CLINICAL AND LABORATORY STANDARDS INSTITUTE®

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# I/LA26-A2

## Performance of Single Cell Immune Response Assays; Approved Guideline—Second Edition



This document contains methods of intracellular cytokine evaluation, major histocompatibility complex multimer quantitation, enzyme-linked immunospot technology, and carboxyfluorescein succinimidyl ester tracking dye staining for the assessment of cellular proliferation. It also provides basic aspects of specimen collection, transport, and preparation; results interpretation; and quality assurance and test validation approaches.

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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# Performance of Single Cell Immune Response Assays; Approved Guideline—Second Edition

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## Abstract

Clinical and Laboratory Standards Institute document I/LA26-A2—*Performance of Single Cell Immune Response Assays; Approved Guideline—Second Edition* describes assays that measure antigen-specific cellular immune responses in the context of clinical trials and in the management of subjects with immune-mediated diseases. Immune therapeutic approaches are being applied in various fields of medicine, including infectious diseases, transplantation, autoimmune disease, cancer, and allergies. Assays are required to measure the cellular effects of such therapeutic approaches.

This guideline focuses on the methods of intracellular cytokine evaluation, major histocompatibility complex multimer quantitation, enzyme-linked immunospot technology, and carboxyfluorescein succinimidyl ester tracking dye staining. The document covers basic aspects of specimen collection, transport, and preparation, in addition to QA and method validation approaches. Data acquisition, data analysis, and reporting aspects for these assays are also summarized.

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## Foreword

The field of immunology continues to evolve from that of a basic science discipline to a major force in medical and laboratory science. With the continued development of new vaccines and the burgeoning application of immune-based therapies and targeted immune interventions in almost every discipline of medical science, a need exists to develop better laboratory tools for measuring antigen-specific immune responses and for monitoring the effects of the various interventions on these immune responses. These complex assays are often performed on cells that have been cryopreserved, which has resulted in performance characteristics that vary greatly from laboratory to laboratory. The recognition of the importance of these assays and their increased use, along with their inherent complexities and variable performance characteristics, requires their standardization if the field is to move forward; the urgency of this need is the impetus for producing this guideline.

It is hoped that such an effort will result in more effective evaluations of new immune interventions and immune-based therapeutic agents in clinical trials and translational research, especially as they are considered for approval by regulatory agencies. In addition, guidance for performance of these cellular immune assays (eg, for T-cell responses) should improve this performance and expedite the evaluation of their role in routine patient monitoring for eventual clinical use.

The document development committee recognizes the large and varied methodology that has evolved for evaluating cellular immune responses. It has chosen to focus its efforts on intracellular cytokine measurements, major histocompatibility complex multimer quantification, enzyme-linked immunospot (ELISPOT) assays, and carboxyfluorescein succinimidyl ester (CFSE) fluorescent staining for the assessment of cell proliferation. As applications using these methods evolve and the methods improve, it is anticipated that new assays for monitoring immune responses will be developed along with new laboratory approaches. As the field advances, the changes will be incorporated in future editions of this guideline.

The second edition of I/LA26 includes the following changes that have been made since the first approved edition:

- The references were revised to include recent experience with each of the assays in terms of their new applications, inclusions in clinical trials, and assay optimizations.
  - For the flow cytometry–based assays, new and more complex gating algorithms (eg, doublet removal, inclusion, exclusion gating) are described, which serve to increase the signal-to-noise ratio and improve the sensitivity and precision of detecting rare events. New figures illustrating the improved gating algorithms are included.
  - Information related to newly available methods of assessing proficiency is included.
  - The features and impact of improved and harmonized ELISPOT assays are reflected, along with more detailed information regarding troubleshooting, and figures illustrating common problems.
- The specimen handling guidelines were revised according to Centers for Disease Control and Prevention recommendations.
- An entirely new section for assessment of antigen-specific proliferation using the tracking dye CFSE was added.
- Additional information was added on pentamers and Dextramers® (or the equivalent), in addition to new information on multimer products in general.

- Information was added on new cell preparation tubes, which contain a premade gel barrier for density gradient separation for the isolation of peripheral blood mononuclear cells with a single centrifugation step.

- Modifications were made to the intracellular cytokine staining section (formerly cytokine flow cytometry), which include polychromatic flow and more versatility in the preparation and storage of samples that reflects the experience with the assays since the last version of this document. These changes are accompanied by new figures.

- Information was added on new fixable stable viability marker dyes compatible with intracellular staining protocols.

- A new appendix (see Appendix B) was added that describes the statistics of rare-event analysis.

- The terminology was revised to reflect current practice.

**Note that the trade name Dextramer® is included throughout 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 I/LA26.**

**Key Words**

Carboxyfluorescein succinimidyl ester tracking dye, CD4 and CD8 T-cells, enzyme-linked immunospot, flow cytometry, intracellular cytokine, major histocompatibility complex multimer

# Performance of Single Cell Immune Response Assays; Approved Guideline—Second Edition

## 1 Scope

This document provides guidance for the performance of single cell immune response assays within the clinical context of infectious diseases (especially HIV), cancer, transplantation, autoimmune disease, and allergies. This guideline focuses on antigen-specific functional assays within CD4 and CD8 T-cell subsets in response to the recognition that markers of immune competency are increasingly required in clinical trials and for the approval of new immune-based therapies by regulatory agencies. The assays in this document include antigen-stimulated intracellular cytokine production measured by flow cytometry, the quantification of antigen-specific CD4 and CD8 T-cells using major histocompatibility complex (MHC) multimers and flow cytometry, antigen-specific cell quantification using the enzyme-linked immunospot (ELISPOT) assay, and lastly the flow cytometric assessment of changes in the level of fluorescence of carboxyfluorescein succinimidyl ester (CFSE)–stained cells as a measure of antigen-induced lymphocyte proliferation. The document covers details of the procedure and data interpretation as well as issues such as specimen collection, transport, sample preparation, QC, test validation, and troubleshooting.

The guideline provides laboratorians with methods for clinical research application in the growing field of immune-based therapy, as well as guidance to pharmaceutical manufacturers in the laboratory evaluation of new products before submission to regulatory agencies. It is also a valuable resource for academic investigators developing these assays for the evaluation of antigen-specific responses in their own research and for coordinating the improved implementation and assessment of these assays within and between laboratories participating in multicenter/multinational clinical trials. Overall, this guideline establishes consensus methods for a rapidly evolving field of single cell immune functional assays.

Clinical applications of single cell response assays have not been approved by the US Food and Drug Administration (FDA) to date.

This guideline:

- Is not intended to be used "as is" for clinical use by diagnostic laboratories; nor is it intended to be a clinical diagnostic procedure manual. It is not intended to be formatted according to CLSI document QMS02[1] for writing clinical laboratory procedures for adoption by diagnostic laboratories.

- Is designed to address the general procedures and those particular components involved in each of the four procedures that have been observed to be important in their successful application and interpretation, and is not intended to provide detailed step-by-step instructions for any specific stimuli or for specific lymphocyte subsets. However, these limitations do not preclude its use as a guide in the development of future clinical laboratory procedures.

- Does not address any specific application within any specific patient population.

## 2 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 blood-borne 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.[2] 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.[3]

# 3 Terminology

## 3.1 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.

The globally preferred terms *preexamination*, *examination*, and *postexamination* are used in I/LA26, and *preanalytical*, *analytical*, and *postanalytical* are included parenthetically after their respective counterparts.

## 3.2 Definitions

**3-amino-9-ethylcarbazole** – a soluble substrate for horseradish peroxidase that generates a colored, insoluble product; **NOTE:** It is often used in enzyme-linked immunospot assays, as well as Western blots.

**accuracy (of measurement)** – closeness of agreement between a measured quantity value and the true value of the quantity intended to be measured (modified from JCGM 200:2012).[4]

**allophycocyanin (APC)** – a fluorescent protein derived from cyanobacteria or red algae that is excited by a red (632 nm) laser (eg, HeNe) on many flow cytometers; **NOTE:** APC is excited maximally at approximately 650 nm with an emission maximum at 660 nm.

**antibody** – specific immunoglobulin formed by B lymphocytes in response to exposure to an immunogenic substance (antigen) and able to bind to this antigen; **NOTE:** The molecule of an immunogenic substance contains one or more parts with a characteristic chemical composition, ie, an epitope.

**antigen** – any substance which, when injected into an animal or human being, elicits an immune response, either cellular, humoral, or both.

**antigen-presenting cells** – cells (primarily dendritic cells, monocytes, and B-cells) that are able to bind and internalize large protein antigens, process them, and then present peptide fragments of these antigens to cytotoxic T-cells and T-helper cells in the context of their major histocompatibility complex Class I and Class II surface molecules, respectively.

**apoptosis** – the process of programmed cell death resulting from specific cell signaling events.

**brefeldin A (BFA)** – a relatively nontoxic but potent protein inhibitor of intracellular protein transport.[5]



 

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

I/LA26-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<br>M29 | | GP41 | H42 | X<br>EP14<br>GP29<br>GP41<br>H42 | QMS02 | | | GP29 | |

### 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.

I/LA26-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<br>H42 | X<br>GP41<br>H42 | X<br>GP41<br>H42 | X<br>GP41<br>H42 | X<br>GP41<br>H42 | X<br>GP41<br>H42 | X<br>H42 | X<br>H42 | |

## Related CLSI Reference Materials*

**EP14-A2** **Evaluation of Matrix Effects; Approved Guideline—Second Edition (2005).** This document provides guidance for evaluating the bias in analyte measurements that is due to the sample matrix (physiological or artificial) when two measurement procedures are compared.

**GP29-A2** **Assessment of Laboratory Tests When Proficiency Testing Is Not Available; Approved Guideline—Second Edition (2008).** This document offers methods to assess test performance when proficiency testing (PT) is not available; these methods include examples with statistical analyses. This document is intended for use by laboratory managers and testing personnel in traditional clinical laboratories as well as in point-of-care and bedside testing environments.

**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.

**H42-A2** **Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition (2007).** 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.

**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.

**QMS02-A6** **Quality Management System: Development and Management of Laboratory Documents; Approved Guideline—Sixth Edition (2013).** This document provides guidance on the processes needed for document management, including creating, controlling, changing, and retiring a laboratory's policy, process, procedure, and form documents in both paper and electronic environments.



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

 


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