CLINICAL AND LABORATORY STANDARDS INSTITUTE®

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H42-A2

Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition

This document provides guidance for the immunophenotypic analysis of non-neoplastic lymphocytes by immunofluorescence-based flow cytometry; sample and instrument quality control; and precautions for acquisition of data from lymphocytes.

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

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For additional information on committee participation or to submit comments, contact CLSI.

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Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition

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Abstract

Clinical and Laboratory Standards Institute document H42-A2—*Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition* was developed to address issues of procedures and quality assurance for clinical applications of flow cytometry. It is designed to aid clinical laboratorians in the development of quality assurance procedures and to establish the foundation for different laboratories using different commercially available instruments to obtain comparable results. Specific topics covered include: specimen collection, transport, and preparation; sample quality control and staining procedures; instrument calibration; sample analysis; and data analysis, storage, and reporting.

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Foreword

Advances in the availability and reproducibility of monoclonal antibody reagents specific for a wide range of cell types, coupled with lower costs for increasingly automated flow cytometers with greater data analysis capabilities, have made flow cytometry the method of choice for immunophenotyping hematopoietic cells in the clinical laboratory. CLSI document H42-A2 represents the effort of the CLSI Working Group on Immunophenotyping of Lymphocytes appointed to establish guidelines for enumeration of lymphocyte subsets and $CD34^+$ hematopoietic stem cells by flow cytometry. In this context, it should be noted that for both types of assays, similar guidelines already have been developed by specific professional organizations or at national levels.[1-10] The current guideline aims to bring the state-of-the-art techniques together in a comprehensive, but readily usable format. It should be recognized that on occasion, national guidelines will override this document where applicable.

H42-A2 is designed to aid clinical laboratorians in the development of quality assurance procedures and to establish the foundation for laboratories using different commercially available instruments to obtain comparable results. This document should help minimize interoperator and interlaboratory variability in the various components of flow cytometry. Specific topics covered include specimen collection, transport, and preparation; sample quality control and staining procedures; instrument calibration; sample analysis; and data analysis, storage, and reporting.

In an effort to create an easy-to-use guideline, the main body of the H42-A2 document was divided into three parts. Part A: *General* includes the Scope, Introduction, Standard Precautions, Overview, Definitions, Safety, and an introductory section on methods for enumeration of absolute cell numbers. Part B: *Enumeration of Lymphocyte Subsets* includes recommendations related to the collection, transport, preparation, and analysis of specimens for enumeration of lymphocyte subsets. Part C: *Enumeration of $CD34^+$ Hematopoietic Stem and Progenitor Cells*, includes specific recommendations for the enumeration of $CD34^+$ stem cells, as well as—for convenience—some of the same general information provided in Part B (e.g., patient information, venipuncture technique, labeling of specimen).

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, 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, CLSI recognizes that harmonization of terms facilitates the global application of standards and deserves immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

In order to align the usage of terminology in this document with that of ISO, the term *accuracy,* in its metrological sense, refers to the closeness of the agreement between the result of a (single) measurement and a true value of a measurand, and comprises both random and systematic effects.

The term *diagnostic sensitivity* is combined with the term *clinical sensitivity,* and correspondingly the term *diagnostic specificity* is combined with the term *clinical specificity*, because in Europe, the term "clinical" often refers to clinical studies of drugs under stringent conditions.

All terms and definitions will be reviewed again for consistency with international use, and revised appropriately during the next scheduled revision of this document.

Key Words

Autofluorescence, CD system, $CD34^+$ hematopoietic stem cells, color compensation, dual-parameter display, flow cytometry, fluorescein isothiocyanate (FITC), fluorescence, forward angle light scatter, gate, histogram, immunoglobulin, immunophenotyping, linear amplification, logarithmic amplification, low angle light scatter, lymphocyte, lymphocyte subsets, 90° light scatter, phycoerythrin (PE), positive procedure control, single-parameter display (histogram), subclass

Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition

PART A: General

1 Scope

The scope of this document is to establish performance guidelines for the identification and enumeration of lymphocyte subpopulations and the enumeration of $CD34^+$ hematopoietic progenitors using immunofluorescence-based flow cytometry (FCM).

The working group recognizes that other, so-called nontraditional methodologies exist or are in development for enumeration of $CD4^+$ T-lymphocytes (e.g., systems using microcapillary sample delivery or nonfluorescent cell detection; see Appendix C). Some of the issues discussed in this document are common to the use of any method for $CD4^+$ T-cell enumeration (e.g., sample collection and transport, safety issues, data reporting, and interpretation). However, issues such as sample preparation, instrument calibration, and quality control differ significantly for nontraditional methodologies and are not discussed in this document.

Presently, there are no universally accepted standards for precision, accuracy, and interlaboratory comparability in lymphocyte enumeration by FCM. General consensus was reached on the basic International Society for Hematotherapy and Graft Engineering (ISHAGE)[9] guidelines for CD34 analysis, and this forms the basis of the technique described herein. 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

Flow cytometry is an established technology that has moved from the research laboratory into the clinical laboratory. The goal of this document is to establish quality assurance procedures that will help ensure precision and accuracy of flow cytometric results appropriate for their use in the clinical laboratory. Since at present, most assays for lymphocyte subset and hematopoietic progenitor enumeration in clinical laboratories are fluorescence-based, this document is limited to specific issues surrounding the use of such systems. Major points of attention include the following:

- potentially biohazardous procedures and appropriate precautions;
- type and frequency of methodologic controls required;
- analysis methods for lymphocyte subset and hematopoietic progenitor identification;
- methods for determination of absolute cell concentrations;
- guidelines for retention of laboratory records; and
- guidelines for definition of laboratory reference intervals.

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 infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the US Centers for Disease Control and Prevention (Garner JS, Hospital Infection Control Practices Advisory Committee. Guideline for isolation precautions in hospitals. *Infect Control Hosp Epidemiol*. 1996;17(1):53-80). For specific precautions for preventing the laboratory transmission of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to the most current edition of CLSI document M29—*Protection of Laboratory Workers From Occupationally Acquired Infections*.

4 Overview

In a previous document, the original subcommittee outlined general principles that are useful for performing FCM on blood samples for lymphocyte subset analysis. Some of these principles are directly applicable to the enumeration of $CD34^+$ hematopoietic stem and progenitor cells. Because the latter analysis involves rare event detection, special procedures not essential for lymphocyte subset enumeration are necessary. This section outlines the most important differences; detailed information is then presented in appropriate sections to follow.

4.1 Goals

The principal goal of blood lymphocyte immunophenotyping is to enumerate antigenically defined lymphocyte subsets. T-cells are the main lymphocyte subset and are defined by their expression of CD3. The major subsets of T-cells are the so-called T-helper cells ($CD3^+$ and $CD4^+$) and the cytotoxic T-cells ($CD3^+$ and $CD8^+$). Smaller lymphocyte subsets include the B-cells ($CD19^+$) and NK cells ($CD3^-$ and $CD56^+$).

$CD34^+$ cell enumeration is used in the evaluation of patients undergoing hematopoietic stem cell and progenitor cell transplants. CD34 is an antigen expressed on hematopoietic stem and progenitor cells.

4.2 Quality Control Procedures

Instrument setup for $CD34^+$ cell enumeration is generally similar to that used for lymphocyte enumeration. The use of the viability dye 7-aminoactinomycin D (7-AAD) necessitates accurate compensation using this dye, and this point is addressed in the instrument setup section (see Appendix A).

4.3 Sample Preparation

Although the methods initially developed for processing blood may be applicable to the study of bone marrow, cord blood, apheresis, or other types of samples, special care is often necessary to ensure that the cell concentration is appropriate for the amounts of antibody used, and methods for RBC lysis do not impact on the viability of cells of interest.

4.4 Reagents

The reagent panels used for lymphocyte subset enumeration are fairly well-standardized and usually include CD45 and/or CD3 as a gating reagent. CD4 and CD8 subsets are routinely reported as a subset of

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 approach is based on the model presented in the most current edition of CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care.* The quality management 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 (i.e., 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 quality system essentials (QSEs) are:

Documents & Records	Equipment	Information Management	Process Improvement
Organization	Purchasing & Inventory	Occurrence Management	Customer Service
Personnel	Process Control	Assessments—External and Internal	Facilities & Safety

H42-A2 addresses the quality system essentials (QSEs) indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessments—External and Internal	Process Improvement	Customer Service	Facilities & Safety
			X H43 I/LA24	H3	X C28 GP5 H3 H20 H43 I/LA24 M29			I/LA24	I/LA24		GP5 GP17 H3 M29

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

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, CLSI/NCCLS document GP26—*Application of a Quality Management System Model for Laboratory Services* defines a clinical laboratory path of workflow which consists of three sequential processes: preexamination, examination, and postexamination. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

H42-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/NCCLS Publications 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
X H3 H43	X H3 H43	X H3 H43	X H3 H20 H43	X H20 H43	X H20 H43	X H43	X H43	

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

Related CLSI/NCCLS Publications*

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

GP5-A2 **Clinical Laboratory Waste Management; Approved Guideline—Second Edition (2002).** Based on US regulations, this document provides guidance on the safe handling and disposal of chemical, infectious, radioactive, and multihazardous wastes generated in the clinical laboratory. While a valuable resource for a wider audience, it is intended for use primarily in the United States.

GP17-A2 **Clinical Laboratory Safety; Approved Guideline—Second Edition (2004).** This document contains general recommendations for implementing a high-quality laboratory safety program, which are provided in a framework that is adaptable within any laboratory.

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. It also includes recommendations on order of draw.

H20-A2 **Reference Leukocyte (WBC) Differential Count (Proportional) and Evaluation of Instrumental Methods; Approved Standard—Second Edition (2007).** Discusses automated differential counters and establishes a reference method based on the visual (or manual) differential count for leukocyte differential counting to which an automated or manual test method can be compared, and an experiment to carry out the comparison. Describes procedures for collecting specimens; preparing blood films and requirements for acceptable wedge and spun films; Romanowsky staining; the formed elements; variant leukocyte forms; and a protocol for examining blood films. Details procedures for determining inaccuracy, and within-run and between-run imprecision; procedures for determining sensitivity/specificity/predictive values of flags; and statistical methods for determining inaccuracy and imprecision.

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.

I/LA24-A **Fluorescence Calibration and Quantitative Measurement of Fluorescence Intensity; Approved Guideline (2004).** This guideline describes the basic principles, reference materials, and laboratory procedures upon which quantitative fluorescence calibration is based.

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.

* Proposed-level documents are being advanced through the Clinical and Laboratory Standards Institute consensus process; therefore, readers should refer to the most current editions.

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