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Fetal Red Cell Detection; Approved Guideline

This document provides guidance for the quantitation of fetal red blood cells in blood and other biologic fluids. The performance characteristics of various flow cytometric and microscopic assays are reviewed, recommendations are made for control usage, and principles for distinction of F cells and fetal red cells are discussed.

A guideline for global application developed through the NCCLS consensus process.

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Fetal Red Cell Detection; Approved Guideline

Abstract

NCCLS document H52—*Fetal Red Cell Detection*, provides guidance for the performance of fetal red blood cell (RBC) counting in blood and other human biologic samples. The various flow cytometric, static cytometry, and microscopic methods for the detection of fetal RBCs are reviewed, with discussion of the calibration, relative imprecision, and limitations of the various assays. Recommendations for the proper use of quality controls for the assays are presented in the context of the medical and diagnostic utility of the various methods. Additional topics discussed include screening assays for fetomaternal hemorrhage and identification of F cells (HbF containing adult red blood cells) for assessment of hemoglobinopathies and other hematopoietic diseases.

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Bruce H. Davis, M.D., Chairholder
Jonathan Ben-Ezra, M.D.
Ralph M. Bohmer, Ph.D.
Michelle Y. Clark-Stuart, MGA/MIS, M.T. (ASCP)
James N. Lowder, M.D.
Walter C. Mahoney, Ph.D.
Ronald A. Sacher, M.D.
Andre van Agthoven, Ph.D.

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Committee Membership

Area Committee on Hematology

Charles F. Arkin, M.D. **Chairholder**	**Boston University Medical Center** **Boston, Massachusetts**

Subcommittee on Fetal Red Cell Detection

Bruce H. Davis, M.D. **Chairholder**	**Maine Medical Center** **South Portland, Maine**
Jonathan Ben-Ezra, M.D.	Medical College of Virginia Hospital Campus of Virginia Commonwealth University Richmond, Virginia
Ralph M. Bohmer, Ph.D.	New England Medical Center Boston, Massachusetts
Michelle Y. Clark-Stuart, MGA/MIS, M.T. (ASCP)	FDA Center for Devices and Rad. Health Rockville, Maryland
James N. Lowder, M.D.	Chimeric Therapies, Inc. Pleasanton, California
Walter C. Mahoney, Ph.D.	Roche Diagnostics Berkley, California
Ronald A. Sacher, M.D.	Georgetown University Medical Center Washington, D.C.
Andre van Agthoven, Ph.D.	Beckman Coulter Immunotech Marseille, France

Advisors

Thomas A. Campbell, Ph.D.	Wallac, Inc. Akron, Ohio
Berend Houwen, M.D., Ph.D.	Beckman Coulter, Inc. Fullerton, California
James F. Leary, Ph.D.	University of Texas Medical Branch Galveston, Texas
Quincy Crider, Ph.D.	Suretech Diagnostic Associates, Inc. St. Louis, Missouri
Richard M. Rocco, Ph.D.	Biometric Imaging, Inc., “A Becton Dickinson Company” Mountain View, California

Jennifer K. McGeary, M.T. (ASCP), M.S.H.A. *Staff Liaison*	NCCLS Wayne, Pennsylvania
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Contents

Foreword

The detection and quantitation of fetal red blood cells (RBCs), like the reticulocyte count, are determined by most clinical laboratories using manual microscopic visual methods. The performance of this assay has been repeatedly shown to have poor precision and interlaboratory correlations, despite the fact that this assay is a major determinant in the therapeutic administration of Rh immune globulin with fetomaternal hemorrhage (FMH).[1-5] Flow cytometric methods suitable for clinical practice have recently been developed using antigenic differences or quantitative assessment of fetal hemoglobin (HbF) to distinguish fetal RBCs from adult RBCs. These methods are more precise, less subjective, and require less of the technologist's time compared to the microscopic or Kleihauer-Braun-Betke assay.[6-14]

The principles, performance variability, and quality control of flow cytometric and static cytometry methods are outlined in this guideline, along with the microscopic Kleihauer-Braun-Betke (KBB) and rosette or agglutination screening assays. Sources of interferences and the relative limitations inherent with each methodology are discussed. Even though the KBB method has limitations in precision, sensitivity, and standardization, it will likely continue to be utilized in many clinical laboratories where a less precise quantitation of FMH satisfies the clinical needs, lacking access to flow cytometry, and for calibration of more automated methods. Hence, this guideline provides methodologic details on the KBB method in an attempt to improve performance.

Many of the methods used to quantitate fetal RBCs for detection of FMH also identify nonfetal or adult RBCs that contain lower levels of HbF, the so-called "F cells." Recognition of F cells was formerly of concern as a source of false-positive results with the KBB assay and in the evaluation of patients with suspected hereditary persistence of HbF (HPFH).[15-18] However, recognition of the therapeutic benefit of increasing levels of F cells in hemoglobinopathies, such as sickle cell anemia, has recently increased the interest in assays that allow quantitation of F cells.[19-21] Additionally, there is evidence that F-cell quantitation may provide prognostic information in myelodysplastic syndrome.[22-24] Accordingly, this proposed guideline provides a discussion of the challenges in standardizing F-cell counting methods, as well as the importance in distinguishing F cells from fetal RBCs. The subject of fetal cell identification for purposes of genetic testing, although a technologically challenging and potentially important area of medical diagnosis,[25] is outside the scope of this document and is not discussed.

Key Words

Erythrocytes, erythropoiesis, F cells, fetal cells, fetal hemoglobin, fetomaternal hemorrhage, flow cytometry, hematopoiesis, hemoglobinopathy, hemolytic disease of the newborn, maternal transfusion, myelodysplastic syndrome, quality control, red cells, reference method, sickle cell anemia

Fetal Red Cell Detection; Approved Guideline

1 Introduction

The motivation for developing this guideline was twofold: the advent of flow cytometric and other automated methods for fetal red blood cell (RBC) detection and the desire to provide a document to assist in the standardization and quality control of these new techniques. The limitations of the manual microscopic visual counting method for fetal RBCs, the Kleihauer-Braun-Betke (KBB) or acid elution technique are well documented,[1-5] but given the therapeutic need for directing Rh immune globulin (RhIG), administration in fetomaternal hemorrhage (FMH) and the lack of alternative methodology, this method has lingered on in clinical practice. The arrival of flow cytometric and static cytometry alternatives now promises to greatly improve the laboratory's ability to more accurately detect and enumerate fetal RBCs in FMH.[6-13] These automated methodologies have the potential to more reliably and reproducibly count F cells/nonfetal RBCs that contain lower levels of hemoglobin F (HbF), for which a clinical need is becoming established.

This guideline reviews the performance characteristics of all available methods for fetal cell detection and enumeration, cites sources of potential interference, and provides recommendations for quality control.

2 Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80), (MMWR 1987;36[suppl 2S]2S-18S), and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management of blood-borne exposure, refer to the most current edition of NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections*.

3 Scope

This document outlines the methodologies currently available and caveats for interpretation utilized in fetal RBC counting. Included are manual microscopic screening and quantitative techniques and more automated flow cytometric and static cytometric methods. Methods to ensure clinically acceptable precision and accuracy in calibration and quality control are outlined. The KBB method is currently designated the Class B reference method in this document, due to the anticipation that flow cytometric methods based upon HbF detection by monoclonal antibodies will gain acceptance as the more appropriate reference method, due to greater accuracy. The relationship to fetal RBC counting results and therapeutic administration of RhIG in the treatment of FMH in Rh_o or D antigen-negative women is also provided. Although the enumeration of F cells is not the primary focus of this proposed guideline, potential methods for F-cell counting are discussed, primarily in an effort to guide the subsequent standardization of this clinically useful measurement.

4 Definitions[a]

Accuracy, *n* - Closeness of the agreement between the result of a measurement and a true value of the measurand/analyte. **NOTES**: a) Usually expressed in the same units as the result, as the difference between the true value and the value, or as a percentage of the true value that the difference represents; expressed this way, the quantity is more correctly termed "inaccuracy"; b) "Accepted reference value" may be used in place of "true value"; c) The difference includes contributions not only from process inaccuracy but also from process imprecision, especially when one determination per specimen is the rule; d) The relevant meaning of the term "accuracy" from the patient's point of view.

Calibration, *n* - 1) The process of testing and adjustment of an instrument, kit, or test system, to provide a known relationship between the measurement response and the value of the substance being measured by the test procedure; 2) The set of operations that establish, under specified conditions, the relationship between values of quantities indicated by a measuring instrument or measuring system, or values represented by a material measure or a reference material, and the corresponding values realized by standards; **NOTES**: a) The result of a calibration permits either the assignment of values or measurements to the indications or the determination of corrections with respect to indications; b) A calibration may also determine other methodological properties, such as the effect of influence quantities; c) A slightly different World Health Organization (WHO) definition is "the set of operations that establish, under traceable conditions, the relationship between values indicated by a measuring instrument or measuring system for an established reference material and the corresponding value of a candidate reference material."

Carry-over, *n* - The discrete amount of analyte carried by the measuring system from one specimen reaction into subsequent specimen reactions, thereby erroneously affecting the apparent amounts in subsequent specimens.

Control material, *n* - A device, solution, or lyophilized preparation intended for use in the quality control process. **NOTES:** a) The expected reaction or concentration of analytes of interest are known within limits ascertained during preparation and confirmed in use; b) Control materials are generally not used for calibration in the same process in which they are used as controls.

F cell, *n* – A non-nucleated erythrocyte or red cell containing hemoglobin F in addition to other hemoglobin types. **NOTE:** It is found in all individuals of all ages, as distinct from fetal red cells which contain hemoglobin F as the sole or predominant hemoglobin type and are only found in the *in utero* fetus, newborn, and pregnant female circulation or fluids containing blood.

Fetal red blood cell, *n* – A nucleated normoblast (red blood cell precursor) or non-nucleated erythrocyte containing hemoglobin F as the predominant hemoglobin type and produced by an *in utero* fetus. **NOTE:** It may be found in maternal circulation, as red cells, which contain hemoglobin F as the predominant hemoglobin type, but distinct from those F cells present in adult circulation in the nonpregnant individual.

Flow cytometry, *n* - A methodologically oriented subdiscipline of analytical cytology that measures cells in suspension in a liquid vehicle as they pass, typically one cell at a time, by a measurement station. **NOTE:** The measurement represents transformations or changes in the output of a detector (or detectors) due to changes in scattered light, absorbed light, light emitted (fluorescence) by the cell, or changes in electrical impedance, as the cell passes through the measuring station.

Fluorochrome, *n* - A chemical compound that has the property of absorbing light at one wavelength and emitting light of a longer wavelength.

[a] Some of these definitions are found in NCCLS document NRSCL8—*Terminology and Definitions for Use in NCCLS Documents.* For complete definitions and detailed source information, please refer to the most current edition of that document.

Linearity, *n* **-** The ability (within a given range) to provide results that are directly proportional to the concentration of the analyte in the test sample*;* **NOTE:** Linearity typically refers to overall system response (i.e., the final analytical answer rather than the raw instrument output).

Miller disc, *n* - An optical micrometer or reticule that is placed into the optical light path of a microscope. **NOTE:** It is designed as an inscribed square containing smaller inner square(s) inscribed with exactly one-ninth the area of the larger outer square. The examiner determines the ratio of one cell type (e.g., reticulocytes) to another cell type (e.g., mature erythrocytes).

Patient sample, *n* - A sample taken from the patient specimen and used to obtain information by means of a specific laboratory test.

Precision, *n* **-** The closeness of agreement between independent test results obtained under prescribed/stipulated conditions; **NOTE:** Precision is not typically represented as a numerical value but is expressed quantitatively in terms of imprecision—the SD or the CV of the results in a set of replicate measurements.

Qualified examiner, *n* – For the purpose of this document, a person with special training and recognized skills in peripheral blood cell morphology, and who is qualified according to the criteria detailed in NCCLS document H20—*Reference Leukocyte Differential Count (Proportional) and Evaluation of Instrumental Methods.*

Reference interval, *n* - The range of test values expected for a designated population of individuals. **NOTE:** For example, 95% of individuals that are presumed to be healthy or normal.

Reference method, *n* **-** A thoroughly investigated method, in which exact and clear descriptions of the necessary conditions and procedures are given for the accurate determination of one or more property values, and in which documented accuracy and precision of the method are commensurate with the method's use for assessing the accuracy of other methods for measuring the same property values, or for assigning reference method values to reference materials*;* **NOTE:** Several categories of reference method exist, including: Class A reference method**,** which is characterized by both sufficient accuracy and precision and by a low incidence of susceptibility to known interferences, so that the stated purpose of the analytical process can be achieved, all of which is demonstrated by direct comparison with the definitive method; Class B reference method**,** which is believed to be of the caliber of a Class A Reference Method, except that the process of evaluation with a definitive method and a certified reference material has not yet been completed; Class C reference method**,** which is believed to be of the caliber of a Class A Reference Method, except that no definitive method is likely to become available.

Sensitivity, *n* **-** The change in response of a measuring system or instrument divided by the corresponding change in the stimulus; **NOTES:** a) A significant scientific dispute exists regarding this term, its underlying concept and its definition, with the opposing view defining Sensitivity in a manner similar to VIM93's definition for Limit of Detection. While ordinarily, a VIM93 citation as given would be sufficient to settle the dispute, a significant case has been made on both sides. Consequently, until the dispute is scientifically settled, the definition above is a standard only if it is clearly stated in the context of its use in a document; b) The sensitivity may depend on the value of the stimulus; c) The sensitivity depends on the imprecision of the measurements of the sample.

Specificity, *n* - The ability of a test or procedure to correctly identify or quantify an entity in the presence of interfering phenomena/influence quantities.

Specimen (patient), *n* - The discrete portion of a body fluid or tissue taken for examination, study, or analysis of one or more quantities or characteristics to determine the character of the whole.

Stabilized blood product, *n* - A material prepared from blood cells treated to prolong its usefulness as a quality control material. **NOTE:** It can lack some of the functional characteristics of blood.

Static cytometry, *n* - A recently introduced clinical technology which differs from flow cytometry in that fluorescently stained cells are optically measured in a fixed volume capillary. **NOTE:** a) In this technique the laser continuously scans down the length of a disposable capillary, and the image of the cells is plotted and counted providing an absolute cell number in a defined volume; b) Gating strategies are similar to flow cytometry and include cell staining intensity, cell size, and color slope with multicolor capability.

Tolerance limits, *n* - Specified limits for allowable error. **NOTE:** Limits should depend on both the effect of the error on the clinical significance of a test and on what is technically achievable.

5 Specimen Collection and Storage

5.1 Specimen Collection Information

5.1.1 Patient Information

A test requisition should accompany all specimens and must include patient demographic information, a unique patient identification number, and should state the reason why the test is requisitioned (e.g., fetal maternal bleed or hereditary persistence of fetal hemoglobin). If the specimen is other than venous blood, it should state the source of the specimen, such as umbilical cord aspiration or amniotic fluid. Pertinent laboratory information such as Rh blood type, hemoglobin electrophoresis, or other hemoglobin analysis should be made available when appropriate and possible.

5.2 Sample Collection Techniques

As a rule, all body fluids, whether they are blood, bone marrow, or other aspirates, should be considered potentially infectious. Biosafety Level 2 (BSL-2) practices should be followed during the collection and handling of body fluids. The date and time of collection of every specimen should be noted. When shipped, care should be taken that shipment temperatures do not exceed room temperature. If measurement of red cell volume is part of the assay, it should be noted that MCV changes over time and increases as a result of lack of oxygen and other metabolic processes.

5.2.1 Venipuncture

Please refer to the most current edition of NCCLS document H3— *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture* for detailed information on the collection of blood specimens by venipuncture.

5.2.2 Skin Puncture Blood Collection

Please refer to the most current edition of NCCLS document H4— *Procedures and Devices for the Collection of Diagnostic Blood Specimens by Skin Puncture* for recommendation on the collection of diagnostic blood specimens by skin puncture.

5.2.3 Other Body Fluids or Tissue Samples

Additional sources of specimens could be useful in the laboratory evaluation of patients with fetal red blood cells present under circumstances where they should not be present or where the quantitation of the fetal RBCs is of clinical importance. Such specimens may include amniotic fluid, bone marrow aspirates, vaginal fluids, or gastric lavage.

5.2.4 Anticoagulants

All specimens should contain an anticoagulant. Ethylenediaminetetraacidic acid (EDTA), acid citrate, dextrose (ACD, solution A), or heparin should be used to avoid the formation of cellular clots. In general, EDTA as either a K_2 or K_3 salt is the anticoagulant of choice.

5.2.5 Labeling of Specimen

The specimen should be labeled with a unique patient identifier as on the requisition form. When multiple specimens from the same patient are collected for analysis, the specimens should have a unique specimen identifier or hazard label. Specific labeling of specimens as infectious is not required, because all specimens should be regarded as potentially infectious and standard precautions must be followed.

5.2.6 Specimen Handling and Packaging

Please refer to the most current local and regional regulations for detailed information on specimen handling and packaging.

5.3 Specimen Integrity

Handling and transportation procedures must maintain the viability of specimens. Specimen integrity is a composite product of the anticoagulant, storage conditions (time and temperature), and sample preparation procedures. Specimens can be maintained at room temperature (18 to 22°C), but for long-term storage (greater than six hours), refrigerated temperatures (4°C) should be preferred to prevent deterioration of the specimen. Specimens designated for overnight shipment should be packaged on wet ice or similar material to ensure near-refrigerated temperatures without the danger of specimen freezing. These are general guidelines based upon the collective experience of the subcommittee. Laboratories should establish specific guidelines for sample collection and storage based upon the assay method and anticoagulants employed. Loss of specimen integrity is judged by the lysis or loss of red cells, which may be determined by cell counting or the increase in free hemoglobin level in the plasma component of the specimen.

5.4 Specimen Evaluation

Specimen evaluation is necessary to ensure specimen integrity as well as proper specimen handling and mixing prior to analysis.

5.4.1 Hemolysis

Hemolysis indicates that the blood has been exposed to conditions that can cause erythrocyte lysis. Visibly hemolyzed specimens should be rejected. Samples exhibiting evidence of hemolysis may still be tested by the laboratory; however, the reported results should note that the presence of hemolysis may present a potential complication.

5.4.2 Clotted Blood

A small blood clot may not influence the outcome of testing, but severely clotted specimens should be rejected. Samples exhibiting evidence of blood clots may still be tested by the laboratory; however, the results should note that the presence of a clotted specimen may present a potential complication.

5.4.3 Partial Draw

When the blood sample is severely underdrawn into a blood collection device, the hypertonic conditions by the anticoagulants present in the tube may be deleterious to the cells. If no hemolysis has occurred, the small volume sample may not affect final results, but should be noted as a potential complication.

5.4.4 Extreme Temperatures

If the specimen was mailed to the laboratory, it may have been exposed to extreme temperatures. If a specimen arrives in the laboratory after prolonged storage (i.e., greater than six hours after blood collection) in a frozen state or at ambient temperature, there should be a suspicion that the sample has been exposed to extremely hot or cold temperatures. Samples exhibiting evidence of such exposure to extreme temperatures may still be tested by the laboratory; however, exposure should be noted for consideration during preparation, analysis, and interpretation. Reported results should note evidence of exposure to extreme temperatures as a potentially complicating issue.

5.4.5 Improper Specimen Labeling

Improperly labeled specimens should be rejected by the laboratory.

5.5 Specimen Storage

If specimen analysis is delayed for more than six hours from the time of sample collection, the specimen should be refrigerated. Specimens stored at refrigerated temperatures (2 to 6°C) may be stable for up to 72 hours. Shipment of specimens to laboratories requiring a prolonged, transient time (e.g., greater than one hour) should be accomplished using containers that allow storage of specimens at refrigerated temperatures (2 to 6°C). However, the manufacturer's stability recommendations for their fetal RBC method should be confirmed by the laboratory. There should be no visible hemolysis, and the red cell counts should be stable (i.e., coefficient of variation, CV ≤ 3 %). These are general guidelines based upon the collective experience of the subcommittee. Laboratories should establish specific guidelines for sample collection and storage based upon the assay method and anticoagulants employed.

6 Methodology

6.1 Rosette Anti-D Qualitative or Screening Method

6.1.1 Principle

The microscopic weak-D (MWD) test or its more sensitive modification, the rosette test, can be used for qualitative detection of fetal-maternal hemorrhage where the infant is D-positive and the mother is D-negative (Rh_0). It is based on the principle that D-positive red cells in the mother's D-negative blood produce a mixed field agglutination in the antiglobulin phase following the addition of anti-D reagent. This test can be enhanced by the further addition of indicator D-positive cells that react with antibody molecules bound to the D-positive cells in the maternal circulation to form visible agglutinates or rosettes around them.[26] The rosette assay is reported to have a sensitivity of ~0.25% fetal D positive cells in a background of D-negative (Rh_0) red cells, which is sufficient to detect a fetomaternal hemorrhage of approximately 6.25 mL of red cells or 12.5 mL of fetal blood.[7]

6.1.2 Procedure

The MWD or its modification, the rosette test, may be performed either with a commercial kit or with another verified protocol.[27]

(1) A specimen of maternal D-negative blood is collected in EDTA and completely mixed.

(2) The cells are washed and a 3% suspension (v/v) in saline is incubated with a serum containing anti-D for 15 minutes at 37°C. Subsequently, the cells are washed to remove all unbound antibody.

(3) In the MWD phase of the test, antihuman globulin is added to the washed cells, centrifuged and examined microscopically for mixed-field agglutination. In the case of the rosette test indicator cells (D-positive cells) and an enhancement reagent are added; the tube is mixed and then centrifuged briefly followed by placement on a slide and microscopic examination for the presence of rosettes or mixed field agglutination.

NOTE: Because mixed field agglutination may be difficult to detect, the rosette procedure uses D-positive red cells as an indicator to demonstrate antibody coating of the D-positive cells that were present in the original maternal blood specimen.

(4) The easily visible rosettes can be viewed under low-power magnification (100x). Although the number of rosettes has a rough relationship to the degree of fetal-maternal hemorrhage, this test and the microscopic weak-D test are qualitative procedures and should be used for screening only. These tests should then be followed by a procedure for enumerating fetal cells such as those described below.

6.1.3 Controls

Each assay batch should include a known negative and positive control sample, which can be either fresh blood or stabilized blood products with known Rh or D antigen status. Negative control samples should be ~3% suspensions of D (Rh_0)-negative red blood cells in saline. Positive control samples should be a similar ~3% suspension of red cells in saline, but containing at least a 0.5% concentration (1/200) of the red blood cells being D positive in a background of D-negative red blood cells. Control samples should be handled and assayed in a similar fashion to the patient sample(s). Assays should be validated regarding the sensitivity level of fetal RBC detection. (See below.)

6.1.4 Interpretation

A negative result is the absence of any rosettes. When enzyme-treated indicator cells are used, occasional rosettes may be seen (up to one rosette per three fields in a negative specimen). When using untreated indicator cells and an enhancing medium, up to six rosettes per five microscopic fields may be seen in a negative test.

The presence of greater numbers of rosettes than these allowable levels is interpreted as a positive result.[27] A positive test indicates that a large fetal-maternal hemorrhage (at least 6.25 mL of red cells or 12.5 mL of fetal blood) has occurred, although the threshold of sensitivity may vary depending upon the assay employed. A number of variables influence the test procedure, and consequently this test should not be used for quantitating the amount of a fetomaternal hemorrhage. Consequently, other quantitative tests should be used for validation of positive results or calculation of additional Rh immune globulin therapy.

6.1.5 Limitations

The MWD or rosette assay is subject to predictable conditions giving possible false-positive or false-negative results. False positivity can occur if the maternal cells are positive for a direct antiglobulin test, as seen with immune-mediated hemolytic anemia. Incomplete washing of the cells after incubation with the anti-D reagent may cause a false-positive result, which would be detected in the negative control sample. False positivity can also occur in a woman who has a weak-D (formerly termed D^u) phenotype rather than D negative and might simulate the occurrence of a massive fetal-maternal hemorrhage.

Similarly, if the infant's cells are weak-D positive, the test may be falsely negative. The presence of ABO antigen incompatibility between mother and fetus may cause a false-negative result even in the presence of a large fetomaternal hemorrhage, if the ABO incompatible fetal red cells are cleared at an accelerated rate from the maternal circulation. These assays are not reliable in the assessment of FMH in conditions other than that of a D-negative mother with a D-positive fetus.

As the rosette and MWD assays are not quantitative, a positive result does not necessarily indicate that additional Rh immune globulin therapy is required. Any positive result with these qualitative assays should be repeated with a quantitative assay, such as the KBB test or flow cytometric assays for FMH. Conversely, a negative result must not be interpreted as indicating that no Rh immune globulin therapy is required, as there is limited sensitivity to the qualitative assays.

6.2 Kleihauer-Braun-Betke (Acid Elution) Test

6.2.1 Principle

The Kleihauer-Braun-Betke (KBB) test, commonly referred to as the Kleihauer Betke or KB assay, is a frequently employed method for enumerating fetal cells in maternal blood and was first described in 1957.[2,28] It is based on the principle that cells containing hemoglobin F are less susceptible to elution of their hemoglobin by weak acids than cells containing hemoglobin A. When red blood cells on a smear are incubated in an acid buffer, the hemoglobin from adult cells is leached into the solution, leaving only ghost-like red cells. In contrast, the red cells containing Hb F retain their hemoglobin and their color following an eosin counterstain. The approximate volume of fetomaternal hemorrhage can be derived from the percentage of fetal cells in the blood film and the maternal blood volume. (See below.)

6.2.2 Procedure

The KBB test, or one of its modifications, may be performed either with a commercial kit or with another verified protocol.[29]

(1) A specimen of whole blood is collected in EDTA or suitable anticoagulant and completely mixed.

(2) A thin smear is made from this fresh blood diluted 1:1 with saline, and is allowed to air dry. The smear is then fixed in an 80% ethanol solution.

(3) The smear is washed in distilled water, and immersed in 0.1 M citric acid buffer pH 3.2 for 5 minutes, or as indicated by the manufacturer.

(4) The slides are then stained in an erythrocin B solution or other similar eosin stain, rinsed, and air-dried.

(5) The slides are examined microscopically and red cells counted in several areas under oil immersion. Counting is performed on non-nucleated erythrocytes. The fetal RBCs stain dark pink or red with a slightly darker central region. Other adult cells vary from a totally washed-out or ghost-cell appearance to less intensely pink-stained F cells, which should be counted as nonfetal RBCs. To obtain reasonable precision, at least 2,000 RBCs should be counted and scored. (See below.)
NOTE: If a slide spinner or other automated slide maker is used to prepare the smears, it must comply with appropriate safety procedures.

6.2.3 Counting Procedures

The level of sensitivity for the KBB must be at least that of the clinical usefulness. Therefore, for institutions that use a 100-µg dose of Rh immune globulin, the analytic level of sensitivity must be at least 0.2% (corresponding to an FMH of 5-mL red cells or 10 mL of blood); for institutions that use a 300-µg

dose of Rh immune globulin, the analytic level of sensitivity must be at least 0.6% (the level to detect an FMH of 15-mL red cells or 30 mL of blood). To achieve adequate accuracy and precision at this level, at least 2,000 cells (or their Miller disc equivalent [See below.]) should be counted. Table 1 shows the variation of precision with the percentage of fetal cells in the patient sample and the number of cells counted.

Table 1. RBC Number to Be Counted for Required Precision*

Desired Coefficient of Variation

Fetal RBC Proportion (%)	2%	5%	10%
0.01	247,500	39,600	9,900
0.02	122,500	19,600	4,900
0.05	47,500	7,600	1,900
0.10	22,500	3,600	900
0.20	10,000	1,600	400
0.50	2,500	400	100

* These RBC numbers are the total RBCs to be counted if a Miller disc is *not* being used. If a Miller disc is used the actual RBCs counted would be 1/9 of these numbers, since the small square in which the red cells are actually counted is 1/9 the size of the larger square. Fetal RBC proportions (%) are listed as percentages of fetal RBC of total RBC population, not SI units.

The blood films are examined under low power (100x) to ensure the uniform distribution of the red cells. The red cells should be closely spaced, but not touching or overlapping. If satisfactory, the film is examined with an oil immersion lens. It is preferable to perform the microscopic counts from several regions of the smear. The total number of red cells counted is recorded, as well as the number of fetal cells detected.

The use of a restrictive eyepiece, such as the Miller disc, may improve the accuracy of the count relative to the actual number of cells counted. If a Miller disc is used, care must be taken to observe the "edge rule" when counting red cells in the small square of the Miller disc. Procedures for using the Miller disc may be found in the most current edition of NCCLS document H44— *Methods for Reticulocyte Counting (Flow Cytometry and Supravital Dyes)*.

6.2.4 Controls

Each assay batch should include known negative and positive control samples, which can be either fresh blood or stabilized blood products with a known concentration of fetal RBCs. Negative control samples should be adult blood or stabilized blood products from a nonpregnant individual and preferably not containing increased F cells. Positive control samples should be a mixture of adult and fetal (or umbilical cord) blood or stabilized blood products containing at least 1% fetal RBCs for a high-positive control sample, and 0.1% to 0.3% fetal RBCs for a low-positive control sample.

6.2.5 Limitations and Known Interferences

Some hematological disorders, such as certain types of thalassemia, sickle cell anemia, and hereditary persistence of fetal hemoglobin (HPFH), may produce a marked increase in so-called F cells in the maternal circulation that has high levels of fetal hemoglobin. In these cases, there may be an apparent increase in fetal cells in the maternal blood due to the difficulty in distinguishing fetal RBCs from F cells. In addition, the degree of elution of adult hemoglobin will vary from patient to patient, and from run to run. Conditions with an increase of Hemoglobin F may produce an increase in the numbers of F cells,

complicating an accurate count of fetal cells. The interobserver variation on the KBB assay has repeatedly demonstrated a high level of imprecision with the CV typically greater than 50%.[1,5,8]

6.3 Flow Cytometric Protocols

6.3.1 Principle

Flow cytometric analysis for fetal RBCs and FMH assessment has been reported utilizing differences in fetal and maternal cells either based upon D-antigen expression,[10-13, 30-32] or the level of Hb F in the cell.[6,9,13,33,34] The use of differences in D-antigen expression is a relatively uncomplicated cell-surface immunophenotypic procedure, but does limit the clinical application to situations where there are antigen differences. The anti-Hb F assay, although more broadly applicable to all clinical situations of FMH, is technically more complicated by fetal hemoglobin's intracellular location. Like other intracellular protein measurements, the anti-Hb F assay allows erythrocytes to be fixed and the cell membrane permeabilized to allow the anti-Hb F antibody to bind. Accordingly, a number of fixation/permeabilization (fix/perm) protocols have been developed. The anti-Hb F assay identifies fetal RBCs on the basis of the higher level of intracellular Hb F relative to adult erythrocytes. This identification can be combined with measurement of the blood group antigen i, a carbohydrate antigen expressed on fetal RBCs and usually absent on adult erythrocytes, which is insensitive to fixation procedures. At the time of this writing, one anti-Hb F method is available in the U.S. for clinical fetal RBC counting in FMH,[6] but it is likely other similar diagnostic assays will achieve the same status. It is not the goal of this document to recommend a specific protocol or commercial kit, but to summarize the methodology and enlighten the user to potential problems.

6.3.2 Anti-Hemoglobin F Methods

6.3.2.1 Fixation

"Fixation" describes the cross-linking of the hemoglobin and other protein molecules within the erythrocytes. This is done so that the cell membrane remains intact and hemoglobin will not leak out of the cell when the cell's membrane is permeabilized prior to incubation with the anti-Hb F antibody. Cross-linkers commonly used to fix erythrocytes include dimethyl 3,3'-dithiobispropionimadate (DTBP), glutaraldehyde, and formaldehyde. Regardless of the fixative, the highest quality of reagent available should be used.

Since glutaraldehyde and formaldehyde are volatile and undergo polymerization in aqueous conditions, many protocols recommend preparing fresh fixation buffer immediately before use and proper storage of stock solutions. Regardless of the fixative used, the biconcave shape of the erythrocytes should be preserved after fixation. This can be confirmed by microscopic examination of the fixed cells. No clumps or fragments should be present. Equally important is that significant cell lysis does not occur, which may preferentially affect adult or fetal cells and alter the original relative proportion of these populations. Cell lysis can be detected by the presence of free hemoglobin in the cell suspension supernatant.

Also, the fixed cells should preferably behave similarly to unfixed cells, in terms of forward- and side-scatter parameters, when analyzed by flow cytometry. This similarity of size can be confirmed by performing the initial fixation steps described in the user's chosen protocol with/without the fixative followed by analysis. Additionally, fixation should not result in an increase in cell aggregates, which is generally avoided by mixing or gentle vortexing during the initial phases of the fixation step of a procedure. All times and wash steps should be identical. Permeabilization cannot be performed during this comparison since the unfixed cells will undergo lysis.

6.3.2.2 Washing

Regardless of the protocol chosen, thorough washing of the erythrocytes before and after fixation/permeabilization may be required. Cells should be handled as gently as possible during pelleting and resuspension. Utilization of automated cell washers, which are commonly used in immunohematology laboratories, can provide uniformity to a procedure and shorten the requisite technical time of a procedure.

6.3.2.3 Permeabilization

Once fixed, the erythrocyte cell membrane has to be permeabilized to allow the intracellular access and binding of the anti-Hb F antibody. Several different permeabilization methods/protocols have been described. These protocols can use organic solvents, such as chilled acetone and methanol, or detergents, such as Triton X-100. Again, the highest quality of reagents available should be used to prepare these solutions, and the erythrocyte morphology should be maintained without loss of cells through lysis.

6.3.2.4 Antibodies

A number of different anti-Hb F antibodies are available from commercial as well as private sources. Although the specificity of most of these antibodies has been well established by ELISA or immunostaining, the user is urged to confirm the specificity of the antibody when used with his/her fixation/permeabilization protocol of choice. Consideration should be given to local regulatory requirements for clinical use of the antibodies and methods.

Antibody concentration should also be optimized prior to implementation. In addition to specificity, optimal antibody concentration should be determined. Both specificity and antibody concentration can be tested and optimized by preparing mixtures of normal adult blood with blood containing high levels of fetal RBCs, such as umbilical cord or neonatal blood. These samples should be of the same ABO and Rh group to minimize any agglutination or hemolysis. The mixtures should be made in the range that simulates the actual expected range of significantly abnormal clinical samples (e.g., 1 to 5% fetal cells). The antibody concentration should be sufficient to ensure antigen saturation even in the presence of high levels of fetal RBCs; otherwise subsaturated binding may result in under-counting of fetal RBCs and the false elevation of F cells. Another concern in determining the optimal antibody concentration is fluorescence quenching of fluorochromes at high antibody concentrations.

Analysis of adult samples, as well as adult samples containing 1% to 5% cord blood, should produce histograms similar to those illustrated in Figure 1. Unstained, fixed cells produce a symmetrical peak that should be positioned fully within the first decade of the log fluorescence scale (Figure 1A). Normal adults possess a small number of fetal hemoglobin-containing cells known as “F cells” (Figure 1B). Levels of F cells can range from <1% to 5% in normal adults. F cells are not as fluorescent as fetal cells, as the Hb F content of these cells is less than fetal RBCs, and manifest themselves in a properly stained histogram as a small “tail” jutting off the fetal hemoglobin-negative peak. Fetal cells, on the other hand, are more fluorescent than F cells and when stained properly, should manifest themselves as a distinct peak separate from the F cells (Figure 1C). An acceptable antibody should produce a histogram similar to Figure 1C; the percentage of positive F cells should correlate to the mixture percentage. Optimal antibody concentration should also be determined by titration (i.e., the concentration of antibody which results in the best separation of F cells from fetal cells as well as the highest fluorescence intensities of the target fetal cells).

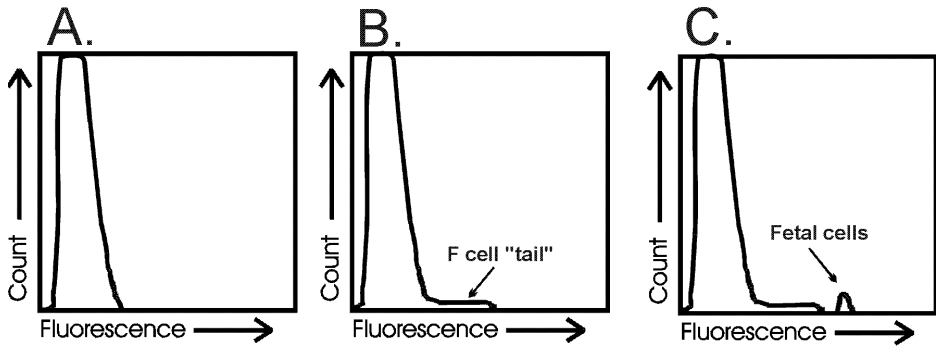

Figure 1. Analysis of Adult Samples and Adult Samples Containing 1% to 5% Cord Blood

Most of the published protocols contain descriptions of the parameters used for analysis. Since a large number of fixed erythrocytes can be produced from a small volume of whole blood, a large number of events (30,000 to 50,000) can be easily acquired. It is this increase in the number of RBCs analyzed that contributes to the increased precision of the flow cytometric methods. (See Table 1.)

6.3.2.5 Limitations

The user should also be sensitive to the following potential pitfalls:

- **Clumping**. There should be no evidence of erythrocyte clumping during fixation. It can be avoided by thorough mixing and avoiding addition of the fixative to pelleted cells.

- **Hemolysis**. Hemolysis should be kept to a minimum during the whole fix/perm procedure. Hemolysis manifests itself as a pinkish color present in the supernatant at any of the steps involved with fixation/permeabilization.

- **Antibody Binding**. Different fixation protocols can affect antibody binding, altering the optimal antibody concentration required to achieve antigen saturation and changes in so-called nonspecific binding (to be discussed below). Antiblood group i antibodies are usually of the IgM isotype and have to be checked for specificity, preferably in the International Workshop on Monoclonal Antibodies Against Human Red Cell and Related Antigens. Many anti-i antibodies are low-affinity antibodies, and suitability for cytometric analysis has to be verified. The i antigen is insensitive to fixation, however; and after a fixation/permeabilization procedure, the presence of intact i antigenicity has to be verified.

- **Autofluorescence**. Aldehyde fixatives, particularly glutaraldehyde, induce autofluorescence in nucleated cells. Hence analysis protocols should be designed to identify and exclude such cells from the RBCs. Inclusion of these cells may cause a false-positive result, since the level of fluorescence can approach that of anti-Hb F stained fetal RBCs and/or F cells. Reticulocytes, due to their high RNA content, may also exhibit increased autofluorescence after fixation, though to a lesser degree than nucleated cells. It is important not to exclude reticulocytes of either adult or fetal origin when performing analysis.

- **Fluorochrome Properties**. Fluorescence properties of the various fluorochromes conjugated to monoclonal antibodies should be understood. Some fluorochromes, such as fluorescein, are pH dependent with regard to their quantum yield upon excitation, so attention to the pH of cell suspension buffers used during flow cytometric analysis is important. Some fluorochromes can exhibit fluorescence quenching at high concentrations or when used in combination with different fluorochromes under conditions allowing for energy transfer.

- **Coincidence during Flow Cytometry Analysis**. The use of high cell concentrations or high-flow rates (greater than 5,000 cells per second for most instruments) can result in particle coincidence (the physical proximity of two cells during the passage through the detection area resulting in being "perceived" as a single particle or cell). High coincidence rates can cause an undercounting of the negative population and potentially result in the artificial elevation of the fetal RBC frequency. Optimization of assays should include the cell concentration range and/or flow rates of flow cytometric analysis to ensure the minimization of coincident events.

- **Separation of F cells and Fetal RBCs**. As with the KBB assay, accurate fetal RBC counting is dependent upon the accurate distinction between F cells and fetal RBCs based upon the cellular level of Hb F. The use of controls containing fetal RBCs to define the fetal RBC fluorescence intensity range is critical to avoid this potential problem. The use of a second fetal marker like blood group i, which is negative on F cells, may also help to distinguish F cells from fetal cells.

6.3.3 Anti-D Antigen Methods

6.3.3.1 Fixation

Fixation of samples can be performed after staining with monoclonal antibodies, but unlike the anti-Hb F procedure, is not critical for the successful identification of fetal RBCs. However, for reasons of convenience or reduction of potential infectious agents, samples can be fixed with 0.5% to 1.0% formaldehyde in buffered salt solutions prior to flow cytometric analysis.

6.3.3.2 Antibody Staining

A number of different anti-D antibodies are available from commercial as well as private sources. Although the specificity of most of these antibodies has been well established, users should be aware of the D antigen specificity, as not all monoclonal antibodies react with all D phenotypes.[35] Different antibody combinations have been employed in the reported assays, including indirect immunofluorescence staining, direct fluorochrome conjugates to the anti-D monoclonal antibody, and biotin conjugates.[11,12,15,30,32] The choice of conjugate can influence both the assay procedure (incubation times, washing procedures, pH requirements, etc.) and the sensitivity and specificity of the method.

6.3.3.3 Limitations

The anti-D flow cytometric method is applicable only to conditions of FMH involving D-negative maternal samples with a D-positive fetus, but in that context can be utilized in a practical sense directing the appropriate utilization of RhIG therapy.[8,35,36] The anti-D method cannot be broadly applied for all individuals with suspected FMH due to the need for a defined maternal and fetal D antigen status. Additionally, the assay may give a false-negative result if the fetal blood is a weak D antigen (formerly termed D^u) or D^I phenotype, particularly if there are less than 1,000 D-antigen sites per cell.[31,35,37,38] There exists the potential for false-negative results in women given recent RhIG injection resulting in antigen epitope masking by the antibody therapy. As with the anti-Hb F assay, RBC aggregation and coincident events during flow cytometric analysis should be avoided, as it will alter the precision and accuracy of the technique.

6.3.4 Controls

The use of controls with flow cytometric methods is important not only for the usual reasons of procedural quality control, but also for defining the region of fluorescence analysis for counting of fetal RBCs in the anti-Hb F methods. Mixtures of fresh adult/cord blood can also be prepared for use as negative and positive controls. Such controls, when washed and suspended in Alsever's solution, are stable at 4°C for two weeks.[6] Alternatively stabilized blood products can be utilized, provided the staining

intensity of the material is identical to fresh fetal RBCs with the antibody utilized in the assay. Each assay batch should include a negative control, a low positive control of 0.1% to 0.3% fetal RBCs to ensure assay sensitivity, and a high positive control of greater than 1% fetal RBCs to ensure adequate antibody saturation. The high positive control can be used to set the analysis region for fetal cell detection with the region having upper and lower limits defined by the fetal RBC population of the control. Controls for the anti-D method have the additional requisite specification that the adult blood or stabilized blood product be D negative and the fetal RBC component be D positive.

Care should be taken to set the flow cytometer threshold so that small particles, such as platelets and cell debris, are excluded. Standard techniques should be used to optimize color compensation between the two fluorescence channels prior to detection of these cells. (See the most current edition of NCCLS document H42— *Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Lymphocytes.)* Also, fixed white blood cells and other nucleated cells tend to exhibit high autofluorescence, which can be identified by collecting data in different fluorescence spectral regions, such as log-green fluorescence (usually FL1) and log-orange fluorescence (FL2). Autofluorescent nucleated cells usually appear positive in both FL1 and FL2. By gating the cells that appear positive in both channels, the contribution of these cells to possible positive results should be gated out or excluded from analysis.[6] Another possible procedural approach to gate out nucleated cells is the use of a nuclear dye, such as LDS 751 or propidium iodide. Cells that are positive in the fluorescence spectra of the dye can then be gated out of the analysis.

Isotype controls can be included with each flow cytometric analysis batch, if one is concerned about nonspecific antibody binding. The use of an isotype control antibody may facilitate the definition of a "positive region" for the anti-D antigen method and has been employed in some anti-Hb F assays, particularly in the quantitation of F cells. If isotype controls are employed in an assay design, care should be taken to match the isotype to the monoclonal reagent used for fetal-RBC or F-cell identification with regard to nonspecific binding properties and the degree of fluorescence labeling of the antibody reagents (so-called F/P ratio). However, the use of a defined negative control sample can also provide similar information to using isotype controls in both the anti-D antigen and anti-Hb F methods.

6.4 Volumetric Capillary or Static Cytometry (VCC)

6.4.1 Principle

Volumetric capillary cytometry (VCC) is a recently introduced clinical technology and type of static cytometry, which differs from flow cytometry in that fluorescently stained cells are optically measured in a fixed-volume capillary.[39] VCC uses a single helium neon laser that excites at 633 nm and measures emission fluorescence with two detectors at 660 and above 690 nm. In this technique, the laser continuously scans down the length of a disposable capillary, and the image of the cells is plotted and counted providing an absolute cell number. Gating strategies are similar to flow cytometry and include cell staining intensity, cell size, and color slope with multicolor capability.

A fetal-cell and F-cell counting procedure has been described using VCC.[40] Following fixation and permeabilization of the red cells with Triton X-100, the fetal RBCs and F cells are detected with the use of a monoclonal anti-Hb F antibody labeled with the red-emitting dye CY-5. The assay takes less than one hour to perform and has a reported imprecision in preliminary studies of less than 10% CV with 0.6% and 1.8% fetal RBCs.

6.4.2 Limitations

As the VCC method represents a variation on the anti-Hb F flow cytometric method, the same limitations apply.

6.5 Enzyme-Linked Antiglobulin Test

6.5.1 Principle

The enzyme-linked antiglobulin test (ELAT) represents another quantitative method for the determination of D antigen-positive RBCs, and thus does not identify whether the cells are of maternal or fetal origin. Suspensions of RBCs are incubated sequentially with anti-D antigen reagent, anti-IgG conjugated with an indicator enzyme, and a colorimetric enzyme substrate. The subsequent reaction is then read with a spectrophotometer and the optical density recorded is proportional to the number of D antigen-positive RBCs present in the original specimen. The imprecision level of the assay has not been reported, but should be similar to other such solid-phase immunoassays with a CV of less than 10%. The method is sensitive enough to identify all women requiring additional RhIG therapy and capable of detecting at least 12.5 mL of fetal Rh-positive whole blood.[41]

6.5.2 Limitations

As with the rosetting test and the anti-D flow cytometric method, the ELAT is suitable for FMH detection in the setting of a D antigen-negative maternal blood and a D antigen-positive fetus. A false-positive result will be obtained with maternal blood of the D^u or weak-D antigen phenotype. There is also a theoretical concern for false-negative results in women given recent RhIG injection and the potential of antigen masking by the therapy. Hemolysis may present a further problem to the assay, although modifications of the assay using glutaraldehyde fixation have been reported.[42]

6.6 Gel Agglutination Technique

6.6.1 Principle

The gel agglutination technique (GAT) is a recently described assay utilizing the same principle as indirect antiglobin blood typing methodologies.[43] The method involves the incubation of test cells with serial dilutions of monoclonal IgG anti-D reagent, centrifugation, and testing the supernatant against D-positive RBCs using a GAT. A positive reaction is detected by the inhibition of agglutination of the D-positive RBCs. The imprecision level of the assay has not been thoroughly studied to date. The assay has reported a sensitivity of 0.2% D-positive RBCs.[43]

6.6.2 Limitations

The GAT appears to be semiquantitative in its performance, but further studies will need to be performed in order to determine if the assay can only substitute for the rosette assay for screening purposes or actually be utilized as a truly quantitative assay. As with all assays utilizing an anti-D antigen reagent, it is not applicable to all situations of FMH. False-positive results will occur with maternal blood of the weak-D antigen phenotype.

7 Quality Control/Quality Assurance

Good laboratory practice requires that controls be run with every batch of patient samples or at least once per day of analysis. Ideal control materials contain the analyte of interest at concentrations that cover the analytical range of the assay or at least reflect the concentrations seen in patient samples. Controls are ideally stabilized whole blood materials that can be used to provide precision monitoring of the analytical method. Whole blood controls, which are stabilized and used for precision monitoring, are an integral part of clinical laboratory testing for a wide variety of hematology-related analytes.[44] At the time of this writing, whole blood stabilized control materials for fetal RBCs or F-cell assays are under commercial development by several companies. Controls may be prepared for short-term use by admixing type-compatible fresh cord blood with normal male adult blood at various concentrations of fetal RBC levels.

These controls are stable at 4 to 8° C for about eight hours or up to two weeks if washed and suspended in Alsever's solution as described in Section 6.3.4.

Recently the College of American Pathologists (CAP) has provided stabilized whole blood challenge controls as part of a twice per year fetal RBC-counting quality assurance program (HBF survey). A similar program is offered in the United Kingdom by the United Kingdom National External Quality Assessment Scheme (UKNEQAS). Some distributors of KBB diagnostic kits offer a proficiency-testing program to their customers. Interestingly, both the CAP and UKNEQAS programs have documented in samples straddling the threshold for additional RhIG therapy that 40% to 60% of labs using the KBB method would recommend overdosing of RhIG and 4% to 11% of labs under-dosing the anti-D preparation. These proficiency-testing samples are compatible with the rosette, flow cytometry, and the KBB methods for measuring fetal RBC levels. Stabilized whole blood controls, when available, could be used for intralaboratory quality assurance programs, as it is clear from the results of the CAP and UKNEQAS surveys that the KBB method, in particular, is subject to a high level of interobserver imprecision, and such material could help improve such performance.

Each laboratory must establish guidelines for acceptable performance with control materials. As a general rule, within-run and between-run CVs using control materials at two different levels should meet or better a 15% imprecision for both fetal-cell and F-cell levels for flow cytometric and VCC methods. The table below provides a guideline on the total number of fetal cells in a maternal peripheral blood sample at various percentages of fetal RBC levels. These calculations are based on the assumption of a total red cell count in the maternal sample of 4×10^{12}/L. Each type of assay must take into consideration the numbers below when expectations are established for precision and sampling numbers in the assay at various fetal RBC levels. (Also see Section 6.2.3 and Table 1.)

Table 2. Fetal Cells Required in Control Material Samples

% Fetal Cells *	Fetal Cells/µL
0.05	2,000
0.10	4,000
0.20	8,000
0.40	16,000

* Fetal RBC proportions (%) are listed as percentages of fetal RBC of total RBC population, not SI units.

8 Analytical Precision/Decision Levels

8.1 Calculation of Fetal-Maternal Hemorrhage Volumes

8.1.1 Fetal RBC Counts

For all quantitative methods, except the KBB assay using a Miller disc or similar ocular counting device, the number of fetal cells and the total number of red cells (including fetal cells) are counted separately. The percentage of fetal cells is calculated as follows:

$$\%\,fetal\ cells\ in\ maternal\ circulation = \left(\frac{Number\ of\ \ fetal\ cells\ counted}{Total\ number\ of\ \ red\ cells\ counted} \right) 100$$

If a Miller disc is used, because the area of the small square is one-ninth that of the large square, the percentage of fetal cells is:

$$\%\ fetal\ cells\ in\ maternal\ circulation = \left(\frac{Total\ \#\ fetal\ cells\ counted\ in\ large\ squares}{Total\ \#\ RBCs\ counted\ in\ small\ squares \bullet 9}\right)100$$

8.1.2 Estimation of the Volume of Fetal-to-Maternal (FMH) Hemorrhage

The dose of Rh-immune globulin necessary to adequately treat an FMH is based upon the calculated size of the transplacental fetal hemorrhage.[3,45-48] The measured size of the FMH may vary depending upon the fetal RBC counting assay employed; in particular the flow cytometric methods tend to give lower results for the fetal RBC percentage in large part due to the increased precision afforded by this methodology.[6,8,49,50] To calculate the quantity of fetal hemorrhage (in mL of fetal blood) into the maternal circulation, the percentage of fetal cells in maternal circulation is multiplied by 50 (using the assumption that the maternal blood volume is 5.0 L).

Quantity of fetal hemorrhage into maternal circulation (mL) = (% fetal cells in maternal circulation) x 50

Examples: If percentage of fetal cells is measured to be 0.6%; FMH=0.6 x 50= 30 mL
If percentage of fetal cells is measured to be 0.2%; FMH=0.2 x 50= 10 mL
If percentage of fetal cells is measured to be 0.04%; FMH=0.04 x 50= 2 mL

The above formula for FMH calculation is used for expressing the FMH in terms of fetal blood volume. If the volume of fetal RBCs is desired, the calculated number should be divided by 2 (using the assumption that the hematocrit of fetal blood is approximately 50%). Although not typically employed in clinical practice, a more purist approach to the calculation of fetal RBC volume could be to correct the amount derived with the above formula by a factor of the measured hematocrit on the maternal blood sample and further increased by 22% (the average increase in mean cell volume of fetal RBCs compared to adult cells).

8.1.3 Calculation of Rh-Immune Globulin Dose

The standard initial dose of RhIG to provide prophylaxis for Rh-immune mediated hemolytic disease of the newborn or to cover an FMH varies based upon regionally defined accepted standards of medical practice or the clinical situation. Recommendations generally range from 50 to 300 µg (micrograms) or from 125 to 500 IU.[3,48] Calculation of therapeutic Rh-immune globulin doses should always be performed after consulting the manufacturer's package insert. Actual doses may vary depending upon the manufacturer and type of preparation, but 5 IU is reported by some preparations as being equivalent to 1 µg. Early studies suggested that 20 µg would provide protection against 1.0 mL of Rh-positive RBCs;[46] other recommendations advocate giving 125 IU for each mL of fetal RBCs, which may be a slightly higher dose.[48]

Additionally, RhIG doses may vary if the therapy is given intravenously, rather than using an intra-muscular injection as the route of administration. Given the geographic variability and lack of an international consensus for RhIG dosing, laboratories should report results only in terms of the fetal RBC percentage for the tested maternal blood sample with or without the calculated FMH size (clearly indicated whether the calculated amount represents fetal RBC or fetal blood volume) and avoid making RhIG dose recommendations.

8.2 Analytical Precision

8.2.1 Clinical Requirements for Precision/Accuracy

As previously mentioned in Section 6.2.1, fetal RBC quantitation is performed for the purpose of determining the amount of bleeding which has occurred during pregnancy or the peripartum period for the

purpose of administering RhIG. Not only is precision of value in this circumstance, but accuracy is also important since a dosing decision is made upon a single observation. The likelihood of D-antigen sensitization is purported to be 3% with 0.1 mL fetal bleeding, increasing to 65% with a 5-mL bleed into the maternal circulation. Accurate detection of 2.5 mL of fetal blood diluted in the entire erythrocyte volume of the mother would be valuable in establishing dosing of the rather limited supply of RhIG preparations. Consensus on initial RhIG dosage ranges from 100 to 300 µg to cover 10- to 30-mL fetal blood volumes of FMH, and manufacturers recommend additional 50-µg doses for each 2.5 mL of fetal blood or 125 IU for each milliliter of fetal RBCs. Consequently, minimal sensitivity of determinations should be 0.2% of fetal RBCs with incremental increases of 0.04% being accurately measured.

The number of positive events counted is the dominant factor influencing the variability (as measured by the coefficient of variation) of the results. Most counting assays are minimally affected by the counting statistics, as the CV of 1,000 counted events is 3.2%. Assuming that the system is able to effectively separate the cells of interest from other events without loss, the ability to count more than a thousand positive events should not be limited by time or volume. Other sources of variability include sample processing and accurate identification of Hb AA RBC (adult cells), Hb AF RBC (F cells), Hgb FF RBC (fetal cells), and debris.

Absolute volume of fetal blood in the maternal blood circulation is the measurement this assay is to achieve. Overestimation, as opposed to underestimation, is the preferable error. The actual blood volume of an individual, particularly a pregnant woman, is difficult to estimate. Variations based upon weight, height, or body surface area would likely contribute 10% or greater additional imprecision. Methods to measure blood volume currently rely upon radioisotope measurements, have associated expense, and are time consuming. Consequently, primary determination of the actual concentrations of fetal cells will not eliminate important sources of error in the fetal maternal hemorrhage volume. Given these sources of unavoidable imprecision, the percentage of fetal cells in the entire erythrocyte population using estimation for the maternal blood volume provides an adequate estimate of FMH.

Replicate specimens at the critical concentrations can provide reproducibility assessments from which confidence intervals may be derived for values in that range. As we are concerned about underestimation, the lower confidence interval is of greatest importance. Assuming that we count 4,000, 10,000, 25,000, 50,000 or 100,000 total RBC events, the anticipated positive events and confidence intervals for each percent are calculated through the critical range of values and shown in Table 3.

Table 3. Relationship of Fetal RBC Frequency, Cell Counts, and Confidence Intervals (CI)

Fetal-Bleed Blood (RBC volume) mL	% Fetal RBC*	95% CI of % Fetal RBC with 4,000 cell count	95% CI of % Fetal RBC with 10,000 cell count	95% CI of % Fetal RBC with 25,000 cell count	95% CI of % Fetal RBC with 50,000 cell count	95% CI of % Fetal RBC with 100,000 cell count
5 (2.5)	0.1	0.002-0.198	0.038-0.162	0.061-0.139	0.072-0.138	0.08-0.120
10 (5)	0.2	0.062-0.338	0.112-0.288	0.145-0.255	0.161-0.239	0.172-0.228
15 (7.5)	0.3	0.131-0.409	0.193-0.407	0.232-0.368	0.252-0.348	0.266-0.334
20 (10)	0.4	0.204-0.596	0.276-0.524	0.322-0.478	0.345-0.455	0.361-0.439
25 (12.5)	0.5	0.281-0.719	0.362-0.638	0.413-0.587	0.438-0.562	0.456-0.544
30 (15)	0.6	0.361-0.839	0.449-0.751	0.504-0.696	0.532-0.668	0.552-0.648

* Fetal RBC proportions (%) are listed as percentages of fetal RBC of total RBC population, not SI units.

As can be seen in Table 3, confidence intervals narrow as greater numbers of erythrocytes are counted. Until 100,000 total events are counted, the confidence intervals of each critical value overlap. Consequently, it is not possible with 95% certainty to distinguish 5-mL intervals of fetal blood volume from each other without counting more than 50,000 cells. However, if the criterion for performance is only to separate 0.2% from 0.6%, any total count greater than or equal to 10,000 is sufficient. Of course, when the variability from other than counting statistics is added, the confidence intervals are likely to broaden. Another way to look at precision and confidence in the value is coefficient of variation (CV). The CV becomes smaller as more total cells are counted and as more positive cells are counted. This is seen in Figure 2. The CV generally accepted as reasonable for a diagnostic test is ten percent. As can be seen from the graph, this is achieved across the clinically important range of 0.2 to 0.75% when 50,000 or more cells are counted.

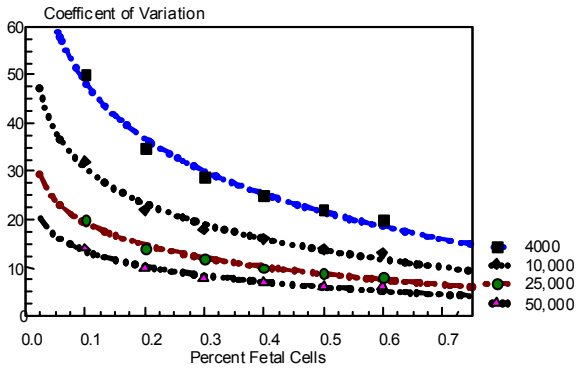

Figure 2. Relationship of Total Cells Counted to CV (ordinate) and the Measured Frequency of Fetal RBC (abscissa) Within Relevant Clinical Range Seen With Fetomaternal Hemorrhage

8.3 Linearity

Mixing experiments using cord blood and normal adult blood can be performed to assess linearity and sensitivity. Dilutional studies that extend throughout the reportable range and lower than the critical range are important in validating an assay. For a fetal RBC counting assay for FMH, linearity should be performed between 0.01% and 10% fetal RBCs.

8.4 Sensitivity

Determination of the sensitivity of the assay, defined in this instance as the lowest value that can be distinguished from zero, can be valuable. Using blood from normal adult donors compared to early-second-trimester pregnant women, one could assess the ability of an assay to differentiate circulating F cells from true fetal erythrocytes. This functional feature of an assay can be tested in several other ways. Continued dilution of the linearity assay is one method. Another method of determining sensitivity involves performing replicate assays on donors previously characterized and validating the confidence intervals. These approaches provide insight into values indistinguishable from zero.

8.5 Carryover

The previous analysis of one sample analyzed by an instrument should have negligible effect on the result of the succeeding sample. Carryover can have either a negative or positive effect on the subsequent samples. Carryover is not usually a problem in flow cytometry assays, but becomes particularly important when looking at rare events (<1%). In particular, care should be taken if a high-positive control sample or cord blood specimen is being analyzed on the same instrument as patient samples for fetal RBC

detection. In routine practice, it is recommended that buffer is rinsed through the flow cytometry instruments between samples and that high controls or fetal blood samples are analyzed as the last sample in the assay batch. Carryover testing can be performed by first running replicate samples with high levels of fetal RBCs (>3%) on the flow cytometer or any other instrument used for counting followed immediately by a series of replicate samples with low levels of fetal RBCs (<0.2%). Determine the carryover percent using the following formula:

$$\text{Carryover (\%)} = \frac{(B_1 - B_3)}{(A_3 - B_1) \cdot 100}$$

Where A_1, A_2, and A_3 are consecutive analyses of the high fetal RBC sample and B_1, B_2, and B_3 are consecutive analyses of the low fetal RBC sample.

Example: Consecutive result of high 4.12, 4.15, 4.14 (A samples) and low 0.18, 0.14, 0.13 (B samples). Carryover (%) = (0.18 – 0.13)/(4.14 – 0.18) x 100 = 1.26%

If an automatic loader or sample stainer is used routinely, it should be evaluated for carryover as well. If all specimens remain within the distribution of zero and no serial reduction in values is observed, carryover is not a problem for the system. The results of the carryover study should also be compared with the stated instrument performance specifications provided by the manufacturer.

9 Sources of Error/Test Interferences

9.1 Nonfetal Fetal Hemoglobin-Containing Cells (F Cells)

Fetal hemoglobin is the main target for the identification of fetal cells in the KBB acid elution assay and in many anti-Hb F antibody-based cytometry assays. F cells are, however, always present in low amounts in normal blood and can impair the specificity of fetal cell detection. Excessive amounts of maternal F cells are found in women with sickle cell and thalassemic syndromes, can be increased up to 25% in normal women in the second trimester of pregnancy, and rise in starvation or other states of ketosis.[16,21,51]

Therapy with cytostatic drugs (e.g., hydroxyurea) can also lead to elevated levels of F cells, and there is an increasing interest in F cell counting for purposes of therapeutic monitoring.[17,20,53-56]

Additionally, F cells are increased in some patients with myelodysplasia, and the quantitation of F cells may be of prognostic significance.[22-24]In fetal hemoglobin-based assays, F cells can only be distinguished from fetal cells by the quantitation of the fetal hemoglobin level, which is lower in the F cells than in fetal RBCs. This quantitation is problematic in the KBB test and can give rise to false-positive results.[5] In cytometry assays, F cells can be distinguished and omitted in the final fetal RBC frequency measurement, provided a positive control sample is employed in the analysis routine.[6]

Other targets for fetal cell identification, independent from fetal hemoglobin, are Rh or D antigen, which can be used in the case of D antigen incompatibility, and the blood group i antigen, which is expressed at a high density on fetal RBC and at a low density on adult RBC. Assays using these markers may be of further help in distinguishing between fetal RBC and maternal F cells.

9.2 Fetal Reticulocytes

A small percentage of fetal red blood cells and F cells are immature reticulocytes.[19,47,52,56] In case of fetal stress however, the percentage of reticulocytes within these cell populations can amount to values as high as 30%. In the KBB test, reticulocytes are resistant to the acid elution procedure and are counted as fetal cells. Also in other tests, utilizing D antigen, Hb F, or blood group i antigen expression, fetal reticulocytes

will behave as fetal RBCs. Care should to be taken in cytometric procedures measuring Hb F after a fixation/permeabilization procedure. Since reticulocytes contain RNA, gating procedures excluding nucleated cells by autofluorescence or nuclear dyes might exclude reticulocytes. Fetal and F cell reticulocytes should not be excluded from the counting of total fetal RBCs or F cells. Manufacturers' instructions should clearly indicate whether fetal reticulocytes are included in the fetal cell population and if so, how this is compensated for or dealt with by the user.

9.3 Hemagglutination

A typical problem in flow cytometry of fetal RBC is hemagglutination. Agglutination usually occurs by the interaction of divalent or other multivalent antibodies with RBCs, leading to cross-linking or agglutination of cells. Agglutinated cells, because of their size and fluorescence properties, will fall outside of the normal range of detection. Fixation of cells with an aliphatic aldehyde or a suberimidate, in combination with the use of directly fluorescent-labeled antibodies, will strongly reduce the occurrence of agglutinated RBC. Some antigens, including the D antigen, are sensitive to some fixation treatments. In the case of using unfixed cells, it is recommended to use modified monovalent fluorescent antibodies.

Mixtures of adult male blood and cord blood are often used when setting up a quality assurance system. Incompatibility of the ABO system of the two sources of blood may induce loss of RBC in cytometric analysis due to agglutination by natural antibodies. To avoid this type of agglutination, it is recommended that the red cells are washed in buffered saline solution or the samples should be processed immediately. For storage of mixed blood, the blood may be washed in neutral-buffered salt solution or any isotonic, neutral pH solution and then resuspended in Alsever's solution, AB serum, or fetal bovine serum, according to the manufacturer's recommendations.

9.4 Other Potentially Interfering Substances

In cytometry assays, elements that bind fluorescent antibodies nonspecifically or have strong autofluorescence can adversely affect the specificity of the assay. In unfixed blood, these elements may be fibrin, platelet aggregates, dead leukocytes, or parasites. In fixed blood, as used in Hb F detection, the DNA-containing leukocytes and nucleated normoblasts become a potential source of interference in cytometry. These elements can be excluded from the analysis by their high autofluorescence or by a DNA/RNA fluorescent stain, emitting at a wavelength different from that used to detect the antibodies.

In the instructions for use of any commercially available fetal RBC detection assay, the manufacturer should indicate the possible interferences and detail to the user how to recognize the variations.

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NCCLS consensus procedures include an appeals process that is described in detail in Section 9.0 of the Administrative Procedures. For further information contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Subcommittee Responses

H52-P: *Fetal Red Cell Detection; Proposed Guideline*

General

1. In general, I would like to propose that a standard nomenclature be used, i.e., "cells containing fetal hemoglobin" rather than fetal cells and "F" cells. This would eliminate any confusion as to whether the cells counted were cells with fetal hemoglobin from a fetus or cells containing fetal hemoglobin of maternal origin.

- **The subcommittee agrees that any attempt to reduce confusion on the part of the reader is important. However, the distinction between fetal red blood cells and F cells is important both from the perspective of medical implication and scientific accuracy.**

2. There are procedures included for methods described in Sections 6.1, 6.2, and 6.3, but not for methods described in Sections 6.4, 6.5, and 6.6.

- **The methods described in Sections 6.4, 6.5, and 6.6 are cited for reasons of completeness as they are either described in the literature or are under development. As these methods are not at present commercially available and/or are not in clinical use to a significant degree, detailed methods are not included by design and more detailed literature is cited for the interested reader.**

3. As an international reference point in Sections 6, 7, and 8, we would suggest that it be clarified that percentages are not displayed as SI ratios.

- **Text has been added to clarify the presentation of data.**

Section 5

4. For FMH evaluation, the guideline does not address when the mother's specimen needs to be collected regardless of what test will be employed for fetal erythrocyte detection. Is it within one hour of delivery?

- **There is no clear international guideline regarding optimal time for maternal blood sampling. Also, sample collection can be influenced by clinical circumstances. The focus of this guideline is on information relating to laboratory practice. It is outside the scope of this document to dictate clinical management. Should a clinical consensus be drafted on the aspect of optimal timing of blood sampling, these additions or changes can be made in a future edition of the document.**

Section 5.3

5. Studies at our institution have shown that samples not processed within two hours of collection may show less than optimal results and may cause nonfetal cells to take on the appearance of intermediate staining "F" cells.

- **Studies by several of the committee members and published studies have documented the stability in retained specimens as indicated in the document regarding fetal RBC counting. However, as there is little information on the effect of processing time on F cell counting by manual methods, this comment will be addressed in preparing the next edition of this guideline. Yet, F-cell counting by KB methods is not to be recommended and this potential source of error serves as another reason for not doing F-cell counts by light microscopic methods.**

Section 6.2

6. In intralaboratory studies using double blind samples, we found that the CVs were much lower than generally reported. It is our opinion after evaluating most kits on the market that there are several that do not perform as well as others. The lack of experience of the testing personnel may contribute to the cause of higher CVs rather than an inherent problem with the KB test itself. A joint effort between NCCLS and the College of American Pathologists to survey participants as to the level of experience, standardization with the Miller disk, and comparative CV assessment between kits is recommended.

- **The subcommittee will forward this comment to the CAP Hematology and Clinical Microscopy Committee. However, similar to the manual reticulocyte count, improving clinical performance is much more likely to be achieved by automated methods, such as flow cytometry, rather then attempting to improve the performance on the subjective KB assay. CAP proficiency surveys on reticulocyte counting have not demonstrated significant improvement in imprecision using the Miller disc; therefore, it is unlikely the use of this ocular assist device would improve fetal RBC counting, which is truly rare event counting.**

Section 6.2.2

7. We have found that the saline often causes the appearance of artifact in the red cells. Several studies within our institution have shown that excellent slides can be prepared without using saline. We suggest that saline not be used.

- **Studies in the published literature, several commercial kits, and several members of the committee successfully employ saline to dilute samples to minimize cell-to-cell contact on the slide, which can induce staining or interpretation artifact. Saline at too low a pH may induce artifact on that basis. Although KBB tests can be done without a 1:1 dilution, superior counting accuracy is achieved using a saline dilution.**

8. We have found that slides must be thoroughly rinsed after the buffer step before being placed into the erythrocin-B stain. This helps maintain the pH of all solutions for optimal elution and staining.

- **Studies in the published literature, several commercial kits, and several members of the committee successfully employ the procedure as written, but the comment in 6.2.2 (3) "..or as indicated by the manufacturer" is intended to give labs the prerogative to optimize the method being used by a laboratory.**

9. In reference to Table 1, it is my opinion after numerous years of experience with this test, that use of the Miller disk should be mandatory. Random counting can only lead to higher CVs.

- **The same impression existed about reticulocyte counting, but CAP proficiency surveys on reticulocyte counting have not demonstrated significant improvement in imprecision using the Miller disc, so it is speculative the use of this ocular assist device would improve fetal RBC counting, which is truly rare event counting. Because currently there is no published, supporting literature, providing mandatory recommendations without documented objective data is contraindicated.**

  As discussed in Section 6.2.3, the only true way to improve precision is through counting more RBCs; this is one of the major reasons that flow cytometric methods improve precision.

10. I suggest that slides be examined under high-dry rather than oil immersion (also suggest using higher power oculars such as 15x, if needed to enlarge cells). Sometimes with oil on the slide and the condenser not set properly, it is possible to have the "ghost" adult cells disappear from view, causing false reporting of cells with fetal hemoglobin only.

- **Studies in the published literature, several commercial kits, and several members of the committee successfully employ the procedure as written. This does not mean that experience technologists cannot perform an equally suitable count with the conditions afforded by "high-dry" lens. A recommendation for the use of 15x oculars would be a nonstandard microscopic set-up and it is the committee's view that it is impractical to recommend a more costly approach. We concur that the condenser should be properly set in an optimal fashion for each microscopic task.**

Related NCCLS Publications*

C24-A2 **Statistical Quality Control for Quantitative Measurements: Principles and Definitions; Approved Guideline—Second Edition (1999).** This document provides definitions of analytical intervals; plans for quality control procedures; and guidance for quality control applications.

C28-A2 **How to Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline (2000).** This document provides guidelines for determining reference values and reference intervals for quantitative clinical laboratory tests.

EP6-P2 **Evaluation of the Linearity of Quantitative Analytical Methods; Proposed Guideline–Second Edition (2001).** This document provides guidance for characterizing the linearity of a method during a method evaluation; for checking linearity as part of routine quality assurance; and for determining and stating a manufacturer's claim for linear range.

H3-A4 **Procedure for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard— Fourth Edition (1998)**. This document provides procedures for the collection of diagnostic specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children. Recommendations for the order of draw are also included.

H4-A4 **Procedures for the Collection of Diagnostic Blood Specimens by Skin Puncture; Approved Standard—Fourth Edition (1999)**. This standard provides detailed descriptions and explanations of proper collection techniques, as well as hazards to patients due to inappropriate specimen collection by skin puncture procedures.

H42-A **Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Lymphocytes; Approved Guideline (1998).** This document provides guidelines for the quality assurance of flow cytometric instrument operation and procedures for immunophenotyping.

H44-A **Methods for Reticulocyte Counting (Flow Cytometry and Supravital Dyes); Approved Guideline (1997)**. This document provides guidance for the performance of reticulocyte counting by flow cytometry. It also includes methods for determining the accuracy and precision of the reticulocyte flow cytometry instrument and a recommended reference procedure.

M29-A2 **Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline—Second Edition; Approved Guideline (2001).** Based on U.S. regulations, this document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

NRSCL8-A **Terminology and Definitions for Use in NCCLS Documents; Approved Standard (1998).** This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSCL).

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

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NCCLS ▼ 940 West Valley Road ▼ Suite 1400 ▼ Wayne, PA 19087 ▼ USA ▼ PHONE 610.688.0100
FAX 610.688.0700 ▼ E-MAIL: exoffice@nccls.org ▼ WEBSITE: www.nccls.org ▼ ISBN 1-56238-452-X