

MM17-A
Vol. 28 No. 9
Replaces MM17-P
Vol. 27 No. 21

Verification and Validation of Multiplex Nucleic Acid Assays; Approved Guideline

This guideline provides recommendations for analytic verification and validation of multiplex assays, as well as a review of different types of biologic and synthetic reference materials.

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



Verification and Validation of Multiplex Nucleic Acid Assays; Approved Guideline

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Abstract

Multiplex assays detect the presence of and discriminate two or more analytes simultaneously in the same sample. The number of commercially available multiplex assays is increasing rapidly, as is the number of laboratory-developed multiplex assays, and these use a variety of technologies and instrument platforms. Multiplex testing provides significant challenges to the laboratory with regards to appropriate verification and validation testing, and especially the acquisition of appropriate control and reference materials to conduct the testing. The complexity of data analysis and reporting of results is increased relative to single-result assays. CLSI document MM17-A—*Verification and Validation of Multiplex Nucleic Acid Assays; Approved Guideline* addresses analytical verification and validation of qualitative and semiquantitative multiplex nucleic acid assays; however, verification and validation of quantitative assays or expression assays are not addressed in this guideline. Topics covered include sample preparation, a general discussion of multiplex methods and technologies, reference and quality control materials, analytic verification and validation, data analysis, and reporting of results. Clinical validation is briefly reviewed. Specific protocols for verification and validation are not given, due to the variety and breadth of multiplex testing; but detailed recommendations, based on the most current guidance documents, for appropriate analytical verification and validation are provided.

Clinical and Laboratory Standards Institute (CLSI). *Verification and Validation of Multiplex Nucleic Acid Assays; Approved Guideline*. CLSI document MM17-A (ISBN 1-56238-661-1). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2008.

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Foreword

Nucleic acid testing is the fastest growing field in laboratory medicine. The first generation of nucleic acid tests concentrated on measuring the presence or the quantification of a single target, often using a single internal control. More recently, the field of multiplex nucleic acid testing has expanded greatly for both laboratory-developed and commercially marketed tests. Applications of multiplex technology occur in many different clinical areas: carrier screening for genetic diseases (eg, cystic fibrosis mutations, Ashkenazi Jewish mutation panels and thrombosis panels); pharmacogenomics (eg, cytochrome P450 genotyping and single nucleotide polymorphisms that discriminate different rates of warfarin metabolism); infectious disease panels (eg, for respiratory pathogens and gastrointestinal tract infections); pathogen genotyping (eg, hepatitis C virus [HCV]); and genetic risk applications (eg, breast cancer prognosis and therapy). These assays use a variety of platforms and technologies, and measure both DNA and RNA targets. While the chemistry of the different technologies applied to multiplex nucleic assays may be different, sample handling, control strategy, data assessment, and reporting of the results are independent of any set of reagents that might be used. For this guideline, multiplex assays are defined as assays where at least two targets are simultaneously detected through a common process of sample preparation, amplification (target or signal), detection, and interpretation. This guideline is limited to a discussion of multiplex assays for genotyping and pathogen detection, and excludes extensive discussion of gene expression assays.

For a multiplex nucleic acid test to reliably achieve its intended use, there must be control of the process from the acquisition of the sample and preparation of the nucleic acid for testing to the evaluation of the data and the reporting of the results. The competition among reactions in multiplex assays may impose more stringent requirements for sample purity, sample input, reagents, and platforms to avoid nonspecific reactions and background signal. In comparison to single analyte assays, multiplex assays require an increased number of controls, more complex performance evaluation/data analysis algorithms, and more complex reporting of results. The availability of sufficient and appropriate control and reference materials to properly verify and validate multiplex nucleic acid tests is a major challenge. This guideline provides recommendations for various aspects of verification and validation of multiplex testing, and also includes a general overview of technologies currently in use for multiplex testing. The types of control and reference materials that may be available for verification, validation, and daily quality control testing for multiplex assays are extensively discussed. Adequate performance evaluation, as well as interpretation and reporting of multiplex testing results, is still evolving, and additional guidance documents from regulatory agencies and guidelines from standards organizations will surely appear; but this guideline provides the best recommendations available at this time. Overall, this guideline is designed to assist laboratories and manufacturers in developing, verifying, validating, and implementing multiplex nucleic acid tests for diagnostic use.

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 challenges 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.

Key Words

Genotyping, laboratory-developed assay, multiplex, multiplex assay, validation, verification

Verification and Validation of Multiplex Nucleic Acid Assays; Approved Guideline

1 Scope

Multiplex assays generate multiple, simultaneous results on a single sample, such that two or more mutations are simultaneously examined in either a single gene or many genes; examples include cystic fibrosis transmembrane conductance regulator (*CFTR*) mutation analysis, a panel of several loci with common mutations in the Ashkenazi Jewish population, cytochrome P-450 genotyping, deep vein thrombosis panels, and hepatitis C virus (HCV) genotyping. Multiplex testing provides significant challenges to the laboratory with regards to appropriate verification and validation. A variety of technologies and instrument platforms are available for performing multiplex assays. It is important to note that acquisition of appropriate biological control materials can be extremely difficult or impossible. Moreover, the complexity of data analysis and reporting of results is increased relative to uniplex assays.

This guideline provides recommendations for analytic verification and validation of qualitative multiplex assays, as well as a review of different types of biologic and synthetic reference materials (RM). Multiplex assays are defined as those in which two or more targets are simultaneously detected through a common process of sample preparation, target or signal amplification, allele discrimination, and collective interpretation. An overview of currently available technologies, as well as recommendations for evaluating new ones, is provided. An extensive review of the design, acquisition, and appropriate use of different types of control materials, including blood samples, residual patient samples, products of whole genome amplification, synthetic oligonucleotide uniplex and multiplex controls, and plasmids, is provided. Current assay formats are used to illustrate proper verification and validation protocols, and appropriate data analysis and result reporting for multiplex assays are described.

This guideline does not address assays that measure individual targets that are then evaluated simultaneously, and is limited to a discussion of analytic validation and verification of qualitative multiplex assays for genotyping and pathogen detection/identification. Extensive discussion of validation and verification of gene expression assays, which may be a subject of a separate guideline, is excluded; however, many aspects of this guideline may also apply to expression assays, such as specimen handling, sample preparation, nucleic acid isolation, amplification and detection technologies, and instrument platforms. This guideline focuses on analytical performance and only includes a general discussion on clinical performance. A general overview of multiplex technologies is provided to the extent of discussing unique aspects and special considerations for multiplex assays, but does not discuss in detail the basic technologies, which are covered in other Molecular Methods guidelines (ie, this guideline does not specifically address many microarray-based detection platforms, which are the subject of a separate CLSI document MM12).¹ See CLSI documents MM1, MM3, and MM12.¹⁻³

2 Introduction

With the complete sequencing of the human genome and ever increasing numbers of viral and bacterial genomes, as well as the development of the associated fields of genomics and pharmacogenomics, there has been a rapid expansion of genotyping assays available in the clinical laboratories. More importantly, genotyping assays are increasingly run as multiplex assays (ie, the simultaneous detection of two or more targets on the same sample in a single reaction). Multiplex assays are used for detection and identification of infectious agents (pathogen panels), identification of genetic disorders (targeted mutation panels), choosing drug therapies and doses (pharmacogenetic panels), and assessing disease progression and prognosis (genetic association panels). Irrespective of the clinical use, all multiplex assays present the clinical laboratory with significant challenges in verification and validation, acquisition of appropriate control materials, data analysis, and reporting. Laboratories can develop assays in-house (“home-brew,

laboratory-developed”) or use commercially available multiplex assays involving a variety of technologies and instrument platforms. With the increase in the availability and use of multiplex assays, a guideline for the development, verification, validation, control, data analysis, and implementation of multiplex assays is needed to assist laboratories and manufacturers. This guideline serves as a roadmap for laboratories and manufacturers when considering multiplex technologies and assays.

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.⁴ 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 CLSI document M29.⁵

4 Terminology

4.1 Definitions

accuracy – closeness of agreement between a test result and the accepted reference value (ISO 3534-1)⁶; **NOTE:** The term accuracy, when applied to a set of test results, involves a combination of random components and a common systematic error or bias component (ISO 3534-1).⁶

acridinium ester – compound that undergoes a light emitting reaction in the presence of a dilute aqueous solution of sodium hydroxide and hydrogen peroxide; the amount of compound can be quantified by measurement of the intensity of the emitted light or rate of photon emission; **NOTE:** The compound reacts with other substances containing primary and secondary aliphatic amines to yield chemiluminescent derivatives.

algorithm – a set of rules or calculations applied to test data that generate an interpretable or reportable result.

allele – **1)** *in genetics*, any of several forms of a gene that is responsible for hereditary variation; **2)** one of the alternate forms of a polymorphic DNA sequence that is not necessarily contained within a gene; **3)** one of the alternative forms of a gene that may occupy a given locus.

allelic ratio – the ratio of a specified allele to the total number of alleles, normally expressed as a fraction; **NOTE 1:** For example, if a specific allele represents 40% of the total alleles found at a given locus, the allelic ratio is 0.4; **NOTE 2:** Allelic ratio is synonymous with allele frequency.

amplicon – the product of PCR; a fragment of DNA that has been synthesized using amplification techniques; **NOTE:** Amplicons will be double-stranded DNA if created by a PCR reaction, and will be primarily single-stranded RNA if created in a nucleic acid sequence-based amplification (NASBA) or transcription-mediated amplification (TMA) reaction.

analyte – component represented in the name of a measurable quantity (ISO 17511).⁷

analytic accuracy – see the definition of **accuracy** above.

Related CLSI Reference Materials*

- EP5-A2** **Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline—Second Edition (2004).** This document provides guidance for designing an experiment to evaluate the precision performance of quantitative measurement methods; recommendations on comparing the resulting precision estimates with manufacturers' precision performance claims and determining when such comparisons are valid; as well as manufacturers' guidelines for establishing claims.
- 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.
- EP7-A2** **Interference Testing in Clinical Chemistry; Approved Guideline—Second Edition (2005).** This document provides background information, guidance, and experimental procedures for investigating, identifying, and characterizing the effects of interfering substances on clinical chemistry test results.
- EP12-A2** **User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline—Second Edition (2008).** This document provides a consistent approach for protocol design and data analysis when evaluating qualitative diagnostic tests. Guidance is provided for both precision and method-comparison studies.
- 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.
- EP15-A2** **User Verification of Performance for Precision and Trueness; Approved Guideline—Second Edition (2005).** This document describes the demonstration of method precision and trueness for clinical laboratory quantitative methods utilizing a protocol designed to be completed within five working days or less.
- EP17-A** **Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline (2004).** This document provides guidance for determining the lower limit of detection of clinical laboratory methods, for verifying claimed limits, and for the proper use and interpretation of the limits.
- GP10-A** **Assessment of the Clinical Accuracy of Laboratory Tests Using Receiver Operating Characteristic (ROC) Plots; Approved Guideline (1995).** This document provides a protocol for evaluating the accuracy of a test to discriminate between two subclasses of subjects where there is some clinically relevant reason to separate them. In addition to the use of ROC plots, the importance of defining the question, selecting the sample group, and determining the "true" clinical state are emphasized.
- 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.
- MM1-A2** **Molecular Diagnostic Methods for Genetic Diseases; Approved Guideline—Second Edition (2006).** This document provides guidance for the use of molecular biological techniques for clinical detection of heritable mutations associated with genetic disease.
- MM3-A2** **Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—Second Edition (2006).** 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.
- MM5-A** **Nucleic Acid Amplification Assays for Molecular Hematopathology; Approved Guideline (2003).** This guideline addresses the performance and application of assays for gene rearrangement and translocations by both polymerase chain reaction (PCR) and reverse-transcriptase polymerase chain reaction (RT-PCR) techniques and includes information on specimen collection, sample preparation, test reporting, test validation, and quality assurance.

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

Related CLSI Reference Materials (Continued)*

- MM6-A** **Quantitative Molecular Methods for Infectious Diseases; Approved Guideline (2003).** This document provides guidance for the development and use of quantitative molecular methods, such as nucleic acid probes and nucleic acid amplification techniques of the target sequences specific to particular microorganisms. It also presents recommendations for quality assurance, proficiency testing, and interpretation of results.
- MM9-A** **Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline (2004).** This document addresses automated, PCR-based, dideoxy-terminator, and primer extension sequencing done on gel- or capillary-based sequencers. Topics covered include specimen collection and handling; isolation of nucleic acid; amplification and sequencing of nucleic acids; interpretation and reporting results; and quality control/assessment considerations as appropriate.
- MM10-A** **Genotyping for Infectious Diseases; Identification and Characterization; Approved Guideline (2006).** This guideline describes currently used analytical approaches and methodologies applied to identify the clinically important genetic characteristics responsible for disease manifestation, outcome, and response to therapy in the infectious disease setting. It also provides guidance on the criteria to be considered for design, validation, and determination of clinical utility of such testing.
- MM12-A** **Diagnostic Nucleic Acid Microarrays; Approved Guideline (2006).** This guideline provides recommendations for many aspects of the array process including: a method overview; nucleic acid extraction; the preparation, handling, and assessment of genetic material; quality control; analytic validation; and interpretation and reporting of results.
- MM13-A** **Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods; Approved Guideline (2005).** This document provides guidance related to proper and safe biological specimen collection and nucleic acid isolation and purification. These topics include methods of collection, recommended storage and transport conditions, and available nucleic acid purification technologies for each specimen/nucleic acid type.
- MM16-A** **Use of External RNA Controls in Gene Expression Assays; Approved Guideline (2006).** This document provides protocols supporting the use of external RNA controls in microarray and QRT-PCR-based gene expression experiments, including preparation of control transcripts, design of primers and amplicons, quality control, use in final experimental or clinical test application, and analysis and interpretation of data obtained. A CLSI-IFCC joint project.
- POCT4-A2** **Point-of-Care *In Vitro* Diagnostic (IVD) Testing; Approved Guideline—Second Edition (2006).** This document provides guidance to users of *in vitro* diagnostic (IVD) devices outside the clinical laboratory, to ensure reliable results comparable to those obtained within the clinical laboratory.