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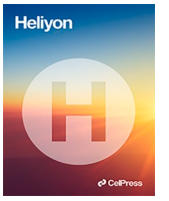

Research article

Simultaneous high throughput genotyping of 36 blood group systems using NGS based on probe capture technology

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A R T I C L E I N F O

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A B S T R A C T

Human blood group antigen has important biological functions, and transfusion of incompatible blood can cause alloimmunization and may lead to serious hemolytic reactions. Currently, serological methods are most commonly used in blood group typing. However, this technique has certain limitations and cannot fully meet the increasing demand for the identification of blood group antigens. This study describes a next-generation sequencing (NGS) technology platform based on exon and flanking region capture probes to detect full coding exon and flanking intron regions of the 36 blood group systems, providing a new high-throughput method for the identification of blood group antigens. The 871 capture probes were designed for the exon and flanking intron sequences of 36 blood group system genes, and synchronization analysis for 36 blood groups was developed. The library for NGS was tested using the MiSeq Sequencing Reagent Kit (v2, 300 cycles) by Illumina NovaSeq, and the data were analyzed by the CLC Genomics Workbench 21.0 software. A total of 199 blood specimens have been sequenced for the 41 genes from 36 blood groups. Among them, heterozygote genotypes were found in the ABO, Rh, MNS, Lewis, Duffy, Kidd, Diego, Gerbich, Dombrock, Globoside, JR, LAN, and Landsteiner-Wiene blood group systems. Only the homozygous genotype was found in the remaining 22 blood group systems. The obtained data in the NGS method shows a good correlation (99.98 %) with those of the polymerase chain reaction-sequence-based typing. An NGS technology platform for 36 blood group systems genotyping was successfully established, which has the characteristics of high accuracy, high throughput, and wide coverage.

1. Introduction

Human blood group antigens are expressed in the red blood cells (RBCs), and most of them are attributed to blood group systems. Blood group antigens are present on multiple proteins and molecules, including molecular transport proteins, metabolism proteins, and vesicles, which are also relevant in transfusion [1,2]. Furthermore, ABO and RhD antigen matching are mandatory for clinical blood transfusion in most countries. However, following the transfusion of ABO- and RhD-compatible blood, patients may produce antibodies by alloimmunization of the blood group antigens [3–6]. Some cases may exhibit hemolytic transfusion reactions due to antigen and antibody reactions. Therefore, accurate identification of the blood group antigens for individuals and selection of compatible blood can effectively ensure transfusion safety for the patients.

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To date, 45 blood group systems containing 362 red cell antigens have been officially nominated by the Red Cell Immunogenetics and Blood Group Terminology of the International Society of Blood Transfusion (ISBT) [7].Various antigens were identified in the different blood group systems. In general, red cell antigens were detected using the serological hemagglutination method, which involves potential limitations. Firstly, only a few antigens can be detected in a single procedure, which imposes a heavy workload for identification for multiple blood group systems. In addition, some blood group antigens or rare blood antigens lack specific antibodies and cannot be detected by serological techniques [6,8–10]. However, previous studies have clarified the genetic and molecular mechanisms of 45 blood group systems and their 362 red cell antigens. Therefore, molecular diagnostics techniques have been employed for the identification of red cell antigens, including polymerase chain reaction specific sequence primer (PCR-SSP), PCR-sequence-based typing (PCR-SBT), BeadChips, Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS), and next-generation sequencing (NGS) [11–15]. Kappler-Gratias S et al. [16] selected reagent RBCs using a high-throughput DNA analysis with BeadChips, which improved the quality of reagent RBCs. The NGS technique has been used for RBC antigen determination, with the concordance rate ranging from 0.982 to 0.994 between NGS and other methods [17–19]. The NGS method presents some advantages, such as the ability to analyze a large number of specimens and genomic regions simultaneously with minimal cost, providing a comprehensive genetic analysis [20]. Fichou Y et al. [21] reported that 18 genes involved in 15 blood systems were analyzed using the NGS technique. Schoeman EM et al. [6] have detected polymorphisms in 28 blood group systems in 28 individuals using targeted exome sequencing. In this study, an NGS method was established to detect exon and flanking intron regions of 36 blood group systems simultaneously based on probe capture technology, enabling the identification of red blood cell antigens across multiple blood group systems.

2. Results

2.1. ABO blood group system analysis using NGS

Among the 199 blood donors, the serological analysis revealed that 60 were type A, 54 were type B, 67 were type O, and 18 were type AB. The genotypes of the specimens were consistent with those of the phenotypes except for one individual with O type. The *ABO*A2.05* allele of the A2 subtype was found in two type A donors. The *ABO*BEL.03* allele was found in one type O donor. In addition, four rare O alleles were detected in four O-type donors, including three alleles already described in the ISBT, namely *ABO*O.01.06*, *ABO*O.01.11*, and *ABO*O.01.71,* and a new O variant allele with c.882C > T (GenBank ID:PP393496). 21 genotypes were found in the study (Fig. 1).

2.2. Rh blood group system analysis

All RhD blood groups were RhD positive, and the most common phenotypes were CCDee and CcDEe, accounting for 51.5 % and 31 %, respectively. Using the serological method, the CcDee, ccDEE ccDEe, and CCDEe phenotypes accounted for 5.5 %, 5.5 %, 5 %, and 1.5 %, respectively. The results of the serological method and NGS technique were in concordance. Among them, two *RHD* copies were found in 187 specimens (*RHD+/RHD* + homozygote) and one *RHD* copy was found in 12 specimens (*RHD+/RHD*-hemizygote). These findings were also confirmed by our PCR-SSP method for the detection of *RHD* zygosity Rhesus box [22].

2.3. Other 34 blood group system analysis results

In addition to the ABO and Rh blood groups, the results of the other 34 blood group systems are shown in Table 1. From the results of the NGS method, only one high-frequency genotype was found in the following blood group systems, including P1PK, Lutheran, Kell, Yt, Xg, Scianna, Colton, Kx, Cromer, Knops, Indian, Ok, Raph, John Milton Hagen, I, Gill, FORS, Rh-associated glycoprotein, FORS, Vel, CD59, and Augustine blood groups. In addition, red blood cell phenotype frequencies among Chinese in this study was compared to reported data in other populations (Table 2) [23–26].

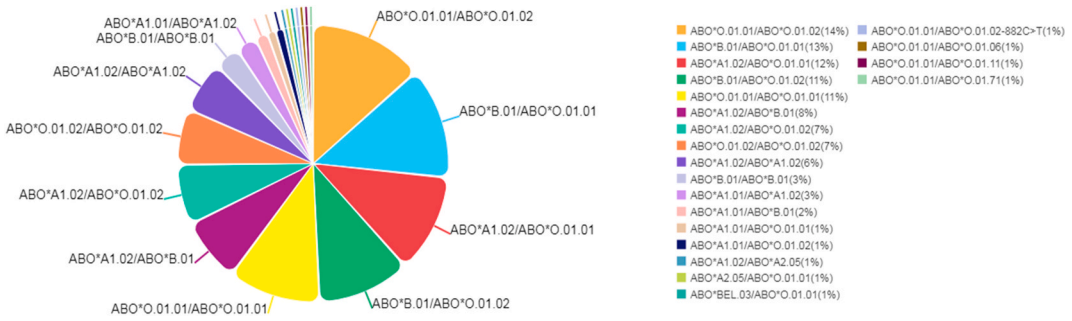

Fig. 1. Results of NGS genotyping of ABO blood group in 199 blood donors. Different colors indicate different genotypes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Genotyping results of 34 blood group systems except for ABO and Rh blood group.

ISBT No.	System name	Gene name	Genotype	Phenotype	Nucleotide change	Exon No.	Predicted amino acid change	Number
002	MNS	*GYPA*M*	*GYPA*M/GYPA*M*	M + N-	c.59C c.71G c.72T	2		52
		*GYPA*N*	*GYPA*M/GYPA*N*	M + N+	c.59Y c.71R c.72K			90
			*GYPA*N/GYPA*N*	M-N+	c.59T c.71A c.72G		p.Ser20Leu p.Gly24Glu	57
		*GYPB*s*	*GYPB*s/GYPB*s*	S-s+	c.143C	4		180
		*GYPB*S*	*GYPB*S/GYPB*s*	S + s+	c.143Y			16
			*GYPB*S/GYPB*S*	S + s-	c.143T		p.Thr48Met	3
003	P1PK	*A4GALT*	*A4GALT*01/ A4GALT*01*	P1+Pk+	c.109A	3		199
			*A4GALT*01.02/ A4GALT*01.02*	P1+Pk+	c.109G		p.Met37Val	0
005	Lutheran	*BCAM*	*LU*B/LU*B*	Lu(a-b+)	c.230G	3		199
			*LU*A/LU*A*	Lu(a+ b-)	c.230A		p.Arg77His	0
006	Kell	*KEL*	*KEL*02/KEL*02*	K-k+	c.578C	6		199
			*KEL*01.01/KEL*01.01*	K + k-	c.578T		p.Thr193Met	0
007	Lewis	*FUT3*	*Le/Le*	Le(a-b+)	c.59T, c.202T	1,2		177
			Le/le^{59}	Le(a-b-)	c.59T > G		p.Leu20Arg	8
			Le/le^{202}	Le(a-b-)	c.202T > C		p.Trp68Arg	14
008	Duffy	*ACKR1*	*FY*A/FY*A*	Fy(a+b-)	c.125G	2		170
			*FY*A/FY*B*	Fy (a+b+)	c.125R			28
			*FY*B/FY*B*	Fy(a-b+)	c.125A		p.Gly42Asp	1
009	Kidd	*SLC14A1*	*JK*A/JK*A*	Jk(a+b-)	c.838G	8		48
			*JK*B/JK*B*	Jk(a-b+)	c.838A		p.Asp280Asn	52
			*JK*A/JK*B*	Jk(a+b+)	c.838R			99
010	Diego	*SLC4A1*	*DI*B/DI*B*	Di(a–b+)	c.2561C	19		185
			*DI*A/DI*B*	Di(a+b+)	c.2561Y		p.Pro854Leu	14
011	Yt	*ACHE*	*YT*A/YT*A*	Yt(a+b-)	c.1057C	2	p.His353Asn	199
			*YT*B/YT*B*	Yt(a-b+)	c.1057A			0
012	Xg	*XG*	*XG*01/XG*01*	Xg^{a}	–			199
			*XG*01 N/XG*01 N*	Xg(a-)	NC_000023.11: g.2748343G > C	5′ UTR	p.0	0
		CD99	*CD99*01/CD99*01*	CD99	–	–		199
			*CD99*01 N.01/ CD99*01 N.01*	CD99^{-}	Exons deletion	3–7	p.0	0
013	Scianna	*ERMAP*	*SC*01/SC*01*	Sc1+	c.169G	4	p.Gly57Arg	199
			*SC*02/SC*02*	Sc2+	c.169A			0
014	Dombrock	*ART4*	*DO*B/DO*B*	Do(a-b+)	–	2	p.Asp265Asn	163
			*DO*A/DO*B*	Do(a+b+)	c.793 R			36
015	Colton	*AQP1*	*CO*01.01/CO*01.01*	Co(a+)	c.134C	1	p.Ala45Val	199
			*CO*02/CO*02*	Co(b+)	c.134T			0
016	Landsteiner-Wiener	*ICAM4*	*LW*A/LW*A*	LW(a+b-)	c.299A	1	p.Gln100Arg	198
			*LW*A/LW*B*	LW (a+b+)	c.299R			1
017	Chido/Rodgers	*C4A, C4B*	/	/	/	/	/	/
018	H	*FUT1*	*FUT1*01/FUT1*01*	H+	c.35C	4	p.Ala12Val	105
			*FUT1*01/FUT1*01.02*	H+	c.35R			83
			*FUT1*01.02/ FUT1*01.02*	H+	c.35T			11
019	Kx	*XK*	*XK*01/XK*01*	Kx+	–	1–3	No protein present	199
			*XK*N.01/XK*N.01*	kx-	Deletion			0
020	Gerbich	*GYPC*	*GE*01/GE*01*	GE:2,3,4	/			198
			/	/	c.290A > G	4	p.Lys97Arg	1
021	Cromer	*CROM*	*CROM*01/CROM*01*	Cr(a+)	c.679G	6	p.Ala227Pro	199
			CROM–01/CROM*–01*	Cr(a–)	c.679C			0
022	Knops	*CR1*	*KN*01/KN*01*	Kn(a+)	c.4681G,4843A	29		199
			*KN*02/KN*02*	Kn(b+)	c.4681A,c.4843G		p.Val1561Met	0
023	Indian	*CD44*	*IN*B/IN*B*	In(a–b+)	c.137G	2		199
			*IN*A/IN*A*	In(a+b–)	c.137C		p.Arg46Pro	0
024	Ok	*BSG*	*OK*01.01/OK*01.01*	Ok(a+)	c.274G	4		199
			*OK*01.–01/OK*01.–01*	Ok(a–)	c.274A		p.Glu92Lys	0
025	Raph	*CD151*	*RAPH*01/RAPH*01*	RAPH:1	c.511C; c.579A	7		199

(*continued on next page*)

Table 1 (*continued*)

ISBT No.	System name	Gene name	Genotype	Phenotype	Nucleotide change	Exon No.	Predicted amino acid change	Number
			*RAPH*01.−01.01/ RAPH*01.−01.01*	RAPH: 1	c.511T c.579G		p.Arg171Cys p.Gly193	0
026	John Milton Hagen	*SEMA7A*	*JMH*01/JMH*01*	JMH+	c.619C,c.1545A c.1545A	6,12		199
			*JMH*01.−02/ JMH*01.−02*	JMHK–	c.619T,c.1545G c.1545G	12	p.Arg207Trp p.Gln515Gln	0
027	I	*GCNT2*	*GCNT2*01/GCNT2*01*	I	c.816G	1C		199
			*GCNT2*01.02/ GCNT2*01.02*	I	c.816C		p.Glu272Asp	0
028	Globoside	*B3GALNT1*	*GLOB*01/GLOB*01*	GLOB:1	c.376G	5		192
			*GLOB*01/GLOB*01.02*	GLOB:1	c.376R		p.Asp126Asn	7
029	Gill	*AQP3*	*GIL*01/GIL*01*	GIL:1	c.710+1G	Intron		199
			*GIL*01 N.01/GIL*01 N.01*	GIL:−1	c.710+1G > A	5	Aberrant splicing	0
030	Rh-associated glycoprotein	*RHAG*	*RHAG*01/RHAG*01*	RHAG:1	–	2		199
			*RHAG*01.−01/ RHAG*01.−01*	RHAG:−1	c.316C > G		p.Gln106Glu	0
031	FORS	*GBGT1*	*GBGT1*01 N.01/ GBGT1*01 N.01*	FORS–		7		199
			*GBGT1*01.01/ GBGT1*01.01*	FORS+	c.887G > A		p.Arg296Gln	0
032	JR	*ABCG2*	*ABCG2*01/ABCG2*01*	Jr(a+)	c.376C	4		195
			*ABCG2*01/ABCG2*01 N.01*	Jr(a+)	c.376Y		p.Gln126Ter	4
033	LAN	*ABCB6*	*ABCB6*01/ABCB6*01*	Lan+	–			197
034	Vel	*SMIM1*	*VEL*01/VEL*01*	Vel+	–			199
			*VEL*01 N.01/VEL*01 N.01*	Vel-	c.64_80delAGCCTA GGGGCTGTGTC	3	p.Ser22Glnfs*?	0
035	CD59	*CD59*	*CD59*01/CD59*01*	CD59.1+				199
			*CD59*01 N.03/ CD59*01 N.03*	CD59.1-	c.266G > A	6	p.Cys89Tyr	0
036	Augustine	*SLC29A1*	*AUG*01/AUG*01*	At(a+)	c.1171G c.1159A			199
			*AUG*02/AUG*02*	At(a−)	c.1171A	12	p.Glu391Lys	0
			*AUG*03/AUG*03*	ATML+	c.1159C	12	p.Thr387Pro	0

2.4. The comparison of the NGS method and Sanger sequencing for blood group systems

To verify the accuracy of the NGS method, 26 antigens of 24 blood group systems were also detected by Sanger sequencing. The comparison results are shown in Table 3. Only one mismatch was observed between the two methods, which occurred in the Dombrock blood group; Do(a-b+) was found in NGS, but Sanger sequencing indicated Do(a+b+). The obtained data from the NGS method showed a good correlation (99.98 %) with those of the Sanger sequencing method.

2.5. Other variations in blood group systems by the NGS method

Among the 199 specimens, other variations were found and listed in Table 4, which were also confirmed with the Sanger sequencing method.

3. Discussion

The serological method is the conventional method to identify blood group antigens. However, with the growing number of discovered antigens, this method cannot fully meet the clinical needs [8]. Molecular biology technology has opened a new era of immunohematology. In recent years, the genetic diagnosis of blood group antigens has become a research hotspot, and many methods have been used for the identification of blood group antigens [27,28]. The NGS technique presents many advantages, including the possibility of analyzing hundreds of individuals and many gene regions simultaneously, as well as the ability to provide a comprehensive genetic analysis. This helps to characterize new alleles and better understand the genetic basis of blood group antigens [10, 29–31]. Jakobsen MA et al. [32] performed an NGS assay, exploring 15 different blood group systems from 72 blood donors of various ethnicities. The results revealed that the NGS method could effectively detect common and rare alleles. Roulis E et al. [33] designed an NGS method for RBC, platelet, and neutrophil antigen-associated genes and tested 51 reference specimens. They demonstrated the feasibility of the NGS method, detecting previously overlooked variants. Furthermore, Paganini J et al. [34] analyzed 9 RBC antigens in 79 individuals using whole-genome sequencing (WGS), reporting the potential of WGS for rare blood antigen screening. Lane WJ et al. [35] also analyzed 12 blood group systems in 110 individuals using WGS technology, and conventional serology and SNP array

Table 2
Red blood cell phenotype frequencies among Chinese in compared to reported data in other populations [23–26].

System name	Phenotype	Frequency(%)				
		Chinese	Koreans	South Indians	Europeans	Africans
MNS	M + N-	26.13	24.75	38.38	28	26
	M + N+	45.23	45.22	48.17	50	44
	M-N+	28.64	30.03	13.43	22	30
	S-s+	90.45	90.68	45.29	45	69
	S + s+	8.04	8.94	39.92	44	28
	S + s-	1.51	0.38	14.77	11	3
P1PK	P1+	100	NA	68.71	79	94
Lutheran	Lu(a+b+)	0	NA	0.19	7.5	NA
	Lu(a+b-)	0	NA	0	0.15	NA
	Lu(a-b+)	100	NA	99.61	92.35	NA
	Lu(a-b-)	0	NA	0.19	Very rare	NA
Lewis	Le(a-b-)	11.06	NA	51.54	6	22
	Le(a-b+)	88.94	NA	61.80	72	55
	Le(a+b-)	0	NA	22.07	22	23
	Le(a+b+)	0	NA	0.57	Rare	Rare
Kell	K + k+	0	NA	1.72	8.8	2
	K-k+	100	NA	98.27	91	98
	K + k-	0	NA	0	0.2	Rare
Duffy	Fy(a+b-)	85.43	85.19	43.19	17	9
	Fy(a+ b+)	14.07	14.39	39.92	49	1
	Fy(a-b+)	0.50	0.41	16.89	34	22
	Fy(a-b-)	0	0	0	Very rare	68
Kidd	Jk(a+ b-)	26.13	22.44	41.07	28	57
	Jk(a+ b+)	49.75	49.67	42.61	49	34
	Jk(a- b+)	24.12	27.89	16.31	23	9
Diego	Di(a–b+)	92.96	88.78	NA	NA	NA
	Di(a+b+)	7.04	10.87	NA	NA	NA
	Di(a+b–)	0	0.35	NA	NA	NA
Dombrock	Do(a+b-)	0	1.07	NA	NA	9
	Do(a+b+)	18.09	19.02	NA	NA	41
	Do(a-b+)	81.91	79.91	NA	NA	50
JR	Jr(a+)	100	100	NA	NA	NA
	Jr(a-)	0	0	NA	NA	NA
Ok	Ok(a+)	100	100	NA	NA	NA
	Ok(a–)	0	0	NA	NA	NA

NA: not available.

methods were used to verify these results, showing 99.2 % concordance. Schoeman EM et al. [6] evaluated the 28 protein-based blood group systems using targeted exome sequencing and found that it could provide an immunohematology reference for laboratory testing compared with whole-genome sequencing.

This study described a new method for detecting 36 RBC blood group systems based on probe capture technology simultaneously. The method used 871 probes to capture the exon and flanking intron sequences of 41 genes from 36 RBC blood group systems. The probes were single-stranded, biotin-modified DNA fragments that are 120bp in length. The method was successfully established after optimizing relevant parameters. This technique goes beyond the detection of only known SNPs in the coding region, covering the exon and flanking intron sequences, avoiding the limitations of previously reported techniques, and maintaining the high throughput scale of blood group genotyping [11,13,14]. At the beginning of our experiment, only 36 blood group systems were named by ISBT. Therefore, the probes only targeted 36 blood group systems. However, 45 blood group systems have been officially named by ISBT now, which can collectively be analyzed by adding probes for the newly discovered blood group systems. To verify the accuracy of the results of the blood group systems based on NGS technology, a PCR-SBT method was developed for 26 antigens. The coincidence rate of the two methods was 99.98 %, and only the Dombrock system showed inconsistent results in one specimen, which requires further analysis. It may because by the capture failure or ineffective coverage by NGS sequencing, then resulting in miss the change of the c.793 base of the *ART4* gene.

In the detection of the ABO blood group, group O is the most common group among Chinese healthy blood donors. The *ABO*-BEL.03* allele was detected in one individual with type O. B antigen expression is very weak in this allele and requires confirmation by the elution test. Therefore, the individual was misjudged as an O type in the conventional serological methods, which poses a certain safety risk in the transfusion process. In addition, a new O variant allele of c.882C > T was found. The genotype of the specimen was *ABO*O.01.01/ABO*O.01.02* heterozygote. Through data analysis, it was found that the c.882T variation and c.771T, c.829A (the polymorphism sites of the *ABO*O.01.02* allele) were in a single NGS read. Therefore, it is clear that the variation was located on the *ABO*O.01.02* allele. However, the variant O allele showed no enzymatic function due to c.261G deletion. Owing to the high homology between *RHD* and *RHCE*, the CLC Genomics Workbench 21.0 software aligned the vast majority of *RHD* reads to *RHCE* or *RHCE* reads with *RHD,* resulting in incorrect typing. Therefore, the results for Rh system genotyping were rechecked by manual analysis and

Table 3
The results comparison between the Sanger sequencing method and the established NGS method.

System name	Genotype	Phenotype	Number (NGS)	Number(Sanger)
MNS	*GYPA*M/GYPA*M*	M+	52	52
	*GYPA*M/GYPA*N*	M + N+	90	90
	*GYPA*N/GYPA*N*	N+	57	57
	*GYPB*s/GYPB*s*	s+	180	180
	*GYPB*S/GYPB*s*	S + s+	16	16
	*GYPB*S/GYPB*S*	S+	3	3
P1PK	*A4GALT*P1.01/A4GALT*P1.01*	P1+	199	199
	*A4GALT*01.02/A4GALT*01.02*	P1+	0	0
Lutheran	*LU*B/LU*B*	Lu(b+)	199	199
	*LU*A/LU*A*	Lu(a+)	0	0
Kell	*KEL**02/KEL**02*	k+	199	199
	*KEL*01.01/KEL*01.01*	K + k-	0	0
Duffy	*FY*A/FY*A*	Fy(a+)	170	170
	*FY*A/FY*B*	Fy (a+ b+)	28	28
	*FY*B/FY*B*	Fy(b+)	1	1
Kidd	*JK*A/JK*A*	Jk(a+ b-)	48	48
	*JK*A/JK*B*	Jk(a+ b+)	99	99
	*JK*B/JK*B*	Jk(a- b+)	52	52
Diego	*DI*B/DI*B*	Di(a–b+)	185	185
	*DI*A/DI*B*	Di(a+b+)	14	14
	*DI*A/DI*A*	Di(a+b–)	0	0
Yt	*YT*A/YT*A*	Yt(a+b-)	199	199
	*YT*B/YT*B*	Yt(a-b+)	0	0
Scianna	*SC*01/SC*01*	Sc1+	199	199
	*SC*02/SC*02*	Sc2+	0	0
Dombrock	*DO*A/DO*A*	Do(a+b-)	0	0
	DO*A/DO*B	**Do(a+b+)**	**36**	**37**
	DO*B/DO*B	**Do(a-b+)**	**163**	**162**
Colton	*CO*01.01/CO*01.01*	Co(a+)	199	199
	*CO*02/CO*02*	Co(b+)	0	0
Landsteiner-Wiener	*LW*A/LW*A*	LW(a+b-)	198	198
	*LW*A/LW*B*	LW(a+b+)	1	1
	*LW*B/LW*B*	LW(a-b+)	0	0
Cromer	*CROM*01/CROM*01*	Cr(a+)	199	199
	CROM–01/CROM*–01*	Cr(a–)	0	0
Knops	*KN**01/KN**01*	Kn(a+)	199	199
	*KN**02/KN**02*	Kn(b+)	0	0
Indian	*IN*B/IN*B*	In(a–b+)	199	199
	*IN*A/IN*A*	In(a+b–)	0	0
Ok	*OK*01.01/OK*01.01*	Ok(a+)	199	199
	*OK*01.–01/OK*01.–01*	Ok(a–)	0	0
Raph	*RAPH*01/RAPH*01*	RAPH:1	199	199
	*RAPH*01.−01.01/RAPH*01.−01.01*	RAPH: 1	0	0
John Milton Hagen	*JMH*01/JMH*01*	JMH+	199	199
	*JMH*01.–02/JMH*01.–02*	JMHK–	0	0
	*JMH*01.–03/JMH*01.–03*	JMHL–	0	0
I	*GCNT2*01/GCNT2*01*	I	199	199
	*GCNT2*01.02/GCNT2*01.02*	I	0	0
Gill	*GIL*01/GIL*01*	GIL+	199	199
	*GIL*01 N.01/GIL*01 N.01*	GIL−	0	0
FORS	*GBGT1*01 N.01/GBGT1*01 N.01*	FORS-	199	199
	*GBGT1*01.01/GBGT1*01.01*	FORS+	0	0
Vel	*VEL**01/VEL**01*	Vel+	199	199
	*VEL*01 N.01/VEL*01 N.01*	Vel–	0	0
CD59	*CD59*01/CD59*01*	CD59.1+	199	199
	*CD59*01 N.01/CD59*01 N.01*	CD59.1-	0	0
Augustine	*AUG*01/AUG*01*	At(a+)	199	199
	*AUG*02/AUG*02*	At(a−)	0	0
	*AUG*03/AUG*03*	ATML+	0	0

interpretation. The NGS analysis and manual analysis showed consistent results with those of serology. A similar situation was encountered during the MNS blood group system typing. *GYPA* and *GYPB* were also highly homologous, resulting in errors in the CLC software analysis, which could be corrected by manual analysis and interpretation. Therefore, the Rh phenotype and MNS phenotype of all samples were determined by manual analysis. For RhD, RhC, E, c, e phenotype, the nucleotide variations of c.48, c.178, c.203, c.307, c.676 of the *RHD* and *RHCE* were analyzed. While for MNS phenotype, nucleotide variations of the c.59, c.71, c.72 of the *GYPA* and the c.143 of the *GYPB* were determined. According to the nucleotide variations in these position, the RhD, RhC,E,c,e, and MNS phenotypes were determined respectively. However, it is need to improve the ability of analysis software and develop a professional

Table 4
Other variations in blood group systems by NGS method.

System name	Nucleotide change	dbSNP	Amino acid change	Number of specimens
Kell	c.526-53G > C	rs8175976	–	1
	c.526-53S	rs8175976	–	12
Duffy	c.199C > T	rs118062001	p.Ieu67Phe	2
	c.199Y	rs118062001	–	19
Kidd	c.812-7T > C(Y)	rs567213799	–	2
Scianna	c.138C > T(Y)	rs150122881	–	1
	c.213G > A(R)	rs200998492	–	1
	c.224A > G(R)	rs199680522	p.Gln75Arg	1
Dombrock	c.624C > T(Y)	rs3088189	–	35
Colton	c.180C > T(Y)	rs11537656	–	9
	c.331A > G (R)	rs758373056	p.Ile111Val	1
	c.377G > A (R)	rs556371635	p.Arg126His	1
Landsteiner-Wiener	c.144C > A (M)	rs35713817	p.Phe48Leu	2
	c.394 + 24G > A(T)	rs145682448	–	6
Knops	c.4577C > A (M)	rs117571325	p.Ser1526Tyr	1
	c.4619A > G (R)	rs17259045	p.Asn1540Ser	3
	c.4677G > A (R)	/	–	1
Ok	c.235G > C(S)	rs775681075	–	1
Raph	c.457-14T > C(Y)	rs199498141	–	1
Gill	c.556G > A(R)	rs760141667	p.Val186Ile	2
	c.710 + 27C > T	rs2231235	–	2
	c. 710 + 27Y	rs2231235	–	18
John Milton Hagen	c.1920G > A(R)	/	–	1
Diego	c.113A > C(M)	rs5035	p.Asp38Ala	27
Indian	c.255C > T(Y)	/	–	53
FORS	c.397G > A(R)	/	p.Glu133Lys	3
	c.707G > A(R)	/	p.Arg236His	24
LAN	c.412A > G	/	p.Met138Val	1
	c.1598T > A	/	p.Phe533Tyr	1
	c.1600_1601insT		p.Gly534fs	

A = Adenine,T = Thymine,C=Cytosine,G = Guanine,S = G or C,Y=C or T,R = A or G,M = C or A.

software for blood group systems in the future.

Only one genotype was found in 22 blood group systems, indicating low polymorphism. All polymorphism sites in the exon and flanking intron regions of 36 blood group systems can theoretically be detected by the NGS method. There are differences in phenotypes of the blood group systems in different countries and regions [23–26]. Interestingly, some null alleles were found. c.376C > T heterozygous variation in the *ABCG2* of the JR blood group system was found in 4 out of the 199 specimens. It was reported that the *ABCG2*01 N.01*(c.376C > T) homozygote can lead to the JR(a-) phenotype, which is a rare blood type. According to the frequency of c.376C > T variation in this study, the frequency of the JR(a-) phenotype is estimated to be approximately 1/10000 in the Chinese population. One specimen had c.1598T > A and c.1600_1601insT variations in exon 10 of the *ABCB6*, resulting in an amino acid change from phenylalanine to tyrosine (p.Phe533Tyr) in position 533, and a frameshift mutation after the amino acid 534 (p. Gly534fs). Whether these variations affect the function of the proteins remains to be further investigated. Other variation sites (Table 4) were also found in this study, but the significance of these variations remains unknown and needs to be further studied.

The NGS technology platform in this study can simultaneously synchronize for the genotyping of 36 blood group systems, with high throughput and high accuracy, providing a new method for the identification of blood group antigens. However, some shortcomings were found and require improvement. This method could not assess the Chido/Rodgers system, which was mainly due to the *C4A* gene and *C4B* gene being highly homologous, and the presence of recombination events. In addition, the NGS technique cannot automatically distinguish Rh and MNS using the CLC software, and a specialized bioinformatics system is required for blood group systems analysis. Finally, up to now, 45 blood group systems have been discovered, and the technique should be updated to include all 45 blood group systems.

4. Conclusion

Blood group genotyping is widely applied to confirm rare phenotypes, prenatal diagnosis, large-scale screening of blood group matching donors, etc. In this experiment, a high-throughput method was established for the simultaneous diagnosis of 36 red blood group systems, which helps to improve the accuracy of identification of RBC antigens. The method allows for the identification of rare blood types and improves the safety and effectiveness of RBC transfusion.

5. Methods

5.1. Blood specimen collection

This study was conducted on 199 healthy whole blood donors in the Blood Center of Zhejiang Province, China. The project was approved by the Ethics Committee of the Blood Center of Zhejiang Province, China (approval number: No.004, dated 13-01-2022). Written informed consent was obtained from all participants. All donors have filled out the health consultation form and completed the required tests, including a health history questionnaire, a brief physical examination, and rapid pre-donation testing according to the guidelines for blood donation in China. Whole blood was collected from eligible donors in an aseptic tube with EDTA anticoagulant after topical disinfection. Then, ABO and Rh blood group phenotyping was performed using serological methods according to our previous reports [12,36].

5.2. Genomic DNA extraction

DNA was extracted from whole blood using MagNA Pure LC DNA Isolation Kits III (Roche-diagnostics Inc, Indianapolis, IN, USA) according to the manufacturer's instructions. The quality and quantity of DNA was detected using an ultraviolet spectrophotometer (MULTISKAN GO, ThermoFisher Scientific, Waltham, MA, USA) and Qubit®dsDNA HS Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). The final DNA concentration was adjusted to 50 ng/μL.

5.3. Probe design

A total of 871 probes (Table S1) for 41 genes related to 36 human blood groups, including *AQP1, AQP3, ABO, ABCG2, ABCB6, ACHE, ACKR1, A4GALT, ART4, BSG, BCAM, B3GALNT1, C4A, C4B, CD59, CD44, CD55, CD99, CD151, CR1, ERMAP, FUT1, FUT3, GCNT2, GYPA, GYPB, GYPC, GYPE, GBGT1, ICAM4, KEL, RHCE, RHAG, RHD, SEMA7A, SLC29A1, SLC4A1, SLC14A1, SMIM1, XG, and XK* were designed using BaitsTools. The probes covered the exon and flanking intron sequences of each gene. The designed probe had a length of 120bp and was synthesized using the CapSeq single DNA synthesis technique. After inspecting the single probe, biotin modification was performed at the 5 'end of the probe and evenly mixed to form the capture probe mixture (WISGEN BIO, Hangzhou, China).

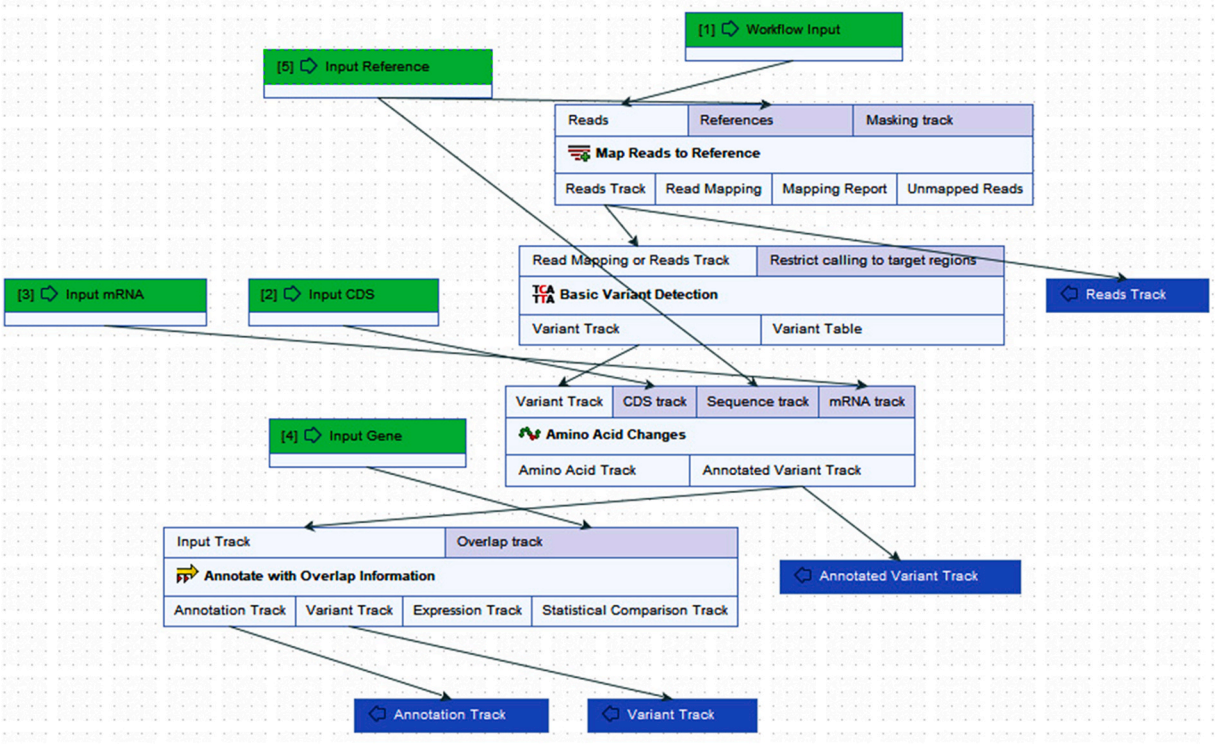

Fig. 2. CLC Genomics Workbench 21.0 software data analysis steps.

5.4. Library preparation and sequencing

The DNA library was constructed using the FS Pro DNA Lib Prep Kit for Illumina (ABclonal Technology, Wuhan, China) according to the manufacturer's instructions. The primary procedures involved fragment DNA, end repair, A-tailing, ligation adaptor, fragment sorting, and library amplification. The initial library and probe mixture were then hybridized. DynabeadsTM M − 270 Streptavidin (ThermoFisher Scientific, Waltham, MA, USA) was added to capture the hybrid DNA, and the elution was discarded to remove the unbound DNA. The capture libraries were then amplified and purified. Subsequently, the final sequencing libraries were obtained and quality-checked using the Aligent 4200 TapeStation Instrument (Agilent Technologies, Santa Clara, CA, USA). The results confirmed the concentration of the fragment distribution, showing a main peak of about 300bp. Sequencing was conducted using an Illumina NovaSeq instrument with PE300 sequencing mode, and 1G sequencing volume for each specimen. The original data were obtained in the fastq format using the software in the NovaSeq instrument.

5.5. Sequencing analysis

The sequencing data were further analyzed using the CLC Genomics Workbench (version 21, Qiagen, Hilden, Germany) according to the manufacturer's instructions. The blood group sequence analysis module was first constructed using CLC Genomics Workbench software, and the raw sequencing data were compared with the conventional reference sequence of each gene from ISBT. Variant analysis was performed on the coding regions of the 36 blood groups-related genes. The alleles of each blood group were predicted by analyzing the variants for each gene (Fig. 2).

5.6. Analysis of the copy number of the RHD gene

The read number of the *RHCE* gene was used as a reference for each variation site as two copies of *RHCE* can be found in all individuals except for very rare conditions. The read frequency at the exon variation sites was used to determine *RHD* heterozygosity. In cases with homozygous *RHD* with 2 copies, the *RHD* genotype was determined to be *RHD+/RHD +* when the frequency of each variation site approached 50 % ($\frac{2\text{RHD copys}}{2\ \text{RHD+2 RHCE}} * 100$).

For individuals exhibiting a hemizygote of 1 copy, a frequency of each variation site of approximately 33 % ($\frac{1\text{RHD copy}}{1\ \text{RHD+2 RHCE}} * 100$) indicated that the *RHD* genotype was *RHD+/RHD-*.

The *RHD* zygosities of all specimens were also analyzed using the PCR-SSP method according to our previous report [22].

5.7. 24 blood group systems were detected using Sanger sequencing

For accuracy validation of the results using NGS, partial polymorphism sites in the 24 blood group systems were detected using Sanger sequencing, including *AQP1, AQP3, ACHE, ACKR1, A4GALT, ART4, BSG, BCAM, CD59, CD44, CD55, CD151, CR1, ERMAP, GCNT2, GYPA, GYPB, GBGT1, ICAM4, KEL, SEMA7A, SLC29A1, SLC4A1, SLC14A1, and SMIM1*. A total of 26 pair primers (Table S2) were designed and amplified by polymerase chain reaction, and the amplicons were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kits according to the manufacturer's instructions (Applied Biosystems, Waltham, MA, USA). The sequences were analyzed using the SeqScape v2.5 software (Applied Biosystems) and each polymorphic site and new variation site of each blood group was recorded.

Ethics declarations

This study was reviewed and approved by the Ethics Committee of the Blood Center of Zhejiang Province with the approval number: No.004, dated 13-01-2022.

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Data availability statement

Data associated with this study is not deposited into a publicly available repository. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Jingjing Zhang: Writing – original draft. **Wenjing Yuan:** Formal analysis. **Xiaozhen Hong:** Methodology. **Yanling Ying:** Writing – review & editing. **Faming Zhu:** Writing – review & editing.

Declaration of competing interest

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33608.

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