A detailed flow cytometric method for detection of low-level in vivo red blood cell–bound IgG, IgA, and IgM

W. Beres, G.M. Meny, and S. Nance

Flow cytometric methods are commonly used to analyze white blood cell surface antigen expression. We developed a flow cytometric method to detect red blood cell (RBC)-bound immunoglobulin (Ig)G, IgA, and IgM. RBCs were washed; incubated with fluorescein isothiocyanate (FITC)-conjugated anti-IgG, -IgA, or -IgM; washed; and analyzed on the flow cytometer. The method was optimized by determining the dilution of FITC-conjugated anti-IgG, -IgA, and -IgM providing the greatest amount of fluorescence when tested with Ig-coated RBCs and the least amount of fluorescence when tested with naive RBCs. Tannic acid was used to prepare Ig-coated RBCs. Cross-reactivity of FITC-conjugated anti-IgG, -IgA, and -IgM with Ig-coated RBCs was evaluated, and a reference range was established. Use of this method may assist in clinical evaluation of patients who present with hemolysis and a negative direct antiglobulin test. *Immunohematology* 2016;32:161–169.

**Key Words:** red blood cell, direct antiglobulin test, autoimmune hemolytic anemia, tannic acid, flow cytometry, immunoglobulins

The tube-based direct antiglobulin test (DAT) is routinely used in clinical laboratories to evaluate red blood cell (RBC)-bound immunoglobulin (Ig). Although flow cytometric methods are commonly used to analyze white blood cell (WBC) surface antigen expression, the flow cytometer is less frequently used by clinical laboratories to detect and analyze RBC-bound IgG, IgA, or IgM. A positive DAT can indicate the presence of autoimmune hemolytic anemia (AIHA), but 5 percent to 10 percent of patients with AIHA are DAT-negative. Therefore, a method that detects low levels of RBC-bound immunoglobulins may be of value in the symptomatic, DAT-negative patient who has no other reason for hemolysis.

Patient samples submitted to an immunohematology reference laboratory (IRL) for evaluation of AIHA are routinely tested by serologic assays, including the DAT. The routine DAT detects as few as 200 molecules of IgG per RBC. A negative DAT in the presence of clinical hemolysis may warrant further study. DAT-negative samples may be further analyzed via special serologic methods, such as washing the patient’s RBCs in cold (4°C) saline, performing the DAT using additional antiglobulin reagents or antisera, such as anti-IgA and anti-IgM, and testing a concentrated eluate from the patient’s RBCs. These special serologic methods were reported to detect RBC-bound IgG in 3 percent to 19 percent of DAT-negative RBC samples from patients suspected to have AIHA.

Flow cytometric methods have been described for detecting low-level RBC-bound IgG in cases of suspected AIHA with a negative DAT. This nonserologic assay was reported to detect IgG on RBCs in 21 percent of DAT-negative RBC samples from patients suspected to have AIHA.

In addition to the detection of IgG, the detection of IgA and IgM may also be important in cases of suspected AIHA with a negative DAT. RBC-based flow cytometry is a rare application of flow cytometry not generally available in hospital laboratories. There are no commercially available tests to detect RBC-bound IgA and IgM. A flow cytometry–based method was developed in our facility to detect RBC-bound IgG, IgA, and IgM using reagents not manufactured or sold for this purpose. Detailed information is presented to permit implementation in a clinical laboratory of a flow cytometric method for detection of RBC-bound IgG, IgA, and IgM. Use of this method may assist in clinical evaluation of patients who present with hemolysis and a negative DAT.

**Materials and Methods**

**Flow Cytometry Startup, Shutdown, and Calibration**

Two flow cytometers (FACSCalibur and FACScan, Becton Dickinson, San Jose, CA) were used for RBC analysis. The manufacturer’s startup, shutdown, maintenance, and care procedures were followed. Before and after use, the flow cytometer was cleaned using a 10 percent bleach solution and deionized water, the waste container was emptied when applicable, and the sheath reservoir was maintained at the appropriate level.

Each day prior to testing, software and special beads (FACSComp with CaliBRITE beads, Becton Dickinson) were used to monitor the flow cytometer performance and provide
automated instrument setup: i.e., check laser alignment, optimally adjust instrument settings, monitor sensitivity, and set compensation. The lyse/wash assay on both flow cytometers was performed with five sets of beads: unlabeled beads, fluorescein isothiocyanate (FITC)-labeled beads, phycoerythrin (PE)-labeled beads, peridinin chlorophyll protein (PerCP)-labeled beads, and allophysocyanin (APC)-labeled beads (only used with the FACSCalibur, Becton Dickinson). After analysis of the beads, a summary report was generated, and all results had to be reported as “pass” before the flow cytometer could be used. PE, PerCP, and APC, although not used in this assay, were evaluated as part of the standard instrument setup procedure implemented in our laboratory.

Prior to use each day, current instrument settings on the flow cytometer, namely, detectors (photodiode and photomultiplier tubes), amplifiers, threshold, and compensation, are checked to ensure that the data plots show all captured RBCs without the use of gates (Fig. 1). The RBCs used in this check are collected in ethylenediaminetetraacetic acid (EDTA) and washed to minimize interference by agglutinated RBCs and WBCs. Using software (CellQuest, Becton Dickinson), slight adjustments to the instrument settings are made, if needed, and saved for use in that day’s testing.

Flow Cytometry Negative Control RBC Preparation

Baseline settings to detect RBC autofluorescence and instrument noise were determined by analyzing RBC samples prepared in the following manner without the addition of FITC-labeled anti-immunoglobulin.1 Residual RBC samples from healthy group O blood donors passing screening and health history for blood donation were used as negative controls. Ten residual donor RBC samples collected on the same day were washed four times in 1× phosphate-buffered saline (Dulbecco’s phosphate-buffered saline [DPBS], Lonza, Walkersville, MD) and pooled together. An aliquot from the pooled donor sample was prepared and tested as the negative control for each test run. The RBCs were washed four times in 1× DPBS, once in 0.6 percent bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO) prepared in the DPBS, and resuspended to a 1 percent concentration in 0.6 percent BSA. The 1 percent RBC suspension was analyzed on the flow cytometer, and integrated markers on the histogram were used to control for autofluorescence and instrument noise (Fig. 2).1

The pooled donor sample was stored in a storage solution (RBC Storage Solution, Immucor, Inc., Norcross, GA) and used as a negative control for up to 30 days after the initial draw date.13 This expiration date was based on a stability study that analyzed five control sets during and up to 37 days after the initial draw date (Table 1).13 In a separate study, over 16 months, 24 pooled donor samples were evaluated 67 times for RBC-bound IgG, IgM, and IgA. The pooled donor samples showed low variance in the percent of RBC-bound

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Fig. 1 Example of a dot plot of pooled donor red blood cells without the addition of antibody to adjust instrument controls.

Fig. 2 Example of a histogram of pooled donor red blood cells (RBCs) without the addition of antibodies. The instrument controls were adjusted to place the RBCs below $10^{-4}$, and the markers were set at the RBC peak.
immunoglobulins detected and provided expected normal results when evaluated up to 30 days after the initial draw date (Table 2).14

Table 1. Stability study analyzing five control sets during and up to 37 days after the initial draw date

<table>
<thead>
<tr>
<th>Pool</th>
<th>Pool age when coated (days)</th>
<th>Time of study (days)</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>37</td>
<td>95.4</td>
<td>83.4</td>
<td>92.4</td>
</tr>
<tr>
<td>B</td>
<td>13</td>
<td>27</td>
<td>69.1</td>
<td>41.3</td>
<td>78.7</td>
</tr>
<tr>
<td>C</td>
<td>13</td>
<td>31</td>
<td>62.6</td>
<td>11.2</td>
<td>81.5</td>
</tr>
<tr>
<td>D</td>
<td>17</td>
<td>28</td>
<td>99.0</td>
<td>65.0</td>
<td>99.4</td>
</tr>
<tr>
<td>E</td>
<td>20</td>
<td>35</td>
<td>95.9</td>
<td>72.8</td>
<td>99.2</td>
</tr>
</tbody>
</table>

Based on data from Beres and Nance.13

Table 2. Evaluation of 24 normal donor pools for RBC-bound immunoglobulin levels

<table>
<thead>
<tr>
<th></th>
<th>% RBC-bound IgG</th>
<th>% RBC-bound IgA</th>
<th>% RBC-bound IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of 24 pools*</td>
<td>1.34</td>
<td>2.74</td>
<td>1.69</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.33</td>
<td>2.79</td>
<td>2.16</td>
</tr>
</tbody>
</table>

Based on data from Beres and Nance.14

*The normal range for these data was determined to be: RBC-bound IgG: 0–5.33 %M2; RBC-bound IgA: 0–11.11 %M2, and RBC-bound IgM: 0–8.17 %M2.

Flow Cytometry: Positive Control RBC Preparation

Specific Ig-coated RBCs were prepared to ensure appropriate reactivity of the specific antisera (e.g., anti-IgG) with Ig-coated RBCs and also to ensure no cross-reactivity with the other coated RBC preparations (e.g., anti-IgG with IgA-coated RBCs and anti-IgG with IgM-coated RBCs). Neither the antisera nor the proteins used to coat the RBCs are manufactured for flow cytometric analysis of RBCs; therefore, these controls are important to include in testing.

In vitro Ig-coated (IgG, IgA, and IgM) RBCs were prepared by tannic acid treatment for use as positive controls.15

The tannic acid method was selected to minimize nonspecific aggregation of Ig-coated RBCs. The pooled donor sample that served as a negative control was used to create the positive control samples 8 to 21 days after collection. The pooled donor sample was washed three times in DPBS to remove any residual tannic acid, and an aliquot was set aside for flow cytometric testing. The tannic acid–treated RBCs were stored in the storage solution (RBC Storage Solution) and used for up to 31 days beyond the donor’s collection date.

The tannic acid–treated RBC aliquots were coated with IgG, IgM, or IgA (ChromPure human IgG whole molecule, ChromPure human IgM [myeloma] whole molecule, or ChromPure human IgA whole molecule, Jackson ImmunoResearch Laboratories, West Grove, PA). Various concentrations of the coating protein were evaluated prior to use to determine the optimal coat concentration (1.9 × 10–6 mg/mL to 0.5 mg/mL), as the concentration can vary between reagent lots and with the antibody used. A 0.02 mg/mL solution of each immunoglobulin was determined to be the optimal coating concentration for the coated proteins. The immunoglobulin coating proteins were prepared in DPBS and heated in a 56°C ±1°C water bath for 1 hour ±5 minutes before mixing with the tannic acid–treated RBCs. A total of 200 μL washed packed tannic acid–treated RBCs were mixed with 500 μL heated immunoglobulin solution. This mixture was incubated in a 37°C ±1°C dry air incubator for 1 hour ±5 minutes. Passive binding of immunoglobulin to RBCs occurs immediately after adding immunoglobulin to the tannic acid–treated donor RBC aliquots.15

The Ig-coated RBCs were washed three times in DPBS and once with 0.6 percent BSA to remove any excess immunoglobulin, and resuspended to a 1 percent concentration in 0.6 percent BSA for use as a positive control. Aliquots of each positive control (IgA-, IgM-, and IgG-coated RBCs) were set aside for testing. Samples were mixed by vortexing and were inspected for agglutination prior to testing. Visual agglutination of RBCs has not been observed when viewed microscopically. The donor pooled sample (negative control), described in the previous section, and the positive control samples prepared from the donor pool were tested in parallel for each run as a complete control set.

The Ig-coated RBCs were stored in the red blood cell storage solution (RBC Storage Solution) and used for 31 days beyond the initial draw date.13 The use of the Ig-coated cells for 31 days was based on a stability study that analyzed five complete control sets during and up to 37 days after the initial draw date.13

Patient and Donor Samples

Residual aliquots of blood donor samples were obtained after completion of infectious disease testing to prepare the complete control set. Ninety residual donor samples, 24 pooled donor samples, and 8 complete control sets are included in this
study. Residual aliquots of 317 patient blood samples referred to the IRL were selected for flow cytometry analysis if their DAT was negative to 3+ (4+ is strongest reaction) with anti-IgG. All blood samples were collected in EDTA and stored between 2°C and 6°C prior to use or up to 31 days past the initial draw date. The protocol was developed and approved by an institutional review board; the samples were tested according to this protocol.

**Direct Antiglobulin Test**

RBCs were washed four times in saline and suspended to a 4 percent suspension. Four test tubes were labeled (Poly, IgG, C3, and Saline) and prepared by adding one drop of 4 percent RBC suspension to each tube followed by one to two drops of anti-human immunoglobulin reagents (anti-IgG, -C3d; anti-IgG; anti-C3bd; Immucor, Inc.) or 0.85 percent saline (Blood Bank Saline, Thermo Scientific, Waltham, MA) to the appropriately labeled test tube. The tubes were mixed and centrifuged at 1000g. The cell buttons were gently resuspended and examined for agglutination. Negative tests were controlled by adding one drop of the appropriate control RBCs, recentrifuged, and examined again for agglutination.16

**Flow Cytometry Test Sample Preparation**

The patient and donor RBCs were washed four times in DPBS, once in 0.6 percent BSA, and resuspended to a 1 percent concentration in BSA. Subsequently, 500 μL of the 1 percent RBC suspensions was aliquoted into four flow cytometry tubes (Falcon® 5 mL polystyrene round-bottom 12 × 75 mm, Corning Inc., Life Sciences, Tewksbury, MA): one tube to control for background and autofluorescence and the other three for antibody testing. A maximum of 10 patient samples, including controls, is recommended for one batch of testing when evaluating for RBC-bound IgG, IgA, and IgM. As the number of samples increases, the amount of time between steps increases, thus extending incubation periods. This limit was based on one technician performing the assay on one flow cytometer not equipped with a loader or high-throughput sampler.

**Flow Cytometry Antibody Labeling**

The flow cytometer detects immunoglobulins (antibodies) on RBCs by the addition of FITC-labeled anti-immunoglobulin. The test samples and a set of the control samples were sensitized with FITC-conjugated antibodies. Samples of 500 μL of the 1 percent RBC suspensions were centrifuged for 60 seconds at 1000g, and the supernatant was removed. Then, 500 μL FITC-conjugated antibodies diluted in 0.6 percent BSA was added to each cell button, and the tubes were vortexed. The RBC and FITC-antibody solution was incubated in the dark, at room temperature, for 45–60 minutes, washed, and resuspended in 500 μL of 0.6 percent BSA. Controls are used together as a set: the in vitro Ig-coated (IgG, IgA, and IgM) samples (positive controls) and the nontreated pooled donor sample (negative control). Therefore, with each test batch, all antisera are tested against a negative control and cells coated with IgG, IgA, and IgM (thus, one coated cell serves as a positive control and the two other coated cells are controls for cross-reactivity). This control set is tested in parallel with each test run. A reagent control sample of tannic acid—only treated RBCs was evaluated during method development, but was discontinued, since nonspecific binding caused by tannic acid treatment was not observed.

**Flow Cytometry Acquisition and Analysis**

Flow cytometry testing was performed on two flow cytometers (FACSCalibur and FACSscan, Becton Dickinson). Prior to use, the automated instrument setup was performed using software and special beads (FACScComp and CaliBRITE beads, Becton Dickinson). The instrument controls were optimized to display cell populations of interest on the data plots, forward and side scatter amps were set to linear mode, and the FL1 and FL2 amps were set to logarithmic mode.17 Gates were established to view all RBCs. Prior to testing, all samples were mixed by vortexing and were visually inspected for agglutination. If RBC agglutination was suspected, the sample was viewed microscopically after the flow cytometry testing to determine false positives; no RBC agglutination was observed by microscopic evaluation in this study. Consequently, only the positive tests were viewed microscopically in the amended procedure. Fifty thousand RBCs are acquired from each sample at optimized settings.

During sample analysis, the relative fluorescence or light scatter intensity is displayed in a single parameter histogram on the x axis and the number of events on the y axis. Marker 1 (M1) is used to separate the native background autofluorescence of RBCs, and Marker 2 (M2) is used to identify the fluorescence emitted by the RBC-bound FITC-labeled antibodies bound to immunoglobulins on the target RBCs (Fig. 2). The results are calculated by subtracting the %M2 value of the washed target RBCs from the %M2 FITC-labeled antibody treated target RBCs. This calculated value is then evaluated against the established normal reference range to determine if the RBC immunoglobulin levels detected are in the normal range.
Results

Optimal FITC-Conjugated Antibody Dilution

Prior to testing, the optimal FITC-conjugated antibody dilution was determined for staining a 500 µL volume of 1 percent RBCs. During method development, antibodies from two manufacturers were evaluated to determine the optimal antibody dilution: the first set of IgG, IgM, and IgA antibodies [FITC-conjugated goat F(ab')2 anti-human IgG, FITC-conjugated goat F(ab')2 anti-human IgM, FITC-conjugated goat F(ab')2 anti-human IgA; Invitrogen, Camarillo, CA; Life Technologies, Carlsbad, CA] were all goat-sourced antibodies. The set from the second manufacturer (FITC-conjugated mouse Fcγ anti-human IgG, FITC-conjugated goat Fcγ anti-human IgM, FITC-conjugated goat anti-human serum IgA; Jackson ImmunoResearch Laboratories) included an IgG antibody that was mouse-sourced.

The optimal FITC-conjugated antibody dilution was determined by comparing the results of the RBCs from the positive and negative control set: i.e., observing results generated from testing various antibody dilutions (e.g., between 1/25 and 1/400) of the in vitro Ig-coated (IgG, IgA, and IgM) positive controls and the untreated pooled donor sample (negative control). The antibody dilution that yielded the highest percentage of RBC-bound Ig detected on the corresponding positive control sample (e.g., IgA-coated RBCs analyzed by anti-IgA) when compared with the other antibody dilutions evaluated, that had no observed cross-reactivity, and that exhibited the greatest percentage of separation between negative and positive control results was chosen as the optimal dilution. It is critical to choose an antibody dilution with a high result when selecting the positive control. The positive control result must be greater than the results of the patient samples evaluated in the test run to ensure that cross-reactivity with the other antibodies tested in this assay is excluded.

Each lot of FITC-conjugated antibody is prepared by the manufacturer and, in general, with different antibody concentrations (mg/mL) and with different ratios of FITC fluorophoros conjugated to the protein (µg/mg). The optimal dilution of each FITC-conjugated antibody is lot-specific and was determined prior to its use to optimize maximum reactivity and to minimize cross-reactivity.

As an example, the optimal dilution was identified as 1/50 for the FITC-conjugated goat F(ab')2 anti-human IgM when compared with the results from the 1/25, 1/100, and 1/200 dilutions (Table 3). The optimal dilution for FITC-conjugated goat anti-human serum IgA (lot 120546, Jackson ImmunoResearch Laboratories) was identified as 1/50 (Table 4), which also had the greatest amount of RBC-bound IgA when compared with the other dilutions. The optimal dilution of the FITC-conjugated goat F(ab')2 anti-human IgG (lot 119255, Jackson ImmunoResearch Laboratories) was identified as 1/300 (Table 5), since this dilution showed minimal cross-reactivity when compared with the other anti-IgG dilutions, and had the greatest percentage of RBC-bound IgG with IgG-coated RBCs for 1/50 and 1/100, and similar for 1/200 and 1/400, making either acceptable for use.

Cross-Reactivity

While evaluating the optimal FITC-conjugated antibody dilution, the antibodies were also evaluated for cross-reactivity. The control set, as described earlier, RBCs from the negative and positive control set (the in vitro Ig-coated [IgG, IgA, and IgM] control), and the untreated pooled donor sample were tested in parallel with each test run. The anti-IgG was evaluated with RBC-coated IgA whole molecule (ChromPure, Jackson ImmunoResearch Laboratories) and IgM (myeloma) whole molecule (ChromPure); the anti-IgA was evaluated with RBC-coated IgG whole molecule (ChromPure) and IgM (myeloma) whole molecule (ChromPure); and the anti-IgM was evaluated with RBC-coated IgG whole molecule (ChromPure) and RBC-bound IgA whole molecule (ChromPure). The IgG-, IgA-, and IgM-coated RBCs were prepared by tannic acid treatment, as described earlier, and were analyzed by flow cytometry by the corresponding antibody (e.g., IgA-coated RBCs analyzed by anti-IgA) to ensure adequate RBC coating. The pooled donor sample used in preparing the Ig-coated RBCs was analyzed as a negative control.

Cross-reactivity was evaluated with one of each IgG-, IgM-, and IgA-conjugated antibody: FITC-conjugated goat F(ab')2 anti-human IgM (Invitrogen; Life Technologies) (Table 3), FITC-conjugated goat anti-human serum IgA (lot 93818, Jackson ImmunoResearch Laboratories) (Table 4), and FITC-conjugated goat F(ab')2 anti-human IgG (Jackson ImmunoResearch Laboratories) (Table 5). Cross-reactivity was tested concurrently along with determining the optimal reagent antibody dilutions. The percent of RBC-bound antibody detected from RBCs coated with the conjugate antibody was within the established normal reference ranges (as described in the following section), and cross-reactivity was not observed. Cross-reactivity with controls was monitored with each batch.
Table 3. Determination of IgM dilution by evaluation of optimal reactivity and cross-reactivity: percentage of RBC-bound IgM detected by anti-human IgM

<table>
<thead>
<tr>
<th>Control set*</th>
<th>1/25</th>
<th>1/50†</th>
<th>1/100</th>
<th>1/200</th>
<th>Unstained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor pool</td>
<td>2.08</td>
<td>0.62</td>
<td>0.27</td>
<td>0.32</td>
<td>0.12</td>
</tr>
<tr>
<td>IgG</td>
<td>3.52</td>
<td>1.41</td>
<td>1.00</td>
<td>0.38</td>
<td>0.15</td>
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<tr>
<td>IgA</td>
<td>3.11</td>
<td>1.26</td>
<td>0.87</td>
<td>0.45</td>
<td>0.15</td>
</tr>
<tr>
<td>IgM</td>
<td>77.58</td>
<td>77.99</td>
<td>75.22</td>
<td>74.33</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Antibody: FITC-conjugated goat F(ab’)_2 anti-human IgM lot: 923278E, Invitrogen; Life Technologies.
*The control set consists of RBCs from the negative and positive control set: the in vitro IgG-coated (IgG, IgA; and IgM) controls and the untreated pooled donor sample.
†From these data, the optimal antibody dilution was determined to be 1/50.
The normal range was determined in Table 6 to be 0–6.00 %M2. Ig = immunoglobulin; RBC = red blood cell.

Table 4. Determination of IgA dilution by evaluation of optimal reactivity and cross-reactivity: percentage of RBC-bound IgA detected by anti-human IgA

<table>
<thead>
<tr>
<th>Control set*</th>
<th>1/25</th>
<th>1/50†</th>
<th>1/100</th>
<th>1/200</th>
<th>Unstained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor pool</td>
<td>1.95</td>
<td>0.91</td>
<td>0.56</td>
<td>0.37</td>
<td>0.06</td>
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<tr>
<td>IgG</td>
<td>6.15</td>
<td>2.39</td>
<td>2.39</td>
<td>1.39</td>
<td>0.08</td>
</tr>
<tr>
<td>IgA</td>
<td>61.46</td>
<td>61.53</td>
<td>53.53</td>
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<td>0.06</td>
</tr>
<tr>
<td>IgM</td>
<td>6.28</td>
<td>2.85</td>
<td>2.28</td>
<td>1.88</td>
<td>0.11</td>
</tr>
</tbody>
</table>

*The control set consists of RBCs from the negative and positive control set: the in vitro IgA-coated (IgG, IgA; and IgM) controls and the untreated pooled donor sample.
†From these data, the optimal antibody dilution was determined to be 1/50.
The normal range was determined in Table 7 to be 0–6.90 %M2. Ig = immunoglobulin; RBC = red blood cell.

Table 5. Determination of IgG dilution by evaluation of optimal reactivity and cross-reactivity: percentage of RBC-bound IgG detected by anti-human IgG

<table>
<thead>
<tr>
<th>Control set*</th>
<th>1/50</th>
<th>1/100</th>
<th>1/200</th>
<th>1/300†</th>
<th>1/400</th>
<th>Unstained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor pool</td>
<td>2.72</td>
<td>0.77</td>
<td>0.64</td>
<td>0.49</td>
<td>0.52</td>
<td>0.18</td>
</tr>
<tr>
<td>IgG</td>
<td>75.01</td>
<td>73.72</td>
<td>80.78</td>
<td>70.86</td>
<td>70.61</td>
<td>0.15</td>
</tr>
<tr>
<td>IgA</td>
<td>5.55</td>
<td>3.39</td>
<td>3.20</td>
<td>1.65</td>
<td>1.36</td>
<td>0.25</td>
</tr>
<tr>
<td>IgM</td>
<td>8.64</td>
<td>5.83</td>
<td>2.71</td>
<td>3.35</td>
<td>2.28</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*The control set consists of RBCs from the negative and positive control set: the in vitro IgG-coated (IgG, IgA; and IgM) controls and the untreated pooled donor sample.
†From these data, the optimal antibody dilution was determined to be 1/300.
The normal range was determined in Table 8 to be 0–7.29 %M2. Ig = immunoglobulin; RBC = red blood cell.

From these data, the optimal antibody dilution was determined to be 1/300.

RBC-Bound Immunoglobulin (IgG, IgA, and IgM) Reference Range

The RBC-bound immunoglobulin reference range or normal range of RBC-bound immunoglobulin was defined to detect positive samples. The ranges were established by determining the amount of RBC-bound immunoglobulin on 30 naive washed individual allogeneic donor blood samples by flow cytometry; 10 samples were evaluated in three separate batches on different days. Following the described protocol, the blood samples were evaluated by each antibody using the lot-specific predetermined optimal dilution to give the RBC-bound immunoglobulin negative range. This step was performed for all antibodies used—anti-IgG, anti-IgA, and anti-IgM—after the optimal antibody dilution and cross-reactivity were determined. The normal range was calculated by using the mean result of the donor samples ±3 standard deviations (Tables 6–8). For the normal range to be valid, the control set (evaluated in the previous section) must be within the newly established normal range. If the control set is outside the normal range, a new optimal dilution must be chosen.

Negative and Positive Control Set Storage Parameters

The pooled donor sample, tannic acid–only treated RBCs, and Ig-coated RBCs were stored in the storage solution (RBC Storage Solution) and used for up to 31 days after the initial draw date. The use of the coated cells for 31 days was based on a stability study that analyzed five control sets during and up to 37 days after the initial draw date. In a separate study, over 16 months, 24 pooled naive donor samples were evaluated 67 times for RBC-bound IgG, IgM, and IgA. The pooled donor samples showed low variance in RBC-bound immunoglobulins and provided expected normal results when evaluated up to 30 days after the initial draw date (Table 2).

Patient and Donor Samples

Preliminary results in our laboratory on 237 patient samples and 20 autologous donor samples examined by flow cytometry (individual donations for self-use) demonstrated RBC-bound IgG on 16 percent of DAT-negative patient samples and 0 percent of autologous donor samples (Table 9). Results on 80 DAT-negative patient samples suspected for AIHA were collected over 4 years (July 2012 to Aug 2016). The samples were examined by flow cytometry, and 28.75 percent were found to have RBC-bound IgG, 32.50 percent had RBC-
Eleven (13.75%) of the DAT-negative samples evaluated had elevated levels of RBC-bound IgG, IgA, and IgM (Fig. 3).

**Discussion**

When implementing a RBC-based flow cytometry method, there are many different points to consider, because these FITC-conjugated antibodies are not standardized by the manufacturers for this method. The optimal FITC-conjugated antibody dilutions must be chosen that result in a low %M2 with negative controls, a high %M2 with positive controls, and no cross-reactivity with other Ig-coated RBCs. The positive control %M2 should be optimized or selected to be greater than the highest %M2 results anticipated to be found in positive patient samples to rule out cross-reactivity for valid tests. The negative control range %M2 should be low enough to detect RBCs coated with low levels of immunoglobulins.

Many flow cytometry operators are trained in WBC applications but are not trained in the study of RBC-based antigen–antibody reactions. A detailed flow cytometer procedure will be of assistance in the laboratory evaluation of patients who present clinically with hemolysis and a negative DAT. Ideally, it should be adaptable to flow cytometers of different models, from different manufacturers, or with different software—with careful validation and controls. The method described in this article is an improvement over subjective manual testing because results are obtained by an automated system and analyzed cell by cell. Our lab studies started with the evaluation of RBC-bound IgG because of its clinical importance and our desire to provide the ability to compare flow cytometry to other methods.

**Table 6.** Evaluation of 30 randomly selected donors over 3 days (normal range): percentage of RBC-bound IgM detected by 1/50 anti-human IgM

<table>
<thead>
<tr>
<th>Donors (N = 30)</th>
<th>Mean RBC-bound IgM detected</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Range*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–10</td>
<td>1.17</td>
<td>1.17</td>
<td></td>
<td></td>
<td>1.17</td>
<td></td>
<td>0–6.00</td>
</tr>
<tr>
<td>11–20</td>
<td>2.02</td>
<td></td>
<td>2.02</td>
<td></td>
<td>2.02</td>
<td></td>
<td>0–6.00</td>
</tr>
<tr>
<td>21–30</td>
<td>2.05</td>
<td></td>
<td></td>
<td>2.05</td>
<td>2.05</td>
<td></td>
<td>0–6.00</td>
</tr>
<tr>
<td>1–30</td>
<td>1.68</td>
<td>1.68</td>
<td>1.44</td>
<td>1.44</td>
<td>1.68</td>
<td></td>
<td>0–6.00</td>
</tr>
</tbody>
</table>

Antibody: FITC-conjugated goat F(ab’), anti-human IgM, lot: 923278E Invitrogen; Life Technologies.

*The normal range was calculated by using the mean of the donor samples ±3 standard deviations.

RBC = red blood cell; Ig = immunoglobulin.

**Table 7.** Evaluation of 30 randomly selected donors over 3 days (normal range): percentage of RBC-bound IgA detected by 1/50 anti-human IgA

<table>
<thead>
<tr>
<th>Donors (N = 30)</th>
<th>Mean RBC-bound IgA detected</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Range*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–10</td>
<td>3.25</td>
<td></td>
<td></td>
<td>3.25</td>
<td>3.25</td>
<td></td>
<td>0–6.00</td>
</tr>
<tr>
<td>11–20</td>
<td>2.76</td>
<td></td>
<td>2.76</td>
<td></td>
<td>2.76</td>
<td></td>
<td>0–6.00</td>
</tr>
<tr>
<td>21–30</td>
<td>1.11</td>
<td></td>
<td></td>
<td>1.11</td>
<td>1.11</td>
<td></td>
<td>0–6.00</td>
</tr>
<tr>
<td>1–30</td>
<td>2.37</td>
<td>2.37</td>
<td>1.51</td>
<td>1.51</td>
<td>2.37</td>
<td></td>
<td>0–6.90</td>
</tr>
</tbody>
</table>


*The normal range was calculated by using the mean of the donor samples ±3 standard deviations.

RBC = red blood cell; Ig = immunoglobulin.

**Table 8.** Evaluation of 30 randomly selected donors over 3 days (normal range): percentage of RBC-bound IgG detected by 1/300 anti-human IgG

<table>
<thead>
<tr>
<th>Donors (N = 30)</th>
<th>Mean RBC-bound IgG detected</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Range*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–10</td>
<td>1.15</td>
<td></td>
<td></td>
<td>1.15</td>
<td>1.15</td>
<td></td>
<td>0–7.29</td>
</tr>
<tr>
<td>11–20</td>
<td>2.25</td>
<td></td>
<td>2.25</td>
<td></td>
<td>2.25</td>
<td></td>
<td>0–7.29</td>
</tr>
<tr>
<td>21–30</td>
<td>2.72</td>
<td></td>
<td></td>
<td>2.72</td>
<td>2.72</td>
<td></td>
<td>0–7.29</td>
</tr>
<tr>
<td>1–30</td>
<td>2.04</td>
<td>2.04</td>
<td>1.75</td>
<td>1.75</td>
<td>2.04</td>
<td></td>
<td>0–7.29</td>
</tr>
</tbody>
</table>


*The normal range was calculated by using the mean of the donor samples ±3 standard deviations.

RBC = red blood cell; Ig = immunoglobulin.

bound IgA, and 40.00 percent had RBC-bound IgM (Table 10). Eleven (13.75%) of the DAT-negative samples evaluated had elevated levels of RBC-bound IgG, IgA, and IgM (Fig. 3).

**Table 9.** Detection of in vivo RBC-bound IgG measured by flow cytometry

<table>
<thead>
<tr>
<th></th>
<th>Number of samples evaluated</th>
<th>Number of samples with elevated levels of RBC-bound IgG</th>
<th>% Samples with RBC-bound IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAT-negative patients</td>
<td>95</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Autologous donors</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Based on data from Beres and Nance.14

RCB = red blood cell; Ig = immunoglobulin; DAT = direct antiglobulin test.

**Table 10.** Detection of in vivo RBC-bound IgG, IgA, and IgM by flow cytometry in DAT-negative patient samples (N = 80)

<table>
<thead>
<tr>
<th>IgG*</th>
<th>IgA*</th>
<th>IgM*</th>
<th>IgG and IgA</th>
<th>IgG and IgM</th>
<th>IgA and IgM</th>
<th>IgG, IgA, and IgM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>9</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>11</td>
<td>42</td>
</tr>
<tr>
<td>(1.25%)</td>
<td>(5.00%)</td>
<td>(11.25%)</td>
<td>(6.25%)</td>
<td>(7.50%)</td>
<td>(7.50%)</td>
<td>(13.75%)</td>
<td>(52.50%)</td>
</tr>
</tbody>
</table>

The samples were examined by flow cytometry; 28.75 percent were found to have RBC-bound IgG, 32.50 percent had RBC-bound IgA, and 40.00 percent had RBC-bound IgM.

*Only one RBC-bound immunoglobulin detected on the sample.

RBC = red blood cell; Ig = immunoglobulin; DAT = direct antiglobulin test.
results with standard tube test results. RBC-bound IgA and IgM detection was studied after the IgG detection procedure was optimized to enable the evaluation of patients with negative DATs and hemolysis of unknown etiology that may be caused by anti-IgA or anti-IgM.

Patient samples submitted to a laboratory for evaluation of AIHA are routinely tested for the presence of cell-bound IgG (and C3) via a tube DAT. If a negative DAT is obtained, flow cytometry testing can be performed after preparing controls, establishing the appropriate dilution of FITC-conjugated antibody, and establishing a reference range. We have provided information for flow cytometry operators to allow for implementation of a flow cytometric method for detection of RBC-bound IgG, IgA, and IgM to assist in clinical evaluation of patients who present with hemolysis and a negative DAT.

**Acknowledgments**

We would like to thank Savita Singh, Karen Weikel, and Abraham Thomas for collecting and providing residual patient and donor samples used for this testing. This study was supported by the American Red Cross Blood Services.

**References**


18. Beres W, McGuire L, Nance S, Meny G. Detection of in vivo red blood cell (RBC) bound IgG measured by flow cytometry (FC) [abstract]. Transfusion 2010;50:166A.

Wendy Beres, BS, Immunohematology Assay Development Associate II (corresponding author), Biomedical Services, American Red Cross, 700 Spring Garden St., Philadelphia, PA 19123, Wendy.Beres@redcross.org; Geralyn M. Meny, MD, MS, MT(ASCP)SBB, Physician Consultant, Grifols Diagnostic Solutions, Inc., Emeryville, CA; and Sandra Nance, MS, MT(ASCP)SBB, Senior Director, Immunohematology Reference Laboratory, Biomedical Services, American Red Cross, Philadelphia, PA.

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