invasive limitations associated with conventional methods, cell-free fetal DNA from from maternal plasma and serum was used for the determination of the fetal D status (Faas et al., 1998; Lo et al., 1998; Bischoff et al., 1999). This development represented an important step towards the routine application of noninvasive fetal blood group diagnosis and possibility of doing direct genetic analysis using free plasma DNA.

1.9 Oman, a multi-ethnic country

Oman is an Arab country bordered by Saudi Arabia to the west, the United Arab Emirates to the northwest and Yemen to the southwest, Oman shares marine borders with Iran and Pakistan. According to Central Intelligence Agency (CIA, 2014), Oman's population consists of African, Arab, South Asian (Pakistani, Sri Lankan, Indian, Bangladeshi), Baluchi ethnic groups.

Many Omanis in the capital city Muscat and coastal areas are of non-Arab origin, namely Baloch, Zanzibari, Al-Lawati and Persian (Al Shaibany, 2010) Al-Lawati and Persian. Lawatis are of South Asian descent and are believed to share similar origins with the India and Pakistan. The Baloch are an Iranian and Pakistani people who mainly speak the Balochi language, a branch of North western Iranian languages. Balochis account for 25% of the Oman population.

The Swahili-Arab people come from east coast of Africa, mainly Zanzibar, Bagamoyo, and Dar es Salaam They are a mixture of Omani and some Yemeni Arabs who travelled to East Africa in the 1700's to establish trade with Europe, primarily in slaves.

These Arabs had children with their African wives and slaves. In the mid-1960s an African revolution against Arabs in East Africa resulted in the killing of tens of thousands of Omani and Yemeni people. It was after this that Sultan Qaboos bin Said (18 November 1940 – 10 January 2020) invited many Omanis living in East Africa back to Oman.

Based on the population distribution and their geographic isolation, it is conceivable that the blood types that are present in these countries may also be found in Oman as well. we anticipate that the RhD and RhCE antigens present a distinct pattern compared to Caucasian populations.

1.10 Scope

The D-negative phenotype accounts for less than 1% of Asian individuals. The very low frequency of the D-negative in Asian populations brings into question the necessity of routinely testing donors and patients for D in this region of the world. The data for the distribution of *RHD* variants and alleles are important for public health care system to implement good and effective population specific transfusion practice and obstetrical management (Thongbut et al., 2019). However, there are limited studies published on the RhD and RhCE antigens for Gulf Arabs broadly or the Omani population specifically, to the best of our knowledge and no published data on *RHD* and *RHCE* genotyping (Al-Riyami et al., 2019). This contrast with studies on African populations, where two *RHD* alleles are mainly responsible for the D negative phenotype that are *RHD*Ψ* (66%) (Singleton et al., 2000) and *RHD-CE-D*^{s} (15%) (Daniels et al., 1998; Faas et al., 1998), while in Iran, the main cause of the D negative phenotype is complete deletion of *RHD* gene (Khosroshahi et al., 2019) . There are no reports of DEL phenotypes among either African or Iranian D negative populations. Given the Omani connection to both African and Iranian populations, we hypothesized that Omanis may have a silent, inactive African *RHD* alleles and/or complete deletion of *RHD* gene as causes of serological D negative but not DEL phenotype.

1.11 Aim and objectives

To begin addressing this hypothesis, we conducted a retrospective study to find out the frequency of ABO/Rh blood group in the Omani population. Evaluation of 3416 Omani donors using serological typing found that 8.4% were D negative, which is in close agreement to the 5–10% reported for Arab Gulf and Iranian populations (see Figure 1.6). Al-Riyami and colleagues conducted a prospective study to find out frequencies of different blood groups in Omani population and found that 10.7% of the population is D negative (Al-Riyami et al., 2019). Some literature shows that *RHD* genotyping should be recommended primarily for D negative C or E positive donors, taking into consideration the results of some authors who found weak D or DEL phenotypes in serologically typed D negative and C/E positive individuals only by *RHD* molecular typing (Gassner et al., 2005; Engelfriet and Reesink, 2006).

The first aim of this study then was to determine the Rh serological backgrounds of the D negative phenotypes in the Omani population. Serological testing relies on Rh reagents that primarily anti-D typing reagents to detect D antigen or reagents to detect the presence or absence of the C, E, c and e antigens. These reagents can either be of high protein derived from human serum of immunized person or monoclonal IgM or monoclonal or polyclonal IgG of low protein derived from human/murine heterohybridoma sources (Rudmann, 2005).

The current consensus laboratory standard guidelines for Rh phenotyping recommended by Central Blood Bank (CBB) Oman does not include confirmation of D status in serological D negative by molecular typing. In the Middle East, most published studies that investigated the frequencies of red blood cell types were based on serology typing with different commercial monoclonal/polyclonal antibodies. Genotyping studies have been limited, and those were generally restricted to patients with haematological diseases, such as

haemoglobinopathies (Chou and Westhoff, 2011; Dhawan et al., 2014). However, serological phenotyping of D has limitations. For instance, the blood of serologically negative donors with weak D antigen expression may be immunogenic and induce the formation of immunogenic alloantibodies in the recipient (Flegel and Wagner, 2002; Noizat-Pirenne et al., 2007). This implies that some of the 8.4% of the Omani populations might have been mistyped as D negative and require *RHD* molecular typing to retype the D status and avoid alloimmunization if transfused to D negative recipients.

In view of these limitations and given the growth/validation/establishment of PCR-based *RHD* genotyping in clinical practice, the second aim of this study was to develop a real-time PCR based assay for the determination of the molecular background of D negative population in Oman. Most of the studies used conventional PCR or TaqMan real-time PCR for the determination of the molecular background of D negative. For instance, Singleton and colleagues used conventional PCR to detect *RHDΨ* pseudogene and *RHD-CE-D*^{s} screening different *RHD* exons (Singleton et al., 2000). Likewise, Wanger and colleagues also used conventional PCR to screen *RHD* exons and introns of more than 8000 whites with antigen D negative and identified many *RHD* alleles such as Hybrid *RHD-CE(3-7)-D*^{s}, *RHD-CE(2-9)-D* and DEL phenotypes *RHD* (K409K), *RHD* (M295I) and *RHD(IVS3+1G>A)* (Wagner, Frohmajer and Flegel, 2001). Studies also used modified PCR based on real-time PCR with allele specific TaqMan probes or with SYBR Green DNA intercalating dyes for *RHD* genotyping of serological D negative samples (Moezzi et al., 2016; Kim et al., 2020). For instance, Khosroshahi and colleagues used melting curve analysis real-time PCR technique with SYBR Green to screen 200 D negative Iranian blood donors for *RHD* exons 5, 7 and 10 and identified weak D type 11 and *RHD-CE(2-9)-D*$_{2}$ alleles in two samples (Khosroshahi et al., 2019). TaqMan based real-time PCR also been useful to determine different *RHD* alleles

in serological D negative phenotype. Mousa and colleagues used TaqMan chemistry to detect *RHD* gene in Tunisian population and were able to detect *RHDΨ* pseudogene, *RHD-CE(3-8)-D*, *RHD-CE-D*^{s}, *RHD-CE(4-7)-D*, weak D type 11, Weak D type 4·0, Weak D type 29 and *RHD-CE(5-7)-D (DBT-1)* (Moussa et al., 2012). The study was based on *RHD* exon 10 detection in the samples initially and further screening for the presence of other *RHD* exons 1 through 5 and 7 through 9. Another study conducted by Gunel and colleagues for the detection of *RHD* gene in amniotic fluid proven TaqMan chemistry to be more sensitive, accurate, and specific for *RHD* gene than SYBR Green I method (Gunel et al., 2011). Though nonspecific reaction products are more likely to develop with SYBR Green protocols, leading to increased background and false positives, however, it is less expensive and more convenient than probe-based methods. A survey of *RHD* alleles was lacking in Omani population. We aimed to unveil the molecular background of *RHD* gene by melting curve analysis for different *RHD* exons using SYBR Green and real-time PCR using TaqMan chemistry. Such knowledge could improve the efficiency which with blood transfusions are delivered to patients with *RHD* molecular variant

Objectives:

1. Perform serological Rh phenotyping for C, E, c and e on D negative donors using antibodies in commercially available kits.

2. Confirm the D status by the indirect anti-human test (IAT). This method enables weak D cohorts to be excluded as weak D are D positive

3. Evaluate anti-D reagent by molecular studies using real-time PCR approaches

4. Study the molecular background of the serological D negative cohort by designing and optimising end-point and real-time PCR assays

5. Analyse samples by melting curve analysis for the presence of *RHD* exons

6. Analyse samples for the presence of specific *RHD* exons with a view to developing a singleplex and multiplex assays for exon-specific real-time PCR detection

7. To compare the molecular background determined based on objectives 4 and 5 above with data from a commercial PCR test ((BAGene test, partial D-TYPE and RH-TYPE).

8. To sequence *RHD* exons to reconfirm the presence of few *RHD* alleles and determine the putative presence of novel polymorphisms in the D negative cohorts

2 CHAPTER TWO

2.1 Materials

The samples for the study were collected in BD Vacutainer K2 EDTA (BD Franklin Lakes NJ USA). Rhesus Blood grouping and phenotyping was done by tube haemagglutination test using commercially available Anti-D IgM/IgG blend (Atlas Medical, UK), Anti-C, Anti-c, Anti-E & Anti-e reagents (BioRad Cressier FR, Switzerland). Direct (DCT) and indirect (IDT) Coombs tube test for D weak detection was done using clear Anti-Human Globulin (Atlas Medical Cambridge, UK). Control ID DiaCell I & II from BioRad, and coombs control cells (Lorne Laboratories Danehill, UK) were used as procedure and washing efficiency controls for IDT and DCT test respectively in D weak detection test. The Rh phenotyping, both DAT and IDT tube method was reconfirmed by ID card gel method using ID-Card ‘’Coombs Anti-IgG’’, ID-Card ‘’RhD + Phenotype’’ (Human), ID-CellStab, Anti-D IgG/IgM blend (Atlas Medical, UK), ID-Diluent 1, ID-Diluent 2, ID-Centrifuge 24, ID-Incubator from Bio-Rad (Cressier FR, Switzerland).

For molecular study of *RHD* exons, DNA sample was prepared using Whatman™ FTA™ Elute Card (GE healthcare Buckinghamshire, UK Limited). DNA extraction was done using 3 mm Uni-Core (GE healthcare Buckinghamshire, UK Limited) for obtaining punch of sample from FTA card and QIAamp® DNA Investigator Kit (Qiagen Manchester, UK) for extraction procedure. DNA concentrations of the extracted samples were recorded using Thermo Scientific™ NanoDrop 1000. Presence of *RHD* exons 1 through 7, 9 and 10 and human growth hormone factor (HGHF) were tested using forward and reverse primers (IDT Leuven, Belgium) (Table 2.1). For TaqMan assay specific primers and probes for exons 5, 7 and 10 and intron 4 were used (Applied Biosystems®, UK) (table2.1). Quantitative PCR was run on Qiagen Rotor-Gene Q with SYBR Green Master Mixes. These were

QuantiNova™ SYBR Green PCR (Qiagen Manchester, UK), qPCRBIO SyGreen (London, UK) and PowerUp™SYBR™Green (Thermo Scientific Vilnius, Lithuania). Quantitative PCR specific DNA products were tested with gel electrophoresis and products were visualised using BioRad ChemiDoc™ MP, Imaging analyser.

PCR-SSP was done using BAGene kits RH-TYPE, partial D-TYPE and Weak D-TYPE from (BAGene,BAG Health Care, Lich,Germany). Samples were prepared using Happy Taq polymerase 5 units/µl and 10x PCR buffer both provided by (BAGene,BAG Health Care, Lich,Germany). The samples were run using BIO-RAD C1000 Touch Thermal Cycler. The products were tested with gel electrophoresis and products were visualised using BioRad ChemiDoc™ MP, Imaging analyser.

For QuantStudio™ 3D Digital PCR System, same primers (IDT Leuven, Belgium) and probes used for real-time PCR (Applied Biosystems®, UK). In SYBR Green assay, SYBR Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO and QuantStudio™ 3D Digital PCR Master Mix both from Applied Biosystems®, UK were used. For TaqMan assay the same Master Mix was used. Sample loading materials, QuantStudio™ 3D Digital PCR Chip, QuantStudio™ 3D Digital PCR Chip Adapters, QuantStudio™ 3D Digital PCR Thermal Pads and QuantStudio™ 3D Digital PCR Tilt Base were from Applied Biosystems®, UK. The PCR was run in ProFlex™ 2x Flat PCR (Applied Biosystems®, UK)

2.2 Methods

2.2.1 Ethical approval

Arabic written consent was obtained from each donor (Appendix 2). The study was approved by Research and Ethical Review & Approve Committee (RERAC) of Ministry of Health Oman (MOH/DGPS/CSR/PROPOSAL_APPROVED /25/2016) and Liverpool John Moores University Research and Ethical Committee (no. 16/PBS/003).

2.2.2 Blood samples collection

Ethylenediaminetetraacetic (EDTA) is widely used as an anticoagulant blood samples for blood groups serology and genotyping as EDTA has DNase inhibition activity and thus protects cell free DNA from degradation (Barra et al., 2015; Basu et al., 2018; Ouchari et al., 2018). Total of 3 ml blood samples was collected from 203 D negative Omani donors between September 2016 and April 2017 using vacutainer K_2EDTA tubes (BD Franklin Lakes NJ USA). The blood donors were from central and regional blood banks in Oman. The specified volume was collected as a part of regular whole blood donations process (450 ml) in the blood bank. The tubes mixed immediately for 5 minutes on a tube roller. Samples were either tested immediately or else stored at 4°C for up to 5 days.

2.2.3 Dry blood sample preparation

Dry blood sample preparation using Whatman™ FTA™ Elute Card (GE healthcare Buckinghamshire, UK) was initiated prior to Rh blood group serological tests. The FTA elute cards are designed for DNA preservation, transport and shipment at room temperature and purification for molecular analysis. The patented chemicals on FTA cards causes cells lysis, proteins denaturation and protect DNA against degradation by nuclease, oxidative, ultraviolet damage and helps in long term storage (da Cunha Santos, 2018). Many studies on different

genes observed no correlation between robustness of PCR and collection time for different samples stored on the FTA cards for up to 1 year (Mas et al., 2007; Peluso et al., 2015)

Each card was used for one biological sample and labelled anonymously with appropriate sample identification. A total of 40 µl Vacutainer K_2EDTA (BD Franklin Lakes NJ, USA) venous blood was transferred in all four wells using sterile aerosol resistant tips and the card was placed to dry at 80^{o}C oven for 30 minutes. The samples were stored at room temperature until used.

2.2.4 Immunohaematological tube test for RhD & RhCE phenotyping

The ABO forward and reverse and RHD grouping was done by central and regional blood banks as part of donor sample processing. Therefore, results of ABO and RHD grouping were provided by blood banks. Both tube and ID-card RhD + phenotype gel methods were used to reconfirm D negative initially done by blood banks and further phenotyping for RhC, c, E and e antigens were done.

BioRad protocol for tube test using specifc monoclonal IgM antibodies, Anti-c (cell line: MS-33), Anti-e (cell line: MS-16, MS-21, MS-63), Anti-C (cell line: MS-24) and Anti-E (cell line: MS-260) (BioRad Cressier FR, Switzerland) and Atlas medical (Cambridge, UK) anti-D IgM/IgG blend were used. Specific Rh antisera if reacted with its corresponding antigen causes agglutination which indicates the presence of Rh antigen (Figure 2.1). Absence of the Rh antigen is indicated by no agglutination (Figure 2.1B). Tube method for Rh phenotyping is still a cost-effective method in transfusion medicine and many transfusion centres in different countries use this method (Siransy Bogui et al., 2014; Gundrajukuppam et al., 2016).

Typically, 3–5% RBCs suspension was prepared according to the manufacturer's instructions. For each sample, five tubes were allocated. One drop of the respective antibody reagent was added into appropriate tube with one drop of RBCs suspension. The tubes were

mixed and incubated for 5 minutes at room temperature, centrifuged at 1000 g for 20 seconds and observed for agglutination macroscopically. Known positive and negative controls were included in accordance with the relevant guidelines of quality assurance. Agglutination grading was recorded according to the Figure 2.2

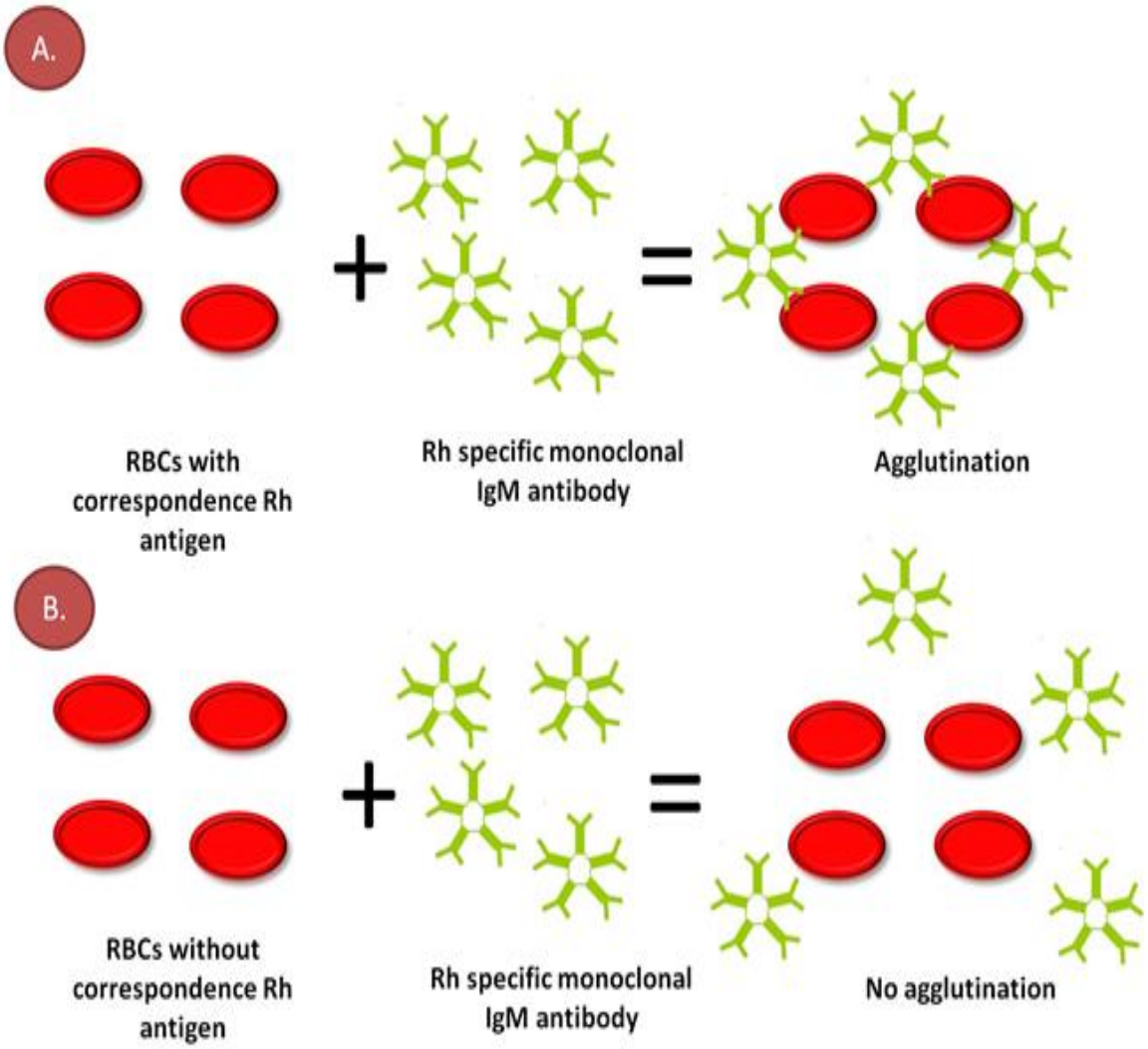


Figure 2.1 Rh IgM monoclonal antibody agglutination reaction pattern.

RBCs with correspondence Rh antigen will react with Rh specific monoclonal IgM antibody and cause agglutination (A), whereas no reaction occur in the absence of correspondence antigen and thus no agglutination (B).

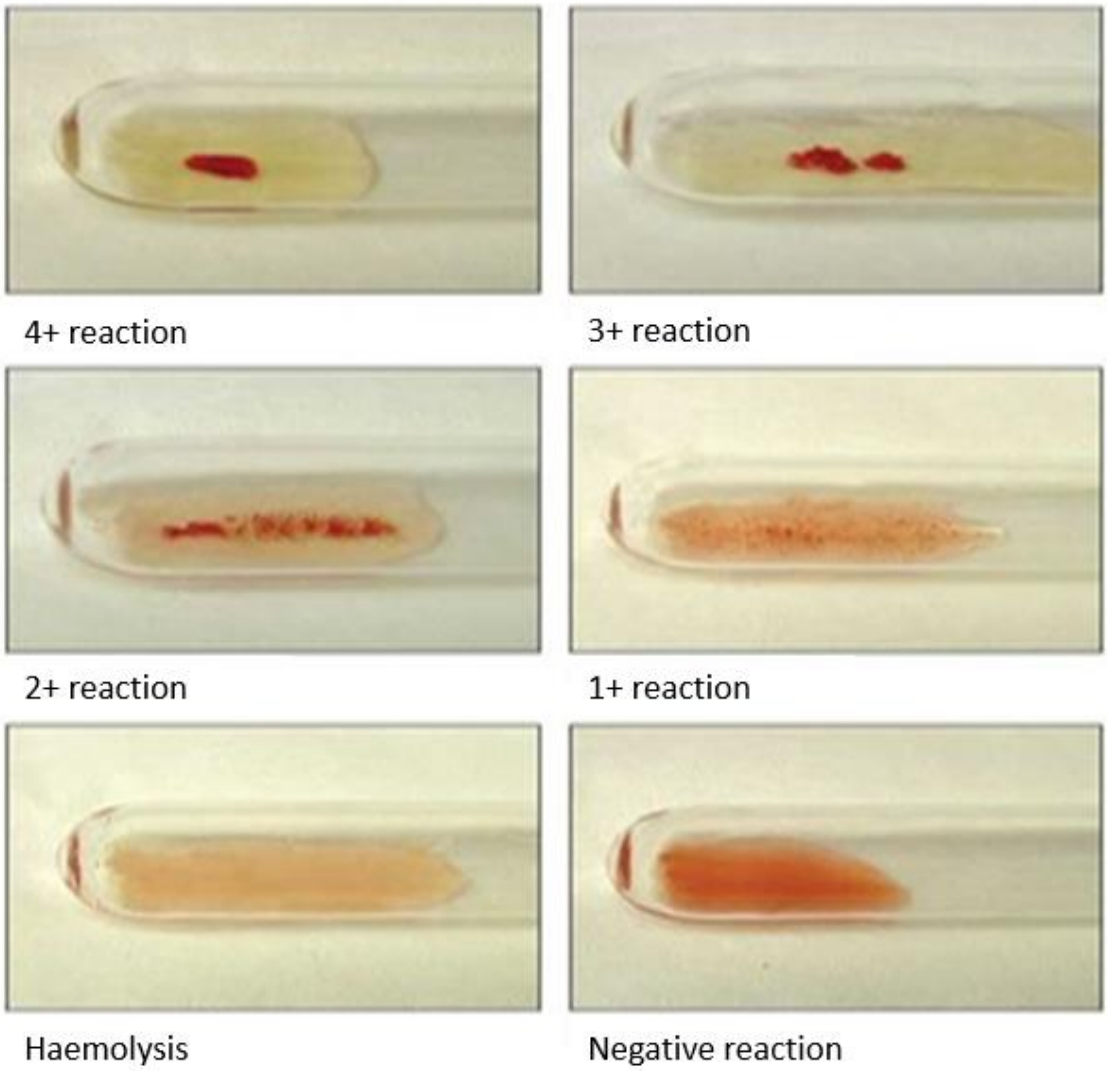


Figure 2.2 Agglutination grading pattern in tube method.

A +4 grading reaction is given for one solid clump. A clear background with few large clumps is graded. A cloudy background with multiple small clumps is graded as +2. A +1 is graded for very cloudy background with very small clumps. A negative reaction shows no clumps with very cloudy background. Adapted from (Harmening, 2012)

2.2.5 Indirect anti human globulin test (IAT)

Serological IAT tube method for the detection of weak D has been in practice in transfusion medicine by both developed and developing countries (Ouchari et al., 2015; Ogasawara et al., 2016; Khosroshahi et al., 2019). An indirect anti human globulin test (IAT) was performed on all negative reactions with anti-D to observe weak D. RBCs with corresponding antigen (D antigen) are sensitized with IgG antibody (anti-D) when incubated at 37^{o}C do not undergo agglutination. Antihuman globulin (AHG) reagent forms a bridge between the Fc portion of IgG antibodies that coat RBCs and cause visible agglutination indicating positive result (Weak D) (Figure 2.3). Direct anti-human globulin test (DAT) was performed as a control to rule out any false positive reaction.

Negative anti-D reactions were incubated at 37^{o}C for 45 minutes and then washed 3 times with isotonic saline. The saline was decanted completely at third wash, one drop of AHG reagent (Atlas Medical Cambridge, UK) was added and centrifuged at 1000 r.p.m for one minute to observe any agglutination. As washing efficiency control, all samples that showed no agglutination were tested with one drop coombs control cells (Lorne Laboratories Danehill, UK) and centrifuged at 1000 r.p.m for one minute to observe agglutination. Procedure control was run using anti-D IgG antibodies with control DiaCell I, II and III (BioRad Cressier FR, Switzerland).

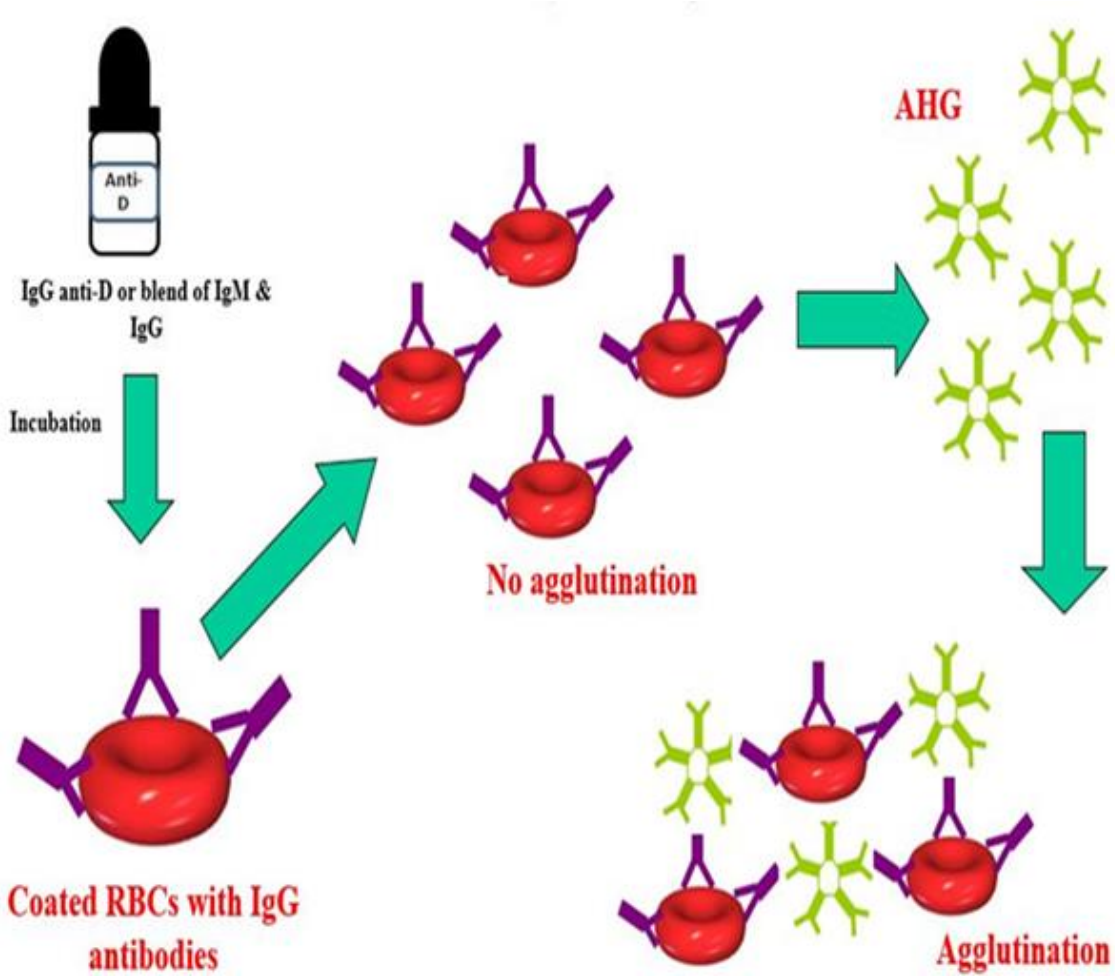


Figure 2.3 Principle of weak D detection by IAT method.

Anti-D IgG antibodies coat the RBCs that have corresponding D antigen. IgG is too small to bring RBCs together and cause agglutination. AHG form as a bridge and bind to IgG antibodies that are already bound to RBCs and cause agglutination.

2.2.6 Immunohaematological ID card method for RhD & RhCE phenotyping

The gel card technique has been shown to be more sensitive than tube method in different transfusion tests (Novaretti et al., 2003; Novaretti et al., 2004; Finck et al., 2013). Recent studies also used this technique to report Rh prevalence and frequencies among different ethnic groups (Neeraj et al., 2015; Zahid et al., 2016; Swelem et al., 2018). Each column contains dextran acrylamide gel matrix, which acts as sieve and suspended in buffer solution. The gel is premixed with Rh antibodies (each column with specific Rh antibody as shown in Figure 2.4). Reaction between correspondence Rh antigen and specific Rh antibody on the column causes agglutination, which indicates the presence of Rh antigen. Large agglutinates remain on the top of gel interference, whereas smaller agglutinates pass partway through the gel, depending on the size. Un-agglutinated RBCs pass to the bass of the column to form a button (Figure 2.5).

For Rh phenotyping, 5% RBCs suspension was prepared in ID-Diluent 1 (BioRad Cressier FR, Switzerland) according to the manufacturer's instructions. ID-Diluent 1 (0.5 ml) was dispensed in a tube and mixed with 50 µl of EDTA whole blood sample. The mixture was incubated for 10 minutes at room temperature and used within 15 minutes. RBCs suspension (12 µl) was added to all microtubes on ID-Card Rh+ phenotype (BioRad Cressier FR, Switzerland). The ID-Card was centrifuged for 10 minutes in the ID-Centrifuge. The agglutination was recorded in each microtube as per the criteria shown in (Figure 2.6).

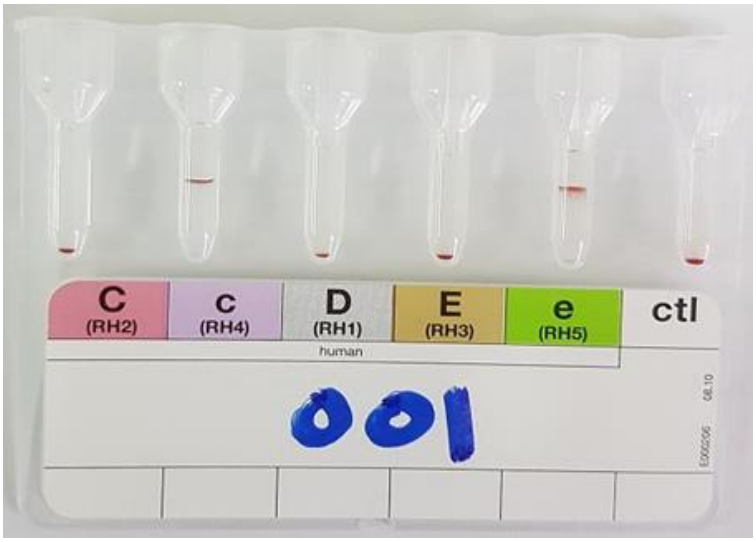


Figure 2.4 BioRad ID-Card gel.

Each column has specific Rh antibody on the top of the matrix. Rh specific antibodies on the top of the matrix react with Rh corresponding antigen on RBCs and cause agglutination (+) that remain on the top of the gel interference matrix (c and e). Un-agglutinated (-) RBCs pass through the matrix towards the bottom C, D and E).

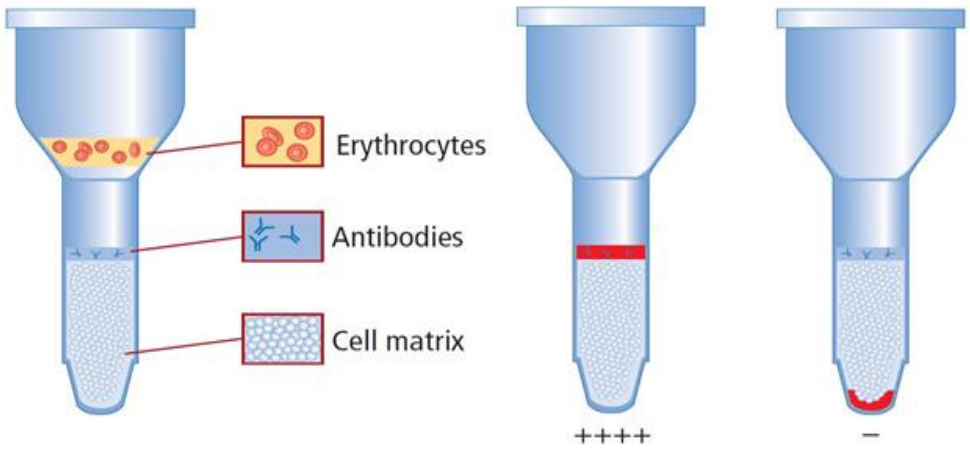


Figure 2.5 ID-card gel technique principle for Rh phenotyping.

Rh specific antibodies react with Rh corresponding antigen and cause agglutination (+) that remain on the top of the gel interference matrix. Un-agglutinated (-) RBCs pass through the matrix towards the bottom. adapted from (Daniels and Bromilow, 2013)

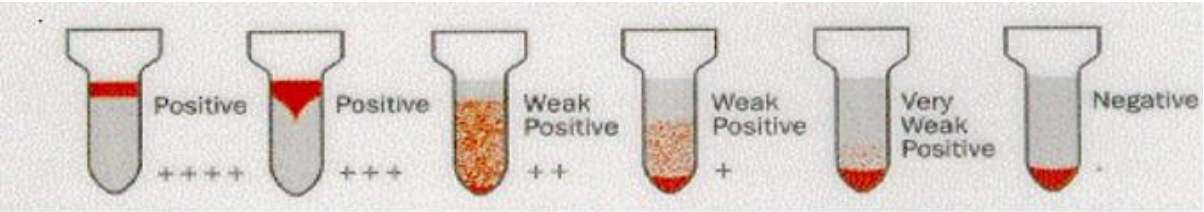


Figure 2.6 Agglutination grading pattern in ID-Card gel.

A 4+ grading is given to solid band of red cells at top of the gel. A 3+ grading is designated when most of red cell band at the top but started to disperse into the gel media. A designation of 2+ is given when agglutinated cells disperse along into the gel media and 1+ designated for most of the cells at the lower half of the gel. A reaction is called weak when two layers, agglutinate cells at the top of the gel matrix with un-agglutinated cells at the bottom as a button. A negative reaction is given when all cells pass through the matrix and form a button at the bottom of the microtube. Adapted from (Nicol, 1998)

The 'No-wash' ID-System (gel column) for the IAT incorporates the AHG within the gel matrix. Sensitised cells react with the AHG on centrifugation, leaving the liquid reactants, including any unbound globulins in the reaction chamber. Cells free from IgG are centrifuged to the bottom of the microtube. No control of negative results was necessary as no washing is required in this technique.

For IAT weak D testing method, suspension of 0.8% RBCs with BioRad ID-Diluent 2 was prepared and 50 µl of the suspension was added in BioRad ID-Card Coombs rabbit Anti-IgG microtube. A total of 50 µl Atlas Medical anti-D IgG/IgM blend (UK) was added to the microtube, incubated for 15 minutes at 37^{o}C and the results were recorded after 10 minutes centrifugation using ID-centrifuge. To avoid any false positive, DAT control on the sample was performed by adding 50 µl of 0.8% RBCs suspension to the BioRad ID-Card Coombs rabbit Anti-IgG microtube, centrifuging the card in ID-centrifuge for 10 minutes and recording any agglutination. A positive weak sample was excluded from the study.

2.2.7 Primer selection, design and bioinformatics

RHD specific primers (IDT Leuven, Belgium) were adapted from different studies (Table 2.1 and Table 2.2). The *RHD* coding sequence were the target for the primer design at first instance to amplify the whole *RHD* exons or to identify the presence of the exons. Normal *RHD* & *RHCE* gene nucleotides sequence were selected from DNA reference number NCBI (NC_000001.11) and Ensembl (ENSG00000187010) websites and the coding sequence of 1254 nucleotides long was identified as follows starting from exon 1 through exon 10 (Black highlight indicates exons and blue highlighting indicates alternating exons):

ATGAGCTCTAAGTACCCGCGGTCTGTCCGGCGCTGCCTGCCCCTCTGGGCCCTAA
CACTGGAAGCAGCTCTCATTCTCCTCTTCTATTTTTTTACCCACTATGACGCTTCC
TTAGAGGATCAAAAGGGGCTCGTGGCATCCTATCAAGTTGGCCAAGATCTGACC
GTGATGGCGGCCATTGGCTTGGGCTTCCTCACCTCGAGTTTCCGGAGACACAGCT
GGAGCAGTGTGGCCTTCAACCTCTTCATGCTGGCGCTTGGTGTGCAGTGGGCAAT
CCTGCTGGACGGCTTCCTGAGCCAGTTCCCTTCTGGGAAGGTGGTCATCACACTG
TTCAGTATTCGGCTGGCCACCATGAGTGCTTTGTCGGTGCTGATCTCAGTGGATG
CTGTCTTGGGGAAGGTCAACTTGGCGCAGTTGGTGGTGATGGTGCTGGTGGAGGT
GACAGCTTTAGGCAACCTGAGGATGGTCATCAGTAATATCTTCAACACAGACTAC
CACATGAACATGATGCACATCTACGTGTTCGCAGCCTATTTTGGGCTGTCTGTGG
CCTGGTGCCTGCCAAAGCCTCTACCCGAGGGAACGGAGGATAAAGATCAGACAG
CAACGATACCCAGTTTGTCTGCCATGCTGGGCGCCCTCTTCTTGTGGATGTTCTGG
CCAAGTTTCAACTCTGCTCTGCTGAGAAGTCCAATCGAAAGGAAGAATGCCGTGT
TCAACACCTACTATGCTGTAGCAGTCAGCGTGGTGACAGCCATCTCAGGGTCATC
CTTGGCTCACCCCCAAGGGAAGATCAGCAAGACTTATGTGCACAGTGCGGTGTT
GGCAGGAGGCGTGGCTGTGGGTACCTCGTGTCACCTGATCCCTTCTCCGTGGCTT
GCCATGGTGCTGGGTCTTGTGGCTGGGCTGATCTCCGTCGGGGGAGCCAAGTACC
TGCCGGGGTGTTGTAACCGAGTGCTGGGGATTCCCCACAGCTCCATCATGGGCTA
CAACTTCAGCTTGCTGGGTCTGCTTGGAGAGATCATCTACATTGTGCTGCTGGTG
CTTGATACCGTCGGAGCCGGCAATGGCATGATTGGCTTCCAGGTCCTCCTCAGCA
TTGGGGAACTCAGCTTGGCCATCGTGATAGCTCTCATGTCTGGTCTCCTGACAGG
TTTGCTCCTAAATCTTAAAATATGGAAAGCACCTCATGAGGCTAAATATTTTGAT
GACCAAGTTTTCTGGAAGTTTCCTCATTTGGCTGTTGGATTTTAA

Primers for *RHD* exon 2 and forward primer for *RHD* exon 10 was designed in house. For primers design, specific nucleotides area on *RHD* gene (May include 5' UTR, part of intron or 3' UTR) was selected and processed in NCBI primer blast online software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to get the primer pair. The region of each primers pair was selected so the amplicon sequence covers *RHD* exon specific coding

sequence above. Multiple Sequence Alignment using Clustal Omega online software (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for alignment of primers with *RHD* and *RHCE* specific regions. Selection priority was given to those primers that showed 100% specificity for *RHD* region and differ at least at 3' region with selected homologues *RHCE* region. NCBI Primer BLAST was used to identify specificity of the designed primers. The amplicon sequence was identified using UCSC genome browser – InSilico-PCR software (http://genome.ucsc.edu/cgi-bin/hgPcr) (Figure 2.7).

NCBI

```
Primer pair 1
Sequence (5'->3')       Length  Tm      GC%     Self complementarity    Self 3' complementarity
Forward primer  TGGGCTTCCTCACCTCGTG     19      61.58   63.16   3.00    3.00
Reverse primer  CTCTATGACCCAGAAGTGAT    20      53.55   45.00   3.00    2.00
Products on target templates
>NC_000001.11 Homo sapiens chromosome 1, GRCh38.p12 Primary Assembly

product length = 190
Features associated with this product:
   blood group Rh(D) polypeptide isoform 1

   blood group Rh(D) polypeptide isoform 2

Forward primer  1         TGGGCTTCCTCACCTCGTG  19
Template        25284609  ..................A.  25284627

Reverse primer  1         CTCTATGACCCAGAAGTGAT  20
Template        25284798  ....................  25284779
```

UCSC genome browser

```
>chr1:25284609+25284798 190bp TGGGCTTCCTCACCTCGAG CTCTATGACCCAGAAGTGAT
TGGGCTTCCTCACCTCGAGtttccggagacacagctggagcagtgtggcc
ttcaacctcttcatgctggcgcttggtgtgcagtgggcaatcctgctgga
cggcttcctgagccagttcccttctgggaaggtggtcatcacactgttca
ggtattgggatggtggctggATCACTTCTGGGTCATAGAG
```

Figure 2.7 NCBI Primer blast and UCSC genome browser result for RHD exon 2.

NCBI Primer BLAST showed the primer pair is specific for *RHD* gene. UCSC genome browser showed the nucleotides sequence covered by primer pair. Yellow highlight indicates forward primer, whereas blue highlight indicates reverse primer. Capital letters indicates place of primers, small letters indicate the nucleotide sequence covered by primer pair.

Table 2.1 Primers used to assess the presence of *RHD* specific exons.

Exon	Primer name	Direction	specificity	Position*	Sequence (5′ to 3′)	Reference
1	re01	F	D	Promoter -149 to -132	atagagaggccagcacaa	(Singleton et al., 2000)
	RHIN1R	R	D + CE	Intron 1 84 to 64	tctgtgcccctggagaaccac	
2	ME2F	F	D	Exon 2 185 to 203	TGGGCTTCCTCACCTCGTG	In-house
	ME2R	R	D	Intron 2 39 to 28	ctctatgacccagaagtgat	
3	ga31	F	D	Exon 3 362 to 383	TTGTCGGTGCTGATCTCAGTGGA	(Wagner, Frohmajer and Flegel, 2001)
	rb21	R	D + CE	Intron 3 28 to 11	aggtccctcctccagcac	
4	ga41	F	D	Exon 4 503 to 527	ACATGATGCACATCTACGTGTTCGC	(Wagner, Frohmajer and Flegel, 2001)
	ga42	R	D	Exon 4 625 to 602	CAGACAAACTGGGTATCGTTGCTG	

F: Forward primer **R:** Reverse primer

Capital letters represent *RHD/RHCE* exonic region whereas as small letters represent non-coding sequence

Table 2.1 Primers used to assess the presence of *RHD* specific exons (contd..)

Exon	Primer name	Direction	specificity	Position	Sequence (5′ to 3′)	Reference
5	R648	F	D	Exon 5 648 to 667	GTGGATGTTCTGGCCAAGTT	(Maaskant-van Wijk et al., 1998)
	Rex5AD2	R	D	Intron 5/exon 5 3 to 787	cacCTTGCTGATCTTACC	
6	ga62	F	D + CE	Exon 6 804 to 826	TTATGTGCACAGTGCGGTGTTGG	(Wagner, Frohmajer and Flegel, 2001)
	ga61	R	D	Exon 6 936 to 916	CAGGTACTTGGCTCCCCCGAC	
7	R973	F	D	Exon 7 973 to 992	AGCTCCATCATGGGCTACAA	(Maaskant-van Wijk et al., 1998)
	R1068	R	D	Exon 7 1068 to 1048	ATTGCCGGCTCCGACGGTATC	
9	re83	F	D + CE	Intron 8 -54 to - 34	gagattaaaaatcctgtgctcca	(Wagner, Frohmajer and Flegel, 2001)
	re94	R	D	Exon 9 1216 to 1193	CTTGGTCATCAAAATATTTAGCCT	
10	MDF10	F	D + CE	Intron 9 -77 to -54	taggctgtttcaagagatcaagc	In house
	rr4	R	D	3′ UTR 1,541 to 1,522	agcttactggatgaccacca	(Wagner, Frohmajer and Flegel, 2001)

*The positions of the synthetic oligonucleotides are indicated relative to their distances from the first nucleotide position of the start codon ATG

F: Forward primer **R:** Reverse primer

Capital letters represent *RHD/RHCE* exonic region whereas as small letters represent non-coding sequence

Table 2.2. Primers used for identifying the presence of *RHD* intron 4 and exons 5, 7 and 10 using TaqMan chemistry

Exon/Intron	Primer name	Position	Sequence (5′ to 3′)	Reference
Intron 4	RHDIn4F	Intron 4 113 to 134	gcccttccatcatgattcattt	(Dovc-Drnovsek et al., 2013)
	RHDIn4R	Intron 4 244 to 223	acaaggaaacaaaggccaagag	
	RHDIn4M	Intron 4 172 to 188	FAM-aagcacttcacagagca-MGB	
Exon 5	RHD ex5F	Exon 5 636 to 654	CGCCCTCTTCTTGTGGATG	(Legler et al., 2007)
	RHD ex5R	Exon 5 717 to 697	GAACACGGCATTCTTCCTTTC	
	RHD ex5Q	Exon 5 656 to 683	VIC-TCTGGCCAAGTTTCAACTCTGCTCTGCT-QSY	
Exon 7	RHD 940S	Exon 7 940 to 960	GGGTGTTGTAACCGAGTGCTG	(Legler et al., 2007)
	RHD 1064R	Exon 7 1064 to 1046	CCGGCTCCGACGGTATC	
	RHD968T	Exon 7 968 to 992	FAM-CCCACAGCTCCATCATGGGCTACAA-TAMRA	
Exon 10	RHDex10F	3'UTR 1314 to 1334	tgcctgcatttgtacgtgaga	(Dovc-Drnovsek et al., 2013)
	RHDex10R	3'UTR 1372 to 1355	cctgcgcgaacattgga	
	RHDex10M	3'UTR 1337 to 1352	FAM-acgctcatgacagcaa-MGB	

2.2.8 DNA extraction

Different biological samples stored on FTA cards such as blood (Wong, Lim and Tan-Siew, 2012; Lipic, Giordullo and Fredericks, 2018) and urine (Srisiri et al., 2017) have been used in clinical research studies for DNA amplification and found to be efficient. Most studies using blood on FTA card, targeted blood parasites and viruses (Nzelu et al., 2016; Qin et al., 2016). Yet, *RHD* genotyping on blood samples extracted from FTA card have not been studied hence; this is the first report to our knowledge.

DNA was extracted from FTA card using QIAamp® DNA Investigator reagent (Qiagen Manchester, UK) which is silica-based membrane extraction method using a spin column. The method is based on cell lysis with lysis buffer to disrupt the cell membrane and protinase K to digest proteins such as nuclease. The lysate combined with alcohol and DNA binds to the silica based membrane. Multiple buffer washing and centrifugation steps ensure removal of proteins and positively charged ions, including magnesium. Pure concentrated DNA is eluted from the membrane by ATE (Tris-EDTA) elution buffer (Figure 2.8).

A 3 mm punch from dry blood on FTA card was placed in 1.5 ml microcentrifuge tube. Sample pre-treated with 280 µl of buffer ATL and 20 µl of protinase K. Following vortexing, samples were incubated at 56^{o}C for an hour with 10 seconds vortexing every 10 minutes. Following brief centrifugation, 300 µl of buffer AL was added, vortexed for 10 seconds and incubated at 70^{o}C for 10 minutes with 10 seconds vortexing every 3 minutes. After brief centrifugation, 150 µl of 100% ethanol was added, mixed by vortexing for 15 seconds and centrifuged briefly. The lysate was transferred to QIAamp MinElute column , centrifuged at 8000 r.p.m for 1 minute and collection tube containing flow-through was discarded. Similar centrifuging and discarding process were repeated in each of the following steps after adding 500 µl AW1 buffer, 700 µl AW2 and 700 µl of 100% ethanol. To dry the membrane completely, tubes were centrifuged at 14000 r.p.m for 3 minutes. The QIAamp

MinElute column were placed in a 1.5 microcentrifuge tube and incubated at room temperature for 10 minutes. ATE elution buffer 50 µl was applied at the centre of the membrane and incubated at room temperature for 5 minutes followed by centrifuging at 14000 r.p.m for 1 minute.

Stangegaard and colleagues were able to extract DNA from dry blood spot on FTA card for up to six additional DNA extractions using the same initial sample (Stangegaard et al., 2013). We adopted the same idea to test representative samples (n = 5) for multiple extraction for up to four times using the same initial sample. QIAamp® DNA Investigator kit was used for subsequent extraction of DNA from the initial sample as described above.

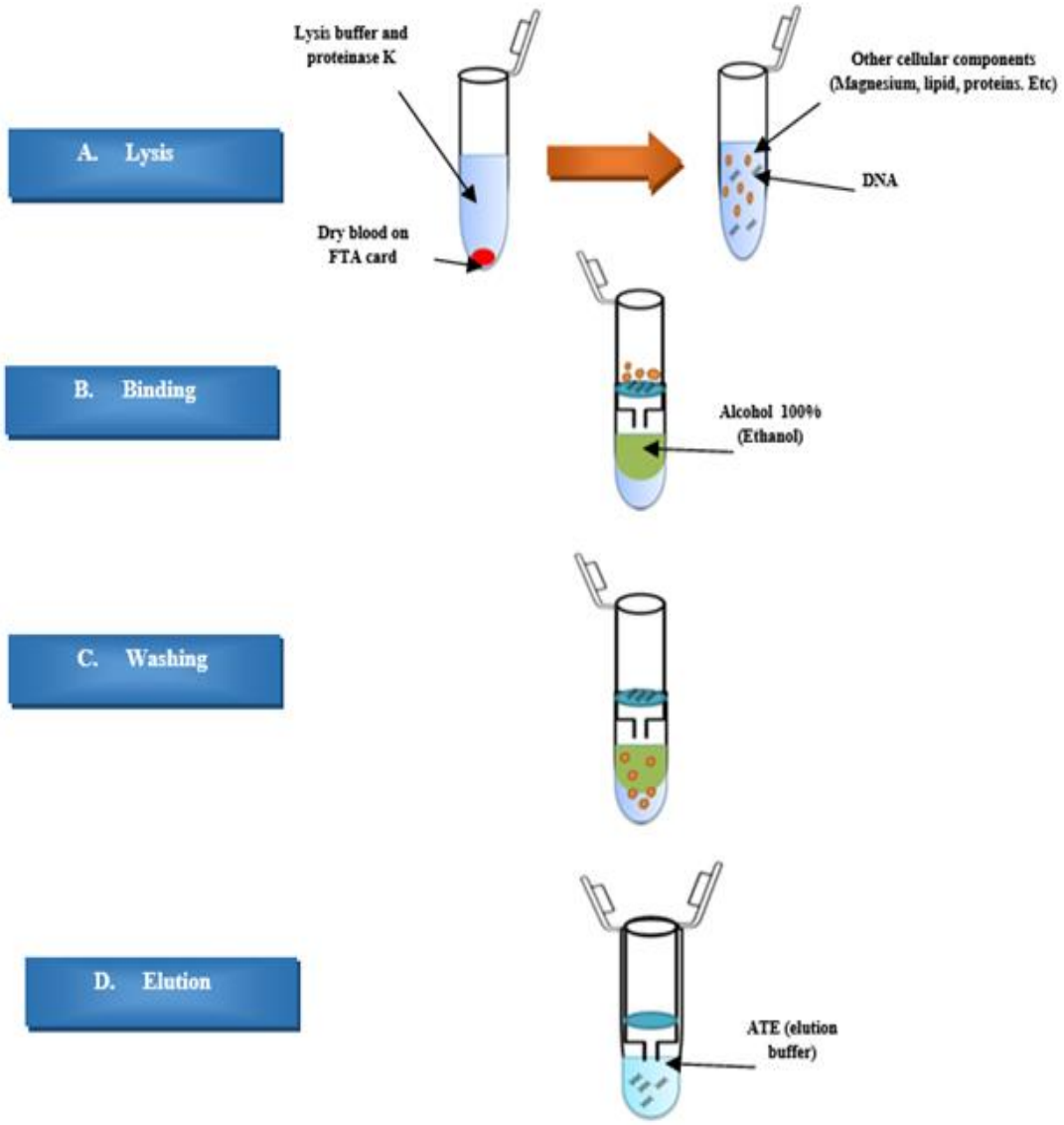


Figure 2.8 Principle of DNA extractions from FTA card using Qiagen investigator kit.

The sample exposed to lysis buffer and proteinase K which causes lysis of the cellular and organelle membrane releasing their contents e.g DNA, magnesium, lipid, proteins..etc (A). Addition of alcohol leads to binding of DNA to the silica based membrane (B). Multiple buffer washing ensures removal of proteins and other cellular components but not DNA which remain bound on the silica membrane (C). DNA is eluted by ATE buffer as it dissolves in water (D).

2.2.9 DNA purity and estimation of DNA concentration and yield

FTA cards are a convenient solution for collecting low volume blood samples such as those from neonates and for long term storage at room temperatures for archival purposes and extended periods. Stangegaard and colleagues studied DNA purity and yield from dry blood spot on FTA for forensic genetic analysis and proved to be possible to extract DNA from these cards in sufficient amount and quality to obtain complete short tandem repeat (STR) using different automated extraction protocols such as QIAcube, QIAsymphony SP and PrepFiler Express kit. The study had also proved that following initial sample pretreatment and DNA extraction, the processed FTA card pieces were useful to up to six additional DNA extractions (Stangegaard et al., 2013). As stated in section 2.2.8 on DNA extraction, for *RHD* genotyping DNA was extracted from dry blood spot on FTA cards using QIAamp® DNA Investigator kit.

The most common technique to determine DNA yield and purity is measurement of absorbance. DNA concentration can be estimated using spectrophotometer under ultraviolet (UV) light at 260 nm due to purine and pyrimidine bases that strongly absorb UV light. However, DNA is not the only molecule that can absorb UV light at 260 nm. Since RNA also has a great absorbance at 260 nm, and the aromatic amino acids present in protein absorb at 280 nm, both contaminants, if present in the DNA solution, will contribute to the total measurement at 260 nm. Additionally, the presence of guanidine will lead to higher 260 nm absorbance. This means that if the A260 number is used for calculation of yield, the DNA quantity may be overestimated.

To evaluate DNA purity, absorbance from 230 nm to 320nm to detect other possible contaminants is required. The most common purity calculation is the ratio of the absorbance at 260 nm divided by the reading at 280 nm. Good-quality DNA will have an A260/A280 ratio of 1.7–2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but

lower ratios indicate more contaminants are present. The ratio can be calculated after correcting for turbidity (absorbance at 340 nm).

Strong absorbance around 230 nm can indicate that organic compounds or chaotropic salts are present in the purified DNA. A ratio of 260 nm to 230 nm can help evaluate the level of salt carryover in the purified DNA. The lower the ratio, the greater the amount of salt is present, for example. As a guideline, the A260/A230 is best if greater than 1.5. A reading at 340 nm will indicate if there is turbidity in the solution, another indication of possible contamination. Therefore, taking a spectrum of readings from 230 nm to 340 nm is most informative.

Thermo Scientific™ NanoDrop 1000 was used to estimate DNA concentration and purity. Nucleic acid application was selected and the device optical surfaces were cleaned using DNA free water. The appropriate constant (dsDNA – DNA 50) for the sample was selected and blank measurement was performed using Qiagen ATE buffer from which the extracted DNA was eluted. Both optical surfaces were cleaned with a clean, dry, lint-free lab wipe. To determine DNA concentration, 1 µl of extracted DNA was dispensed onto the lower optical pedestal and the lever arm was closed. The nucleic acid concentration and purity ratios (260/280 and 260/230) were calculated automatically by the software and presented as data and spectral output. DNA yield was calculated by multiplying total sample volume by DNA concentration.

2.2.10 Molecular analysis of *RHD* gene by real-time PCR

During DNA amplification in real-time PCR, light passes through monochromator that transmits a mechanically selectable narrow band of wavelengths of light chosen from a wider range of wavelengths that is dependent upon fluorophore used. Upon excitation at the appropriate wavelength, the fluorophore (DNA binding dye or probe) on the sample with

DNA emits fluorescence that can be captured as data in photodetector every cycle and translated as real-time PCR graph (Arya et al., 2005; Valasek and Repa, 2005).

For SYBR Green chemistry, this DNA binding dye binds only to major groove double stranded DNA (dsDNA) but not single stranded (Rapley, 2010). SYBR Green does not emit fluorescence when it is in free form but fluorescence signal is emitted as it binds to dsDNA (Mirmajlessi and Loit, 2016) . During amplification detection in real-time, SYBR Green emits green light at 520 +/- 10 nm that can be detected by excitation at 470 +/- 10 nm.

For TaqMan chemistry, qPCR technique uses a probe as a fluorophore. Each TaqMan probe has a fluorophore molecule such as 6-carboxyfluorescein (6-FAM) or 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) and a quencher such as tetramethylrhodamine (TAMRA), QSY and Minor Groove Binding (MGB) but unable to florescent at this stage. As the DNA polymerase extend DNA from primer template it cleaves the probe bound to DNA template releasing the fluorophore that emits light at specific wavelength to be detected. Different fluorophores used in TaqMan probes have different maximum emission and excitation wavelength. Table 2.2 shows fluorophores used in real time PCR for current study. With a choice of many excitation sources and detection filters combined (up to 6 in Rotor-Gene® Q) with a short, fixed optical in thermocyclers, it is possible to detect DNA amplification in real-time.

Table 2.2 Absorbance, excitation and emission of different fluorescence dye used in real-time PCR in current study

Dye filter	Absorbance (nm)	Excitation (nm)	Emission (nm)
6-FAM	495	470	520
VIC	538	470	558
SYBR	495	470	630

Real-time PCR can also be used as qualitative indicator for desired DNA amplicon which is known as dissociation curve or melting curve analysis. Fluorescence intensity remains high in low temperature and as the temperature is increased, the dsDNA starts to dissociate decreasing the intensity (Figure 2.9A). The melting point temperature is calculated from the intensity change in a given interval from fluorescence derivative (df) in relation to temperature derivative (dt) (Figure 2.9B).

The real-time PCR was used to investigate the presence and mutation in *RHD* gene exons 1 through 10 using *RHD* specific and sequence-specific primers (SSP). The D positive sample was used as a control for the amplification of exons and melting curve analysis of PCR products.

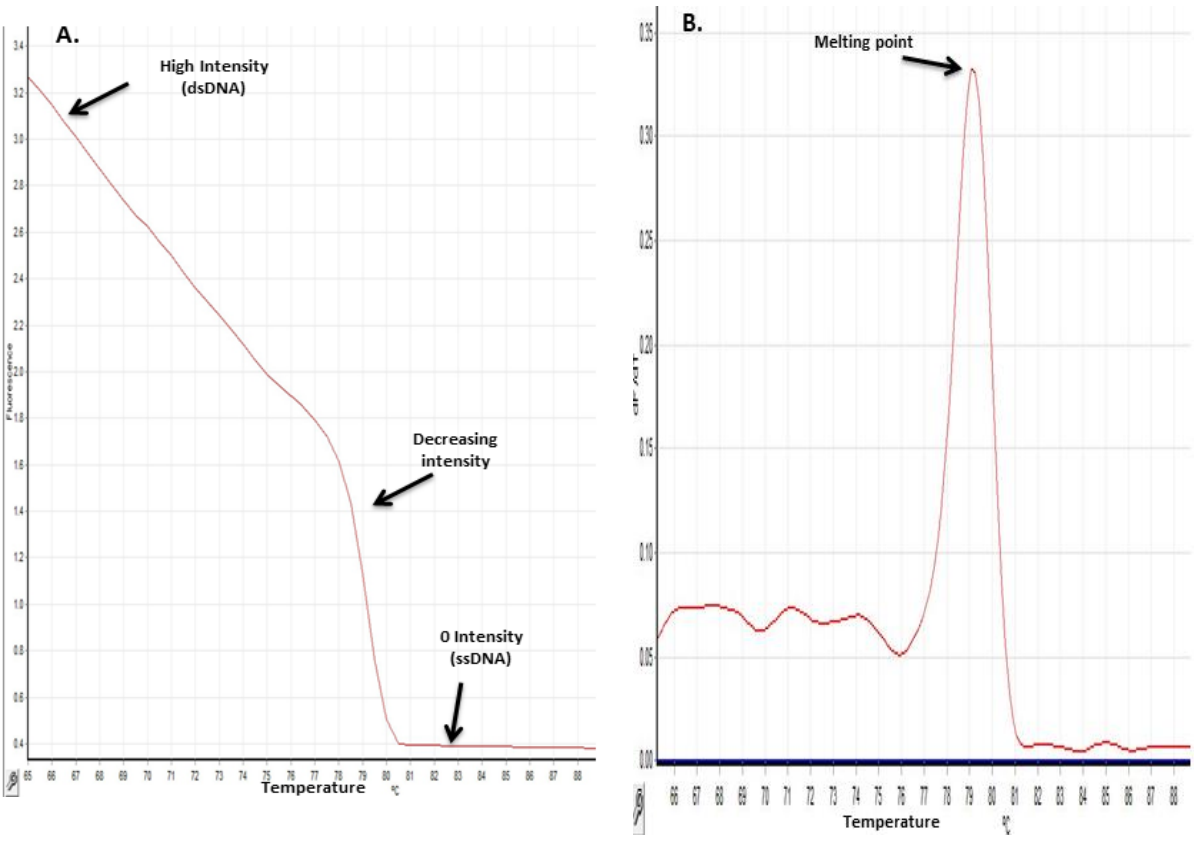


Figure 2.9 Dissociation and melting curve analysis in real-time PCR.

The fluorescence intensity remains high in dsDNA. As the temperature increases, dsDNA starts to dissociate decreasing the intensity until no more dsDNA at zero intensity represented as dissociation curve (A). The fluorescence intensity changes in a given interval for fluorescence derivative (df) in relation to temperature derivative. This change is calculated and appears as melt point peak represented as melting curve (B).

2.2.11 Screening for *RHD* deletion

All DNA samples were screened for the presence of *RHD* exon 10 to detect the deletion or presence of *RHD* gene by using a real-time PCR SYBR Green and TaqMan chemistry. For SYBR Green chemistry, MDF10 and rr4 and primers for *RHD* exon 10 were used, with HGHF and HGHR human growth hormone primers (IDT Leuven, Belgium) as positive controls. PCR was performed in a total reaction volume of 10 μL. All *RHD* exons amplifications were programmed on a thermal cycler (Rotor-Gene Q; Qiagen) unless stated otherwise and the samples ran in duplicates. The cycling conditions used were as follows: Hold at 95°C for 3 minutes and then 40 cycles of : 5 seconds at 95°C and 10 seconds at 60°C. All PCR amplifications were carried out in duplicate in 72 well reaction rotor. Melting curve analysis in all reactions was performed between 60 and 95°C with a temperature increment of 0.5°C/s unless stated otherwise. The melting curves were sketched using the instrument's data analysis software (Rotor-Gene Q Pure Detection, version 2.3.1; Qiagen) and evaluated visually. The PCR products size were visualised by electrophoresis on a 2% agarose gel and then sent to eurofins or GENEWIZ for Sanger sequencing.

For TaqMan chemistry, real-time PCR was performed from FTA extracted DNA with concentration between 20 and 30 ng, 400 nM of each primer and QuantiNova Probe Mater Mix (Qiagen, UK) in a total reaction volume of 10 μL. The cycling conditions used were as follows: denaturation at 95°C for 3 min and then 40 cycles of 5 seconds at 95°C and 15 seconds at 60°C acquired on green channel.

2.2.12 Further molecular characterisation of *RHD* exons

All samples positive for *RHD* exon 10 were further investigated for the presence of *RHD* exons 1 through 7 and 9 by real-time simplex PCR with SYBR Green chemistry. Amplification reactions were set up in a total volume of 10 µL using the QuantiNova SYBR Green PCR Master Mix (Qiagen). The *RHD* primers and their concentrations stated in Table 2.3. An amount between 20 and 30 ng of the extracted blood DNA was used for amplification.

RHD intron 4, exons 5 and 7 were also investigated by a real-time simplex PCR with TaqMan chemistry. *RHD* specific intron4/exon5/exon7 simplex PCR (10 µl reactions) consisted of QuantiNova Probe Master Mix (Qiagen), primers and probes previously described by Leglar et al. (2007) and Drnovšek et al. (2013) and 2 µl of template. Primer pairs and their concentration stated in Table 2.4. After initial incubation at 95°C for 3 min, 40 cycles of two-step cycling, denaturation at 95℃ for 5 seconds and annealing and extension at 60℃ for 15 seconds was performed. All reaction were acquired on green channel except exon 5, which acquired on yellow channel.

Table 2.3 Primers and their concentrations and cycling condition used to amplify *RHD* specific exons 1 through 9

***RHD* exon**	**Primer* pair**	**Primer conc (nM)**	**real-time PCR Cycling condition****		
			Denaturation	**Annealing**	**Extension[d]**
1	re01 and RHIN1R	500	95°C (5 s)[a]	60°C (15 s)	
2	ME2F and ME2R	100	95°C (5 s)[a]	60°C (15 s)	
3	ga31 and rb21	200	95°C (15 s)[b]	70°C (20 s)	72°C (30 s)
			95°C (15 s)[c]	60°C (20 s)	72°C (30 s)
4	ga41 and ga42	200	95°C (15 s)[b]	70°C (20 s)	72°C (30 s)
			95°C (15 s)[c]	60°C (20 s)	72°C (30 s)
5	R648 and Rex5AD2	400	95°C (5 s)[a]	60°C (15 s)	
6	ga62 and ga61	100	95°C (15 s)[b]	70°C (20 s)	72°C (30 s)
			95°C (15 s)**c**	60°C (20 s)	72°C (30 s)
7	R973 and R1068	400	95°C (5 s)[a]	60°C (15 s)	
8					
9	re83 and re94	400	95°C (15 s)[b]	70°C (20 s)	72°C (30 s)
			95°C (15 s)[c]	60°C (20 s)	72°C (30 s)

[a] 40 cycles [b] Touch-down 10 cycles with 1°C decrease in annealing temperature in each cycle [C] 30 cycles [d] Acquired on green channel s: seconds

*Prior to the cycling, there was a hold step at 95oC for 3 min

**The primer concentrations are final concentrations, not stock solutions

Table 2.4 Primers for *RHD* TaqMan real-time PCR used for further molecular characterization of *RHD* gene in Omani population

Primer / Probe	Label	RHD region	Primer / Probe concentration (nM)
RHDIn4F	None	Intron 4	800
RHDIn4F	None	Intron 4	800
RHDIn4M	FAM-MGB[d]	Intron 4	300
RHD ex5F	None	Exon 5	200
RHD ex5R	None	Exon 5	200
RHD ex5Q	VIC-QSY[e]	Exon 5	50
RHD 940S	None	Exon 7	500
RHD 1064R	None	Exon 7	500
RHD968T	FAM-TAMRA[d]	Exon 7	250
RHDex10F	None	Exon 10	800
RHDex10R	Note	Exon 10	800
RHDex10M	FAM-MGB[d]	Exon 10	300

[d] Acquired on green channel [e] Acquired on yellow channel

2.2.13 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to check that real-time PCR product gave the desired size of *RHD* specific exon amplicon and to relate that with melting temperature (T_m) curve. Agarose gel 2–2.5% (w/v) from (Sigma-Aldrich, USA) was prepared as follows: for each 100 ml, 2 to 2.5 g of agarose was added to 100 ml 1xTBE buffer (Lonza, Belgium) and microwaved at medium heat for 3-4 minutes until the agarose dissolved. The solution was cooled down till 60°C, poured into UV-transparent gel tray with inserted comb and allowed to solidify for 30 minutes. The gel was then placed into the tank filled with 1xTBE buffer. FastRuler™ Low Range DNA Ladder (ThermoFisher, UK) was loaded in the first lane as a marker (50-1500 bp). The marker or sample was loaded by adding 4 µl of the DNA and 1 µl of Novel Juice DNA stain (Sigma-Aldrich, USA). The electrophoresis was run at 80V for 45 minutes. The gel was viewed with BioRad ChemiDoc™ MP, Imaging analyser under ultra violet light (ethidium bromide channel).

2.2.14 *RHD* exons sequencing

As this is the first *RHD* molecular study on Omani population, PCR amplicons for exons 1 through 10 were sequenced to find out possible novel mutation(s) in *RHD* gene coding sequence. It also confirmed the specificity of the selected primers to the *RHD* gene specific exon and not *RHCE* gene exons. Non-purified PCR products were sent to Eurofins and GENEWIZ for Sanger sequencing using primers stated in Table 2.1. Both forward and reverse primers (5 µM) and DNA templates (20 µl) were submitted in a separate 1.5 mL locked Eppendorf tubes. DNA sequencing data were analysed using Chromas (version 2.6) software (Technelysium Ply Ltd). NCBI blast was used to perform sequence alignment between each primer pair sequencing result and human reference gene as shown in Figure

2.10. Mutation within the coding sequence was reconfirmed with both primers (forward and reverse). Sequencing result of each primer then aligned using online Clustal Omega software to reconfirm the mutation location as shown in Figure 2.11.

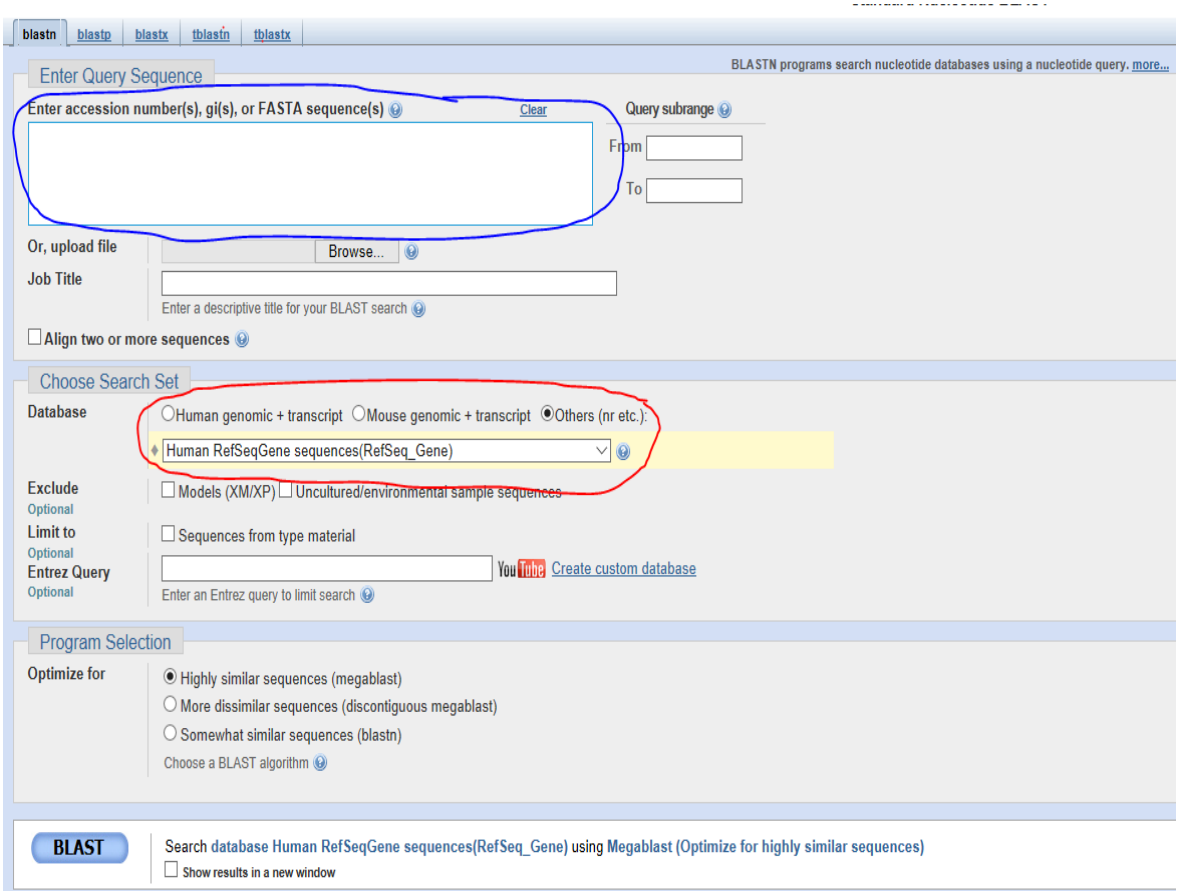


Figure 2.10 NCBI BLAST method used to compare Sanger sequencing results with reference gene.

DNA sequence result was entered in the box (highlighted blue) and the database was selected as Human RefSeqGene sequences (RefSeq_Gene) highlighted red. The results of the sequence was then compared with the reference sequence gene after clicking on blast.

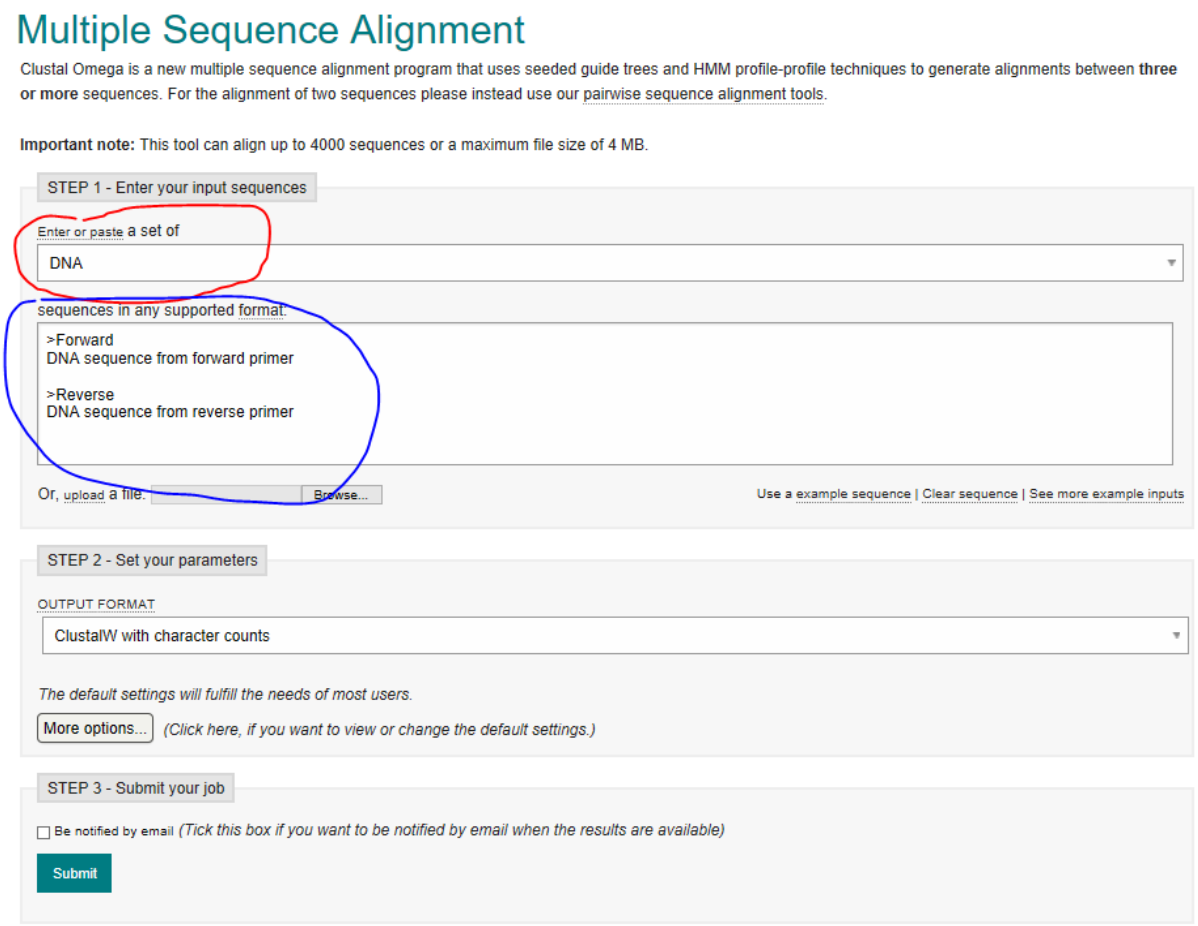


Figure 2.11 *RHD* exon DNA sequence alignment of forward and reverse primers

Type of nucleic acid is selected (Red highlight), any sequence to be analysed was labelled with symbol > and name (Blue highlight) and sequence was added in the box (Blue highlight). The data was analysed after clicking on submit button.

2.2.15 Molecular analysis of *RHD* gene commercial BAGene SSP kit

The BAGene kits are used to determine blood group specificities of donors, recipients and pregnant women on a molecular genetic basis. A study conducted by Bakanary and colleagues using BAGene RH-TYPE kit revealed the importance of genotyping in transfusion management of chronically transfused patients as the classical hemagglutination assays may be inaccurate (Bakanay et al., 2013). We used Rh-TYPE and partial D-TYPE type kits (BAGene,BAG Health Care, Lich,Germany) to determine different *RHD* alleles in D negative Omani population and reconfirm the presence of *RHD* exons detected by real-time PCR stated in section 2.2.12. The kit also reconfirms the serological RhCE phenotyping by molecular method to detect *C,c*, *E* and *e* alleles. Extracted DNA from dried blood on FTA card was used as a template.

The test procedure is performed by using the Sequence Specific Primers (SSP)-PCR (Figure 2.12). This method is based on the fact that primer extension, and hence successful PCR, relies on an exact match at the 3'-end of both primers. As a result, the amplification is obtained only if the primers entirely match the target sequence. The kits consist of PCR plates with chambers with pre-aliquoted and dried reaction mixes containing allele-specific primers, internal control primers and deoxyribonucleotide triphosphates (dNTPs). The master mix for RH-TYPE and partial D-TYPE was prepared according to manufacturer instructions (Table 2.5) with 10x PCR buffer (included in the kit), DNA template concentration between 20 and 30 ng, Happy Taq Polymerase (BAGene,BAG Health Care, Lich,Germany) and ddH2O.

BioRad C1000 Touch™ thermal cycler was used for the assay with the following cycling condition: denaturation at 96°C for 5 min; 5 cycles of denaturation at 96°C for 10 seconds and annealing and extension at 70°C for 60 seconds; 10 cycles of denaturation at 96°C for 10 seconds, annealing for 50 seconds at 65°C and extension for 45 seconds at 72°C; 25 cycles of denaturation at 96°C for 10 seconds, annealing for 50 seconds at 61°C and

extension at 72°C for 45 seconds and final extension at 72°C for 5 minutes. The product of the amplification is visualized by 2.5% (w/v) agarose gel electrophoresis as describe in section 2.2.13.

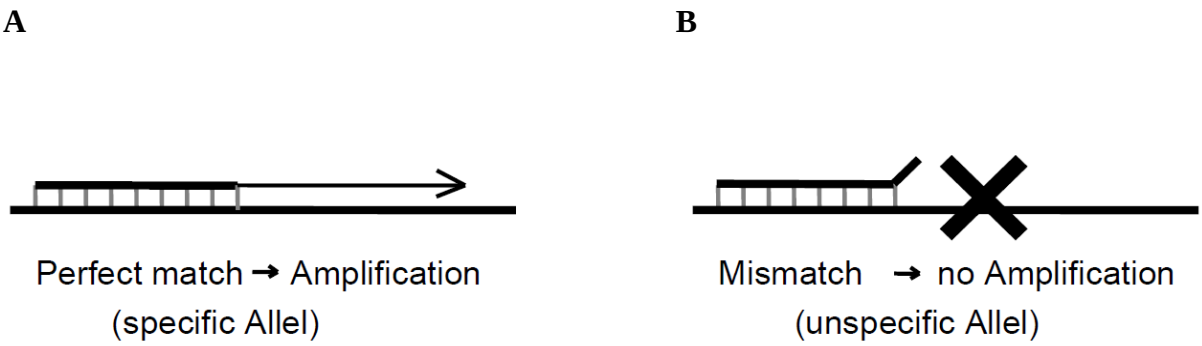


Figure 2.12 Principle of the BAGene commercial diagnostic assay for RH-TYPE and partial D-TYPE.

A perfect match with Sequence specific primers leads to primer extension and thus amplification (A), whereas, mismatch prevents extension and thus amplification (B).

Table 2.5 Master Mix preparation to run BAGene RH-TYPE and partial D-TYPE in-vitro test

BAGene SSP Kit	Number of (mixes)	ddH_2O (µl)	10x PCR (µl)	DNA template	Happy Taq 5 U/µl (µl)	Total volume (µl)*
RH-TYPE	13	123.6	15.6	15.6	1.3	156
Partial D-TYPE	15	142.6	18	18	1.4	180

*For one sample

2.2.16 Molecular analysis of *RHD* gene by QuantStudio™ 3D digital PCR

Digital PCR builds on the same principle of the qPCR enabling an absolute value with much higher precision compared to standard PCR. It starts with the partition of reaction mix into thousands of independent PCR replicates. Some partitions will include the PCR template and others do not. Partitions that include DNA template will amplify the DNA at detectible level that can be directly proportional to the total number of molecules enabling an absolute count to be established (Hudecova, 2015). The reactions are run on integrated fluidic circuits (chips). These chips have integrated chambers and valves for partitioning samples and reaction reagents. Following identification of positive and negative wells a Poisson model is used to calculate the probability of a given reaction receiving 0, 1, 2 or more copies. This correction factor enables all molecules to be counted yielding to an absolute value.

2.2.16.1 *RHD* exons 5, 7 and 10 detection using SYBR Green I dye

TaqMan® assays are recommended by Applied Biosystems® as a proven and validated chemistry for digital PCR, with over 8 million assays available. Since SYBR Green dye

based detection is widely used for target quantification in traditional real-time PCR, they have developed a protocol for using SYBR Green I dye for target quantification on the QuantStudio™ 3D system. This protocol is meant as a starting point for optimization with primer sets that were previously validated for SYBR Green dye based detection.

SYBR Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO (Applied Biosystems®, UK) was diluted with 100% DMSO (Applied Biosystems®, UK) to make 1000X stock solution aliquots. For digital PCR work, stock solution was further diluted to 20X with TE buffer, pH 8.0 (Qiagen, UK). Previously validated real-time PCR forward and reverse primers for *RHD* exons 5 (R648 and Rex5AD2), *RHD* exon 7 (R973 and R1068) and *RHD* exon 10 (MDF10 and rr4) were used with same concentration as indicated in table 2.1. Amplification reactions were set up in a total volume of 14.5 µL using the QuantStudio™ 3D Digital PCR Master Mix (Applied Biosystems®, UK) as the setup shown in Table 2.6. The reaction mix (14.5 µL) was loaded onto the QuantStudio™ 3D Digital PCR chip using the chip loader, and ran using the standard thermal cycling conditions recommended by the manufacturer (Table 2.7). For the optimization of annealing temperature, temperature gradients 56 °C, 58 °C and 60 °C were used.

Table 2.6 Master mix preparation for QuantStudio™ 3D Digital PCR using SYBR Green I dye

Material	Volume per chip (µl)	Final concentration
QuantStudio™ 3D Digital PCR Master Mix	7.25	1X
20X SYBR Green I dye in TE buffer, pH 8	1.45	2X
Forward and reverse primer	Variable	Variable*
DNA template	Variable	20-30 ng
ddH_2O	Bring volume up to 14.5	

*Primer concentration used for specific *RHD* exon in real-time PCR

Table 2.7 Thermal cycler condition used to amplify *RHD* exons 5, 7 and 10 for QuantStudio™ 3D Digital PCR using SYBR Green I dye

	PCR protocol					Cover Temperature
	Stage 1	Stage 2		Stage 3		
Temperature	96ºC	60ºC*	98ºC	60ºC*	10ºC	
Time	10 min	2 min	30 sec	2 min	∞	70ºC
Cycles	1x (Hold)	39x		1x (Hold)		

*This temperature was adjusted to 56ºC and 58ºC for optimization process.

We analysed the fluorescent data from the cdPCR with QuantStudio® 3D AnalysisSuite™ online software. A known D positive buccal extracted DNA was used as positive control. The threshold was set manually based on NTC and applied to all samples in each batch. Based on this, positive and negative droplets were determined. DNA concentration (copies/µl) was then determined by the QuantStudio® 3D AnalysisSuite™ online software by use of Poisson statistics (95% confidence interval) for each sample.

2.2.17 Comparison between 3 different SYBR Green master mixes for the detection of *RHD* exon 10 using direct dry blood on FTA card

Several attempts on direct amplification of DNA from biological samples has been going on. Mercier and colleagues successfully amplified *HLA DQ A* gene using whole blood sample without the need of extraction process (Mercier et al., 1990). Kline and colleagues evaluated the performance of a short tandem repeat multiplex using dried whole blood stains preserved on FTA card without extraction and found these cards are useful media for DNA study (Kline et al., 2002). However, there are no studies tested direct PCR using FTA card on RHD blood group genotyping.

A total of 40 µl K_2EDTA blood sample per well was applied on Whatman FTA card (GE Healthcare) and dried at 80^{o}C for 30 minutes. The samples were stored at room temperature until used. For sample preparation; 3 mm disc was punched out of sample in FTA card and placed in 1.5 microcentrifuge tube. Quick washing and lysis process were followed as described previously. The samples were washed with 20 µl of ddH_2O at 50^{o}C for 3 minutes. Tris-EDTA buffer pH 8.0 (Qiagen, UK) was added after water removal and the samples were incubated for 5 minutes at 98^{o}C in a heating block. Lysate was transferred to 0.5 ml tube to be used in PCR.

A total of 14 samples previously known for being positive for *RHD* exon 10 were tested. Real-time PCR was performed in a total volume of 20 µl consisting of either 1x Qiagen (QuantiNova SYBR®Green Master Mix), PCRBIOSYSTEMS (qPCRBIO SyGreen Mix) or Applied Biosystems (PowerUp™ SYBR™ Green Master Mix), 400nM of each primer MDF10 and rr4 (Table 2.1) and 4 µl of lysate (DNA template). The reaction was carried out in Rotor-Gene Q thermocycler (Qiagen). Thermocycler was run for 40 cycles under the following condition: initial denaturation at 95^{o}C for 3 min, denaturation at 95^{o}C for

5 sec, annealing and extension at 60°C for 20 sec. For direct real-time PCR, a total volume of 20 µl (4 µl of DNA) in 40 cycles using thermal cycler Rotor-Gene Q (Qiagen, UK) was used. Melting curve analysis was performed between 65 and 95°C with a temperature increment of 0.5°C/second. Curves were sketched using Rotor-Gene Q data analysis software. Melting curve peak as *RHD* exon 10 amplicon was confirmed with a 381 bp band in 2% gel electrophoresis. The sensitivity of each master mix to detect *RHD* exon 10 by direct method without DNA extraction was calculated. Real-time PCR result for *RHD* exon 10 from extracted DNA used as a gold standard method for this comparison.

2.2.18 Statistical and data analysis

Convenience sampling method were used for collecting D negative donors' samples. For serological methods, data were statistically described in terms of frequencies (number of cases) and percentages when appropriate. The results were expressed as percentages by dividing total positive cases for the particular positive antigen by total number of samples (*n* = 203) and multiplied by 100. Data were managed and analysed using GraphPad Prism 8 (USA). Confidence level chosen was 95% and confidence interval (C.I) was calculated using https://www.surveysystem.com website. The C.I value was calculated based on unpublished data of D negative (8.4%) which estimates about 209000 out of 2.5 million of Omani population with D negative. The sample size of 203 with estimated D negative Omani population gave 6.87 C.I level with 95% confidence level. The calculated frequency range of 95% C.I is based on frequency percentage ± 6.87. Rh haplotype frequency was estimated using Hardy-Weinberg equation. The following equation was used (r is *r* haplotype, p is *r´* haplotype and q is *r´´* haplotype, square is diplotype):

$$p^2 + 2pq + 2pr + q^2 + 2qr + r^2 = 1$$

In order to find out the haplotype frequency, dce (r) phenotype was calculated (total dce phenotype divided by total samples). This was considered diplotype frequency (r^2 in the equation). Square root of the r^2 was considered the *dce* haplotype frequency (r). Likewise, other haplotypes frequency were calculated and the values were added in the equation as to reconfirm total of all values were equal to 1 (please see Appendix 3 for the detailed calculation). All other statistical analysis (mean, standard deviation, standard error, *t*-test, chi-square test and *p* value) were performed using GraphPad Prism 8 application unless stated otherwise. The distribution of antigens/phenotypes between this study and studies of other regional populations was compared using the chi-square test. A $p < 0.05$ was considered statistically significant. Male to female ratio was calculated by dividing number of males by number of females. To find out Rh phenotype's distribution difference between D negative Omani male and female, frequency and *p* value was calculated. Frequency was calculated by dividing specific phenotype by $n = 203$ and multiplied by 100. Statistical *p* value using frequency value of different Rh phenotypes was calculated. As we used two categorical variables (gender and Rh phenotype), chi-square test for 2x5 contingency table was used to find out *p* value.

To compare Omani population with other populations for a specific Rh major antigen (C, c, E or e) and Rh haplotype, frequency of Rh major antigen/Rh haplotype of each population was recorded from the literature. Statistical *P* value using these frequencies was calculated to find out significant difference between the populations compared to Omanis. As we used two categorical variables (population and Rh major antigen/Rh haplotype), chi-square test for 2x2 contingency table was used to find out *P* value.

For DNA multiple extraction (up to 4 extractions) from initial sample ($n = 5$ independent samples), the average DNA concentration and purity (260/280) was compared

between each extraction round. Mean standard deviation and standard error was calculated and two standard error bar charts were plotted. As the comparison has one categorical variable (extraction round) and one numerical variable (DNA concentration or DNA purity), we implemented *t*-test to compare these variables to get the *P* value. *P* value < 0.05 was considered significant.

For molecular genotyping, all *RHD* exon 10 positive was calculated by number and the percentage calculated by dividing total positive cases for *RHD* exon 10 by total number of samples and multiplied by 100. The result of ASP-PCR for the detection of *RHDΨ* pseudogene, *RHD-CE-D*^{s} hybrid gene, and *RHD* deletion in serological D negative blood samples allowed us to calculate experimental allele frequencies for each molecular background. Assuming consistency with Hardy-Weinberg equilibrium, genotype and allele frequencies of inactive *RHD* genes and *RHD* deletion was calculated using Bernstein formula (Touinssi et al., 2009) - detailed calculations can be seen in Appendix .

The sensitivity assessment for the evaluation of different SYBR Green master mixes ability to detect *RHD* exon 10 from direct dry blood sample on FTA card without DNA extraction method was calculated as follows:

$$Sensitivity\ (\%) = \frac{\text{True positive}}{\text{True positive} + \text{False negative}}\ x\ 100$$

In cdPCR using SYBR Green, *RHD* exon 10 DNA concentration (copies/µl) and sample volumes with different dilutions correlation was calculated using statistical Anova test and significance was accepted at $p < 0.05$. Using TaqMan chemistry, DNA concentration (copies/µl) and sample volumes was calculated using Anova test and significance was accepted at $p < 0.05$. All statistical analysis was performed using GraphPad Prism 8 (USA).

3 CHAPTER THREE: Serological RhCE phenotyping & molecular background of D negative Omani population

3.1 Introduction:

The D antigen of the Rh blood group system is the most important protein due to its involvement in HDFN and in HTR. According to recent data, transfusion with a unit or more D positive blood unit leads to the production of anti-D in 20-30% of D negative individuals (Frohn et al., 2003; Yazer and Triulzi, 2007; Gonzalez-Porras et al., 2008). Therefore, to prevent anti-D alloimmunization, exposure of D negative individuals to D positive RBCs should be avoided by appropriate transfusion strategies, and routine administration of anti-D immunoglobulin to D negative women during the third quarter of pregnancy and after delivery of a D positive infant.

Studies shown that detection of weak D, DEL phenotype or partial D may be missed by standard serologic methods including IAT and may cause anti-D immunization when transfused to D negative recipients. Garratty calculated that at least 120 weak D or Del donors, typed D negative serologically, are transfused to D negative recipients annually in Southern California (Engelfriet and Reesink, 2006). In another study by Flegel and colleagues on 46133 serologically D negative donors, the *RHD* genotyping showed that 96 samples had *RHD* gene, half of which harboured Del phenotype (Flegel, von Zabern and Wagner, 2009). Moussa and colleagues study realized that a partial D sample type DBT was mistyped as D negative by serological tests (Moussa et al., 2012).

The D negative phenotype is characterized by a high molecular diversity which explains the discrepancies found between serologic and molecular methods. *RHD* genotyping is very efficient for managing donors and patients carrying any of the various molecular types of weak D, DEL phenotype and partial D. in addition, it overcomes the limitations of serological methods that are unable to differentiate between *RHD* variants and mistyped D

status. Moreover, it also unveils the type of molecular *RHD* genotyping of D negative (silent/inactive or full deletion). Unpublished data of frequency of D negative phenotype was estimated at 8.4% in Oman.

Oman has a high prevalence of hereditary blood disorders such as SCD and thalassemia (Al-Riyami et al., 2001). Alkindi and colleagues investigated the rates of alloimmunization in Omani SCD and thalassemia patients and found that the rate of alloimmunization in SCD was 31.6%, whereas in patients with thalassaemia it was 20%. Antibodies to E, e, C, c, D, K, S, Fy^a, Kp^a, Jk^a and C^w were observed; 85% of the patients were also immunised with Rh and Kell antigens (Alkindi et al., 2017). Another study conducted by Al-Dughaishi and colleagues on D negative Omani pregnant women revealed that, the prevalence of D negative was 7.3%. and the rate of D negative alloimmunization was 10%, and anti-D was the most common antibody detected (Al-Dughaishi et al., 2016). Therefore, a comprehensive *RHD* alleles study would be very useful to unveil D status and reduce the risk of anti-D alloimmunization in D negative recipients. *RHD* allele study was lacking in Omani population, hence this propelled us to conduct such study.

Daar and colleagues demonstrated multicentric origin of sickle mutation in Oman with haplotypes originated by gene flow from sub-Sahara west Africa, East Africa (Zanzibar) and Arab-India haplotype (Iran, Iraq, Pakistan and India) (Daar et al., 2000). Based on this, likewise we hypothesized molecular background of *RHD* negative in Oman would be multicentric origin either inactive/silent due to *RHDΨ* pseudogene and *RHD-CE-D*^{S} as seen in African (Moussa et al., 2012) and full deletion as seen in Iran (Khosroshahi et al., 2019). The aim of the current study was to detail *RHD* molecular background on apparently D negative individuals in Omani donors. The first step in the study was to reconfirm D status of 205 D negative samples and phenotype them for C,c,E and e antigens by two different methods (tube and gel card). The second step was to test all these samples for weak D by two

different serological methods (tube and gel card). Samples positive for weak D testing (*n* = 2) were excluded from the study. The last step of the study consisted of using real-time PCR to confirm the D status of the samples (*n* = 203) that were typed as D negative after being confirmed by AHG testing.

3.2 Serological determination of ABO/RhCE phenotypes on D negative Omani donors

RhD and RhCE phenotyping by direct gel card serological test was done on 205 D negative Omani blood donors. All D negative samples with direct method were further tested for positivity by IAT weak D gel card test which excluded two samples as being weak D. ABO phenotyping were done by each regional blood bank as routine donor testing. ABO group distribution and frequencies for 203 samples (excluding *n* = 2, positive for weak D) were as follows: O (*n* = 115, 56.7%) > A (*n* = 49, 24.1%) > B (*n* = 30, 14.8%) > AB (*n* = 9, 4.4%) (Figure 3.1).

All direct serological tests (*n* = 203, excluding weak D) showed the following Rh phenotypes and probable genotypes: C-c+E-e+ (*rr*, *n* = 158, 77.8%), C+c+E-e+ (*r'r*, *n* = 38, 18.7%), C-c+E+e+ (*r″r* , *n* = 5, 2.5%), C+c-E-e+ (*r'r'*, *n* = 1, 0.5%), C-c+E+e- (*r″r″*, *n* = 1, 0.5%) (Table 3.1). Both weak D samples showed C-c+E-e+ phenotype and R_0r probable genotype (excluded from the study). The frequency of Rh phenotype and probable genotypes for males and females was also compared. Distribution of RhCE phenotypes is similar in both the genders, whereas the distribution of probable genotypes showed *rr* > *r'r* > *r''r* > *r'r'* > *r''r''* for male and *rr* > *r'r* > *r'r'* > *r''r''* = *r''r* for female (Table 3.2). The frequency of C, c, E and e were 20%, 99%, 3% and 99% respectively (Figure 3.2). Haplotype frequencies were: (*dce*, 0.89), (*dCe*, 0.1) and (*dcE*, 0.01) (Figure 3.3). Representative images are presented in in Figure 3. 4 (A-F), showing RhD and RhCE phenotyping by gel technique.

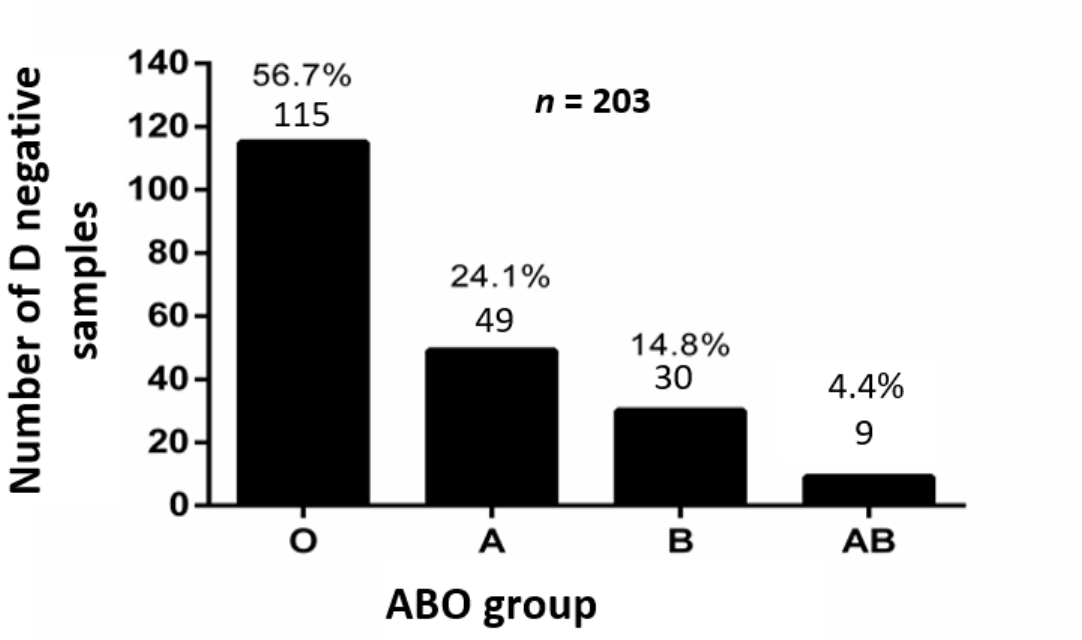


Figure 3.1 ABO distribution among D negative Omani donors.

ABO phenotyping on 203 blood samples obtained during routine blood donation in Oman. Testing was performed by the respective blood banks using BioRad DiaClon ABO/Rh ID-Card technique. The most frequent ABO group was O followed by A, B and AB.

Table 3.1 Rh phenotypes and probable genotype frequencies in D negative Omani donors (*n* = 203)

		Probable genotype				
Antigens	**Phenotype**	**Wiener**	**Fisher race**	**Number**	**Frequency %***	**95% C.I****
D-C-E-c+e+	rr	*rr*	dce/dce	158	77.8	70.9 – 84.7
D-C+E-c+e+	r'r	*r′r*	dCe/dce	38	18.7	11.8 – 25.6
D-C-E+c+e+	r"r	*r″r*	dcE/dce	5	2.5	< 9.4
D-C-E+c+e-	r"r"	*r″r″*	dcE/dcE	1	0.5	< 7.4
D-C+E-c-e+	r'r'	*r′r′*	dCe/dCe	1	0.5	< 7.4
	Total			*n* = 203	100	

*The specific Rh phenotype frequency percentage was calculated by dividing Rh phenotype by 203 and multiply by 100.

**Confidence interval. C.I was calculated using https://www.surveysystem.com website. The C.I value was calculated based on unpublished data of D negative (8.4%) which estimates about 209000 out of 2.5 million Omani population with D negative. The sample size of 203 with an estimated D negative Omani population gave 6.87 C.I level with a 95% confidence level. The calculated range of 95% C.I is based on a frequency percentage ± 6.87.

Table 3.2 Rh phenotype distribution between D negative Omani male and female blood donors (*n* = 203)

	Phenotype*					
Gender	r''r''	rr	r'r	r''r	r'r'	Total
Male	1 (0.6%)	137 (77.2%)	34 (18.9%)	5 (2.8%)	1 (0.6%)	178
Female	0 (0%)	21 (84%)	3 (12%)	0 (0%)	1 (4%)	25
p-value**	***p* = 0.3772**					**203**

*Frequency was calculated by dividing specific phenotype by *n* = 203 and multiplied by 100

***p* value was calculated using Prism GraphPad software (USA). A chi-square test for 2x5 contingency table was used.

There is no statistical difference between Omani male and female donors with regard to Rh phenotype distribution

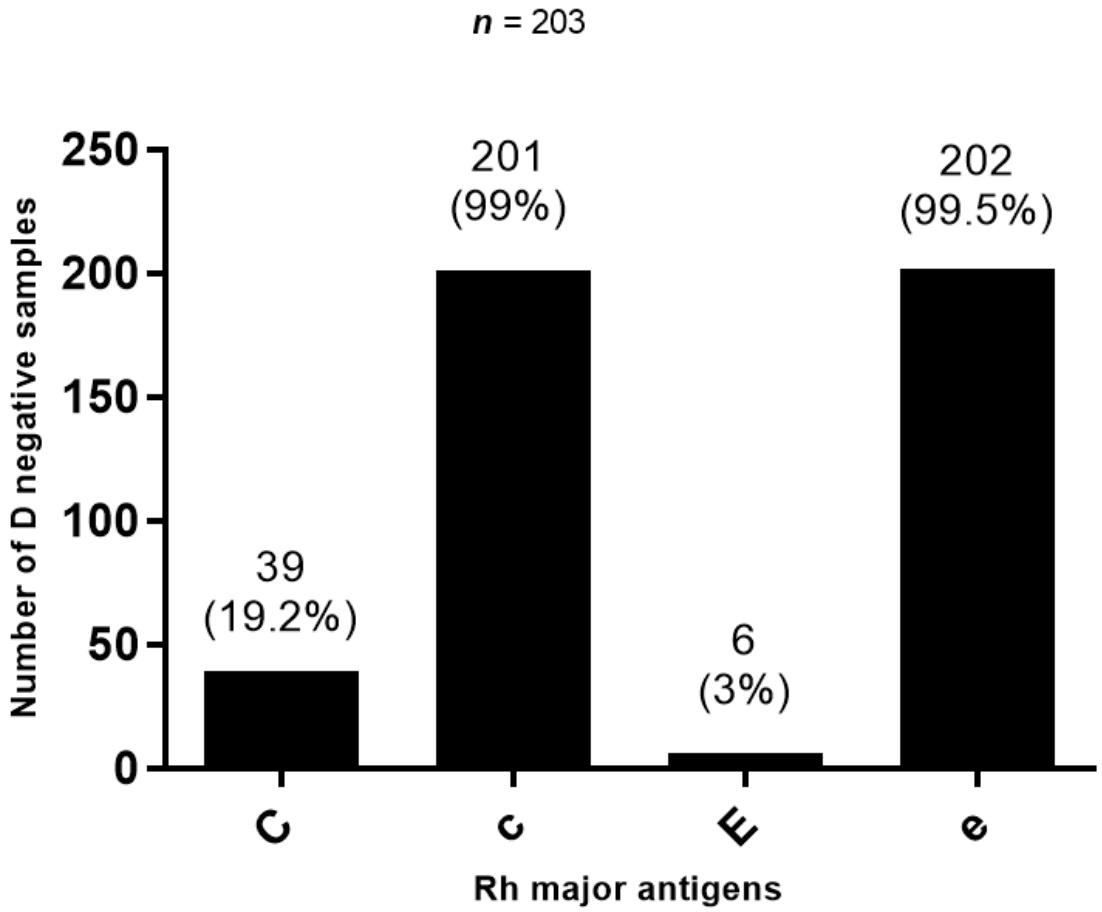


Figure 3.2 Rh phenotype frequencies in D negative Omani blood donors.

Rh phenotyping was performed on 203 blood donors using Bio-Rad RhD + Phenotype ID-Card system. The most common antigen found was e followed by c, C and E.

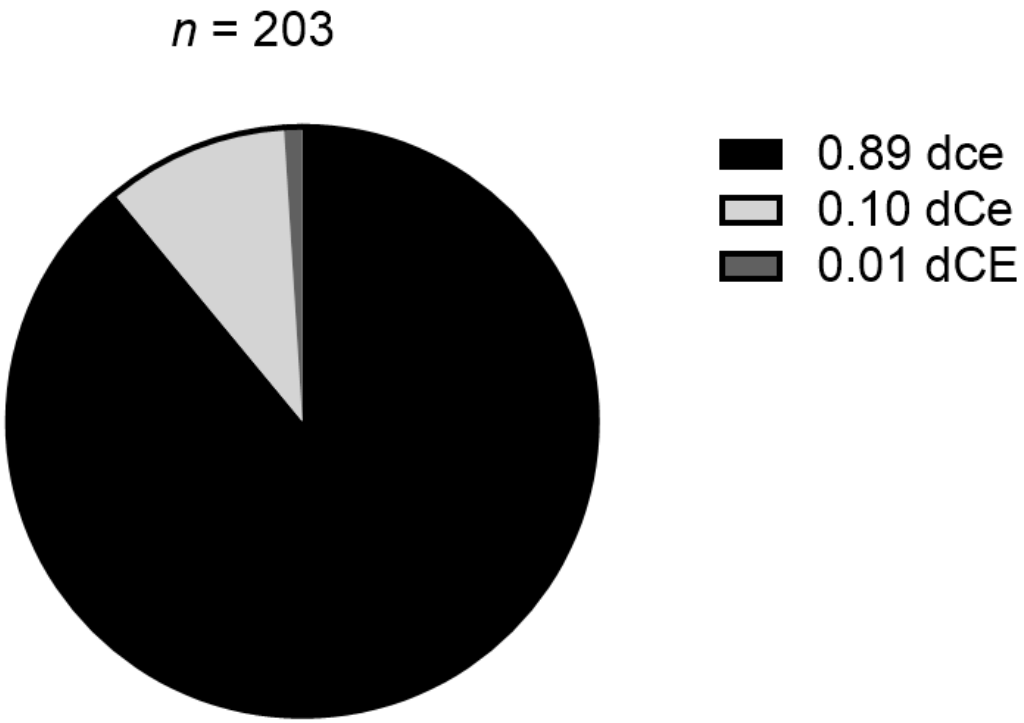


Figure 3.3 Rh haplotype frequencies in D negative Omani blood donors.

The haplotype frequencies were determined in 203 blood samples obtained during routine blood donation to Omani blood banks using Hardy-Weinberg equation. The haplotypes order was *dce* > *dCe* > *dcE*.

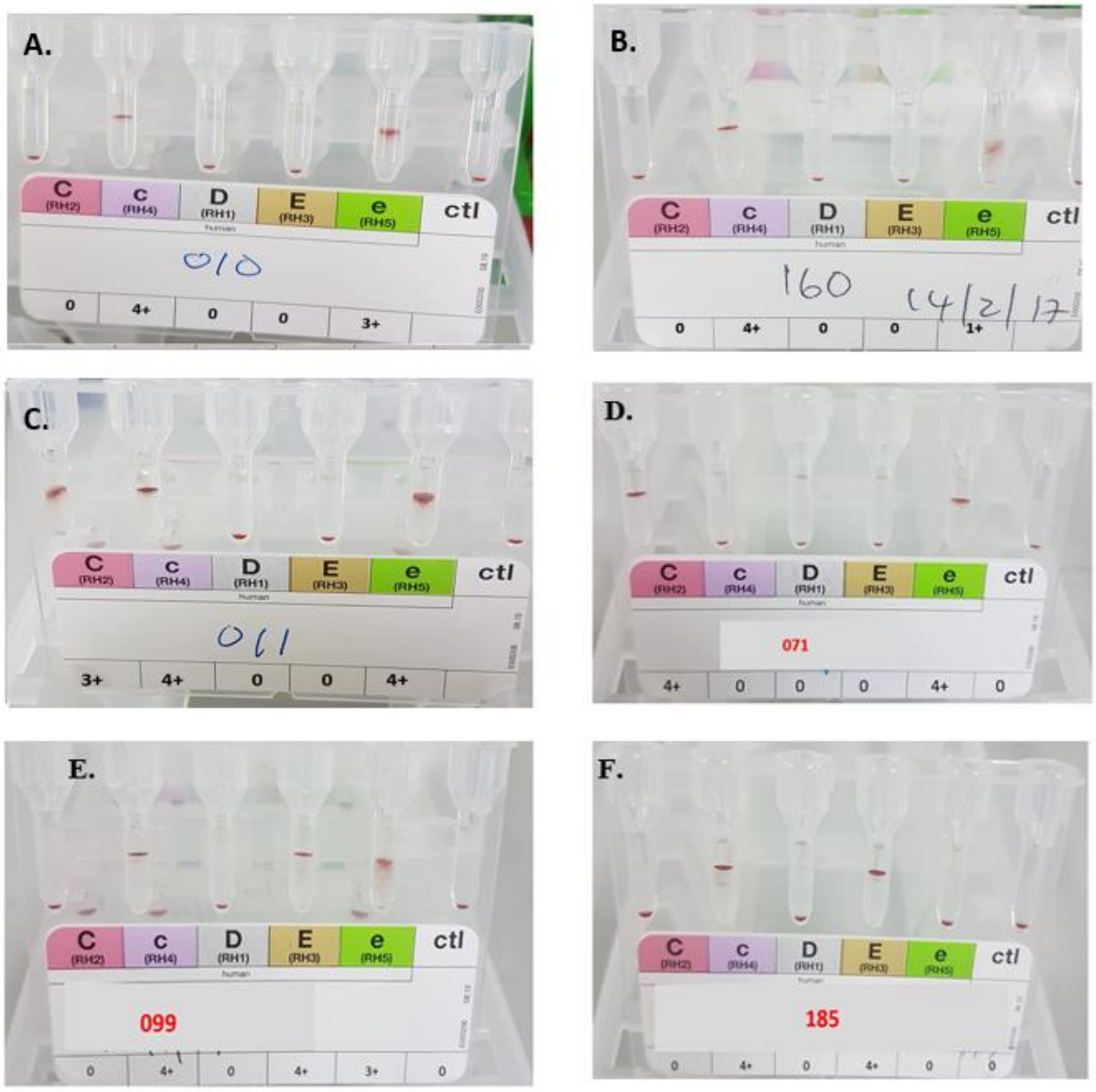


Figure 3.4 Rh phenotyping by BioRad gel card technique.

Representative samples (*n* = 6) shows Rh phenotyping. Agglutination indicates presence of the Rh antigen on RBCs and thus phenotyping & probable genotype. Agglutination with only c and e suggests D-C-E-c+e+ phenotype with *rr* probable genotype (A & B). A probable genotype *r'r* and D-C+E-c+e+ phenotype is recorded for samples giving agglutination with C, c & e (C), whereas a probable genotype *r'r'* and D-C+E-c-e+ phenotype is recorded for any agglutination with C & e only (D). Agglutination with c, E and e suggests D-C-E+c+e+ phenotype with *r''r* probable genotype (E), whereas agglutination with only c and E suggests D-C-E+c+e- phenotype with *r''r''* probable genotype (F). (0): no agglutination (–) negative; (+): agglutination – positive; (1-4): agglutination grading.

3.3 Serological determination of weak D on D negative Omani donors

Weak D testing was performed on 205 samples by IAT to determine any D positive sample not detected by direct serological method. The tube technique was negative for all. In comparison, gel card method gave negative IAT for all samples (Figure 3.5) except sample 001 and sample 209 (excluded in the study), which suggests C-c+D+E-e+ phenotype and R_0r probable genotype (Figure 3.6). DAT was used as a control to rule out any false positive weak D.

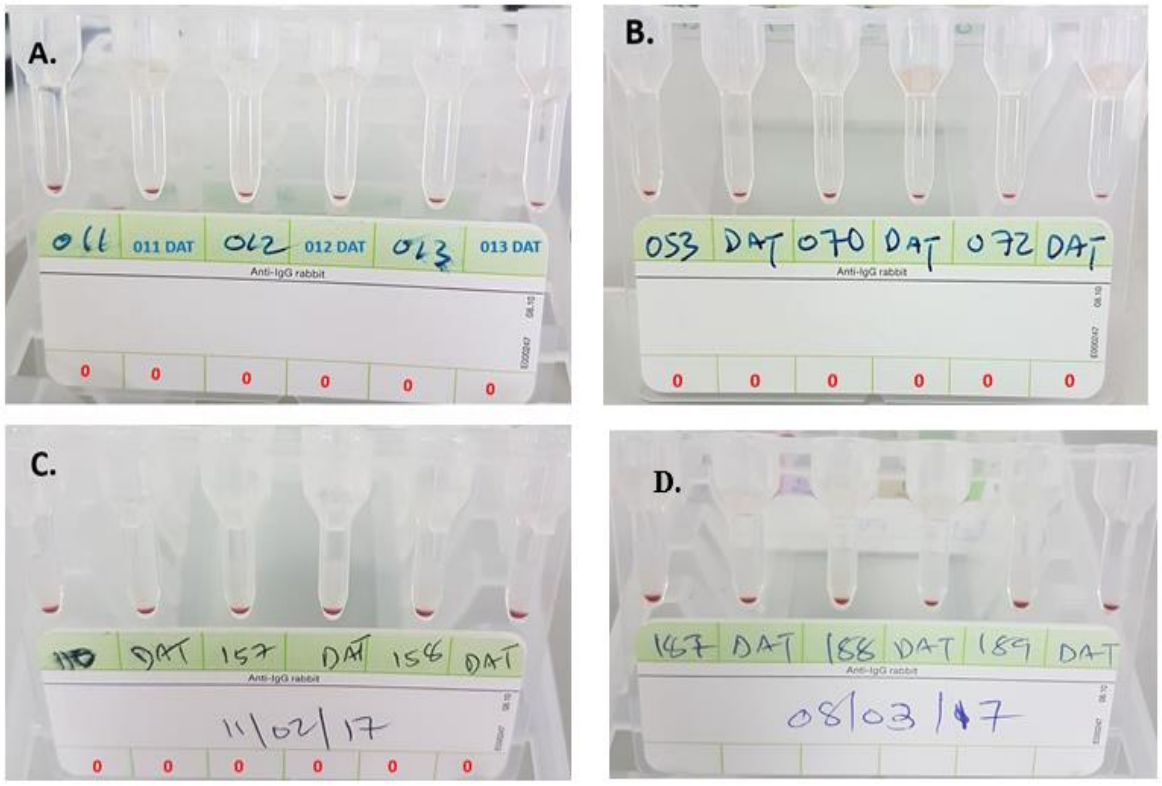


Figure 3.5 IAT testing for weak D.

Representative samples (n = 12) shows weak D test with DAT control results. Weak D testing performed using Bio-Rad anti-IgG (rabbit) ID-card. Representative samples showed no agglutination, which indicates negative weak D test and thus may confirm serological D negative (A-D). DAT test was performed for each sample as a control to rule out false positive results. (0: No agglutination – negative). Total of 203 out of 205 samples showed no agglutination.

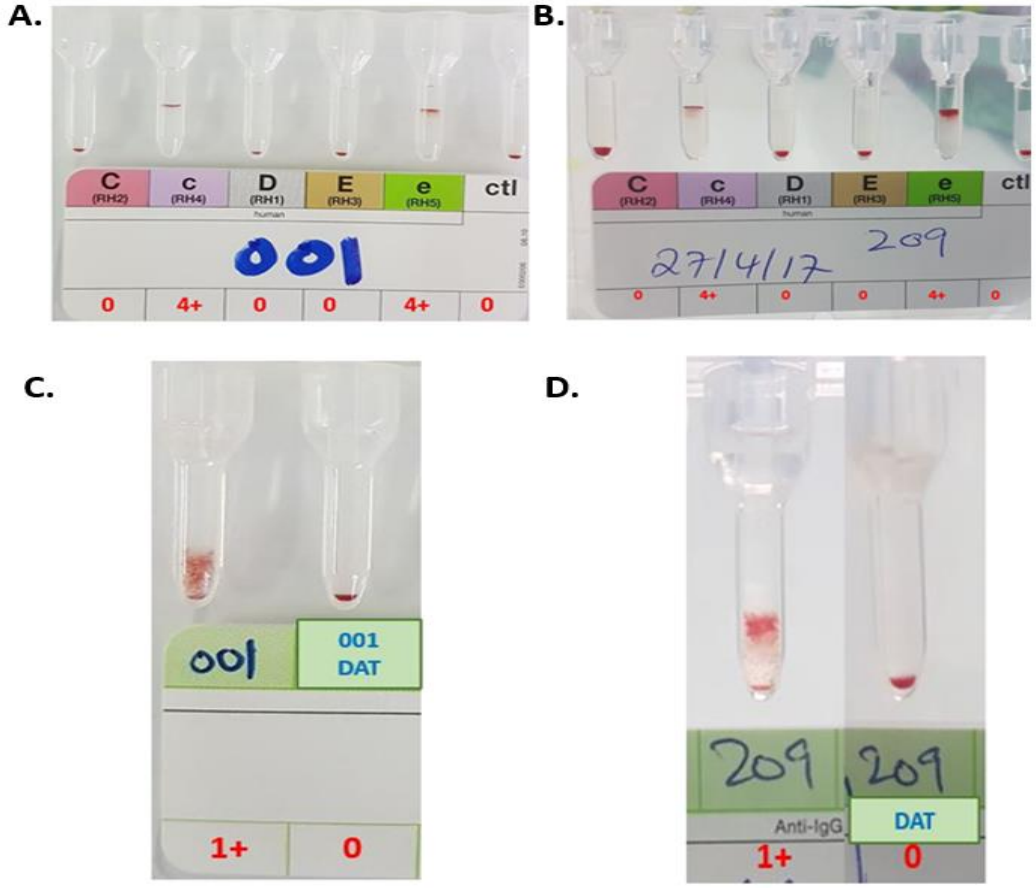


Figure 3.6 Rh phenotype and serological determination of weak D by IAT for samples 001 & 209.

Rh phenotyping was performed using BioRad RhD + Phenotype ID card, whereas ICT weak D testing done using BioRad coombs anti-IgG ID card. Both samples showed 4+ agglutination with c & e only, which suggested D-C-E-c+e+ phenotype and *rr* probable genotype initially (A & B). However, weak D testing by ICT gel card technique showed +1 agglutination for both samples, which determined weak D presence and thus D positive with DAT control negative result to rule out any false positive weak D (C & D).This suggests re-phenotyping to D+C-E-c+e+ with probable genotype R_0r.

3.4 Serological Rh phenotype of D negative Omanis compared to other populations

The D negative ratio for Omani male to female was 7.1:1. This ratio showed no significance compared to 4.2:1 ratio observed in Gana [$p = 0.172$] (Opoku-Okrah C, Amidu N and Amoah-Sakyi S, 2008). However, it showed significant difference ($p < 0.001$) from observed ratio 1.27:1 in a study conducted by Muhammed et al, 2016 in Pakistan (Muhammad et al., 2016).

As stated in section 3.2, current study showed no significant difference between D negative male and female Omani blood donors with regard to Rh phenotyping ($p = 0.224218$) [Table 3.2]. The commonest phenotype and probable genotype noticed was C-c+E-e+ (*rr*). This finding is similar to the result observed in two Arabian countries, Tunisian population (Moussa et al., 2012; Sassi et al., 2014) and Saudi Arabia (Al-Shiakh. et al., 1997).

As seen in Figure 3.2, among D negative blood donors the most common antigen found to be e followed by c, C and E. This is similar to that in other Arab countries UAE, Saudi Arabia, Morocco and India (Al-Shiakh. et al., 1997; Taha, 2012; Makroo et al., 2014; Zahid et al., 2016). Predominantly, there are no significant differences between these frequencies compared to the most other Arab countries, Iran and India; nonetheless, significant differences have been observed in C and E antigens (Al-Shiakh. et al., 1997; Moussa et al., 2012; Makroo et al., 2014). Rate of C positive in the total populations was 19.7% and showed significant difference compared to 9.52% in Saudi Arabia, 33.5% in India and 6.7% in Tunisia ($p < 0.001$) whereas the rate of E positive was 3% which determined significant difference in comparison to India ($p < 0.001$). Antigen c positivity found to be 98.5% compared to 93.6% in UAE with significant difference [$p < 0.05$] (Taha, 2012). Frequencies of major Rh antigens in D negative Omani population compared to other populations are provided in (Table 3.3).

Table 3.3 Rh major antigens frequencies in different D negative ethnic populations

Rh antigen	Current study	Saudi Arabia (Al-Shiakh. et al., 1997)	India (Makroo et al., 2014)	UAE (Taha, 2012)	Morocco (Zahid et al., 2016)	Iran (Keramati et al., 2011)	Tunisia (Moussa et al., 2012)
C	40 (19.7%)	62 (9.5%)***	1269 (33.5%)***	13 (21%)	20 (15.2%)	7 (13.7%)	30 (6.7%)***
E	6 (3%)	12 (1.8%)	68 (1.8%)***	2 (3.2%)	1 (0.8%)	1 (2%)	16 (3.6%)
c	201 (98.5%)	639 (98.2%)	3736 (98.7%)	58 (93.6%)*	131 (100%)	51 (100%)	448 (100%)**
e	203 (99%)	647 (99%)	3762 (99.3%)	61 (98.4%)	131 (100%)	51 (100%)	448 (100%)
Total	*n = 205*	*n = 651*	*n = 3773*	*n = 62*	*n = 131*	*n = 51*	*n = 448*

*$p < 0.05$ **$p < 0.01$ ***$p < 0.001$

p value calculated using GraphPad prism 8 (USA). 2 x 2 contingency table.

p value compares Omanis with other populations for a specific indicated Rh antigen. Rh frequency for the c and e antigens show no statistical difference compared to the listed countries with exception of UAE and Tunisia for c antigen. Saudi Arabia, India and Tunisia show statistical difference in the frequency of C antigen, whereas only India shows difference with frequency of E antigen.

As stated in section 3.2, the most common haplotype observed in current study was *r* followed by *r'* and *r″*. Rh haplotypes frequency order observed in current study was similar in other D negative populations. In our study *r* haplotype frequency was 0.887 which is almost proportionate with frequency in UAE, Morocco and Iran, whereas it showed significant difference with frequency in Saudi Arabia and India ($p < 0.05$) (Table 3.4)

Table 3.4 Rh Haplotype frequencies in different D negative ethnic populations

Haplotype	Current Study	Saudi Arabia* (Al-Shiakh. et al., 1997)	India* (Makroo et al., 2014)	UAE (Taha, 2012)	Morocco (Zahid et al., 2016)	Iran (Keramati et al., 2011)
r	0.887	0.944	0.809	0.88	0.916	0.918
r'	0.1	0.046	0.178	0.071	0.08	0.072
r''	0.013	0.006	0.004	0.049	0.004	0.01
r_y	0	0	0.011	0	0	0

* $p < 0.05$

p value calculated using GraphPad prism 8 (USA) 9 – 2 x 4 contingency table

p value compares Omanis with other populations for Rh haplotypes. Rh haplotypes frequency for the c showed no statistical difference compared to the listed countries with exception of Saudi and India

3.5 DNA extraction for molecular study

DNA was extracted using QIAamp® DNA Investigator protocol on 3 mm discs taken from the centre of FTA cards spotted with 40 µl of whole human blood. We observed an average of 18 ng/µl with a minimum of 2 ng/µl of genomic DNA recovery (n = 203).

Representative samples (n = 5) were tested for subsequent extraction for 4 times to determine the possibility of obtaining DNA from 3 mm FTA card disc. We observed an average of 14 ng/µl of genomic DNA recovery from extraction one, which increased to 36 ng/µl after extraction two and reduced to 29 ng/µl after extraction three. All representative samples gave no DNA yield with extraction 4, which suggests repeated extraction of piece of FTA cards indicated that the DNA bound to the FTA membrane was not fully eluted in the first extraction but cannot be reused after three extractions (Figure 3.7). Combining the DNA extracts (Figure 3.8) obtained from the three rounds of extractions increase the yield. The average yield observed from 5 samples was 85 ng/µl in 75 µl volume.

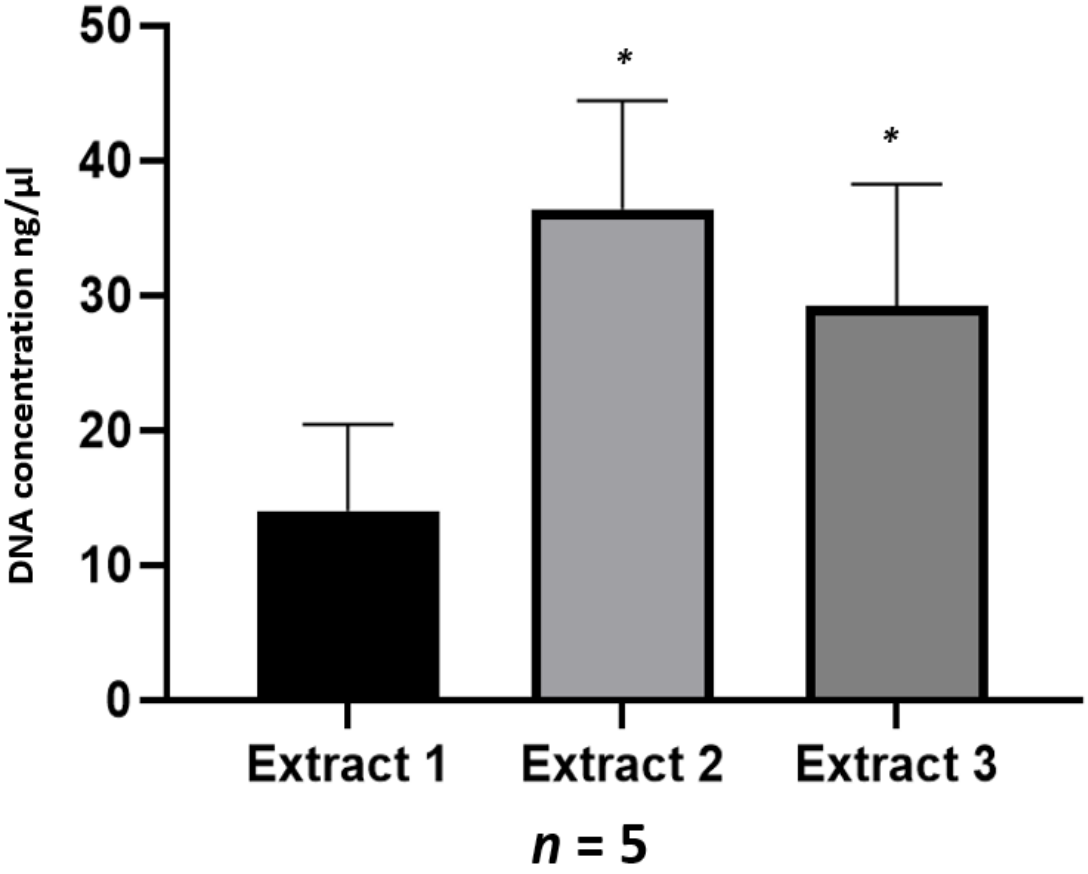


Figure 3.7 Bar plot of mean DNA concentration from repeated DNA extraction on FTA card.

Qiagen Investigator Kit protocol was used for the extraction process. The bar graph shows mean DNA concentration (standard error bar) of representative samples (n = 5) for multiple extraction for up to four times using the same initial sample. The mean values are 14, 36 and 29 from extraction 1, 2 and 3 respectively and no yield was observed from extraction 4. Highest yield can be obtained from second extraction, whereas no yield could be obtained after three extractions. Asterisk (*) indicates significant difference ($p < 0.05$) between groups compared to extract 1 using *t*-test.

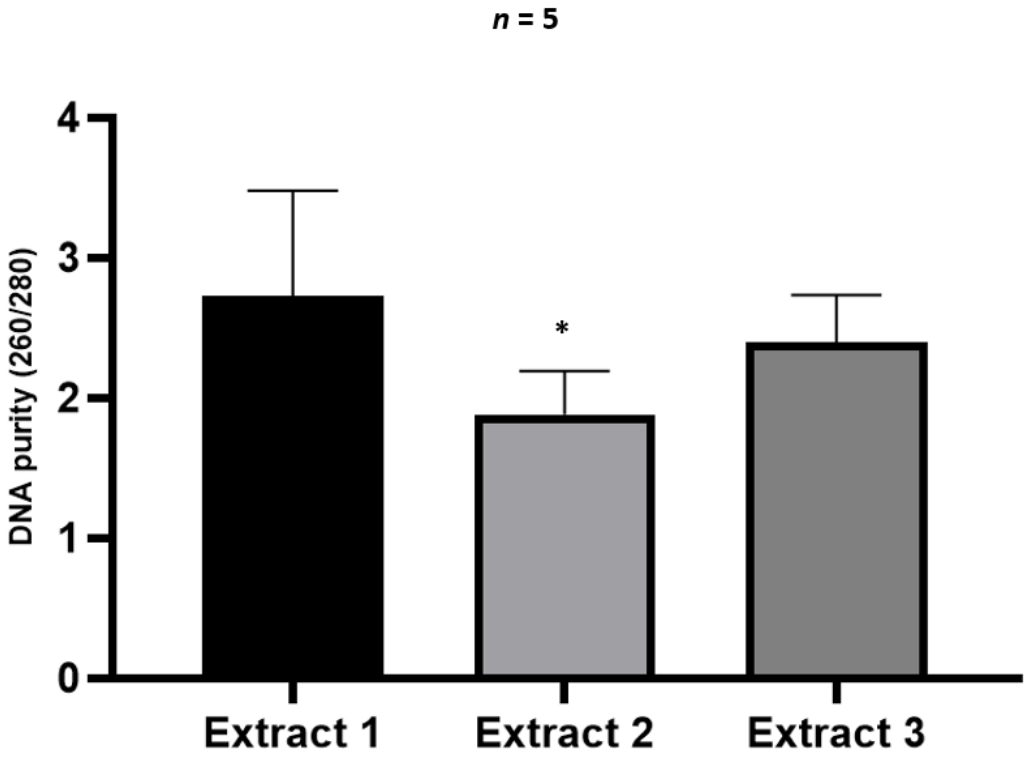


Figure 3.8 Bar plot of mean DNA purity from repeated DNA extraction on FTA card.

The mean, standard deviation, standard error and bar graph calculated and plotted using GraphPad Prism 8. Statistical *t*-test and *p* value calculated using GraphPad Prism 8. The bar graph shows mean DNA purity (standard error bar) of representative samples (n = 5) for multiple extraction for up to four times using the same initial sample. The mean DNA purity values are 2.72, 1.88 and 2.4 from extraction 1, 2 and 3 respectively and no yield was observed from extraction 4. Asterisk (*) indicates significant difference ($p < 0.05$) between other extraction groups compared to extract 1 using *t*-test. A significant difference was found between extract 1 and 2, where extract 2 gives the best DNA purity compared to extract 1 as it falls within recommended normal range of 1.8-2.0.

3.6 RHD exon 10 analysis

All 203 samples were tested for the presence of *RHD* gene by SYBR Green qPCR technique to amplify *RHD* exon 10 initially. Among the studied samples, 179 (88.18%) showed no amplification of the *RHD* exon 10, suggesting a *RHD* deletion (*del*) molecular background, in accordance with the serological results. This categorized serological D status as true D negative. The remaining 24 (11.82%) samples showed a positive amplification for *RHD* exon 10, indicating a *RHD* genetic polymorphism (Table 3.5). Total of 50% of the 24 samples that indicated polymorphism had C or E antigen as per serological results.

The amplification presence was indicated by Ct and confirmed by melting curve which showed 2 peaks; the first between 78.75°C and 79.00°C and the second between 81.25°C and 81.50°C (Figure 3.9). Negative samples showed no Ct value and no melting curve peak (Figure 3.9). Samples amplification data from the Rotor-Gene Q was reconfirmed by 2% agarose gel electrophoresis which showed a single expected product size of 392 bp (Figure 3.9). For *RHD* exon 10 positive samples, a further reconfirmation of the product and exon presence was done by Sanger sequencing where the BLAST showed 100% similarity with normal *RHD* gene (Figure 3.10) suggesting the primers had amplified the desired gene region.

The sequencing result of the 24 samples showed no single nucleotide mutation (SNP), deletion or insertion, which suggested a normal coding sequence for exon 10 in Omani population (Figure 3.10). All *RHD* exon 10 positive samples with SYBR Green chemistry were retested with TaqMan chemistry along with 30 representative *RHD* exon 10 negative samples and found to be consistent with SYBR Green results.

Table 3.5 Rh phenotype distribution according to the existence of *RHD* exon 10

Molecular background	Rh Phenotype of serological D negative samples (*n* = 203)				
	ddccee	ddCcee	ddCCee	ddccEe	ddccEE
	n = 158 (77.8 %)	*n* = 37 (18.2 %)	*n* = 2 (1 %)	*n* = 5 (2.5 %)	*n* = 1 (0.5 %)
RHD deletion* *n* = 179 (88.12%)	146	27	1	4	1
RHD polymorphism** *n* = 24 (11.82%)	12	10	1	1	0

**RHD* gene deletion was considered based on the absence of *RHD* exon 10

** *RHD* gene polymorphism was considered based on the presence of *RHD* exon 10

The table shows Rh phenotype distribution according to the molecular background in D negative Omani donors based on SYBR Green qPCR for *RHD* exon 10.

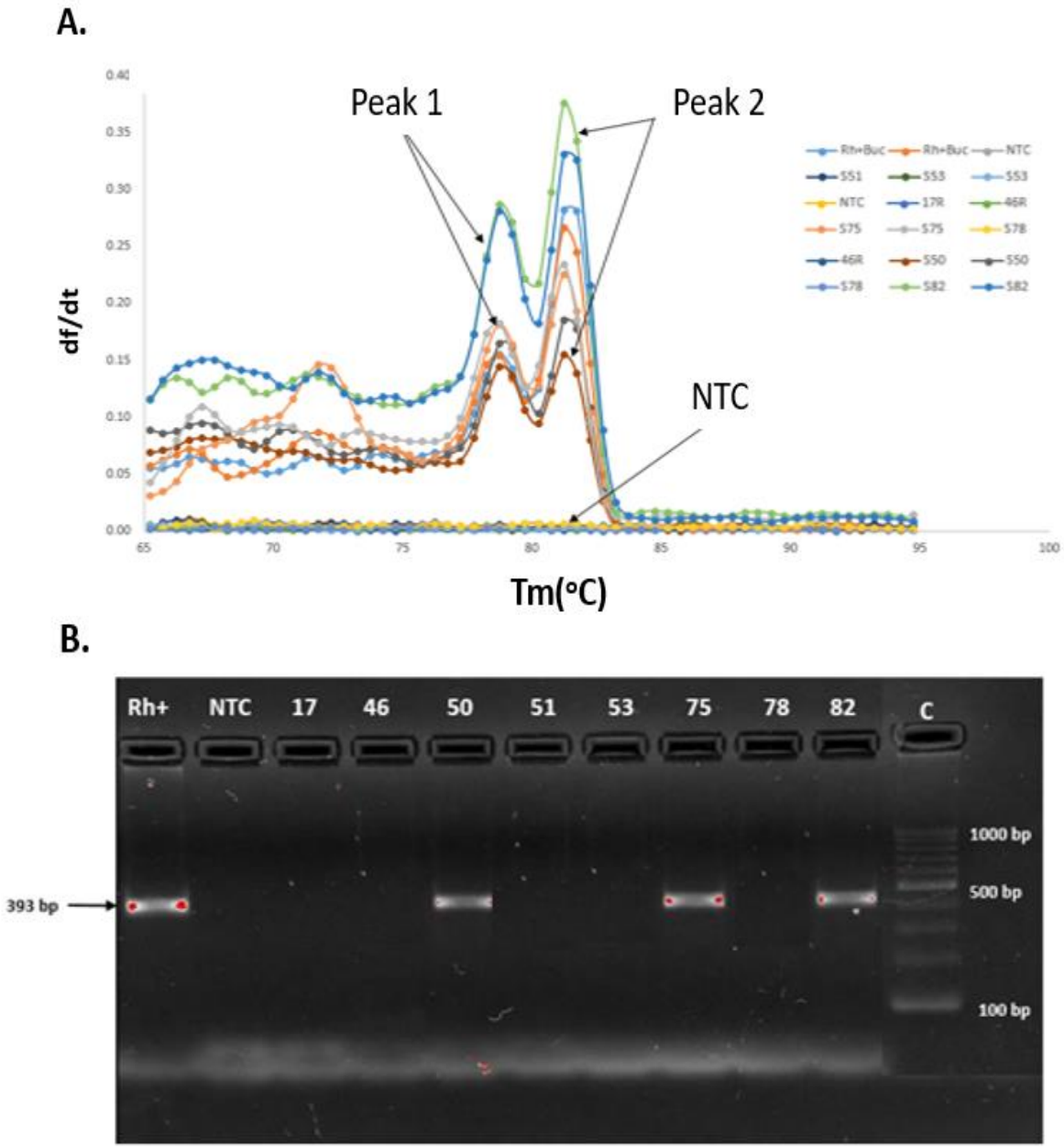


Figure 3.9 Melting curve peak versus gel electrophoresis for *RHD* exon 10 amplicon.

Shows results for representative samples (n = 9). All samples were run in duplicates with a positive control and non-template control (NTC). Two peaks were observed in all 9 samples, first peak (78.75°C-79.00°C) and second peak (81.25°C-81.50°C), indicating *RHD* exon 10 amplification with possible second amplicon (contamination), whereas no peak was observed in the no template control NTC (A). DNA ladder marked as (C). *RHD* positive control showed specific PCR product for the *RHD* exon 10 *(lane* marked Rh+, 393 bp). A negative NTC showed no product *(lane* marked NTC). All representative *RHD* exon 10 positive samples showed 393 bp product *(lane*s 50, 75 and 82) compared to peaks presence in melting view. Negative samples *(lane*s 17, 46, 51, 53 and 78) showed no *RHD* exon 10, no peak in melting curve and thus a negative genotyping result (B). correlation between two peaks in melting curve (A) and one band in gel view (B) rule out contamination.

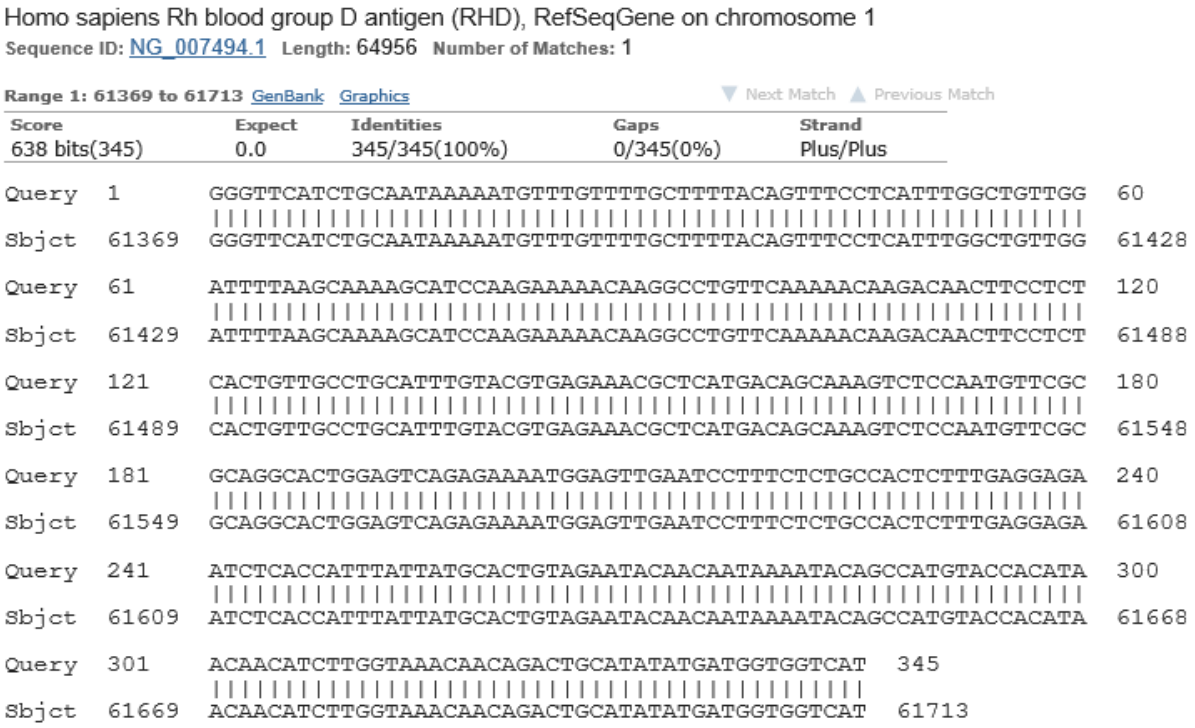


Figure 3.10 Representative nucleotides BLAST result of *RHD* exon 10 amplicon.

The BLAST was performed using NCBI BLAST online software. Query is the sample nucleotides sequence determined from Sanger sequencing. Subject is the nucleotide sequence in RefSeqGene that matches the most with query sequence. The BLAST showed 100% identity to D antigen reference gene on chromosome 1 with no SNP.

3.7 Molecular analysis of serological D negative samples and positive RHD exon 10

3.7.1 Correlation between melting curve analysis and gel electrophoresis of *RHD* exons 1 through 9

In order to determine the molecular background of the 24 *RHD* exon 10 positive samples, further molecular investigation was done by testing *RHD* exons 1 through 9. QuantiNova SYBR Green PCR master mix was used with specific primers listed in Table 2.1 as per the method described in section 2.2.12. The melting curve temperature was determined of each *RHD* exon and further correlated and reconfirmed by agarose gel electrophoresis as shown in Table 3.6. We observed all *RHD* exons melting curve temperature were close to each other (between 81.25 and 87.50^{o}C) except *RHD* exon 9 (75.25-75.75^{o}C) which suggests melting curve analysis of SYBR Green chemistry is not a good tool to discriminate between *RHD* exons using melting curve analysis in general.

Table 3.6 *RHD* exons 1 through 9 melting curve temperature and product size in agarose gel electrophoresis

***RHD* exon**	**Melting curve temperature (°C)**		**Agarose (bp)**	**Remarks**
	Peak 1	**Peak 2 (if any)**		
1	84.25-84.50*	86.25-86.50*	381	Figure 3.11
2	87.10-87.50*		190	Figure 3.12
3	82.25-82.75*		154	Figure 3.13
4	82.75		123	Figure 3.14
5	82.25		157	Figure 3.15
6	87.25		133	Figure 3.16
7	81.25-81.75*		96	Figure 3.17
9	75.25-75.75*		119	Figure 3.18

Asterisk (*) indicates the melting temperature range from minimum to maximum for the respective *RHD* exon amplicon

The table shows *RHD* exons 1 through 7 and exon 9 melting curve temperature (n = 24) determined for SYBR Green chemistry compared to product size in agarose gel electrophoresis. *RHD* exon 9 shows a unique melting temperature curve compared to *RHD* exons 1 through 7 which determined overlapping curves.

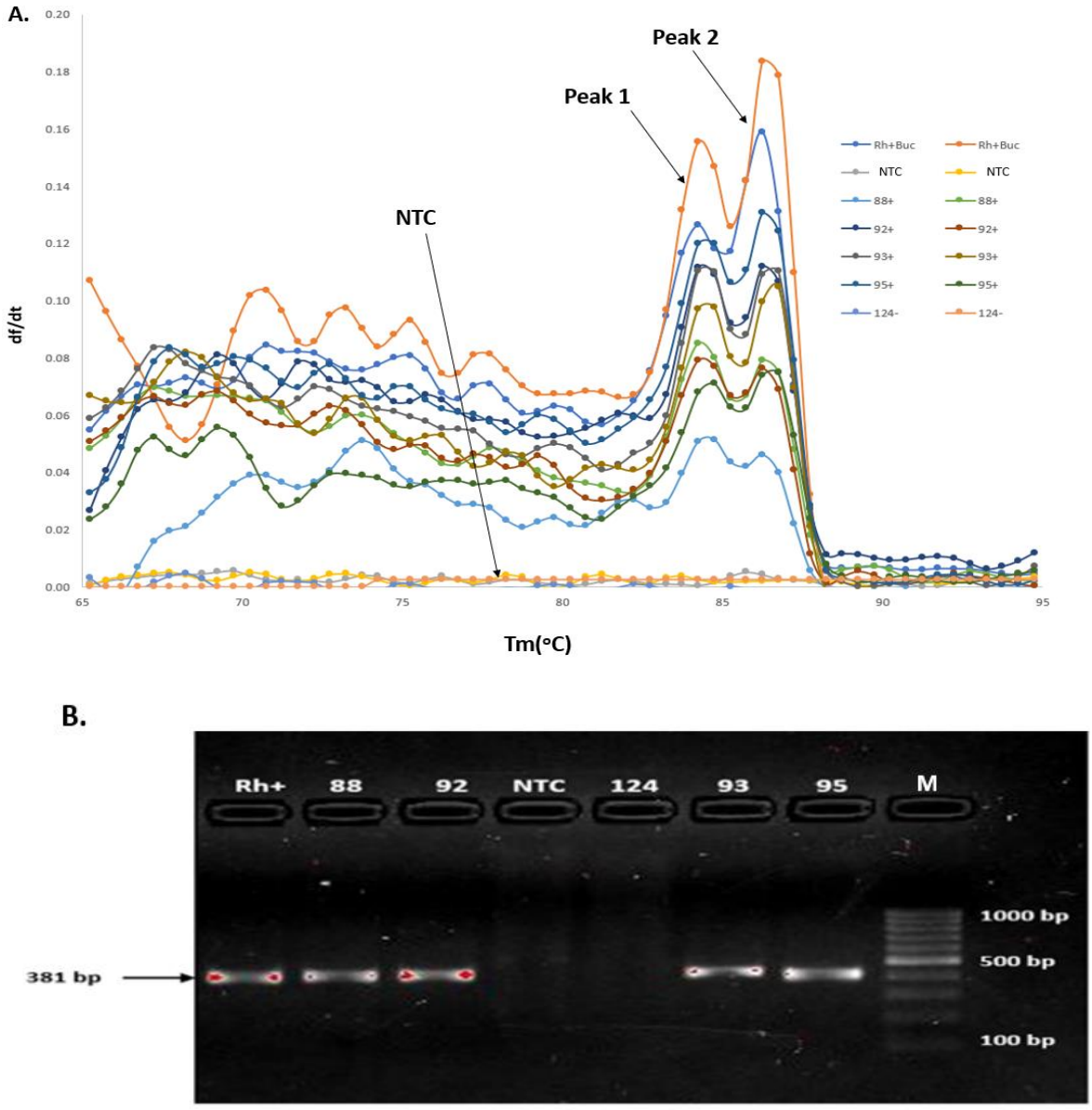


Figure 3.11 Melting curve temperature analysis versus gel electrophoresis for *RHD* exon 1.

Shows results for representative samples (n = 5). All samples were run in duplicates with a positive control and non-template control (NTC). In (A) y axis is the intensity change in a given interval from fluorescence derivative (df) in relation to temperature derivative (dt) df/dt and x axis is the temperature in ^{o}C. Two peaks were observed in 4 samples, first peak (83.50°C-84.0°C) and second peak (84.25°C-84.50°C), indicating *RHD* exon 1 amplification with possible second amplicon (contamination), whereas no peak was observed in the no template control NTC and sample 124 (A). DNA ladder marked as (M). RhD positive control showed specific PCR product for the *RHD* exon 1 *(lane* marked Rh+, 381 bp). A negative NTC showed no product *(lane* marked NTC). All representative *RHD* exon 1 positive samples 88, 92, 93 and 95 showed 381 bp product compared to peaks presence in melting analysis. Negative sample 124 *(lane*s 124) showed no *RHD* exon 1, no peak in melting curve

and thus a negative genotyping result (B). correlation between two peaks in melting curve (A) and one band in gel view (B) rule out contamination.

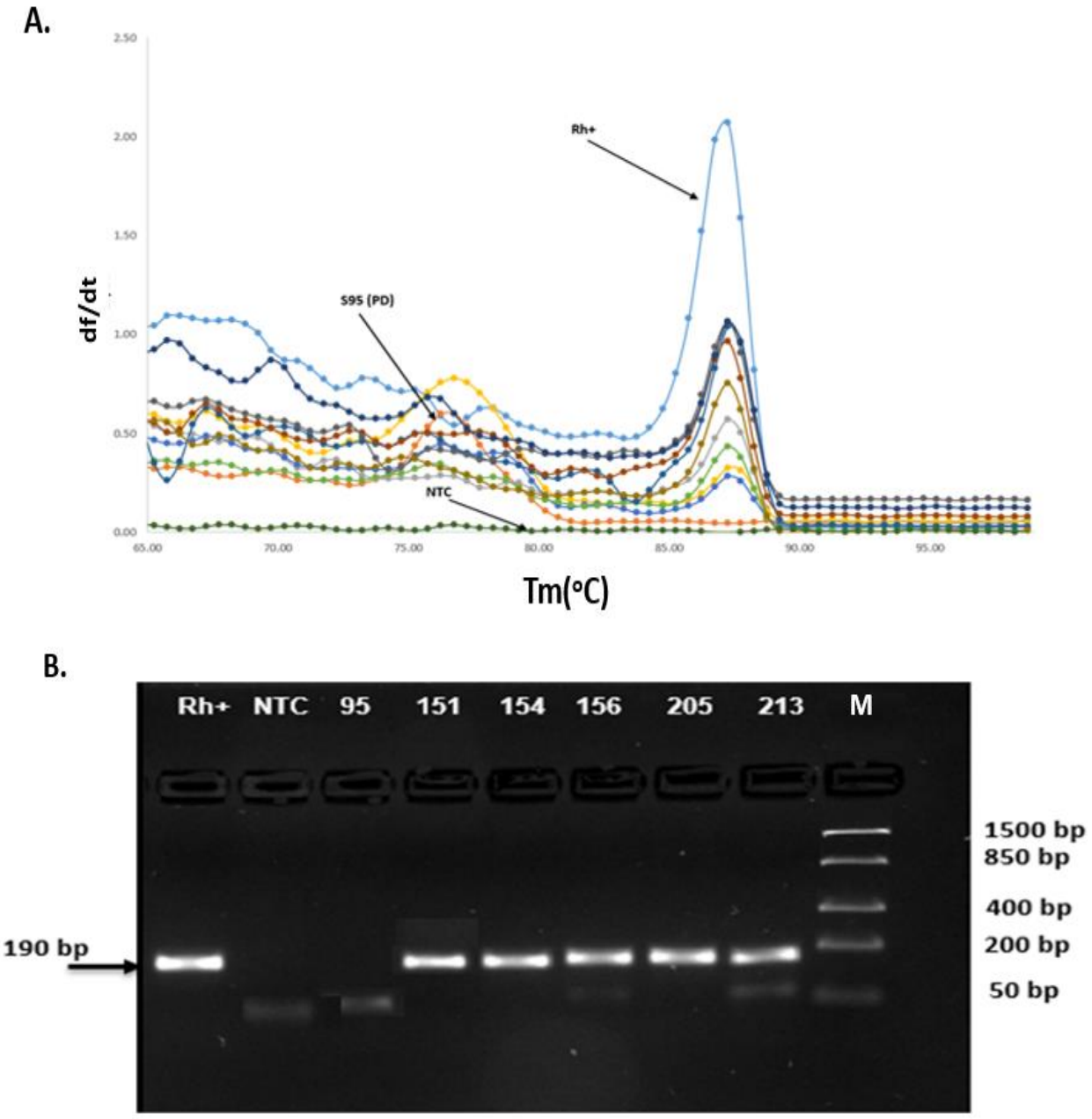


Figure 3.12 Melting curve temperature analysis versus gel electrophoresis for *RHD* exon 2.

Shows results for representative samples (n = 6). The melting curve analysis showed one peak for all representative positive samples (Rh+, 151, 154, 156, 205 and 213), between 87.10°C and 87.50°C. Sample S95 showed primer dimer (PD) peak at 76.0°C (A). Rh positive control showed one band of 190 bp and NTC showed no band. All representative positive samples, showed band of 190 bp band (samples 151, 154, 156, 205 and 213) that reflects the melting curve between 87.10 and 87.50, whereas negative sample 95 showed no

band at 190 bp but a band under 50 bp that suggests primer dimer (B). DNA ladder marked as (M).

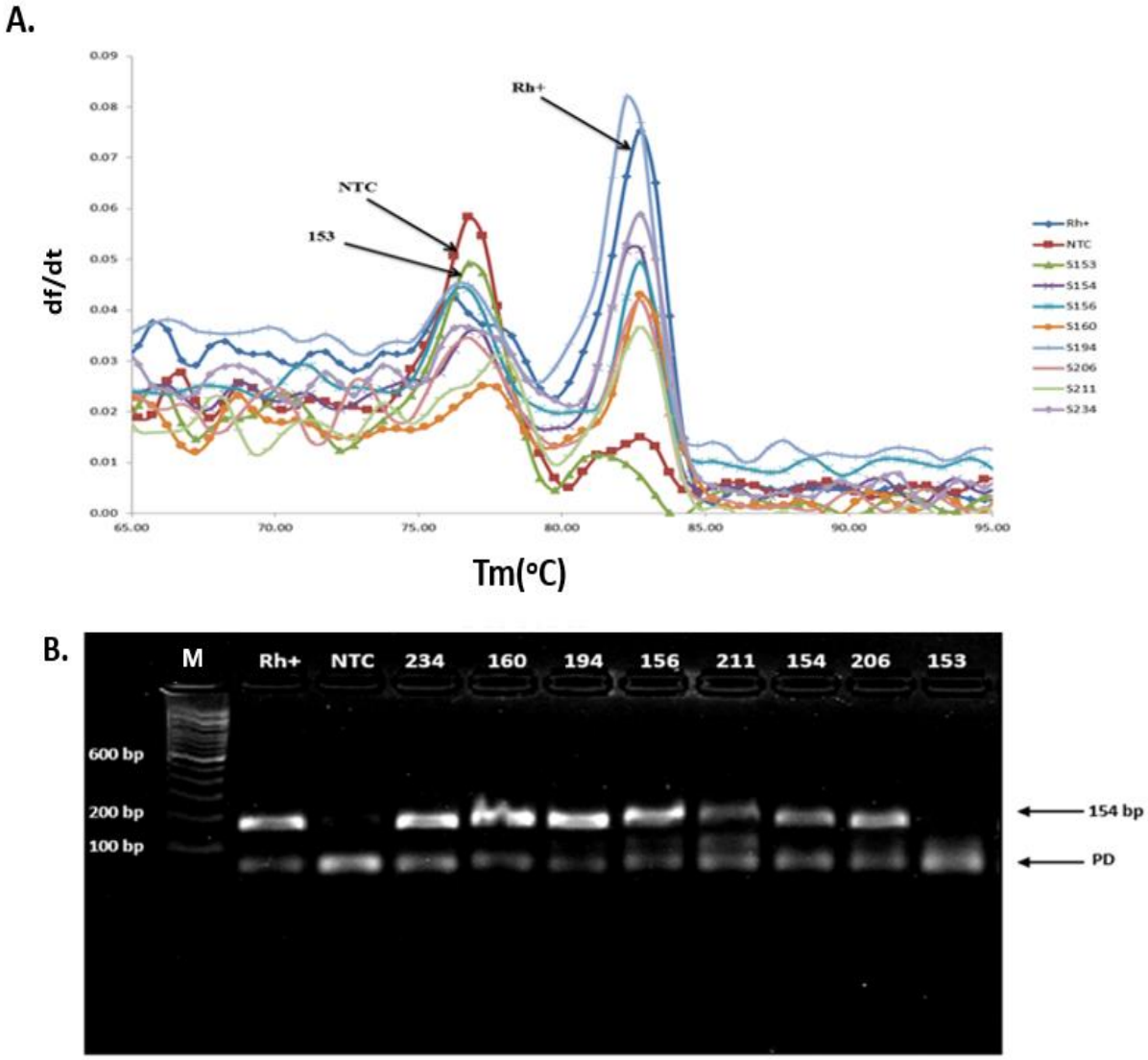


Figure 3.13 Melting curve temperature analysis versus gel electrophoresis for *RHD* exon 3.

Shows results for representative samples (*n* = 9). Rh positive control showed two peaks at 82.75°C and primer dimer at 76.0°C and NTC showed a peak at 76.75°C indicating primer dimer (A). All representative positive samples (S154, S156, S160, S206, S211 and S234) showed two peaks, peak one between 82.25°C and 82.75°C and peak two as primer dimer whereas, negative samples (S153) showed primer dimer (PD) peak at 76.75°C (A). Rh positive control showed two bands; first as desired 190 bp and second < 50 bp (PD) and NTC showed one band as well (PD) (B). All representative positive samples, showed two bands of 190 bp band PD (samples 234, 160, 194, 156, 211, 154 and 206), whereas negative sample (153) showed one PD band (B). DNA ladder marked as (M). Product size 190 bp showed as a

peak between 82.25°C and 82.75°C in melting curve graph, whereas, PD under 50 bp is a primer dimer indicated as a peak between 76.0°C and 76.75°C.

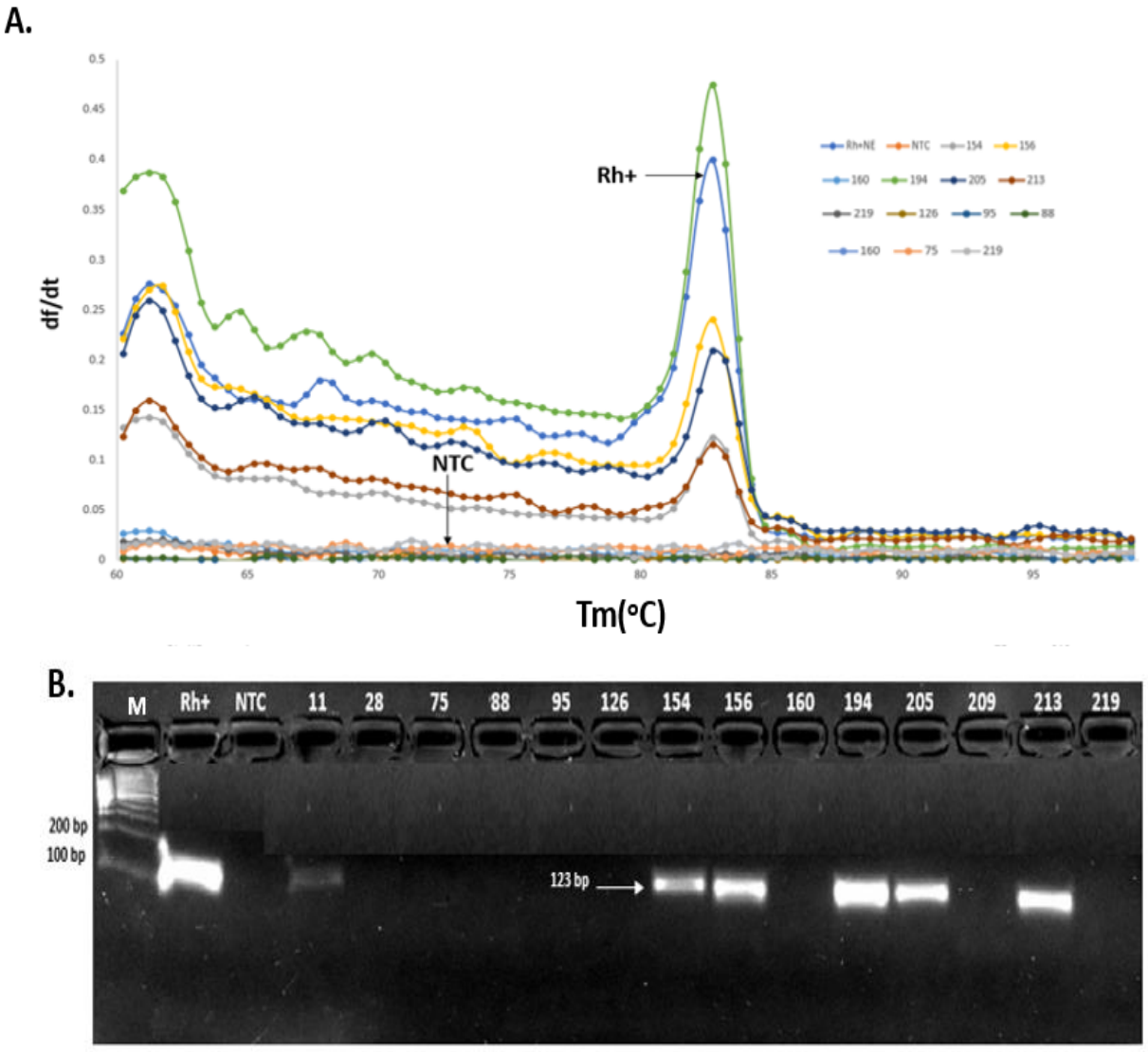


Figure 3.14 Melting curve temperature analysis versus gel electrophoresis for *RHD* exon 4.

Shows results for representative samples (*n* = 15). Rh positive control showed one peak at 82.75°C whereas NTC showed no peak (A). All representative positive samples (S154, S156, S194, S205 and S213) showed melting curve peak at 82.75°C, whereas negative samples showed no peak (S28, S75, S88, S95, S126, S160, S209 and S219) (A). Rh positive control showed one band of 123 bp and NTC showed no band (B). All representative positive samples, showed a band of 123 bp (samples 154, 156, 194, 205 and 213) (B) that reflect the only peak at 82.75°C in melting curve analysis, whereas negative samples 28, 75, 88, 95, 126, 160, 209 and 219 showed no band. DNA ladder marked as (M)

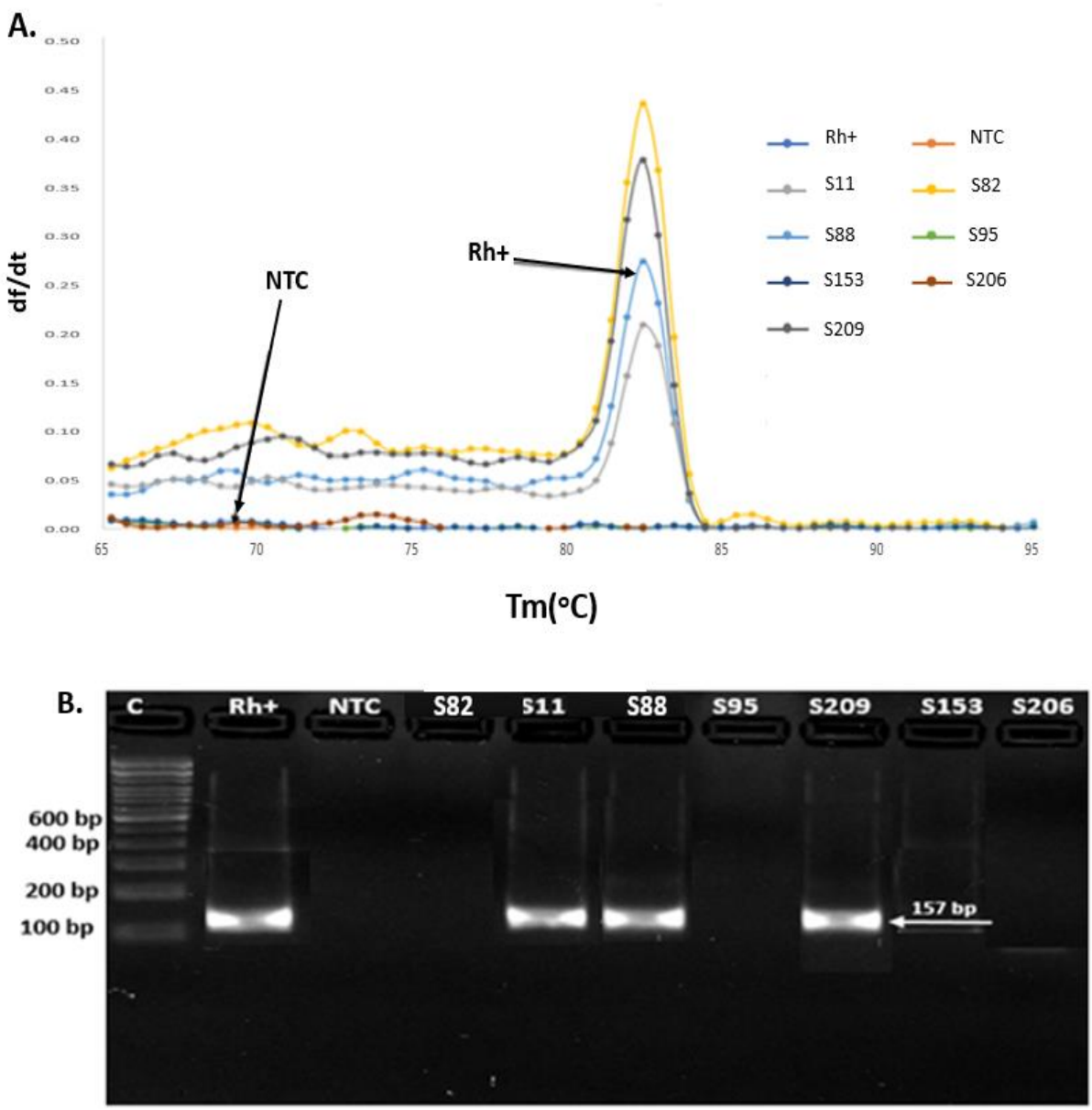


Figure 3.15 Melting curve temperature analysis for *RHD* exon 5.

Shows results for representative samples (*n* = 8). Rh positive control showed one peak at 82.25℃ whereas NTC showed no peak (A). All representative positive samples showed melting curve temperature at 82.25℃ (S11, S88 and S209), whereas negative samples showed no peak (S82, S95, S153 and S206) (A). Rh positive control showed one band of 157 bp and NTC showed no band (B). All representative positive samples, showed a band of 157 bp samples S11, S88, and S209) that reflect the only peak at 82.25℃ in melting curve graph, whereas negative samples S82, S95, S153 and S206) showed no band (B). DNA ladder marked as (C)

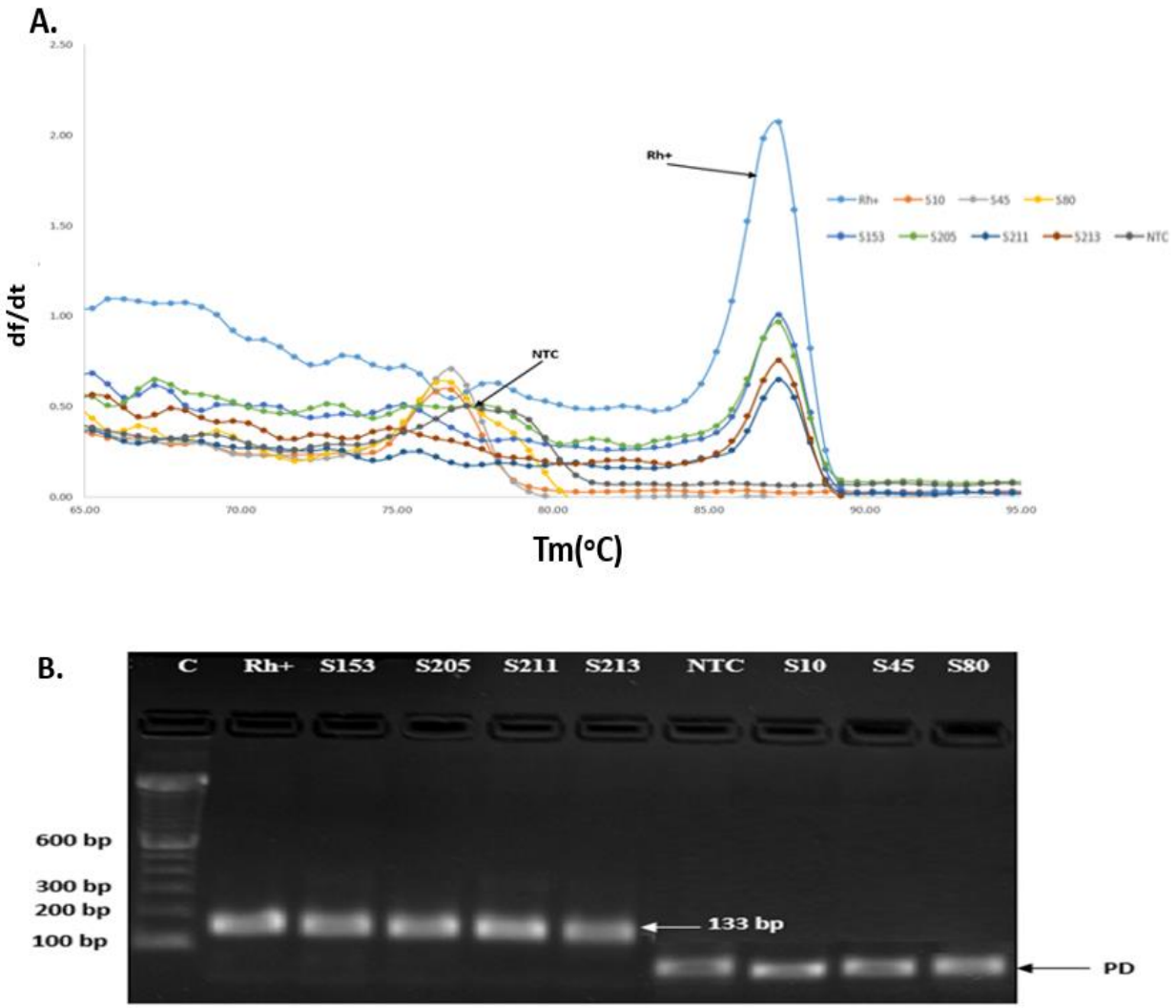


Figure 3.16 Melting curve temperature analysis for *RHD* exon 6.

Shows results for representative samples (*n* = 8). Rh positive control showed one peak at 87.25℃ whereas NTC showed a peak at 76.25℃ (A). The melting curve analysis also showed one peak at 87.25℃ for all representative positive samples (S153, S205, S211 and S213) suggesting existence of RHD exon 6, whereas negative samples showed a peak at 76.25℃ (S10, S45 and S80) that suggests primer dimmer (A). In gel view, Rh positive control showed a band of 133 bp, whereas NTC showed one band <50 bp (primer dimer - PD) (B). All representative positive samples, showed single 133 bp band (samples S153, S205, S211 and S213), whereas negative samples S10, S45 and S80 showed one PD band (B). DNA ladder marked as (C). Product size 133 bp showed as a peak of 87.25°C in melting curve graph, whereas, PD under 50 bp is a primer dimer indicated as a peak between 76.25°C.

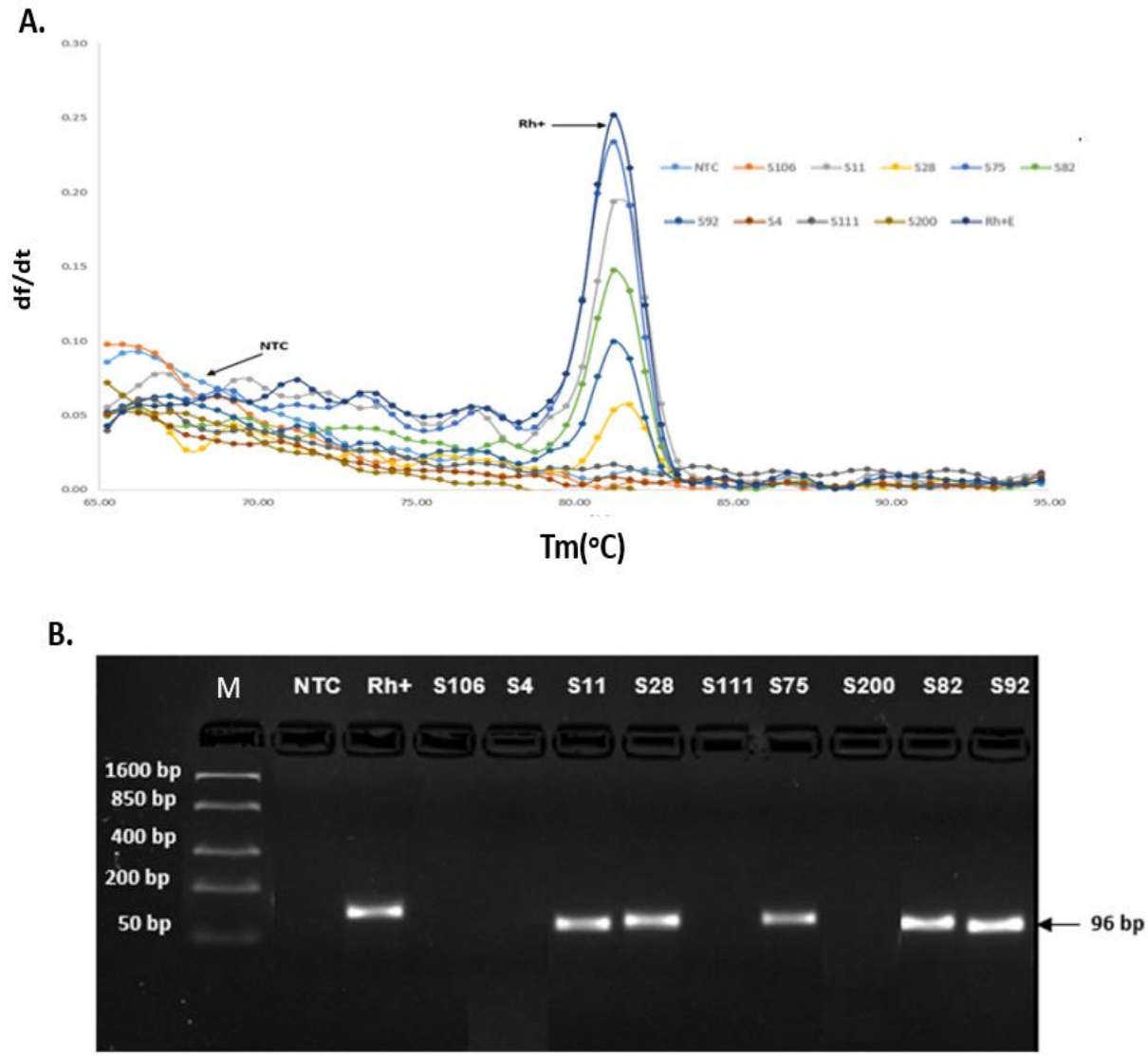


Figure 3.17 Melting curve temperature analysis versus gel electrophoresis for *RHD* exon 7 using SYBR Green chemistry.

Shows results for representative samples (*n* = 10). Rh positive control showed one peak at 81.25℃ whereas NTC showed no peak (A). The melting curve analysis also showed one peak between 81.25℃ and 81.75℃ for all representative positive samples (samples S11, S28, S75, S82 and S92) which suggests amplification of *RHD* exon 7, whereas negative samples showed no peak (samples S106, S4, S111 and S200) which suggests absence of *RHD* exon 7 (A). Rh positive control showed one band of 96 bp and NTC showed no band (B). All representative positive samples, showed a band of 96 bp (samples S11, S, 28, S75, S82, and S292) that reflect the only peak between 81.25℃ and 81.75℃ in melting curve graph confirming the presence of *RHD* exon 7 specific amplicon, whereas negative samples S106, S4, S111 and S200 showed no band (B). DNA ladder marked as (M).

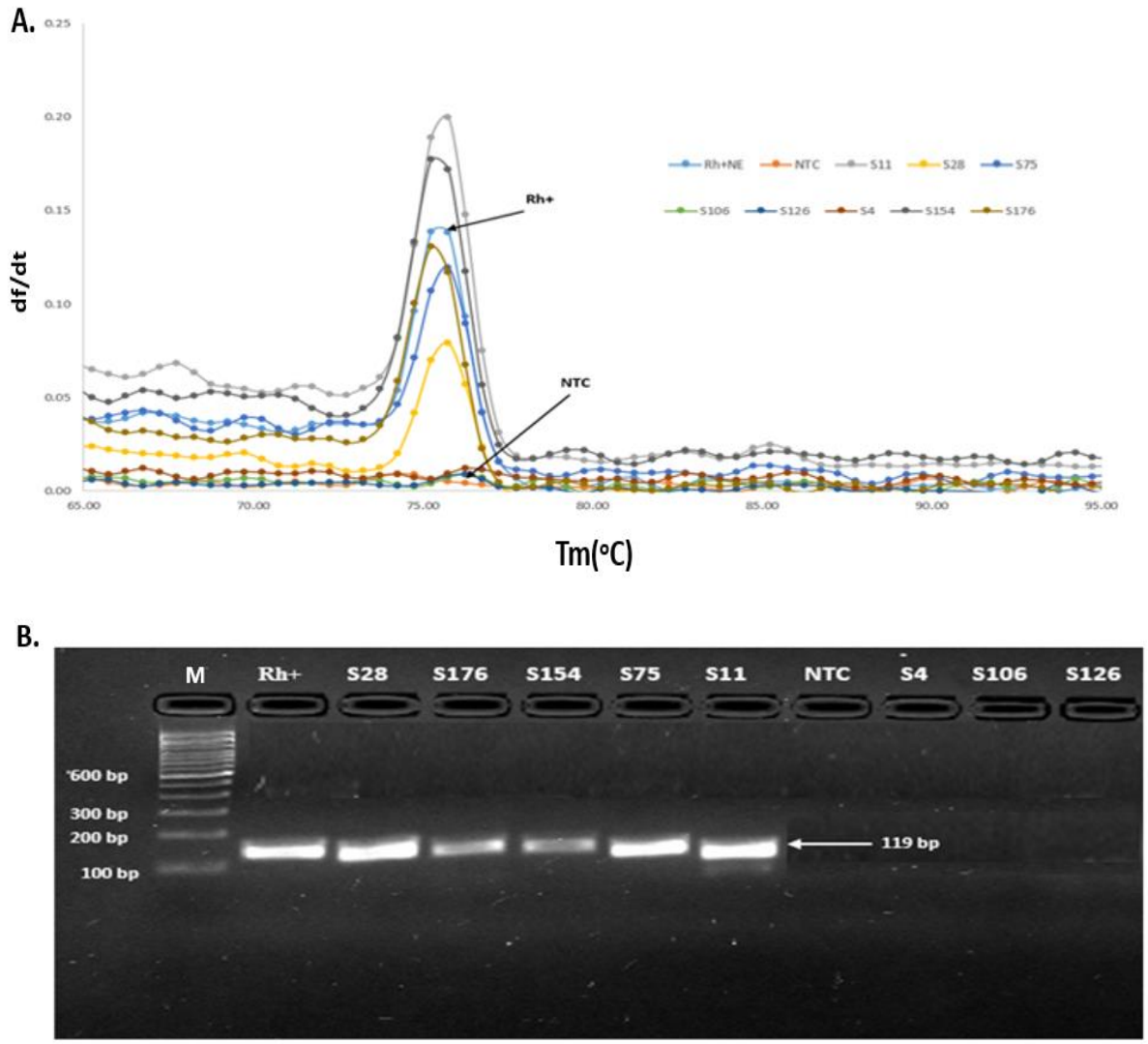


Figure 3.18 Melting curve temperature analysis versus gel electrophoresis for *RHD* exon 9.

Shows results for representative samples (*n* = 9). Rh positive control showed one peak at 75.75℃ whereas NTC showed no peak (A). The melting curve analysis also showed one peak between 75.25℃ and 75.75℃ for all representative positive samples (S11, S154, S176, S75 and S28) suggesting existence of *RHD* exon 9, whereas negative samples showed no peak (S4, S106, and S126) suggesting *RHD* exon 9 absence (A). Rh positive control showed one band of 119 bp and NTC showed no band (B). All representative positive samples, showed a band of 119 bp (samples S28, S176, S154, S75 and S11) that reflect the only peak between 75.25℃ and 75.75℃ in melting curve graph confirming the presence of *RHD* exon 9 specific amplicon, whereas negative samples S106, S4, and S126 showed no band (B). DNA ladder marked as (M).

3.7.2 *RHD* exons and possible allele determination

Based on singleplex real-time PCR and primers used in Table 2.1, melting curve analysis with gel electrophoresis for *RHD* exons 1 through 7 and *RHD* exon 9 showed three patterns (Table 3.7). Two samples showed DFR partial type of D. Six samples were positive for *RHD* exons 1, 2, 3, 6, 7 and 9 and negative for exons 4 and 5 suggesting the presence of *RHD-CE (4-5)-D* hybrid gene. One sample amplified all *RHD* exons except exons 3 through 5 suggesting *RHD-CE(3-5)-D* hybrid gene. Nine samples amplified all *RHD* exons except exon 5 suggesting homozygous *RHDΨ* gene or DVa or heterozygous *RHDΨ* / DVa presence. One sample showed *RHD-CE(3-6)-D* and one sample showed amplification of all *RHD* exons suggesting weak D. All the 8 samples that showed hybrid gene expressed C antigen. Three samples were inconclusive. Table 3.7 and Table 3.8 shows the different *RHD* alleles noted.

Table 3.7 Molecular background pf D negative Omani cohort by singleplex real-time PCR results with different exon specific primers (sample wise).

Phenotype	Sample	Ex 1	Ex 2	Ex 3	Ex 4	Int 4	Ex 5	Ex 6	Ex 7	Ex 8	Ex 9	Molecular background
ddCcee	11	+	+	+	**0**	**0**	+	+	+	NT	+	*DFR*
ddCcee	28	+	+	+	**0**	**0**	**0**	+	+	NT	+	*RHD-CE(4-5)-D*
ddCcee	75	+	+	+	**0**	**0**	**0**	+	+	NT	+	*RHD-CE(4-5)-D*
ddCcee	82	+	+	+	+	+	+[a]	+	+	NT	+	*Weak D*
ddccee	88	+	+	+	**0**	+	+	+	+	NT	+	*DFR*
ddccee	92	+	+	+	+	+	**0**	+	+	NT	+	*RHDΨ*
ddccee	93	+	+	+	+	+	**0**	+	+	NT	+	*RHDΨ*
ddCcee	95	+	**0**	+	**0**	**0**	**0**	+	+	NT	+	*?*
ddccee	106	+	+	+	**0**	**0**	**0**	**?**	**0**	NT	**0**	*?*
ddccee	126	+	+	+	**0**	**0**	**0**	+	+	NT	**0**	*?*
ddCcee	151	+	+	+	**0**	**0**	**0**	+	+	NT	**0**	*RHD-CE(4-5)-D*
ddCcee	153	+	+	**0**	**0**	**0**	**0**	+	+	NT	+	*RHD-CE(3-5)-D*
ddccee	154	+	+	+	+	+	**0**	+	+	NT	+	*RHDΨ/DVa*
ddccee	156	+	+	+	+	+	**0**	+	+	NT	+	*RHDΨ/DVa*
ddccee	160	+	+	+	**0**	**0**	+	+	+	NT	+	*DFR*
ddCcee	176	+	+	+	+	+	**0**	+	+	NT	+	*RHDΨ/DVa*
ddccee	194	+	+	+	+	+	**0**	+	+	NT	+	*RHDΨ/DVa*
ddccee	205	+	+	+	+	+	**0**	+	+	NT	+	*RHDΨ/DVa*
ddccee	206	+	+	+	+	+	**0**	+	+	NT	+	*RHDΨ/DVa*
ddCcee	211	+	+	+	**0**	**0**	**0**	+	+	NT	+	*RHD-CE(4-5)-D*
ddccee	213	+	+	+	+	+	**0**	+	+	NT	+	*RHDΨ/DVa*
ddCcee	219	+	+	+	**0**	**0**	**0**	+	+	NT	+	*RHD-CE(4-5)-D*
ddCcee	230	+	+	+	**0**	**0**	**0**	**0**	+	NT	+	*DVI.2*
ddccEe	234	+	**0**	+	+	+	**0**	+	+	NT	+	*RHDΨ/DIIIb*

+: Positive **0:** Negative **NT:** Not tested **Ex:** *RHD* exon **Int:** *RHD* Intron

? : Inconclusive a: No amplification with primers R648 and Rex5AD2 for SYBR Green, but amplified with TaqMan primers RHDex5F and RHDex5R

Table 3.8 Molecular background of D negative Omani cohort by singleplex real-time PCR results with different exon specific primers (phenotype wise).

Phenotype	Number (*n*)	Ex 1	Ex 2	Ex 3	Ex 4	Int 4	Ex 5	Ex 6	Ex 7	Ex 8	Ex 9	Molecular background
ddCcee	1	+	+	+	**0**	**0**	+	+	+	NT	+	*DFR*
ddccee	2	+	+	+	**0**	**0**	+	+	+	NT	+	
ddCcee	5	+	+	+	**0**	**0**	**0**	+	+	NT	+	*RHD-CE(4-5)-D*
ddccee	8	+	+	+	+	+	**0**	+	+	NT	+	*RHDΨ*
ddCcee	1	+	+	+	+	+	**0**	+	+	NT	+	
ddCcee	1	+	+	+	+	+	+	+	+	NT	+	*Weak D*
ddCcee	1	+	**0**	+	**0**	**0**	**0**	+	+	NT	+	*?*
ddCcee	1	+	+	**0**	**0**	**0**	**0**	+	+	NT	+	*RHD-CE(3-5)-D*
ddccee	1	+	+	+	**0**	**0**	**0**	**?**	**0**	NT	**0**	*?*
ddccee	1	+	+	+	**0**	**0**	**0**	+	+	NT	**0**	*?*
ddCcee	1	+	+	+	**0**	**0**	**0**	+	+	NT	+	*DV1.2*
ddccEe	1	+	**0**	+	+	+	**0**	+	+	NT	+	*RHDΨ/DIIIb*
Total	24											

+: Positive **0:** Negative **NT:** Not tested **Ex:** *RHD* exon **Int:** *RHD* Intron

? : Inconclusive

3.7.3 Determination of mutation in *RHD* coding sequence

All the 24 samples listed in Table 3.7 were sequenced for *RHD* exons 1 through 7 and *RHD* exon 9 to identify any mutations within the coding sequence of *RHD* gene using the primers stated in the Table 2.1. In addition, sequencing also confirmed the amplification of the target *RHD* exon and eliminated the possibility of *RHCE* gene interference and false positive results. None of the positive samples for *RHD* exon 1, exon 2, exon 5, exon 7 and exon 10 showed any SNP, whereas exons 3, 4, 6 and 9 showed mutation in coding sequence with few at intronic region. *RHD* exon 4 also showed (IVS3-19 dupl 37) typical for the presence of *RHDΨ* pseudogene in 10 samples.

For *RHD* exon 3, out of 24 positive samples, two samples (75 and 211) showed combined mutations c.455A>C (rs_17418085) and IVS3+9 T>C (rs_113795383) in the coding sequence and intronic region respectively using forward primer (Figure 3.19). However, reverse primer could not reconfirm c.455A>C mutation as it showed a normal A nucleotide in the same position. This would suggest heterozygous mutation but allelic dropout due to either degraded DNA or less DNA quantity in the sample. Reverse primer could not even reconfirm IVS3+9 T>C mutation as the region where it is located was very near to the reverse primer and Sanger sequencing result could not cover that specific area (Figure 3.20).

Intron 3/Exon 4 specific primers for *RHDΨ* showed 10 samples (Samples 92, 93, 154, 156, 176, 194, 205, 206, 213 and 234) having 37 bp insertion with c.609 G>A mutation. This suggests and confirms the presence of African *RHD* genotype responsible for D negative phenotype (Figure 3.21).

For exon 6, out of 24 positive samples, a total of 11 samples showed a novel heterozygous mutation (c.928 A>G) using forward primer (Figure 3.22). NCBI BLAST confirmed the mutation at *RHD* gene region (Figure 3.23). Reverse primer could not

reconfirm this mutation as the SNP location was within the reverse primer sequence and Sanger sequencing result using this reverse primer could not cover that specific area for sequencing (Figure 3.24).

For exon 9, out of 24 positive samples, two samples (S82 and S230) showed rs_28669938 heterozygous mutation (IVS8-31 T>C) in intron 8 region using reverse primer (Figure 3.25 A and B). NCBI BLAST for the sequenced DNA demonstrated replacement of nucleotide T with C in these samples compared to wild type *RHD* gene (Figure 3.25 C). Forward primer could not reconfirm mutation IVS8-31 T>C as the good sequencing data analysis started at base 26 using this primer and IVS8-31 T>C SNP is at base 22 which hindered the sequencing result.

Based on singleplex real-time PCR results stated in sections 3.6 and 3.7.2 and sanger sequencing results in this section, molecular background of serological D negative was divided into four general categories. These are; absence of hybrid and pseudogene (*del/del*), presence of pseudogene and absence of hybrid (*RHDΨ/RHDΨ or RHDΨ/del*), presence of hybrid and absence of pseudogene (*RHD-CE-D/RHD-CE-D* or *RHD-CE-D/del*) and presence of hybrid and pseudogene (*RHDΨ/RHD-CE-D*) summarised in Table 3.9.

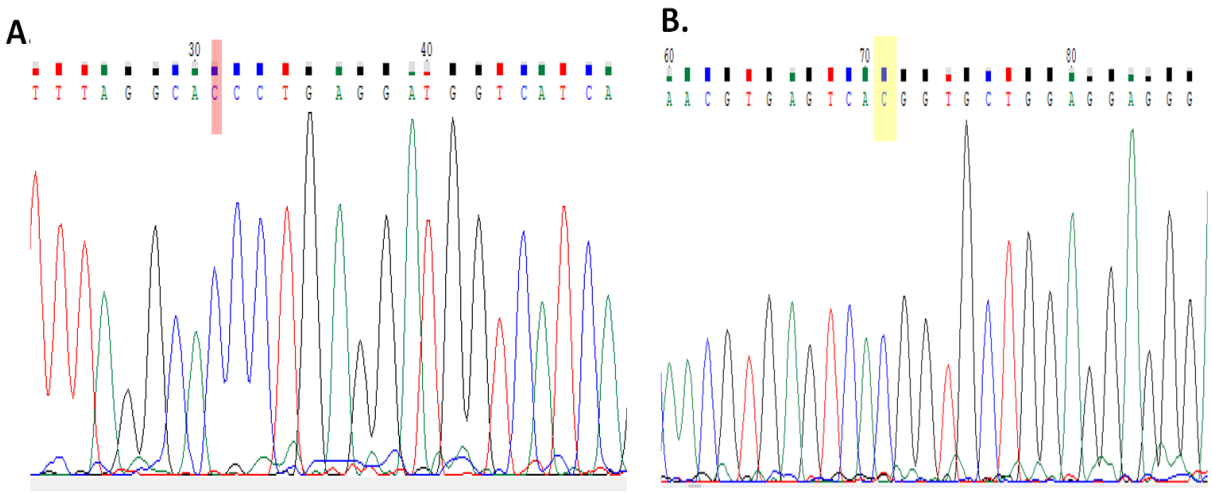


Figure 3.19 Validation of *RHD* exon 3 SNPs using DNA sequencing

Representative Sanger sequencing images for PCR products obtained from two samples (S75 and S211) showing (A) the c.455 A>C SNP and (B) the IVS3+9 T>C SNPs in *RHD* exon/intron 3. NCBI blast was used to perform sequence alignment between each primer pair sequencing result and human reference gene as seen in (C). The reference human genome (*GRCh38*) for *RHD* exon 3 showing the c.455 A>C SNP (highlighted in pink) and IVS3+9 T>C SNP (highlighted in yellow).

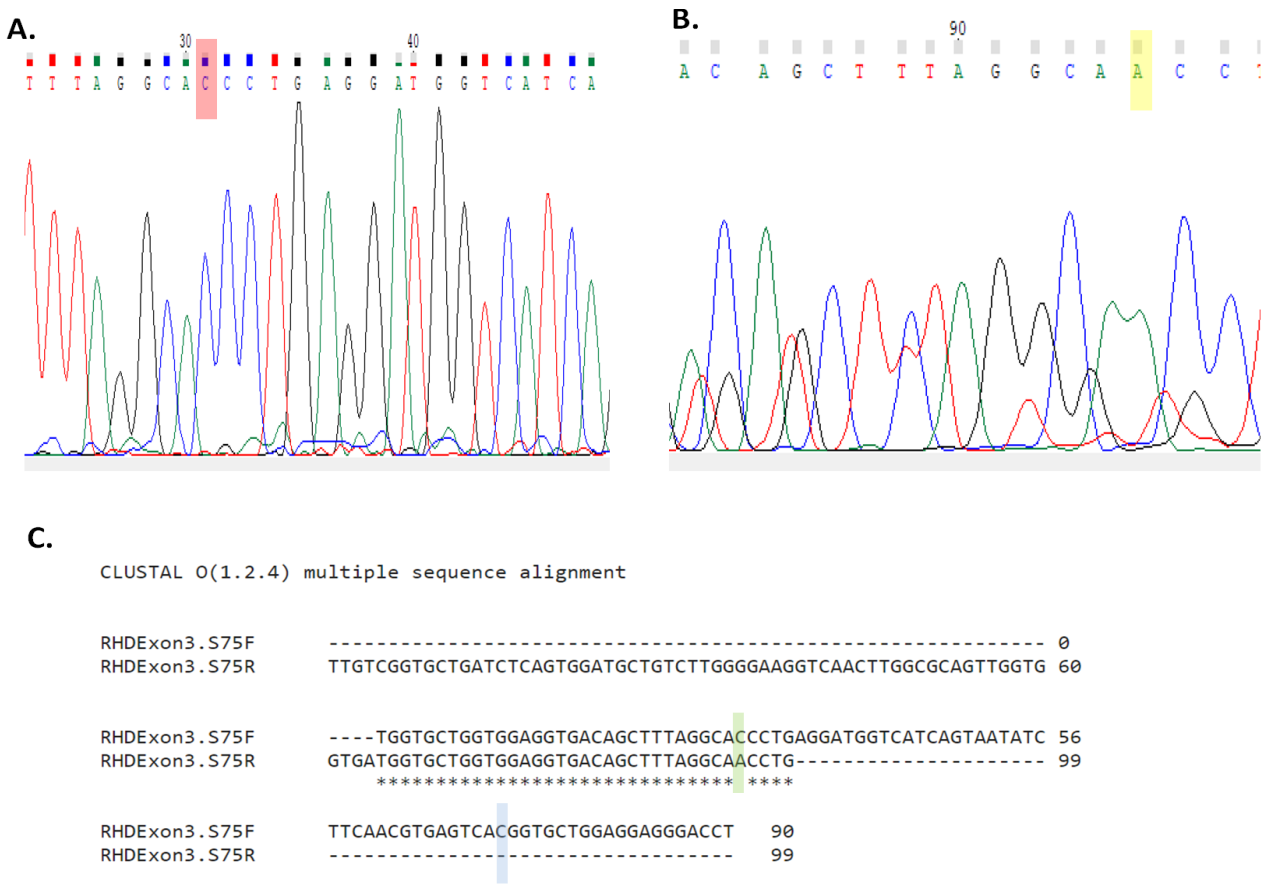


Figure 3.20 Combined SNP (c.455 A>C) and (IVS3+9 T>C) in *RHD* exon

Representative Sanger sequencing images for PCR products obtained from two samples (S75 and S211) showing homozygous mutation c.455 A>C highlighted in pink (A) with forward primer, whereas reverse primer showed wild type A nucleotide highlighted in yellow (B). Clustal Omega was used to perform sequence alignment between each primer pair sequencing result as seen in (C). Alignment of the sequencing for both primers showed reverse primer could not confirm forward primer result highlighted in green and could not cover the intronic region where second mutation IVS3+9 T>C is located and highlighted in blue (C).

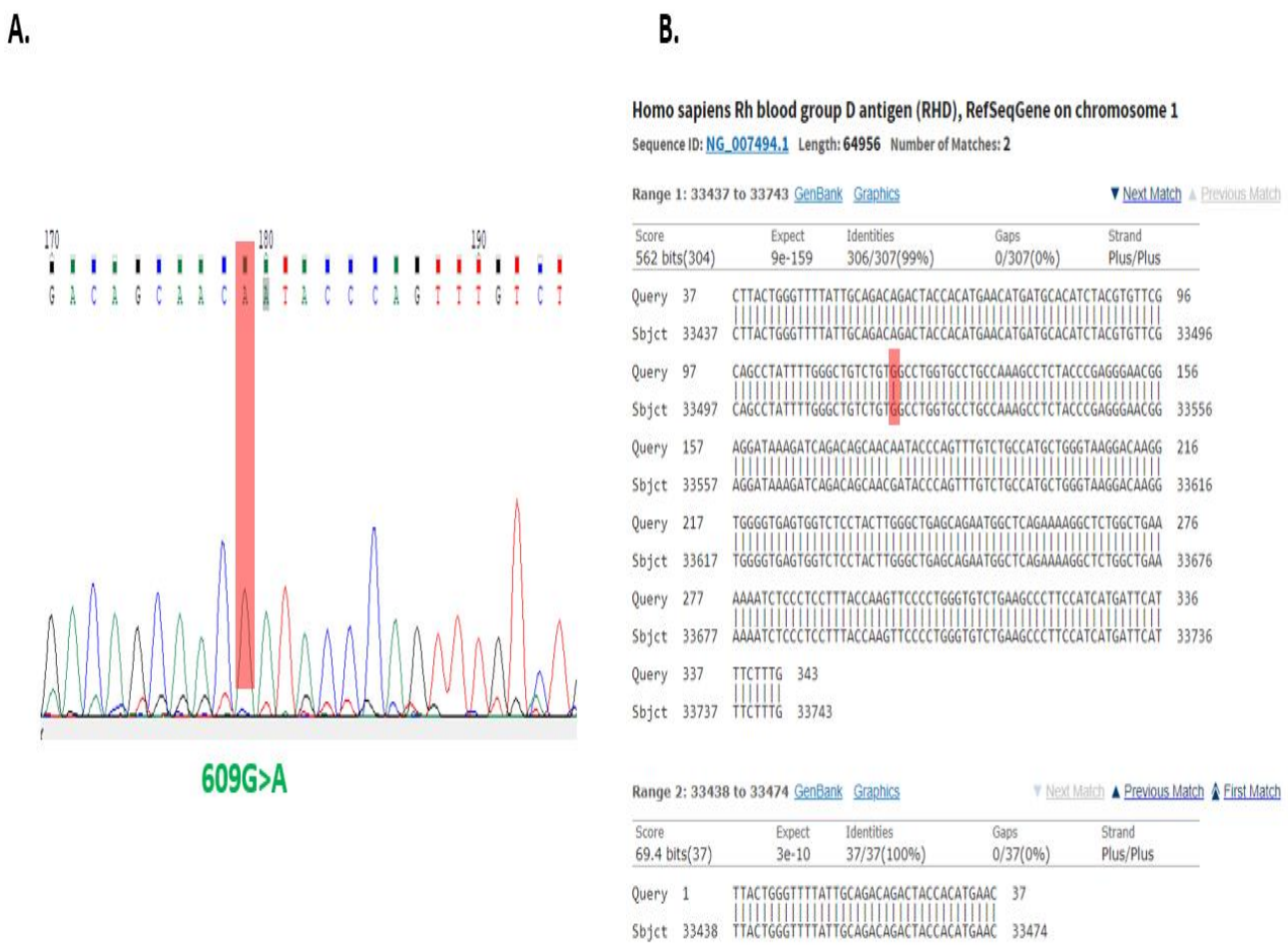


Figure 3.21 *RHDΨ* allele in Omani population

Representative image for *RHDΨ* allele found in Omani population. *RHD* exon 4 sequencing showed c.609G>A mutation (A). NCBI blast was used to perform sequence alignment between each primer pair sequencing result and human reference gene as seen in (B). The reference human genome (GRCh38) for *RHD* intron 3/exon 4 showing the 37 bp insertion and c.609G>A SNP highlighted in pink (B).

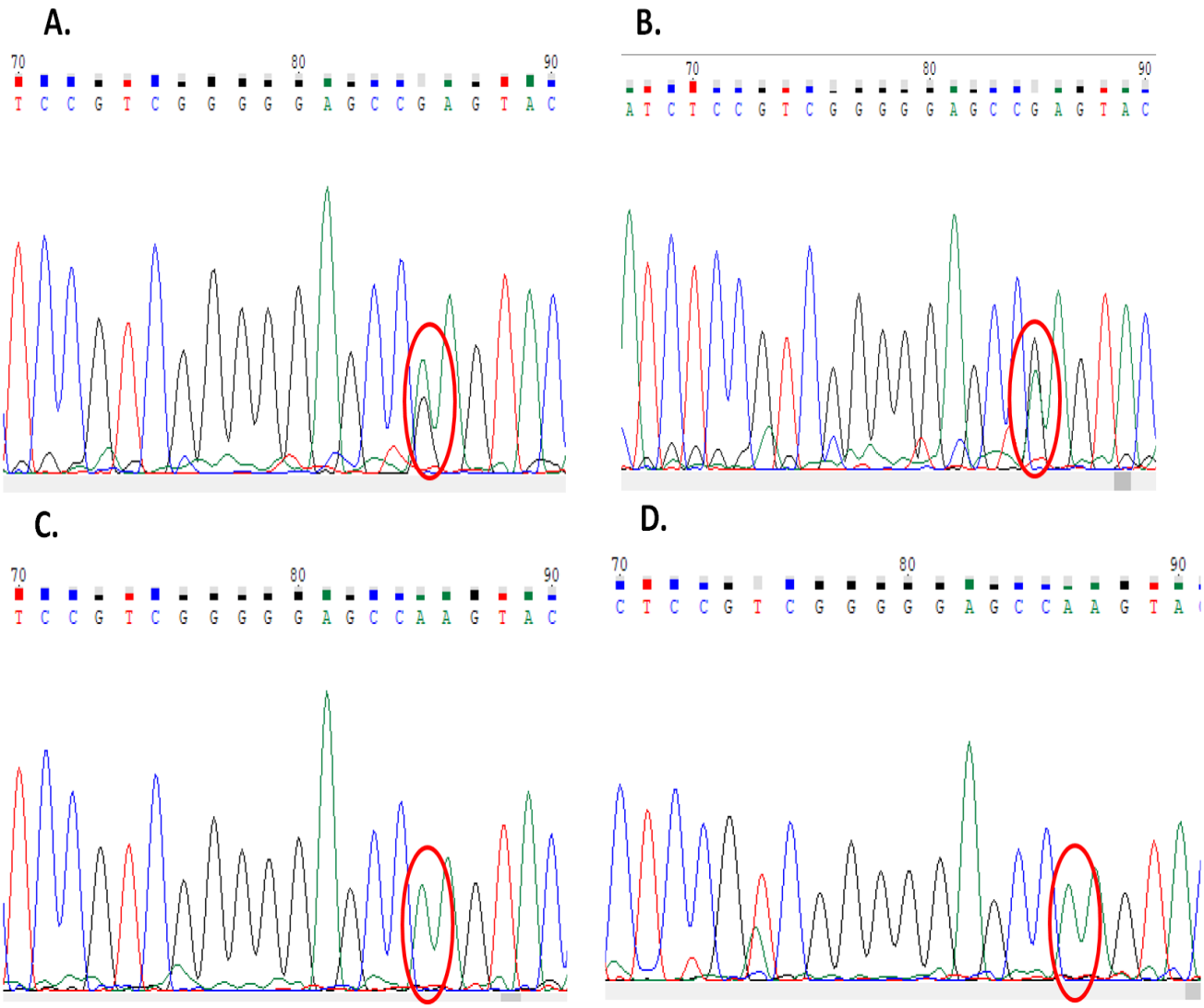


Figure 3.22 A novel heterozygous SNP (c.928 A>G) in *RHD* exon 6 in Omani population
Representative images for a novel heterozygous (c.928 A>G) and normal samples. Forward primer target sequence showed two peaks (for nucleotides A and G) that indicated a novel heterozygous (c.928 A>G) mutation (*n* = 11) circled red (A and B), whereas normal samples (*n* = 13) did show only one normal nucleotide A peak (C and D).

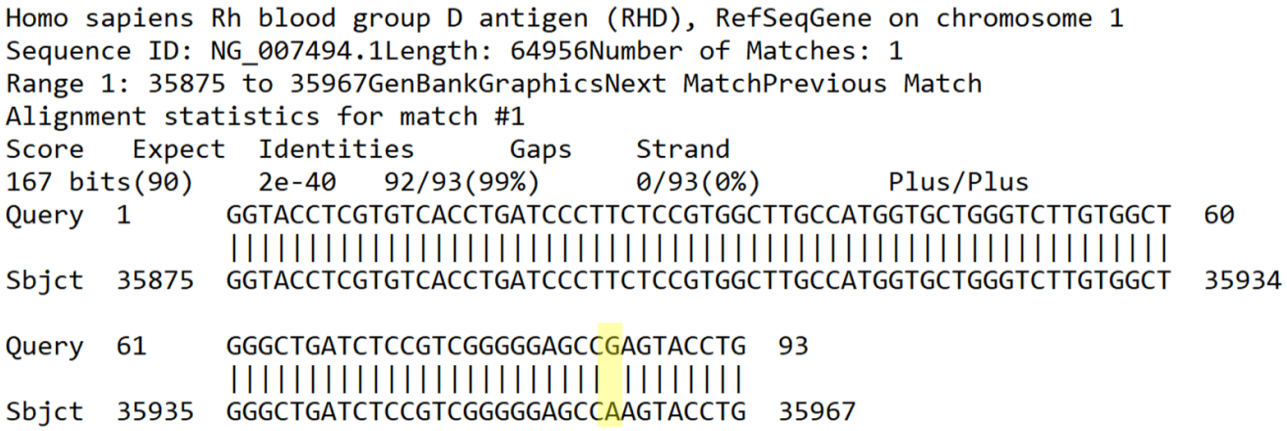
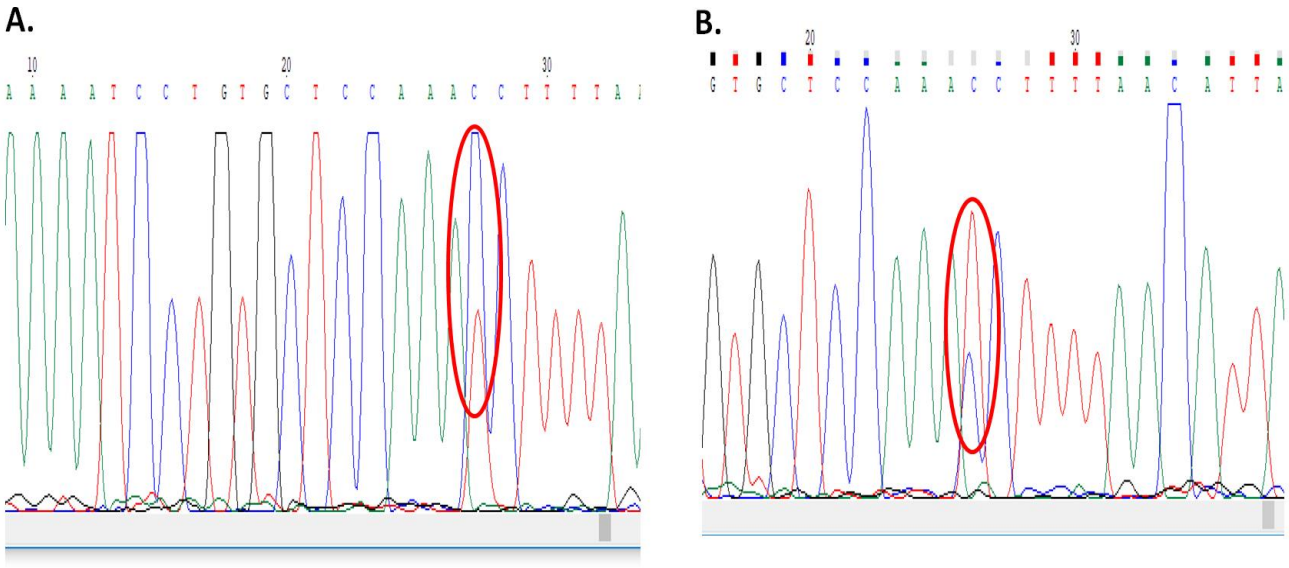


Figure 3.23 NCBI BLAST for the novel heterozygous mutation (c.928 A>G).

NCBI blast was used to perform sequence alignment between each primer pair sequencing result and human reference gene. The reference human genome (*GRCh38*) for *RHD* exon 6 showing (c.928A>G) highlighted in yellow.

Figure 3.24 Forward and reverse primers sequence alignment for *RHD* exon 6.

CLUSTAL O was used to perform sequence alignment between forward and reverse primer. Alignment of the sequencing for both primer sequences showed reverse primer could not confirm forward primer that showed novel mutation (c.928A>G) highlighted in yellow.



C.

```
Homo sapiens Rh blood group D antigen (RHD), RefSeqGene on chromosome 1
Sequence ID: NG_007494.1Length: 64956Number of Matches: 1
Range 1: 54343 to 54420GenBankGraphicsNext MatchPrevious Match
Alignment statistics for match #1
Score    Expect  Identities      Gaps    Strand
139 bits(75)    3e-32   77/78(99%)      0/78(0%)        Plus/Plus
Query  1      TGAGATTAAAAATCCTGTGCTCCAAACCTTTTAACATTAAATTATGCATTTAAACAGGTT  60
              ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  54343  TGAGATTAAAAATCCTGTGCTCCAAATCTTTTAACATTAAATTATGCATTTAAACAGGTT  54402

Query  61     TGCTCCTAAATCTTAAAA  78
              ||||||||||||||||||
Sbjct  54403  TGCTCCTAAATCTTAAAA  54420
```

Figure 3.25 Validation of *RHD* intron8/exon 9 SNPs using DNA sequencing

The figure shows SNP (IVS8-31 T>C) found on *RHD* intron 8/exon 9 for samples S82 (A) and S230 (B). Sanger sequencing result showed heterozygous mutations, C circled red for sample 82 (A) and sample 230 (B). NCBI blast was used to perform sequence alignment between each primer pair sequencing result and human reference gene as seen in C. The reference human genome (GRCh38) for *RHD* intron 8/exon 9 showing IVS8-31 T>C (highlighted in yellow) (C).

Table 3.9 Presence of the *RHD* deletion (del), *RHD* pseudogene (*RHDΨ*), and *RHD-CE-D* hybrid gene in the serological D negative Omani cohort

Phenotype	Sample	real-time PCR exon amplification with sequencing	Possible molecular background
ddCcee	11	No amplification of *RHD* exon 4 with c.928A>G heterozygous SNP in exon 6	*RHD-CE(4)-D/RHD-CE(4)-D*
ddCcee	28	No amplification of *RHD* exon 4 and 5 with c.928A>G heterozygous SNP in exon 6	*RHD-CE(4-5)-D/del* or *RHD-CE(4-5)-D/ RHD-CE(4-5)-D*
ddCcee	75	No amplification of *RHD* exon 4 and 5 Heterozygous SNP c.455A>C and IVS3+9 T>C in exon 3 & intron 3 respectively. Heterozygous SNP c.928A>G in exon 6	*RHD-CE(4-5)-D/del* or *RHD-CE(4-5)-D/ RHD-CE(4-5)-D*
ddCcee	82	Heterozygous (IVS8-31 T>C) in intron 8 region	*Weak D*
ddccee	88	No amplification of *RHD* exon 4	*RHD-CE(4)-D/del* or *RHD-CE(4)-D/ RHD-CE(4)-D*
ddccee	92	No amplification of *RHD* exon 5* with 37 bp insertion in *RHD* Intron3/exon4 and c.609 G>A mutation in exon 4. In addition, c.928A>G heterozygous SNP in exon 6	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	93	No amplification of *RHD* exon 5* with 37 bp insertion in *RHD* Intron3/exon4 and c.609 G>A mutation in exon 4	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddCcee	95	No amplification of *RHD* exons 2, 4 and 5	Inconclusive *RHD-CE-D /del* or *RHD-CE-D /RHD-CE-D*
ddccee	106	No amplification of *RHD* exons 4 through 7 and *RHD* exon 9	*RHD-CE(4-9)/del* or *RHD-CE(4-9)-D/RHD-CE(4-9)-D*
ddccee	126	No amplification of *RHD* exons 4, 5 and 9 with c.928A>G heterozygous SNP in exon 6	Inconclusive *RHD-CE-D /del* or *RHD-CE-D /RHD-CE-D*
ddCcee	151	No amplification of *RHD* exons 4, 5 and 9	Inconclusive *RHD-CE-D /del* or *RHD-CE-D /RHD-CE-D*

Phenotype	Sample ID	real-time PCR exon amplification with sequencing	Possible molecular background
ddCcee	153	No amplification of *RHD* exons 3 through 5	*RHD-CE(3-5_-D /del* or *RHD-CE(3-5)-D /RHD-CE(3-5)-D*
ddccee	154	No amplification of *RHD* exon 5* with 37 bp insertion in *RHD* Intron3/exon4 and c.609 G>A mutation in exon 4	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	156	No amplification of *RHD* exon 5* with 37 bp insertion in *RHD* Intron3/exon4 and c.609 G>A mutation in exon 4. In addition, c.928A>G heterozygous SNP in exon 6	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	160	No amplification of *RHD* exons 4 with c.928A>G heterozygous SNP in exon 6	*RHD-CE(4)-D/del* or *RHD-CE(4)-D/ RHD-CE(4)-D*
ddCcee	176	No amplification of *RHD* exon 5* with 37 bp insertion in *RHD* Intron3/exon4 and c.609 G>A mutation in exon 4	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	194	No amplification of *RHD* exon 5* with 37 bp insertion in *RHD* Intron3/exon4 and c.609 G>A mutation in exon 4	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	205	No amplification of *RHD* exon 5* with 37 bp insertion in *RHD* Intron3/exon4 and c.609 G>A mutation in exon 4. In addition, c.928A>G heterozygous SNP in exon 6	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	206	No amplification of *RHD* exon 5* with 37 bp insertion in *RHD* Intron3/exon4 and c.609 G>A mutation in exon 4	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddCcee	211	No amplification of *RHD* exon 4 and 5 Heterozygous SNP c.455A>C and IVS3+9 T>C in exon 3 & intron 3 respectively. Heterozygous SNP c.928A>G in exon 6	*RHD-CE(4-5)-D/del* or *RHD-CE(4-5)-D/ RHD-CE(4-5)-D*
ddccee	213	No amplification of *RHD* exon 5* with 37 bp insertion in *RHD* Intron3/exon4 and c.609 G>A mutation in exon 4. In addition, c.928A>G heterozygous SNP in exon 6	*RHDΨ/del* or *RHDΨ/ RHDΨ*

Phenotype	Sample	real-time PCR exon amplification with sequencing	Possible molecular background
ddCcee	219	No amplification of *RHD* exon 4 and 5	*RHD-CE(4-5)-D/del* or *RHD-CE(4-5)-D/ RHD-CE(4-5)-D*
ddCcee	230	No amplification of *RHD* exon 4 through 6. Heterozygous (IVS8-31 T>C) in intron 8 region	*RHD-CE(4-6)-D/del or RHD-CE(4-6)-D/ RHD-CE(4-6)-D*
ddccEe	234	No amplification of *RHD* exon 2 and 5*. with 37 bp insertion in *RHD* Intron3/exon4 and c.609 G>A mutation in exon 4. In addition, c.928A>G heterozygous SNP in exon 6	Hemizygous *RHD-CE(2)-D and RHDΨ/del*

Detection of Exons 4 and 5 was carried out according to (Singleton et al., 2000) and (Maaskant-van Wijk et al., 1998) respectively. *RHD* exon 4 and 5 positivity and negativity distinguish *RHD* from *RHD* pseudogene (*RHDΨ*).

* No amplification of *RHD* exon 5: The forward primer 3' specific for wild type c.654 in exon 5 and do not amplify mutation G>C (M218I) associated with *RHDΨ*

3.7.4 Further determination of *RHD* allele by BAGene kits for Rh

The RhCE serological phenotyping and *RHD* molecular background results indicated in Table 3.7 was further reconfirmed by SSP-PCR BAGene partial D-TYPE and BAGene RH-TYPE. Testing samples with BAGene kits further explored the type of *RHD* alleles involved in Omani population.

Singleplex real-time PCR results in Table 3.7 were used to support few inconclusive results. The molecular background of serological D negative samples that possess *RHD* exon 10 (n = 24) were re-evaluated D classified as true serological D negative or false serological D negative. The true serological D negative indicated two patterns that are; presence of 37 bp insertion in exon 4 that is unique for *RHDΨ* (n = 8, representative Figure 3.26) and presence of *RHDΨ* in cis to partial D without *RHCE* hybrid (n = 2, Figure 3.27 & Figure 3.28). D positive (serological false D negative, n = 9) indicated patterns that are; *RHD-CE-D^s/RHD-CE(4)-D* (n = 1, Figure 3.29), *RHD-CE(5)-D* in cis to *weak D DAR2.00* (n = 1, Figure 3.30), partial D without *RHCE* hybrid *(DIIIb)* in cis to *weak D type 45* (n = 1, Figure

3.31), *RHD-CE(4)-D* in cis to *weak D DAR2.00* (*n* = 1, Figure 3.32), *weak D type 4.2* in cis to *weak D type 41* (*n* = 1, Figure 3.33), *RHD-CE(4)-D* in cis to *RHD-CE(8-9)-D (n* = 1, Figure 3.34), *RHD-CE(4)-D* in cis to *weak D type 4.2* (*n* = 2, Figure 3.35), *DVI.2 (RHD-CE(4-6)-D* in cis or trans to *DEL(IVS8-31T>C)* (*n* = 1, Figure 3.36). Total of 5 samples were unclassified as no bands were detected with multiple *RHD* exons. Table 3.10 summarizes the findings of SSP-PCR BAGene partial D-TYPE. Therefore, total of 189 samples were classified as serological true D negative, whereas 9 samples were reclassified as D positive and 5 samples were unclassified. Genotype and allele frequencies of inactive *RHD* genes and *RHD* deletion from 189 D negative individuals is summarized in Table 3.11. Figure 3.39 summarizes the serological and molecular methods used in order to reclassify the D status.

Table 3.10 BAGene partial D assay to determine *RHD* allele in serological D negative an *RHD* exon 10 positive Omani population

BAGene tube Mix		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Possible *RHD* allele[a]
Phenotype	**Sample**	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D7/8**	**D9**	**D10**	**D2**	**D6**	**D7**	**D7**	**D8**	
ddCcee	11	+	+	+	0	+	+	+	+	+	+	0	0	0	0	0	*RHD-CE-D^{s}/DFR-1*
ddCcee	28	+	+	+	0	+	+	+	0	0	+	0	0	0	0	0	Unclassified
ddCcee	75	+	+	0	0	+	+	+	+	0	+	0	0	0	0	0	Unclassified
ddCcee	82	+	+	+	+	0	+	+	0	+	+	0	0	0	0	0	*DBS-0 in cis to DAR2.00*
ddccee	88	+	+	+	0	+	+	+	0	+	+	0	0	0	0	0	*DFR-1 in cis to DAR2.00*
ddccee	92	+	+	+	0	+	+	+	+	+	+	0	0	0	0	0	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	93	+	+	+	0	0	+	+	0	+	+	0	0	0	0	+	*RHDΨ* in cis to *DAU-5 and DAR2.00*
ddCcee	95	+	0	+	+	+	+	+	+	0	+	0	0	0	0	0	*DIIIb* in cis to *weak D type 45*
ddccee	106	+	+	+	0	0	0	+	0	0	+	0	0	0	0	0	Unclassified
ddccee	126	+	+	+	+	+	+	+	0	0	+	0	0	0	0	0	*Weak D type 4.2* in cis to *weak D 41*
ddCcee	151	+	+	+	0	+	+	+	0	0	w+	0	0	0	0	0	*DFR* in cis to *RHD-CE(8-9)-D*
ddCcee	153	+	+	+	0	0	+	+	0	0	+	0	0	0	0	0	Unclassified
ddccee	154	+	+	+	0	+	+	+	+	+	+	0	0	0	0	0	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	156	+	+	+	0	+	+	+	+	+	+	0	0	0	0	0	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	160	+	+	+	0	+	+	+	0	w+	+	0	0	0	0	0	*DFR* in cis to *weak D 4.2*
ddCcee	176	+	+	+	0	+	+	+	NR	+	+	0	0	0	0	0	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	194	+	+	+	0	+	+	+	+	+	+	0	0	0	0	0	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	205	+	+	+	0	+	+	+	+	+	+	0	0	0	0	0	*RHDΨ/del* or *RHDΨ/ RHDΨ*

Table 3.10 BAGene partial D assay to determine *RHD* allele in serological D negative an *RHD* exon 10 positive Omani population (Cont…)

ddccee	206	+	+	+	0	+	+	+	+	+	+	0	0	0	0	0	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddccee	211	+	+	+	0	+	+	+	0	+	+	0	0	0	0	0	*DFR in cis to weak D 4.2*
ddccee	**213**	+	+	+	0	+	+	+	+	+	+	0	0	0	0	0	*RHDΨ/del* or *RHDΨ/ RHDΨ*
ddCCee	**219**	+	+	+	0	0	?	+	0	?	+	0	0	0	0	0	Unclassified
ddCcee	**230**	+	+	+	0	0	0	+	+	+	+	0	0	0	0	0	*DVI.2* in cis or trans to *DEL(IVS8-31T>C)*
ddccEe	**234**	+	**0**	+	**0**	+	+	+	+	+	+	0	0	0	0	0	*RHD*DIIIb* in cis to *RHDΨ*

[a] Reported in correlation with real-time singleplex PCR and RH-TYPE BAGene.

NR: Not reliable as it showed negative for both target product and control

?: inconclusive

w: weak reaction

Table 3.11 Genotype and allele frequencies of inactive *RHD* genes and *RHD* deletion from 188 D negative individuals*

Genotype			Allele	
Molecular background	Experimental number	Experimental frequency (%)	Type	Experimental frequency
Absence of hybrid (*RHD-CE-D^{S}*)** and pseudogene (*del/del*)	179	94.701	*RHD* deletion (*del*)	0.9732
Presence of pseudogene and absence of (*RHD-CE-D^{S}*) hybrid (*RHDΨ* / *RHDΨ* or *RHDΨ* /*del*)	10	5.291	Pseudogene (*RHDΨ*)	0.0268
Total	189	100		1

*Assuming consistency with Hardy-Weinberg equilibrium, the Bernstein's formula was applied to define allele frequencies of *RHD* gene deletion, hybrid *RHD-CE-D^{s}* gene, and *RHDΨ* pseudogene. Because only two genotypes were observed the deviation value (D) was 0 (Appendix 3).

**(*RHD-CE-D^{S}*) genotype and allele frequency was not calculated as it was observed only in one sample that was in trans to partial D hybrid allele and the sample was reclassified as D positive.

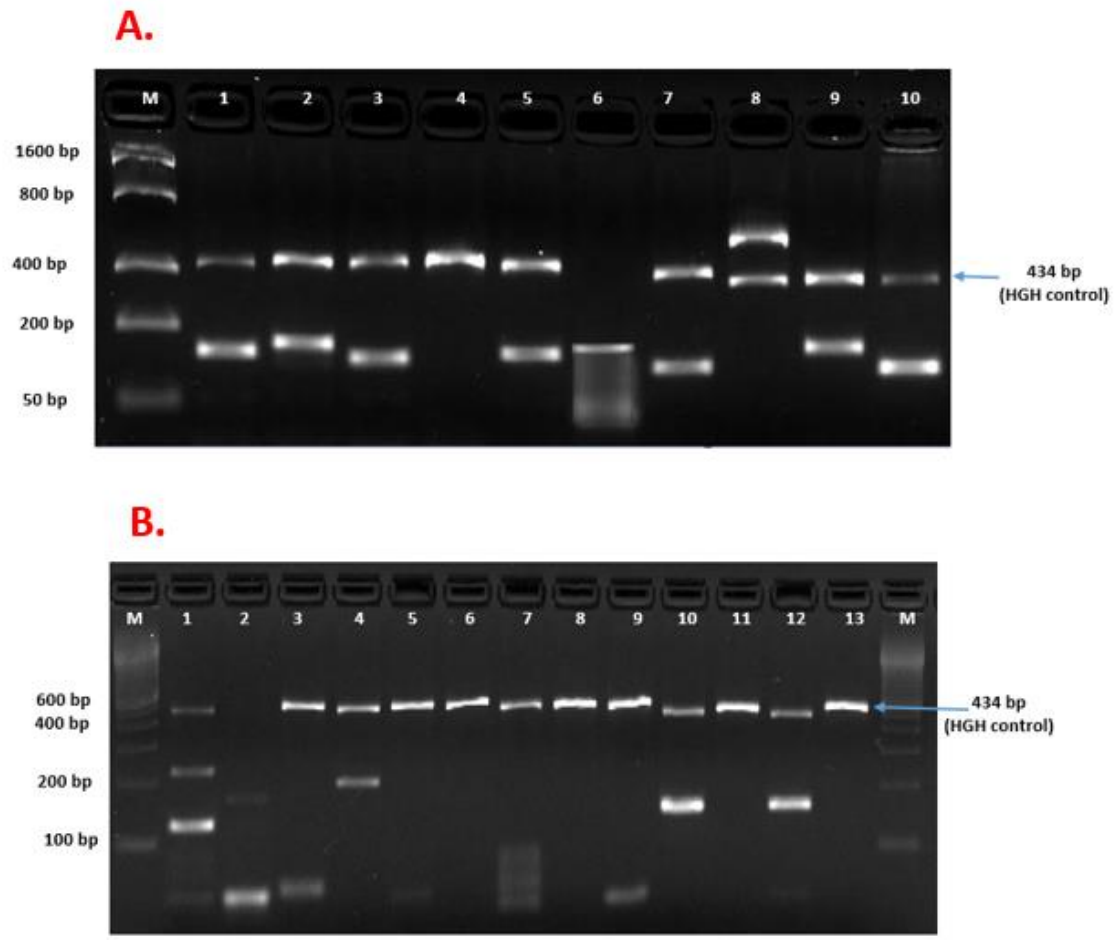


Figure 3.26 Representative image for a possible hemizygous or homozygous *RHDΨ* allele detected in Omanis

The gel images are for sample 156 as to represent all other samples that showed similar pattern. Partial D-TYPE gel analysis shows lanes 1-10 for *RHD* exons 1-10 respectively (A). The gel (A) showed amplification of *RHD* exon 1 (134 bp), 2 (146 bp), 3 (118 bp), 5 (132 bp), 6 (132 bp), 7 (120 bp), exon 8 (673 bp), exon 9 (184 bp) and exon 10 (132 bp). This suggests either *RHD* exon 4 deletion/hybrid or existence of *RHDΨ*. RH-TYPE gel view (B) showed bands 224 bp and 123 bp amplification for exons 4 and 7 respectively *(lane* 1), 154 bp specific for *RHDΨ (lane* 2), 220 bp for negative K409K *DEL RHD (lane* 4), 145 bp for *Rhc* gene *(lane* 11) and 155 bp for *Rhe* gene *(lane* 13) for which it suggests Rhccee with presence of *RHDΨ*. The gel did not show any band in lane 3 for *RHDΨ* negative which suggests the absence of normal *RHD* exon 4 and therefore *RHDΨ/ del* or *RHDΨ/ RHDΨ* allele. The gel did not show any band for DEL *RHD* (K909), *DEL RHD* (M295I), *DEL RHD (IVS3+1G>A)* and *Cde*^{s} in lanes 5, 6, 7 and 8 respectively suggesting absence of these alleles. Human growth hormone control amplicon of 434 bp was detected in all lanes. Lane M denotes 100 bp DNA ladder

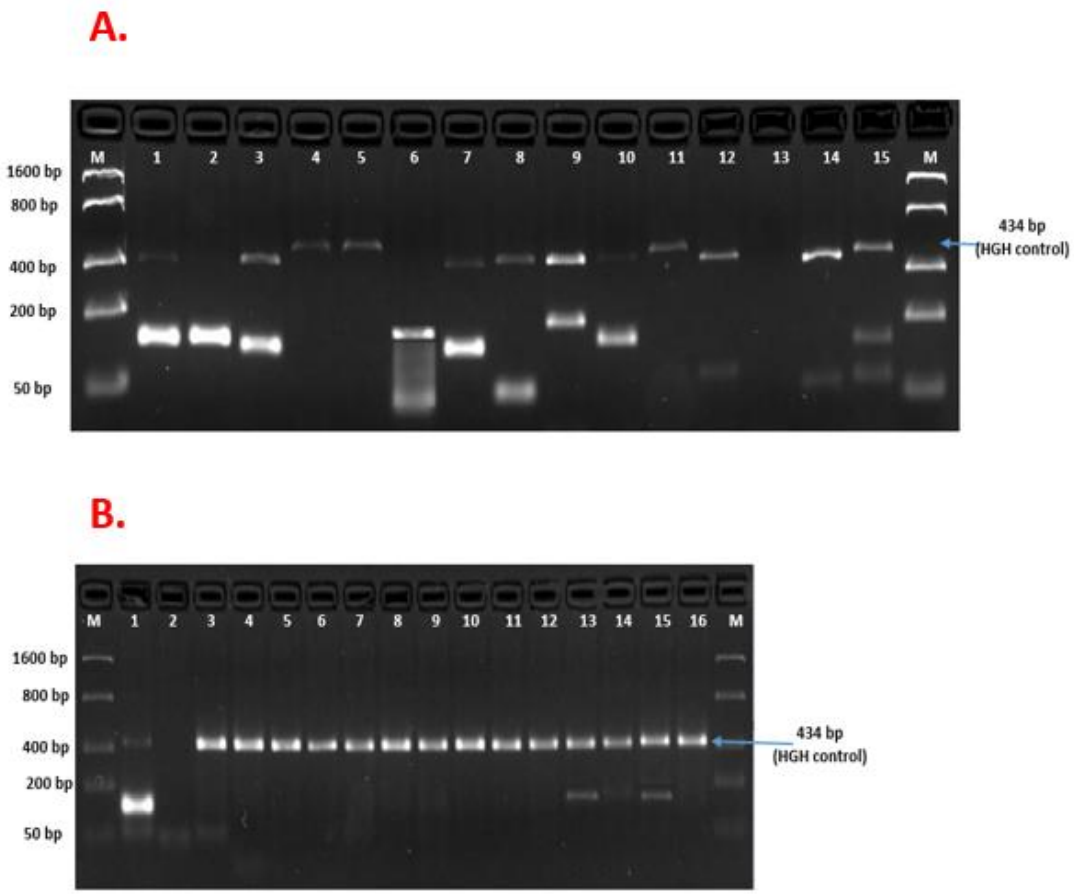


Figure 3.27 Inconclusive but possible *DAU5* allele detection on sample 93.

Partial D-TYPE gel analysis shows lanes 1-10 for *RHD* exons 1-10 respectively (A). This shows amplification of exon 1 (134 bp), 2 (146 bp), 3 (118 bp), 6 (132 bp), 7 (120 bp), exon 9 (184 bp) and exon 10 (132 bp). A missing band of *RHD* exon 4 in lane 4 (135 bp) suggests either DFR or *RHDΨ* existence. A missing band of 132 bp *(lane* 5) and 673 bp *(lane* 8) for *RHD* exons 5 and 8 respectively suggests DAR2.00 (weak D type 4). No bands seen in lane 11 through 14 for unique partial D except lane 15 which showed 140 bp specific for *DAU-5*. This could exclude the existence of *DAR2.00* supporting *DAU-5*. RH-TYPE gel view (B) showed 123 bp amplification for exons 7 *(lane* 1), 145 bp for *Rhc* gene *(lane* 13) and 155 bp for *Rhe* gene *(lane* 15) for which it suggests Rhccee. Lane 2 is not reliable, as the control did not appear thus *RHDΨ* is not excluded. The gel did not detect *RHDΨ* negative which suggests no wild type *RHD* exon 4, *DEL RHD* (K909) negative, *DEL RHD* (K909), *DEL RHD* (M295I), *DEL RHD* (IVS3+1G>A) and *Cde*^{s} in lanes 3 through 8 respectively. Both gel views have Human Growth Hormone control of 434 bp in all lanes. Human growth hormone control amplicon of 434 bp was detected in all lanes. Lane M is FastRuler™ LowRange DNA Ladder.

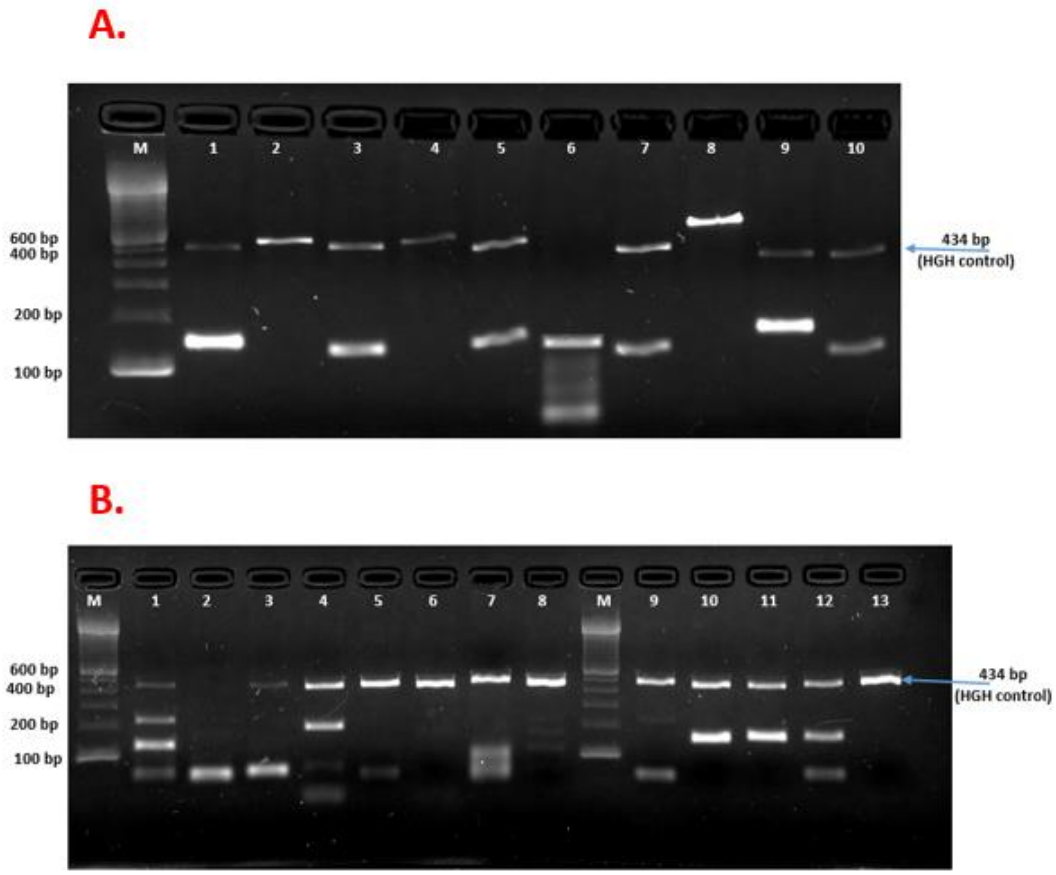


Figure 3.28 Possible Hemizygous *RHDΨ* and *RHD*DIIIb* (Sample 234).

Partial D-TYPE gel view shows lanes 1-10 for *RHD* exons 1-10 respectively (A). This shows amplification of exon 1 (134 bp), exon 3 (118 bp), exon 5 (132 bp), exon 6 (132 bp), exon 7 (120 bp), exon 8 (673 bp), exon 9 with (184 bp) and exon 10 (132 bp) for lanes 1, 3, 5, 6, 7, 8, 9 and 10 respectively. Missing band of 146 bp in lane 2 for exon 2 suggests partial D type DIIIb and a missing band of 135 bp for exon 4 *(lane* 4) may suggest *DFR* or *RHDΨ*. RH-TYPE gel view (B) showed 123 bp and 224 bp amplification for exons 7 and intron4/exon4 respectively *(lane* 1) which suggests the presence of *RHD* exon 4 and excludes partial D DFR involvement. No control band appeared in l*ane* 3, therefore this lane result for *RHDΨ* is not reliable and therefore, presence of *RHDΨ* allele is not excluded. Missing band of 140 bp for the wild type *RHD* exon 4 suggests possible hemizygous *RHDΨ*. The gel also showed 220 bp for DEL RHD (K909) negative *(lane* 4) which suggests the presence of wild type RHD allele in intron9/exon9 boundary and excludes K409K DEL phenotype. A 145 bp, 157 bp and 155 bp for *Rhc*, *RhE* and *Rhe* respectively *(lane*s 10, 11 and 12 respectively) which suggests *RhccEe*. The gel did not detect *RHDΨ* negative, *DEL RHD* (K909) negative, *DEL RHD* (K909), *DEL RHD* (M295I) and *Cde*^{s} in lanes 3 through 8 respectively. *Lane* M denotes 100 bp DNA ladder. Both gel views have human growth hormone control of 434 bp in all lanes

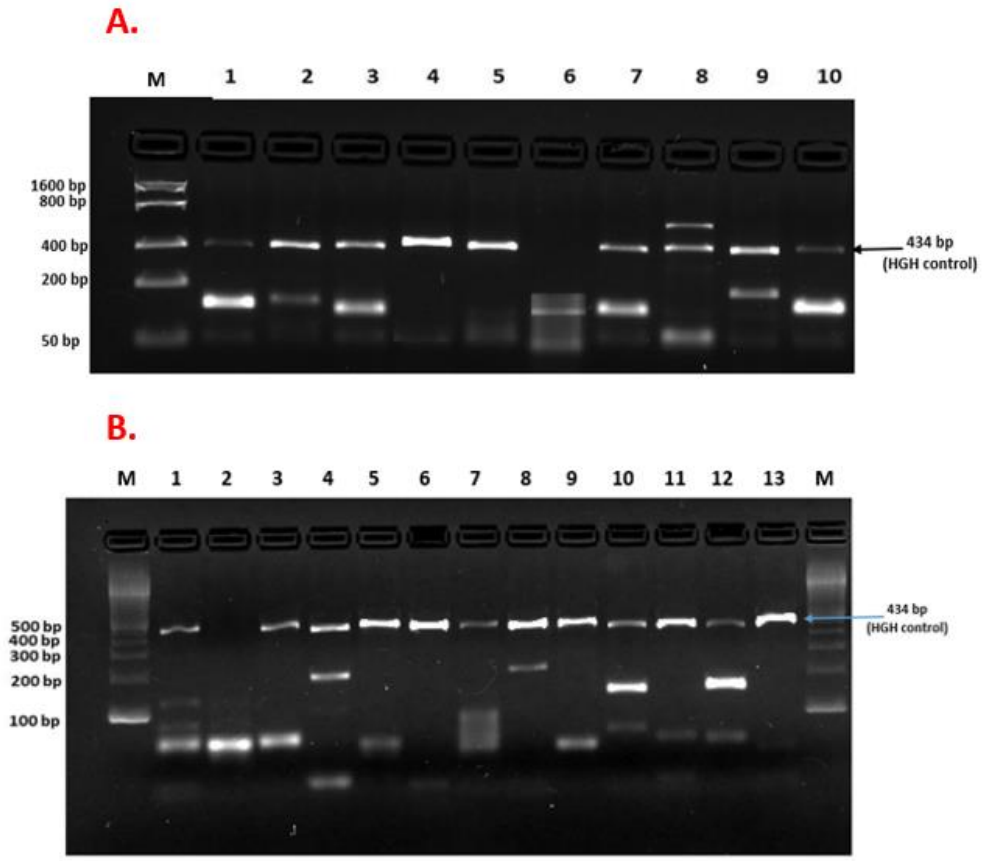


Figure 3.29 *RHD* (*C)ce^{s}/DFR-1* allele detection on sample 11.

BAGene partial D-TYPE gel analysis shows lanes 1-10 for *RHD* exons 1-10 respectively (A). The gel view shows amplification of *RHD* exon 1 (134 bp), 2 (146 bp), 3 (118 bp), 6 (132 bp), 7 (120 bp), Intron 7/Exon 8 (673 bp), exon 9 (184 bp) and exon 10 (132 bp). The absence of *RHD* exon 4 suggests hybrid *RHCE(4)* or *RHDΨ* presence. BAGene RH-TYPE gel view (B) showed 123 bp amplification for exon 7 *(lane* 1), 220 bp for negative K409K *DEL RHD (lane* 4), 215 bp for *Cde^{s} (lane* 8), 145 bp for *Rhc* allele *(lane* 10) and 155 bp for *Rhe* allele *(lane* 12) and no RhC allele band (*lane* 9) which conflicts with serological phenotyping shown in Figure 3.4C . *Lane* 8 suggests existence of hybrid *RHD-CE-D^{s}*. The gel did not detect *RHDΨ, RHD* exon 4, *DEL RHD* (K909), *DEL RHD* (M295I), *DEL RHD* (IVS3+1G>A) in lanes 2, 3, 5, 6 and 7 respectively which confirms existence of *RHCE(4)* hybrid and no *RHDΨ*. Both partial D-Type and RH-Type gel views confirmed *RHD* exon 4 deletion in *lane* 4 and *lane* 3 respectively suggesting hybrid *RHD-CE(4)-D* called DFR existence. Therefore, the D status can be reclassified as D positive. *Lane* M denotes 100 bp DNA ladder. Both gel views have human growth hormone control of 434 bp in all lanes

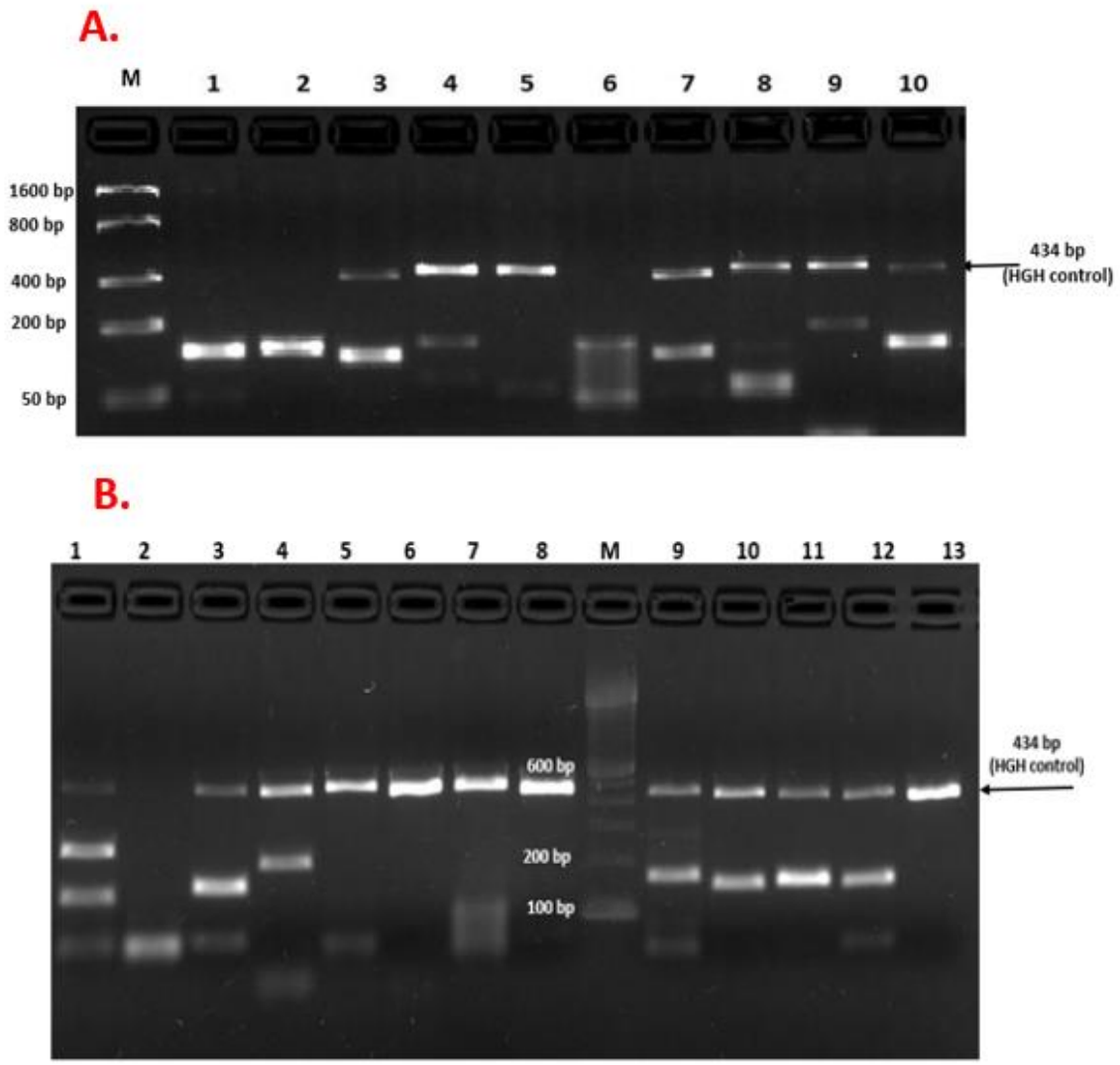


Figure 3.30 *DBS-0* in cis to *DAR2.00* allele on sample 82.

BAGene partial D-TYPE gel analysis shows lanes 1-10 for *RHD* exons 1-10 respectively (A). This shows amplification of exon 1 (134 bp), 2 (146 bp), 3 (118 bp), 4 (135 bp), 6 (132 bp), 7 (120 bp), exon 9 (184 bp) and exon 10 (132 bp). No amplification bands of exon 5 (132 bp) and intron 7/exon 8 (637 bp) in lanes 5 and 8 respectively suggests DAR2.00. BAGene RH-TYPE gel view (B) showed bands 224 bp and 123 bp amplification for exons 4 and 7 respectively *(lane* 1), 140 bp band for *RHDΨ* negative *(lane* 3) which suggests the presence of normal *RHD* exon 4, 220 bp for negative K409K *DEL RHD (lane* 4), 162 bp for *RhC* gene *(lane* 10), 145 bp for *Rhc* gene *(lane* 11), 157 bp for *RhE* gene *(lane* 12) and 155 bp for *Rhe* gene *(lane* 13) for which it suggests RhCcEe. The gel did not detect *RHDΨ*, *DEL RHD* (K909), *DEL RHD* (M295I), DEL *RHD (IVS3+1G>A)* and *Cde*^{s} alleles in lanes 2, 5, 6, 7 and 8 respectively. Therefore, the D status can be reclassified as D positive. Both gel views have human growth hormone control of 434 bp in all lanes. *Lane* M is FastRuler™ LowRange DNA ladder.

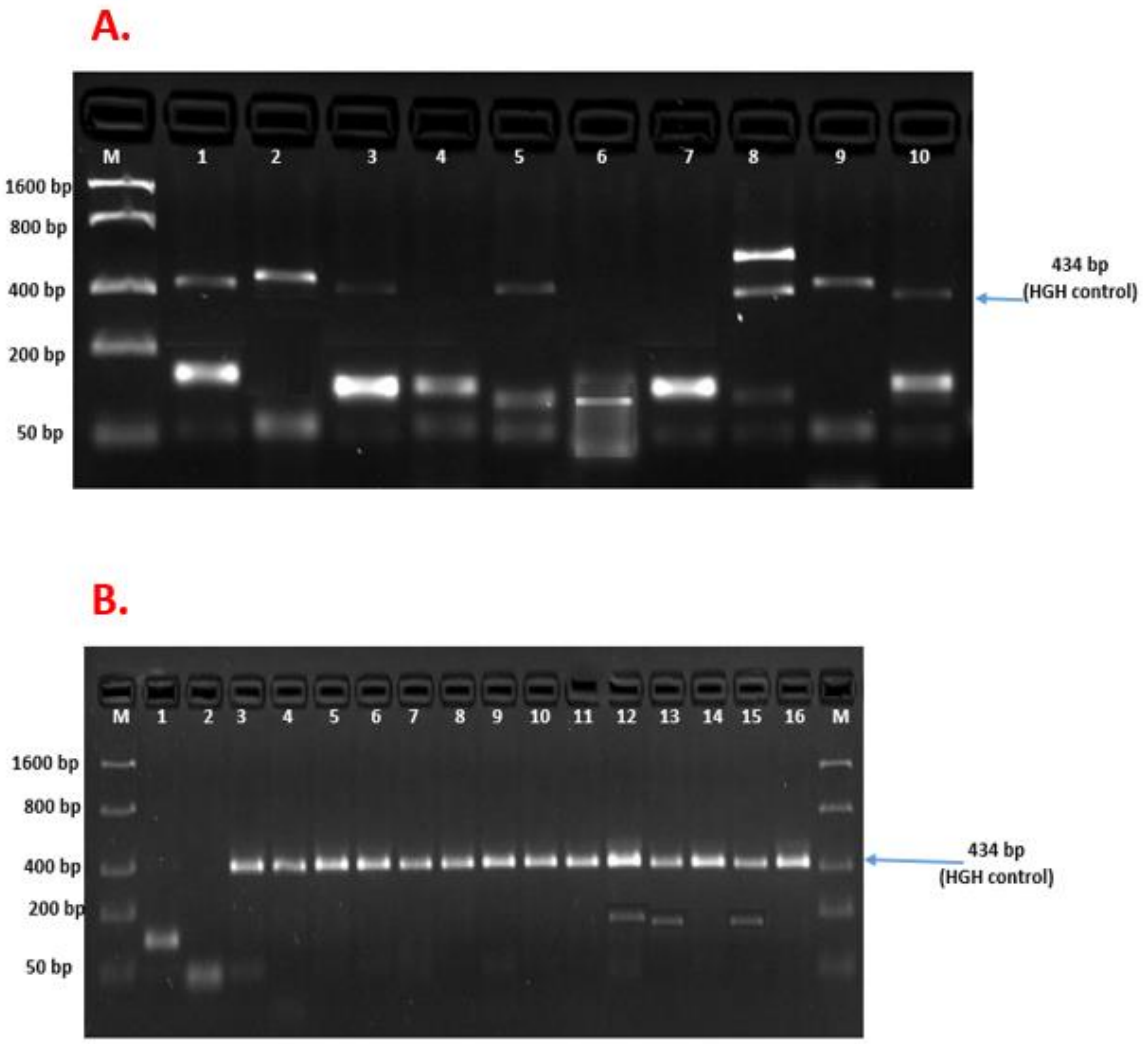


Figure 3.31 Inconclusive but possible *DIIIb* hybrid allele (sample 95).

Partial D-TYPE gel analysis shows lanes 1-10 for *RHD* exons 1-10 respectively (A). This shows amplification of exon 1 (134 bp), exon 3 (118 bp), exon 4 (135), exon 5 (132 bp), exon 6 (132 bp), exon 7 (120 bp), exon 8 (673 bp), and exon 10 (132 bp). Missing band (146 bp) for *RHD* exon 2 *(lane* 2) suggests partial D type DIII. This accompanied with missing band in *lane* 9 for *RHD* exon 9 which may suggest weak D type 41 or 45 involvement as well. RH-TYPE gel view (B) showed 123 bp amplification for *RHD* exons 7 *(lane* 1), 262 bp for *RhC* allele *(lane* 12), 145 bp for *Rhc* allele *(lane* 13) and 155 bp for *Rhe* allele *(lane* 15) for which it suggests RhCcee. Lane 2 is not reliable, as the control did not appear for *RHDΨ* detection. The gel did not detect *RHDΨ* negative, *DEL RHD* (K909) negative, *DEL RHD* (K909), *DEL RHD* (M295I), *DEL RHD* (IVS3+1G>A) and *Cde*^{s} in lanes 3 through 8 respectively. The result for RH-TYPE is inconclusive. Both gel views have human growth hormone control of 434 bp in all lanes. *Lane* M is FastRuler™ LowRange DNA ladder.

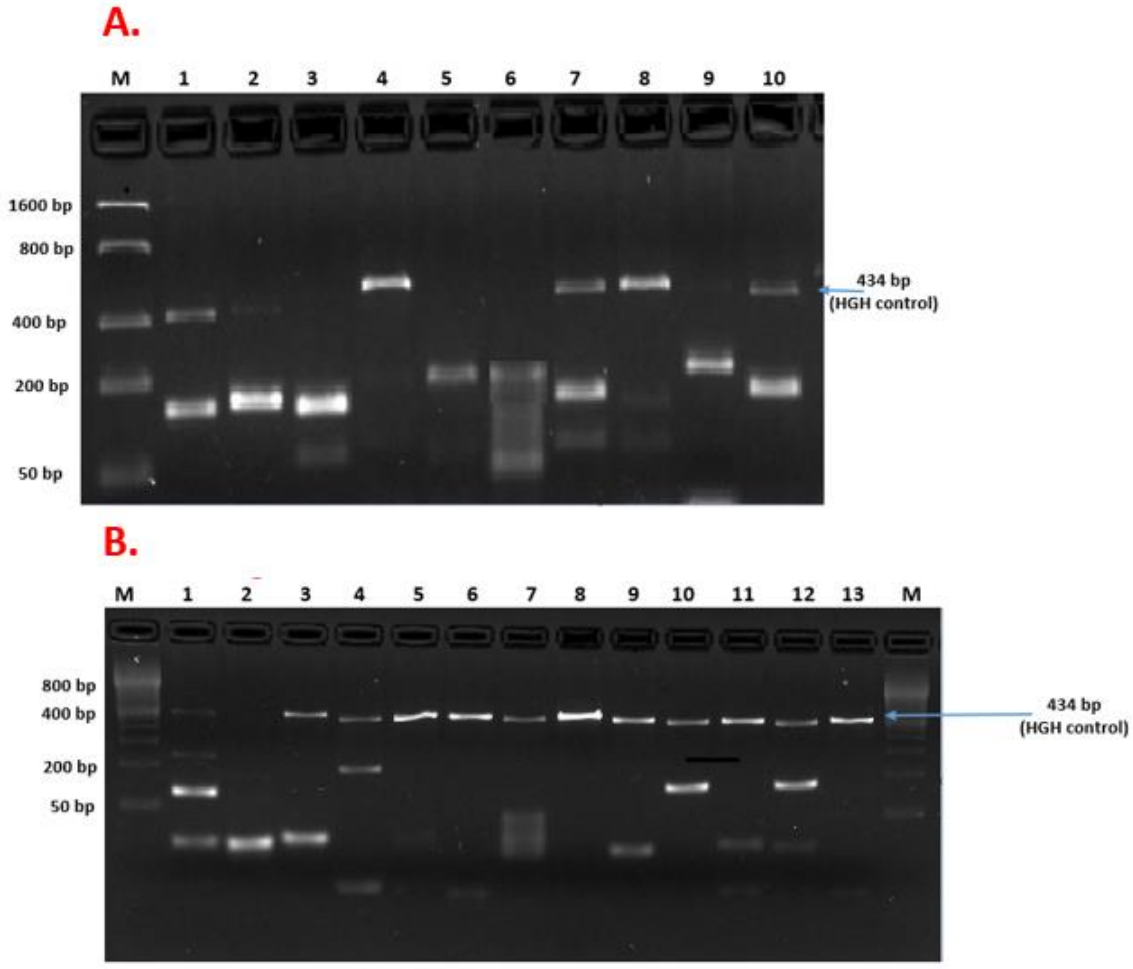


Figure 3.32 A possible *DFR* partial D in cis to *DAR2.00* seen in sample 88

BAGene partial D-TYPE gel analysis shows lanes 1-10 for *RHD* exons 1-10 respectively (A). This shows amplification of *RHD* exon 1 (134 bp), 2 (146 bp), 3 (118 bp), 5 (132 bp), 6 (132 bp), 7 (120 bp), exon 9 (184 bp) and exon 10 (132 bp). No amplification of exon 4 and 8 suggests hybrid *RHD-CE(4)-D* in cis to *DAR 2.00*. BAGene RH-TYPE gel view (B) showed bands 224 bp and 123 bp amplification for exons 4 and 7 respectively *(lane* 1) which suggests the existence of *RHD* exon 4. The gel view also showed 220 bp for negative K409K *DEL RHD (lane* 4), 145 bp for *Rhc* allele *(lane* 11) and 155 bp for *Rhe* allele *(lane* 13) for which it suggests Rhccee. The gel did not detect *RHDΨ* negative 140 bp band *(lane* 3) for *RHD* exon 4 which conflicts with results of lane 1 that detects 224 bp band for exon 4 and may suggest existence of hybrid *RHCE(4)*. The gel did not detect *RHDΨ*, *DEL RHD* (K909), *DEL RHD* (M295I), *DEL RHD* (IVS3+1G>A) and Cde^{s} alleles in lanes 2, 5, 6, 7 and 8 respectively. Both gel views have Human Growth Hormone control of 434 bp in all lanes. *Lane* M denotes 100 bp DNA ladder. Both gel views have human growth hormone control of 434 bp in all lanes. *Lane* M is FastRuler™ LowRange DNA ladder.

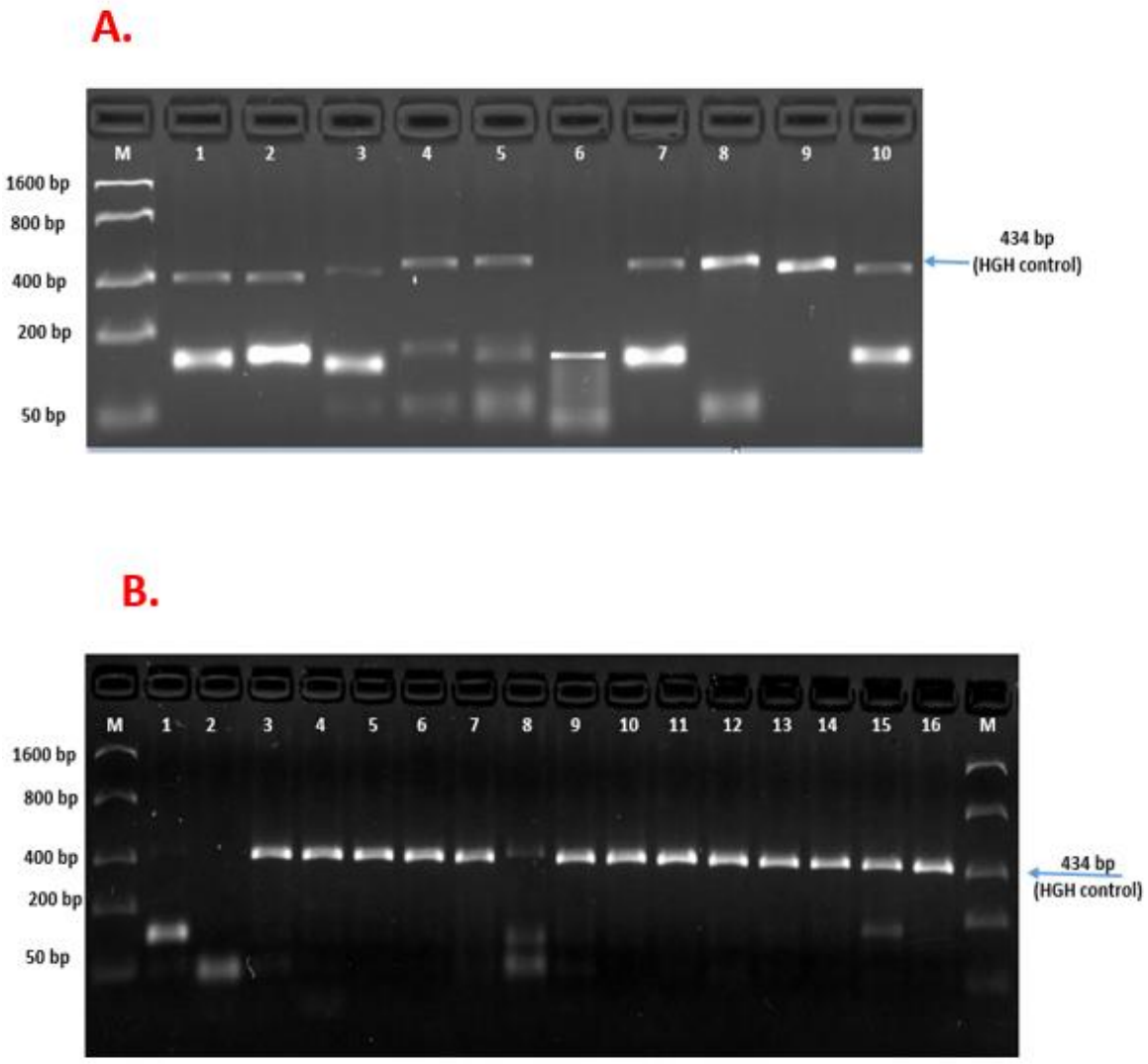


Figure 3.33 Possible weak D 4.2 in cis to weak D type 41 (sample 126).

Partial D-TYPE gel analysis shows lanes 1-10 for *RHD* exons 1-10 respectively (A). This shows amplification of exon 1 (134 bp), exon 2 (146 bp), exon 3 (118 bp), exon 4 (135), exon 5 (132 bp), exon 6 (132 bp), exon 7 (120 bp) and exon 10 (132 bp). Missing bands 673 bp *(lane* 8) and 184 bp *(lane* 9) of exon 8 and 9 respectively suggests either *RHD-CE(8-9)-D* or *weak D type 41* in cis with *weak D 4.2*. RH-TYPE gel view (B) showed 123 bp amplification for *RHD* exons 7 *(lane* 1), and 155 bp for *Rhe* gene *(lane* 15). *Rhc* gene band of 145 bp *(lane* 13) was expected to be seen in correlation with serological c phenotype for *Rhccee* genotype. Possibility of *RHD-CE(8-9)-D* is excluded as this allele is seen in *Cde* haplotype. *Lane* 2 is not reliable, as the control did not appear for *RHDΨ* detection. The gel did not detect *RHDΨ* negative, *DEL RHD* (K909) negative, *DEL RHD* (K909), *DEL RHD* (M295I), *DEL RHD* (IVS3+1G>A) and *Cde*^{s} in lanes 3 through 8 respectively. The result for RH-TYPE is inconclusive and the Rh genotype could not be confirmed and compared with serological phenotype. Both gel views have human growth hormone control of 434 bp in all lanes. *Lane* M is FastRuler™ LowRange DNA ladder.

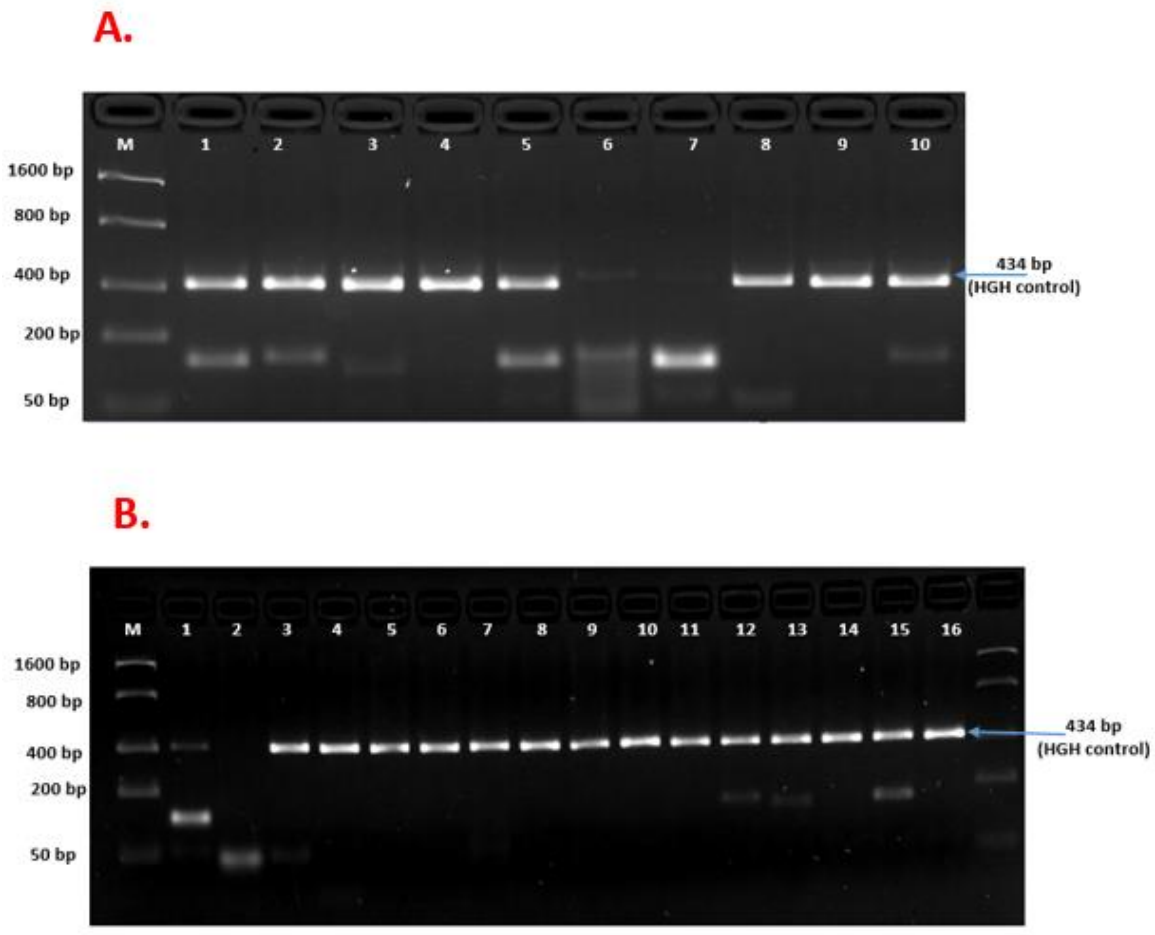


Figure 3.34 A possible hemizygous *DFR* in cis to *RHD-CE(8-9)-D* (Sample 151).

Partial D-TYPE gel analysis shows lanes 1-10 for *RHD* exons 1-10 respectively (A). This shows amplification of exon 1 (134 bp), exon 2 (146 bp), exon 3 (118 bp), exon 5 (132 bp), exon 6 (132 bp), exon 7 (120 bp) and exon 10 (132 bp). Missing band of 135 bp *(lane* 4) for exon 4 suggests either *DFR* or *RHDΨ* existence. Missing bands of 673 bp and 184 bp of exons 8 and 9 respectively suggests *RHD-CE(8-9)-D*. RH-TYPE gel view (B) has missing band of exon 4 (224 bp) and showed 123 bp amplification for exons 7 *(lane* 1) which suggested exon 4 deletion and replacement with *RHCE* exon 4. A band of 162 bp for *RhC* allele *(lane* 12), 145 bp for *Rhc* allele *(lane* 13) and 155 bp for *Rhe* allele *(lane* 15) also seen which suggests *RhCcee* genotype. Lane 2 is not reliable, as the control did not appear for *RHDΨ* detection but since there is *RHD* exon 4 deletion it predicted to be not present. The gel did not detect *RHDΨ* negative due to *RHD* exon 4 deletion. Missing bands for *DEL RHD* (K909) negative, *DEL RHD* (K909), *DEL RHD* (M295I), DEL *RHD* (IVS3+1G>A) and Cde^{s} alleles in lanes 3 through 8 respectively. *Lane* M denotes 100 bp DNA ladder. Both gel views have human growth hormone control of 434 bp in all lanes.

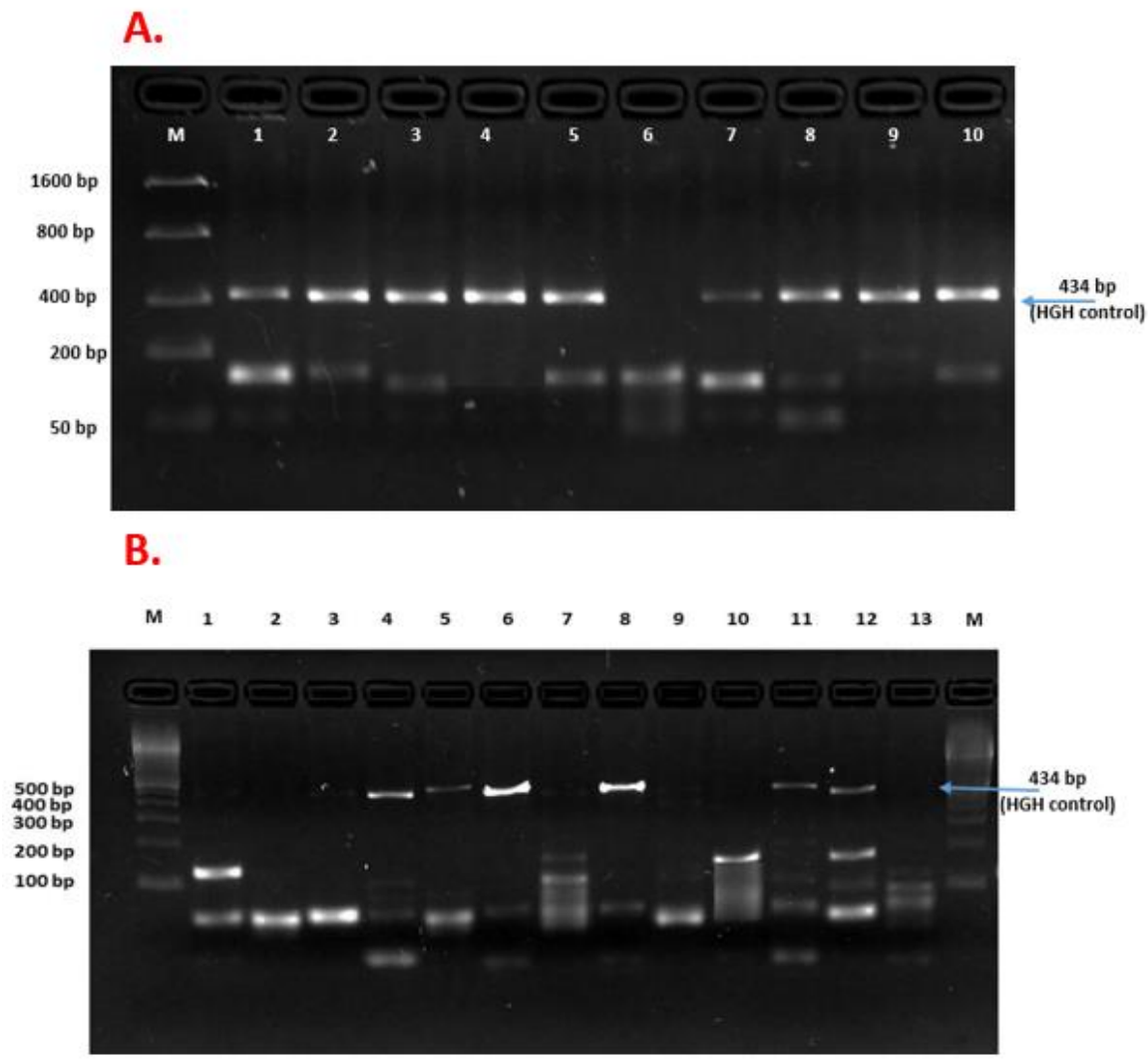


Figure 3.35 A possible hemizygous *RHD*DFR1* and *weak D 4.2* (Sample 160).

Partial D-TYPE gel analysis shows lanes 1-10 for *RHD* exons 1-10 respectively (A). *Lane* M is FastRuler™ LowRange DNA Ladder in (A). This shows amplification of exon 1 (134 bp), exon 2 (146 bp), exon 3 (118 bp), exon 5 (132 bp), exon 6 (132 bp), exon 7 (120 bp), exon 9 with weak band (184 bp) and exon 10 (132 bp). Missing band of 135 bp *(lane* 4) for exon 4 suggests DFR or *RHDΨ*. Missing band of 673 bp for exons 8 *(lane* 8) may suggest *weak D 4.2* allele presence. RH-TYPE gel view (B) showed 123 bp amplification for exons 7 *(lane* 1), multiple bands in *lane* 7 (contamination indicator) and 145 bp for *Rhc* allele *(lane* 10) and 155 bp for *Rhe* allele *(lane* 12). Lane 2 is not reliable, as the control did not appear for *RHDΨ* detection but since there is missing *RHD* exon 4 it predicted to be not present. The gel did not detect *RHDΨ* negative due to missing *RHD* exon 4. The gel did not detect negative allele for *DEL RHD (K409K)*, *DEL RHD (K409K)*, *DEL RHD* (M295I) and Cde^{s} in lanes 3 through 8 respectively. *Lane* M denotes 100 bp DNA ladder in (B). Both gel views have human growth hormone control of 434 bp in all lanes.

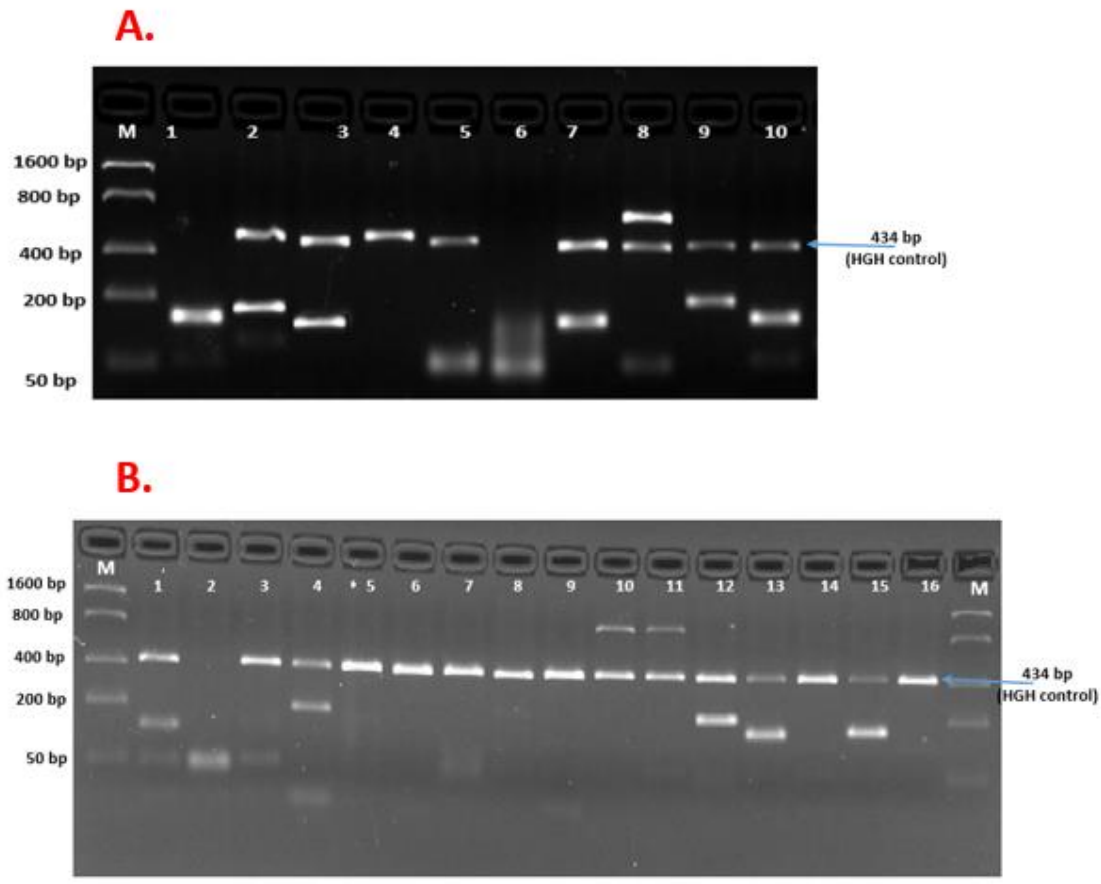


Figure 3.36 A possible partial D type DVI.2 (Sample 230)

Partial D-TYPE gel analysis shows lanes 1-10 for *RHD* exons 1-10 respectively (A). This shows amplification of exon 1 (134 bp), exon 2 (146 bp), exon 3 (118 bp), exon 7 (120 bp), exon 8 (673 bp), exon 9 (184 bp) and exon 10 (132 bp). Deletion of exons 4-6 *(lane*d 4-6) suggests DVI.2. RH-TYPE gel view (B) showed 123 bp amplification for exons 7 *(lane* 1), 220 bp for *DEL RHD* (K909) negative *(lane* 4), 940 bp for H and U box *(lane*s 10 and 11 respectively), 162 bp for *RhC* allele *(lane* 12), 145 bp for *Rhc* allele *(lane* 13) and 155 bp for *Rhe* allele *(lane* 15). Lanes 2 and 3 did not appear for *RHDΨ* positive and negative due missing exon 4. No bands seen in *DEL RHD (K909)*, *DEL RHD (M295I)*, *DEL RHD (IVS3+1G>A*), *Cde*^{s} and DAR1 in lanes 5 through 9 respectively, which suggests missing of these alleles. *Lane* M denotes 100 bp DNA ladder. Both gel views have human growth hormone of 434 bp in all lanes.

3.7.5 Sensitivity evaluation of different SYBR Green master mix for the direct real-time PCR from Blood preserved on FTA card

We also evaluated sensitivity of three different SYBR Green master mix suppliers, Qiagen (QuantiNova SYBR®Green Master Mix), PCRBIOSYSTEMS (qPCRBIO SyGreen Mix) and Applied Biosystems (PowerUp™ SYBR™ Green Master Mix) were evaluated for the direct detection of *RHD* exon 10 from dry blood on FTA card without extraction of DNA. The previously identified *RHD* exon 10 positive samples (n = 14) were used in this analysis. We found that qPCRBIO SyGreen Mix performed the best with sensitivity of 71% (n = 10) followed by PowerUp™ SYBR™ Green Master Mix with 43% (n = 6) and QuantiNova SYBR®Green Master Mix with only 29% (n = 4) (Figure **3.37**). We also observed dissociation curve appearance for different supplier's master mix and observed that qPCRBIO SyGreen mix gives a single melt point peak compared to other two with anomalous peaks (Figure 3.38).

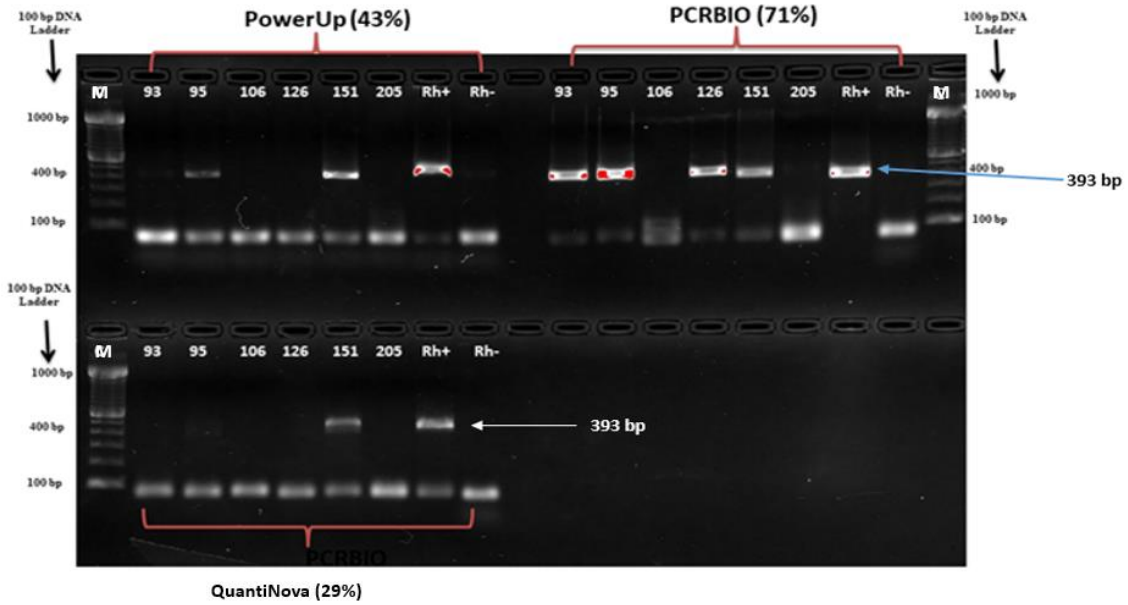


Figure 3.37 Sensitivity of three different SYBR Green master mixes.

This is a representative image that compares three different SYBR Green master mixes for the direct detection of *RHD* exon 10 from dry blood spot on FTA card without DNA extraction (n = 7 in this representative image, originally 14 samples used). M *lane* is 100 bp DNA ladder. Rh+ and Rh- are positive and negative controls respectively. All the samples (93, 95, 106, 126, 151, 205 and Rh+) were tested previously from extracted DNA and should have given 393 bp product for *RHD* exon 10. All suppliers master mixes were able to give 393 bp product but with different sensitivity. qPCRBIO SyGreen gave the best sensitivity (71%), followed by PowerUp™ SYBR™ Green (43%) and QuantiNova SYBR®Green (29%).

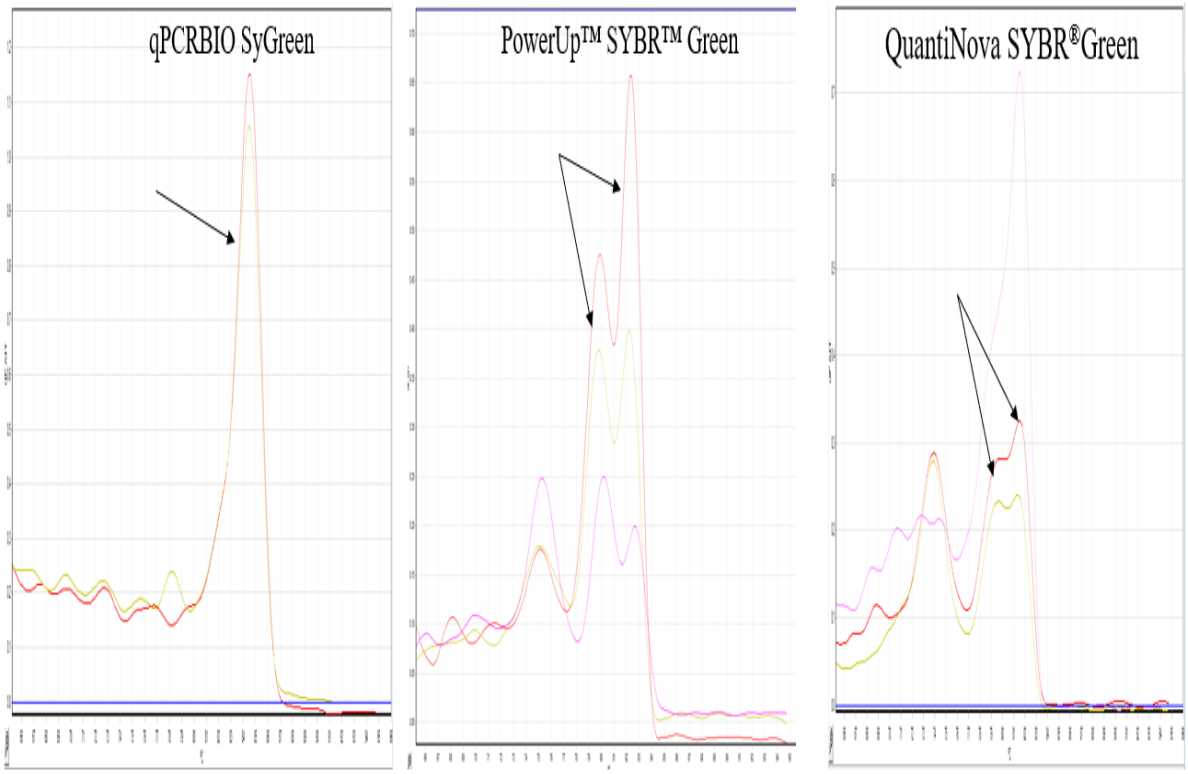


Figure 3.38 Melting curve peak appearance using three different master SYBR Green master mixes

Representative images showing melting curve peaks for *RHD* exon 10 amplicon using qPCRBIO SyGreen, PowerUp™ SYBR™ Green and QuantiNova SYBR Green master mixes. Master mix qPCRBIO SyGreen gave a single peak compared to PowerUp™ SYBR™ Green and QuantiNova SYBR Green master mixes that gave anomalous peaks (pointed with arrows).

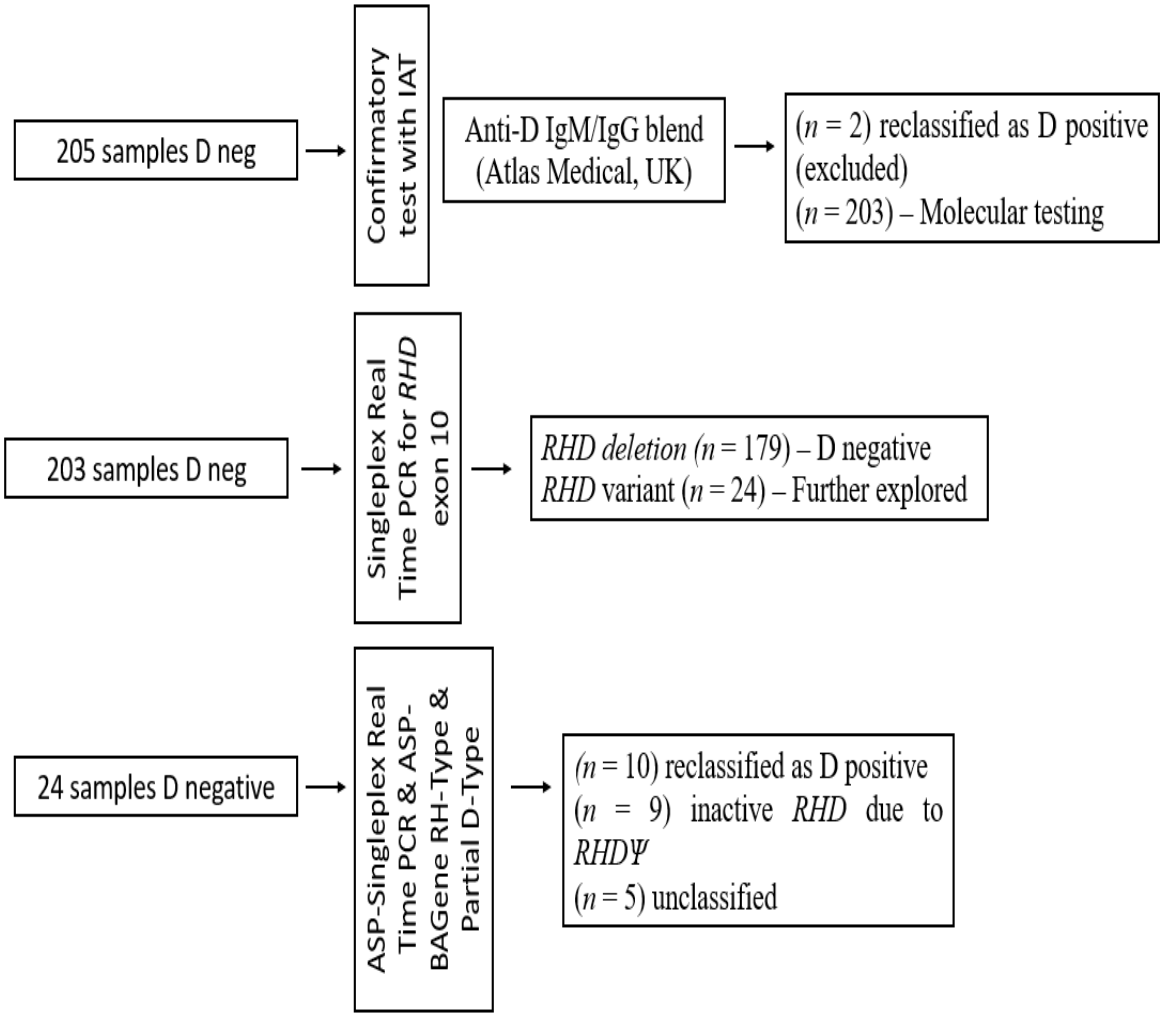


Figure 3.39 Flowchart for the serological and molecular confirmatory D tests performed for samples in current study.

A total of 205 D negative samples were collected and D status was further reconfirmed by IAT method using anti-D IgM/IgG blend (Atlas,UK) which excluded (*n* = 2) from the study as both samples were reclassified to D positive. The remaining 203 samples were tested for the existence of *RHD* exon 10 using ASP real-time PCR which divided the samples into two categories; *RHD* complete deletion (*n* = 179) and *RHD* variant (n = 24). *RHD* variant samples (*n* = 24) were further explored by ASP-Singleplex real-time PCR and BAgene ASP-PCR for RH-Type and partial D-Type which reclassified (*n* = 10) as D positive. Total of (*n* = 9) were classified to be true D negative due to inactive *RHDΨ* existence and (*n* = 5) remained unclassified.

3.8 Discussion:

This study was done on serological D negative Omani population to explore the Rh common antigens (C, c, E and e) by molecular phenotyping. There is evidence that some RBC units with weak D or DEL phenotype may escape detection by standard serologic methods including the IAT method (Sassi et al., 2014). The D negative phenotype is characterized by a high molecular diversity which explains the discrepancies found between serologic and molecular methods. Therefore, we also studied the molecular background of the serological D negative phenotype to further explore the actual D status. To the best of our knowledge, there are no published data on Rh common blood group antigens and haplotypes frequencies for D negative Omani individuals. Seven Omani regional transfusion centres associated with the CBB in capital were involved in the collection of samples.

The D− C− c+ E− e+ phenotype was the most frequent phenotype observed in the D negative samples. These results were in accordance with the previous study in other Arab populations (Moussa et al., 2012; Khosroshahi et al., 2019) and in Caucasians population (Avent et al., 1997c). The negativity of IAT test for samples that has been reclassified later as D positive by molecular method may be explained by the low sensitivity of the Anti-D IgM/IgG blend (Atlas Medical, UK) used. The negativity could also exist due to more than one D variants allele in cis to each other (hemizygous) or in trans so both haplotypes will show qualitative or/and quantitative effect on D antigen.

Melting curve analysis of *RHD* exons 1 through 7 and *RHD* exons 9-10 showed T_m that are too close to each other's (Table 3.6) to be separated by real-time PCR SYBR Green chemistry. This makes it difficult to make a multiplex assay for *RHD* exons detection in order to be analysed by Tm. A future study with high resolution melt (HRM) could be an option to explore further this melting curve analysis by SYBR Green I (Farrar, 2015) or Eva Green chemistry (Jiang, 2019).

Total of 11.82% of D negative samples from Omanis expressed *RHD* variant allele. The molecular results showed that D negative results are highly associated with complete deletion of *RHD* gene with frequency of 0.878 (Table 3.5). This frequency is lower than those reported in Caucasian population (Singleton et al., 2000; Touinssi et al., 2009) and Iran (Khosroshahi et al., 2019) but higher than those reported in East Asian (Luettringhaus et al., 2006; Ji et al., 2017). In contrast, there was a high African contribution associated to the presence of *RHDΨ* pseudogene either alone, identified in eight samples or in combination identified in two samples, whereas *RHD-CE-D* variants *(C)ce*^{s} was identified in combination in one sample. *RHD* gene deletion (18%), *RHD-CE-D* (15%) and *RHDΨ* (66%) are the main causes of the D negative phenotype among black Africans (Singleton et al., 2000). The identified African contribution frequency of 0.06 in Omani population was higher than those reported in North African Tunisian population (Moussa et al., 2012; Sassi et al., 2014), but lower than those reported in West African Mali population (Wagner et al., 2003).

In line with the *RHD* genotyping results that showed African origin alleles, we observed one sample (Sample 11) with hybrid *RHD-CE-D (C)ce*^{s}, but with complete *RHD* exons 6 and 7 (Figure 3.29, partial D gel view), specific amplicon of IVS2 (Figure 3.29, RH-TYPE gel view lane no.8) for the detection *Cde*^{s} and no *C* allele detection (Figure 3.29), RH-TYPE gel view lane no.9). Existence of *RHD* exons 6 and 7 is unlikely to be found in hemizygous and homozygous types 1 and 2 of *RHD* hybrid gene *(C)ce*^{s} (Wagner, Frohmajer and Flegel, 2001; Fasano et al., 2010). The presence of another partial hybrid *RHD-CE(4)-D* allele haplotype is a possible reason to combine with that and mask missing of *RHD* exons 6 and 7 accompanied with *(C)ce*^{s}. BAGene partial D type detected deletion of *RHD* exon 4 which suggests partial D DFR-1 is combined with *(C)ce*^{s} to give possible *Cde*^{s}*/cDe* (Figure 3.40). Other types of DFR are excluded as they are reported

with *C* allele existence (http://www.rhesusbase.info/), which is not possible in this case to be as trans haplotype. This is due to *c* allele existence (confirmed by serological and molecular methods). Therefore, this sample can be reclassified as D positive. Individual with this diplotype may be at risk of producing anti-C if transfused with C positive unit, as *(C)ce*^{s} haplotype has alerted C and not compensated with another C in trans (Fasano et al., 2010). The *Ccde*^{s} allele of the *RHD* gene is typically linked to the *ce* allele of the *RHCE* gene. Because there is no *Ce* allele, the kits are not supposed to detect any *C* allele signal, and that explains no reaction in mix 9 (Figure 3.29, RH-TYPE gel view, *lane* 9) with positive weak C in serological test. Due to the presence of another haplotype with *ce* allele, serological phenotyping of c and e remained strong and not weak as seen partial in *(C)ce*^{s}.

The presence of C antigen with weak e antigen on BioRad gel card (Figure 3.4C) supported by the absence of *C* allele in BAGene kit suggests the presence of VS antigen with partial C but not V antigen. This also suggests presence of an additional substitution Gly336Cys, in addition to Leu245Val. V and VS antigens should be tested for this sample in future to confirm the finding.

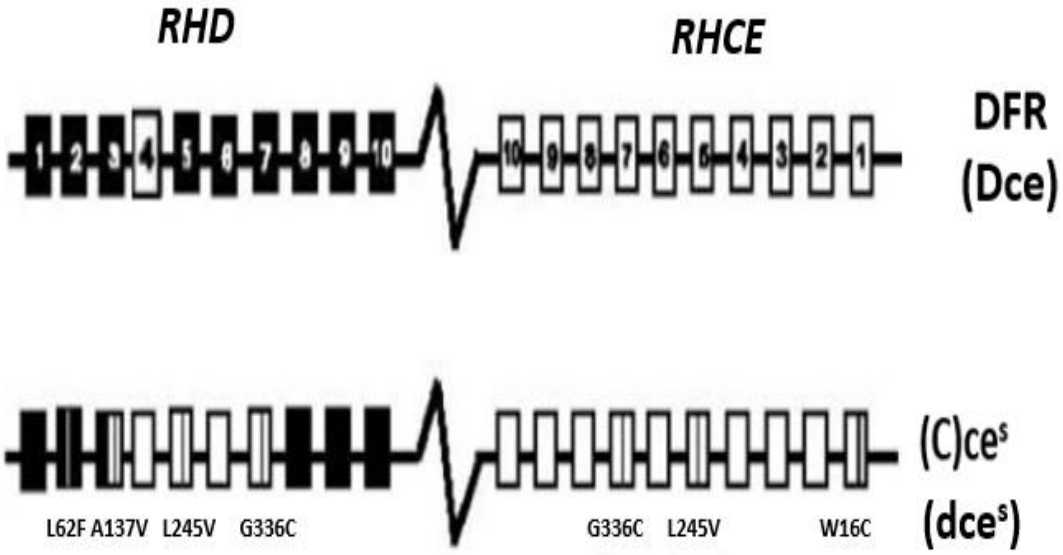


Figure 3.40 Possible *RH* genotype of *DFR*/*Cde*^{s} on sample 11.

The 10 exons of the *RHD* (black box) & *RHCE* (white box) genes are symbolized by squares and numbered 1 to 10. Sample 11 genotype possibly composed of the variant *DFR* (*Dce*) haplotype (top panel) and *(C)ce*^{s} (*dce*^{s}) haplotype (bottom panel). In *DFR*, *RHD* exon 4 is replaced by hybrid *RHCE* exon 4. In *(C)ce*^{s}, the variant amino acid substitutions are shown by positions and amino acids involved, relative to the common *RH* alleles. The figure was adapted from (Fasano et al., 2010) and modified accordingly.

We also observed a case (Sample 82) of possible novel DBS-0 like allele in cis to DAR weak D 4.2 (Figure **3.41**). BAGene partial D has detected no amplification with mix 5 and 8 (Figure 3.30, *lane* 5 and 8, partial D-Type gel view). DBS appears as a CDe haplotype (RhesusBase website) with hybrid RHcE [226P] (5:667-5:712) (Avent et al., 1996a). The hybrid gene has *RHD* exons 1-4, part of *RHCE* exon (5:667-5:712) and *RHD* exons 6 through 10. Both BAGene partial D-Type SSP and SYBR Green I singelplex real-time PCR failed to amplify exon 5 as the allele specific primers used target location 697 bp (G nucleotide) on RHD exon 5 underlined which is replaced by RHCE (C nucleotide) with hybrid RHCE involvement and thus explains negative result. Taq Man real-time PCR primers (RHDex5F and RHDex5R) amplified *RHD* exon 5 as they target different location on *RHD* exon 5 that did not overlap with specific *RHCE* nucleotides in *RHCE* hybrid. Serological result was found to be ddCcee phenotype, however a discrepancy was found compared to molecular genotyping by BAGene RH-TYPE that showed amplification of *C, c*, *E* and *e RH* alleles (Figure 3.30, RH-TYPE gel view Lanes 10 through 13) suggesting *RHCcEe* instead. Many studies compared between genotyping and serological phenotyping of RHCE blood group to explain discrepancies seen between both.

As stated in section 1.5 , *RHCE* gene encodes for C/c and E/e antigens. RhC/c rises from single base substitution(C307T) CCT (RhC) for serine or TCT (Rhc) for proline in exon 2 at nucleotide (nt) 307. There are other polymorphisms in exon 1 but not shown to be involved in RhC/c serology. The *RHC* allele has identical sequence to the *RHD* allele in exon 2 and thus the use of ASP directed toward the nt307 polymorphism cannot discriminate between *RHC* and *RHD* alleles. A combination of two ASPs can be used; one that targets nt307 for *RHc* allele and other that targets nt48 (C48G) in exon 1 specific for *RHC* allele in the presence of both *RHD* and *RHc* alleles. Hyland and colleagues found 100% of individuals with *C* allele have expected nt48 cytosine, so did 5.2% of individuals

with *c* allele (Hyland et al., 1994). In another study on black Africans, 74% of Rhcc individuals had the nt48 cytosine (Avent et al., 1997a). Therefore, possibility of false positive for the detection of *RHC* allele is high among African ancestry using these primers.

Poulter and colleagues described *RHC* specific insertion in intron 2 of *RHCE* gene and designed PCR-RFLP test that detects this allele (Poulter, Kemp and Carritt, 1996). Avent described and defined *RHC* allele specific intron 2 sequence to be insert of 109 bp and developed ASP for the typing of Rh C/c (Avent, 1998). This unique 109 bp insertion has been correlated with RhC serology in two different studies that evaluated more than 600 individuals and found to be in complete concordance (Avent et al., 1997a; Flegel et al., 1998).

Zhou and colleagues studied the comparison between genotyping and serological phenotyping in RhCE blood group and found the results of *E/e* and *c* alleles genotyping were correct with PCR-SSP and accordant with that of phenotyping. However, using the C48G polymorphism in exon 1 of *RHCE* to genotype *C* allele would result in false positive resulting from *c* allele mutation at this locus, and using the 109 bp insertion to genotype *C* allele gene would result in false negative because of the absence of the 109 bp (Zhou et al., 2008). Existence of hybrid *RHCE* (5:667-5:712) that carries p.A226P specific for E phenotype explains the amplification observed in (Figure 3.30, RH-TYPE gel view, *lane* 12) for E *allele*.

Avent and colleagues found that mRNA transcript by RT-PCR and amplification of genomic DNA has failed to identify any *RHCE* gene or transcript that encodes RhE in DBS (Avent et al., 1996a). However, they found partial E in serological test, where in this case it came to be negative. BAGene partial D-Type also showed possible intron 7/exon 8 missing or have allelic mutation at primers binding site, suggesting combination of another

DAR (weak D 4.2) allele on the same haplotype. Sample 82 would need further investigations, which could reveal a new *DBS-0* like allele in this sample. Our studied sample is reclassified as D positive sample since partial D *DBS-0* allele was detected and to be removed from the D negative donor pool, but should be studied further in future at messenger RNA (mRNA) level. As the sample showed *RHD*DAR* allele, this suggests inheritance with *RHCE*ceAR* or *RHCE*ceEK* (Hemker et al., 1999) which requires confirmation in future.

Another thought, DVI type 1 gives an incorrect E genotype signal derived from the *RHD* gene. The *RHD-CE-D* hybrid of DVI type 1 is always linked to an *RHCE* with E genotype & phenotype. Hence, the sample has no E antigen, therefore cannot have DVI type 1. The other 3 DVI type (2 to 4) all come with C antigen & phenotype also not an explanation for the sample. The solution of the observed discrepancy will require establishing *the RHD-RHCE* haplotype in sufficient detail in future.

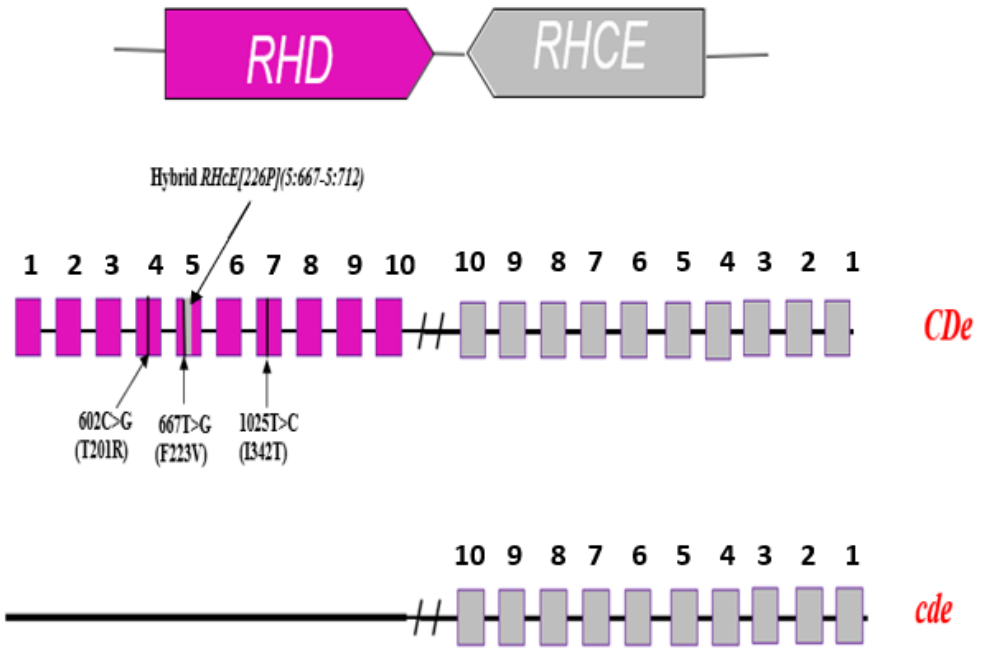


Figure 3.41 Possible *DBS-0 allele* in cis to *DAR 2.00* (novel *DBS-0* like allele) on sample 82.

The 10 exons of the *RHD* (pink box) & *RHCE* (grey box) genes are symbolized by squares and numbered 1 to 10. The grey box within *RHD* box indicates hybrid *RHcE* involved in *DBS-0* partial D allele. The black line within *RHD* exon denotes for missense mutation involved in *DAR 2.00*. Sample 82 genotype possibly composed of the variant *DBS-0* in cis to *DAR 2.00* (DCe) haplotype (top panel) and complete *RHD* gene deletion (bottom panel). A possible novel *DBS-0* like allele is composed of hybrid RHcE[226P](5:667-5:712) and 602C>G, 667T>G and 1025T>C missense mutations in *RHD* exons 4, 5 and 7 respectively (top panel).

Sample 88, was observed to have no exon 4 with real-time PCR and BAGene partial D-Type PCR-SSP. This is an indication of the existence of DFR partial D type. Many partial D DFR types has been categorized with similar molecular background. *DFR type 1* (*ISBT RHD*DFR1*) characterized by hybrid *RHD-CE(4:505-4:514)-D* with multiple haplotypes (Rouillac et al., 1995), *DFR type 2* (*ISBT RHD*DFR2*) with hybrid *RHD-CE(4)-D* in CDe haplotype, DFR type 3 (*ISBT RHD*DFR3*) with 539G>C (G180A) and

RHD-CE(4:505-4:514)-D with CDe haplotype (von Zabern and Flegel, 2007) and *DFR* type 4 (*ISBT RHD*DFR1*) with hybrid *RHD-CE(4:505-4:514)-D* in haplotype CDe. According to serological finding, sample 88 phenotype is ddccee (No C antigen), therefore *DFR type 1* is most probably the phenotype. BAGene partial D-Type PCR-SSP failed to show the amplification of exon 4 due to the primer that targets base pair 505, which is substituted in this case with *RHCE* hybrid nucleotides. This was reconfirmed by BAGene RH-TYPE as mix 1 showed 224 bp band for the amplification of exon 4/intron4 as it targets a different location in exon 4 away from hybrid *RHCE (4:505-4:514)*. In contrast, BAGene RH-TYPE failed to amplify exon 4 in mix 3 that target exon 4 at the area where *RHCE (4:505-4:514)* exist which confirms the finding. BAGene partial D-Type PCR-SSP failed to show in band with mix 8 which suggests *DAR 2.00* allele involvement in the same haplotype. Therefore, existence of *DFR-1* in cis to *DAR 2.00* in one haplotype and complete *RHD* deletion in another haplotype (Figure 3.42) could explain serological D negative phenotype. This sample therefore, is most probably *Dce/dce* genotype and should be reclassified as D positive. Similar to sample 82, this sample also showed *RHD*DAR* allele, which suggests inheritance with *RHCE*ceAR* or *RHCE*ceEK* that requires confirmation in future.

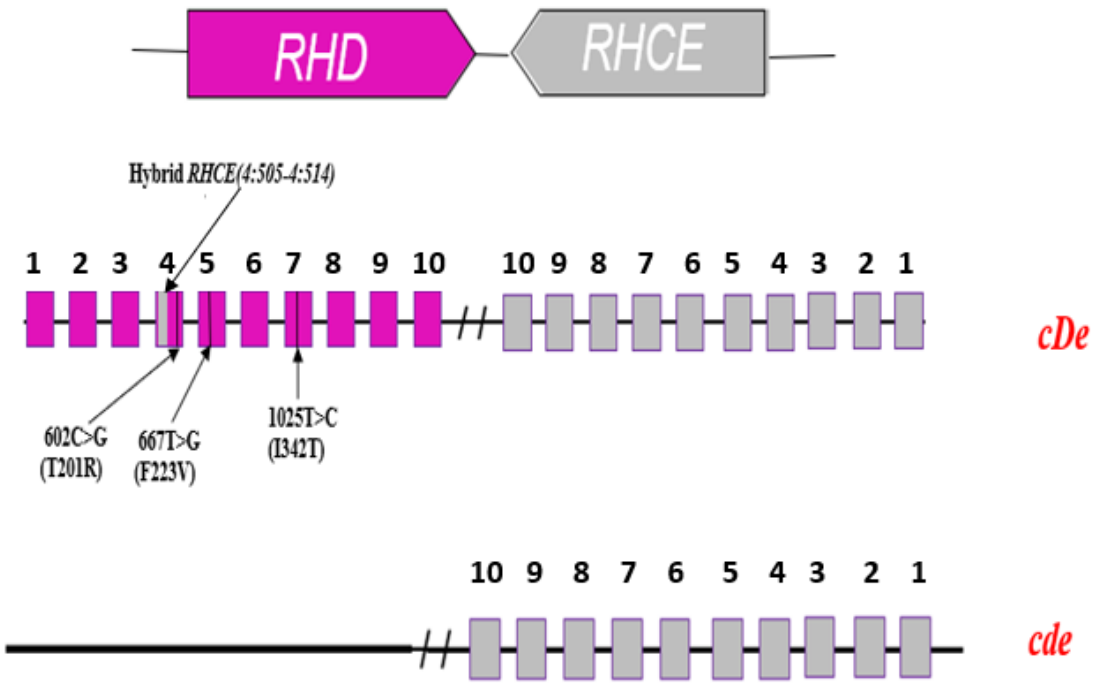


Figure 3.42 Possible *DFR-1* in cis to *DAR 2.00* in sample 88.

The 10 exons of the *RHD* (pink box) & *RHCE* (grey box) genes are symbolized by squares and numbered 1 to 10. The grey box within RHD box indicates hybrid *RHCE* involved in *DFR-1* partial D allele. The black line within *RHD* exon denotes for missense mutation involved in *DAR 2.00*. Sample 88 genotype possibly composed of the variant *DFR-1* in cis to *DAR 2.00* (cDe) haplotype (top panel) and complete *RHD* gene deletion (bottom panel). *DFR-1* in cis to *DAR weak D 4.2* is composed of hybrid *RHCE* (4:505-4:514) and 602C>G, 667T>G and 1025T>C missense mutations in *RHD* exons 4, 5 and 7 respectively (top panel). The hybrid *RHCE* (4:505-4:514) is unique to *DFR* partial D type.

We also observed another African origin hemizygous / homozygous *RHDΨ* in eight samples. This allele causes 37 bp insertion in *RHD* exon Intron3/exon4. For real-time PCR, primers RHDΨ-F and RHDΨ-R adapted from (Singleton et al., 2000) designed in a way to amplify existence of exon 4 in general with or without duplication of 37 bp to give a product of 381 bp for a normal type *RHD* exon 4 (without 37 bp insertion) and 418 bp for *RHDΨ*. Sequence specific Primers (SSP) for exon 5 adapted from (Legler et al., 2007) designed in a way so the forward primer 3' specific for wild type c.654 in exon 5 and do not amplify mutation G>C (M218I) associated with *RHDΨ*. Therefore, amplification of

exon 4 and no amplification of exon 5 suggests *RHDΨ*. Samples that suggested hemizygous / homozygous *RHDΨ* in real-time PCR showed amplification of exons 1 through 10 except exon 5. This observation was further confirmed by BAGene partial-D and BAGene RH-TYPE. BAGene partial D-Type for *RHDΨ* presence showed deletion of exon 4 (Figure 3.28, *lane* 4) only. The SSP in mix 4 *(lane* 4 in partial D-TYPE gel view) in the kit targets the area of exon 4 and 37 bp insertion due to *RHDΨ* interferes with primer target region and thus explains the failure to amplify exon 4. BAGene RH-TYPE reconfirmed the finding by showing amplification in mix 1 and 2 (Figure 3.28, *lane* 1 and 2, RH-TYPE gel view) for exon 4/intron 4 (222 bp) and *RHDΨ* respectively, whereas mix 3 *(lane* 3) did not show any amplification for exon 4 that is specific for normal *RHD* exon 4 without 37 bp insertion.

The Omani population is heterogeneous, with mixed ethnicity tracking their ancestral roots to neighboring countries via tribal migrations and trading contacts. The main ethnicities are Arab, Baluchi, Sindi and African. Arabs migrated from what was known as Arabia since the 9th century BC onward and settled in Oman. Some Omani African originated from Africa, due to the historical trade between Oman, Zanzibar and Mombasa favoured by the Indian Oceans monsoons. Oman was a Portuguese colony from 1508 to 1741, and when the Portuguese were forced out, Oman became an empire that expanded to Zanzibar. This lasted until 1861, when Zanzibar was separated from Omani control. Omani African started coming back to Oman, as a result of that (Suha, 2015; Suha et al., 2015). This might explain the existence of African *RHD* alleles (*RHDΨ* and *RHD-CE-D^{s})* in Omani population.

As stated in Table 3.7, sample 93 showed amplification of exon 4 and no amplification of exon 5 in real-time PCR which suggests existence of *RHDΨ* gene. In contrast, BAGene partial D-Type gel view in Figure 3.27 showed no amplification in mix

tubes for *RHD* exon 4, 5 and 8 *(lane*s 4, 5 and 8 respectively), which supported the possibility of *RHDΨ* gene existence along with other *RHD* alleles. It also showed amplification of all other exons in addition to mix 15 *(lane* 15) that which 140 bp band and confirmed the existence of 1136C>T (T379M) in exon 8 that is unique for partial D *DAU* allele. No amplification in tubes 5 and 8 *(lane*s 5 and 8 respectively) suggest existence of *DAR 2.00* allele, whereas no amplification in tube mix 5 with amplification in mix 15 *(lane* 15) suggests *DAU*. Allele specific primers used in BAGene partial D-Type for mix 5 targets *RHD* exon 5 nucleotide 697 that is substituted in both *DAU* and *DAR2.00* alleles and this explains the failure to amplify. BAGene RH-TYPE showed unreliable results with *RHD* alleles detection (no amplification) but confirmed presence of *RHce* alleles. Sanger sequencing from the real-time PCR product of exon 4 confirmed the existence of 37bp insertion with c.609G>A point mutation as previously observed (Singleton et al., 2000). The results are consistent with *RHDΨ* in cis to DAU and DAR2.00 as one haplotype, which is in agreement with Rhce phenotype in this sample. Exon 8 deletion was excluded in this case, due to detection of *RHD* allele 1136C>T found in exon 8 (detected by BAGene partial-D kit). This sample is possibly *RHDΨ* Hemizygous and the insertion of 37 bp makes the gene inactive and therefore phenotype exist as D negative in serology. Therefore, sample 93 has inactive *RHD* gene and can be classified as D negative with possible *dce/dce* genotype (Figure 3.43). The existence of *DAU* and *DAR* may be associated with *RHCE*ceMO* (Westhoff et al., 2013b) and *RHCE*ceAR* or *RHCE*ceEK* variants respectively.

Sample 95 phenotypes as RhCcee with no amplification of exon 2, 4 and 5 with real-time PCR and no amplification of exon 2 and 9 with BAGene partial D-Type. The absence of amplification of exon 9 in BAgene and its existence in real-time PCR excludes the possibility of *RHD* (delEx9) allele presence. According to the BAGene worksheet

instructions, present of weak D types 41 and 45 may cause missing reaction with exon 9. In real-time PCR, using allele specific reverse primers re94 stated in Table 2.1 targets nucleotide 1193 (mutation site for weak D type 41 in exon 9) which amplified the exon suggesting no mutation at this site. Therefore, weak D type 45 (1195G>A) is possibly the case in this sample but not weak D type 41. However, further confirmation with BAGene Weak D-TYPE would unmask the type. The missing amplification in real-time PCR for exon 2 is due to specificity of the 3´ forward primer ME2F and probably forward primer in BAGene kit to detect 203 base pair in *RHD* exon, which is different in *RHCE* and thus suggests hybrid *RHCE(2)* called *DIIIb* partial D. Missing reaction in exon 5 probably is due to mutation at location c.654, which is allele specific primer target of forward primer RHDex5F, whereas BAGene allele specific primer target location of exon 5 is 697 and this explains amplification reaction in the mix 5. Therefore, this sample is possibly *DIIIb* in cis to *weak D 45* and reclassified as D positive.

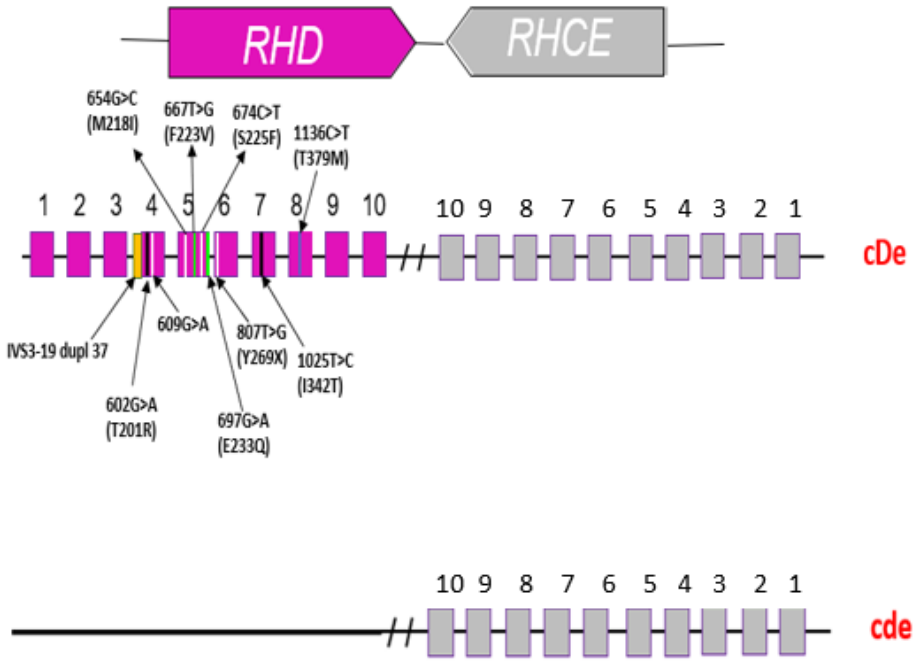


Figure 3.43 Possible hemizygous *RHDΨ* in cis to *DAU-5* and *DAR2.00* in sample 93.

The 10 exons of the *RHD* (pink box) & *RHCE* (grey box) genes are symbolized by squares and numbered 1 to 10. The orange box within *RHD* box 4 indicates 37 bp insertion in *RHD* Intron3/exon4 unique for *RHDΨ*. The white line within *RHD* exons denotes for missense mutation involved in *RHDΨ* gene. The black line within *RHD* exons denotes for missense mutation involved in DAR2.00. The blue line with *RHD* exon 8 denotes for unique missense mutation seen in *DAU-5*. The green lines within *RHD* exon 5 denote the mutual missense mutation between *DAU-5* and *DAR2.00*. Sample 93 genotype possibly composed of the variant *RHDΨ* in cis to *DAU-5* and *DAR2.00* (cDe) haplotype (top panel) and complete *RHD* gene deletion (bottom panel). One haplotype is possible *RHDΨ* in cis to *DAU-5* and *DAR2.00* (Top panel). *IVS3-19 dupl 37* and 609G>A was confirmed with Sanger sequencing. Exon 5 mutations c.654G>C and 667T>G was assumed to be in as exon 5 primers for Taq-Man and SYBR Green chemistry target these alleles respectively and showed negative. Exon 5 for c.674C>T was not tested. BAGene confirmed the presence of 697G>A and 1136C>T alleles specific for *DAU*. The other haplotype is complete gene deletion (bottom panel). The inactivation of the gene due to *IVS3-19dupl 37* is the cause for D negative phenotype.

Sample 126 phenotypes as Rhccee, showed missing reaction with exons 8 and 9 in BAGene partial D-Type and missing reaction with exon 9 in real-time PCR. In both methods missing reaction suggests *RHD(delEx9)*, *RHD*D-CE(8-9)-D* or weak D type 41. Both *RHD(delEx9)* and *RHD*D-CE(8-9)-D* were excluded as they come with CDe and Cde respectively and *C* antigen is negative in this case. Both real-time PCR forward ASP used for the detection of exon 9 in this study and BAGene ASP primer in mix 9 target c.1193, this could explain failure to amplify exon 9 in both reactions. A missing reaction in mix 8 with BAGene partial-D Type suggests existance of DAR1.00 (weak D 4.2) as well. Therefore, this is possibly a case of *weak D 4.2* in cis to *weak D type 41* with probably a *Dce/dce* genotype and can be classified as D positive. Existence of *weak D 4.2* may be associated with a *RHCE*ceAR* allele (Prisco Arnoni et al., 2016).

Sample 151 with RhCcee phenotype showed no amplification of exon 4, 8 and 9 with BAGene partial D-Type (Figure 3.34, *lane* 4, 8 and 9 respectively), whereas no amplification with exon 4, 5 and 9 with real-time PCR. RH-TYPE gel view showed no amplification of exon 4 in *lane* 1 and 3 which further confirmed the deletion of exon 4 and no amplification in *lane* 4 (K409K negative) which confirms *RHD* exon 9 deletion / *RHCE* hybrid. The findings are suggestive of *DFR* as all exon 4 primers in BAgene and real-time PCR failed to amplify. Exon 5 deletion in real-time PCR with amplification in BAGene suggests mutation existence in exon 5 in addition to DFR, which needs further study. Exon 9 deletion in both may confirm *RHD-CE(8-9)-D* allele presence. Presence of *C* allele is in consistent with DFR and *RHD-CE(8-9)-D* combination in one haplotype. Though both alleles are reported to be CDe haplotype, however *RHD-CE(8-9)-D* existence may explain D negative phenotype in the serology as observed in previous studies (Wagner, Frohmajer and Flegel, 2001). Therefore, this sample may be hemizygous *DFR* in cis to *RHD-CE(8-9)-D* and categorized as D negative due to the presence of hybrid *RHCE(8-9)*.

Sample 160 (Figure 3.35) with Rhccee phenotype with weak e (Figure 3.4B) showed amplification of exons 1, 2, 3, 5, 6, 7, 9 and 10 with RH partial D-Type. Real-time PCR showed no amplification with exon 4 only (Exon 8 not tested). Combining missing exon 4 in both methods suggests *DFR-1* as no C antigen exists. As exon 8 band *(lane* 8) was also not seen, *weak D 4.2* is predicted to be in cis to *DFR-1* as one haplotype. BAGene RH-TYPE gel view showed no amplification of 224 bp band for exon 4, no amplification in *lane* 2 and 3 all for exon 4 which further confirms deletion of *RHD* exon 4 and possibility of hybrid *RHCE(4). Lane* 7 has a band of 143 bp which confirms DEL *RHD* (IVS3+1G>A). Exon 9 amplification in real-time PCR suggests no hybrid or deletion of exon 9. Sanger sequencing from real-time PCR exon 3 did not show *DEL RHD (IVS3+1G>A),* which suggested a contamination band seen in RH-TYPE gel. *Lane* 8 did not show any band to suggest involvement of *(C)ce*^{s}, due to the presence of weak e in serology test. Therefore, this sample can be classified as D positive. Weak e with *weak D type 4.2* may be associated with *RHCE*ce.01* (Fichou et al., 2015). This needs further testing and evaluation at molecular level.

Sample 230 serological phenotyping appeared to be ddCcee. Both real-time PCR and BAGene partial D-Type showed missing exons 4, 5 and 6 (Figure 3.36, *lane* 3, 5 and 6 respectively) suggesting partial D type DVI.2. RH-TYPE gel view confirmed deletion of exon 4 by missing reaction band of 244 bp in *lane* 1, missing reaction in lane 2 and missing reaction in *lane* 3 (Figure 3.36). Sanger sequencing of exon 9 from real-time PCR product revealed existence of heterozygous DEL(IVS8-31T>C) (Figure 3.25). The presence of heterozygosity suggests *RHD* gene is not completely deleted on the other haplotype. Present of antigen C is consistent with DEL and DVI.2 existence, however, molecular basis of zygosity determination would be a good approach to further explore the

case. Therefore, this case is classified as D positive with a haplotype DCe and probable DCe/dce or DCe/Dce genotype where *DVI.2* in cis or trans to *DEL(IVS8-31T>C)*.

Sample 234 with serological phenotype ddccEe showed missing reaction in mix 2 and mix 4 (Figure 3.28, l*ane* 2 and 4) with BAGene partial D-Type and missing reaction with exon 2 and 5 in real-time PCR. This suggested existence of *RHDΨ* and DIIIb partial D in one haplotype (Figure 3.44). This is consistent with appearance of E in partial D DIIIb which was retyped to DIII type 7 (Lomas-Francis et al., 2012). Missing reaction in mix 2 with BAGene is specific for *DIIIb*, RH-TYPE gel view showed 2 bands in mix 1 which suggests existence of exon 4, but failed to amplify *RHDΨ* as control did not appear as well in mix 2 but also failed to prove non-existence of *RHDΨ* in mix 3, (Figure 3.28, *lane* 1, 2 and 3, RH-TYPE gel view). However, Sanger sequencing of exon 4 amplified by real-time PCR showed IVS3-19 dupl 37 and 609G>A hemizygous which confirms the finding. Therefore, this is a D negative sample caused by inactive *RHDΨ* allele with a probable DcE/dce genotype.

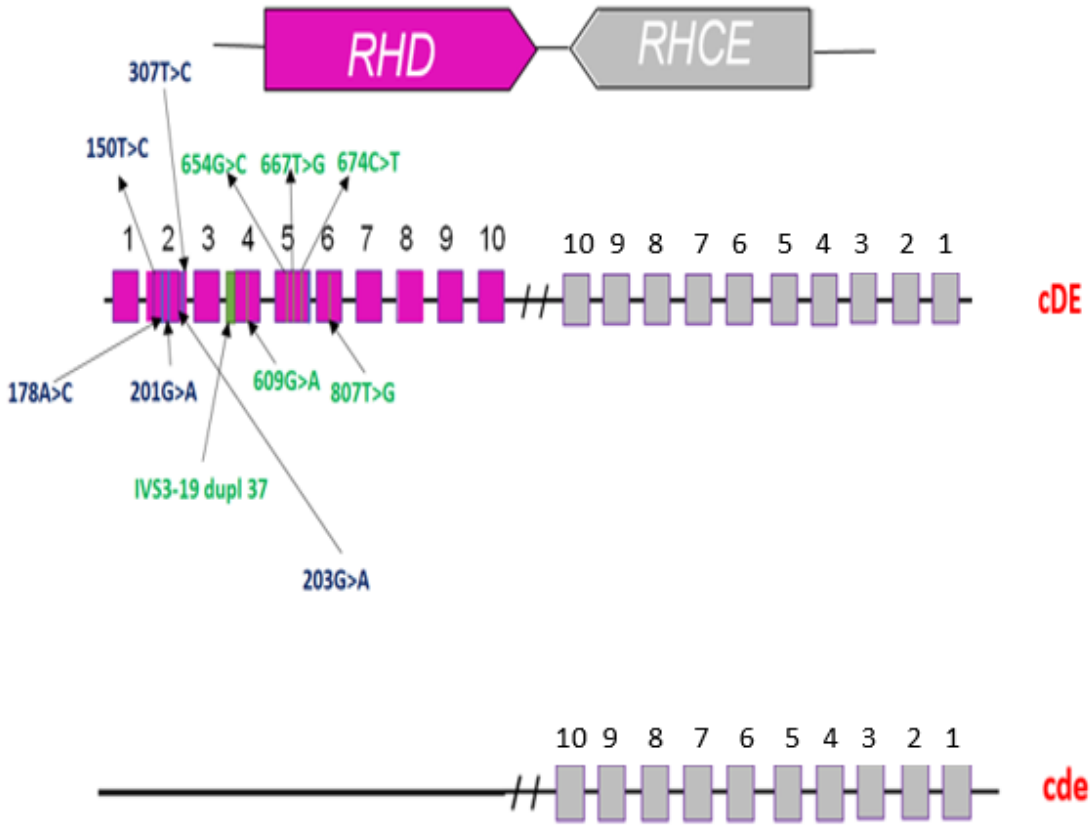


Figure 3.44 Possible *RH* genotype in sample 234.

The 10 exons of the *RHD* (pink box) & *RHCE* (grey box) genes are symbolized by squares and numbered 1 to 10. The green box within *RHD* box 4 indicates 37 bp insertion in *RHD* Intron3/exon4 unique for *RHDΨ*. The green line within *RHD* exons denotes for missense mutation involved in *RHDΨ* gene. The blue line within *RHD* exons denotes for missense mutation involved in *DIIIb*. The haplotype (cDE) is possibly have *DIIIb* in cis to *RHDΨ* (Top panel) with complete *RHD* deletion in other haplotype (bottom panel – *cde*). IVS3-19 dupl 37 and 609G>A was confirmed with Sanger sequencing. Exon 5 mutations c.654G>C and 667T>G was assumed to be in as exon 5 primers for Taq-Man and SYBR Green chemistry respectively target these alleles and showed no amplification. Exon 5 for c.674C>T was not tested. BAGene confirmed the presence of *DIIIb* by missing reaction for exon 2. The other haplotype is complete gene deletion. The inactivation of the gene due to IVS3-19 dupl 37 is the cause for D negative phenotype and thus cdE haplotype as DIIIb appear with E.

In PCR, failure of amplification of one of two alleles at any single target locus may be due to either sequence independent factors or allele-specific sequence variations (Blais et al., 2015). Use of extracted DNA from degraded specimen as a template for DNA typing results in less efficient PCR amplification. Additionally, using trace amounts of extracted DNA for the template poses the risk of allele drop-out. Allelic drop-out can even be observed in undegraded DNA sample with enough amount of extracted DNA (Tsukada et al., 2008). Therefore, inability of primer pairs used in sequencing of *RHD* exon 3 to reconfirm heterozygosity of c.455A>C mutation (see section 3.7.3) may be due to allelic drop-out raised from trace amount of extracted or possibly degraded DNA.

Overall *RHD* deletion was the commonest cause for serological D negative in Omani population (*n* = 179, 87.8%), followed by existence of African ancestry *RHDΨ* (*n* = 10, 4.43%) that makes *RHD* gene inactive. The existence of different D variants such as weak D and partial D in cis to each other with *RHD* deletion in other haplotype and low sensitivity of anti-D IgM/IgG blend (Atlas, UK) may explain the failure to detect these variants and reclassify them as D positive in serology. PCR are useful for the identification of common Rh variants, but are not assertive in excluding the presence of variant alleles, even with multiplex reactions (Dezan et al., 2017). Therefore, digital PCR based assay could be very effective to unveil the correct molecular background.

4 CHAPTER FOUR: *RHD* genotyping using QuantStudio™ 3D Digital PCR System

4.1 Introduction:

Detection of specific DNA sequence by PCR test is essential in research and diagnostic laboratories. The first generation of PCR analyses were based on qualitative detection of DNA amplicons that were visualised by agarose gel electrophoresis. The advent of second-generation so called real-time PCR was based on quantitation by monitoring the progression of amplification after each cycle using fluorescence probes. However, in order to estimate unknown concentration in real-time PCR external calibrators or normalization to endogenous controls are required. Imperfect amplification efficiencies affect cycle threshold (C_t) values which in turn limits the accuracy of this technique for absolute quantitation (Hindson et al., 2011). Next to the well-established method real-time PCR (qPCR); a new quantification strategy, digital PCR (dPCR), has been developed (Bustin et al., 2009; Huggett et al., 2013). This new approach is based on portioning of the measured sample into thousands of uniform droplets (separate reactions) which are then amplified and analysed individually.

Several dPCR platforms are available and generally they can be divided in two groups: droplet digital PCR (ddPCR) such as Bio-Rad's QX100/QX200 and chip-based digital PCR (cdPCR) such as Thermo Fisher Scientific QuantStudio™ 3D. Currently, traditional PCR and quantitative real-time PCR (qPCR) is the popular choice for specific nucleic acid detection including *RHD* genotyping. However, studies on *RHD* genotyping using ddPCR have been in practice as well. Svobodova and colleagues implemented ddPCR for fetal *RHD* genotyping from maternal plasma, compared it with real-time PCR and found that the precision of both methods equalized with decreasing concentrations of tested DNA samples (Svobodova et al., 2015). Another similar study conducted by

Sillence and colleagues demonstrated ddPCR to be greater sensitivity than real-time PCR (Sillence et al., 2015). Droplet digital PCR has also been used for the determination of *RHD* zygosity as qPCR and Rhesus box PCR have limitations particularly for black African populations as some individuals have differences in the hybrid box (Matheson and Denomme, 2002; Grootkerk-Tax et al., 2005). Sillence and colleagues extracted DNA from blood samples and found that ddPCR is highly accurate method to rapidly define *RHD* zygosity (Sillence et al., 2017).

Extracted DNA from blood sample and cell free DNA (cfDNA) has been used for *RHD* genotyping using cdPCR. Yet, determination of *RHD* genotyping using DNA sample extracted from dry blood spot on FTA card has not been studied. In our study, cdPCR was implemented for *RHD* genotyping using DNA sample extracted from dry blood spot on FTA card which we believe is the first with this kind of sample. We hypothesized that dPCR will rapidly amplify extracted DNA from dry blood spot on FTA card. The study was divided into two parts. In the first part, *RHD* exons 5, 7 and 10 amplification were observed using SYBR Green chemistry. The method was optimized by using temperature gradient (56^{o}C, 58^{o}C and 60^{o}C) experiment. In the second part of our study, *RHD* exon 5 amplification was observed using Taq-Man chemistry.

4.2 Target quantification of RHD exons 5, 7 and 10 using QuantStudio™ 3D Digital PCR System using SYBR Green chemistry

We evaluated the ability of the QuantStudio™ 3D Digital PCR System to detect and quantify *RHD* exons 5, 7 and 10 concentration extracted from dried blood on FTA card using SYBR Green and TaqMan chemistry. The manufacturer's recommended precision range is <10% but our detection precision was >15%, which suggests DNA purity issues with FTA extracted DNA or DNA concentration (For exon 10, Table 4.1 and Table 4.2, for exon 5, Table 4.3 and Table 4.4, for exon 7, Table 4.5 and Table 4.6). For optimization we performed a temperature gradient study on ten D positive samples, with annealing temperatures (60^{o}C, 58^{o}C and 56^{o}C) and compared buccal DNA with DNA extracted from dry blood spot on FTA card. In these experiments, we focussed on *RHD* exons 5, 7 and 10 (because we had TaqMan probes for the detection of these exons) with a total DNA volume of 1 µl (Figures 4.1 to 4.9). The optimum annealing temperature and primer pair concentration should give the best separation of positive and negative droplets. No good separation was observed between amplified (D positive) and non-amplified reaction compared to buccal DNA. The optimum annealing temperature for SYBR Green assay for *RHD* exons 5, 7 and 10 was found to be 56^{o}C as per buccal extracted DNA data.

Once the optimum annealing temperature had been determined (56^{o}C), further study was performed to increase sample volume and thus DNA concentration to detect and quantify *RHD* exon 10. We tested 10 known *RHD* exon 10 positive samples (DNA concentration between 20 and 30 ng/µl) previously tested with real-time PCR. We found that even increasing the volume and concentration of DNA did not improve the precision (> 15%) and detection of the *RHD* gene, which suggested inhibitors effect.

To test for inhibitors effect, the same *RHD* exon 10 positive (*n* = 10) samples diluted 1 in 4 with final sample volumes 1µl, 2.5µl and 5µl to decrease the effect of inhibitors if any. We found that even diluting the samples did not improve the precision (> 15%) and detection of the *RHD* exon (Figure 4.10), which suggested purity issues with FTA extracted samples.

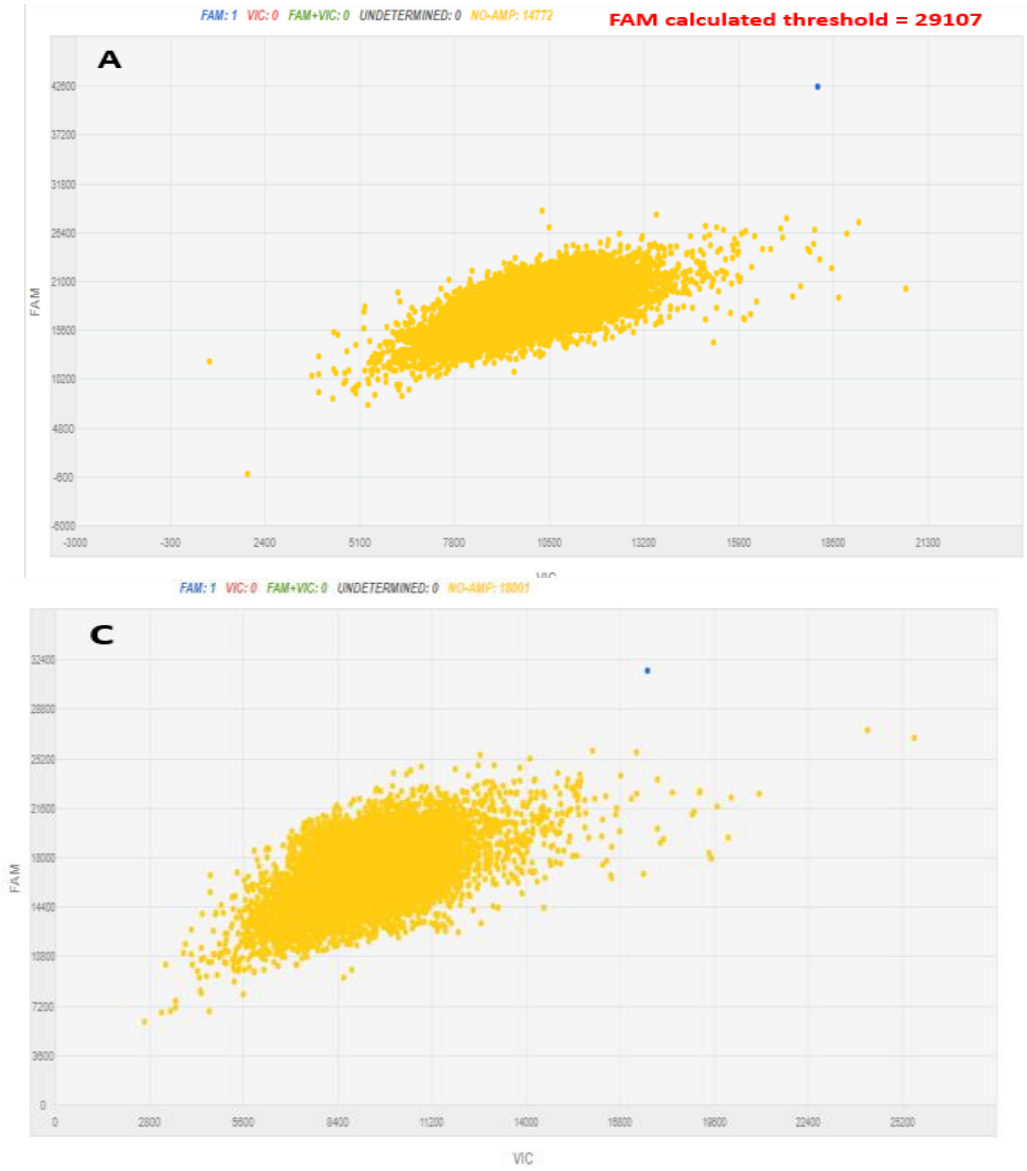


Figure 4.1 Data quality for *RHD* exon 10 with annealing temperature 60°C as displayed in QuantStudio™ 3D AnalysisSuite™ Cloud Software.

Scattered plot views for NTC (A), *RHD* exon 10 buccal DNA extracted (B) and *RHD* exon 10 extracted from dry blood spot on FTA card (C) with SYBR Green I read in the FAM™ channel. Yellow dots correspond to no amplification whereas blue dot correspond to amplification of target exon using FAM channel. As per NTC plot view with non-amplified wells, the adjusted FAM threshold is 29107 (A). Only plot view of buccal extracted *RHD* exon 10 (B) show two populations: the larger yellow population corresponds to the non-amplified wells with lower fluorescence, and the very smaller blue population corresponds to the amplified wells with significantly higher fluorescence.

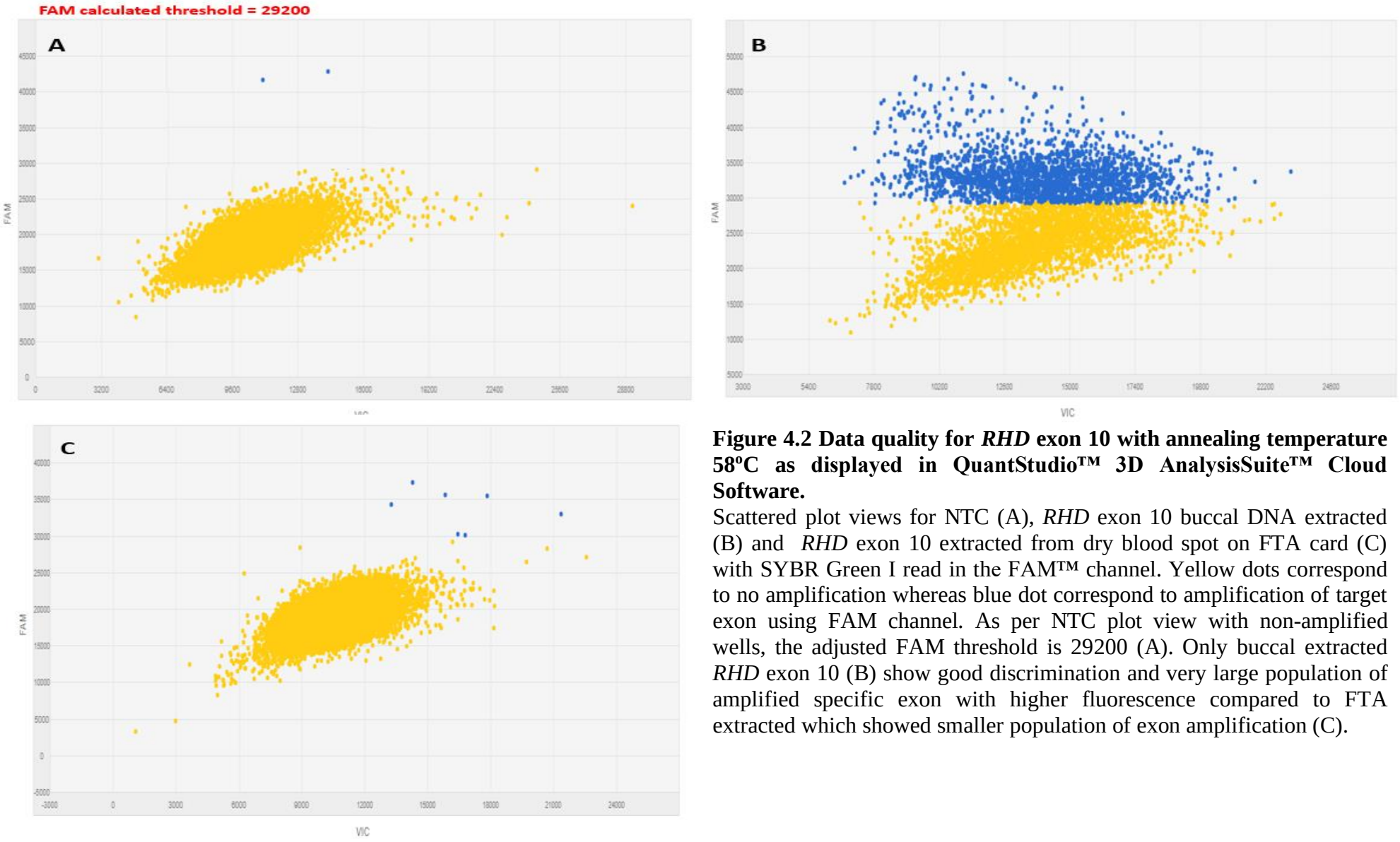


Figure 4.2 Data quality for *RHD* exon 10 with annealing temperature 58ºC as displayed in QuantStudio™ 3D AnalysisSuite™ Cloud Software.

Scattered plot views for NTC (A), *RHD* exon 10 buccal DNA extracted (B) and *RHD* exon 10 extracted from dry blood spot on FTA card (C) with SYBR Green I read in the FAM™ channel. Yellow dots correspond to no amplification whereas blue dot correspond to amplification of target exon using FAM channel. As per NTC plot view with non-amplified wells, the adjusted FAM threshold is 29200 (A). Only buccal extracted *RHD* exon 10 (B) show good discrimination and very large population of amplified specific exon with higher fluorescence compared to FTA extracted which showed smaller population of exon amplification (C).

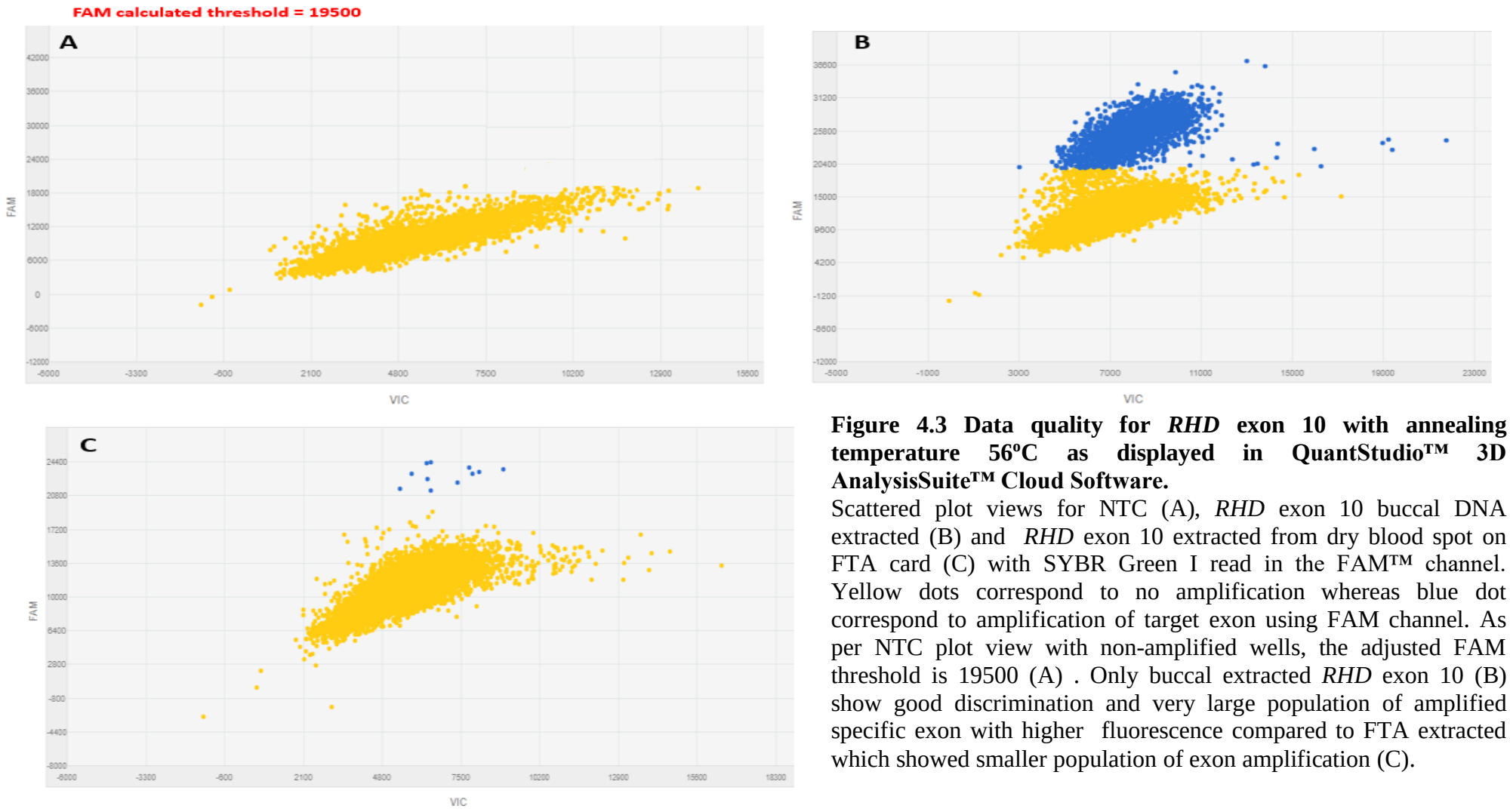


Figure 4.3 Data quality for *RHD* exon 10 with annealing temperature 56ºC as displayed in QuantStudio™ 3D AnalysisSuite™ Cloud Software.
Scattered plot views for NTC (A), *RHD* exon 10 buccal DNA extracted (B) and *RHD* exon 10 extracted from dry blood spot on FTA card (C) with SYBR Green I read in the FAM™ channel. Yellow dots correspond to no amplification whereas blue dot correspond to amplification of target exon using FAM channel. As per NTC plot view with non-amplified wells, the adjusted FAM threshold is 19500 (A) . Only buccal extracted *RHD* exon 10 (B) show good discrimination and very large population of amplified specific exon with higher fluorescence compared to FTA extracted which showed smaller population of exon amplification (C).

Table 4.1 SYBR Green I assay summary of results for *RHD* exon 10 quantification extracted from buccal cells, with statistical analysis performed on AnalysisSuite™ software.

Annealing temperature	Copies / µl	CI[a] (copies/µl)	Precision[b] (%)	Chips[c]
56ºC	191.97	184.25 -- 200.01	4.9	2
58ºC	2618.1	2529.4 -- 2710	3.5	1
60ºC	1.18	0.699 -- 1.992	68.85	1

[a] Lower and upper 95% confidence interval for quantity of sample in copies/µl.

[b] Calculated precision (%) for the data group, defined as size of the confidence interval for distinguishing between two sample concentrations at a given confidence level.

[c] Number of chips containing the data group. Multiple chips can be combined to improve the calculated precision.

Table 4.2 SYBR Green I assay summary of results for *RHD* exon 10 quantification extracted dried blood on FTA card, with statistical analysis performed on AnalysisSuite™ software

Annealing temperature	Copies / µl	CI[a] (copies/µl)	Precision[b] (%)	Chips
56ºC	1.899	1.283 -- 2.81	48	1
58ºC	0.556	0.265 -- 1.166	110	1
60ºC	< 0.5	< 0.5	610	1

[a] Lower and upper 95% confidence interval for quantity of sample in copies/µl.

[b] Calculated precision (%) for the data group, defined as size of the confidence interval for distinguishing between two sample concentrations at a given confidence level.

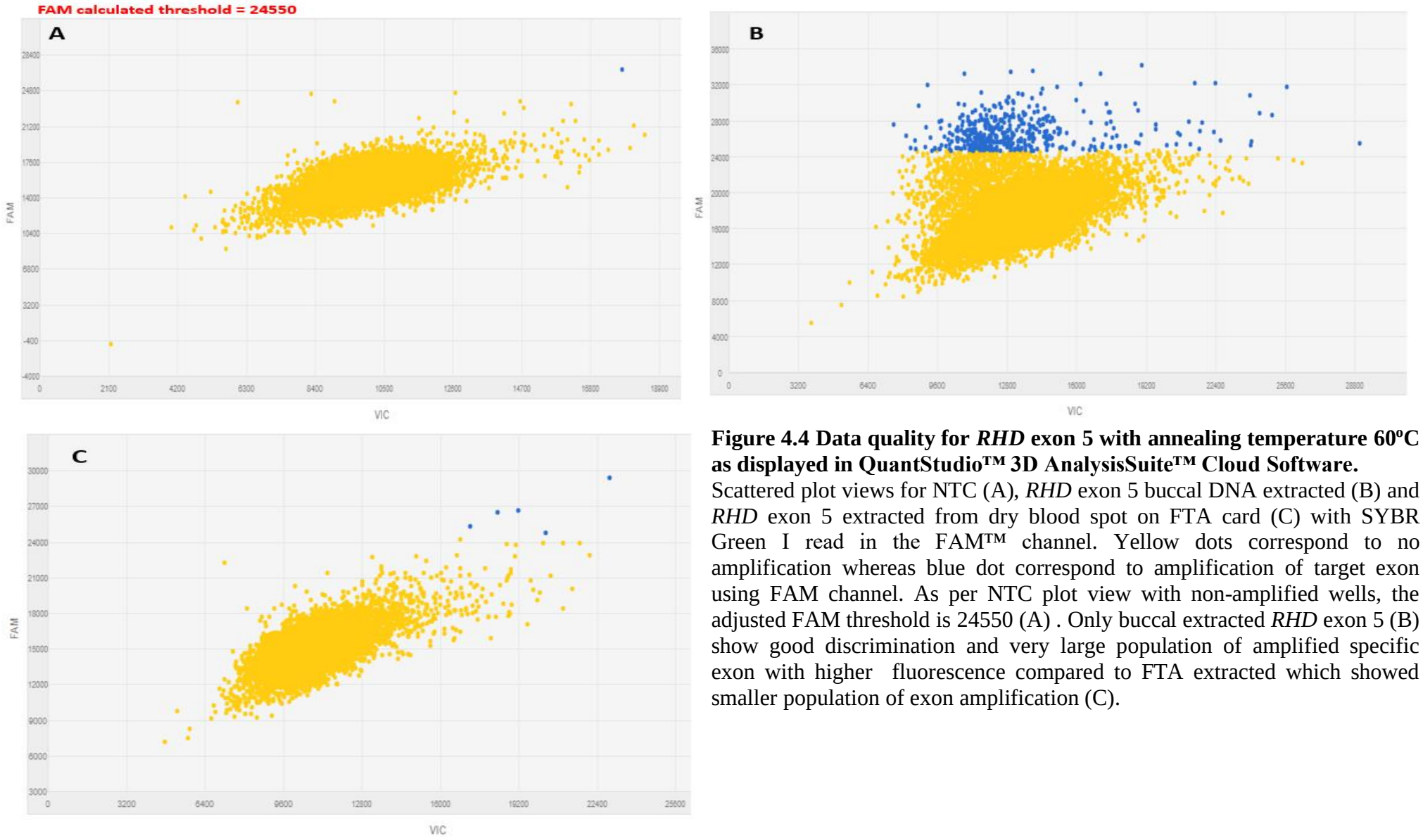


Figure 4.4 Data quality for *RHD* exon 5 with annealing temperature 60ºC as displayed in QuantStudio™ 3D AnalysisSuite™ Cloud Software.
Scattered plot views for NTC (A), *RHD* exon 5 buccal DNA extracted (B) and *RHD* exon 5 extracted from dry blood spot on FTA card (C) with SYBR Green I read in the FAM™ channel. Yellow dots correspond to no amplification whereas blue dot correspond to amplification of target exon using FAM channel. As per NTC plot view with non-amplified wells, the adjusted FAM threshold is 24550 (A) . Only buccal extracted *RHD* exon 5 (B) show good discrimination and very large population of amplified specific exon with higher fluorescence compared to FTA extracted which showed smaller population of exon amplification (C).

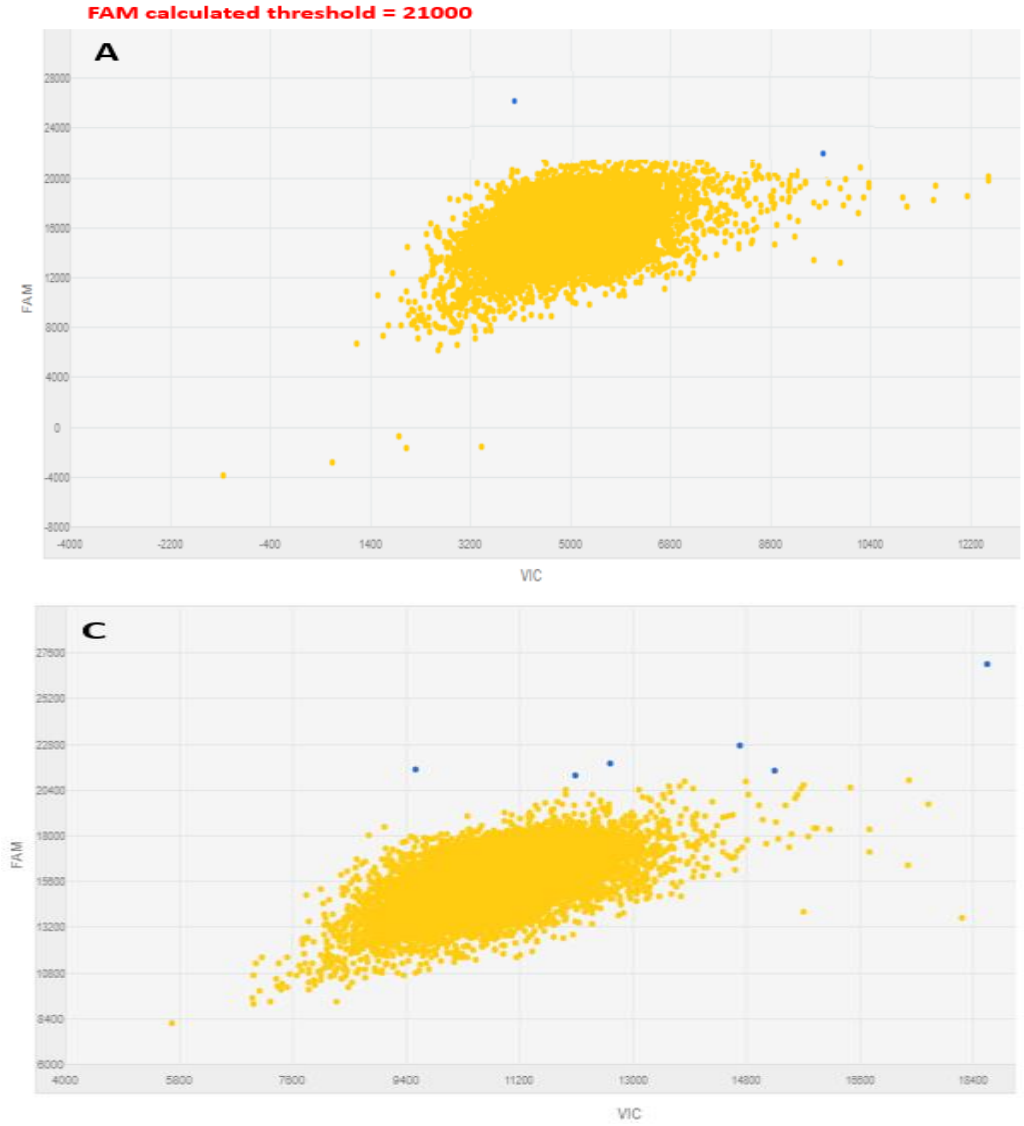


Figure 4.5 Data quality for RHD exon 5 with annealing temperature 58oC as displayed in QuantStudio™ 3D AnalysisSuite™ Cloud Software.

Scattered plot views for NTC (A), *RHD* exon 5 buccal DNA extracted (B) and *RHD* exon 5 extracted from dry blood spot on FTA card (C) with SYBR Green I read in the FAM™ channel. Yellow dots correspond to no amplification whereas blue dot correspond to amplification of target exon using FAM channel. As per NTC plot view with non-amplified wells, the adjusted FAM threshold is 21000 (A) . Only buccal extracted *RHD* exon 5 (B) show good discrimination and very large population of amplified specific exon with higher fluorescence compared to FTA extracted which showed smaller population of exon amplification (C).

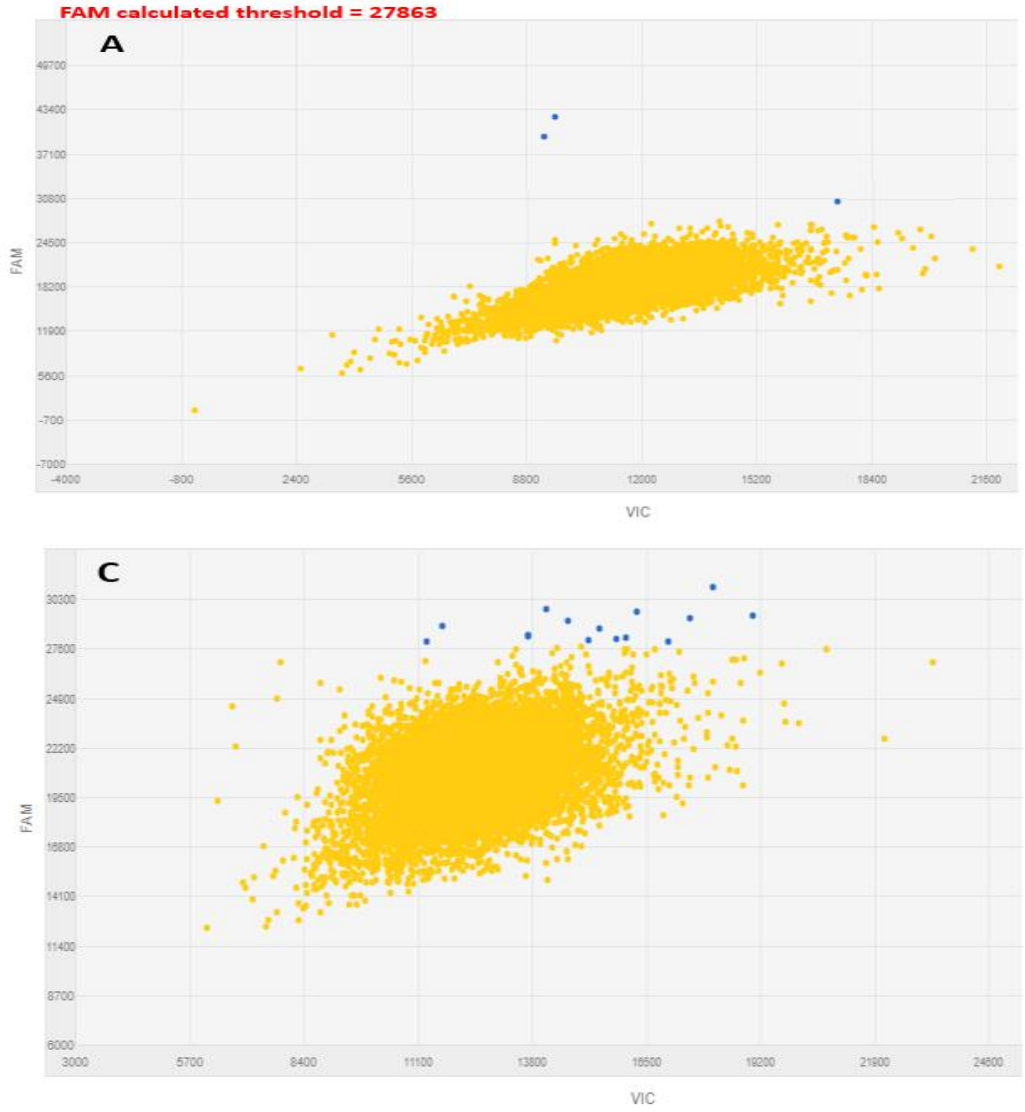


Figure 4.6 Data quality for *RHD* exon 5 with annealing temperature 56ºC as displayed in QuantStudio™ 3D AnalysisSuite™ Cloud Software.

Scattered plot views for NTC (A), *RHD* exon 5 buccal DNA extracted (B) and *RHD* exon 5 extracted from dry blood spot on FTA card (C) with SYBR Green I read in the FAM™ channel. Yellow dots correspond to no amplification whereas blue dot correspond to amplification of target exon using FAM channel. As per NTC plot view with non-amplified wells, the adjusted FAM threshold is 21000 (A) . Only buccal extracted *RHD* exon 5 (B) show good discrimination and very large population of amplified specific exon with higher fluorescence compared to FTA extracted which showed smaller population of exon amplification (C).

Table 4.3 SYBR Green I assay summary of results for *RHD* exon 5 quantification extracted from buccal cells, with statistical analysis performed on AnalysisSuite™ software.

Annealing temperature	Copies / µl	CI[a] (copies/µl)	Precision[b] (%)	Chips
56ºC	49.695	45.705 -- 54.033	8.7	1
58ºC	41.946	38.362 -- 45.866	9.3	1
60ºC	37.79	34.353 -- 41.57	10	1

[a] Lower and upper 95% confidence interval for quantity of sample in copies/µl.

[b] Calculated precision (%) for the data group, defined as size of the confidence interval for distinguishing between two sample concentrations at a given confidence level.

Table 4.4 SYBR Green I assay summary of results for *RHD* exon 5 quantification extracted dried blood on FTA card, with statistical analysis performed on AnalysisSuite™ software

Annealing temperature	Copies / µl	CI[a] (copies/µl)	Precision[b] (%)	Chips
56ºC	1.552	0.936 -- 2.575	65.9	1
58ºC	1.115	0.558 -- 2.229	100	1
60ºC	0.564	0.235 -- 1.355	140	1

[a] Lower and upper 95% confidence interval for quantity of sample in copies/µl.

[b] Calculated precision (%) for the data group, defined as size of the confidence interval for distinguishing between two sample concentrations at a given confidence level.

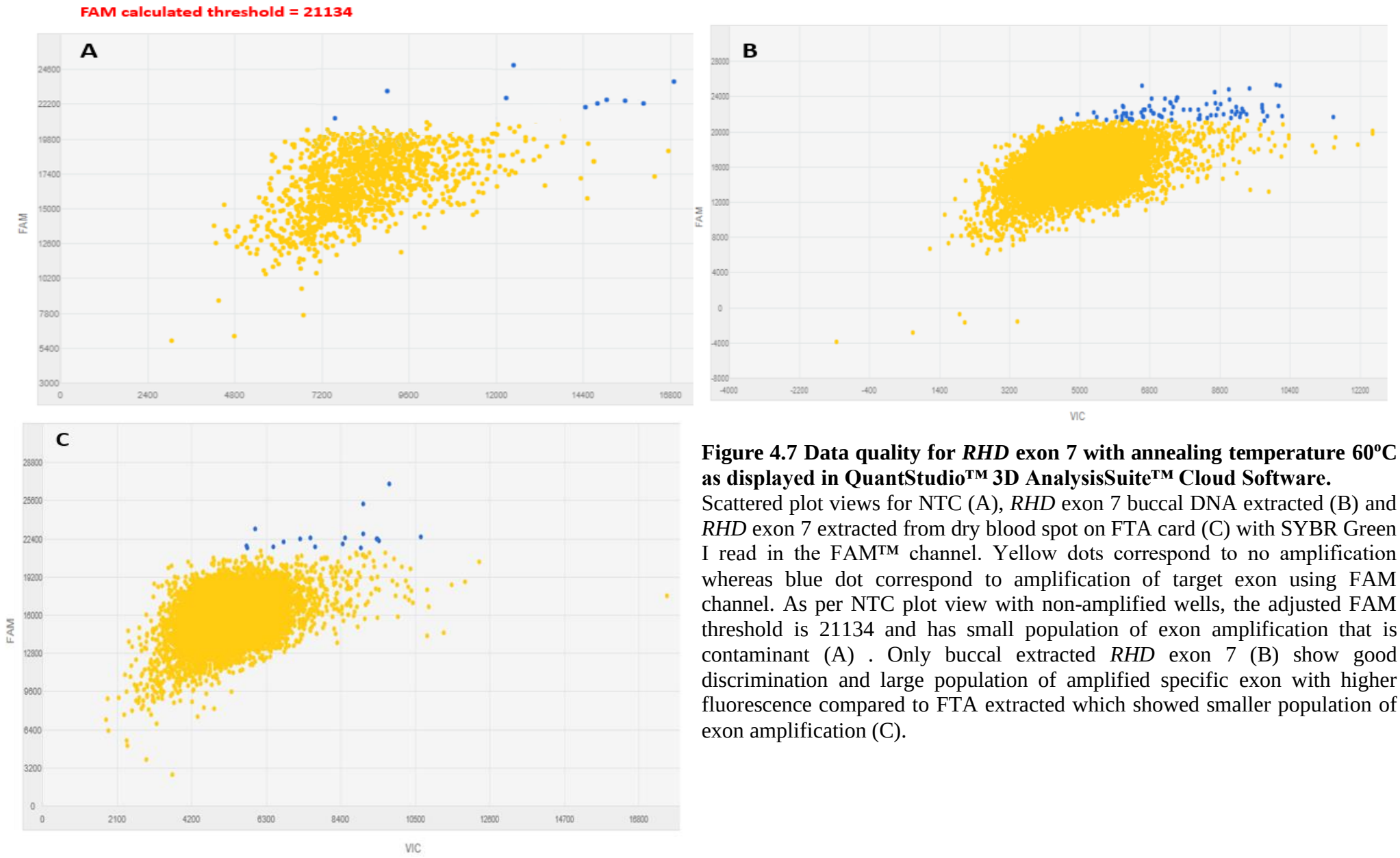


Figure 4.7 Data quality for *RHD* exon 7 with annealing temperature 60ºC as displayed in QuantStudio™ 3D AnalysisSuite™ Cloud Software.
Scattered plot views for NTC (A), *RHD* exon 7 buccal DNA extracted (B) and *RHD* exon 7 extracted from dry blood spot on FTA card (C) with SYBR Green I read in the FAM™ channel. Yellow dots correspond to no amplification whereas blue dot correspond to amplification of target exon using FAM channel. As per NTC plot view with non-amplified wells, the adjusted FAM threshold is 21134 and has small population of exon amplification that is contaminant (A) . Only buccal extracted *RHD* exon 7 (B) show good discrimination and large population of amplified specific exon with higher fluorescence compared to FTA extracted which showed smaller population of exon amplification (C).

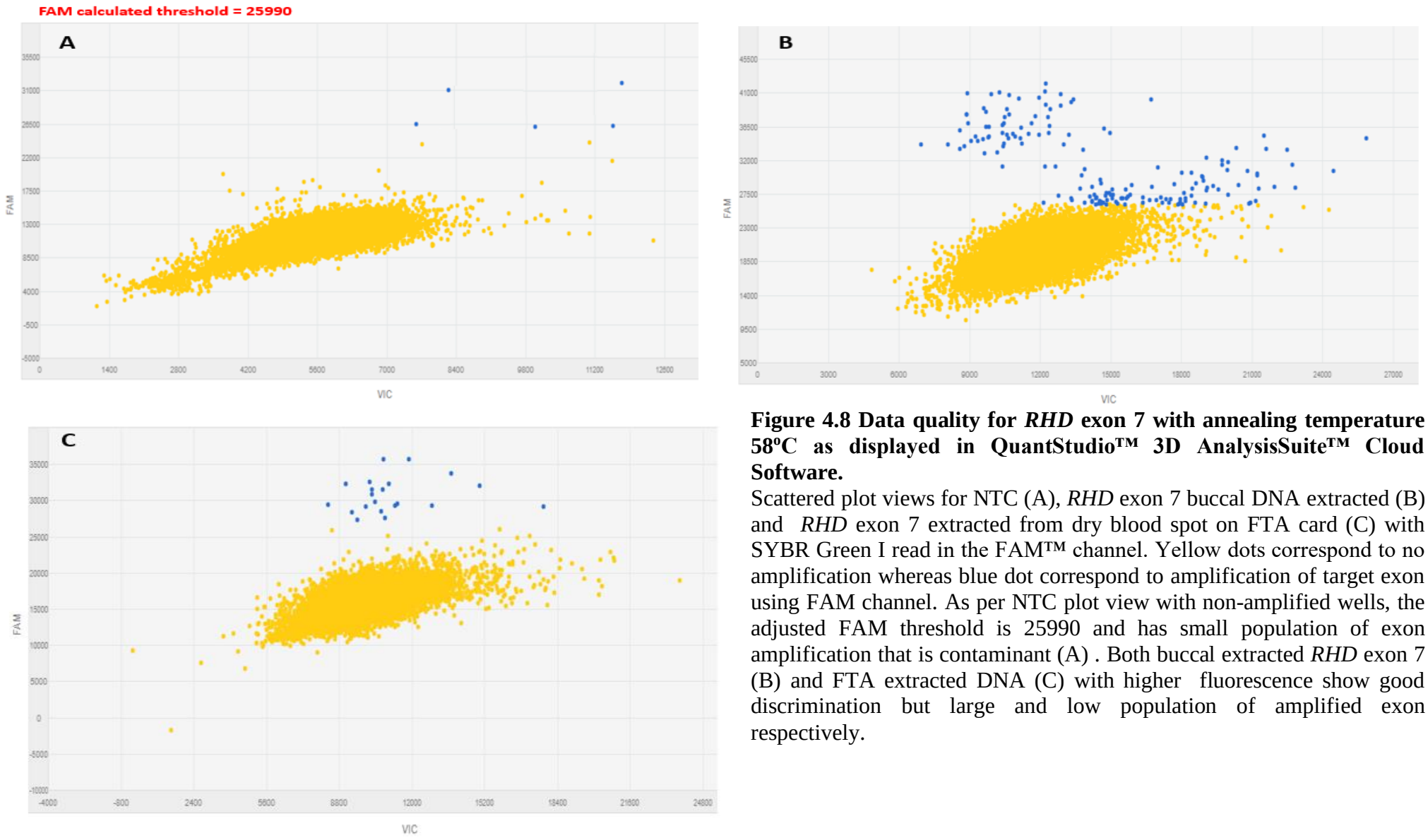


Figure 4.8 Data quality for *RHD* exon 7 with annealing temperature 58ºC as displayed in QuantStudio™ 3D AnalysisSuite™ Cloud Software.

Scattered plot views for NTC (A), *RHD* exon 7 buccal DNA extracted (B) and *RHD* exon 7 extracted from dry blood spot on FTA card (C) with SYBR Green I read in the FAM™ channel. Yellow dots correspond to no amplification whereas blue dot correspond to amplification of target exon using FAM channel. As per NTC plot view with non-amplified wells, the adjusted FAM threshold is 25990 and has small population of exon amplification that is contaminant (A) . Both buccal extracted *RHD* exon 7 (B) and FTA extracted DNA (C) with higher fluorescence show good discrimination but large and low population of amplified exon respectively.

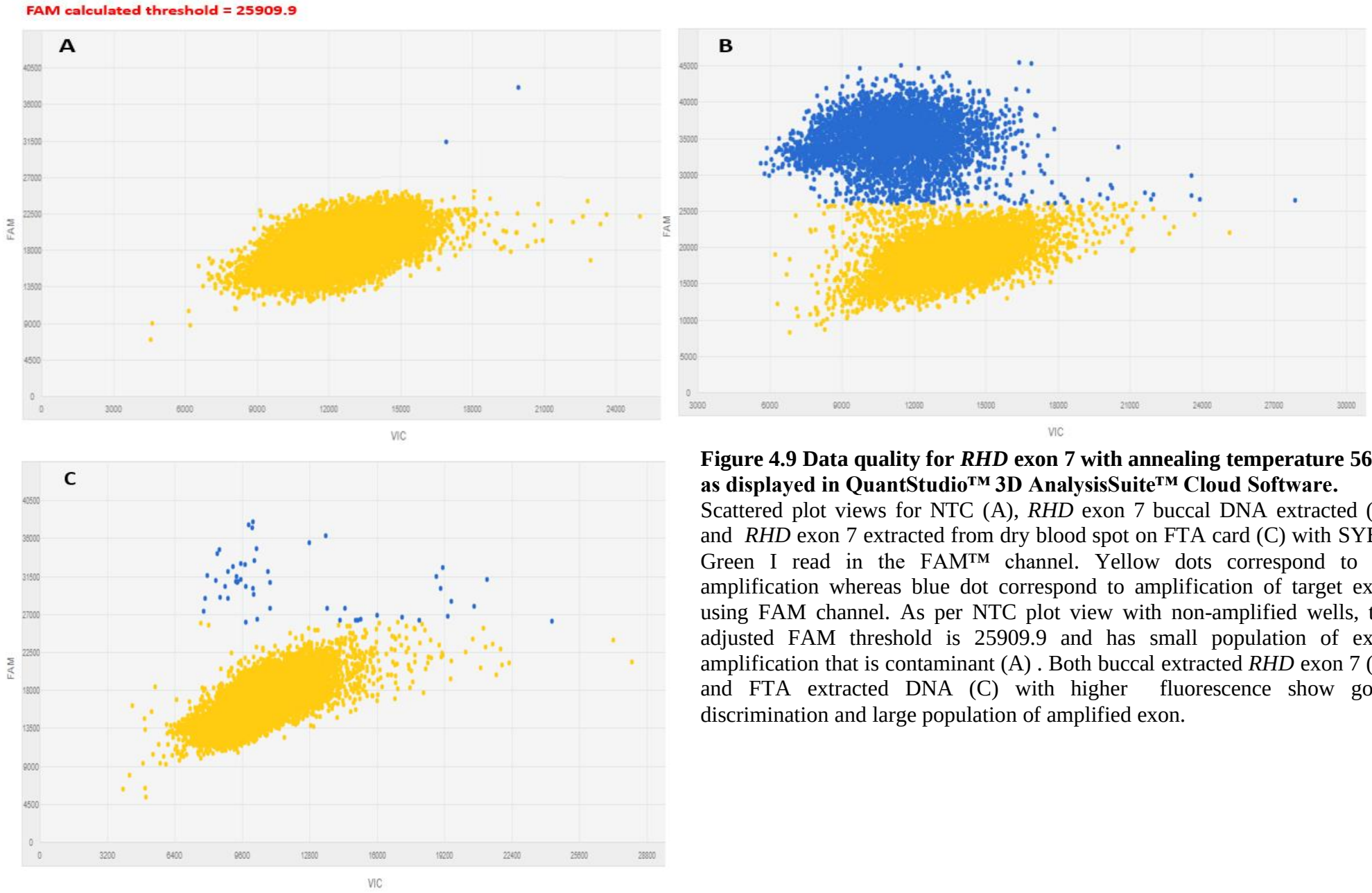


Figure 4.9 Data quality for *RHD* exon 7 with annealing temperature 56ºC as displayed in QuantStudio™ 3D AnalysisSuite™ Cloud Software.
Scattered plot views for NTC (A), *RHD* exon 7 buccal DNA extracted (B) and *RHD* exon 7 extracted from dry blood spot on FTA card (C) with SYBR Green I read in the FAM™ channel. Yellow dots correspond to no amplification whereas blue dot correspond to amplification of target exon using FAM channel. As per NTC plot view with non-amplified wells, the adjusted FAM threshold is 25909.9 and has small population of exon amplification that is contaminant (A) . Both buccal extracted *RHD* exon 7 (B) and FTA extracted DNA (C) with higher fluorescence show good discrimination and large population of amplified exon.

Table 4.5 SYBR Green I assay summary of results for *RHD* exon 7 quantification extracted from buccal cells, with statistical analysis performed on AnalysisSuite™ software

Annealing temperature	Copies / µl	CI[a] (copies/µl)	Precision[b] (%)	Chips
56ºC	170.493	165.26 – 175.89	9.2	1
58ºC	14.564	12.536 -- 16.919	16.2	1
60ºC	5.838	4.611 -- 7.391	26.3	1

[a] Lower and upper 95% confidence interval for quantity of sample in copies/µl.

[b] Calculated precision (%) for the data group, defined as size of the confidence interval for distinguishing between two sample concentrations at a given confidence level.

Table 4.6 SYBR Green I assay summary of results for *RHD* exon 7 quantification extracted dried blood on FTA card, with statistical analysis performed on AnalysisSuite™ software

Annealing temperature	Copies / µl	CI[a] (copies/µl)	Precision[b] (%)	Chips
56ºC	4.741	3.593 -- 6.255	31.9	1
58ºC	1.595	1.04 -- 2.446	53.4	1
60ºC	1.552	1.127 -- 2.839	58.7	1

[a] Lower and upper 95% confidence interval for quantity of sample in copies/µl.

[b] Calculated precision (%) for the data group, defined as size of the confidence interval for distinguishing between two sample concentrations at a given confidence level.

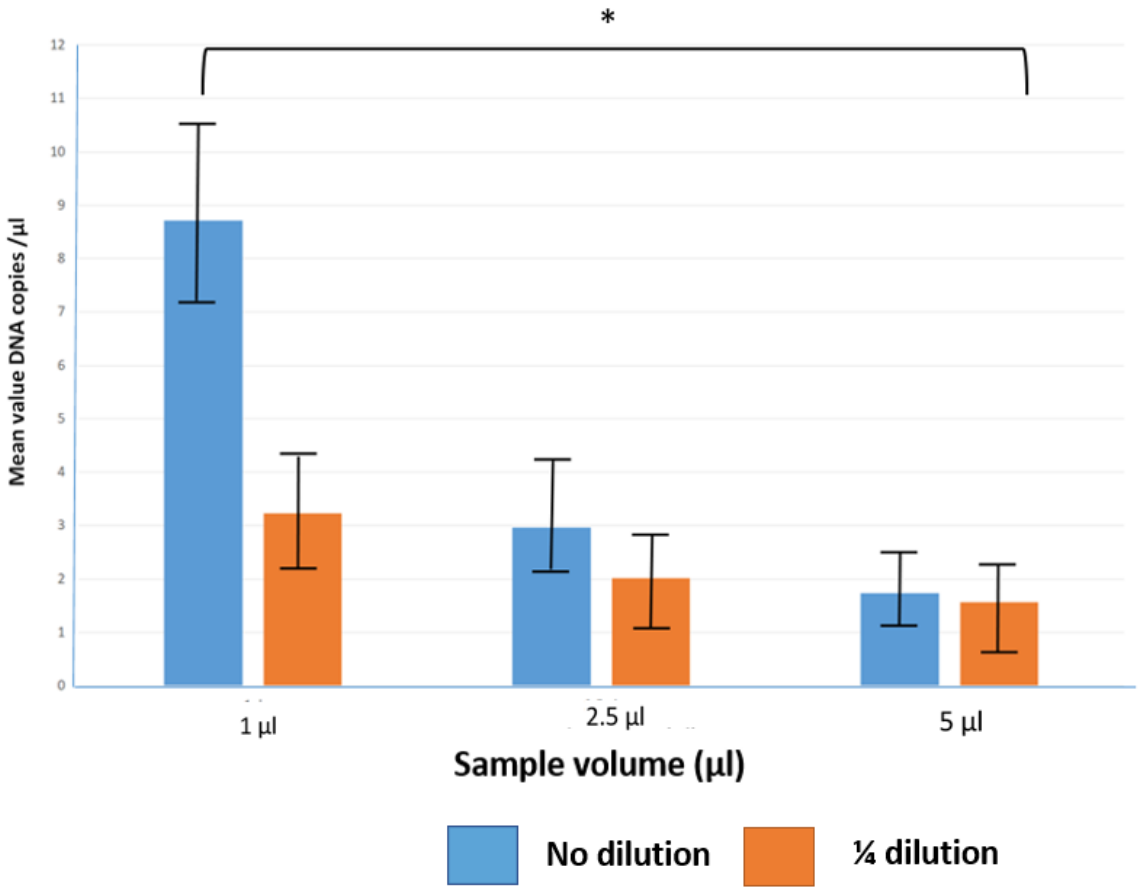


Figure 4.10 inhibitor effect study on DNA samples extracted from dry blood spot on FTA card

Analysis of *RHD* exon 10 DNA copies with different sample volume tested by QuantStudio™ 3D Digital PCR System using SYBR Green chemistry. The line inside of the box indicates 95% confidence interval range for the mean value of each sample volume (n = 10). The undiluted sample bar graph shows decrease in the mean copies of the DNA as the DNA concentration and sample volume increase which may suggest inhibitor effect. The 1 in 4 diluted samples bar graph show decrease in the mean copies of the DNA as the sample volume increase which excluded inhibitor effect as a cause. (*) The mean value of DNA copies and different sample volumes (undiluted) versus ¼ dilution showed no significant difference (p = 0.2).

4.3 Target quantification of RHD exons 5 using QuantStudio™ 3D Digital PCR System with TaqMan chemistry

We also evaluated the ability of the QuantStudio™ 3D Digital PCR System to detect and quantify *RHD* exons 5 extracted from dried blood spot on FTA card using TaqMan chemistry and specific primers and probe with concentration as indicated in Table 2.3. Annealing temperature 60^{o}C was used as recommended by the manufacturer. We found that the detection precision is above the range recommended by the manufacturer (< 10% precision) which again suggests DNA purity issues with FTA extracted DNA. We tested known D positive sample extracted from FTA card (Figure 4.11) versus buccal extracted with a 1 µl sample volume. No good separation was observed between amplified (D positive) and non-amplified reaction compared to buccal extracted DNA for the same.

Further study was performed to increase sample volume and thus DNA concentration to detect and quantify *RHD* exon 5. We tested (n = 5) *RHD* exon 5 positive samples (DNA concentration between 20 and 30 ng/µl) previously tested with real-time PCR. We found that though increasing the volume and concentration of DNA caused increase of mean DNA copies (Figure 4.12), it did not improve the precision (> 15%) and detection of the exon (Table 4.7), which again suggested DNA purity issues.

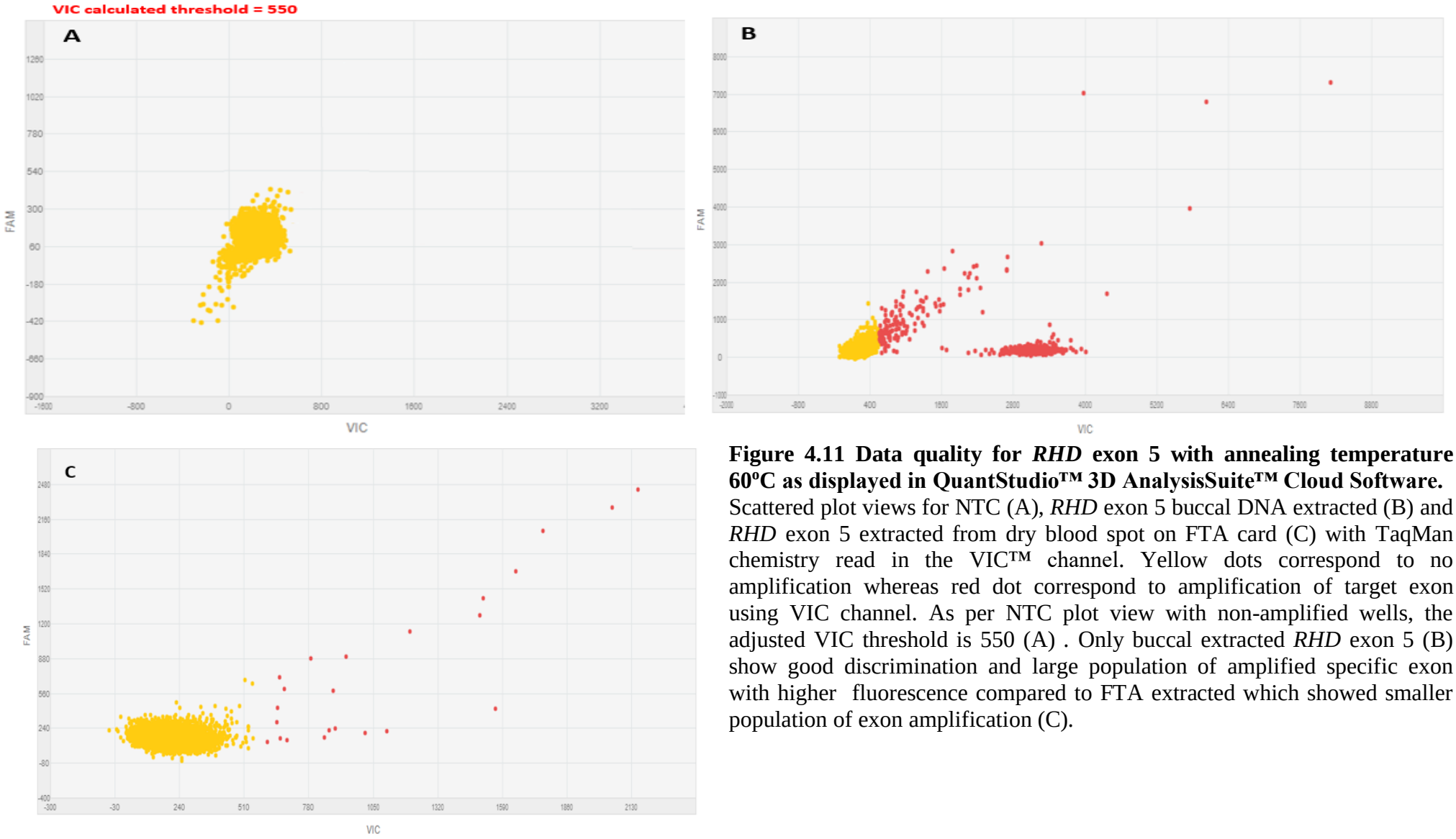


Figure 4.11 Data quality for *RHD* exon 5 with annealing temperature 60°C as displayed in QuantStudio™ 3D AnalysisSuite™ Cloud Software. Scattered plot views for NTC (A), *RHD* exon 5 buccal DNA extracted (B) and *RHD* exon 5 extracted from dry blood spot on FTA card (C) with TaqMan chemistry read in the VIC™ channel. Yellow dots correspond to no amplification whereas red dot correspond to amplification of target exon using VIC channel. As per NTC plot view with non-amplified wells, the adjusted VIC threshold is 550 (A) . Only buccal extracted *RHD* exon 5 (B) show good discrimination and large population of amplified specific exon with higher fluorescence compared to FTA extracted which showed smaller population of exon amplification (C).

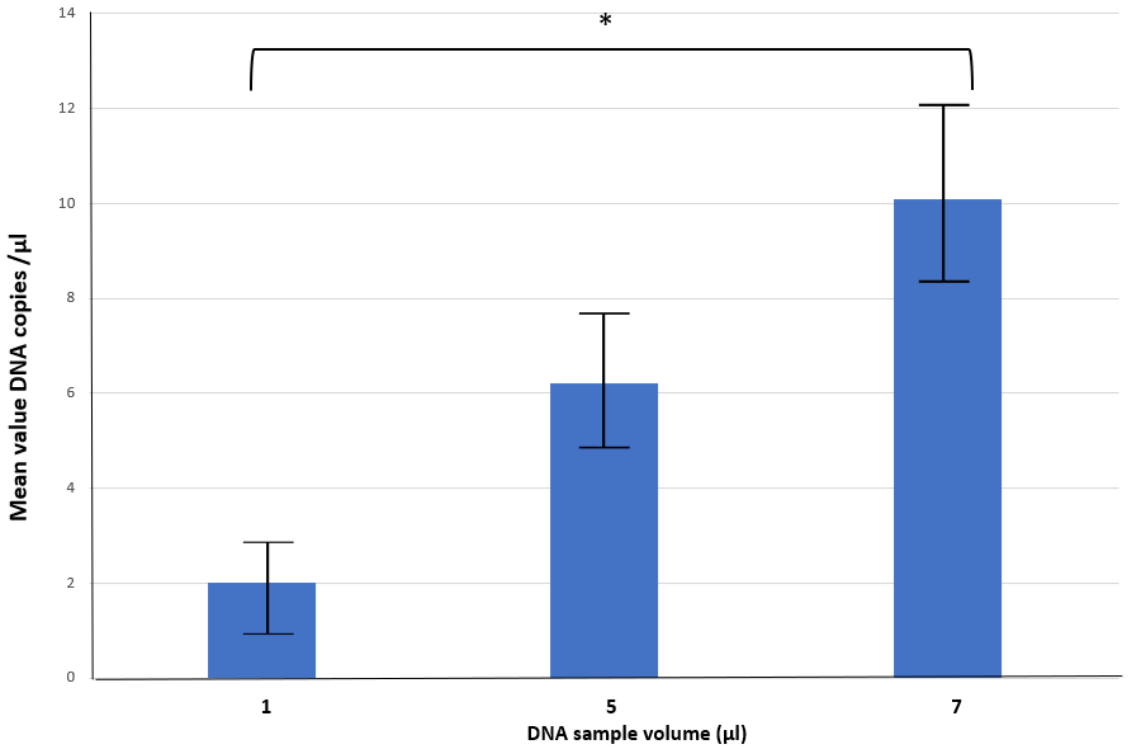


Figure 4.12 Effect of sample volume on mean DNA copies extracted from from dry blood spot on FTA card

RHD exon 5 DNA copies with different DNA sample volumes (1, 5 and 7 ul) tested by QuantStudio™ 3D Digital PCR System using TaqMan chemistry. Y axis represent mean DNA copies/µl of *RHD* exon 5 for different volumes of DNA samples (n = 5). The line inside of the box indicates 95% confidence interval range for the mean value of each sample volume. The mean DNA copies increases as the sample volume increases. (*) The mean value of DNA copies and different sample volumes showed significant difference (p < 0.0001). Though there is statistical difference, however the precision is above the recommended manufacturer percentage (>10%) which suggests DNA purity issues (Table 4.7).

Table 4.7 TaqMan assay summary of results for *RHD* exon 5 with statistical analysis performed on AnalysisSuite™ software

Sample Type / Volume (µl)	Copies / µl	CI[a] (copies/µl)	Precision[b] (%)	Chips
Buccal / 1 µl	47.778	44.123 -- 51.735	8.3	1
Dry blood FTA/ 1 µl	2.014	1.338 -- 3.031	50.5	5
Dry blood FTA/ 5 µl	6.212	4.939 -- 7.814	25.8	5
Dry blood FTA/ 7 µl	10.094	8.447 -- 12.063	19.51	5

[a] Lower and upper 95% confidence interval for quantity of sample in copies/µl.

[b] Calculated precision (%) for the data group

4.4 Discussion

Digital PCR has been proved to be an accurate quantitative PCR method that is replacing the conventional real-time PCR. Here, we studied if cdPCR rapidly amplify *RHD* gene exons from DNA extracted from dry blood spot on FTA cards. Without doubt, the most appropriate technique would be the assessment of known D positive sample (buccal extracted) and compare it with FTA extracted. A *dce* (D negative) sample was required to be involved in this experiment as well. As the study ethical approval was to collect dry blood on FTA card from Omani population and due to the limitation, time constraints and other ethical issues; we were unable to collect a blood sample from a D negative *dce* Caucasian. The most frequent cause for the absence of the antigen D in Caucasians is the lack of the whole *RHD* gene. This can be an excellent candidate sample for a negative control in this study as it could have been of an aid to define D specificity of the primers in temperature gradient experiment.

The recommended, proven and validated chemistry for digital PCR is TaqMan® Assays. Since SYBR Green dye based detection is widely used for target quantification in traditional real-time PCR, we have tested SYBR Green I dye for *RHD* exons 5,7 and 10 target quantification on the QuantStudio™ 3D system. The optimization study with temperature gradient worked quite well in determining the best annealing temperature for the detection of *RHD* exons 5, 7 and 10 which came out to be 56^{o}C. This is because the buccal extracted D positive sample gave the best precision (<10%) that is recommended by the manufacturer with this temperature (Table 4.1, Table 4.3 and Table 4.5). In contrast, this precision was above 10% with DNA sample extracted from dry blood spot on FTA card (Table 4.2, Table 4.4 and Table 4.6). Digital PCR technology can be used for extremely low-target quantitation from variably contaminated samples (Racki et al., 2014), therefore DNA concentration and inhibitors as causes for lower quantification accuracy in this study were excluded. To support what we stated, we conducted inhibitor effect test by diluting the FTA extracted DNA samples 1 in 4 (to dilute the effect of inhibitors if any) and compared it with undiluted sample. Our study showed that inhibitors (if any) could not affect the quantitation (DNA copies / µl) as no significant difference were observed p = 0.2, which suggests no inhibitors interference at all or cdPCR result is not affected by inhibitors.

We have also tested TaqMan® Assays for *RHD* exons 5 target quantification on the QuantStudio™ 3D system. As seen with SYBR Green dye–based detection, buccal extracted D positive DNA sample gave the best precision (<10%) that is recommended by the manufacturer compared to FTA card extracted DNA that showed >10% (Table 4.7). This again suggests purity issues with FTA extracted DNA. Though there is significant difference (p < 0.0001) between DNA copies/µl and the increasing volume of DNA

(Figure 4.12), however lower quantification accuracy was observed with precision >10% which further supports the purity issue.

These results demonstrate that the QuantStudio™ 3D system may be not compatible with SYBR Green dye– based digital PCR and TaqMan® Assay based digital PCR for the quantitation of *RHD* gene extracted from dry blood spot on FTA card.

5 CHAPTER FIVE: Discussion

The limitations of serology can be overcome by molecular typing. In order to evaluate the contribution of RH systematic genotyping and its implication in transfusion practice, a genotyping of D negative blood donors was studied in Omani population for the first time. We aimed to explore the molecular background of serological D negative cohort by different methods such as real- time PCR Tm curve analysis, ASP singleplex real-time PCR and ASP-PCR using BAGene kits. The outcome showed that the existing IAT method with anti-D reagent used to find out D variants such as weak D and partial D demonstrated to be quite ineffective for the classification of D status based on serological data in transfusion practice in Oman.

We aimed to develop a multiplex real-time PCR method for the detection of *RHD* exons based on Tm analysis curve study. We studied and recorded Tm curve analysis of each *RHD* gene exon with exception of exon 8 in real-time PCR with SYBR Green chemistry. We found Tm study to be ineffective for the study of molecular background of serological D negative cohort. We observed Tm curve analysis of *RHD* exons to be too close to each other's (Table 3.6). This makes it difficult to develop a multiplex assay for *RHD* exons detection in order to be analysed by Tm.

Molecular study using ASP singleplex real-time PCR and BAGene ASP-PCR reclassified a total of 4.4% (n = 9, out of 203 samples tested) of serological D negative as D positive (section 3.7.4 and Table 3.10). From the molecular background details collected for Omani cohort, it is clear that Omanis may possess multiple *RHD* gene alleles that make existing ASPs and related techniques quite challenging. For example, molecular study using BAGene partial D-Type kit failed to classify about 21% (n = 5, out of 24 samples tested) of D variants (section 3.7.4 and Table 3.10). In addition, BAGene kit with support

of singleplex real-time PCR determined more than one D variant may exist in a haplotype which further makes the available diagnostic kit quite challenging to explore the molecular background of serological D negative in Omanis.

We adopted a strategy described previously to detect deletion or presence of *RHD* gene in serological D negative Omani cohort by screening of *RHD* exon 10 (Moussa et al., 2012; Sassi et al., 2014). Based on this we were able to classify 88.18% (*n* = 179, out of 203 samples) of the samples as D negative due to complete *RHD* gene deletion (see section 3.6). However, the most common D negative haplotype in all populations is caused by the deletion of the whole *RHD* gene with the concomitant presence of the hybrid Rhesus box (Flegel, 2011). In the *RHD* positive haplotype, the *RHD* gene is flanked by two highly homologues DNA segments called Rhesus boxes, that are located 5´upstream Rhesus box and 3´downstream Rhesus box of *RHD* gene. In D negative (Caucasians), the *RHD* gene composed of 5´ end of the upstream Rhesus box and 3´end of downstream Rhesus box that results in hybrid Rhesus box (Prager, 2007). Therefore, serological D negative, *RHD* exon 10 negative samples in this study should be tested in future for the presence of only hybrid Rhesus box in order to reconfirm the complete *RHD* gene deletion.

As discussed in chapter three, 11.82% (*n* = 24, out of 203 samples) of the cohort showed D variants. These D variants included homozygous or hemizygous *RHDΨ*, *RHD-CE-D*^{s} in trans to partial, combination of *RHD* alleles (weak D and partial D) on the same chromosome (in cis to each other's) and combination of *RHD* alleles on both chromosomes (in trans to each other's). The BAGene RH-Type diagnostic kit failed to determine zygosity of few *RHD* alleles such as *RHDΨ/RHD*. The D zygosity test would further confirm the existence of *RHD* gene on one chromosome or both. The hybrid Rhesus box may also be used for D zygosity testing. Our future work with these samples is to use PCR-SSP that allows the determination of *RHD* zygosity (homozygosity or hemizygosity of D)

by the amplification of the downstream Rhesus box (DD), or by the hybrid Rhesus box (dd), or by the downstream and the hybrid Rhesus box (Dd), respectively. For *RHD* alleles, which cannot be determined serologically (D negative), a discrepancy between the serological test result and genotyping may occur. The positive detection of the downstream Rhesus box shows the presence of an *RHD* allele (serological D positive), except *RHDΨ* homozygous and hemizygous respectively. The reaction hereby is negative although an *RHD* allele is present. In addition, the result with a genetically modified downstream Rhesus box may be also false negative, although the specimen is serologically D positive. Thus, with a serologically D positive result and positive PCR for the hybrid Rhesus box, the outcome is *Dd*. It is *DD* when a negative PCR result for the hybrid Rhesus box occurs.

Our body work detected partial D alleles that are due to hybrid *RHD-CE-D* (section 3.7.4). Due to combination of two *RHD* alleles (for partial D) on one chromosome, the BAGene partial-D diagnostic kit failed to confirm possible existence of hybrid *RHCE*. BAGene RH-Type also detected *RHD-CE-D*^{s} African allele in cis to a partial D, in contrast to singleplex real-time PCR which did not test for *RHD-CE-D*^{s}. The primers used for singlepex real-time PCR detected the alleles that are specific for *RHD* gene specific allele on the exon. However, we did not confirm the existence of the *RHCE* hybrid on *RHD* gene by using primers that are specific for *RHCE* gene as well. Therefore, our potential future work is to perform *RHCE* molecular testing for these samples to confirm the existence of hybrid *RHCE*. In addition, characteristic SNPs are also needed for all tested samples to determine the novel (if any) *RHCE* allele linked to the detected *RHD* allele in Omani cohort, thus constituting *RH* haplotypes. For example, *RHD-CE-D*^{s} would need demonstration of the *RHCE*(Leu245Val) and *RHD*(Asn152Thr) substitutions characteristic of *Cde*^{s} as well as missing *RHD* exons 3 through 7 that are replaced with *RHCE* exons 3 through 7. There are many other *RHCE* variants that are inherited with altered *RHD*,

includes; *RHCE*ceAR* inherited with *RHD*DAR* (Hemker et al., 1999), *RHCE*ceTI* is frequently in cis to *RHD*DIVa-2* (Westhoff et al., 2013a), *RHCE*ceMO* is often found with *RHD*DAU0* (Westhoff et al., 2013b) and *RHCE*ce48C,733G,1006T* is usually associated to *RHD*DIIIa* (Westhoff et al., 2010).

Anti-D monoclonal antibodies to Rh low frequency antigens to detect specific partial D are unavailable. It has been difficult to define the precise population frequencies of the various RH haplotypes because of the inability to differentiate between homo or hemizygous individuals and prediction of the Rh genotype based on serological data and common haplotype in the population would be incorrect in these cases (Sillence et al., 2017). Rh genotyping overcomes the limitation associated with serological method as it can detect low frequency antigens by wide range of PCR based molecular methods to detect predefined nucleotides and DNA region. However, as of the designed methods, these cannot detect the novel variants and alleles (Avent et al., 2015). Sequencing of *RHD* and *RHCE* coding regions by Sanger method may unveil the search for Rh variants, however the results may be difficult to interpret due to deletions, inclusions and hybrid alleles (Stabentheiner et al., 2011). Recent advances in NGS technologies offer a more comprehensive DNA sequence based approach to determine Rh variants (Fichou et al., 2014; Fichou et al., 2016). Due to possible multiple *RHD* alleles that were predicted on one chromosome in a few samples from Omani cohort, Sanger sequencing results that were difficult to interpret in some cases, *RHD* exon 8 that was not studied and unclassified molecular background (n = 5), NGS would be one of the best options to unveil possible novel *RHD* alleles in Omani cohort.

Many studies used NGS different technologies for blood group genotyping. Dezan and colleagues and Chou and colleagues used exome sequencing for the identification of Rh variants (Chou et al., 2017; Dezan et al., 2017). However, the sequence sharing

between wild type *RHD* and *RHCE* exons 8 and 10 (Figure 1.4) makes it difficult to analyse the data. A long-range PCR (LR-PCR) method was used by Hyland and colleagues to amplify *RHD* exon 2 through 7 (Hyland et al., 2017), which still cannot unveil the remaining *RHD* exons 1, 8, 9 and 10 genotyping. LR-PCR can be used to amplify DNA to over 30 kb by the use of modified polymerases such as SequalPrep polymerase (Invitrogen, Carlsbad, CA), AccuPrime Taq DNA Polymerase (Invitrogen, Carlsbad, CA), PrimeSTAR GXL polymerase (TaKaRa Bio, Shiga, Japan), LA Taq Hot Start Version Polymerase (TaKaRa Bio, Osaka, Japan), KAPA long Range HotStart DNA polymerase (KAPA Biosystems, Wobum, MA) and QIAGEN LongRange PCR Polymerase (Hilden, Germany) (Jia et al., 2014). These polymerases are of high fidelity with enhanced DNA binding that results in accurate long fragment DNA amplification. The blend of *Taq* DNA polymerase is combined with a small amount of proofreading polymerase that repairs DNA mismatches incorporated at the 3' end of the growing strand, allowing *Taq* polymerase to continue to elongate the DNA much further than it would otherwise, resulting in longer DNA amplification. These technical advances have brought the speed and simplicity of PCR to genomic mapping and sequencing and have facilitated studies in molecular genetic including NGS. Tounsi and colleagues were able to establish a reference *RHD* allele using long-range PCR and NGS that amplified the complete *RHD* gene by sequencing full *RHD* gene including promoter, introns, and all exons (Tounsi, Madgett and Avent, 2018).

Providing Rh antigen matched RBCs is considered the best prophylaxis to avoid alloimmunization caused by Rh antibodies, especially in Beta thalassaemia major and SCD cases. However, this can be compromised by existence of *RHD* & *RHCE* variants if not disclosed at the start of prophylaxis. As Oman has a high prevalence of hereditary blood disorders such as SCD and thalassemia (see section 3.1) and our study detected multiple *RHD* alleles that needs further study (see section 3.7.4), we aim to do a D zygosity test and

adopt Tounsi and colleagues *RHD* alleles reference NGS method as a future follow up work as well for this body work.

Conclusion

Overall, the serological limitation of phenotyping RHD in Omani cohort has proved to be existing. We used dry blood spot on FTA card as a source for DNA sample for *RHD* genotyping. We observed that *RHD* exons 4, 5 and probably exon 8 with *RHD* intron 7 are the regions where most of the polymorphism detected in Omani cohort. We demonstrated *RHD* positive haplotype in serological D negative Omanis and found that these positive haplotypes are either inactive *RHD* gene (*RHDΨ*) giving true serological D negative or partial D / weak D alleles giving false serological D negative. We also observed *RHD* negative haplotype with complete *RHD* gene deletion that gives true serological D negative. The study could not unveil molecular background of few samples that are predicted to possess *RHD* haplotype. BAGene kits may not be suitable choice for *RHD* genotype screening in Omani population due to multiple *RHD* alleles existence.

Though real-time PCR using SYBR Green and Taq-Man chemistry was successful to amplify different *RHD* exons, however; Melting curve analysis by SYBR Green chemistry has not been successful to distinguish different *RHD* exons and to be recommended for multiplexing study. The nature of extracted DNA from dry blood on FTA card has also not been successful to be used with most sophisticated PCR technology for testing *RHD* genotyping as dPCR could not robustly amplified *RHD* exons on DNA samples extracted from dry blood spot on FTA card which reflects the poor quality of extracted DNA sample.

Further study may unmask novel *RHD* alleles that should add a contribution to transfusion science and safety practice. *RHCE* genotyping would be a potential future work with these samples in order to link type of *RHCE* allele(s) involved with detected *RHD* alleles in Omani cohort. D zygosity and next generation sequencing would be the methods of choice to overcome PCR *RHD* genotyping limitations associated in this study. Our

future work aim will be to adopt established referenced *RHD* allele genotyping by NGS to unveil the complete *RHD* gene screening test to help clinicians in their current practice to further improve blood transfusion safety in Oman.

Future work

Our future work on these samples will be focused mainly on three assays: a complete *RHD* allele genotyping by NGS, finding out D zygosity and exploring *RHCE* alleles involved in detected *RHD* alleles. These will provide more understanding of molecular background of D negative phenotype in Omanis. The outcome of these assays should suggest a better management of the donors and the patients genotyped with weak D or partial D who can receive D positive blood units.

Though our study of *RHD* genotyping unveiled *RHD* alleles involved in serological D negative Omani cohort, however it is uncertain of possibility of having two or more *RHD* alleles on the same haplotype (cis). To confirm the existence of multiple *RHD* alleles, a complete *RHD* genotyping; previously described LR-PCR and NGS technology could be adopted as a potential future work (Tounsi, Madgett and Avent, 2018). A fresh EDTA sample from the participants who showed serological D negative but *RHD* positive haplotype (n = 24) would be ideal to avoid DNA purity issues as observed in this study with extracted DNA from dry blood spot on FTA card. Genomic DNA will be extracted using Qiagen investigator kit or any available DNA extraction kit. DNA concentration and purity will be determined using Thermo Scientific™ NanoDrop 2000. Six sets of *RHD* specific primers previously published by Tounsi and colleagues will be used to amplify the *RHD* gene in 6 LR-PCR amplicons. LR-PCR, Library construction and NGS will run as described by the authors.

We predicted complete *RHD* gene deletion (*del/del*) based on the absence of *RHD* exon 10, but; we were uncertain if the presence of different weak D/partial D *RHD* alleles are homozygous [*RHD allele(s)/RHD allele(s)*] or hemizygous [*RHD allele(s)/del*]. In both cases, D zygosity testing will be very helpful to unveil that. As a future work, all samples in this study (n = 203) will be tested for the D zygosity using either BAGene D-Zygosity

type kit or running sequence specific amplification PCR using previously described primers that detect hybrid Rhesus box as a marker for *RHD* deletion and/or downstream Rhesus box as marker for the existence of *RHD* gene (Wagner and Flegel, 2000).

Once zygosity and complete *RHD* genotyping determined, *RHCE* testing would be very useful to determine *RHCE* alleles that are involved with *RHD* alleles detected in Omani cohort. Sequence specific amplification PCR for the specific detection of the 10 *RHCE* exons from genomic DNA will be carried out and sequencing of each product with sequencing primers will be performed according to previous description (Wagner, Ladewig and Flegel, 2003). In the long term, these efforts may translate into practice in transfusion clinics to benefit patients in Oman.

Bibliography

Al-Dughaishi, T., Al Harrasi, Y., Al-Duhli, M., Al-Rubkhi, I., Al-Riyami, N., Al Riyami, A., Pathare, A.V. and Gowri, V. (2016) Red Cell Alloimmunization to Rhesus Antigen Among Pregnant Women Attending a Tertiary Care Hospital in Oman. *Oman Med J*, 31 (1), 77-80.

Al-Riyami, A.A., Suleiman, A.J., Afifi, M., Al-Lamki, Z.M. and Daar, S. (2001) A community-based study of common hereditary blood disorders in Oman. *East Mediterr Health J*, 7 (6), 1004-1011.

Al-Riyami, A.Z., Al-Marhoobi, A., Al-Hosni, S., Al Mahrooqi, S., Schmidt, M., O'Brien, S. and Al-Khabori, M. (2019) Prevalence of Red Blood Cell Major Blood Group Antigens and Phenotypes among Omani Blood Donors. *Oman Med J*, 34 (6), 496-503.

Al-Shiakh., I.H., Zaidi., Z.A., Islam., S., Qaduri., M.D. and Jama, A.A. (1997) Frequency of various rh antigens in Dammam Eastern provience, Saudi Arabia *Saudi M J*, 19 (3), 265-268.

Al Shaibany, S. (2010) *Omanis flocking to Zanzibar, their ancestral home* [online]

Available at: https://www.thenational.ae/world/mena/omanis-flocking-to-zanzibar-their-ancestral-home-1.498003

[Accessed: August 2015]

Alimba, C.G., Adekoya, K.O. and Oboh, B.O. (2010) Prevalence and gene frequencies of phenylthiocarbamide (PTC) taste sensitivity, ABO and Rhesus factor (Rh) blood groups, and haemoglobin variants among a Nigerian population. *Egypt. J. Med. Hum*, 11 (2).

Alkindi, S., AlMahrooqi, S., AlHinai, S., AlMarhoobi, A., Al-Hosni, S., Daar, S., Fawaz, N. and Pathare, A. (2017) Alloimmunization in Patients with Sickle Cell Disease and Thalassemia: Experience of a Single Centre in Oman. *Mediterr J Hematol Infect Dis*, 9 (1), e2017013.

Arce, M.A., Thompson, E.S., Wagner, S., Coyne, K.E., Ferdman, B.A. and Lublin, D.M. (1993) Molecular cloning of RhD cDNA derived from a gene present in RhD-positive, but not RhD-negative individuals. *Blood*, 82 (2), 651-655.

Argall, C.I., Ball, J.M. and Trentelman, E. (1953) Presence of anti-D antibody in the serum of a Du patient. *J Lab Clin Med*, 41 (6), 895-898.

Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N. and Patel, H.R. (2005) Basic principles of real-time quantitative PCR. *Expert Rev Mol Diagn*, 5 (2), 209-219.

Avent, N., Poole, J. and Singleton, B. (1999) *Studies of two partial Ds: DMH and DOL*.

Avent, N.D. (1998) Antenatal genotyping of the blood groups of the fetus. *Vox Sang*, 74 Suppl 2, 365-374.

Avent, N.D., Daniels, G.L., Martin, P.G., Green, C.A., Finning, K.M. and Warner, K.M. (1997a) Molecular Investigation of the Rh C/c Polymorphism. *Transfus Med*, 7, 18.

Avent, N.D., Finning, K.M., Liu, W. and Scott, M.L. (1996a) Molecular biology of partial D phenotypes. *Transfus Clin Biol*, 3 (6), 511-516.

Avent, N.D., Liu, W., Jones, J.W., Scott, M.L., Voak, D., Pisacka, M., Watt, J. and Fletcher, A. (1997b) Molecular analysis of Rh transcripts and polypeptides from individuals expressing the DVI variant phenotype: an RHD gene deletion event does not generate All DVIccEe phenotypes. *Blood*, 89 (5), 1779-1786.

Avent, N.D., Liu, W., Warner, K.M., Mawby, W.J., Jones, J.W., Ridgwell, K. and Tanner, M.J. (1996b) Immunochemical analysis of the human erythrocyte Rh polypeptides. *J Biol Chem*, 271 (24), 14233-14239.

Avent, N.D., Madgett, T.E., Halawani, A.J., Altayar, M.A., Kiernan, M., Reynolds, A.J. and Li, X. (2015) Next-generation sequencing: academic overkill or high-resolution routine blood group genotyping? , 10 (S1), 250-256.

Avent, N.D., Madgett, T.E., Lee, Z.E., Head, D.J., Maddocks, D.G. and Skinner, L.H. (2006) Molecular biology of Rh proteins and relevance to molecular medicine. *Expert Rev Mol Med*, 8 (13), 1-20.

Avent, N.D., Martin, P.G., Armstrong-Fisher, S.S., Liu, W., Finning, K.M., Maddocks, D. and Urbaniak, S.J. (1997c) Evidence of genetic diversity underlying Rh D-, weak D (Du), and partial D phenotypes as determined by multiplex polymerase chain reaction analysis of the RHD gene. *Blood*, 89 (7), 2568-2577.

Avent, N.D. and Reid, M.E. (2000) The Rh blood group system: a review. *Blood*, 95 (2), 375-387.

Avent, N.D., Ridgwell, K., Tanner, M.J. and Anstee, D.J. (1990) cDNA cloning of a 30 kDa erythrocyte membrane protein associated with Rh (Rhesus)-blood-group-antigen expression. *Biochem J*, 271 (3), 821-825.

Bakanay, S.M., Ozturk, A., Ileri, T., Ince, E., Yavasoglu, S., Akar, N., Uysal, Z. and Arslan, O. (2013) Blood group genotyping in multi-transfused patients. *Transfus Apher Sci*, 48 (2), 257-261.

Barra, G.B., Santa Rita, T.H., de Almeida Vasques, J., Chianca, C.F., Nery, L.F. and Santana Soares Costa, S. (2015) EDTA-mediated inhibition of DNases protects circulating cell-free DNA from ex vivo degradation in blood samples. *Clin Biochem*, 48 (15), 976-981.

Basu, D., Datta, S.S., Montemayor, C., Bhattacharya, P., Mukherjee, K. and Flegel, W.A. (2018) ABO, Rhesus, and Kell Antigens, Alleles, and Haplotypes in West Bengal, India. *Transfus Med Hemother*, 45 (1), 62-66.

Beckers, E.A., Faas, B.H., Ligthart, P., Simsek, S., Overbeeke, M.A., von dem Borne, A.E., van Rhenen, D.J. and van der Schoot, C.E. (1996) Characterization of the hybrid RHD gene leading to the partial D category IIIc phenotype. *Transfusion*, 36 (6), 567-574.

Bischoff, F.Z., Nguyen, D.D., Marquez-Do, D., Moise, K.J., Jr., Simpson, J.L. and Elias, S. (1999) Noninvasive determination of fetal RhD status using fetal DNA in maternal serum and PCR. *J Soc Gynecol Investig*, 6 (2), 64-69.

Blais, J., Lavoie, S.B., Giroux, S., Bussieres, J., Lindsay, C., Dionne, J., Laroche, M., Giguere, Y. and Rousseau, F. (2015) Risk of Misdiagnosis Due to Allele Dropout and False-Positive PCR Artifacts in Molecular Diagnostics: Analysis of 30,769 Genotypes. *J Mol Diagn*, 17 (5), 505-514.

Blunt, T., Daniels, G. and Carritt, B. (1994) Serotype switching in a partially deleted RHD gene. *Vox Sang*, 67 (4), 397-401.

Burton, N.M. and Daniels, G. (2011) Structural modelling of red cell surface proteins. *Vox Sang*, 100 (1), 129-139.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*, 55 (4), 611-622.

Callender, S.T. and Race, R.R. (1946) A serological and genetical study of multiple antibodies formed in response to blood transfusion by a patient with lupus erythematosus diffusus. *Ann Eugen*, 13 (Pt 2), 102-117.

Cartron, J.P. (1994) Defining the Rh blood group antigens. Biochemistry and molecular genetics. *Blood Rev*, 8 (4), 199-212.

Cherif-Zahar, B., Bloy, C., Le Van Kim, C., Blanchard, D., Bailly, P., Hermand, P., Salmon, C., Cartron, J.P. and Colin, Y. (1990) Molecular cloning and protein structure of a human blood group Rh polypeptide. *Proc Natl Acad Sci U S A*, 87 (16), 6243-6247.

Cherif-Zahar, B., Le Van Kim, C., Rouillac, C., Raynal, V., Cartron, J.P. and Colin, Y. (1994) Organization of the gene (RHCE) encoding the human blood group RhCcEe antigens and characterization of the promoter region. *Genomics*, 19 (1), 68-74.

Cherif-Zahar, B., Raynal, V. and Cartron, J.P. (1997) RH gene structure: reassignment of two exon-exon junctions. *Blood*, 89 (12), 4661-4662.

Chou, S.T., Flanagan, J.M., Vege, S., Luban, N.L.C., Brown, R.C., Ware, R.E. and Westhoff, C.M. (2017) Whole-exome sequencing for RH genotyping and alloimmunization risk in children with sickle cell anemia. *Blood Adv*, 1 (18), 1414-1422.

Chou, S.T. and Westhoff, C.M. (2011) The role of molecular immunohematology in sickle cell disease. *Transfus Apher Sci*, 44 (1), 73-79.

Conroy, M.J., Bullough, P.A., Merrick, M. and Avent, N.D. (2005) Modelling the human rhesus proteins: implications for structure and function. *Br J Haematol*, 131 (4), 543-551.

da Cunha Santos, G. (2018) FTA Cards for Preservation of Nucleic Acids for Molecular Assays: A Review on the Use of Cytologic/Tissue Samples. *Arch Pathol Lab Med*, 142 (3), 308-312.

Daar, S., Hussain, H.M., Gravell, D., Nagel, R.L. and Krishnamoorthy, R. (2000) Genetic epidemiology of HbS in Oman: multicentric origin for the betaS gene. *Am J Hematol*, 64 (1), 39-46.

Daniels, G. (2002) Human blood groups.

Daniels, G. (2013a) Rh and RHAG Blood Group Systems. In: (ed.) *Human Blood Groups.* Wiley-Blackwell.

Daniels, G. (2013b) Variants of RhD--current testing and clinical consequences. *Br J Haematol*, 161 (4), 461-470.

Daniels, G. and Bromilow, I. (2013) *Essential Guide to Blood Groups*. Wiley.

Daniels, G.L., Anstee, D.J., Cartron, J.P., Dahr, W., Issitt, P.D., Jorgensen, J., Kornstad, L., Levene, C., Lomas-Francis, C., Lubenko, A. and et al. (1995) Blood group terminology 1995. ISBT Working Party on terminology for red cell surface antigens. *Vox Sang*, 69 (3), 265-279.

Daniels, G.L., Faas, B.H., Green, C.A., Smart, E., Maaskant-van Wijk, P.A., Avent, N.D., Zondervan, H.A., von dem Borne, A.E. and van der Schoot, C.E. (1998) The VS and V blood group polymorphisms in Africans: a serologic and molecular analysis. *Transfusion*, 38 (10), 951-958.

Daniels, G.L., Fletcher, A., Garratty, G., Henry, S., Jorgensen, J., Judd, W.J., Levene, C., Lomas-Francis, C., Moulds, J.J., Moulds, J.M., Moulds, M., Overbeeke, M., Reid, M.E., Rouger, P., Scott, M., Sistonen, P., Smart, E., Tani, Y., Wendel, S., Zelinski, T. and International Society of Blood, T. (2004) Blood group terminology 2004: from the International Society of Blood Transfusion committee on terminology for red cell surface antigens. *Vox Sang*, 87 (4), 304-316.

Daniels. G. (2008) *Human Blood Groups*. 2nd ed. UK: Blackwell Science.

Dean, L. (2005) Blood groups and red cell antigens.

Denatale, A., Cahan, A., Jack, J.A., Race, R.R. and Sanger, R. (1955) V, a new Rh antigen, common in Negroes, rare in white people. *J Am Med Assoc*, 159 (4), 247-250.

Dezan, M.R., Ribeiro, I.H., Oliveira, V.B., Vieira, J.B., Gomes, F.C., Franco, L.A.M., Varuzza, L., Ribeiro, R., Chinoca, K.Z., Levi, J.E., Krieger, J.E., Pereira, A.C., Gualandro, S.F.M., Rocha, V.G., Mendrone-Junior, A., Sabino, E.C. and Dinardo, C.L. (2017) RHD and RHCE genotyping by next-generation sequencing is an effective strategy to identify molecular variants within sickle cell disease patients. *Blood Cells Mol Dis*, 65, 8-15.

Dhawan, H.K., Kumawat, V., Marwaha, N., Sharma, R.R., Sachdev, S., Bansal, D., Marwaha, R.K. and Arora, S. (2014) Alloimmunization and autoimmunization in transfusion dependent thalassemia major patients: Study on 319 patients. *Asian J Transfus Sci*, 8 (2), 84-88.

Dovc-Drnovsek, T., Klemenc, P., Toplak, N., Blejec, T., Bricl, I. and Rozman, P. (2013) Reliable Determination of Fetal RhD Status by RHD Genotyping from Maternal Plasma. *Transfus Med Hemother*, 40 (1), 37-43.

Dunsford, I. and Aspinall, P. (1951) The Rh chromosome CwdE (Ryw) occurring in three generations. *Nature*, 168 (4283), 954-955.

EL Wafi, M., EL Housse, H., Nourichafi, N., Bouisk, K., Benajiba, M. and Habti, N. (2016) Prevalence of weak D phenotype among D negative C/E+ blood donors in Morocco. *Int J Blood Transfus Immunohematol*, 6, 3-7.

Engelfriet, C. and Reesink, H.W. (2006) Testing for weak D. *Vox Sang*, 90 (2), 140-153.

Faas, B.H., Beckers, E.A., Wildoer, P., Ligthart, P.C., Overbeeke, M.A., Zondervan, H.A., von dem Borne, A.E. and van der Schoot, C.E. (1997) Molecular background of VS and weak C expression in blacks. *Transfusion*, 37 (1), 38-44.

Faas, B.H., Beuling, E.A., Christiaens, G.C., von dem Borne, A.E. and van der Schoot, C.E. (1998) Detection of fetal RHD-specific sequences in maternal plasma. *Lancet*, 352 (9135), 1196.

Farrar, J.S., Wittwer, C.T. (2015) *Molecular Diagnostics*. 3rd ed.

Fasano, R.M., Monaco, A., Meier, E.R., Pary, P., Lee-Stroka, A.H., Otridge, J., Klein, H.G., Marincola, F.M., Kamani, N.R., Luban, N.L., Stroncek, D. and Flegel, W.A. (2010) RH genotyping in a sickle cell

disease patient contributing to hematopoietic stem cell transplantation donor selection and management. *Blood*, 116 (15), 2836-2838.

Fichou, Y., Audrezet, M.P., Gueguen, P., Le Marechal, C. and Ferec, C. (2014) Next-generation sequencing is a credible strategy for blood group genotyping. *Br J Haematol*, 167 (4), 554-562.

Fichou, Y., Le Marechal, C., Scotet, V., Jamet, D. and Ferec, C. (2015) Insights into RHCE Molecular Analysis in Samples with Partial D Variants: the Experience of Western France. *Transfus Med Hemother*, 42 (6), 372-377.

Fichou, Y., Mariez, M., Le Marechal, C. and Ferec, C. (2016) The experience of extended blood group genotyping by next-generation sequencing (NGS): investigation of patients with sickle-cell disease. *Vox Sang*, 111 (4), 418-424.

Fichou, Y., Parchure, D., Gogri, H., Gopalkrishnan, V., Le Marechal, C., Chen, J.M., Ferec, C., Madkaikar, M., Ghosh, K. and Kulkarni, S. (2018) Molecular basis of weak D expression in the Indian population and report of a novel, predominant variant RHD allele. *Transfusion*, 58 (6), 1540-1549.

Filosa, L., Beley, S., Chiaroni, J., Bailly, P. and Silvy, M. (2016) New silent and weak D alleles: molecular characterization and associated antigen density. *Transfusion*, 56 (8), 2154-2155.

Finck, R., Lui-Deguzman, C., Teng, S.M., Davis, R. and Yuan, S. (2013) Comparison of a gel microcolumn assay with the conventional tube test for red blood cell alloantibody titration. *Transfusion*, 53 (4), 811-815.

Flegel, W.A. (2006) Molecular genetics of RH and its clinical application. *Transfus Clin Biol*, 13 (1-2), 4-12.

Flegel, W.A. (2007) The genetics of the Rhesus blood group system. *Blood Transfus*, 5 (2), 50-57.

Flegel, W.A. (2011) Molecular genetics and clinical applications for RH. *Transfus Apher Sci*, 44 (1), 81-91.

Flegel, W.A., von Zabern, I. and Wagner, F.F. (2009) Six years' experience performing RHD genotyping to confirm D- red blood cell units in Germany for preventing anti-D immunizations. *Transfusion*, 49 (3), 465-471.

Flegel, W.A. and Wagner, F.F. (1996) The frequency of RHD protein variants in Caucasians. *Transfus Clin Biol*, 3 (10s), (abstr).

Flegel, W.A. and Wagner, F.F. (2002) Molecular biology of partial D and weak D: implications for blood bank practice. *Clin Lab*, 48 (1-2), 53-59.

Flegel, W.A., Wagner, F.F., Muller, T.H. and Gassner, C. (1998) Rh phenotype prediction by DNA typing and its application to practice. *Transfus Med*, 8 (4), 281-302.

Flores-Bello, A., Mas-Ponte, D., Rosu, M.E., Bosch, E., Calafell, F. and Comas, D. (2018) Sequence diversity of the Rh blood group system in Basques. *Eur. J. Hum. Genet.*, 26 (12), 1859-1866.

Frohn, C., Dumbgen, L., Brand, J.M., Gorg, S., Luhm, J. and Kirchner, H. (2003) Probability of anti-D development in D- patients receiving D+ RBCs. *Transfusion*, 43 (7), 893-898.

Gardener, G.J., Legler, T.J., Hyett, J.A., Liew, Y.W., Flower, R.L. and Hyland, C.A. (2012) Anti-D in pregnant women with the RHD(IVS3+1G>A)-associated DEL phenotype. *Transfusion*, 52 (9), 2016-2019.

Gassner, C., Doescher, A., Drnovsek, T.D., Rozman, P., Eicher, N.I., Legler, T.J., Lukin, S., Garritsen, H., Kleinrath, T., Egger, B., Ehling, R., Kormoczi, G.F., Kilga-Nogler, S., Schoenitzer, D. and Petershofen, E.K. (2005) Presence of RHD in serologically D-, C/E+ individuals: a European multicenter study. *Transfusion*, 45 (4), 527-538.

Gonzalez-Porras, J.R., Graciani, I.F., Perez-Simon, J.A., Martin-Sanchez, J., Encinas, C., Conde, M.P., Nieto, M.J. and Corral, M. (2008) Prospective evaluation of a transfusion policy of D+ red blood cells into D- patients. *Transfusion*, 48 (7), 1318-1324.

Granier, T., Beley, S., Chiaroni, J., Bailly, P. and Silvy, M. (2013) A comprehensive survey of both RHD and RHCE allele frequencies in sub-Saharan Africa. *Transfusion*, 53 (11 Suppl 2), 3009-3017.

Grootkerk-Tax, M.G., Maaskant-van Wijk, P.A., van Drunen, J. and van der Schoot, C.E. (2005) The highly variable RH locus in nonwhite persons hampers RHD zygosity determination but yields more insight into RH-related evolutionary events. *Transfusion*, 45 (3), 327-337.

Gundrajukuppam, D.K., Vijaya, S.B., Rajendran, A. and Sarella, J.D. (2016) Prevalence of Principal Rh Blood Group Antigens in Blood Donors at the Blood Bank of a Tertiary Care Hospital in Southern India. *J Clin Diagn Res*, 10 (5), EC07-10.

Gunel, T., Kalelioglu, I., Surmeli, Y., Turken, B., Ermis, H. and Aydinli, K. (2011) Comparison of real-time polymerase chain reaction assay methods for detection of RHD gene in amniotic fluid. *J Nat Sci Biol Med*, 2 (2), 193-197.

Haer-Wigman, L., Ji, Y., Loden, M., de Haas, M., van der Schoot, C.E. and Veldhuisen, B. (2013) Comprehensive genotyping for 18 blood group systems using a multiplex ligation-dependent probe amplification assay shows a high degree of accuracy. *Transfusion*, 53 (11 Suppl 2), 2899-2909.

Harmening, D. (2012) *Modern blood banking & transfusion practices*. Philadelphia: F.A. Davis.

Hartel-Schenk, S. and Agre, P. (1992) Mammalian red cell membrane Rh polypeptides are selectively palmitoylated subunits of a macromolecular complex. *J Biol Chem*, 267 (8), 5569-5574.

Hemker, M.B., Ligthart, P.C., Berger, L., van Rhenen, D.J., van der Schoot, C.E. and Wijk, P.A. (1999) DAR, a new RhD variant involving exons 4, 5, and 7, often in linkage with ceAR, a new Rhce variant frequently found in African blacks. *Blood*, 94 (12), 4337-4342.

Hillyer, C.D. (2007) *Blood Banking and Transfusion Medicine: Basic Principles & Practice*. Churchill Livingstone/Elsevier.

Hindson, B.J., Ness, K.D., Masquelier, D.A., Belgrader, P., Heredia, N.J., Makarewicz, A.J., Bright, I.J., Lucero, M.Y., Hiddessen, A.L., Legler, T.C., Kitano, T.K., Hodel, M.R., Petersen, J.F., Wyatt, P.W., Steenblock, E.R., Shah, P.H., Bousse, L.J., Troup, C.B., Mellen, J.C., Wittmann, D.K., Erndt, N.G., Cauley, T.H., Koehler, R.T., So, A.P., Dube, S., Rose, K.A., Montesclaros, L., Wang, S., Stumbo, D.P., Hodges, S.P., Romine, S., Milanovich, F.P., White, H.E., Regan, J.F., Karlin-Neumann, G.A., Hindson, C.M., Saxonov, S. and Colston, B.W. (2011) High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal Chem*, 83 (22), 8604-8610.

Hou, L., Yan, Q.D. and Tian, L. (2017) A novel nonsense mutation in RHAG gene responsible for Rhnull phenotype in a Chinese individual. *Transfus Apher Sci*, 56 (2), 220-222.

Howe, J.G. and Stack, G. (2017) Structural and functional impacts of amino acid substitutions that create blood group antigens: implications for immunogenicity. *Transfusion*, 57 (3), 541-553.

Hudecova, I. (2015) Digital PCR analysis of circulating nucleic acids. *Clin Biochem*, 48 (15), 948-956.

Huggett, J.F., Foy, C.A., Benes, V., Emslie, K., Garson, J.A., Haynes, R., Hellemans, J., Kubista, M., Mueller, R.D., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T. and Bustin, S.A. (2013) The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. *Clin Chem*, 59 (6), 892-902.

Hyland, C.A., Millard, G.M., O'Brien, H., Schoeman, E.M., Lopez, G.H., McGowan, E.C., Tremellen, A., Puddephatt, R., Gaerty, K., Flower, R.L., Hyett, J.A. and Gardener, G.J. (2017) Non-invasive fetal RHD genotyping for RhD negative women stratified into RHD gene deletion or variant groups: comparative accuracy using two blood collection tube types. *Pathology*, 49 (7), 757-764.

Hyland, C.A., Wolter, L.C., Liew, Y.W. and Saul, A. (1994) A southern analysis of Rh blood group genes: association between restriction fragment length polymorphism patterns and Rh serotypes. *Blood*, 83 (2), 566-572.

Ipe, T.S., Wilkes, J.J., Hartung, H.D., Westhoff, C.M., Chou, S.T. and Friedman, D.F. (2015) Severe hemolytic transfusion reaction due to anti-D in a D+ patient with sickle cell disease. *J Pediatr Hematol Oncol*, 37 (2), e135-137.

The ISBT Working Party on Terminology for Red Cell Surface Antigens. (1990) *Vox Sang*, 58 (2), 152-169.

Issitt, P.D. and Telen, M.J. (1996) Introduction of the term “partial D”. 36 (8), 761-762.

Ji, Y.L., Luo, H., Wen, J.Z., Haer-Wigman, L., Veldhuisen, B., Wei, L., Wang, Z., Ligthart, P., Loden-van Straaten, M., Fu, Y.S., van der Schoot, C.E. and Luo, G.P. (2017) RHD genotype and zygosity analysis in the Chinese Southern Han D+, D- and D variant donors using the multiplex ligation-dependent probe amplification assay. *Vox Sang*, 112 (7), 660-670.

Jia, H., Guo, Y., Zhao, W. and Wang, K. (2014) Long-range PCR in next-generation sequencing: comparison of six enzymes and evaluation on the MiSeq sequencer. *Scientific Reports*, 4 (1), 5737.

Jiang, M., Li, J., Zhou, J., Xing, C., Xu, J., & Guo, F. (2019) High-resolution melting analysis for rapid and sensitive MYD88 screening in chronic lymphocytic leukemia. *Oncology Letters,*, 18, 814-821.

Jones, J., Scott, M.L. and Voak, D. (1995) Monoclonal anti-D specificity and Rh D structure: criteria for selection of monoclonal anti-D reagents for routine typing of patients and donors. *Transfus Med*, 5 (3), 171-184.

Kacem, N., Jemni-Yacoub, S., Chiaroni, J., Bailly, P. and Silvy, M. (2015) Paternal RHD zygosity determination in Tunisians: evaluation of three molecular tests. *Blood Transfus*, 13 (1), 59-65.

Keramati, M.R., Shakibaei, H., Kheiyyami, M.I., Ayatollahi, H., Badiei, Z., Samavati, M. and Sadeghian, M.H. (2011) Blood group antigens frequencies in the northeast of Iran. *Transfus Apher Sci*, 45 (2), 133-136.

Khosroshahi, B.N., Oodi, A., Namjou, S., Gholamali, T. and Amirizadeh, N. (2019) RHD Genotyping by Molecular Analysis of Hybrid Rhesus box in RhD-Negative Blood Donors from Iran. *Indian J Hematol Blood Transfus*, 35 (1), 119-124.

Kim, B., Lee, S.T., Kim, S., Choi, J.R. and Kim, H.O. (2018) Application of Multiplex Ligation-Dependent Probe Amplification Assay for Genotyping Major Blood Group Systems Including DEL Variants in the D-Negative Korean Population. *Ann Lab Med*, 38 (1), 32-38.

Kim, T.Y., Hong, Y.J., Kim, M.J., Kim, H., Kim, T.S., Park, J.S., Park, K.U. and Han, K.S. (2020) Recommendations Regarding Practical DEL Typing Strategies for Serologically D-Negative Asian Donors. *Transfus Med Hemother*, 47 (1), 88-93.

Kline, M.C., Duewer, D.L., Redman, J.W., Butler, J.M. and Boyer, D.A. (2002) Polymerase chain reaction amplification of DNA from aged blood stains: quantitative evaluation of the "suitability for purpose" of four filter papers as archival media. *Anal Chem*, 74 (8), 1863-1869.

Kormoczi, G.F., Gassner, C., Shao, C.P., Uchikawa, M. and Legler, T.J. (2005) A comprehensive analysis of DEL types: partial DEL individuals are prone to anti-D alloimmunization. *Transfusion*, 45 (10), 1561-1567.

Kulkarni, S., Parchure, D.S., Gopalkrishnan, V. and Madkaikar, M. (2018) Screening for DEL phenotype in RhD negative Indians. *J Clin Lab Anal*, 32 (3).

Lacey, P.A., Caskey, C.R., Werner, D.J. and Moulds, J.J. (1983) Fatal hemolytic disease of a newborn due to anti-D in an Rh-positive Du variant mother. *Transfusion*, 23 (2), 91-94.

Landsteiner, K. and Wiener, A.S. (1940) An Agglutinable Factor in Human Blood Recognized by Immune Sera for Rhesus Blood. 43 (1), 223-223.

Le van Kim, C., Mouro, I., Cherif-Zahar, B., Raynal, V., Cherrier, C., Cartron, J.P. and Colin, Y. (1992) Molecular cloning and primary structure of the human blood group RhD polypeptide. *Proc Natl Acad Sci U S A*, 89 (22), 10925-10929.

Legler, T.J., Liu, Z., Mavrou, A., Finning, K., Hromadnikova, I., Galbiati, S., Meaney, C., Hulten, M.A., Crea, F., Olsson, M.L., Maddocks, D.G., Huang, D., Fisher, S.A., Sprenger-Haussels, M., Soussan, A.A. and van der Schoot, C.E. (2007) Workshop report on the extraction of foetal DNA from maternal plasma. *Prenat Diagn*, 27 (9), 824-829.

Levine, P. and Stetson, R.E. (1939) AN UNUSUAL CASE OF INTRA-GROUP AGGLUTINATION. *JAMA*, 113 (2), 126-127.

Lipic, S.G., Giordullo, L.M. and Fredericks, J.D. (2018) A novel FTA™ elute card collection method that improves direct DNA amplification from bloodstained concrete. *Sci. Justice*, 58 (5), 303-307.

Lo, Y.M., Hjelm, N.M., Fidler, C., Sargent, I.L., Murphy, M.F., Chamberlain, P.F., Poon, P.M., Redman, C.W. and Wainscoat, J.S. (1998) Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med*, 339 (24), 1734-1738.

Lomas-Francis, C., Halter Hipsky, C., Velliquette, R.W. and Reid, M.E. (2012) DIII Type 7 is likely the original serologically defined DIIIb. *Transfusion*, 52 (1), 39-42.

Lucie, O., Sophie, R., Aurélie, V., Olivia, A., François, G., Laïla Allach El, K., France, L., Dominique, V., Thierry, B., Vassilis, T. and Juliette, N. (2016) La PCR digitale, une nouvelle approche pour analyser l'ADN fœtal à partir du sang maternel : application à la détermination du génotype RHD fœtal. *Annales de Biologie Clinique*, 74 (3), 269-277.

Luettringhaus, T.A., Cho, D., Ryang, D.W. and Flegel, W.A. (2006) An easy RHD genotyping strategy for D- East Asian persons applied to Korean blood donors. *Transfusion*, 46 (12), 2128-2137.

Lukacevic Krstic, J., Dajak, S., Bingulac-Popovic, J., Dogic, V. and Mratinovic-Mikulandra, J. (2016) Anti-D Antibodies in Pregnant D Variant Antigen Carriers Initially Typed as RhD. *Transfus Med Hemother*, 43 (6), 419-424.

Maaskant-van Wijk, P.A., Faas, B.H., de Ruijter, J.A., Overbeeke, M.A., von dem Borne, A.E., van Rhenen, D.J. and van der Schoot, C.E. (1998) Genotyping of RHD by multiplex polymerase chain reaction analysis of six RHD-specific exons. *Transfusion*, 38 (11-12), 1015-1021.

Makroo, R., Gupta, R., Bhatia, A. and Rosamma, N.L. (2014) Rh phenotype, allele and haplotype frequencies among 51,857 blood donors in North India. *Blood Transfus*, 12 (1), 36-39.

Mas, S., Crescenti, A., Gasso, P., Vidal-Taboada, J.M. and Lafuente, A. (2007) DNA cards: determinants of DNA yield and quality in collecting genetic samples for pharmacogenetic studies. *Basic Clin Pharmacol Toxicol*, 101 (2), 132-137.

Matheson, K.A. and Denomme, G.A. (2002) Novel 3'Rhesus box sequences confound RHD zygosity assignment. *Transfusion*, 42 (5), 645-650.

Mercier, B., Gaucher, C., Feugeas, O. and Mazurier, C. (1990) Direct PCR from whole blood, without DNA extraction. *Nucleic Acids Res*, 18 (19), 5908.

Merrick, M., Javelle, A., Durand, A., Severi, E., Thornton, J., Avent, N.D., Conroy, M.J. and Bullough, P.A. (2006) The Escherichia coli AmtB protein as a model system for understanding ammonium transport by Amt and Rh proteins. *Transfus Clin Biol*, 13 (1-2), 97-102.

Mirmajlessi, S.M. and Loit, E. (2016) General Principles of Real-Time PCR: A Technology for Quantitative Detection of Phytopathogens. *J. Med. Biol. Eng*, 5 (1), 49-52.

Moezzi, L., Keshavarz, Z., Ranjbaran, R., Aboualizadeh, F., Behzad-Behbahani, A., Abdullahi, M., Ramezani, A., Samsami, A. and Sharifzadeh, S. (2016) Fetal RHD Genotyping Using Real-Time Polymerase Chain Reaction Analysis of Cell-Free Fetal DNA in Pregnancy of RhD Negative Women in South of Iran. *Int J Fertil Steril*, 10 (1), 62-70.

Mourant, A.E. (1945) A New Rhesus Antibody. *Nature*, 155 (3940), 542-542.

Mouro, I., Colin, Y., Cherif-Zahar, B., Cartron, J.P. and Le Van Kim, C. (1993) Molecular genetic basis of the human Rhesus blood group system. *Nat Genet*, 5 (1), 62-65.

Mouro, I., Colin, Y., Sistonen, P., Le Pennec, P.Y., Cartron, J.P. and Le Van Kim, C. (1995) Molecular basis of the RhCW (Rh8) and RhCX (Rh9) blood group specificities. *Blood*, 86 (3), 1196-1201.

Mouro, I., Le Van Kim, C., Rouillac, C., van Rhenen, D.J., Le Pennec, P.Y., Bailly, P., Cartron, J.P. and Colin, Y. (1994) Rearrangements of the blood group RhD gene associated with the DVI category phenotype. *Blood*, 83 (4), 1129-1135.

Moussa, H., Tsochandaridis, M., Chakroun, T., Jridi, S., Abdelneji, B., Hmida, S., Silvy, M., Bailly, P., Gabert, J., Levy-Mozziconacci, A. and Jemni-Yacoub, S. (2012) Molecular background of D-negative phenotype in the Tunisian population. *Transfus Med*, 22 (3), 192-198.

Muhammad, T., Hamid, R., Maqsood, A., Muhammad, U.S., Hussain, S., Aamir, H. and Nadia, R. (2016) Determination of Weak "D" Antigen among Rhesus Negative Pakistani Blood Donors. . *Ann Pak Inst Med Sci*, 12, 131-135.

Neeraj, G., Deepak, K.S., Reena, T. and Bharat, S. (2015) Phenotype Prevalence of Blood Group Systems (ABO, Rh, Kell) in Voluntary, Healthy Donors-Experience of a Tertiary Care Hospital in Delhi, North India. *J Blood Disord Transfus*, 6 (4), 1-4.

Nicholson, G., Lawrence, A., Ala, F.A. and Bird, G.W. (1991) Semi-quantitative assay of D antigen site density by flow cytometric analysis. *Transfus Med*, 1 (2), 87-90.

Nicol, G. (1998) *Diamed ID microtyping system combined forward and reverse group test CF-02*. 1st ed. Melbourne, Australia Diamed Australia Pty Ltd.

Noizat-Pirenne, F., Verdier, M., Lejealle, A., Mercadier, A., Bonin, P., Peltier-Pujol, F., Fialaire-Legendre, A., Tournamille, C., Bierling, P. and Ansart-Pirenne, H. (2007) Weak D phenotypes and transfusion safety: where do we stand in daily practice? *Transfusion*, 47 (9), 1616-1620.

Novaretti, M.C., Jens, E., Pagliarini, T., Bonifacio, S.L., Dorlhiac-Llacer, P.E. and Chamone, D.A. (2003) Comparison of conventional tube test with diamed gel microcolumn assay for anti-D titration. *Clin Lab Haematol*, 25 (5), 311-315.

Novaretti, M.C., Jens, E., Pagliarini, T., Bonifacio, S.L., Dorlhiac-Llacer, P.E. and Chamone, D.A. (2004) Comparison of conventional tube test technique and gel microcolumn assay for direct antiglobulin test: a large study. *J Clin Lab Anal*, 18 (5), 255-258.

Nuchnoi, P., Thongbus, J., Srisarin, A., Kerdpin, U. and Prachayasittikul, V. (2014) Clinical and laboratory update on the DEL variant. *Lab Med*, 45 (4), 285-290.

Nzelu, C.O., Cáceres, A.G., Guerrero-Quincho, S., Tineo-Villafuerte, E., Rodriquez-Delfin, L., Mimori, T., Uezato, H., Katakura, K., Gomez, E.A., Guevara, A.G., Hashiguchi, Y. and Kato, H. (2016) A rapid molecular diagnosis of cutaneous leishmaniasis by colorimetric malachite green-loop-

mediated isothermal amplification (LAMP) combined with an FTA card as a direct sampling tool. *Acta Tropica*, 153, 116-119.

Ogasawara, K., Sasaki, K., Isa, K., Tsuneyama, H., Uchikawa, M., Satake, M. and Tadokoro, K. (2016) Weak D alleles in Japanese: a c.960G>A silent mutation in exon 7 of the RHD gene that affects D expression. *Vox Sang*, 110 (2), 179-184.

Okubo, Y., Seno, T., Yamano, H., Yamaguchi, H., Lomas, C. and Tippett, P. (1991) Partial D antigens disclosed by a monoclonal anti-D in Japanese blood donors. *Transfusion*, 31 (8), 782.

Okubo, Y., Yamaguchi, H., Tomita, T. and Nagao, N. (1984) A D variant, Del? *Transfusion*, 24 (6), 542.

Okuda, H., Kawano, M., Iwamoto, S., Tanaka, M., Seno, T., Okubo, Y. and Kajii, E. (1997) The RHD gene is highly detectable in RhD-negative Japanese donors. *J Clin Invest*, 100 (2), 373-379.

Okuda, H., Suganuma, H., Kamesaki, T., Kumada, M., Tsudo, N., Omi, T., Iwamoto, S. and Kajii, E. (2000) The analysis of nucleotide substitutions, gaps, and recombination events between RHD and RHCE genes through complete sequencing. *Biochem Biophys Res Commun*, 274 (3), 670-683.

Olsson, M.L., Michalewska, B., Hellberg, A., Walaszczyk, A. and Chester, M.A. (2005) A clue to the basis of allelic enhancement: occurrence of the Ax subgroup in the offspring of blood group O parents. *Transfus Med*, 15 (5), 435-442.

Ooley, P. (2015) *Standards for Blood Banks and Transfusion Services*. 30th ed. Bethesda, MD: American Association of Blood Banks.

Opoku-Okrah C, Amidu N and Amoah-Sakyi S (2008) Detection of Weak D (Du) Phenotype among Rh-D Negative Males and Females in Kumasi, Ghana. *JST*, 28 (3), 34-40.

Ostgard, P., Fevang, F. and Kornstad, L. (1986) Anti-D in a 'D positive' mother giving rise to severe haemolytic disease of the newborn. A dilemma in antenatal immunohaematological testing. *Acta Paediatr Scand*, 75 (1), 175-178.

Ouchari, M., Romdhane, H., Chakroun, T., Abdelkefi, S., Houissa, B., Hmida, S. and Yacoub, S.J. (2015) Weak D in the Tunisian population. *Blood Transfus*, 13 (2), 295-301.

Ouchari, M., Srivastava, K., Romdhane, H., Jemni Yacoub, S. and Flegel, W.A. (2018) Transfusion strategy for weak D Type 4.0 based on RHD alleles and RH haplotypes in Tunisia. *Transfusion*, 58 (2), 306-312.

Peluso, A.L., Cascone, A.M., Lucchese, L., Cozzolino, I., Ieni, A., Mignogna, C., Pepe, S. and Zeppa, P. (2015) Use of FTA cards for the storage of breast carcinoma nucleic acid on fine-needle aspiration samples. *Cancer Cytopathol*, 123 (10), 582-592.

Pham, B.-N., Peyrard, T., Juszczak, G., Dubeaux, I., Gien, D., Blancher, A., Cartron, J.-P., Rouger, P. and Le Pennec, P.-Y. (2009a) Heterogeneous molecular background of the weak C, VS+, hrB–, HrB– phenotype in black persons. *Transfusion*, 49 (3), 495-504.

Pham, B.N., Peyrard, T., Juszczak, G., Auxerre, C., Godin, S., Bonin, P., Rouger, P. and Le Pennec, P.Y. (2009b) Alloanti-c (RH4) revealing that the (C)ce s haplotype encodes a partial c antigen. *Transfusion*, 49 (7), 1329-1334.

Poulter, M., Kemp, T.J. and Carritt, B. (1996) DNA-based rhesus typing: simultaneous determination of RHC and RHD status using the polymerase chain reaction. *Vox Sang*, 70 (3), 164-168.

Prager, M. (2007) Molecular genetic blood group typing by the use of PCR-SSP technique. *Transfusion*, 47 (1 Suppl), 54S-59S.

Prisco Arnoni, C., Guilhem Muniz, J., de Paula Vendrame, T.A., de Medeiros Person, R., Roche Moreira Latini, F. and Castilho, L. (2016) RHCE variants inherited with altered RHD alleles in Brazilian blood donors. *Transfus Med*, 26 (4), 285-290.

Qin, Y., Zhang, H., Marlowe, N., Fei, M., Yu, J., Lei, X., Yu, L., Zhang, J., Cao, D., Ma, L. and Chen, W. (2016) Evaluation of human papillomavirus detection by Abbott m2000 system on samples collected by FTA Elute™ Card in a Chinese HIV-1 positive population. *J CLIN VIROL*, 85, 80-85.

Quantock, K.M., Lopez, G.H., Hyland, C.A., Liew, Y.W., Flower, R.L., Niemann, F.J. and Joyce, A. (2017) Anti-D in a mother, hemizygous for the variant RHD*DNB gene, associated with hemolytic disease of the fetus and newborn. *Transfusion*, 57 (8), 1938-1943.

Quinley, E.D. (2011) *Immunohematology : principles and practice*. Baltimore, Md: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Race, R.R. (1948) The Rh genotypes and Fisher's theory. *Blood*, 3 (2), 27-42.

Race, R.R., Taylor, G.L., Cappell, D.F., McFarlane. and Marjory, N. (1944) Recognition of a Further Common Rh Genotype in Man. *Nature*, 153 (3871), 52-53.

Racki, N., Dreo, T., Gutierrez-Aguirre, I., Blejec, A. and Ravnikar, M. (2014) Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples. *Plant Methods*, 10 (1), 42.

Rapley, R. (2010) Molecular biology, bioinformatics and basic techniques. In: Walker, J. and Wilson, K. (ed.) *Principles and Techniques of Biochemistry and Molecular Biology.* 7 ed. Cambridge: Cambridge University Press. pp. 138-194.

Raud, L., Ferec, C. and Fichou, Y. (2017) From genetic variability to phenotypic expression of blood group systems. *Transfus Clin Biol*, 24 (4), 472-475.

Reid, M.E. and Denomme, G.A. (2011) DNA-based methods in the immunohematology reference laboratory. *Transfus Apher Sci*, 44 (1), 65-72.

Ridgwell, K., Eyers, S.A., Mawby, W.J., Anstee, D.J. and Tanner, M.J. (1994) Studies on the glycoprotein associated with Rh (rhesus) blood group antigen expression in the human red blood cell membrane. *J Biol Chem*, 269 (9), 6410-6416.

Ridgwell, K., Spurr, N.K., Laguda, B., MacGeoch, C., Avent, N.D. and Tanner, M.J. (1992) Isolation of cDNA clones for a 50 kDa glycoprotein of the human erythrocyte membrane associated with Rh (rhesus) blood-group antigen expression. *Biochem J*, 287 (Pt 1), 223-228.

Rosenfield, R.E., Allen, F.H., Jr., Swisher, S.N. and Kochwa, S. (1962) A review of Rh serology and presentation of a new terminology. *Transfusion*, 2, 287-312.

Rouillac, C., Colin, Y., Hughes-Jones, N.C., Beolet, M., D'Ambrosio, A.M., Cartron, J.P. and Le Van Kim, C. (1995) Transcript analysis of D category phenotypes predicts hybrid Rh D-CE-D proteins associated with alteration of D epitopes. *Blood*, 85 (10), 2937-2944.

Rudmann, S.V. (2005) *Textbook of blood banking and transfusion medicine*. Philadelphia: Elsevier/Saunders.

Sandler, S.G., Chen, L.N. and Flegel, W.A. (2017) Serological weak D phenotypes: a review and guidance for interpreting the RhD blood type using the RHD genotype. *Br J Haematol*, 179 (1), 10-19.

Sandler, S.G., Flegel, W.A., Westhoff, C.M., Denomme, G.A., Delaney, M., Keller, M.A., Johnson, S.T., Katz, L., Queenan, J.T., Vassallo, R.R., Simon, C.D. and College of American Pathologists Transfusion Medicine Resource Committee Work, G. (2015) It's time to phase in RHD genotyping for patients with a serologic weak D phenotype. College of American Pathologists Transfusion Medicine Resource Committee Work Group. *Transfusion*, 55 (3), 680-689.

Sassi, A., Ouchari, M., Houissa, B., Romdhane, H., Abdelkefi, S., Chakroun, T. and Jemni Yacoub, S. (2014) RHD genotyping and its implication in transfusion practice. *Transfus Apher Sci*, 51 (3), 59-63.

Schöfl, G., Lang, K., Quenzel, P., Böhme, I., Sauter, J., Hofmann, J.A., Pingel, J., Schmidt, A.H. and Lange, V. (2017) 2.7 million samples genotyped for HLA by next generation sequencing: lessons learned. *BMC Genomics*, 18 (1), 161.

Scott, M.L. (2004) The complexities of the Rh system. *Vox Sang*, 87 Suppl1, 58-62.

Shao, C.P. (2010) Transfusion of RhD-positive blood in "Asia type" DEL recipients. *N Engl J Med*, 362 (5), 472-473.

Shao, C.P., Maas, J.H., Su, Y.Q., Kohler, M. and Legler, T.J. (2002) Molecular background of Rh D-positive, D-negative, D(el) and weak D phenotypes in Chinese. *Vox Sang*, 83 (2), 156-161.

Sillence, K.A., Halawani, A.J., Tounsi, W.A., Clarke, K.A., Kiernan, M., Madgett, T.E. and Avent, N.D. (2017) Rapid RHD Zygosity Determination Using Digital PCR. *Clin Chem*, 63 (8), 1388-1397.

Sillence, K.A., Roberts, L.A., Hollands, H.J., Thompson, H.P., Kiernan, M., Madgett, T.E., Welch, C.R. and Avent, N.D. (2015) Fetal Sex and RHD Genotyping with Digital PCR Demonstrates Greater Sensitivity than Real-time PCR. *Clin Chem*, 61 (11), 1399-1407.

Simmons, R.T. and Krieger, V.I. (1960) Anti-Rh (D) antibodies produced by isoimmunization in an Rh positive woman of the unusual genotype (CDue/CdE). *Med. J. Aust*, 2, 1021-1022.

Singleton, B.K., Green, C.A., Avent, N.D., Martin, P.G., Smart, E., Daka, A., Narter-Olaga, E.G., Hawthorne, L.M. and Daniels, G. (2000) The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in africans with the Rh D-negative blood group phenotype. *Blood*, 95 (1), 12-18.

Siransy Bogui, L., Dembele, B., Sekongo, Y., Abisse, S., Konate, S. and Sombo, M. (2014) Phenotypic Profile of Rh and Kell Blood Group Systems among Blood Donors in Cote d'Ivoire, West Africa. *J Blood Transfus*, 2014, 309817.

Smythe, J.S., Avent, N.D., Judson, P.A., Parsons, S.F., Martin, P.G. and Anstee, D.J. (1996) Expression of RHD and RHCE gene products using retroviral transduction of K562 cells establishes the molecular basis of Rh blood group antigens. *Blood*, 87 (7), 2968-2973.

Southcott, M.J., Tanner, M.J. and Anstee, D.J. (1999) The expression of human blood group antigens during erythropoiesis in a cell culture system. *Blood*, 93 (12), 4425-4435.

Srisiri, K., Jaroenwattana, R., Panvisavas, N. and Bandhaya, A. (2017) Optimisation of DNA recovery and analysis of urine samples stored on FTA® card. *Forensic Science International: Genetics Supplement Series*, 6, e520-e522.

Stabentheiner, S., Danzer, M., Niklas, N., Atzmuller, S., Proll, J., Hackl, C., Polin, H., Hofer, K. and Gabriel, C. (2011) Overcoming methodical limits of standard RHD genotyping by next-generation sequencing. *Vox Sang*, 100 (4), 381-388.

Stangegaard, M., Borsting, C., Ferrero-Miliani, L., Frank-Hansen, R., Poulsen, L., Hansen, A.J. and Morling, N. (2013) Evaluation of four automated protocols for extraction of DNA from FTA cards. *J Lab Autom*, 18 (5), 404-410.

Storry, J.R., Castilho, L., Chen, Q., Daniels, G., Denomme, G., Flegel, W.A., Gassner, C., de Haas, M., Hyland, C., Keller, M., Lomas-Francis, C., Moulds, J.M., Nogues, N., Olsson, M.L., Peyrard, T., van der Schoot, C.E., Tani, Y., Thornton, N., Wagner, F., Wendel, S., Westhoff, C. and Yahalom, V. (2016) International society of blood transfusion working party on red cell immunogenetics and terminology: report of the Seoul and London meetings. *ISBT Sci Ser*, 11 (2), 118-122.

Storry, J.R., Castilho, L., Daniels, G., Flegel, W.A., Garratty, G., Francis, C.L., Moulds, J.M., Moulds, J.J., Olsson, M.L., Poole, J., Reid, M.E., Rouger, P., van der Schoot, E., Scott, M., Smart, E., Tani, Y., Yu, L.C., Wendel, S., Westhoff, C., Yahalom, V. and Zelinski, T. (2011) International Society of Blood Transfusion Working Party on red cell immunogenetics and blood group terminology: Berlin report. *Vox Sang*, 101 (1), 77-82.

Stratton, F. (1946) A new Rh allelomorph. *Nature*, 158, 25.

Suha, M., Hassan., (2015) *Toward prevention of Hemoglobinopathies in Oman*.

Suha, M.H., Al Muslahi, M., Al Riyami, M., Al Balushi, A., Egbert, B. and (2015) Haplotypes, sub-haplotypesand geographical distribution in Omani patients with sicklecell disease. *Thalassemia Reports*, 5:4739, 6-11.

Svobodova, I., Pazourkova, E., Horinek, A., Novotna, M., Calda, P. and Korabecna, M. (2015) Performance of Droplet Digital PCR in Non-Invasive Fetal RHD Genotyping - Comparison with a Routine Real-Time PCR Based Approach. *PLoS One*, 10 (11), e0142572.

Swelem, O., Goubran, F., Younis, S. and Kamel, N. (2018) ABO, RH phenotypes and kell blood groups frequencies in an Egyptian population. *Hematol Transfus Int J*, 6 (2), 71-75.

Szulman, A., Nardozza, L.M., Barreto, J.A., Araujo Junior, E. and Moron, A.F. (2012) Investigation of pseudogenes RHDPsi and RHD-CE-D hybrid gene in D-negative blood donors by the real time PCR method. *Transfus Apher Sci*, 47 (3), 289-293.

Taha, J.Y. (2012) Rh Antigen and Phenotype Frequency in Kalba Region, UAE *Bahrain Medical Bulletin*, 34 (1), 1-5.

Thongbut, J., Raud, L., Férec, C., Promwong, C., Nuchnoi, P. and Fichou, Y. (2019) Comprehensive Molecular Analysis of Serologically D-Negative and Weak/Partial D Phenotype in Thai Blood Donors. *Transfus Med Hemother*.

Tilley, L., Green, C., Poole, J., Gaskell, A., Ridgwell, K., Burton, N.M., Uchikawa, M., Tsuneyama, H., Ogasawara, K., Akkok, C.A. and Daniels, G. (2010) A new blood group system, RHAG: three antigens resulting from amino acid substitutions in the Rh-associated glycoprotein. *Vox Sang*, 98 (2), 151-159.

Tippett, P. (1986) A speculative model for the Rh blood groups. *Ann Hum Genet*, 50 (Pt 3), 241-247.

Tippett, P. and Sanger, R. (1977) Further observations on subdivisions of the Rh antigen D. *Ärztl.Lab*, 23, 476–480.

Touinssi, M., Chapel-Fernandes, S., Granier, T., Bokilo, A., Bailly, P. and Chiaroni, J. (2009) Molecular analysis of inactive and active RHD alleles in native Congolese cohorts. *Transfusion*, 49 (7), 1353-1360.

Tounsi, W.A., Madgett, T.E. and Avent, N.D. (2018) Complete RHD next-generation sequencing: establishment of reference RHD alleles. *Blood Adv*, 2 (20), 2713-2723.

Tsukada, K., Nojiri, M., Kurasawa, Y. and Kasahara, K. (2008) Case study of dissolving allele dropout. *Forensic Science International: Genetics Supplement Series*, 1 (1), 132-134.

Unger, L.J., Wiener, A.S. and Katz, L. (1959) Studies on blood factors RhA, RhB, and RhC. *J Exp Med*, 110, 495-510.

Valasek, M.A. and Repa, J.J. (2005) The power of real-time PCR. *Adv Physiol Educ*, 29 (3), 151-159.

Vege, S. and Westhoff, C.M. (2019) Chapter 26 - Rh and RhAG Blood Group Systems. In: Shaz, B. H., Hillyer, C. D. and Reyes Gil, M. (ed.) *Transfusion Medicine and Hemostasis (Third Edition).* Elsevier. pp. 149-155.

von Zabern, I. and Flegel, W.A. (2007) IVS5-38del4 deletion in the RHD gene does not cause a DEL phenotype: relevance for RHD alleles including DFR-3. *Transfusion*, 47 (8), 1552-1555.

Wagner, F.F. (2019) Getting comfortable with RH blood group system terminologies and databases. *ISBT Science Series*, 14 (1), 24-31.

Wagner, F.F. and Flegel, W.A. (2000) RHD gene deletion occurred in the Rhesus box. *Blood*, 95 (12), 3662-3668.

Wagner, F.F., Frohmajer, A. and Flegel, W.A. (2001) RHD positive haplotypes in D negative Europeans. *BMC Genet*, 2, 10.

Wagner, F.F., Gassner, C., Muller, T.H., Schonitzer, D., Schunter, F. and Flegel, W.A. (1999) Molecular basis of weak D phenotypes. *Blood*, 93 (1), 385-393.

Wagner, F.F., Ladewig, B. and Flegel, W.A. (2003) The RHCE allele ceRT: D epitope 6 expression does not require D-specific amino acids. *Transfusion*, 43 (9), 1248-1254.

Wagner, F.F., Moulds, J.M., Tounkara, A., Kouriba, B. and Flegel, W.A. (2003) RHD allele distribution in Africans of Mali. *BMC Genet*, 4, 14.

Wagner, T., Kormoczi, G.F., Buchta, C., Vadon, M., Lanzer, G., Mayr, W.R. and Legler, T.J. (2005) Anti-D immunization by DEL red blood cells. *Transfusion*, 45 (4), 520-526.

Wallace, M., Lomas-Francis, C., Beckers, E., Bruce, M., Campbell, G., Chatfield, S., Nagao, N., Okubo, Y., Opalka, A., Overbeeke, M., Scott, M. and Voak, D. (1997) DBT: a partial D phenotype associated with the low-incidence antigen Rh32. *Transfus Med*, 7 (3), 233-238.

Wang, Q.P., Dong, G.T., Wang, X.D., Gu, J., Li, Z., Sun, A.Y., Shao, C.P., Pan, Z.L., Huang, L.H., Xie, W.X., Sun, G.M., Chen, J.J., Pei, H., Yang, X.J. and Shan, P.N. (2014) An investigation of secondary anti-D immunisation among phenotypically RhD-negative individuals in the Chinese population. *Blood Transfus*, 12 (2), 238-243.

Westhoff, C.M. (2004) The Rh blood group system in review: a new face for the next decade. *Transfusion*, 44 (11), 1663-1673.

Westhoff, C.M. (2007) The structure and function of the Rh antigen complex. *Semin Hematol*, 44 (1), 42-50.

Westhoff, C.M., Vege, S., Halter-Hipsky, C., Whorley, T., Hue-Roye, K., Lomas-Francis, C. and Reid, M.E. (2010) DIIIa and DIII Type 5 are encoded by the same allele and are associated with altered RHCE*ce alleles: clinical implications. *Transfusion*, 50 (6), 1303-1311.

Westhoff, C.M., Vege, S., Halter Hipsky, C., Hue-Roye, K., Copeland, T., Velliquette, R.W., Horn, T., Lomas-Francis, C. and Reid, M.E. (2013a) RHCE*ceTI encodes partial c and partial e and is often in cis to RHD*DIVa. *Transfusion*, 53 (4), 741-746.

Westhoff, C.M., Vege, S., Horn, T., Hue-Roye, K., Halter Hipsky, C., Lomas-Francis, C. and Reid, M.E. (2013b) RHCE*ceMO is frequently in cis to RHD*DAU0 and encodes a hr(S) -, hr(B) -, RH:-61 phenotype in black persons: clinical significance. *Transfusion*, 53 (11 Suppl 2), 2983-2989.

Whitlock, S. (2010) *Immunohematology for medical laboratory technicians*. Clifton Park, NY: Delmar, Cengage Learning.

Wiener, A.S. (1943) Genetic Theory of the Rh Blood Types. 54 (3), 316-319.

Wiener, A.S. and Unger, L.J. (1962) Further observations on the blood factors Rh-A, Rh-B, Rh-C and Rh-D. *Transfusion*, 2, 230-233.

Wong, H.Y., Lim, E.S. and Tan-Siew, W.F. (2012) Amplification volume reduction on DNA database samples using FTA Classic Cards. *Forensic Sci Int Genet*, 6 (2), 176-179.

Yazer, M.H. and Triulzi, D.J. (2007) Detection of anti-D in D- recipients transfused with D+ red blood cells. *Transfusion*, 47 (12), 2197-2201.

Zahid, H., Yahyaoui, A., Uwingabiye, J., El Khazraji, A., Labrini, F., Hadef, R. and Messaoudi, N. (2016) henotype frequencies of Rh and Kell Blood Group Systems in Blood Transfusion department of Avicenna Military Hospital, Marrakech, Morocco. *Int. j. med. health res*, 2 (1), 1-10.

Zhou, H.Y., Zhang, Y.Z., Meng, Q.B., Bai, X.H., Wang, C.R., Cao, Q. and Lan, J.C. (2008) [Comparison between genotyping and serological phenotyping in RhCE blood group]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*, 25 (1), 66-69.