CONTENTS

1 Original Report
Comparative evaluation of gel column agglutination and erythrocyte magnetized technology for red blood cell alloantibody titration
A. Dubey, A. Sonker, and R.K. Chaudhary

7 Original Report
High-resolution melting analysis as an alternative method for human neutrophil antigen genotyping

14 Review
Kell and Kx blood group systems
G.A. Denomme

20 Original Report
A simple approach to screen rare donors in Brazil

24 Original Report
Proposed criterion for distinguishing ABO mosaics from ABO chimeras using flow cytometric analysis

29 Review
Kidd blood group system: a review
J.R. Hamilton

36 Announcements

40 Advertisements

44 Instructions for Authors

46 Subscription Information
Jean Metzinger painted in the Divisionist mode inaugurated by Seurat as “Chromoluminarism” in the late 19th century, with its small patches of separated color and its aims of maximal brightness. But Metzinger brought to the style a faceted geometry that anticipated cubism. In *Femme au Chapeau* (Woman with a Hat), which he completed in 1906, we can see in the woman’s eponymous headgear and also in her clothing, face, and features, contrapuntal to their implied curvature, small cubes that embody symmetry and mathematical order. The mosaic quality of Metzinger’s composition typified his work and ties into the Oda et al. article in this issue of *Immunohematology*.

David Moolten, MD
Comparative evaluation of gel column agglutination and erythrocyte magnetized technology for red blood cell alloantibody titration

A. Dubey, A. Sonker, and R.K. Chaudhary

Antibody titration is traditionally performed using a conventional test tube (CTT) method, which is subjected to interlaboratory variations because of a lack of standardization and reproducibility. The aim of this study is to compare newer methods such as gel column technology (GCT) and erythrocyte magnetized technology (EMT) for antibody titration in terms of accuracy and precision. Patient serum samples that contained immunoglobulin G (IgG) red blood cell (RBC) alloantibodies of a single specificity for Rh or K antigens were identified during routine transfusion service testing and stored. Titration and scoring were performed separately by different laboratory personnel on CTT, GCT, and EMT. Testing was performed a total of three times on each sample. Results were analyzed for accuracy and precision. A total of 50 samples were tested. Only 20 percent of samples tested with GCT showed titers identical to CTT, whereas 48 percent of samples tested with EMT showed titers identical to CTT. Overall, the mean of the titer difference from CTT was higher using GCT (+0.31) compared with that using EMT (+0.13). Precision shown by CTT was 30 percent, EMT was 76 percent, and GCT was 92 percent on repeat testing. GCT showed higher titer values in comparison with CTT but was found to be the most precise. EMT titers were comparable to CTT, and its precision was intermediate. Further studies to validate this method are required. Immunohematology 2015;31:1–6.

Key Words: titration, alloantibody, conventional test tube, gel column technology, erythrocyte magnetized technology

In 1990, Lapierre et al. introduced a column agglutination method, which gained popularity because of its standardized performance, technical ease, stable end point, and the versatility of the method. This method is presently used worldwide and has been reported to be more sensitive for detection and identification of RBC alloantibodies. However, on performing the titration studies with gel column technology (GCT), researchers have found no linear correlation and several-fold higher titers in comparison with CTT. This may lead to overestimation of the antibody's strength and, hence, clinical decisions toward more invasive interventions for patients who develop the antibody.

A recent introduction in the field of transfusion medicine is erythrocyte magnetized technology (EMT) (Fig. 1). This method is based on the adsorption of paramagnetic particles in the presence of an externally applied magnetic field on the membrane of the RBCs. Thus, after contact with antibodies, reactive and nonreactive magnetized RBCs are rapidly pulled...
to the bottom of the well when placed on a magnetic plate. A final phase of shaking reveals positive or negative reactions, with no need for centrifugation (Fig. 2). The method has been found to be highly reliable for evaluating ABO grouping, Rh phenotyping, K typing, and antibody detection. To the best of our knowledge, no major study has been conducted so far for evaluating antibody titration using EMT.

This study was conducted to determine which method (GCT versus EMT) is a better substitute for replacing the age-old, gold standard of CTT for titration studies in terms of accuracy and precision. Precision serves as a more useful indicator of the method’s ability to reproducibly predict a rise in titer and not simply a variation observed as a result of individual technique.

Materials and Methods

The study was conducted at a transfusion medicine department of a tertiary care hospital and research center in North India. Blood samples that contained immunoglobulin G (IgG) RBC alloantibodies of a single specificity for Rh or K antigens were identified during routine transfusion service patient testing. For this study, only samples with antibodies that had strength of at least 2+ by CTT were included. Sera were separated from clotted blood samples and stored at −18°C in three separate aliquots. They were thawed immediately before testing.

Testing was performed separately by different trained laboratory personnel using CTT, GCT (LISS/Coombs ID-card Bio-Rad Laboratories, DiaMed GmbH, Cressier, Switzerland), and EMT (QWALYS 3, Diagast, Loos, France) to remove the operator bias. Sample identity was also blinded. Titration and scoring for each serum sample was performed a total of three times. On each test day, a new master dilution was prepared and one aliquot of serum sample was tested by all three methods. For comparison of the accuracy, CTT was considered the reference method, and results obtained on the first testing of a sample were used. Results of all three trials for a particular sample were analyzed to determine precision.

Serum Dilution

Serial twofold dilutions were made in normal saline. Pipette tips were changed after the transfer of each dilution. Using the same master dilution tube for all three methods reduces the likelihood of variation in titer and score relevant to the preparation of serial twofold dilutions.

Reagent RBCs

Reagent RBCs for CTT and GCT were prepared from donors who were homozygous for the allele encoding the corresponding antigen for the alloantibody being titrated using commercial antisera (DiaClon Bio-Rad Laboratories, DiaMed GmbH). A suspension was prepared in normal saline for CTT (2%) and GCT (0.8%) using the same reagent RBCs. For EMT, three vials of premagnetized group O test RBCs (HemaScreen I, II, III), which were supplied by the manufacturer with the antibody detection kit (ScreenLys, Diagast), were used. As per the anti-gram, the reagent RBC panel cells carrying apparently double dose expression of the cognate antigen were selected for the titration study.

Titration by CTT

Titration was performed using 12 test tubes following the standard procedure in the AABB Technical Manual. Briefly, 100 μL diluted serum was placed into each test tube, and 100 μL of 2 percent cell suspension was then added. The tubes were incubated at 37°C for 60 minutes. After washing four times with saline, two drops of anti-IgG (AHG; Eryclon Tulip Diagnostics, Goa, India) were added to each tube. The tubes were centrifuged and read for macroscopic agglutination. In-house prepared check cells were added to all tubes showing negative reaction and checked for agglutination to ensure the integrity of the AHG test results.

Titration by GCT

Titration was performed following the procedure used for antibody detection and identification. Briefly, 50 μL of 0.8 percent reagent RBC suspension was added to the gel column, followed by 25 μL diluted serum sample. After a standard
15-minute incubation at 37°C, the gel cards were centrifuged and the reactions were immediately graded.

**Titration by EMT**

Titration using EMT was also performed following the procedure used for antibody detection, in which each dilution was tested as an individual sample. The machine first dispensed 60 μL of a high-density solution (NanoLys) that prevents contact between the patient’s serum and the anti-IgG coated on the plates (ScreenLys). Next, 60 mL diluent, 15 mL patient’s serum, and 15 mL test RBCs (1% suspension) were dispensed. The plate was incubated at 37°C for 20 minutes and then placed on the magnetic shaker. Sensitized cells migrated through the NanoLys solution and reacted with the coated anti-IgG at the bottom of the wells. Positive reactions appear as a cellular layer, and negative reactions appear as a dot at the bottom of the well (Fig. 2). The results reported by the instrument were graded from negative to 4+ depending on the intensity of the reaction as per the literature provided by the manufacturer.

**Scoring and Titration End Points**

For all the methods, the titer was reported as the reciprocal of the highest dilution of serum at which 1+ agglutination was observed, and the strength of reactions was scored as described in the AABB Technical Manual.  

**Statistics**

Results are presented as numbers and percentages. For calculating the mean differences in titer by two methods, all titer values were converted to a log value (the logarithm of the titer value to the base 2). The arithmetic mean of these differences was then calculated.

For determining the precision, the differences were calculated between the lowest and highest values of the three test values of titers and scores. If the results of testing three times show variation, the differences were termed Δ value titer and Δ value score, respectively.

![Table 1. Difference in titers by GCT and EMT in comparison with CTT](image)

<table>
<thead>
<tr>
<th>Difference in titers (vs. CTT)</th>
<th>GCT [n (%)]</th>
<th>EMT [n (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identical</td>
<td>10 (20%)</td>
<td>24 (48%)</td>
</tr>
<tr>
<td>Twofold Higher</td>
<td>23 (46%)</td>
<td>16 (32%)</td>
</tr>
<tr>
<td>Lower</td>
<td>3 (6%)</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>Threefold Higher</td>
<td>8 (16%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Lower</td>
<td>2 (4%)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Fourfold Higher</td>
<td>4 (8%)</td>
<td>—</td>
</tr>
<tr>
<td>Lower</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

GCT = gel column technology; EMT = erythrocyte magnetized technology; CTT = conventional test tube.

**Results**

In the present study, 50 samples containing antibodies of six different specificities were subjected to titration with CTT, GCT, and EMT. Considering CTT as the reference method, difference in titers obtained by GCT and EMT are shown in Table 1. Only 20 percent of the samples tested with GCT showed titers identical with CTT, whereas 48 percent of the samples tested with EMT showed identical titers with CTT. In four samples, the titers with GCT were fourfold higher than those with CTT. In no sample were the titers by EMT that much higher than those with CTT (i.e., fourfold).

The mean difference in titers, calculated by converting the titer values to log (base 2) values for each antibody, is shown in Table 2. Overall, the mean difference of titer and score from CTT was higher with GCT (+0.31, 10.62) compared with that using EMT (+0.13, 4.88). The mean difference of titer between

![Table 2. Comparison of the mean difference in titer and score](image)

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Number</th>
<th>Mean difference in titer</th>
<th>Mean difference in score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GCT–CTT</td>
<td>EMT–CTT</td>
<td>GCT–CTT</td>
</tr>
<tr>
<td>Anti-D</td>
<td>32</td>
<td>+0.38</td>
<td>+0.12</td>
</tr>
<tr>
<td>Anti-E</td>
<td>2</td>
<td>+0.30</td>
<td>+0.15</td>
</tr>
<tr>
<td>Anti-e</td>
<td>6</td>
<td>−0.10</td>
<td>−0.05</td>
</tr>
<tr>
<td>Anti-C</td>
<td>7</td>
<td>+0.26</td>
<td>+0.17</td>
</tr>
<tr>
<td>Anti-c</td>
<td>1</td>
<td>+0.30</td>
<td>0</td>
</tr>
<tr>
<td>Anti-K</td>
<td>2</td>
<td>+0.45</td>
<td>+0.15</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>+0.31</td>
<td>+0.13</td>
</tr>
</tbody>
</table>

GCT = gel column technology; CTT = conventional test tube method; EMT = erythrocyte magnetized technology.
Table 3. Precision of various methods in terms of titer

<table>
<thead>
<tr>
<th>Difference in titer</th>
<th>CTT</th>
<th>GCT</th>
<th>EMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ0</td>
<td>15 (30%)</td>
<td>46 (92%)</td>
<td>38 (76%)</td>
</tr>
<tr>
<td>Δ1</td>
<td>28 (56%)</td>
<td>4 (8%)</td>
<td>11 (22%)</td>
</tr>
<tr>
<td>Δ2</td>
<td>5 (10%)</td>
<td>—</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Δ3</td>
<td>2 (4%)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*A represents the highest difference in titer.
CTT = conventional test tube; GCT = gel column technology; EMT = erythrocyte magnetized technology.

Table 4. Precision of various methods in terms of score

<table>
<thead>
<tr>
<th>Difference in score*</th>
<th>CTT</th>
<th>GCT</th>
<th>EMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ0–2</td>
<td>12 (24%)</td>
<td>39 (78%)</td>
<td>32 (64%)</td>
</tr>
<tr>
<td>Δ3–4</td>
<td>20 (40%)</td>
<td>9 (18%)</td>
<td>12 (24%)</td>
</tr>
<tr>
<td>Δ5–6</td>
<td>9 (18%)</td>
<td>2 (4%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Δ6–7</td>
<td>5 (10%)</td>
<td>—</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Δ8–9</td>
<td>2 (4%)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Δ&gt;10</td>
<td>2 (4%)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*A represents the highest difference in score.
CTT = conventional test tube; GCT = gel column technology; EMT = erythrocyte magnetized technology.

GCT and CTT was highest for anti-K (+0.45), followed by anti-D. The mean difference of score between EMT and CTT was highest for anti-C (+0.17). The mean difference of score from CTT was highest for anti-D for both GCT (12.19) and EMT (7.06).

Reproducibility of the methods was compared in terms of precision. Comparing the differences when repeating both the titration and scoring values (Tables 3 and 4), the highest precision was observed with GCT, followed by EMT, and was lowest with CTT. In terms of titer, absolute precision was seen with GCT in 92 percent of cases, EMT in 76 percent of cases, and CTT in 30 percent of cases. Highest precision in terms of score (Δ0–2) was seen with GCT in 78 percent of cases, EMT in 64 percent of cases, and CTT in 24 percent of cases.

Discussion

CTT is recommended for performing antibody titration, but this method is subject to technical variables that can affect the results substantially. This study was conducted to establish whether titrations with newer methods such as GCT and EMT correlate with CTT and to compare the precision of various methods.

There are no previous studies on antibody titration by EMT; however, a few studies have been conducted to evaluate antibody titration by EMT for ABO-Rh testing, antibody detection, and phenotyping, in which it demonstrated a reliable performance with a high sensitivity and specificity. EMT’s comparison with GCT demonstrated similar performance in detecting clinically relevant antibodies together with a noteworthy reduction in the number of antibodies without clinical importance. This previous information led us to evaluate the EMT against GCT as a suitable method for antibody titration.

The titration of antibodies by the three methods in the present study showed variable results. On comparison of the titers, it was observed that titers obtained by EMT correlate better with CTT than GCT. In nearly half of the samples (48%), the titers obtained by CTT and EMT were identical. However, with GCT, 46 percent of the samples were found to have twofold higher titers than CTT, and 8 percent had titers as much as fourfold higher in comparison with CTT. Many studies have been conducted in the past for comparing the titrations by CTT and GCT and have reported variable findings. Novaretti et al. found a strong variability in anti-D titration by GCT in all 79 samples tested. The observed differences were as high as threefold in 5 sera, fourfold in 21, fivefold in 30, sixfold in 20, sevenfold in 2, and eightfold in 1. Thus, these authors have concluded that GCT should not be used for anti-D to monitor fetuses at risk for hemolytic disease of the fetus and newborn (HTFN).

Bromilow et al. found that 31 of 34 samples with Rh, K, Duffy, and Kidd alloantibodies had a higher titer score by GCT than CTT. Of 82 samples with Rh alloantibodies, Steiner et al. reported that only 20 (24%) had significantly higher titers by GCT compared with CTT. In their evaluation of 27 samples with non-Rh antibodies, the two methods performed equivalently, generating titers within two serial dilutions for all samples. A previous study from India reported that GCT is more sensitive than CTT for antibody detection, but significantly higher titers were found in 22 (26.5%) of 83 samples tested. The authors thus concluded that titers obtained by GCT should not be relied on for clinical management of HDFN.

GCT has also been evaluated for monitoring titers of anti-A and anti-B in patients undergoing ABO-incompatible kidney transplantation performed by Shirey et al. They have found identical titer values by GCT and CTT in 26 of 50 samples, and no sample’s titer values varied more than one dilution between the two methods. Other researchers have reported that anti-A and anti-B titers obtained by GCT were less variable between institutions and demonstrated better clinical correlation compared with titers by CTT in similar transplant programs.

In the present study, the titer values for most of the Rh antibodies (D, E, C, c) were approximately one tube higher by GCT compared with CTT. For anti-e, the average titer values...
by both GCT and EMT were lower than those by CTT. The
largest difference between the methods was observed with
samples for anti-K, in which the titer values by GCT were 1.5
dilutions higher compared with CTT. Both CTT and EMT
had equal sensitivity in cases containing anti-c. Overall, the
mean titer by GCT was 1.1 dilutions higher and those by
EMT were 0.35 dilutions higher than CTT. A recent study was
conducted by Finck et al. to determine whether GCT yields
comparable results with the CTT method in titrating Rh and K
alloantibodies. For most alloantibodies titrated (anti-E, anti-e,
and anti-c), the GCT generated titer values were less than one
dilution higher than the value by CTT. The GCT system was
found to be slightly less sensitive than CTT for anti-D, giving
titer results that were on average 0.09 dilutions lower. Samples
with anti-K tended to generate higher titer values in CTT.

Titer values alone are said to be misleading without
evaluating the strength of agglutination as well. The observed
strength of agglutination is assigned a number, and the sum
of these numbers for all tubes in a titration study represents
the score, which is another semiquantitative measurement
of antibody reactivity. The arbitrarily assigned threshold for
significance in comparing scores is a difference of 10 or more.
In the present study, the mean difference in scores of GCT and
CTT was more than 10 and, hence, significant. On the other
hand, the mean difference in scores of EMT and CTT was
4.88, thus indicating that the strength of reaction with these
two methods does not differ significantly.

Antibody titration has long been found difficult to
standardize and to reproduce precisely. This finding is
exemplified by the AABB recommendation that antenatal
evaluations of maternal antibodies should be performed on
previously frozen serum samples in parallel with a current
specimen to minimize the possibility that changes in the titer
result from differences in method and in the skill of the testing
technologist. Such duplicate testing mitigates the problem
of imprecision within a laboratory. The disparity in titration
results is also reported in proficiency testing samples provided
in the antibody titration survey of the College of American
Pathologists. Results from different laboratories were
grouped according to the method used, and the variations
extended over five or more dilutions (i.e., a 32-fold difference,
for both anti-D and anti-A).

On comparing the precision of various methods in terms
of both titer and score in the present study, GCT was found to
have the best reproducibility. This method reported identical
titers in 92 percent of cases and identical scores in 78 percent
of cases. CTT was found to be the least precise, with few cases
having titer difference of three dilutions and a score difference
of more than 10. EMT had intermediate precision, with
identical titers in 76 percent and identical scores in 64 percent
of the cases. Judd et al. suggested that reproducible grading
is well known to be problematic, even among technical staff
in a single laboratory, and this apparently is a major source of
discrepancy when reading titers. An international study was
conducted by AuBuchon et al. in which they reported that
the gel card method at the AHG phase (1+ end point) showed
reduced variance compared with tube-based methods. This
high precision of GCT may be attributed to the clear-cut
grading of the results.

There were several limitations in this study. First, we
could not perform a clinical correlation of the titer values.
Second, the reagent RBCs for antibody titration by EMT were
procured commercially from the manufacturer, although
their phenotype was identical to the in-house RBCs used
for CTT and GCT. Third, testing by EMT was performed
on an automated platform, whereas the other methods were
performed manually. Finally, the manufacturers of GCT and
EMT do not include their application for antibody titration in
the product literature, which thereby constitutes off-label use
of these methods.

In conclusion, the titers obtained by GCT were very high
and not dependable for clinical monitoring of patients. Titers
obtained by EMT were somewhat similar to CTT, yet their
reliability for clinical application remains to be determined.
Precision is another mandate for determining the suitability
of a method for antibody titration. GCT has been proven best
in this regard owing to clear-cut interpretation of the results.
The reproducibility by EMT was comparable with that of GCT
in most cases. Given its intermediate accuracy and precision,
EMT may be adopted for performing antibody titration in
settings where it is currently in use after further validation of
its performance in studies with larger sample numbers.

Acknowledgments

This study was conducted at the Department of
Transfusion Medicine, Sanjay Gandhi Post Graduate Institute
of Medical Sciences, Lucknow, India.

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High-resolution melting analysis as an alternative method for human neutrophil antigen genotyping


Human neutrophil antigen (HNA)-typed granulocyte panels are widely used to screen for the presence of HNA antibodies and to determine antibody specificity. Many laboratories screen donors for HNA genotypes using low-throughput methods such as allele-specific polymerase chain reaction (PCR), PCR–restriction fragment–length polymorphism, and multiplex PCR. In the present study, we used a high-resolution melting (HRM) analysis to determine HNA genotypes. For the HRM analysis, purified genomic DNA samples were amplified via PCR with HNA-specific primers. Nucleotide substitutions in genes encoding HNAs were differentiated on the basis of the HRM curves, and the results of HRM and DNA sequencing analyses were determined to be in complete agreement. The gene frequency of HNA-1a, -1b, -1c, -3a, -3b, -4a, -4b, -5a, and -5b in the Japanese population was consistent with the previous reports. Our results suggest that HRM analysis can be used for genotyping HNA antigens determined by single nucleotide substitutions. Immunohematology 2015;31:7–13.

Key Words: neutrophil, HNA, antibody, genotyping, high-resolution melting analysis

Evidence indicates that leukocyte antibodies are one of the primary causes of nonhemolytic transfusion reactions, particularly in transfusion-related acute lung injury (TRALI).1–4 Human leukocyte antigen (HLA) class I,2,5 HLA class II,6–9 and human neutrophil antigen (HNA)10–15 antibodies have been associated with nonhemolytic transfusion reactions. Therefore, it is important to detect such antibodies in blood components used for transfusion.

HNAs have been classified into five systems (HNA-1, HNA-2, HNA-3, HNA-4, and HNA-5), and differences between these HNA polymorphisms can result in several alloimmunization responses. HNA-1 comprises the following antigens: HNA-1a, HNA-1b, HNA-1c, and HNA-1d; these are specifically expressed on neutrophils. Antibodies against HNA-1 are frequently thought to be the cause of alloimmune neutropenia (ANN), autoimmune neutropenia, and TRALI.16–18 HNA-2 is represented by a single antigen and is expressed on neutrophils in Caucasian (97%), African American (95%), and Japanese (88%) populations.19,20 Polymorphism of this antigen has not been reported. Antibodies against this antigen are associated with ANN, autoimmune neutropenia, febrile transfusion reactions, and TRALI.12,21–23 HNA-3, comprising HNA-3a and HNA-3b, is expressed on granulocytes, lymphocytes, platelets, endothelial cells, kidney, spleen, and placental cells.24 Alloantibodies to HNA-3a are associated with occasional cases of febrile transfusion reactions,25 ANN,26 and serious cases of TRALI.24–27 HNA-4 and HNA-5 antigens reside on the subunits of the β-2 integrin family (CD11a and CD11b, respectively). HNA-4 is expressed on granulocytes, monocytes, and NK cells, whereas HNA-5 is expressed on all leukocytes.17 Moreover, alloantibodies against HNA-4a can cause ANN, but those specific for HNA-5a have not been clinically associated with neutropenia.29

The detection of antibodies against HNAs primarily relies on cell-based assays, the granulocyte immunofluorescence test (GIFT), and granulocyte agglutination test (GAT). The International Society of Blood Transfusion Working Party on Granulocyte Immunobiology recommends GIFT and GAT as reference methods for detecting HNA antibodies.30 Although cells could be stored for a week following fixation for use with GIFT, they were difficult to use in high-sensitivity flow cytometry analysis because normal human sera revealed high background reactivity to neutrophils.

Genotyping via high-resolution melting (HRM) analysis can be used to rapidly predict the HNA antigen status of cells that could be used as panel cells in GIFT and GAT. In the field of genotyping, HRM analysis sensitively and specifically detects a single nucleotide change in a gene.31 Further, HRM analysis is a simpler and more rapid genotyping method compared with allele-specific polymerase chain reaction (PCR), PCR–restriction fragment–length polymorphism, and multiplex PCR.32 In the present study, we used HRM analysis to genotype for HNAs.
Materials and Methods

Blood Samples and DNA Preparation

Whole blood anticoagulated with ethylenediaminetetraacetic acid was collected from healthy blood donors and used as leukocyte samples for genotyping HNAs. DNA was prepared using the Qiasymphony instrument and Qiasymphony DNA Mini kit (Qiagen, Hilden, Germany). This research project was approved by the ethics committee of the Japanese Red Cross Society Blood Service Headquarters.

Primer Selection

We performed HNA-1, HNA-3, HNA-4, and HNA-5 genotyping using a PCR-HRM method. HNA-1a, HNA-1b, and HNA-1c were encoded by FCGR3B*,1, FCGR3B*,2, and FCGR3B*3 (GenBank accession number: NC_000001.10), respectively. Because FCGR3B highly resembles FCGR3A," which encodes FcγR3a, we first amplified FCGR3B-specific DNA to avoid the contamination of FCGR3A DNA before performing PCR-HRM analysis for HNA-1 genotyping; we developed a pair of primers [5-GGACATATGGGGGAGAAT-3, called “FCGR3B forward” (nucleotide position in FCGR3B, 6616–6633), and 3-GAGTCCTGACACTTCTTG-5, called “FCGR3B reverse” (nucleotide position in FCGR3B, 7604–7622)] that were designed to amplify the FCGR3B fragment, including the five polymorphic sites described subsequently but not the FCGR3A fragment. FCGR3B*1 differs from FCGR3B*2 at five nucleotide positions, and a single nucleotide polymorphism (SNP) differentiates FCGR3B*2 from FCGR3B*3 (Table 1). HNA-1a differs from HNA-1b at five nucleotide positions (141, 147, 227, 277, and 349), which results in four amino acid residue changes at positions 36, 65, 82, and 106 in the membrane-distal domain of the glycoprotein. Furthermore, HNA-1b differs from HNA-1c at nucleotide position 266 alone, resulting in the substitution of Ala to Asp at position 78 (Table 1). Recently, HNA-1d was proposed as an additional allele of the HNA-1 system. Reil et al. performed epitope mapping experiments using human embryonic kidney cells that express different recombinant variants of FcγRIIIb. We designed several primer sets to amplify the polymorphic sites of HNA-1, HNA-3, HNA-4, and HNA-5. The primer sequences are shown in Table 2. These primers were synthesized using standard phosphoramidite chemistry (Life Technologies, Carlsbad, CA).

PCR Amplification of FCGR3B Fragment Prior to HNA-1 Genotyping

Because nucleotide sequences of FCGR3A and FCGR3B are very similar, we developed a PCR pre-amplification system for HNA-1 to avoid the amplification of the FCGR3A fragment prior to HNA-1 genotyping. Each PCR contained 1 µL genomic DNA, 1 µL forward primer (5 µmol/L), 1 µL reverse primer (5 µmol/L), 8.5 µL RNase-free water (Qiagen), and 12.5 µL PrimeSTAR Max DNA polymerase premix (Takara, Seta, Japan) in a final volume of 25 µL. PCR amplification was performed with initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 10 seconds at 95°C, at 55°C for 5 seconds, and at 72°C for 10 seconds. Amplicons were purified using Diffinity RapidTip2 (Sigma, Deisenhofen, Germany).

PCR Amplification for Genotyping of HNA-1 to -5

This assay used the Type-it HRM Kit (Qiagen). Each PCR contained 1 µL DNA, 1 µL forward primer (5 µmol/L), 1 µL reverse primer (5 µmol/L), 8.5 µL RNase-free water (Qiagen), and 12.5 µL of a 2× HRM PCR master mix (Qiagen). The purified amplicons described earlier were used as templates for the genotyping of HNA-1, and genomic DNA was used for the genotyping of the other HNAs. The final reaction volume was 25 µL. A Roter-Gene Q (Qiagen) instrument was used. PCR amplification was performed with initial denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 10 seconds, at 52°C for 30 seconds, and at 72°C for 10 seconds, with data acquisition during the 72°C step.

Table 1. Alleles of FcγRIIIb with location of single nucleotide polymorphisms and resulting amino acid changes

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Allele</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>141*</td>
</tr>
<tr>
<td>HNA-1a</td>
<td>FCGR3B*01</td>
<td>AGG (p.Arg36)</td>
</tr>
<tr>
<td>HNA-1b</td>
<td>FCGR3B*02</td>
<td>AGC (p.Ser36)</td>
</tr>
<tr>
<td>HNA-1c</td>
<td>FCGR3B*03</td>
<td>AGC (p.Ser36)</td>
</tr>
<tr>
<td>HNA-1d</td>
<td>FCGR3B*02</td>
<td>AGC (p.Ser36)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>147*</td>
</tr>
<tr>
<td>HNA-1a</td>
<td>FCGR3B*01</td>
<td>CTG (p.Leu38)</td>
</tr>
<tr>
<td>HNA-1b</td>
<td>FCGR3B*02</td>
<td>CTT (p.Leu38)</td>
</tr>
<tr>
<td>HNA-1c</td>
<td>FCGR3B*03</td>
<td>CTT (p.Leu38)</td>
</tr>
<tr>
<td>HNA-1d</td>
<td>FCGR3B*02</td>
<td>CTT (p.Leu38)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>227*</td>
</tr>
<tr>
<td>HNA-1a</td>
<td>FCGR3B*01</td>
<td>AAC (p.Asn65)</td>
</tr>
<tr>
<td>HNA-1b</td>
<td>FCGR3B*02</td>
<td>AGC (p.Ser65)</td>
</tr>
<tr>
<td>HNA-1c</td>
<td>FCGR3B*03</td>
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<td>266*</td>
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<td>FCGR3B*02</td>
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<td>277*</td>
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<tr>
<td>HNA-1a</td>
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<td>FCGR3B*03</td>
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<tr>
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<td>GTC (p.Val106)</td>
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<td>ATC (p.Ile106)</td>
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</table>

*The underlined letters correspond to the position of the single nucleotide polymorphisms.

Note that for HNA-1d, amino acid positions and nucleotide positions were estimated according to the reactivity of the antisera against HNA-1d as reported by Reil et al."
Creation of Positive Control Plasmid for Genotyping
Synthetic DNA fragments (Fig. 1A–D) were cloned into the pCR2.1 TOPO plasmid (Life Technologies, Carlsbad, CA) and served as positive controls.

HRM Analysis
For HRM analysis, amplified samples bound to the fluorescent dye were heated from 65°C to 95°C. The temperature was increased by 0.1°C/second at each step using the Rotergene Q and covered the full range of expected melting points. HRM data were analyzed using the Rotergene Q software. Fluorescence intensity values were normalized between 0 percent and 100 percent by defining linear baselines before and after the melting transition of each sample. The fluorescence of each acquisition was obtained from HRM curves and was

Table 2. DNA sequences of primers used in polymerase chain reaction and high-resolution melting analyses

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
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</thead>
<tbody>
<tr>
<td>FCGR3B</td>
<td>forward</td>
<td>GGCACATATGGGGACGACAT</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>GAGCTCAGTCGAACCTTTTC</td>
</tr>
<tr>
<td>FCGR3B</td>
<td>forward</td>
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<td>(NC_000001.10)*</td>
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<tr>
<td>SLC4A2</td>
<td>forward</td>
<td>HNA-3 forward1</td>
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<td>(NC_000019)*</td>
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<td>ITGAM</td>
<td>forward1</td>
<td>HNA-4 forward1</td>
</tr>
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<td></td>
<td>GAGATAGTTGGCTGCAACACC</td>
</tr>
<tr>
<td>ITGAL</td>
<td>forward1</td>
<td>HNA-5 forward1</td>
</tr>
<tr>
<td>(NC_000016.9)*</td>
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<td>GGCACCGACAGTCCCTCAG</td>
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</table>

*GenBank accession numbers.

HNA genotyping using real-time PCR and HRM

Fig. 1. Outlines of high-resolution melting analysis for HNA-1 to HNA-5 genotyping. The structures of responsible exons of each human neutrophil antigen (HNA), the sequences of synthetic DNA fragments used in high-resolution melting (HRM) analysis as positive controls, and the location of each single nucleotide polymorphism (SNP) of HNA alleles are presented. Arrows indicate the primers were used. The regions with the SNPs in different alleles are shown below each synthetic DNA fragment. Polymerase chain reaction (PCR) amplification regions of HNA-1 (A), HNA-3 (B), HNA-4 (C), and HNA-5 (D) are shown.
calculated as the percentage of fluorescence between the top and bottom baselines of each acquisition temperature, with a confidence threshold of 80 percent of the controls.

**Statistical Analysis**

Genotype and allele frequencies were calculated by the counting method. The validity of the Hardy-Weinberg equilibrium was tested by calculating the expected number of subjects for each genotype. Agreement of the observed and expected genotypes, based on the Hardy-Weinberg equilibrium, was determined using the $\chi^2$ test. The level of statistical significance was set at $p < 0.05$.

**Results**

**HRM Analysis for Genotyping of HNAs**

In the field of DNA-based genotyping, HRM analysis was developed as a novel method for detecting a single nucleotide change in a gene. Before performing HRM analysis, the target sequence is amplified in the presence of a double-stranded DNA-binding fluorescent dye, and the melting temperature (Tm) is increased from a lower to a higher temperature for the HRM analysis. Differences in the gene sequences between the heterozygous and homozygous genotypes lead to differences in Tm. Heterozygous genotypes tend to have lower Tm than homozygous genotypes, and, consequently, the overall HRM curves will shift to the left. The differences in the HRM curves were determined using the Roter-Gene Q software.

To confirm validity of HRM analysis for genotyping HNA-1, we amplified the control plasmids of each HNA-1 allele (a/a, b/b, c/c, a/b, a/c, and b/c) and analyzed them using the Roter-Gene Q software. Because four nucleotide substitutions are involved in HNA-1 polymorphism, we set up a PCR system to categorize the “1a,” “1b,” and “1c” alleles based on the SNPs at positions 266 and 277. Figure 2A presents the representative HRM curves of synthesized DNA samples containing 1a, 1b, or 1c sequences. The melting curve shift for each of the synthetic DNA samples was estimated using Roter-Gene Q software, and these curves successfully defined each HNA-1 genotype. Subsequently, we used these curves as standard allotype-specific curves. With regard to HNA-1 genotyping, we first pre-amplified HNA-1–specific PCR amplicons and used these amplicons as templates for HNA-1 genotyping to avoid amplification of FCGR3A, whose DNA sequence highly resembles that of HNA-1. The absence of contamination of FCGR3A in the amplicons was confirmed using 15 representative genomic DNA samples derived from blood donors based on the following two points: (1) the amplicons revealed only FCGR3B-specific bands on agarose gel electrophoresis, and (2) subsequent DNA sequence analysis revealed that the amplicons were derived from FCGR3B. After excluding the possibility of contamination of FCGR3A DNA, we genotyped these 15 genomic DNA samples using the PCR-HRM method. Following analysis of all samples, samples 6, 7, and 2 were determined as HNA-1a/1a, HNA-1b/1b, and HNA-1a/1b, respectively (data not shown). The results of HRM analysis were in complete agreement with the sequencing data. Using the two different sets of HRM analysis, we successfully determined the genotypes of the three HNA-1 alleles.

Then, we analyzed HNA-3 (a/a, b/b, and a/b), HNA-4 (a/a, b/b, and a/b), and HNA-5 (a/a, b/b, and b/c). The HRM curves of HNA-3 (Fig. 3A), HNA-4 (Fig. 3B), and HNA-5 (Fig. 3C) clearly identified the individual genotypes using the synthetic DNA samples, and the results for 15 blood donors completely coincided with the DNA sequence analyses (data not shown). Therefore, HRM analysis successfully detected all the HNA polymorphisms.

**Frequencies of HNA Genotypes Among Japanese Blood Donors**

Having demonstrated that HRM analysis can be used to determine HNA genotypes, we subsequently genotyped 500 Japanese individuals for HNA-1, HNA-3, HNA-4, and HNA-5 using HRM analysis and calculated the genotype frequency (Table 3) and the allele frequency (Table 4) of the
The results reported here, using a different method, are consistent with these findings (Tables 3 and 4). The frequency of HNA-1d has not been reported previously. The HNA-1 system has three FCGR3B alleles. FCGR3B*01 encodes only one antigen (HNA-1a), while FCGR3B*02 and FCGR3B*03 encode two antigens each (HNA-1b and HNA-1d, and HNA-1b and HNA-1c, respectively). Because the occurrence of HNA-1c was not observed in this study, the frequency of HNA-1d was similar to that of HNA-1b.

Occasionally, the contamination of PCR products interferes with genotype testing. HRM analysis determines genotypes of individual test samples using a real-time PCR instrument with a sample cap piercing feature, which eliminates a potential source of contamination.

The frequency of HNA-1 among the Japanese population considerably differs from that of Caucasians, who express HNA-1b more frequently than HNA-1a. Additionally, the

<table>
<thead>
<tr>
<th>Table 3. HNA genotypes among Japanese blood donors</th>
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<td>HNA-3</td>
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<td>HNA-4</td>
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<tr>
<td>HNA-5</td>
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</tbody>
</table>

HNA = human neutrophil antigen.

<table>
<thead>
<tr>
<th>Table 4. HNA alleles among Japanese blood donors</th>
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</thead>
<tbody>
<tr>
<td><strong>HNA</strong></td>
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<tr>
<td>HNA-1a</td>
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<tr>
<td>HNA-1b</td>
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<tr>
<td>HNA-1c</td>
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<tr>
<td>HNA-3a</td>
</tr>
<tr>
<td>HNA-3b</td>
</tr>
<tr>
<td>HNA-4a</td>
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<tr>
<td>HNA-4b</td>
</tr>
<tr>
<td>HNA-5a</td>
</tr>
<tr>
<td>HNA-5b</td>
</tr>
</tbody>
</table>

HNA = human neutrophil antigen.

HNA system in Japan. The deviation of the observed numbers of genotypes from the expected numbers on the basis of the Hardy-Weinberg equilibrium was not statistically significant (Table 3). The occurrence of HNA-1 to -5 was similar to that reported elsewhere.23-35

**Discussion**

HRM is a very attractive, advanced, fast, and cost-effective SNP genotyping technology based on the analysis of the melting profile of PCR products, using intercalating fluorescent dyes to monitor the transition from double-stranded to single-stranded (melted) DNA. This method was used to confirm HLA genotypic identity between unrelated individuals before allogeneic hematopoietic stem-cell transplantation.32,36 Subsequently, several blood group antigens, including some in the Duffy, Kidd, and Diego blood group systems, were also genotyped using HRM analysis.37 Further, SNPs in the genes encoding human platelet antigens 1–6 and 15 were analyzed using HRM analysis.38 Thus, HRM analysis is a useful method for genotyping SNPs. In the present study, we applied HRM analysis to HNA genotyping and clearly genotyped and distinguished homozygous from heterozygous HNA alleles (Fig. 3).

Knowledge of HNA frequency is important for predicting the risk of alloimmunization to HNA. Previous studies had reported the frequency of HNA-1 to HNA-5 (except
frequency of HNA-3b seems to be higher in the Japanese population than in Caucasians.\textsuperscript{14,39,60} This observation suggests a higher risk of alloimmunization for individuals homozygous for HNA-3b by exposure to the HNA-3a antigen during transfusion or pregnancy. Detection of antibodies against HNA-3a was infrequent in Japanese patients with TRALI, however. Therefore, additional factors specific for the Japanese population may elicit antibodies against HNA-3a.

Acknowledgments

We thank the laboratory staff of the Japanese Red Cross Kinki Block Blood Center for preparing blood samples and extracting genomic DNA.

References

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**Manuscripts**

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The Kell and Kx blood group systems are expressed as covalently linked molecules on red blood cells (RBCs). The Kell blood group system is very polymorphic, with 35 antigens assigned to the system. The expression of Kell glycoprotein on RBCs is not critical to the erythrocyte function. However, the expression of Kx is critical to normal morphology, and null mutations are associated with the McLeod neuroacanthocytosis syndrome. The immunogenicity of the K antigen is second only to the D antigen, and alloantibodies to Kell antigens can cause transfusion reactions and hemolytic disease of the fetus and newborn. Kell alloantibodies in pregnancy are known to suppress erythropoiesis, which can result in serious disease despite low amniotic bilirubin levels and low antibody titers. Late-onset anemia with reticulocytopenia is thought to be attributable to the continual suppression of erythropoiesis from residual alloantibody in the infant. Alloimmunization to XK protein is rare, and expressed polymorphisms have not been reported. Together these two blood group systems share an integral relationship in transfusion medicine, neurology, and musculoskeletal biology. *Immunohematology* 2015;31:14–19.

**Key Words**: blood group systems, Kell, Kx

Kell glycoprotein is a single-pass type II transmembrane moiety expressed on red blood cells (RBCs). Kell is highly polymorphic, expressing 7 sets of 15 antithetical antigens (one set is triallelic), 2 low prevalent antigens, and 18 high prevalent antigens. The first alloantibody was discovered in 1946 as the result of hemolytic disease of the fetus and newborn (HDFN). The name of the antigen, “Kell”, was taken from the last name of the woman whose serum contained the antibody. The maternal antibody reacted with paternal and infant RBCs and reacted retrospectively with RBCs from their firstborn child. An incidence of 9 percent among random blood donors was indicative of a new blood group antigen. A few years later, the antithetical antigen was identified. Together, they were known as Kell/Cellano antigens, which are now named K/k or KEL1/KEL2. To this day, reference to the K antigen often is confused by the inappropriate use of the term “Kell” because it is also the name of the blood group system.

The Kell and Kx blood group systems are discussed together because their antigens are expressed on the surface of RBCs as covalent-linked moieties (Fig. 1). An eloquent historical perspective of the landmark discoveries and relationships between Kell glycoprotein and XK protein was reviewed in Redman and Lee. Suffice it to say that the discovery of these two blood group systems relied on RBC serological, biochemical, immunohistochemistry, and molecular observations. The fact that Kell glycoprotein is particularly immunogenic in humans...
provided multiple sources of the alloantibodies and antigen-negative RBCs.

It is important to note that although Kell and XK are covalently linked molecules on RBCs, they are not necessarily expressed together in other tissues. Kell is expressed in testis, brain, and muscle. XK is found in muscle, heart, and brain. Expressed sequence tagged analyses show that KEL is expressed in cDNA libraries from bone marrow, macrophages, spleen, and brain. XK was detected in cDNA from the same libraries and was detected in peripheral nervous tissue, and eye. Interestingly, KEL, but not XK, is detected in 8- and 9-week-old embryo libraries.\(^7\)–\(^9\)

Kell glycoprotein shares sequence homology with M13 family neutral zinc-dependent endopeptidases, and has been demonstrated to have endothelin-converting enzyme-1 activity. RBCs expressing Kell glycoprotein can cleave big endothelin 3 into its bioactive peptide. The expression of Kell glycoprotein on RBCs does not appear to be critical to red cell membrane structure or function, however.\(^10\) The Kell glycoprotein forms part of a surface membrane complex with glycophorin C and D because it has been demonstrated that Kell antigens are weakly expressed with the Ge:-3 phenotype or when glycophorin C/D are absent (Leach phenotype).\(^11\)

The Kx blood group system is a multi-pass transmembrane moiety and contains one antigen, the XK protein. It is predicted to traverse the plasma membrane 10 times and, because of this structure, is thought to be a membrane transporter.\(^12\) This protein is biologically important because the absence of XK protein results in RBC morphological changes called acanthocytosis and leads to the midlife onset of neuromuscular abnormalities known as the McLeod neuroacanthocytosis syndrome.\(^13\)

**Kell and XK Proteins**

Kell glycoprotein has 732 amino acids and contains five N-glycosylation sites, with the threonine to methionine substitution at position 193 resulting in a loss of one N-glycosylation site. Therefore, Kell glycoprotein expressing a methionine at position 193 (K antigen) migrates faster in sodium dodecyl sulfate polyacrylamide gel electrophoresis than the version with threonine at that position. Kell glycoprotein has 15 cysteine residues on the exofacial domain. The Cys72 forms a disulfide bond with Cys347 of the XK protein. It is because of several internal cysteine–cysteine bonds that Kell antigens are sensitive to disulfide bond reducing agents (2-aminoethyl-isothiouronium bromide and dithiothreitol). The metalloendopeptidase activity is attributable to a conserved HxxLH motif (histidine-glutamate-leucine-leucine-histidine in Kell glycoprotein) at amino acid positions 581–585. The three-dimensional structure of Kell glycoprotein has been modeled on the crystal structure of neutral endopeptidase 24.11. Lee and coworkers noted that most of the Kell antigens are the result of amino acid changes in the non-conserved exofacial globular domain.\(^14\)

The expression of Kell glycoprotein is weaker than normally observed when it contains a glutamate at position 281 (i.e., Kp\(^b\) antigen). In other words, a reduced amount of K antigen can be shown when RBCs are Kp(a+)\(^15\)\(^,\)\(^16\) although K antigen expression is unaffected. The reason for this observation is that K antigen contains an arginine at position 281, i.e., Kp\(^b\) antigen. It was noted that K and Kp\(^b\) would not be observed in cis moiety because both are low prevalence antigens. The probability is exceedingly rare for a non-sister chromatic exchange or sequential mutation at both nucleotide positions to create this haplotype. But the Kp\(^b\)-associated reduced expression was confirmed with the characterization of a KEL*1,3 allele.\(^17\) Kell glycoprotein has been reported to be weakly expressed in the presence of autoantibodies showing Kell specificity. However, it is important to evaluate the phenomenon carefully because of the immunoglobulin M (IgM) autoantibody masking of Kell antigens, as shown by Zimring and colleagues. Their eloquent study also showed that antibodies to Kp\(^b\) sterically hinder the binding of anti-K to its cognate epitope.\(^18\)\(^,\)\(^19\)

The XK protein comprises 444 amino acids and has no known polymorphisms leading another blood group antigen. The moiety is not glycosylated.\(^15\) RBCs are deemed to have the McLeod phenotype when they lack Kx antigens and weakly express Kell antigens and when mild hemolysis is observed in the patient with or without acanthocytosis. The McLeod phenotype may be part of the McLeod neuroacanthocytosis syndrome in which neurological and musculoskeletal abnormalities are also present.\(^13\)\(^,\)\(^20\)

**Kell and XK Genes**

The gene responsible for the expression of Kell glycoprotein was cloned in 1991. Lee and coworkers used a short oligonucleotide probe deduced from a tryptic peptide of the proposed glycoprotein to screen a λgt cDNA library. Later in 1995, Lee reported that KEL was organized into 19 exons and spanned approximately 21.5 kilobasepair. Genetic linkage analysis with prolactin-inducible protein by Zelinski et al. mapped KEL to chromosome 7q32-36. Lee showed that KEL maps to 7q33.\(^1\)\(^,\)\(^21\)\(^,\)\(^22\)
The distinguishing feature of KEL is that it is predicted to be a type II single transmembrane spanning protein; the N-terminal is on the cytoplasmic side of the plasma membrane. The metalloendopeptidase studies were performed on the basis of sequence homology with neutral endopeptidases and the fact that the positions of many of the cysteines are conserved. Lee determined the molecular basis of KEL1/KEL2 and, with that publication, the ability to predict fetal inheritance of KEL1 and hemolytic disease using amniotic fluid–derived DNA. The molecular basis for Js+/Js− was reported in that same year. The molecular basis for Kpα, Kpβ, and Kpγ followed in 1996. Table 1 summarizes the molecular features for the Kell blood group system antigens.

The lack of Kell expression has lead to a number of nucleotide changes responsible for the Kell-null phenotype. The nucleotide changes result in alternative splice sites, amino acid substitutions deleterious to expression, nucleotide insertions and deletions causing frameshifts, and termination codons. In addition, several nucleotide changes result in the reduced expression of Kell glycoprotein, termed Kmod phenotype. The amino acid change for KEL13 not only causes the loss of the high prevalence antigen but also reduces the expression of Kell glycoprotein.

**Kell and Kx Antigens**

The principle antigens K/k, Kpα/Kpβ, and Js+/Js− are invariably included in commercially available reagent RBC panels (Table 1). The K antigen is considered the most immunogenic among the minor blood group antigens, with the exception of the D antigen, which is nearly always matched in RBC transfusions. In fact, the K antigen has been given an immunogenicity index of 1.0 by Tormey and Stack, and is the antigen with which all other minor blood group antigens are compared for the purpose of ranking immunogenicity (save for the D antigen). The frequency of anti-K in pregnancy also attests to its immunogenicity, although a significant proportion of anti-K in pregnancy is the result of the transfusion of K+ RBCs to K− women prior to pregnancy, since K antigen matching is not mandatory. Approximately 15–20 percent of RBC alloimmunizations seen in pregnancy are caused by anti-K. The immunogenicity of K antigen may be HLA-related. The frequency of HLA-DRB1*11 and HLA-DRB1*13 are statistically higher in anti-K alloimmunized patients versus matched controls. This observation led Chiaroni et al. to conclude that the immune response to K is partially attributable to preferred association of HLA type for antigen presentation.

**Table 1. Molecular features, nucleotide polymorphisms, and antigens of the Kell blood group system**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Exon</th>
<th>rs#</th>
<th>Antigen(s)</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>577C&gt;T</td>
<td>6</td>
<td>8176058</td>
<td>KEL2/KEL1 (K/k)</td>
<td>Thr193Met</td>
</tr>
<tr>
<td>577T&gt;A</td>
<td>6</td>
<td>61729031</td>
<td>KEL1* (Kmod)</td>
<td>Thr193Ser</td>
</tr>
<tr>
<td>841C&gt;T</td>
<td>8</td>
<td>876059</td>
<td>KEL4/KEL3 (Kpα/Kpβ)</td>
<td>Arg281Trp</td>
</tr>
<tr>
<td>842G&gt;A</td>
<td>8</td>
<td>—</td>
<td>KEL21 (Kpα−)</td>
<td>Arg281Gln</td>
</tr>
<tr>
<td>1790T&gt;C</td>
<td>17</td>
<td>8176038</td>
<td>KEL7/KEL6 (Js+/Js−)</td>
<td>Leu597Pro</td>
</tr>
<tr>
<td>905T&gt;C</td>
<td>8</td>
<td>—</td>
<td>KEL11/KEL17</td>
<td>Val902Ala</td>
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<tr>
<td>539G&gt;C</td>
<td>6</td>
<td>61729039</td>
<td>KEL14/KEL24</td>
<td>Arg180Pro</td>
</tr>
<tr>
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<td>6</td>
<td>—</td>
<td>KEL14 (KEL−14)</td>
<td>Arg180His</td>
</tr>
<tr>
<td>538C&gt;T</td>
<td>6</td>
<td>—</td>
<td>KEL14/KEL24 (KEL−14,−24)</td>
<td>Arg180Cys</td>
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<tr>
<td>742C&gt;T</td>
<td>8</td>
<td>61728832</td>
<td>KEL25/KEL28 (VLAN+/VONG+)</td>
<td>Arg248Gln</td>
</tr>
<tr>
<td>743G&gt;A</td>
<td>8</td>
<td>61729040</td>
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</tr>
<tr>
<td>875G&gt;A</td>
<td>8</td>
<td>201698610</td>
<td>KEL31/KEL38 (KLYR+)</td>
<td>Arg292Gln</td>
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<tr>
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<td>13</td>
<td>—</td>
<td>KEL10 (Ut+)</td>
<td>Glu494Val</td>
</tr>
<tr>
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<td>15</td>
<td>—</td>
<td>KEL12</td>
<td>His548Arg</td>
</tr>
<tr>
<td>986T&gt;C</td>
<td>9</td>
<td>—</td>
<td>KEL13 (Kmod)</td>
<td>Leu929Pro</td>
</tr>
<tr>
<td>388C&gt;T</td>
<td>4</td>
<td>184131044</td>
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<td>Arg130Trp</td>
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<tr>
<td>389G&gt;A</td>
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<td>KEL18</td>
<td>Arg130Gln</td>
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<tr>
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<td>—</td>
<td>KEL19</td>
<td>Arg492Gln</td>
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<tr>
<td>965C&gt;T</td>
<td>9</td>
<td>—</td>
<td>KEL22</td>
<td>Ala322Val</td>
</tr>
<tr>
<td>1145A&gt;G</td>
<td>10</td>
<td>—</td>
<td>KEL23</td>
<td>Gln382Arg</td>
</tr>
<tr>
<td>1217G&gt;A</td>
<td>11</td>
<td>—</td>
<td>KEL26 (TOU)</td>
<td>Arg406Gln</td>
</tr>
<tr>
<td>745G&gt;A</td>
<td>8</td>
<td>61729042</td>
<td>KEL27 (RAZ)</td>
<td>Gln249Lys</td>
</tr>
<tr>
<td>1868G&gt;A</td>
<td>17</td>
<td>—</td>
<td>KEL29 (KALT)</td>
<td>ARg263Lys</td>
</tr>
<tr>
<td>913G&gt;A</td>
<td>8</td>
<td>—</td>
<td>KEL30 (KTM)</td>
<td>Asp305Asn</td>
</tr>
<tr>
<td>1271C&gt;T</td>
<td>11</td>
<td>—</td>
<td>KEL32 (KUCI)</td>
<td>Ala424Val</td>
</tr>
<tr>
<td>1283G&gt;T</td>
<td>11</td>
<td>—</td>
<td>KEL33 (KANT)</td>
<td>Arg426Leu</td>
</tr>
<tr>
<td>758A&gt;G</td>
<td>8</td>
<td>—</td>
<td>KEL34 (KASH)</td>
<td>Tyr253Cys</td>
</tr>
<tr>
<td>780G&gt;T</td>
<td>8</td>
<td>18</td>
<td>KEL35 (KELP)</td>
<td>Leu260Phe</td>
</tr>
<tr>
<td>1391T&gt;C</td>
<td>12</td>
<td>190890637</td>
<td>KEL36 (KETI)</td>
<td>Thr464Ile</td>
</tr>
<tr>
<td>877C&gt;T</td>
<td>8</td>
<td>—</td>
<td>KEL37 (KUL)</td>
<td>Arg293Trp</td>
</tr>
</tbody>
</table>

The prevalence of Js+/Js− antigens in people of African ancestry (20% and 80%, respectively) is unique to this racial population. The prevalence of Js(a+b−) is approximately 1 percent, and therefore anti-Js− alloimmunization is observed.
occasionally in transfusion recipients, including chronically transfused patients with sickle cell disease. These patients pose particular challenges when requiring Js(b−) blood. Transfusing institutions and blood centers must rely on continued surveillance of suitable donors and on the American Rare Donor Program. On the other hand, the prevalence of K antigen does not create challenges when providing compatible blood.

Nearly all Kell blood group system antigens are caused by single nucleotide polymorphisms leading to single amino acid substitutions. The K−35 phenotype is the result of two amino acid substitutions at positions 260 and 675.

**Kell and Kx Antibodies**

Kell blood group system antibodies are usually IgG, but can also be IgM. As a result, anti-K has been manufactured as IgM monoclonal antibodies with sufficient avidity to be used as phenotyping reagents. The antibodies can cause acute and delayed hemolytic transfusion reactions, and autoantibodies with Kell specificities have been reported. As stated previously, alloantibodies to this blood group system cause HDFN. It is likely that all Kell blood group system antibodies suppress erythropoiesis. Antibodies to the XK protein would react similarly using in vitro techniques. Antibodies to the XK protein are not found naturally occurring.

Many single nucleotide polymorphisms lead to the K₀ (i.e., the complete absence of Kell glycoprotein) phenotype, and thus it is theoretically possible for alloimmunization to occur. The antibody produced by K₀ transfusion recipients is anti-Ku (K5). The serum of alloimmunized K₀ persons represents antibodies to the Kell glycoprotein in much the same way as polyclonal anti-D or anti-U; it contains antibodies to multiple epitopes expressed on the Kell glycoprotein. Anti-Ku is exceedingly rare—there are only ~100 K₀ phenotypes described worldwide. Persons with marked reduction in the expression of Kell antigens, i.e., the Kmod phenotype, are at risk of forming antibodies to the epitope(s) expressed on wild-type Kell that they lack. Kmod variant is not mutually compatible, although the risk of alloimmunization of Kmod RBC transfusions to a Kmod recipient is lower because of the lower dose of antigen. Theoretically, K₀ transfusion recipients are not tolerant of Kmod transfusions, but the immunogenicity of Kmod RBCs is unknown.

When the XK protein is absent (McLeod phenotype), transfusion recipients are at risk of forming anti-Kx along with anti-Km (K20). It is generally reported that major chromosomal deletions that include XK and the gene responsible for chronic granulomatous disease (CGD) make anti-Kx+Km. The McLeod phenotype without CGD results in anti-Km alloimmunization. Exceptions, however, have been reported of anti-Kx without anti-Km in XK−CGD deletions, and anti-Kx+Km in McLeod neuroacanthocytosis syndrome without CGD.

**Hemolytic Disease of the Fetus and Newborn**

HDFN attributable to Kell blood group system antibodies has unique clinical features. The antibody titers do not correlate with disease severity nor are amniotic fluid bilirubin levels consistent with disease severity. The disease appears much more severe than titers indicate. A critical titer of 8 for intervention is recommended—however, middle cardiac vein Doppler echocardiography has changed the management of HDFN. Therefore, it is assumed that HDFN can occur earlier in gestation, when RBCs begin to form. For example, maternal anti-K in the fetal circulation can bind to the fetal K antigen expressed on early erythroid progenitor cells, which are thus removed by the fetal mononuclear phagocytic system. In vitro studies by Daniels and coworkers showed that peripheral blood mononuclear cells from cord blood (a source of CD34+ and early hematopoietic progenitor cells) grown in a semi-solid agar to enumerate erythroid cells (a source of hematopoietic progenitor cells) grown in CD34+ and early hematopoietic progenitor cells) grown in a semi-solid agar to enumerate erythroid colony-forming and burst-forming units. They showed that anti-K suppressed erythropoiesis in an antibody dose-dependent manner. Therefore, both phagocytosis of early erythroid progenitor cells and suppressed erythropoiesis contribute to disease severity that does not correlate with titers or evidence of RBC destruction. The suppressive effect of anti-K goes beyond gestation. Residual alloantibody in the newborn can continue to ongoing suppression of erythropoiesis. Suppressed erythropoiesis is supported by the lack of reticulocytosis that is normally observed in
newborns. Infants who are supported by allogeneic top-up or exchange transfusions may appear well a few days after delivery, but are at risk of late-onset anemia as the antibodies continue to cause RBC destruction (as they would with anti-D) along with suppression of erythropoiesis. The result is a risk of late-onset anemia within a couple of weeks as the transfused RBCs are sequestered and the infant’s marrow is unable to produce sufficient RBCs.

Conclusions

Antibodies to the Kell and Kx blood group systems provide serological challenges in immunohematology. The K antigen is among the most immunogenic antigen, and the expression of a covalent-linked complex with the XK protein and the non-covalent interactions with glycoporphin C underscore the importance of Kell and XK in membrane integrity and cellular function. The transfusion of K+ RBCs to women of childbearing potential remains controversial given the significant incidence of anti-K HDFN. Kell blood group antibodies do more than bind to their cognate antigen, which makes the Kell-XX blood group systems clinically relevant and scientifically challenging. The antibody-mediated intracellular signaling events responsible for suppressed erythropoiesis remain to be characterized. The prevention of anti-K HDFN by passive immunization in a similar way as anti-D is unlikely practical. Identification of immunodominant epitopes and a better understanding of T-cell tolerance induction may lead to a new era of immunotherapy for the prevention of HDFN.

References


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**For information** concerning the National Reference Laboratory for Blood Group Serology, including the American Rare Donor Program, contact Sandra Nance, by phone at (215) 451-4362, by fax at (215) 451-2538, or by e-mail at Sandra.Nance@redcross.org

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A simple approach to screen rare donors in Brazil


Providing blood units for patients with an antibody to a high-prevalence antigen or with multiple common antibodies is a constant challenge to the blood banks. Finding a compatible donor requires extensive screening, which incurs a large amount of investment. In this article, we share our experience of organizing a rare donor inventory with limited resources, we include the strategy used for finding rare donors, and we share the difficulties found during the implementation of the approach and the results obtained. ImmunoHematology 2015;31:20–23.

Key Words: rare donor, high prevalence antigen, phenotyping, genotyping

Rare donor identification began to get attention in the 1960s when the American Red Cross and American Association of Blood Banks began compiling a rare donor database. Both institutions realized that ensuring blood supply for patients with antibodies to high prevalence antigens or with multiple common antibodies was a big challenge. Thereafter, rare donor programs were created in some countries and, in 1998, a national American program was formed (the American Rare Donor Program) to manage and supply rare blood, which currently provides approximately 1800 units per year in the United States. Concomitantly, in 1965, the International Society of Blood Transfusion (ISBT) established the International Panel of Rare Blood Donors gathered by the International Blood Group Reference Laboratory in Bristol, U.K. Consequently, since 1984, when the ISBT Working Party on Rare Donors was formed, the provision of an effective exchange program has been discussed worldwide. In 2004, the ISBT Working Party on Rare Donors reported that the International Panel of Rare Blood Donors held 4000 rare donors registered from 24 countries. Other national rare donor programs were also established—for example, the French program conducted by the French National Reference Laboratory for Blood Groups and the Israeli program headquartered by the Magen David Adom National Blood Services. Unfortunately, however, there are still some countries without a national program.

Currently, we are starting discussions in Brazil to establish a national panel of rare donors. Implementation steps include the screening of large numbers of donors, the education of blood center personnel, the development of a computer system, recruitment of donors, and maintenance of the rare units. Since 2011, Colsan, a blood bank in São Paulo, has implemented a process to screen for rare blood types, trying to set a reliable, useful, and efficient strategy to find rare blood donors. Taking into account the limitations we have related to financial support and the use of different methods, we developed an approach to converge resources and strengthen the efforts that aim to find the best combination of tests to successfully screen large numbers of donors, reduce costs, and increase the chances of encountering rare phenotypes and genotypes.

Different serology methods, as well as DNA typing, are being used to screen rare donors. As serology has some limitations attributable to the scarcity of commercial and potent antisera to type rare antigens, molecular protocols for large-scale genotyping have emerged as a tool to overcome these restrictions.

Because the definition of what can be considered as rare blood differs between countries, the first step is to establish which antigens should be screened. It is very difficult to obtain a negative example of a high prevalence antigen when it only occurs in less than 1 in 1000 individuals. Then, finding donors whose red cells are negative for multiple common antigens can also be a challenge. Therefore, a donor whose red cells lack multiple common antigens is also considered a rare donor.

In a multi-ethnic population such as we find in Brazil, the prevalence of the different blood group antigens varies significantly, and it is necessary to have a range of rare blood types available. According to the prevalence of rare blood groups in the Brazilian population, we established an approach to search for S–s–, k–, Rhnull, r’r’, r”r”, Di(b–), Vel–, Wr(b–), Co(a–), Yt(a–), Js(b–), Kp(b–), Jo(a–), and Hy– donors. Additionally, we searched for R2R2 donors with a combination of negative clinical antigens. This approach includes serologic and molecular screening in repeated donors, in D– donors, and in donors with RhCE variants. We herein share our approach, the difficulties we experienced during implementation, and the results obtained after approximately 3 years of the program.
Screening of rare donors in Brazil

Materials and Methods

Rare Donor Screening Strategy

Screening for Rare Phenotypes in Repeat Donors

Repeat donors, including group O donors with at least two prior donations, are selected for screening by serology as well as by DNA typing.

1. Serologic Screening: We first perform a cross-match between the donor’s red blood cells (RBCs) and plasmas containing antibodies, such as anti-K, anti-Di, anti-s, and anti-E by gel test in an automated instrument (Wadianna, Grifols, Barcelona, Spain). To optimize the searching and to reduce costs, we mix plasmas with anti-K and anti-Di and perform the tests on the pool. If the test is positive, K and Di are individually typed. All the positive results are confirmed with commercial sera.

The aim of this serologic strategy is to find donors with k–, S–s–, and Di(b–) phenotypes and R2R2 donors with an interesting combination of antigen-negative results. Figure 1 shows the four scenarios obtained with this strategy and the further steps performed with serologic and molecular protocols.

2. Molecular Screening: Taking into account the lack of commercial antisera to type the majority of high-prevalence antigens and the limitations of conventional polymerase chain reaction (PCR), we developed a SNaPshot protocol to identify rare donors, as previously described by Latini et al.12

Briefly, SNaPshot is a mini-sequencing assay that permits analysis of several single nucleotide polymorphisms (SNPs) from numerous donors in a short period of time. The protocol we developed identifies alleles of the Diego, Colton, Cartwright, KEL, Dombrock, and VEL blood group systems. All samples previously typed by serology, such as Di(a+) and/or K+, R,r, R,R, R,R, or R,r are selected for this molecular analysis.

Screening of Rare Phenotypes in D-negative Donors

All D– donors are typed for C, E, and K by hemagglutination in microplates using an automated instrument (Neo, Immucor, Birkenfeld, Germany). This approach identifies rare Rh phenotypes such as r′r′, r′r′, and Rhnull and also the k– phenotype in the samples typed as K+.

Molecular Screening for RhCE Variants

Molecular screening for RhCE variants is performed in repeat donors of group O African descendants and in donors with altered expression of C, c, E, and e. We also perform molecular searching for CE variants in donors with altered expression of D because RhD variants have been shown to be associated with RhCE variants. In these cases, we select samples with weak D expression for immediate spin with two monoclonal anti-D reagents (IgM RUM-1 and blend D175+D415) on an automated instrument (Neo, Immucor).

Because the most important RhCE variants are those silencing the high prevalence antigens, hr and hr, our strategy includes screening the following alleles: RHCE*ceAR, RHCE*ceEK, RHCE*ceAG, RHCE*ceMO, RHCE*ceBI, RHCE*ceSM, RHCE*ceCF, and RHCE*ce (RHCE*ce733G) that cause the hr– and/or hr– phenotypes. To identify these variants, we first use a PCR–restriction fragment–length polymorphism (PCR-RFLP) for detection of the SNPs 712A>G, 254C>G, 667G>T and 733C>G. Depending on the results obtained, we then use a strategy to identify the variant, as shown in Figure 2.

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Fig. 1. This figure shows the four scenarios obtained by serologic screening. (A) All K+ RBCs are typed for k to identify k– phenotypes. (B) s– and K– RBCs are typed for S and all S–s– samples are genotyped for GYPB to identify U– and U+phenotypes. (C) All Di(a+) and K– samples are typed for RhCE. R,r, R,R, R,r, and R,R,R RBCs are molecular typed by SNaPshot. (D) All E+ K– RBCs are typed for e, and all R,R,R samples are typed for other clinically relevant antigens.
We also perform molecular tests to identify RhD variants in all samples with RhCE variants, based on previously published protocols.

**Results**

Identified rare donors are included in our rare donor registry. Table 1 presents the results obtained in our 3 years of experience of building our rare donor inventory. To all donors included in this inventory, we send a folder with a simple explanation of their rare blood type and instructions regarding their next donation. We also ask that they keep their contact information updated.

Additionally, we also phenotype and genotype all the clinical relevant antigens and freeze RBC aliquots in liquid nitrogen. Phenotype information on rare donors is submitted to the database housed in our blood center. The database allows the identification of the donors and facilitates searching for a requested phenotype. The steps of the process are schematically described in Figure 2.

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**Fig. 2.** Flowchart used to identify hr$^S$- and hr$^B$- donors. The investigation starts with the use of polymerase chain reaction–restriction fragment–length polymorphism to detect the single nucleotide polymorphisms 712A>G, 254C>G, 667G>T, and 733C>G. According to the results, we perform the analyses indicated in the flowchart to identify the RHCE variants.

The inventory of donors with RhCE variants in the homozygous state or in the heterozygous state with Ce or cE haplotypes allows for RH molecular matching to patients with sickle cell disease, preventing Rh alloimmunization and delayed hemolytic transfusion reactions. Six of our 11 donors with RhCE variants were related to RhD variants; all hr$^{-}$ linked to weak D type 4.2.2 and all hr$^{-}$ linked to weak D type 4.0.

**Discussion**

The strategy presented herein has allowed us to search for rare donors and to create a rare donor inventory at our institution. After the complete implementation of the molecular laboratory, the investment is approximately $2.00 per sample for serologic screening and $13.00 per sample for SNaPshot screening for RHCE variants. The search for D- donors is part of our laboratory routine; the additional k typing is the equivalent of $0.70 per sample. The development of the SNaPshot method has helped increase throughput and reduce costs. Therefore, this strategy to find rare donors, based on a focused search, is cost-effective.

**Table 1. Rare donor inventory**

<table>
<thead>
<tr>
<th>Screening</th>
<th>Total number of donors screened</th>
<th>Phenotypes and predicted phenotypes from genotypes</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serologic screening</td>
<td>4500</td>
<td>U$^{+}$-</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U$^{-}$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>k$^{-}$</td>
<td>5</td>
</tr>
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<td>Molecular screening</td>
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<td>Yt(a-b+)</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Co(a-b+)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kp(a+b-)</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
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<td>r$^r$</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>k$^{-}$</td>
<td>2</td>
</tr>
<tr>
<td>RhCE variants</td>
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<td>hr$^S$-</td>
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<tr>
<td></td>
<td></td>
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<td>3</td>
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</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>38</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^{*}$U$^{-}$ and U$^{+}$ were determined by polymerase chain reaction–allele specific and restriction fragment–length polymorphism.14
as compared with other strategies using microarrays and may help other blood centers to build their rare donor inventory.

The benefit of this investment is documented when an alloimmunized patient with a rare phenotype requires a rare unit of blood and is successfully transfused with a compatible unit. In a period of 3 years, we received 18 requests for rare units, and 8 of these were fulfilled using our rare donor inventory. Applying this approach, we intend to meet the needs of the patients with a rare phenotype in our institution and help other blood centers. Nevertheless, we still have some limitations to ensure that blood is made available to specific patients. Although we observed a great collaboration of the donors when recruited, some of these may be unable to donate when required. Freezing blood would be a good option, but this procedure is currently a challenge in Brazil, since the blood bag and the glycerol solution used for freezing are not licensed by our regulatory agency.

Limitations of the Approach

Our strategy for searching for rare donors does not include screening for D− −, Ge−2, and K0 phenotypes, which have already been found in the Brazilian population, but our future screening activities will focus on the identification of such donors. Another unfavorable point of this strategy is the long duration of the whole process. In general, when we find a rare donor, the RBC unit has already been transfused and we need to wait until the next donation to freeze RBC aliquots and to perform complete phenotyping.

Conclusion

It is widely accepted that a rare donor program is important to ensure a safe transfusion. In our experience, we realized that the implementation of a rare donor program requires efforts from both technical and administrative areas. On the other hand, we show herein that a simple and focused strategy can help fulfill the requests for units of rare blood for patients with antibodies against high-prevalence antigens or patients with multiple antibodies. Exchanges of experience and collaboration between different centers, through a national program, will allow us to ensure that blood will be made available to patients with rare blood phenotypes.

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Proposed criterion for distinguishing ABO mosaics from ABO chimeras using flow cytometric analysis


Differentiation of ABO mosaics from chimeras is performed using flow cytometry (FCM) analysis. Although mosaics and chimeras have been distinguished by presence or absence of clear resolution using FCM analysis, the lack of quantitative metrics and definitive criteria for this differentiation has made some cases difficult to differentiate. In this study, therefore, we attempted to establish a definitive and quantitative criterion for this differentiation. When FCM histogram gates for group “A” or “B” antigen-negative and -positive red blood cells (RBCs) were set such that group O RBCs were classified as 99 percent negative and group A or B RBCs as 99 percent positive, the percentages of RBCs in the middle region of six chimeras and 23 mosaics (12 A mosaics and 11 B mosaics) were 0.1–0.6 percent and 7.0–19.0 percent, respectively. This result suggested that ABO mosaics and chimeras can be unambiguously differentiated when the cutoff point of the intermediate region is set to 1 percent. *Immunohematology* 2015;31:24–28.

Key Words: ABO, mosaic, chimera, flow cytometry

The differentiation of ABO subgroups is usually determined serologically on the basis of the following: (1) agglutination tests with human polyclonal anti-A, anti-B, and anti-A\textsubscript{1} lectin; (2) identification of anti-A or anti-B in serum; and (3) ABO transferase activity. ABO mosaics and chimeras are usually differentiated from other subgroups using these tests.\textsuperscript{1–5} Consequently, mosaics and chimeras are differentiated by coil planet centrifugation (CPC)\textsuperscript{6} or flow cytometry (FCM) methods.\textsuperscript{7–9} Genotyping is useful as a supplementary tool to confirm the final differentiation of these ABO subgroups.\textsuperscript{1,2} Although ABO mosaics and chimera can be easily differentiated from other subgroups, it is difficult to differentiate them from each other using standard serologic methods.\textsuperscript{3,4} and only CPC and FCM methods are available for this purpose. The coil planet centrifuge is no longer manufactured and is thus difficult to obtain. Furthermore, distinguishing ABO mosaics from chimeras using the FCM method is based simply on the presence or absence of clear resolution of positive and negative peaks. This lack of quantitative metrics has made some cases difficult to differentiate because there are no definitive criteria for this determination. In addition, a quantitative FCM method appears promising for diagnosing early relapse or rejection after hematopoietic progenitor cell transplantation using ABO-incompatible cells.\textsuperscript{10} Therefore, in the present study, we developed a quantitative criterion for FCM-based differentiation of ABO mosaics from chimeras by setting a gate between the “A” or “B” antigen-negative and -positive red blood cell (RBC) populations in the FCM histogram and then determining the proportion of cells in the middle gate. Quantitative tests such as this FCM system should be applied to other qualitative serologic tests.

Materials and Methods

Blood Samples

Twelve A mosaic samples, 11 B mosaic samples, and 6 ABO chimeras were obtained from volunteer blood donors in the Kinki area of Japan. These samples were previously identified as mosaics or chimeras based on the following: (1) agglutination tests with monoclonal anti-A, anti-B, anti-A\textsubscript{1} lectin, and anti-H lectin; (2) identification of anti-A or anti-B in serum; and (3) ABO transferase activity in plasma. Consequently, the possibility of other subgroups including A\textsubscript{1}, A\textsubscript{2}, A\textsubscript{3}, and A\textsubscript{x} were ruled out.\textsuperscript{1,2} Similarly, B, B\textsubscript{3}, and B\textsubscript{x} were ruled out. In addition, their phenotypes as mosaics or chimeras were confirmed by the CPC method, which can detect ABO chimerism as low as 0.1 percent.\textsuperscript{6} A flow chart outlining the classification of ABO mosaics, chimeras, and other subgroups is presented in Figure 1. We did not perform a family study to determine if any of the donors were related. It is likely that most of the donors were unrelated, however, because they all had different family names and lived in widely scattered regions.

ABO Genotyping

Genomic DNA was prepared from 200 μL ethylenediaminetetraacetic acid (EDTA) whole blood using a collection system (Quick whole blood kit, KURABO Industries, Osaka, Japan). The polymerase chain reaction–reverse sequence specific oligonucleotide (PCR-rSSO) method was performed.
to detect single nucleotide polymorphisms (SNPs) on ABO alleles. Amplicons labeled with fluorescence were separately amplified by PCR from ABO exons 6 and 7 using an automated system (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA,) and the group-specific reagent (Genosearch ABO reagent, Medical & Biological Laboratories, Nagano, Japan). We detected SNPs on ABO alleles using a florescent system (Luminex System 200, Hitachi Solutions, Tokyo, Japan). In addition, we confirmed the DNA sequence of exon 7 on both A and B alleles by direct PCR sequencing. We first amplified exon 7 on both alleles using the primers GA22 and GA23 (Table 1). Each PCR contained 2.5 μL genomic DNA, 1 μL GA22, 1 μL GA23, 35.25 μL sterile water (distilled deionized sterile water, Nippon Gene Co. Ltd., Toyama, Japan), 5 μL of a dNTP mix at a concentration of 2 mmol/L of each dNTP (GeneAmp Applied Biosystems), 5 μL of a 10× buffer (PCR buffer, Applied Biosystems), and 0.25 μL of a Taq amplifier in a concentration of 250 U at 5 U/μL (AmpliTaq Gold, Applied Biosystems). PCR amplification was performed with initial denaturation at 96°C for 2 minutes, followed by 35 cycles at 96°C for 1 minute, 62°C for 1 minute, and 72°C for 4 minutes. For DNA sequencing analysis, the PCR fragments, which were purified, (QIAquick PCR purification kit, QIAGEN, Hilden, Germany), were fluorescently labeled with primer GA62 or GA03 (Table 1) using a cycle sequencing kit (BigDye Terminator v1.1, Applied Biosystems). The DNA sequences

**Table 1.** Primers used for amplification and direct sequencing of ABO gene fragments

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Primer sequence (5′-3′)</th>
<th>Direction</th>
<th>Primer location</th>
<th>Amplified region</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA22</td>
<td>CTAAAACCAAGGGCGGGAGG</td>
<td>reverse</td>
<td>3′-UTR</td>
<td></td>
</tr>
<tr>
<td>GA23</td>
<td>GAAAGGATGTCTCTGTTGTTA</td>
<td>forward</td>
<td>exon 6</td>
<td>exon 6-7</td>
</tr>
<tr>
<td>GA62</td>
<td>TCAAGGACAGCGAGGGAAACG</td>
<td>forward</td>
<td>intron 6</td>
<td>exon 7</td>
</tr>
<tr>
<td>GA03</td>
<td>TGCTGGAGGTGCACGCTAC</td>
<td>forward</td>
<td>exon 7</td>
<td>exon 7</td>
</tr>
</tbody>
</table>
were measured using a genetic analyzer (ABI PRISM 3130×L, Applied Biosystems). The loci c.261 and c.297 in exon 6 and c.467, c.526, c.547, c.646, c.657, c.681, c.703, c.771, c.784, c.796, c.802, c.803, c.829, c.871, c.930, c.1006, c.1054, and c.1060 in exon 7 were analyzed, and the results indicated that all of the samples that we could analyze were either A102 or B101 (Table 2).

Preparation of Cell Samples for FCM Analysis

A 10-µL RBC suspension (whole blood collected in K$_2$EDTA) was washed three times in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) and fixed by mixing with 50 μL of 0.25% glutaraldehyde (Nacalai Tesque, Inc., Kyoto, Japan) for 15 minutes at 5°C. The RBC suspension was subsequently washed four times using PBS containing 0.2% fetal bovine serum (FBS) (EIDIA Co. Ltd., Tokyo, Japan) and adjusted to a 0.25% cell suspension. Thereafter, 25 μL of this suspension was mixed with 50 μL monoclonal anti-A or anti-B (Bioclone Anti-A and Bioclone Anti-B, Ortho-Clinical Diagnostics, Tokyo, Japan) for 30 minutes at 5°C. After washing twice with PBS containing 0.2% FBS (PBS/FBS), a tagged antibody (goat anti-mouse IgG-FITC, BD Biosciences, San Jose, CA, USA) was added for 30 minutes at 5°C. After a final wash with PBS, the cells’ fluorescence was measured by FCM (FACSCalibur, BD Biosciences).

### Table 2. Summarized results of nucleotide substitutions in ABO exons 6 and 7

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Position</th>
<th>Exon 6</th>
<th>Exon 7</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>261</td>
<td>297</td>
<td>467</td>
</tr>
<tr>
<td>A mosaic 1</td>
<td></td>
<td>G</td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td>A mosaic 2</td>
<td></td>
<td>—</td>
<td>—</td>
<td>T</td>
</tr>
<tr>
<td>A mosaic 3</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A mosaic 4</td>
<td></td>
<td>—</td>
<td>—</td>
<td>T</td>
</tr>
<tr>
<td>A mosaic 5</td>
<td></td>
<td>—</td>
<td>—</td>
<td>T</td>
</tr>
<tr>
<td>A mosaic 6</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A mosaic 7</td>
<td></td>
<td>—</td>
<td>—</td>
<td>T</td>
</tr>
<tr>
<td>A mosaic 8</td>
<td></td>
<td>—</td>
<td>—</td>
<td>T</td>
</tr>
<tr>
<td>A mosaic 9</td>
<td></td>
<td>—</td>
<td>—</td>
<td>T</td>
</tr>
<tr>
<td>A mosaic 10</td>
<td></td>
<td>—</td>
<td>—</td>
<td>T</td>
</tr>
<tr>
<td>A mosaic 11</td>
<td></td>
<td>—</td>
<td>—</td>
<td>T</td>
</tr>
<tr>
<td>A mosaic 12</td>
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<td>—</td>
<td>T</td>
</tr>
<tr>
<td>B mosaic 1</td>
<td></td>
<td>—</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>B mosaic 2</td>
<td></td>
<td>—</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>B mosaic 3</td>
<td></td>
<td>—</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>B mosaic 4</td>
<td></td>
<td>—</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>B mosaic 5</td>
<td></td>
<td>—</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>B mosaic 6</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B mosaic 7</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B mosaic 8</td>
<td></td>
<td>—</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>B mosaic 9</td>
<td></td>
<td>—</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>B mosaic 10</td>
<td></td>
<td>—</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>B mosaic 11</td>
<td></td>
<td>—</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>Chimera 1</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chimera 2</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chimera 3</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Chimera 4</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chimera 5</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chimera 6</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*GenBank accession no. AF134412. NT = not tested; ND = not determined.

Setting the Gate for FCM

Using control RBCs (O and A$_1$ or B groups), the G1 gate was set so that group O RBCs were 99 percent negative for A or B antigens, and the G3 gate was set so that group A$_1$ or B RBCs were 99 percent positive for A or B antigens. When these gates were applied, only 0.1–0.3 percent of group O control RBCs showed nonspecific binding to anti-A or anti-B and only 0.1–0.4 percent of group A$_1$ or B RBCs were false negative. The G2 gate was set as the region between G1 and G3 (Fig. 2). The proportion of cell population detected in the G2 gate was calculated as follows: \[ \frac{G2}{G1 + G2 + G3} \times 100 \].

Calculation of the Heterogeneity of Chimera Samples from a Standard Calibration Curve

The following artificial mixtures of A$_1$ or B and O RBCs were prepared [(A$_1$ or B) / (A$_1$ or B + O) × 100] and analyzed using FCM: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 percent. A standard calibration curve of (G2 + G3) / (G1 + G2 + G3) was generated and used to estimate the ratios of cells in the chimera samples.

Results

The percentages of 23 ABO mosaics and 6 ABO chimeras detected in the G2 gate are presented in Tables 3 and 4. The mean percentages of mosaics and chimeras in the G2 gate were 13.1 percent (range: 7.0–19.0%) and 0.2 percent (range: 0.1–0.6%), respectively.
Distinguishing ABO mosaics from chimeras

Although the number of samples was small, the G2 values of mosaics were significantly higher than the values of chimeras ($p < 0.001$: Mann-Whitney $U$ test). When the cutoff point was set to 1 percent to differentiate mosaics from chimeras, all analyzed cases were differentiated accurately and unambiguously.

We then attempted to estimate the proportion of the two different types of RBCs in the chimera samples using a standard linear curve that defines the relationship between the mixing ratio of artificial chimera samples and their values in the G3 gate. We obtained a linear curve, with the known concentrations on the $y$-axis and the measured variable on the $x$-axis (data not shown). The estimated ratios were almost consistent with the G1 and G3 gate values (Table 4).

**Discussion**

In the present study, we successfully established a clear and simple criterion for FCM analysis to differentiate ABO mosaics from ABO chimeras by setting the FCM histogram gates (G1 and G3) for group “A” and/or “B” antigen-negative and -positive RBCs. We set these gates such that 99 percent negative RBCs and 99 percent positive RBCs were included in gates G1 and G3, respectively. The value of 99 percent set the cutoff point to 1 percent to differentiate mosaics from chimeras. This value allowed us to unambiguously differentiate both types of RBCs. Furthermore, we attempted to determine heterogeneity based on data from artificial chimera samples with a known number of RBCs of each blood group.

After having established the proposed criteria, we performed serologic ABO blood group testing on 777,617
donors as a routine test from December 2013 to October 2014. Among the samples from all donors, nine samples were suspected to be mosaic or chimera based on serologic analyses; subsequent CPC analyses revealed that seven of these samples were mosaics and the remaining two samples were chimeras. When a validation test was performed using these nine samples, our proposed FCM criterion clearly discriminated between these mosaics and chimeras.

Although FCM analysis is widely used to distinguish ABO mosaics from ABO chimeras and hence our qualitative FCM system may not have a powerful impact on the differentiation of ABO mosaics and chimeras, we hope that it contributes to the introduction of more quantitative tests in the field of serologic blood typing.

Our FCM system has a drawback. Because it is necessary to set the G3 gate wide enough for a substantial number of mosaic RBCs to be counted in the G3 population, chimera RBCs in G2 should exhibit relatively high expression of A or B antigens. Therefore, A2 chimeras (e.g., A2 + O), the RBCs of which express only a limited amount of A or B antigens, cannot be distinguished from “A” mosaics. Although A2 is a rare blood group in Asian populations and is not a major problem in Asia, the possibility of finding an A2 blood type should be carefully considered when analyzing RBCs from people of European or African descent, because A2 is found in 8–10 percent of these populations.13

### Table 4. ABO chimera samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Constituent</th>
<th>Positive antigen</th>
<th>% of gate</th>
<th>Estimated chimera ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive antigen</td>
<td>G1</td>
<td>G2</td>
</tr>
<tr>
<td>Chimera 1</td>
<td>B + AB</td>
<td>A</td>
<td>82.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Chimera 2</td>
<td>O + B</td>
<td>B</td>
<td>70.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Chimera 3</td>
<td>A + AB</td>
<td>B</td>
<td>56.1</td>
<td>0.6</td>
</tr>
<tr>
<td>Chimera 4</td>
<td>A + AB</td>
<td>B</td>
<td>95</td>
<td>0.1</td>
</tr>
<tr>
<td>Chimera 5</td>
<td>O + B</td>
<td>B</td>
<td>91.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Chimera 6</td>
<td>O + A</td>
<td>A</td>
<td>29.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Mean</td>
<td>—</td>
<td>—</td>
<td>71.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Constituent = red blood cell ABO group.

### References

The Kidd blood group system has been recognized as clinically important in red blood cell (RBC) serology since its identification in 1951. Forty years later, the JK glycoprotein was determined to be a product of SCL14A1 and was identical to the urea transport protein UT-B produced by HUT11A. The functional role of the protein as a urea transporter in RBCs and kidney has been well documented. The polymorphism responsible for the antithetical antigens Jk\(^a\) and Jk\(^b\) was identified in 1994 as c.838G>A (p. Asp280Asn). Recent discoveries have expanded the system to include 23 variant alleles recognized by the International Society of Blood Transfusion that silence the protein expression and 7 variant alleles presumably producing weak or partial JK antigens. Null phenotypes have been identified in individuals of several populations including those of African, Indian, and Chinese decent, in addition to the well-documented findings in the Polynesian and Finnish populations. This review will examine the historical information about the antigens and antibodies of the JK system as well as catalog the variations of the JK gene.

**Key Words:** Kidd, blood group system, JK, SLC14A1, RBC antigens

The Kidd blood group system (ISBT009) was the ninth blood group system identified. It was described in 1951 when an unknown antibody detected in the plasma of the propositus, Mrs. Kidd, caused hemolytic disease of the fetus and newborn (HDFN) in her infant son.\(^1\) His initials, JK, became the symbol that now represents this blood group system. The antithetical antigen, Jk\(^b\), was identified in 1953 when investigation of a transfusion reaction identified a new antibody.\(^2\) Discovery of the Jk(a–b–) phenotype followed in 1959.\(^3\) It is of interesting historical note that Dr. Mary Crawford, pediatrician and blood group serologist, discovered inheritance patterns in her family that suggested a silent JK allele while investigating her own Lu(a–b–) phenotype.\(^4\) Basic characteristics of the antigens and antibodies of this blood group system have been well established; however, much information has been obtained from molecular analysis of the JK protein.

### Antigens and Inheritance

The JK blood group system consists of three antigens: Jk\(^a\), Jk\(^b\), and Jk3. Jk\(^a\) and Jk\(^b\) are inherited as codominant autosomal characteristics and are commonly found on red blood cells (RBCs) of most population groups.\(^5\) The three resulting phenotypes, however, exhibit varying frequencies among different population groups (Table 1). Jk3 has a very high prevalence and is found on all RBCs carrying either Jk\(^a\) or Jk\(^b\). The null phenotype, Jk(a–b–), identifies those RBCs that are Jk:–3. This phenotype occurs rarely, but has a greater frequency in individuals of Polynesian or Finnish descent upon inheritance of two silent alleles. The phenotype frequency in Polynesian populations is 0.1–1.4 percent, with frequency variations seen in different Polynesian tribes.\(^6\) In Finns, the Jk(a–b–) phenotype frequency is approximately 0.03 percent.\(^7\) A second mechanism for the Jk(a–b–) phenotype is the inheritance of a dominant suppressor gene that leads to the apparent lack of JK antigens on the cell surface.\(^8\) In reality, small amounts of Jk\(^a\), Jk\(^b\), and Jk3 can be demonstrated when sensitive methods such as adsorption/elution are used. The suppressor null phenotype is designated In(Jk) and has been identified in two families of Japanese ancestry.

### Table 1. Phenotypes in the Kidd blood group system

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>White</th>
<th>Black</th>
<th>Asian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jk(a+b–), Jk:3</td>
<td>26</td>
<td>52</td>
<td>23</td>
</tr>
<tr>
<td>Jk(a+b+), Jk:3</td>
<td>50</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Jk(a–b+), Jk:3</td>
<td>24</td>
<td>8</td>
<td>27</td>
</tr>
<tr>
<td>Jk(a–b–), Jk:–3</td>
<td>Rare</td>
<td>Rare</td>
<td>Rare except Polynesians (0.9)</td>
</tr>
</tbody>
</table>

Modified from Fung et al.\(^5\)

The JK antigens have been detected on fetal RBCs as early as 7–11 weeks’ gestation and are fully developed at birth.\(^9\) The Kidd antigen density has been shown to be approximately 14,000 copies per cell as determined on Jk(a+b–) RBCs.\(^10\) The antigens are not destroyed by treatment with proteolytic enzymes or sulfhydryl compounds. Variations in the expression of both Jk\(^a\) and Jk\(^b\) have been described and will be discussed in further detail subsequently.

### Genetics and Antigen Biochemistry

The gene encoding the JK glycoprotein, SLC14A1, is a member of the solute carrier family of genes. It is located on the long arm of chromosome 18 (18q11-q12) and is organized...
into 11 exons. Exons 1–3 are not translated; exons 4–11 code for the mature JK glycoprotein.\textsuperscript{11,12}

The human erythrocyte urea transport gene, \textit{HUT11}, was identified in 1994\textsuperscript{13} and later was assigned to the same position on chromosome 18 as the JK blood group locus.\textsuperscript{14} The encoded protein was demonstrated on all RBCs except Jk(a–b–) cells, a finding that linked the urea transport protein to the JK locus. Subsequently, it was identified that JK renamed \textit{SLC14A1}, encoded for a related but slightly different polypeptide \textit{HUT11A}.\textsuperscript{15} These discoveries resulted in an understanding of the functional aspects of the Kidd antigens.

The mature glycoprotein resulting from JK contains 389 amino acids with a molecular weight of 45 kDa. Hydrophobicity studies predict that the protein is organized into five extracellular loops with 10 membrane-spanning regions\textsuperscript{13,16} (Fig. 1). The third extracellular loop is significantly larger than the other four and contains the protein’s only external glycosylation site at amino acid position 211. This N-linked sugar chain has been shown to also carry ABO antigens.\textsuperscript{16} The N-terminal and C-terminal regions of the protein are both intracellular.

The Jk\textsuperscript{a}/Jk\textsuperscript{b} polymorphism is defined by three single nucleotide polymorphisms (SNPs). An SNP is a variation at a single nucleotide position in the DNA. The antigen-defining SNP occurs in exon 9 at nucleotide position 838.\textsuperscript{37} At this position, the JK*01(JK*A) allele contains nt838G and encodes for aspartic acid at position 280; JK*02 (JK*B) contains nt838A, encoding for asparagine at this position. The p.Asp280Arg substitution is found in the fourth extracellular loop of the JK glycoprotein. The other two SNPs, which cause no change in the amino acid sequence, are at position 588 of exon 7 and position –46 in intron 9. The JK*01 allele is characterized by nt588A and IVS9–46a. The JK*02 allele contains nt588g, IVS9–46g. These changes are summarized in Figure 2. The molecular basis of \textit{In(Jk)} and Jk3 remain unknown, although the \textit{In(Jk)} suppressor is not linked to the JK locus.\textsuperscript{8}

![Fig. 1. Depiction of JK glycoprotein in RBC membrane. Features include ten membrane spanning regions, five extracellular loops, glycosylation site on third extracellular loop, epitopes of Jk\textsuperscript{a} and Jk\textsuperscript{b} in the fourth extracellular loop, and intracellular N-terminus and C-terminus.](image1)

![Fig. 2. Arrangement of JK showing the JK*01/JK*02 polymorphisms. Open rectangles are non-coding exons. Shaded rectangles are coding exons. Arrowhead indicates the missense substitution in exon 9 defining the JK polymorphism. The two remaining changes are silent.](image2)

Sequencing studies have identified a number of molecular variations that result in the Jk(a–b–) phenotype. These changes occur on both the JK*01 and JK*02 alleles and affect the normal expression through exon deletions, intron changes that cause splice site mutations, and nucleotide substitutions. Most variants are, in fact, SNPs in the gene’s coding region that lead to missense mutations or premature stop codons. Two genetic variants are responsible for the majority of Jk(a–b–) phenotypes. The more frequent variant is found in the Polynesian population and has been assigned the allele designation \textit{JK*02N.01}. At position –1 of intron 5 of the \textit{JK*02} allele, a G>A nucleotide substitution (c.342-1G>A) causes a splice site mutation that leads to the skipping of exon 6.\textsuperscript{11} The resulting truncated protein is not transported to the RBC membrane. In addition to Polynesians, this genetic variant has been reported in Vietnamese,\textsuperscript{18} Chinese,\textsuperscript{19,20} Thai, Filipino, and Indonesian\textsuperscript{21} individuals—suggesting a historical relationship between these groups. One Jk(a–b–) Asian Indian individual possesses the same 342-1G>A SNP, although it was found on the background of a JK*01 allele (nt838G).\textsuperscript{22}

The second most frequent Jk(a–b–) genetic variant occurs in the Finnish population. A nucleotide substitution c.871T>C on a JK*02 background results in a serine to proline change at amino acid position 21.\textsuperscript{7} Other null variants have been
identified in single probands or in a small number of families. The Jk(a−b−) genetic variants are summarized in Table 2 based on the International Society of Blood Transfusion (ISBT)-assigned allele name. Of interest are the variants identified in African American and Asian Indian individuals, since these groups have not historically been associated with the Jknull phenotype. A Jk*01 allele with c.561C>A change was described by Horn et al. that illustrates the variation in frequency in different population groups of African descent. The original propositus was an African American individual, but no additional examples of this variant were identified in testing of an additional 1000 African American blood donors. In contrast, the c.561C>A SNP was identified in 7 of 1174 Brazilian blacks (1:164) who were heterozygous for

Table 2. Jknull alleles

<table>
<thead>
<tr>
<th>Allele name†</th>
<th>Exon (intron) location</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Ethnic group</th>
<th>JK antibody produced‡</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jk01N01</td>
<td>Del 4 &amp; 5</td>
<td>c.1-?_341+ ?del</td>
<td>Initiation Met absent, exons 4–5 skipped</td>
<td>English, Tunisian, Bosnian</td>
<td>Jk3</td>
<td>Irshaid et al.,23 Lucien et al.,24 Wester et al.18</td>
</tr>
<tr>
<td>Jk01N02</td>
<td>5</td>
<td>c.202C&gt;T</td>
<td>p.Gln68Ter</td>
<td>Caucasian</td>
<td>Jk3</td>
<td>Wester et al.18</td>
</tr>
<tr>
<td>Jk01N03</td>
<td>7</td>
<td>c.582C&gt; G</td>
<td>p.Try194Ter</td>
<td>Swiss</td>
<td>Jk3</td>
<td>Irshaid et al.23</td>
</tr>
<tr>
<td>Jk01N04</td>
<td>10</td>
<td>c.956C&gt;T</td>
<td>p.Thr319Met</td>
<td>African American</td>
<td>None</td>
<td>Wester et al.18</td>
</tr>
<tr>
<td>Jk01N05</td>
<td>7</td>
<td>c.561C&gt;A</td>
<td>p.Tyr187Ter</td>
<td>African American, Brazilian black</td>
<td>Jk3</td>
<td>Horn et al.25</td>
</tr>
<tr>
<td>Jk01N06</td>
<td>Intron 5</td>
<td>c.342-1G&gt;A</td>
<td>p.(Arg114_Thr156del), exon 6 skipped</td>
<td>Asian Indian</td>
<td>Jk3</td>
<td>Ekman et al.22</td>
</tr>
<tr>
<td>Jk01N07</td>
<td>8</td>
<td>c.723delA</td>
<td>Ile262Ter</td>
<td>Not reported</td>
<td>Jk4</td>
<td>Crews et al.26</td>
</tr>
<tr>
<td>Jk01N08</td>
<td>9</td>
<td>c.866A&gt;G</td>
<td>Asn269Ser</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Moulds et al.27</td>
</tr>
<tr>
<td>Jk01N09</td>
<td>4</td>
<td>c.27_50del</td>
<td>Val10-Arg17del</td>
<td>African American</td>
<td>Jk4</td>
<td>Burgos et al.28</td>
</tr>
<tr>
<td>Jk01N10</td>
<td>Intron 8</td>
<td>c.811+5G&gt;A</td>
<td>p.Ala270fs exon 8 skipped</td>
<td>Chinese</td>
<td>None</td>
<td>Guo et al.29</td>
</tr>
<tr>
<td>Jk02N01</td>
<td>Intron 5</td>
<td>c.342-1G&gt;A</td>
<td>p.(Arg114_Thr156del), exon 6 skipped</td>
<td>Polynesia, Vietnamese, Chinese, Thai, Filipino, Indonesian</td>
<td>Jk3</td>
<td>Lucien et al.,11 Irshaid et al.,12 Lui et al.,13 Yan et al.,14 Lin and Lung-Chih 21</td>
</tr>
<tr>
<td>Jk02N02</td>
<td>Intron 5</td>
<td>c.342-1G&gt;C</td>
<td>p.(Arg114_Thr156del), exon 6 skipped</td>
<td>Chinese</td>
<td>none</td>
<td>Meng et al.30</td>
</tr>
<tr>
<td>Jk02N03</td>
<td>5</td>
<td>c.222C&gt;A</td>
<td>Asn74Lys</td>
<td>Chinese, Taiwanese</td>
<td>not reported</td>
<td>Lui et al.,13 Guo et al.29</td>
</tr>
<tr>
<td>Jk02N04</td>
<td>Intron 7</td>
<td>c.663+1G&gt;A</td>
<td>Leu223fs exon 7 skipped</td>
<td>French</td>
<td>not reported</td>
<td>Lucien et al.11</td>
</tr>
<tr>
<td>Jk02N05</td>
<td>8</td>
<td>c.723delA</td>
<td>Ile262fs</td>
<td>Hispanic</td>
<td>none</td>
<td>Wester et al.18</td>
</tr>
<tr>
<td>Jk02N06</td>
<td>9</td>
<td>c.871T&gt;C</td>
<td>p.Ser291Pro</td>
<td>Finnish</td>
<td>Jk3</td>
<td>Sidoux-Walter et al.,7 Irshaid et al.13</td>
</tr>
<tr>
<td>Jk02N07</td>
<td>9</td>
<td>c.896G&gt;A</td>
<td>p.Gly299Glu</td>
<td>Chinese</td>
<td>not reported</td>
<td>Lui et al.,13 Guo et al.29</td>
</tr>
<tr>
<td>Jk02N08</td>
<td>10</td>
<td>c.956C&gt;T</td>
<td>p.Thr319Met</td>
<td>Asian Indian, Pakistani</td>
<td>Jk3</td>
<td>Wester et al.18</td>
</tr>
<tr>
<td>Jk02N09</td>
<td>4</td>
<td>c.191G&gt;T</td>
<td>p.Arg64Gln</td>
<td>African American</td>
<td>Jk4</td>
<td>Billingsley et al.,31 Gaur et al.32</td>
</tr>
<tr>
<td>Jk02N10</td>
<td>4</td>
<td>c.194G&gt;A</td>
<td>p.Gly65Asp</td>
<td>Not reported</td>
<td>Not reported</td>
<td>St-Louis et al.33</td>
</tr>
<tr>
<td>Jk02N12</td>
<td>6,7</td>
<td>c.437T&gt;C, c.499A&gt;G</td>
<td>p.Leu146Pro, p.Met167Val</td>
<td>Chinese</td>
<td>None</td>
<td>Guo et al.29</td>
</tr>
</tbody>
</table>

JK*01 or JK*02 designates the source allele.

† Allele name is assigned by International Society of Blood Transfusion (ISBT) Working Party on Blood Group Antigens.
‡ JK antibodies identified in original or subsequent propositi as listed in one or more cited references.

Consult the ISBT Web site (www.isbtweb.org) or recent publications for newly described alleles.
561C/A and 1 individual who was homozygous for 561A/A and typed Jk(a−b−). Another variant, c.191G>T in JK*02, has recently been reported in an African American population at a frequency of approximately 1:400.31

Genetic variations predicted to encode weak or partial antigen expression have also been identified. Investigations of the propositi were generally initiated when antigen-typing discrepancies were seen with various sources of antisera or the propositus had produced an apparent alloantibody to the JK antigen detected either by a serological typing or predicted by the genotype at nt838 (Jk\textsuperscript{a} or Jk\textsuperscript{b}). The first allele reported was a c.130G>A substitution described by Wester et al. that led to a weakened expression of Jk\textsuperscript{b}.34 Variant alleles are listed in Table 3. The antigens produced by these variant alleles would be termed partial following the convention of the D weak versus partial alleles. Until alloantibody production demonstrates that a glycoprotein produced is structurally different from the commonly found form, identified variants are considered to code for weaker than normal antigen expression.

Recognition of the relative likelihood of certain alternative alleles is important in designing JK genotyping assays. In addition to the c.838G>A polymorphism, assays should be designed to interrogate other regions of the JK gene where variations affecting antigen expression have been identified. This approach was used by Wester et al. to design a polymerase chain reaction multiplex screening assay to more effectively identify the genetic variants most commonly encountered in their investigations.38 For example, consideration of the c.561C>A variant or the c.191G>T variant would be important in screening assays for Brazilian or American blacks, respectively. Assays based on known SNPs, however, will not detect previously unknown genetic variations. When a screening test does not identify variant alleles, sequencing of exons 4–11 is required to avoid false results.

### Function

The JK protein is expressed on RBCs and in the endothelium of the descending vasa recta and epithelial surfaces of the kidney inner medulla.39 In renal function, this transporter protein is important in regulating urea as part of the mechanism for urine concentration and water conservation.40 The erythrocyte JK glycoprotein serves to facilitate rapid urea transport across the RBC membrane.41 This mechanism is thought to ensure red cell structural stability as the cells pass through the renal medulla via the vasa recta. Individuals of the Jk(a−b−) phenotype have been shown to have suboptimal urine concentrating ability.42 Other than this deficit, no other clinical sequelae have been associated with the Jk\textsubscript{weak} phenotype, suggesting the presence of compensatory mechanisms. The lack of urea transport in the RBCs is illustrated by the resistance of Jk(a−b−) RBCs to lysis in 2M urea.43 RBCs having normal JK phenotypes will lyse within 30 seconds as the urea is transported into the cells, followed by a rapid osmotic influx of water. Because of the lack of urea transport and therefore no water uptake, Jk(a−b−) cells remain intact after 2 minutes. This characteristic has been used to perform mass screening for Jk(a−b−) individuals.

The JK protein has also been isolated from human colon,44 as well as brain, thymus, heart, lung, liver, small intestine, bone marrow, urinary tract, bladder, prostate, pancreas, skeletal muscle and spleen, and testes.45 The exact purpose of the urea transporters in non-erythroid, non-renal tissues remains speculative. No JK antigens have been found on lymphocytes, monocytes, granulocytes, or platelets.9

### Table 3. JK partial or weak alleles

<table>
<thead>
<tr>
<th>Allele name\textsuperscript{1}</th>
<th>Exon (intron) location</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Ethnic group</th>
<th>JK antibody produced\textsuperscript{1}</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JK*01W.01</td>
<td>4</td>
<td>c.130G&gt;A</td>
<td>p.Glu44Lys</td>
<td>Caucasian, Asian, Chinese</td>
<td>-Jk\textsuperscript{b}; -Jk3</td>
<td>Wester et al.,\textsuperscript{34} Whorley et al.\textsuperscript{35}</td>
</tr>
<tr>
<td>JK*01W.02</td>
<td>7</td>
<td>c.551T&gt;C</td>
<td>p.Trp171Arg</td>
<td>African American</td>
<td>None</td>
<td>Whorley et al.\textsuperscript{35}</td>
</tr>
<tr>
<td>JK*01W.03</td>
<td>4</td>
<td>c.28G&gt;A</td>
<td>p.Val10Met</td>
<td>African American</td>
<td>-Jk\textsuperscript{a}</td>
<td>Deal et al.\textsuperscript{36}</td>
</tr>
<tr>
<td>JK*01W.04</td>
<td>5</td>
<td>c.226G&gt;A</td>
<td>p.Val76ile</td>
<td>African American</td>
<td>-Jk\textsuperscript{a}</td>
<td>Deal et al.\textsuperscript{36}</td>
</tr>
<tr>
<td>JK*01W.05</td>
<td>8</td>
<td>c.742G&gt;A</td>
<td>p.Ala248Thr</td>
<td>American Indian</td>
<td>Not reported</td>
<td>Gaur et al.\textsuperscript{32}</td>
</tr>
<tr>
<td>JK*02W.01</td>
<td>7</td>
<td>c.548C&gt;T</td>
<td>p.Ala183Val</td>
<td>African American</td>
<td>None</td>
<td>Whorley et al.\textsuperscript{35}</td>
</tr>
<tr>
<td>JK*02W.02</td>
<td>8</td>
<td>c.718T&gt;A</td>
<td>p.Trp240Arg</td>
<td>African American</td>
<td>Not reported</td>
<td>St-Louis et al.\textsuperscript{37}</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Allele name is assigned by International Society of Blood Transfusion (ISBT) Working Party on Blood Group Antigens.

\textsuperscript{2}JK antigen identified in original or subsequent propositi as listed in one or more cited references.

Consult the ISBT Web site (www.isbtweb.org) or recent publications for newly described alleles.
Antibodies

The antibodies to the three antigens of the JK system have been well documented in multiple studies and case reports as responsible for immediate and delayed transfusion reactions as well as HDFN. In contrast to the frequent severity of reactions to incompatible transfusion, cases of HDFN caused by Kidd antibodies are relatively mild. The antibodies are predominately caused by RBC stimulation, although the literature contains two examples of apparently naturally occurring anti-Jk. Most examples are IgG1 and IgG3, although some antibody examples can contain IgG2, IgG4, or IgM fractions. Anti-Jk and -Jk are well known for their rapid and significant drop in titer to levels that are difficult to detect by routine serological methods. Double-dose Jk(a+) or Jk(b+) test cells or enzyme-treated cells may be necessary to detect weak antibody reactivity. Some Kidd antibodies can only be detected in antiglobulin tests in which serum and polyspecific antihuman globulin are used to detect complement binding. In other reported examples, manual hexadimethrine bromide (polybrene) or solid-phase tests were required to detect reactivity of anti-Jk. If Kidd antibodies are not detected, the subsequent transfusion of antigen-positive RBCs results in a rapid anamnestic response, with the rising titer frequently resulting in a delayed transfusion reaction. Studies at the Mayo Clinic showed that 29 percent of the delayed hemolytic or delayed serologic transfusion reactions between August 1999 and June 2007 involved JK system antibodies. The overwhelming majority of these were attributable to anti-Jk. The severe immediate or delayed hemolytic transfusion reactions described with JK system antibodies were thought to be caused by the complement binding of IgG class antibodies. This was refuted, however, in studies by Yates et al. that demonstrated complement binding was only present in those antibodies that had a direct agglutinating component or were reactive in indirect antiglobulin tests using anti-IgM. Multiple examples of JK autoantibodies have been reported in the literature. The case reports show that the majority have had autoanti-Jk specificity and have presented with immune hemolysis. In many cases, an underlying autoimmune disease was present. Autoanti-Jk and autoanti-Jk3 are also reported. One report described an autoanti-Jk that appeared during a course of methyldopa therapy. The literature also contains descriptions of JK autoantibodies whose reactivity required the presence of paraben compounds found in commercial low-ionic-strength saline reagents, and were apparent autoantibodies with mimicking specificity. It is interesting to postulate that some previously reported immune autoantibodies may have been caused by unrecognized variant Jk or Jk on the RBCs of individuals whose RBCs type as antigen-positive. Reactivity with autologous cells in these cases might have been a temporary finding as the immune system refined the specificity of the alloantibody.

Role in Renal Transplantation

Location of the JK antigens on renal cells raises intriguing questions about the impact of Kidd system antibodies on renal graft survival in kidney transplants. Several case reports suggest a role for this. Hamilton et al., Holt et al., and Rourk et al. report cases of cadaveric transplants to patients with negative antibody screens. Two to ten years after transplant and during a period where each patient was noncompliant with immunosuppressive regimes, acute graft rejection occurred simultaneously with the appearance of a Kidd system antibody. One case reported by Holt et al. described a patient who suffered a hemolytic reaction attributable to anti-Jk following a post-transplant transfusion. This was then followed by hyperacute rejection of the transplanted organ. In two cases where previous exposure to foreign RBCs had occurred, the appearance of the JK antibodies was presumed to be an anamnestic response. In the one case with no apparent RBC stimulation, the antigen-positive transplanted organ was postulated as providing the primary immunization stimulus. Nevertheless, even with prior RBC stimulus, primary immunization by the transplanted kidney should not be excluded. Leure et al. demonstrated in 370 kidney transplants that mismatch of the recipient/graft at the JK locus was associated with a higher frequency of interstitial inflammation observed on kidney biopsy when compared with those recipient/graft pairs that were matched at the JK locus although overall graft survival was not influenced.

Summary

Although recognized at the serological level in its most basic form for many decades, the Kidd blood group system...
has been shown to be increasingly complex. The knowledge of this system has increased as the tools for investigating both phenotypic and genotypic variation have expanded. Future studies would be expected to result in a more complete understanding of the products of the variant alleles that are currently known as well as provide new information on the basis of Jk3 and In(Jk) phenotype. What remains unchanged is the clinical significance of Kidd system antibodies and the importance of recognizing them in order to provide safe blood components for transfusion.

Acknowledgments

The author acknowledges Robert Ratner for preparation of Figure 2 and Connie Westhoff for assistance with genotyping data.

References

21. Lin M, Lung-Chih Y. Frequencies of the JKnull (IVS5-1g>a) allele in Taiwanese, Fujian, Filipino and Indonesian populations (letter). Transfusion 2008;48:1768.


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**For further information, contact:***  
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**For further information, contact:**  
**Neutrophil Serology Laboratory** (651) 291-6797  
Randy Schuller (651) 291-6758  
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American Red Cross Biomedical Services  
Neutrophil Serology Laboratory  
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• Conduct research in transfusion medicine

Who are SBBs?
Supervisors of Transfusion Services
Supervisors of Reference Laboratories
Quality Assurance Officers
Managers of Blood Centers
Research Scientists
Technical Representatives
LIS Coordinators
Consumer Safety Officers
Reference Lab Specialists

Why become an SBB?
Professional growth
Job placement
Job satisfaction
Career advancement

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• Sit for the examination based on criteria established by ASCP for education and experience.

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Additional information can be found by visiting the following Web sites: www.ascp.org, www.caahep.org, and www.aabb.org

Contact the following programs for more information:
A. For describing an allele which has not been described in a peer-reviewed publication and for which an allele name or provisional allele name has been assigned by the ISBT Working Party on Blood Group Allele Terminology (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/blood-group-terminology/blood-group-allele-terminology/)

B. Preparation
   1. Title: Allele Name (Allele Detail)
      ex. RHCE*01.01 (RHCE*ce48C)
   2. Author Names (initials and last name of each (no degrees, ALL CAPS)

C. Text
   1. Case Report
      i. Clinical and immunohematologic data
      ii. Race/ethnicity and country of origin of proband, if known
   2. Materials and Methods
      Description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer’s name, city, and state. Do not use patient names or hospital numbers.
   3. Results
      Complete the Table Below:

      | Phenotype | Allele Name | Nucleotide(s) | Exon(s) | Amino Acid(s) | Allele Detail | References |
      |-----------|-------------|---------------|---------|---------------|---------------|------------|
      | e weak    | RHCE*01.01  | 48G>C         | 1       | Trp16Cys      | RHCE*ce48C    | 1          |

      Column 1: Describe the immunohematologic phenotype (ex. weak or negative for an antigen).
      Column 2: List the allele name or provisional allele name.
      Column 3: List the nucleotide number and the change, using the reference sequence (see ISBT Blood Group Allele Terminology Pages for reference sequence ID).
      Column 4: List the exons where changes in nucleotide sequence were detected.
      Column 5: List the amino acids that are predicted to be changed, using the three-letter amino acid code.
      Column 6: List the non-consensus nucleotides after the gene name and asterisk.
      Column 7: If this allele was described in a meeting abstract, please assign a reference number and list in the Reference section.

   4. Additional Information
      i. Indicate whether the variant is listed in the dbSNP database (http://www.ncbi.nlm.nih.gov/snp/); if so, provide rs number and any population frequency information, if available.
      ii. Indicate whether the authors performed any population screening and if so, what the allele and genotype frequencies were.
      iii. Indicate whether the authors developed a genotyping assay to screen for this variant and if so, describe in detail here.
      iv. Indicate whether this variant was found associated with other variants already reported (ex. RHCE*ce48C,1025T is often linked to RHD*DIVa-2)

D. Acknowledgments

E. References

F. Author Information
   List first name, middle initial, last name, highest degree, position held, institution and department, and complete address (including ZIP code) for all authors. List country when applicable.
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Before submitting a manuscript, consult current issues of *Immunohematology* for style. Number the pages consecutively, beginning with the title page.

II. SCIENTIFIC ARTICLE, REVIEW, OR CASE REPORT WITH LITERATURE REVIEW
A. Each component of the manuscript must start on a new page in the following order:
   1. Title page
   2. Abstract
   3. Text
   4. Acknowledgments
   5. References
   6. Author information
   7. Tables
   8. Figures

B. Preparation of manuscript
   1. Title page
      a. Full title of manuscript with only first letter of first word capitalized (bold title)
      b. Initials and last name of each author (no degrees; all CAPS), e.g., M.T. JONES, J.H. BROWN, AND S.R. SMITH
      c. Running title of ≤40 characters, including spaces
   2. Abstract
      a. One paragraph, no longer than 300 words
      b. Purpose, methods, findings, and conclusion of study
   3. Key words
      a. List under abstract
   4. Text (serial pages): Most manuscripts can usually, but not necessarily, be divided into sections (as described below). Survey results and review papers may need individualized sections
      a. Introduction — Purpose and rationale for study, including pertinent background references
      b. Case Report (if indicated by study) — Clinical and/or hematologic data and background serology/molecular
      c. Materials and Methods — Selection and number of subjects, samples, items, etc. studied and description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer's name, city, and state. Do not use patient's names or hospital numbers.
      d. Results — Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable
      e. Discussion — Implication and limitations of the study, links to other studies; if appropriate, link conclusions to purpose of study as stated in introduction
   5. Acknowledgments: Acknowledge those who have made substantial contributions to the study, including secretarial assistance; list any grants.
   6. References
      a. In text, use superscript, Arabic numbers.
      b. Number references consecutively in the order they occur in the text.
   7. Tables
      a. Head each with a brief title; capitalize the first letter of first word (e.g., Table 1. Results of…) use no punctuation at the end of the title.
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      a. Figures can be submitted either by e-mail or as photographs (5 ×7” glossy).
      b. Place caption for a figure on a separate page (e.g. Fig. 1 Results of…), ending with a period. If figure is submitted as a glossy, place first author’s name and figure number on back of each glossy submitted.
      c. When plotting points on a figure, use the following symbols if possible:

   9. Author information
      a. List first name, middle initial, last name, highest degree, position held, institution and department, and complete address (including ZIP code) for all authors. List country when applicable. Provide e-mail addresses of all authors.

III. EDUCATIONAL FORUM
A. All submitted manuscripts should be approximately 2000 to 2500 words with pertinent references. Submissions may include:
   1. An immunohematologic case that illustrates a sound investigative approach with clinical correlation, reflecting appropriate collaboration to sharpen problem solving skills
   2. Annotated conference proceedings

B. Preparation of manuscript
   1. Title page
      a. Capitalize first word of title.
      b. Initials and last name of each author (no degrees; all CAPs)
   2. Text
      a. Case should be written as progressive disclosure and may include the following headings, as appropriate
         i. Clinical Case Presentation: Clinical information and differential diagnosis
         ii. Immunohematologic Evaluation and Results: Serology and molecular testing
         iii. Interpretation: Include interpretation of laboratory results, correlating with clinical findings
         iv. Recommended Therapy: Include both transfusion and nontransfusion-based therapies
         v. Discussion: Brief review of literature with unique features of this case
         vi. Reference: Limited to those directly pertinent
         vii. Author information (see II.B.9.)
         viii. Tables (see II.B.7.)

IV. LETTER TO THE EDITOR
A. Preparation
   1. Heading (To the Editor)
   2. Title (first word capitalized)
   3. Text (written in letter [paragraph] format)
   4. Author(s) (type flush right; for first author: name, degree, institution, address [including city, state, Zip code and country]; for other authors: name, degree, institution, city and state)
   5. References (limited to ten)
   6. Table or figure (limited to one)

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