
This document provides guidance for the performance of reticulocyte counting by flow cytometry. It includes methods for determining the trueness and precision of the reticulocyte flow cytometry instrument and a recommended reference procedure.

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NCCLS document H44-A2—Methods for Reticulocyte Counting (Automated Blood Cell Counters, Flow Cytometry, and Supravital Dyes); Approved Guideline—Second Edition provides guidance for the performance of reticulocyte counting by flow cytometry and automated hematology instruments. This guideline addresses methods for determining the precision and trueness of the flow cytometer and blood cell counters based upon principles of focused flow dynamics, along with recommendations for calibration and quality control. A description of the new methylene blue (NMB) method, a method against which the test instrument can be compared, is also included. Additional topics discussed include reference intervals and use of related reticulocyte parameters, i.e., the immature reticulocyte fraction (formerly termed “reticulocyte maturation index”) and reticulocyte hemoglobin content (CHr).


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Committee Membership

Area Committee on Hematology

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

Bruce H. Davis, M.D.
Vice-Chairholder
Maine Medical Center Research Institute
Scarborough, Maine

J. David Bessman, M.D.
Boston University Medical Center
Galveston, Texas

Berend Houwen, M.D., Ph.D.
Beckman Coulter, Inc.
Brea, California

Frank M. LaDuca, Ph.D.
International Technidyne Corporation
Edison, New Jersey

Ginette Y. Michaud, M.D.
Center for Devices and Radiologic Health
Food and Drug Administration
Rockville, Maryland

Onno W. van Assendelft, M.D., Ph.D.
Centers for Disease Control and Prevention
Atlanta, Georgia

Advisors

Dorothy M. Adcock, M.D.
Esoterix Coagulation
Aurora, Colorado

Eugene L. Gottfried, M.D.
Orinda, California

J. Heinrich Joist, M.D., Ph.D.
St. Louis University Health Sciences Center
St. Louis, Missouri

John A. Koepke, M.D.
Durham, North Carolina

Francis Lacombe, M.D., Ph.D.
Hôpital Haut-Lévêque
Pessac, France

Jack Levin, M.D.
VA Medical Center
San Francisco, California

Samuel J. Machin, MB, Ch.B, FRCPATH, FRCP
The University College London Hospitals
London, United Kingdom

Richard A. Marlar, Ph.D.
Denver VA Medical Center
Denver, Colorado

Diane I. Szamosi, M.A., M.T.(ASCP)SH
Greiner Bio-One, VACUETTE
North America, Inc.
Monroe, North Carolina

Luc Van Hove, M.D., Ph.D.
Abbott Laboratories
Abbott Park, Illinois

Staff

Tracy A. Dooley, M.L.T.(ASCP)
Staff Liaison
NCCLS
Wayne, Pennsylvania

Jennifer K. McGearry, M.T.(ASCP), M.S.H.A.
Project Manager
NCCLS
Wayne, Pennsylvania

Donna M. Wilhelm
Editor
NCCLS
Wayne, Pennsylvania

Melissa A. Lewis
Assistant Editor
NCCLS
Wayne, Pennsylvania
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Foreword

The reticulocyte count is one of the last common hematology measurements still being performed using manual/visual methods. Flow cytometric methods have been developed that significantly reduce the time it takes a technologist to perform this measurement. In addition, flow cytometric methods for reticulocyte counting have been shown to be more precise than manual/visual methods. These automated methods also afford the ability to determine other reticulocyte-specific parameters, such as the immature reticulocyte fraction (IRF) based upon RNA content of these cells which further improve the diagnostic evaluation of anemic patients.

The inter- and intralaboratory variables of these flow cytometric methods are becoming better understood. This document provides practical guidelines that assist the technologist in counting reticulocytes using flow cytometers and automated blood counters with flow cytometric measurement principles. In addition, quality control/assurance methods are given that help ensure that the procedure is being performed with precision and trueness.

The new methylene blue method outlined in the guideline serves as the comparative procedure, if required, for the automated reticulocyte flow cytometric methods. This manual/visual method may also be used as the back-up method or as the primary reticulocyte counting method in laboratories that do not have the preferred flow cytometric method available.

H44-A2 was developed through the cooperation of the NCCLS Area Committee on Hematology and the International Council for Standardization in Haematology (ICSH). By the cooperative development of a single document, the area committee and the expert panel believe that this guideline avoids duplication and advances the international harmonization of this important hematology measurement.

A Note on Terminology

NCCLS, as a global leader in standardization, is committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. NCCLS recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in NCCLS, ISO, and CEN documents; and that legally required use of terms, regional usage, and different consensus timelines are all obstacles to harmonization. In light of this, NCCLS recognizes that harmonization of terms facilitates the global application of standards and is an area of immediate attention. Implementation of this policy is an evolutionary and educational process that begins with new projects and revisions of existing documents.

In keeping with NCCLS’s commitment to align terminology with that of ISO, the following terms are used in H44: Accuracy in its metrological sense, refers to the closeness of agreement between the result of a (single) measurement and a true value of a measurand, thus comprising both random and systematic effects; Trueness is used in this document when referring to the closeness of the agreement between the average value from a large series of measurements to a true value of a measurand.

For the sake of introduction and to avoid confusion, the subcommittee has chosen to include the following ISO terms parenthetically in the text with the U.S. terms: Measuring range is combined with Reportable range when referring to a set of values of measurands for which the error of a measuring instrument is intended to lie within specified limits; and Reference measurement procedure is combined with Reference method when referring to a thoroughly investigated measurement procedure shown to have an uncertainty of measurement commensurate with the intended use, especially in assessing the trueness of other measurement procedures for the same quantity and in characterizing reference materials.
Users of H44-A2 should understand, however, that the fundamental meanings of the terms are identical in many cases, and to facilitate understanding, the terms are defined along with explanatory notes in the guideline’s Definitions section. All terms and definitions will be reviewed for consistency with international use, and revised appropriately during the next scheduled revision of this document.

**Key Words**

Anemia diagnosis, erythrocyte, erythropoiesis, flow cytometry, immature reticulocyte fraction, new methylene blue (NMB), red cell, reference method (reference measurement procedure), reticulocyte, reticulocyte hemoglobin content

1 Scope

This document outlines the essential factors that affect automated reticulocyte counting using flow cytometric principles. The designated Class C comparative method (see Section 4, Definitions) is the new methylene blue (NMB) staining of blood collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant. Methods to ensure acceptable precision and trueness of the calibration and quality control of the automated reticulocyte counting methods are outlined. Procedures to develop appropriate reference ranges are also outlined. Methods used to express the relative and absolute maturation of reticulocytes are discussed, primarily in an effort to guide in the standardization of these additional clinically useful reticulocyte parameters.

### 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 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.

2 Introduction

Reticulocyte counts have been used for many years to estimate the erythropoietic activity of the bone marrow. While in the past this has been done using manual/visual methods, several flow cytometric and image-analysis methods are now available.1 Interlaboratory studies of flow cytometric methods have shown some intermethod bias in reticulocyte counts, as well as maturation estimates, generated by flow cytometers employing different reagents and instrumentation.2

The production of more precise and true reticulocyte data is necessary before these data can be more generally applied to improve patient care. Methods to harmonize the various reticulocyte technologies are provided in this guideline. These include methods to calibrate, and also to control this analysis.

Although primarily directed at automated or flow cytometric reticulocyte counting, portions of this document are also applicable for the manual/visual and image analysis methods for reticulocyte enumeration. Additionally, this document addresses principles and quality control of the automated reticulocyte parameters related to reticulocyte maturation, termed “immature reticulocyte fraction (IRF)” which is available on many of the automated blood counters using multiparameter flow cytometric principles.
3 Principle

Reticulocytes that are stained with one of a number of different supravital stains (e.g., NMB), fluorochromes (e.g., thiazole orange), or monoclonal antibodies (e.g., anti-CD71) are counted in a flow cytometer or automated blood cell counter that was specially designed, or otherwise modified or adjusted, for this procedure.

4 Definitions

Accuracy (of measurement) – Closeness of the agreement between the result of a measurement and a true value of the measurand (VIM93); NOTE: See the definition of Measurand, below.

Battlement pattern – A method of studying a blood film in which the slide is moved from side to side (or end to end) over acceptable examination areas; NOTE: The cumulative examination pathway resembles the battlement of a castle.

Bias – The difference between the expectation of the test results and an accepted reference value (ISO 3534-1).

Calibration – 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 (VIM93); NOTE: According to the U.S. Code of Federal regulations, calibration is 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 (42CFR493.1217).

Carry-over – The discrete amount of analyte carried by the measuring system from one sample reaction into subsequent sample reactions, thereby erroneously affecting the apparent amounts in subsequent samples.

Control material – A device, solution, or lyophilized preparation, intended for use in the quality control process. NOTES: a) Control material may include a pool of collected human or animal specimen, or artificially derived material; b) The expected reaction or concentration of analytes of interest are known within limits (e.g., mean ± SD) ascertained during preparation and confirmed in use; c) Control materials are generally not to be used for calibration in the same process in which they are used as controls.

Diagnostic sensitivity – The proportion of patients with a well-defined clinical disorder whose test values are positive or exceed a defined decision limit (i.e., a positive result and identification of the patients who have a disease); NOTES: a) The clinical disorder must be defined by criteria independent of the test under consideration; b) The term Clinical sensitivity was formerly used in this document; c) See the Note on Terminology in the Foreword.

Flow cytometry – 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 of 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 – A chemical compound that has the property of absorbing light at one wavelength and emitting light of a longer wavelength.
**Immature reticulocyte fraction** – A quantitative expression of the maturation state of the entire reticulocyte population in the peripheral blood; **NOTES:** a) This has been expressed in terms of mean fluorescence intensity using thiazole orange on multiparameter flow cytometry instruments and as a fractional expression of the subpopulation of the reticulocytes having the highest fluorescence RNA intensity or RNA content; b) Previously called Reticulocyte maturation index.

**Imprecision** – Dispersion of independent results of measurements obtained under specified conditions; **NOTE:** It is expressed numerically as the Standard deviation or Coefficient of variation of the results in a set of replicate measurements.

**Linearity** – The ability (within a given range) to provide results that are directly proportional to the concentration [amount] 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.

**Measurand** – particular quantity subject to measurement (VIM93); **NOTE:** This term and definition encompass all quantities, while the commonly used term “analyte” refers to a tangible entity subject to measurement. For example, “substance” concentration is a quantity that may be related to a particular analyte.

**Miller disc** – An optical micrometer or reticule that is placed in the optical light path of a microscope; **NOTES:** a) It is designed with a large square that contains a smaller square exactly one-ninth the area of the large square (see Figure 1A); b) Using techniques as described in Section 7.3, the examiner determines the ratio of one cell type (e.g., reticulocytes) to another cell type (e.g., mature erythrocytes); c) The disc was invented by Dr. JW Miller of the National Institutes of Health (NIH).

**Precision** – The closeness of agreement between independent test results obtained under stipulated conditions; **NOTE:** Precision is not typically represented as a numerical value but is expressed quantitatively in terms of imprecision—the standard deviation (SD) or the coefficient of variation (CV) of the results in a set of replicate measurements (ISO 3534-1).

**Qualified examiner** – 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** – The range of test values expected for a designated population of individuals, typically a population of healthy, age-matched individuals; **NOTES:** a) For example, 95% of individuals that are presumed to be healthy or normal; b) For measurements having a Gaussian or log-normal distribution only, this may be represented by the population mean plus and minus two standard deviations.

**Reference method** – 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 trueness and precision of the method are commensurate with the method’s use for assessing the trueness of other methods for measuring the same property values, or for assigning reference method values to reference material; **NOTES:** a) This definition is analogous to a Reference measurement procedure in ISO terminology. According to ISO, a reference measurement procedure is a thoroughly investigated measurement procedure shown to have an uncertainty of measurement commensurate with the intended use, especially in assessing the trueness of other measurement procedures for the same quantity and in characterizing reference materials (adopted from ISO/DIS 15193).

**Reportable range** – The range of test values over which the relationship between the instrument, kit, or system's measurement response is shown to be valid; **NOTES:** a) For this document, the range of values...
(in units appropriate for the analyte) over which the acceptability criteria for the method have been met; that is, where errors due to nonlinearity, imprecision, or other sources are within defined limits; b) The reportable range of the assay should be established prior to beginning the clinical evaluation; c) This is similar to the VIM definition for Measuring range or Working range, i.e., a set of values of measurands for which the error of a measuring instrument is intended to lie within specified limits.3 See Measurand defined, above. Also see the Note on Terminology in the Foreword.

Reticulocyte (retic) – 1) Transitional red cells between nucleated red cells and the so-called mature erythrocyte; 2) An erythrocyte that, when stained with a supravital dye (e.g., NMB), contains stainable nucleic acids (i.e., cellular RNA) or when stained with fluorescent reagents with specificity of binding to nucleic acids shows increased cellular fluorescence; NOTES: a) To be identified as a reticulocyte by visual microscopy, the cell must contain two or more distinct blue-staining granules (NMB-reticulocyte) that are visible without requiring fine focus microscope adjustment on the individual cell to confirm their presence; b) The granules should be away from the cell margin to avoid confusion with Heinz bodies.9

Reticulocyte hemoglobin content (CHr) – Reticulocytes, similar to the mature erythrocyte, contain hemoglobin as the primary oxygen-carrying molecule; NOTE: Measurement of the hemoglobin content can be performed and quantitated in terms of mean picogram of hemoglobin per cell.

Sample (patient) – A sample taken from the patient specimen and used to obtain information by means of a specific laboratory test.

Sensitivity – The change in response of a measuring system or instrument divided by the corresponding change in the stimulus (modified from VIM93-5.10);3 Notes: a) The sensitivity may depend on the value of the stimulus;3 b) The sensitivity depends on the imprecision of the measurements of the sample.

Specificity – The ability of a measurement procedure to measure solely the measurand;10 NOTE: a) Specificity has no numerical value in this context; b) See Measurand above.

Specimen (patient) – 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 – 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 – A recently introduced 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.11

Supravital dye – A dye used to stain living cells already removed from an individual.

Tolerance limits – 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.

Trueness (of measurement) – The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value; (ISO 3534-1);4 NOTE: Trueness is usually expressed numerically by the statistical measure bias that is inversely related to trueness. See also Accuracy, Bias, above.
5  Specimen Collection and Storage

5.1  Patient Information

A test requisition should accompany all specimens and must include patient demographic information, a unique patient identification number, and should ideally state the reason why the test is requisitioned. 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 should be made available when appropriate and possible.

5.2  Specimen Collection Techniques

As a rule all body fluids, whether they be 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 mean cell volume (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  Anticoagulants

Whole blood collected into EDTA (final concentration 3.7 to 5.4 µmol/mL or 1.5 to 2.2 mg/mL) is the acceptable specimen. EDTA is available as di/tripotassium and disodium salts. Other anticoagulants, such as heparin or citrate, have not been thoroughly evaluated for reticulocyte counting. NCCLS document H1—Evacuated Tubes and Additives for Blood Specimen Collection gives details about EDTA as an anticoagulant. An accurate red cell count should be determined on the same sample being used for the reticulocyte count, either using a flow cytometer or a hematology analyzer.

5.2.4  Labeling of the 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.

5.2.5  Specimen Handling and Packaging

Please refer to the most current government regulations for detailed information on specimen handling and packaging. For additional information, see the most current version of NCCLS document H18—Procedures for the Handling and Processing of Blood Specimens.
5.3 Sample Integrity

Handling and transportation procedures must maintain the viability of samples. Sample integrity is a composite product of the anticoagulant, storage conditions (time and temperature), and sample preparation procedures. Samples 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 sample. Samples designated for overnight shipment should be packaged on wet ice or similar material to ensure near-refrigerated temperatures without the danger of sample 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 Sample Evaluation

Sample evaluation is necessary to ensure sample integrity as well as proper sample 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 samples should be rejected. Samples exhibiting some 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 (not evident upon visual inspection) may not influence the outcome of testing, but severely clotted samples 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 sample may present a potential complication or may not reflect the true reticulocyte level in the patient.

5.4.3 Partial Draw

When the blood specimen 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 sample was mailed to the laboratory, it may have been exposed to extreme temperatures. If a sample 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

It is recommended that the reticulocyte count be performed promptly after the collection of the blood specimen, or alternatively, that the specimen be stored in such a way that it remains stable until the test is performed. When the original specimen is kept at room temperature, reticulocyte counting should be done within six hours after blood collection. Under such conditions, special storage conditions are not required.

With most reticulocyte methods, there is an apparent in vitro maturation and subsequent disappearance of some of the reticulocytes. This maturation is both time- and temperature-dependent and can be expressed as the number of reticulocytes that mature and are therefore not counted after the delay in analysis. This loss can be up to 20% per 24 hours at room temperature. However, this may vary with the counting system being used. Presently, it is not recommended that any inhibitors of such maturation be added to the sample.

If sample analysis is delayed, the sample should be refrigerated. Samples stored at 2 to 6 °C may be stable for up to 72 hours. However, the manufacturer’s stability recommendations for their reticulocyte method should be confirmed by the laboratory. There should be no visible hemolysis, and the red cell count should be stable (i.e., coefficient of variation [CV] ≤ 3%).

6 Correlation Method—New Methylene Blue Visual Microscopy

The NMB staining method has been recommended as the comparability method for reticulocytes. However, being a manual and subjective method, there are inherent limitations in the imprecision level, reproducibility, and sensitivity of the NMB method. Initial calibration and/or verification of reticulocyte flow cytometers should be carried out by counting patient samples stained with NMB or another previously calibrated, automated reticulocyte counting method and covering the entire range of reticulocyte counts claimed to be accurately enumerated by the instrument. The NMB procedures should be done based upon a minimum count of 2,000 cells and must be done by previously qualified examiners whose ability was verified using the method outlined in NCCLS document H20—Reference Leukocyte Differential Count (Proportional) and Evaluation of Instrumental Methods.

6.1 Stain

The supravital dye, NMB (basic blue 24, color index (CI) 52030) is the stain of choice. It should have a dye content of approximately 90%. Azure B, a purified component of polychrome methylene blue, has been found to be equally acceptable as reticulocyte stain. It has some advantages of purity and the dye does not precipitate on blood films.

Add 0.1 gram of NMB crystals to 100 mL of iso-osmotic phosphate buffer; pH 7.4 (18 mL of 150 mmol/L NaH₂PO₄ and 82 mL of 150 mmol/L Na₂HPO₄). After preparation, the solution should be shaken at intervals over 24 hours. Store in a brown glass bottle at 2 to 6 °C. At this temperature, the dye solution has a shelf life of about one month. Before use, an appropriate volume must be filtered through filter paper to remove any precipitated dye and any other particulate matter.

6.2 Slide Preparation

A specimen of whole blood collected in EDTA is mixed by gentle inversion ten times or until the complete resuspension of cells is accomplished. Equal volumes of NMB stain and blood are mixed in a glass or plastic test tube. In case of anemic or polycytemic blood, the proportion of dye to blood should be adjusted accordingly.
After staining at ambient temperature for three to ten minutes, the stain and blood mixture should be well mixed just before placing a drop of blood on the glass slide. Films covering about two-thirds of the surface are made on glass microscope slides and allowed to dry rapidly in warm air. (Please refer to the most current version of NCCLS document H20—Reference Leukocyte Differential Count (Proportional) and Evaluation of Instrumental Methods.) If a slide spinner is used to prepare the films, it must comply with all applicable safety requirements. (See the Standard Precautions statement after Section 1.)

6.3 Counting Procedure

The blood films are examined under low power (100x magnification) 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 moving from field to field in a battlement pattern. The total number of red cells to be counted varies with the reticulocyte percentage as well as the desired precision. Table 1 (Section 7.4) shows the number of red cells to be counted to obtain the necessary level of precision.

If a Miller disc is employed, it is important, particularly with higher reticulocyte counts, to obey the “edge rule” when counting red cells (as well as reticulocytes) in the small square of the Miller disc. The small square may be either in the center or at a corner of the large square. The “edge rule” states that red cells touching the lower, and also the right, side of the square should not be counted. If the edge rule is ignored, a significant bias will be found between the Miller disc count and the count done without the use of the Miller disc (i.e., the count without the use of a Miller disc is about 30% higher). Three examples of this are given below. In actual practice, many more than one field would be counted. For clarity, these examples have canceled out the data from \( n \) fields, and the cells are ideally spaced.

If a blood sample has a true reticulocyte count of 1.2%, the calculation of the reticulocyte percentage in Figure 1A will be:

\[
\frac{\text{Retics (Large Square)}}{\text{Red Cells (Small Square)}} \times 100 = \frac{1}{9 \times 9} \times 100 = 1.2\%
\]

![Miller Disc Reticulocyte Count with Ideal Red Cell Distribution](image)

- Any reticulocytes in the small square are to be counted as red cells.
If the Miller disc is shifted so that there are many red cells touching the square sides and if the edge rule is ignored (incorrect use of Miller disc):

\[
\text{Retics (Large Square)} \times 100 \\
\text{Red Cells (Small Square\textsuperscript{b})} \times 9 \\
= \frac{1}{16 \times 9} \times 100 \\
= 0.7\% \\
\]

\[\text{Figure 1B. Incorrect Use of Miller Disc}\]

If the same field as in Figure 1B is counted and the edge rule is imposed (correct use of Miller disc):

\[
\text{Retics (Large Square)} \times 100 \\
\text{Red Cells (Small Square\textsuperscript{b})} \times 9 \\
\text{including two edges only} \\
= \frac{1}{9 \times 9} \times 100 \\
= 1.2\%\textsuperscript{c} \\
\]

\textsuperscript{b} Any reticulocytes in the small square are to be counted as red cells.

\textsuperscript{c} This result agrees with the correct result in Figure 1A.
Figure 1C. Correct Use of Miller Disc

### 6.4 Calculations

If a Miller disc is not used, the number of reticulocytes and the number of red cells (including reticulocytes) are tallied separately. The percentage of reticulocytes is calculated as follows:

\[
\text{Retics} \, (\%) = \frac{\text{Total Number of Reticulocytes}}{\text{Total Number of Red Cells} + \text{Retics}} \times 100 \quad (2)
\]

If a Miller disc is used, because the area of the small square equals one-ninth that of the large square, the reticulocyte percentage is calculated as follows:

\[
\text{Retics} \, (\%) = \frac{\text{Total Number of Retics in 20 large squares}}{\text{Total Numbers of RBCs in 20 small squares} \times 9} \times 100 \quad (3)
\]

To determine the absolute number of reticulocytes, multiply the percent (ratio) of reticulocytes by the red cell count determined on the same blood sample using an adequately calibrated and controlled hematology instrument or, if available, from the reticulocyte analyzer itself.

\[
\text{Abs. Retic Count} \, (\times 10^9/L) = \frac{\text{RBC} \, (\times 10^{12}/L) \times \text{Retics} \, (\%)}{10^2} \quad (4)
\]

Due to the above-described potential for counting errors and the potential for counting bias using the Miller disc, it is recommended that such counting methods not be employed for calibration or linearity verification of automated reticulocyte counting methods unless a minimum of 1,000 red cells has been counted.
7 Specifications for Automated Blood Counters and Flow Cytometers to Be Used for Reticulocyte Counting

Automated reticulocyte counting systems based upon flow cytometric principles or static cytometry methods used for reticulocyte counting should measure a specific cellular property, which makes possible the discrimination of reticulocytes from mature red cells. Discrimination from other cells present in peripheral blood, such as leukocytes, erythroblasts, and platelets, is also necessary. At the present time, discrimination is achieved by means of the staining of reticulocyte RNA with fluorochromes or supravital dyes, in association with measurements of size and internal structure. Binding of fluorochromes to RNA makes the reticulocytes fluorescent; mature red cells have a low level of autofluorescence due to the porphyrin ring in the hemoglobin molecule. The fluorescence of stained reticulocytes should be high enough to permit the discrimination from autofluorescent red cells. Binding of supravital stains, such as new methylene blue or oxazine 750, makes the reticulocytes capable of absorbing more light than unstained red cells. The same measurements are used for the differentiation of mature reticulocytes with average RNA content from immature reticulocytes with increased RNA, i.e., the immature reticulocyte fraction (IRF). All dyes should be studied for interfering conditions, such as commonly used medications that might quench signals (e.g., anthracyclines) or pathological conditions (e.g., malaria infection, Heinz bodies, or Howell-Jolly bodies).

7.1 Reportable Range (Measuring Range)

Reticulocyte counters should provide precise and true reticulocyte measurements over the entire reportable range (measuring range). This includes samples with near-zero reticulocytes (such as after myeloablative chemotherapy) as well as samples with very high reticulocyte counts (such as in hemolytic anemias, or following vitamin B₁₂ treatment of megaloblastic anemia). Similarly, the proportion of immature reticulocytes should be accurately measured in the same ranges (especially at the lowest reticulocyte counts, because it is such samples where the clinical utility of these measurements is maximal, as these parameters are early indicators of a normal or abnormal erythropoietic regeneration).

7.2 Sample Preparation for Flow Cytometry

Blood samples are prepared according to the methods recommended by the instrument manufacturer. To obtain acceptable reticulocyte counts, strict adherence to such recommendations is mandatory.

7.3 Method(s) of Operation

The methods of operation must be clearly described in the operator’s manual, even in the case of fully automated methods. The following points require detailed explanation:

- the system used to separate the relevant cell populations (red cells plus reticulocytes) from irrelevant cell populations, such as leukocytes, erythroblasts, and platelets;
- the system used to discriminate unstained red cells from stained reticulocytes;
- the system used to quantify immature reticulocytes;
- recommended methods for calibration and quality control of all reportable reticulocyte parameters; and
- methods and warnings used to identify potentially interfering substances or cellular elements.
7.4 Instrument Settings and Calibration

Optical and electronic components of the instrument should be set according to the manufacturer’s directions. Instrument settings should provide the maximum intensity with minimum variability of scatter, fluorescence, and absorption signals. Optical alignment and signal amplification settings can require the use of artificial particles of standardized size and density, as well as of fluorescent microbeads.

Technical aspects, such as adjustment of photomultiplier voltages, laser output, electronic gains, and signal to noise discrimination, should be correctly set and periodically checked according to the manufacturer’s specifications as a part of the internal quality control procedure. Regular (i.e., semiannual or following major instrument service work) assessment of the linearity of reticulocyte counting methods should be performed to verify the trueness of the reportable range (measuring range) of the automated instrument.

Instrument calibration is the adjustment of an analytic process under specified conditions to obtain accurate measurement results. Calibration should be performed in accordance with the method specified by the manufacturer. If on-site instrument calibration is required, use at least three fresh blood samples for the calibration, covering the reportable range (measuring range) of the instrument’s reticulocyte counts. For initial calibration, a more precise reticulocyte count (CV ~2%) is required; for routine quality control purposes, lesser precision (CV ~5%) is probably adequate.

The approximate numbers of red cells required to be counted for levels of imprecision of 2, 5, and 10% at various reticulocyte proportions are given in Table 1. Even for a reference method (reference measurement procedure) the table shows that acceptable precision cannot be readily achieved when the reticulocyte count is in the range of 1 to 2%.

Table 1. RBC Number to Be Counted for Required Precision

<table>
<thead>
<tr>
<th>Retic Percentage</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2%</td>
</tr>
<tr>
<td>0.01</td>
<td>247,500</td>
</tr>
<tr>
<td>0.02</td>
<td>122,500</td>
</tr>
<tr>
<td>0.05</td>
<td>47,500</td>
</tr>
<tr>
<td>0.10</td>
<td>22,500</td>
</tr>
<tr>
<td>0.20</td>
<td>10,000</td>
</tr>
<tr>
<td>0.50</td>
<td>2,500</td>
</tr>
</tbody>
</table>

*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, but the level of imprecision is not the same and likely higher than that achieved by counting ninefold the cells without the ocular assist device.

Three NMB-stained blood films are prepared on each sample, two for reticulocyte counting by prequalified examiners, the third for use in arbitration of the counts, should it be required. These films should be single-blind coded by the coordinator for distribution to the participating prequalified examiners. Each reference analysis should count a minimum of 2,000 red cells or up to the required number as noted in Table 1. Results, identified by code number, should be submitted to the coordinator for evaluation.

The coordinator should determine the mathematical mean value from duplicate reticulocyte counts done by a minimum of two prequalified examiners. In the event that disagreement beyond predictable limits occurs between the two analysts, the arbitrator should perform one additional reticulocyte count for verification of the results. If the arbitrator’s count falls between the other two, retain the result; otherwise,
take the mean of the two closest counts. Reticulocyte percent values should be converted to reticulocyte count number by use of an RBC count done on a well-calibrated and controlled cell counter.

8 Performance Testing of Automated Blood Counters and Flow Cytometers to Be Used for Reticulocyte Counting

Performance tests for automated instruments for reticulocyte measurements should include assessment of precision, carry-over, comparability, linearity, trueness, sample aging, and sources of interference. The assessment should include the direct flow cytometric measurement, which usually is the proportion of stained cells relative to the total red cell population, as well as the proportion of immature reticulocytes relative to the total reticulocyte population. All the analytical aspects can be evaluated using the ICSH guidelines for the evaluation of blood cell analyzers\textsuperscript{18} using samples at all the levels of reticulocyte counts that can occur in clinical practice. The evaluation should also include samples with abnormal cells or other blood components that can interfere with reticulocyte counts, such as high levels of Howell-Jolly bodies, malaria or other intracellular parasites, lymphocytosis, thrombocytosis, or erythroblastosis.

8.1 Comparability (Correlation) Method

Trueness can be established by the acceptance of values obtained with another previously calibrated method (see Section 6) and comparing the test instrument to these “reference” values. Because there is, as yet, no consensus value for “truth” (including the NMB method), it is more appropriate to refer to this as a method of comparability, correlation, or agreement. Comparability has been defined as the ability of the instrument to produce results that agree satisfactorily with those obtained by routine procedures\textsuperscript{19}. For reticulocyte counting, the means of duplicate analyses by the comparability method are compared with mean values of duplicate analyses by the test (instrument) method.

Instrument calibration should be done using the manufacturer’s specifications at the time of initial installation. Recalibration, or validation of accuracy and linearity, should be done periodically, as indicated by the instrument manufacturer or as established by the laboratory, whenever major preventive maintenance or critical parts replacement is done that can influence test performance, or whenever quality control studies or proficiency testing indicates a potential problem with instrument performance. All calibration and recalibration procedures and adjustments, and all calibration verification or validation, should be documented completely with written records.

Calibration of reticulocyte-related parameters, such as the immature reticulocyte fraction (IRF), is even more problematic due to the lack of commercial calibrators or standards. The variability in IRF among the various reticulocyte staining methods is due to difference in both reagent and data analysis routines.\textsuperscript{19} However, as a guiding principle manufacturers should seek a calibration that allows the reference range on normal adult samples to detect low and high IRF values, so decreased and increased erythropoietic activity in patients can be identified.

8.1.1 Diagnostic Sensitivity Studies

Sample selection, as noted in this section, is intended for the initial examination of device accuracy by determination of agreement with the comparability method. Samples collected for use in the abbreviated accuracy validation procedure may be selected randomly from routine samples, including neonatal samples.

Additional samples should be selected on the basis of clinical history, disease state, or based on routine laboratory results that adequately represent the entire claimed performance range. It is recommended that a minimum of 26 blood samples be examined, with approximately 50% representative of normal range samples and the remainder representative of abnormal range samples (25% decreased and 25% increased...
reticulocyte counts). Pediatric reference ranges are higher than adults, particularly in infants and newborns.

The reference or normal samples should be selected by qualification of the donor as having no detectable hematological abnormality (red cell count, hemoglobin and indices, leukocyte count, platelet count), being free of recent illnesses, and not having donated blood within the last two months.

Abnormal samples should be selected from patients with known or suspected hematological conditions that would have either elevated or depressed reticulocyte counts. Elevated reticulocyte count samples can be obtained from patients with hemolytic anemias or reactive erythrocytosis. Depressed reticulocyte count samples can be found using samples from patients with aplastic anemia or postcytotoxic chemotherapy.

All samples used in this accuracy validation protocol should be stored according to instructions provided in the section on patient specimen collection and handling (see Section 5).

8.1.2 Calculation of Short-Term Imprecision

Determine the absolute differences between the duplicate determinations. Insert these differences ($d_i$) into formula (4), and make sure to divide the data into the tertiles according to the recommended ranges for reticulocyte samples (Section 8.1.1).

Samples should represent as wide as possible a range of reportable values. A minimum of ten samples in each tertile will provide a useful estimate of standard deviation ($SD$). Higher values of $n$ will give more robust estimates of $SD$.

The formula for short-term standard deviation ($SD_s$):

$$SD_s = \sqrt{\frac{\sum_{i=1}^{n} d_i^2}{2n}}$$  \hspace{1cm} (5)

where: $n =$ number of samples; and $d_i =$ difference between duplicates for sample $i$.

Calculate the short-term imprecision at the three levels of reticulocyte counting using formula (4):

Convert $SD$ to Coefficient of Variation (CV) using Equation (6):

$$CV (\%) = \frac{SD}{X_a} \times 100$$  \hspace{1cm} (6)

Note that $X_a$ is the mean of all values, not the mean of the differences.

To utilize this method effectively, each laboratory should establish acceptable limits of reproducibility throughout the range of samples that will be tested. For example, establishment of an acceptable limit for duplicate analysis replication for a 200 x $10^9$/L reticulocyte count might be unacceptably wide for a reticulocyte count of 40 x $10^9$/L.

8.1.3 Procedure

Before beginning the device accuracy validation procedure, it is important to complete the precision study (see Section 8.1.2) with acceptance of test results, because accuracy studies can be invalidated by a failure
of the precision test procedures. This procedure requires careful examination of the same samples by both
the comparative method and the automated method being validated. It is advisable that the laboratory
establish a scheme for analysis of the samples by testing, using both methods within six hours of the time
of specimen collection, particularly during comparison of IRF parameters.

The automated reticulocyte counting method should follow the device manufacturer’s recommended
analytic procedures. Each sample should be analyzed in duplicate, while determining the mathematical
mean for comparison with the reference method (reference measurement procedure). Concurrent use of
these data for definition of instrument imprecision is noted in Section 8.1.2. This method may also be
used for the periodic validation of instrument performance by testing a limited number of samples.

8.1.4  Accuracy

8.1.4.1  Schema for Data Analysis for Accuracy

Because there are two possibilities for error, reference and test, the variances for each of them are
calculated.

• Calculation of the variance of the reference method (reference measurement procedure) (Var$_R$):

$$Var_R = \frac{(p_R \times q_R) \times n_R}{n_R} \quad (7)$$

• Calculation of the variance of the flow cytometer (Var$_{FC}$):

$$Var_{FC} = \frac{(p_{FC} \times q_{FC}) \times n_{FC}}{n_{FC}} \quad (8)$$

where, $p_R$ and $p_{FC}$ are reticulocyte percents
$q_R = (1 - p_R)$ and $q_{FC} = (1 - p_{FC})$
$n_R$ and $n_{FC}$ are the total number of red cells and reticulocytes counted.

Example: An example of this calculation is provided below. The example uses a theoretical model based
on an instrument counting 30,000 cells and the reference method (reference measurement procedure)
counting 4,000 cells. The reticulocyte value $\div 100$ is 1%.

$$Var_R = \frac{0.01 \times 0.99}{4000} = 0.00000247$$

$$Var_{FC} = \frac{0.01 \times 0.99}{30000} = 0.00000033$$

A combined variance of reference and flow cytometer methods is calculated by adding individual
variances together given that the reticulocyte percents are obtained independently by each method. The
square root of this sum represents the combined standard error of test and reference.

$$SE_p = \sqrt{Var_R + Var_{FC}} \quad (9)$$

Combining the variances of the test and reference methods (reference measurement procedures) above:

$$SE_p = \sqrt{0.00000027 + 0.00000033} = 0.00167332$$
The reticulocyte count is a binomial distributed variable that can be approximately distributed as normal when both quantities $p_R \times n_R$ and $q_R \times n_R$ are greater than 5. This requirement is generally satisfied since the total number of red cells and reticulocytes counted is relatively large.

The margin of error for comparing test and reference methods (reference measurement procedures) is calculated as,

$$E = \pm SE_p \times z_{\alpha/2},$$

where $z_{\alpha/2} = 1.96$ for the 95% confidence level. The 95% confidence limits for a reticulocyte percent = 1% are,

$$0.01 \pm E = 0.01 \pm 0.00167332 \times 1.96 = 0.01 \pm 0.00327$$

Lower limit = 0.0067 or 0.67%  
Upper limit = 0.0133 or 1.33%

Thus, if the reticulocyte percent obtained by the reference methods (reference measurement procedures) is 1% then the test method can be as low as 0.67% and as high as 1.33%. If the value obtained by the test method is outside these limits, then the assumption of accuracy fails for that particular reticulocyte level.

When a number of $n$ blood samples are used for comparing two methods, the simultaneous margin of error for all comparisons is:

$$E = \pm SE_p \times z_{\alpha/(2n)},$$

Where $z_{\alpha/(2n)} = 3.10$ for the simultaneous 95% confidence level and $n=26$. Z-values for the simultaneous 95% confidence intervals and different number of samples are given in the table below.

<table>
<thead>
<tr>
<th>Number of Samples</th>
<th>26</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
<th>100</th>
<th>150</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>z-value for simultaneous 95% confidence intervals</td>
<td>3.10</td>
<td>3.14</td>
<td>3.23</td>
<td>3.29</td>
<td>3.34</td>
<td>3.38</td>
<td>3.42</td>
<td>3.45</td>
<td>3.48</td>
<td>3.59</td>
<td>3.66</td>
</tr>
</tbody>
</table>

An array of reticulocyte values with reference counts of 4,000 cells, instrumental counts of 30,000 cells, and 26 blood samples is given in Table 2.
Table 2. Accuracy Estimation: Paired Sample Method

<table>
<thead>
<tr>
<th>Retic %</th>
<th>p</th>
<th>q</th>
<th>Variance n_R = 4,000</th>
<th>Variance n_FC = 30,000</th>
<th>SE_p</th>
<th>Lower Limit</th>
<th>Upper Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.001</td>
<td>0.999</td>
<td>0.000000025</td>
<td>0.00000003</td>
<td>0.00053</td>
<td>-0.0007</td>
<td>0.0027</td>
</tr>
<tr>
<td>1</td>
<td>0.010</td>
<td>0.990</td>
<td>0.000000248</td>
<td>0.000000033</td>
<td>0.00167</td>
<td>0.0048</td>
<td>0.0152</td>
</tr>
<tr>
<td>2</td>
<td>0.020</td>
<td>0.980</td>
<td>0.000000490</td>
<td>0.00000065</td>
<td>0.00236</td>
<td>0.0127</td>
<td>0.0273</td>
</tr>
<tr>
<td>3</td>
<td>0.030</td>
<td>0.970</td>
<td>0.000000728</td>
<td>0.00000097</td>
<td>0.00287</td>
<td>0.0211</td>
<td>0.0389</td>
</tr>
<tr>
<td>4</td>
<td>0.040</td>
<td>0.960</td>
<td>0.000000960</td>
<td>0.00000128</td>
<td>0.00330</td>
<td>0.0298</td>
<td>0.0502</td>
</tr>
<tr>
<td>5</td>
<td>0.050</td>
<td>0.950</td>
<td>0.00001188</td>
<td>0.0000158</td>
<td>0.00367</td>
<td>0.0386</td>
<td>0.0614</td>
</tr>
<tr>
<td>6</td>
<td>0.060</td>
<td>0.940</td>
<td>0.00001410</td>
<td>0.0000188</td>
<td>0.00400</td>
<td>0.0476</td>
<td>0.0724</td>
</tr>
<tr>
<td>7</td>
<td>0.070</td>
<td>0.930</td>
<td>0.00001628</td>
<td>0.0000217</td>
<td>0.00429</td>
<td>0.0567</td>
<td>0.0833</td>
</tr>
<tr>
<td>8</td>
<td>0.080</td>
<td>0.920</td>
<td>0.00001840</td>
<td>0.0000245</td>
<td>0.00457</td>
<td>0.0658</td>
<td>0.0942</td>
</tr>
<tr>
<td>9</td>
<td>0.090</td>
<td>0.910</td>
<td>0.00002048</td>
<td>0.0000273</td>
<td>0.00482</td>
<td>0.0751</td>
<td>0.1049</td>
</tr>
<tr>
<td>10</td>
<td>0.100</td>
<td>0.900</td>
<td>0.00002250</td>
<td>0.0000300</td>
<td>0.00505</td>
<td>0.0843</td>
<td>0.1157</td>
</tr>
</tbody>
</table>

*Lower limit values that are negative can be replaced with zero.

8.1.4.2 Graphic Display

A graph of the data in Table 2 is prepared where the abscissa (the horizontal, or X-axis) represents the mean of duplicate reference observations; the ordinate (the vertical, or Y-axis) represents the mean of results obtained from duplicate analyses on the flow cytometer (see Figure 2). Superimposed on this graph is the envelope representing 95% confidence limits of the binomial.

![Graph of data](image)

Figure 2. Comparison of Instrument Performance with Reference Method (Reference Measurement Procedure)

8.1.4.3 Interpretation of Graphic Display

Assuming that the only source of error is due to binomial counting error (i.e., to chance alone), no more than 5% of the values should fall outside of the binomial confidence bands. If more than 5% of the values fall outside the binomial confidence bands, then the data should be examined for other sources of error.

To verify the calibration of the instrument with a laboratory reference method (reference measurement procedure), regression analysis can be used. Calculation of 95% confidence intervals of the predicted values from the regression line can be used to estimate bias.
Reference should be made to any standard statistical textbook for the following additional statistical procedures:

(a) **Correlation Coefficient (r):**

\[
r = \frac{n \sum xy - (\sum x)(\sum y)}{\sqrt{[n \sum x^2 - (\sum x)^2][n \sum y^2 - (\sum y)^2]}}
\]  

(b) **Linear Regression Statistic:**

\[
y = mx + b
\]

(c) **Paired t-Test Statistic:**

Calculate, \(d_i = x_i - y_i\) for each sample

\[
\bar{d} = \frac{\sum_{i=1}^{n} d_i}{n}
\]

\[
SD = \sqrt{\frac{\sum_{i=1}^{n} (d_i - \bar{d})^2}{n-1}}
\]

\[
t = \frac{\bar{d}}{SD} \sqrt{n}
\]

The difference between methods is statistically significant if |t| > \(t_{a/2, n-1}\).

### 8.2 Internal Consistency Testing

Because automated reticulocyte counting methods enumerate 30 (or more) times as many cells as the usual manual methods, reproducibility of the flow cytometric method is expected to be better. The confidence intervals for the true reticulocyte proportion for various sample sizes are calculated as follows:

\[
P \pm z_{a/2} \sqrt{\frac{(p \times q)}{n}}
\]

The usual manual reticulocyte count differentiates 1,000 red cells. If a sample contains 1% reticulocytes, it is presumed that ten reticulocytes would be counted. The 95% confidence limits for this reticulocyte count would be 0.4 to 1.6%.

If, however, this same 1% reticulocyte count sample were analyzed on a flow cytometer that counts 30,000 events (cell counts), 300 reticulocytes are counted. The expected performance for this example would be 0.9 to 1.1%.
The status of instrument precision should be verified at the time of initial installation, after service that could affect hydraulic or pneumatic operation, and after routine maintenance. A schedule should be established by each laboratory for precision validation depending on frequency of instrument use, work load, and previous performance history.

8.2.1 Sample Selection

It is suggested that, for the initial instrument performance validation, precision be examined at three reticulocyte count values. Five samples are selected in each range; low (≤10 x 10⁹/L), normal (~60 x 10⁹/L), and high (≥200 x 10⁹/L) reticulocyte count samples. The counts of these samples can be approximated by preliminary analysis with the automated method.

8.2.2 Procedure

Count each sample of the study samples in duplicate, and make sure each determination is an independent test. For example, if preliminary sample preparation is required, be sure to do two entirely separate procedures starting with the anticoagulated blood sample.

8.3 Specificity of Testing

Specificity of reticulocyte analysis can be impaired by other cellular material containing the nucleic acids RNA and/or DNA, which can stain with the fluorescent dyes used in some reticulocyte methods. Specific cellular and noncellular elements that can adversely affect the method specificity are included in Table 3. Of these potential interferents, the most common problems are due to platelet clumps, leukocyte fragments (e.g., aged blood samples or leukemic samples), nucleated red cells, Howell-Jolly body-containing red cells, and intracellular parasites, such as malaria. Any RNA- or DNA-containing elements within the cells can be stained by the dyes/fluorochromes and, thus, be enumerated by the fluorescence-detection methods of the flow cytometer potentially as reticulocytes.

Falsely depressed reticulocyte counts can be observed with the presence of cold agglutinins if the analyzer concurrently performs a red cell count. Interferents typically affect the placement of discriminators or gates, which separate cell populations.

Instrument manufacturers should clearly indicate any potential interferents in their device support material. Analytic procedures for use of these devices should alert the user to the occurrence of these interferents, whenever possible, by use of flags triggered by the failure of appropriate gate settings.

Table 3. Known or Potential Interferents

<table>
<thead>
<tr>
<th>Cellular Elements</th>
<th>Cellular Inclusions</th>
<th>Miscellaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet clumps</td>
<td>Howell-Jolly bodies</td>
<td>Autofluorescence with porphyria and some drugs</td>
</tr>
<tr>
<td>Basophilic stippling</td>
<td>Heinz bodies</td>
<td>(e.g., anthracyclines or fluorescein for fundus</td>
</tr>
<tr>
<td>Giant platelets</td>
<td>Pappenheimer bodies</td>
<td>angiography)</td>
</tr>
<tr>
<td>Leukocytes and</td>
<td>Parasites (malaria, babesia)</td>
<td>Abnormal red cells</td>
</tr>
<tr>
<td>leukocyte fragments</td>
<td></td>
<td>Paraproteins</td>
</tr>
<tr>
<td>Nucleated erythrocytes</td>
<td></td>
<td>Cold agglutinins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Platelet/erythrocyte coincidence</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hemolysis</td>
</tr>
</tbody>
</table>
8.4 Sensitivity of Testing

Sensitivity of flow cytometric reticulocyte analysis is the ability of the method to detect and reliably quantitate low percentages of reticulocytes. It is dependent upon trueness, precision, carry-over, and method bias. All four of these test criteria must be tested and found acceptable before determining the sensitivity of the device.

Sensitivity must be tested at the limit of clinical performance requirements. This is a complex issue, because the exact definition of a reticulocyte is a function of the assay, i.e., the slope and intercept for regression of the test system and reference method (reference measurement procedure) data may not be 1 and 0. Thus, the numerical limit to which a particular system might need to perform may be different. This value should be obtained from the accuracy analysis. For example, if the test system correlates linearly with the NMB method with a slope of 0.5 and intercept of 0.1, and the previously established performance limit for the NMB method is 0.1%, then the limit for the test system would be 0.15%. Because the recommended performance level for automated reticulocyte counting includes samples between zero and 1.0% reticulocytes (see Section 9.1), and because it is unlikely that the nature of the relationship between the test and comparability methods will be known with any certainty below 1.0% reticulocytes due to limitations of the comparability method, the test system should be tested for linearity and sensitivity independent of the comparative method chosen.

The equations derived from accuracy analysis can be used to determine precision at the level of the assay equivalent to 0.1% NMB reticulocytes. Precision is determined using the methods and equations in Section 8.1.2. Determination of the actual required limit of clinical performance can only be made after reference ranges have been established and after extensive experience with the test system. This becomes a system of successive approximations. For example, in a laboratory performing reticulocyte assays on bone marrow transplant patients, it might be desirable, and necessary, to detect reticulocytes between the 0.01 and 0.1% levels. This might only become known after having analyzed samples falling below the known sensitivity of the instrument, and it would then be routinely tested so that the instrument performed in the range necessary for the laboratory.

Because it is the lowest point on the linearity scale, sensitivity is also related to linearity. (Please refer to the most current version of NCCLS document EP6—Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach for additional information.) To test linearity and investigate sensitivity, the following procedure should be used. Schimenti, Lacerna, and Maston, et al give examples of the use of this procedure in reticulocyte counting.

1. Select one sample with a normal, or moderately elevated, reticulocyte count (~150 x 10^9/L) and label it “Sample H.” Select a second sample with a reticulocyte count that is at the lowest level of reticulocyte counts routinely assayed in the laboratory (~5 x 10^9/L) and label it “Sample L.” The lowest available sample should be used. Do not use samples with cold agglutinins. A good source of low reticulocyte blood is the post-treatment blood from bone marrow transplant patients that will be assayed for platelet and granulocytes. Typically, at the nadir of bone marrow activity, the reticulocyte count is ~0.1% or less.

2. Centrifuge both blood samples and remove the plasma. Wash with physiological buffered saline (PBS), then resuspend in ten volumes of PBS or any compatible buffered salts solution (~50 mL). This step permits accurate pipetting of nonviscous fluid with variable-volume, air-displacement pipettes. It is also necessary to remove any traces of incompatible plasma.

3. Set up seven tubes labeled 0, 10, 25, 50, 75, 90, and 100. Pipette 0, 0.10, 0.25, 0.50, 0.75, 0.90, and 1.00 mL of dilute Sample H into tubes 0, 10, 25, 50, 75, 90, and 100, respectively; then pipette 1.00, 0.90, 0.75, 0.50, 0.25, 0.10, and 0 mL of dilute Sample L into tubes 0 through 100, respectively. Mix each tube thoroughly, centrifuge, remove PBS, and then add 0.060 mL (60 µL)
of ABO-compatible plasma (or fetal bovine serum) to each tube. Mix thoroughly, then aliquot the appropriate amount from each tube to the test assay system.

(4) After obtaining the results, calculate the expected absolute values using Equations 15 and 16 and the expected percentage reticulocytes using Equation 17.

\[
E_{ar} = ah_r + (1 - a)l_r
\]

\[
E_{at} = ah_t + (1 - a)l_t
\]

\[
E_{%r} = \frac{E_{ar}}{E_{at}} \times 100
\]

where:

- \( E_{ar} \) = the expected absolute reticulocyte count (x \( 10^9 \)/L);
- \( a \) = the fraction of the high reticulocyte blood in the tube (e.g., 0, 0.1, 0.25...1.00);
- \( h_r \) = the absolute reticulocyte count of Sample H (high count sample);
- \( l_r \) = the absolute reticulocyte count of Sample L;
- \( E_{at} \) = the expected absolute total RBC count (x \( 10^9 \)/L);
- \( h_t \) = the absolute total RBC count of Sample H;
- \( l_t \) = the absolute total RBC count of Sample L; and
- \( E_{%r} \) = the reticulocyte percentage.

For example, if Sample L has \( 5 \times 10^{12} \)/L RBC and \( 5 \times 10^9 \)/L reticulocytes (0.1% reticulocytes), and Sample H has \( 4.0 \times 10^{12} \)/L RBC and \( 150 \times 10^9 \)/L (3.75% reticulocytes), then Table 4 shows the results of calculations using Equations 15 through 17. The expected absolute reticulocyte counts are then used as the independent variable and the measured values as the dependent variable (the 0 and 100 tube counts are taken to be the truth). Linear regression is performed. Linearity and sensitivity can be checked by examining the residuals, slope and intercept, and \( r^2 \) value. One can expect randomly distributed residuals and \( r^2 > 0.98 \). If there is considerable nonrandom distribution of the residuals and if \( r^2 \) is lower than \( <0.98 \), then Sample L may be below the level of sensitivity. The test can be repeated with more dilutions to determine where deviation from linearity occurs. At that point, the sensitivity of the test system has been estimated.

**NOTE:** On instruments where absolute counts are not performed, it is necessary to perform an independent absolute red cell count and convert percent reticulocyte counts to absolute reticulocyte counts.

**Table 4. Example of Linearity Data**

<table>
<thead>
<tr>
<th>Fraction of High Retic Blood (a)</th>
<th>Expected Retic (E_{ar})</th>
<th>Expected RBC (E_{at})</th>
<th>%Retic (E_{%r})</th>
<th>Measured Retic</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>5.0</td>
<td>5,000</td>
<td>0.10</td>
<td>5</td>
</tr>
<tr>
<td>0.10</td>
<td>19.5</td>
<td>4,900</td>
<td>0.40</td>
<td>20</td>
</tr>
<tr>
<td>0.25</td>
<td>41.3</td>
<td>4,750</td>
<td>0.87</td>
<td>35</td>
</tr>
<tr>
<td>0.50</td>
<td>77.5</td>
<td>4,500</td>
<td>1.72</td>
<td>80</td>
</tr>
<tr>
<td>0.75</td>
<td>113.8</td>
<td>4,250</td>
<td>2.68</td>
<td>120</td>
</tr>
<tr>
<td>0.90</td>
<td>135.5</td>
<td>4,100</td>
<td>3.30</td>
<td>140</td>
</tr>
<tr>
<td>1.00</td>
<td>150.0</td>
<td>4,000</td>
<td>3.75</td>
<td>150</td>
</tr>
</tbody>
</table>

**NOTE:** Counts are \( 10^9 \)/L. Example data have been synthesized but are similar to measured data.

*The data from Table 4 is graphed in Figure 3, Panel A. One example of possible nonlinear data (synthesized) is shown in Figure 3, Panel B. The synthesized data in B are for a high reticulocyte count of 60, low reticulocyte count of 5, and 10% intervals of dilution. Dashed lines are the best fit lines.
8.5 Testing for Carry-Over

The effect of one sample upon the immediately succeeding sample result should be minimal, with any carry-over being of no clinical significance in a properly operating instrument. Carry-over can exert a negative dilution effect of diluent from a rinse cycle transported into the first sample analysis, a negative effect of a low-sample analysis on a succeeding higher result, or a positive effect of an elevated sample on a succeeding lower result. Reticulocyte instrument analysis is tested for carry-over by testing the effect of an elevated sample on a succeeding low-sample result. To be most sensitive, this test should examine the directly measured parameter containing the largest data set. In the case of flow cytometers, this is represented by the RBC event (count) data. Carry-over is expressed as the percent effect of one sample upon succeeding analysis.

The status of sample carry-over should be verified at the time of initial installation, after service that could affect hydraulic or pneumatic operation, after routine maintenance, and then periodically validated during normal use.

There are two forms of carry-over that can affect data in subsequent analyses. The first is carry-over of reagents, which are used to create the characteristic signature of the particles under analysis; the second is a distinct form of carry-over, i.e., particulate or cell carry-over.

The reagent carry-over is methodology- and instrument-specific. Clearly, instruments that use fluorescent dyes must have test procedures to ensure that dye carry-over does not affect the measured intensity of the mature RBC population in a subsequent unstained sample if unstained samples are required to control the assay. In those cases where the assay is internally controlled, it is necessary to demonstrate that dye carry-over does not affect other assay types run subsequent to reticulocyte analysis. Systems using nonfluorescent technologies must demonstrate that reagent carry-over does not affect assays of other types run on the same instrument.

In closed systems in which absolute counts are being reported, samples used for examination of carry-over should be selected for their elevated and depressed RBC counts and not for reticulocyte count values. It is suggested that the elevated RBC count sample be selected with the value above $6 \times 10^{12}/L$ and the low RBC count sample be selected with the value below $2 \times 10^{12}/L$. If necessary, a low sample may be created by dilution of red cells with autologous plasma, and an elevated sample can be made by centrifugation of a sample and removal of a portion of the plasma.
Analyze an elevated RBC count sample three times consecutively, followed by three consecutive analyses of the low RBC count sample. Determine carry-over percent by use of the following statistical formula:

\[
\text{Carry-over} \ (\%) = \frac{(j_1 - j_3)}{(i_1 - i_3)} \times 100
\]  

(18)

where \(i_1, i_2, \text{ and } i_3\) are consecutive analyses of the elevated RBC sample and \(j_1, j_2, \text{ and } j_3\) are consecutive analyses of the low RBC sample.

**Example:** Consecutive results from analysis of high 6.25, 6.28, 6.31 (i) and low 0.90, 0.87, 0.86 (j) samples.

\[
\text{Carry-over} = \frac{(0.90 - 0.86)}{(6.31 - 0.86)} \times 100 = 0.73\%
\]

Compare the results of this carry-over testing procedure with the stated instrument performance specifications.

9 Quality Control and Quality Assurance

Quality control (QC) is an organized system for continuously monitoring the analytical processes to ensure true and precise data. This involves collection and treatment of the specimens, the analytical process, and reporting the results. (Please refer to the most current version of NCCLS document C24—Statistical Quality Control for Quantitative Measurements: Principles and Definitions for additional information.) This document is limited to the QC of the analytic process. Quality control is divided into long-term QC and short-term QC.

QC analyses should be run at an interval within which the trueness and precision of a testing system is expected to be stable, but it must not exceed a period of 72 hours. It is recommended that QC material(s) be run once within each eight hours of operation. The manufacturer's recommendations regarding the inclusion of controls and calibrators (if available) should be followed. In addition, the QC procedure should include a minimum of two levels of control material, preferably in both the abnormal and normal ranges of reportable patient values.

9.1 Short-Term Quality Control Procedures

9.1.1 Red Cell Samples

One or more patient samples may be selected from the first analytical run after assurance of acceptable instrument performance by use of the long-term control procedure. Samples should be reanalyzed (and restained, if required) throughout the day or 72-hour period to determine that there was no significant shift (>3 SD) in instrument performance. All samples used for this purpose should be stored at 2 to 6 °C between runs, and they should be warmed to room temperature immediately before each analysis. However, repeated warming cycles of such material should be investigated by either the manufacturer or laboratory, as no such data exists in the published literature.

Whole blood (human or rabbit) collected in citrate phosphate dextrose (CPD) has been proposed as a quality control. A reticulocyte count is determined shortly after collection. Aliquots are stored at 2 to 6 °C and are counted at regular intervals for up to three weeks. The reticulocyte count decreases during storage, apparently at a predictable rate. However, this control system has not yet been widely evaluated for effectiveness. Several commercial reticulocyte control preparations are also available from various vendors and use should follow the manufacturer’s instruction. The advantages of the commercial control
material include a longer stability than whole blood material, independently assigned assay values, and the potential to be included in interlaboratory QC programs.

9.1.2 NMB-Stained Blood Films

For QC purposes, two blood films from a sample with a normal or, preferably, an elevated, reticulocyte count are prepared according to the method outlined in Section 6. Using this method, 1,000 consecutive red cells are examined for blue-staining granules, i.e., reticulocytes. The mean percent of reticulocytes of both blood films is determined.

The reticulocyte counting system is performing acceptably if the blood film reticulocyte counts are within the limits listed in Table 5. These limits are comparable to intervals calculated by the binomial method.

Table 5. 95% Confidence Limits of NMB Reticulocyte Count (Adapted with permission from Rümke CL. The statistically expected variability in differential leukocyte counting. In: Koepke JA, ed. Differential Leukocyte Counting. Skokie, IL: College of American Pathologists; 1978:39-45.)

<table>
<thead>
<tr>
<th>Instrument Count (%)</th>
<th>Tolerance Limits (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0-1</td>
</tr>
<tr>
<td>1</td>
<td>0-2</td>
</tr>
<tr>
<td>2</td>
<td>1-4</td>
</tr>
<tr>
<td>3</td>
<td>2-5</td>
</tr>
<tr>
<td>4</td>
<td>2-6</td>
</tr>
<tr>
<td>5</td>
<td>3-7</td>
</tr>
<tr>
<td>6</td>
<td>4-8</td>
</tr>
<tr>
<td>7</td>
<td>5-9</td>
</tr>
<tr>
<td>8</td>
<td>6-10</td>
</tr>
<tr>
<td>9</td>
<td>7-11</td>
</tr>
<tr>
<td>10</td>
<td>8-13</td>
</tr>
</tbody>
</table>

9.2 Long-Term Quality Control Procedures

Although the long-term QC procedures are intended to monitor and ensure the stability of instrument performance, the process is dependent upon availability of suitable control material. Long-term QC is usually done by multiple analyses over many days of an aliquot of stabilized or stored blood, most often from a commercial source. Use of only one method is necessary to detect instrument performance drift and ensure consistent instrument operation over an extended time period.

The long-term process controls described below employ either commercially available control materials or controls produced in the laboratory.

9.2.1 Stabilized Control Materials

Control material that is stable for one month or longer should be measured at least once at the beginning of each work run in which the instrument accuracy and precision is demonstrated to be stable. Additionally, the long-term control material should be analyzed after instrument service that affects hydraulic, pneumatic, or electronic operation; after periodic maintenance; and each time a new reagent is introduced.

Commercially available materials for use in hematology analyses, including reticulocyte counting, are supplied with predetermined assay target and limit values. It is important for laboratories to verify the
assigned target values before use when assays are provided for their instrument systems, or to establish values that are specific for their laboratories and instrument systems when predetermined assay targets are not provided.

It is important to know the method(s) used by the manufacturer for assigning reticulocyte counts to “controls with assigned values.” Biases have been noted, apparently due to incorrect use of the Miller disc.17, 23

In addition, all QC protocols should encompass any additional parameter(s) that are reported by the test instrument, including reticulocytes (percent), RBC count, immature reticulocyte fraction, and any parameter unique to the test device.

Control material target value determination should be done with a properly calibrated and maintained instrument. Analyze each level of the material twice a day (preferably at different times during the workday) for a minimum of five days to establish the target value. A minimum of ten data points is required. The target value should not be determined on less than ten data points or from analyses done in less than five days. The control target value is then determined by calculation of the arithmetic mean determined from the ten or more analyses.

Establishment of the performance limits for the control material is accomplished in three phases. The first phase establishes the tentative target value from the initial ten analyses. In the second phase, concurrent with the determination of the target value (mean of ≥10 values), calculate ±1, ±2, and ±3 SD ranges around the mean. These tentative control limits, or acceptable range (±2 SD), are then used until sufficient additional data are collected. In the third phase, the tolerance limits are recalculated around the mean of 20 or more values. This new set of control limits is then used for the remainder of the life of the particular lot of control material.

Another method for development of control limits is the use of historical data to establish a set of permanent limits that will be used with each new lot of control material. Control material manufacturers usually produce each lot of material with values near the same target value. Consistency of these target values allows the laboratory to use historical ±2 SD limits to establish a set of stable limits that can be used continuously, unless the target value changes significantly or the instrument performance is altered.

10 Reticulocyte Reference Intervals and New Reticulocyte Parameters

Reference intervals for each system (automated reticulocyte counting instrument/dye) can be different and, therefore, should be established for each instrument/dye combination. Differences in reference ranges between the reference method (reference measurement procedure) and flow cytometric methods can be anticipated if there is a bias in the slope and/or in the linear regression intercept (see Section 8.1.4). Each laboratory using flow cytometric reticulocyte counts should establish reference ranges for its particular method. If a reference range has not been established for the comparative method, it is appropriate for the laboratory to do so in conjunction with the instrument reference interval study. The same samples may be used for determination of both reference intervals, and the data may additionally be used for the accuracy validation study (see Section 8.1).

10.1 Reference Population

At least 100 normal adults between the ages of 18 and 50 years, and equally divided between men and women, are required for the determination of reference ranges. For confirmation of a system reference range, at least 40 normal adult samples are required. (See the most current version of NCCLS document C28—How to Define and Determine Reference Intervals in the Clinical Laboratory.) These persons should have a normal blood count, including red and white cell parameters. Studies by several subcommittee members have noted small but significant differences between the reference ranges of men
and women. Pediatric reference ranges, particularly in the ages of <2 years, will differ from adult reference ranges, but establishment of laboratory specific ranges in the various pediatric age groups may not be practical or feasible due to ethical constraints.

The data are almost invariably log-normally distributed. Therefore, the central 95% of results, not a mean ±2 SD, should be taken as the reference range. Results are expressed as the number of reticulocytes x 10⁹/L. Reference ranges should be expressed in terms of absolute reticulocyte count. Reporting reticulocyte counts solely as a percentage is archaic and not acceptable clinical practice.

10.2 Reticulocyte Reference Intervals

Due to methodologic differences and the lack of absolute reticulocyte standards, reference ranges are dependent upon the dye or fluorochrome being used (see Table 6). Reference ranges for reticulocytes stained with fluorochromes tend to be somewhat higher than those done with vital dyes. Therefore, each laboratory should determine, or at least confirm, its own reference ranges. Also, studies have shown a significant biological diurnal variability of the reticulocyte count. This variability is approximately ±20% of the mean reticulocyte count over a period of four weeks.

Table 6. Tentative Reticulocyte Reference Intervals², ³

<table>
<thead>
<tr>
<th>Fluorochrome/Dye</th>
<th>Mean</th>
<th>95% Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>New methylene blue,</td>
<td>48</td>
<td>20-110</td>
</tr>
<tr>
<td>manual/visual</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>50</td>
<td>20-85</td>
</tr>
<tr>
<td>Auramine O²⁶</td>
<td>50</td>
<td>27-95</td>
</tr>
<tr>
<td>Thiazole orange</td>
<td>70</td>
<td>15-135</td>
</tr>
<tr>
<td>Thioflavin T Adults</td>
<td>65</td>
<td>30-100</td>
</tr>
<tr>
<td>Newborns</td>
<td>130</td>
<td>65-230</td>
</tr>
<tr>
<td>Oxazine 750</td>
<td>75</td>
<td>20-135</td>
</tr>
<tr>
<td>Cell Dyn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK520²⁶</td>
<td>55</td>
<td>26-84</td>
</tr>
</tbody>
</table>

*This table also contains unpublished data from several committee members. All data is given as number of reticulocytes x 10⁹/L and is rounded to the nearest 5, except as referenced. Results are from adult males and females with no statistically significant differences between sexes observed.

10.3 Immature Reticulocyte Fraction (Reticulocyte Maturation Index)

Automated reticulocyte counting has provided laboratories with improved precision in reticulocyte enumeration. It has also increased an interest in the clinical utility of reporting an additional reticulocyte parameter quantitating the immaturity level of a reticulocyte population. Attempts by the laboratorians to describe and quantitate reticulocyte maturation can be traced back to the work of Ehrlich.²⁷ However, visual methods to quantitatively describe the reticulocyte maturational stage in the peripheral blood are usually variations of the work of Heilmeyer²⁸ and are not sufficiently reproducible or precise to routinely offer in clinical laboratory practice.²⁹ Flow cytometry reticulocyte counting instruments provide the ability to analyze with acceptable precision the distribution of staining intensity (or similar physical or chemical measures) of the RNA-binding or visualization reagents, which is directly proportional to the amount of cellular RNA. Because the population of reticulocytes most recently released from the bone marrow contains the highest concentration of RNA, quantitative assessment of the distribution of the highly fluorescent or highly stained reticulocytes can provide a sensitive indicator of erythropoietic bone marrow activity.
Since the last edition of this document, consensus on the terminology measuring reticulocyte maturity has been reached through effort of the International Society for Laboratory Hematology (ISLH). Davis and Bigelow introduced the term “reticulocyte maturity index (RMI),” expressed in arbitrary mean fluorescence intensity units, in early studies using thiazole orange (TO) flow cytometry methodology. Various other terms have been used in the literature to describe reticulocyte immaturity measurements. These terms include “RNA index,” “RNA content,” “highly fluorescent reticulocytes,” and “immature reticulocyte fraction.” Since the last edition, both manufacturers and clinical practice have accepted the term proposed by the ISLH consensus process, “immature reticulocyte fraction (IRF).” Other instruments have been developed that measure the percentage and number of reticulocytes that fall into arbitrarily defined regions of the data presentation histogram from which an IRF, comparable to other methods, can be derived.

Since the last edition of this document, the U.S. Food and Drug Administration (FDA) has cleared the IRF for diagnostic use on at least three manufacturer’s instruments, and clinical use has progressively increased. However, because instrument and reagent performances and reference ranges differ, individual laboratories should establish or verify manufacturer’s suggested reference ranges based on their own performance characteristics. The IRF is a promising new hematology parameter that needs further integration into clinical practice with cooperation between manufacturers, clinicians, laboratorians, and standards-setting organizations. In practice it has effectively replaced the previous practice of adjusting the reticulocyte count based upon the level of anemia. The utility of the IRF has been validated in the literature for a variety of clinical applications, as summarized in the table below.

Table 7. Clinical Utility of Immature Reticulocyte Fraction

| Monitor Bone Marrow or Stem Cell Regeneration post BMT or ChemoRx | Timing for Stem Cell Harvests Following Growth Factor or Cytotoxic Drug Therapy |
| Monitor Neonatal Transfusion Needs and Prognosis in Anemia of AIDS and Prematurity | Verify Aplastic Anemia |
| Monitor Renal Transplant Engraftment (Epo Production) | Monitor Bone Marrow Toxic Insults from Drugs (e.g., AZT) |
| Classification of Anemias | Monitor Erythropoietin Therapy: Renal Failure, AIDS, Infants, Myelodysplasia, Blood Donation |
| Monitor Efficacy of Anemia Therapy | Evaluate Normochromic Anemias of Various Etiologies |
| Detection of Aplastic Crisis in Hemolytic Anemias | Detection of Occult or Compensated Hemorrhage or Hemolysis |

10.4 Reticulocyte Hemoglobin Content (CHr)

The reticulocyte hemoglobin content (CHr) is reported to approximate the frequency of hypochromic reticulocytes in blood. Some automated analyzers are capable of measuring the mean corpuscular volume of reticulocytes (MCVr). At present, only one manufacturer offers measurement of CHr but other related parameters have also been reported to correlate with iron deficiency or hypochromic red cells, such as the RBC-Y and Ret-Y measurements on another automated reticulocyte analysis instrument. Recent publications indicating measurement of hypochromatic reticulocytes has clinical utility in identifying states of functional iron deficiency may drive a clinical demand for expanded automated measurements of reticulocyte parameters equivalent to the CHr or Ret-Y. If clinical utility of these parameters is confirmed with continued experience, this will drive the need for quality control and calibration of measurements of hemoglobin levels in reticulocytes.
References

24. Package inserts: Retic-Chex7 Streck Laboratories, Omaha, NE; Retic-I7 R&D Systems, Minneapolis, MN.


NCCLS consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information, contact the Executive Offices or visit our website at www.nccls.org.

Summary of Delegate Comments and Area Committee Responses


Key Words

1. Please add the following key words: RBC-Y, RET-Y, and % hypochromic red cells.

   • These terms are not accepted key words for literature searches. Two of the terms are specific to one manufacturer’s instrument and are not accepted medical terminology. “Hypochromic red cells” is too specific a descriptor for a key word search; this terminology would be included in key word searches for “red cells” and/or “anemia.” Therefore, the suggested additions have not been incorporated.

Section 3, Principle (Formerly Section 4)

2. In this paragraph the examples have changed from the previous publication: “Auramine O” to “Thiazole Orange” and “Transferrin” to “Anti-CD71.” Why is that? Auramine O is still a valid example and should be included. Is Anti-CD71 a better example than Transferrin? We suggest including the original and new examples. The new title does not limit the document to flow cytometers and, therefore, the use of Thiazole Orange should be extended.

   • Both Thiazole Orange and Auramine O are listed as examples. CD71 is the current accepted term for what was previously referred to as “transferrin receptor.”

Section 4, Definitions (Formerly Section 5)

3. Accuracy  b) “Accepted reference values” is a new term that is introduced and needs a definition. Also, d) contains an incomplete sentence.

   • The definition of “accuracy” has been revised.

4. The previous version of this document (H44-A) used the term “replicate measurements” instead of the newly introduced phrase “independent test results obtained under prescribed/stipulated conditions.” Which term follows the NCCLS definition? That is the one to use.

   • The definition that appears in this version of the guideline is current and accepted.

5. A definition of “reference method” should be included.

   • The definition has been added.

6. Please add the following definitions:
   a)  % Hypochromic red cell (% Hypo) is a percentage of red blood cells with lower than normal hemoglobin content.
   b)  RBC-Y is the mean value of the forward light scatter histogram of mature red blood cells.
   c)  RET-Y is the mean value of the forward light scatter histogram of mature reticulocytes.

   • See the response to Comment 1.

7. A definition of the term “immature reticulocyte fraction” should be added to this section.

   • A definition has been added to the document.

8. A reference should be included for the term “static cytometry.”

   • A reference has been included.
Section 5.1, Patient Information (Formerly Section 6.1)

9. Obtaining the “reason why the test is requisitioned” will be difficult and impossible to obtain. We suggest that this statement be deleted.

- The text has been modified to read, “…ideally should state the reason why the test is requisitioned.” In some countries, such as the U.S., this information is required to justify billing claims, so deletion of the statement would not be appropriate.

Section 5.2, Specimen Collection Techniques (Formerly Section 6.2)

10. The last sentence belongs in Section 6.3.

- The rationale for the suggested modification is not clear. The text has been maintained in Section 6.2.

Section 5.2.5, Specimen Handling and Packaging (Formerly Section 6.2.5)

11. Please include reference to NCCLS document H18—Procedures for the Handling and Processing of Blood Specimens for completeness.

- A reference to the H18 guideline has been added as recommended.

Section 5.4, Sample Evaluation (Formerly Section 6.4)

12. Is this section necessary since there are other NCCLS documents that address this issue?

- The subcommittee believes this section is a necessary component of the guideline. Other NCCLS documents contain general information on Sample Evaluation, and Section 5.4 provides details specific to reticulocyte analysis.

Section 6, Correlation Method-New Methylene Blue Visual Microscopy (Formerly Section 7) and Section 8.1, Comparability (Correlation) Method (Formerly Section 9.1)

13. The terms “correlation” and “comparability” have been substituted for “reference,” which was in the previous publication. Why is this? According to Clin Lab Haem. 1998;20:77-79, ICSH calls this a proposed reference method. Shouldn’t the NCCLS document state this as proposed reference method and not use the terms correlation or comparable?

- The current ICSH committee does not favor NMB as a reference method (reference measurement procedure), as the documented imprecision and subjectivity of the method yield it unacceptable, particularly in light of the existence of methods demonstrating superior performance.

Section 8.1.1, (Diagnostic Sensitivity Studies (Formerly Section 7.1.1)

14. It was recommended previously that a minimum of 20 blood samples be examined. Now it is changed to 26. Please provide a reference for the change.

- The change from a minimum of 20 blood samples to a minimum of 26 blood samples is based upon a statistical recommendation, not experimental evidence. References are not available, nor needed, to support the change.

Section 8.1.3, Procedure (Formerly Section 7.1.3)

15. The method to evaluate reticulocyte counting by flow cytometry is complete and well described. However, it is difficult to perform all of the steps for confirmation of accuracy and precision of automated analyzers as they are too time consuming.

- This section is intended for instrument evaluations and periodic validation of instrument performance if calibration problems are suspected. This section is not intended to provide recommendations for routine or daily quality control functions.

16. Table 2. Accuracy Estimation: Paired Sample Method: It appears that there are several differences from the previous table in H44-A. For example, the Retic % was .01 in the original table and now it is 0.1. Under the SE p column, the numbers in A2 appear to have a two decimal place difference from the table in A1. The 95% SE p has been deleted in the table in A2. The 95% Low and High in the original table have been substituted for Upper and Lower Limit in the table A2. Please correct if necessary and/or explain the changes.
Changes in this version of the guideline are based upon statistical consultation, indicating that 26 samples is the preferred “N” to verify a normal range. This sample size will allow for a confidence interval of 95%. Other changes were made to correct inaccuracies in the previous version of the document.

Section 8.5, Testing for Carry-Over (Formerly Section 7.5)

17. This needs a reference. Is it an ICSH procedure? We recommend that other comparable methods be added after Section 11.4 since there are other alternatives to monitoring functional iron deficiency than just CHr.

This section is based upon intuitive mathematics with definitions and explanations provided. Some additional methods related to reticulocyte analysis have been incorporated in Section 10.3. Other methods for functional iron deficiency determination are not based upon reticulocyte analysis and are therefore, outside of the scope of this document.

Section 10.4, Reticulocyte Hemoglobin Content (CHr) (Formerly Section 11.4)

18. The RBC-Y/RET-Y has been reported to be used as sensitive and specific indicators of functional iron deficiency. According to the literature, these two values seem to equate with red cell/reticulocyte hemoglobin content. Please add the following references:


The references have been added as suggested.

19. % Hypo has been reported to be used as sensitive and specific indicators of functional iron deficiency in clinical studies. Please add the following reference:


The text in Section 10.4 has been modified and the suggested reference has been added.
The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS document *HS1—A Quality System Model for Health Care*. The quality system approach applies a core set of “quality system essentials (QSEs),” basic to any organization, to all operations in any healthcare service’s path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager’s guide. The quality system essentials (QSEs) are:

- **Documents & Records**
- **Equipment**
- **Information Management**
- **Process Improvement**
- **Organization**
- **Purchasing & Inventory**
- **Occurrence Management**
- **Service & Satisfaction**
- **Personnel**
- **Process Control**
- **Assessment**
- **Facilities & Safety**
- **Path of Workflow**

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, *GP26-A2* defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytical, analytical, and postanalytical. All clinical laboratories follow these processes to deliver the laboratory’s services, namely quality laboratory information.

*H44-A2* addresses the clinical laboratory path of workflow steps indicated by an “X.” For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the following page.

<table>
<thead>
<tr>
<th>Preanalytic</th>
<th>Analytic</th>
<th>Postanalytic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Assessment</td>
<td>Test Request</td>
<td>Specimen Collection</td>
</tr>
<tr>
<td>Specimen Transport</td>
<td>Specimen Receipt</td>
<td>Testing Review</td>
</tr>
<tr>
<td>Laboratory Interpretation</td>
<td>Results Report</td>
<td>Post-test Specimen Management</td>
</tr>
</tbody>
</table>

Adapted from NCCLS document *HS1—A Quality System Model for Health Care*. 

![Table of Path of Workflow](image-url)
Related NCCLS Publications∗

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


GP5-A2  Clinical Laboratory Waste Management; Approved Guideline—Second Edition (2002). Based on U.S. regulations, this document provides guidance on safe handling and disposal of chemical, infectious, radioactive, and multihazardous wastes generated in the clinical laboratory.

H1-A5  Tubes and Additives for Venous Blood Specimen Collection; Approved Standard—Fifth Edition (2003). This standard contains requirements for venous blood collection tubes and additives including heparin, EDTA, sodium citrate, and heparin compounds used in blood collection devices.

H3-A5  Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard—Fifth Edition (2003). This document provides procedures for the collection of diagnostic specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children.

H4-A4  Procedures and Devices for the Collection of Diagnostic Blood Specimens by Skin Puncture; Approved Standard—Fourth Edition (1999). A consolidation of H4-A3 and H14-A2, this standard provides detailed descriptions and explanations of proper collection techniques, as well as hazards to patients from inappropriate specimen collection by skin puncture procedures.

H18-A2  Procedures for the Handling and Processing of Blood Specimens; Approved Guideline—Second Edition (1999). This guideline addresses multiple factors associated with handling and processing specimens, as well as factors that can introduce imprecision or systematic bias into results.

H20-A  Reference Leukocyte Differential Count (Proportional) and Evaluation of Instrumental Methods; Approved Standard (1992). This standard describes automated differential counters and establishes a reference method (reference measurement procedure) based on the visual (or manual) differential count for leukocyte differential counting, to which an automated or manual test method can be compared.

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