

An automatable format for accurate immunohematology testing by flow cytometry

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

BACKGROUND: Current immunohematology testing methods have limitations including cost, throughput, and adaptability to automation. Furthermore, current automated and semiautomated workstations cannot accommodate many other tests relevant to blood transfusion.

STUDY DESIGN AND METHODS: Authentic clinical samples from hospitalized patients were tested for ABO group, D type, and presence of RBC alloantibodies by column agglutination technology (CAT), standard tube methods, and a recently developed flow cytometry (FC) technique. Included were challenging samples with rouleaux, autoantibodies, mixed-field reactions, and weak antibodies. Antibody staining of RBCs for FC was initially performed in test tubes and subsequently in microtiter filter plates interfaced with a vacuum manifold.

RESULTS: When antibody staining was performed in tubes, FC testing determined the correct ABO group and D type for 99.1 percent of 222 clinical samples, as compared to accuracies of 91.9 percent for CAT and 95.0 percent for standard tube testing. FC testing also detected 99.5 percent of clinically relevant RBC alloantibodies in 239 patient samples, as compared to 98.9 percent for CAT and 94.7 percent for LISS-IAT. Using the FC filter plate technique, 104 of 109 samples (95.4%) were correctly typed for ABO and D (the remaining five samples were read as "no type determined" due to RBC and serum testing discrepancies), and RBC alloantibodies of the IgG and IgM classes were correctly identified in 98.3 percent of samples.

CONCLUSIONS: Optimized FC testing methods that are comparable in accuracy to standard CAT and tube methods are described. When used with filter plates, this methodology should allow rapid and cost-effective immunohematology testing of both patient and donor samples in an automated workstation format. The same workstation should support automation of other pre-transfusion assays that can be analyzed by FC.

Before RBC transfusion, blood samples from donors and recipients are tested to determine their ABO group and D type, to detect unexpected RBC alloantibodies, and to confirm cross-match compatibility. These immunohematology assays represent a subset of pretransfusion testing, which also includes infectious disease serology, counting of residual WBCs in filtered blood components, platelet cross-matching, and additional assays.

Routine immunohematology testing has historically been performed by centrifuging RBCs and antibodies in test tubes followed by a visual determination of the extent of RBC agglutination. Although standard tube testing is still considered by many to be the de facto "gold standard" for immunohematology, this assay is labor intensive, not amenable to automation, and the results are operator dependent. With a decline in laboratory technical staff trained to perform standard tube testing, this assay is becoming increasingly impractical.

Column agglutination technology (CAT) (ID-Micro Typing System, Ortho-Clinical Diagnostics, Raritan, NJ)¹ and solid-phase RBC adherence assays (SPRCA) (Capture-R, Immucor, Norcross, GA)² overcome some deficiencies of standard tube testing. They eliminate tedious washing

ABBREVIATIONS: CAT = column agglutination technology; FC = flow cytometry; NTD = no type determined; SPRCA = solid-phase RBC adherence assays; TAT = turn-around time.

From the Transfusion Medicine Program, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia.

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

This work was supported by the NIH (R41 HL66751), Georgia Research Alliance Faculty Research Commercialization Program, and the Resource Forum New Enterprise Award.

Received for publication November 25, 2002; revision received February 20, 2003, and accepted February 24, 2003.

TRANSFUSION 2003;43:918-927.

steps (CAT), have semiobjective readouts, and have been commercialized as automated or semiautomated workstations. However, their reagent and disposable costs are significantly greater than for standard tube testing, throughput can be slow and not suited for emergency testing, and their performance in detecting weak antibody-antigen interactions has been questioned.³ Moreover, these methodologies are not adaptable to many other pretransfusion tests.

We sought an alternative technology for immunohematology testing that is accurate, rapid, cost effective, and compatible with other assays. We describe here the basis for a multifunctional immunohematology workstation that uses flow cytometry (FC) for assay readout. FC has previously been used for specialized immunohematology applications including quantitation of RBC antigen density,^{4,5} detection of antigen variants,⁶ and quantitation of antibody binding to RBCs.⁷ This technology has also been used for quantitation of residual WBCs after filter WBC reduction,^{8,9} detection of fetomaternal hemorrhage,¹⁰ platelet cross-matching,¹¹⁻¹³ and other applications.¹⁴⁻¹⁶ Despite this wide range of applications, use of FC for standard immunohematology testing has been hampered by the propensity of large RBC agglutinates to form during antibody staining.^{4,16,17} Because FC requires sampling of individual cells through small-bore apertures, RBC agglutinates can have adverse effects, including blocking of the flow cell. In the present report, we describe an approach that minimizes agglutination during the staining procedure and thus allows accurate detection of RBC-antibody interactions by FC. This technique should make automated FC pretransfusion testing feasible on a cost-effective and high-throughput basis.

MATERIALS AND METHODS

Samples, reagents, and supplies

Samples were obtained from discarded blood specimens submitted to the Emory University Hospital Blood Bank.

This protocol was approved by the Emory University Human Investigations Committee. Standard blood typing reagent antibodies and RBCs were obtained from Ortho-Clinical Diagnostics (Raritan, NJ) or Immucor (Norcross, GA). PE-labeled secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Directly fluoresceinated anti-D was prepared by labeling anti-D containing human gammaglobulin fraction (WinRho SDF; Cangene, Winnipeg, Canada) with FITC using a commercially available kit (Pierce Biotechnology, Rockford, IL). Antibody and RBC working dilutions were determined through empirical studies and were made in 0.9-percent normal saline. A variety of 96-well microtiter filter plates were tested, although most studies utilized plates with 0.2- μ m polyvinylidene difluoride filters (Corning, Acton, MA). The bottom of each well is perforated and covered by a filter such that the application of vacuum beneath the plate draws fluid through the filter, while material larger than the nominal pore size of the filter is retained in the microtiter well.

Flow cytometry staining

The fluorescent staining protocol for FC is summarized in Table 1. In initial studies, RBC-antibody manipulations were performed in round-bottom 12 \times 75-mm polystyrene or 10 \times 75-mm glass tubes. For all assays, except as noted below, a minimum of 20,000 events were acquired on a flow cytometer (Becton-Dickinson FACScan, San Jose, CA).

ABO RBC grouping

Patient RBCs were added to two tubes, followed by the addition of mouse anti-A to one tube and anti-B to the other (BioClone Anti-A and Anti-B murine monoclonal IgM blends; 1-in-100 to 1-in-1000 dilutions from stock; Ortho-Clinical Diagnostics). For some studies, purified mouse IgM was used as an isotype control (Sigma-Aldrich; St. Louis, MO). After incubation, tubes were centrifuged at

TABLE 1. FC immunohematology protocols for tube and filter plate tests

Step	Assay			
	RBC A,B	RBC Rh(D)	Serum α -A,B	Alloantibodies
1. Add RBCs	2% patient RBCs (25 μ L)	2% patient RBCs (25 μ L)	3% A, B, O RBCs (30 μ L)	3% screening RBCs (25 μ L)
2. Add primary Ab	Mse α -A or α -B (50 μ L)	FITC Hum α -D (50 μ L)	Patient plasma (50 μ L)	Patient plasma (50 μ L)
3. Add potentiator	NA	20% PEG (100 μ L)	NA	20% PEG (100 μ L)
4. Incubate	RT \times 2 min	37°C \times 5 min	NA	37°C \times 5 min
5. Wash	Saline \times 4 (200 μ L)	Saline \times 4 (200 μ L)	Saline \times 4 (200 μ L)	Saline \times 4 (200 μ L)
6. Add secondary Ab	PE- α -Mse IgM (100 μ L)	NA	PE- α -Hum IgM (100 μ L)	PE- α -Hum IgG (100 μ L)
7. Incubate	RT \times 5 min	NA	RT \times 5 min	RT \times 5 min
8. Wash	Saline \times 2 (200 μ L)	NA	Saline \times 2 (200 μ L)	Saline \times 2 (200 μ L)
9. Disperse RBCs		Vigorous pipetting and vortexing (as necessary)		

1000 × g for 20 seconds to pellet RBCs. The supernatants were discarded, saline was added, and the samples were centrifuged again for a total of four washes. PE-labeled antimouse IgM was added, the RBC pellets were dispersed by vigorous repetitive pipetting and vortexing, and the mixtures were incubated followed by two washes. Any remaining RBC aggregates were dispersed as above. Forward- and side-scatter gates were set to select intact RBCs for analysis (generally >90% of all events). Fluorescence in FL-2 channel was used to quantitate PE-labeling of RBCs. Replicate negative controls were performed, and the results were used to determine the threshold for positive reactions.

D typing

Patient RBCs were added to two tubes followed by either FITC-conjugated human anti-D (1 in 100) or saline (control). PEG (molecular weight 3350; Sigma-Aldrich) was added as a potentiator. The mixtures were incubated, washed four times, and analyzed using the FL-1 channel to quantitate FITC-labeled RBCs.

ABO serum testing

Group A, B, or O reagent RBCs were added to three separate tubes followed by patient plasma (or serum). The mixtures were washed four times, the pellets were resuspended in PE-labeled antihuman IgM, and after a 5-minute incubation the samples were washed and acquired in the FL-2 channel.

RBC alloantibody testing

RBCs from a three-cell screening panel were added to three tubes, followed by patient plasma and/or serum and PEG. The mixtures were incubated and washed, and a PE-conjugated antihuman IgG antiserum was added before FC.

Filter plate technique

Assays were performed similarly when 96-well microtiter filter plates were used, except RBCs were washed by manually applying vacuum to individual wells using a Bio-Dot apparatus (Bio-Rad Laboratories, Hercules, CA). Typically, only a fraction of the pressure (–1 to –5 inches Hg) generated by standard vacuum systems was needed to rapidly aspirate fluid through the filter. At the conclusion of each vacuum step, RBCs were readily dislodged from the filter surface for subsequent manipulation. Loss or fragmentation of RBCs occurred infrequently and did not affect results. After staining, RBCs were transferred to 12 × 75-mm tubes for manual data acquisition and

analysis by FC. Occasionally (<5% of samples) there were insufficient RBCs to acquire 20,000 events, and 10,000 were acquired instead. Because RBC agglutination was minimal in this assay configuration, pipetting and vortexing were not necessary before FC. For the present studies, all sample transfer steps, as well as tube and plate labeling, were performed manually. Sample identities were confirmed by an independent observer to eliminate transcription and identification errors. This methodology is the subject of a patent application (Serial No. 09/773 826).

CAT and standard tube testing

Gel-column cards were used to perform forward and reverse ABO grouping and alloantibody screens (Ortho-Clinical Diagnostics). Standard tube testing was performed according to usual procedures, including alloantibody detection by the LISS-IAT.¹⁸ All samples were tested and interpreted without knowledge of results from other assays.

RESULTS

FC methods for ABO grouping and D typing

For the initial FC studies, antibody staining and washing were performed in round-bottom test tubes. Samples with unambiguous ABO grouping and D typing results (by CAT) were first used to optimize FC assay parameters, including RBC concentration, antibody titer, use of potentiators, incubation time and temperature, and washing conditions. Despite these efforts, RBC agglutinates frequently formed in the tubes, although they could usually be dispersed. In some cases residual agglutinates occluded the cytometer flow cell, and these samples could not be analyzed. Nonetheless, the resulting staining protocol (Table 1) produced encouraging results (Fig. 1). To derive quantitative cut-off values for objectively scoring samples as positive or negative, replicate control reactions were performed: 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. The MFIs for each of 10 ABO control reactions were averaged to yield a value of 2.26 and a SD of 0.23. Subsequent reactions were automatically scored as negative if their MFI was less than 2.72 (average MFI + 2 × SD) and positive if it was greater than 4.56 (average + 10 × SD); intermediate values (approx. 15% of samples tested) required visual interpretation. The cut-off values remained essentially unchanged when monitored over time with different control samples. Table 2 lists the MFIs for the assays shown in Figs. 1 through 5.

Tube-based FC methods were then used for ABO grouping and D typing of 222 randomly selected samples,

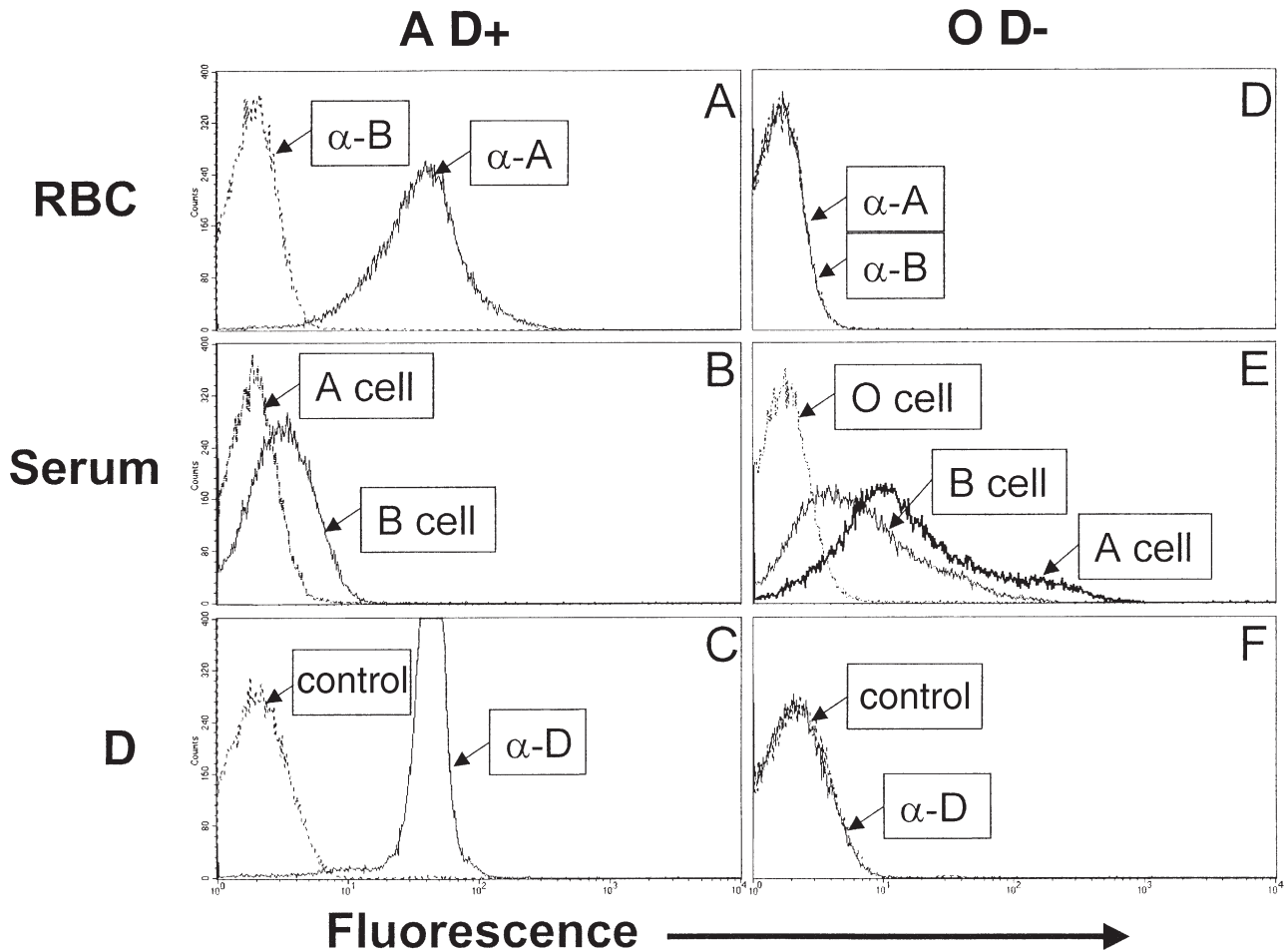


Fig. 1. Representative results for ABO grouping and D typing by FC when RBCs were stained in tubes. Samples from a group A, D+ (A–C) and a group O, D– (D–F) patient were subjected to RBC (A, D) and serum testing (B, E) as well as typing for D (C, F). For each panel, fluorescence intensity is displayed 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.

which were also tested by CAT and standard tube methods. Of the 222 samples, 202 (91.0%) typed identically by each test. The remaining 20 samples gave discordant results that were subsequently resolved by further analysis including testing for unexpected antibodies, prolonged incubation for reverse types, A1 typing, and testing for weak D (Table 3). For nine of these samples, no type was determined (NTD) by CAT due to disagreement between the forward and reverse types. CAT also failed to identify two mixed-field reactions and a weak D, while incorrectly identifying six A1-negative blood samples as having mixed-field reactions. Overall, 2 of the 20 discrepant samples were typed correctly by CAT, for an accuracy of 204 out of 222 (91.9%). For standard tube testing, 9 of the 20 samples yielded the correct results for an overall accuracy of 211 out of 222 (95.0%). In contrast, 18 of the 20 samples were typed correctly by FC, while the two remaining samples were NTD due to weak alloantibodies. The accuracy of FC testing was 99.1 percent

(220/222 samples). Thus, FC performed as well as CAT and standard tube testing for ABO grouping and D typing of typical blood samples and better than other methodologies when samples had abnormalities such as rouleaux, autoantibodies, weak alloantibodies, and mixed-field reactions.

FC methods for RBC alloantibody testing

Using test tubes for sample preparation, specimens containing strong alloantibodies (3+ and 4+ by CAT) were used to develop a protocol for RBC alloantibody detection by FC (Table 1). A total of 239 random patient samples were then tested: 188 samples with previously identified RBC alloantibodies and 51 samples without detectable alloantibodies. The results were compared to those obtained by CAT and LISS-IAT. The set of alloantibodies examined had a wide range of clinically relevant specificities including D (20 examples), E(33), C(5), c(5), K(24),

TABLE 2. MFI for Figs. 1–5

Figure	Sample	Rgt 1	MFI	Rgt 2	MFI	Rgt 3	MFI	Interpretation
1A	RBC	α -A	45.21	α -B	1.86	NA	NA	Group A
1B	Serum	A cell	2.18	B cell	4.05	NA	NA	Group A
1C	RBC	α -D	33.85	Cont	1.89	NA	NA	D+
1D	RBC	α -A	1.56	α -B	1.54	NA	NA	Group O
1E	Serum	A cell	14.82	B cell	6.06	O cell	1.68	Group O
1F	RBC	α -D	2.12	Cont	2.01	NA	NA	D–
2A	Serum	SC1	6.60	SC2	3.82	SC3	4.26	α -C
2B	Serum	SC1	4.21	SC2	9.06	SC3	8.53	α -Fy ^a
2C	Serum	SC1	6.61	SC2	4.51	SC3	6.38	α -Jk ^a
2D	Serum	SC1	30.64	SC2	49.93	SC3	3.38	α -D
3A	RBC	α -A	2.26	α -B	13.64	NA	NA	Group B
3B	Serum	A cell	13.22	B cell	2.84	NA	NA	Group B
3C	RBC	α -D	47.43*	Cont	2.82	NA	NA	D-mf
3D	RBC	α -A	1.76	α -B	1.74	NA	NA	Group O
3E	Serum	A cell	4.96	B cell	5.01	O cell	2.58	Group O
3F	RBC	α -D	2.23	Cont	1.71	NA	NA	D–
4A	Serum	SC1	6.19	SC2	3.20	SC3	3.17	α -C
4B	Serum	SC1	3.94	SC2	6.87	SC3	4.24	α -Jk ^a
4C	Serum	SC1	12.10	SC2	20.07	SC3	2.62	α -D
5A	Serum	A cell	1.75	B cell	22.48	NA	NA	Group A
5B	Serum	A cell	31.62	B cell	15.96	O cell	1.86	Group O
5C	Serum	SC1	152.02	SC2	289.49	SC3	3.4	α -D
5D	Serum	A cell	1.91	B cell	58.23	NA	NA	Group A
5E	Serum	A cell	38.80	B cell	14.24	O cell	1.85	Group O
5F	Serum	SC1	67.09	SC2	110.00	SC3	2.50	α -D

* MFI for right-shifted fluorescence peak.

Fy^a(15), Jk^a(6), Jk^b(3), and S(2). Antibody strengths ranged from weak to 4+. A total of 58 samples had multiple (2–4) alloantibodies. Representative results are shown in Fig. 2. Seventeen additional samples with room temperature-reactive antibodies of less clinical relevance (anti-M, anti-N, anti-Le^a, and anti-Le^b) were also included.

The average MFI for negative alloantibody controls was 4.04 (SD = 0.44). Of 51 samples negative for alloantibodies by CAT, 49 were also negative by LISS-IAT and FC. The remaining two samples, negative by CAT, in fact contained alloantibodies detected by LISS-IAT (1+ and 4+ strength) and FC methods. Of 188 samples with alloantibodies detected by CAT, 177 also tested positive by LISS-IAT and FC, while 11 samples were discordant. Of the 11 samples (with specificities including D, C, Jk^a, and K), 10 were negative by LISS-IAT, weak+/1+ in CAT, but were readily detectable with FC. The 11th sample had a weak (1+) anti-K detected by both CAT and LISS-IAT but not FC. For the 58 samples with multiple alloantibodies, all were detected by both CAT and FC. Thus, FC detected a greater percentage (189/190, 99.5%) of clinically relevant alloantibodies than LISS-IAT (94.7%) and about the same percentage as CAT (98.9%). The detection of room

TABLE 3. Analysis of 20 samples with discrepant ABO/Rh(D) typing results

Sample abnormality (number of samples)	Correct result?		
	Gel	Tube	Flow
NTD: rouleaux (2 samples)	*	*	Yes
NTD: cold autoantibody (3)	*	*	Yes
NTD: weak alloantibodies (4)	Yes (2/4)	*	Yes (2/4)
NTD: extraneous A+ forward reaction (1)	*	Yes	Yes
NTD: positive D control-autoantibody (1)	*	Yes	Yes
Mixed-field: D-recent transfusion (1)	*	*	Yes
Mixed-field: A-recent transfusion (1)	*	Yes	Yes
Mixed-field: A1-negative (6)	*	Yes	Yes
Weak D (1)	*	*	Yes

* Invalid or incorrect result on initial testing.

temperature-reactive alloantibodies was more variable. The FC technique, using antihuman IgM or IgG secondary antibodies, detected 6 of 10 anti-M, 2 of 2 anti-N, 2 of 4 anti-Le^a, and 0 of 1 anti-Le^b alloantibodies.

Development of FC methodologies compatible with automation

These studies confirmed that FC could detect antibody-RBC interactions with accuracy and sensitivity comparable to commercially available methodologies. However, they also confirmed that RBC agglutinates formed in test tubes during staining can occlude the cytometer

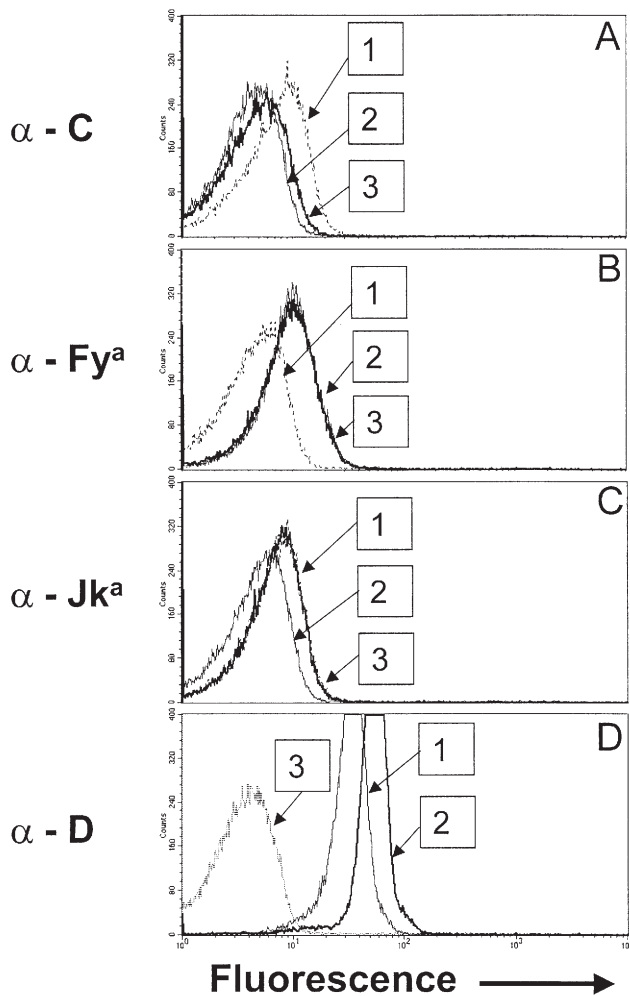


Fig. 2. Representative results for RBC alloantibody screens performed by the FC tube method. (A–D) Samples from four patients with anti-C, anti-Fy^a, anti-Jk^a, and anti-D are displayed. Screening cell phenotypes: Cell 1: D+ C+, Fy(a– b+), Jk(a+ b–); Cell 2: D+ C–, Fy(a+ b–), Jk(a– b+); Cell 3: D– C–, Fy(a+ b+), Jk(a+ b–).

and prevent analysis, as was previously described.^{4,16,17} Because of this drawback, alternate approaches of preparing RBCs for FC were investigated.

Automated laboratory systems frequently employ disposable microtiter filter plates and vacuum stations for sample washing. We examined a variety of filter plates as substitutes for test tubes and determined that plates containing 0.2- μ m PVDF filters optimally preserved RBC integrity during vacuum washing. These plates were then used for ABO grouping and D typing as described above (Table 1) except RBCs were washed by vacuum. Ten control reactions again showed a tight distribution with an averaged MFI of 2.08 and a SD of 0.21. The results from representative randomly selected samples are shown in Fig. 3. Of 83 samples with strong (3+ or 4+) forward and reverse typing reactions by CAT, 82 (99%) typed correctly

by FC. The remaining sample tested NTD due to an extraneous weakly positive reaction. An additional 26 samples were tested that had abnormalities including weak reverse typing reactions (<2+ by CAT), mixed-field reactions, or rouleaux. A total of 22 typed correctly by FC, while 4 were NTD due to weak reverse reactions that were not detected; of these, two samples were also NTD by CAT. Overall, 104 of 109 samples (95.4%) typed correctly by FC when stained in filter plates, while the remaining samples were flagged as NTD for further analysis. None of the samples was incorrectly typed by FC. CAT showed 98.2 percent accuracy and a 1.8 percent NTD rate.

The filter-plate method was also used to screen for unexpected alloantibodies in 175 samples containing 238 alloantibodies and 50 samples without alloantibodies. Representative results for FC are shown in Fig. 4. The average MFI for negative controls was 3.43 (SD = 0.65). Of the 175 samples that were positive by CAT, 169 (96.6%) were also positive by FC, while 160 (91.4%) were positive by LISS-IAT. Among the six samples that were negative by FC, one was a weakly reactive anti-E, two were 1+ anti-Kell, and three were anti-M. These three anti-M were subsequently detected when the staining procedure was modified to include both antihuman IgG and IgM secondary antibodies. Of 50 samples that tested negative by CAT, 49 were also negative by LISS-IAT and FC. However, one sample had detectable alloantibodies by LISS-IAT and FC. When the one positive sample that tested negative by CAT is included (n = 176 total positive samples), the accuracy rates of FC, CAT, and LISS-IAT were 98.3, 99.4, and 91.5 percent, respectively. Thus, in this study set, FC performed comparably to CAT and better than LISS-IAT.

Although the FC results were similar whether washing was performed in tubes or in microtiter filter plates, the extent of RBC agglutination was significantly different. While samples washed in tubes were vigorously pipetted and vortexed, RBC agglutinates remained in up to 10 percent of samples and produced irregular histograms (Fig. 5A–C). Approximately 1 percent of samples had large enough agglutinates to occlude the cytometer. In contrast, there were no visible RBC agglutinates in filter plates even though no efforts were made to disperse the RBCs (Fig. 5D–F). The difference in agglutination is likely due to the fact that centrifugation in tubes collects RBCs into a tight pellet, facilitating the formation of agglutinates. The vacuum technique separates and distributes RBCs over the entire surface area of the filter during washing steps, preventing significant agglutination. There were no consistent differences in the resulting fluorescence intensity with the two methods.

DISCUSSION

Although manual tube-based immunohematology testing remains the standard in many laboratories, this technique

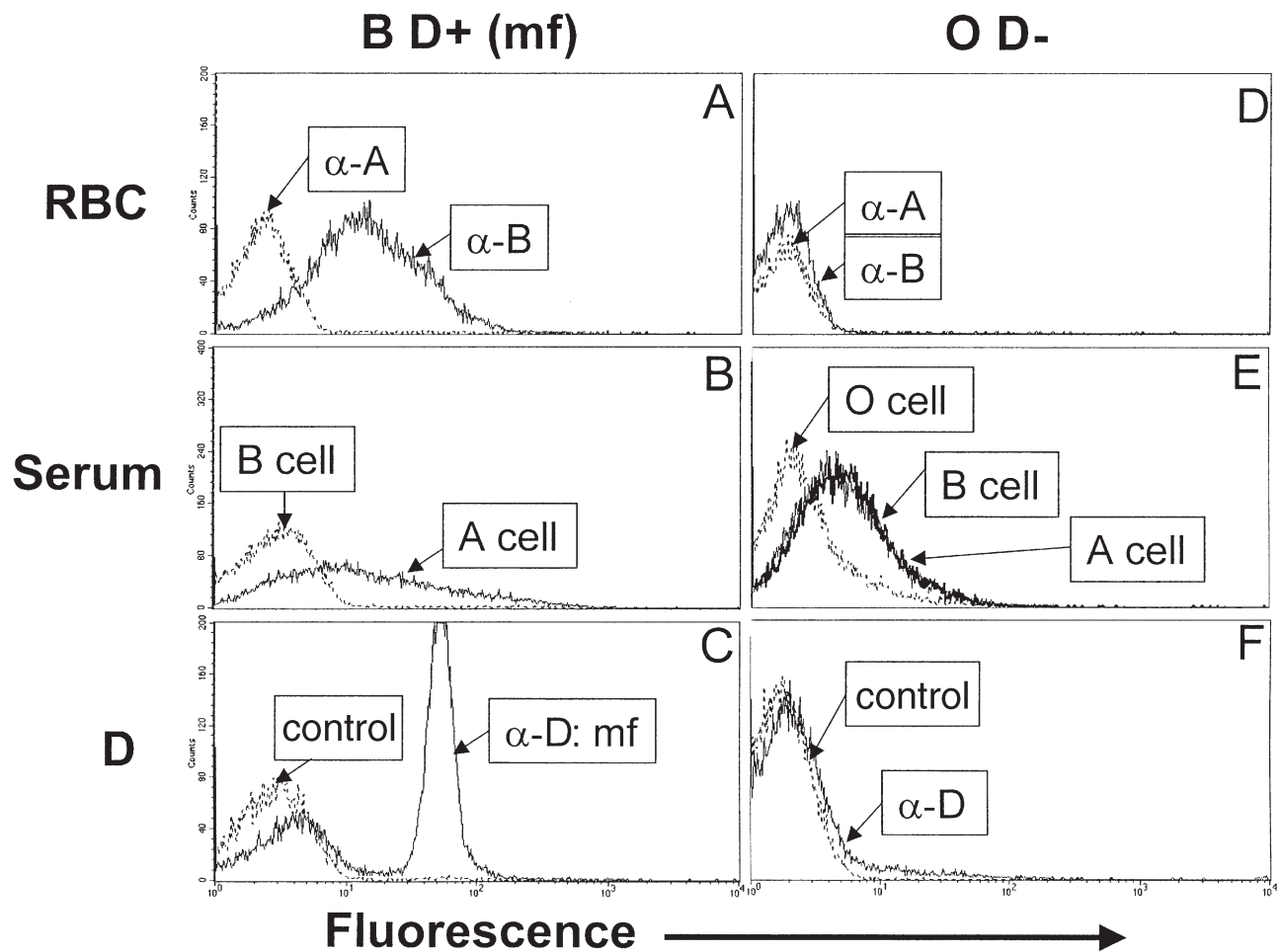


Fig. 3. Representative results for ABO grouping and D typing by FC using filter plates. Blood samples from a group B, D+ (A–C) and a group O, D– (D–F) patient were subjected to RBC (A, D) and serum testing (B, E) as well as typing for D (C, F). Note that the group B, D+ patient was recently transfused with group B, D– RBCs. When this sample was analyzed for D (C, —) a mixed-field (mf) reaction was observed: the left-most peak coincided with the negative control (cont, -----) and was composed of the transfused D– cells; the right-shifted population was positive for D and represents the patient’s RBCs.

is labor intensive, is not suited to automation, and requires highly skilled technologists for accurate results. Alternative methodologies have been commercialized to address these deficiencies, including CAT, SPRCA, and amphitheater plate-mediated hemagglutination (Olympus PK7200, Olympus of America, Melville, NY). Other novel methods, such as a microplate-based agglutination assay¹⁹ and UV-visible spectrophotometric typing,^{20,21} are also in development.

The most important requirement for any immuno-hematology assay must be accuracy. Standard tube testing, CAT, SPRCA, and amphitheater hemagglutination have shown sufficient accuracy to obtain FDA approval for commercialization. Herein, we demonstrate that the accuracy of FC testing compares very favorably with that of standard tube testing and CAT. When samples were stained in tubes before FC, ABO group and D type were

correctly determined for 99.1 percent of clinical samples. By comparison, accuracy rates of CAT and standard tube testing were 91.9 and 95.0 percent for the same sample set, respectively. FC also detected 99.5 percent of clinically relevant alloantibodies, while CAT and LISS-IAT identified 98.9 percent and 94.7 percent, respectively. To overcome the necessity of dispersing RBC agglutinates before FC, we optimized a vacuum filtration method that eliminated the need for vigorous vortexing and pipetting or caustic agents like glutaraldehyde.^{4,16,17,22} Using the filter plate FC technique, 104 of 109 samples (95.4%) were correctly typed for ABO and D, whereas the remaining five samples were read as NTD. Although the 4.6-percent NTD rate was higher than when samples were stained in tubes, it is still less than the 6-percent rate mandated by recent FDA guidance.²³ More recently, technical modifications have reduced the NTD rate to less than 1 percent (unpublished

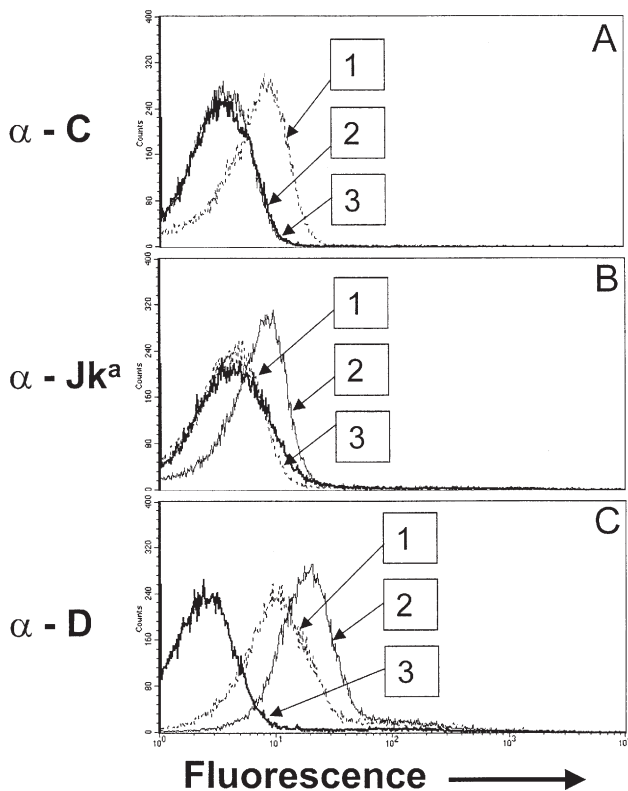


Fig. 4. Representative results for RBC alloantibody screens performed by FC using filter plates. (A–C) Samples from three patients with anti-C, anti-Jk^a, and anti-D are displayed. The same samples shown in Panels A and C were also stained in tubes before FC, as shown in Fig. 2A, D, respectively. Screening-cell phenotypes: Cell 1: D+ C+, Jk(a- b+); Cell 2: D+ C-, Jk(a+ b-); Cell 3: D- C-, Jk(a- b+).

data). Further improvements might result through the use of anti-IgG in combination with anti-IgM for ABO serum testing. Using the filter plate FC method, 98.3 percent of alloantibody-containing samples were correctly identified, compared to 99.4 percent with CAT and 91.5 percent with LISS-IAT. Importantly, these comparisons were made using authentic clinical samples with abnormalities including weak alloantibodies, rouleaux, autoantibodies, and mixed-field reactions, which are not uncommon in hospitalized patients. In contrast, other recently described methods have only used samples from healthy donors, and none of these methods has been applied to routine alloantibody identification.^{19–22} We have not yet examined assay performance for pretransfusion cross-matches, but we anticipate that accuracy will be similar to that seen for ABO serum testing (“immediate spin” cross-match) and RBC alloantibody testing (“Coombs” cross-match).

After accuracy, other considerations include cost, turn-around time (TAT), and compatibility with additional pretransfusion tests. Despite the high labor costs of standard tube testing, many facilities continue to rely on

this methodology because of the offsetting inexpensive equipment and reagents. However, the cost benefits of standard tube testing may not be realizable due to a shortage of qualified technologists. For this reason, many facilities have found it necessary to implement automation, even though automated devices have higher associated capital and disposables costs. Currently available immunohematology workstations range in capital cost from \$60,000 to \$350,000.³ We estimate that an automated FC testing platform would be in the middle of this range. However, unlike methods such as CAT and SPRCA, the FC technology utilizes relatively inexpensive disposables and reagents and thus over time may be less expensive to implement.

Assay TAT is important in the hospital transfusion-service setting, particularly if emergency testing is performed. The TAT to determine a blood type and test for RBC alloantibodies is approximately 30 minutes for CAT and 45 minutes for SPRCA. Based on current protocols (Table 1), we estimate that an FC workstation could perform these assays on a single sample in approximately 20 minutes. When multiple samples are batched, we estimate 20 samples can be tested in 45 minutes. These times may be reduced through further assay optimization.

Although the assays in the present report were performed manually, we are currently investigating several commercially available liquid-handling platforms that can automate all of the steps in this procedure for high-throughput testing. Unlike other technologies, this automated system could serve as a platform for a variety of pretransfusion tests. For example, FC has been used to quantitate residual WBCs after filtration,^{8,9} detect antibody binding to platelets,^{11–13} detect bacteria,²⁴ identify feto-maternal hemorrhage,^{10,25,26} and as an alternative to conventional serologic methods to detect pathologic antibodies in serum (such as antiviral antibodies).²⁷ FC can also be used for determination of RBC survival after transfusion,^{28,29} quantitation of RBC blood group antigen density,^{4,5} and determining expression of blood group antigen variants.⁶ With appropriate reagents, all of these assays should be compatible with an automated FC workstation.

In conclusion, we have described an accurate FC methodology for ABO grouping, D typing, and testing for unexpected RBC alloantibodies. Although the studies described herein were performed with samples from hospitalized patients, this technology should also be applicable to testing donor samples. We are currently developing a fully automated prototype incorporating this technology and estimate that the resulting workstation will be competitive with current commercially available devices in terms of cost and TAT. In addition, through the use of FC, this workstation should be compatible with other pretransfusion tests, including residual WBC counting and detection of bacterial contamination.

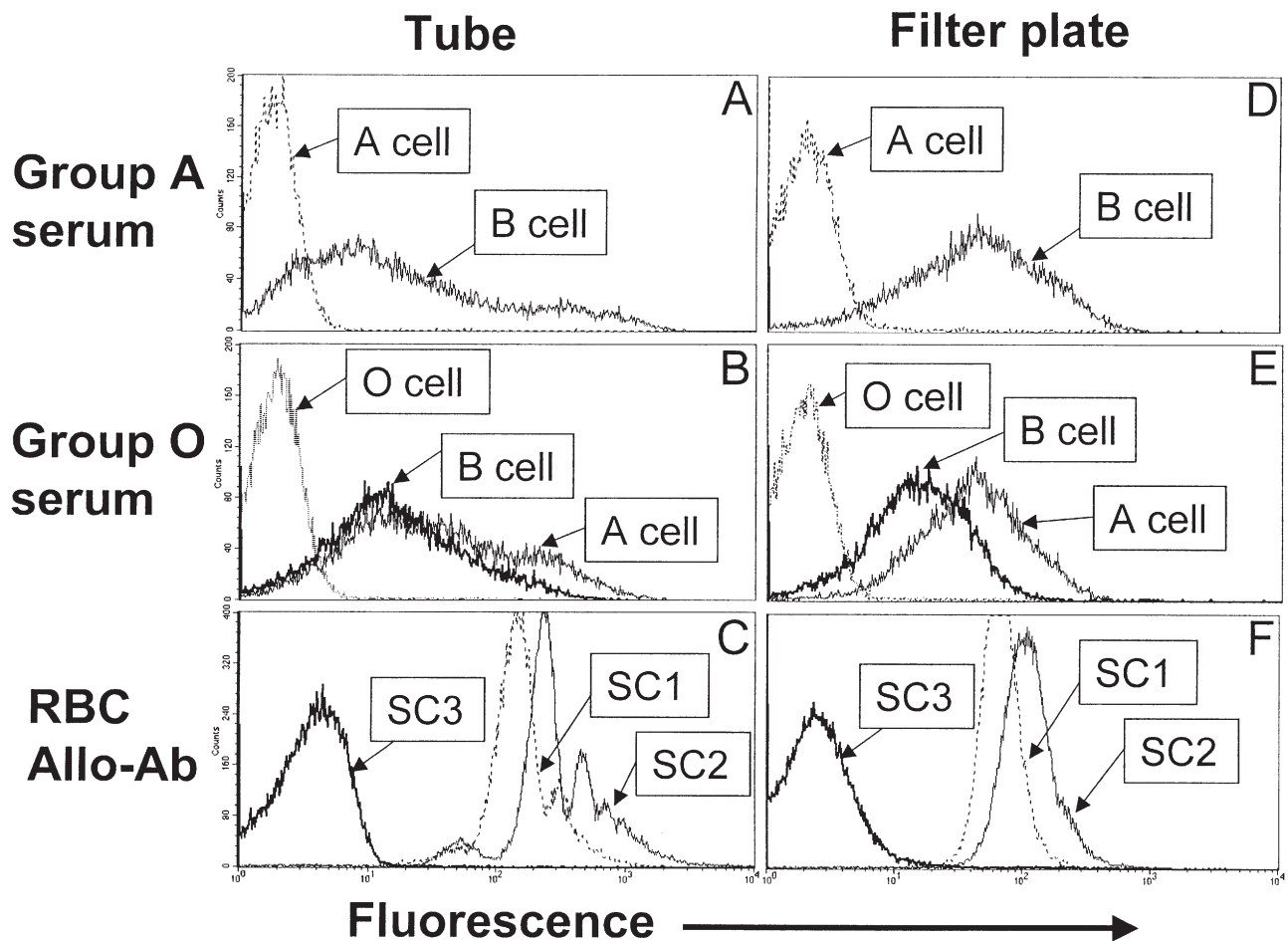


Fig. 5. Comparison of RBC agglutination and staining intensity in test tubes and filter plates. A group B (A, D) and group O (B, E) sample was subjected to serum testing for anti-A and -B using tubes (A, B) or filter plates (D, E). A sample with an anti-D was likewise tested in parallel in tubes (C) and plates (F). Samples stained in tubes were vigorously pipetted and vortexed before FC analysis, whereas those stained in plates were not. Note that although the results in tubes and filter plates are similar, staining in tubes can produce asymmetric curves with intensely stained right-shifted subpopulations that represent RBC agglutinates. In contrast, symmetric peaks result when the same samples are stained in filter plates. Screening cell (SC) phenotypes: SC1: D+; SC2: D+; SC3: D-.

REFERENCES

- Lapierre Y, Rigal D, Adam J, et al. The gel test: a new way to detect red cell antigen-antibody reactions. *Transfusion* 1990;30:109-13.
- Sinor LT. Advances in solid-phase red cell adherence methods and transfusion serology. *Transfus Med Rev* 1992;6:26-31.
- Voak D. The status of new methods for the detection of red cell agglutination (letter; comment) *Transfusion* 1999;39:1037-40.
- Berneman ZN, van Bockstaele DR, Uyttenbroeck WM, et al. Flow-cytometric analysis of erythrocytic blood group A antigen density profile. *Vox Sang* 1991;61:265-74.
- van Bockstaele DR, Berneman ZN, Muylle L, et al. Flow cytometric analysis of erythrocytic D antigen density profile. *Vox Sang* 1986;51:40-6.
- Hasekura H, Ota M, Ito S, et al. Flow cytometric studies of the D antigen of various Rh phenotypes with particular reference to Du and Del. *Transfusion* 1990;30:236-8.
- Lynen R, Krone O, Legler TJ, et al. A newly developed gel centrifugation test for quantification of RBC-bound IgG antibodies and their subclasses IgG1 and IgG3: comparison with flow cytometry. *Transfusion* 2002;42:612-8.
- Dzik WH, Ragosta A, Cusack WF. Flow-cytometric method for counting very low numbers of leukocytes in platelet products. *Vox Sang* 1990;59:153-9.
- Dzik S, Moroff G, Dumont L. A multicenter study evaluating three methods for counting residual WBCs in WBC-reduced blood components. *Nageotte hemocytometry*,

- flow cytometry, and microfluorometry. *Transfusion* 2000;40:513–20.
10. Nance SJ, Nelson JM, Arndt PA, et al. Quantitation of fetal-maternal hemorrhage by flow cytometry. A simple and accurate method. *Am J Clin Pathol* 1989;91:288–92.
 11. Gates K, MacPherson BR. Retrospective evaluation of flow cytometry as a platelet crossmatching procedure. *Cytometry* 1994;18:123–8.
 12. Skogen B, Christiansen D, Husebekk A. Flow cytometric analysis in platelet crossmatching using a platelet suspension immunofluorescence test. *Transfusion* 1995;35:832–6.
 13. Levin MD, de Vries W, de Veld J, et al. Platelet-bound immunoglobulins before and after platelet transfusion: measurement of in vivo binding. *Br J Haematol* 1999;104:397–402.
 14. Lazarus AH, Wright JF, Blanchette V, Freedman J. Analysis of platelets by flow cytometry. *Transfus Sci* 1995;16:353–61.
 15. Freedman J, Lazarus AH. Applications of flow cytometry in transfusion medicine. *Transfus Med Rev* 1995;9:87–109.
 16. Garratty G, Arndt PA. Applications of flow cytofluorometry to red blood cell immunology. *Cytometry* 1999;38:259–67.
 17. Sharon R, Fibach E. Quantitative flow cytometric analysis of ABO red cell antigens. *Cytometry* 1991;12:545–9.
 18. Brecher ME, ed. AABB technical manual. 14th ed. Bethesda: American Association of Blood Banks, 2002.
 19. Spindler JH, Kluter H, Kerowgan M. A novel microplate agglutination method for blood grouping and reverse typing without the need for centrifugation. *Transfusion* 2001;41:627–32.
 20. Narayanan S, Orton S, Leparc GF, et al. Ultraviolet and visible light spectrophotometric approach to blood typing: objective analysis by agglutination index. *Transfusion* 1999;39:1051–9.
 21. Narayanan S, Galloway L, Nonoyama A, et al. UV-visible spectrophotometric approach to blood typing II. Phenotyping of subtype A2 and weak D and whole blood analysis. *Transfusion* 2002;42:619–26.
 22. Takahashi J, Seno T, Nakade T, et al. Detection and quantitation of ABO RBC chimerism by a modified coil planet centrifuge method. *Transfusion* 2002;42:702–10.
 23. Food and Drug Administration. Guideline for quality assurance in blood establishments (July 11, 1995). Rockville, MD: CBER Office of Communication, Training, and Manufacturers Assistance, 1995.
 24. Shapiro HM. Multiparameter flow cytometry of bacteria: implications for diagnostics and therapeutics. *Cytometry* 2001;43:223–6.
 25. Lloyd-Evans P, Kumpel BM, Bromelow I, et al. Use of a directly conjugated monoclonal anti-D (BRAD-3) for quantification of fetomaternal hemorrhage by flow cytometry (comment). *Transfusion* 1996;36:432–7.
 26. Davis BH, Olsen S, Bigelow NC, Chen JC. Detection of fetal red cells in fetomaternal hemorrhage using a fetal hemoglobin monoclonal antibody by flow cytometry. *Transfusion* 1998;38:749–56.
 27. Drouvalakis KA, Neeson PJ, Buchanan RR. Detection of anti-phosphatidylethanolamine antibodies using flow cytometry. *Cytometry* 1999;36:46–51.
 28. Issitt PD, Valinsky JE, Marsh WL, et al. In vivo red cell destruction by anti-Lu6. *Transfusion* 1990;30:258–60.
 29. Zeiler T, Muller JT, Hasse C, et al. Flow cytometric determination of RBC survival in autoimmune hemolytic anemia. *Transfusion* 2001;41:493–8. 