Transfusion Medicine Reviews 27 (2013) 2–9

Contents lists available at SciVerse ScienceDirect

Transfusion Medicine Reviews

journal homepage: www.tmreviews.com

Original Articles

Matrix-Assisted Laser Desorption/Ionisation, Time-of-Flight Mass Spectrometry–Based Blood Group Genotyping—The Alternative Approach

Christoph Gassner [a,*], Stefan Meyer [a], Beat M. Frey [a], Caren Vollmert [b]

[a] Blood Transfusion Service Zurich, SRC, Department of Molecular Diagnostics &Cytometry (MOC), 8952-Schlieren, Switzerland
[b] Sequenom GmbH, Hamburg, Germany

A B S T R A C T

Although matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry (MALDI-TOF MS) has previously been reported for high throughput blood group genotyping, those reports are limited to only a few blood group systems. This review describes the development of a large cooperative Swiss-German project, aiming to employ MALDI-TOF MS for the molecular detection of the blood groups Rh, Kell, Kidd, Duffy, MNSs, a comprehensive collection of low incidence antigens, as well as the platelet and granulocyte antigens HPA and HNA, representing a total of 101 blood group antigens, encoded by 170 alleles, respectively. Recent reports describe MALDI-TOF MS as a technology with short time-to-resolution, ability for high throughput, and cost-efficiency when used in genetic analysis, including forensics, pharmacogenetics, oncology and hematology. Furthermore, Kell and RhD genotyping have been performed on fetal DNA from maternal plasma with excellent results. In summary, this article introduces a new technological approach for high throughput blood group genotyping by means of MALDI-TOF MS. Although all data presented are preliminary, the observed success rates, data quality and concordance with known blood group types are highly impressive, underlining the accuracy and reliability of this cost-efficient high throughput method.

Blood group genotyping began in 1990, when Yamamoto and colleagues defined the molecular genetic basis of the histo-blood group ABO system. In 1992, Le van Kim and colleagues amplified for the first time RhD-specific polymerase chain reaction (PCR) products [1,2]. Typical target applications are (i) genotyping apparently RhD negative, genetically *RHD* positive individuals, (ii) detailing blood donors' antigenic profiles even from post-transfusion blood mixture samples, (iii) clarifying rare blood group variants, and (iv) screening for blood donors with (very) rare antigenic constellations [3–6].

Twenty years after the first genotyping attempts, 328 recognized blood group antigens, of which 284 are comprised within 30 blood group systems, have been described on the molecular level [7]. Most antigens differ from their antithetic partner by Single Nucleotide Polymorphisms (SNPs) in their genomic sequence, or may technically be addressed accordingly. The Blood Group Antigen Gene Mutation Database currently lists 1,334 alleles in 42 genes [8]. These refer to authenticated blood group antigens falling into one of the 4 ISBT classifications: systems, collections, and low- and high-incidence antigens, respectively [9]. Molecular blood group typing has co-evolved with available SNP typing and Next Generation Sequencing technologies [10]. Several reviews focus on blood group–related genotyping approaches [5,11–13].

The following review is intended to describe the current status of a large cooperative project, aiming to develop and implement a high through-put alternative, the matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry (MALDI-TOF MS) blood group genotyping, at the Blood Transfusion Service of the Swiss Red Cross in Zurich, Switzerland, together with Sequenom GmbH, Hamburg, Germany.

MALDI-TOF MS for Genetic Analysis

Matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry was initially introduced in proteomics applications, while the full potential for DNA analysis was demonstrated in 1995 [14].Optimized for the detection of nucleic acids the MALDI-TOF MS MassARRAY system is currently applied for SNP genotyping (including insertions and deletions), somatic mutation screening, quantitative gene expression and copy number variation analysis, and DNA methylation detection [15–21]. Due to the short amplicon length and minimal DNA amount requirements, even formalin-fixed and paraffin-embedded tissue derived or ancient DNA samples can be processed. The system has been used successfully for instance in forensics, oncology, pharmacogenetics, and hematology, including β-thalassemia genotyping [22–30]. Non-Mendelian mixtures of DNAs, eg, fetal DNA in maternal plasma, or somatically mutated DNA in cancerous tissue are technically challenging to detect due to the preponderance of maternal or wild-type sequences, respectively.

* Corresponding author. Tel.: +41 58 272 5195; fax: +41 44 731 9010.
E-mail address: c.gassner@zhbsd.ch (C. Gassner).

0887-7963/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.tmrv.2012.10.001

MALDI-TOF MS has been successfully employed in genotyping fetal, paternally inherited, SNPs [31]. Quantitative sensitivity of 5% and less has been shown [16,32].

SNP genotyping starts with a multiplex PCR amplification of the genomic DNA, followed by an allele-specific single base extension of a primer that anneals directly adjacent to the SNP of interest. Resulting single-stranded, nucleic acid oligomer analytes of 15 TO 30 bp in length (4,300–9,000 Dalton range) will be deposited on a silicon chip with pre-spotted matrix crystal (eg, 3-hydroxy picolinic acid) containing patches. The analyte/matrix co-crystals are then irradiated by a laser inducing their desorption and ionization. The +1 positively charged molecules accelerate into a flight tube towards a detector. Separation occurs by the TOF, which is proportional to the mass of the individual molecules. Low mass molecules arrive in a shorter time than those of higher masses, and molecules of different masses are thereby separated. After data processing, a spectrum is produced with relative intensity on the y-axis and mass/charge on the x-axis (Fig 1). The main advantage of the method is that it directly measures the mass of the molecules of interest, without using any surrogate, such as fluorescence [19]. The platform supports multiplexed reactions up to a plex level of 40+ assays (SNPs) per reaction, acquires and interprets data quickly, gives a quantitative output and is highly sensitive [33]. MALDI-TOF MS SNP genotyping is accurate, highly automatable and fast, with a capacity of up to 150,000 SNPs per day. Beside this high throughput 384 well format, there is also a system of low-density 96-well chip format available. While the former is well suited for the analysis of up to several 1000 samples a day, the latter is optimal for an amount of up to several 100 samples per day. That is, due to its flexibility, investigators can screen medium to large sets of nucleic acid biomarkers affordably and simultaneously across medium to large numbers of samples [34].

Requirements for Blood Group Genotyping and Rationales for MALDI-TOF MS

Focusing on a set of SNPs, blood group genotyping is rapidly evolving, and several MALDI-TOF MS approaches have been reported. Within the HÉMA Québec blood donor screening, Génome Québec developed a blood group antigen panel including M/N, S/s, RHD/d, RHE/e, K/k, Kpa/b, Fya/b, and HPA-1. While the original design was on GenomeLab SNP stream technology, Génome Québec changed to Sequenom's MassARRAY platform developing a 40 antigen panel (personal communication Phillips, M.S., 2012) [35]. In addition, Sequenom's MassARRAY platform has been used for the genotyping of human platelet antigens, eg, HPA-1 to 5 and 15 [12,36]. In comparison to other methods, the authors give a positive report on short time-to-resolution, ability for high throughput, and cost-efficiency [36]. Furthermore, the high throughput capability and efficiency of MALDI-TOF MS has been examined for the non-invasive

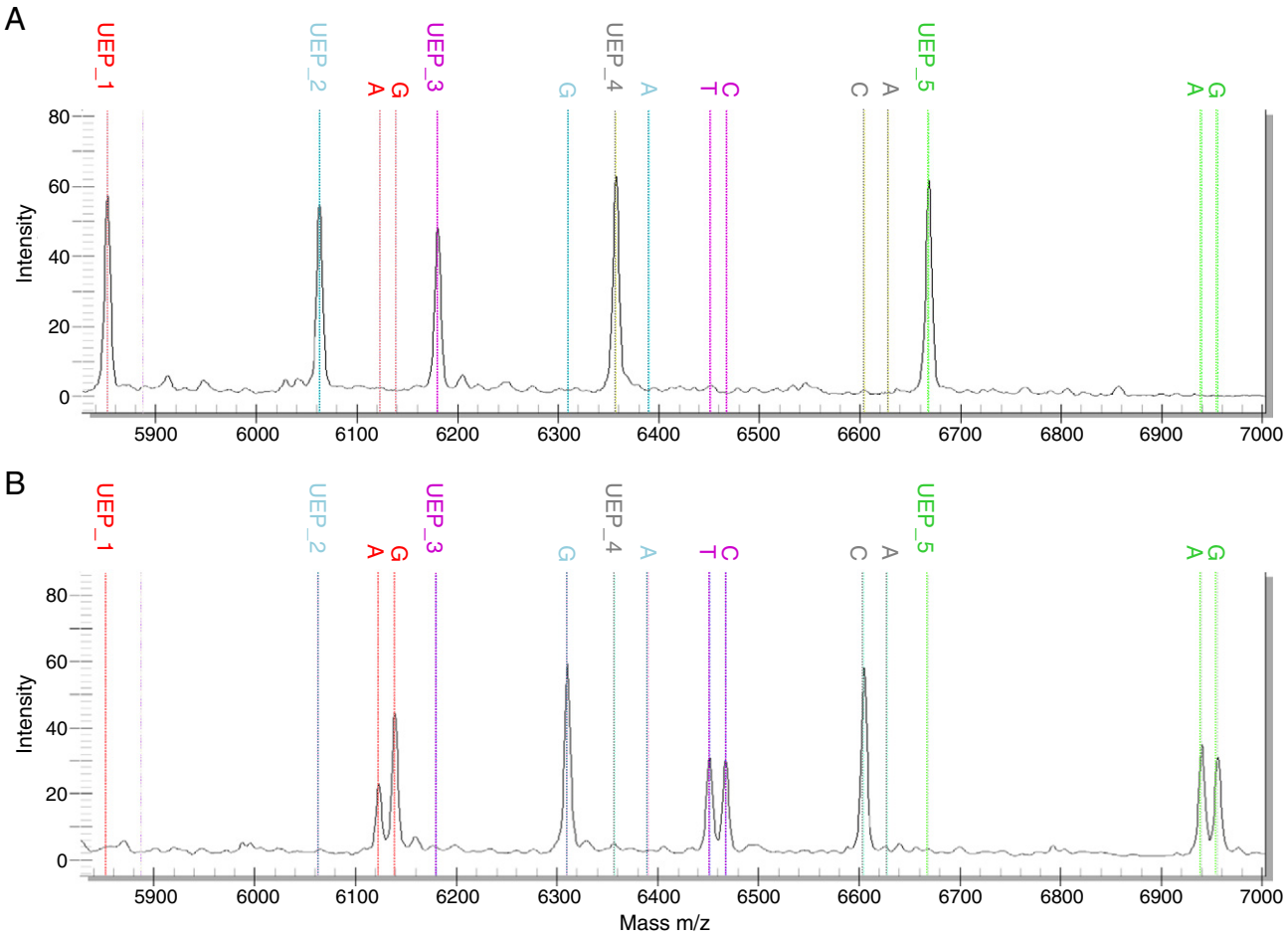

Fig 1. MALDI-TOF MS genotyping. Mass spectrogram. MALDI–TOF mass spectrum of five SNP sites (out of 14) in different genes of the HPA/HNA module from a negative control sample (H_2O) (A) and one exemplary DNA sample (B). For every SNP the calculated mass of the UEP (UnExtended Primer before single base extension) and the masses of the corresponding extended primers are marked by dashed lines (colour-coded per SNP assay); as expected in the water sample only peaks of UEPs were detectable. In contrast, the mass spectrum of the DNA sample revealed either one or two extension products depending on homozygosity (SNP2, SNP4) or heterozygosity (SNP3, SNP5) of the SNP at this position, respectively. Strikingly, compared to the two expected equal peak areas of the heterozygous SNP3 and 5, the mass spectrum of SNP1 showed an uneven peak area quotient. This is caused by a generic background amplification of *FCGR3A* (2 Guanosine) and a G>A SNP at the same position in *FCGR3B*.

determination of the fetal RhD and Kell status of fetal DNA in maternal plasma [37–41].

With respect to the requirement for blood group genotyping *donor- and recipient-specific* approaches need to be distinguished. In recipient typing, typically, only individual samples are analyzed at varying time-points, due to the urgency and impact of the results on transfusion decisions. In contrast, genotyping of donors aims to type as many blood group systems and donors' samples as possible, (i) to identify rare blood group constellations, and (ii) to generate donor data sets. These sets represent an unprecedented multiplicity of blood group information, and may therefore be used for "in silico matching" (or "dry matching") [42]. This theoretically allows broadest matching of donors'/recipients' genotype/phenotype before transfusion and will improve transfusion safety. Consequently, it may reduce new immunizations and circumvent hazards by preformed alloantibodies in recipients' circulation.

In general, analytic accuracy represents a striking difference between donor- and recipient-specific genotyping with respect to the required resolution level. For instance, null-alleles remain unidentified since they cannot be detected, and donor typing would accept these, because such heterozygous "pretender-results" would phenotypically behave as homozygous. So, there is no harm to the patient, if being transfused according to its heterozygous phenotype. However, given the high level of analytic resolution by MALDI-TOF MS, including maximal number of null, and other variant alleles per blood group system investigated, genotype misinterpretation can be strongly reduced if not fully avoided.

Currently, recipient typing requirements are satisfactorily addressed by rapid, flexible and comprehensive PCR-SSP approaches in the Zurich institute. For donor typing, however, MALDI-TOF MS genotyping ideally complements the current typing strategy due to its distinctive high throughput capability, allowing both, rare donor identification and generation of single donor data sets for a foreseeable in silico matching [42].

Even in the future, recipient- and donor blood group genotyping may require two independent and technologically different platforms to address the different needs accordingly. However, MALDI-TOF MS–based blood group genotyping may represent the one technological high throughput platform, optimally covering all requirements for donor specific blood group genotyping. In its 384-well plate version, limitations may rather result from insufficient DNA sample number preparation, an under-scaled PCR cycling production line, or limited man-power, then from the analytical capacity of the mass spectrometer itself.

Throughput depends on (i) the number of DNAs investigated, (ii) the number of SNPs these DNAs are investigated for and (iii) the time needed for that. A single multiplex reaction, eg, covering 13 SNPs specific for K/k, $Jk^{a/b}$, $Fy^{a/b/x/null}$, MN, Ss, HPA-$1^{a/b}$, $Kp^{a/b}$, $Lu^{a/b}$, LU14/18, $Yt^{a/b}$, and $Co^{a/b}$, would allow for genotyping of approximately 3,840 DNAs per day (10 times 384 different DNAs). The below described comprehensive blood group genotyping panel for 107 SNPs, logically arranged for specific blood groups in 5 modules including a total of 10 multiplex reactions, allows typing of 384 DNA samples per day (384 different DNAs typed with 10 different multiplex reactions each). Turn-around time is one working day (6-8 hours). Alternatively, individual steps of the lab process can also be run over night, or the process can be interrupted at any time (eg, after amplification, SAP treatment, elongation, and resin purification), and resumed at any later time point. Products are stable for up to 6 months if stored in sealed microtiter plates at −20°C.

Another requirement is *flexibility*. The MALDI-TOF MS platform does not need fixed formats like DNA-chips, and users are therefore free to select and customize modules of their interest. These can be combined as well as be processed independently; eg, genotyping of Kp(a+b−), or Yt(a−b+) is independent of Jk, or MNSs. Additionally, the platform allows easy integration of (new) blood group SNPs, or creation of new combinations using provided assay design software tools, following good laboratory practice guidelines, and national legislation requirements. Even current research-use-only modules already serve perfectly for identification of rare donors, which may then be confirmed by certified applications.

In contrast, recipient blood group genotyping requires a different kind of flexibility. In general, requirements such as excessively variable sample numbers per day, dependency on time-to-result and highly individual demands for different genetic blood group specificities seem not to be compatible with high-throughput oriented microtiter-plate formats. However, given a certain daily sample number and replacing the recipient-specific genetic demands with a more generalized process, eg, using the same general panel for every recipient, MALDI-TOF MS may readily accept this challenge.

Finally there are requirements for the *costs of genotypes*. For Europe list prices of the full 96-well and full 384-well format system are €250,000 and €350,000, respectively. Thereby, the 384 well format system automatically includes the option to use the 96-well format, which allows to adapt the system configuration to high and low throughput needs within minutes. While these complete hardware costs for MALDI-TOF MS genotyping are relatively high, consumables and personal costs are comparably affordable, which is an advantage in high throughput approaches. Consumables list price per multiplex reaction will be € 10. This would result in a consumables price per sample of €10 for a highly comprehensive analysis covering *KEL*(Kell), *SLC14A1*(Jk) and *DARC*(Fy), or €0.90 per antigen couple of the "rare" module investigating low incidence antigens (Table 1). Alternatively, €70 will be charged for the full panel of 10 multiplex reactions (Table 1), also including the MNS module.

Blood Group Genotyping by MALDI-TOF MS, Standard Operation Procedure for All Modules

Written informed consent for molecular blood group genotyping was obtained for all donor samples investigated in the project. The "Kantonale Ethikkommission Zurich" confirmed the proposed project as no subject to ethical authorization according to the cantonal and national Swiss legislation.

A total of 170pre-typed DNAs, including rare alleles such as weak *RHD* type 15 (*RHD*15*), *RHD*psi (*RHD*04N.01*), *KEL2*(IVS3+1g>a) null (*KEL*02N.06*), or rare genotypes, such as *KEL*17 (*KEL*02.17*), *FY**X (*FY*02M.01*), or *IN*1 (*IN*01*) homozygous samples, were used as positive control DNAs for verification and validation of 87% of all assays included in the various modules. In case of unavailability of natural control DNAs, eg, *SC*2 (*SC*02*), *LW*B (*LW*07*), and 22 others (13% of all assays), artificial DNA fragments were synthesized by standard PCR using one mutated and one regular amplification primer, covering the prospective diagnostic site.

For routine samples, beside manual DNA preparation using Nucleon BACC 3 reagents (Gen-Probe Life Sciences Ltd, Manchester, UK), automated DNA preparation has been implemented, applying the Chemagen magnetic bead technology (Perkin Elmer, Baesweiler, Germany) in its automated 96-well microtiter plate format. Preparation was done from 0.2 ml and 8 ml EDTA anticoagulated blood, for the automated and manual protocol, resulting in approximately 8 μg (in 100 μl eluate) and 400 μg total genomic DNA, respectively. Sequenom recommends using 20 ng DNA per multiplex reaction, resulting in a total amount of 200 ng for the full panel including 10 multiplexes. In order to avoid time-consuming UV quantification of every single DNA, a standard of 0.4-μL eluate (averaging a total of 33 ng per multiplex reaction) of automatically prepared DNAs were tested, and drop-out rates were compared to results of manually prepared and UV quantitated DNAs with 50 ng per multiplex reaction using the 17 SNP module KEL-JK-FY. Total DNA drop-out rates, calculated as number of DNAs without any valid results, were 1 of 760 (0.13%) and 4 of 756 (0.53%), respectively. Total SNP drop-out rate,

Table 1
Blood group specificities detected in the 6 different modules

Module name	Genes	Positions on genes, (trivial) (allele) names, additional information	n MPX	n SNPs	n alleles	n antigens
KEL-JK-FY			1	15	21	12
	KEL	K/k, Kp, Js, eg, KEL(ISVS3+1g>a)null & 5 others, KEL(1719C>T)mod		9	11	7
	SLC14A1	Jk, JK(IVS5-1g>a)null & 2 others, JK(582C>G)		3	6	3
	DARC	Fy, -67T>C, FYX		3	4	2
	GYG2, AMXY	cross-ID-control		2	2	0
MNSs §	*GYPA, GYPB, GYPE*	currently under development	2	11	15	10
"Rare antigens"			2	22	34	45
	KEL	K/k, Kp, Js, KEL11/17		4	5	8
	LU	Lu, LU8/14, Au		3	4	6
	Band 3	Di, Wr		2	3	4
	ACHE	Yt		1	2	2
	AQP1	Co		1	2	2
	CR-1	Kn, McC, Sl		3	4	6
	ART4	Do, Hy, Jo		3	4	6
	ICAM4	LW		1	2	2
	ERMAP	SC		1	2	2
	DAF	Cr, Tc		2	4	5
	CD44	In		1	2	2
	GYG2, AMXY, ABO	cross-ID-control, ABO positions: 261, 802, 803		5	6	0
RH	*RHCE, RHD*		5	49	~79	17
	RHC, RHc , RHCw	122, 201, 307, i2		4	3	4
	RHE, Rhe	676 generic and on RHCE only		2	2	2
	RHD exons	−132, i1+18, 455, 514, 787, 916, 968, i7-327, 1048, 1170, 1193, 1359		12	~45	1
	RHD categories & partials	VII, DFL, DOL, DVL-2, V(697A), weak type 4.0-3, 11, 15, DNB, DAU		11	11	9
	RHD weaks	1, 1.1, 2, 3, 5, 17		6	6	0
	RHD DELs	delA147, IVS3+1g>a, IVS3+2T>A, K409K, X418L		5	5	1
	RHD nulls	W16X, Dces type 1 & 2, RHD-CE(2–9)-D 2 subtypes, RHDpsi, Y401X		9	7	0
	GYG2, AMXY, ABO	cross-ID-control, ABO positions: 261, 803		5	5	0
HPA-HNA	eg, *ITGB3, FCGR3b, SLC44A2*		1	13	25	23
	HPA	HPA-1 to 6, 15		7	14	14
	HNA	HNA-1, 3 to 5		6	9	9
	AMXY	cross-ID-control		1	2	0
		redundancy of "rare" to "KEL-JK-FY"		−3	−4	−6
		total different blood (platelet, granulocyte) groups	11	107	170	101
		total different cross-ID-controls		5	6	n.a.

calculated as the number of SNPs without any valid results, were 20 of 12,920 (0.15%) and 136 of 12,852 (0.53%), respectively. Therefore, laborious single DNA UV quantification of automatically prepared DNAs seems to be dispensable.

Sequenom's Assay Design software was used to select PCR primer sets that uniquely amplify only the region of interest, and validate the primers within a multiplex for unintended cross-reactions with paralog genes and other regions of the genome. All SNPs were genotyped following the standard Sequenom MassARRAY iPLEX Pro genotyping protocol and using the Sequenom MassARRAY Analyzer 4 platform. Data analysis was performed by Sequenom's TYPER 4.0 software. While Microsoft Excel software (Microsoft, Redmond, WA) is currently used to translate genotyping data into blood group information, an R-based interpretation software tool is under development, which will be integrated into Sequenom's TYPER 4.0 Analyzer software.

MALDI-TOF MS Genotyping Specific for Kell-Kidd-Duffy, MNSs, Rare Antigens, Rh, and HPA/HNA

All blood group specificities were arranged into 6 modules, 2 specific for Rh, and 1 each for Kell-Kidd-Duffy, MNSs, rare antigens, and HPA/HNA as detailed in Table 1. Every module includes either assays for genetic ABO, or gender determination, or both, which serve to link DNA samples to the respective donor phenotype data: "crosslink ID control." This represents a powerful tool to exclude qualitative sample mix-up. All data reported are preliminary and current status of validation is described where appropriate; however, further validation is ongoing for all modules.

KEL, *JK* and *FY* Genotyping and the Attraction of Null-alleles

Analysis of K, k, Jk^a, Jk^b, Fy^a, Fy^b, Fy^x and Fy^{null} (GATA-1) may be done by typing 5 SNPs only. However, null-alleles have been reported and their detection should be considered, even for high throughput donor typing, given that compound heterozygotes of rare alleles with null-alleles can be expected and additional costs are marginal [43,44]. Such individuals would add to the collection of rare donors, since behaving like phenotypically homozygous ones. Therefore, the most frequent *KEL* and *JK* null-alleles, beside specificities for Kp and Js, were additionally included in the developed multiplex (Table 1). During assay verification and validation, a 100% concordance of genotyping results with pre-typed controls was observed. Among another 1,520 donor DNA genotypes, comparison with serology of KEL, Jk and Fy showed 2 discrepancies for Jk and one for Fy, respectively. But, erroneous serological results and novel null, or weak alleles may not be excluded at this stage. In detail, allele positive individuals for Fy^x (*FY*02M.01* n = 36), Fy^{null}(GATA-1) (*FY*02N.01* n = 13), Kp^a (*KEL*02.03* n = 36), Js^a (*KEL*02.06* n = 3) and surprisingly one KEL(G573G)mod(*KEL*02M.05*, second report of this allele on the European continent), have been identified among the investigated Caucasian population of the Zurich area [43].

Genotyping the Polygenetic "rare" Low Incidence Blood Group Antigens

The two multiplex reactions for rare blood group genotyping are aimed to detect a total of 34 different alleles, encoded by

24 different SNPs found on 11 different genes plus 5 crosslink ID-control specificities, 3 for ABO and 2 for gender (Table 1).

In accordance with current ISBT terminology, typing of 24 bi-allelic SNPs results in 34 different alleles, instead of 48. For instance, one wild-type allele (eg, *KEL*02*) simultaneously encodes several antigens (eg, k, Kp^b, Js^b, KEL11), and is therefore counted as one instead of four alleles [45]. Due to independency and flexibility purpose, the rare module shares 3 SNPs with the KEL-JK-FY module. All genetically predefined control DNAs were concordantly typed. Allele encoding negativity for the Dombrock antigens Jo^a on *ART4*, uncommon among Caucasians, became only apparent among the 28 control samples with presumably black African ancestry. In summary, all specificities expected were concordantly typed either on natural (n = 21) or artificial control DNAs (n = 1, SC:2, *SC*02*).

Until now 3040 DNAs of the Zurich area have been typed using the "rare module", and rare homozygous carriers were identified for the following antigens K (n = 4), Kp^a (n = 1), Lu^a (n = 5), LU14 (n = 2), Yt^b (n = 14), and Co^a (n = 2), respectively. Observed allele frequencies in the investigated Caucasian population are concordant with those reported (Table 2A) [46]. However, beside plausibility testing of allele-frequencies, direct comparisons to phenotypic results or to an independent, phenotypically validated genotyping method is mandatory for validation.

Two Modules for *RH* Genotyping, and A Pleasant *rhd*zygosity Surplus

Typing for *RH* is done in two modules, "broad" and "variant", with a total of five multiplex reactions and on 49 "SNP" positions to detect a total of 79 different RH alleles (Table 1). Three multiplex reactions are used for the broad Rh antigen determination, such as RhD positivity and negativity, and RhC/c, and E/e entities, and two multiplex reactions for the detection of the variant Rh antigens, such as categories, partials, Dels and weak D types caused by single point mutations (Table 1).Typing on natural and artificial control DNAs gave the expected results for all specificities investigated. *RHD* exon specific SNPs represent sequence-specific positions on the *RHD* gene in relation to the highly homologous and generically co-amplified *RHCE* gene. *RHD* negatives of the homozygous deletion type [47]are clearly negative for all *RHD* exon specific SNPs.

This exon screening strategy also allows for reliable *RHD* gene copy number (*RHD*zygosity) detection of every individual sample by calculating the *RHD*/*RHCE* MALDI-TOF MS peak area quotient resulting in the differentiation of *RHD* hetero- and homozygous individuals, at multiple genetic positions, respectively (Table 1, Fig 2).This is not only of interest in order to detect *RHD*zygosity, which is a problematic serological task, but also to identify *RHD-CE-D* hybrid alleles, masked by regular *RHD* genes in compound heterozygous individuals. Unexpectedly, a total of 5

Table 2
Results of the "rare" module investigating low incidence antigens and *HPA* and *HNA* genotyping

Table 2A

antigen	ISBT allele	observed "rare" allele frequency (%), MALDI-TOF MS	n observed "rare" homozygous carriers (in 3.040)	n expected "rare" homozygous carriers (in 3.040)
K	*KEL*01*	0.0395	4	4.7
Kp^a	*KEL*02.03*	0.0107	1	0.3
Js^a	*KEL*02.06*	0.0008	0	0.0
KEL17	*KEL*02.17*	0.0013	0	0.0
Lu^a	*LU*01*	0.0370	5	4.1
LU14	*LU*02.14*	0.0093	2	0.3
LU19	*LU*02.19*	0.3182	313	303.9
Di^a	*DI*01*	0.0002	0	0.0
Wr^a	*DI*02.03*	0.0003	0	0.0
Yt^b	*YT*02*	0.0648	14	12.6
Co^b	*CO*02*	0.0333	2	3.3
Kn^b	*KN*02*	0.0283	5	2.4
McC^b	*KN*01.06*	0.0003	0	0.0
Vil	*KN*01.07*	0.0018	0	0.0
Do^a	*DO*01*	0.4061	487	495.8
Hy neg	*DO*02.–04*	0.0007	0	0.0
Jo^a neg	*DO*01.–05*	0.0000	0	0.0
LW^b	*LW*07*	0.0040	0	0.0
SC:2	*SC*02*	0.0000	0	0.0
Cr^a neg	*CROM*–01*	0.0002	0	0.0
Tc^b	*CROM*01.03*	0.0002	0	0.0
Tc^c	*CROM*01.04*	0.0008	0	0.0
In^a	*IN*01*	0.0000	0	0.0

Table 2B

Group system	Allele	Allele-frequency MALDI-TOF MS	Allele-frequency Switzerland	Allele-frequency Austria	Allele-frequency Germany
HPA	1b	0.147	0.191	0.148	0.161
	2b	0.090	0.109	0.082	0.090
	3b	0.334	0.407	0.388	0.414
	4b	0.000	0.000	0.000	0.000
	5b	0.106	0.066	0.108	0.083
	15b	0.490	n.a.	0.500	n.a.
HNA	1b	0.621	n.a.	n.a.	0.601
	1c	0.033	n.a.	n.a.	0.008
	3b	0.207	n.a.	n.a.	0.256
	4b	0.117	n.a.	n.a.	0.092
	5b	0.276	n.a.	n.a.	0.269

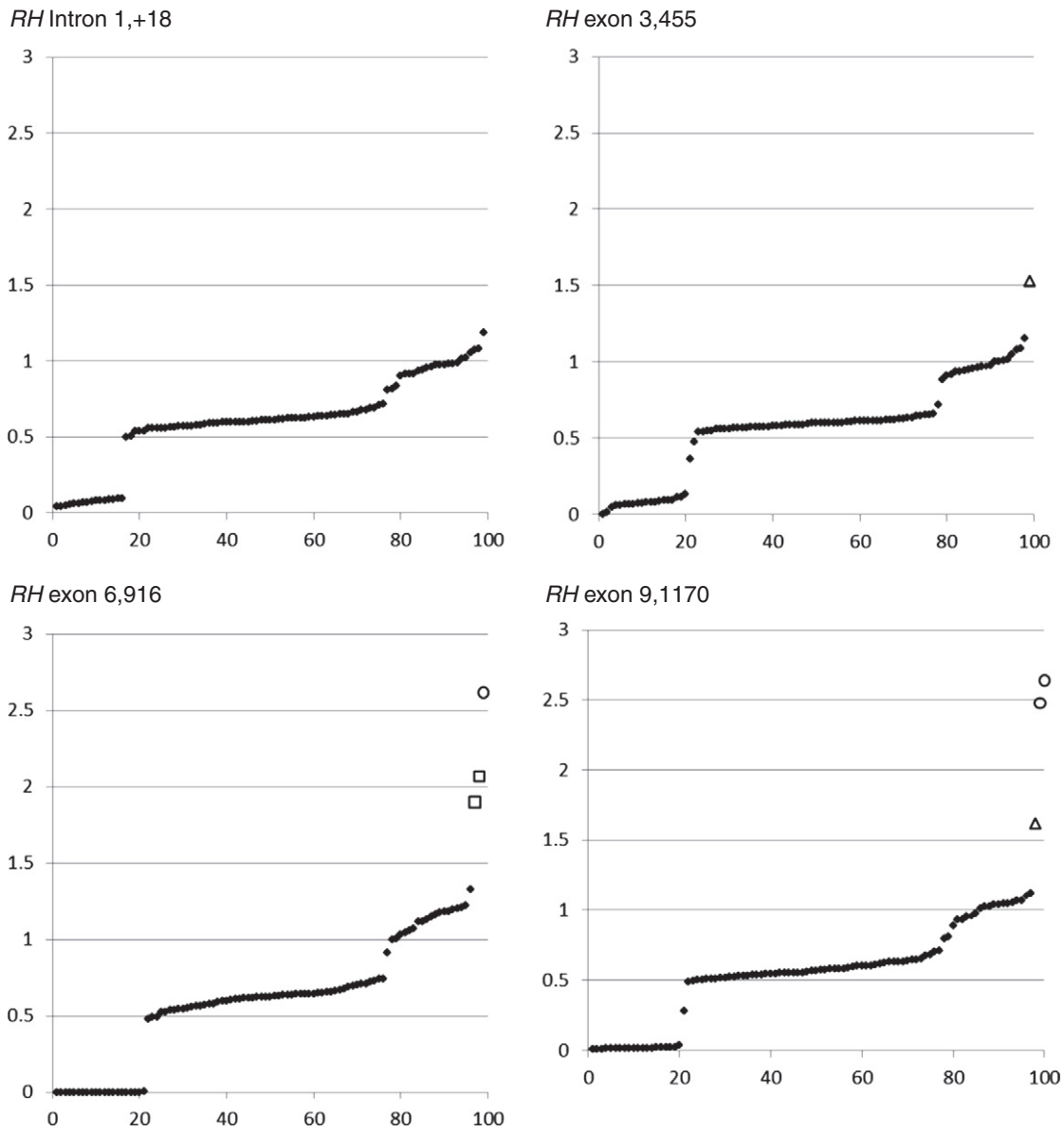

Fig 2. *RHD/RHCE* MALDI-TOF MS area quotient. *RHD/RHCE* MALDI-TOF MS peak area quotient of 100 control DNA samples (x-axis) at 4 out of a total of 12 investigated gene positions in intron 1(+18), and exons 3 (cd nt 455), 6 (cd nt 916), and 9 (cd nt 1170). Theoretical values for single samples are 0.0 in *RHD* gene negatives, and 0.5, and 1, representative for *RHD* gene hetero- and homozygous individuals, respectively(y-axis). Although these theoretical values do not exactly match for every group, clear cut differences may be identified for the vast majority of samples at the 4 genetic positions shown. Interestingly, samples with *RHD/RHCE* quotients higher than 1.0 and apparently approximating values of 1.5 in exons 3 and 9 in one *RHD* positive sample of Caucasian origin (open triangles), 2.0 in exon 6 in two *RHD* weak type 4.2 samples (*RHD*09.01*, open squares), and even 2.5 in exon 6 (1 sample), and exon 9 (2 samples), both samples with known positivity for *RHD*psi (*RHD*04N.01*, open circles), were observed.

samples of the original control panel consisting of 100 DNAs showed higher *RHD/RHCE* peak area quotients than 1 (Fig 2). Most likely, these results indicate mutant *RHCE* alleles, rather than suggesting complete *RHD* gene duplications on only one haplotype and need further investigation.

Final *RH* modules were tested on 186RhD positive routine DNAs and 4 ring test samples of the 5th international ISBT Workshop on molecular blood group genotyping in 2012, which were typed independently in Zurich and Hamburg [48]. Compared to other commercially available products the 2 *RH* modules are comparable, or even more comprehensive. Allele coverage easily fulfils requirements for routine *RHD* genotyping of donors and even recipients. In addition, this system is highly customizable to individual needs due to its flexible modular design and independent assay design tools. The Rh system is deemed to include the largest number of antigens and the most complex genetics of the 30 known blood group systems, although MNS may become a veritable competitor in the future [49]. However, with the successful analysis of the complex *RH* genetics, MALDI-TOF MS genotyping has proven its principle capability as a well suited platform for molecular blood group typing.

HPA and HNA Genotyping for Platelet and Granulocyte Antigen Analysis

This module allows the parallel investigation of samples forHPA-1 to −6 and HPA-15 as well as for HNA-1 and −3 to −5 in one multiplex reaction. All results were concordant with pre-typed controls. Of 1,520 platelet donor samples of the Zurich and Basel area, HPA allele frequencies are well comparable to data reported for Austrian while HNA allele frequencies are similar to those observed in Germany (Table 2B) [50,51].

MALDI-TOF MS Blood Group Genotyping: A Swiss Project

Mainly in order to ensure the supply of rare donor blood to the Swiss population, the whole project aims to type 36,000 Swiss DNAs with the "rare module". Two thirds of the donors are expected from the Zurich area, whereas the other third may be provided by the other 12 Swiss transfusion institutions. Additional typing of *HPA* and *HNA* for 3,000, and of *KEL, JK, FY, MNS* and *RH* with the presented modules for 6,000 donors will be included, respectively. A rough total of 1.5 million "SNPs", or 125,000 multiplex reactions, or 326 times a 384-well plate run, will be investigated in total until the end of July 2014.

Outlook and Conclusion

This manuscript introduces a new technological approach for high throughput blood group genotyping using MALDI-TOF MS. Besides the 5 modules presented above, a sixth module is currently under development. It comprises detection for MN-, Ss-, Vw-, Mg-, Hut-, He-, Mt(a)-antigens, and the detection of homo- and heterozygous *GYPB* deletions, hereby identifying hemi-, or homozygous U negative individuals. Although all presented data are preliminary and needs further validation, the observed success rates, data quality and concordance with known blood group types are highly impressive, underlining the accuracy and reliability of this cost-efficient high throughput method.

Acknowledgments

We thank Sonja Sigurdardottir and Nadine Trost for excellent technical assistance, and Susanne Müller, Heiko Meyer and Edvin-Munk for critical comments and review. Two samples of the exceedingly rare blood group phenotype In(a+b−) were kindly provided by Joyce Pool, IBGRL Bristol, UK. 200 DNA samples for HPA/HNA and Kell, Kidd and Duffy genotyping and 190 samples for "rare" genotyping were kindly provided by Andreas Buser, Blutspende SRK Basel, Basel, Switzerland.

Declaration of financial support and conflict of interest

This article introduces a new technological approach for high throughput blood group genotyping by means of MALDI-TOF MS using Sequenom's MassARRAY technology. The development of this technological approach is the result of a cooperative effort between the Blood Transfusion Service Zurich of the Swiss Red Cross (SRC) in Zurich, Switzerland, and the company Sequenom GmbH, Hamburg, Germany.

Aim of this project is to develop and implement MALDI-TOF MS as a high throughput alternative for the molecular detection of blood groups at the Blood Transfusion Service Zurich, SRC, Switzerland.

Financial support for this project is granted from the Humanitarian Foundation of the SRC, the Blood Transfusion Service Zurich, SRC, Switzerland, the Swiss blood transfusion umbrella organization Blutspende SRK Schweiz, Bern, Switzerland, and the company Sequenom GmbH, Hamburg, Germany.

The authors Christoph Gassner, Stefan Meyer, Beat M. Frey of this article are employed at the Blood Transfusion Service Zurich, SRC, Switzerland, and do not declare any conflicts of interest. Caren Vollmert is employed at Sequenom GmbH, Hamburg, Germany.

References

[1] Yamamoto F, Clausen H, White T, Marken J, Hakomori S. Molecular genetic basis of the histo-blood group ABO system. Nature 1990;345:229-33.
[2] Le van Kim C, Mouro I, Cherif-Zahar B, Raynal V, Cherrier C, Cartron JP, et al. Molecular cloning and primary structure of the human blood group RhD polypeptide. Proc Natl Acad Sci U S A 1992;89:10925-9.
[3] Wagner FF, Frohmajer A, Flegel WA. RHD positive haplotypes in D negative Europeans. BMC Genet 2001;2:10.
[4] Rozman P, Dovc T, Gassner C. Differentiation of autologous ABO, RHD, RHCE, KEL, JK, and FY blood group genotypes by analysis of peripheral blood samples of patients who have recently received multiple transfusions. Transfusion 2000;40:936-42.
[5] Denomme GA, Johnson ST, Pietz BC. Mass-scale red cell genotyping of blood donors. Transfus Apher Sci 2011;44:93-9.
[6] Jungbauer C, Hobel CM, Schwartz DW, Mayr WR. High-throughput multiplex PCR genotyping for 35 red blood cell antigens in blood donors. Vox Sang 2012;102:234-42.
[7] Storry JR, Castilho L, Daniels G, Flegel WA, Garratty G, Francis CL, et al. International Society of Blood Transfusion Working Party on red cell immunogenetics and blood group terminology: Berlin report. Vox Sang 2011;101:77-82.
[8] Patnaik SK, Helmberg W, Blumenfeld OO. BGMUT: NCBI dbRBC database of allelic variations of genes encoding antigens of blood group systems. Nucleic Acids Res 2012;40:D1023-9.
[9] ISBT. Committee on Terminology for RBC Surface Antigens. http://ibgrl.blood.co.uk/isbt%20pages/isbt%20terminology%20pages/terminology%20home%20page.htm 2012.
[10] Stabentheiner S, Danzer M, Niklas N, Atzmuller S, Proll J, Hackl C, et al. Overcoming methodical limits of standard RHD genotyping by next-generation sequencing. Vox Sang 2011;100:381-8.
[11] Anstee DJ. Red cell genotyping and the future of pretransfusion testing. Blood 2009;114:248-56.
[12] Garritsen HS, Xiu-Cheng Fan A, Lenz D, Hannig H, Yan Zhong X, Geffers R, et al. Molecular Diagnostics in Transfusion Medicine: in Capillary, on a Chip, in Silico, or in Flight? Transfus Med Hemother 2009;36:181-7.
[13] Gassner C. Molecular blood group diagnostics. Transfus Med Hemother 2009;36:154-5.
[14] Tang K, Fu D, Kotter S, Cotter RJ, Cantor CR, Koster H. Matrix-assisted laser desorption/ionization mass spectrometry of immobilized duplex DNA probes. Nucleic Acids Res 1995;23:3126-31.
[15] Millis MP. Medium-throughput SNP, genotyping using mass spectrometry: multiplex SNP genotyping using the iPLEX(R) Gold assay. Methods Mol Biol 2011;700:61-76.
[16] Fumagalli D, Gavin PG, Taniyama Y, Kim SI, Choi HJ, Paik S, et al. A rapid, sensitive, reproducible and cost-effective method for mutation profiling of colon cancer and metastatic lymph nodes. BMC Cancer 2010;10:101.
[17] Docherty SJ, Davis OS, Haworth CM, Plomin R, Mill J. DNA methylation profiling using bisulfite-based epityping of pooled genomic DNA. Methods 2010;52:255-8.
[18] Ding C, Cantor CR. A high-throughput gene expression analysis technique using competitive PCR and matrix-assisted laser desorption ionization time-of-flight MS. Proc Natl Acad Sci U S A 2003;100:3059-64.
[19] Ragoussis J, Elvidge GP, Kaur K, Colella S. Matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry in genomics research. PLoS Genet 2006;2:e100.
[20] Bradic M, Costa J, Chelo IM. Genotyping with Sequenom. Methods Mol Biol 2011;772:193-210.
[21] Meyer K, Ueland PM. Use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for multiplex genotyping. Adv Clin Chem 2011;53:1–29.
[22] Blievernicht JK, Schaeffeler E, Klein K, Eichelbaum M, Schwab M, Zanger UM. MALDI-TOF mass spectrometry for multiplex genotyping of CYP2B6 single-nucleotide polymorphisms. Clin Chem 2007;53:24-33.
[23] Cerezo M, Cerny V, Carracedo A, Salas A. Applications of MALDI-TOF MS to large-scale human mtDNA population-based studies. Electrophoresis 2009;30:3665-73.
[24] Xiu-Cheng Fan A, Garritsen HS, Tarhouny SE, Morris M, Hahn S, Holzgreve W, et al. A rapid and accurate approach to identify single nucleotide polymorphisms of mitochondrial DNA using MALDI-TOF mass spectrometry. Clin Chem Lab Med 2008;46:299-305.
[25] Mengel-Jorgensen J, Sanchez JJ, Borsting C, Kirpekar F, Morling N. MALDI-TOF mass spectrometric detection of multiplex single base extended primers. A study of 17 y-chromosome single-nucleotide polymorphisms. Anal Chem 2004;76:6039-45.
[26] Jaremko M, Justenhoven C, Abraham BK, Schroth W, Fritz P, Brod S, et al. MALDI-TOF MS and TaqMan assisted SNP genotyping of DNA isolated from formalin-fixed and paraffin-embedded tissues (FFPET). Hum Mutat 2005;25:232-8.
[27] Mendisco F, Keyser C, Hollard C, Seldes V, Nielsen AE, Crubezy E, et al. Application of the iPLEX(TM) Gold SNP genotyping method for the analysis of Amerindian ancient DNA samples: Benefits for ancient population studies. Electrophoresis 2011;32:386-93.
[28] Looi ML, Sivalingam M, Husin ND, Radin FZ, Isa RM, Zakaria SZ, et al. Multiplexed genotyping of beta globin mutations with MALDI-TOF mass spectrometry. Clin Chim Acta 2011;412:999–1002.
[29] Liao HK, Su YN, Kao HY, Hung CC, Wang HT, Chen YJ. Parallel minisequencing followed by multiplex matrix-assisted laser desorption/ionization mass spectrometry assay for beta-thalassemia mutations. J Hum Genet 2005;50:139-50.
[30] Vogel N, Schiebel K, Humeny A. Technologies in the whole-genome age: MALDI-TOF-based genotyping. Transfus Med Hemother 2009;36:253-62.
[31] Li Y, Wenzel F, Holzgreve W, Hahn S. Genotyping fetal paternally inherited SNPs by MALDI-TOF MS using cell-free fetal DNA in maternal plasma: influence of size fractionation. Electrophoresis 2006;27:3889-96.
[32] Vivante A, Amariglio N, Koren-Michowitz M, Ashur-Fabian O, Nagler A, Rechavi G, et al. High-throughput, sensitive and quantitative assay for the detection of BCR-ABL kinase domain mutations. Leukemia 2007;21:1318-21.
[33] Oeth P, del Mistro G, Marnellos G, Shi T, van den Boom D. Qualitative and quantitative genotyping using single base primer extension coupled with matrix-

assisted laser desorption/ionization time-of-flight mass spectrometry (MassARRAY). Methods Mol Biol 2009;578:307-43.
[34] Cantor CR. Company profile: Sequenom, Inc. Pharmacogenomics 2012;13:529-31.
[35] Montpetit A, Phillips MS, Mongrain I, Lemieux R, St-Louis M. High-throughput molecular profiling of blood donors for minor red blood cell and platelet antigens. Transfusion 2006;46:841-8.
[36] Garritsen HS, Fan AX, Bosse N, Hannig H, Kelsch R, Kroll H, et al. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for genotyping of human platelet-specific antigens. Transfusion 2009;49:252-8.
[37] Grill S, Banzola I, Li Y, Rekhviashvili T, Legler TJ, Muller SP, et al. High throughput non-invasive determination of foetal Rhesus D status using automated extraction of cell-free foetal DNA in maternal plasma and mass spectrometry. Arch Gynecol Obstet 2009;279:533-7.
[38] Bombard AT, Akolekar R, Farkas DH, VanAgtmael AL, Aquino F, Oeth P, et al. Fetal RHD genotype detection from circulating cell-free fetal DNA in maternal plasma in non-sensitized RhD negative women. Prenat Diagn 2011;31:802-8.
[39] Li Y, Finning K, Daniels G, Hahn S, Zhong X, Holzgreve W. Noninvasive genotyping fetal Kell blood group (KEL1) using cell-free fetal DNA in maternal plasma by MALDI-TOF mass spectrometry. Prenat Diagn 2008;28:203-8.
[40] Tynan JA, Angkachatchai V, Ehrich M, Paladino T, van den Boom D, Oeth P. Multiplexed analysis of circulating cell-free fetal nucleic acids for noninvasive prenatal diagnostic RHD testing. Am J Obstet Gynecol 2011;204:251.e1-6.
[41] Zhong XY, Holzgreve W. MALDI-TOF MS in prenatal genomics. Transfus Med Hemother 2009;36:263-72.
[42] Denomme GA, Flegel WA. Applying molecular immunohematology discoveries to standards of practice in blood banks: now is the time. Transfusion 2008;48:2461-75.
[43] Kormoczi GF, Wagner T, Jungbauer C, Vadon M, Ahrens N, Moll W, et al. Genetic diversity of KELnull and KELel: a nationwide Austrian survey. Transfusion 2007;47:703-14.
[44] Lucien N, Sidoux-Walter F, Olives B, Moulds J, Le Pennec PY, Cartron JP, et al. Characterization of the gene encoding the human Kidd blood group/urea transporter protein. Evidence for splice site mutations in Jknull individuals. J Biol Chem 1998;273:12973-80.
[45] ISBT. Terminology for blood group alleles. http://ibgrl.blood.co.uk/ISBTPages/AlleleTerminology/Allele-Terminology.htm 2012.
[46] Daniels G. Human Blood Groups. Blackwell Science; 2002.
[47] Wagner FF, Flegel WA. RHD gene deletion occurred in the Rhesus box. Blood 2000;95:3662-8.
[48] Daniels G, van der Schoot CE, Olsson ML. Report of the fourth International Workshop on molecular blood group genotyping. Vox Sang 2011;101:327-32.
[49] Flegel WA. Molecular genetics and clinical applications for RH. Transfus Apher Sci 2011;44:81-91.
[50] Immuno-Polymorphism-Database. IPD - HPA Database. http://www.ebi.ac.uk/ipd/hpa/2012.
[51] Hauck B, Philipp A, Eckstein R, Ott S, Zimmermann R, Dengler T, et al. Human neutrophil alloantigen genotype frequencies among blood donors with Turkish and German descent. Tissue Antigens 2011;78:416-20.