Laboratory methods for Rh immunoprophylaxis: a review
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The recommended dose of Rh immune globulin for postpartum Rh immunoprophylaxis is based on an estimation of the volume of the fetomaternal hemorrhage, if any, measured as the percent of fetal RBCs in a sample of the D– mother’s blood. Laboratory methods for distinguishing fetal from maternal RBCs have been based on their different blood types (D+ versus D–) or predominant hemoglobin content (hemoglobin F versus hemoglobin A). We conducted a review of the medical literature describing laboratory methods for detecting and quantifying fetal RBCs in maternal blood samples. We also used data collected for the College of American Pathologists Fetal RBC Detection Surveys to determine which laboratory methods are used currently in hospitals in the United States. The rosette screen is used widely for identifying D– mothers who may require additional doses of Rh immune globulin for postpartum immunoprophylaxis. As the rosette screen targets the D antigen, it is not suitable for detecting a fetomaternal hemorrhage in D+ mothers or when the D type of the fetus or newborn is D– or unknown. The acid-elution (Kleihauer-Betke) assay is a sensitive laboratory method for quantifying a fetomaternal hemorrhage, but it is tedious, often inaccurate, and difficult to reproduce. Flow cytometry, using anti-D or anti-hemoglobin F reagents, offers a more precise quantification of fetal RBCs in maternal blood. However, flow cytometry services for this function are available in relatively few hospital laboratories in the United States because of logistic and fiscal impediments. Immunohematology 2010;26:92–103.

Key Words: Rh immunoprophylaxis, Rh immune globulin, rosette screen, Kleihauer-Betke assay, flow cytometry, fetomaternal hemorrhage, hemolytic disease of the newborn

In the United States, standard practice for determining the postpartum dose of Rh immune globulin (RhIG) begins with a laboratory measurement of the percent of fetal RBCs in a sample of maternal blood followed by a calculation of the estimated volume of the fetomaternal hemorrhage (FMH) (mL of RBCs).1–6 Most hospital laboratories perform an initial qualitative assay for fetal RBCs in the mother’s blood using the rosette screen for D+ fetal RBCs.7 If the rosette screen is positive, most laboratories perform a quantitative measurement of the percent of fetal RBCs in a sample of the mother’s blood using the Kleihauer-Betke (K-B) acid-elution assay.7 Evaluations of the various versions of the K-B acid-elution assay consistently conclude that the assay is imprecise and lacks reproducibility for quantifying fetal RBCs in maternal blood.5,8,9 Flow cytometry (FC) offers a more precise and reproducible methodology for quantifying fetal RBCs in a sample of maternal blood, but logistical and fiscal factors have impeded widespread implementation of FC assays for this purpose in hospitals in the United States.4,10 Recognizing the technical limitations of the sequential rosette screen and K-B acid-elution assay, the editors of the AABB Technical Manual recommend routinely adding one additional vial of RhIG to the number calculated using the standard formula for determining the postpartum dose of RhIG (one vial of 300 µg of anti-D per 15 mL of RBC FMH).5 Ramsey, writing for the College of American Pathologists (CAP) Transfusion Medicine Resource Committee, analyzed the results submitted by nearly 1600 laboratories that participated in the CAP Proficiency Testing survey for Fetal RBC Detection to determine the methods used to calculate the dose of RhIG.3 He reported that in three of the four calculation exercises in this survey, 20 to 30 percent of participating laboratories underestimated the necessary dose of RhIG.3 Ramsey concluded that laboratories performing quantification of FMHs should review their procedures and training for calculating RhIG dosage.

The following review supplements Ramsey’s analysis of methods for calculating the dose of RhIG and provides an updated review of laboratory methods for quantifying the number of fetal RBCs in a sample of maternal blood. For this review, we focused on those laboratory methods reported by 1911 laboratories participating in the CAP’s 2009 HBF-01 Fetal RBC Detection Survey.7 We confirmed the availability of commercially marketed test kits that have been cleared by the US Food and Drug Administration (FDA) for clinical use in the United States by a search of Internet Web sites. There is no standard format for comparing the performance of different laboratory assays for detecting and quantifying fetal RBCs. For any given volume of an FMH, the percent of fetal RBCs in a sample of maternal blood will vary in proportion to the mother’s RBC volume. For purposes of this review, we selected the detection of 0.6 percent fetal RBCs in a sample of maternal blood to represent an assay’s ability to detect a 30-mL FMH, as proposed by Bayliss and colleagues* and by Sebring and Polesky.9 This degree of sensitivity is critical, because standard practice in the United States is to calculate the dose of RhIG based on the premise that one 300-µg vial of RhIG will suppress D alloimmunization by an FMH of 30 mL (or 15 mL of RBCs).1–6 Failure to detect a 30-mL FMH could result in inadequate dosing of RhIG.3

Laboratory Methods of Historical Interest
Microscopic Weak D Test
If a microscopic weak D test (formerly known as the Dw test) is performed on a sample of blood from a D– mother after
a large FMH (>30 mL), the presence of D+ fetal RBCs may be inferred by observing mixed-field agglutination. Based on this observation, the microscopic weak D test was used historically by many laboratories as a screen for an FMH. As recently as 1980, the majority of laboratories participating in CAP’s Immunohematology Survey reported using the microscopic weak D test as a screen for an FMH. Using this test as a screen, 12.2 percent of nearly 2000 laboratories obtained a false-negative result on a proficiency sample that contained approximately 0.6 percent D+ RBCs. In an evaluation by Riley and colleagues, the microscopic weak D test failed to detect 25 percent of simulated FMHs prepared as admixtures of 0.5 percent D+ cord RBCs in D– adult RBCs. In an evaluation by Sebring, the microscopic weak D test did not reliably detect a simulated 30-mL FMH in an admixture of D+ and D– RBCs. Nance and Garratty and Wenz and Apuzzo attempted to improve the sensitivity by adding polyethylene glycol (PEG) to the serologic reactants. In a direct comparison, Bayliss and colleagues found that the addition of PEG did not offer a significant advantage over the conventional microscopic weak D test.

More-sensitive laboratory assays are currently available to screen for an FMH. The microscopic weak D test is no longer recommended as a screen for an FMH.

**Enzyme-Linked Antiglobulin Test (ELAT)**

In 1982, Ness and Riley and colleagues described an enzyme-linked antiglobulin test (ELAT) that targeted D+ RBCs and was reported to be capable of detecting a simulated 12.5-mL FMH, based on testing for D+ cord RBCs admixed with D– RBCs. Ness and colleagues also reported the results of using their ELAT to quantify FMHs in 789 consecutive D– postpartum mothers who had delivered D+ newborns. There were 117 (14.8%) samples from mothers who had a detectable FMH, including 8 (1%) who had an FMH greater than 30 mL and required more than one vial of RhIG. They reviewed each case for high-risk features that might predict an FMH and concluded that there were neither maternal nor newborn characteristics that reliably predicted an FMH. Greenwalt and colleagues developed a modified ELAT for quantifying D+ RBCs in the circulations of D– mothers. By stabilizing RBCs using 0.05 percent glu taraldehyde, they decreased in vitro hemolysis, which had been troublesome in other assays. They reported that their ELAT was capable of detecting as small a volume as 2 mL of D+ cord RBCs admixed in 1600 mL of D– adult RBCs.

In a personal communication, Dr. Paul Ness informed the authors of this review that “... although the ELAT for FMH provided accurate clinical information that enabled the laboratory to both screen for FMH and quantify the unusually large FMH that was detected, standardized tests were not developed and licensed by the FDA. In the absence of a standardized licensed test, we discontinued the routine use of the test in our laboratory and believe that it is not being performed in other laboratories for similar reasons” (personal communication, Paul Ness, August 2010).

**Qualitative Screens for Fetal RBCs in Maternal Blood**

**Rosette Screen for D+ RBCs**

In 1982, Sebring and Polesky described a modified microscopic weak D test in which ficin-treated indicator RBCs enhanced the detection of D+ fetal RBCs by forming aggregates (rosettes). Sebring and Polesky’s rosette screen was adapted from a generic model described by Helderviert and Sokol, who used “incomplete antibodies” to blood group antigens to identify minor populations of RBCs admixed in major populations. In Sebring and Polesky’s rosette screen, a sample of maternal D– RBCs was examined microscopically after adding reagent anti-D and ficin-treated R, indicator RBCs. If D+ RBCs were present, the D+ ficin-treated indicator RBCs formed aggregates (rosettes) around the anti-D–coated fetal RBCs (Fig. 1). Sebring and Polesky reported that 100 percent of 20 technologists participating in a study of the rosette screen identified a 0.6 percent admixture of D+ cord RBCs in D+ adult RBCs, corresponding to a 30-mL FMH. In contrast, only 17 of 20 (85%) technologists detected the 0.6 percent admixture using the microscopic weak D test. Sebring and Polesky subsequently published a letter to the editor of Transfusion alerting readers

![Fig. 1. The rosette screen. One drop of the manufacturer's reagent-reduced, alkylated, and buffered IgG anti-D in bovine albumin (Fetal Bleed Screening Test, Immucor/Gamma) was added to one drop of a 3% suspension of Immucor’s positive control RBCs (0.6% group O D+ RBCs in 99.4% of group O D– RBCs) and incubated at 37°C for 15 minutes. After washing the RBCs four times and decanting the supernatant, one drop of indicator anti-D and ficin-treated R, indicator RBCs. If D+ RBCs were present, the D+ ficin-treated indicator RBCs formed aggregates (rosettes) around the anti-D–coated fetal RBCs (Fig. 1). Sebring and Polesky reported that 100 percent of 20 technologists participating in a study of the rosette screen identified a 0.6 percent admixture of D+ cord RBCs in D+ adult RBCs, corresponding to a 30-mL FMH. In contrast, only 17 of 20 (85%) technologists detected the 0.6 percent admixture using the microscopic weak D test. Sebring and Polesky subsequently published a letter to the editor of Transfusion alerting readers.
that the rosette screen was not sufficiently sensitive to detect a 30-mL FMH if the Rh phenotype of the cord RBCs was a weak D. The rosette screen was quickly recognized to be more accurate and reliable than the microscopic weak D test for detecting a significant FMH in a D– mother.26 Commercially marketed kits are available in the United States (Fetal Bleed Screening Test; Immucor/Gamma, Norcross, GA). Another widely used kit (FetalScreen; Ortho-Clinical Diagnostics, Raritan, NJ) was recalled from the market in January 2005 and is no longer available. In April 2009, the FDA cleared FetalScreen II/Fetal Maternal Hemorrhage Screening Test (Alba Bioscience, Edinburgh, UK). FetalScreen II kits are currently marketed in the United States by Ortho-Clinical Diagnostics, Raritan, New Jersey. The rosette screen will detect an FMH as small as 10 mL of D+ whole blood.45 Of 1911 participants in the CAP 2009 HBF-01 Fetal RBC Detection Survey who reported results for qualitative screening for FMH, 1907 (99.8%) used the Fetal Bleed Screening Test (Immucor/Gamma) kit.7

A positive rosette screen result provides (only) qualitative evidence of a large-volume FMH that may require additional doses of RhIG. Retesting the mother’s blood sample using a quantitative method is necessary to determine the percentage of D+ fetal RBCs in the maternal blood sample, which is the basis for estimating the volume of the FMH. Because the targeted analyte is the blood group antigen D, not hemoglobin F (HbF), the rosette screen is not a suitable assay for detecting FMHs in all women. Using a standard table of blood group genotype frequencies,27 we calculated that approximately 40 percent of pregnant Caucasian D– mothers will be carrying a D– fetus. Therefore, by its design—targeting D+ RBCs in D– mothers’ blood—the rosette screen will not detect approximately 40 percent of significant FMHs in D– mothers, if the rosette screen is (mis)applied to screen for an FMH in a D– mother. Also, the rosette screen cannot be used in D+ mothers to test for an FMH, because maternal D+ RBCs will be agglutinated.

**Gel Agglutination Cards**

Salama and colleagues28 and David and colleagues29 adapted a brand of gel agglutination cards (DiaMed, Cressier sur Morat, Switzerland), which are marketed in Europe but not in the United States, to detect D+ fetal RBCs in D– maternal blood samples. They evaluated the card assay’s performance by adding monoclonal IgG anti-D to admixtures of D+ and D– RBCs (0.05–0.5% D+ RBCs) and testing for agglutination using DiaMed anti-IgG gel cards. The results were compared with those for detecting fetal RBCs by a standard acid-elution staining method for HbF. The authors reported that their gel agglutination card assay had comparable sensitivity with the acid-elution staining assay and detected all admixtures of D+ RBCs that were greater than 0.2 percent.28,29 Ben-Harroush and colleagues30 analyzed blood samples from 118 D– postpartum women for fetal RBCs by both gel agglutination and flow cytometry (anti-HbF), and reported that the gel agglutination test failed to detect FMHs reliably in the range of 0.1 to 10.0 mL. Subsequently, Agaylan and colleagues31 described a particle gel immunoassay using superparamagnetic particles coated with monoclonal anti-D. The antibody-coated particles were isolated by a magnetic particle concentrator and placed in the reaction chamber of a DiaMed gel card for centrifugation and reading. They reported that this version of the gel agglutination card assay was capable of detecting as few as 0.3 percent of D+ RBCs admixed in D– RBCs.31

Although a version of the gel agglutination card assay is marketed as a screen for FMH (ID-FMH Screening Test, DiaMed), there are few data evaluating its use for this function. None of the 1911 laboratories submitting results in CAP’s 2009 HBF-01 Fetal RBC Detection Survey Participant Report indicated using a gel agglutination card assay to screen for FMH. However, CAP participants are primarily located in the United States, and, therefore, statistics from this survey do not reflect use elsewhere. Both versions of the gel agglutination assay use a commercially marketed gel card, offering the option of a simple, convenient, and standardized alternative to the rosette method for screening for an FMH. However, these gel agglutination cards are not commercially marketed or cleared by the FDA for clinical use in the United States. Further comparative testing is required before their role as an initial screen for FMHs can be determined.

**Quantitative Methods for Measuring Fetal RBCs in Maternal Blood Samples**

**K-B Acid-Elution Assay**

In 1957, Kleihauer, Braun, and Betke32 described an acid-elution and staining assay for quantifying small populations of fetal RBCs in postpartum samples of maternal blood. Their assay was based on a prior observation by Korber,33 who reported that HbF was more resistant to alkali denaturation than HbA. Kleihauer, Braun, and Betke32 described a quantitative method for screening for an FMH. However, these gel agglutination cards are not commercially marketed or cleared by the FDA for clinical use in the United States. Further comparative testing is required before their role as an initial screen for FMHs can be determined.

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ethanol, immersing the slides in citric acid–sodium phosphate buffer (which elutes all hemoglobins except HbF), and staining the dried smears using hematoxylin and eosin (Fig. 2). The percentage of darkly stained fetal RBCs among the lightly stained adult RBCs is determined by microscopic examination. In 1962, Shepard and colleagues applied the K-B acid-elution assay to study the distribution of HbF in fetal RBCs, as well as in RBCs from adults with sickle cell anemia, thalassemia, aplastic anemia, and hereditary persistence of HbF. The descriptions by Shepard and colleagues are among the earliest of “F cells” (adult RBCs containing HbF), although the term “F cell” was not introduced until 13 years later by Boyer and colleagues. Shepard and colleagues also confirmed prior observations that the proportion of HbA and HbF in newborns’ RBCs varies widely and that the percentage of HbF in newborns’ RBCs correlates better with the gestational age than with birth weight. Clayton and colleagues evaluated the optimal technical conditions for performing the K-B acid-elution assay. They observed that the citric acid–phosphate buffer must be prepared at precisely pH 3.2 for optimal elution, which was performed after adding methylene blue to the citric acid–phosphate buffer at 50ºC and cooling it to 37ºC for a 15-minute elution. Using this modification, Clayton et al. reported accurate detection of cord RBCs in dilutions as high as 1:100,000 in adult RBCs, which calculates to an FMH of approximately 0.5 mL or one drop of fetal blood in the entire maternal circulation.

Modified K-B Acid-Elution Assay: Commercially Marketed Reagents and Kits

Commercially marketed kits of reagents are available in the United States for performing the K-B acid-elution assay using the original 37ºC elution (Fetal-Hemoglobin–Differential Stain Kit Modified Kleihauer Technique, Eng Scientific, Inc., Clifton, NJ; Fetal Hemoglobin Kit, Sigma-Aldrich, St. Louis, MO). Also, kits are available that substitute the original 37ºC elution for a more convenient room temperature elution (Fetal Cell Stain Kit, Simmler, Inc., High Ridge, MO; Fetal Hemoglobin–Differential Staining Kit/Room Temperature Procedure, Eng Scientific, Inc.: Fetal Hemoglobin/For the Identification of Fetal Erythrocytes in the Presence of Adult Red Cells, Sure-Tech Diagnostic Associates, St. Louis, MO). Of 1010 laboratories which participated in CAP’s 2009 HBF-01 Fetal RBC Detection Survey and reported results for the K-B acid-elution assay, 976 (96.6%) used one of these commercially marketed kits, and 34 (3.4%) reported using an in-house acid-elution assay.

Modified K-B Acid-Elution Assay: Automated Detection

A significant limitation of the K-B acid-elution assay is the imprecision of only one observer’s subjective interpretation of the different shades of color of stained RBCs, as well as the relatively small number of RBCs that can be counted manually by even the most dedicated technologist. The consequence is a wide variation in results reported by different observers of the same blood smears. To address this issue, Cupp and colleagues, Medearis and colleagues, Ravkin and Temov, and Pelikan and colleagues proposed automated laboratory systems designed to increase the number of RBCs scanned and improve the objectivity of end point determinations. The Pelikan automated computer-assisted microscopy system consisted of an automated scanning stage, microsetting motor controls, and bright-field analysis using green and red absorption filters. The system counted the percentage of fetal RBCs in 1517 low-power fields (10× microscope objective) with superior precision compared with the results reported by investigators scanning 400 high-power fields (40× microscope objective). Pelikan and colleagues compared results for admixtures of fetal and adult RBCs and reported superior determination of fetal RBCs in the range from 0.0001 to 0.001 percent compared with the standard manual K-B acid-elution method, but comparable results in the range of 0.01 to 1.0 percent.
Modified K-B Acid-Elution Assay: Standardizing Enumeration

Several modifications of the K-B acid-elution assay have been proposed to standardize or otherwise improve counting the relatively few darkly stained fetal RBCs among the relatively large number of lightly stained adult RBCs. Woodrow and Finn\(^5\) proposed standardizing the number of fetal RBCs counted by designating a specified number of low-power fields to be examined. Finn and colleagues\(^5\) standardized the number of differentially stained RBCs counted on the blood smear in a defined time, and Jones\(^5\) proposed standardizing the volume (2 mL) for preparing the blood smear using a mechanical spreader. Howarth and colleagues\(^5\) reported that performing acid elution on only half of the blood smear improved the count of RBCs on the blood smear.

Alternative Elution Assay (Elute HbF, not HbA)

Kabat\(^4\) described an alternative elution and staining method based on Itano’s prior observation that HbA is less soluble in concentrated salt solutions compared with HbF.\(^5\) After a peripheral blood smear has been eluted in concentrated potassium phosphate buffer, adult RBCs containing HbA stain bright red, whereas fetal RBCs appear as clear ghosts (i.e., the reverse of the K-B acid-elution assay). Kabat\(^4\) used this elution assay to study the concentrations of HbA and HbF in fetal RBCs during the switch in synthesis of hemoglobinins in human development. We are not aware that this assay has been applied in clinical laboratories for the detection of fetal RBCs in maternal blood.

Modified K-B Acid-Elution Assay: Limitations

Nearly all investigators evaluating the various versions of K-B acid-elution assays report a wide range of inter-observer variation, as well as poor reproducibility of results.\(^3,8,9\) Also, although most infants cease to produce high levels of HbF by 6 months of age, healthy-appearing adults, as well as adults with certain inherited hemoglobinopathies or acquired diseases of hematopoiesis, produce RBCs containing varying percentages of HbA and HbA2 and a small subset of RBCs containing HbF (F cells).\(^3,56-59\) The percent age of F cells in adults is genetically determined, and considerable variation has been observed.\(^8\) In most adults, HbF represents less than 0.6 percent of the total Hb.\(^57\) Most normal adults (85%) have 0.3 to 4.4 percent F cells.\(^5\) Women have higher percentages of F cells than age-matched men, raising the possibility that there is an X-linked factor in the control of Hb synthesis.\(^9\) Probably, the most problematic F cells for purposes of calculating the dose of RhIG after a D− mother delivers a D+ newborn are those F cells that increase during pregnancy.\(^60,66\) In approximately 25 percent of pregnant women, HbF starts to increase after 8 weeks’ gestation and may reach 7 percent by 32 weeks.\(^65,66\)

**Flow Cytometry Anti-D Method**

In an effort to move beyond the limitations of the rosette screen and K-B acid-elution assays, investigators have proposed several applications of FC technology as an alternative to quantifying fetal RBCs in maternal blood. Initial FC assays targeted the D antigen on fetal RBCs, distinguishing D+ fetal RBCs from D− maternal RBCs using polyclonal or monoclonal anti-D reagents.\(^46,67-74\) Nance and colleagues\(^67\) compared the results of testing mixtures of D+ cord RBCs and D− adult RBCs by the rosette screen, K-B acid elution assay, and an FC (anti-D) assay. They reported that results by FC (anti-D) were more accurate, reproducible, and sensitive.\(^67\) They also tested postpartum blood samples from 56 D− women by the K-B acid-elution assay and FC (anti-D) assay. They observed that when significant FMHs did occur (greater than or equal to 0.6% of fetal RBCs), FC (anti-D) results were consistently lower than those measured by the K-B acid-elution assay. Well-controlled FC (anti-D) assays are capable of detecting 0.1 percent of D+ RBCs admixed in D− RBCs.\(^3\) An FC (anti-D) reagent (Quant-Rho FITC anti-D, Alba Bioscience Limited, Edinburgh, UK) has been FDA cleared [510 (k)] for clinical use in the United States (Quotient Biodiagnostics, Newtown, PA). Figures 3A and 3B illustrate how monoclonal anti-D reagents can be applied to detect FMHs by FC. Figure 3A is a histogram illustrating the result of testing a blood sample prepared to simulate an FMH by mixing 1.5 percent of cord RBCs in adult RBCs and testing using Quanta-Rho FITC. Figure 3B is a histogram illustrating the result of testing the same mixture of cord and adult RBCs and testing using Chemicon’s monoclonal anti-D (Millipore, Billerica, MA).

![Fig. 3.](image)

**Fig. 3.** (A) Histogram illustrating application of FC to quantify simulated FMHs using a mixture of 1.5% D+ cord RBCs in D− adult RBCs (Fetal-trol, Trillium Diagnostics) stained using monoclonal anti-D (Quant-Rho FITC). Adult RBCs (left) are separated from cord RBCs (right). (B) Illustrates the same mixture of 1.5 D+ cord RBCs in D− RBCs stained with monoclonal anti-D (Chemicon anti-D, Millipore, Billerica, MA).
Anti-HbF Method

FC assays that target D+ fetal RBCs are a logical and sensitive strategy for identifying D− postpartum mothers who may require additional doses of RhIG. However, assays that target the D antigen cannot be applied to detection of FMHs in D+ mothers or in cases in which the D type of the fetus is D− or not known. For these reasons, FC assays have been developed that target fetal HbF, thereby combining the broader clinical applicability of a test for HbF-containing RBCs with the increased sensitivity of an FC assay. Davis and colleagues developed a rapid FC assay for routine clinical use for detecting FMHs using a fluorescein isocyanate-conjugated hybridoma anti-HbF. The authors were able to process five maternal blood samples and controls in less than 1 hour using this method. The assay had good correlation with the K-B acid-elution assay ($r^2 = 0.86$) and superior precision with a CV of less than 15 percent for blood samples with greater than 0.1 percent fetal RBCs. Their analysis of 150 blood samples from non-pregnant adults, including persons with increased F cells as a result of hemoglobinopathies and hereditary persistence of HbF, gave a mean value of 0.02 percent of fetal RBCs. Autofluorescent leukocytes, often a problem with other FC methods, were excluded by gating. Davis and colleagues evaluated their assay using mixtures of cord and adult RBCs and concluded that their FC (anti-HbF) assay was practical, more precise, and technically superior to the K-B acid-elution assay. This method required several washing steps, but was subsequently modified to eliminate the time-consuming washes. Reagents for this assay are marketed in Europe (QuikQuant, Trillium Diagnostics, Brewster, ME), but not in the United States. Radel and colleagues developed an FC (anti-HbF) method that also included an FC determination of the D type of RBCs using a monoclonal anti-D. This combination method for detecting HbF-containing RBCs and determining the D type expands on the fixation-permeation FC (anti-HbF) method and FC quantification of an FMH in a single assay. Chen and colleagues described an FC (anti-HbF) reference method to quantify F cells in pertinent disease states. Although this method is intended to quantify adult F cells, not fetal RBCs, its technical precision may offer an improved approach to distinguishing fetal RBCs from F cells in postpartum women whose dosing for RhIG is complicated by disease-related increased F cells. The authors are aware of only one FDA-cleared, commercially marketed FC (anti-HbF) reagent kit (Fetal Hemoglobin Kit, Caltag Laboratories, Burlingame, CA) (Fig. 4).

There is no consensus among proponents of FC assays concerning the relative advantages and disadvantages of using anti-D versus anti-HbF reagents for quantifying an FMH in a D− mother. Regarding ease of use, the availability of fluorochrome-conjugated anti-D reagents overcomes the time-consuming aspects of early two-step methods for reacting RBCs with polyclonal anti-D, followed by fluorochrome-conjugated anti-IgG. On the other hand, anti-HbF reagents require RBCs to be fixed and permeabilized to permit interaction with intracellular HbF before staining, a more time-consuming process than reacting anti-D reagents with D on the surface of the RBC membrane. All reports of FC (anti-D) and FC (anti-HbF) methods that we identified using PubMed and reviewed for this article claim improved sensitivity compared with the K-B acid-elution method. However, we were not able to identify a study that directly compared the sensitivities of the FC (anti-D), FC (anti-HbF), and K-B acid-elution assays. Davis and colleagues reported that their FC (anti-HbF) method had a satisfactory linear correlation with the

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**Fig. 4.** (A) Histogram illustrating the application of FC to quantify a simulated FMH using monoclonal anti-HbF (Fetal Hemoglobin Kit, Caltag Laboratories) and adult RBCs (negative control without admixture of adult RBCs with cord RBCs; Fetalrol, Trillium Diagnostics). Adult RBCs (left) are detected, as is the normal content of adult F cells (middle region), but no cord RBCs are visualized (right region). (B) This sample was prepared to simulate the minimally detectable FMH (0.21% cord RBCs), in which fetal RBCs (right region) are readily distinguished from adult F cells (middle region). (C) This sample was prepared to simulate a significant FMH (1.50% cord RBCs).
K-B acid-elution assay in the range of 0 to 1.6 percent. They noted that, as with the K-B acid-elution assay, a greater number of F cells can contribute to an apparent increase in the number of fetal RBCs using their FC (anti-HbF) assay, although still yielding values of less than 0.1 percent fetal RBCs and not contributing to a clinically significant level of false-positive results. Thus, a major advantage of the FC (anti-HbF) method—provided it is adequately controlled—is that F cells can be distinguished and omitted in the final fetal RBC frequency measurement. An FDA-cleared control product is available in the United States (Fetaltril, Trillian Diagnostics) for documenting and monitoring the performance of test methods used to determine fetal RBCs in maternal blood samples. This product consists of three assayed mixtures of D– adult RBCs and D+ cord RBCs in the ranges of 0 to 0.02 percent (negative), 0.1 to 0.3 percent (low positive), and 0.79 to 1.60 percent (high positive) cord RBCs. Kennedy and colleagues conducted a retrospective audit of test results on blood samples from 14 women with suspected FMHs. The samples had been tested routinely by K-B acid-elution assay, as well as by FC using conjugated FITC anti-D monoclonal Ig and by directly conjugated PE anti-HbF. They observed that the K-B acid-elution assay potentially overestimated FMHs in comparison to FC, and that FC (anti-HbF) labeling potentially underestimated the volume of massive FMHs compared with FC (anti-D). They followed up with a study of simulated FMHs consisting of mixtures of D+ cord RBCs in adult D– RBCs. In this study, FC (anti-HbF) underestimated the percentage of cord RBCs compared with FC (anti-D), but only in the subset of samples containing at least 1 percent cord RBCs. There was no significant difference in results for samples containing 0.06 percent or less fetal RBCs. Dziegiel and colleagues had a fortuitous opportunity to address the concern raised by Kennedy and colleagues, i.e., that the FC (anti-HbF) method underestimates large FMHs, when they applied their FC (anti-HbF) assay to blood samples from a postpartum woman with an estimated 314-ml FMH. In that study, Dziegiel and colleagues found close agreement of their measurement of fetal RBCs using FC (anti-HbF) and using FC with anti-Fy\textsuperscript{a}, anti-s, and anti-Jk\textsuperscript{b} to separate fetal and maternal RBC populations. They concluded that there was no systematic deviation of measurements by FC (anti-HbF) compared with their reference blood group method.

Discussion

According to the Participant Summary for CAP’s 2009 HBF-01 Fetal RBC Detection, only 44 of 1054 (4.2%) participating laboratories reporting results for quantifying fetal RBCs used FC and 1010 (95.8%) used the K-B acid-elution assay. All 44 laboratories reporting FC results used anti-HbF reagents. Most participant laboratories (99.8%) used the commercially marketed rosette screen (Fetal Bleed Screening Test, Immucor/Gamma) as their initial qualitative screen. Most laboratories using the K-B acid-elution assay (96.6%) reported using one of the commercially marketed kits. Despite multiple evaluations reporting false-positive results associated with the rosette screen, this assay remains the most widely used in the United States for initial screening for an FMH. The rosette screen is sensitive, convenient, and relatively quick, taking approximately 15 minutes to prepare and interpret a maternal blood sample. Blood bank technologists are accustomed to evaluating the end point of microscopic mixed-field agglutination. There are multiple options for selecting an FDA-cleared commercially marketed kit for reagent kits for quantifying fetal RBCs using modified K-B acid-elution assays. The conclusions of all published evaluations that we reviewed, as well as the opinions of our laboratory’s technologists who perform the K-B acid-elution assay, are that the assay has significant technical limitations. Nevertheless, the K-B acid-elution assay remains the standard quantitative assay of fetal RBCs in the United States.

Despite the evidence that FC can offer improved accuracy and reproducibility of results for quantifying FMHs, few hospitals in the United States use FC assays for routine management of postpartum Rh immunoprophylaxis. The primary reason in our own hospital and, we believe, in most others is the requirement for availability of FC services around the clock to ensure that all postpartum D– mothers receive a laboratory-based determination for the dose of RhIG before they are discharged from the hospital. Although clinical, accreditation, and regulatory requirements allow hospitals to administer RhIG as late as 72 hours after delivery, many patients are discharged on short notice and well before 72 hours after delivery. Even if a hospital has FC services for other clinical applications, few hospitals have technically proficient personnel support for round-the-clock FC services. In contrast, the relatively low-technology requirements for quantifying fetal RBCs by the K-B acid-elution assay generate a numerical result (albeit questionably accurate). Thus, the K-B acid-elution assay has remained the standard of practice for quantifying FMHs in the United States for more than five decades. To accommodate the recognized imprecision of the K-B acid-elution assay, a standard of practice has evolved to routinely add one additional vial of RhIG to the calculated dose. This formula for calculating the dose of RhIG—including the addition of the compensatory extra vial—is incorporated in the RhIG Dose Calculator, which is recommended by CAP’s Transfusion Medicine Resources Committee and posted on the CAP Web site (www.cap.org: CAP Home / Committees and Leadership / Transfusion Medicine Resource Committee / Transfusion Medicine Topic Center / RhIG Dose Calculator; accessed August 18, 2010). D– women with diseases known to be associated with an increased percentage of F cells (sickle cell anemia, thalassemia, hereditary persistence of HbF, and certain other diseases of hemato poiesis) require special management for postpartum Rh immunoprophylaxis. The detection of D+ fetal RBCs by the rosette screen is not compromised by the presence of an
increased number of maternal D– F cells. However, many technologists have difficulty separating disease-related F cells and fetal RBCs by the K-B acid-elution assay. For those D– postpartum women with increased F cells, FC assays using either anti-D or anti-HbF offer a more precise method for determining the dose of RhIG (Fig. 3). Some, but not most, hospitals managing deliveries and postpartum Rh immunoprophylaxis for women with sickle cell disease and other diagnoses known to be associated with increased numbers of F cells may have FC services readily available. The question arises, “What laboratory methods are available to ensure an adequate dose of RhIG for those women with an increased percentage of F cells?” An estimated 0.3 to 1.0 percent of all obstetrical deliveries result in an FMH greater than 30 mL and require more than one vial of RhIG for adequate Rh immunoprophylaxis. However, the likelihood that a D– mother receiving only one vial of RhIG will become alloimmunized to D and form anti-D is only 0.07 percent of all D– women who receive RhIG. Thus, despite technical limitations, the risk of failure of Rh immunoprophylaxis (alloimmunization) is very low. Nevertheless, the possibility of an FMH in a woman with increased F cells requiring additional doses of RhIG should be addressed. Recognizing that an inadequate dose of RhIG is likely to be completely adsorbed by D+ fetal RBCs in the maternal circulation, Bowman notes that after administration of an adequate dose of RhIG, passive anti-D should be present in the maternal circulation. Following this rationale, some physicians advocate measuring anti-D titers after administration of RhIG to assess the need for additional doses of RhIG. Mollison and colleagues consider this practice “unsound in principle,” as even when the D antigen concentration is low, not all anti-D is bound to RBCs. This opinion is maintained by Klein and Anstee, who revised and edited the most recent edition of Mollison’s textbook. Ness and Salamon studied anti-D titers in 30 recipients of RhIG and found no correlation between the titers and the volume calculated for FMHs. Measuring anti-D after an intramuscular dose of RhIG is an unreliable indicator of the adequacy of the dose of RhIG, and the practice is discouraged. The most specific assay for determining the adequacy of the dose of RhIG in a D– postpartum mother is one that reflects clearance of fetal D+ RBCs from the mother’s circulation. Mollison and colleagues recommend testing for clearance of D+ fetal RBCs using the rosette screen. Because the rosette screen is specific for D and is sufficiently sensitive to detect an FMH of 10 mL, we recommend this method for determining adequacy of the dose of RhIG when FC (anti-D or anti-HbF) is not available.

In summary, FC for quantifying FMHs and determining the dose of RhIG remains an underutilized technology in hospitals in the United States. During the four decades since Herzenberg and others advocated using fluorescent-activated cell-sorting technology for laboratory diagnosis, our colleagues in hematology have used FC to improve the precision of diagnoses for a wide range of hematologic diagnoses. A recent editorial in the American Journal of Clinical Pathology, “Beyond Gating: Capturing the Power of Flow Cytometry,” illustrates how effectively other disciplines have captured the promise of this technology. The present review reveals that those of us who are concerned with the laboratory basis of Rh immunoprophylaxis have not yet taken full advantage of the opportunities that FC offers. Analyzers with flow capabilities, reagent antibodies, and control reagents for FC are becoming increasingly available in the United States, making round-the-clock service more accessible (Fig. 5). We encourage industry to develop and market FDA-cleared...
reagents for FC applications for quantifying fetal RBCs for Rh immunoprophylaxis. We encourage directors of hospital and reference laboratories to look positively to the opportunities of FC for quantifying FMHs.

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References

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