

Improved method for fluorescence cytometric immunohematology testing

John D. Roback, Sheilagh Barclay, and Christopher D. Hillyer

BACKGROUND: A method for accurate immunohematology testing by fluorescence cytometry (FC) was previously described. Nevertheless, the use of vacuum filtration to wash RBCs and a standard-flow cytometer for data acquisition hindered efforts to incorporate this method into an automated platform.

STUDY DESIGN AND METHODS: A modified procedure was developed that used low-speed centrifugation of 96-well filter plates for RBC staining. Small-footprint benchtop capillary cytometers (PCA and PCA-96, Guava Technologies, Inc.) were used for data acquisition. Authentic clinical samples from hospitalized patients were tested for ABO group and the presence of D antigen (n = 749) as well as for the presence of RBC alloantibodies (n = 428). Challenging samples with mixed-field reactions and weak antibodies were included. Results were compared to those obtained by column agglutination technology (CAT), and discrepancies were resolved by standard tube methods. Detailed investigations of FC sensitivity and reproducibility were also performed.

RESULTS: The modified FC method with the PCA determined the correct ABO group and D type for 98.7 percent of 520 samples, compared to 98.8 percent for CAT (p > 0.05). No-type-determined (NTD) rates were 1.2 percent for both methods. In testing for unexpected alloantibodies, FC determined the correct result for 98.6 percent of 215 samples, compared to 96.3 percent for CAT (p > 0.05). When samples were automatically acquired in the 96-well plate format with the PCA-96, 98.7 percent of 229 samples had correct ABO group and D type determined by FC, compared to 97.4 percent for CAT (p > 0.05). NTD rates were 0.9 and 2.6 percent, respectively. Antibody screens were accurate for 99.1 percent of 213 samples with the PCA-96, compared to 99.5 percent for CAT (p > 0.05). Further investigations demonstrated that FC with the PCA-96 was better than CAT at detecting weak anti-A (p < 0.0001) and alloantibodies.

CONCLUSIONS: An improved method for FC immunohematology testing has been described. This assay was comparable in accuracy to standard CAT

techniques, but had better sensitivity for detecting weak antibodies and was superior in detecting mixed-field reactions (p < 0.005). The FC method demonstrated excellent reproducibility. The compatibility of this assay with the PCA-96 capillary cytometer with plate-handling capabilities should simplify development of a completely automated platform.

Testing before transfusion includes immunohematology assays to determine the ABO group and D type of blood donors and recipients, as well as tests to identify unexpected RBC alloantibodies and to confirm cross-match compatibility. Historically, these assays have been performed by visually scoring antibody-mediated RBC agglutination in test tubes following centrifugation. Column agglutination technology (CAT) (ID-micro typing system, Ortho-Clinical Diagnostics, Raritan, NJ)¹ and solid-phase RBC adherence assays (Capture-R, Immucor, Inc., Norcross, GA)³ have been developed to automate immunohematology testing. Recently, we reported a fluorescence cytometry (FC) method for immunohematology testing.² This FC assay

ABBREVIATIONS: CAT = column agglutination technology; FC = fluorescence cytometry; NTD = no type determined.

From the Transfusion Medicine Program, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia; and Transfusion & Transplantation Technologies, Inc., Dunwoody, Georgia.

Address correspondence to: J.D. Roback, MD, PhD, Emory University, WMB 2307, 1639 Pierce Drive, Atlanta, GA 30322; e-mail: jroback@emory.edu.

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used a standard-flow cytometer and demonstrated accuracy comparable to CAT and tube testing. Additionally, it had advantages including the possibility that an automated FC immunohematology workstation could be used for other blood bank tests such as infectious disease serology, counting residual WBCs in filtered blood, and PLT cross-matching.

In the process of developing an automated platform, two significant improvements are now reported. First, vacuum filtration was replaced with a novel low-speed centrifugation method that better maintains RBC integrity thus improving assay reproducibility and performance. Second, we demonstrate accurate detection of RBC-antibody interactions with two small-footprint benchtop capillary cytometers: the PCA, a manually operated instrument, and the recently introduced PCA-96, which can automatically acquire samples in a 96-well plate format (Guava Technologies, Inc., Hayward, CA). With these improved methods, herein we demonstrate the feasibility, accuracy, sensitivity, and reproducibility of a fully automated FC workstation for use in the blood center and transfusion service environments.

MATERIALS AND METHODS

Samples, reagents, and supplies

Anonymized samples were obtained from discarded blood specimens submitted to the Emory University Hospital Blood Bank (Atlanta, GA). All samples used for determination of ABO group and D type and approximately one-half of the samples used for alloantibody testing were obtained after 4 days of refrigerated storage and tested within 24 hours. The remainder of the specimens for alloantibody testing were obtained from archived samples stored in aliquots at -20°C . This protocol was approved by the Emory University Human Investigations Committee. Standard blood typing reagent antibodies and RBCs were obtained from Ortho-Clinical Diagnostics or Immucor, Inc.. The donkey anti-human IgM ($\text{Fc}_{5\mu}$ -specific), goat anti-human IgG (Fc_{γ} -specific), and goat anti-mouse IgM (μ -chain-specific) secondary antibodies used for this

study were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All secondary antibodies were used as affinity-purified, PE-labeled $\text{F}(\text{ab}')_2$ fragments. Antibody and RBC working dilutions were determined through empirical studies and were made in 0.9 percent normal saline. The 96-well microtiter filter plates used for the majority of these studies contained $0.45\text{-}\mu\text{m}$ polypropylene filters (UniFilter; Whatman, Clifton, NJ); other commercially available filter plates also worked adequately in similar studies (data not shown).

Capillary cytometry

The fluorescent staining protocol for FC is summarized in Table 1. A minimum of 10,000 to 20,000 events were acquired on the cytometers. When using the PCA, samples were pipetted into 1.5-mL microcentrifuge tubes that were then manually inserted into the cytometer. With the PCA-96, multiple samples were automatically acquired from 96-well plates. For these studies, all sample transfer steps, as well as tube and plate labeling, were performed manually.

ABO RBC grouping

Patient RBCs were added to three wells of the filter plate, followed by the addition of mouse anti-A to the first well, anti-B to the second (BioClone anti-A and anti-B murine monoclonal IgM blends, Ortho-Clinical Diagnostics), and mouse preimmune IgM antibody to the third well as an isotype control (Sigma-Aldrich, St. Louis, MO). After incubation, the plates were centrifuged at $400\times g$ for 60 seconds. These centrifugation conditions dispersed the RBCs over the filter surface with minimal agglutination. The RBC-free supernatant was removed by pipetting, saline wash solution was added, and the samples were centrifuged. This procedure was repeated for a total of four washes. PE-labeled anti-mouse IgM was added and the mixtures were incubated followed by two washes. MFIs collected in the PM-1 channel of the cytometer were used to quantitate PE-labeled RBCs. Samples that were repro-

TABLE 1. Staining protocol with filter plates and PCA or PCA-96

Assay step	RBC A, B	RBC D	Serum α -A, B	Alloantibodies
1. Add RBCs	2% patient RBCs (25 μL)	2% patient RBCs (25 μL)	3% A, B, O RBCs (30 μL)	3% screening RBCs (13 μL)
2. Add primary antibody	Murine α -A or α -B (50 μL)	Human α -D (50 μL)	Patient plasma (50 μL)	Patient plasma (25 μL)
3. Add potentiator				20% PEG (50 μL)
4. Incubate	RT* \times 2 min	RT \times 2 min	RT \times 2 min	37°C \times 5 min
5. Wash	Saline \times 4 (200 μL)	Saline \times 4 (200 μL)	Saline \times 4 (200 μL)	Saline \times 4 (200 μL)
6. Add secondary antibody	PE- α -murine IgM (100 μL)	PE- α -human IgM (100 μL)	PE- α -human IgM (100 μL)	PE- α -human IgG (100 μL)
7. Incubate	RT \times 5 min	RT \times 5 min	RT \times 5 min	RT \times 5 min
8. Wash	Saline \times 2 (200 μL)	Saline \times 2 (200 μL)	Saline \times 2 (200 μL)	Saline \times 2 (200 μL)
9. Acquire				

* RT, room temperature.

ducibly negative or positive (3+ to 4+) by CAT and tube testing were used as negative and positive controls for FC testing, respectively. Negative controls were run in replicates and the results were used to determine the threshold for positive reactions. Similar control procedures were used for all FC assays. No efforts were made to disperse the RBCs before acquisition on the PCA or PCA-96.

D typing

The same procedure was used, except patient RBCs were added to two wells followed by either human IgM anti-D or saline (control) and PE-labeled anti-human IgM was used as the secondary antibody.

ABO plasma testing

Group A, B, or O reagent RBCs were added to three separate wells, followed by patient plasma. The mixtures were washed four times, and PE-labeled anti-human IgM was added. After incubation the samples were washed and acquired as above.

RBC alloantibody testing

RBCs from a three-cell screening panel were added to three wells followed by patient plasma and PEG solution (molecular weight 3350, Sigma-Aldrich, St. Louis, MO). Addition of PEG was found to speed the formation of complexes between patient IgG antibodies and screening cells. The mixtures were incubated and washed and a PE-conjugated anti-human IgG antiserum was added before FC analysis.

CAT and standard tube testing

Gel-column cards were used to perform forward and reverse ABO grouping and alloantibody screens with manufacturer's methodologic instructions (Ortho-Clinical Diagnostics). Standard tube testing was performed according to usual procedures including alloantibody detection by the LISS-IAT and PEG-IAT.⁴ All samples were tested and interpreted without knowledge of results from other assays.

Statistical analysis

The chi-square test was used, with significance demonstrated at the 0.05 level.

RESULTS

Antibody staining of RBCs in microtiter filter plates

We previously reported the use of 96-well microtiter filter plates for RBC staining before FC.² During washing steps,

negative vacuum pressure was applied to the plate to separate the RBCs (with attached antibodies) from the washing solution. Under the proper conditions, RBCs evenly spread over the filter surface, minimizing agglutination and thus improving FC data acquisition. During attempts to automate this method, however, different samples sometimes required vacuum application for different amounts of time. If the vacuum time was too short washing was incomplete, whereas application of vacuum for excessive time led to overdrying of the RBCs. In either case, assay reproducibility was compromised (data not shown).

In the course of those studies, it was found that brief (60 sec) low-speed centrifugation (400 × g) evenly pelleted the RBCs over the filter surface without aggregation, even in the presence of agglutinating antibodies (Fig. 1). The RBCs adhered to the filter sufficiently well for the supernatant to be removed by pipette with minimal RBC loss. The adherent RBCs were readily resuspended with saline during washing steps. These centrifugation conditions were insufficient to force fluid through the filters. If the centrifugation time or speed were significantly reduced, RBCs were not adequately pelleted and were lost when the supernatant was removed by pipette. In contrast, centrifugation at markedly higher speeds or for longer times compacted the RBCs at the edge of the well leading to large agglutinates in the presence of some antibodies. No centrifugation conditions could be identified that worked

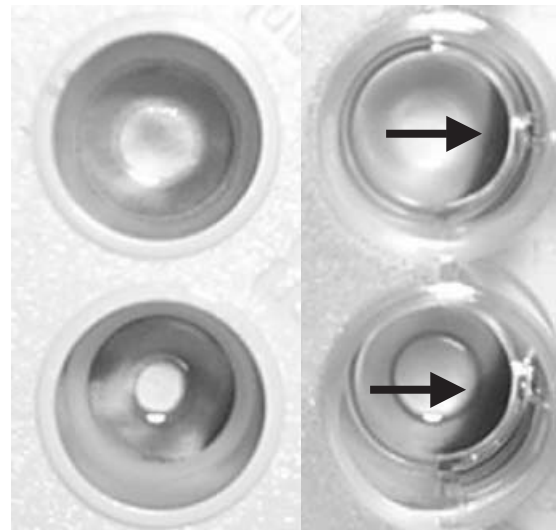


Fig. 1. Examples of staining reactions where the same samples were centrifuged in filter plates (left column) and standard plastic microtiter plates (right column) under otherwise identical conditions. Note that the RBCs formed crescent-shaped pellets in standard plates (arrows), enhancing antibody-mediated agglutination. In contrast, RBCs were well dispersed in filter plates, with minimal accompanying agglutination.

adequately with standard 96-well microtiter plates (without the filters; Fig. 1), indicating that interactions between the RBCs and filter membrane were important to the success of this procedure. This method is the subject of a patent application.

Immunohematology testing with the PCA

The modified washing procedure was incorporated into a staining protocol similar to that previously described (Table 1).² Because the PCA system is not designed to detect FITC fluorescence, the protocol was modified by replacing FITC-labeled anti-D with unlabeled human IgM anti-D followed by a PE-labeled secondary antibody. Typically, eight samples could be stained for ABO group, D type, and alloantibody screen in 30 to 45 minutes. Blood samples from hospitalized patients (n = 520) were tested for ABO group and D type by staining followed by manual data acquisition on the PCA. Samples were also tested in parallel by CAT, which was the original clinical test of record for these patient samples. Discrepant results were resolved by tube testing. Among the samples were 84 (16%) with previously documented reverse typing reaction strengths of 2+ or less, 10 (2%) from HPC transplant recipients, 85 (16%) with mixed-field reactions, and 14 (2.7%) with autoantibodies.

Trained technologists blinded to sample identity scored FC results by manual visual analysis of the resulting histograms. A total of 513 of 520 samples (98.7%) were correctly typed by FC, compared to 98.8 percent by CAT ($p > 0.05$). Representative histograms are shown in Fig. 2. (MFI for the assays shown in Figs. 2-5 are shown in Table 2.). One weak D typing reaction was not detected by FC, but was detected by CAT. Because this was the first weak D reaction encountered during the study, this sample was used to further optimize the D typing protocol. Testing was repeated with sequential twofold increases in anti-D titer until the weak D antigen was readily detected. The resulting concentration of anti-D was used for all subsequent samples. Six samples had no type determined (NTD) by both methods (1.2%). The NTD result was usually due to the absence of the expected isohemagglutinins. The PCA detected 84 of 85 (98.8%) mixed-field reactions compared

to 71 of 85 (83.5%) for CAT ($p < 0.005$). The FC method also detected five cold autoantibodies that were not identified by CAT.

These two methods were next used to detect alloantibodies in 215 patient samples, 50.7 percent of which had previously documented antibodies. The set of alloantibodies had a wide range of clinically relevant specificities including D (n = 10 examples), E (n = 32), K (n = 26), and Fy^a (n = 13). Antibody strengths ranged from "weak" to 4+. Seventeen samples had multiple 2-4 alloantibodies. Five additional samples with room-temperature-reactive anti-M were also included. The overall accuracy of FC with the PCA was 98.6 percent (212 of 215 samples). Representative histograms are shown in Fig. 3. FC did not detect two alloantibodies, one with E specificity and the other a high-titer low-avidity antibody of undetermined specificity. One sample also had an extraneous positive reaction by FC; this sample did not have alloantibodies by LISS-IAT.

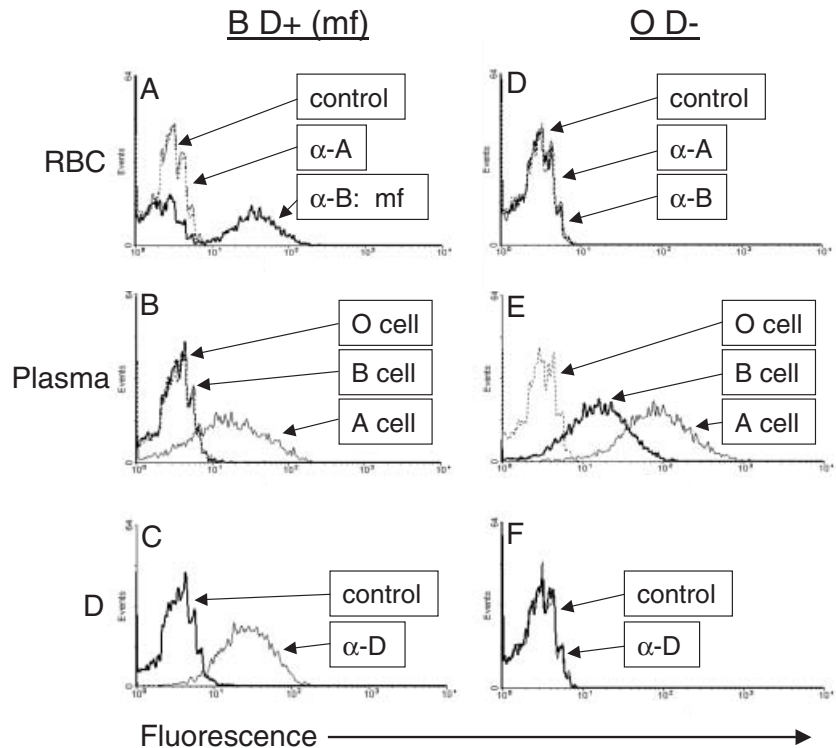


Fig. 2. Representative results for ABO grouping and D typing by FC with the Guava. Samples from a group B, D+ (A-C) and a group O, D- (D-F) patient were subjected to RBC (A, D) and plasma testing (B, E) as well as typing for D (C, F). For each panel, fluorescence intensity is displayed on the x-axis (log scale) and the number of RBCs with a given level of fluorescence is on the y-axis. Samples were scored as positive or negative as described in the text. Note that the group B, D+ patient was recently transfused with Group O, D+ RBCs. During RBC testing a mixed-field (mf) reaction was observed with anti-B antibodies (A, bold line): the leftmost peak coincided with the negative control (control, dotted line) and was composed of the transfused O, D+ cells; the right-shifted population was positive for B antigen and represents the patient's RBCs.

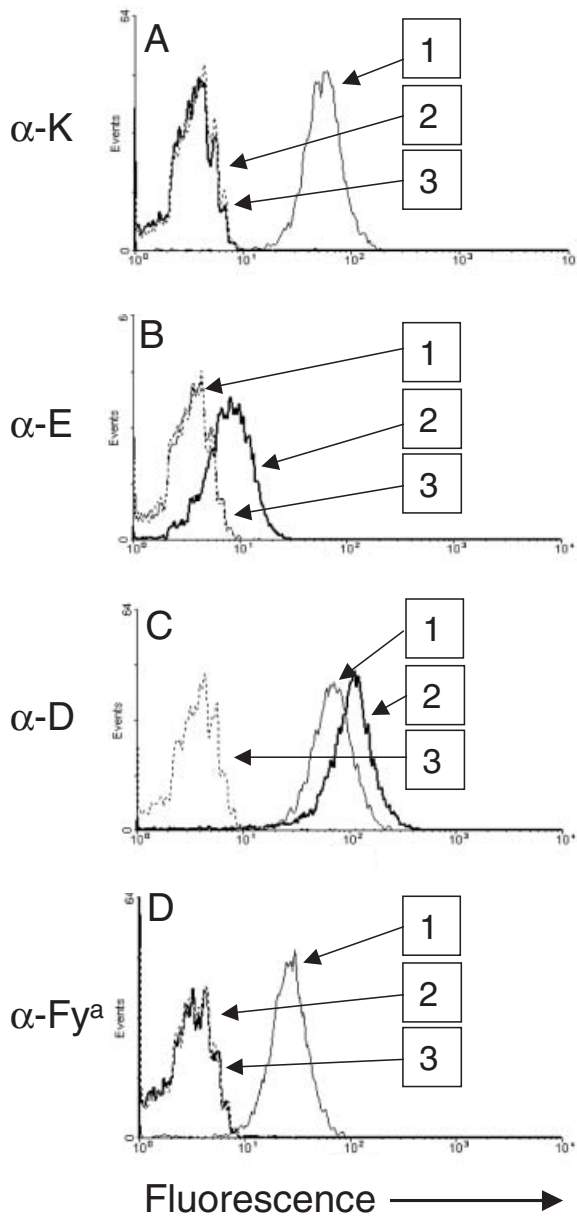


Fig. 3. Representative results for RBC alloantibody screens with the PCA. Results from samples from four patients with anti-K, anti-E, anti-D, and anti-Fy^a alloantibodies are displayed in A-D, respectively. Screening cell phenotypes: Cell 1, D+E-, Fy(a+), K+ Cell 2, D+E+, Fy(a-), K-; Cell 3, D-E-, Fy(a-), K-.

All five anti-M were readily detected, even with secondary anti-human IgG. CAT did not detect eight alloantibodies that were detected by FC, for an overall accuracy of 207 of 215 samples (96.3 percent; $p > 0.05$ vs. FC). One of the antibodies that was not detected by CAT was subsequently identified as anti-K by LISS-IAT. Although the seven remaining antibodies were also detected by LISS-IAT, insufficient volume remained in the samples to determine antibody specificity.

Data acquisition with the automated PCA-96

An additional 229 samples were tested for ABO group and D type by FC with the recently introduced PCA-96 cytometer, which has 96-well microtiter plate handling capabilities. Typically, eight samples were simultaneously stained in a 96-well plate for ABO and D and the plates were then automatically acquired on the PCA-96 without operator intervention. FC accurately typed 226 of 229 samples (98.7%) compared to 223 of 229 samples by CAT (97.4 percent; $p > 0.05$) (Fig. 4). Two samples were NTD by FC (0.9%) compared to 6 NTD by CAT (2.6%). One of the 229 samples was problematic for both FC and CAT. The patient's blood type was A_{subgroup}B+ with an anti-A₁. CAT did not detect the anti-A₁ and thus "incorrectly" typed the patient as AB+, rather than typing accurately as NTD. In contrast, FC missed the A_{subgroup} antigen but detected the anti-A₁, thus incorrectly typing the patient as B+. In subsequent studies the titer of anti-A used for FC was further optimized to detect this A_{subgroup} antigen. There were three extraneous mixed-field reactions detected by FC that were not confirmed by tube testing; CAT missed four authentic mixed-field reactions.

The FC method with the PCA-96 was then used to identify alloantibodies in 213 patient samples, 56.3 percent with previously identified antibodies. FC was accurate for 211 of 213 samples (99.1%) (Fig. 5), compared to 212 of 213 for CAT (99.5 percent; $p > 0.05$). FC missed two anti-E that were detected by LISS-IAT, but not by PEG-IAT. CAT detected a reagent-dependent antibody in one sample that was appropriately negative by LISS-IAT.

Further analysis of FC assay performance

Based on these promising results, we initiated a more detailed analysis of FC assay performance with the PCA-96. Voak⁵ has discussed a number of methods to validate the sensitivity of new immunohematology assays that can be grouped into three general approaches: 1) testing of weak antibodies with reagent RBCs heterozygous or homozygous for the corresponding antigen (this method also examines requirements for antigen dosage on screening RBCs); 2) endpoint titration of anti-D, -K, and -Fy^a alloantibodies; and 3) detection of weak ABO incompatibilities with group B plasma and A₂B RBCs. With assays that use microtiter plates, such as the present FC method, reproducibility can be evaluated by replicate testing of weak reactions throughout different well positions of the microplate.⁵ Although Voak⁵ also discussed the importance of performing clinical trials at several sites, this evaluation is beyond the scope of the present study.

Anti-E, -K, -Jk^a, -c, and -Fy^a alloantibodies, all of which were 2+ by both LISS-IAT and CAT methods, were tested against screening RBCs that were heterozygous or homozygous for the corresponding antigens with FC. As expected, reactions were stronger (higher MFIs) with

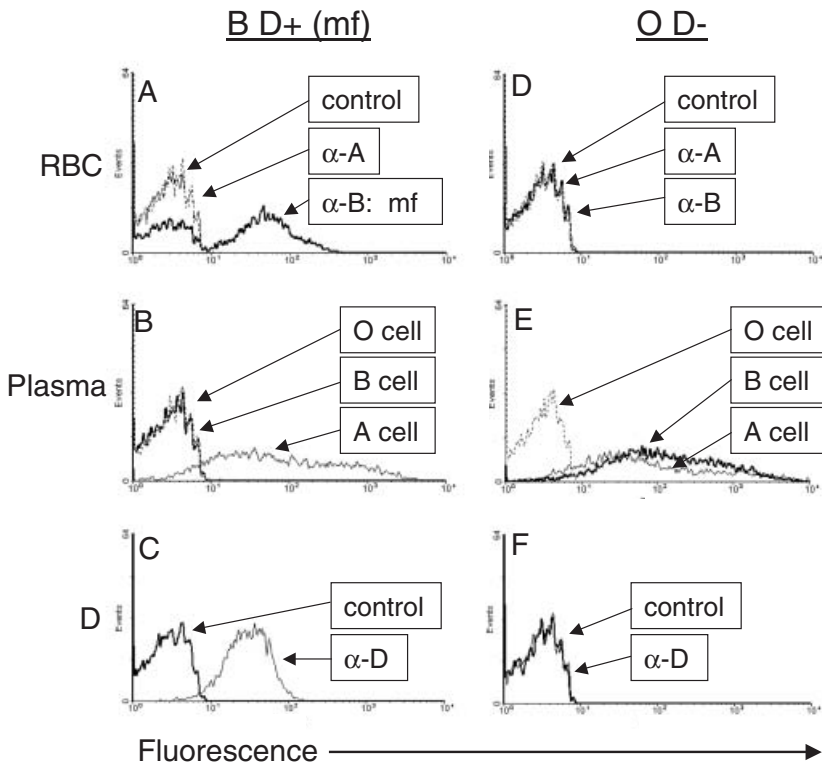


Fig. 4. Representative results for ABO grouping and D typing with automated sample acquisition on the PCA-96. Blood samples from a Group B, D+ (A-C) and a Group O, D- (D-F) patient were subjected to RBC (A, D) and plasma testing (B, E) as well as typing for D (C, F). Note that although the patients are different from those represented in Fig. 2, this Group B, D+ patient also received Group O, D+ transfusions, resulting in a mixed-field reactions when RBCs were tested with anti-B.

homozygous compared to heterozygous RBCs (anti-K was only tested with heterozygous cells). All heterozygous cells were clearly positive, with MFIs between 2.4- and 17.7-times greater than when the antibodies were tested in parallel with antigen-negative RBCs (data not shown). To better examine assay sensitivity, each antibody was retested with a panel of 8 to 10 different heterozygous screening cells. All antibodies were readily detected, as illustrated by representative data in Fig. 6. Despite the fact that a panel of different screening cells was used, the MFIs were highly reproducible. The average of the MFIs for the 8 to 10 antigen-heterozygous screening cells (\pm SD; range) were anti-Jk^a, 12.8 (\pm 4.2; 6.8-18.5); anti-E, 38.0 (\pm 4.7; 29.2-42.4); anti-K, 22.1 (\pm 3.1; 18.6-28.8); anti-Fy^a, 16.3 (\pm 3.8; 9.8-22.0); and anti-c, 5.8 (\pm 1.6; 3.93-8.03). The MFIs of the antigen-negative cells ranged from 2.04 to 2.53 and thus did not overlap the range for the antigen-heterozygous cells. All of the antigen-heterozygous cells would have yielded the correct test result with a semiautomated algorithm described previously where samples are scored as negative if their MFI is less than the average + (2 \times SD) for negative controls (3.53) and positive if greater than the

average + (10 \times SD) for negative controls (9.13) and require visual interpretation in the intermediate range (3.53-9.13).² By use of this approach, 35 of the samples scored positive, 0 scored negative, and 11 required visual interpretation.

To directly compare the sensitivity of FC with CAT for alloantibody screens, patient plasmas containing anti-Fy^a, -D, or -K alloantibodies were subject to sequential 1-in-2 dilutions and tested in parallel with the two techniques. By use of screening cells with homozygous antigen expression, anti-Fy^a was detected up to a 1:256 dilution with FC compared to 1:128 by CAT (read both manually and by automated reader). The anti-D was detected at 1:4 by FC, but only at a 1:2 dilution or undiluted when CAT was read manually or by automated reader, respectively. By use of heterozygous screening cells, anti-K was detected at 1:32 by the PCA-96, but only 1:4 by CAT with either manual or automated reading. Thus, FC was usually as sensitive, and in one case more sensitive, than CAT in detecting weak alloantibodies in diluted plasma samples.

To compare assay sensitivity for cross-matching, 54 archived plasma samples from Group B patients were tested against incompatible RBCs from a single A₂B donor. Consistent with previous studies,⁶ only 21 of 54 samples (39%) were incompatible by CAT, compared to 50 of 54 samples (93%) by immediate spin. FC with the PCA-96 with the standard reverse typing protocol (Table 1) detected 51 of 54 incompatibilities (94%). Thus, FC and saline tube testing were less likely to miss weak ABO mismatch than CAT ($p < 0.0001$).

To evaluate the reproducibility of FC, a plasma sample containing a 2+ anti-E alloantibody (strength determined by CAT and LISS-IAT) was tested with E+ (homozygous) and E- reagent RBCs in replicates of 16 each. The 32 reactions were performed simultaneously and were distributed uniformly throughout the microtiter plate. The average MFI for the positive samples was 56.7, with a SD of 9.1 (CV, 16.1%) (Fig. 7A). For the E- RBCs, the average MFI was 2.4 (SD = 0.05) with a CV of 2 percent (Fig. 7B). Reproducibility was further tested with a plasma sample with a "weak" (by CAT and LISS-IAT) anti-Fy^a. The sample was frozen in 10 aliquots, which were then thawed and tested on 10 sequential days. Each replicate was clearly positive (representative data in Fig. 7C and D). The average MFI (and SD) for the antigen-negative RBCs was

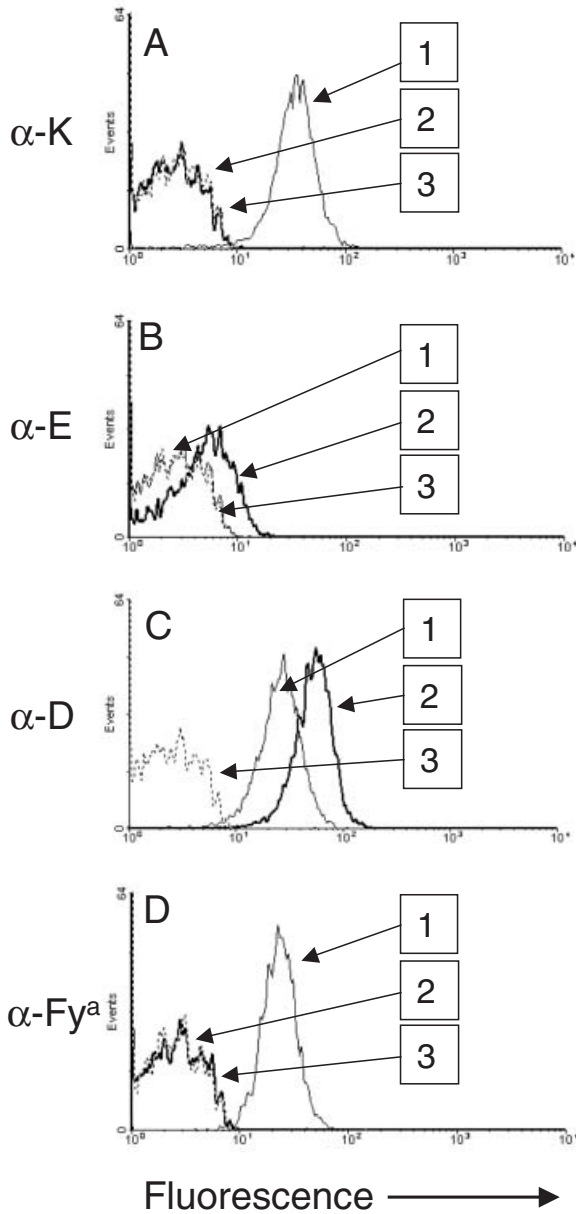


Fig. 5. Representative results for RBC alloantibody screens analyzed on the PCA-96. Samples from four patients with anti-K, anti-E, anti-D, and anti-Fy^a alloantibodies are displayed in A-D, respectively. These samples are from different patients from those shown in Fig. 3. Screening cell phenotypes: Cell 1, D+E-, Fy(a+), K+ Cell 2, D+E+, Fy(a-), K-; Cell 3, D-E-, Fy(a-), K-.

3.4 (±0.2; range, 3.0-3.8), whereas the value for the antigen-positive screening cell was 4.7 (±0.3; range, 4.0-5.0). These ranges are artificially expanded because on days where the positive samples had the highest (or lowest) MFIs the negative samples also showed their highest (or lowest) MFIs, consistent with subtle differences in laser setup or cytometer performance from day to day. Thus, these exaggerated ranges are not relevant to the typical

testing situation where positive and negative samples run on the same day are compared against one another. Nevertheless, with this weak anti-Fy^a, the MFI for all antigen-positive samples was clearly greater than 3.8 (the average MFI of the negative samples + (2 × SD) over the 10-day period). In summary, the FC method demonstrated good well-to-well and day-to-day reproducibility, which were problems previously associated with other immunohematology testing systems.⁵

Following testing by CAT, reactions are relatively stable, allowing the gel columns to be reevaluated up to 5 days later by another worker, although this feature is rarely clinically utilized. Likewise, the histograms resulting from our FC testing can be stored digitally and reevaluated indefinitely. To determine whether antibody-stained RBCs are sufficiently stable to be reacquired on the cytometer at a later time if necessary, samples were stained and analyzed with the standard FC procedure, stored overnight, and then acquired on the cytometer a second time approximately 18 hours later. All positive and negative reactions were clearly distinguished when acquired initially and at 18 hours later. As expected, there were decreases in fluorescence staining intensities. Positive reactions showed an average decrease in MFI of 24.9 percent (±6.0%), compared to a 12.3 percent (±2.9%) decrease for negative samples. The stability of antibody-stained RBCs should facilitate reevaluation of blood typing reactions for problematic samples as well as protocols for batch testing of samples.

DISCUSSION

One of the primary impediments to the use of FC for blood typing is the propensity of RBCs to aggregate in the presence of agglutinating antibodies.⁷⁻⁹ RBC aggregates cannot be analyzed in standard cytometers, which are designed to accommodate single-cell suspensions. Although our filtration method² prevented agglutination by spreading RBCs over the filter surface, it proved difficult to automate. For reasons that are not clear, different samples stained in the same plate sometimes filtered at different rates under identical vacuum pressure. Slowly filtering samples were not adequately washed, whereas RBCs were left dry in rapidly filtering samples. Both situations compromised assay accuracy, sensitivity, and reproducibility.

As an alternative to vacuum, fluid can be expressed from filter plates under centrifugal force. This approach was found to be no more effective than vacuum for our assay. Nevertheless, these studies led to the serendipitous discovery that low-speed centrifugation, although not expressing fluid from the wells, did evenly distribute the RBCs over the filter surface without agglutination such that the supernatant could be quantitatively removed by pipetting without significant RBC loss. Optimal centrifugation settings were found to be 400 × g for 60 seconds, although modest variations were also acceptable. No con-

TABLE 2. MFIs for Figs. 2-5

Figure	Sample	Reagent 1	MFI	Reagent 2	MFI	Reagent 3	MFI	Interpretation
2A	RBCs	α -A	2.64	α -B	38.24*	Control	2.59	Group B, m†
2B	Plasma	A cell	14.83	B cell	3.18	O cell	3.16	Group B
2C	RBCs	α -D	25.39	Control	3.34			D+
2D	RBCs	α -A	2.62	α -B	2.60	Control	2.54	Group O
2E	Plasma	A cell	80.58	B cell	15.07	O cell	2.76	Group O
2F	RBCs	α -D	2.61	Control	2.67			D-
3A	Plasma	SC1‡	52.15	SC2	3.31	SC3	3.46	α -K
3B	Plasma	SC1	3.52	SC2	11.84	SC3	3.18	α -E
3C	Plasma	SC1	60.76	SC2	92.01	SC3	3.55	α -D
3D	Plasma	SC1	24.69	SC2	2.91	SC3	2.89	α -Fy ^a
4A	RBCs	α -A	2.46	α -B	53.90*	Control	2.50	Group B, mf
4B	Plasma	A cell	68.62	B cell	2.56	O cell	2.65	Group B
4C	RBCs	α -D	28.81	Control	2.53			D+
4D	RBCs	α -A	2.62	α -B	2.58	Control	2.55	Group O
4E	Plasma	A cell	69.16	B cell	120.40	O cell	2.62	Group O
4F	RBCs	α -D	2.61	Control	2.60			D+
5A	Plasma	SC1	31.63	SC2	2.30	SC3	2.35	α -K
5B	Plasma	SC1	2.41	SC2	4.41	SC3	2.34	α -E
5C	Plasma	SC1	24.99	SC2	51.71	SC3	2.24	α -D
5D	Plasma	SC1	22.87	SC2	2.29	SC3	2.23	α -Fy ^a

* MFI for right-shifted fluorescence peak.

† mf = mixed-field.

‡ SC = screening cell.

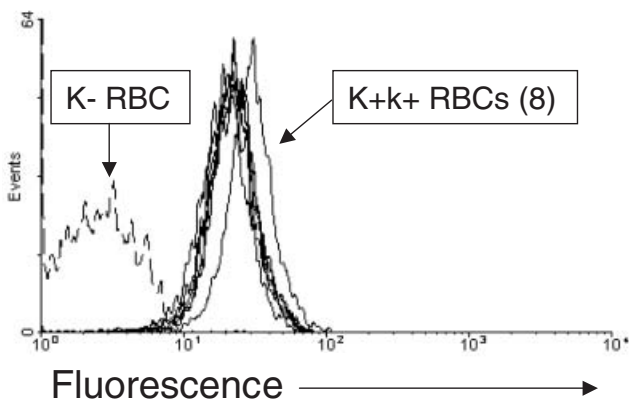


Fig. 6. A representative plasma sample containing a 2+ anti-K was tested against a K- screening cell, as well as eight different heterozygous K+k+ screening cells, and read on the PCA-96. All nine reactions are displayed on the same histogram. Note that the eight K+k+ screening cells have similar staining intensities (average MFI, 22.1 + 3.1; CV, 14.2%), and all are clearly distinguishable from the K- cell samples (MFI, 2.3).

ditions were found that could similarly pellet RBCs with typical plastic microtiter plates without filters, because RBCs either were not pelleted or were compacted at the edge of the well. These findings suggest that interactions between the RBCs and filter material are critical to the success of this technique. Over the course of testing more than 1000 samples, this washing method has proven to be extremely reliable and reproducible, without the sample-to-sample variations seen with the vacuum filtration approach.

The second improvement is the modification of FC typing to be compatible with the latest generation of benchtop cytometers. The PCA has approximately the footprint of a laptop computer (8.5 inches high x 12.56 inches wide x 14.25 inches deep) and can perform forward-scatter and two-color fluorescence analysis with 580- and 675-nm filters (cytometer can be seen at <http://www.guavatechnologies.com/main/index.cfm>). In contrast to standard-flow cytometers that utilize a continuous stream of sheath fluid for sample injection, the PCA is a capillary cytometer. It uses a precision syringe with stepper motor to accurately measure and deliver a sample through a capillary for analysis. Although the ability to measure sample volume was not needed for the present studies, we are taking advantage of this feature in studies with the PCA for other blood bank tests that require cell quantitation. The more recently introduced PCA-96 has similar optics to the PCA but has, in addition, an integrated microtiter plate handler allowing 96 samples to be automatically acquired without operator intervention. Because the microplate interface is similar to typical ELISA readers, and compatible with robotic liquid handlers, we are now integrating the PCA-96 into a completely automated workstation.

The results of these studies show that the FC method with filter plate centrifugation and capillary cytometers works as well as CAT for immunohematology testing. FC with the PCA accurately determined ABO group and D type for 98.7 percent of 520 samples, compared to 98.8 percent for CAT. Similarly, ABO and D testing with the PCA-96 was accurate for 98.7 percent of 229 samples, compared to 97.4 percent for CAT. NTD rates for FC were

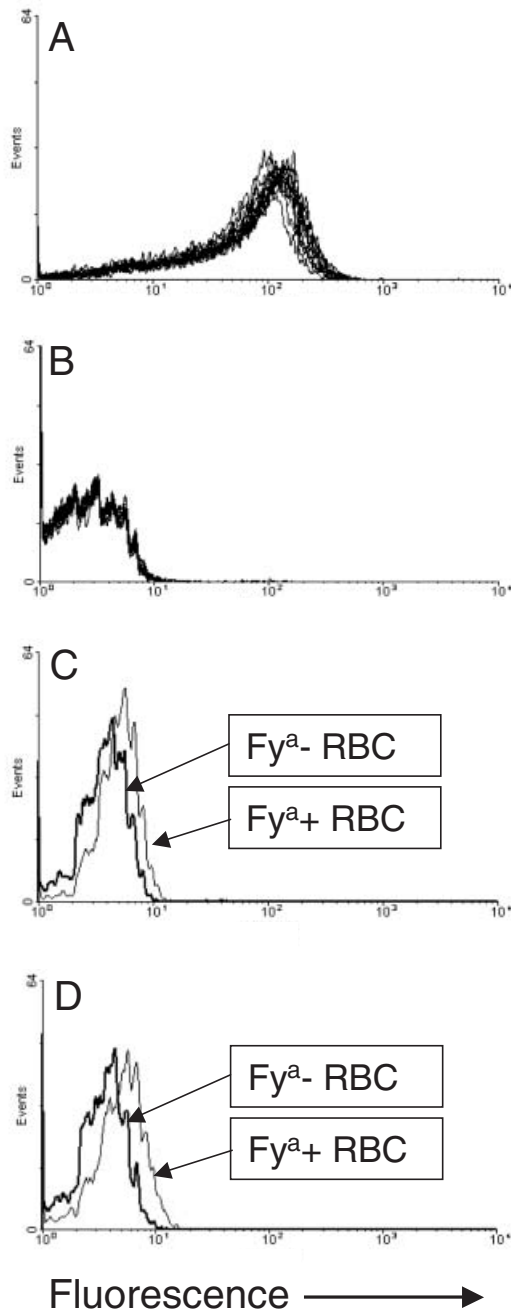


Fig. 7. FC assay reproducibility was investigated in two ways. A plasma sample containing a 2+ anti-E was tested against homozygous antigen-positive and antigen-negative RBCs in replicates of 16 each. The 32 reactions were randomly distributed throughout a single 96-well plate for simultaneous staining and acquisition. The positive (A) and negative (B) reactions are overlaid in the histograms. In addition, a plasma sample containing a weak anti-Fy^a antibody was frozen in 10 aliquots that were then thawed on sequential days and assayed against an antigen-positive and antigen-negative screening cell. Representative data from the aliquots thawed on the first (C) and 10th (D) days are shown. These data demonstrate good well-to-well (A, B) and day-to-day (C, D) reproducibility of the FC method.

1.2 percent (PCA) and 0.9 percent (PCA-96), which are well below the 6 percent rate mandated by recent FDA guidance.¹⁰ FC accurately performed alloantibody screens for 98.6 percent of 215 samples (PCA) and 99.1 percent of 213 samples (PCA-96). The accuracy of CAT with the same specimens was 96.3 and 99.5 percent, respectively. Although there were no differences between the performance of FC and CAT in the above assays, FC was superior to CAT for detecting mixed-field reactions ($p < 0.005$).

The antibody concentrations used for ABO grouping and D typing were determined from initial studies as the lowest concentrations that could sensitively detect A, B, and D antigens in a small panel of patient and reagent RBCs. This approach was based on the hypothesis that higher concentrations may lead to more agglutination. During the course of testing patient samples, FC did not accurately identify one weak D (PCA) and one A_{subgroup} (PCA-96) antigen. Although missing this weak A_{subgroup} antigen during clinical testing would not have led to an ABO-mismatched transfusion, these samples were nonetheless used as an opportunity to further optimize antibody concentrations. The resulting increased antibody concentrations could readily detect these weak antigens and were incorporated into our protocol as the standard method. It is likely that over the course of future work, further minor modifications will further improve accuracy.

We subjected the optimized FC assay, with the PCA-96, to a series of studies described by Voak⁵ to monitor immunohematology assay performance. These studies demonstrated that: 1) FC can sensitively detect moderate strength (2+) anti-E, -K, -Jk^a, -c, and -Fy^a alloantibodies with both homozygous and heterozygous target RBCs; 2) the reproducibility of FC in detecting interactions between these alloantibodies and antigen-heterozygous screening cells was excellent, even when comparing different cells against one another; 3) with endpoint dilution of alloantibody-containing samples FC was as sensitive, and in one case more sensitive, than CAT; 4) FC was as accurate as immediate spin tube testing, and markedly more accurate than CAT in detecting weak ABO-mismatches between Group B plasma samples and A₂B RBCs; and 5) FC showed good well-to-well and day-to-day reproducibility, even with plasma samples with weak alloantibodies. Although FC was more sensitive than CAT in some assays, the results taken in aggregate demonstrate that this increased sensitivity does not lead to numerous extraneous reactions. Antibody-stained RBCs were also found to be relatively stable, which would allow samples to be stained in large batches, stored, and then acquired without a significant decrement in assay performance. This form of batch processing may prove useful in the blood center environment.

Manual visual interpretation was used to score positive and negative results in the present studies. A simple

alternative method² that would allow computer interpretation of most results is to use the replicate negative control reactions performed on the same day as the patient samples: IgM isotype antibody for ABO RBC testing, reagent O RBCs for ABO serum testing, removal of primary antibody for D typing, and normal serum for RBC alloantibody testing. Because these control reactions are highly reproducible, averaging the MFIs for a panel of replicate controls yields quantitative cutoff values. In these studies, typical average (\pm SD) MFIs for ABO RBC testing, serum testing, D typing, and alloantibody testing were 2.72 (\pm 0.05), 2.98 (\pm 0.06), 2.76 (\pm 0.05), and 2.13 (\pm 0.7), respectively. In these preliminary studies, reactions could be accurately scored as negative if their MFI was less than the average negative control MFI + ($2 \times$ SD) and positive if it was greater than the average control MFI + ($10 \times$ SD) (data not shown). Intermediate values (usually less than 15% of samples) required visual interpretation.

In summary, we have demonstrated accurate and sensitive immunohematology testing by FC including assay optimizations that present a significant advance over our previous work.² Although these studies were performed with samples from hospitalized patients, this technology should also be applicable to testing donor samples in blood centers. We are currently constructing a fully automated platform with a robotic pipetter, automated centrifugation, the PCA-96 cytometer, and computerized data analysis and interpretation. The availability of this automated workstation will allow us to address other important considerations, including capital equipment costs, disposable and reagent costs, sample turnaround time and throughput, and reproducibility of assay performance in other transfusion service and blood center environments. Furthermore, the availability of this workstation will allow us to investigate complete automation of other pretransfusion tests, including quantitation of residual WBCs after filtration, PLT cross-matching, bacterial detection, and quantitation of fetal-maternal hemorrhage, all of which have been previously performed by traditional flow cytometry.¹¹⁻¹⁹

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