Development of a Novel South African Rare Donor Red Cell Genotyping Database

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AUTHOR'S DECLARATION

This study represents original work by the author. It has not been submitted to any other tertiary institution. Where the work by others is made use of, this has been duly acknowledged.

The research described in this dissertation was carried out in the Specialised Laboratory Services (SLS) Immunohaematology department at the South African National Blood Services (SANBS) under the supervision of Mr Ravi Reddy (COO-SANBS) and Mr Kuben Vather (Senior Manager, SLS, SANBS) and under the supervision of Dr Rosaley Prakaschandra and Dr Pavitra Pillay in the department of Biomedical and Clinical Technology, Faculty of Health Sciences, Durban University of Technology.

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DEDICATION

I dedicate this work to:

My parents, siblings and their families for their love, support and patience.

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LIST OF DEFINITIONS

DEFINITION	MEANING
Alleles	Pairs of genes located at the same site on chromosome pairs.
Alloantibody	Formation of an antibody to an antigen following exposure to an antigen that is recognised as foreign.
Alloimunization (red cell)	Formation of an antibody in response to a foreign antigen that stimulates the complement cascade and destruction of red cells. In blood transfusion donor antigens can be introduced in a patient by blood transfusion. In a pregnant mother, foreign antigens can be introduced by foetal antigens inherited from the father.
Blood	Blood in the human body is comprised of red blood cells which carry oxygen throughout the body, white blood cells which fight infections, platelets which are cells that help stop bleeding and plasma which is a yellowish liquid that carries nutrients, hormones, and proteins throughout the body.
Blood Group	Antigens found on the surface of red cells in a person and usually defined by an antibody found in the plasma of another person.
Blood Transfusion	Is the process of receiving blood or blood products into one's circulation intravenously for various medical conditions to replace lost components of the blood.
Deletion	Where nucleotide should be present however is deleted.
Duplication	A nucleotide occurs twice where it normally occurs once.
Gene	The basic unit of heredity, made of DNA. Each gene occupies a specific location on a chromosome.
Genome	A genome is an organism's complete set of DNA, including all of its genes.
Genotyping (red cell)	A molecular method that uses DNA to determine the genes that code for red cell antigens.
Haemovigilance report	A formal programme that will look-back, investigate and report all events related to the transfusion of blood and blood products that caused an adverse reaction in the patient. The report is released annually.
High-frequency/ prevalence antigen	Antigens that occur in 99 – 99.9% of the population.
Insertion	Nucleotide insertion – one nucleotide is inserted in place of another naturally occurring nucleotide.

Inversion	Two side-by-side occurring nucleotides swop their original positions with each other.
Low-frequency/ prevalence antigen	Antigens that occur in <1% of the population.
Nucleotide	Building blocks of DNA, alleles, genes. Four nucleotides only called Adenine, Thymine, Cytosine, Guanine.
Null	Null phenotype – the gene is present however due to a gene mutation, the expression of the red cell antigens are suppressed.
Phenotyping	In blood transfusion, phenotyping is a serological method of adding serum and cells with the resultant absence or presence of haemagglutination that indicate the absence or presence of red cell antigens.
Red cell antigens	Antigens that are found on the surface of red blood cells.
Transfusion Medicine	Field of clinical medicine in the discipline of Haematology that is related to Blood Transfusion.
Translocation	DNA nucleotides move their entire position from the original location (locus) to another position on the gene.

LIST OF ABBREVIATIONS

AABB	American Association of Blood Banks
BI	Business Intelligence – type of IT program
BGMUT	Blood Group Antigen Gene Mutation
ExAC	Exome Aggregation Consortium
HDFN	Haemolytic Disease of the Foetus and Newborn
HTR	Haemolytic Transfusion Reaction
IRDP	International Rare Donor Panel
ISBT	International Society of Blood Transfusion
NCBI	National Center for Biotechnology Information
RBC	Red blood cell
SANBS	South African National Blood Service
SCD	Sickle Cell Disease
SNP	Single Nucleotide Polymorphism
WPBTS	Western Province Blood Transfusion Service
1000 genome	Thousand genome database

ABSTRACT

INTRODUCTION

More effective blood management can be implemented by issuing genotype-matched blood with a reduction in transfusion reactions and haemolytic disease of the newborn, since serological phenotyping methods for the detection of red cell antigens, while simple and easy to perform is limited by biological interference factors. Targeted rare donor screening using the frequency of rare types found amongst the ethnic groups as a guide will assist increase rare donor inventory. Currently, no such inventory exists in South Africa. The aim of this study was to develop a database to analyse and interpret red cell genotyping data using a novel molecular based red cell genotyping method at the South African National Blood Service (SANBS).

AIMS AND OBJECTIVES

The purpose of this study was to determine the prevalence of red cell genotypes and predicted phenotypes amongst the ethnic groups of blood donors at SANBS using the IDCORE^{XT} red cell genotyping assay. This allowed for the identification of high and low prevalence antigens from commonly occurring red cell antigens, the probability of finding specific blood types depending on the prevalence in the population. The rare blood genotypes were identified and were used to establish a novel rare donor red cell genotyping database for SANBS and South Africa using a Business Intelligence (BI) IT program.

METHODOLOGY

Red cell genotyping data of 323 donors tested by means of the IDCORE^{XT} assay from January 2015 to August 2016 was analysed using BIDS^{XT} software. The ABO group, Rh types and ethnicity of 278 donors were imported to a Business Intelligence (BI) IT program after excluding seven invalid results and 38 confirmatory genotyping results and described. The Power BI programme was then used to exclude 44 serologically known rare donors prior to completing prevalence studies. High and low prevalence genotypes and predicted phenotypes for the remaining 234 donors were tabulated to ascertain the lowest percentage of positive or negative antigens in order to establish the rare blood types in each of the 10 blood groups per sample. Using Power BI, 161 rare blood types were identified from a total of 2340 genotyping results (234 samples, 10 blood groups) and was combined with the 44 serological known donors to develope the final rare donor red cell genotyping database comprised of 205 red cell genotyping results..

RESULTS

The study population (n=278) included more males (61%) than females (39%) and the distribution of donors amongst the four major ethnic groups in South Africa was 48.9% White, 37.7% Black, 8.7% Indian and 4.7% Coloured similar to the current donor population in South Africa. Group O+ was the most prevalent blood group found in 76% of the study population. The most prevalent

Rh phenotype was Ro (cDe/cDe) found in the majority of Black donors (60%, 50/84). The White donors showed the most Rh genetic variability with R_1R_2 found in White donors only (10%, 13/124) and R_1R_1 was most prevalent in Indians (57%, 12/21). The final rare donor red cell genotyping database comprised 205 donors and 13 high-frequency antigens were found: HrB-, HrS-, k-, Js(b-), Kp(b-), Jk(b-), Fy(b-), s-, S-s-U-, Joa-, Hy-, Yt(a-) and Lu(b-). Five positive low-frequency antigens were identified: Cw+, Js(a+), Kp(a+), K+ and Uvariant. The rare Rh phenotype, R_zR_1 (CDE/CDe) was found in one Coloured donor.

CONCLUSION

This was the first study to determine the prevalence of red cell genotypes and predicted phenotypes amongst a subset of South African blood donors. In addition to new antigens identified and contributing to the international red cell genotyping database of rare donors, this study has now established a benchmark for similar studies to be completed on a larger scale.

CHAPTER 1: INTRODUCTION

1.1 Rationale

Antigens found on the surface of red blood cells determine the blood type of each individual which is important information required for identifying the most suitable donor-patient matched blood for blood transfusion (Daniels, 2013). Blood transfusions due to red cell antigen mismatch between donor and patient can result in alloimmunisation which is the formation of red cell antibodies to the donor red cell antigens that the patient lacks. The clinical implication of alloimmunisation is mild to fatal Haemolytic Transfusion Reactions (HTRs) (Suddock and Crookston, 2018; Daniels, Hadley and Soothill, 2002). In neonates, alloimmunisation is capable of causing Haemolytic Disease of the Foetus or Newborn (HDFN). Haemolytic disease of the foetus and newborn occurs when the mother lacks an antigen that the newborn has inherited from the father thus developing antibodies. Subsequent pregnancies by the mother are affected because the antibodies cross the placenta and destroy foetal cells causing severe anaemia and in some cases are fatal (Reid, Rios and Yazdanbakhsh, 2000; Daniels, Hadley and Soothill, 2002). In addition, finding compatible blood for patients with antibodies to rare negative high-frequency antigens is challenging due to the limited number of rare blood donors found worldwide as listed by the International Rare Donor Panel (IRDP) (Kaur and Jain, 2012). Therefore, the accurate identification of red cell antigens is critical as patients requiring blood already may have severe underlying clinical conditions and a transfusion reaction or HDFN due to red cell antibody must be avoided.

The current routine method of red cell antibody detection relies on serological phenotyping methods. There are however limitations due to:

- Inconclusive serology results caused by interference of donor red blood cells in patients receiving multiple blood transfusions (Daniels, 2013);
- The presence of autoantibodies in patients with autoimmune diseases where the reaction of the patient's serum and patient cells are positive and serological test results are falsely positive (Daniels, 2013); and
- Lack of available and/or expensive rare polyclonal or monoclonal antisera (Daniels, 2013).

Red cell genotyping, using molecular diagnostics assays such as the Grifols IDCORE^{XT} kit, overcomes the limitations associated with serology (Goldman, Nuria and Castilho, 2015; Keller, 2015; Lopez et al., 2018). Red cell genotyping assays utilise patients' DNA to provide information about the genes that code for the red cell antigens found on the surface of the red blood cells and thus infers the red cell phenotype. While genotyping has its advantages over serology such as

testing multiple blood groups in one test and high-throughput testing over a shorter time period, it is also limited to the allele coverage of the commercially designed kit and therefore may miss some rare blood types and may not detect novel alleles (Lopez et al., 2018; Keller, 2015).

Although red cell genotyping was introduced more than 20 years ago in developed countries, it was only in the last few years that this technology became available and affordable as a service in South Africa (Govender, Vather and Naidoo, 2015). For this reason, the rare and unique red cell genotypes in South African blood donors have not been defined at a genetic level until recently. This dissertation is based on the implementation of this new technology at the South African National Blood Service (SANBS). The study design employed was a retrospective, descriptive analysis of red cell genotyping data obtained over the study period of 1st January 2015 to 31st August 2016. The purposive sampling method resulted in a final study population of 323 donors.

The aim of this study was to develop the first rare donor red cell genotyping database at SANBS. To develop the database, Objective 1 was to determine the prevalence of red cell genotypes in a subset of South African blood donors that were randomly selected for screening but also included some donors purposively selected as per the laboratory algorithm of testing for rare blood donors. The samples were tested using the reference laboratory toolbox of tests which included the IDCORE^{XT} red cell genotyping assay run on the Luminex 200IS instrument (Finning et al., 2016). This red cell genotyping assay was selected as it tests for the genes of ten blood group systems in one test and the Luminex 200IS instrument was already in use for HLA and HPA typing at the SANBS.

Objective 2 was to determine the 2 allele/antigen prevalence and the frequency of rare genes as red cell antigens are inherited either as homozygous or heterozygous, using the direct counting method as per the AABB Technical Manual (Brecher, 2008). The importance of this objective was that it will allow SANBS to contribute their most frequent and least frequent rare blood types to the IRDP where other countries have their rare blood type frequencies listed (Poole, 2006). It is evident from published literature that certain countries have more of a particular rare blood type than other countries based on their population diversity and this information assists in locating rare blood units from countries worldwide who may have a larger inventory of the rare blood type (Poole, 2006). An example of this is the discovery in South Africa of the hrB- and HrB- (Rh:-34) rare blood type in the serum of a Black SA women and the hrS- and rare HrS- (RH:-18) blood type in a Black SA male (Moores, 1991; Daniels, 2005). Therefore, there are more prevalent rare types in South African Blacks in comparison to other countries where there is no African ancestry (Moores, 1991; Daniels, 2013). The anti-Hr antibodies are capable of causing severe HTRs and HDFN hence the critical nature of accurately determining the rare genotypes and to avoid wastage of rare units where not required (Pham et al., 2009; Strobel, 2008).

Since this was the first rare donor red cell genotyping database based on a subset of South African donors, the baseline criteria such as gender, ethnicity, ABO group and Rh type were described. The different rare blood types and the volume/frequency of each rare blood type found in this study was compared to other countries. Blood types not rare in South Africa but rare in other parts of the world, were also compared.

1.2 Summary of Chapters

Chapter 1.0 – Introduction: In this chapter the importance of matching donor-patient red cell antigens and the clinical implications that result from incompatibility are described. The rationale is explained and the gaps in the current serological testing methods are highlighted. The chapter includes a brief overview of the sample design, aim and objectives of the study.

Chapter 2.0 – Literature Review: This chapter contains a summary of the current and original literature describing similar or related studies completed in South Africa, Africa and internationally. The laboratory methods including serology and molecular diagnostics for red cell antibody detection are compared. The molecular background of the various blood groups and the mechanisms that result in the various polymorphisms are discussed. In addition, the benefits and limitations of the diagnostic methods for red cell genotyping are listed. The clinical significance and benefits to the patients that the red cell genotyping methods offer are highlighted. Implementation of the rare donor red cell genotyping databases amongst other countries internationally are reviewed.

Chapter 3.0 – Materials and Methods: This chapter describes the materials and methods used in the design and development of the rare donor red cell genotyping database. A detailed description of the process of blood donor collection, testing and then referral to the Immunohaematology Reference Laboratory is completed. The red cell genotyping IDCORE^{XT} assay operated on the Luminex 200IS with analysis and interpretation of results on BIDSXT is shown by the use of flow diagrams. The chapter includes a diagrammatic representation of the final study population with the application of the data inclusion and exclusion criteria. Final data review and analysis using the BI IT program is described.

Chapter 4.0 – Results: In this chapter the study findings are described using bar graphs, pie graphs, tables and descriptive statistics such as the direct counting method. The algorithmic approach used to analyse the data is described.

Chapter 5.0 – Discussion: The discussion begins with interpretation of the donor demographics of gender, ethnicity, ABO group and Rh type across the four major ethnicities in South Africa. This is followed by a description and discussion of the outcomes of Objective 1 prevalence studies in comparison to similar studies performed internationally. Objective 2 separated the common or

more frequent blood types from rare blood types in order to develop the rare donor red cell genotyping database. A description of the trends in prevalence patterns between ethnicities in South Africa in comparison to similar ethnicities internationally is presented. The strengths and weaknesses of the study are highlighted.

Chapter 6.0 – Conclusion: Main conclusions based on the study findings are reported. The implications of the study outcomes for South Africa and future studies that the present study may allow are alluded to.

CHAPTER 2: LITERATURE REVIEW

This chapter describes the circumstances under which the limitations of serology cannot be overcome and resolved in the absence of molecular based testing. This includes the clinical implications of alloimmunisation as detailed in haemovigilance reports. The molecular basis of red cell antigens underpinned by the various molecular mechanisms such as deletions, insertions, and single nucleotide polymorphisms are described. The predominant red cell antigens per blood group and prevalence of red cell antigens per major ethnic groups worldwide are stated. In addition, the benefits and limitations of the diagnostic methods for red cell genotyping are discussed. The clinical, scientific and operational benefits of the red cell genotyping method is highlighted. Lastly, there is an overview of similar international studies of rare blood types and rare donor databases.

2.1 Background

2.1.1 Red Cell Antigens and HTR and HDFN – Limitation to Serology

In transfusion medicine, numerous blood group red cell antigens are considered clinically significant as they can result in an antigen-antibody reaction in patients receiving blood from donors that have an antigen that the patient lacks (Alter and Klein, 2008). The resultant formation of alloantibodies called alloimmunisation can cause adverse events, acute or delayed HTRs or HDFN (Strobel, 2008). An acute HTR occurs within 24 hours following transfusion of incompatible red blood cells and therefore strict measures in blood transfusion are followed as stated in the Clinical Guidelines for Use of Blood Products in South Africa (SANBS/WPBTS, 2014). The HTRs can range from being mild, moderate, severe or fatal as indicated in the haemovigilance reports (SANBS/WPBTS, 2016a).

The most clinically significant red cell antigens are from the ABO and Rh blood group systems with ABO-incompatible blood transfusions resulting in severe transfusion reactions and even death (Reid, Lomas-Francis and Olsson, 2012). This is then followed by the antigens of the Kell, Kidd, Duffy, MNS, Diego, Colton and other blood group systems (Reid, Lomas-Francis and Olsson, 2012). The majority of the red cell antigens can be easily detected by serological phenotyping methods which are simple and inexpensive. However, the phenotyping method using serum samples or red blood cells as per the AABB technical manual is associated with several limitations, as discussed hereunder (Brecher, 2008).

2.1.2 Red Cell Antigens in Chronic Clinical Conditions – Limitation to Serology

The prevalence of Sickle Cell Disease (SCD) in South Africa was previously reported as low. The sickle cell gene was found in South African Indians (originating from South East-Asia), and sickle cell-C and sickle cell-beta thalassaemia in Whites and the heterozygous HbS sickle cell trait (originating in East Africa) was almost absent (<0.2%) in Black ethnic groups. However, SCD in South Africa has significantly increased in the last 10 years due to an increase in immigrants with SCD arriving from the rest of Africa specifically to the Western Cape (Wonkam et al., 2012). Sickle cell disease has since become a major burden on health resources as SCD patients require lifelong treatment including several blood transfusions (Wonkam et al., 2012). The multiple blood transfusion in turn triggers the formation of many alloantibodies and many of these patients eventually require rare blood units for treatment. The multiple transfusions also cause inconclusive serology results due to interference of mixed donor cell populations hence molecular genotyping becomes more essential (Fasano and Chou, 2016).

It is also known that patients who have autoimmune diseases such as autoimmune haemolytic anaemia (AIHA), Haemophilia, Systemic Lupus Erythematosus and other autoimmune conditions have circulating autoantibodies that cause false positive serology results which may mask true underlying clinically significant antibodies (Quist and Koepsell, 2015).

2.1.3 Red Cell Antigens Negative for High-Frequency Antigens – Limitation to Serology

Serological tests are limited when testing for the negative high-frequency antigens (rare blood types) because there is scarcity in supply of the rare antisera required for testing. Rare commercial antisera where available is expensive and is therefore not cost-effective for large-scale screening for rare blood types (Wilkinson, 2016). The absence of high-frequency antigens also causes false positive pan-reactive reactions across the full panel of cells used for testing. The direct antiglobulin test between negative serum and patient cells should be negative but is positive in these cases indicating sensitisation of patient cells (Daniels, 2013; Brecher, 2008; Reid, Lomas-Francis and Olsson, 2012). The standard serological tests are therefore inconclusive.

At SANBS, there are no other specialised serological tests that are routinely completed such as absorption-elution tests or Western blotting as described in the AABB technical manual as these are not practical and are time consuming to complete (Brecher, 2008). Due to lack of adequate skill in these specialised tests as well as the strain on available laboratory resources at the SANBS reference laboratory in resolving complex serology cases, these specialised laboratory tests are no longer part of routine laboratory testing.

2.2 Haemovigilance/SHOT (Serious Hazards of Transfusion) Reports

Due to the limitations of serology, the SANBS in certain circumstances such as blood shortages had no option but to issue least incompatible units to patients on numerous occasions, resulting in the manifestation of adverse clinical events (SANBS/WPBTS, 2016a). SANBS issues a haemovigilance report annually that reports on the causes of adverse reactions, the investigations and the outcomes. According to the 2016 haemovigilance report, there were 4 870 donor adverse events, 986 transfusion related adverse events and 14 cases of transfusion-related patient deaths. Of the 14 cases of transfusion-related deaths, four were in babies < one year, four in patients > 55 years and the remaining in cases of severe trauma such as gunshot injury, major surgery or complications in minor surgery. The hospitals ruled that 7 of the 14 cases were not related to blood transfusion, however due to insufficient details, clinical information and lack of post transfusion testing the probable cause of incompatible blood product or allommunisation could not be excluded in the remaining 7 mortality cases (SANBS/WPBTS, 2016a).

At SANBS, adverse events including acute and delayed HTRs are thoroughly investigated to determine if incompatible units have been transfused and to ensure that confirmed technical errors are not repeated. The haemovigilance report of 2016 highlighted that fact that due to the heavy burden placed on state hospitals and clinics in South Africa, accurate and detailed clinical records are not always kept updated and several cases of adverse events due to blood transfusion may be unreported, and even when reported on the SANBS transfusion reaction forms, vital information was missing (SANBS/WPBTS, 2016a).

A similar programme in the United Kingdom named the SHOT reports, in 2016 reported 264 near miss ABO-incompatible transfusions that were stopped before they caused harm and three cases of ABO incompatibility, two of which resulted in serious morbidity but no deaths (Bolton-Maggs, 2016). In the SHOT 2015 report one case of ABO incompatibility due to hospital error resulted in death and therefore one of the action items of SHOT was to focus on reducing laboratory errors in testing (Bolton-Maggs, 2015). However, a more serious consequence for the staff member in the United Kingdom who issued the ABO-incompatible unit was legal action due to it being common knowledge that ABO-incompatible transfusions are fatal and extra caution must always be heeded. In South African no such legislation exists; however, at SANBS dismissal is actioned where incompatible blood transfusion due to blood-bank error results in patient death.

2.3 Molecular Immunohaematology

The introduction of molecular DNA-based test methods provided a solution to overcome the gap created by the limitations of serology and the requirement to keep cases of alloimmunisation to a minimum (Wilkinson, 2016). The mapping of the human genome provided knowledge on the molecular backgrounds and polymorphisms of the blood groups that enabled the development of DNA-based assays for red cell genotyping (Anstee, 2009: 141).

2.3.1 Molecular Basis of Red Cell Antigens

The red cell genotyping method is based on the identification of single nucleotide polymorphisms (SNPs) (Daniels, 2005; Reid, 2018). A single nucleotide polymorphism occurs when there is a variation in a single DNA nucleotide and normally occurs throughout a person's DNA (Reid, 2018). These SNP variations are found in the DNA within a gene or in a regulatory region near a gene. The genetic mechanisms that result in polymorphisms are nucleotide substitutions, insertions, deletions and gene conversions that determine what and how red cell antigens are expressed (Daniels, 2005).

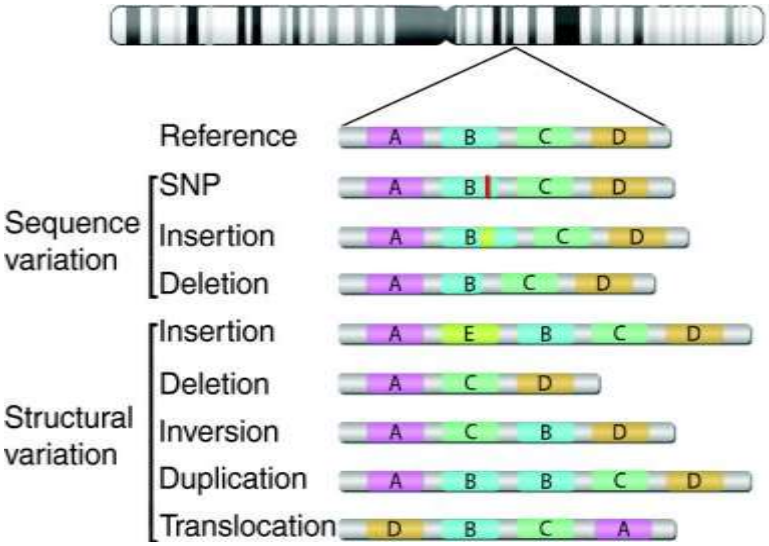


Figure 2.1: Schematic description of mechanisms resulting in genetic variants
Source: Rahim et al. (2008)

As depicted in Figure 2.1, there are several molecular mechanisms that are responsible for polymorphisms that are classed as either sequence variation or structural variation. The most common polymorphisms found in molecular immunohaematology are SNPs where one nucleotide (Adenine, Cytosine, Thymine, Guanine) is replaced by another nucleotide. This may result in a change in a protein that could lead to a premature stop codon, or a mutation in the regulatory region or promoter region in genes therefore affecting the expression of red cell antigens (Rahim et al., 2008). In the current study, the researcher referred a discordant genotyping/serology result

for red cell sequencing confirmation that showed a novel polymorphism in the regulatory region of the RHCE gene that was thought to suppress the expression of RHCE antigens (Fennell et al., 2017). This variation results in the RHCE antigens not being identified with certain phenotyping reagents (polyclonal vs monoclonal reagents) which could potentially result in an incompatible blood transfusion even if the crossmatch is compatible.

Other mechanisms include nucleotide insertions and deletions called indels (Rahim et al., 2008). Structural variations (Figure 2.1) caused by nucleotide inversions, duplications and translocations could introduce recombination between closely-linked genes (such as the RHD and RHCE genes) that result in hybrid genes/proteins (Rahim et al., 2008). Another example of gene mutations affecting blood group antigen expression is in null phenotypes where the relevant blood group gene is present but no red cell antigens are expressed (Rahim et al., 2008). Matching the relevant SNPs to red cell antigens and genes provides a means to accurately predicting blood group phenotypes from genomic DNA with red cell genotyping. Appendix 4 lists the SNPs that the IDCORE^{XT} assay in this study identifies for the 10 blood group genotypes and predicted phenotypes (Progenika Grifols, 2014).

2.3.2 Molecular Basis per Blood Group

There are currently 36 blood group systems that have been identified (Daniels, 2013). A description of the main features of six main blood groups – ABO, Rh, Kell, Kidd, Duffy and MNS are described hereunder (Daniels, 2013; Dean, 2005).

ABO

The ABO blood group comprises the A, B, AB and O antigens. The molecular basis is that the ABO gene indirectly encodes the ABO blood group antigens by encoding transferases which catalyse the synthesis of the antigens (Reid, Lomas-Francis and Olsson, 2012; Dean, 2005). The A/B polymorphism arises from several SNPs in the ABO gene (Dean, 2005; Daniels, 2013). The O allele encodes an inactive glycosyltransferase that leaves the ABO antigen precursor (the H antigen) unmodified (Daniels, 2013). The system of glycoproteins and glycolipids have unknown function (Dean, 2005). ABO typically causes an acute haemolytic reaction (Strobel, 2008), but HDFN is very mild. The frequency of antigens/phenotypes in the ethnic groups of the general population are as below (Dean, 2005):

- Caucasians: group O, 44%; A, 43%; B, 9%
- Blacks: group O, 49%; A, 27%; B, 20%
- Asians: group O, 43%; A, 27%; B, 25%

Rh

The Rh blood group comprises D, C, E, c, e which are the main antigens (Daniels, 2013; Dean, 2005; Reid, Lomas-Francis and Olsson, 2012). The molecular basis are two genes, namely, the RHD gene that codes for the D antigen and the RHCE gene that codes for the C/c, E/e antigens. The entire deletion of the RHD gene is RH negative (dd) (Daniels, 2013). The C/c polymorphism arises from four SNPs that cause four amino acid changes (Dean, 2005). The E/e polymorphism arises from a single SNP (676G → C) (Dean, 2005). The proteins have an unknown function (Dean, 2005). Rh antibodies cause delayed HTRs and is the most common cause of HDN (Strobel, 2008). Frequency of antigens/phenotypes are as described below (Dean, 2005):

- D: 85% Caucasian, 92% Blacks, 99% Asians
- C: 68% Caucasians, 27% Blacks, 93% Asians
- E: 29% Caucasians, 22% Blacks, 39% Asians
- c: 80% Caucasians, 96% Blacks, 47% Asians
- e: 98% Caucasians, 98% Blacks, 96% Asians
- Rh haplotype DCe: most common in Caucasians (15%), Native Americans (44%) and Asians (70%)
- Rh haplotype Dce: most common in Blacks (44%)
- Rh D-negative phenotype: most common in Caucasians (15%), less in Blacks (8%), rare in Asians (1%) (Dean, 2005)

Kell

The Kell blood group system comprises the K, k, Kp^a, Kp^b, Js^a, Js^b antigens. The molecular basis is the KEL gene. The two codominant alleles, K and k, result from a SNP (698C → T) (Daniels, 2005; Dean, 2005). Kell arises from glycoproteins with enzymatic functions, although the specific functions are unknown (Dean, 2005). The Kell antibodies, especially anti-K, cause severe HTRs and sever foetal anaemia (Strobel, 2008). The frequency of antigens/phenotypes are as follows (Dean, 2005):

- K-k+: 91% Caucasians, 98% Blacks
- K+k-: 0.2% Caucasians, rare in Blacks
- K+k+: 8.8% Caucasians, 2% Blacks
- Kp(a-b+): 97.7% Caucasians, 100% Blacks
- Js (a-b+): 100% Caucasians, 80% Blacks (Dean, 2005)

Kidd

The Kidd blood group system comprises the Jk^a, Jk^b and Jk3 (Jk^{a-b-}) antigens. The SLC14A1 gene encodes the Kidd glycoprotein. The SLC14A1 gene has two major codominant alleles, Jk^a and Jk^b which result from a SNP (838G → A). The function of the glycoprotein is to transport urea. Kidd antibodies are a common cause of delayed HTRs and only causes mild or no HDFN. The frequency of the antigens/phenotypes are as follows (Dean, 2005):

- Jk^a: 77% Caucasians, 92% Blacks, 73% Asians
- Jk^b: 74% Caucasians, 49% Blacks, 76% Asians
- Jk^3: 100% in most populations, >99% Polynesians
- Jk(a-b-): rare in most populations, found in 0.9% Polynesians (Dean, 2005)

Duffy

The Duffy blood group system comprises the Fy^a and Fy^b antigens. The FY gene encodes the two codominant alleles, FYA and FYB, that result from the SNP (125G → A). The homozygous-33T→C in the FYB allele results in the Fy^{a-b-} (FY3) (Daniels, 2013; Dean, 2005). The function of the Duffy antigens is to bind cytokines during inflammation. The absence of Duffy antigens in individuals, Fy^{a-b-} phenotype, confers resistance to *P. Vivax* (Dean, 2005). Duffy antibodies cause mild to severe delayed HTRs and only causes mild HDFN (Strobel, 2008). The frequency of the antigens/phenotypes are as follows (Dean, 2005):

- Fy^a: 66% Caucasians, 10% Blacks, 99% Asians
- Fy^b: 83% Caucasians, 23% Blacks, 18.5% Asians
- Fy^3: 100% Caucasians, 32% Blacks, 99.9% Asians. The Duffy null phenotype Fy (a-b-) called Fy^3 is very rare in Caucasians but is found in 68% Blacks (Dean, 2005)

MNS

The MNS blood group comprises the M, N, S, s U, Mia antigens. The molecular basis are two genes called GYPA and GYPB. The GYPA has two codominant alleles, M and N, that result from three SNPs (59C→T, 71G→A, 72G→T). The codominant alleles of GYPB, S and s, result from one SNP (143C→T) (Dean, 2005). Glyocophorins A and B carry the MNS antigens and may also function as receptors for cytokines and pathogens including the malaria parasite *Plasmodium falciparum* (Dean, 2005). Transfusion reactions and HDFN are uncommon but potentially severe (Strobel, 2008; Reid, 2018). The frequency of antigens/phenotypes are as follows (Dean, 2005):

- M: 78% Caucasians, 74% Blacks
- N: 72% Caucasians, 755 Blacks
- S: 55% Caucasians, 31% Blacks
- s: 89% Caucasians, 93% Blacks
- M+N+S+s- phenotype is found in 4% Caucasians and M-N+S+s- in 1% Caucasians, 2% Blacks
- M+N-S-s-, M+N+S-s- and M-N+S-s- are rare in Caucasians but found in +/- 0.5% Blacks (Dean, 2005).

2.3.3 Identifying Red Cell Antigens – Types of Red Cell Genotyping Assays

The molecular basis of each blood group system has been described in 2.3.2 above. This molecular background was then applied in the development of several red cell genotyping assays. Depending on the application of red cell genotyping for different purposes, the various types of assays had their own advantages and disadvantages. Table 2.1 describes a few of these assays based on the throughput of testing achieved.

The low throughput assays such as BAGene and Fluogene kits are based on the principle of using sequence specific primers (SSP) and therefore is suitable for applications where only a specific blood group antigen is suspected and requires confirmation (Prager, 2007; Innotrain, 2017). As per Table 2.1, the BAGene kit only allows one sample to be tested per run and all steps are manual. Interpretation of final results from observing bands on an agarose gel may be subjective and vary from one user to the next (Prager, 2007). The Fluogene kit also only allows one or three samples to be tested depending on the blood group, however the pipetting can be automated on a PiU (pipetting unit) if purchased by the laboratory or the process remains manual (Moonsamy, Govender and Vather, 2017). These assays therefore are only suitable for resolving complex or inconclusive serology results or confirming a suspected rare blood type.

Sanger sequencing as described in Table 2.1 allows for the testing of 1 - 96 samples, however it has been termed a medium throughput assay as it can only be used for targeted sequencing (McBean, Hyland and Flower, 2014). Therefore, this assay would suit researchers screening for one particular rare blood type for example GYPB deletions (U negative) (Moonsamy, Govender and Vather, 2017). The advantage is that specific primers can be designed as required and tested. However, the instrument (ABI Prism 3500) is expensive and requires many consumables and reagents which results in an expensive final cost per test (McBean, Hyland and Flower, 2014).

The high-throughput assays described in Table 2.1 are the HemoIDChip, BeadChip and IDCORE^{XT} assays (McBean, Hyland and Flower, 2014; Goldman, Nuria and Castilho, 2015). These assays allow many blood groups systems per sample to be identified by a single test and 1- 96 tests can be completed per run. The initial cost of the instrument is expensive, and the cost of the kits are expensive, however, it is the most cost-effective solution in a laboratory to batch test routine samples and mass-scale screening for rare donors (Lopez et al., 2018). There is the benefit of time and staff cost saving when compared to complex serology cases that require several specialised serological tests (McBean, Hyland and Flower, 2014).

Table 2.1: Low, medium and high-throughput red cell genotyping assays

	Low Throughput	Medium Throughput	High-Throughput
	BAGene PCR-SSP Kit (Prager: 2007) **Fluogene PCR-SSP Kit** (Innotrain, 2017)	**Sanger DNA sequencing** (McBean, Hyland and Flower, 2014: 147-151)	**HemoID, BeadChip** (McBean, Hyland and Flower, 2014: 147-151) **Grifols ID COREXT** (Progenika Grifols IDCORE^{XT}: 2014)
Benefits	Low costs Ease of performance Small scale screening	Processes 1 - 96 samples Rapid analysis Sequence specific	Semi-automated Rapid, high-throughput testing – (10 blood group systems, 37 antigens-IDCORE^{XT}), (12 blood groups, 46 antigens – HemoID)
Limitations	Labour intensive – 1 sample per run Time intensive – manual steps Difficult to automate – running gels Fluogene – automated with PiU	Not suitable for mass screening Time consuming High cost of instrument	High cost of instrument and kits

Source: Prager (2007), Innotrain (2017), McBean, Hyland and Flower, (2014), Progenika Grifols (2014)

The development of red cell genotyping assays progressed rapidly with the release of more sophisticated test methods using high tech instruments to offer more expansive red cell genotyping coverage. Newer methods include the TaqMan OpenArray on the QuantStudio (ThermoFisher), MALDI-TOF multiplex PCR on the MassArray with HemoID panels (Agena Bioscience), sequencing using SNaPshot on the ABI Prism (ThermoFisher) (Keller, 2015).

The Grifols IDCORE^{XT} kit was selected for this study due to the benefits of this kit as listed in Table 2.1 above. An added benefit was that no capital outlay was required for the Luminex 200IS platform as it was already available at SANBS for other applications.

2.4 Applications and Benefits of Red Cell Genotyping

2.4.1 Hospital Transfusion Setting

Genotyping patient's assists in providing antigen negative or matched RBC (red blood cell) products (Keller, 2015). It has been reported that genotyping has the potential to benefit patients requiring blood for clinical conditions such as pregnant women at delivery, trauma patients, cardiac surgery, cancer patients and patients requiring chronic blood transfusions such as SCD and thalassaemia patients (Rujirojindakul and Flegel, 2014; Reid, Rios and Yazdanbakhsh, 2000; Anstee, 2009). In pregnant mothers, Rh genotyping provides vital information on predicting the risk of HDN if the Rh positive mother is pregnant with a Rh negative father. This information also assists clinicians in deciding on the use of Rh immunoglobulin prophylaxis (Finning et al., 2014; Flegel, von Zabern and Wagner, 2009).

2.4.2 Donor Centres

Genotyping of RBC products will assist in providing matched products for special patient populations or antigen-negative products for patients with alloantibodies (Keller, 2015; Progenika Grifols, 2014). However, in South Africa, the blood donor centres are not equipped with staff, skills and instruments to perform red cell genotyping. The blood centres will therefore forward complex serology cases to what is called the Reference laboratory. The Reference laboratory at SANBS is a specialized laboratory that is equipped and staff are skilled to perform specialized tests such as advanced serology techniques or molecular red cell genotyping. However, if emergency blood is required during the period while the Reference laboratory completes testing then the blood donor centres may have to issue least incompatible units for transfusion upon approval from a clinician (SANBS/WPBTS, 2016a).

2.4.3 Reference Laboratory

The benefits of genotyping in the Reference laboratory are multi-fold:

- Genotyping allows the resolution of complex serology cases without the need for numerous specialised tests (Avent et al., 2009).
- Inconclusive serology results due to interference of mixed donor cells or autoantibodies are resolved (Fasano and Chou, 2016; Quist and Koepsell, 2015).
- Panel cell and screen cell donors are genotyped to ensure accurate coverage of antigens in red cell antibody screening and identification reagents (Keller, 2015).
- Mass-scale screening for rare red cell antigens in the absence of expensive commercial reagents or limited resources (Noumsi et al., 2014a).
- Identification of weak antigen expression that serology reagents may miss due to strength of certain monoclonal reagents.
- Identification of variant antigen expression that result in the formation of alloantibodies if antigen-negative blood is not transfused, for example, patients who are e+ with anti-e (Keller, 2015; Moulds, Noumsi and Billingsley, 2015).
- Building a database of red cell genotypes to study the trends and patterns amongst the various ethnic groups in South Africa including predicting or verifying if certain Rh types are related to rare types as this will direct more targeted screening efforts (Storry, 2017).
- Comparison of South African red cell genotypes to genotypes in other countries to determine the uniqueness if any in different populations (Govender, Niekerk and Vather, 2015, Poole, 2006).
- Building a dedicated rare donor red cell genotyping database (which was the aim of this study for South Africa) (Moller et al., 2016, Avent et al., 2009).
- The sourcing of compatible blood amongst rare donors will be made simpler as knowing the frequency distribution of rare alleles worldwide will prevent delays and HTRs will be avoided if antigen negative units are found timeously (Reid, 2000).

2.5 Red Cell Genotyping – a preliminary South African Perspective based on Serology/Phenotyping

In South Africa, earlier studies (1951 – 1985) determined blood group gene frequencies based on different serological test methods (Moores, 1991). Although there has been rapid advancement in test methods, technologies and increased sensitivity and specificity, the baseline frequencies identified by Moores in South Africa will be used as a reference for comparison to the current study data. According to Moores, in the Rh group that was discovered by Landsteiner and Weiner in 1940, the rare e+hrS- is associated with the Rh gene complex R_o (cDe/cDe) and R_2 (cDE/cDE) and the rare e+hr^B- have Rh backgrounds of R_o (cDe/cDe), R_2 (cDE/cDE), r'n (Cde/Cde) and r (cde/cde) (Moores and Smart, 1991). The haplotype frequencies showed that Indian donors had the highest R_1R_1 (Cde), Whites the highest rr (cde), R_2R_2 (cDE), r"r" (cdE) and Black donors the highest R_o (cDe). The C^w phenotype was closely associated with the haplotype DCe in White and Indian donors (Moores, 1991). The K-k+ gene frequency in the Coloured ethic group was 3% compared to K- in which was 97%, while Kp(a+b+) was 0.4% versus Kp^{a-} which was 96.4%. The M antigen was highest in Indians and the N antigen was highest in Whites. The Lu^a phenotype was found in equal percentage in White and Black donors and not identified in Indian donors. The frequency of Fy(a+) phenotype and Fy^a gene was found to be highest in Indian and lowest in Black donors. The Fy(a-b-) was commonly found in the malaria areas of Northern Natal which is populated mainly by the Black race group and hence they have the Fy (a-b-) phenotype. Indians have more Jk^a than Jk^b (Moores, 1991).

The ethnic groups in South Africa are defined as Black, White, Indian, Coloured. However internationally, the ethnicities are termed Caucasian, African American, Asian, Other (Dean, 2005). From serology-based prevalence studies it is evident that South African Whites are similar to Caucasians, and South African Blacks and Coloureds are similar to African Americans and Indians similar to Asians and in some cases Caucasians (Daniels, 2013; Reid, 2018).

2.6 Red Cell Genotyping – Rare Blood Types

2.6.1 Definition of Rare Blood Type

There is no consensus amongst the various countries as to the level of incidence which is used to describe 'rare' blood types. America terms an incidence of 1/1000 (0.1%) as 'rare' and 1/10 000 (0.01%) as 'very rare'. France uses an incidence of 4/1000 (0.4%) and Japan has two categories of rare; Category I rare types occurring in 1/1000 and Category II rare types occurring in 1/100 (Flickinger, 2016; Peyrard, 2016; Tani, 2016b).

There are three instances where the term rare blood type is used in transfusion medicine. The first is a rare blood type that is described as being negative for a high prevalence antigen. By definition a red cell antigen is defined as being high prevalence if it is found in 99 – 99.9% of the population. In the < 1% of the population who lack this antigen, it would be difficult to source antigen-negative compatible blood for transfusion as the majority of the general population is positive for this antigen which, if transfused, will cause an acute or delayed transfusion reaction (Woodfield et al., 2004; Tani, 2016b; Strobel, 2008). The transfusion reaction will be due to the development of clinically significant red cell antibodies to antigens that they lack.

To a lesser extent, rare is also defined by South Africa and many other countries as those low prevalence red cell antigens that are positive (Daniels, 2013). A low prevalence red cell antigen is an antigen that is positive or present in < 1% of the population, therefore sourcing antigen positive compatible blood becomes a challenge as 99% of the population are negative for the antigen (Tilley et al., 2010; Storry, Reid and Yazer, 2010). In South Africa, sourcing compatible blood for patients that are positive for low-frequency antigens is not difficult as targeted screening of units is completed. However, during periods of blood shortages, this screening of units is restricted.

The third group of blood types that are defined by the term rare is where there is a lack of multiple common antigens. Antigens that occur in a high percentage of the population, for example > 10%, are termed common. These common antigens can in some cases be absent or negative in patients. Where a patient is negative for many common antigens simultaneously, then sourcing antigen-negative blood types across the various blood groups may be challenging and is therefore termed rare (Poole, 2006; Woodfield et al., 2004).

2.6.2 Rare Blood and Ethnicity

There have been several possibilities that have been investigated in determining the link between rare blood types and ethnicity in various countries. The theories include the founder effect, spontaneous mutations, genetic selection based on pathogens, gender and impact of migration (Storry, 2017).

The founder effect describes whether there is a genetic inheritance in a particular group of people which has been passed down to several generations of family relatives. Founder effects have been suggested in the Amish people in Northern Sweden who have the 'p' - PP1Pk– phenotype and – Jr(a–) in the Roma people in Slovakia, Japan (Storry, 2017; Anstee, 2010; Benidt et al., 2010). The rare Kx phenotype is a high-frequency antigen from the Kell blood group systems. Spontaneous mutations example the PP1Pk phenotype have been found in Europe (Hellberg et al., 2013). The Fy^{a-b} phenotype found in African populations in areas endemic for malaria is thought to have formed in response to infection caused by the malaria parasite *Plasmodium vivax* and this phenotype confers resistance to these parasites (Roche et al., 2017; Storry, 2017). The Kx mutation occurs due to a mutation on the gene found on the short arm of Chromosome X in males. Anti-Kx causes HTRs (Daniels, Hadley and Soothill, 2002). In South Africa, specifically in the Western Cape, due to the immigration of people from West Africa who have chronic diseases such as SCD and thalassemia, there has been an increase in the requirement for rare blood types (Storry, 2017, Fasano and Chou, 2016).

2.6.3 Serological Rare Blood Types – South Africa

In 2012, there were 20 rare blood types prevalent in South Africa based on serological results (Smart, ISBT 2006, 210-212, Olsen C 2012).

The rare high-frequency negative antigens listed for South Africa are: HrS-, Rh:-34, Js(b-), U-, Kn(a-), Lan-, Lu (b-), Bombay O_h, Jk(a-b-), Yt(a-), Co(a-), k-, Kp(b-), Vel-, Adult I-, Ge-, In(b-) and K_o. The positive for low-frequency antigens are: Henshaw+, In(a+), M_1+, St(a+), Wr(a+), Dantu+, Mi III+, STEM+, Js(a+), V+, VS+ and Kp(a+). The most challenging rare types to find in South Africa are Ge-, Lan-, PP1Pk-, Jk(a-b-) and Lu:-5 (Govender, Niekerk and Vather, 2017). Most of the positive for low-frequency antigens are not actively screened for routinely due to lack of reagents. Genotyping will assist in the ability to screen for more negative for high-frequency and positive for low-frequency antigens. The last class of rare, negative for common multiple antigens in South Africa was not captured on the rare donor file and only screened for when blood was required for patients. This would can now be actively monitored using the rare donor red cell genotyping database developed in the present study.

In 2016-2017, the number of serologically identified rare blood types dropped from 20 to 16 due to the lapse and aging of four previously active rare donors (Govender, Niekerk and Vather, 2017). The number of rare blood types that have been identified serologically was and still is restricted to the supply of bulk and expensive rare antisera for testing and screening. For this reason, historically SANBS only tested specific groups of the donor population for a particular rare type that was associated with that ethnicity. Whether rare types existed in other ethnicities, and in ethnically mixed individuals, and whether a rare type was accompanied by other blood group rare types, have been unknown up until now.

2.6.4 Serological Rare Blood Types – Internationally

There are 27 countries that list their rare blood types on the IRDP maintained by the International Blood Group Reference Laboratory in the United Kingdom. The IRDP originated 50 years ago with nearly 8000 rare donors listed as of 2016 (Thornton, 2016).

The most common rare phenotype listed on the rare donor files in various countries are as follows:

- k- in Belgium, Brazil, Canada, France, Spain, Switzerland and America.
- Fy(a-) in China.
- Fy(b-) in Japan.
- Fy(a-b-) in the Netherlands, France, Canada and Switzerland.
- YT(a-) in Israel, Italy, Canada, Switzerland and America.
- Bombay O_h rare in Singapore and Iran.

Other rare phenotypes listed on donor files internationally are: Lu(b-), Lu(a-b-), Jk(a-b-), r'r', Co(a-), Di(b-), In(Lu), Ko, D- -, Lan-, Vel -, k-, Kp(b-), Jk(a-b-), U-, rare Rh, Js(b-) (Vanhonsebrouck and Najdovski, 2016; Castilho, 2016; Goldman and St Croix, 2016; Peyrard, 2016; Muñiz-Diaz et al., 2016; Hustinx et al., 2016; Woodfield et al., 2004). Table 2.2 lists the difficult to obtain high-frequency-negative-rare blood types per country.

Table 2.2: Rare blood types that are difficult to obtain

Country	Difficult to obtain blood types
Austria	Rare Rh Phenotypes with mixtures of antibodies
Brazil	McLeod, K_0, Lan-, U-, RH:-29, RH:-17
Finland	Vel-, Ge:-2
France	D-U-, hrS-, hrB-, Js(b-), RH:-46, Rhnull, Jr(a-), Co(a-b-)
Germany	Fy(a-b-), In(b-), Ge:-2,-3
China	Di(b-), Fy(a-b-), Jk:-3
Oman, India	D- -, In(b-), Co(a-b-)
Israel	p, Jr(a-), Oh, K_0, U-, Vel-, Lan-
Italy	Sc:-1, LW(a-), K_0, Jk:-3, U-, Di(b-)
Japan	Ge-, En(a-), MkMk, Lan-
Netherlands	D-U-, Fy(a-b-), D-Lu(a-b-), At(a-), Cr(a-)
New Zealand	D- -, K_0, McLeod, p, Ge:-2, Js(b-)
Spain	Yt(a-), Co(a-), Js(b-), Lan-, Ge-, I-, Jr(a-)
Switzerland	Kp(b-), Vel-, Pk, Jk:-3, D- -, K_0, Lan-
United Kingdom	Rhnull, Sc:-1. P1k, Ge:-2,-3, D- -, McLeod, U-
United States	At(a-), En(a-), Hy-, K_0,Cr(a-), Ge:-2, In(b-), Lan-, Di(b-),Ge:-2, Jk:-3, Lu(a-b-), hrS-E-, Gy-, K:-2 Vel-, Wes(b-)

Source: Nance (2009)

The common multiple-antigen-negative sets are as follows (Alcantara, Chay and Ang, 2016):

- R_1R_1, Jk(a-) or Jk(b-) and s- or R_1R_1, Fy(a-) or Fy(b-) and s- or R_2R_2 or rr in Singapore.
- r'r', K-, and r''r'', K- or R_1R_1, k- and R_2R_2, k- and rr, k- in the United Kingdom (Thornton, 2016).
- America shares a similarity with Singapore with a few more common antigens in their list as follows – R_1R_1, K-, Jk(a-) or Jk(b-), Fy(a-) or Fy(b-) and S-s- or R_2R_2, R_oR_o or rr with the same combinations. In addition, R_1R_1, R_2R_2 or rr with K- and Fy(a-b-) (Woodfield et al., 2004).

The SANBS does not list combinations of common multiple-antigen-negative units on their rare donor file. The reason for this is that with approximately 800 000 blood units collected annually, it is not difficult to locate common multiple-antigen-negative units from amongst the 84 blood banks spread throughout the country.

2.7 Rare Donor or Red Cell Genotyping Databases

This section will explain how databases were developed internationally using which particular red cell genotyping method, the clinical and laboratory benefits, and how the databases are maintained. It is evident from the literature that each country has its own unique setup based on their diverse population.

2.7.1 Rare Donor/Red Cell Genotyping Databases – South Africa

No red cell genotyping database currently exists in South Africa at SANBS or the Western Province Blood Transfusion Service (WPBTS), these being the only two blood transfusion institutions who can genotype blood donors and patients requiring blood. Limited red cell genotyping literature originating from South African has been published, the main item being the doctoral thesis of Phyllis Moores predicting genotypes based on serological testing (Moores, 1991). Prior to the introduction of molecular tests in South Africa, many samples were outsourced internationally for confirmations or studies and data was published as part of international publications (Singleton et al., 2000).

In South Africa, there are no published red cell genotyping studies other than two presentations by the researcher (Govender, Vather and Naidoo 2015). The first presentation described the validation and implementation of the IDCORE^{XT} kit for red cell genotyping of 10 blood group systems in a single test at the SANBS. The second presentation described three case studies where red cell genotyping assisted to resolve some serological discrepancies but also drew attention to the limitation of the kit in that the allele coverage is limited to 29 polymorphisms representing 37 antigens.

2.7.2 Rare Donor/Red Cell Genotyping Databases – Africa

Granier et al. (2013) performed red cell genotyping on 347 individuals from sub-Saharan Africa to determine the type and frequency of RHD and RHCE alleles using a Bioarray multiplex PCR assay. It was found that the frequency of partial RH1 (D) and RH5 (e) was less than expected, and the aberrant RHD and RHCE alleles were similar irrespective of location and ethnicity. The study concluded that in the transfusion setting, patients must be tested for partial C and donors for RH54 (DAK) (Granier et al., 2013).

2.7.3 Rare Donor/Red Cell Genotyping Databases – Internationally

Internationally several large databases have been developed. The first big project was the BloodGen Project of 2003 – 2009 published by Avent et al. (2009) that described the genotyping of almost 36 000 donors from various ethnic groups in countries across Europe (Avent et al.,

2009). This collaborative effort across laboratories in Europe led to the development of the BloodChip assay that was developed to address the gap of restricted rare allele coverage in genotyping kits. Another suggestion was to genotype individuals at birth for inborn errors of metabolism accompanied by red cell and HLA genotyping (Avent et al., 2009; Singleton et al., 2000).

Another study by Avent et al, 2009 was about the comprehensive genotyping of blood donors and vulnerable patient groups in the European Union. One valuable suggestion that was concluded was to genotype RhD- blood group units on a mass-scale to remove weak D, partial D, and DEL units that have caused alloimmunisation. This followed the finding that initially D- mothers, when retested by *RHD* genotyping were normal RhD+ individuals who had been mistyped either by serologic or by clerical errors.

Flegel Gottschall, and Denomme (2015) completed a population-based study in 2010 and the database was integrated into the blood supply chain by providing hospitals with online access to a web portal in 2013 in the states of Bethesda and Wisconsin. The outcome was that hospital transfusion services could locate antigen-negative units from their local inventories. These new efficiencies allowed better quality of transfusion support to patients especially when emergency supply of blood was required. Interestingly, serologic screening for antigens was phased out within a year of implementing red cell genotyping, providing a glimpse of where the future of phenotyping by serology lies versus genotyping.

In Los Angeles, America, a study by Noumsi et al. (2014a) described the benefit of high-throughput blood group genotyping in increasing the capacity to identify rare blood donors and building a rare donor database (Noumsi et al., 2014a). The researchers found that genotyping was more effective than routine serology in identifying rare donors (ratio=1.64) and more than 79% of the rare donors would have gone unrecognised with serology. In addition, genotyping was more effective in identifying suitable donors (38 hrB- identified) for alloimmunised patients with complex RH variants (r's patients with "e-like" antibody). Upon identification, rare donors were included in an internal "rare donor database", and a total of 1 109 blood units were collected (Noumsi et al., 2014a).

In a multicentre study in Europe completed by Finning et al. (2016), 258 samples were evaluated with the IDCORE^{XT} assay which highlighted a number of low-frequency antigen variants and uncommon phenotypes such as Co^{a}−, C^{W}+, Di^{b}−, Jo^{a}−, Lu^{b}−, Mi^{a}+ and *RHCE*CeRN* (1 sample); k-, Kp^{a}+, r's, GYPB*S_null(230T), GYPB*deletion, GYPB*S_null (IVS5+5T) and RHCE*ceAR (2-5 samples); Co^{b}+, Di^{a}+, K+, Js^{a}+, hrB-, Lu^{a}+, Yt^{a}-, FY*X and RHCE*ce[733G, 1006T] (6-15 samples); Do^{b}-, Yt^{b}+, s-, V+, VS+, FY*B_GATA and RHCE*ce [733G] (> 15 samples). The European study mimics the findings found by the researcher in the current study on a subset of South African blood donors and this will be described in the Results and Discussion chapters.

2.7.4 Public Rare Donor Databases – Human Gene and Genome Databases

(i) Erythrogene and 1000 Genome Databases

According to Moller et al. (2016), Storry and Olsson et al. developed two databases named the Erythrogene and Thousand (1000) Genomes databases. The 1000 Genomes Project was a collaboration between US, UK, China and Germany where the genomes of over 1 000 unidentified individuals from around the world was sequenced. The goal of the 1000 Genomes Project was to provide a resource of almost all variants, including SNPs and structural variants.

The Erythrogene database was then developed to complete an in-depth analysis of the extensive variation in 36 blood group systems identified by the 1000 Genomes Project. There were 210 412 alleles from 43 blood group-related genes imported from data from 2 504 individuals sequenced. Erythrogene is available as a search engine for blood group genes, displaying the coding sequences and the protein sequences (Moller et al., 2016).

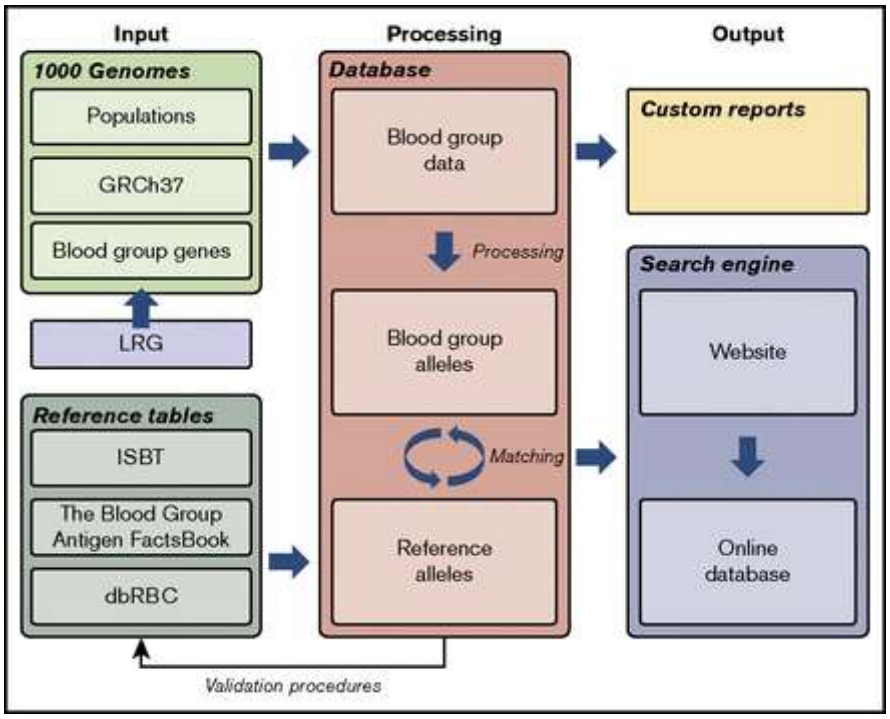


Figure 2.2: **Graphic working model of the Erythrogene project**
Source: Moller et al. (2016)

(ii) Exome Aggregation Consortium database (ExAC)

The ExAC database was developed by investigators who combined the exome sequencing data of 60 706 individuals sequenced as part of population genetic or disease-specific studies. This database describes exon and flanking intron data, blood group gene variants, and the allele frequencies of a variant in a given population (Kobayashi et al., 2017). A new low prevalence Kell antigen was found in the database (Scharberg et al., 2017).

(iii) Blood Group Antigen Gene Mutation Database (BGMUT)

The BGMUT contains the sequence variations from 1 251 alleles of 40 gene loci that together are known to affect the antigens of 30 human blood groups. The BGMUT is a part of the dbRBC resource of the National Center for Biotechnology Information (NCBI). The database is limited as population data is not always provided and there are irregular updates (Patnaik, Helmberg and Blumenfeld, 2014; Patnaik, Helmberg and Blumenfeld, 2012).

(vi) ISBT database

The International Society of Blood Transfusion website provides allele tables, nomenclature, phenotypes, and SNP information, but no population data and no references.

There are many databases developed internationally, most originating from research projects undertaken by Masters and PhD students. The countries lagging in creating similar such databases are South Africa and the rest of Africa. The researcher in this current study aims to start development of this database as a pilot for replication of this study on a larger scale.

The literature and more importantly the gap in the literature for red cell genotyping in South Africa in relation to the rest of the world, particularly developed versus less developed countries, is the justification for this study. Chapter 3 is a description of the materials and methods that were utilised to achieve the objectives of this study based on the aim to develop a rare donor red cell genotyping database.

CHAPTER 3: MATERIALS AND METHODS

This chapter is an overview of the study design, sampling methods, analytical test methods and data analysis that was completed in order to develop a rare donor red cell genotyping database.

The SANBS is the national blood transfusion service in 8 of the 9 provinces in South Africa, and began implementing red cell genotyping as a new test in the reference laboratory assay toolbox in 2014 (Govender, Vather and Naidoo, 2015). Apart from the WPBTS who supply blood to Western Cape, there is no other institution that performs red cell genotyping in the field of blood transfusion in South Africa. Therefore, this research study was designed to explore the red cell genotypes and predicted phenotypes of 10 blood group systems in a subset of blood donors at SANBS who had samples referred to the reference laboratory for testing and screening.

Objective 1 was achieved by transferring the genotyping data onto a Business Intelligence PowerBI IT program to determine the prevalence of red cell genotypes and inferred phenotypes of 10 blood group systems in the study population. This objective was in line with many other countries that have completed similar studies following implementation of red cell genotyping as seen from a study completed amongst South Texas donors (Aranda et al., 2015).

Objective 2 was accomplished by using PowerBI to filter the high prevalence genotypes from the low prevalence genotypes and was used to define frequent red cell genotypes from the rare blood genotypes. The study protocol that was followed is described below.

3.1 Study Design

This was a retrospective, exploratory study that was undertaken at the SANBS Immunohaematology Reference Laboratory from the period January 2015 to August 2016. A non-probability convenience sampling method was used as it allowed the researcher the freedom to use data from donors irrespective of age, race, and ethnicity as the probability of selecting any specific population is unknown in this methodology (Zikmund et al., 2013). Convenience sampling, also referred to as haphazard or accidental sampling, is a sampling procedure used to obtain data that is most conveniently available (Zikmund et al., 2013).

Initially, exploratory research was conducted on those donors that were randomly screened by red cell genotyping. In addition, samples that could not be resolved serologically as well as serologically known rare donors that had donated during the study period were also genotyped. The total sample size was 323 donors.

Ethics approval was obtained by the Durban University of Technology Institutional Research Ethics Committee (IREC) (Appendix 1) and the SANBS HREC and IREC (Appendix 2). Permission to utilise the red cell genotyping data from SANBS was granted prior to commencing the study (Appendix 3). Consent for laboratory testing was obtained at the point of donation with the completion of the donor questionnaire by donors prior to blood donation and collection. There was no bias based on age, gender, race, geographical location or other selection criteria as blood was collected across the eight provinces served by the SANBS.

Bulk data was anonymised by the researcher using unique study numbers with no date of births or other identifiers used, thus maintaining the confidentiality of the donor/patient results. The original raw data and red cell genotyping reports were filed and stored in a locked cupboard and all spreadsheets were password-protected.

3.2 Study Population – Subjects and Sampling

3.2.1 Blood Collection Process

Units of whole blood samples were collected in EDTA anticoagulation blood tubes from voluntary blood donors who presented themselves at blood donor clinics throughout South Africa. At the time of blood donation, potential blood donors completed a blood donor questionnaire that made them eligible to donate blood according to SANBS blood donation acceptance criteria (SANBS, 2016b). The process of collecting blood was by the venepuncture method and was collected by qualified nurses/phlebotomists who followed all precautions to maintain sterility of the process and safety and comfort of the patients as per the Standards of Practice for Blood Transfusion in South Africa (SANBS, 2016b).

All blood units were transported by a strictly controlled cold chain management system to two main Inventory laboratories located at Constantia Kloof in Gauteng and Pinetown in KwaZulu-Natal (SANBS, 2016b). The blood units were received by the inventory laboratories where the units were separated into their components (red cells, white cells, platelets, plasma) and entered on the laboratory information system called Meditech. Based on the information on Meditech, the laboratory staff tagged repeat blood donors with their known ABO and RhD type and any special markers such as “Rare Unit” or for “Reference laboratory”. Units tagged with special markers were stored in a refrigerator in the Inventory laboratory specifically for Reference laboratory where staff collected blood units and any blood samples daily.

As per normal blood collection protocol, the two tubes of blood also drawn during blood collection in addition to the 450ml unit of blood were forwarded to the Donation Testing Department for viral testing of HIV, Hepatitis B, Hepatitis C and Syphillis (SANBS, 2016b). Testing could only resume in other departments once the sample were released as viral and syphilis negative.

Over the 20-month study period of January 2015 – August 2016, a total 323 blood samples were received by the Immunohaematology Reference Laboratory for red cell genotyping. The molecular testing process was followed as per SANBS procedures.

3.3 Molecular Test Procedure

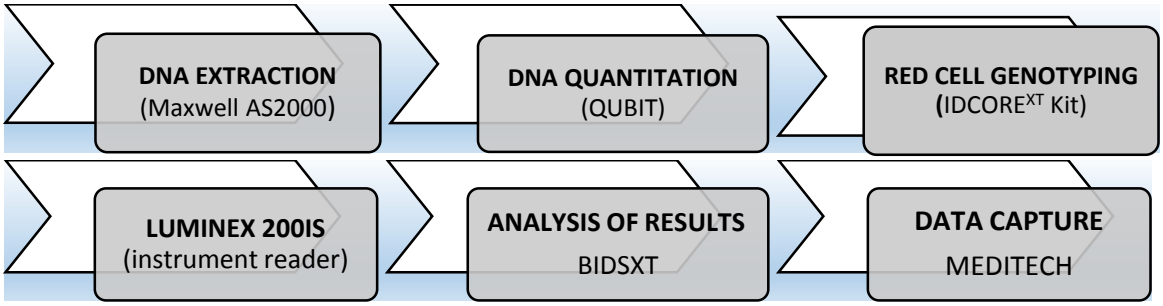


Figure 3.1: Molecular red cell genotyping workflow

As per Figure 3.1, the molecular red cell genotyping procedure followed involved completion of six steps that are described more in detail below.

3.3.1 DNA Extraction

DNA extraction is the process of obtaining DNA from white cells present in blood. DNA was extracted from whole blood samples using the Promega Maxwell 16 Cell SEV (standard elution volume) kit on the Maxwell AS2000 instrument.

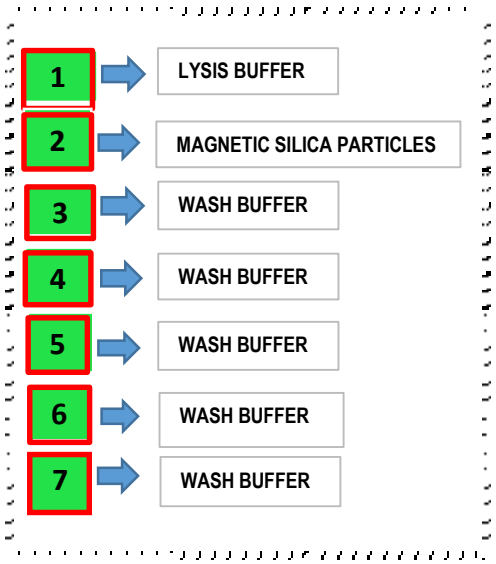


Figure 3.2: Maxwell 16 7-well cartridge
Source: Promega (2012)

As illustrated in Figure 3.2, the Maxwell 16 cartridge contains seven wells with pre-dispensed reagents. To Well 1 of the cartridge, 200ul of whole blood was added, a plunger was inserted in Well 8 and 50ul elution buffer added to each elution tube (Promega, 2012). The extraction steps were then automated on the Maxwell AS2000 instrument. The lysis buffer in Well 1 dissolved the cell components to release the DNA from within the nucleus. The sample was then transferred to Well 2 where the silica bound the DNA and the unbound material was removed by several wash steps from Wells 3 – 7. At the final step, the plunger tip moved the silica-bound DNA to the elution tube where DNA was eluted from the silica magnetic particles then stored at -20ºC (Promega, 2014). The Maxwell Cell DNA SEV Purification kit allowed 48 extractions per kit and the Maxwell AS2000 instrument could extract 16 samples per run.

3.3.2 DNA Quantification

Following DNA extraction, the yield and purity of DNA was measured by a process called DNA quantification on the Nanodrop 1000 spectrophotometer (Thermofisher Scientific, 2007). The Nanodrop uses surface tension to hold the DNA in place between two measurement pedestals (upper and lower pedestal). The Nanodrop was initially calibrated by blanking the spectrophotometer with 1ul of nuclease free water. Thereafter 1ul volume of DNA sample was pipetted on the lower pedestal, the arm was lowered and the sample read. The DNA concentration was measured in ng/ul units and the purity was the ratio of the 260nm/280nm wavelength readings. The sample pedestal was cleaned with lint-free paper between readings to prevent cross contamination between samples (ThermoFisher Scientific, 2007). Only those DNA samples that had the required DNA concentration of between 100 – 400ng and a purity of 1.63 – 2.1 were genotyped (Progenika Grifols, 2014).

3.3.3 Red Cell Genotyping – IDCORE^{XT} Assay

The ID CORE^{XT} is a qualitative kit that produces a simultaneous multiplex PCR reaction for 10 blood group systems in a single test. The kit covers the red cell antigens/alleles from the RHCE, Kell, Kidd, Duffy, MNS, Diego, Dombrock, Colton, Cartwright and Lutheran blood group systems comprising 29 polymorphisms determining 37 red cell antigens (Goldman, Nuria and Castilho, 2015).

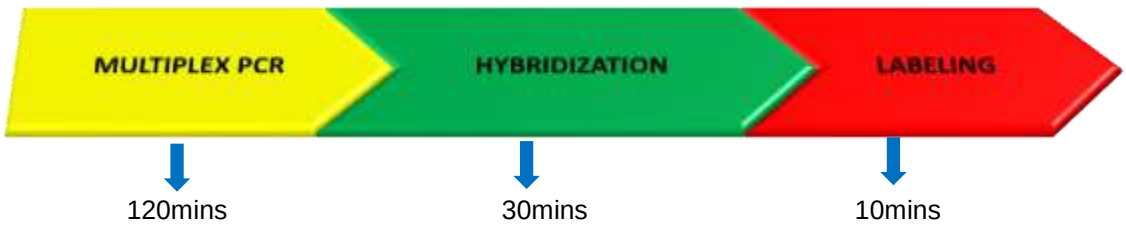


Figure 3.3: IDCORE^{XT} Assay on the Luminex 200IS Workflow
Source: Progenika Grifols (2014)

The completion of the IDCORE^{XT} assay involved the three steps as illustrated in Figure 3.3. Run worklists were created on the BIDSXT software that was used in conjunction with the IDCORE^{XT} kit and the volume of each reagent was automatically calculated based on the number of DNA samples tested (Appendix 5 – BIDSXT Run Worksheets). A negative water control was added to each IDCORE^{XT} run to ensure that there was no PCR contamination. The DNA that was diluted to the required concentration of 100 – 400ng was amplified using the supplied master-mix provided in the IDCORE^{XT} kit. The TAQ enzyme was the catalyst for the multiplex PCR reaction on the Applied Biosystems 9700 thermocycler over a duration of 90 minutes. The PCR products were denatured and hybridised to fluorescent colour-coded oligonucleotide (allele-specific) probes. A list of the SNPs that are coated to the probes and are specific for identification of the genes and inferred antigens are found as per Appendix 4 (IDCORE^{XT} Assay Kit Coverage). The hybridised DNA was then labelled by addition of a fluorescent conjugate at 56°C on the thermocycler for 10 minutes (Goldman, Nuria and Castilho, 2015).

3.3.4 Luminex 200IS

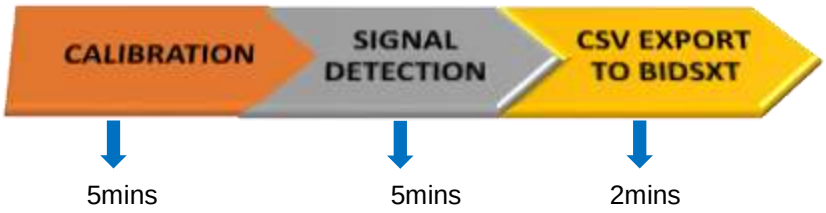


Figure 3.4: Luminex 200IS workflow
Source: Luminexcorp (2013)

The Luminex 200IS instrument is based on Luminex xMAP technology where two lasers detect fluorescent labelled probes as they pass through a flow channel (Luminexcorp, 2013). As per the workflow described in Figure 3.4, the lasers were calibrated with calibration microspheres and then verification microspheres to confirm that the lasers were aligned. Once calibration was successful then the fluorescent signal generated by the IDCORE^{XT} assay was quantitative and detected as median fluorescent intensity (mfi) readings. The mfi readings translated into values on .csv extension files.

3.3.5 Analysis of Results on BIDSXT

Raw data generated from the IDCORE^{XT} runs were processed on Progenika Grifols BIDSXT Analysis Software to obtain a genotype and a predicted phenotype for each red cell antigen covered by the assay. Post each IDCORE^{XT} run, .csv extension files of runs were automatically exported from the Luminex 200IS to the BIDSXT software. The BIDSXT software used a series of built-in algorithms to determine the red cell genotypes and predicted phenotypes per blood group system per sample based on a pattern of probe reactions.

3.3.5.1 Quality Control and Traceability

All testing and analysis of results were performed by personnel trained and competent in the procedure, including the researcher. Each user had individual usernames to login to the software to maintain traceability of the staff performing the various runs. During each run, the BIDSXT software registered the kit reagent and TAQ enzyme lot numbers and expiry dates. Run worksheets were automatically generated with predefined reagent volumes for each step in the procedure (Appendix 5 – Run Worksheets). Further quality control measures included the running of a negative quality control sample which was distilled water per run and the laboratory is also part of an external quality control programme that assesses the ability of staff to consistently produce accurate red cell genotypes. The BIDSXT software also manages the performance of positive and/or negative controls to monitor trends and patterns.

3.3.5.2 Analysis Parameters

Interpretation of the raw data results was completed on BIDSXT by initial assessment of two analysis parameters. Acceptance of valid genotyping results was determined by ensuring that all negative controls had no genotypes; the mfi readings were > 1000 thus ensuring that sufficient initial DNA was present and the beads counted were > 30, thus ensuring that enough probes were detected to make genotype calls (Appendix 6 - BIDSXT Median Fluorescent Intensity |(MFI) Mean and Bead Counts).

The third visual indicator of run acceptance criteria was indicated by colour coded reactions once the .csv files from Luminex were automatically exported to the BIDSXT software. The coded reactions are explained below:

- Valid red cell genotyping results were indicated by a green circle ○
- Inconclusive results were indicated by a yellow triangle △
- Invalid results were indicated by a red cross ✖

As per Appendix 7 (IDCORE^{XT} results on BIDSXT), the predicted phenotypes were expressed by a '+' sign to indicate normal antigen expression and a '0' to show no antigen expression detected. Variable or partial antigen expression where applicable was indicated by a numerical key in parentheses as '+$^{(2)}$' or '+$^{(4)}$. Where a NC (No Call) appeared in a result that passed minimum criteria of acceptance for validity, this was an indicator of an allele that was not detected by the kit and a possible new allele.

The next step in the analysis was the analysis of the red cell genotypes and predicted phenotypes based on the SNPs that were recognised in each sample. The interpretation of the results was completed as follows using the allele RHCE*CeCw as an example in Appendix 4. As per the ISBT nomenclature, the RHCE*CeCw alleles that resulted from the polymorphism RHCE

c122A>G (which is interpreted as, at position 122 on the RHCE gene, there is a nucleotide change from the normal A with a G) were named RHCE*02.08.01. The RHCE*CeCw genotype codes for the CW antigen and the ISBT nomenclature for this phenotype is RH8. Due to the fact that the ISBT nomenclature is still being integrated into standard practice, it is not consistently applied in the laboratory. As per Appendix 4, the 10 blood groups and pattern of reactivity with SNPs determined the red cell genotype and predicted red cell antigens in the study samples.

Analysis of 323 red cell genotyping tests was completed with seven sample test results being excluded due to mfi readings not reaching > 1000. This was due to low DNA yields and the DNA purity falling outside the 1.6 – 1.8 purity range that was probably caused by interfering substances in the samples which were not adequately removed during extraction such as proteins and RNA (Salazar et al., 1998). During testing, the Luminex 200IS instrument experienced software communication problems with the BIDSXT software in a few runs due to network issues at SANBS. Upon resolving the network error, the .csv files generated by Luminex 200IS were imported into an analysis software programme for analysis. Once red cell genotyping results were analysed then confirmed by a second user on BIDSXT, they were referred for data capture on the laboratory information system (Meditech) according to the SANBS laboratory procedure for result entry.

3.3.6 Data Capture/ Result Entry

3.3.6.1 Meditech

Due to the exclusion of the seven invalid results, 316 red cell genotyping results that had been confirmed on BIDSXT were referred for data capture on the laboratory information system, Meditech. As part of the Reference laboratory algorithm of testing, 38 rare genotypes that were identified during testing were confirmed by completing genotyping on a second unit for donors that had donated again during the study period, which also served to verify the reproducibility of the assay. The 38 duplicate results for the donors with a known rare genotype were not captured on Meditech as these were confirmations. The antigens and genotypes per blood group were manually entered on Meditech for 278 samples. A second qualified person including the researcher verified that the results were captured accurately. There were six results that had a few minor data capture errors that were corrected and then re-verified.

3.4 Data Analysis

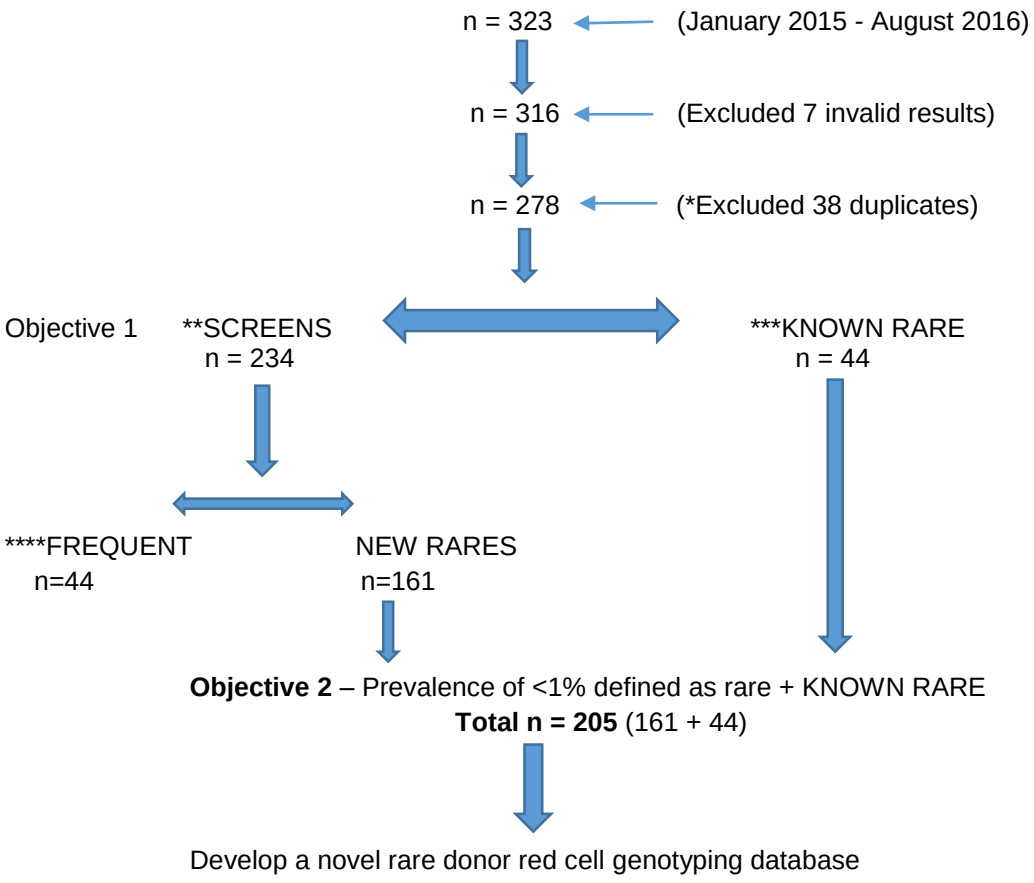
3.4.1 Data Import to the Business Intelligence (BI) programme

For the purposes of this study, a BI IT programme was used to create a database initially of all the red cell genotyping results generated during January 2015 – August 2016. The BI is run by

a PowerBI software programme. A pilot run of 30 samples was imported from Meditech to the BI system. The programme pulled all the information through, although certain filters had to be created to optimise the way the data was formatted. To ensure that no further errors occurred during the importing of results, two pathways were used. The first was that all red cell genotyping results entered on Meditech under the test code RC_GENO was imported into BI by using the filter to recognise RC_GENO only. The second pathway used was directly from BIDSXT where a feature allowed all 278 results to be generated on an excel spreadsheet which was imported to BI. The results of these two pathways were validated by the researcher ensuring that the results imported by both paths correlated 100%.

In addition, the corresponding serology data were available for the samples tested were also imported from the reference laboratory ACCESS program to the same BI database. The reasons for importing the serology data was to obtain the baseline ABO group and Rh type and also to establish whether there were any discrepancies between the molecular and serology methods which was the procedure followed by many similar studies (Noumsi et al., 2014b; Aranda et al., 2015). Prior to detailed data analysis, the patient details, date of birth and laboratory numbers were deleted and samples were allocated study numbers beginning with Sample 1 to Sample 278. This was done in order to ensure that the results in this study were anonymised and could not be traced to blood donors or patients. Bulk data analysis of the 278 samples was completed according to the pathway described in Figure 3.5 below.

3.4.2 Algorithmic Approach to Analysis of Data



* duplicates were confirmation of rare blood types upon a second blood unit donation as per Reference laboratory testing algorithm for the red cell genotyping test

** based on red cell genotyping

*** based on serology

**** FREQUENT is used to describe commonly occurring alleles

Figure 3.5: Algorithmic data analysis breakdown

As explained in 3.3.6.1 above and illustrated in Figure 3.5, seven invalid results and 38 duplicate results were excluded. Of the remaining 278 red cell genotyping results, 44 samples were from serologically KNOWN RARE donors and 234 were samples that were newly screened (SCREENS) by red cell genotyping. Filters were applied to determine the prevalence of red cell genotypes from the 234 samples only on BI. Once this was completed, the data was further filtered to separate the NEW RARE genotypes (n=161) from frequent genotypes (n=44) with the final total of 161 samples that was used to start the new rare donor red cell genotyping database. The serologically KNOWN RAREs and the NEW RAREs was then used to start the rare donor red cell genotyping database.

Initially, a stratified descriptive analysis of the data was completed using pie graphs, bar graphs and tables to describe the ethnicity, ABO group and Rh type from the data of the 278 donors as these are the basic details required prior to blood transfusion. For this study, the sample population distribution was described across the four major ethnic groups of the South African

donor population being Indian, Black, Coloured and White South Africans. There are 4 major ABO groups found in the general population – Group A, Group B, Group O and Group AB and each are either one of two Rh types, Rh positive or Rh negative. Therefore, the study data was described for 8 main types, A-, A+, B-, B+, O-, O+, AB-, AB+.

3.4.2.1 Prevalence of Cell Red Genotypes and Predicted Phenotypes (Objective 1)

Objective 1 was completed by using the BI to first exclude (using filters) the 44 serologically KNOWN RAREs. The prevalence of red cell genotypes and predicted phenotypes was then completed on the remaining 234 red cell genotypes termed SCREENS as per Figure 3.5. The 44 KNOWN RAREs were excluded from the prevalence study as it would have falsely elevated the number of rare blood group genotypes.

The direct counting method as described by the AABB technical manual as quantifying the number of positive reactions versus negative reactions was applied to the data (Brecher, 2008). The BI software programme through a series of filtering data was used to calculated the positive and negative reactions of the antigens and related genotypes as listed below:

- RHCE (C, c, E, e, Cw, V, VS, hrB, hrS)
- Kidd (Jk^a, Jk^b)
- Kell (K, k, Kp^a, Kp^b, Js^a, Js^b)
- Duffy (Fy^a, Fy^b)
- MNS (M, N, S, s)
- Diego (Di^a, Di^b)
- Colton (Co^a, Co^b)
- Cartwright (Yt^a, Yt^b)
- Lutheran (Lu^a, Lu^b)
- Dombrock (Do^a, Do^b)

The prevalence calculations were tabulated and then used to complete Objective 2 of the study which involved the separation of high- from low-prevalence genotypes and predicted phenotypes so that the rare blood types could be defined.

3.4.2.2 Rare Blood Types and the Frequencies as Defined in this Study (Objective 2)

For the purposes of this study, newly identified rare donors were classified into three types of donors. The first rare type comprised donors who showed the absence of genotypes that are positive in > 99% of the study population and are termed ‘negative for high-frequency antigens’ (Peyrard, 2016; Thornton, 2016). Genotypes that were positive in < 1% of the population termed ‘positive for low-frequency antigens’ was the second rare type (Woodfield et al., 2004). The third

rare types were defined as those donors that showed the absence of multiple common antigens (Flickinger, 2016; Peyrard, 2016; Tani, 2016b).

There were 15 samples that were discordant when the red cell genotypes with the predicted phenotypes were compared to the serological phenotypes. These 15 samples were outsourced internationally to be confirmed by a third method based on red cell sequencing technology called the Grifols BloodChip assay (Finning et al. 2016: 160-167).

While the frequency of rare blood types is known internationally, this information is not available at a molecular level in South Africa and this study of a subset of the South African donor population aims to start this rare donor red cell genotyping database comprising 10 blood groups and 37 red cell antigens. Rare blood types in South Africa have been defined at a serological level that was based historically on screening specific ethic groups.

CHAPTER 4: RESULTS

This chapter provides a detailed description of the results obtained in the development of a rare donor red cell genotyping database on a subset of South African blood donors. Although red cell genotyping was implemented more than 20 years ago in developed countries, it only became affordable to provide the genotyping assay on the Luminex 200IS platform at SANBS in 2014 (Govender, Vather and Naidoo, 2015).

Objective 1 involved the determination of the cell antigen prevalence predicted by red cell genotyping, similar to studies completed internationally (Aranda et al., 2015). The prevalence calculations were completed on 234 of 278 donors as the 47 known rare donors were excluded to prevent skewness of prevalence of rare red cell antigens. A filter was applied using the PowerBI software program on BI to exclude the 44 KNOWN RAREs from the 234 SCREENs (refer to METHODS). Objective 2 was completed by separating the frequently occurring genotypes and predicted phenotypes from the rare phenotypes. For the purposes of this study as explained in Chapter 2, the rare genotypes were defined as those red cell genotypes that were present or absent in < 1% of the study population (Tani, 2016b; Flickinger, 2016; Peyrard, 2016). The frequency of the rare genotypes across the ethnic groups in this study was completed and compared to other countries to assess the likelihood of certain countries having higher numbers of a particular rare blood type due to their population diversity (Woodfield et al., 2004; Peyrard, 2016; Thornton, 2016).

4.1 Introduction

At the end of the 20-month study period (January 2015 – August 2016), 323 red cell genotyping tests were completed.

Seven genotyping results were excluded due to the DNA concentration not falling within the 100 – 400ng range and DNA purity not being between 1.63 – 2.1, the ideal being between 1.7 – 1.9 (Thermofisher Scientific, 2007 #33). The insufficient pure DNA resulted in the minimum fluorescent intensity (mfi) being lower than 1000 mfi and caused an invalid genotyping result (Progenika Grifols, 2014).

Of the remaining 316 valid red cell genotyping results, 38 results were a duplicate and, in a few cases, a triplicate result for the same donor. This was due to the reference laboratory algorithm for red cell genotyping where the serologically defined rare donors have to be confirmed twice from two separate blood donations.

Due to the 45 genotyping results being excluded, the remaining total of 278 genotyping results were eligible for inclusion in this study. The 278 red cell genotypes and predicted phenotypes were captured on the laboratory information system (Meditech) and verified by staff trained and found competent to the procedure including the researcher. The program created on the SANBS BI system for this study was used to extract the data for the 278 genotypes and predicted phenotypes from Meditech and details such ethnicity, ABO group and Rh types from the laboratory ACCESS database (described in Chapter 3). The samples were anonymised by the issuing of study numbers of Sample 1 to Sample 278. In addition, the serologically known rare blood donors were tagged as KNOWN RAREs and the remaining samples as SCREENS for the purposes of differentiation in this study.

4.2 Donor Demographics

An overview of the gender, ethnicity, ABO group and Rh type was completed on the study population (n=278) as this is routinely vital information that is always provided prior to blood transfusion and described in similar studies (SANBS, 2016b; Aranda et al., 2015).

4.2.1 Gender

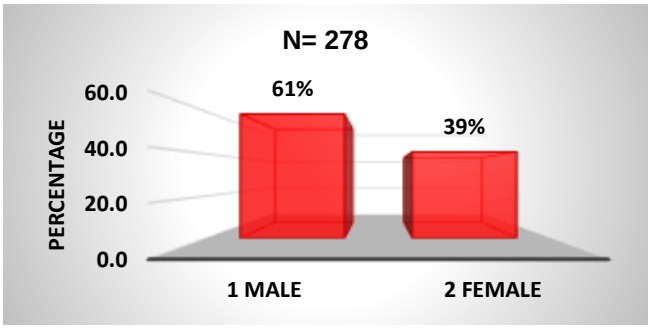


Figure 4.1: Gender distribution of the study population (N=278)

As shown in Figure 4.1, there were more males (61% n=169) than females (39% n=109) in the study.

4.2.2 Ethnicity

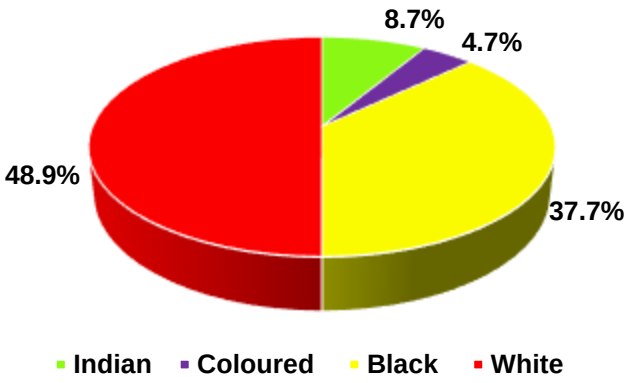
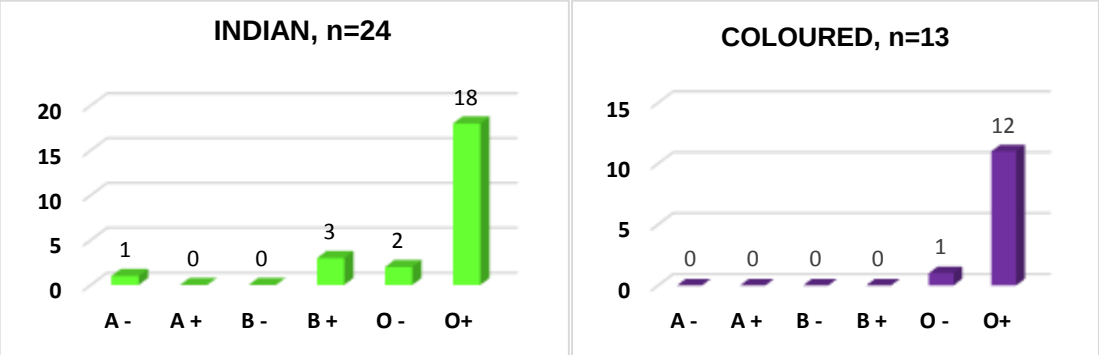


Figure 4.2: Distribution of South African ethnic groups amongst the study population (N=278)

There was a higher percentage of White (49%, n=136) and Black (38%, n=105) South Africans with minor representation of the Indian (9%, n=24) and Coloured (5%, n=13) ethnicities (Figure 4.2).

4.2.3 ABO Group and RhD type

Each blood donor had an ABO and RhD type completed by serological phenotyping prior to transfusion of the blood units. The RhD blood type is defined as either negative (-) or positive (+) per ABO group (Daniels, 2013). The ABO group comprised Group A, Group B and Group O (Daniels, 2013). The fourth blood group, Group AB is missing as there were no Group AB donors that were forwarded for red cell genotyping during the study period.



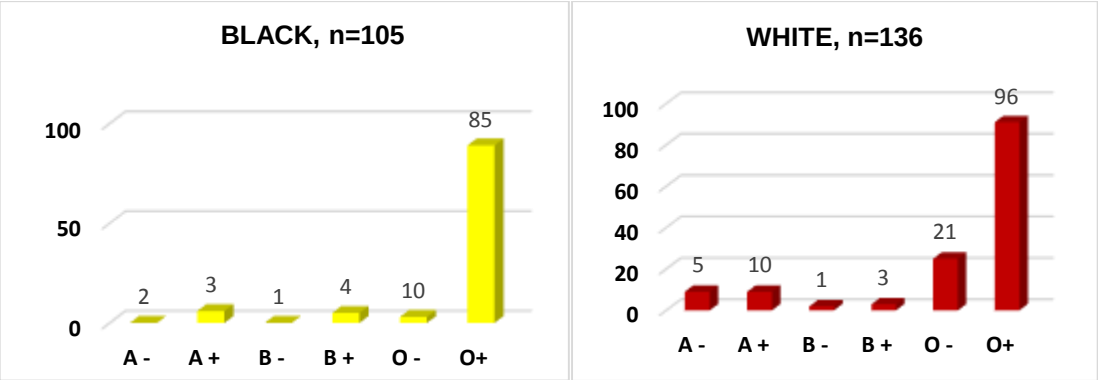


Figure 4.3: ABO/RhD blood groups

Figure 4.3 shows the ABO group and RhD type for donors across the four major ethnic groups of South Africa. Of the 278 donors, 76% (n=211) were Group O+ and 12% (n=34) were Group O-. The remaining 12% comprised Group A+, A-, B+ and B-.

4.3 Exclusion of KNOWN RAREs

Prior to inititating prevalence studies, 44 serologically defined rare donors had donated blood as part of the South African Rare Donor Program. As per the reference laboratory algorithm of testing, all rare donors must be genotyped and therefore were referred for red cell genotyping during the study period. The results of the 44 donors were excluded from the prevalence studies as these would have erroneously inflated the revalence of rare red cell genotypes and predicted phenotypes in the general donor population. The list of rare red cell antigens found in the 44 rare donors are illustrated in Figure 4.4 (Govender, Niekerk and Vather, 2017, Woodfield et al., 2004).

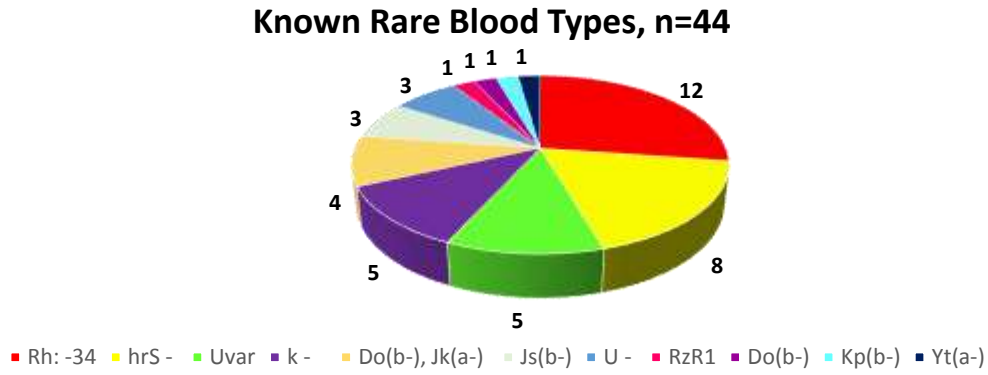


Figure 4.4: Serologically known rare antigen/s

As shown in Figure 4.4, 12 rare red cell antigens were found amongst the 44 rare donors with the most common rare types being the Rh: -34 (n=12) and hrS- (n=8).

4.4 Prevalence of Red Cell Genotypes and Predicted Phenotypes Based on the IDCORE^{XT} Assay (Study Objective 1)

Once the 44 serologically known rare donors were excluded, the prevalence study was based on 234 red cell genotyping results and the distribution amongst the ethnic groups was 53% (n=124) White, 36% (n=84) Black, 9% (n=21) Indian and 2% (n=5) Coloured. The prevalence of red cell genotypes and predicted phenotypes of the 10 blood groups RHCE, Kell, Kidd, Duffy, MNS, Diego, Dombrock, Colton, Cartwright and Lutheran covered by the IDCORE^{XT} assay was calculated (Lopez et al., 2018). The high and low prevalence of the various red cell genotypes and predicted phenotypes per blood group system are reported on below.

4.4.1 Rh Blood Group

The IDCORE^{XT} assay does not cover the RHD gene only the RHCE gene of the Rh blood group system. However, the RhD type is the most critical information required in all established blood transfusion protocols due to links between RhD/RHCE antigens and rare red blood types as well as ethnicity that has been published widely in the literature (Pham et al., 2009; Rahim et al., 2008; Reid et al., 2012). Therefore, the combination of RhD serology results in combination with the RHCE predicted phenotypes will be described first followed by red cell genotyping results of the RHCE gene.

4.4.1.1 RhD and RHCE Predicted Phenotypes

The RhD type and RHCE was reported as per the routine protocols at SANBS using the Fisher, Wiener and Race nomenclature as shown in Figure 4.5 (Daniels, 2013; Reid, 2018; Dean, 2005). The distribution of the Rh subtypes is also shown in Figure 4.5.

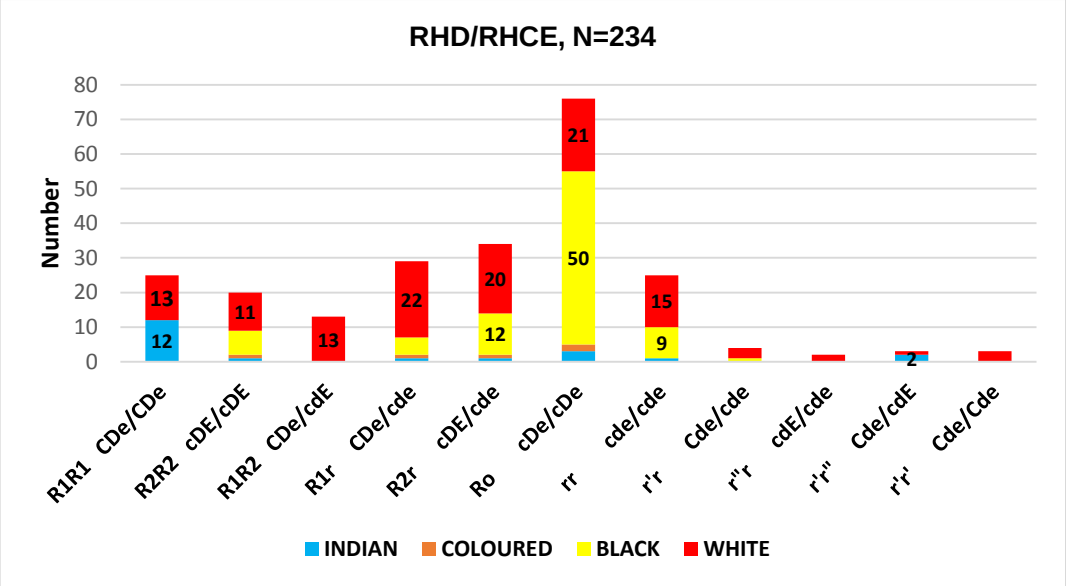


Figure 4.5: Description of the Rh blood group system amongst the 4 major ethnic groups

The predominant phenotype R_o cDe/cDe was found in 60% (50/84) of Black donors. The most prevalent phenotyes amongst the Whites was 18% (22/124) with R_1r CDe/cde in, 17%, (21/124) with R_o cDe/cDe and 16% (20/124) with R_2r cDE/cde. The Rh type R_1R_1 CDe/CDe was predominant in 57% (12/21) of Indian donors (Figure 4.5). The 5 Coloured donors were too small a sample number to note any defining conclusions on the most prevalent Rh phenotypes.

4.4.1.2 Two-allele Frequency of RhD/RHCE Phenotypes

The RHCE alleles can be inherited as RHCE*Ce, *cE, *ce or the very rare *CE and these may be in combinations of being homozygous (*ce/*ce) or heterozygous (*Ce/ce) (Daniels, 2013). The 2-allele frequency calculation using the direct dounting method was applied to the data from the 234 genotyping results (Brecher, 2008) as shown in Table 4.1. The RHCE*ce alleles were most prevalent in 60% of Coloured, 81% of Black and 48% of White donors. The homozygous RHCE*Ce allele was most common in 64% of Indian donors and in 29% of White donors. The relevance of the predominant RHCE alleles per ethnic group serving as a trigger to a potential rare blood type is explained in Chapter 5.

Table 4.1: RHCE phenotype frequency using the direct counting method (AABB Technical Manual)

	INDIAN (n=21)	**COLOURED** (n=5)	**BLACK** (n=84)	**WHITE** (n=124)
RHCE*Ce	27 (64%)	1 (10%)	6 (4%)	71 (29%)
RHCE*cE	5 (12%)	3 (30%)	26 (15%)	58 (23%)
RHCE*ce	10 (24%)	6 (60%)	136 (81%)	119 (48%)
2 allele	n=42	n=10	n=168	n=248

Methodology source: Brecher (2008)

4.4.1.3 RHCE IDCORE^{XT} genotypes

The RhD and RHCE genes are > 90% homologous and highly polymorphic which results in many variants of the RHCE genes as well as the formation of RhD/RHCE hybrids. The RHCE genetic variability is evident from the 28 different genotypes identified by the IDCORE^{XT} assay as shown in Table 4.2. The most common RHCE genotype in Black and White donors was the RHCE*ce (homozygous), and the most common in the Indian donors was the RHCE*Ce. This was consistent with the findings of the direct counting method presented in in 4.4.1.2. Five RHCE genotypes were found in equal proportion amongst the Coloured donors but is too small a sample number to draw definitive conclusions. The most genetic variability was found amongst the Black donors as is evident from the genotypic coverage on Table 4.2. Although there were no genotypes that were absent in < 1% of the population (negative high-frequency antigen) amongst the Black donors due to n=84 donors only, the researcher identified the rare genotype RHCE*ceAR, RHCE*ce[712G] in Table 4.2. In addition, 6% of the Black donors showed the formation of RHD/RHCE hybrids. The hybrids were shown by the r's haplotype RHD*r's-

RHCE*ce[733G,1006T]. There was a lesser degree of RHCE genetic variability amongst the White donors with 92% of the donors displaying the normal RHCE antigen profiles. There were however 2 donors who were positive for the rare low-frequency *CeCW antigen found in < 1% of the study population.

Table 4.2: Prevalence of RHCE IDCORE^{XT} red cell genotypes

	Indian n=21	***Coloured n=5***	***Black n=84***	***White n=124***	***TOTAL n=234***
*RHCE*ce*	2 (10%)	1 (20%)	21 (25%)	32 (26%)	56 (23.9%)
*RHCE*Ce*	12 (57%)			17 (14%)	29 (12.4%)
*RHCE*ce, RHCE*cE*	1 (5%)	1 (20%)	6 (7%)	19 (15%)	27 (11.5%)
*RHCE*ce, RHCE*Ce*	1 (5%)	1 (20%)	3 (4%)	21 (17%)	26 (11.1%)
*RHCE*cE*	1 (5%)	1 (20%)	7 (8%)	11 (9%)	20 (8.5%)
*RHCE*Ce, RHCE*cE*	2 (10%)			14 (11%)	16 (6.8%)
*RHCE*ce, RHCE*ce[733G]*			13 (15%)	2 (1.6%)	15 (6.4%)
*RHCE*ce[733G,1006T]*	1 (5%)		6 (7%)	1 (0.8%)	8 (3.4%)
*RHCE*ce, RHCE*ce[733G,1006T]*			5 (6%)		5 (2.1%)
*RHCE*ce[733G], RHCE*ce[733G,1006T]*	1 (5%)		3 (4%)	1 (0.8%)	5 (2.1%)
*RHCE*cE, RHD*r's-RHCE*ce[733G,1006T]*			2 (2%)	1 (0.8%)	3 (1.3%)
*RHCE*ce, RHD*r's-RHCE*ce[733G,1006T]*			3 (4%)		3 (1.3%)
*RHCE*ce[733G]*		1 (20%)	2 (2%)		3 (1.3%)
*RHCE*Ce, RHCE*ce[733G]*			1 (1%)	1 (0.8%)	2 (0.9%)
*RHCE*ceAR, RHCE*cE*			2 (2%)		2 (0.9%)
*RHCE*cE, RHCE*ce[712G]*			2 (2%)		2 (0.9%)
*RHCE*Ce, RHCE*ce[712G]*			1 (1%)		1 (0.4%)
*RHCE*Ce, RHCE*ce[733G,1006T]*				1 (0.8%)	1 (0.4%)
*RHCE*cE, RHCE*ce[733G,1006T]*				1 (0.8%)	1 (0.4%)
*RHCE*ce, RHCE*ceAR*			1 (1%)		1 (0.4%)
*RHCE*cE, RHCE*ce[733G]*			1 (1%)		1 (0.4%)
*RHCE*Ce, RHCE*CeCW(CW+)*				1 (0.8%)	1 (0.4%)
*RHCE*ce, RHCE*CeCW (CW+)*				1 (0.8%)	1 (0.4%)
*RHCE*ce[712G]*			1 (1%)		1 (0.4%)
*RHCE*ce[733G], RHD*r's-RHCE*ce[733G,1006T]*			1 (1%)		1 (0.4%)
*RHCE*ceAR, RHCE*ce[712G](E-, hrS-)*			**1 (1%)**		1 (0.4%)
*RHCE*ceAR, RHCE*ce[733G,1006T]*			1 (1%)		1 (0.4%)
*RHCE*ceAR, RHCE*ce[733G]*			1 (1%)		1 (0.4%)

4.4.2 Kell IDCORE^{XT} genotypes and predicted phenotypes

The Kell blood group system comprises six main red cell antigens K, k, Kp^a, Kp^b, Js^a and Js^b (Daniels, 2013). As presented in Table 4.3, the most frequent red cell genotype detected in 88.5% of the study population was the KEL*k_KPB_JSB with predicted phenotype k+ Kpb+ Jsb+ found in 95% (20/21) of Indian donors, 100% (5/5) Coloured donors, 89% (74/84) Black donors and 87% (108/124) White donors.

In contrast samples that showed the presence of the k-, Kpb- and Jsb- antigens were described as containing negative high-frequency- antigens with a prevalence of 0.4% homozygous

RHCE*K_KPB_JSB (k-), 0.9% KEL*k_KPA_JSB (homozygous Kp^{b-}) and 1.7% homozygous KEL*k_KPB_JSA (Js^{b-}).

The KEL*K genotype or k- phenotype and KEL*KPA or Kp^{b-} were specific to the White ethnic group while the KEL*JSA genotype or Js^{b-} phenotype was found in the Black donors and an Indian donor. One White donor had a Js(a-) phenotype. In addition, there were three NC results found in 4% (n=3) of Black donors which indicates a possible new or novel allele that was not part of the allele coverage of the IDCORE^{XT} kit.

Table 4.3: Prevalence of Kell IDCORE^{XT} red cell genotypes and predicted phenotypes

KELL Blood Group	***K***	***k***	***Kp^a***	***Kp^b***	***Js^a***	***Js^b***	***N=234***	***%***
*KEL*k_KPB_JSB*	0	+	0	+	0	+	207	88.5% (95%I,100%C, 88%B, 87%W)
*KEL*K_KPB_JSB, KEL*k_KPB_JSB*	+	+	0	+	0	+	12	5.1% (9.8%W)
*KEL*k_KPB_JSA(Js^{b-})*	0	+	0	+	+	0	4	1.7 (5%, 3.6%B)
*KEL*k_KPB_JSB, KEL*k_KPB_JSA*	0	+	0	+	+	+	4	1.7 (4.8%B)
*KEL*k_KPA_JSB(Kp^{b-})*	0	+*(2)	+	0	0	+ *(2)	2	0.9 (1.6%W)
*KEL*K_KPB_JSB(k-)*	+	0	0	+	0	+	1	0.4 (0.8%W)
*KEL*k_KPB_JSB, KEL*k_KPA_JSB (Js^{a-})*	0	+	+	+	0	+	1	0.4 (0.8%W)

Key: W – White, B – Black, I – Indian, C – Coloured. *(2) – partial expression as defined by the IDCORE^{XT} assay.
Note: 3 x No call (NC) results in 3 (3.6%) Black donors (results in bold)

4.4.3 Kidd IDCORE^{XT} Genotypes and Predicted Phenotypes

The Kidd blood group system has two antigens, Jk^a and Jk^b encoded by the JK*A and JK*B genes respectively (Daniels, 2013). As per Table 4.4, the percentage distribution of Kidd genotypes was 45% homozygous JKA (Jk^{a+b-}) and 42% heterozygous JK*A, JK*B (Jk^{a+b+}). The JK*B (Jk^{a-b+}) was found in only 1 Indian, 1 Coloured and 4 Black donors but in 19% (n=23) of White donors. There was one rare JK*A, JK*B_null (IVS5-1a) genotype found in a White donor, but the clinical significance is unknown. The rare Jk(a-b-) (JK3) was not found in this study.

Table 4.4: Prevalence of IDCORE^{XT} Kidd system red cell genotypes

KIDD BLOOD GROUP		**INDIAN** n=21	**COLOURED** n=5	**BLACK** n=84	**WHITE** n=124	**TOTAL** N=234
JK*A	*Jk(a+b-)*	10 (48%)	1 (20%)	51 (61%)	44 (35%)	106 (45.3%)
JK*A, JK*B	*Jk(a+b+)*	10 (48%)	3 (60%)	29 (35%)	56 (45%)	98 (41.9%)
JK*B	*Jk (a-b+)*	1 (4%)	1 (20%)	4 (4%)	23 (19%)	29 (12.4%)
JK*A, JK*B_NULL(IVS5-1A) Jk(a+b-)		0	0	0	1 (1%)	1 (0.4%)

4.4.4 Duffy IDCORE^{XT} Genotypes and Predicted Phenotypes

The Duffy blood group system is comprised of the Fy^{a} and Fy^{b} antigens (Daniels, 2013). As per Table 4.5, the two common Duffy blood group genotypes were FY*A, FY*B and FY*B_GATA in 56% of the donors followed by FY*B and FY*B in 30% of the donors. Heterozygous FY*A. FY*B was found in majority of the White and Indian donors. A polymorphism found in the promoter region of the FY*B gene resulted in the FY*B_GATA genotype that was prevalent in 28% of donors, with the majority being amongst the Black donors. The FY*B_GATA has the Fy^{a-b-} predicted phenotype however is not the same as the rare Fy^{a-b-} and this differentiation will be explained in Chapter 5. The FY*B[265T]_FY*X genotype was found at a very low prevalence (0.9% and 0.4%).

Table 4.5: Prevalence of IDCORE^{XT} Duffy system red cell genotypes

		INDIAN n=21	COLOURED n=5	BLACK n=84	WHITE n=124	N=234 (%)
FY*A, FY*B	*Fy (a+b+)*	11 (52%)	2 (40%)	4 (5%)	50 (40%)	67 (28.6%)
FY*B_GATA	*Fy (a-b-)*	1 (5%)	2 (40%)	56 (67%)	6 (4.8%)	65 (27.8%)
FY*B	*Fy (a-b+)*	3 (14%)	-	4 (5%)	34 (27%)	41 (17.5%)
FY*A	*Fy (a+b-)*	6 (29%)	-	1 (1%)	23 (19%)	30 (12.8%)
FY*B, FY*B_GATA	*Fy (a-b+)*	-	1 (10%)	17 (20%)	7 (6%)	25 (10.7%)
FY*A, FY*B_GATA(1)	*Fy(a+b-)*	-	-	2 (2%)	1 (0.8%)	3 (1.3%)
FY*A, FY*B[265T]_FY*X	*Fy(a+*b$^{+(2)}$)*	-	-	-	2 (1.6%)	2 (0.9%)
FY*B, FY*B[265T]_FY*X	*Fy(a-b+)*	-	-	-	1 (0.8%)	1 (0.4%)

*b$^{+(2)}$ – the (2) indicates partial expression of the Fy^{b} in the IDCORE^{XT} assay

4.4.5 MNS IDCORE^{XT} Genotypes and Predicted Phenotypes

The IDCORE^{XT} assay covers six antigens (M, N, S, s, U, Mia) of the MNS blood group system encoded by the GYPA and GYPB gene. The GYPA genotypes encoding the M and N antigens are reported separately from the GYPB genotypes encoding the S, s, U, Mia (Daniels, 2013; Daniels, 2005) by IDCORE^{XT}. As per Table 4.6, the heterozygous genotype GYPA*M, GYPA*N (M+, N+) is the most prevalent genotype found in 52% of the donors at almost equal proportion amongst the four ethnic groups. The homozygous GYPA*M genotype was found in 32% of donors and the GYPA*N in 16% of donors with the only striking result being that the M- was found in only one Indian donor.

In Table 4.7, the homozygous GYPB*s genotype and heterozygous GYPB*S, GYPB*s genotype was found in 55% and 29% of donors respectively and was found in majority of the donors in the various ethnic groups - 57% Indian, 60% Coloured, 57% Black and 54% White South Africans.

The homozygous GYPB*S was found in 10% of donors. The GYPB*deletion and GYPB*S_null (IVS5+5T) and GYPB*S_null 230T and was found in < 1% and 1.4% of the donors respectively. There were five donors who had displayed a NC result in the MNS blood group system only and

these were outsourced for further molecular characterisation by red cell sequencing using an assay called the BloodChip (McBean, Hyland and Flower, 2014).

Table 4.6: Prevalence of IDCORE^{XT} MN red cell genotypes

	INDIAN n=21	**COLOURED** n=5	**BLACK** n=84	**WHITE** n=124	**N=234 (%)**
GYPA*M, GYPA*N (M+, N+)	11 (52%)	2 (40%)	39 (46%)	69 (56%)	121 (52%)
GYPA*M (M+, N-)	9 (43%)	1 (20%)	28 (33%)	37 (30%)	75 (32%)
GYPA*N (M-, N+)	1 (5%)	2 (40%)	16 (19%)	18 (14%)	38 (16%)

Table 4.7: Prevalence of IDCORE^{XT} Ss red cell genotypes

	INDIAN n=21	**COLOURED** n=5	**BLACK** n=84	**WHITE** n=124	N=234
GYPB*s	12 (57%)	3 (60%)	48 (57%)	67 (54%)	130 (55%)
GYPB*S, GYPB*s	5 (24%)	1 (20%)	23 (27%)	39 (31%)	68 (29%)
GYPB*S	4 (19%)	1 (20%)	6 (7.4%)	13 (10%)	24 (10%)
GYPB*s, GYPB*S_null (230T) (U^{var})	-	-	2 (2.4%)	1 (1%)	3 (1.4%)
GYPB*Deletion (S-s-U-)	-	-	1 (1.2%)	1 (1%)	2 (0.9%)
GYPB*S_Null(IVS5+5T) (U^{var})	-	-	1 (1.2%)	1 (1%)	2 (0.9%)

Note: 5 (1.9%) No call results were found in 3 (3.6%) Black and 2 (2%) White

4.4.6 Diego, Dombrock, Colton, Cartwright and Lutheran IDCORE^{XT} genotypes and predicted phenotypes

The Diego, Dombrock, Colton, Cartwright and Lutheran blood groups are the remaining five blood groups that are covered by the IDCORE^{XT} assay. The prevalence of red cell genotypes is shown in Table 4.8 below.

The Diego blood group system is comprised of the DI*A and DI*B genotypes. As per table 4.8, only DI*B genotype was present in all ethnic groups.

The Dombrock blood group system antigens are comprised of combinations of the DO*A, DO*B, DO*HY and DO*JO. As per Table 4.8, the homozygous DO*B and heterozygous DO*A, DO*B are the most prevalent Dombrock antigens found in 47% and 33% of the population respectively. The remaining Dombrock antigens are found in < 10% of the study population. The homozygous and heterozygous DO*A_JO genotype was found in < 1% of total study population and only in the Black and White donors. There was slightly more variability of the Dombrock antigens amongst the Black ethnic group.

As per Table 4.8, the most prevalent Colton red cell genotype was CO*A (96%) across the four ethnicities. The heterozygous CO*A, CO*B was found in 4% of the total donors made up of 8% of White donors only.

In the Cartwright blood group system, the YT*A (96%) was most prevalent across the four ethnicities and the remaining 4% was the heterozygous YT*A, YT*B genotype that was found in the Indian and White ethnic groups.

In the Lutheran system, the LU*B (94%) genotype was the most prevalent red cell type genotype with small numbers of heterozygous LU*A, LU*B found in the Coloured, Black and White ethnic groups.

Table 4.8: Prevalence of the IDCORE^{XT} Diego, Dombrock, Colton, Cartwright and Lutheran red cell genotypes

	INDIAN (n=21)	COLOURED (n=5)	BLACK (n=84)	WHITE (n=124)	Prevalence (n=234)
Diego ***DI*B***	21 (100%)	5 (100%)	84 (100%)	124 (100%)	100.00%
Dombrock ***DO*B***	12 (57%)	4 (80%)	46 (55%)	48 (38.7%)	110 (47%)
DO*A, DO*B	5 (24%)	-	18 (21.2%)	54 (43.7%)	77 (33%)
DO*A	4 (19%)	1 (20%)	2 (2.4%)	20 (16%)	27 (12%)
DO*B, DO*A_JO	-	-	7 (8.3%)	1 (0.8%)	8 (3.0%)
DO*B, DO*B_HY	-	-	6 (7.1%)	-	6 (2.4%)
DO*A, DO*B_HY	-	-	2 (2.4%)	-	2 (0.9%)
DO*A_JO	-	-	1 (1.2%)	1 (0.8%)	2 (0.9%)
DO*A, DO*A_JO	-	-	1 (1.2%)	-	1 (0.4%)
DO*B_HY	-	-	1 (1.2%)	-	1 (0.4%)
Colton ***CO*A***	21 (100%)	5 (100%)	84 (100%)	117 (92%)	225 (96%)
CO*A, CO*B	-	-	-	10 (8%)	10 (4%)
Cartwright ***YT*A***	18 (85%)	5 (100%)	87 (100%)	112 (95%)	225 (96%)
YT*A, YT*B	3 (15%)	-	-	7 (5%)	10 (4%)
Lutheran ***LU*B***	20 (100%)	5 (83%)	82 (94%)	111 (94%)	94.37%
LU*A, LU*B	-	1 (17%)	5 (6%)	7 (6%)	5.63%

Based on the prevalence data obtained in Objective 1, Objective 2 sought to determine the 2-allele/antigen prevalence and the frequency of rare genes using the direct counting method and the BI system.

4.5 Frequency of Rare Genotypes and Predicted Antigens

Using the direct counting method and the BI IT program, the researcher applied filters to the prevalence data of the 234 donors that were included in the prevalence studies (Brecher, 2008).

The first filter applied was the search for negative high-frequency antigens including those listed on the IRDP of other countries. The second filter was to search for the positive low-frequency antigens. The third filter was to search the combinations of common multiple-antigen-negative units. The outcome of this process was that 44 donors were termed FREQUENT and 161 donors were termed NEW RAREs (see Figure 3.5, Chapter 3). The high number of new rare blood types

being identified during screening of donors was due to the extensive coverage of the IDCORE^{XT} assays across 10 blood groups system and 37 red cell antigens (Noumsi et al., 2014a).

4.6 Development of the Novel Rare Donor Red Cell Genotyping Database

The novel rare donor red cell genotyping database was developed on the BI system by importing the red cell genotypes and predicted phenotypes of the 161 NEW RAREs and the 44 serologically KNOWN RAREs.

4.6.1 Gender, ABO Group and Rh Type of the Rare Donors on the Rare Donor Database

As was completed on the initial study, the Gender, ABO group and Rh type for N=205 donors were described amongst the four main ethnic groups, as seen in in Table 4.9.

Table 4.9: Gender, ABO and Rh Type of Rare Donors on the rare donor database

		INDIAN	*COLOURED*	*BLACK*	*WHITE*	*N=205 (%)*
Gender						
	Male	10	1	40	73	124 (60%)
	Female	5	3	36	37	81 (40%)
ABO Group						
	Group O+	10	4	59	77	150 (74%)
	Group O-	1	0	7	16	24 (11.5%)
	Group A+	-	-	3	10	13 (6%)
	Group A-	1	-	2	5	8 (4%)
	Group B+	3	-	4	2	9 (4%)
	Group B-	-	-	-	1	1 (0.5%)
Rh group	R_o	2	2	41	18	63 (31%)
	R_1r	1	1	7	18	27 (13%)
	R_1R_1	10	-	-	13	23 (11%)
	rr	1	0	8	13	22 (11%)
	R_2R_2	-	1	7	10	18 (9%)
	R_2r	-	-	-	18	18 (9%)
	R_1R_2	1	-	-	12	13 (6%)
	r'r	-	-	2	6	8 (4%)
	r'r'	-	-	-	3	3 (1.5%)
	r'r''	1	-	-	1	2 (1%)
	r''r	-	-	-	1	1 (0.5%)
	R_zR_1	-	1	-	-	1 (0.5%)

The rare donor database comprised more males (60%) than females (40%) (Table 4.9). As expected from the initial studies the Group O, RHD + was the most common in 74% of the population. The Ro phenotype was present in 31% of donors and was most common amongst Black donors. With the exception of R_1R_1 found in the majority of Indian (n=10) donors, the remaining Rh positives phenotypes (R_o, R_1r, R_1R_1, R_2R_2, R_1R_2) were distributed amongst 48% of

the White donors. The two lowest frequency alleles are r"r (cdE/cde) and R_zR_1 (CDE/CDe) which were found in 0.5% (n=1) Black and 1 Coloured donor respectively.

4.6.2 The Frequency of Rare Genotypes and Predicted Phenotypes Found on the Rare Donor Database

The established frequency of the rare genotypes and predicted phenotypes of the 20 different rare types as compared to rare blood types found on the IRDP and on the South African Rare Donor panel is tabulated in Table 4.10.

Table 4.10: Gene Frequencies of the negative high-frequency antigens in the new rare donor red cell genotyping database and rare Rh subtypes

	PHENOTYPE	GENOTYPE	ETHNICITY	FREQUENCY
Rh	cDe/cDe R_0	RHCE*ce, RHCE*ce	2I, 2C, 41B, 18W	31% (n=63)
	E-HrB -	RHCE*ce[733G,1006T], RHD*r's-RHCE*ce[733G,1006T](30)	7B	3% (n=7)
	Cde/Cde r'r'	RHCE*Ce, RHCE*Ce	3 W	2.5% (n=5)
	E-HrS-	RHCE*ceAR, RHCE*ce[712G] or RHCE*ceAR	3B, 1C	2% (n=4)
	Cde/cdE r'r''	RHCE*Ce, RHCE*cE	1 I, 1W	1% (n=2)
	cdE/cde r''r	RHCE*cE, RHCE*ce[712G](15)	1W	0.5% (n=1)
	CDE/CDeRzR1	RHCE*CE, RHCE*Ce	1C	0.5% (n=1)
	CDE/CDE RzRz	-	-	-
	cdE/cdE r''r''	-	-	-
	D--	-	-	-
	Rhnull	-	-	-
Kell	k-	KEL*K_KPB_JSB #	4W	2% (n=4)
	Js(b-)	KEL*k_KPB_JSA	3B, 1I	2% (n=4)
	Kp(b-)	KEL*k_KPA_JSB	2W	1% (n=2)
	Ko	-	-	-
	Kp(a-b-)	-	-	-
Kidd	Jk(a-b-)	-	-	-
	Jk(a+b-)	JK*A, JK*B_null(IVS5-1a)	1W	0.5% (n=1)
Duffy	Fy(a+b-)	FY*A	5I, 1B, 22W	14% (n=28)
	Fy(a-b-)	-	-	-
MNS	s-	GYPB*S	3I, 6B, 12W	10% (n=21)
	S-s-U-	GYPB*deletion	1W, 1B	1% (n=2)
Diego	Di(a+b-)	-	-	-
Dombrock	Joa-	DO*A_JO	1B, 1W	1% (n=2)
	Hy-	DO*B_HY	1W	0.5% (n=1)
Colton	Co(a-b+)	-	-	-
Cartwright	Yt(a-)	YT*B	1I	0.5% (n=1)
Lutheran	Lu (b-)	LU*A	1W	0.5% (n=1)
	Lu(a-b-)	-	-	-

As can be seen in Table 4.10, there are 13 high-frequency antigens listed in the rare donor genotyping database, namely, E-HrB-, E-HrS-, k-, Js(b-), Kp(b-), Jk(b-), Fy(b-), s-, S-s-U-, Joa-, Hy-, Yt(a-) and Lu(b-) (Jungbauer, 2009).

The following Rh subtypes were added to the rare donor database based on published literature citing these rare types across many countries: R_o, r'r', r''r, r''r, and R_zR_1 (Jungbauer, 2009). This study shows a higher frequency of R_o found in 31% of the donors across the four ethnicities but with the majority being found amongst Black donors. Rare types did show patterns of specificity between certain ethnicities and this will be explained in Chapter 5.

Table 4.11: Gene Frequencies of the positive low-frequency antigens in the new rare donor red cell genotyping database and rare Rh subtypes

	PHENOTYPE	GENOTYPE	ETHNICITY	FREQUENCY
Rh	Cw+	RHCE*Ce, RHCE*CeCW	2W	1% (n=2)
		RHCE*ce, RHCE*CeCW(12)		
Kell	Js(a+)	KEL*k_KPB_JSB, KEL*k_KPB_JSA	7B	3% (n=7)
	Kp(a+)	KEL*k_KPA_JSB	2W	1% (n=2)
	K+	KEL*K_KPB_JSB	1W	0.5% (n=1)
MNS	Uvariant	GYPB*s, GYPB*S_null(IVS5+5t)	7B, 6W	6% (n=13)
		GYPB*s, GYPB*S_null(230T)		
		GYPB*S_null(IVS5+5t)		
		GYPB*S_null(230T), GYPB*S_null(IVS5+5t)		
		GYPB*S_null(IVS5+5t)		
		GYPB*S, GYPB*S_null(IVS5+5t)		

As per Table 4.11, there were 5 donors positive for low-frequency antigens found in the rare donor red cell genotyping database from the Rh, Kell and MNS systems.

Chapter 5 will cover the interpretation of the data in comparison to other similar studies completed in countries in Africa and internationally.

CHAPTER 5: DISCUSSION

This was the first comprehensive red cell genotyping study completed at SANBS and in South Africa that included coverage of 10 blood group systems and 37 red cell antigens from RHCE, Kell, Kidd, Duffy, MNS, Dombrock, Colton, Diego, Cartwright and Lutheran blood groups as identified by the IDCORE^{XT} assay (Finning et al., 2016). This makes the current study findings of interest scientifically, operationally and clinically to the SANBS. In addition, these results add to the international body of knowledge of rare blood types prevalent in South African ethnic groups.

The results of this study revealed that 69% (n=161) of 234 donors screened were defined as rare and together with the 44 serologically known rare donors formed the final rare donor red cell genotyping database (n=205). Although 69% of rare types were identified and were confirmed from a small study group of 234 (278 including 44 donors), this was not unusual as a similar study completed in the United States by Noumsi et al. (2014b) showed that 79% of rare red cell genotypes went unrecognised by serology (Noumsi et al., 2014b). In another multicentre study with collaboration between four European countries (United Kingdom, Ireland, Italy and Spain), Finning et al. (2016) showed that > 80% of 258 donors tested by IDCORE^{XT} were rare.

In comparison to the European study by Finning et al. (2016), 3 of the 13 negative high-frequency antigens, Js(b-), Kp(b-) and Jk(b-) were unique to the Black and White SA donors respectively and 1 positive low-frequency antigen Kpa+ was found in 2 White donors. Unique to the European study was the Dib-, RHCE*CeRN, Dia-, Mia+, Cob+, Lua+, Dob+ (Finning et al., 2016). Although there has been suggested similarities between SA White donors and Caucasians/Europeans, it is clear from these findings that some uniqueness exists between the two regions. More studies will be required to determine the root of these differences at a genetic level.

There were six rare unusual Rh subtypes at the following frequencies 31% R_0, 2.5% r'r', 1% r'r", 0.5% r"r and 0.5% R_zR_1 (Woodfield et al., 2004). The R_zR_1 found in this study is a rare Rh subtype for South Africa. A study completed by Sharma et al. (2010) reported R_zR_1 to be found in 6% Native Americans and was present in 2.2% of Indians in a similar study carried out in Central India. This could indicate that the one Coloured donor from South Africa could be due to an Indian and Black ethnic union.

Five rare positive low-frequency antigens identified were 6% Uvariant, 3% Js(a+), 1% Cw+, 1% Kp(a+) and 0.5% K+, which is consistent with the phenotype frequencies amongst the different ethnicities (Moller et al., 2016; Poole, 2006; Sharma et al., 2010; Dean, 2005).

In addition, 11 donors had 9 sets of common multiple-antigen-negative combinations and these were similar to antigen combinations found in populations amongst the Asians, Europeans, Americans (Alcantara, Chay and Ang, 2016; Tani, 2016b; Thornton, 2016; Woodfield et al., 2004).

There were 11 negative high-frequency antigens listed as rare on the IRDP and covered by the IDCORE^{XT} kit, that were not found in this study (Thornton, 2016). These rare types are also listed on the South African rare donor file however were not prevalent in this study. This finding prompts more targeted screening efforts for these 11 antigens especially with Jk(a-b-), Co(a-) and K_0 where rare donors were active but may have lapsed during the study period (Govender, Niekerk and Vather, 2017). There were seven negative high-frequency antigens defined as rare that are not covered by the IDCORE^{XT} assay, namely, Kn(a-), Lan-, Bombay O_h, Vel-, Adult I-, Ge- and In(b-), which highlights the need to identify further molecular based assays that will cover these blood groups (Govender, Niekerk and Vather, 2017; Thornton, 2016; Poole, 2006; Progenika Grifols, 2014).

The positive for low-frequency antigens that are defined as rare on the IRDP that are identified by the IDCORE^{XT} assay are the five listed on Table 4.11 and that are not covered are: He+, In(a+), M_1+, St(a+), Wr(a+), Dantu+, Mi III+ and STEM+. The SANBS reference laboratory did have active rare donors for all of the eight rare antigens not covered by IDCORE^{XT}, but they have since lapsed and therefore another focus area for targeted screening and molecular assays with the adequate coverage is necessary for future studies.

Donors of ABO Group A (10%), Group B (4.5%) and Group O (85.5%) were found in the rare donor red cell genotyping database. The majority of Group O was found in White donors, 77% O+ and 15% O- and Black donors 59% O+ and 7% O-. The normal distribution of Group O RhD types according to previous years SANBS internal records of donor population statistics is White (85% Group O+, 15% Group O-) and Black (98% O+, 2% O-). The reason for the skewness in the Black donors was due to purposive sampling as directed by the reference laboratory algorithm of testing over the study period. Further the majority of Group O blood units was because the SANBS focuses their efforts in obtaining the commonly occurring Group O+ blood donations as this can be issued to males and females not of child-bearing age in cases of emergency's. Group O- females of child-bearing age can only receive Group O- blood (SANBS, 2016b).

The donors were of four major ethnicities: 54% White, 37% Black, 7% Indian and 2% Coloured (SANBS, 2016). The proportion of of donors amongst the ethnic groups was similar to the SANBS blood donor population statistics which are 47% White, 41% Black, 6% Indian and 6% Coloured but is not comparable to the general South African population comprised of 79.7% Whites, 8.9% Coloureds, 8.9% Whites and 2.5% Indians (SANBS, 2016). Most countries internationally have ethnicites termed as African American, Cauasian, and Asian that are similar to South Africa

ethnicities as explained below (Patin et al., 2017; Avent et al., 2009; Flegel, von Zabern and Wagner, 2009).

The South African Black Bantu-speaking people originated from West Africa then moved to South Africa and genetically adapted with the local South African populations (Patin et al., 2017). The Bantu also have been linked to the ancestry of African Americans and therefore Black South Africans may share similar blood group types to African Americans (Patin et al., 2017; Tishkoff et al., 2009). Although the Coloured South Africans orignate from unions between White and Black ethnicities, the Coloured people first developed from mixed African ancestries (Tishkoff et al., 2009). For this reason, the Coloured and Black South Africans show similar phenotypes.

The White South Africans originated from the early Dutch settlers in Cape Town from 1652 and hence the frequencies of rare types found in Whites in South Africa are comparable to Europeans/Causasians (Ramerini, 1998).

The Indian South Africans originated from the thousands of settlers brought from India to work as sugar-cane farmers in 1860. Makroo et al. (2013), concluded that the phenotype prevalence in India is statistically different from the Causasian, Black and Chinese populations but more similar to Cauasians than the other two. Thus the Indian population share some similarities with Cauasians who also share similarities with the White South Africans and these similarities between the Indian and White donors at SANBS has been observed phenotypically (Moores, 1991). Similarly, the Black, Coloured and African American share phenotypes and this has been observed phenotypically in South Africa (Moores, Vaaja and Smart, 1991; Patin et al., 2017).

The Rh subtypes reflected the most Rh genetic variability amongst the White donors with O-subtypes being present more in White donors and R_0 more prevalent in Black donors. Black donors who were Rh negative rr subtype had similar rare phenotypes to study findings in other countries with populations of African ancestry (Tishkoff et al., 2009; Nance, 2009).

The most frequent Fy(b-) negative high-frequency antigen was the homozygous FY*A genotype found in 33% Indian and 20% White which is similar to the Asian population, and has been reported as the most frequent rare type listed on Japan's Rare Donor program (Tani, 2016a; Dean, 2005). The FY*A is present usually in 83% Whites and therefore the one White donor who had the FY*B, FY*B[265T]_FY*X and Fy(a-) predicted phenotype was unusual (Dean, 2005; Daniels, 2005). The Fy(a-b-) rare phenotype was not identified in the current study, and may be attributed to the purposive sampling techniques used in the study. However, if the study included samples from blood donors from malaria endemic regions especially in the Western Cape where migrants travel from malaria areas, this phenotype would likely have been present (Dean, 2005).

The s- antigen was the next most frequent rare type on the rare donor database identified against the IRDP. However, this does not feature on the South African rare donor panel as it is not difficult to source for transfusion (Poole, 2006; Govender, Niekerk and Vather, 2017). However, its status as a rare genotype on the rare donor genotyping database will remain so that countries such as Spain and Japan who list them on their rare donor files can request units when required (Tani, 2016b; Muñiz-Diaz et al., 2016).

The S-s-U- phenotype predicted by the GYPB*deletion are rare in Caucasians and found mainly in African Americans: the present study reports one White and one Black donor each with this deletion in the rare donor database (Dean, 2005; Poole, 2006). Although these mutations are found in conserved regions of the genes (conserved regions are regions that are not susceptible to mutations or polymorphisms) and are passed down to subsequent generations hence remain specific to certain ethnicities, it is possible that the White donor may have this deletion due to a parent or grandparent that was from the Black or Coloured genetic background (Daniels, 2005). Interestingly, the Uvariant positive low-frequency antigen GYPB*S_null (230T) and GYPB*S_null (IVS5+5t) on the rare donor database is also found in the Black and White donors suggesting that the polymorphism that occurred originally only amongst Blacks are now also being found as a variant amongst White donors (Moonsamy, Govender and Vather, 2017). Due to the null GYPB*S genotype, the gene is present but with no antigen expressed on the red cell, as a result, the Uvariant type may be missed and incompatible blood may be issued, causing formation of Anti-U antibodies (Lopez et al., 2018).

Although there were no genotypes that were absent in < 1% of the population amongst the Black donors, the researcher identified that the rare genotype RHCE*ceAR, RHCE*ce[712G] genotype which is was associated with the rare HrS- (RH:-18) phenotype (Moller et al., 2016). In addition, 3% of Black donors showed the formation of RHD/RHCE hybrids and the r's haplotype RHCE*ce(733G, 1006T), RHD*r's-RHCE*(733G,1006T) consistent with the HrB (RH:-34) rare type (Daniels, 2013; Moulds, Noumsi and Billingsley, 2015). The E-HrB- and E-HrS- originated and were first identified in South Africa due to alloantibody formation in a Coloured woman and black man respectively (Moores, Vaaja and Smart, 1991). However with the advent of molecular genotyping, there was a debate of the hrB- (RH:-31) versus the HrB- (RH:-34) phenotypes as it was proven that only the RH:-34 requires rare blood because E+, RH:-31 can be given common E+ (R_2R_2) blood (Pham et al., 2009). Currently serology methods at SANBS cannot differentiate hrB- (Rh:-31) from the rare HrB- (Rh:-34); however, the presence of the r's genotype on IDCORE^{XT} is a good indication of the HrB- (Lopez et al., 2018). A similar differentiation of the hrS- and the rare HrS- is possible based on specific genotypes on the IDCORE^{XT} assay.

The rare negative high-frequency antigens k-, Kp(b-), Jk(b-), Jo(a-), Hy- and Lu(b-) were found only in White donors on the rare donor genotyping database and this is consistent with Europeans and Caucasians in American and Europe (Muñiz-Diaz et al., 2016; Avent et al., 2009; Hustinx et

al., 2016). The Js(b-) is normally found amongst Black donors (Reid, 2018) and this is the case in the rare donor genotyping database in the present study. However, one Indian donor also showed the Js(b-) phenotype which is unusual and could be due to a Black relative or could be a pattern which has not been monitored in Indians. This finding will be monitored in future rare donor screening using genotyping (Kahar and Patel, 2014). In addition, the Yt(a-) was found in only one Indian and this has not been found in prevalent types in central India (Kahar and Patel, 2014).

The Cw+, Kp(a+), K+ and Uvariant was observed amongst White donors and Uvariant and Js(a+) amongst Black donors.

Many countries list on their rare donor files, sets of common multiple-antigen-negative combinations that make sourcing of blood for patients difficult (Woodfield et al., 2004). The SANBS previously did not list any sets of common multiple-antigen-negative antigens on the rare donor file because with a large donor population of approximately +/-800 000 blood units collected annually, it is not difficult to locate these units amongst the 84 blood banks found nationally. There were nine different combinations of common multiple-antigen-negative antigens found amongst three Indian, three Black, and five White (Alcantara, Chay and Ang, 2016; Tani, 2016b; Thornton, 2016; Woodfield et al., 2004). These combinations will be newly reported on the South African rare donor file in line with reporting by other countries (Alcantara, Chay and Ang, 2016; Tani, 2016b; Thornton, 2016; Woodfield et al., 2004).

During the study, there were eight NC results – three of the Kell blood group and five of the GYPB gene. Due to all other run criteria being met, these samples were referred to Progenika Grifols for red cell sequencing on the BloodChip for identification of possible new alleles not covered by the IDCORE^{XT} SNPs (Avent et al., 2009).

5.1 Strengths of this Study

This was the first comprehensive red cell genotyping study undertaken at SANBS and for South Africa. The IDCORE^{XT} assay is a comprehensive red cell genotyping assay covering 10 of the most common blood groups systems: RHCE, Kell, Kidd, Duffy, Dombrock, Colton, Cartwright, MNS, Lutheran and Diego. The assay covers 29 polymorphisms that are used to identify the genes that encode 37 red cell antigens (Finning et al., 2016). In addition, the assay is designed to run on the Luminex 200IS platform where two runs of 96 samples each can be completed daily therefore there is high-throughput testing potential for mass screening of donors (Progenika Grifols, 2014).

The extensive coverage of 10 blood groups systems in a single test translates to cost efficiencies in laboratory testing in comparison to the high costs of resolving complex serology cases and

high-throughput testing (Noumsi et al., 2014a). A second advantage is that common multiple-antigen-negative units are easier to locate. Red cell genotyping covering 10 blood group systems simultaneously in one test provides an additional benefit in that “rare donors” may be rare across more than one blood group system and this was discovered in the present study.

The database will become part of the routine algorithm of testing in the Immunohaematology Reference Laboratory at SANBS for the resolution of inconclusive or complex serology cases. and will be available for reference internationally for sourcing of rare blood. This will provide information on which rare donors molecular genotyping background will best match the patients blood type requirements.

Genotyping will be used to test and provide suitable antigen-negative units to patients who have developed alloantibodies. This will translate to a decrease in the number of transfusion reaction cases as well as haemolytic disease of the newborn (Keller, 2015).

Donors will be chosen based on their genotypes in order to locate donors for production of screen and panel cells used in the blood banks for serological red cell screening and antibody identification.

Wastage of rare donor inventory will be avoided if true rare types are confirmed by genotyping where serology is limited. The HrB-, HrS- units will be saved, and the Uvariants and RH variants will be discerned. The FY*X can be easily identified genotypically.

5.2 Weaknesses of the Study

Although 278 was a small number in relation to ± 800 000 blood donors that donate annually, high-throughput red cell genotyping has not being implemented at SANBS due to high costs. However, similar studies completed by Finning et al. (2016) showed that a study population of 258 participants that were genotyped with the IDCORE^{XT} assay was sufficient to identify numerous low incidence and uncommon antigens.

The sample numbers representing the Coloureds were too low to draw any conclusions however the rare RzR1 (CDE,CDe) was found in one Coloured person. For this reason, more screening of the Coloured ethnicity is required.

CHAPTER 6: CONCLUSION

The aim of this study was to develop the first rare donor red cell genotyping database to determine the prevalence of RBC genotypes and predicted phenotypes in the South African donor population. The study aim was completed and the final rare donor database is comprised of the molecular red cell genotpying data of 205 donors. Where prevously there was no formal database and limited published literature regarding red cell genotyping data amongst the four major ethnicities, this study has resulted in a database that can be used as a benchmark to replicate the study on a larger scale.

The Rh subtype R_zR_1 was found in one Coloured donor, however this donor has lapsed due to relaocation and therefore further investigation into this rare subtype is warranted. The other rare Rh subtype which is defined as the rarest rare blood type worldwide is the Rh_{null} (Woodfield et al., 2004). More focused efforts will therefore be directed to screening for unusal Rh subtypes.

There are rare blood types defined serologically and listed on the South African and IRDP that are not covered by the IDCORE^{XT} study (Govender, Niekerk and Vather, 2017; Thornton, 2016). In order to further expand the coverage of the rare donor red cell genotpying database to make it more comprehensive, continuous surveying of the market for commercial assays that cover these antigens is required. Alternatively, a more sensitive method will be red cell next generation sequencing (NGS) that can be used to sequence specific taget sequences of the entire gene.

Based on the data reported in this study, more effective and targeted screening for antigen-negative units for patients and rare donor screening to increase the pool of rare donors can be implemented. This can include using the BI system to flag Group O+ Black donors with a R_o or r'r subtype for screening for the HrS- and HrB- rare blood types. White donors can be selected for screening of the k- rare blood type. The rare donor red cell genotyping database has highlighted several trends of rare blood types being associated with specific ethnicites and this will assist in finding appropriate antigen-matched blood for patients.

The value derived from the database is that screening by red cell genotyping can assist in finding donors with multiple combinations of antigens that can be used to manufacture red cell screening and identification panels for testing patients blood. This will be of great benefit to the SANBS reagents laboratory which manufactures serology testing reagents for SANBS, external stakeholders and for supply to other pharmaceutical and blood transfusion centres in Africa.

There is no longer a requirement to randomly screen donors for rare blood types as red cell genotyping has provided certain triggers and has confirmed patterns from serology along with new triggers of possible rare types amongst a specific ethnicity with certain Rh subtypes.

Therefore, screening by genotyping can be sucessfully applied to the different requirements associated with rare blood, that is, for patients, donors, rare blood types, and sourcing of units for manufacture of reagents.

The competitiveness of various commercial companies to provide faster, more cost-effective genotyping kits with multiplexing ability and high-throughput volumes has made it possible to introduce mass-scale genotyping. This allows SANBS the opportunity to contribute on a large scale to international databases or even create a database of thousands of donors. A more adequate representation of donors of all ethnicities can be covered, a weakness that was highlighted in this study.

Red cell genotyping offers many practical advantages, many of which have been implemented already at SANBS since the introduction of molecular testing, and some of which can still be easily implemented.. Ten years from today, or even sooner, it is possible that SANBS will implement genotyping of all donors upon second blood donation so that phenotype matched blood becomes a reality on a larger scale. The challenge facing blood tranfusion services worldwide is the replacement of routine phenotyping with red cell genotyping.

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APPENDIXES

Appendix 1: Durban University of Technology (DUT) Institutional Research Ethics Committee (IREC) Study Approval Letter

DUT DURBAN UNIVERSITY OF TECHNOLOGY

Institutional Research Ethics Committee
Research and Postgraduate Support Directorate
2nd Floor, Berwyn Court
Gate 1, Steve Biko Campus
Durban University of Technology

P O Box 1334, Durban, South Africa, 4001

Tel: 031 373 2375
Email: lavishad@dut.ac.za
http://www.dut.ac.za/research/institutional_research_ethics

www.dut.ac.za

14 February 2017

IREC Reference Number: **REC 130/16**

Ms L Govender
59 Foresthaven Drive
Foresthaven
Phoenix
4068

Dear Ms Govender

Development of a Novel South African Rare Donor Red Cell Genotyping Database

I am pleased to inform you that Full Approval has been granted to your proposal REC 130/16.

The Proposal has been allocated the following Ethical Clearance number **IREC 011/17**. Please use this number in all communication with this office.

Approval has been granted for a period of two years, before the expiry of which you are required to apply for safety monitoring and annual recertification. Please use the Safety Monitoring and Annual Recertification Report form which can be found in the Standard Operating Procedures [SOP's] of the IREC. This form must be submitted to the IREC at least 3 months before the ethics approval for the study expires.

Any adverse events [serious or minor] which occur in connection with this study and/or which may alter its ethical consideration must be reported to the IREC according to the IREC SOP's. In addition, you will be responsible to ensure gatekeeper permission.

Please note that any deviations from the approved proposal require the approval of the IREC as outlined in the IREC SOP's.

Yours Sincerely

Professor J K Adam
Chairperson: IREC

DUT DURBAN UNIVERSITY OF TECHNOLOGY
2017 -02- 14
INSTITUTIONAL RESEARCH ETHICS COMMITTEE
P O BOX 1334 DURBAN 4000 SOUTH AFRICA

Appendix 2: South African National Blood Service (SANBS) Human Research Ethics Committee (HREC) Approval Letter

SOUTH AFRICAN NATIONAL BLOOD SERVICE NPC

Human Research Ethics Committee

SANBS

OHRP Number : IORG0006278
FWA Registration Number : IRB00007553
SA NHREC Registration Number : REC-270606-013

Secretariat: Tel: 011 761 9135 | Fax: 011 761 9137 | Cell: 083 708 0569 | Email: veronica.pepping@sanbs.org.za

To: Lavendri Govender
SANBS
E-mail: lavendri.govender@sanbs.org.za

Dear Lavendri,

DATE OF COMMITTEE MEETING:	**6 June 2016**
PROJECT TITLE:	**Development of a Novel South African Rare Donor Red Cell Genotyping Database**
DECISION OF THE COMMITTEE:	**Approved**
CLEARANCE CERTIFICATE NO:	**2016/06**

- Execution of the study must be compliant with applicable guidelines and policies.
- Any amendment, extension or other modifications to the protocol must be submitted to this Ethics Committee for approval prior to implementation.
- The Committee must be informed of any serious adverse event, planned and unplanned termination of the study.
- A progress report should be submitted yearly for long-term studies and a final report at completion of both short term and long term studies.
- Kindly refer to the SANBS HREC clearance certificate number on all future correspondence on this study to the HREC secretariat.
- This approval is valid for 5 years from the date stated above.

COMMITTEE GUIDANCE DOCUMENTS:

- International Conference on Harmonization (ICH) Good Clinical Practices (GCP) Guideline (ICH, 1996), Ethics in Health Research: Principles, Structures and Procedures (SA Department of Health, 2004); Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa (SA Department of Health, 2016); Ethical Principles for Medical Research Involving Human: Declaration of Helsinki (World Medical Association, 2013); Reviewing Clinical trials: A Guide For Ethics Committees (Karlberg and Speers, 2010)

13 December 2016

CHAIRPERSON: Prof J.N. Mahlangu **DATE**

Appendix 3: South African National Blood Service (SANBS) Letter of Permission to utilise data for study

Head Office or Zone
1 Constantia Boulevard
Constantia Kloof
Ext 22
1709

Postal Address
P/Bag X 14
Weltevreden Park
1715

Tel: +27 31 7196953 Fax: +27 86 646 5293

Email: Lavendri.govender@sanbs.org.za www.sanbs.org.za

Toll Free: 0800 11 9031

SANBS

Registration No. 2000/026390/08

Accreditation Site Facility Number: B0000

28th January 2016

Attention: Mr Trevor Vroom - Chief Executive Officer (CEO), SANBS

RE: Letter of Permission

I, Lavendri Govender, pay number LG4929 do hereby request permission to pursue a Master's Degree qualification at Durban University of Technology (DUT). I am currently a Senior Biomedical Scientist of Molecular Research and Development in the Specialised Laboratory Services (SLS) Department at SANBS.

In 2015, I evaluated a new test called Red Cell Genotyping on the Luminex platform in the Immunohaematology Reference laboratory department of SLS which was subsequently implemented as part of routine testing in the Reference laboratory. The main function of this test is to perform Red Cell Genotyping on our Rare Donors and in line with this testing, I have chosen the following Master's Research topic.

Title of Proposed Research - Creation of a Novel South African Rare Donor Red Cell Genotyping Database

Duration - January 2016 to January 2017

Supervisors - Dr. Charlotte Ingram, Medical Director, SANBS
Kuben Vather, Senior Manager, SLS

I do hereby request permission to complete my Master's degree using SANBS Red Cell Genotyping data. This Letter will form part of the requirements to obtain permission from the Durban University of Technology Ethics committee and Human Research Ethics Committee (HREC). Following a successful application, permission will then be requested from SANBS ethics committee.

Thank you, your signature below will serve as permission granted for continuation of Master's degree.

29/1/16
DATE

Trevor Vroom
Chief Executive Officer (CEO)
South African National Blood Services
+27 11 761 9111
Trevor.vroom@sanbs.org.za

sanas

Appendix 4: IDCORE^{XT} Assay Kit Polymorphisms

Blood Group	Polymorphism	ISBT Name	IDCOREXT Allele	ISBT Phenotype	IDCOREXT Antigen
Rh	RHCE c.122A>G	RHCE*02.08.01	RHCE*CeCW	RH:8	CW (RH8)
		Ref.1	RHCE*ceCW	N/A	CW (RH8)
		Ref.2	RHCE*CECW	N/A	(RH31)
	RHCE:c.307T>C	RHCE*01	RHCE*ce	RH:4	c (RH4)
		RHCE*03	RHCE*Ce		
	RHCE:c.335+3039ins109	RHCE*02	RHCE*Ce	RH:2	C (RH2)
		RHCE*04	RHCE*CE		
	RHCE.c.676G>C	RHCE*01	RHCE*ce	RH:5	e (RH5)
		RHCE*02	RHCE*Ce		
		RHCE*03	RHCE*Ce	RH:3	E (RH3), hrS (RH19), hrB (RH31)
		RHCE*04	RHCE*CE		
	RHCE*c.712A>G, RHCE*c.733C>G, RHCE*c.1006G>T, RHD-CE-D hybrid	RHCE*01.04	RHCE*ceAR	RH:10, RH:-19	V (RH10), hrS(RH19)
		RHCE*01.05/08/09	RHCE*ce[712G]	RH:-19	hrS (RH19)
		RHCE*02.02	RHCE*CeFV	n/a	n/a
		RHCE*03.03	RHCE*cEFM	n/a	hrS (RH19), hrB (RH31)
		RHCE*01.20.01/02	RHCE*ce[733G]	31	hrB(RH31)
		RHCE*01.20.03/05	RHCE*ce[733G,1006T]	RH:20, RH:-31	VS (RH20), hrB(RH31)
		Ref.3	RHCE*CeVG	n/a	n/a
		Ref.4	RHCE*cE[712G,733G]	n/a	hrS (RH19), hrB (RH31)
		Ref.5	RHCE*Ce[733G]	n/a	n/a
		RHCE*01.20.03/05	RHCE*r's-RHCE*ce[733G,1006T]	RH:20, RH:-31	VS (RH20), hrB(RH31)
	RHCE*c.676G>C, RHCE*c.712A>G, RHCE*c.733C>G, RHCE*c.1006G>T	Ref.6	RHCE*CE-D[5, 7]-CE	n/a	C (RH2)
					E (RH3)
					c (RH4)
					e (RH5)
					CW (RH8)
					V (RH10)
					hrS (RH19)
					VS (RH20)
					hrB (RH31)
Kell	KEL*c.578T>C	KEL*01.01	KEL*K_KPB_JSB	KEL:1	K (KEL1)
		KEL*02	KEL*k_KPB_JSB	KEL:2	k (KEL2)
	KEL*c.841T>C	KEL*02.03	KEL*k_KPA_JSB	KEL:3, KEL:-4	Kpa (KEL3), Kpb (KEL4)
	KEL:c.1790C>T	KEL*02.06	KEL*k_KPB_JSA	KEL:6, KEL:-7	Jsa (KEL6), Jsb (KEL7)
Kidd	SLC14A1:c342-1G>A	JK*02N.01	JK*B_null(IVS-1a)	JK:-3	Jkb (JK2)
		JK*01N.06	JK*A_null(IVS5-1a)	JK:-3	Jka (JK1)
	SLC14A1:c838-1G>A	JK*01	JK*A	JK:01	Jka (JK1)
		JK*02	JK*B	JK:02	Jkb (JK2)
	SLC14A1:C871T>C	JK*02N.06	JK*B_null(871C)	JK:-3	Jkb (JK2)
Duffy	FY:c.1-67T>C	FY**01N.01	FY*A_GATA	Fy(a-b-)	Fya (FY1)
		FY*02N.01	FY*B_GATA	Fy(a-b-)	Fyb(FY2)
	FY:c.125G>A	FY*01	FY*A	FY:1	Fya (FY1)
		FY*02	FY*B	FY:2	Fyb(FY2)
	FY:c.265C>T	FY*01W	FY*A[265T]	Fya+w	Fya (FY1)
		FY*02W	FY*B[265T]_FY*X	Fyx	Fyb(FY2)
MNS	GYPA:c.[59C>T]	GYPA*01	GYPA*M	MNS:1	M (MNS1)
		GYPA*02	GYPA*N	MNS:2	N (MNS2)
	GYPB:c.143T>C	GYPB*03	GYPB*S	MNS:3	S (MNS3)
		GYPB*04	GYPB*s	MNS:4	s (MNS4)
	GYPB:c.230C>T	GYPB*03N.01	GYBP*S_null(230T)	MNS:-3, MNS:5W	S (MNS3), U (MNS5)
	GYPB:c.270+5G>T	GYPB*03N.03	GYBP*S_null(IVS+5t)	MNS:-3, MNS:5W	S (MNS3), U (MNS5)
	GYPB-A-B hybrid	GYP.501	GYP.Mur	MNS:7	Mia (MNS7)
	GYPB:c.143T>C, GYPB:C230C>T, GYPB:c270+5G>T	GYPB*01N	GYPB*deletion	MNS:-3,-4,-5	S (MNS3), s (MNS4), U (MNS5), Mia (MNS7)
Diego	DI:c.2561T>C	DI*01	DI*A	DI:1	Dia (DI1)
		DI*02	DI*B	DI:2	Dib (DI2)
Dombrock	DO:c.793A>G	DO*01	DO*A	DO:1	Doa (DO1)
		DO*02	DO*B	DO:2	Dob (DO2)
	DO:c.323G>T	DO*02.-04	DO*B_HY	DO:-4	Hy (DO4)
	DO:c.350C>T	DO*01.-05	DO*A_JO	DO:-5	Joa(DO5)
Colton	CO:c.134C>T	CO*01.01	CO*A	CO:1	Coa (CO1)
		CO*02	CO*B	CO:2	Cob (CO2)
Cartwright	YT:c.1057C>A	YT*01	YT*A	YT:1	Yta (YT1)
		YT*02	YT*B	YT:2	Ytb (YT2)
Lutheran	LU:c.230A>G	LU*01	LU*A	LU:1	Lua (LU10
		LU:02	LU*B	LU:2	Lub (LU2)

Appendix 5: BIDSXT RUN WORKSHEETS

Batch #: 2015_07_30_0001
Comments:

AMPLIFICATION

	1	2	3	4	5	6	7	8	9	10	11	12
A	01508385 SOR2212_14 ID CORE XT											
B	01508385 SOR1301_15 ID CORE XT											
C	LOUWJM00100 SOR1148 ID CORE XT											
D	FOURIS00060 SOR0164 ID CORE XT											
E												
F												
G												
H												

PRODUCT Kit # Tests #	ID CORE XT 0202010004 4		
REAGENTS	**LOT #**	**Volume (µl)**	**Check**
ID CORE XT PCR Master Mix	0202000009	90.0	
HotStarTaq DNA Polymerase (5 U/µL)	145037701	2.0	

Guidelines

- Switch on the thermal cycler.
- Vortex the XT PCR Master Mix. Spin down both the XT PCR Master Mix and the HotStarTaq® DNA Polymerase.
- Set up the PCR reaction following the table above.
- Vortex and spin down the PCR reaction and dispense 20 µL per sample in each well.
- Add 5 µL of each DNA sample per well.
- Seal the PCR plate, centrifuge gently and place it on the thermal cycler block.
- Start ID XT PCR program in the thermal cycler.

Batch #: 2015_07_30_0001
Comments:

HYBRIDIZATION

	1	2	3	4	5	6	7	8	9	10	11	12
A	01508385 SOR2212_14 ID CORE XT											
B	01508385 SOR1301_15 ID CORE XT											
C	LOUWJM00100 SOR1148 ID CORE XT											
D	FOURIS00060 SOR0164 ID CORE XT											
E												
F												
G												
H												

PRODUCT Kit # Tests #	ID CORE XT 0202010004 4		
REAGENTS	**LOT #**	**Volume (µl)**	**Check**
ID CORE XT Beads Master Mix	0202000008	184.0	

Guidelines

- Switch on the thermal cycler and prepare the Luminex® System.
- Vortex Beads Master Mix for 30 sec.
- Dispense 46 µl per sample into the hybridization plate. (Avoid bubble formation and vortex Beads Master Mix after each 8 samples).
- Spin down the PCR plate.
- Add 4 µL of PCR product to each well and mix gently by pipetting up and down several times.
- Seal the plate with the corresponding film.
- Place the plate and the silicone compression mats on the thermal cycler block.
- Start the ID XT HYB program in the thermal cycler

Batch #: 2015_07_30_0001
Comments:

LABELING

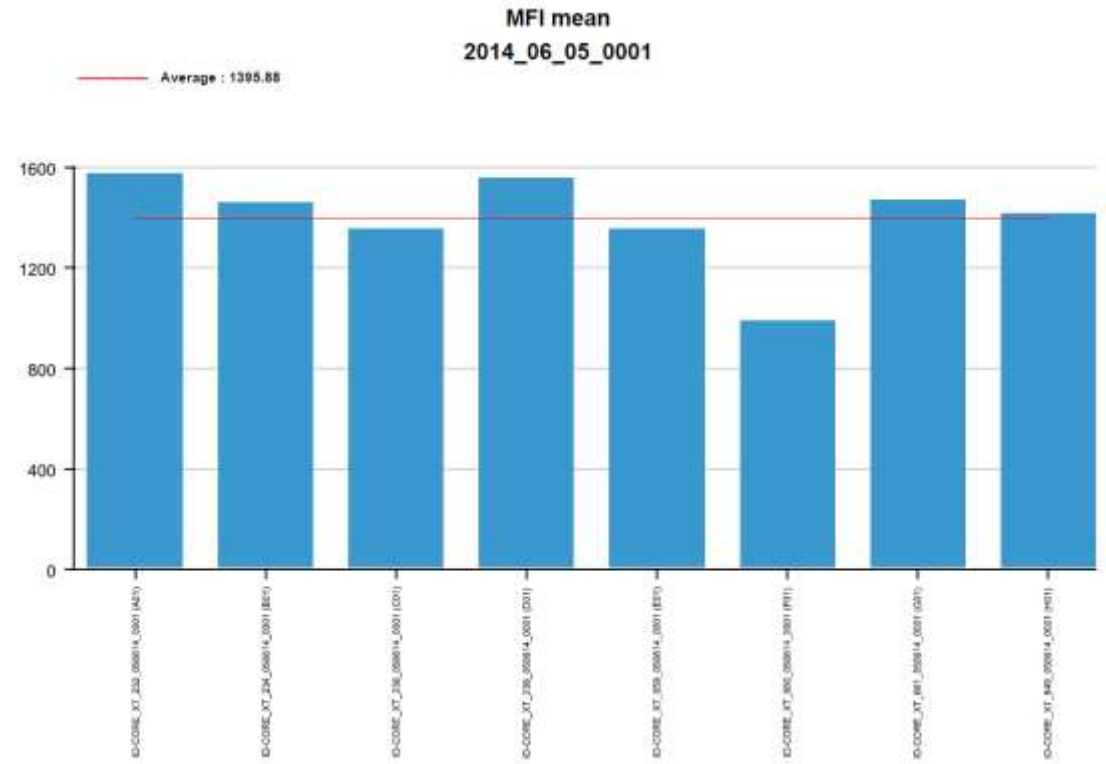
	1	2	3	4	5	6	7	8	9	10	11	12
A	01508385 SOR2212_14 ID CORE XT											
B	01508385 SOR1301_15 ID CORE XT											
C	LOUWJM00100 SOR1148 ID CORE XT											
D	FOURIS00080 SOR0164_ ID CORE XT											
E												
F												
G												
H												

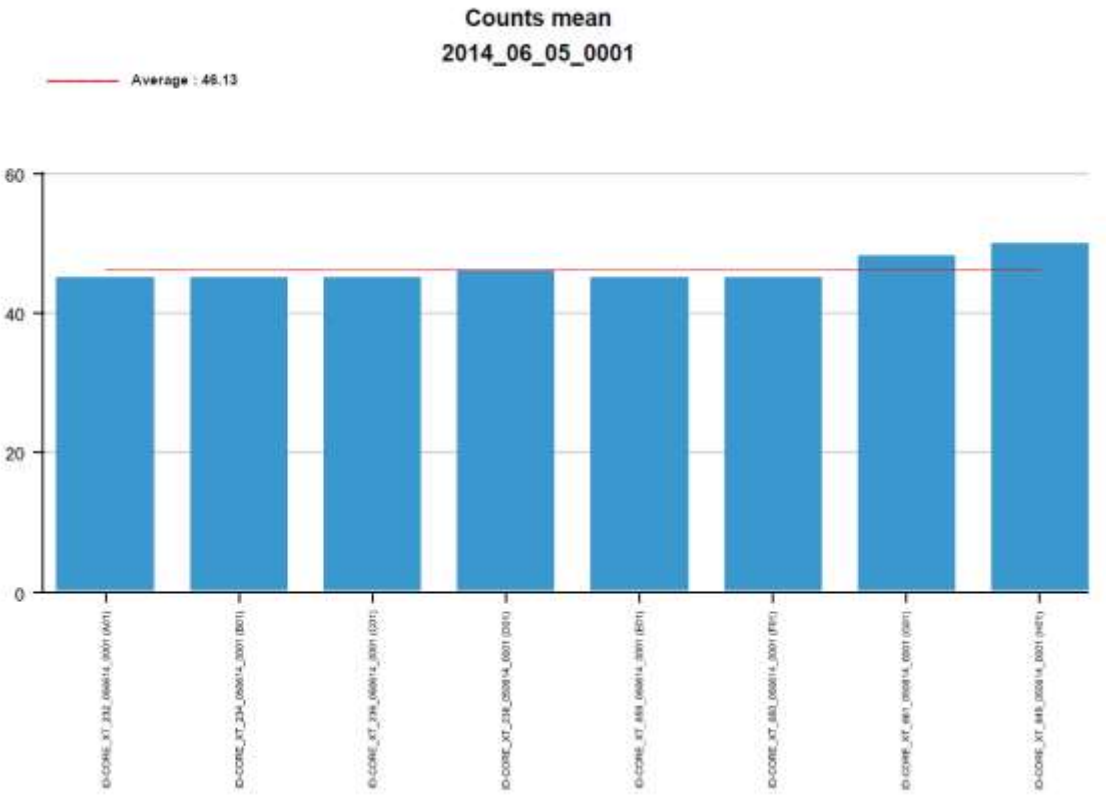
PRODUCT	ID CORE XT		
Kit #	0202010004		
Tests #	4		
REAGENTS	**LOT #**	**Volume (µl)**	**Check**
SAPE Dilution Buffer	0000000029	348.0	
SAPE	0000000040	18.4	

Guidelines

- Vortex and spin down the SAPE.
- Prepare the labeling mix as shown in the table above.
- At the 52 ºC hold step in the ID XT HYB program, open the thermal cycler lid .
- Dispense 80 µL of the labeling mix into each well of the hybridization plate and mix gently by pipetting up and down once.
- Place the plate in the Luminex® system inmediately after labeling.

Appendix 6: BIDSXT Median Fluorescent Intensity |(MFI) Mean and Bead Counts





Appendix 7: IDCOREXT Red Cell Genotyping Result

IDCOREXT

Red Blood Cells typing report

Date: 22/09/2016 15:00:00 *User:* Grifols

Patient name:	Undefined	*Ordered by:*	
		Ordering institution:	
Patient #:		*Test performed:*	ID CORE XT
Sample Id.:	SOR1920_14	*Software version:*	1.4.2
Sample type:	DNA	*Lot #:*	0201040022
Accessioning date:	23/02/2015	*Enzyme lot #:*	145037701

Blood Group System	Alleles Assayed	Genotype Result	Antigens (ISBT Phenotype)	Predicted Phenotype Result
Rh	*RHCE*ce* *RHCE*Ce* *RHCE*cE* *RHCE*CE* *RHCE*CeCW* *RHCE*ceCW* *RHCE*CECW* *RHCE*ceAR* *RHCE*CeFV* *RHCE*CeVG* *RHCE*cEFM* *RHCE*ce[712G]* *RHCE*ce[733G]* *RHCE*ce[733G,1006T]* *RHCE*CE-D[2, 5, 7]-CE* *RHCE*cE[697G,712G,733G]* *RHD*r's-RHCE*ce[733G,1006T]*	*RHCE*ce, RHCE*ce[733G]*	C (RH:2)	0
			E (RH:3)	0
			c (RH:4)	+
			e (RH:5)	+
			CW (RH:8)	0
			V (RH:10)	+
			hrS (RH:19)	+
			VS (RH:20)	+
			hrB (RH:31)	+
Kell	*KEL*K_KPB_JSB* *KEL*k_KPB_JSB* *KEL*k_KPA_JSB* *KEL*k_KPB_JSA*	*KEL*k_KPB_JSB*	K (KEL:1)	0
			k (KEL:2)	+
			Kpa (KEL:3)	0
			Kpb (KEL:4)	+
			Jsa (KEL:6)	0
			Jsb (KEL:7)	+
Kidd	*JK*A* *JK*B* *JK*B_null(871C)* *JK*B_null(IVS5-1a)*	*JK*A*	Jka (JK:1)	+
			Jkb (JK:2)	0
Duffy	*FY*A* *FY*B* *FY*B_GATA* *FY*B[265T]_FY*X*	*FY*B, FY*B_GATA*	Fya (FY:1)	0
			Fyb (FY:2)	+
MNS	*GYPA*M* *GYPA*N*	*GYPA*M, GYPA*N*	M (MNS:1)	+
			N (MNS:2)	+
	*GYPB*s* *GYPB*S* *GYPB*Mur* *GYPB*deletion* *GYPB*S_null(230T)* *GYPB*S_null(IVS5+5t)*	*GYPB*s*	S (MNS:3)	0
			s (MNS:4)	+
			U (MNS:5)	+
			Mia (MNS:7)	0

Blood Group System	Alleles Assayed	Genotype Result	Antigens (ISBT Phenotype)	Predicted Phenotype Result
Diego	*DI*A* *DI*B*	*DI*B*	Dia (DI:1)	0
			Dib (DI:2)	+
Dombrock	*DO*A* *DO*B* *DO*B_HY-* *DO*A_JOA-*	*DO*B*	Doa (DO:1)	0
			Dob (DO:2)	+
			Hy (DO:4)	+
			Joa (DO:5)	+
Colton	*CO*A* *CO*B*	*CO*A*	Coa (CO:1)	+
			Cob (CO:2)	0
Cartwright	*YT*A* *YT*B*	*YT*A*	Yta (YT:1)	+
			Ytb (YT:2)	0
Lutheran	*LU*A* *LU*B*	*LU*B*	Lua (LU:1)	0
			Lub (LU:2)	+

Legend
+: Normal antigen expression 0: Undetectable antigen expression NC: No Call, result inconclusive UN: Unknown, highly unlikely. The antigen expression is not known.
Notes

Observations
REVIEW AND APPROVAL
Grifols | Grifols

ASSAY LIMITATIONS
- The predicted phenotype generated by the ID CORE XT test is inferred only from the alleles described in the Intended Use. - ID CORE XT only detects genotypes defined by alleles reported in the literature. - ID CORE XT may not detect alleles containing unreported mutations at primer binding sites or at probe-binding sites. In such rare cases this may lead to erroneous genotype calls. - Variants in other genes, post transcriptional or epigenetic events are not measured by the test. Therefore, in rare cases, the predicted phenotype may differ from the phenotype detected by serology.