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Molecular Typing of Blood Cell Antigens

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Chapter 5

Parallel Donor Genotyping for 46 Selected Blood Group and 4 Human Platelet Antigens Using High-Throughput MALDI-TOF Mass Spectrometry

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Abstract

Most blood group antigens are defined by single nucleotide polymorphisms (SNPs). Highly accurate MALDI-TOF MS has proven its potential in SNP genotyping and was therefore chosen for blood donor oriented genotyping with high-throughput capability, e.g., 380 samples per day. The Select Module covers a total of 36 SNPs in two single-tube reactions, representative of 46 blood group and 4 human platelet antigens. Using this tool, confirmatory blood group typing for RhD, RhCE, Kell, Kidd, Duffy, MN, Ss, and selected rare antigens is performed on a routine basis.

Key words Blood group gene, Blood group genotyping, High-throughput SNP typing, MALDI-TOF MS, Kell, Kidd, Duffy, MNSs, Rare blood group antigens, Blood donor

1 Introduction

1.1 Blood Groups and Single Nucleotide Polymorphisms

Today, over 300 blood group antigens have been characterized [1, 2]. Nearly all were observed in patient samples using serological techniques [3]. The analysis of the molecular basis for blood group expression has identified single nucleotide polymorphisms (SNPs) as the major causative DNA variation between these antigens. Almost all common SNPs have only two alleles, and the variation may encode for two different amino acids within the coding regions, typically leading to the two representatives of two antithetic blood group antigens as exemplified for Colton a and b, respectively [4].

Since it is relevant for all blood group systems and their respective genes, insertions and deletions of single or multiple nucleotides (nts) represent the second common mechanism for the molecular determination of blood group antigens. Positioned within coding regions, these "indel" variations lead to altered reading frames, usually preventing physical presence, or catalytic activity of the

respective peptides as exemplified for by the famous deletion of G at coding nucleotide (cdnt) 261, inactivating the ABO glycosyl-transferase, thereby encoding the ABO O phenotype [5]. In genotyping, short insertions and deletions may technically be addressed according to SNPs. Many of these SNPs, representing intra-genetic variation, are entered in specialized databases, comprehensively documented and selectively retrievable by their unique reference SNP (rs) ID number [6].

However, genetic single nt variations between paralogs, e.g., two genes as a result of gene duplication, usually coding for proteins with a similar function and/or structure, are not considered as SNPs. Typical examples are the genes *RHD/RHCE* and *GYPA/GYPB* of the RhD/RhCE and MNSs blood group systems, respectively [7, 8]. Genetic dissimilarity between such highly homologous genes may be limited to 3.6 % of all cdnt (45 dissimilarities of 1,254 cdnt), as exemplified for the two genes *RHD* and *RHCE*, encoding the peptides specific for RhD and Rhce, respectively [7]. Again, molecular diagnosis of such inter-genetic variation is technically similar to common SNP detection and widely used since decades [9]. Dependent on the existence of paralogous loci, hybrid-genes represent the third common molecular mechanism for blood group antigen determination. Representative examples for such results of gene conversion events may be encountered within the Rh and MNSs blood group systems, occasionally [8, 10, 11].

1.2 Errors in Predicting Blood Group Phenotypes from Genotypes

SNP based genotyping of blood groups, is surprisingly accurate, considering the fact that most techniques rely on the exclusive detection of one single SNP for the prediction of a whole peptide (antigen, phenotype), usually consisting of several 100 amino acids, encoded by three times as many cdnts. However, minimizing the pleiority of potential reasons for technical failures applying robust techniques, e.g., MALDI-TOF MS, still the genetic background itself represents some risks for the correct prediction of blood group phenotypes deduced from genotypes. Most, if not all such "genotyping errors" may be explained by rarely occurring genetic variants of commonly known alleles, and therefore rather be interpreted as highly specific "indicators" further adding to the genetic complexity, than as profane "errors".

At least two different genetic backgrounds may cause such genetically reasoned "genotyping errors." One is the whole world of unexpressed alleles, the other a growing number of very rare mutant alleles, interacting specifically with primer binding sites nearby the diagnosed SNP of interest [12]. Blood group null phenotypes, e.g., found as ABO O, K_0, Jr-, or Lan negative phenotypes, are well recognized to be encoded by many more alleles, than only by a single one, solely responsible for the respective null phenotype [13–17].

1.3 Different Requirements in Donor Versus Recipient Genotyping

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 repetitive donors is time insensitive and aims to type as many blood group systems and donors' samples as possible. Also requirement for analytic accuracy represents a striking difference between donor- and recipient-specific genotyping: For instance, unconsidered null-alleles remain unidentified, 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. In recipients on the other hand, such untrue heterozygous "pretender-results" could potentially lead to fatal transfusion reactions and would genetically best be addressed by a technique currently unavailable, e.g., the affordable whole blood group genome within 2 h. However, recipients' whole blood group genomes within a timely manner will probably stay illusive for the foreseeable future, whereas donors' SNP based blood group genotypes are readily available.

1.4 "High-Throughput" Genotyping

Although a variety of different blood group genotyping methods [1, 3, 18] have been claimed to possess capability for "high-throughput," there are no commonly accepted numerical characteristics for this figure. Impressive sample throughput may also be generated using pooling strategies, in order to identify certain analytes of relatively low frequencies, e.g., such as viruses, or rare blood group alleles [19]. However, efficiency of such strategies is directly dependent on the frequency and number of analytes investigated in parallel, and needs to be optimized based on mathematical models. Currently and on the other hand, Next-Generation Sequencing, may well be used for the generation of an excessive number of SNP genotypes from a single, or a small number of samples and as such be qualified of being capable for high throughput. Still, this type of information may not fulfil present-day requirements for transfusion services.

Consequently, capability for high-throughput may be expressed considering (1) the type and (2) wanted number of samples, (3) the number of SNPs of interest and still, though of minor impact (*see* **Note 1**) (4) the time interval needed for their analysis. Using matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) blood group genotyping, a single multiplex reaction, e.g., covering 16 SNPs allows for genotyping of approximately 3,840 blood donor DNAs per day (10 times 384 different DNAs), or roughly 60,000 SNP genotypes within 1 day. In its 384-well microtiter plate version, throughput limitations may rather result from a lack of donor numbers (*see* **Note 2**), insufficient DNA sample number extraction, under-scaled PCR cycling production line, or limited manpower, then

from the analytical capacity of the mass spectrometer itself. In fact, in our institute, the average throughput need for the described "Select Module" is far below the described maximal throughput capacity and lies at some 10,000 samples per year. A typical daily typing batch size consists of 384 DNA samples, simultaneously analyzed by two single multiplexes, covering the described 36 SNPs of interest (Table 1). As a result, approximately 14,000 SNPs are genotyped per day, which to our opinion, may still be considered as "high-throughput".

1.5 Core Analytical Procedures of MALDI-TOF MS

The core analytical procedures of MALDI-TOF MS based SNP genotyping are shown in Fig. 1. As claimed by the provider, multiplexing levels, e.g., simultaneous amplifications of polymerase chain reaction (PCR) fragments of up to 40 genomic target DNA sequences seems to be feasible. However, the presented Select Module consists of two multiplexes, with amplifications allowing the simultaneous genotyping of 23 and 13 SNPs, representative of gender determination plus 30 blood group in 1, and 16 blood group and 4 human platelet antigens in the other, respectively (Table 1). Amplification is followed by the degradation of dNTPs and 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 15–30 base-pairs (bp) in length (4,300–9,000 Da range) are then desalted applying anion-exchange resin material and transferred to a silicon chip with pre-spotted matrix crystal (e.g., 3-hydroxy picolinic acid) containing patches.

Laser-irradiation induces the desorption and ionization of the analytes causing the +1 positively charged molecules to accelerate into a vacuum flight tube towards a detector. Separation occurs by the time of flight (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. 2). The method directly measures the mass of the molecules of interest, without using any surrogate, such as fluorescence.

1.6 General Applications of MALDI-TOF MS Based Genotyping

MALDI-TOF MS was initially introduced in proteomics applications, while the full potential for DNA analysis was demonstrated in 1995 [20]. The special and advantageous features of mass spectrometry qualify the technology adapted for genotyping as an advanced system for automated high-throughput analysis of SNPs [21]. MALDI-TOF MS also proved its ability in the reliable quantification of certain alleles and genetic variants in non-Mendelian mixtures of DNAs, e.g., fetal DNA in maternal plasma, or somatically mutated DNA in cancerous tissue [22–24]. Reproducibly, the respective sensitivity limits were shown to be as low as 5 % [23, 25]. The advantage of short amplicon lengths and minimal

Table 1
Detected blood group antigens included in the two multiplexes of the MALDI-TOF MS based Select Module

ISBT#	Blood group	Gene (HGNC)	On	Allele name 1	Allele name 2	nt position	nt 1	nt 2	Amino a.	Antigens ct	Allele ct	SNP ct.	plex	rs #
001	ABO A, B, O2/O1	ABO	9q	[ABO*G261]	[ABO*delG261]	261	G	delG	fsThr88Pro	1	2	1	2	no rs
002	M/N	GYPA	4q	GYPA*01	GYPA*02	59	C	T	Ser20Leu	2	2	1	2	rs7682260
002	S/s	GYPB	4q	GYPB*03	GYPB*04 (wt)	143	C	T	Thr48Met	2	2	1	1	rs7683365
002	S, s/U–	GYPA/GYPB	4q	GYPA	GYPB	i1+15914	G	C	–	1	1	1	2	no rs
002	M, N/ Henshaw	GYPA/GYPB	4q	GYPA*01, 02	GYPB*06.01	59	Y	G	Leu20Trp	1	1	–	2	no rs
004	RhD+/RhD–	RHD/RHCE	1p	RHD*01 (wt)	RHD*01N.01	455	A	[C]	Asn152[Thr]	2	2	1	1	no rs
004	RhD+/RhD–	RHD/RHCE	1p	RHD*01 (wt)	RHD*01N.01	787	G	[A]	Gly263[Arg]	–	–	1	1	no rs
004	RhD+/RhD–	RHD/RHCE	1p	RHD*01 (wt)	RHD*01N.01	1362	A	[T]	–	–	–	1	1	no rs
004	RhD+/RhD–	RHD/RHCE	1p	RHD*01 (wt)	RHD*04N.01	504–541	–	ins 37bp	–	–	1	1	1	no rs
004	RhD+/RhD–	RHD/RHCE	1p	RHD*01 (wt)	RHD*01N.06	1006	G	C	Gly336Gly	–	1	1	1	no rs
004	RhD+/RhD–	RHD/RHCE	1p	RHD*01	RHCE*all (wt)	1362	[A] T	T	–	–	–	–	1	no rs
004	Rhc/RhC	RHCE	1p	RHCE*01 (03) (wt)	RHCE*02 (04)	i2+3095	–	ins 109bp	–	2	2	1	2	no rs
004	Rhc/RhC	RHCE	1p	RHCE*01 (03) (wt)	RHCE*02 (04)	307	C	T	Ser103Pro	–	–	1	2	rs676785
004	RhC, Rhc/ RhCʷ	RHCE	1p	RHCE*all	RHCE*02.08	122	A	G	Gln41Arg	1	1	1	1	rs138268848
004	Rhe/RhE	RHCE	1p	RHCE*01 (02)	RHCE*03 (04)	676	G	C	Pro226Ala	2	2	1	2	rs609320
005	Luᵃ/Luᵇ	BCAM	19q	LU*01	LU*02 (wt)	230	A	G	His77Arg	2	2	1	1	rs28399653

(continued)

Table 1
(continued)

ISBT#	Blood group	Gene (HGNC)	On	Allele name 1	Allele name 2	nt position	nt 1	nt 2	Amino a.	Antigens ct.	Allele ct.	SNP ct.	plex	rs #
006	K/k	*KEL*	7q	*KEL*01.1*	*KEL*02 (wt)*	578	T	C	Met193Thr	2	2	1	2	rs8176058
006	K,k/K_{mod}	*KEL*	7q	*KEL*01M.01*	*KEL*02 (wt)*	578	G	C	Arg193Thr	–	1	–	2	rs8176058
006	Kp^a/Kp^b	*KEL*	7q	*KEL*02.03*	*KEL*02 (wt)*	841	T	C	Trp281Arg	2	1	1	2	rs8176059
006	Js^a/Js^b	*KEL*	7q	*KEL*02.06*	*KEL*02 (wt)*	1790	C	T	Pro597Leu	2	1	1	1	rs8176038
006	K, k/K_0	*KEL*	7q	*KEL*02 (wt)*	*KEL*02N.06, 02N.01*	i3+1g>m	G	M	–	1	2	1	1	no rs
008	Fy^a/Fy^b	*DARC*	1q	*FY*01, or FY*A*	*FY*02, or FY*B*	125	G	A	Gly42Asp	2	2	1	2	rs12075
008	Fy^b/Fy^x	*DARC*	1q	*FY*wt*	*FY*02M*	265	C	T	Arg89Cyt	–	1	1	2	no rs
008	$Fy^{a,b}$/Fy null	*DARC*	1q	*FY*wt*	*FY*02N.01*	P-67t>c	T	C	–	1	1	1	1	no rs
009	Jk^a/Jk^b	*SLC14A1*	18q	*JK*01, or JK*A*	*JK*02, or JK*B*	838	G	A	Asp280Asn	2	2	1	1	rs1058396
009	$Jk^{a,b}$/Jk null	*SLC14A1*	18q	*JK*wt*	*JK*02N.01, 01N.06, 02N.02*	i5-1g>m	G	M	–	1	3	1	1	rs78937798
009	$Jk^{a,b}$/Jk null	*SLC14A1*	18q	*JK*wt*	*JK*01N.03*	582	C	G	Tyr194Stop	–	1	1	1	rs34756616
010	Di^a/Di^b	*SLC4A1*	17q	*DI*01*	*DI*02 (wt)*	2561	T	C	Leu854Pro	2	2	1	1	no rs
010	Wr^a/Wr^b	*SLC4A1*	17q	*DI*02.03*	*DI*02 (wt)*	1972	A	G	Glu658Lys	2	2	1	1	rs75731670
011	Yt^a/Yt^b	*ACHE*	7q	*YT*01 (wt)*	*YT*02*	1057	C	A	His353Asn	2	2	1	1	rs1799805
013	SC:1, SC:2	*ERMAP*	1p	*SC*01 (wt)*	*SC*02*	169	G	A	Gly57Arg	2	2	1	1	rs56025238
014	Do^a/Do^b	*ART4*	12p	*DO*01*	*DO*02 (wt)*	793	A	G	Asn265Asp	2	2	1	1	no rs

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015	Coᵃ/Coᵇ	AQP1	7p	CO*01.01 (wt)	CO*02	134	C	T	Ala45Val	2	2	1	1	rs28362692
016	LWᵃ/LWᵇ	ICAM-4	19p	LW*05 (wt)	LW*07	299	A	G	Gln100Arg	2	2	1	1	rs77493670
023	Inᵃ/Inᵇ	CD44	11p	IN*01	IN*02 (wt)	137	G	C	Arg46Pro	2	2	1	1	rs121909545
n.a.	Vel+/Vel−	SMIM1	1p	[SMIM1*Vel+]	[SMIM1*Vel−]	64–80	–	del 17bp	–	1	2	1	2	no rs
n.a.	[HPA-1a/b]	ITGB3	17q	[ITGB3*001] (HPA-1a) (wt)	[ITGB3*002] (HPA-1b)	176	T	C	Leu59Pro	2	2	1	2	rs5918
n.a.	[HPA-5a/b]	ITGA2	5q	[ITGA2*001] (HPA-5a) (wt)	[ITGA2*002] (HPA-5b)	1600	G	A	Glu534Lys	2	2	1	2	rs10471371
n.a.	[Female]/[male]	GYG2/ paralog	Xp/ Yp	[GYG2*Xfemale] (wt)	[GYGpar*Ymale]	i2+3291	C	[A]	–	–	2	1	1	no rs

Blood groups are ordered according to their official International Society of Blood Transfusion (ISBT) numbers. For the human platelet antigens (HPA), and gender genotyping, no ISBT numbers are available. Every line is representative of one antithetic antigen pair. Values in square brackets are no blood groups (column "blood group"), or do not represent official ISBT allele terminology (columns "allele name"), or are single nucleotide (nt) variations between two paralog genes (column "nt" and amino a.). Gene names are given according to the HUGO Gene Nomenclature Committee (HGNC). Nt values are given in International Union of Pure and Applied Chemistry (IUPAC) notation. Amino a. for amino acid, "ct." for count, and "rs" for reference SNP. (wt) indicates the more frequent allele, occurring among Caucasians. "i" stands for intron, "ins" for an insertion, "del" for a deletion, and "bp" for base pairs. Beside citations given, additional important information regarding blood group polymorphisms was retrieved from NCBI dbSNP and OMIM [6, 40]
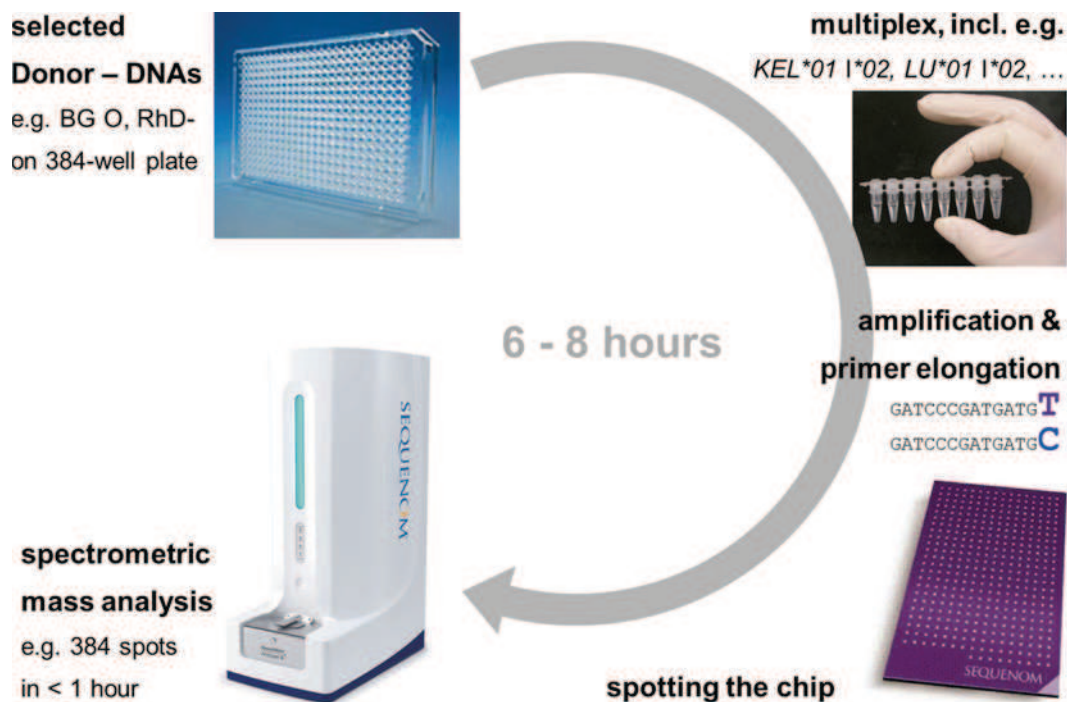
Fig. 1 Schematic representation of the MALDI-TOF MS based high-throughput blood group genotyping procedure applying the Select Module

requirement for template DNA amounts further expand the range of application to forensics, pathology (formalin-fixed and paraffin-embedded tissue) and the analysis of DNA methylation patterns [26–29].

1.7   MALDI-TOF MS Based Blood Group Genotyping of Donors

To date, MALDI-TOF MS based genotyping has been successfully applied in "non-invasive" determination of fetal blood group *RHD* and *KEL*01* status [30, 31]. Apart from those reports and starting in 2006, at least four different groups reported on their results using MALDI-TOF MS for donor genotyping of blood groups, or human platelet antigens (HPA), so far [24, 32–36]. MALDI-TOF MS analysis in comparison to PCR using Sequence Specific Priming (PCR-SSP) and a novel real-time PCR high-resolution melt curve (HRM) analysis, delivered 100 % concordance rates for all investigated HPA and blood group Indian genotypes, respectively [33, 35]. A third group of researchers reported about polymorphisms linked to 22 different blood groups, that both the error rate of the MALDI-TOF MS assay, as measured by the strand concordance rate, and the no-call rate were very low (0.1 %) [32]. With respect to donor genotyping, all authors positively commented on the ability of MALDI-TOF MS based methodology for automation, high throughput, and cost efficiency.
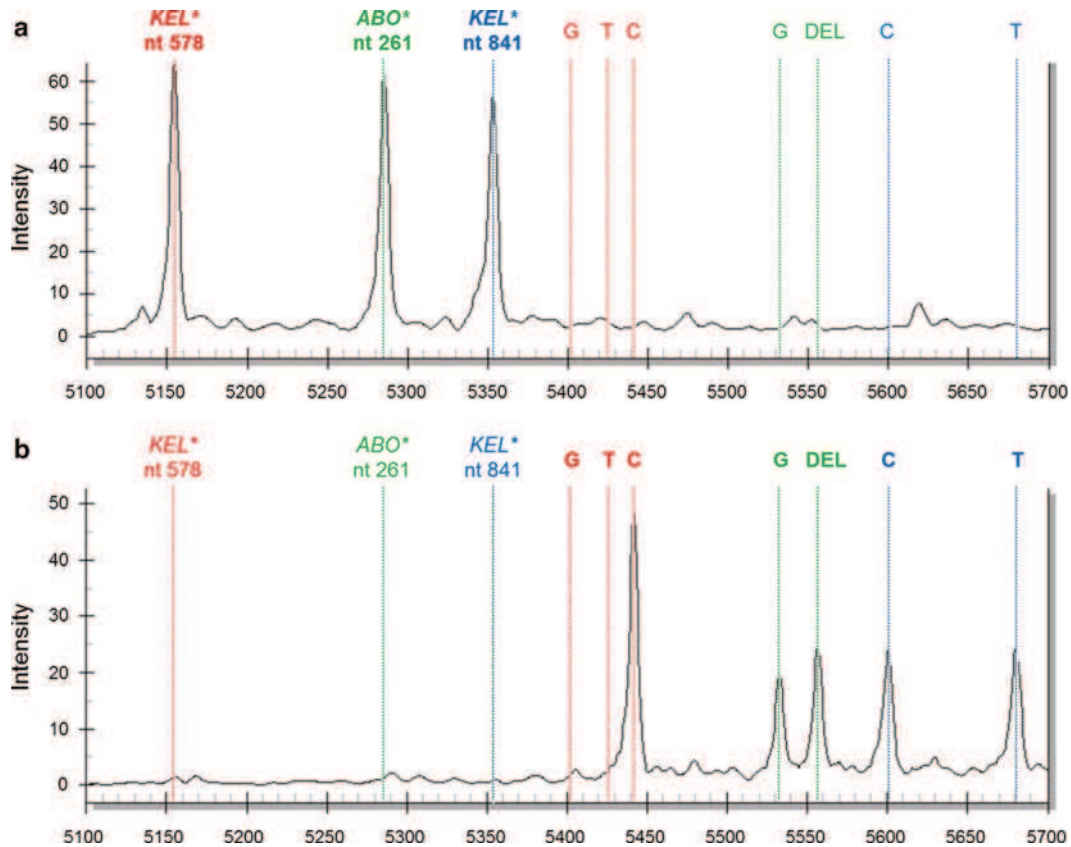
Fig. 2 MALDI–TOF mass spectrum of three SNPs (out of 13), specific for *KEL*01* vs. *02* (e.g., K vs. k, plus *KEL*01M.01*), [*ABO*G261*] vs. [*ABO*delG261*], and *KEL*02* vs. *KEL*02.03* (e.g., Kp[a] vs. Kp[b]) in multiplex 2 of the Select Module from a negative control sample (no-template control, H_2O) (**a**), and an exemplary DNA sample (**b**). For every SNP the calculated mass of the unextended primer (UEP) before base extension reaction (**a**) and the masses of the corresponding extended primers (**b**) are marked by *dashed lines* (*colour-coded* per SNP assay). As expected in the no template control sample (**a**) only peaks of UEPs were detectable. The mass spectrum of all SNPs showed either one or two extension products in homozygous (*KEL** coding nt 578 CC), or in an approximate 1:1 ratio in heterozygous (*ABO** coding nt 261 G/del, *KEL** coding nt 841 CT) genotypes of the respective SNP (**b**). For SNP details, also refer to Table 1

Above mentioned key statements were confirmed by our own experience using MALDI-TOF MS in genotyping of 170 alleles predictive of 101 blood group and platelet antigens [34]. The method also allowed for reliable differentiation of *RHD* hetero- and homozygous individuals, at multiple genetic positions in individual samples by calculating the *RHD/RHCE* MALDI-TOF MS peak area quotient [34]. A more detailed analysis of 4,000 Swiss blood donor samples delivered genotype/phenotype concordance rates between 99.93 and 100 % for the blood groups Kell, Kidd, and Duffy, respectively. Genotyping proved its practicability in the daily routine setting and qualitatively outperformed serology. Technology was ideal for time-insensitive donor genotyping and allowed for a broad range of throughput needs. We therefore

suggested that, from a technological point of view, serotyping should be replaced by genotyping for donors' blood groups encoded by *KEL*, *SLC14A1*, and *DARC* [36]. Currently, 6,000 blood group MN and Ss phenotypes from Swiss donors are compared to their MALDI-TOF MS derived genotyping data. Again, robustness, accuracy, reproducibility, and phenotype-predictive value of MALDI-TOF MS based genotyping are impressive (unpublished data, manuscript in preparation). Therefore, we concluded, that 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 [34].

1.8 MALDI-TOF MS Based Blood Group Genotyping Using "Select Module"

Obviously, there are several reasons to take advantage of MALDI-TOF MS in high-throughput blood group genotyping. 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. Of note, this flexibility of the MALDI-TOF MS technology is unique among all other genotyping methods and based on the simple fact, that amplification and elongation primers are the only variable determinant of all (!) sorts of applications based on SNP detection. Therefore, we defined a new combination of certain blood group specific SNPs, specifically tailored to our needs for blood donor genotyping. This new "Select Module" should allow for (i) routine genotyping the most transfusion-relevant and additional blood group antigens (*see* **Note 2**), e.g., such as K/k, Jk$^{a/b}$, or MN and Ss, the (ii) simultaneous genetic prediction of rare blood group phenotypes, e.g., such as Kp^b and Vel negativity, (iii) minimizing costs for this purpose and (iv) consider additional advantages arising from genetic typing.

The presented Select Module finally consists of two multiplexes, allowing the parallel genotyping of 23 and 13 SNPs in two single tubes per DNA sample, respectively (Table 1). Beside given specificities addressing (i) and (ii) given above, the Select Module includes a low resolution *RHD* genotyping capability, rather thought as a tool for aspects of confirmation, than considered for comprehensive *RHD* gene analysis. With respect to (iv) given above, specificities for HPA-1 and HPA-5 were included in order to identify potential platelet donors with rare platelet antigen constellations. And, serving as a security measure, assays specific for the cdnt deletion G261 of the *ABO* gene and an inter-genetic single nt variation between *GYG2* on chromosome X and a highly homologous sequence on chromosome Y, allowed for the prediction of blood group O and gender, respectively [5, 37]. Both genotypes were then routinely compared to the respective existing phenotypic pre-values of every donor in order to exclude erroneous qualitative serial sample mix-up, e.g., bottom to top, or left to right flips of the 96-, or 384-well microtiter plates handled in the course of the analytical procedure.

Validation of the Select Module was done on 1,520 previously genotyped individual donor DNA samples and showed full concordance, e.g., identical results for all 54,720 SNP genotypes tested when comparing the results of the previously published methods to the new Select Module [34, 36]. Costs consist of material costs of € 1.20 (US$ 1.65, August 2014) and 2.1 min of labour per DNA extraction (using Chemagen technology, PerkinElmer, Baesweiler, Germany) and material costs of € 8.75 (US$ 12.00, August 2014) and 2.4 min of labour per two multiplexes (complete typing), not including overhead, amortization costs for hardware and yearly maintenance fees. Since published guidelines of the national blood transfusion service of the Swiss Red Cross prescribe duplicate testing of donors' blood groups on an independent second sample [38], the Select Module is considered as highly attractive alternative in comparison to a second typing done by serology. Further validation is underway in order to fulfil Swiss national requirements for an approval of the Select Module as an officially accepted method for second testing of confirmatory character, in the course of the routinely performed blood group typing in duplicate.

2 Materials

2.1 Genomic DNA

1. 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 (PerkinElmer, Baesweiler, Germany) in its automated 96-well microtiter plate format.

2. Preparation was done from 0.2 mL and 6 mL EDTA anticoagulated blood (see **Note 3**), for the automated and manual protocol, resulting in approximately 8 μg (in 100 μl eluate) and 400 μg total genomic DNA, respectively (see **Note 4**).

3. For the automated method, all individual donor samples, e.g., 3 mL EDTA anticoagulated blood were barcoded. The "chemagen 96-deep well microtiter input plate" and respective data files were generated using standard operation procedures and pipetting robots by Tecan (Tecan Group AG, Maennedorf, Switzerland), or Hamilton (Hamilton AG, Bonaduz, Switzerland) linked to our in-house blood management software.

2.2 Thermal Cyclers

Four Veriti DX 384-well thermal cyclers (0.02 mL, Applied Biosystems by Life Technologies, Zug, Switzerland) (see **Note 5**).

2.3 Hardware and Software for Mass Spectrometry

1. All hardware and software for mass spectrometry were provided by Sequenom GmbH, Hamburg, Germany a division of Sequenom Inc., San Diego, USA. Mass spectrometry package included:

2. MassARRAY® Analyzer 4, with capacity for the analysis of up to two times 384 SpectroCHIP®s per load.

3. MassARRAY® Nanodispenser RS-1000.

4. Server for data management and software MassARRAY® Genotyper 4.v.

5. Delivery of the 384-well microtiter plate format system additionally includes a complete lay out for a 96-well microtiter plate format procession.

6. Alternatively, a pure 96-well microtiter plate format for reduced costs may be considered and will also abrogate the need for two Liquid Handler Stations.

7. Additional equipment was two MassARRAY® Liquid Handler Stations (Matrix).

8. Additional software needed was MassARRAY Quantitate Gene Expression 3.v* also including the software tool MassArray Assay Design mentioned later in this article (*see* **Note 6**).

2.4 Consumables for Mass Spectrometry

1. Complete MassARRAY iPLEX Pro Genotyping Reagent Set including all reagents for the genotyping 10 x 384 DNA samples with 1 multiplex (Sequenom GmbH, Hamburg, Germany).

2. Amplification and elongation primers of adequate quality (*see* **Note 7**) were provided by Metabion (Metabion International AG, Planegg, Germany).

2.5 Water, Purity Requirements

Usage of ultrapure water is highly recommended for all washing and flushing steps performed by the Liquid Handler Stations and the Nanodispenser during the whole typing procedure. Volumes needed range between 2 and 5 l per 2×384 well plate run. Water should have a resistivity of 18.2 M$\Omega \times$cm and was purified in-house using a Milli-Q Direct system (Merck Millipore, Zug, Switzerland).

3 Methods

This section can hardly describe all the details needed to be considered and conducted in performing a high-throughput MALDI-TOF MS Select Module genotyping on multiples of 384 DNA samples per run. However, the following paragraphs provide a good overview of the work-flow (Fig. 1) and procedures performed and add further details. Readers with increased thirst for knowledge regarding this may wish to contact the manufacturer and further consult detailed standard operation procedure manuals for this purpose. All SNPs were genotyped following the standard Sequenom MassARRAY iPLEX Pro genotyping procedure.
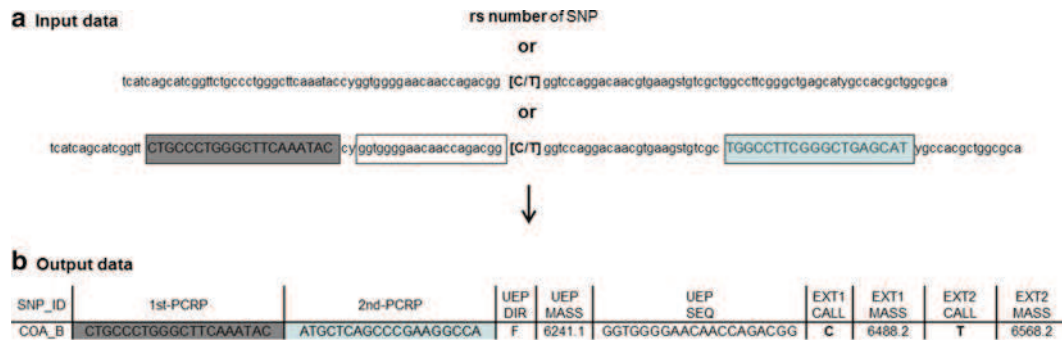
Fig. 3 Data in- and output using Assay Designer software exemplified for blood group Colton a/b polymorphism specific SNP of the Select Module multiplex reaction 1. (**a**) Data entry either requires only a list of rs numbers (*upper option* represented as "rs"), or DNA sequence given in IUPAC notation, spanning approximately 50–300 base-pairs (bp) flanking sequence of SNP given with the variation and in *square brackets* (*middle and lower options*). Sequence given in *lowercase letters* allows for an unrestricted calculation of amplification primer binding sites (*middle option*), *uppercase letter* (*shaded in dark and light grey*) constrict calculation of primer binding sites to a preferred region, or even a genetically required exact position (*lower option*). Binding site of the unextended primer (*unshaded box*, *lower option*) will be determined by the software, but the user is free to demand forward or reverse orientation if required (not shown in figure). (**b**) The output file delivers the DNA sequence of the amplification (first-primer, second-primer) and unextended primers (UEP), the orientation of UEP (forward or reverse) and the mass of the unextended and each version of the extended primers, respectively

3.1 Assay Design

1. Use the assay design software (TYPER 4.0, Sequenom) and online assay design tools (MySequenom, Sequenom) to select PCR amplification primer pairs, which uniquely amplified only the genomic region of interest, considering all database retrievable allelic variants.

2. The software continue to group the pairs into multiplexes and to validate all pairs within one multiplex for undesired cross-reactions among each other, with paralog genes and other homologous regions of the genome.

3. Finally, the software choose orientation and sequence of the extension primers, and the expected molecular weights of all expected elongated products, representing the 2–4 alleles potentially present at each SNP position and calculated mass distribution for compatibility with the mass analysis window.

4. In- and output files are comparably prosaic with respect to their generation, content, and handling as shown in Fig. 3. The output file of the Assay Designer is formatted for direct use further downstream in the genotyping process under the Assay Editor function of the TYPER 4.0 software.

3.2 Primer Mixes for Multiplex Amplification and Elongation

Of note, flexibility of the MALDI-TOF MS technology is unique among all other genotyping methods and based on the simple fact, that amplification and elongation primers are the only variable determinant of all sorts of applications based on SNP detection.

Downstream genotyping is widely facilitated using specific primer mix concentrates for (1) the amplification and (2) elongation reactions, respectively.

1. Both types of concentrated primer mixes should ideally be prepared ahead of the actual typing procedure in multiples of amounts needed for single typing runs and kept frozen at –20 °C for up to at least 6 months.

2. These batches of primer mixes should be controlled for their claimed specifications using a panel of predefined DNAs with a maximal reproduction of genotypes available.

3. For amplification, concentration of all primers in the final reaction volume of a 4.8 μL multiplex reaction is 100 nM.

4. Concentration of the elongation primers is much higher and varies, depending on the expected mass of the elongated product. The larger the product, or the respective unextended primer, the higher is its concentration in the final reaction volume of 8.6 μL elongation reaction and typically lies between 590 and 1,180 nM.

5. Primer orders need to account for the high quality requirements of the elongation primers (*see* **Note 7**), and appropriate synthesis scales. The micromolar amounts of elongation primers needed for MALDI-TOF MS genotyping are a relevant cost factor, whereas costs for amplification primers are insignificant.

3.3 Prearrangements of DNAs, Amplification, and SAP Treatment

1. Use four Chemagen 96-well microtiter output plates as matrices and pre-PCR Liquid Handler Stations as automated 96-well pipettors to generate working plates for the appropriate dilution of 384 different DNAs, and the final transfer to two 384-well microtiter amplification plates (2 h). Air-dry the DNAs overnight.

2. All enzymes, reagents and consumables needed for genotyping are provided within the complete MassARRAY iPLEX Pro Genotyping Reagent Set. Prepare the two amplification cocktails for the two multiplexes each with a calculated excess of 30 % in two separate tubes.

3. Aliquot the amount of three times 150 μL to each well of rows 1, and 7, and from there dispense five times 25 μL to each remaining empty well of a 96-well V-bottom plate using an automated 8-channel pipettor.

4. Transfer the final 4.8 μL to each 384-well of the amplification plate using the pre-PCR Liquid Handler Station (1 h).

5. Perform amplification of each DNA for both Select Module multiplexes on the two different 384-well amplification plates, also taking advantage of a "hot start" thermocycling procedure. Use two pre-PCR Veriti DX 384-well thermocyclers to facilitate parallel work-flow of the two 384-well plates (cycling:

2 h 45 min). From here on, carry out all steps in the post-PCR area.

6. Transfer the shrimp alkaline phosphatase (SAP) reaction mixture to all wells, again using automated pipettors and the post-PCR Liquid Handler Station. Non-incorporated dNTPs are enzymatically dephosphorylated by SAP, which ensures exclusive single nt extension in the next step. SAP reactions are carried out in the two post-PCR Veriti DX 384-well thermal cyclers (45 min).

3.4 Elongation of Unextended Primers, Spotting of Chip

1. The extension reactions calculated with an excess of 54 % contain buffer, termination mix, *Thermus aquaticus* Polymerase (TAQ) enzyme, and extension primers. The use of ddNTPs instead of dNTPs ensures exclusive single nt extension of the unextended primers (UEP 2 h 30 min).

2. Resulting single-stranded, nucleic acid oligomer analytes of 15–30 bp in length (4,300–9,000 Da range) are then desalted applying anion-exchange resin material (45 min) and transferred by the MassARRAY® Nanodispenser RS-1000 to a silicon chip with pre-spotted matrix crystal (e.g., 3-hydroxy picolinic acid) containing patches.

3. The Nanodispenser simultaneously aspirates 24 samples by 24 grooved needles just by capillary force. It delivers the impressively low volume of only 10 nL per sample with high accuracy. The dispensed volume is regulated, only by adjusting the speed of the needles the chip is hit with (30 min).

4. After spotting, chip is transferred into the vacuum chamber of the mass spectrometer.

3.5 Mass Spectrometry and Data Generation

1. Complete data flow is documented. In brief, data arrangement of the original four "chemagen 96-well microtiter output plates" is done using the Plate Editor function of the TYPER 4.0 software and results in the final 384-well plate file. The Assay Editor function of the TYPER 4.0 software holds all relevant "output files" of the Assay Designer (*see* Subheading 3.1), thereby providing specific information for each multiplex to be linked to each plate file. Finally, the 384-well plate file is linked to the chip using the software tool Chip Linker.

2. The mass spectrometry system is equipped with an N2 laser with 337-nm wavelength (pulse max energy of 100 microJ and 0.5 ns pulse width) for use with matrix components absorbing light of this wavelength. Every single spot of the chip, e.g., all analytes of one multiplex and one individuals' DNA (1 spot), are analyzed by 15 laser pulses (3 positions, 5 pulses each) and mathematically processed further downstream.

3. Spotting and laser operation are controlled by additional control spots. Ahead of sample analysis, calibration is performed using a 3-oligo mass/amount calibrant and water.

4. Data analysis is performed using Assay Analyser function of the TYPER 4.0 software, allowing for a comprehensive analysis of every single SNP, every single sample, e.g., peak area for all allele-specific analytes in any given assay, and systemic performance of every single run, e.g., call and extension rate.

5. A comprehensive mass-spectrum, visualizing all detected peaks of every SNP of every single DNA sample is provided (Fig. 2). TYPER 4.0 output files have a standardized format and may therefore easily be converted into other files, or used for selective data retrieval.

6. In order to translate TYPER 4.0 derived SNP genotyping data into predicted blood group phenotypes, a computer spreadsheet program (Microsoft Excel, Microsoft, Redmond, WA) fulfilled all our needs satisfactorily. This way, data were "humanized" and could be used for comparisons to their serological pre-values, statistical analysis, served as documents and offered a way for the transfer to our in-house blood management system (*see* **Note 8**).

4   Notes

1. Alternatively, individual steps of the lab process can also be run over night, or the process can be interrupted at any time (e.g., 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.

2. In Switzerland, the minimal mandatory immune-hematological blood group antigen determination for every erythrocyte concentrate includes duplicate serotyping for ABO and RhD on two independent samples before their blood is transfused for the first time. Started in 2012, additionally all RhD negative donors are molecularly tested once for the presence of the *RHD* gene before the first usage of their blood, mandatorily [19, 39]. Expanded phenotyping for RhC/c, RhCw, RhE/e, Kell, Kidd, Duffy, MN, and Ss phenotypes is only done depending on the regional requirements, influenced by the local presence of "high-end" medicine, and usually covers between 20 and 80 % of all donors. Only donors selected for expanded phenotyping are also considered as candidates for genotyping by the MALDI-TOF MS based Select Module.

3. Dependent on the number of DNAs per time interval expected, EDTA blood may also be stored at 4 °C for up to 2 weeks before extraction. Alternatively, collection might take place externally and last for longer than 2 weeks. In this case, EDTA blood may simply be frozen at –20 °C, before shipped collectively using

cool-packs, or frozen ice. However, care should be taken, that collection tubes withstand the freezing/thawing procedure without being ruptured. In both cases, EDTA blood needs thorough stirring before transfer to the "chemagen 96-deep well microtiter input plate", best achieved using commercially available "head-over-head" mixers. Still, samples having been stored at –20 °C might need time-consuming labor for the manual removal of coagulated material with wooden sticks.

4. For MALDI-TOF MS analysis, approximately 20–30 ng of genomic DNA per each of the two multiplex reactions were used. Due to the high concentration conformity of the automatically extracted DNAs, laborious single DNA UV quantification was abandoned and replaced by the simplified usage of a fixed volume of 0.4 µL of the "chemagen eluate" per multiplex [34].

5. Four Veriti DX 384-well thermal cyclers used in our laboratory are labelled for in vitro diagnostic use, with a list price of € 13,800 each (US$ 19,400, September 2011). In order to maintain validity of the in vitro diagnostic use label, hardware requires inspection by the manufacturer on a yearly basis. In our department for Molecular Diagnostics and Research (MOC), barrier between pre- and post-amplification area is represented by a 10 m² room, serving as a gate. Air conditioning in this room and the post-amplification area has a separate circle and accounts for (1) appropriate cooling, in order to discharge thermal heat produced by the thermal cyclers located the gate, and (2) a slight atmospheric underpressure. The two doors for entering the room, or leaving it towards the post-amplification area, may only be opened alternatively and never simultaneously. This way, potential PCR product contaminations will always be directed towards the "uncritical" post-amplification area.

6. The mass spectrometry package was provided by Sequenom GmbH, Hamburg, Germany a division of Sequenom Inc., San Diego, USA. In 2014, parts of Sequenom Inc. have been acquired and are now firming as Agena Bioscience, San Diego, USA. The hardware and software package included a MassARRAY® Analyzer 4, with capacity for the analysis of up to 2×384 SpectroCHIP®s per load, a MassARRAY® Nanodispenser RS-1000, a server for data management and software MassARRAY® Genotyper 4.v* with a total list price of € 349,000.00 (US$ 502,200.00, June 2011). Additional equipment was 2×MassARRAY® Liquid Handler Stations (Matrix) with a list price of € 53,500 each (US$ 77,000, June 2011). Additional software was the MassARRAY Quantitate Gene Expression 3.v* including the MassARRAY Assay Design tool with a list price of € 39,500 (US$ 56,800, June 2011).

According to the manufacturer, yearly maintenance of all hardware components is mandatory.

7. Self-evidently, manufacturing process of single elongation primers needs to account for oligonucleotides of unique length. Mass detection of elongated primers with contaminant adducts of +1 or –1 nts in length would seriously worsen single peak-quality of the spectrum.

8. Currently, our IT department is on its way to establish a direct transfer from TYPER 4.0 (raw) data to our in-house blood management system.

Acknowledgements

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