Extended Donor Typing by Pooled Capillary Electrophoresis: Impact in a Routine Setting

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Summary

Background: PCR with sequence-specific priming using allele-specific fluorescent primers and analysis on a capillary sequencer is a standard technique for DNA typing. We aimed to adapt this method for donor typing in a medium-throughput setting. **Methods:** Using the Extract-N-Amp PCR system, we devised a set of eight multiplex allele-specific PCR with fluorescent primers for Fy^a/Fy^b, Jk^a/Jk^b, M/N, and S/s. The alleles of a gene were discriminated by the fluorescent color; donor and polymorphism investigated were encoded by product length. Time, cost, and routine performance were collated. Discrepant samples were investigated by sequencing. The association of new alleles with the phenotype was evaluated by a stepwise statistical analysis. **Results:** On validation using 312 samples, for 1.1% of antigens (in 5.4% of samples) no prediction was obtained. 99.96% of predictions were correct. Consumable cost per donor were below EUR 2.00. In routine use, 92.2% of samples were successfully predicted. Discrepancies were most frequently due to technical reasons. A total of 11 previously unknown alleles were detected in the Kell, Lutheran, and Colton blood group systems. In 2017, more than 20% of the RBC units prepared by our institution were from donors with predicted antigen status. A steady supply of Yt(a–), Co(a–) and Lu(b–) RBC units was ensured. **Conclusions:** Pooled capillary electrophoresis offers a suitable alternative to other methods for extended donor DNA typing. Establishing a large cohort of typed donors improved transfusion support for patients.

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Introduction

In the past decade, molecular phenotype prediction has increasingly been replacing serologic antigen determination. Systematic donor typing allows to supply units much better matched to the recipient than conventional approaches starting with antigen determination only if there is an acute need for typed units [1, 2]. A large number of methods have been developed that allow high-throughput typing of blood donors for many antigens [3–12]. These methods differ in the antigens predicted, the number of rare alleles covered, and the cost per antigen and per donor. Attempted high-accuracy prediction of a donor's phenotype [3, 11] contrasts with approaches that expect a serologic confirmation on release [8].

The driving force for large-scale molecular typing differs between populations. In countries with a large number of patients of African descent, the correct identification of 'African' *RHCE* alleles and Dombrock phenotypes is a major challenge and convincing argument to switch to molecular typing [12, 13], because serologic typing is unsatisfactory. In some European countries like Germany, such alleles are nearly absent, and compatible units can usually be identified by serologic methods. In these countries, the major challenge is the provision of blood for patients with antibodies to the high-prevalence antigens Kp^b, Lu^b, Yt^a or Vel [14], and for patients with multiple alloantibodies [15, 16] to the clinical relevant antigens outside ABO and RH. Most of these antigens may be easily determined by serology; hence, any molecular method has to compete with serologic approaches.

A decade ago, we developed a simple multiplex PCR approach that allowed to screen for donors who are negative for high-frequency antigens at less than USD 3.00 per donor [17]. In order to extend this method to the more polymorphic antigens of the Duffy and Kidd blood group system, we adapted our approach to capillary gel electrophoresis with fluorescent dye-labeled sequence-specific primers. Here we present our experience with this improved method.

Material and Methods

Blood Samples

EDTA blood samples were collected for blood group determination from blood donors donating at the German Red Cross Blood Donation Service NSTOB (North-central Germany). The donors consented for scientific use of their samples.

DNA Isolation

DNA was isolated from EDTA blood samples using the Extract-N-Amp Blood PCR kit (Sigma-Aldrich, Taufkirchen, Germany) with slight modifications as reported previously [17].

Multiplex PCR

PCR amplification was performed using the Extract-N-Amp Blood PCR mix with primers and concentrations shown in table 1. Eight distinct primer sets were used that resulted in slightly different product sizes. In addition to primers, MgCl₂ was added to the PCR mixture to a final extra of 1.2 mmol/l. Reactions were initially performed in a volume of 5 µl including 0.5 µl DNA preparation; during routine use reaction size was reduced to a total volume of 3.9 µl. PCR consisted of 6 min initial denaturation at 94 °C, 10 cycles of 15 s at 94 °C and 45 s at 65 °C followed by 29 cycles of 20 s at 94 °C, 30 s at 65 °C, and 1 min at 72 °C and a final 15 min at 72 °C.

Analysis of Pooled Samples by Capillary Electrophoresis

16 PCR products, each obtained with a different primer set, were mixed and cleansed using Nucleo Spin Gel- and PCR Clean up Kits (Macherey-

Nagel, Düren, Germany). 5 µl of amplificate were analyzed using an ABI 310 capillary sequencer (Thermo-Fisher, Darmstadt, Germany). Applied Biosystems GeneScan 500 LIZ dye size standard (Thermo-Fisher) was used as size marker. Donor-specific antigen status was derived from the ratio of blue and green fluorescence or red and yellow fluorescence (for Lu antigens) at the reaction- and gene-specific product sizes. In our hands, the exact position of the amplicons could differ from the calculated by a few base pairs (bp) and even varied from run to run. Therefore, peak positions calculated by the Gene mapper 4.0 Software were exported to an Excel sheet that included formulas for an advanced identification of the peaks based on the expected peak position and the position of neighboring peaks. In short, for each SNP, first the position of the whole group was identified allowing for a few bp offset, and then within each group the peaks were assigned to the respective samples. Analysis included warnings for low signal intensity, off-scale peaks, and borderline fluorescence ratios. Automated analysis was checked by visual inspection of the electropherograms.

IT Solution for Unit Release

An IT solution to use the obtained data as blood unit sorting algorithm was established: The data were linked to the donor registry. For each donation, it was checked on release whether historical data indicated the probable presence of an useful phenotype (e.g. negative for Kp^b or ccD.EE and negative for Fy^a and Jk^a). These units were diverted to a 'laboratory inventory' and kept for 1–4 weeks depending on the rarity of the assumed phenotype. If units of this phenotype were demanded, they were confirmed by serology or used without confirmation for preventive matching. If unused, they were rescheduled to general purpose transfusion when the hold phase was finished.

Table 1. PCR primers used

Name	Sequence*	Product size, bp	Use	Typical concentration, µmol/l
Lua2	NED-cctgcatctcagccgaggctaTgt		Lu ^a specific	0.05
Lub2	ROX-cctgcatctcagccgaggcAagAc		Lu ^b specific	0.04
118b	aaggaccagagagagagagactga	120	Lu reverse set 1	0.04
122b	gagaaaggaccagagagagagagactga	124	Lu reverse set 2	0.04
126c	cagagagaaaggaccagagagagagagactga	128	Lu reverse set 3	0.05
130	gctgcagagagaaaggaccag	132	Lu reverse set 4	0.05
134	aggagctgcagagagaaaggacc	136	Lu reverse set 5	0.05
138	agccaggagctgcagagagaaag	140	Lu reverse set 6	0.05
142	gcagagccaggagctgcagag	144	Lu reverse set 7	0.05
146	agaggcagagccaggagctgcag	148	Lu reverse set 8	0.05
M3F	FAM-gaagaAggtgaagtgtgcattgccacac		M specific	0.10
N3-H2	HEX-gaagaAggtgaagtgtgcattgccacct		N specific	0.04
mnbv6	gggatgtgagAggaattgtcttttgca	154	GYPA reverse set 1	0.09
mnbv604	tTTTgggatgtgagAggaattgtcttttgca	158	GYPA reverse set 2	0.14
mnbv608	TTTTtTTTgggatgtgagAggaattgtcttttgca	162	GYPA reverse set 3	0.14
mnbv612	TTTTtTTTcagggatgtgagAggaattgtcttttgca	166	GYPA reverse set 4	0.14
mnbv616b	TTTCTTTtCTTctcagggatgtgagAggaattgtcttttgca	170	GYPA reverse set 5	0.14
mnbv620b	TTTTTCTTTcTcttagctcagggatgtgagAggaattgtcttttgca	174	GYPA reverse set 6	0.14
mnbv624b	TtTCTCTTTCTTTcTcttagctcagggatgtgagAggaattgtcttttgca	178	GYPA reverse set 7	0.14
mnbv628c	tCTTtTCTCTTTCTTTcTcttagctcagggatgtgagAggaattgtcttttgca	182	GYPA reverse set 8	0.14
coa5	FAM-TgacaccttcacgtgtcctggaTcg		Co ^a specific	0.03
cob5	HEX-cgacaccttcacgtgtccttgacca		Co ^b specific	0.03
Co186	agcggctctcaggccaagccc	188	Co reverse set 1	0.04
Co190	TTTcagcggctctcaggccaagccc	190	Co reverse set 2	0.07
Co194n	TATcTTTcagcggctctcaggccaagccc	194	Co reverse set 3	0.07
Co198	tgagcaccggcagcggctc	198	Co reverse set 4	0.07
Co202	gaattgagcaccggcagcgg	202	Co reverse set 5	0.07

Table 1. continued on next page

Table 1. Continued

Name	Sequence*	Product size, bp	Use	Typical concentration, $\mu\text{mol/l}$
Co206	gaggggaattgagcaccggcag	206	Co reverse set 6	0.09
Co210	ctcagaggggaattgagcaccgg	210	Co reverse set 7	0.12
Co214	agctctcagaggggaattgagcacc	214	Co reverse set 8	0.18
gs4	FAM-gtacagtgaacgatggacaagttgtcAca		S specific set 1,3,5,7	0.24
kS4-H2	HEX-gtacagtgaacgatggacaagttgtcTcg		s specific set 1,3,5,7	0.19
gSI	FAM-TctTgtacagtgaacgatggacaagttgtcAca		S specific set 2,4,6,8	0.36
kSI-H2	HEX-TctTgtacagtgaacgatggacaagttgtcTcg		s specific set 2,4,6,8	0.29
S212	attactaatggaagactgacacattacctcataaattgtt	220 224	GYPB reverse set 1,2	0.34
S220	atactgaaattactaatggaagactgacacattacct	230 235	GYPB reverse set 3,4	0.23
S228b	aaaagtagatactgaaattactaatggaagactgacacattac	239 243	GYPB reverse set 5,6	0.39
S236	gggtcattaaaagtagatactgaaattactaatggaaga	248 252	GYPB reverse set 7,8	0.23
YtaF	FAM-ccactagttacctgcaggTTgtg		Yt ^a specific	0.32
YtbH	HEX-ccactagttacctgcaggcTgtt		Yt ^b specific	0.08
Yt260	agaggcTcgTcgaggcc	261	Yt reverse set 1	0.25
Yt264.2	tgTgagaggcTcgTcgagg	258	Yt reverse set 2	0.33
Yt268	ggcatgTgagaggcTcgTcg	262	Yt reverse set 3	0.33
Yt272c	TgtgggcatgTgagaggcTcgTcg	265	Yt reverse set 4	0.4
Yt272c(-2)	TTTTTgtgggcatgTgagaggcTcgTcg	270	Yt reverse set 5	0.4
Yt 280c	tgTgccacgggtgTgcatgggagag	273	Yt reverse set 6	0.33
yt284b	acTctgTgccacgggtggcatgg	277	Yt reverse set 7	0.33
Yt288b.2	TTtTctTctgTgccacgggtggcatgg	281	Yt reverse set 8	0.33
Fya6	FAM-ggTagctgtctcagggttggcTc		Fy ^a specific	0.43
Fyb6	HEX-ggcagctgtctccaggAtggcat		Fy ^b specific	0.10
fy297	cttcaggctccctgcttctg	298	Fy reverse set 1	0.53
fy301	ggctcttcaggctccctg	298	Fy reverse set 2	0.53
fy305	ctccggctcttcaggctc	302	Fy reverse set 3	0.53
fy309	cctgctccggctcttcag	308	Fy reverse set 4	0.53
fy313	tggtctgctccggctcttca	311	Fy reverse set 5	0.53
fy317	ggactgttctgctccgg	316	Fy reverse set 6	0.53
fy321	cccaggactgttctgctc	320	Fy reverse set 7	0.44
fy326b	cttcccaggactgttctgctc	325	Fy reverse set 8	0.44
jkfa4	FAM-gttgaaaccccagagtcTaaagtagatgtc		Jk ^a specific	0.05
jkfb4-H2	HEX-gttgaaaccccagagtcTaaagtagatgtt		Jk ^b specific	0.06
jk333b	actctctcTcactcatgtgcctg	335	JK reverse set 1	0.07
jk339	tcctactctctcTcactcatgtg	339	JK reverse set 2	0.10
jk343	gccatctactctctcTcact	343	JK reverse set 3	0.07
jk347	gTgagccatctactctctcc	347	JK reverse set 4	0.07
jk351	tcctgTgagccatctactctcc	351	JK reverse set 5	0.10
jk355	attttcctgTgagccatctactctcc	355	JK reverse set 6	0.08
jk359	gTgcattttctgTgagccatctac	359	JK reverse set 7	0.07
jk363b	caggTggcattttctgTgagccatctac	363	JK reverse set 8	0.07
kpa1107F	FAM-ctcttctgtcaatctccatcactAcat		Kp ^a specific	0.18
kpb	HEX-ctcttctgtcaatctccatcactTAc		Kp ^b specific	0.17
kp372b	gcaggacaaccagtcgatggcg	372	Kp reverse set 1	0.29
kp 376b	gcaagcaggacaaccagtcgatgg	376	Kp reverse set 2	0.29
kp380	gcttgcaagcaggacaaccagtcg	380	Kp reverse set 3	0.29
kp384b	gtcgcttgcaagcaggacaaccag	384	Kp reverse set 4	0.35
kp388b	tgaatgtcgcttgcaagcaggacaacc	388	Kp reverse set 5	0.35
kp392b	gggtgtgaatgtcgcttgcaagcagg	392	Kp reverse set 6	0.29
kp396b	catcggtgtgaatgtcgcttgcaagc	396	Kp reverse set 7	0.29
Kp400b	gggacatcggtgtgaatgtcgcttg	400	Kp reverse set 8	0.35

*Big letters denote mismatched nucleotides.

Fig. 1. Outline of the workflow. **Panel A** Schematic workflow of the pooled capillary electrophoresis: From eight donors, a crude DNA preparation was prepared using Extract-N-Amp system. For each sample, two multiplex PCR were performed, eight different variants of the multiplex PCR were used that yielded slightly different product sizes. PCR amplicons of these 2 × 8 multiplex PCR were pooled and analyzed by capillary electrophoresis. **Panel B** For each sample and polymorphism, a specific product size was expected that could show fluorescence for one, the other or both alleles. The fluorescence ratio of the two allele-specific colors was analyzed. **Panel C** Visualization of the fluorescence ration distribution (left panel) and the fluorescence ratio versus mean fluorescence (right panel) allowed to define the regions for heterozygosity (yellow), and borderline results (blue and green) used for the automated analysis of the fluorescence ratio.

Validation

After establishing the reaction conditions in preliminary experiments, 312 samples were tested and the predicted results compared to results obtained with routine serologic methods. Discrepant results were further investigated by repeat serology, repeat pooled capillary electrophoresis, and, if the discrepancy persisted, sequencing.

Sequencing

Sanger sequencing was done using BigDye sequencing kit (Thermo-Fisher). The standard approach for sequencing from genomic DNA was chosen. One or several exons were amplified from genomic DNA and sequenced using the primers also used for amplification or internal sequencing primers. For KEL, the primers of Lee et al. [18] were used.

Statistical Analysis of Mutations in Discrepant Samples

A possible causative role of a polymorphism detected in a sample displaying a discrepant phenotype was evaluated as follows:

- Polymorphisms in known null alleles and alleles with destroyed start codon, stop codons in the coding sequence, or frameshift mutations were considered to be causative due to their structure, if a predicted antigen was not present.

- A mutation interfering with primer binding was considered to be causative due to its structure, if the discrepancy was caused by a false-negative antigen prediction.
- Polymorphisms known to be highly associated with the polymorphism investigated were considered unlikely to be causing a discrepancy. The only example was c.586G>A in BCAM that is highly associated with the Lu^a-expressing LU*01 allele (c.230 G>A) [19].
- The residual samples were counted and the observed mutations evaluated in a sequential manner with correction for multiple testing according to Bonferoni-Holm [20] considering the known allele distribution found in large databases.

For each polymorphism observed, its frequency among non-Finnish Europeans was extracted from the ExAC database (exac.broadinstitute.org) [21]. The cumulative frequency of the encountered and all less frequent polymorphisms was calculated. This cumulative frequency was used to calculate the probability that any polymorphism at least as rare was observed among the collated samples (2n alleles) by chance. If the probability was less than 0.01, the polymorphism was considered most likely associated with the discrepant phenotype, if it was less than 0.05, it was considered likely associated or causative. The samples explained by such polymorphism were removed from the set of samples under consideration, and the calculation was repeated with the residual samples until no sample or no polymorphism with likely association was left.

Hands-On Time and Consumable Cost

Hands-on time and consumable cost were collated and estimated according to the technicians' experience.

Snapshot of Units Available in the Blood Service

Snapshots of the number of available units in our Blood Service were taken from our SAP-based inventory management system (transaction BSD/MM_Chargenbest).

Results

Antigen Prediction by Pooled Capillary Electrophoresis

A medium-throughput donor typing method was developed

based on PCR amplification of crude DNA with fluorescence-labeled allele-specific primers, product length-based barcoding, and analysis by capillary electrophoresis. DNA amplification was performed in two multiplex reactions, one containing primers to predict Co^a/Co^b, Jk^a/Jk^b, Lu^a/Lu^b, and M/N, the other those for Fy^a/Fy^b, Kp^a/Kp^b, S/s, and Yt^a/Yt^b. Each reaction contained sequence-specific primers for both alleles that were 5' labeled with different fluorescence dyes (usually FAM and HEX with the exception of Lu using NED and ROX). Eight variations of these multiplex PCR reactions were developed that yielded PCR products differing by a size of 4 bp from the next shorter version. Thus, eight donor samples were amplified in parallel using eight different versions of the multiplex PCR. All products were pooled, purified, and analyzed in

Table 2. Validation of antigen prediction

System	Molecular analysis		Serologic analysis	
	prediction*	n	confirmed	discrepant serology
JK	Jk(a+b-)	88	88	
	Jk(a+b+)	151	151	
	Jk(a-b+)	72	72	
	no call	1		Jk(a+b-): 1
FY	Fy(a+b-)	54	54	
	Fy(a+b+)	154	149	Fy(a+b-): 5 [§]
	Fy(a-b+)	98	98	
	Fy(a?b+)	2		Fy(a-b+): 1; Fy(a+b+): 1
	no call	4		Fy(a-b+): 2; Fy(a+b+): 2
MN	M+N-	99	99	
	M+N+	151	150	M+N-: 1 [§]
	M-N+	58	58	
	M+N?	1		M+N+: 1
	no call	3		M+N-: 2; M+N+: 1
Ss	S+s-	28	28	
	S+s+	148	148	
	S-s+	129	128	S+s+: 1 [‡]
	S?s+	1		S-s+: 1
	no call	6		S+s+: 4; S-s+: 2
Co	Co(a+b-)	284	284	
	Co(a+b+)	24	24	
	Co(a-b+)	0	0	
	Co(a+b?)	2		Co(a+b-): 2
	no call	2		Co(a+b+): 1; Co(a+b-): 1
LU	Lu(a+b-)	0	0	
	Lu(a+b+)	24	24	
	Lu(a-b+)	286	286	
	Lu(a?b+)	1		Lu(a-b+): 1
	no call	1		Lu(a-b+): 1
YT [†]	Yt(a+b-)	285	285	
	Yt(a+b+)	23	23	
	Yt(a-b+)	1	1	
	No call	3		Yt(a+): 3
Kp	Kp(a+b-)	0	0	
	Kp(a+b+)	6	6	
	Kp(a-b+)	302	302	
	Kp(a?b+)	1		Kp(a-b+): 1
	no call	3		Kp(a-b+): 3

*One antigen not predicted is indicated by an '?', both antigens reported as 'no call'.

[†]Antigen Yt^b was not checked by serology.

[§]Fy^b antigen missed by serology in FY^a allele.

[§]Non-systematic misprediction: repeat molecular prediction correct.

[‡]GYPB*04/GYPB*24 sample.

Fig. 2. Molecular basis of a false-negative antigen S prediction in the Mit allele. The electropherogram of the Mit+ sample (upper sequence) and a control Ss sample (lower sequence) is shown in a region encompassing the primer binding site. The asterisks indicate the 143C/T polymorphism discriminating S and s, the caret the 161G/A polymorphism characterizing the Mit allele. The A of the Mit allele introduces an additional mismatch of the primer which likely prevents efficient primer binding.

a single capillary electrophoresis run (fig. 1). Obtained fluorescence signals were assigned to sample and polymorphisms according to the product size, and allele status was deduced from the ratio of fluorescences that corresponded to the different alleles. The pooling prior to capillary run increased analysis throughput by a factor of 8. Considering a run time of 50 min, the maximal throughput using a single capillary machine was limited to 230 samples per day.

Validation

A comparison of prediction and serologic typing was attempted for 312 donor samples representing 4,992 antigens predicted by pooled capillary electrophoresis. As no suitable anti-Yt^b was available, serologic typing was restricted to Co^a, Co^b, Fy^a, Fy^b, Jk^a, Jk^b, Kp^a, Kp^b, Lu^a, Lu^b, M, N, S, s, and Yt^a (table 2), resulting in a set of 4,680 serologic antigen typings. 23 allele pairs could not be predicted in 12 samples due to low signal, in addition 8 antigens were not predicted because of borderline fluorescence ratios in 7 samples. Two samples yielded both failures due to low signal and borderline ratio, resulting in a total of 54 antigens (including 3 Yt^b) that could not be predicted in 17 samples, corresponding to a rate of 1.1% failed antigen predictions and 5.4% of samples with at least one failed antigen prediction.

Among the evaluable 4,629 antigen predictions, 4,622 (99.8%) were identical to the serologic result. There were 7 discrepancies: In 5 cases, antigen Fy^b was predicted positive, but determined negative by routine serology. These 5 cases were found to be due to the presence of a Fy^x allele known to express a weak Fy^b antigen and considered serologic failures. In one sample, the S antigen was missed in a GYPB*24 allele (Mit-positive allele), because the additional polymorphism in GYPB*24 interfered with primer binding (fig. 2). Finally, 1 sample was predicted as N-positive but found to be N-negative in serology. Repeat molecular testing yielded the correct prediction, and the discrepancy was categorized as random failure due to technical causes. In conclusion, 4,627 of the 4,629 antigen predictions were correct (99.96%), which corresponded to 98.87% of all antigens attempted to predict.

Fig. 3. Inventory management. Use of historical typing data for patient care. On release, an algorithm checks whether antigens historically determined by serology or predicted by pooled capillary electrophoresis are known. If such antigens are known, the antigen pattern is compared to 'suitable' antigen patterns. Units with such patterns are kept in a laboratory inventory for 1–4 weeks depending on the rarity of the expected phenotype. If used for indications necessitating antigen negative units, the predicted antigen status is confirmed by serology. If no matching patient is found, units are used for general purpose transfusions.

Hands-On Time and Consumable Cost

Hands-on time and consumable cost was collated (table 3, hands-on time) and compared to a previously developed molecular typing solution based on agarose gel electrophoresis [17]. While the hands-on time per donor for the set-up was slightly lower than in the gel method, visual proofreading of the results incurred an increase of total hand-on time by 49%. Likewise, product cleanse-up and capillary electrophoresis was more expensive than agarose gel electrophoresis resulting in a 25% increase of cost per donor. Considering that the capillary electrophoresis yielded 16 predictions, while the gel method only yielded 4, the cost per antigen was about a third of the old method.

Routine Use of Pooled Capillary Electrophoresis

Until December 2017, a total of 136,120 donor samples representative of more than 100,000 donors were tested. The system was

Table 3. Hands-on time and price estimate: comparison to agarose electrophoresis

Capillary electrophoresis		Agarose electrophoresis	
process	hands-on time, min	process	hands-on time, min
DNA Extraction	20	DNA Extraction (including additional mixing step)	28
PCR set-up and start	20	PCR set-up and start	21
Post-PCR workup	45		
Electrophoresis set-up	5	electrophoresis set-up	37
Electrophoresis maintenance	5		
Documentation	5	documentation	14
Time per 96 donors	160	time per 91 donors	102
Time per donor	1.04	time per donor	1.12
Proofreading of results	60		
Time per donor (including proofreading)	1.67*		
Time per antigen	0.10	time per antigen	0.28
Consumable/chemical	Cost per test, EUR	Consumable/chemical	Cost per test, EUR
Extract-N-Amp Blood Kit (quarter standard volume)	0.76	Extract-N-Amp Red Blood Kit (half standard volume)	0.76
Oligonucleotides	0.03	oligonucleotides	0.04
Product cleanse-up	0.26		
Capillary electrophoresis†	0.20	agarose electrophoresis	0.05
Tubes, plates and tips	0.38	tubes and filter tips	0.44
Total	1.63	total	1.3
Antigen predictions per test	16	antigen predictions per test	4
Cost per antigen	0.10	cost per antigen	0.33

*The time for the preparation of the PCR mixes and for collecting and preparing the samples is not included.

modified several times, and Co testing was temporarily dropped to allow the inclusion of other systems. A total of 287 Yt(a-), 145 Co(a-), 96 Lu(b-) and 12 Kp(b-) donors were identified. Data predicted by pooled capillary electrophoresis were pooled with historic serologic data and used to identify units with an 'expected' suitable antigen pattern that were diverted to a special 'laboratory inventory' and kept for 1–4 weeks (fig. 3).

The prediction rate was re-evaluated after 34,768 samples (table 4): It dropped to 92.9%, Fy and Yt being the most unstable systems. The main cause of failure was low signal, followed by a borderline fluorescence ratio. Rarely, off-scale peaks (fluorescence too strong for correct color compensation) prevented analysis. For practical purposes, a donor was considered successfully typed if a prediction of Fy^a and Fy^b was present.

From January 1, 2017 to December 31, 2017, a total of 20,861 donor samples were successfully typed, and the 'success rate' was 91% (based on successful Fy typing). During this year, 188,309 (28%) of 670,799 RBC units were from typed donors, 42,731 (23% of typed units, 6% of all units) of these fitted a 'rule' and were stored in the laboratory inventories. This number represents a slight overestimate of the efficiency of the typing project, because some units were selected due to historical serologic typing information. As a result of the computer-controlled guidance of unit use, we were able to maintain a stock of about 10 Lu(b-), 25 Yt(a-) and 15 Co(a-) non-cryoconserved units in our blood service (table 5).

Table 4. Antigen prediction rate in the first 34,768 samples and causes of failure

	Predicted, %	Failed to predict		
		Low signal, %	Offscale, %	Ratio, %
Jk ^a	95.4	4.3	0.0	0.3
Jk ^b	95.1	4.3	0.0	0.6
Fy ^a	86.4	11.5	0.0	2.1
Fy ^b	87.7	11.5	0.0	0.8
M	95.1	3.8	0.6	0.5
N	94.1	3.8	0.6	1.5
S	91.6	7.3	0.2	0.9
s	91.6	7.3	0.2	0.9
Co ^a	97.3	2.5	0.2	0.0
Co ^b	96.5	2.5	0.2	0.9
Lu ^a	97.8	1.6	0.0	0.6
Lu ^b	98.3	1.6	0.0	0.1
Yt ^a	86.7	13.0	0.0	0.3
Yt ^b	86.2	13.0	0.0	0.7
Kp ^a	92.8	6.7	0.0	0.5
Kp ^b	93.3	6.7	0.0	0.0
Mean	92.9	6.3	0.1	0.7

Analysis of Mispredictions

In 2017, 62 antigen predictions turned out to be incorrect (2009–2017: 433 predictions). While this number represents only 0.14% of units scheduled for the laboratory inventory, a realistic

Table 5. Availability of rare types

Phenotype	Snapshots				
	April 20, 2009	September 8, 2015	February 22, 2016	September 29, 2017	March 13, 2018
Vel–					
All	1	2	2	–	3
O RhD–	–	–	–	–	1
Kp(b–)					
All	–	1	–	1	2
O RhD–	–	–	–	–	1
Lu(b–)					
All	2	12	11	10	12
O RhD–	–	1	3	4	3
Yt(a–)					
All	–	20	21	37	25
O RhD–	–	2	1	6	2
Co(a–)					
All	–	29	14	18	14
O RhD–	–	3	3	1	2
k–					
All	–	11	19	21	32
O RhD–	–	3	4	5	4

rate of discrepancies cannot be derived from the data, because many units were used for general-purpose transfusion and not checked by serology. Most mispredictions were due to low-quality runs not identified by antigen calling (2017: 65%; all years: 49%) or an attempted manual interpretation of a result that could not be automatically called (2017: 27%; all years: 35%).

Systematic Serologic Typing of Donors Predicted to Be Kp(a+b+), Co(a+b+) or Lu(a+b+)

In order to detect null alleles, samples predicted to be Kp(a+b+), Co(a+b+), or Lu(a+b+) were checked by serology yielding 8,355 Lu^a typings (Lu^b 8,965; Kp^a 2,468; Kp^b 2,565; Co^a 1,497; Co^b 1,520). A discrepant result reproduced on re-testing was obtained for Lu^a in 1 proband (plus 1 proband detected during routine re-typing; Lu^b 10; Kp^a 7, Kp^b 2, Co^a 2, Co^b 2). These numbers compared to 10 Kp(b–), 96 Lu(b–) and 145 Co(a–) donors correctly predicted as Kp(a+b–), Lu(a+b–) and Co(a+b–), respectively.

Molecular Causes of Discrepant or Weakened Antigen Expression

Rare genotypes detected in donors with discrepant or weakened antigen expression are collated in table 6. Several new alleles with weakened or absent antigen expression were found: 1 for Kell, 2 for Colton, and 8 for Lutheran. Adsorption/elution was done in selected cases only and found to be unreliable: 1 sample with a known KEL mod allele was found negative for Kp^a on adsorption/elution. False-negative predictions due to mutations interfering with primer binding were usually detected during routine use and alleles with weak or absent antigen expression by systematic testing of predicted heterozygotes.

For KEL, 2 negative serologic Kp^b results were caused by the *KEL*02N.17* and by the previously unknown *KEL*02N/M. (1658C>T)* allele. No adsorption/elution result was available for that donor; hence the allele could represent a KEL null or KEL mod allele. Furthermore, 8 false-positive Kp^a predictions were caused by the *KEL*02M.13* allele [22].

Ten donors predicted to be Lu^a/Lu^b were Lu(b–) by serology. We identified four previously unknown alleles with missense mutations: *LU*02.(c.529C)*, *LU*02.-13.(c.997A)*, *LU*02.(c.711T, 714T, 733A)*, and *LU*02.(c.1612A)*. In addition, we detected two silent *LU*01*-derived alleles, and one allele with weak expression of Lu^b antigen. No cause could be identified in 3 donors and possibly associated alterations in 2 donors. Most alleles identified in these donors were previously unknown and carried missense mutations, suggesting that they could represent *LU* mod rather than *LU* null alleles. Adsorption/elution was done for two alleles only and was positive in one.

For Co^a, 2 donors predicted to be Co^a/Co^b were Co(a–). One carried a mutation in the promoter; in the other one no cause could be identified. In addition, 3 donors had weak or almost absent Co^b expression, but a probably causative mutation was found in only 1 of them.

Discussion

We developed a low-cost molecular typing system to screen our donor population for rare antigen constellations. Key features included a simplified DNA isolation approach, simultaneous analysis of several samples by pooled capillary electrophoresis, and the de-

Table 6. Molecular structures identified in samples with reproducible discrepancy between prediction and serology

Samples (n)	Phenotype		Donor	protein	antigens	snp number	ExAC frequency*	deduced genotype	evidence for causality / association
	predicted	found							
Glycophorin B									
False-negative antigen prediction									
3	S-s+	S+s+	c.143C/T; c.161G/A	48 Met / Thr 54 Arg / His	S / s Mit+	rs7683365 rs370332485	0.3802; 0.0002869	GYPB*04/ GYPB*24	disrupted primer binding
1	S-s+	S+s+	c.143C/T; c.161G/C	48 Met / Thr 54 Arg / Pro	S / s unknown	rs7683365	0.3802; absent	GYPB*04/ GYPB*03 , (c.161C)†	disrupted primer binding
1	S+s-	S+s+	c.143C/T; c.163T/C ; c.251 G/C	48 Met / Thr; 55 Phe / Leu 84 Ser/ Thr	S / s unknown unknown	rs7683365 rs755129621 rs1132783	0.3802; 0.00008228 0.232	GYPB*04/ GYPB*03 , (c.163C,251C) or GYPB*03(c.163C)	disrupted primer binding
Lutheran									
Lu ^b - (including Lu ^b mod)									
2	Lu(a+b+)	Lu(a+b-)	c.230 G/A; c.586 G/A; c.1615 A/G	77 Arg / His 196 Val / Ile; 539 Thr / Ala	Lu ^b +/Lu ^a +; unknown; Au ^a +/Au ^b +	rs28399653 rs28399654 rs1135062	0.0382; 0.03308; 0.29932	LU*01.19.(c.586A)/ Lu*02	no cause identified [§] ;
1	Lu(a+b+)	Lu(a+b-)	c.230 G/A; c.586 G/A	77 Arg / His 196 Val / Ile	Lu ^b +/Lu ^a +; unknown	rs28399653 rs28399654	0.0382; 0.03308;	LU*01.(c.586A)/ Lu*02	no cause identified [§] ;
1:	Lu(a+b+)	Lu(a+b-)	c.-4036_-4029del (het)	Non-coding; 77 Arg / His; 196 Val / Ile; 539 Thr / Ala	unknown; Lu ^b +/Lu ^a +; unknown; Au ^a +/Au ^b +	- rs28399653 rs28399654 rs1135062	NC; 0.0382; 0.03308; 0.29932	LU*01.19.(c.586A)/ Lu*02(-4036_-4029del)†	the deletion is 63 bp 5' of the BCAM promoter
1	Lu(a+b+)	Lu(a+b-) elution neg	c.230 G/A; c.529 G/C ; c.586 G/A; c.1615 A/G	77 Arg / His 177 Gly / Arg 196 Val / Ile; 539 Thr / Ala	Lu ^b +/Lu ^a +; unknown; unknown; Au ^a +/Au ^b +	rs28399653 rs777617369 rs28399654 rs1135062	0.0382; W: 0.0001225 0.03308; 0.29932	LU*01.19.(c.586A)/ Lu*02.(c.529C)†	p < 0.01
1	Lu(a+b+)	Lu(a+b-)	c.230 G/A; c.711 C/T c.714 C/T c.733 G/A	77 Arg / His (silent) (silent) 245 Glu / Lys	Lu ^b +/Lu ^a +; -; -; unknown	rs28399653 rs3810140 rs3810140 rs762681823	0.0382; 0.06594; 0.06590 0.000015603	LU*01 / Lu*02.c (711T, 714T, 733A)†	p < 0.05
2	Lu(a+b+)	Lu(a+b-)	c.230 G/A; c.586 G/A; c.997 G/A ; c.1671 C/T c.1742 A/T	77 Arg / His 196 Val / Ile; 333 Gly / Arg ; (silent) 581 Gln / Leu	Lu ^b +/Lu ^a +; unknown; unknown; -; Lu:13 / -13	rs28399653 rs28399654 rs147593462 rs28399658 rs28399659	0.0382; 0.03308; W:0.00002477 0.04170; 0.04632	LU*01.(c.586A)/ Lu*02.-13.(c.997A)†	p < 0.01

Table 6. continued on next page

Table 6. *Continued*

Samples (n)	Phenotype		Donor polymorphisms	protein	antigens	snp number	ExAC frequency*		evidence for causality / association
	predicted	found					non-Finnish Europeans	deduced genotype	
1	Lu(a+b+)	Lu(a+b-)	c.230 G/A; c.1612 G/A ; c.1615 A/G	77 Arg / His 538 Gly / Ser ; 539 Thr / Ala	Lu ^b + / Lu ^a +; unknown; Au ^a + / Au ^b +	rs28399653 rs145626518 rs1135062	0.0382; 0.0005108; 0.29932	LU*01.19 / Lu*02. (c.1612A) [†] or LU*01 / Lu*02.10. (c.1612A) [†]	unsure: p > 0.05
1	Lu(a+b+)	Lu(a+b-) elution pos	c.100_105del [‡] ; c.230 G/A; c.586 G/A; c.1615 A/G	34_35delArgLeu [‡] 77 Arg / His; 196 Val / Ile; 539 Thr / Ala	LU:12 / LU:-12; Lu ^b + / Lu ^a +; unknown; Au ^a + / Au ^b +	– rs28399653 rs28399654 rs1135062	0.001679; 0.0382; 0.03308; 0.29932	LU*01.19.(c.586A) / Lu*02.-12.1	unsure: p > 0.05
Lu ^b weak									
1	Lu(a+b+)	Lu(a+b+w)	c.230 G/A; c.586 G/A; c.1615 A/G	77 Arg / His 196 Val / Ile; 539 Thr / Ala	Lu ^b + / Lu ^a +; unknown; Au ^a + / Au ^b +	rs28399653 rs28399654 rs1135062	0.0382; 0.03308; 0.29932	LU*01.19.(c.586A) / Lu*02	no cause identified [§] ;
1	Lu(a+b+)	Lu(a+b+w)	c.230 G/A; c.1183 C/T ; c.1615 T/A	77 Arg / His 395 Arg / Cys ; 539 Thr / Ala	Lu ^b + / Lu ^a +; unknown; Au ^a + / Au ^b +	rs28399653 rs761656868 rs1135062	0.0382; 0.00003004; 0.29932	LU*01.19 / Lu*02.(c.1183T) [†]	p < 0.05
1	Lu(a+b+)	Lu(a+b+w)	c.230 G/A; c.586 G/A; c.1495 C / T	77 Arg / His 196 Val / Ile; 499 Arg / Trp	Lu ^b + / Lu ^a +; unknown; LU:26/-26	rs28399653 rs28399654 rs148391498	0.0382; 0.03308; 0.0007812	LU*01.19.(c.529C) / Lu*02.-26	unsure: p > 0.05
Lu ^a -									
1	Lu(a+b+)	Lu(a-b+)	c.230 G/A; c.586 G/A; c.937 G/C c.1615 T/A	77 Arg / His 196 Val / Ile; 313 Val / Leu 539 Thr / Ala	Lu ^b + / Lu ^a +; unknown; unknown; Au ^a + / Au ^b +	rs28399653 rs28399654 rs199762962 rs1135062	0.0382; 0.03308; W:0.000008293 0.29932	LU*01.19.(c.937T) [†] / Lu*02	p < 0.01
1	Lu(a+b+)	Lu(a-b+)	c.230 G/A; c.1722 A/T c.1742 A/T	77 Arg / His 574 Lys / Asn 581 Gln / Leu	Lu ^b + / Lu ^a +; unknown; Lu:13 / -13	rs28399653 – rs28399659	0.0382; absent 0.04632	LU*01.(c.586A) . (c.1722T) [†] / Lu*02.-13	p < 0.01
False-negative antigen prediction									
1	Lu(a+b-)	Lu(a+b+)	c.230 G/A; c.240 G/A	77 Arg / His; (silent)	Lu ^b + / Lu ^a +; –	rs28399653 rs779970171	0.0382; W:0.00002868	LU*01.19 / LU*02.(c.240A)	disrupted primer binding
Kel									
Kpb- (including mod phenotype)									
1	Kp(a+b+)	Kp(a+b-)	c.841 C/T; c.1658 C/T	281 Arg / Trp; 553 Pro / Leu	Kp ^b + / Kp ^a +; unknown	rs8176059 rs1431256906	0.010544; absent	KEL*02.03 / KEL*01.01. (c.1658T) [†]	p < 0.01
1	Kp(a+b+)	Kp(a+b-)	c.841 C/T; c.1546 C/T	281 Arg / Trp; 516 Arg / Stop	Kp ^b + / Kp ^a +; K null	rs8176059 rs8176034	0.010544; 0.00007494	KEL*02.03 / KEL*02N.17	known null allele

Table 6. continued on next page

Table 6. *Continued*

Samples (n)	Phenotype		Donor	protein	antigens	snp number	ExAC frequency*	
	predicted	found					non-Finnish Europeans	deduced genotype
Kpa- (including mod phenotype)								
6	Kp(a+b+)	Kp(a-b+) Elution negative	c.257 G/A; c.841 C/T	86 Arg / Gln; 281 Arg / Trp	K mod; Kp ^{b+} / Kp ^{a+}	rs777011308 rs8176059	0.00001501; 0.010544	known mod allele
1	Kp(a+b+)	Kp(a-b+)	c.257 G/A; c.841 C/T; c.1680 A/C	86 Arg / Gln; 281 Arg / Trp; (silent)	K mod; Kp ^{b+} / Kp ^{a+} -	rs777011308 rs8176059 rs8176036	0.00001501; 0.010544; 0.04956	known mod allele
Colton								
Co ^a - (including mod phenotype)								
1	Co(a+b+)	Co(a-b+)	c.-101 C/G; c.134 C/T	Non-coding; 45 Ala / Val	-; Co ^{a+} / Co ^{b+} ;	rs28362692	NC; 0.03976	promoter, possibly trans-cription factor binding inhibited
1	Co(a+b+)	Co(a-b+)	c.134 C/T; c.494 G/A	45 Ala / Val; 165 Gly / Asp	Co ^{a+} / Co ^{b+} ;	rs28362692 rs28362731	0.03976 0.04309	no cause identified [§] ;
Co ^b - (including mod phenotypes)								
1	Co(a+b+)	Co(a+b-) Elution pos	c.134 C/T	45 Ala / Val	Co ^{a+} / Co ^{b+}	rs28362692	0.03976	no cause identified
Co ^b weak								
1	Co(a+b+)	Co(a+wb+)	c.134 C/T	45 Ala / Val	Co ^{a+} / Co ^{b+}	rs28362692	0.03976	no cause identified
1	Co(a+b+)	Co(a+b+w) Elution pos	c.134 C/T; c.502 G/A	45 Ala / Val; 168 Ala / Thr	Co ^{a+} / Co ^{b+} ;	rs28362692 -	0.03976 absent.	p < 0.01
False-negative antigen prediction								
1	Co(a-b+)	Co(a+)	c.134 C/T; c.135 G/A	45 Ala / Val; (silent)	Co ^{a+} / Co ^{b+} ;	rs28362692 rs781356338	0.03976 0.0004542	yes: c.135G>A interferes with primer binding
NNC = Not covered (polymorphism outside the scope of ExAC, e.g. in the promoter); absent = no single case worldwide observed in ExAC despite coverage of the region. *Frequency of the mutation among non-Finnish Europeans according to the ExAC database (http://exac.broadinstitute.org). If the mutation was absent among non-Finnish Europeans but present worldwide, the worldwide frequency was given with a prefixed W. The mutations possibly causing the discrepancy are indicated by bold letters. [†] Previously unknown allele. [§] The shown polymorphisms are frequent and unlikely to cause the discrepant phenotype.								

liberate allowance of a relevant failure rate as well as the exclusion of the complicated blood group systems ABO and RH. Implementation of our typing approach on a routine basis resulted in major improvements of RBC supply for patients with anti-Yt^a or anti-Lu^b.

Current approaches for high-throughput molecular blood group antigen prediction in blood donors [3–11] differ relevantly with respect to typing effort, cost, and quality of the results. On one hand, sophisticated approaches attempted a highly reliable determination of the blood type even in many rare phenotypes [3, 11]. Other approaches were optimized for high throughput [8] or low cost [23] and relied on serologic confirmation on release [8, 9]. Our approach may represent the extreme version of low-cost, low-quality testing: we deliberately allowed for a 10% ‘no call’ rate and did not bother with rare alleles in order to be able to screen a large donor cohort with limited financial resources.

The antigen prediction was focused on the antigens clinically most relevant in our population, excluding ABO, Rh phenotype and K that had to be determined for unit release. Therefore, we complemented predictions for Fy^a/Fy^b, Jk^a/Jk^b, S/s, and M/N by a screening approach covering the rare phenotypes most relevant for our population: Kp(b–), Lu(b–), Yt(a–) [14], and Co(a–). We could not include a prediction of the Vel– phenotype that is caused by a 17 bp deletion [24], because no information on its molecular basis was available at the start of our typing project. The antigens Co^b, Lu^a, Yt^b, and Kp^a were included for technical reasons to balance for Co^a, Lu^b, Yt^a, and Kp^b.

Already in 2008, we showed that molecular typing from crude DNA preparations was useful to screen donors for rare phenotypes. The pooled capillary electrophoresis extended this concept, keeping the simplified DNA isolation method based on the Extract-N-Amp system but replacing agarose gel by capillary electrophoresis. Pooling of 8 samples in a single capillary run resulted in a cost reduction by about 66% and increased throughput by up to 8-fold. In contrast to other donor typing approaches using pooling [25], product length encoding of the sample number obviated the need for a separate pool resolution. Compared to the agarose gel approach, the number of predicted antigens was quadrupled, resulting in a lower cost per antigen of EUR 0.10 (USD 0.13). The semi-automated analysis of the data allowed the inclusion of all antigen predictions into our donor database and paved the way for an IT-controlled unit guidance system.

The simplistic approach came at a cost: the major antigens (evaluated as successful Fy^a/Fy^b prediction) were successfully predicted in only about 90% of donors (table 4), despite the results being checked manually. Without the manual check, the prediction rate would have been even worse. Furthermore, there was a certain rate of mispredictions resulting in antigen discrepancies on serologic confirmation. These problems often occurred in samples with generally low signals, with technical problems during data interpretation being the main cause of error. Systematic mistyping of alleles was a rarity (table 6) and negligible compared to technical failures; hence we did not perceive it necessary to include additional polymorphisms for antigen prediction. Considering that the

observed discrepancies are accumulated from 4 laboratories serving an area with approximately 8 million inhabitants, each laboratory encountered such a problem one to two times a month. Most technicians did not consider this discrepancy rate a practical problem and rather valued the wide availability of units for which a 99% correct antigen status was known.

It should be realized that our approach is critically dependent on the availability of the commercial ‘Extract-N-Amp’ kit. While there are other enzymes suitable for direct PCR, a change of the DNA isolation and PCR chemistry would incur relevant redesign of the method.

In very rare phenotypes like Kp(b–), compound heterozygotes with one null allele may underlie a relevant proportion of the antigen-negative donors [11]. We tried to verify this proposal by systematic serologic testing of samples predicted to be heterozygous for Kp^a/Kp^b, Lu^a/Lu^b, or Co^a/Co^b. The yield was limited: In addition to 11 Kp(b–) donors predicted by single polymorphism typing, we found 2 serologically Kp(b–) donors among 2,565 predicted to be Kp^a/Kp^b. For Lu^b, in addition to 96 donors predicted to be Lu(b–), 10 serologically Lu(b–) donors were found among 8,965 predicted Lu^a/Lu^b. For Co^a, 145 Co(a–) donors found by single polymorphism prediction compared to a single additional Co(a–) donor detected by serologically typing of 1,497 predicted heterozygous donors. A total of 11 previously unknown alleles was found, many of which carried missense mutations suggesting that they may represent mod rather than null alleles. Even if many of them were seen in a single sample only, the statistical significance of the association could usually be proven by a combination of SNP frequency data taken from the ExAC database [21] with a sequential statistical testing approach [20]. For Lu and Co, in a relevant number of samples no cause for the diminished or absent antigen expression was detected, suggesting that regions outside the coding region are of relevance for Co and Lu expression. While serologic typing of predicted heterozygous donors was an interesting tool to find possible null alleles, it had minor impact on the number of identified antigen-negative donors.

Our large-scale typing program was complemented by an IT solution that allowed to select suitable donors and to guide units with predicted interesting phenotypes into the ‘laboratory inventory’ by a check of the predicted antigen pattern to a set of ‘rules’. The importance of a suitable IT integration of the typing process has been stressed by Perreault et al. [26] and becomes obvious considering the number of available k– units: For many years before the start of the project, RhD– K+ donors were systematically tested for antigen k, but without a working IT solution, we were unable to establish a stock useful in emergency situations.

In conclusion, our strategy allowed us to apply this approach to 20% of our RBC units (i.e. more than 120,000 units/year) and obviated the need for several rounds of serologic antigen screening when a patient with multiple antibodies needed blood: For a patient needing 4 Jk(a–) Fy(a–) units, in older times 64 units were tested for Jk^a, the negative units for Fy^a, and the double-negative units were cross-matched. Nowadays, 4 units are cross-matched and tested in parallel. The demand for Yt(a–), Co(a–), and Lu(b–)

RBC units can now be fulfilled in the majority of cases. The typing project thus decisively improved the transfusion support for allo-immunized patients.

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Authors Contributions

FFW invented the approach, did the initial primer design, established the data extraction, and analyzed the data. AD performed PCE during the development phase and analyzed discrepant samples. RB performed PCE, monitored the efficiency of the method during routine use, optimized PCR conditions and designed PCR primers in the later phase of the project. THM decided to start the typing project and continuously supported its further development, including the demand for a useful IT integration.

Disclosure Statement

Nothing to disclose.

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