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# 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.

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



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*Advancing Quality in Health Care Testing*

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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.

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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).<sup>1</sup> See CLSI documents MM1, MM3, and MM12.<sup>1-3</sup>

### 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,