

MM3-A2
Vol. 26 No. 8
Replaces MM3-P2
Vol. 25 No. 11

Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline— Second Edition

This guideline addresses topics relating to clinical applications, amplified and nonamplified nucleic acid methods, selection and qualification of nucleic acid sequences, establishment and evaluation of test performance characteristics, inhibitors, and interfering substances, controlling false-positive reactions, reporting and interpretation of results, quality assurance, regulatory issues, and recommendations for manufacturers and clinical laboratories.

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



*(Formerly NCCLS)
Providing NCCLS standards and guidelines,
ISO/TC 212 standards, and ISO/TC 76 standards*

Clinical and Laboratory Standards Institute

Providing NCCLS standards and guidelines, ISO/TC 212 standards, and ISO/TC 76 standards

Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) is an international, interdisciplinary, nonprofit, standards-developing, and educational organization that promotes the development and use of voluntary consensus standards and guidelines within the healthcare community. It is recognized worldwide for the application of its unique consensus process in the development of standards and guidelines for patient testing and related healthcare issues. Our process is based on the principle that consensus is an effective and cost-effective way to improve patient testing and healthcare services.

In addition to developing and promoting the use of voluntary consensus standards and guidelines, we provide an open and unbiased forum to address critical issues affecting the quality of patient testing and health care.

PUBLICATIONS

A document is published as a standard, guideline, or committee report.

Standard A document developed through the consensus process that clearly identifies specific, essential requirements for materials, methods, or practices for use in an unmodified form. A standard may, in addition, contain discretionary elements, which are clearly identified.

Guideline A document developed through the consensus process describing criteria for a general operating practice, procedure, or material for voluntary use. A guideline may be used as written or modified by the user to fit specific needs.

Report A document that has not been subjected to consensus review and is released by the Board of Directors.

CONSENSUS PROCESS

The CLSI voluntary consensus process is a protocol establishing formal criteria for:

- the authorization of a project
- the development and open review of documents
- the revision of documents in response to comments by users
- the acceptance of a document as a consensus standard or guideline.

Most documents are subject to two levels of consensus—"proposed" and "approved." Depending on the need for field evaluation or data collection, documents may also be made available for review at an intermediate consensus level.

Proposed A consensus document undergoes the first stage of review by the healthcare community as a proposed standard or guideline. The document should receive a wide and thorough technical review, including an overall review of its scope, approach, and utility, and a line-by-line review of its technical and editorial content.

Approved An approved standard or guideline has achieved consensus within the healthcare community. It should be reviewed to assess the utility of the final document, to ensure attainment of consensus (i.e., that comments on earlier versions have been satisfactorily addressed), and to identify the need for additional consensus documents.

Our standards and guidelines represent a consensus opinion on good practices and reflect the substantial agreement by materially affected, competent, and interested parties obtained by following CLSI's established consensus procedures. Provisions in CLSI standards and guidelines may be more or less stringent than applicable regulations. Consequently, conformance to this voluntary consensus document does not relieve the user of responsibility for compliance with applicable regulations.

COMMENTS

The comments of users are essential to the consensus process. Anyone may submit a comment, and all comments are addressed, according to the consensus process, by the committee that wrote the document. All comments, including those that result in a change to the document when published at the next consensus level and those that do not result in a change, are responded to by the committee in an appendix to the document. Readers are strongly encouraged to comment in any form and at any time on any document. Address comments to Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, PA 19087, USA.

VOLUNTEER PARTICIPATION

Healthcare professionals in all specialties are urged to volunteer for participation in CLSI projects. Please contact us at customerservice@clsi.org or +610.688.0100 for additional information on committee participation.

MM3-A2
ISBN 1-56238-596-8
ISSN 0273-3099

Volume 26 Number 8

Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—Second Edition

Frederick S. Nolte, PhD
Judy C. Arbique, ART(CSMLS), CLS(NCA)
Franklin R. Cockerill, III, MD
Peter J. Dailey, PhD, MPH
David Hillyard, MD
Sherrol McDonough, PhD
Richard F. Meyer, PhD
Roxanne G. Shively, MS

Abstract

Nucleic acid methods for the detection and characterization of microorganisms in clinical specimens are now firmly established in laboratory medicine. These methods offer opportunities for clinical laboratories to provide more rapid and accurate results, and have changed the practice of clinical microbiology and infectious diseases. Clinical and Laboratory Standards Institute (CLSI) document MM3-A2—*Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—Second Edition* addresses topics relating to clinical applications, amplified and nonamplified nucleic acid methods, selection and qualification of nucleic acid sequences, establishment and evaluation of test performance characteristics, inhibitors, and interfering substances, controlling false-positive reactions, reporting and interpretation of results, quality assurance, regulatory issues, and recommendations for manufacturers and clinical laboratories.

Clinical and Laboratory Standards Institute (CLSI). *Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—Second Edition*. CLSI document MM3-A2 (ISBN 1-56238-596-8). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2006.

The Clinical and Laboratory Standards Institute consensus process, which is the mechanism for moving a document through two or more levels of review by the healthcare community, is an ongoing process. Users should expect revised editions of any given document. Because rapid changes in technology may affect the procedures, methods, and protocols in a standard or guideline, users should replace outdated editions with the current editions of CLSI/NCCLS documents. Current editions are listed in the CLSI catalog, which is distributed to member organizations, and to nonmembers on request. If your organization is not a member and would like to become one, and to request a copy of the catalog, contact us at: Telephone: 610.688.0100; Fax: 610.688.0700; E-Mail: customerservice@clsi.org; Website: www.clsi.org



(Formerly NCCLS)
Providing NCCLS standards and guidelines,
ISO/TC 212 standards, and ISO/TC 76 standards

This publication is protected by copyright. No part of it may be reproduced, stored in a retrieval system, transmitted, or made available in any form or by any means (electronic, mechanical, photocopying, recording, or otherwise) without prior written permission from Clinical and Laboratory Standards Institute, except as stated below.

Clinical and Laboratory Standards Institute hereby grants permission to reproduce limited portions of this publication for use in laboratory procedure manuals at a single site, for interlibrary loan, or for use in educational programs provided that multiple copies of such reproduction shall include the following notice, be distributed without charge, and, in no event, contain more than 20% of the document's text.

Reproduced with permission, from CLSI publication MM3-A2—*Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—Second Edition* (ISBN 1-56238-596-8). Copies of the current edition may be obtained from Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

Permission to reproduce or otherwise use the text of this document to an extent that exceeds the exemptions granted here or under the Copyright Law must be obtained from Clinical and Laboratory Standards Institute by written request. To request such permission, address inquiries to the Executive Vice President, Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

Copyright ©2006. Clinical and Laboratory Standards Institute.

Suggested Citation

(Clinical and Laboratory Standards Institute. *Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—Second Edition*. CLSI document MM3-A2 [ISBN 1-56238-596-8]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2006.)

Proposed Guideline

March 1994

Approved Guideline

December 1995

Proposed Guideline—Second Edition

April 2005

Approved Guideline—Second Edition

February 2006

ISBN 1-56238-596-8

ISSN 0273-3099

Committee Membership

Area Committee on Molecular Methods

Roberta M. Madej, MS, MT
Chairholder
Roche Molecular Systems, Inc.
Pleasanton, California

Zhimin Cao, MD, PhD
 New York State Dept. of Health
 Albany, New York

Maurizio Ferrari, MD
 International Federation of Clinical
 Chemistry
 Milan, Italy

Frederick S. Nolte, PhD
 Emory University Hospital
 Atlanta, Georgia

Timothy J. O'Leary, MD, PhD
 Biomedical Laboratory Research
 and Development Service
 Department of Veterans Affairs
 Washington, D.C.

Carolyn Sue Richards, PhD,
 FACMG
 Oregon Health Sciences University
 Portland, Oregon

Uwe Scherf, PhD
 FDA Center for Devices and
 Radiological Health
 Rockville, Maryland

Michael A. Zoccoli, PhD
 Celera Diagnostics
 Alameda, California

Advisors

Dale H. Altmiller, PhD
 O.U. Medical Center
 Edmond, Oklahoma

Lee Ann Baxter-Lowe, PhD
 University of California, San
 Francisco
 San Francisco, California

Mark Evans, PhD
 American Medical Association
 Chicago, Illinois

Cristina Gianchetti, PhD
 Gen-Probe
 San Diego, California

Leslie Hall, MMSc
 Mayo Clinic
 Rochester, Minnesota

Robert B. Jenkins, MD, PhD
 Mayo Clinic
 Rochester, Minnesota

Alan L. Landay, PhD
 Rush-Presby.-St. Lukes Medical
 Center
 Chicago, Illinois

Mario Pazzagli, PhD
 University of Florence
 Florence, Italy

Richard S. Schifreen, PhD, DABCC
 Mirus Bio Corp.
 Madison, Wisconsin

Laurina O. Williams, PhD, MPH
 Centers for Disease Control and
 Prevention
 Atlanta, Georgia

Janet L. Wood, MT(ASCP)
 BD Diagnostic Systems
 Sparks, Maryland

Subcommittee on Molecular Diagnostic Methods for Infectious Diseases

Frederick S. Nolte, PhD
Chairholder
Emory University Hospital
Atlanta, Georgia

Judy C. Arbique, ART(CSMLS),
 CLS(NCA)
 Arbique-Rendell Onsite Training &
 Consulting
 Halifax, Nova Scotia, Canada

Franklin R. Cockerill, III, MD
 Mayo Clinic/Mayo Foundation
 Rochester, Minnesota

Peter J. Dailey, PhD, MPH
 Roche Molecular Systems,
 Discovery Research
 Alameda, California

David Hillyard, MD
 ARUP
 Salt Lake City, Utah

Sherrol McDonough, PhD
 Gen-Probe Incorporated
 San Diego, California

Richard F. Meyer, PhD
 Centers for Disease Control and
 Prevention
 Atlanta, Georgia

Roxanne G. Shively, MS
 FDA Center for Devices and
 Radiological Health
 Rockville, Maryland

Staff

Clinical and Laboratory Standards
 Institute
 Wayne, Pennsylvania

John J. Zlockie, MBA
Vice President, Standards

Lois M. Schmidt, DA
Staff Liaison

Donna M. Wilhelm
Editor

Melissa A. Lewis
Assistant Editor

Contents

Abstract..... i

Committee Membership..... iii

Foreword..... vii

1 Scope..... 1

2 Introduction..... 1

3 Standard Precautions..... 1

4 Terminology..... 2

 4.1 Definitions 2

 4.2 Abbreviations and Acronyms 6

5 Applications 7

 5.1 Utility of Molecular Diagnostic Tests for Infectious Diseases 8

 5.2 Screening or Initial Testing..... 9

 5.3 Confirmatory and Supplemental Testing 9

6 Specimen Collection, Transport, and Processing..... 10

7 Contributors to False Negatives and Controls 10

 7.1 Detection of Inhibitors and Interfering Substances..... 11

 7.2 Inhibitory Samples 12

8 Methods 12

 8.1 Physical and Chemical Methods for Nucleic Acid Detection..... 13

 8.2 Detection Formats..... 15

 8.3 Nucleic Acid Amplification Technologies 17

 8.4 Real-Time PCR Instruments 19

9 Selection and Qualification of Nucleic Acid Sequences..... 20

 9.1 Target Region 20

 9.2 PCR Primer Sequence Selection 21

 9.3 Hybridization Probe Sequence Selection..... 22

 9.4 Fluorescent Resonance Energy Transfer (FRET) Probes 23

 9.5 Probe and Primer Forms and Purity..... 24

10 Establishment and Evaluation of Performance Characteristics of Molecular Diagnostic Tests..... 25

 10.1 Limit of Detection (Analytical Sensitivity) 25

 10.2 Analytical Specificity 26

 10.3 Precision..... 26

 10.4 Cutoff Values 26

 10.5 Diagnostic Sensitivity 27

 10.6 Diagnostic Specificity 27

 10.7 Predictive Values 28

 10.8 Diagnostic Accuracy..... 28

 10.9 Diagnostic Value..... 28

Contents (Continued)

10.10	Test Limitations	29
10.11	Implementation of FDA-Cleared Tests.....	29
11	Quality Assurance.....	29
11.1	Laboratory Design and Practices	30
11.2	Instruments.....	31
11.3	Quality Assurance (QA) During Development of Molecular Diagnostic Tests	31
11.4	Control Materials	32
11.5	Selecting Organism Strains for Analytical Studies.....	34
11.6	Preparing Nucleic Acid Controls	34
11.7	Types of Testing During Assay Development.....	35
11.8	Quality Assurance (QA) for Implementation of Molecular Diagnostic Tests	42
12	Proficiency Testing.....	43
13	Controlling False-Positive Nucleic Acid Target Amplification Reactions.....	43
13.1	Reagents and Solutions.....	44
13.2	Laboratory Practice.....	44
13.3	Selection and Preparation of Controls	44
13.4	Amplification Product Inactivation Methods.....	45
14	Reporting of Results	47
14.1	Organism and Nucleic Acid Target	47
14.2	Equivocal Results	47
14.3	Reference Range	48
14.4	Critical Results.....	48
14.5	Test Limitations	48
14.6	Interpretation.....	48
14.7	Clarifying Statements	48
15	Recommendations for Manufacturers and Clinical Laboratories	49
15.1	Regulatory Requirements	49
15.2	Recommendations to Assay Developers.....	49
15.3	Recommendations for Clinical Laboratories	50
15.4	Selection of Referral Laboratories	50
	References.....	52
	Additional References.....	56
	Appendix. Nucleic Acid Amplification Technologies.....	57
	Summary of Consensus/Delegate Comments and Committee Responses.....	67
	The Quality System Approach.....	72
	Related CLSI/NCCLS Publications	73

Foreword

MM3—*Molecular Diagnostic Methods for Infectious Diseases* was published as an approved guideline in 1995. It was the first of what was to be many Clinical and Laboratory Standards Institute (CLSI) guidelines in the area of molecular diagnostics, and the first consensus guideline published in what was then the new field of molecular microbiology. Molecular microbiology, simply put, is the application of nucleic acid methods to the diagnosis and management of patients with infectious diseases. The field has advanced enormously since the publication of the first approved edition of MM3 and is now an integral part of laboratory medicine.

New developments in the enabling technology, increased understanding of its strengths and limitations, and pervasive clinical applications required a complete rewriting of MM3-A. The second approved edition of MM3 bears little resemblance to its predecessor in organization and content, and relies heavily on other relevant, recently published CLSI documents.

Note that the following trade names are included in this document: ATCC^{®a} and GenBank^{®b}. It is CLSI policy to avoid using trade names unless the products identified are the only ones available, or they serve solely as illustrative examples of the procedure, practice, or material described. In this case, the subcommittee believes the trade names are important descriptive adjuncts to the document.

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

In keeping with CLSI's commitment to align terminology with that of ISO, the following terms are used in MM3: *accuracy* is used in this document when referring to the closeness of the agreement between the result of a measurement and a true value of the measurand, and *trueness* is used when referring to the closeness of the agreement between the average value from a large series of measurements and to a true value of a measurand; the measurement of trueness is usually expressed in terms of *bias*; *repeatability* has replaced the term *within-run precision* where appropriate, when describing the closeness of agreement between results of successive measurements of the same measurand carried out under the same conditions of measurement; *measuring range* has replaced *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. The ISO terms *diagnostic sensitivity* and *diagnostic specificity* have replaced *clinical sensitivity* and *clinical specificity*. In Europe, for the most part, the term *clinical* is applied to the evaluation of medical products used on or in patients, or when referring to clinical studies of drugs, under much more stringent conditions.

^a ATCC is a registered trademark of the American Type Culture Collection.

^b GenBank is a registered trademark of the United States Department of Health and Human Services.

Users of MM3-A2 should understand, however, that the fundamental meanings of the terms are similar and to facilitate understanding, terms are defined along with explanatory notes in the guideline's Definitions section.

Key Words

Development, implementation, methods, molecular microbiology, nucleic acid amplification, quality assurance, reporting, verification

Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline— Second Edition

1 Scope

This guideline describes general principles for the development, evaluation, and application of tests designed for direct detection of microorganisms in clinical specimens and for identification of microorganisms grown in culture. The document provides evidence-based recommendations, where appropriate. The following content areas are addressed: clinical applications; amplified and nonamplified nucleic acid methods; selection and qualification of nucleic acid sequences; establishment and evaluation of test performance characteristics, inhibitors, and interfering substances; controlling false-positive reactions; reporting and interpretation of results; quality assurance; regulatory issues; and recommendations for manufacturers and clinical laboratories.

This guideline is intended for use by clinical laboratories, test developers and manufacturers, and regulatory agencies. It is not intended to be a compilation of successful protocols for detection/characterization of microorganisms, but rather to describe general principles for the development, evaluation, and application of these tests. The readers are directed to the *Manual of Clinical Microbiology*, 8th edition, and *Molecular Microbiology: Diagnostic Principles and Practice* from ASM Press, Washington, DC, for more information on specific applications.

This guideline should be used in conjunction with the most current editions of the following related CLSI/NCCLS documents: MM6—*Quantitative Molecular Methods for Infectious Diseases*; MM9—*Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine*; MM10—*Genotyping for Infectious Diseases: Identification and Characterization*; MM13—*Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods*; and MM14—*Proficiency Testing (External Quality Assessment) for Molecular Methods*.

2 Introduction

Nucleic acid-based methods for detection and identification of microorganisms are commonly used in clinical laboratories. However, their inherent complexity and unparalleled analytical sensitivity require special attention to the assay design, use of controls, and laboratory practice. The diagnostic industry has not kept pace with the medical demand for these tests, and in many cases, in-laboratory-developed nucleic acid tests have become the standard of care. Due to the number of different in-laboratory-developed assays used, molecular diagnostic methods for infectious diseases often lack standardization.

Although molecular diagnostic methods are becoming more pervasive in clinical laboratories, efforts should continue to increase the understanding of the strengths and limitations of these new methods. These methods often may enhance diagnostic capabilities. However, the results should be interpreted within the clinical context in which they are used, and on the basis of individual laboratory performance.

This document presents consensus guidelines for method development, verification, and validation. It is a guide to practical implementation of molecular tests in the clinical laboratory and to the assessment of their clinical utility. It also provides recommendations to assay developers in clinical laboratories and industry. This guideline is also intended to serve as a resource for the relevant regulatory agencies.

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