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Establishing a regional registry of genotyped blood donors for extended match transfusions

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ABBREVIATIONS

CD – Cluster of Differentiation

DNA – Deoxyribonucleic Acid

EDTA – Ethylenediaminetetraacetic Acid

HAV – Hepatitis A Virus

HBV – Hepatitis B Virus

HCV – Hepatitis C Virus

HIV – Human Immunodeficiency Viruses

ISBT – International Society of Blood Transfusion

NTC – No Template Control

PCR – Polymerase Chain Reaction

RBC – Red Blood Cell

rpm – revolutions per minute

SNP – Single Nucleotide Polymorphism

SSP – Sequence Specific Priming

As the use of first-person singular is highly unusual in scientific writing, first person plural may have been used in a few instances where this author claims full responsibility for the conclusions drawn.

1 INTRODUCTION

1.1 The Human Blood Group Systems

A human blood group is a human red blood cell surface antigen with known inherited variabilities, which induce antibody-formation[1]. Since those antibodies can induce immunological reactions, they are a known obstacle in transmitting blood and its derived products from one human to another[2].

Table 1: *Common definitions as used in transfusion medicine. Text in quotation marks is a direct quote from the cited source*

Name	Definition
Blood group system	"One or more antigens controlled at a single gene locus, or by two or more very closely linked homologous genes with little or no observable recombination between them"[1]
Blood type	Varying definitions, generally synonymous to a single blood group category[3]
Rare blood type	No general definition. See 1.2 for details
Antigen	A red cell surface molecule, in most cases a glycoprotein which must be inherited and "defined serologically by the use of a specific antibody"[1]

With the discovery of the ABO blood group system by Landsteiner[4] the role of blood transfusions in medicine was transformed. His and subsequent discoveries made hitherto unpredictable and often deadly transfusion reactions avoidable and treating with blood a viable option rather than a deadly experiment.

The discovery and characterization of the Rhesus system or rather the D antigen in the middle of the past century[5, 6] helped reduce the incidence of hemolytic disease of the newborn.

Today, there are 36 blood group systems recognized by the International Society of Blood Transfusion[7]. Each of those systems has, by definition[1], defined human alloantibodies and contains one or multiple alleles that are encoded on the same gene. In addition, most if not all of the known blood group systems have been implicated in clinically relevant transfusion related adverse events or hemolytic disease of the

newborn. Notably not included in this definition is an antithetical relationship between the different elements of a blood group. A single individual can therefore have several blood types of the same blood group. Antigens within a blood group can, however, have an antithetical relationship. Indeed, blood groups comprise mostly antithetical antigen groups of varying numbers. Antigen in this context means a target for a specific antibody, rather than an entire protein, as included in the above definition.

1.2 Rare Blood Types

There is currently no consensus on what defines a rare blood type, as opposed to a common one. While there are national definitions (e.g. an allele prevalence of <4‰ in France[8]) no international definition has been established[9]. Rare blood groups or more specifically, rare blood types, are noteworthy because the absence of the antithetical high frequency antigen in affected individuals causes three problems:

1. Antibody formation against high frequency antigens can lead to transfusion reactions, especially for recipients of multiple transfusions[8].
2. Those same antibodies can in some cases cross the placental barrier and lead to hemolytic disease of the newborn[10].
3. Lastly, recipients without a rare blood type and a history of transfusions from a donor with a rare blood type are at risk for antibody formation against the low frequency antigen[9].

Indeed, new blood types are usually discovered by the detection of their corresponding antibodies. For instance, Lu14, a high frequency antigen of the Lu blood group was discovered when its agglutinating antibody was found in the blood of a patient who had undergone multiple transfusions[11].

Each of the three groups outlined above is at risk for complications following blood transfusions such as transfusion reactions or hemolytic anemia induced by autoantibody formation[12].

To reduce the risk of these complications, most transfusing institutions employ a variety of tests to detect existing alloantibodies. These include determining a recipients blood type regarding the most significant blood group antigens (generally ABO, c, C, D, e and E) as well as a variety of tests designed to detect preexisting antibodies against donor red cells[13, 14].

This second step is commonly known as cross-matching.

This common screening strategy greatly reduces the incidence of transfusion reactions arising from rare blood types (as well as common ones, which is, however, not subject of this document). Since low frequency blood types are only detected once antibodies against them have been formed, no measures against antibody formation which specifically target rare blood types are being taken in standard transfusion practice.

1.3 Genetic Basis of Clinically Important Blood Group Systems

In clinical practice, the ABO (or AB0, as was it was originally called by Landsteiner[4]) blood group system is generally considered to be the most important one[15]. This is because ABO mediated transfusion reactions are generally associated with widespread hemolysis and associated complications.
Case series of such transfusion reactions show a fatality rate of approx. 20% where more than 50ml of incompatible blood were transfused[16].
The ABO system is, immunologically speaking, based on an oligosaccharide chain on the surface of red cells. The specific makeup and variety of these chains are determined by a number of enzymes, whose coding locations make up the basis of the ABO blood group system[17].
All ABO blood types need a pre-existing oligosaccharide named H onto which specific monosaccharides are added to produce either the A or B phenotype. Individuals lacking the enzymes necessary for producing the A or B specific saccharides are blood type O as they produce antibodies against both.
In contrast, persons lacking the H molecule produce antibodies against it and consequently all H positive blood. This is known as the Bombay phenotype[18].

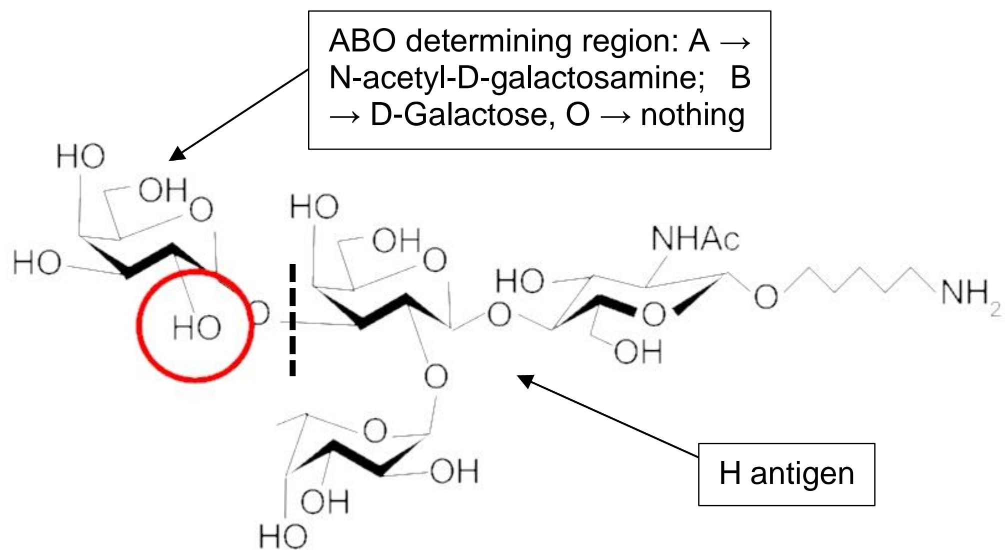

Figure 1: *B antigen with determining elements of the ABO system marked and annotated*

Source: Ihadpieforlunch (https://commons.wikimedia.org/wiki/File:AP-Biology_Final_Project.svg), Cut and reformatted to include only one blood type, annotations added by Jan Portegys, https://creativecommons.org/licenses/by-sa/4.0/legalcode Access Date: 25.02.2019

The ABO blood goup system is encoded by the *ABO* gene. For the most common phenotypes A_1, A_2, B and O the molecular basis is given by only a few DNA sequence variations in exon 6 and 7 of the ABO gene (**Figure 2**). However, for the rarer phenotypes such as A_3, A_w, B_3 and others, many different ABO gene variants have been described.

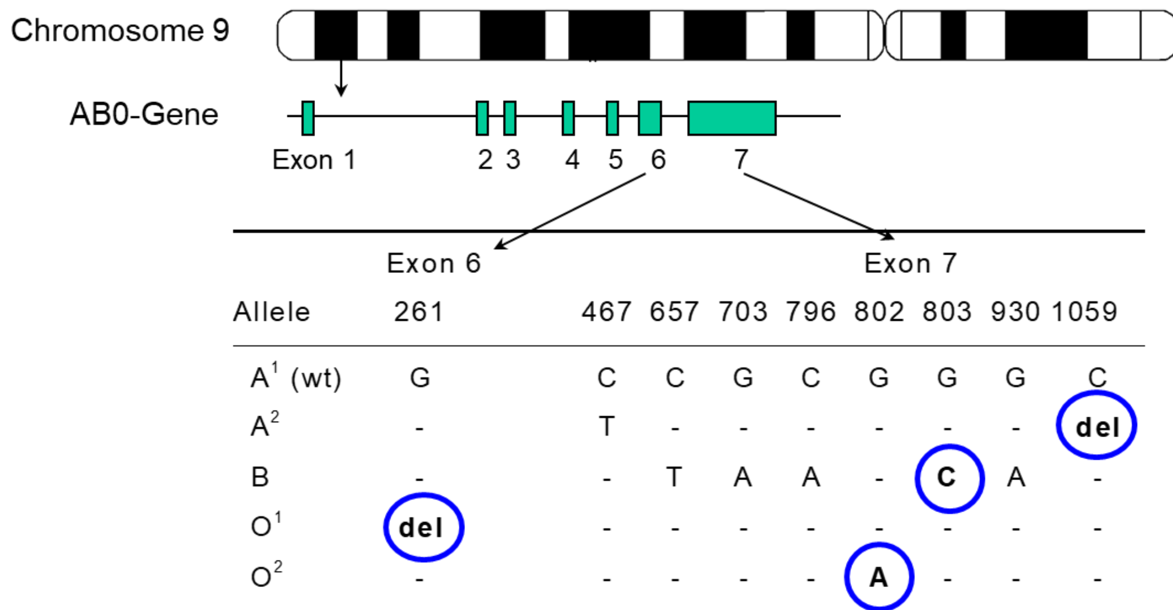

***Figure 2**: Structure of the ABO gene and location of the major sequence variations.*
Source: Bugert P., Teaching materials of the Institute for Transfusion Medicine and Clinical Immunology of Mannheim Medical Faculty, Heidelberg University, Acquired with permission 13.03.2019

Another blood group system, the Rh system, is considered clinically important as well[19]. Almost every blood donation service determines at least one component of the Rhesus system in its donors. Due to its involvement in hemolytic disease of the newborn and many transfusion reactions it has been studied extensively in the past century, to the point where its genetics and clinical implications are well known[20]. The Rh system is based on three proteins[19]:

- The D antigen is based on the presence or absence of the RhD protein and is encoded by the *RHD* gene on chromosome 1 (***Figure 3***). There are several mutations leading to D non-expression (d phenotype) with differing prevalence based on the screened population.
- The Cc and Ee antigen pairs are both encoded by the neighboring *RHCE* gene on chromosome 1 (***Figure 3***) and expressed into these 4 antigens of the RhCE protein. There are many missense mutations described causing various low and high frequency antigens.
- Both antigens are only expressed on the red cell surface if the RhAG antigen is also present. RhAG has no transfusion relevant polymorphism. Its gene is located on chromosome 6. RhAG absence can, however lead to non-expression of Rh antigens (Rh_{null} phenotype).

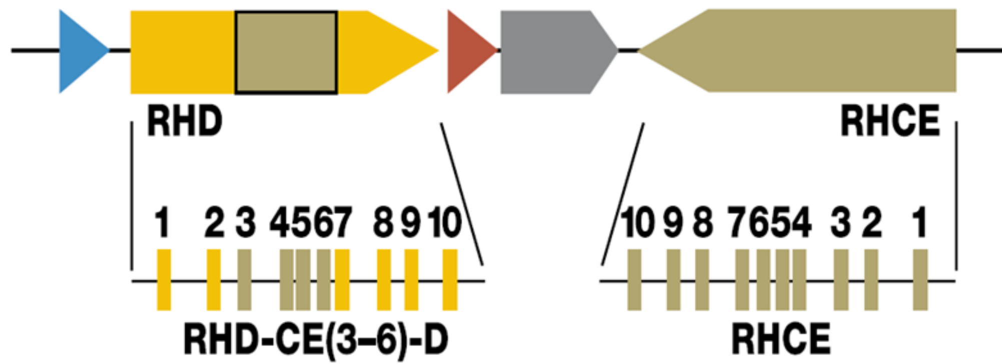

Figure 3: *Structure of the RHD-RHCE gene region on chromosome 1.*

Source: Bugert P., Teaching materials of the Institute for Transfusion Medicine and Clinical Immunology of Mannheim Medical Faculty, Heidelberg University, Acquired with permission 13.03.2019

As both ABO and Rh phenotypes are hard to screen on a genetic basis and serotyping is routinely performed for both, they do not present promising candidates for genetic screening studies.

On the other hand, several blood group systems are based on small genetic variations and associated with relevant transfusion reactions. As examples, the MNS, KEL and FY systems will be examined.

The MNS blood group system is represented by the Glycophorin A and B proteins of the red cell surface. As a very complex system it includes a large number of antigens which are determined by many SNPs in the *GYPA* and *GYPB* genes[21]. Clinically speaking, transfusion reactions have been described against many antigens of the MNS system[21]. The most relevant antigens are M (MNS1) and N (MNS2) encoded by *GYPA* as well as S (MNS3) and s (MNS4) encoded by *GYPB*.

Table 2: *Relevant antigens of the MNS system adapted from the ISBT table for the MNS system*[22]

Phenotype	Allele name	Nucleotide change	Exon	Predicted amino acid change	Gene
MNS:1 or M+	*GYPA*01* or *GYPA*M*				GYPA
MNS:2 or N+	*GYPA*02* or *GYPA*N*	c.59C>T; c.71G>A; c.72T>G	2	p.Ser20Leu; p.Gly24Glu	
MNS:4 or s+	*GYPB*04* or *GYPB*s*				GYPB
MNS:3 or S+	*GYPB*03 or GYPB*S*	c.143C>T	4	p.Thr48Met	

KEL denotes another blood group system whose antigens are genetically differentiated by several SNPs[23]. It is based on the CD238 endopeptidase and contains, apart from the K/k antigen pair, a number of other clinically relevant antigens, including Kp(a/b), Js(a/b) and Côte/Wk(a)[24]. Each of these antigen pairs is associated with transfusion reactions.
K/k is associated with an insidious type of hemolytic disease of the fetus where antibody levels do not correlate with disease activity[25].

Table 3: *Relevant antigens of the KEL system adapted from the ISBT table for the KEL system*[26]*. Note that KEL*02 also encodes KEL4, KEL7 and KEL11 (=Côte)*

Phenotype	Allele name	Nucleotide change	Exon	Predicted amino acid change	Gene
KEL:1,−2 or K+ k−	KEL*01.01	c.578C>T	6	p.Thr193Met	KEL
KEL:2 or k+	KEL*02				
KEL:3,−4,−21 or Kp(a+b–c–)	KEL*02.03	c.841C>T	8	p.Arg281Trp	
KEL:6,−7 or Js(a+b–)	KEL*02.06	c.1790T>C	17	p.Leu597Pro	
KEL: −11,17	KEL*02.17	c.905T>C	8	p.Val302Ala	

Lastly, Duffy (FY[7]) is a blood group system with high relevance for centers with donors or patients of African ethnicity. The DARC glycoprotein, encoded by the ACKR1 gene is a chemokine receptor with a proven role in the transmission of malaria[27].

Consequently, there is a high proportion of ethnically African donors which are not positive for either of the two alleles of the Fy(a/b) dimorphism[28].

***Table 4**: Relevant antigens of the FY system adapted from the ISBT table for the FY system*[29]

Phenotype	**Allele name**	**Nucleotide change**	**Exon**	**Predicted amino acid change**	**Gene**
FY:1 or Fy(a+)	*FY*01* or *FY*A*	c.125A>G	2	p.Asp42Gly	ACKR1
FY:2 or Fy(b+)	FY*02 or FY*B				
Fy(a−b−) erythroid cells only	FY*02N.01	c.-67T>C	Promoter	p.0	

1.4 Extended Match Transfusions and Maternal Screening

Under the assumption that avoiding antibody formation caused by rare blood types can contribute to a reduction in transfusion related adverse events, especially in chronically transfused patients[30], several strategies have been employed to reduce immunization:

Multiple methods have been suggested to reduce antibody formation regardless of incompatibility between donor and recipient[31]. These include suppressing recipient immune response (e.g. splenectomy, immunosuppression) as well as treating blood products to reduce the immune system's reaction to them (e.g. leukoreduction, shorter storage before transfusion).

While there is some evidence that these may be effective, avoiding antigen exposure in the first place should be more effective. Prospective trials have been undertaken in this regard and showed a greatly reduced incidence of alloantibodies when trying to match donor and recipient blood type closer than ABO and D matching only[30, 32, 33]. There is also some retrospective data[34].

This transfusion strategy is commonly known as extended matching. There is no definitive international definition for what extended matching entails.

Most trials have included at least K, C and E as an additional phenotype to be matched[32], other trials included k, Fy^a, Fy^b, Jk^a, Jk^b, M, N, S and s[34]. One trial used a "perfect match" approach and included C, c, E, e, K, k, Kp(a), Kp(b), Js(a), Js(b), Fy(a), Fy(b), Fyb(w), Jk(a), Jk(b), M, N, S, s, Do(a), Do(b), Hy, Jo, LW(a), LW(b), Co(a), Co(b), Lu(a), Lu(b), Di(a), Di(b), Sc1and Sc2[30].
As this strategy leads to an increase in cost of matching compared to ABO, D testing and cross-matching only, it has mostly been tested on patients at risk of alloimmunization (mainly those suffering from either thalassemia or sickle cell anemia[35]). Even for these groups there is still no general consensus as to the overall effect of these measures[36].

Data regarding other groups is even more scarce. A prospective trial on pregnant women found no advantage for extended match transfusions[37], as immunization in this group is most likely triggered by fetal antigens rather than transfusions.

Keeping in mind this background, extended matching is still undoubtedly necessary to avoid transfusion reactions against a rare blood type or because the recipient has a rare blood type and developed antibodies against a common blood type[38]. Here extended matching is however understood to mean matching for already existing antibodies which do not target the more common antibody inducing antigens.

1.5 Genotype Screening of Blood Donors and Patients

When determining which blood to provide to which patient two main methods can be used to determine the blood type of both donor and recipient.

Firstly, serological determination of blood group antigens, also known as serotyping has been the go-to method for blood group determination in the past and is still in use as the standard method today[13, 39, 40]. In principle, serotyping involves detecting either the blood type defining antigen present in a tested person's blood or circulating antibodies against it using antigen-antibody reactions. For a detailed overview of the methods used see e.g. Mujahid et. al[41].

Serotyping is available for every blood group known, as alloantibodies are necessary to define a blood group. As the still foremost method used to determine the phenotype of a transfusion recipient, serotyping has several advantages:

1. Since it uses antigen antibody reactions it emulates the physiological mechanism of many transfusion reactions.
2. Stringent and controlled standards are in place to ensure valid results, potentially increasing patient safety[14, 42, 43].
3. Advances in serologic testing allow for fast results from serological testing, ensuring both fast as well as large scale serotyping is possible[41, 44].

Even with these advantages there are limitations to this method[45]. Namely, even modern automated systems are limited in terms of speed necessary to complete any single serologic reaction. In addition to this fact, the need for specific antibodies and standardized RBCs necessary for serotyping cannot always be fulfilled or the antibodies at hand are lacking in terms of either sensitivity or specificity[46, 47].

Secondly, genotyping can be used to determine the status of coding mutations for many blood types.

Genotyping is the use of molecular assays *“to detect the presence or absence of a specific variant (or variants) in DNA”* [48]. As many blood groups are defined by a single genetic variation (e.g. a SNP)[49], it is easy to determine the probable blood type of any individual by determining his or her genotype in regard to these variations. This does of course not exclude the presence of so-called null alleles[48], that is, a mutation downstream or upstream of the tested SNP which has not been tested and suppresses the expression of the blood group one would expect with the tested SNP.

One example of this drawback is the Fy(a-b-) phenotype already briefly mentioned in section 1.3. It is present in approx. 50%-90% of humans of African descent[28, 50]. Here, a promoter mutation stops the expression of an otherwise wild-type FY allele[51] which stops the expression of a chemokine receptor which in turn decreases susceptibility to plasmodium vivax[52].

Screening solely for the Fy(a/b) coding SNP would therefore lead one to wrongly characterize these persons as Fy(a), Fy(b) or expressing both antigens rather than Fy(a-b-).

Genotypic screening is nevertheless helpful in many circumstances: Genomic DNA is easy to extract and relatively stable, thus facilitating long term storage and easy

processing[53, 54]. Genotyping is also possible where serology is hindered by external factors[55]. One clinically relevant example of this is fetal genotyping in D- negative mothers. Here, isolation of fetal DNA in maternal blood can non-invasively precede an indicated anti-D prophylaxis and stop alloimmunization as well as hemolytic disease[56]. Standardization has also started to affect genotyping, thus decreasing risk to the patient[57].

Advances in PCR and processing technique have lead to a large increase in speed, making genotyping the fastest method available to determine the probable blood type of any individual as well as large groups of donors[58].

1.6 High and Medium-Throughput Screening

With the ability to quickly determine the genotypical status of any individual came the idea to use it to screen large numbers of potential blood donors.

One use of this technique is the recruitment of rare blood donors[38]. Since genotyping is not dependent on specific and often limited antisera to determine the probable blood type of a donor and can be used on large numbers of samples at the same time it has been the method of choice for this undertaking[59].

For this reason mass donor screening has gained traction in the past years as a method used in transfusion medicine[60].

There are several genotyping methods that can be used[58]: In general one can differentiate between:

- Low and medium throughput methods which are hard to automate or limited in scope even when automated. Examples include PCR-SSP, where a PCR is performed using two forward or reverse primers, each specific to one allele[61] and a nonspecific primer of the missing type: either reverse or forward. These systems are considered reliable as well as proven[62]. Even with the limited throughput available, mass scale typing is still possible, just more work intensive.
- High-throughput screening utilizes highly automatable techniques that can be scaled up to process thousands of donors or multiple blood groups at the same time. Commercially available systems are able to process up to 116 SNPs in one donor in less than a day or 12 SNPs for 384 samples in 36 hours[58]. While the cost per sample is comparatively low, especially when factoring in personnel

costs[63], specialized systems have to be procured up front and a large number of donors has to be available to guarantee efficient use of such a system.

As the method used in this study, a further examination of the TaqMan™ PCR method seems warranted. First described in 1991[64], the method is based on the cleavage of a DNA probe.

When a specific DNA sequence is present in a sample, a probe can bind to this tested for sequence during PCR. The 5'-3' exonuclease activity of the Taq-polymerase employed in PCR the cleaves the probe thus releasing a fluorescent dye (e.g. FAM© or VIC©) from the 5'-end that was repressed by a quencher bound to the 3'-end of the probe. This creates a detectable fluorescence signal which corresponds to the number of cleaved probe molecules and thus the amount of product. A homozygous sample will, in the same number of PCR cycles and up to a point, generate approx. double the number of separated probes and thus a higher fluorescence compared to a heterozygous sample, leading to a higher signal.

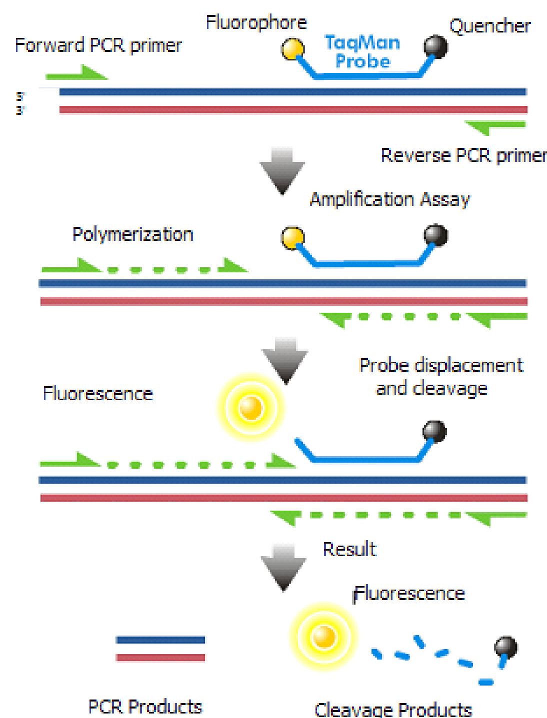

Figure 4: *TaqMan™ working principle. The diagram shows the probed sequence being present. Step one would not take place otherwise. Reproduced from: User: Braindamaged (https://commons.wikimedia.org/wiki/File:Taqman.png), „Taqman“, marked as public domain. Accessed 25.02.19*

The individual approach to mass donor screening differs from setting to setting. While large scale phenotyping studies have been performed in the past[44] and some blood

types, such as ABO are still determined phenotypically[58], genetic screening has become the go-to method for rarer or less immunogenic antigens[56].
National donor databases use mostly high-throughput methods as they have the required number of donors available[65]. Concerted efforts at regional centers to genotype a large number of registered blood donors also make use of high-throughput technology[60].
Low-throughput screening is still in use to determine the genotype of individual recipients, especially where antibodies or former transfusions make serotyping unreliable[58, 66].

1.7 Hypotheses, Aims and Problems

The Institute for Transfusion Medicine and Clinical Immunology of Mannheim Medical Faculty, Heidelberg University is located within the Rhein-Neckar metropolitan region. The area is a region with an ethnically diverse population[67] when compared to the rest of Germany[68]. Consequently, rare blood types must be provided on a regular basis, especially since the higher ratio of patients of African and Mediterranean descent is associated with a higher incidence of thalassemia and sickle cell anemia both of which require frequent transfusions[69].
Since screening for rare blood once the need arises is often a slow and arduous process[38] it is instead usually provided by either

1. Local calling up of or requisitioning available blood products from known rare blood donors. This approach is limited by the low number of locally registered rare blood donors since they are until now not routinely screened for. Further drawbacks include the limited stability of packed RBCs as well as the quality degradation inherent in long term storage[70].
2. Regional requisitioning or international procurement of blood products. This approach is limited by logistical considerations and furthermore only shifts the problem to a third party.

To combat this problem our blood center decided to implement a rare-donor screening program. A large number of donors would be genotyped for several rare blood groups and the results saved in a searchable database to provide rare-blood products on demand. As this was a local approach several hurdles had to be overcome to make screening a feasible undertaking:

1. As commercial high-throughput methods are suited for as many as 100 donors a day[63], which is considerably more donors than was expected to be recruitable and would result in an unaffordable up-front cost, alternative screening methods had to be explored.
2. As a further cost-saving measure and since commercial kits were not available for a few of the blood groups selected for screening, existing commercial testing kits had to either be modified or new methods had to be developed internally.
3. Since no one genotyping method has absolute reliability and the results would be used to guide patient care (though not without additional verification by e.g. serotyping) verification procedures had to be established.

While the end goal was the deployment of rare-donor information to our blood center additional hypotheses were to be tested. Namely:

1. Is there any significant difference in blood-type makeup between our donor pool and a Caucasian, or more specifically, European reference population?
2. Are our screening assays reliable in predicting the blood type of our donors when compared to serotyping?

2 MATERIALS AND METHODS

2.1 Donors

The aim of this study was to establish a screening process which could be applied to the routine testing of every blood donor. Every blood donor at the Institute for Transfusion medicine, as opposed to those participating in blood drives outside the main building of the institute, was automatically enrolled in the study. This included whole-blood donation, plasma separation and granulocyte separation.
Participants consisted of all walk-in blood donors between June 2015 and December 2016.
Walk-in here refers to blood donors who actively visited our institute to donate blood as opposed to participating in a blood drive.

2.1.1 Acquisition and Consent

Before being allowed to donate blood, all donors were, besides being cleared by a specialist physician for transfusion medicine, required to consent to their blood being used for scientific purposes. This was achieved using a standardized consent form[71].
To participate in the extended donor screening, blood donation had to be approved beforehand as DNA extraction was performed from blood drawn in the process of donation. This ensured that consent had been given before any blood was drawn.
There was no monetary or other compensation for participation in the screening program.
There was non-monetary (food, small-value coupons for local businesses, raffles for sporting tickets etc.) and in some cases, such as plasma separation, monetary compensation for donating blood.
This method was approved by an ethics committee of Mannheim Medical Faculty, Heidelberg University.

2.1.2 Demographics

Overall, we tested 2084 donors. The male to female sex ratio was 1.52 (60.4% male). Median age was 32 years with an average age of 36.32 years.

Based on the routine screening protocol in use at our institution, all donations were tested for HIV, HBV, HCV, HAV and Parvovirus B19[72]. For privacy reasons the results of the pathogen testing were not released to our group.

The following blood types were tested serologically, as part of the normal blood donation process, and the results released to us:

- ABO
- CcDEe
- K (but not k)

The precise nature of those tests is not subject of this dissertation and will consequently not be explained in detail. However, all methods are approved for routine testing for clinical purposes and validated independently several times a year.

First-time in-house donors, who represented 20.91% of our collective were tested in this way after their blood sample had already been released to us. Consequently, their test results were not known at the time of screening.

Table 5: *demographic characteristics of the screened donor population*

Age (years)	**% of all donors**
<20	6.70%
20-29	37.80%
30-39	15.90%
40-49	17.20%
50-59	14.50%
>60	7.90%
Sex	**% of all donors**
M	60.40%
F	39.60%

2.2 Screening and Confirmation Testing

2.2.1 DNA Isolation

Blood collection and DNA isolation were not performed by the author but by donation center staff and laboratory staff respectively. For clarity purposes a short breakdown of the procedure is provided here:
During donation a blood sample of up to 3 ml EDTA blood was collected from a reservoir of blood attached to the single use donation kit used by our institute. The primary purpose of this reservoir is to hold the first few ml of blood taken during a donation. This is done to decrease potential bacterial and other contamination of the collected blood (i.e. by discarding the portion of the blood having been in contact with the skin). Additionally, all routine testing (e.g. HIV, Hepatitis B and typing) is performed on these few milliliters of blood.
After receiving the sample, DNA extraction was performed using a commercial DNA extraction kit (Invisorb® Blood Mini HTS 96 Kit, Stratec Biomedical AG, Berlin, Germany).

The following custom protocol was used:
200 µl of EDTA blood were pipetted in 95 of 96 wells (excluding well H12 which was later to be used as NTC) of a collection plate and treated with 200µl proprietary lysis buffer. After mixing and 5-minute incubation at 56°C 20µl of proprietary Proteinase K solution were applied.
This was followed by another incubation at 56° C for 5minutes. This lysis step was followed by a binding step using 200µl proprietary binding buffer and a subsequent short centrifuge spin at 200g. Afterwards, the lysate was transferred to another 96 well binding plate which was then incubated at room temperature for one minute. The combined collection plate and binding plate were then centrifugated at 3500 rpm for 20 minutes to transfer lysate to the collection plate. After this the DNA was purified using three washing steps each consisting of:

- Discarding of liquid in collection plate
- Addition of either 600µl proprietary "wash buffer I" (step 1) or 700µl "wash buffer II" to the collection plate
- Centrifugation at 3500 rpm for 5 minutes

The collection plate was then emptied and centrifuged for another 10 minutes at 3500 rpm.
The DNA was then eluted by placing the collection plate on top of a 0.5 ml elution plate and adding 250µl Elution Buffer D. After 10 minutes of incubation at room temperature and 5 minutes' centrifugation at 3500 rpm the DNA eluate was to be found inside the elution plate.

2.2.2 TaqMan™ Based Screening

In total, 21 SNPs or mutations other than SNPs were investigated. These code for a variety of blood groups antigens. A breakdown of the screened loci and their associated entry in the NCBI database of genetic variation (dbsnp)[73] is provided in the following table:

Table 6: *Polymorphisms screened via PCR-SSP*
rs 2143918 has a 100% association with P phenotypes except in rare African donors[74]

Gene	Allele	associated blood type	dbsnp
A4GALT	(+)2857 T	P1 (not coding)	rs2143918
	(+)2857T/G	P1(not coding)	
	(+)2857G	P2 (not coding)	
ABCB6	LAN (574C)	LAN (+)	rs149202834
	LAN (574T/C)	LAN (+)	
	LAN (574T)	LAN (-)	
	LAN (1942C)	LAN (+)	rs376664522
	LAN (1942T/C)	LAN (+)	
	LAN (1942T)	LAN (-)	
ACHE	YT1	Yt(a)	rs1799805
	YT1/2	Yt(a/b)	
	YT2	Yt(b)	
AQP1	CO1	Co(a)	rs28362692
	CO1/2	Co(a/b)	
	CO2	Co(b)	
ART4	DO1	Do(a)	rs11276
	DO1/2	Do(a/b)	
	DO2	Do(b)	
BCAM	LU1	Lu(a)	rs28399653
	LU1/2	Lu(a/b)	
	LU2	Lu(b)	
	LU8	Lu8	rs28399656

	LU8/14	Lu8/14	
	LU14	Lu14	
	LU18	Au(a)	rs1135062
	LU18/19	Au(a/b)	
	LU19	Au(b)	
ACKR1	FY1	Fy(a)	rs12075
	FY1/2	Fy(a/b)	
	FY2	Fy(b)	
	(-)67T	Fy	rs2814778
	(-)67T/C	Fy	
	(-)67C	Fy(0)	
ERMAP	SC1	Sc1	rs56025238
	SC1/2	Sc1/2	
	SC2	Sc2	
GPA	MNS1	M	rs7687256 and rs7658293
	MNS1/2	MN	
	MNS2	N	
KEL	KEL1	K	rs8176058
	KEL1/2	K/k	
	KEL2	k	
	KEL3	Kp(a)	rs8176059
	KEL3/4	Kp(a/b)	
	KEL4	Kp(b)	
	KEL6	Js(a)	rs8176038
	KEL6/7	Js(a/b)	
	KEL7	Js(b)	
	KEL11	Côte	rs61729034
	KEL11/17	Côte/Wk(a)	
	KEL17	Wk(a)	
SMIM1	64-80ins17	Vel(+)	rs566629828
	64-80del17/ins17	Vel(+)^{w} (most likely)	
	64-80del17	Vel(-)	
SLC14A1	JK1	Jk(a)	rs1058396
	JK1/2	Jk(a/b)	
	JK2	Jk(b)	
SLC4A1	DI1	Di(a)	rs2285644
	DI1/2	Di(a/b)	
	DI2	Di(b)	
	DI3	Wr(a)	rs75731670
	DI3/4	Wr(a/b)	
	DI4	Wr(b)	

Initially a 96-well plate was prepared using the 1µl of DNA eluate from step 2.2.1. per well. This eluate was diluted by adding 6,3µl of distilled water/well. Well 96 (position H12) contained no DNA and was used as a NTC.
A mixture of 7.6µl/well of proprietary Hot Rox high Rox master mix (BIORON GmbH, Ludwigshafen, Germany) and a varying amount of primer-probe mix was prepared in batches sufficient for 96 to 384 wells. The exact amount and content of the Primer-Probe mix were specific to each assay. Generally, however, a modification of a proprietary protocol using prepared primer/probe mixes was utilized.
Primer probe mixes were commercially sourced (Life Technologies, Darmstadt, Germany) except in the cases of the MN and Vel(-) defining mutations where individually prepared mixes were used. The Vel(-) mix was already described before this project[75]. The primers and probes for MN are listed in **Table 7**.

Table 7: *sequences for MN TaqMan™-PCR primers and probes*

<table>
<tr><td>Blood group system (SNPs)</td><td>Antigens</td><td>PCR-Primer (5’-3’ sequence)</td><td>TaqMan™ MGB-Probes</td></tr>
<tr><td rowspan="2">MNS (rs7687256, rs7658293)</td><td>MNS1 (M)</td><td rowspan="2">forward: CTCAGTCACCTCGTTCTTAATC
reverse: GGCAAGAATTCCTCCATAGTAG</td><td>6-FAM-CACTGGTGTGGCAA</td></tr>
<tr><td>MNS2 (N)</td><td>VIC-CACTGAGGTGGCAA-MGB</td></tr>
</table>

Validation of essays (see 3.1.1) lead to the amount of primer/probe mixes listed in **Table 8** being used during the screening process.

Table 8: *primer/probe mix amounts used per well*

Assays	amount primer-probe mix per well
Vel, MN	0.375 µl
Yt(a/b), Co(a/b), Lu 8/14	0.187 µl
all other assays	0.093 µl

***Table 9**: Ingredients for 100µl primer/probe mix as used for the MN and Vel assays*

Ingredient	Amount
Primers	9 µl each
Probes	2 µl each
Distilled water	78 µl

After adding the prepared solution to each of the wells, TaqMan™ PCR was performed using a ABI 7000 real-time PCR cycler (Applied Biosystems, Darmstadt, Germany). TaqMan™-PCR was performed using fluorescence measurement prior to starting PCR (pre-read) and after completition of PCR (post-read).
The selected PCR program was the standard genotyping program of the utilized cycler and can be seen in **Figure 5**

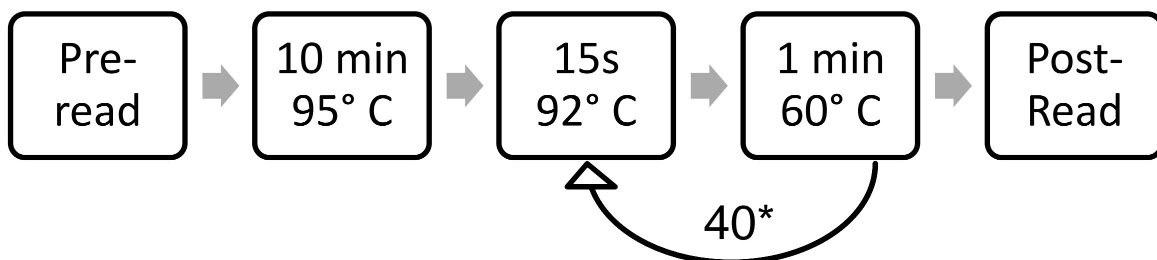

***Figure 5**: PCR program for TaqMan™ PCR*

Readouts were assessed on a graphical interface which charted post-read fluorescence levels after subtracting pre-read fluorescence levels. Samples were rated as homozygous when they showed fluorescence levels consistent with the NTC for one allele as signified by 6-Carboxyfluorescein (FAM©) fluorescence level, i.e. they were rated as negative for that allele, and greater than the NTC for the other allele, as signified by VIC© fluorescence level or vice versa.
Samples were rated as heterozygous when both FAM© and VIC© fluorescence levels were greater than those of the NTC. As 95 samples were tested at the same time, samples belonging to one genotype formed "clouds" on the results scale. Thus, reactions that did not perform as expected could be identified even if their fluorescence differed from the NTC.

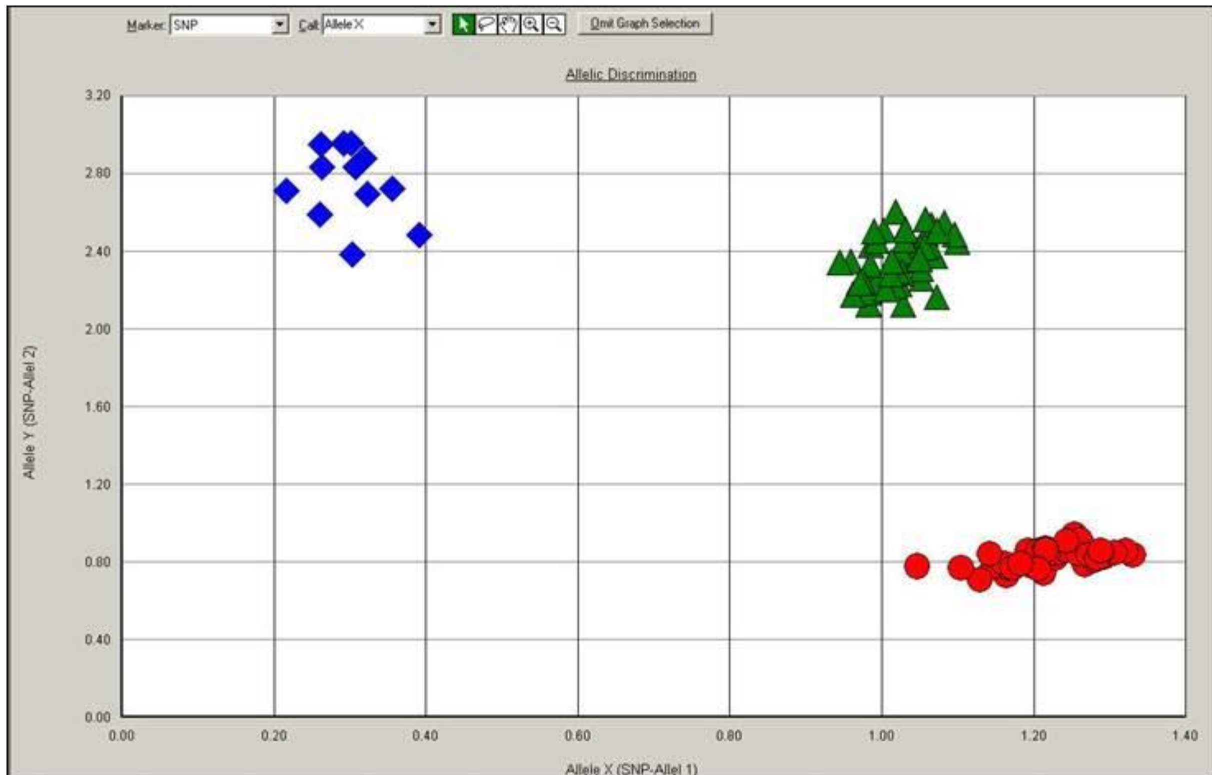

***Figure 6**: Representative readout of our TaqMan© PCR system. Each symbol group represents one of the sample groups outlined above with green triangles representing heterozygous samples.*

A representative read-out can be seen in **Figure 6**. All results were archived as both fluorescence values pre- and post-read as well as differences.

Plausible results were entered into a Microsoft Access© (Microsoft Corporation, Redmond, WA, USA) database. Where appropriate, SSP-PCR based confirmation testing was performed as specified in section 2.2.3.

2.2.3 SSP-PCR Based Confirmation Testing

Whenever a sample was rated as belonging to a rare blood type associated genotype, PCR-SSP based confirmation testing was performed. Additionally, PCR-SSP was performed whenever a readout in TaqMan™ PCR testing was considered not plausible.

For PCR-SSP part of the HBB gene (β-globin) was co-amplified as an internal control PCR fragment (536 bp) using primers β-glob-F (5'-GGTTGGCCAATCTACTCCCAGG-3') and β-glob-R (5'-GCTCACTCAGTGTGGCAAAG-3'). Genomic DNA (10 to 50 ng) was added to 10 µl PCR reaction mix including each 1µM of Allele specific forward- or

reverse- and the complementary Gene specific primer (see **Table 10**), 0.2 µM each internal control primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 µM each dNTP, and 0.5 units Taq DNA polymerase (Bioron GmbH; Ludwigshafen, Germany).

Table 10: *PCR primers for PCR-SSP*

Gene	Associated antigen	PCR-SSP Primer	
A4GALT	(+)2857 T	specific	CACATCTTTCCTGGGAAGGAATT
	(+)2857T/G	reverse	CACAAAGAACCTGGCTTCTCG
	(+)2857G	specific	CACATCTTTCCTGGGAAGGAATG
ABCB6	LAN (574C)	specific	TTTAGCCTGTGGGTGCTGC
	LAN (574T/C)	reverse	AGGTGTACCTGGCTCCTTTC
	LAN (574T)	specific	TTTAGCCTGTGGGTGCTGT
ACHE	YT1	specific	CATCAACGCGGGAGACTTCC
	YT1/2	reverse	GGGAGGACTTCTGGGACTTC
	YT2	specific	CATCAACGCGGGAGACTTCA
AQP1	CO1	specific	GGGGAACAACCAGACGGC
	CO1/2	reverse	GCACGGAAGATGCTGATCTGG
	CO2	specific	GGGGAACAACCAGACGGT
ART4	DO1	specific	GTTGACCTCAACTGCAACCAGTT
	DO1/2	forward	CTCCACATCCCTCCTGAAAG
	DO2	specific	GACCTCAACTGCAACCAGTC
BCAM	LU1	specific	TCTCAGCCGAGGCTAGGT
	LU1/2	forward	CCAGGGAGACCCATAACAAG
	LU2	specific	TCTCAGCCGAGGCTAGGC
	LU8	specific	CTCTCCCAGAGGGCTACAT
	LU8/14	reverse	GAGGTCAAAGGCCAGCACAG
	LU14	specific	GATCTCTCCCAGAGGGCTACAA
ACKR1	FY1	specific	CTTCCCAGATGGAGACTATGG
	FY1/2	reverse	GGCACCACAATGCTGAAGAG
	FY2	specific	CTTCCCAGATGGAGACTATGA
	(-)67T	specific	CCTCATTAGTCCTTGGCTCTTAT
	(-)67T/C	reverse	CAAAGGGAGGGACACAAGAG
	(-)67C	specific	CCTCATTAGTCCTTGGCTCTTAC
ERMAP	SC1	specific	CTCTCTCCCTCTGGCCCG
	SC1/2	reverse	CCCTTATATTCCGGCATCAGATC
	SC2	specific	CTCTCTCCCTCTGGCCCA
GPA	MNS1	specific	CATATCAGCATTAAGTACCACTGGT
	MNS1/2	reverse	GGCAAGAATTCCTCCATAGTAG
	MNS2	specific	CATATCAGCATTAAGTACCACTGAG

KEL	KEL1	specific	GACTCATCAGAAGTCTCAGCA
	KEL1/2	forward	GGGAGATGGAGATGGAAATGG
	KEL2	specific	GACTCATCAGAAGTCTCAGCG
	KEL3	specific	CTTGTCAATCTCCATCACTTCAT
	KEL3/4	reverse	AGGGCACTAGGAGGAAGAAG
	KEL4	specific	CTTGTCAATCTCCATCACTTCAC
	KEL6	specific	TGCCTGGGGGCTGCCC
	KEL6/7	reverse	GGCCCTTGACACTTGCATAC
	KEL7	specific	TGCCTGGGGGCTGCCT
	KEL11	specific	GGCAAGCTCTTCCAGATGGT
	KEL11/17	reverse	CCTTAGAGGAGGGACACAAAG
	KEL17	specific	GGCAAGCTCTTCCAGATGGC
SMIM1	64-80ins17	specific	GCAGCAGGGACGGAGTCA
	64-80del17/ins17	reverse	CCAAAGGCTGCGGTTTGCTG
	64-80del17	specific	GCAGCAGGGACGGAGTCC
SLC14A1	JK1	specific	TCTTTCAGCCCCATTTGAGG
	JK1/2	reverse	AGACAGCAAGTGGGCTCAAG
	JK2	specific	GTCTTTCAGCCCCATTTGAGA
SLC4A1	DI1	specific	GGGTGGTGAAGTCCACGCT
	DI1/2	reverse	TCCTGCCTGCCCTAGTTCTG
	DI2	specific	GGTGGTGAAGTCCACGCC
	DI3	specific	CACTGGGCTTGCGTTCCG
	DI3/4	reverse	TGGGAGAATGCCAGGGAAAG
	DI4	specific	CACTGGGCTTGCGTTCCA

The cycling conditions were: 2 min initial denaturation at 95 °C, followed by 10 cycles with 20 s denaturation at 95 °C and 1 min annealing/extension at 65 °C, followed by 20 cycles with 20 s denaturation at 95 °C, 1 min annealing at 61 °C and 30 s extension at 72 °C. Amplification products were separated on 2 % agarose gels containing GelRed DNA stain (Biotium). Results were documented using a UV gel documentation device (UVP Inc., Upland, CA, USA).

When discrepant results were obtained between PCR-SSP and TaqMan™ PCR, PCR-SSP results were given precedence over TaqMan™ results. Results were entered into the same database as the TaqMan™ results. Additionally, all gel photographies were stored digitally for permanent documentation.

2.3 Analysis and Statistics

All measured genotype distributions were compared to an ideal Hardy-Weinberg equilibrium based on the allele frequencies obtained during screening. Any deviations from such an equilibrium could point to a test which is unable to effectively discern heterozygous from homozygous samples. A chi² test and G-test was used to effect this comparison using SAS v.9.4 (SAS Institute Inc., Cary, NC, USA).
The obtained results for each genotype were compared to the expected genotype distribution obtained via Formula 1.

$$n_{a1} = {f_{a1}}^2 * 2048 \; ; \; n_{hz} = 2 * f_{a1} * f_{a2} * 2048$$

Formula 1: *Expected genotype numbers used for chi² comparison testing where* n *represents a number of expected donors with* a *genotype and* f *represents an allele frequency.* a *represents any allele (with* 1 *and* 2 *being the antithetical alleles) while* hz *denotes a heterozygous genotype.*

Additionally, all allele frequencies were compared to the allele frequencies obtained by the ExAC exome sequencing project where possible[76]. Here, the white, non-Finnish subgroup of the ExAC collective was used as the reference. As before, a chi² test and G-test was utilized.
In addition, Yates correction was applied to tables with cell counts <5.
Allele numbers determined during tested were compared to expected allele numbers based on the ExAC data which were obtained by multiplying the allele frequencies by 4168.

The significance level for all comparisons was set at α=0.05

The frequency data for each comparison was entered manually after calculation of frequency data in Excel (Microsoft Corporation, Redmond, WA, USA). The following code was used:

***Figure 7**: Code used for Chi² testing against ExAC data. || denote individually changed values*

```
data |library|.|SNP| ;
      input  Allele $  Source $  Count @@;
      datalines;
|Allele 1| Study |frequency| |Allele 1| ExAC |frequency|
|Allele 2| Study |frequency| |Allele 2| ExAC |frequency|
;
run ;
TITLE1 '|SNP|';
proc freq data=|library|.|SNP|;
      tables Allele*Source / CHISQ;
      weight Count;
run;
```

***Figure 8**: Code used for Chi² testing for distribution probability. || denote individually changed values*

```
data |library|.|SNP|;
      input  Genotype $  Source $  Count @@;
      datalines;
|Allele 1| Study |frequency| |Allele 1| expec |frequency|
|Allele 1| Study |frequency| |Allele 1| expec |frequency|
hz Study |frequency| hz expec |frequency|
;
run ;
TITLE1 '|SNP|';
proc freq data=|library|.|SNP|;
      tables Genotype*Source / CHISQ;
      weight Count;
run;
```

3 RESULTS

3.1 Validation of Assays

To validate both our PCR-SSP as well as TaqMan™ assays both methods were tested with samples whose phenotype had been confirmed serologically. All in all, 8 donors with each at least one known rare blood type were selected. When their rare blood types were not specifically tested they formed the presumably wild type controls to the rare blood types other donors were positive for.

3.1.1 TaqMan™ Assays

TaqMan™ screening was able to clearly differentiate between both types of homozygous donors and heterozygous donors in all cases. However, there were no serotyped samples available for validation of SNPs rs61729034 and rs376664522. Consequently, it was only possible to determine that the assay was able to determine the much more frequent of the 2 genotypes and did not falsely identify any validation sample as belonging to the rare non-available genotype.

To facilitate a lower use of laboratory materials all assays were subsequently repeated with lower amounts of primer probe mixes until the amounts listed in **Table 8** were deemed the minimum amount of materials that could be used to distinguish between the different groups with a reasonable amount of certainty.

3.1.1 PCR-SSP Methods

All PCR-SSP methods were successfully validated for one allele per specific primer. A representative result can be seen in **Figure 9**. The validation limitations outlined above apply to the PCR-SSP validation as well.

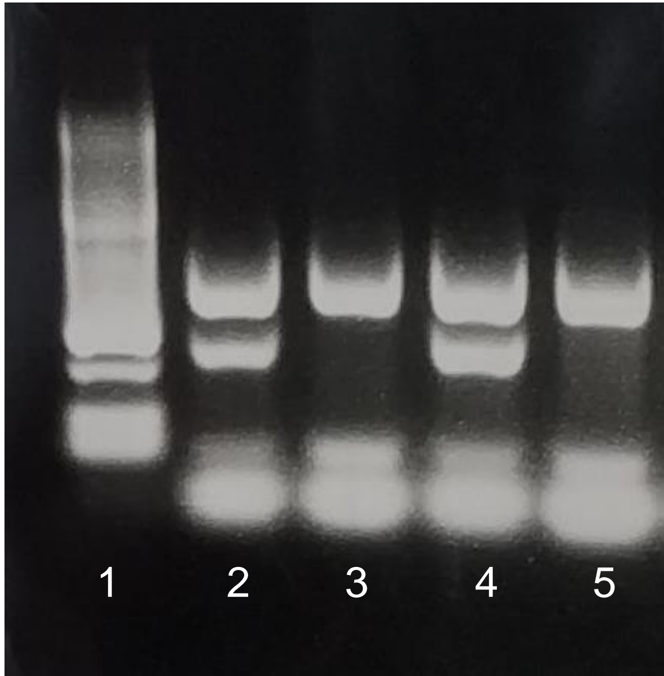

Figure 9 : *representative PCR-SSP result. Pictured are two samples being tested for the rs61729034 SNP which codes for the Côte/Wk(a) polymorphism. Well 2 and 4 carry the specific primer for Côte and show amplification in the expected bp range while well 3 and 5 carry the specific primer for Wk(a) and show no amplification. All wells, excepting the ladder, showed amplification of the internal control (longest visible product in each well) Both samples were therefore identified as homozygous for the Côte coding genotype.*

3.2 Screening Results

Initial screening was completed between August 2015 and May 2017 and included the donors listed in section 2.1.

3.2.1 Allele Distribution

Using the approach declared in 2.2 the allele distribution listed in **Table 11** was obtained. All screened donors could be identified as belonging to one of the screened genotypes.

Table 11: *Allele distribution obtained during initial screening*

dbsnp	associated Antigen	associated blood type	No. of donors	% of all donors	screened donors
rs2143918	(+)2857 T	P1	554	26.58%	2084
	(+)2857T/G	P1	1012	48.56%	
	(+)2857G	P2	518	24.86%	
rs149202834	LAN (574C)	LAN (+)	2064	99.04%	2084
	LAN (574T/C)	LAN (+)	20	0.96%	
	LAN (574T)	LAN (-)	0	0.00%	
rs376664522	LAN (1942C)	LAN (+)	2084	100.00%	2084
	LAN (1942T/C)	LAN (+)	0	0.00%	
	LAN (1942T)	LAN (-)	0	0.00%	
rs1799805	YT1	Yt(a)	1848	88.68%	2084
	YT1/2	Yt(a/b)	226	10.84%	
	YT2	Yt(b)	10	0.48%	
rs28362692	CO1	Co(a)	1931	92.66%	2084
	CO1/2	Co(a/b)	151	7.25%	
	CO2	Co(b)	2	0.10%	
rs11276	DO1	Do(a)	319	15.31%	2084
	DO1/2	Do(a/b)	1029	49.38%	
	DO2	Do(b)	736	35.32%	
rs28399653	LU1	Lu(a)	4	0.19%	2084
	LU1/2	Lu(a/b)	153	7.34%	
	LU2	Lu(b)	1927	92.47%	
rs28399656	LU8	Lu8	1987	95.35%	2084
	LU8/14	Lu8/14	96	4.61%	
	LU14	Lu14	1	0.05%	
rs1135062	LU18	Au(a)	1001	48.03%	2084
	LU18/19	Au(a/b)	932	44.72%	
	LU19	Au(b)	151	7.25%	
rs12075	FY1	Fy(a)	401	19.24%	2084
	FY1/2	Fy(a/b)	999	47.94%	
	FY2	Fy(b)	684	32.82%	
rs2814778	(-)67T	Fy	2031	97.46%	2084
	(-)67T/C	Fy	47	2.26%	
	(-)67C	Fy(0)	6	0.29%	
rs56025238	SC1	Sc1	2067	99.18%	2084
	SC1/2	Sc1/2	17	0.82%	
	SC2	Sc2	0	0.00%	
rs7687256, rs7658293	MNS1	M	589	28.26%	2084
	MNS1/2	MN	1075	51.58%	

	MNS2	N	420	20.15%	
rs8176058	KEL1	K	7	0.34%	2084
	KEL1/2	K/k	171	8.21%	
	KEL2	k	1906	91.46%	
rs8176059	KEL3	Kp(a)	1	0.05%	2084
	KEL3/4	Kp(a/b)	35	1.68%	
	KEL4	Kp(b)	2048	98.27%	
rs8176038	KEL6	Js(a)	0	0.00%	2084
	KEL6/7	Js(a/b)	1	0.05%	
	KEL7	Js(b)	2083	99.95%	
rs61729034	KEL11	Côte	2078	99.71%	2084
	KEL11/17	Côte/Wk(a)	6	0.29%	
	KEL17	Wk(a)	0	0.00%	
rs566629828	64-80ins17	Vel(+)	2003	96.11%	2084
	64-80del17/ins17	Vel(+)w	80	3.84%	
	64-80del17	Vel(-)	1	0.05%	
rs1058396	JK1	Jk(a)	564	27.06%	2084
	JK1/2	Jk(a/b)	999	47.94%	
	JK2	Jk(b)	521	25.00%	
rs2285644	DI1	Di(a)	2083	99.95%	2084
	DI1/2	Di(a/b)	1	0.05%	
	DI2	Di(b)	0	0.00%	
rs75731670	DI3	Wr(a)	0	0.00%	2084
	DI3/4	Wr(a/b)	3	0.14%	
	DI4	Wr(b)	2081	99.86%	

3.2.2 Comparison with Serological Results

The K antigen status is routinely typed by serological methods. For 1920 of the genotyped donors the K antigen status was known: 1753 were K- and 167 were K+. Results for the KEL1/2 associated SNP were compared to the serologically typed K antigen status. The genotypes of the 1920 donors completely matched with the K antigen status, i.e. all KEL2 homozygotes were K- and all KEL1 homozygotes and KEL1/2 heterozygotes were K+.

3.2.3 Comparison with Known Allele Frequencies

The frequencies listed in **Table 11** were each converted into allele frequencies using Formula 2.

$$f = \frac{n_a + 0.5 * n_{hz}}{n_d}$$

Formula 2: *conversion formula to allele frequencies.* f *denotes the allele frequency,* n_a *denotes the number of donors homozygous for a certain allele,* n_{hz} *the number of heterozygous donors and* n_d *the total number of donors*

Afterwards a Chi^2 test was performed comparing the allele frequencies of each SNP to those listed in the ExAC database[76].

Frequencies were unavailable for rs566629828 as this mutation is not located within the exome. Since the laboratory this publication originates from had previously screened a larger number of our own donors for this mutation[75] the frequency obtained in this study was instead compared against this data.
The same applies to rs2814778, though no comparison data from this laboratory was available. Therefore our data was compared against the European super population of the 1000 genomes project[77].
As rs7687256 and rs7658293 were not properly represented in the ExAC database or dbsnp they were instead compared against historical data for MN serotypes in English blood donors[78].

There were significant differences, here defined as a $p<0.05$ between our population and the comparison population in two cases. The rs2814778 SNP encoding the Fy(0) phenotype showed a significantly higher frequency of the variant allele (59 vs. 25 expected). Also, the variant allele of the rs28399656 SNP encoding the Lu14 antigen was significantly more frequent in our population (98 vs. 61 expected).

3.3 Probability Analysis

The probability analysis as outlined in 2.3 revealed a $p>0.05$ in all but one case. The one exception was rs2814778.

The rs2814778 SNP showed a larger number of homozygous Fy0 donors (6 vs.0.4 expected) leading to a p of ~0.04 or ~0.03 with a G-test as several cell numbers were smaller than 5.

4 DISCUSSION

4.1 Validity of Methods

Given that all validation testing could be performed as expected and showed the employed methods to be working, some notable observations must be kept in mind:

- As noted, some PCR-SSPs could not be validated on positive samples as none were available for those rare mutations.
- This naturally applies to the appropriate TaqMan™©-PCR assays as well.

Concerning those mutations, namely rs61729034 and rs376664522 a different approach seems warranted for each.

The test for rs61729034 was, during the performed screening, able to identify several samples positive for the rare Wk(a) coding mutation. These results were confirmed using PCR-SSP and thus verified using a second testing method. All donors identified were positive for the antithetical Côte allele coding mutation. The measured frequencies were in line with comparison data and not skewed towards a non-Hardy-Weinberg distribution.
All in all, this was judged as enough evidence to accept the measured results as valid for the purpose of the study aim even without definitive verification with a positive sample which had been serologically typed beforehand. Should any differences show between the measured genotype and the serologically determined phenotype of our donors, this assumption will have to be revisited.

The rs 376664522 SNP on the other hand, represents a very rare mutation leading to a Lan(-) phenotype. To date, this mutation was described only in a few donors[79] but was present in the original study describing the Lan(-) mutation[80]. ExAC showed a frequency of below 0,1‰ in the European subpopulation which was also the highest frequency measured in any subpopulation[76] in this study. We were unable to validate our assay, as material positive for this mutation was not available for validation. Given however, that no donors positive for this mutation in our study of approx. 2000 donors is a very probable result it was decided to accept this limitation of our study.

As a further point, the assays for rs12075 and rs2814778 were not correlated with each other. As rs2814778 codes for a mutation which renders rs12075 null[27], some results for the latter SNP do not represent the accurate genotype. Since the aim of this study was to recruit potential rare blood donors and not to definitely type blood donors, this limitation was accepted. Before clinical use of all our results, serotyping will have to be performed.

While the Chi² test is generally regarded as unsuitable for cell values smaller than 5, the test was nevertheless applied to such values. At the same time a G-Test, which is considered to be superior for cell values <5[81] was performed and showed no difference in significance . That is, no two significances in both tests for the same cell values were on different sides of the 0.05 significance threshold.

4.2 Characterization of The Rhein-Neckar Blood Donor Population by Blood Group Alleles

Upfront, a European reference population was chosen as a comparison reference for our study population. This was done because our region, while comparatively rich in immigrants[67], is populated mostly by humans of European ethnicity. Given that most immigrants are from European countries and Germany itself has a lower proportion of immigrants than our region[68], one might potentially argue that our donor population is more uniformly European than the average German donor population.
Before this background, the almost non-existent deviations of our population from our reference population are a strong argument for our population being indeed very similar to, if not characteristic of, an “average” European blood donor population.
This, of course, only applies insofar as blood group antigen distribution is concerned. Nevertheless, some deviations from the expected distribution could be measured.

4.3 Deviations from Known Frequencies of Blood Group Genotypes

Somewhat unexpectedly, there were differences between the study population and the reference population used for comparison. Regarding those differences, several explanations can be entertained as possible.

The difference in rs2814778 positive donors could theoretically be a quirk of our local population but this seems unlikely given the nearly 3-fold increase in Fy0 mutation carrying blood donors. More likely, our study population includes a few donors of African ethnicity.
Fy0 is a mutation which confers resistance to plasmodium vivax[51] and is consequently a high incidence antigen in African populations, with our comparison reference listing a frequency of 96% in African populations as opposed to less than 1% in European populations[77].
Thus, even as few as the six homozygous donors in our population can lead to statistical significance.
As transfusion reactions to Duffy positive blood have been reported in the past[82] it is imperative to be able to provide Duffy negative blood to patients with this genotype when needed.

On the other hand, the difference in rs28399656 seems harder to explain. Lu14 is an antigen which differs in a single amino acid from its antithetical Lu8 antigen[83]. It has, as far as was possible to find out by this author, only been researched upon in the context of its discovery and regarding its low tendency to cause transfusion reactions[84]. According to the gnomAD database the prevalence of the rs28399656 minor allele is different between ethnic groups (**Table 12**) with the highest in Europeans (~0.015) and the lowest in Africans (0.001)[76].

Table 12: *Population frequencies of the rs28399656 minor allele in the gnomAD database[76]. Screenshot of the database web interface.*

Population	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
European (non-Finnish)	1890	125576	26	0.01505
Ashkenazi Jewish	144	10198	0	0.01412
Other	87	7102	0	0.01225
European (Finnish)	234	23038	3	0.01016
Latino	236	35266	0	0.006692
South Asian	93	30492	1	0.003050
African	26	24168	0	0.001076
East Asian	0	19688	0	0.000
Total	**2710**	**275528**	**30**	**0.009836**

In our blood donor cohort, we found a higher frequency of 0.0235 for the minor allele. Consequently, the difference in prevalence is statistically and potentially clinically significant but does not appear to result from any underlying ethnical or other differences in our population.

Interestingly, the minor allele was found with a frequency of 4.5% in a recent genome wide association study of Estonian patients[85]. A large enough influx of donors from that region might potentially lead to the higher prevalence of Lu14 positive donors in our population. However, this seems unlikely as a very large number of such “Estonian” donors would be needed. On the other hand, the minor allele might be underrepresented in the gnomAD database for unknown reasons.

4.4 Usability of TaqMan™ Based Screening in A Clinical Context

To perform the screening mentioned in this work, 97 laboratory days, excluding validation and repetition of non-performing assays, were needed.

Given an average working time of around 90 minutes per laboratory day (excluding cycler time) this amounts approx. 146 hours of routine work or 7 hours per 95 donors screened for all alleles. When incorporating experience gained while repeatedly

performing the same steps and time saved if one works 8-hour days as opposed to setting up all equipment anew every day for 90 minutes of work, it seems reasonable to assume there is a lot of time to be saved vs. the actual 7 hours needed.

By using PCR-SSP only for validation testing one can save the time usually needed for postprocessing of cycled samples. This advantage has been noted before[86] and is one of the reasons why Taq-Man genotyping is considered faster than standard PCR.

Cost-wise the materiel cost per assay is indeed higher than would be the case with PCR-SSP only. Our proposal incurred material costs (excluding new machines) of approx. 25.000 €, which is higher than what would be needed to finance a SSP only screening study.
This, of course, assumes no extra cost for the significantly longer processing time of such a hypothetical study. Processing time is at least doubled with SSP. This is because the additional step of pipetting processed samples onto agarose gel is hard, though not impossible[87], to automate, especially with the budget of a facility tasked with processing regional samples only. Consequently, this last step must be performed manually to retain a financial advantage which increases working time and thus the potential cost if personnel costs are considered as well.
It should be noted, however, that approx. half of the materials ordered for the screening study were not used up during experimentation. These savings, resulting from our changes to the recommended TaqMan™ procedure, can now be invested into the continuation of our genomic screening project.

In conclusion, partly automated screening of 21 blood type defining mutations for approx. 2000 blood donors could be performed at a material cost of less than 13€ per donor and less than 7 hours of working time per 95 donors. These costs represent very conservative estimates and might be considerably (~20-50%) lower with experienced laboratory staff. Whether this is a clinically usable method needs to be decided at an individual level at each institution wishing to institute such a program.

4.5 Possible Workflow for Routine Screening of Blood Donors

Drawing from the lessons learned during the execution of this screening project, a workflow was designed to facilitate an easier screening of blood donors using the method described above. Main aims were to

1. Decrease time to process samples with lower regard given to completing a complete genotypical profile for a single donor first.
2. Conserve reagents by creating larger batches of PCR mix for a single processing step.
3. Reliably confirm rare results.
4. Be able to access and store donor data quickly, securely and reliably. This applies not only to the researchers involved in our screening project but clinicians at the point of care as well.

To achieve those goals the following process (Figure 10) was devised. Using the workflow already established for the screening study our research group can efficiently determine the genotype of many donors.

As for donor selection criteria, it was decided to continue recruiting in-house donors into the extended screening project, thereby continuing to exclude participants in external blood drives. While one might theorize that donors who voluntarily approach blood donation of their own accord, as opposed to participating in blood drives at work or in societal contexts, might do so to gain incentives, our institute does not offer any of the incentives identified as potentially decreasing blood safety in recent literature[88]. Thus, it is hoped that our recruitment approach increases adherence to donation without decreasing recipient safety by requiring donor initiative to be included in our study.

The IT department of our institution is currently working on integrating the acquired data into the point of care system of our blood bank and donor center staff. This way, one can easily access the rare blood type genotype profile of any donor. So far, any specific request for donors of a specific genotype has to be manually answered by our working group. In the future it will be possible to search for rare screening results without this detour.

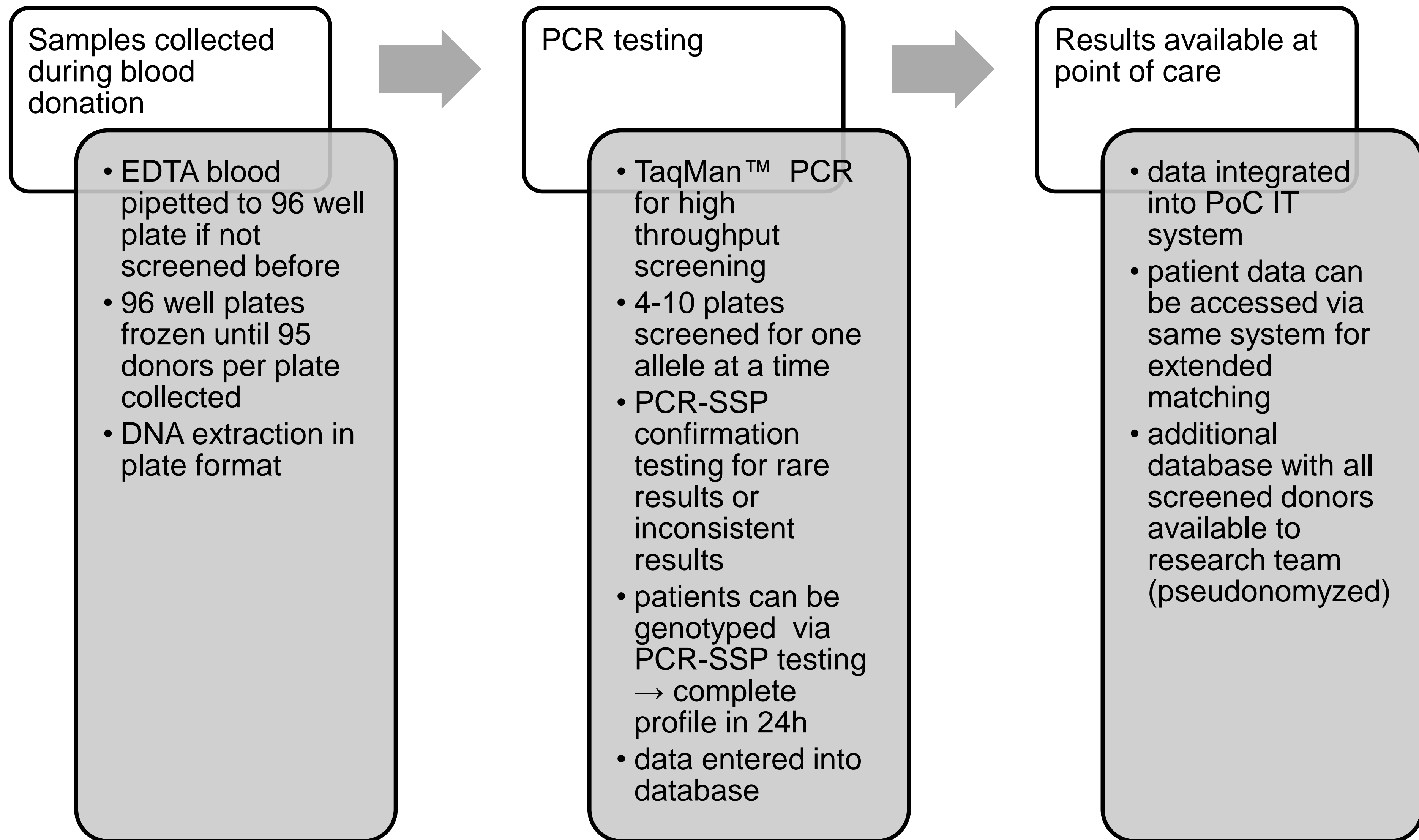

Figure 10: Screening process for new blood donors PoC = point of care

4.6 Possible Impact on Clinical Practice

As outlined in section 1.4, extended genetic blood group profiles of blood donors can be used for a variety of clinical tasks. The most common use is extended genetic matching of recipient and donor before a blood transfusion. In this regard, extended matching can be compared to serological extended matching.

As mentioned before extended matching is generally effective in reducing antibody formation in patients receiving multiple blood transfusions[30, 32-34].

Nevertheless, this is a purely mechanical outcome parameter and therefore not necessarily associated with either outcome or quality of life.

Recent reviews were unable to show a decreased incidence in transfusion reactions even in patients matched via serological extended matching. This was due to the low amount of evidence available as opposed to negative study results.[36] Guidelines for those diseases which are most associated with frequent transfusions do nevertheless recommend extended matching e.g. in thalassemia patients[89].

Therefore, while there is no strong evidence for extended matching and no prospective evidence for genetic extended matching[36] in terms of outcome, it is necessary to be able to provide extended matching for patients with relevant diseases to deliver the recommended standard of care.

As discussed in section 1.7., utilizing extended matching on a regional level can reduce the three most common problems with the necessary provisioning of matched concentrates, especially those of rare antigen status namely:

- Having to start screening donors with every rare blood request leads to prohibitive time increases.
- Storing rare blood for longer time spans increases the risk of degradation[70].
- Escalating to the national or superregional level shifts the burden of providing blood to authorities which might not be able to carry it.

In addition, donors with low prevalence antigens can potentially lead to immunizations versus low prevalence antigens in recipients and cause transfusion reactions later on. This phenomenon has been described for D negative donors and Kp(b) positive donors tested with deficient antisera[56].

This study thus outlines a way to provide reliable and extensive extended matching on a regional level, addressing these problems.

4.7 Conclusion and Outlook

In conclusion, the aims of our donor screening study could be reached for the most part.

Our methods proved to be reliable as far as comparisons with serological results were concerned. While there is still the issue of null alleles as mentioned in section 1.5 the stated aim of our screening, namely to identify potential donors with rare blood types could be reached.
This greatly reduces the necessary effort to serotype such rare donors. Instead of serotyping several hundred to thousand donors a dedicated screening of a handful of potential donors can be undertaken.

The second aim of this study, to identify deviations from known blood group distributions was also successful. The found deviations regarding the Fy(a- b-) status of several of our donors hint towards the need to be able to call on such donors to be able to meet the transfusion needs of our regional population which is liable to be made up of at least some Fy negative donors.

As for our economic goals, namely modifying existing off-the-shelf kits to reduce costs, a simple solution was found in verifying the kits for use with a fraction (0,25-0,5) of the intended amount of primer-probe mix. While certainly not newsworthy or new, simple solutions such as this one should be kept in mind when trying to reduce costs.

Establishing a regional rare donor registry using genotyping studies was thus possible below estimated cost. Consequently, our institute has started to implement this

approach for routine use with all new in-house blood donors. Small and medium sized blood donation centers should take these results, which have already been published[90] to heart when considering whether such a program is feasible.

5 SUMMARY

Providing appropriately matched red blood cell concentrates for patients in need of transfusions is one of the main tasks of transfusion medicine.

Donors and recipients with rare blood groups, especially those outside of the routinely screened ABO, Rhesus and Kell systems are at an increased risk for developing or causing transfusion reactions because their blood types are not routinely tested for and matched RBCs are rarely available at a regional level.

Being able to provide blood products matched for these rare blood types is especially important for patients with an increased need for transfusions over a long period e.g. those with thalassemia and sickle cell anemia. This standard of care is required by current guidelines.

This project screened more the 2000 blood donors for their genotype in 21 coding locations for the

P1/P2, Lan(-), Yt(a)/Yt(b), Co(a)/Co(b), Do(a)/Do(b), Lu(a)/Lu(b), Lu8/Lu14, Au(a)/Au(b), Fy(a)/ Fy(b), Fy(0), Sc1/Sc2, M/N, K/k, Kp(a)/Kp(b), Js(a)/Js(b), Côte/Wk(a), Vel(+)/Vel(+)w/Vel(-), Jk(a)/Jk(b), Di(a)/Di(b) and Wr(a)/Wr(b) blood types. TaqMan®PCR and PCR-SSP assays could be validated for all loci and screened genotype could be determined for all donors.

Statistical analysis and comparison with a European reference population from a large exome screening study (where available) showed no significant difference between the screened donors and the reference except for two loci which code for the Fy(0) type with high differences in worldwide distribution and the Lu14 antigen with little associated data so far.

A cost-effective screening process could be established for the screening of further donors for the blood types named above.

By integrating this screening process into the routine workflow for in-house donors at the Institute for Transfusion Medicine and Immunology rare blood type blood can now be provided on a regional level.

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