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February 2004

H44-A2

Methods for Reticulocyte Counting (Automated Blood Cell Counters, Flow Cytometry, and Supravital Dyes); Approved Guideline—Second Edition

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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.

A guideline for global application developed through the Clinical and Laboratory Standards Institute consensus process.

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Methods for Reticulocyte Counting (Automated Blood Cell Counters, Flow Cytometry, and Supravital Dyes); Approved Guideline—Second Edition

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Abstract

Clinical and Laboratory Standards Institute 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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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.[1] 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.

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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

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Methods for Reticulocyte Counting (Automated Blood Cell Counters, Flow Cytometry, and Supravital Dyes); Approved Guideline—Second Edition

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.

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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)[3]; **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).[4]

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);[3] **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).[5]

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.

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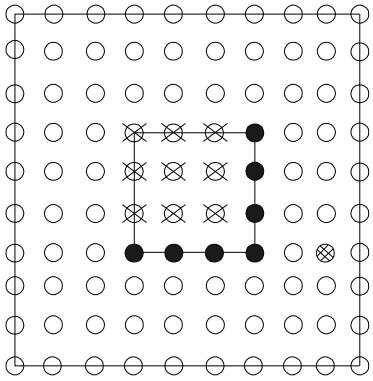

● = red cells not counted
⊗ = red cells counted
⊗ = reticulocyte

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:

Retics (%) =

$$\frac{\text{Total Number of Reticulocytes}}{\text{Total Number of Red Cells + Retics}} \times 100 \quad (2)$$

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:

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.

Abs. Retic Count ($x10^9$/L) =

$$\text{RBC } (x10^{12}/L) \times \text{Retics (\%)} \times 10^{-2} \quad (4)$$

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.

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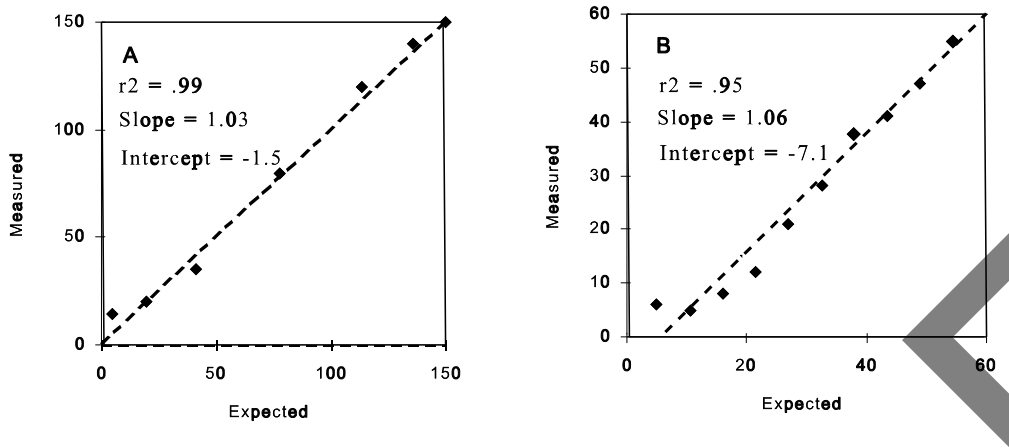

Figure 3. Linearity

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 x 10^{12}/L and the low RBC count sample be selected with the value below 2 x 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.

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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.

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

EP6-A **Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline (2003).** This document provides guidance for characterizing the linearity of a method during a method evaluation; for checking linearity as part of routine quality assurance; and for determining and stating a manufacturer's claim for linear range.

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.

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