Molecular typing for the Indian blood group associated 252G>C single nucleotide polymorphism in a selected cohort of Australian blood donors

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Background. The Indian blood group antigens, In^a and In^b, are clinically significant in transfusion medicine. However, antisera to type these antigens are difficult to obtain. The In^b antigen is a high frequency antigen present in all populations, while the frequency of the antithetical In^a ranges from 0.1% in Caucasians up to 11% in Middle Eastern groups. This antigen polymorphism is encoded by the single nucleotide polymorphism (SNP) 252G>C in *CD44*. The aim of this study was to establish and compare two genotyping methods to measure the frequency of the *IN*A* and *IN*B* alleles in a blood donor cohort.

Materials and methods. Donor blood samples (n=151) were genotyped by a novel real-time polymerase chain reaction (PCR) high-resolution meltcurve (HRM) analysis and a custom matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) assay. Samples with the rare *IN*A* allele were further investigated by nucleotide sequencing, red cell agglutination, and flow cytometry techniques.

Results. In this study group, 149 *IN*B* homozygous and 2 *IN*A/B* heterozygous samples were detected with 100% concordance between HRM and MALDI-TOF MS methods. For PCR HRM, amplicon melting alone did not differentiate *IN*A* and *IN*B* alleles (class 3 SNP), however, the introduction of an unlabelled probe (UP) increased the resolution of the assay. Sequencing confirmed that the two non-homozygous samples were *IN*A/B* heterozygous and phenotyping by red cell agglutination, and flow cytometry confirmed both In^a and In^b antigens were present as predicted.

Discussion. Genotyping permits conservation of rare antisera to predict blood group antigen phenotype. In PCR UP-HRM the *IN*A* and *IN*B* alleles were discriminated on the basis of their melting properties. The In^a frequency in this selected donor population was 1.3%. Application of genotyping methods such as these assists in identifying donors with rare blood group phenotypes of potential clinical significance.

Keywords: Indian blood group genotyping, high-resolution melting analysis, MALDI-TOF MS.

Introduction

The *CD44* gene, located on chromosome 11p13 consists of 20 exons, encoding numerous isoforms of the CD44 molecule[1,2]. The CD44 isoform present on hematopoietic cells is CD44s[1,3] and approximately 2,000-5,000 copies of this molecule are expressed on mature red blood cells[4]. The CD44 glycoprotein carries antigens of the Indian (IN) blood group system[5], namely: In^a, In^b, INFI, and INJA[6-9]. The In^a and In^b are antithetical antigens and have been associated with a single nucleotide polymorphism (SNP) from guanine (encoding In^b) to cytosine (encoding In^a) at nucleotide 252 in exon 2 of *CD44*. This SNP results in an amino acid substitution Arg46Pro of the CD44 molecule[10].

The In^a and In^b antigens are efficient immunogens and antibodies to these polymorphisms are clinically significant[11-14]. Anti-In^a and anti-In^b antibodies have been associated with immediate and delayed haemolytic transfusion reactions[7,13,15]. The In^b antigen is a high frequency antigen expressed in all populations. While In^a is expressed in 0.1% of Caucasians[4], it is present in 2.9% of Indians, 10.6% of Iranians, and 11.8% of Arab populations living in Mumbai (Bombay, India)[7,16]. These studies on the population frequency of In^a and In^b were performed over 30 years ago[7,11,16,17].

The antisera required for typing the In^a and In^b antigens are rare, and difficult to obtain. The SNP associated with the In^a and In^b antigens has been defined; however, commercially-available genotyping systems such as BLOODchip v4.1 (Progenika, Vizcaya, Spain) and Human Erythrocyte Antigen (HEA) BeadChip Kit (BioArray Solutions Ltd, Warren, NJ, USA) do not incorporate this polymorphism.

The aim of this study was to apply two DNA methods to genotype the *IN*A* and *IN*B* alleles and to investigate the frequency of In^a and In^b in a selected population of Australian blood donors. We describe a novel high-resolution meltcurve (HRM) analysis method and a custom matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) assay to genotype the *IN*A* and *IN*B* alleles. Molecular typing results were validated by comparing data obtained between genotyping methods, with phenotype determined by standard serological techniques, and Sanger DNA sequencing.

Materials and methods

Donor selection and DNA preparation

Blood samples collected into EDTA (n=151) were retrieved from volunteer blood donors who presented at collection sites where the population includes South Asian and West Asian (Middle Eastern) individuals. This study had ethics approval from the Human Research Ethics Committee, Australian Red Cross Blood Service.

Two additional samples, In(a+b–) and In(a–b+), were used as controls in genotyping experiments. The In(a+b–) and In(a–b+) control samples were phenotyped by the Blood Service Red Cell Reference Laboratories. The In(a+b–) control was kindly provided by the Sydney Red Cell Reference Laboratory.

Genomic DNA was extracted from whole blood using the EZ1 DNA Blood kit in an automated BioRobot EZ1 Workstation (QIAGEN, Doncaster, VIC, Australia) in accordance with the manufacturer's instructions.

Polymerase chain reaction unlabelled probe high-resolution meltcurve analysis

HRM analysis is a post-polymerase chain reaction (PCR), DNA analysis method used for genotyping and scanning DNA sequence variants[18]. HRM uses intercalating dyes that fluoresce when bound to double-stranded (ds) DNA only. Following PCR, dsDNA is melted by gradually increasing the temperature. As dsDNA dissociates into single-stranded products, total fluorescence is reduced and monitored over time[19]. The standard HRM method is performed by amplicon melting only. A variation of this method is the addition of an unlabelled probe to improve specificity of the assay to detect the target polymorphism during denaturation[20-23].

The primers and probe used in this method were designed in reference to GenBank accession number M59040. Oligonucleotide sequences are listed in Table I. The Indian HRM assay primers amplify a 104-bp product. The UP is a complete match to *IN*B* and is therefore a single-base mismatch to *IN*A*. It has an amino-C6 modification on the 3'-end to prevent polymerase extension during PCR[24]. The primers and probe were ordered from Sigma-Aldrich (Sigma-Aldrich, Castle Hill, NSW, Australia).

The PCR reaction volume was 20 µL and contained 150 nM forward primer (limiting), 750 nM reverse primer (excess), 750 nM unlabelled probe, 20 ng of genomic DNA, and 12.5 µL of HRM PCR master mix (QIAGEN Type-it HRM PCR kit). This assay utilises an asymmetric PCR technique resulting in the generation of excess single-stranded template available for hybridisation of the probe.

The PCR was performed with an initial activation step of 95 °C for 5 minutes followed by 40 cycles of 95 °C for 10 seconds and an annealing/extension step of 60 °C for 30 seconds. Fluorescence data were acquired on the Green Channel during PCR. After the last cycle, samples were held at 95 °C for 1 minute prior to lowering the temperature to 65 °C. Melting was then performed by steadily raising the temperature at increments of 0.5 °C every 2 seconds from 65 °C to 90 °C. The rate of change in fluorescence was monitored on the HRM channel.

Both PCR and HRM steps were performed using real-time PCR equipment, Rotor-Gene 6000 and Rotor-Gene Q, 5plex HRM platform (QIAGEN). Rotor-Gene Q Series Software was used for data analysis. A confidence threshold was set at 90% for genotype call assignment.

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

MassARRAY (Sequenom Inc., San Diego, CA, USA) is a SNP genotyping platform which utilises MALDI-TOF MS technology. SNP genotyping using MassARRAY was performed following the manufacturer's recommendations and involves target-specific PCR amplification followed by target-specific primer single base extension[25].

Table I - Primer and probe sequences used in molecular typing for 252C>G associated with *IN*A/B*.

Method	Oligonucleotide	Sequence
HRM assay	IN-Ex2 forward primer	5'–CGCTTTGCAGGTGTATTCC–3'
	IN-Ex2 reverse primer	5'–ATTGTGGGCAAGGTGCTATT–3'
	IN-33F probe	5'–ATGGTCGCTACAGCATCTCTC**G**GACGGAGGCCG–3'
Exon 2 sequencing	Forward primer9	5'– TGTTAACCAGGCTGGTCTTGAG–3'
	Reverse primer9	5'– AGTTCTAAGCCCAGCTGCCTG–3'

The nucleotide base underlined in the IN33-F probe sequence is a complete match to *IN*B* and a single mismatch to *IN*A*.

MassARRAY provides customisation via the design of amplification and extension primers specific for the SNP of interest[26,27] using Assay Design 4 software. During the primer extension stage an oligonucleotide primer anneals immediately adjacent to the SNP being genotyped[25] and is extended a single base, incorporating the nucleotide at the SNP position. During MALDI-TOF MS, samples are individually irradiated with a short laser pulse which causes the matrix and DNA fragments to desorb, ionise and be accelerated in an electric field[25]. The time-of-flight and mass of each extension primer is calculated from the time of arrival at the detector, allowing the nucleotide at the SNP to be determined[25] in an automated fashion using Typer 4.0 software.

In this study, SNP genotyping was performed following the Sequenom MassARRAY iPLEX Pro genotyping protocol. Primer sequences are available upon request from Sequenom, Inc.

Preparation of the polymerase chain reaction product for DNA sequencing

Primers published by Poole *et al.*[9] targeting exon 2 of *CD44* were used to amplify a 430-bp product. The FastStart High Fidelity PCR System, dNTPack (Roche Diagnostics, Castle Hill, NSW, Australia) PCR kit was used to make up 50 µL of master mix according to the manufacturer's recommendation.

Briefly, the PCR started at 95 °C for 2 minutes followed by 35 cycles at 95 °C for 30 seconds, 65 °C for 30 seconds, 72 °C for 1 minute, and a final extension step at 72 °C for 7 minutes on a Veriti Dx Thermal Cycler (Applied Biosystems, Life Technologies, VIC, Australia). PCR products were purified using a MinElute PCR Purification Kit (QIAGEN) according to the manufacturer's protocol. Purified PCR products were sent to the Australian Genome Research Facility (AGRF), The University of Queensland, QLD, Australia, for DNA sequencing. Data were analysed using Chromas Lite 2.01 software.

Low ionic strength solution-indirect antiglobulin testing

Red cells of all 151 samples were washed in phosphate-buffered saline and resuspended in low ionic strength solution (LISS). Samples were tested in DiaMed Card LISS/Coombs (Bio-Rad, NSW, Australia) using a standard protocol for indirect antiglobulin testing (IAT). Samples were incubated with anti-In^b at 37 °C for 15 minutes and then centrifuged using the DiaMed-ID Micro Typing System (Bio-Rad) at 910 rpm for 10 minutes. Samples were examined for agglutination. The anti-In^b human polyclonal antiserum was from the Serum, Cells, And Rare Fluids (SCARF) exchange programme.

A limited supply of anti-In^a human polyclonal antiserum was used to perform standard LISS-IAT, as described above, on samples found to possess the *IN*A* allele. Anti-In^a antiserum was kindly provided by Joyce Poole and Nicole Thorton of the International Blood Group Reference Laboratory, Bristol, UK.

Flow cytometry

A flow cytometry experiment was performed on samples positive for the In^a antigen by LISS-IAT. Cell suspensions were incubated with anti-In^a and anti-In^b (the same antisera were used in the LISS-IAT) in separate tubes at 37 °C for 30 minutes. Cells were washed in phosphate-buffered saline and incubated with PE Mouse Anti-Human IgG (Cat No. 555787, BD Biosciences, Franklin Lakes, NJ, USA) at room temperature in the dark for 30 minutes. Following secondary antibody incubation, cells were washed and resuspended in phosphate-buffered saline. Samples were analysed using a FACS CANTO II flow cytometer and FACSDiva software (BD Biosciences).

Results

Development of the polymerase chain reaction unlabelled probe high-resolution meltcurve assay

The PCR products derived from *IN*A/A* and *IN*B/B* control samples were denatured between 65.0 °C and 90.0 °C, and generated two distinct domains: an amplicon melting domain from 80.5 °C to 87.0 °C and a probe melting domain from 69.0 °C to 80.5 °C (Figure 1a).

In the amplicon melting domain, the two control samples produced identical melting temperatures (T_m) at 84.0 °C. Melt profiles in this domain showed indistinguishable melt profiles masking the single nucleotide difference between *IN*A* and *IN*B*. Data in this domain were unsuitable for genotyping analysis.

In the probe melting domain, control samples displayed distinct melting behaviour. The T_m was 73.4 °C for the *IN*A/A* control and 77.0 °C for the *IN*B/B* control, a temperature shift of 3.6 °C. These unique melt patterns supported the assignment of homozygous *IN*A/A* and *IN*B/B* genotypes to the two control samples In(a+b–) and In(a–b+) respectively. These melt profiles were then used to assign genotype to our sample cohort.

Polymerase chain reaction unlabelled probe high-resolution meltcurve genotyping analysis

The melt profiles of 149 (149/151) unknown samples matched the *IN*B/B* genotype profile with a percentage confidence of at least 98% (Figure 1b). Two samples (2/151) produced peaks at 73.3 °C and 76.6 °C, in the probe T_m melting domain of melting curves for *IN*A* and *IN*B*, respectively (Table II).

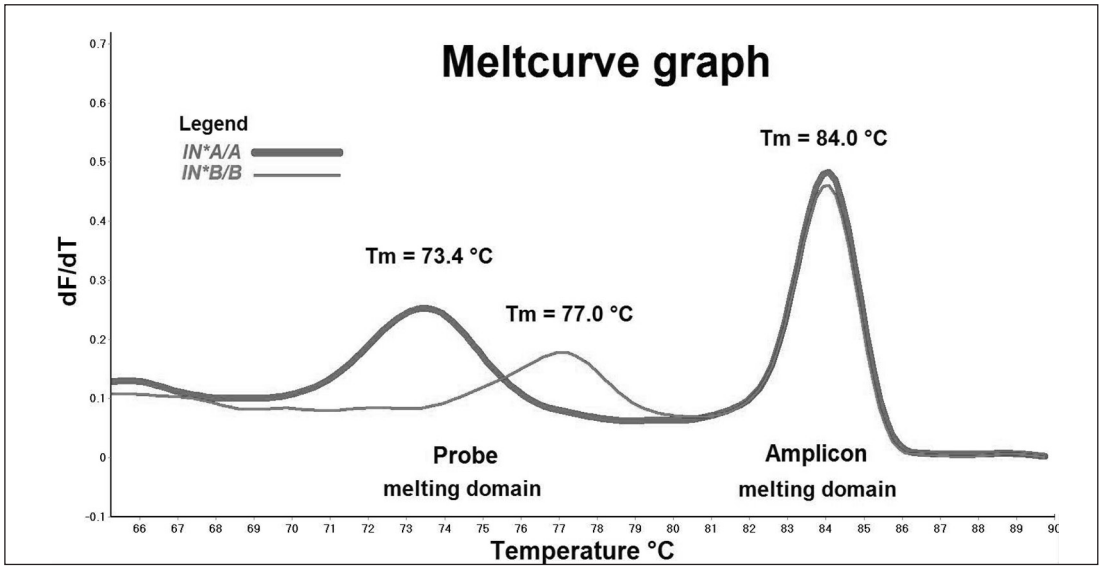


Figure 1a - Representative melt profiles of samples homozygous for *IN*A/A* (thick line) and *IN*B/B* (thin line). Meltcurve graph plots the rate of change in fluorescence (*y*-axis) against temperature (*x*-axis).

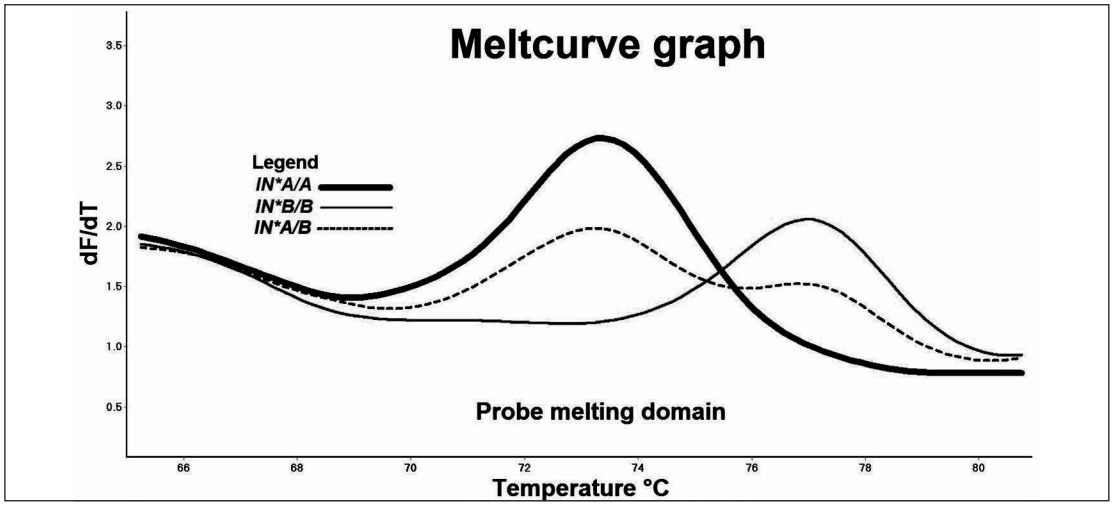


Figure 1b - Representative melt profile of *IN*A/B* heterozygous sample (dash) produced double peaks in the probe melting domain at 73.3±0.3 °C and 76.6±0.2 °C compared to the single peaks produced by *IN*A* (T_m=73.4 °C) and *IN*B* (T_m=77.0 °C) homozygous samples.

Table II - Average T_m of homozygous *IN*A/A* and *IN*B/B* and donor cells: mean T_m and two standard deviations (SD) were calculated from 30 replicate assays.

Sample	Melting domain T_m Probe±2SD	Melting domain T_m Amplicon±2SD
Control *IN*A/A*	73.4±0.3 °C	84.0±0.3 °C
Control *IN*B/B*	77.0±0.3 °C	84.0±0.3 °C
Donor 1 *IN*A/B*	73.3±0.3 °C and 76.6±0.2 °C	83.8±0.2 °C
Donor 2 *IN*A/B*	73.3±0.3 °C and 76.6±0.3 °C	83.8±0.2 °C

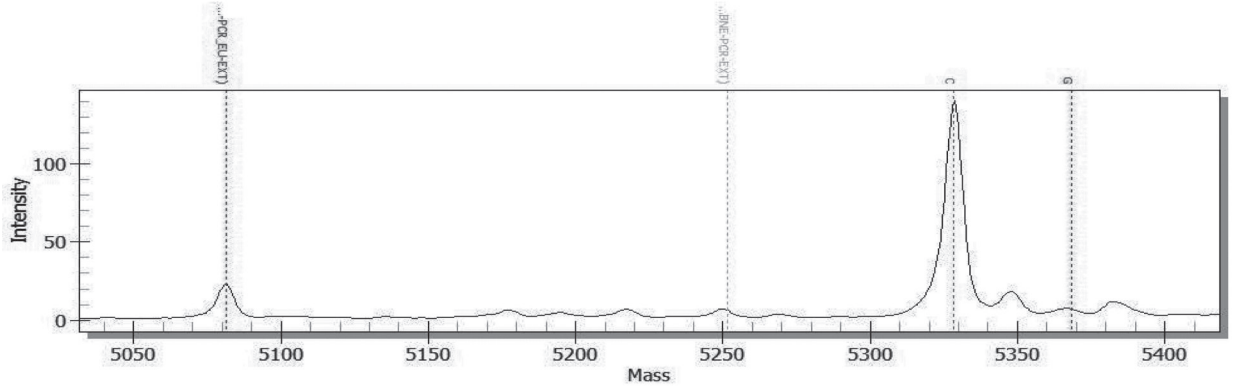
Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry genotyping analysis

On analysis the In(a+b–) control produced a single peak with a mass of 5328.5 Dalton (Da) consistent with the cytosine SNP (Figure 2a). The In(a–b+) control produced a single peak of 5368.5 Da consistent with the guanine SNP (Figure 2b). One hundred and forty-nine samples produced single peaks with a mass of 5368.5 Da and were therefore genotyped as homozygous for guanine (*IN*B/B*). Two samples produced peaks at both 5328.5 Da and 5368.5 Da indicating they were heterozygous for cytosine and guanine (*IN*A/B*) (Figure 2c). MALDI-TOF MS genotyping results of the control samples and all 151 unknown samples

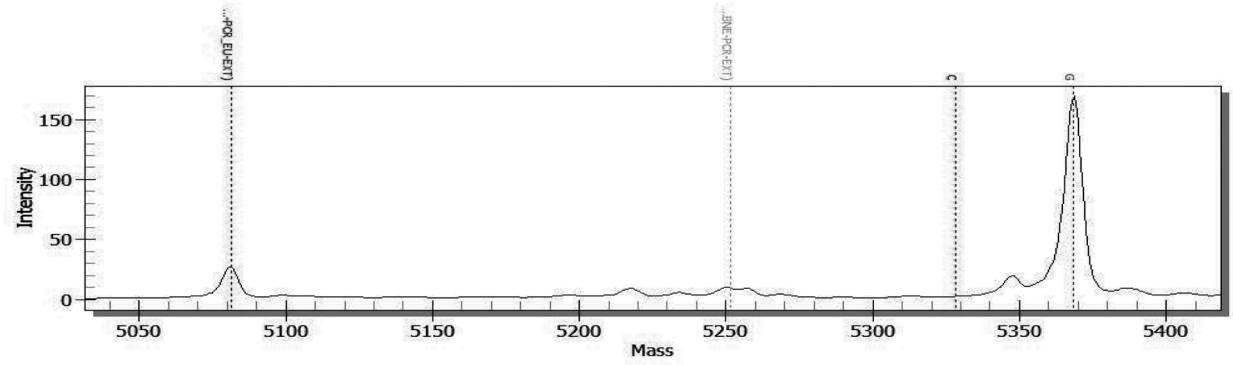
were fully concordant with genotypes determined by unlabelled probe (UP)-HRM.

DNA sequencing

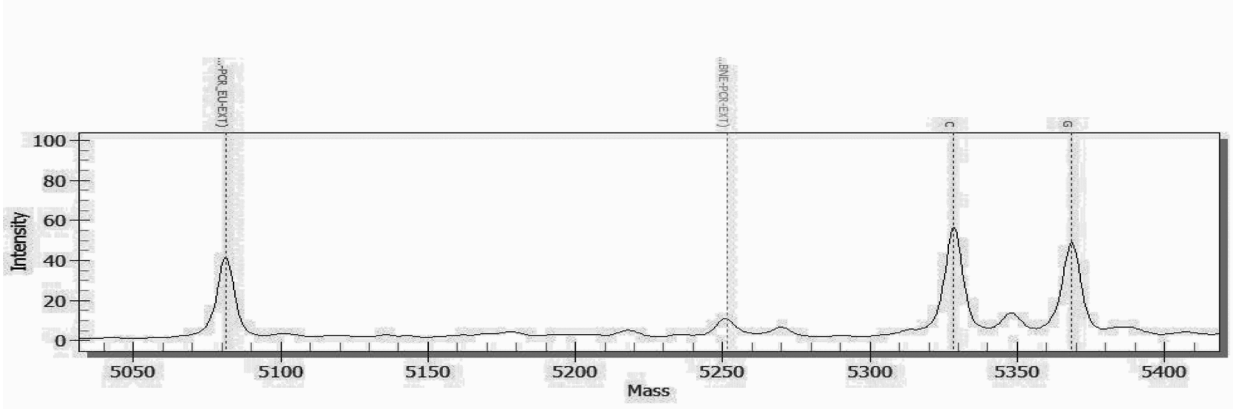
Sequencing data of the two homozygous controls and the two identified *IN*A/B* heterozygous samples were aligned with the reference allele M59040 using the Basic Local Alignment Tool (BLAST) on the NCBI database website (http://www.ncbi.nlm.nih.gov). Chromatograms (Figure 3) show that a cytosine base for *IN*A/A* was substituted by a guanine base in *IN*B/B*, whereas for the two heterozygous *IN*A/B* samples, both cytosine and guanine were detected in the same position.



a - MALDI-TOF MS spectrum of control *IN*A/A*. A single peak at 5328.5 Da is consistent with the cytosine SNP.



b - MALDI-TOF MS spectrum of control *IN*B/B*. A single peak at 5368.5 Da is consistent with the guanine SNP.



c - MALDI-TOF MS spectrum of control *IN*A/B* samples. Heterozygous samples produce peaks at 5328.5 Da and 5368.5 Da consistent with the presence of both cytosine and guanine SNP.

Figure 2 - Representative MALDI-TOF MS spectra. Genotypes are determined by plotting peak intensity (*y*-axis) against mass (Da) (*x*-axis).

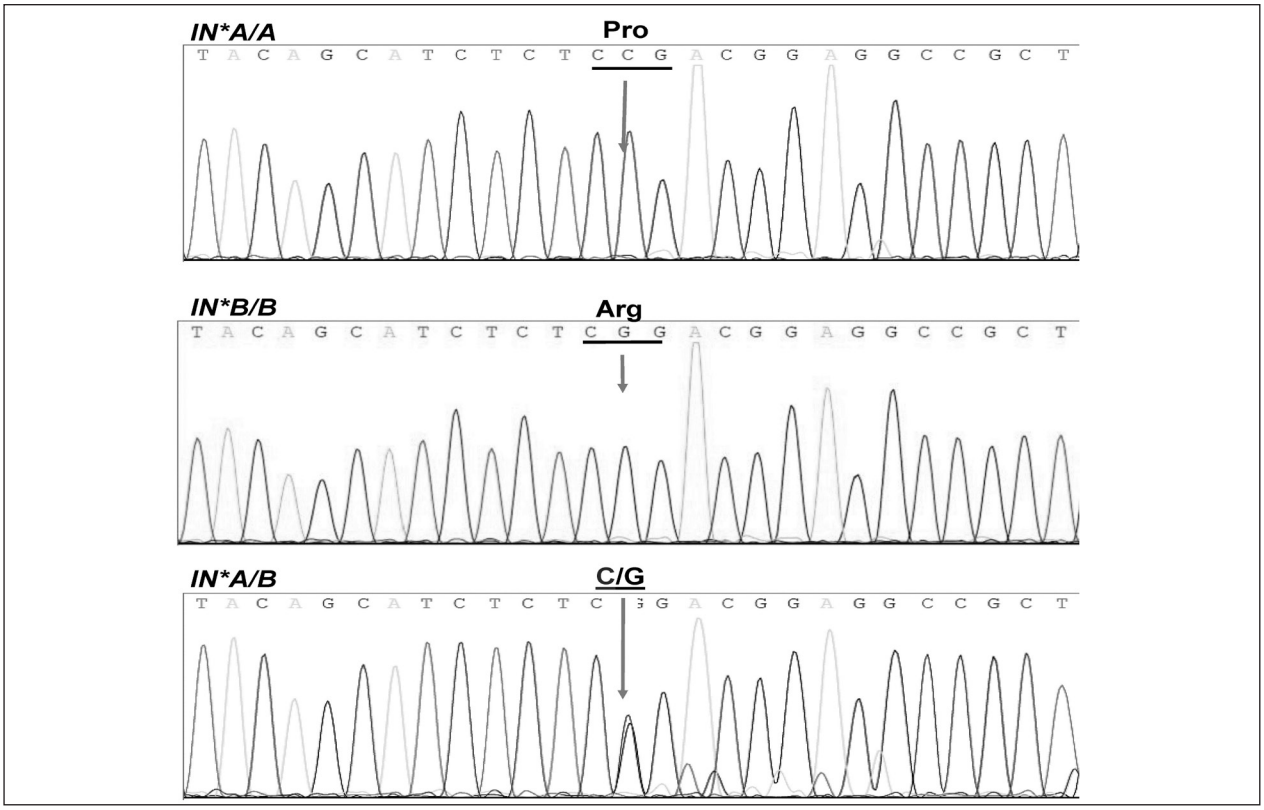


Figure 3 - Nucleotide sequencing chromatograms showing the 252C>G nucleotide polymorphism.
The arrow indicates the location of cytosine (C) in *IN*A/A* to form proline and guanine (G) in *IN*B/B* to form arginine. Both C and G are co-incident for the heterozygous samples *IN*A/B*.

Table III - Mean fluorescence intensity (MFI) ratio of control In(a+b–), control In(a–b+), and two identified In(a+b+) red cells measured with anti-In^a and anti-In^b. Due to the limited supply of antisera, the MFI ratio values supplied in this table were taken from a single experiment only.

Sample	MFI ratio using anti-In^a	MFI ratio using anti-In^b
Control In(a+b–)	4.9	1.0
Control In(a–b+)	1.0	4.7
Donor 1 In(a+b+)	2.7	1.8
Donor 2 In(a+b+)	3.2	4.2

Serology and In^a frequency in the selected population

All 151 samples were agglutinated by anti-In^b antiserum, indicating the presence of the In^b antigen.

Red blood cells of the two samples genotyped as *IN*A/B* were further tested using anti-In^a antiserum. Both cells were positive for agglutination, confirming the phenotype In(a+b+). The frequency of In^a was 1.3% in this selected group.

Flow cytometry

Flow cytometry was performed with anti-In^a and anti-In^b antisera (Table III). To calculate mean fluorescence intensity (MFI) ratios, we divided the MFI value of the sample by the MFI value of the negative control. In(a+b–) and In(a–b+) cells were used as negative controls for anti-In^b and anti-In^a flow cytometry assays, respectively. Negative controls were given an MFI ratio of 1.0. The two donor samples identified as *IN*A/B* gave MFI values between those of the control cells, further confirming that these donor cells express In^a and In^b antigens. The MFI ratios of the two heterozygous samples differed in their reactivity to each particular antisera, possibly because of variable expression of antigens on the cell surface. Due to the limited supply of antisera, it was not possible to repeat this assay.

Discussion

In this study on the detection of In^a blood group antigen in the Australian population, this antigen was detected in 1.3% of a cohort of donors. This rate is intermediate between the 0.1% occurrence in Caucasians[4] and the high rate observed for Middle East groups[16]. Two genotyping methods, one novel PCR-based HRM assay and a commercial MassARRAY system, detected two (2/151) blood donors exhibiting the *IN*A* SNP along with the *IN*B* SNP. Serology testing using the rare anti-In^a confirmed heterozygosity for the In^a antigen in these samples. The concordance rate

between genotyping systems and serotyping for the high frequency In^b was 100%.

The design of both the genotyping assays here was based on the SNP from guanine (encoding In^b) to cytosine (encoding In^a) at nucleotide 252 in exon 2 of *CD44*. This is the first application of HRM genotyping for Indian antigens although standard HRM analysis by amplicon melting has been applied to other blood groups[28]. However, our experience with the HRM technique showed that standard amplicon melting alone is insufficient to detect a nucleotide change between "C" and "G". The amplicon T_m difference in homozygotes with class 3 (C::G) polymorphisms is very small, usually <0.4 °C as class 3 SNP are a simple inversion of a complementary base pair[19]. To overcome this complexity, we introduced an unlabelled probe into the reaction mix. This addition improved the specificity of our HRM genotyping assay by generating distinct melt profiles for samples homozygous for *IN*A* or *IN*B*. The novel, closed-tube, real-time PCR method does not require post-amplification sample processing compared to *IN*A/B* genotyping methods by DNA sequence typing[9] and gel electrophoresis[29]. The PCR UP-HRM described here is a medium-throughput assay capable of genotyping 32 samples in duplicate in 96 minutes. This assay is suitable for referral testing in specialised laboratories.

In contrast, the MassARRAY system has the advantage of being a high-throughput system capable of performing large-scale screening of up to 4,000 samples per day. The MassARRAY system which utilises MALDI-TOF MS technology was adapted through the design of primers for the *IN*A* and *IN*B* SNP. Sequenom Inc. in collaboration with the Blood Transfusion Service Zurich of the Swiss Red Cross are currently developing and evaluating a MassARRAY module for genotyping rare red blood cell polymorphisms, including *IN*A* and *IN*B*[30]. Gassner *et al.*[30] reported a zero allele frequency for *IN*A* (*IN*01*) among 3,040 donors in this Swiss study. This is in contrast to the 1.3% found here in our population. The present study is limited by the sample size and the fact that it is confined to one Metropolitan area. Interestingly, 2% of the Australian population identify as having Indian ancestry[31]. Joshi and Vasantha described serological screening of 2,000 blood units to find two compatible In(a+b–) cells for transfusion to a patient with anti-In^b antibody[32], demonstrating that serological typing to find donors with rare phenotypes is challenging when antisera are rare and in limited supply[33]. As the antisera supply was limited in this study, serological typing for In^a was restricted to the two samples genotyped as *IN*A/B* (by both PCR UP-HRM and MassARRAY). Serology by agglutination and flow cytometry studies confirmed that the In^a antigen was present and the flow cytometry findings were consistent with single-dose gene expression. In future studies, flow cytometry may be preferred as less antisera is required compared to that necessary for red cell agglutination. Additionally, future studies could apply either genotyping methods described here to a larger number of samples to reliably establish the *IN*A/B* allele frequency within the Australian population.

In conclusion, the frequency of ethnically-associated blood group antigens is expected to vary as the population demographic changes. The Australian Red Cross Blood Service has an active policy to recruit donors from different backgrounds to prepare to meet patients' needs in the future. Serological typing to find donors with uncommon phenotypes is challenging especially when antisera are rare and in limited supply[33]. Genotyping methods will play a growing role in defining rare alleles such as *IN*A* in the Australian blood donor panel and direct serology to confirm blood donors with the rare In(a+b–) phenotype. As evidenced by the finding of *IN*A/B* in this sample group, PCR UP-HRM and MassARRAY are viable methods for the detection of the *IN*A* and *IN*B* alleles. Molecular typing is a useful adjunct for Indian typing when antisera are limited in supply.

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This study was approved by the Australian Red Cross Blood Service Ethics Committee (reference number 2010#07).

Authorship contribution

All Authors contributed in writing and reviewing the manuscript. Genghis H. Lopez and Rhiannon S. McBean contributed equally in designing the experiments, performing molecular testing and data analysis. Darryl L. Irwin provided expert advice on the MALDI-TOF MS technology and assay design. Brett Wilson and Yew-Wah Liew performed phenotyping tests and analysed

serological data. Catherine A. Hyland and Robert L. Flower supervised the study design and reviewed molecular and serological data.

Disclosure and competing interests statement

The Authors from the Australian Red Cross Blood Service have no competing interests. Darryl L. Irwin is the Applications and Technology Director of Sequenom Inc. Asia Pacific.

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