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H52-A2

Red Blood Cell Diagnostic Testing Using Flow Cytometry; Approved Guideline— Second Edition

This guideline addresses the diagnostic red blood cell (RBC) assays performed as fluorescence-based assays on a flow cytometry platform; including testing procedures for fetomaternal hemorrhage detection, paroxysmal nocturnal hematuria screening, membrane defect anemia testing for hereditary spherocytosis, and nucleated RBC counting. Points of validation and quality control, and caveats of interpretation are also discussed.

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

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Red Blood Cell Diagnostic Testing Using Flow Cytometry; Approved Guideline—Second Edition

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Abstract

Clinical and Laboratory Standards Institute document H52-A2—*Red Blood Cell Diagnostic Testing Using Flow Cytometry; Approved Guideline—Second Edition* addresses the diagnostic RBC assays performed as fluorescence-based assays on a flow cytometry platform. Preferred and alternative testing procedures for fetomaternal hemorrhage detection, paroxysmal nocturnal hematuria screening, membrane defect anemia testing for hereditary spherocytosis, and nucleated RBC counting are reviewed. Preferred testing methods, points of validation and QC, and caveats of interpretation are discussed from the perspectives of laboratory practitioners, diagnostic test developers, and regulators. Where appropriate, this guideline integrates current statements of other relevant organizations, such as the International Council for Standardization in Haematology.

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Foreword

The recommendations contained herein address both methods in daily use in diagnostic clinical flow cytometry (FCM) and methods for verification or calibration of other assays, including automated cell counting instruments.

Presently, there are no universally accepted standards for precision, accuracy, and interlaboratory comparability in FCM. The recommendations provided in this document reflect the committee's understanding of best practices at the time of publication and in accordance with the present guidelines of the International Council for Standardization in Haematology.

This document replaces the first edition of the approved guideline, H52-A, which was published in 2001. Several changes were made in this edition; chief among them is the revision of the document scope from restricted to fetomaternal hemorrhage (FMH) testing methods to a broader scope of all diagnostic assays on RBCs using FCM. These changes reflect both the expansion of diagnostic FCM testing using RBCs and the clinical need to provide guidelines for testing methods not previously covered by CLSI documents. Specifically, this revision expands the discussion of FMH testing to include preferred testing methodology relating to the diagnosis of paroxysmal nocturnal hemoglobinuria and nonimmune membrane-associated hemolytic anemias (hereditary spherocytosis, hereditary pyropoikilocytosis, and ovalocytosis). Additional diagnostic tools to further evaluate anemic conditions by the reliable quantitation of adult F-cells and nucleated RBCs are also included.

Key Words

Anemia, diagnostic testing, erythrocyte, fetomaternal hemorrhage, flow cytometry, hemolytic anemia, red blood cells

Note that the trade name Triton™ X-100 is included in Section 9.5.4.1, and the trade name ECD® (PE/Texas Red®) is used in Appendix A, Section A2.3 of this document. It is Clinical and Laboratory Standards Institute's policy to avoid using a trade name unless the product identified is the only one available, or it serves solely as an illustrative example of the procedure, practice, or material described. In this case, the document development committee and consensus committee believe the trade name is an important descriptive adjunct to the document. In such cases, it is acceptable to use the product's trade name, as long as the words, "or the equivalent" are added to the references. It should be understood that information on this product in this guideline also applies to any equivalent products. Please include in your comments any information that relates to this aspect of H52.

Red Blood Cell Diagnostic Testing Using Flow Cytometry; Approved Guideline—Second Edition

1 Scope

This document establishes performance guidelines for applying the science of flow cytometry (FCM) to RBC diagnostic testing. It provides guidelines for:

- Specimen collection, handling, and storage
- Procedures for calibrating instruments
- Procedures for QC of blood samples

Specific sections are dedicated to:

- Paroxysmal nocturnal hemoglobinuria (PNH)
- Diseases of RBC shape, including hereditary spherocytosis (HS)
- Detection of fetomaternal hemorrhage (FMH)
- Detection of nucleated RBCs (NRBCs)

This document is intended for use by laboratory practitioners, *in vitro* diagnostic (IVD) device manufacturers concerned with quality laboratory medicine practice, and regulators responsible for clearance of new diagnostic devices and quality laboratory medicine practice.

While there are many other RBC applications, particularly in the area of blood transfusion science or immunohematology, they will not be addressed in this guideline. In addition, it is beyond the scope of this document to establish general performance criteria and reference intervals. Therefore, it is each laboratory's responsibility to establish instrument performance criteria and staining characteristics for its own specific reagents.

2 Introduction

FCM is an established technology in both the research and clinical laboratory. Recently, several methodologies that allow for precise identification and enumeration of fetal RBCs in the maternal circulation and of membrane surface marker defects in PNH have been introduced into the routine clinical laboratory. The original osmotic fragility (OF) test for the detection of HS has been replaced in many large centers by the simpler and more reproducible eosin-5-maleimide (EMA) binding test. The laborious sugar water and Ham tests have been replaced by direct measurement of decreased or defective phosphatidylinositol-linked proteins by FCM for the diagnosis of PNH. Finally, the detection of NRBCs using a nuclear dye and the pan leukocyte marker CD45 allows accurate enumeration of NRBCs in those samples in which hematology analyzers may have difficulty doing so.

The goal of this document is to describe methodologies and QA procedures that will help ensure precision and accuracy of flow cytometric results appropriate for their use in the clinical laboratory. This document should be used in conjunction with other guidance documents, particularly the 2013 International Council for Standardization in Haematology/International Clinical Cytometry Society (ICSH/ICCS) Validation of Cell-based Fluorescence Assays: Practice Guidelines.¹⁻⁵

Major points of attention include:

- Potentially biohazardous procedures and appropriate precautions
- Sample preparation techniques particular to each RBC procedure
- Reagent panels employed and rationale for selection
- Types of methodological controls required and the necessary frequency of their use
- Guidelines for interpretation and reporting of data

3 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are 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 all known infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of bloodborne pathogens. The Centers for Disease Control and Prevention address this topic in published guidelines that address the daily operations of diagnostic medicine in human and animal medicine while encouraging a culture of safety in the laboratory.⁶ For specific precautions for preventing the laboratory transmission of all known infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all known infectious diseases, refer to CLSI document M29.⁷

4 Terminology

4.1 A Note on Terminology

CLSI, as a global leader in standardization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. CLSI 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 CLSI, International Organization for Standardization (ISO), and European Committee for Standardization (CEN) documents; and that legally required use of terms, regional usage, and different consensus timelines are all important considerations in the harmonization process. In light of this, CLSI’s consensus process for development and revision of standards and guidelines focuses on harmonization of terms to facilitate the global application of standards and guidelines.

For consistency with accepted international usage, the terms *preexamination*, *examination*, and *postexamination* are adopted in place of their US counterparts *preanalytical*, *analytical*, and *postanalytical*, respectively. However, the US counterparts are included parenthetically.

In addition, CD235a was adopted throughout the guideline. Users should note that CD235a antigen is synonymous to glycophorin A.

4.2 Definitions

autofluorescence – intrinsic fluorescence of unstained cells, generally caused by pyrimidines and flavin nucleotides; **NOTE:** The level of autofluorescence is a function of the excitation wavelength and varies with the cell type analyzed and/or the state of cellular activation. Cultured cell lines, neutrophils, and macrophages usually demonstrate higher levels of autofluorescence with 488 nm excitation, and proportional lower autofluorescence with excitation at longer wavelengths. Autofluorescence of RBCs is significantly lower than that of WBCs, and instrument settings may need to be adjusted accordingly.

The Quality Management System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management 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. The quality management system approach applies a core set of “quality system essentials” (QSEs), basic to any organization, to all operations in any health care service’s path of workflow (ie, operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager’s guide. The QSEs are as follows:

- Organization
- Customer Focus
- Facilities and Safety
- Personnel
- Purchasing and Inventory
- Equipment
- Process Management
- Documents and Records
- Information Management
- Nonconforming Event Management
- Assessments
- Continual Improvement

H52-A2 addresses the QSE indicated by an “X.” For a description of the other documents listed in the grid, please refer to the Related CLSI Reference Materials section on the following page.

Organization	Customer Focus	Facilities and Safety	Personnel	Purchasing and Inventory	Equipment	Process Management	Documents and Records	Information Management	Nonconforming Event Management	Assessments	Continual Improvement
		GP41 M29		GP41	H43	X GP41 GP42 H20 H43 H44					

Path of Workflow

A path of workflow is the description of the necessary processes to deliver the particular product or service that the organization or entity provides. A laboratory path of workflow consists of the sequential processes: preexamination, examination, and postexamination and their respective sequential subprocesses. All laboratories follow these processes to deliver the laboratory’s services, namely quality laboratory information.

H52-A2 addresses the clinical laboratory path of workflow steps indicated by an “X.” For a description of the other documents listed in the grid, please refer to the Related CLSI Reference Materials section on the following page.

	Preexamination			Examination			Postexamination		
	Examination ordering	Sample collection	Sample transport	Sample receipt/processing	Examination	Results review and follow-up	Interpretation	Results reporting and archiving	Sample management
GP41	X GP41 GP42	X GP41	X GP41	X GP41	X GP41	X GP41	X		
H43	H43 H44	H43 H44	H43 H44	H20 H43 H44	H20 H43 H44	H20 H43 H44	H43 H44	H43 H44	H44

Related CLSI Reference Materials*

- GP41-A6** **Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard—Sixth Edition (2007).** This document provides procedures for the collection of diagnostic specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children.
- GP42-A6** **Procedures and Devices for the Collection of Diagnostic Capillary Blood Specimens; Approved Standard—Sixth Edition (2008).** This document provides a technique for the collection of diagnostic capillary blood specimens, including recommendations for collection sites and specimen handling and identification. Specifications for disposable devices used to collect, process, and transfer diagnostic capillary blood specimens are also included.
- H20-A2** **Reference Leukocyte (WBC) Differential Count (Proportional) and Evaluation of Instrumental Methods; Approved Standard—Second Edition (2007).** This document is a reference method for the evaluation of automated differential counters, based on the visual differential count.
- H43-A2** **Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition (2007).** This document provides performance guidelines for the immunophenotypic analysis of neoplastic hematolymphoid cells using immunofluorescence-based flow cytometry; for sample and instrument quality control; and precautions for acquisition of data from neoplastic hematolymphoid cells.
- H44-A2** **Methods for Reticulocyte Counting (Automated Blood Cell Counters, Flow Cytometry, and Supravital Dyes); Approved Guideline—Second Edition (2004).** 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.
- M29-A3** **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005).** Based on US regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.

* CLSI documents are continually reviewed and revised through the CLSI consensus process; therefore, readers should refer to the most current editions.

Sample



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