Blood group genotyping: the power and limitations of the Hemo ID Panel and MassARRAY platform

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Matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS), is a sensitive analytical method capable of resolving DNA fragments varying in mass by a single nucleotide. MALDI-TOF MS is applicable to blood group genotyping, as the majority of blood group antigens are encoded by single nucleotide polymorphisms. Blood group genotyping by MALDI-TOF MS can be performed using a panel (Hemo ID Blood Group Genotyping Panel, Agena Bioscience Inc., San Diego, CA) that is a set of genotyping assays that predict the phenotype for 101 antigens from 16 blood group systems. These assays involve three fundamental stages: multiplex target-specific polymerase chain reaction amplification, allele-specific single base primer extension, and MALDI-TOFMS analysis using the MassARRAY system. MALDI-TOF MS–based genotyping has many advantages over alternative methods including high throughput, high multiplex capability, flexibility and adaptability, and the high level of accuracy based on the direct detection method. Currently available platforms for MALDI-TOF MS–based genotyping are not without limitations, including high upfront instrumentation costs and the number of non-automated steps. The Hemo ID Blood Group Genotyping Panel, developed and optimized in a collaboration between the vendor and the Blood Transfusion Service of the Swiss Red Cross in Zurich, Switzerland, is not yet widely utilized, although several laboratories are currently evaluating the MassARRAY system for blood group genotyping. Based on the accuracy and other advantages offered by MALDI-TOF MS analysis, in the future, this method is likely to become widely adopted for blood group genotyping, in particular, for population screening. ***Immunohematology* 2015;31:75–80.**

Key Words: blood group genotyping, matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS), single base primer extension, high-throughput genotyping

Matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS), is a sensitive analytical method for the analysis of biomolecules, initially described for proteomics analyses in 1988.[1,2] Proteomics applications of MALDI-TOF MS have greatly diversified and now include evaluation of the expression of specific proteins or peptides in clinical samples for diagnosis of conditions including cancers, ischemic stroke, diabetes, and major depression.[3,4] The identification of microorganisms based on peptide and protein biomarkers is another widely utilized application of MALDI-TOF MS.[5,6] The analysis of nucleic acids by MALDI-TOF MS was first described in 1995 when the ability of MALDI-TOF MS to resolve DNA fragments varying in mass by a single nucleotide was demonstrated by Tang et al.[7]

Genotyping assays using MALDI-TOF MS can be separated into three distinct stages: multiplex target-specific polymerase chain reaction (PCR) amplification, allele-specific single base primer extension, and MALDI-TOF MS analysis. For single base primer extension, primers are designed to anneal adjacent to the single nucleotide polymorphism (SNP) of interest, and the reaction occurs in the presence of terminating nucleotides (similar to the use of dideoxynucleotides in Sanger sequencing) to limit the extension to one nucleotide. This generates allele-specific terminated extension products of defined mass, dependent on the nucleotide incorporated at the polymorphic base. The DNA extension products are then analyzed by MALDI-TOF MS. The power of MALDI-TOF MS analysis rests in the ability of the platform to resolve the intrinsic physical property of the analyte, the molecular mass. This principle makes MALDI-TOF MS an excellent tool for approaching high-throughput analysis of variants, including SNPs, and insertions/deletions, as well as somatic mutation screening, quantitative gene expression and copy number variation analysis, and DNA methylation detection.[8]

MALDI-TOF MS is an attractive method for blood group genotyping because the majority of blood group antigens are encoded by SNPs. The application of MALDI-TOF MS to broad red blood cell (RBC) genotyping was first described by Gassner et al. at the Blood Transfusion Service of the Swiss Red Cross in Zurich, Switzerland, in a large cooperative project with Sequenom, Hamburg, Germany (now Agena Bioscience).[9] The aim of this large cooperative project was to develop and implement a high-throughput blood group genotyping assay utilizing MALDI-TOF MS technology. As an outcome of this project, the Hemo ID Blood Group Genotyping Panel (Hemo ID Panel) was developed and is now a commercially available MassARRAY-based blood group genotyping assay (Agena

Bioscience Inc., San Diego, CA). The Hemo ID Panel predicts the phenotype of 101 antigens, including RBC antigens from 16 blood group systems and 23 platelet and neutrophil antigens. MALDI-TOF MS has also been validated for blood group genotyping of maternal blood samples, for prediction of fetal D and K antigen status, although this application is outside the scope of this review.[10,11]

The aim of this review is to describe blood group genotyping utilizing MALDI-TOF MS with a focus on the methods, strengths, limitations, and implementation of this approach.

Materials and Methods

Overview of the Hemo ID Panel

The Hemo ID Panel genotypes 170 alleles to predict phenotypes for 101 antigens. The Hemo ID Panel can be used in full for comprehensive analysis or separated into six modules that can be purchased and run individually for targeted genotyping. The modules currently available are the Kell, Kidd, and Duffy; MNS; Rare Blood Groups; RHD-RHCE Broad (RHD/C/E B); RHD Variant; and HPA and HNA modules. The variants genotyped in each module are described in detail by Gassner et al. and in the *Hemo ID Blood Group Genotyping Panel User Guide,* supplied by Agena Bioscience with the purchase of a Hemo ID Panel kit.[9,12]

The Hemo ID Panel genotyping procedure is described by Gassner et al. and detailed step-by-step in the user guide.[9,12] The Hemo ID Panel kits contain all primers and reagents required for genotyping, including nucleotides, buffers, enzymes for PCR and post-PCR processing steps, and 96- or 384-well plate launching pads (SpectroCHIPs, Agena Bioscience Inc.). Genotyping utilizing the full Hemo ID Panel requires 10 multiplex reactions (wells) per sample, each containing 2 μL of genomic DNA (gDNA) at a concentration of 10 ng/μL. In our experience, using the 384-well SpectroCHIPs, 33 samples can be run in addition to recommended blanks and assay controls.

MassARRAY Genotyping

MassARRAY genotyping can be divided into eight steps. Steps 1 to 6 involve analyte preparation and proceed from extraction of gDNA to spotting of PCR extension products onto a SpectroCHIP; steps 7 and 8 involve analyte analysis (Table 1). As with any genotyping method, this procedure should be performed in PCR containment facilities to minimize risk of contamination from PCR products. The user guide details containment recommendations and where each procedural step should be conducted.

Table 1. The Hemo ID Panel workflow and required equipment

Step	Description	Required equipment
	Analyte preparation	
1	Obtain gDNA and prepare working dilutions	DNA extraction system; spectrophotometer
2	Multiplex PCR using Hemo ID PCR primers	Thermal cycler
3	SAP inactivation	Thermal cycler
4	Extension PCR using Hemo ID extension primers	Thermal cycler
5	Resin conditioning	360° plate rotator
6	Dispense extension products onto SpectroCHIP	MassARRAY Nanodispenser RS1000
	Analyte analysis	
7	MALDI-TOF MS analysis	MassARRAY Analyzer 4
8	Report generation	Typer v4.0 software

PCR = polymerase chain reaction; SAP = shrimp alkaline phosphatase; MALDI-TOF MS = matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry. Additional standard laboratory equipment is also required, including plate centrifuges, vortex, and microfuge. Genotyping should be performed in PCR containment facilities to minimize PCR contamination risk.

MassARRAY Genotyping: Analyte Preparation

Step 1 requires isolation of gDNA, from fresh blood or tissue, frozen tissue, or cell lines, by standard extraction methods. Following isolation of gDNA, a working dilution of 10 ng/μL is prepared. Once gDNA samples are prepared, MassARRAY genotyping begins with the generation of DNA fragments by multiplex target-specific PCR amplification (step 2). Following generation of DNA fragments, excess nucleotides are removed by shrimp alkaline phosphatase (SAP) inactivation (step 3) and allele-specific single base primer extension is performed (step 4). During the primer extension stage, an oligonucleotide primer anneals immediately adjacent to the SNP of interest in the presence of the complementary terminating nucleotide. This ensures extension is limited to incorporation of a single nucleotide complementary to the variant at the SNP position. Following this extension, conditioning is performed using an ion-exchange resin to remove excess salts that can interfere with the quality of the mass spectra (step 5). In step 6, the DNA extension products (analytes) are dispensed onto a SpectroCHIP using the RS1000 Nanodispenser (Agena Bioscience Inc.). SpectroCHIPs are pre-spotted with 384 or 96 spots of matrix; once dispensed, the DNA extension products (analytes) and matrix co-crystalize.

MassARRAY Genotyping: Analyte Analysis

In step 7, the SpectroCHIP is inserted into the MassARRAY Analyzer for MALDI-TOF MS analysis. During MALDI-TOF

MS, the analyte/matrix co-crystals are irradiated with a short laser pulse, which causes the matrix and DNA fragments to desorb, ionize, and be accelerated in an electric field towards a detector. Data acquisition for time of flight (TOF) determinations takes 3 to 5 seconds per sample and involves 20 to 50 laser shots per sample, such that a 384-well plate is analyzed within 30 minutes. The TOF is calculated from the time of arrival at the detector and is proportional to the mass of the individual molecules. Low-mass molecules arrive in a shorter time than higher mass molecules, allowing resolution of the nucleotide incorporated onto the extension primer at the polymorphic site. After data acquisition, a spectrum is produced with relative intensity on the *y*-axis and mass/charge on the *x*-axis. An example of the spectrum acquired for genotyping of the alleles associated with the Diego blood group antigens, Di^a and Di^b, is shown in Figure 1.

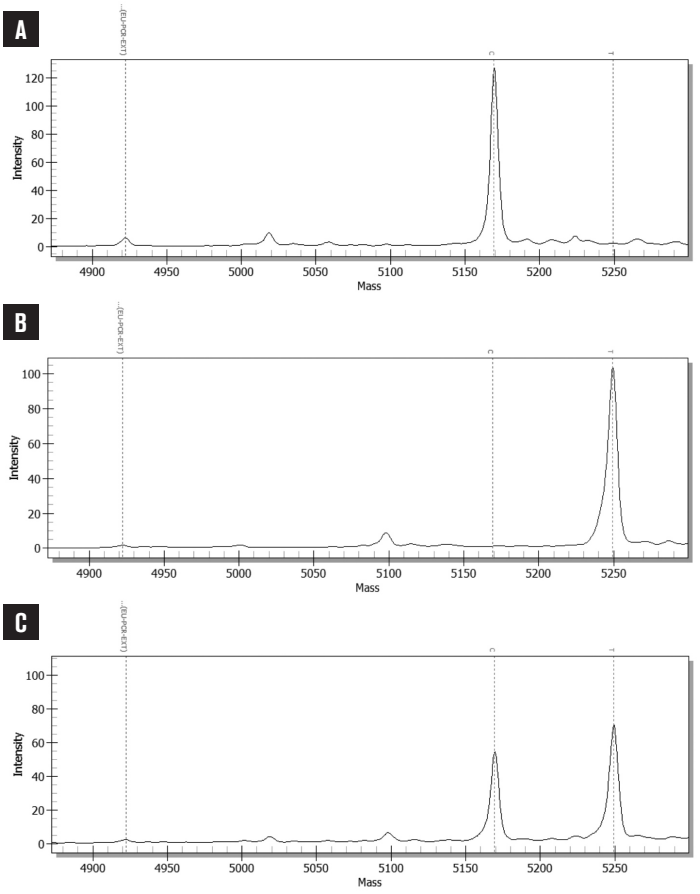

Fig. 1. Matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) mass spectra plotting peak intensity (*y*-axis) against mass (daltons) (*x*-axis). MassARRAY Typer software analyzes the allele peak intensities to determine genotype calls. The example mass spectra here are taken from an assay genotyping the Diego blood group alleles *DI*A* and *DI*B*. (**A**) Mass spectrum of a *DI*B/B* sample showing a single peak of 5169 Da indicating homozygosity for cytosine. (**B**) Mass spectrum of a *DI*A/A* sample showing a single peak at 5249 Da indicating homozygosity for thiamine. (**C**) Mass spectrum of a *DI*A/B* sample showing peaks at both 5169 Da and 5249 Da indicating heterozygosity for cytosine and thiamine.

MassARRAY Genotyping: Software and Report Generation

When setting up a plate for analysis, the user assigns samples and Hemo ID Panel modules to plate wells using software (Typer, Agena Bioscience Inc.). The modules are defined by the Hemo ID Module Definition Files (available for download), which provide all necessary specifications to the software, including extension primer sequences and mass, as well as the expected products and mass. Acquired data are processed by Hemo ID Report software, which analyzes the allele peak intensities to determine genotyping calls. The software interprets one, or a combination of multiple, genotyping calls to predict phenotype. A report is then generated detailing genotype and the associated predicted phenotype (step 8). In addition to data acquisition and analysis, the Hemo ID Report software includes quality-control monitoring and allows for extensive customization of reports.

Strengths and Limitations

Strengths of MassARRAY Genotyping

MassARRAY-based genotyping has many strengths, ranging from the properties of MALDI-TOF MS technology, to throughput and flexibility. First, MALDI-TOF MS results are determined based on the detection of an intrinsic physical property—the molecular mass of the analyte. This differs from alternative SNP genotyping methods that rely on differential hybridization to allele-specific probes and indirect methods such as fluorescence detection to determine genotype. For example, indirect genotyping methods often detect either a "red" or "green" fluorescent signal, which is correlated to the presence or absence of an allele to determine genotype. MALDI-TOF MS offers increased accuracy and specificity over indirect methods, as genotypes are determined directly by detection of a peak at the known mass of an allele; there is no requirement for labelling of extension products. Although available indirect methods have proved to be robust and are widely used, the potential for false negatives or false positives is higher than for MALDI-TOF MS–based genotyping.[8,13–15] The robustness of the MassARRAY platform and Hemo ID Panel have been demonstrated by Sequenom and independently by Meyer et al. with interlaboratory reproducibility studies.[16,17] Sequenom reported a rate of 100 percent concordance between genotype and phenotype for 47 samples across three collaborating laboratories. In the study by Meyer et al., 760

samples were tested in two collaborating laboratories for a total of 11,400 SNPS with resulting genotype call rates differing by only 0.5 percentage points between the two laboratories (99% and 98.5% call rates), and the genotyping concordance was 100 percent.[17]

The optimal application of MALDI-TOF MS–based genotyping is high-throughput, as the technology supports very highly multiplexed reactions. MALDI-TOF MS assays are robust up to the 40× multiplex level.[18] In principle, the MassARRAY system has the capacity to genotype over 100,000 SNPs per day. For blood group genotyping, Meyer et al. reported that one technician could genotype 760 gDNA samples with the Hemo ID Kell, Kidd, and Duffy module (which genotypes 15 SNPs) in 8 hours with 2.5 hours of hands-on time, excluding gDNA extraction and data processing.[17]

Finally, the MassARRAY system with the Hemo ID Panel offers adaptability and flexibility, as genotyping is based on the principle of single base extension. This means assays can be easily adapted by adding/removing PCR and extension primers, as no fixed format DNA chips are required. For example, when the genetic basis of the Vel blood group antigen was described in 2013,[19] the associated polymorphism was incorporated into the Hemo ID Panel by the manufacturer within 4 weeks. The Hemo ID Panel also offers flexibility, as the multiple genotyping modules can be purchased and run as a comprehensive genotyping assay, or tested individually to allow targeted genotyping. If further flexibility or customization is desired, design of custom MassARRAY assays is a straightforward process. Custom design of assays is performed with the online MassARRAY Assay Design Suite (ADS) software (https://www.agenacx.com) and involves the input of polymorphisms of interest, followed by the automatic generation of PCR and extension primers and multiplex reactions. Following custom design, kits containing all required primers and reagents for MassARRAY genotyping are available from Agena Bioscience.

Limitations of MassARRAY Genotyping

MALDI-TOF MS genotyping has some limitations, including high upfront instrument acquisition costs and the number of non-automated steps. First, the cost of instrumentation required for MassARRAY genotyping is relatively high in comparison with other commercially available genotyping systems. MassARRAY genotyping requires equipment standard to most laboratories, including thermocylers and plate centrifuges, in addition to two specialized pieces of equipment: a nanodispenser and mass spectrometer (Table 1). The list price for the complete MassARRAY 4, 384-well format system is ~$295,000, and a 96-well format system costs ~$200,000. All figures provided are in U.S. dollars and are current as of September 2015. Once instrumentation has been purchased, the cost of genotyping on the MassARRAY system is comparable to or less expensive than, other methods. Per-sample cost for analysis on the complete Hemo ID Panel would cost ~$110 in consumables on the 384-well format system, or ~$135 on the 96-well format system. Considering the complete Hemo ID panel provides predicted phenotypes for 101 antigens, this calculates to less than $1.35 per antigen, regardless of the format utilized.

As discussed, the MassARRAY system has high-throughput genotyping capacity. In combination with the high upfront equipment costs, this may be considered a limitation by small-sized laboratories, as the cost-to-benefit ratio of low-throughput testing only, such as referred patient samples, may be difficult to justify.

A further limitation is the number of hands-on steps involved in the MassARRAY genotyping workflow. Hands-on steps include the preparation and pipetting of mastermixes for the PCR steps (steps 2 and 4), preparation and pipetting of the SAP inactivation mastermix (step 3), and the addition of resin to reaction wells (step 5). In general, workflows with multiple hands-on steps have the potential to introduce human error—for example, by incorrectly calculating the quantity of mastermix components, or by missing wells during pipetting.[20] Additionally, workflows with multiple hands-on steps reduce the reproducibility of assays and increase the risk of amplicon contamination. Most of these steps are amenable to liquid handling robotics available from Agena Bioscience, but this is an additional expense.

Furthermore, the MassARRAY system and the Hemo ID Panel, at the time of writing, are not cleared by any regulatory body and are therefore for research use only, which may be considered a limitation in comparison with longer-established systems (BloodChip Reference Progenika Biopharma S.A., Grifols, Bizkaia, Spain), which has obtained CE marking, or the recently U.S. Food and Drug Administration–approved human erythrocyte antigen typing system (PreciseType™ HEA Molecular BeadChip™ Test, Bioarray Solutions, Immucor, Warren, NJ).

Implementation

The Hemo ID Panel has only recently been developed and is not yet widely utilized. As mentioned, this assay was developed and validated in part by Gassner et al. at the Blood Transfusion Service of the Swiss Red Cross in Zurich,

Switzerland.[9] Gassner et al. validated the Hemo ID Panel by comparing genotype with available phenotype data, including samples with known variants.[9] The Blood Transfusion Service of the Swiss Red Cross has now implemented large-scale testing using the Hemo ID Panel with several aims, including the genotyping of 36,000 Swiss donors using the Rare Blood Groups module.[9,17] The most recent report from this laboratory described the genotyping of 4000 samples using the Kell, Kidd, and Duffy modules with over 99.7 percent genotype/phenotype concordance overall for the clinically significant blood group antigens K/k, $Kp^{a/b}$, $Jk^{a/b}$, and $Fy^{a/b}$.[17] The majority of discrepancies were caused by erroneous serology profiles attributable to weakly expressed antigens, with additional discrepancies caused by the presence of rare or novel null alleles within the sample set.[17] Based on their experience to date, Meyer et al. concluded that MALDI-TOF MS–based genotyping proved practical in a routine laboratory setting and qualitatively outperformed serology.[17]

At the Australian Red Cross Blood Service, our experience with MassARRAY genotyping has been in relation to typing reagent RBC donors and other selected donors. We first compared the performance of the Hemo ID Panel with two established genotyping platforms (BloodChip Reference, Progenika, and HEA and RHD BeadChip, BioArray Solutions). This involved genotyping of 300 extensively phenotyped reagent RBC donors. A phenotype genotype discrepancy rate of 7.0 percent was detected, and all discrepancies identified were in clinically significant blood group systems.[21] In all cases of discrepancy, homozygosity for either D, Fy^a, or Fy^b was inferred based on serology, but genotyping revealed variants encoding weak or null antigen expression.[21] Overall, this study highlighted the utility of genotyping, specifically for identifying donors carrying SNPs associated with weak and null antigen expression, which routine serology did not detect. Furthermore, in this study, genotyping by all three platforms achieved sensitivity and specificity of 100 percent on the sample set tested, providing evidence that the MassARRAY system and the Hemo ID Panel are equally accurate when compared with the established genotyping systems.[21]

In subsequent studies, the flexibility offered by the MassARRAY genotyping platform was explored. These studies were undertaken in collaboration with Agena Bioscience to develop custom-designed assays for genotyping of certain ethnically associated blood group polymorphisms. For all of these pilot genotyping studies, MassARRAY genotyping data were compared with historical serology and in-house genotyping results. When further analysis was necessary, samples were tested by Sanger sequencing.

First, we designed and validated a MassARRAY-based assay to genotype the SNP associated with In^a and In^b, as antisera for phenotyping these antigens are extremely limited.[22] In addition, currently available commercial platforms have not incorporated this blood group polymorphism into their tests. For 151 samples, the MassARRAY results were 100 percent concordant with in-house genotyping and serologic testing results, illustrating the potential to extend and develop this method for other antigens.[22]

Second, we designed a MassARRAY-based assay for genotyping the SNP 2561C>T of *SLC4A1,* associated with the Diego blood group antigens, Di^a and Di^b (Figure 1). It is important to note that adjacent to the *DI*A/B* polymorphism is a silent SNP, c.2562G>A. Our laboratory has an established high-resolution melt analysis assay for genotyping of *DI*A/B,* and this adjacent SNP has the potential to confound genotyping results. We included an additional extension primer in our *DI*A/B* MassARRAY assay to allow genotyping of c.2562G>A as proof-of-principle to explore the application of genotyping multiple SNPs within a single amplicon. We incorporated inosine in the extension primer design, as inosine displays some bias in base pairing, but is less destabilizing than mismatches involving the four standard bases (A/C/G/T). This allowed the extension primer to span position c.2561, regardless of the *DI*A/B* genotype, to genotype the SNP at position c.2562. This investigation highlights the complexity of genotyping and the requirement to reference SNP databases during assay design to ensure all reported variants are considered. In this case, a MassARRAY assay that overcame this difficulty was developed, and MassARRAY results were 100 percent concordant with in-house genotyping and serological testing results for 151 samples.

Although the majority of blood group antigens are encoded by SNPs, two clinically significant blood group systems, Rh and MNS, include numerous hybrid alleles. The MassARRAY platform uses exon scanning to characterize hybrid alleles within the Hemo ID Panel, particularly for the Rh blood group system. Our third custom assay focused on genotyping hybrid glycophorins within the MNS blood group system, which are of interest in the ethnically diverse Australian population. For the complex MNS hybrid glycophorins, a genotyping assay was developed to classify published *GYP(B-A-B)* hybrid alleles, including the recently characterized *GYP*KIP*.[23]

At present, MassARRAY-based genotyping for blood group polymorphisms has only been reported by Gassner et al. and Meyer et al. from the Blood Transfusion Service of the Swiss Red Cross and McBean et al. and Lopez et al. at the Australian Red Cross Blood Service.[9,17,21,22] In the International

Society for Blood Transfusion (ISBT) Molecular Blood Group Genotyping Workshop in 2014, four laboratories reported using a MALDI-TOF MS–based genotyping platform, and we anticipate that future data from these trials will provide further evidence of the strengths and limitations of the MALDI-TOF MS approach.

In conclusion, MALDI-TOF MS technology offers many advantages, including the high level of accuracy based on the direct detection of an intrinsic physical property of the analyte, high-throughput, high-multiplex capacity, and potential for flexibility and adaptation. In the future, MALDI-TOF MS–based genotyping has the potential to become widely adopted, in particular for donor screening for an extended array of blood group polymorphisms.

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