

Quantitative Molecular Methods for Infectious Diseases; Approved Guideline

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.

A guideline for global application developed through the NCCLS consensus process.



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Quantitative Molecular Methods for Infectious Diseases; Approved Guideline

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Abstract

Recognizing the increased use of quantitative molecular methods for determining the concentration of microorganisms in patients, NCCLS document MM6 has been created to provide 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, and presents recommendations for quality assurance, proficiency testing, and interpretation of results.

Issues specific to the *quantitation* of nucleic acid in diagnostic testing and monitoring, particularly in viral diseases include: an update on technologies used in molecular quantitation; specimen handling and preparation; standards, calibrators and reference materials; analytical and clinical verification/validation; reporting and interpreting results; clinical utility; and recommendations for manufacturers and clinical laboratories.

NCCLS. *Quantitative Molecular Methods for Infectious Diseases; Approved Guideline*. NCCLS document MM6-A (ISBN 1-56238-508-9). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2003.

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Suggested Citation

(NCCLS. *Quantitative Molecular Methods for Infectious Diseases; Approved Guideline*. NCCLS document MM6-A [ISBN 1-56238-508-9]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2003.)

Proposed Guideline

December 2001

Approved Guideline

October 2003

ISBN 1-56238-508-9
ISSN 0273-3099

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Foreword

In recent years, a number of sensitive and precise methods for quantitation of nucleic acid have been developed. Quantitation of viral nucleic acids in clinical specimens has proven useful in the diagnosis, prognosis, and management of patients with viral disease. The measurement of HIV-1 viral load is now part of the standard of care of HIV-infected patients. The measurement of viral load in other chronic viral infections for which therapy is suboptimal is likely to become the standard of care in the future.

NCCLS document [MM3](#)– *Molecular Diagnostic Methods for Infectious Diseases* has been seminal in catalyzing discussions pertaining to development, performance, and interpretation of qualitative molecular assays. It is the hope of this subcommittee that this document will do the same for quantitative molecular methods in infectious diseases. Other relevant NCCLS documents include [EP5](#), [EP6](#), [EP7](#), [EP9](#), [EP10](#) and [GP10](#) (see the Related NCCLS Publications and the Related Projects in Development sections in the back of this document.) While these are excellent resources, a guideline is needed to provide information specific for nucleic acid quantitation.

This document is intended for manufacturers or laboratories that develop assays, laboratories that perform assays, clinicians that use the results to diagnose or manage patients, and agencies that regulate their use.

Note that the following trade names are included in the Appendix of this document: Invader[®] Assays, Cleavase[®], eSensor[™], and FRET[™] cassette. It is NCCLS 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 and area committee believe the trade names are important descriptive adjuncts to the document. In such cases, it is acceptable to use the products' trade names, as long as the words, "or the equivalent" are added to the references. It should be understood that information on these products in this guideline also applies to any equivalent products. Please include in your comments any information that relates to this aspect of MM6-A.

While MM6-A thus describes a number of available laboratory approaches that can be used for laboratory implementation and quality assurance of quantitative molecular methods for infectious diseases, MM6-A neither requires nor recommends the use of any one specific approach. Since one or more of these technologies and/or procedures described herein may be patented, those who choose to use a specific technology or procedure should determine if a patent exists, and comply with the requirements of the patent holder. Please include in your comments any information that relates to this aspect of MM6-A.

A Note on Terminology

NCCLS, 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. NCCLS 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 NCCLS, 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, NCCLS 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 NCCLS's commitment to align terminology with that of ISO, the following terms are used in MM6: *Trueness* is used in this document 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;

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; *Measurement procedure* has replaced the term *Analytical method* for a set of operations, used in the performance of particular measurements according to a given method; *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.

At this time, however, in order to avoid confusion, the subcommittee has chosen *not* to replace the U.S. term *Calibrator* with the ISO terms *Primary standard* or *Secondary standard*. The subcommittee has also chosen not to replace *Clinical evaluation*, *Clinical sensitivity*, and *Clinical specificity* with the ISO terms *Diagnostic evaluation*, *Diagnostic sensitivity*, and *Diagnostic specificity* due to user nonfamiliarity and for the sake of practicality of the guideline. 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.

Users of MM6-A 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

Amplification, nucleic acid, probe, quantitation, signal, target

Quantitative Molecular Methods for Infectious Diseases; Approved Guideline

1 Scope

This guideline is to be used in conjunction with NCCLS document [MM3](#)—*Molecular Diagnostic Methods for Infectious Diseases*. Issues specific to the *quantitation* of nucleic acid in diagnostic testing and monitoring, particularly in viral diseases, distinguish this document from MM3. Specific topics in this document include an update on technologies used in molecular quantitation; specimen handling and preparation; standards, calibrators and reference materials; analytical and clinical verification/validation; reporting and interpreting results; and clinical utility. This document does not address *in situ* molecular testing, or genetic microbial resistance testing.

Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be 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 any pathogen and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996; Vol 17;1:53-80), (MMWR 1987;36[suppl 2S]2S-18S), and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management of blood-borne exposure, refer to the most current edition of NCCLS document [M29](#)—*Protection of Laboratory Workers from Occupationally Acquired Infections*.

2 Introduction

Nucleic acid testing for infectious agents poses unique issues; quantitation adds complexity. With the advent of standardized quantitative kits and the increase in quantitative in-house-developed (“home-brew”) testing, a guideline for the development, verification, assessment, and implementation of these assays is warranted. During the development of this guideline, the clinical use of quantitative molecular assays was for viral diseases. Therefore, this document addresses assays used to monitor disease progression, therapy efficacy, and the emergence of active disease in chronic viral infections, but the principles can also be applied to other infectious agents and disease processes.

3 Terminology

3.1 Definitions

Accuracy - Closeness of the agreement between the result of a measurement and a true value of the measurand (VIM93)¹; **NOTES:** “Accepted reference value” may be used in place of “true value”; See **Trueness**, below.

Analytical sensitivity - *In quantitative testing*, the change in response of a measuring system or instrument divided by the corresponding change in the stimulus (modified from VIM93)¹; **NOTES:** a) The sensitivity may depend on the value of the stimulus; b) The sensitivity depends on the imprecision of

the measurements of the sample; c) *In qualitative testing*, **Analytical sensitivity** is defined as the test method's ability to obtain positive results in concordance with positive results obtained by the reference method; d) If the true sensitivity of a device is better than the reference method, its apparent specificity will be less and the level of apparent false-positive results will be greater.

Analytical specificity – *In quantitative testing*, the ability of a measurement procedure to determine only the component it purports to measure or the extent to which the assay responds only to all subsets of a specified analyte and not to other substances present in the sample; **NOTE:** *For qualitative or semiquantitative tests*, **Analytical specificity** is defined as the method's ability to obtain negative results in concordance with negative results obtained by the reference method.

Bias - The difference between the expectation of the test results and an accepted reference value (ISO3534-1)²; **NOTE:** In general, the deviation/difference is based on replicate measurement using an accepted (definitive, reference, or designated comparison) method and the method being tested, and expressed in the units of the measurement or as a percentage.

Calibrator - A material or device of known, or assigned quantitative characteristics (e.g., concentration, activity, intensity, reactivity, responsiveness) used to adjust the output of a measurement procedure or to compare the response obtained with the response of a test specimen and/or sample; **NOTE:** The terms **Primary standard** and **Secondary standard** are used by WHO and ISO to refer to calibration materials. See the terms defined below.

Clinical sensitivity - The proportion of patients with a well-defined clinical disorder whose test values are positive or exceed a defined decision limit (i.e., a positive result and identification of the patients who have a disease). **NOTES:** a) The clinical disorder must be defined by criteria independent of the test under consideration; b) The term **Clinical sensitivity** (U.S.) is equivalent to **Diagnostic sensitivity** (Europe).

Clinical specificity -The proportion of subjects who do not have a specified clinical disorder whose test results are negative or within the defined decision limit; **NOTE:** The term **Clinical specificity** (U.S.) is equivalent to **Diagnostic specificity** (Europe).

Coefficient of variation, CV – For a non-negative characteristic, the ratio of the standard deviation to the average; **NOTE:** It is a measure of relative precision; It is often multiplied by 100 and expressed as a percentage.

Homogeneous/Kinetic “real-time” assay – Simultaneous amplification of the target and detection of the product in which data collection occurs throughout the process.

Imprecision – Dispersion of independent results of measurements obtained under specified conditions; **NOTE:** It is expressed numerically as **Standard deviation** or **Coefficient of variation**.

Laboratory-developed tests - Assays that are researched, built, prepared, and verified within the laboratory institution that employs it for patient testing (“home-brew,” “in-house developed”).

Limit of detection, LOD – *In Molecular Methods and Quantitative Molecular Methods*, the lowest concentration of analyte that can be consistently detected (typically, in $\geq 95\%$ of samples tested under routine clinical laboratory conditions) and in a defined type of sample (see Section 7.5.2); **NOTE:** This concentration must yield an assay value that can be reproducibly distinguished from values obtained with samples that do not contain the analyte.

Limits of quantitation, LOQ - *In Molecular Methods and Quantitative Molecular Methods*, the lowest and highest concentrations of analyte that can be detected with acceptable precision and accuracy, under

routine clinical laboratory conditions, in a defined type of sample (see Section 7.5.3); **NOTES:** a) Acceptable precision and trueness depend on technological limitations and on the clinical use for the assay; b) These concentrations establish the measuring range for the assay.

Measurand – Particular quantity subject to measurement (VIM93)¹; **NOTE:** This term and definition encompass all quantities, while the commonly used term “analyte” refers to a tangible entity subject to measurement. For example, “substance” concentration is a quantity that may be related to a particular analyte.

Measurement procedure - A set of operations, described specifically, used in the performance of particular measurements according to a given method (VIM93)¹; **NOTE:** Formerly, the term **Analytical method** was used in this document.

Measuring range - A set of values of measurands for which the error of a measurement procedure is intended to lie within specified limits (VIM93)²; **NOTES:** a) For this document, the range of values (in units appropriate for the analyte [measurand]) over which the acceptability criteria for the method have been met; that is, where errors due to nonlinearity, imprecision, or other sources are within defined limits; b) Formerly, the term **Reportable range** was used in this document.

Negative predictive value – *In the context of this guideline*, the likelihood that a quantitative assay result, below one or more defined thresholds, does not represent patients for whom a medical decision would be made (when the assay is used for managing patients known to have the disease that is associated with the quantified analyte) or patients who do not have the disease (when the assay is used for diagnosis); **NOTE:** $NPV = \text{True negative results} / (\text{true negative results} + \text{false negative results})$.

Positive predictive value - *In the context of this guideline*, the likelihood that a quantitative assay result, above one or more defined thresholds, represents patients for whom a medical decision would be made (when the assay is used for managing patients known to have the disease that is associated with the quantified analyte) or patients with the disease or a particular state of the disease (when the assay is used for diagnosis); **NOTE:** $PPV = \text{True positive results} / (\text{true positive results} + \text{false positive results})$.

Precision - The closeness of agreement between independent test results obtained under stipulated conditions (ISO 3534-1)²; **NOTES:** a) Precision is expressed quantitatively in terms of imprecision—the SD or the CV of the results in a set of replicate measurements; See **Imprecision**, above; b) Precision does not define **Accuracy**.

Primary standard – A standard that is designated or widely acknowledged as having the highest metrological qualities and whose value is accepted without reference to other standards of the same quantity (VIM93-6.3).¹

Reference material/Reference preparation, RM - A material or substance, one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials (VIM93).¹

Repeatability - Closeness of the agreement between results of successive measurements of the same measurand carried out under the same conditions of measurement (VIM93)¹; **NOTE:** Formerly, the term **Within-run precision** was used in this document.

Reproducibility - Imprecision under reproducibility conditions (ISO3534-1)²; See **Reproducibility conditions, Precision**; **NOTE:** Formerly, the term **Total imprecision** was used in this document.

Reproducibility conditions – Conditions where test results are obtained with the same method on identical test items under different settings, e.g., in different laboratories, with different operators, using different equipment (ISO3534-1).²

Secondary standard – A standard whose value is assigned by comparison with a primary standard of the same quantity (VIM93).¹

Specificity - The ability of a test or procedure to correctly identify or quantify an entity in the presence of interfering phenomena/influence quantities; According to ISO 17511³, the ability of a measurement procedure to measure solely the measurand; **NOTES:** a) Specificity has no numerical value in this context; b) See **Measurand** above.

Standard deviation, SD, σ – A measure of variability/dispersion that is the positive square root of the population variance.

Trueness - The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value (ISO3534-1)²; **NOTE:** Trueness is usually expressed numerically by the statistical measure bias that is inversely related to trueness. See also **Accuracy, Bias**, above.

Validation - Confirmation through the provision of objective evidence, that requirements for a specific intended use or application have been fulfilled; (ISO 9000)⁴; **NOTES:** a) WHO defines validation as the action {or process} of proving that a procedure, process, system, equipment, or method used works as expected and achieves the intended result (WHO-BS/95.1793)⁵; b) The components of validation are quality control, proficiency testing, validation of employee competency, instrument calibration, and correlation with clinical findings.

Verification - Confirmation through the provision of objective evidence that specified requirements have been fulfilled. (ISO 9000)⁴; **NOTE:** A one-time process completed to determine or confirm test performance characteristics before the test system is used for patient testing.

Viral load - The total amount of virus, reflecting replication and clearance, in an infected individual. Quantitation of viral nucleic acid in blood is commonly used as a marker of viral load. This may be appropriate for some viruses (e.g., HIV), but not for others.

3.2 Abbreviations and Acronyms

A	adenosine
bDNA	branched DNA
bp	base pair
3SR	self-sustained sequence replication
C	cytidine
CAP	College of American Pathologists
cDNA	complementary DNA
CFU	colony-forming unit
CLIA '88	Clinical Laboratory Improvement Amendments of 1988
CMS	Center for Medicare & Medicaid Services (formerly HCFA)
CMV	cytomegalovirus
cpm	counts per minute
CV	coefficient of variation
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dpm	disintegrations per minute
dTTP	deoxythymidine 5' triphosphate

dUTP	deoxyuridine 5' triphosphate
EIA	enzyme immunoassay
FDA	U.S. Food and Drug Administration
G	guanosine
HBV	hepatitis B virus
HCFA	Health Care Financing Administration (former name of CMS)
HCV	hepatitis C virus
HIV-1	human immunodeficiency virus type 1
HPLC	high-performance liquid chromatography
HPV	human papilloma virus
HSV	herpes simplex virus
IU	International Units
LCR/LAR	ligase chain reaction/ligase amplification reaction
MDV-1	midvariant-1 reporter probe
mRNA	messenger RNA
NAB	nucleic acid-based
NASBA	nucleic acid sequence-based amplification
PAG	polyacrylamide gel
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PFU	plaque-forming unit
QA	quality assurance
QBR	Q-beta replicase
QC	quality control
RNA	ribonucleic acid
ROC	receiver–operator curve
rRNA	ribosomal RNA
RT–PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SDA	strand displacement amplification
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
T	thymidine
Taq	<i>Thermus aquaticus</i>
TAS	transcription-based amplification
TMA	transcription mediated amplification
tRNA	transfer RNA
U	uridine
UNG	uracil-N-glycosylase
UV	ultraviolet

4 Update on Technologies Used in Molecular Quantitation

A variety of nucleic acid amplification techniques can be used to quantitate the amount of a specific nucleic acid target in a sample. These methods can be divided into three general types: target amplification, probe amplification, and signal amplification. For more detailed information and graphical representation of some of these methods, please refer to the appendix and the most current version of NCCLS document [MM3](#)— *Molecular Diagnostic Methods for Infectious Diseases*.

5 Specimen Handling and Preparation for Quantitation of Nucleic Acid

Appropriate specimen handling, including controlled specimen collection and transport conditions, is critical to ensure specimen integrity and the accuracy of quantitative nucleic acid detection. Inappropriate specimen handling can result in nucleic acid degradation, which can lead to inaccurate quantitation. This may affect prognostic and therapeutic test accuracy.

From a safety standpoint, the handling of specimens for transport and storage should proceed with the same precautions taken for any potential infectious materials, following current blood-borne pathogen and infectious disease guidelines.

5.1 Specimen Collection

Written instructions for specimen collection should be kept in the laboratory procedure manual and provided to those who collect the specimens.

Guidelines for proper collection and labeling of specimens and test requisition forms are available in the most current edition of NCCLS document [H3—Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture](#). It is strongly recommended that trained personnel collect specimens.

5.1.1 Timing of Specimen Collection

Since quantitative molecular testing is often performed to assess patient response to specific therapy, proper timing relative to administration of the therapy is important. For HIV patients, current guidelines recommend that pretherapy specimens should be tested to establish a baseline value, calculated as the average of at least two consecutive specimens tested approximately two weeks apart. Samples should be taken at the appropriate time with consideration for the biological responses of the organism in order to correctly measure the magnitude of response.

Recommendations for other infectious disease pretherapy and therapy monitoring sampling will be generated from therapeutic clinical trials and practice committee guidelines.

5.1.2 Sample Type and Quantity of Specimen

Sample type and the quantity of specimen should be specified. Quantitative molecular tests have been applied to a wide variety of specimen types. Appropriate selection of specimen type depends on a variety of factors, including the site of infection and the agent being assayed.

The format of quantitative molecular tests can greatly impact the volume of specimen required. Tests that are performed on multiple aliquots of specimens may require substantially more specimen material than single-point tests. The multipoint titration (i.e., competitive) approach to quantitation often requires six or more reactions, each including an aliquot of the specimen.

5.1.3 Collection/Transport Devices

Collection/transport devices must not irreversibly bind nucleic acids, nor can they interfere with amplification or detection.

If specimen collection/transport devices contain media or stabilization reagent, a dilution effect may have to be factored in when calculating nucleic acid load.

5.2 Specimen Transport and Storage

The specimen must not be exposed to conditions during transport and storage that result in degradation of the target nucleic acids. There may be limitations on time between specimen collection and processing, and on storage and transport temperature. These limitations may vary between specimen type, analyte (RNA versus DNA), and microorganism, and must be determined by the manufacturer or, in the case of laboratory-developed tests, by the laboratory. RNA is highly susceptible to degradation by ubiquitous enzymes, and can be more difficult to recover than DNA. Quantitative molecular methods that monitor messenger or genomic RNA (in the case of RNA viruses) must take this instability into consideration.⁶

From a safety standpoint, the handling of specimens for transport and storage should proceed with the same precautions taken for any potentially infectious materials, following current biosafety guidelines for biomedical laboratories, as well as appropriate regulatory guidelines.^{7,8}

The laboratory must provide instructions for proper specimen handling and transport conditions to those who collect and transport specimens. Special handling requirements should also be provided to courier services. The laboratory must establish criteria for the rejection of specimens that have been handled improperly. Specimens in poor condition, or with evidence of improper handling should not be tested. Rather, the laboratory should notify the requester that the specimen was rejected, and the reason for not performing the test. When using a courier service, it is particularly important for the laboratory to note for every specimen: 1) date of collection; 2) date shipped; 3) date received by the laboratory; and 4) approximate temperature of specimen when received. Monitoring this specimen information will help to assure (but cannot absolutely guarantee) that the specimen was handled properly. Laboratories should maintain a log in which specimen transport problems and specimen rejections are noted for quality assurance purposes.

Because of the relative cost and complexity of quantitative molecular tests, laboratories often batch specimens prior to testing. The storage of batched specimens must follow test requirements.

5.3 Specimen Processing

One of the most significant potential sources of inaccuracy and variability in quantitative molecular tests is the specimen processing/nucleic acid extraction step. The methods used for preparing the nucleic acids must be fully optimized and quality controlled. This is particularly important for RNA, which is less stable than DNA. Methods that are optimal for RNA preparation may not work as well for DNA preparation.

5.3.1 Nucleic Acid Extraction

Methods of nucleic acid extraction and important considerations have been discussed in the most current edition of NCCLS document [MM3](#)—*Molecular Diagnostic Methods for Infectious Diseases*. The method should be as simple as possible, to avoid specimen contamination or loss of nucleic acids.

The stability of extracted nucleic acids may vary depending on the method of extraction. After extraction, nucleic acids not immediately assayed must be stored by strictly following the manufacturer's guidelines. In the case of laboratory-developed tests, storage conditions of processed specimens must be determined for the assay by the laboratory.

For nucleic acid extraction procedures that involve precipitation of nucleic acids, a carrier such as glycogen or irrelevant sequence of nucleic acid may be added to increase total nucleic acid yield and consistency of yield. Loss of nucleic acids during extraction may be controlled by adding an internal calibrator to each specimen prior to extraction.

It is important to verify the consistency of a nucleic acid extraction procedure for quantitative tests. This can be accomplished by testing control specimens with known copy number of nucleic acid, or adding a known amount of internal calibrator to negative specimens. Ideally, the internal calibrator should be in a form equivalent to that of the target nucleic acid in specimens (e.g., free nucleic acid versus intact organism).

Steps should be taken to assure that residual nucleic acid is minimized if, during the verification phase of development, it has been shown to interfere with the assay or compromise the integrity of the results generated. The extraction procedure (manual or automated) should also be evaluated to ensure that specimen-to-specimen carry-over of nucleic acid does not occur.

5.3.2 Inhibitors/Interfering Substances

Specimen inhibitors to varying degrees may adversely affect the different quantitative molecular methods. Additionally, inhibition can vary among specimen types.

An ideal nucleic acid extraction procedure would inactivate or remove all substances that interfere with detection of the target sequence. However, given the abundance and variety of potential inhibitors, the complexity of specimen matrices, and the need to balance the removal of inhibitors versus loss of nucleic acid, this is not practical. Incorporation of an internal calibrator in specimens allows for the control of the effect of inhibitors, so those assays are able to tolerate the presence of some inhibition. [See Section 6.3.2](#) for further discussion on the use of internal calibrators.

6 Standards, Calibrators, and Reference Materials

For many analytes or pathogens, a reliable standard is not available. To *quantitate* an analyte accurately, one requires an assay capable of generating a reproducible relationship between the amount of “input” target and the “output” signal. An analytical **quantitative standard** is a measured amount of “target” or “modified target” which can be used to ensure that the constituents of the analytical process are functioning as expected.

6.1 Quantitated Analytical Standards

6.1.1 Natural Analytical Standards

Nonsynthetic standards consist of known quantities of the analyte as it occurs naturally in the test matrix, purified from the test matrix, or derived by culture. Examples of such materials are intact virus particles, bacteria naturally containing the target in their genome or plasmids, or intracellular RNA or DNA produced during infection. Such materials may be physically quantified by microscopy (light or electron), titered by culture, or (in the case of cellular RNA and DNA) characterized and quantified by other physical or biochemical means. Steps should be taken to assure that residual nucleic acid is minimized if, during the verification phase of development, it has been shown to interfere with the assay or compromise the integrity of the results generated.

6.1.2 Synthetic Standards: DNA and RNA

Synthetic standards do not consist of the analyte as it occurs naturally in the test matrix (i.e., intact virus particles, bacteria, or intracellular RNA or DNA produced during infection). These can be in the form of DNA (either single- or double-stranded) or RNA, which can be readily and accurately quantified by several physical and biochemical methods. Because of its defined nature and composition, such material can function as a representation of the unit of measure required for an assay to generate a standard curve external to a test sample or as an internal calibrator. Synthetic analytical DNA standards can include synthesized DNA in the form of oligonucleotides; single-stranded DNA produced by cloning into

recombinant phage; double-stranded DNA produced by cloning into vectors such as plasmids or unrelated organisms; and DNA fragments of a specified size, composition, and nucleic acid sequence produced by cleavage of larger DNA molecules followed by purification to uniformity. Synthetic RNA standards may be produced through *in vitro* transcription or by using recombinant phage containing cloned sequences of interest.

6.2 Reference Materials

In response to the difficulty in standardizing clinical molecular methods and the necessity to normalize quantitated results, the World Health Organization (WHO) is producing “standards” that are quantified during worldwide testing with relevant methods of the day. There are currently three registered WHO virologic “standards” for molecular testing, and work is active on a fourth. These standards were primarily developed for blood screening but have provided useful comparative information for some diagnostics. They are also being used for calibration and comparison of standards from individual countries.

The first such WHO standard for HCV (96/790) is available as vials of lyophilized material containing 50,000 IU (international units) for each 0.5 mL fill (or 100,000 IU/mL). The mean concentration of this genotype 1 HCV material was determined to be 5.14 log₁₀ genome equivalents/mL by testing it in several laboratories with different methods.^{9,10} Similarly a standard has been established for HIV (97/656). It is a lyophilized preparation of genotype B HIV-1 in a plasmapheresis donation that had been diluted in defibrinated plasma. After testing determined a mean of 4.79 log₁₀ genome equivalents/mL it was given a value of 100,000 IU/mL.¹¹ The HBV standard (97/746) has been given a value of 1,000,000 IU/mL. This lyophilized material is a 1:500 dilution of the Eurohep standard (reference 1, HBV subtype adw). The overall mean titer of this standard was 6.42 log₁₀ genome equivalents/mL.¹²

Other “standards” in consideration are: parvovirus B19 and hepatitis A virus. Once established, further studies are conducted to calibrate other genotypes, subtypes, and existing standards and reference materials to them. Users of the materials should consider the limitations stated above. Further information can be found by contacting UK NIBSC (National Institute of Biological standards and Control) via website (www.nibsc.ac.uk).

6.3 Calibrators

A calibrator is an essential component of a quantitative molecular assay. The calibrator may be in the amplification reaction along with the target sequence (i.e., an internal calibrator), or several concentrations of it may be run in parallel reactions (as external calibrators), to generate an external calibration curve. In either case, the signal result from the specimen is compared to that of one or more calibrators. The amount of target in the original specimen is then calculated based upon that comparison. The different manner in which each technique performs this calculation is discussed in the appendix.

For accurate quantitation, efficiencies of amplifying calibrator and target molecules must be similar. To maximize the likelihood that this will occur, the calibrator should be the same size and have a similar nucleotide composition as the target. When quantifying RNA by using RT-PCR (reverse transcriptase polymerase chain reaction), efficiencies of both the reverse transcription and amplification steps can affect accuracy. When the calibrator is amplified independently of the target molecule, as with an external calibrator, inhibitors or other factors in the specimen that may alter amplification efficiency are not taken into account.

Calibrators are often inappropriately designated “quantitative standards.” The term “quantitative standard,” as used by some assay developers, does not describe material that is a true standard but rather a working standard or calibrator. (See Section 3.1, Definitions.) For the purposes of this document, the term

“calibrator” will be used, unless the material is a quantitated analytical standard as defined in [Section 6.1](#). Similarly, the term “calibration curve” will be used instead of “standard curve.”

6.3.1 External Calibrators

Each external calibrator contains a measured amount of target or modified target that is tested in the same assay run as the specimen, but is not present in the same well or tube as the specimen. Different levels of the target may be run to create an external calibration curve. Signal results of the specimen are then compared to those of the calibrators and the amount of target determined. The benefit of the external curve is that external calibrators do not compete with the target for assay substrates. External calibrators also allow the use of natural analytical calibrators that exactly match the target of interest. Synthetic materials can also be used to develop external calibrators that represent the sequence of the target actually being measured. The disadvantage of external calibration is that factors such as reaction efficiency, inhibition, or other conditions can affect the determined amount of target in the specimen but might not affect the calibrators, thereby causing an inaccurate result.

6.3.2 Internal Calibrators

An internal calibrator contains a measured amount of nucleic acid that is modified from target in specimens, thereby enabling the detection process to differentiate between products generated from calibrator and specimen during the amplification process. An internal calibrator may be a constructed nucleic acid that utilizes the same primer binding sites as the target molecule in specimens, but with a deletion or insertion that is internal to the primer binding sites. This allows the calibrator to be differentiated from the target on the basis of size or sequence. Alternatively, a portion of calibrator sequence, internal to the primer binding sites, may be rearranged to allow separate detection of the target and calibrator by using specific probes.

An advantage of using an internal calibrator is that it allows for, but generally does not distinguish between, detection of inhibitors and recognition of nucleic loss during extraction. When added prior to specimen extraction, internal calibrators will undergo the same assay process as the specimen itself. In this way, factors such as inhibition and process efficiencies can be controlled for; however, when the process fails, such calibrators cannot determine whether the failure occurred during extraction or amplification. On the other hand, it is possible that the calibrators may out-compete any target in the specimen for substrates, resulting in a lower quantified result or no result at all. To minimize the effect of a calibrator on the amplification of the target, the length of the calibrator should be the same as or slightly longer than the target. In addition, proportions of nucleotide bases in amplicons should be the same for calibrator and target. Because there must be some sequence variation to enable specific detection of internal calibrators, natural analytical materials do not make good internal calibrators.

When amplifying nucleic acids from cells or tissues, host genes (e.g., β -globin or actin) may be used as the internal control. However, the number of copies of these genes may be much more abundant in the sample than the target gene, thus impacting amplification efficiency and ultimately the accuracy of quantitation.

7 Analytical Verification

For the purposes of this document, the term “verification” will be used to denote those activities that are performed prior to the routine implementation of a procedure in the clinical laboratory. Consistent with the effort to align terminology with that of ISO, the term verification will be used to denote those activities that provide confirmation that specified requirements have been met (i.e., analytically). The term validation will denote those activities providing confirmation that requirements for specific intended use have been met (i.e., clinically).

7.1 General Considerations

Before a new or improved quantitative molecular method is implemented, its performance characteristics under laboratory conditions must be assessed. This evaluation must be performed regardless of whether the method is manual or automated; and whether reagents have been prepared in the laboratory or by a manufacturer. As with any other laboratory test, the selection and evaluation of a new quantitative molecular method requires critical judgment of the performance as well as practicality characteristics.

The work performed prior to the routine implementation of a clinical test can be divided into analytical verification and clinical validation. Analytical verification provides information about the performance characteristics of the test. On the other hand, clinical validation provides information about the clinical utility with respect to the intended use of the particular assay. Clinical validation will be discussed in detail in subsequent sections. This section provides guidance for developing and executing analytical verification of quantitative molecular methods.

An important purpose of quantitative molecular assays is to monitor changes in the level of a specific analyte for diagnosis, during disease progression or during treatment. As part of analytical verification, laboratories must determine the assay's analytical sensitivity, specificity, accuracy, precision, and measuring range of the overall process to determine when changes in the quantity of the analyte are due to inherent test error before clinical relevance is considered. Personnel involved in the evaluation and verification of a quantitative molecular assay should become familiar with the final assay procedure, experimental protocol, and any devices used in the assay before starting the evaluation. Routine quality control procedures should be carried out during the entire verification process.

7.2 Precision

7.2.1 General Considerations

Every measurement procedure has an inherent random variation. This inherent random variation is termed “random error.” Random error is present in all observations resulting from dispersion of the observations or measurement around their mean. Terms that are commonly used to refer to random error are precision, imprecision, reproducibility, and repeatability. Precision will be used throughout this document to refer to random error. Precision of an assay refers to the reproducibility of the overall process. Precision refers to the agreement between replicate measurements of the same material. Awareness of the precision of the assay allows for the determination of what represents a change in the status of a patient/individual versus expected fluctuations in laboratory determinations.

Nucleic acid amplification methods are typically much more sensitive than methods used routinely in clinical laboratories to measure other analytes. As a consequence, the measurements made with these assays may be less precise. The unparalleled sensitivity of these methods is best appreciated when the analyte concentration is expressed in molarity. For example, HIV-1 viral load assays capable of reliably measuring samples containing 400 copies/mL (approximately 10^{-19} M) may have coefficients of variation ranging from 20 to 40%.

7.2.2 Factors to Be Considered

The objective of the precision experiment should be to determine the total overall process imprecision, from nucleic acid extraction to detection and quantitation. There are different components of the overall process that might contribute differently to the reproducibility of the process. It is important to design the experiment in a way that all the contributory factors to imprecision are measured. Repeatability is a measurement of the variability when the same specimen is analyzed during one analytical run. Reproducibility is determined under stipulated conditions: Between-run precision is a measurement of the variability when the same specimen is analyzed during more than one run on the same day. Between-day

precision is a measurement of the variability when the same material or patient specimen is analyzed over a period of more than one day. Precision testing should also include more than one lot or batch of materials (between-batch precision).

7.2.2.1 Test Material/Matrix

Test material and its matrix should be chosen carefully to mimic patient specimens as closely as possible. In addition, enough test material should be available to last for the entire precision experiment. If a characterized patient specimen is not available, the test material can be created by spiking highly purified analyte into a pool of patient specimens known to be negative for the specific analyte.

7.2.2.2 Concentrations

It is recommended that at least two concentrations of the test material be used for the entire precision experiment. Concentrations that span a significant portion of the measuring range, as well as those close to the medical decision level, should be selected whenever possible.

7.2.2.3 Quality Control Materials

The laboratory should have enough quality control materials so that a single lot or batch of these materials can be used for monitoring assay performance throughout the precision experiment.

7.2.2.4 Number of Runs, Days, and Operators

It is necessary to obtain sufficient data so calculation of overall imprecision of the quantitative molecular method reflects the true imprecision of the overall process. Examples and forms for a one per day run procedure are described in Appendix C of the current edition of NCCLS document [EP5—Evaluation of Precision Performance of Clinical Chemistry Devices](#).

7.2.2.5 Experimental Design

An experimental design should be developed prior to testing. It should include all routinely used controls and duplicate testing of each specimen. If controls are out of range or other operating difficulties occur, the run must be rejected and an additional run should be performed. It is recommended that patient samples are included in the precision experiment to mimic actual-size runs.

7.2.2.6 Data Collection and Recording

Examples of data collection and recording sheets can be found in the current edition of NCCLS document [EP5—Evaluation of Precision Performance of Clinical Chemistry Devices](#).

7.2.2.7 Data Evaluation

Determining whether a change in a test value is significant depends on the precision of the overall system and the shape of the distribution of values about the mean. If the values follow a normal distribution, parametric tests are used for data analysis. If values do not follow a normal distribution, nonparametric tests should be applied or the data should be transformed to comply with parametric criteria. Formulas for the statistical computation of within-run imprecision and for comparison with manufacturer's claims or other performance criteria can be found in the current edition of NCCLS document [EP5—Evaluation of Precision Performance of Clinical Chemistry Devices](#).

7.3 Linearity and Measuring Range

7.3.1 General Considerations

Linearity is the measure of the degree to which a curve approximates a straight line; the linear range is the span of analyte concentrations for which the final output value of the system is directly proportional to the analyte concentration, with acceptable trueness and precision. Measuring range is similar, but without the requirement for direct proportionality; the boundaries of the measuring range are the lower and upper limits of quantitation. While reportable and linear ranges may coincide, one or both ends of the measuring range could extend beyond one end or the other of a linear range (i.e., trueness and precision are acceptable, but the relationship between analyte concentrations and assay output values is nonlinear).

Determination of linearity and measuring range needs to be performed for the overall system, from nucleic acid extraction to detection and quantitation. Understanding the relationships between the analyte and the overall system allows for the determination of usable ranges. Evaluation of linearity performance of a quantitative measurement procedure allows for determination of the linear range and for the limits of quantitation, which establish the measuring range.

7.3.2 Experimental Approach

Linear and measuring ranges are determined by testing different concentrations of analyte in a linearity experiment. Plotting system output values against analyte concentrations will result in a curve. The degree to which the plotted curve conforms to a straight line is an indication of the system's linearity. To determine the overall system linearity and measuring range with accuracy, it is recommended that at least four concentrations of analyte be measured in duplicate. The testing should be replicated at least twice. The matrix used for preparation of the test material must be similar to the patient specimens that will be analyzed.

7.3.2.1 Concentration

Concentrations of analyte in the test material should encompass the estimated minimum and maximum that could be accurately measured. If the results of the experiment fail to show any linear relationship between test material concentration and overall output, a new test material should be assessed. The concentration of analyte in this new test material should be chosen more appropriately.

7.3.2.2 Preparation of Test Material

Test material should be prepared by combining different amounts of sample material that contain the appropriate analyte and sample material previously shown to lack the analyte. For detailed sample preparation and value assignment methods please refer to the current edition of NCCLS document [EP6—Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach](#).

7.3.2.3 Data Collection and Recording

It is convenient to collect and record data on worksheets specifically designed for testing replicates at different analyte concentrations. Examples of such worksheets can be found in the current edition of NCCLS document [EP6—Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach](#).

7.3.2.4 Data Evaluation

7.3.2.4.1 Preliminary Data Examination

When the linearity experiments are complete, data should be arranged in ascending or descending order according to analyte concentration. A preliminary observation of the data should be carried out to determine the presence of obvious errors. If obvious errors exist, the appropriate experiments should be repeated.

7.3.2.4.2 Plotting Data and Visual Evaluation

A simple plot of the data could aid in identification of outliers. After preliminary examination for obvious errors and outliers, a plot of the logarithmically transformed data should be performed. (Parametric statistics can only be used with normally distributed data, which is achieved through logarithmic transformation.) The x-axis plots the input analyte concentrations, while the y-axis plots the output or calculated concentrations of analyte in the test material.

7.3.2.4.3 Statistical Analysis

A recommended approach for the evaluation of linearity is detailed in the current edition of NCCLS document [EP6](#)— *Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*.

7.4 Trueness

7.4.1 General Considerations

Trueness refers to the ability of a method to determine the true value of a particular analyte. Generally, there is a method with established accuracy that serves as the “gold standard” for determining the real value. Some molecular methods have proven to be more sensitive than current “gold standard” methods. This poses a problem, because one can only determine trueness within the measuring range of the “gold standard” method.

In the absence of suitable “gold standard” methods, some laboratories have developed or purchased reference materials for analytical verification and for monitoring daily performance. Approaches to creating a quantitated analytical reference material include using one or more independently established methods to determine the analyte concentration; however, each method may yield a different value.

The term “bias” may be used to describe the lack of agreement. This disagreement can also be termed “systematic error,” since the difference in the true value versus the determined test value will be skewed to one direction.

7.5 Limit of Detection, Limit of Quantitation

7.5.1 General Considerations

Analytical sensitivity of a quantitative molecular method refers to the analyte change in response of a measurement system by a corresponding change of the analyte. In practice, however, the limit of detection or limit of quantitation is determined more frequently.

7.5.2 Limit of Detection

Analytical sensitivity should be determined by testing serial dilutions of an appropriate number of samples containing known concentrations of analyte representing well-characterized strains or genotypes of the pertinent microorganism(s). The highest dilution that is consistently detected will yield the limit of detection (LOD). The LOD is the lowest concentration of analyte that can be consistently detected (e.g., in $\geq 95\%$ of samples tested under routine laboratory conditions) and in a defined type of sample. This should be determined for each type of sample that will be tested in the clinical laboratory. Whenever possible, results should be compared with those from another established method.¹³

7.5.3 Limits of Quantitation

Quantitative molecular assays have a lower and an upper limit for precise and accurate quantitation of analyte in patient specimens. The lower and upper limits of quantitation are, respectively, the lowest and highest concentrations of analyte that are determined with acceptable precision and trueness; “acceptable” is defined by clinical application(s) of the assay. These upper and lower limits establish the measuring range of the quantitative molecular assay. Determination of the measuring range is described in [Section 7.3](#).

7.5.4 Genetic Variability

Inaccurate or false-negative results may arise if reagent nucleic acids are not sufficiently homologous to all subtypes that should be detected. Genetic variation can occur, especially in viruses, such that the primers do not adequately hybridize to the target sequence, leading to inaccurate quantitation. Therefore, primers can be synthesized with degenerate (e.g., inosine) bases. Alternatively, several primer sets may be simultaneously used in the reaction to optimize the detection of variants or subtypes.

7.6 Analytical Specificity

7.6.1 General Considerations

Analytical specificity is the ability of a method to detect and quantify only the analyte it purports to measure. Analytical specificity should be determined for the complete method, from nucleic acid extraction to detection and quantitation.

7.6.2 Cross-Reactivity

Lack of cross-reactivity with closely related substances is one aspect of specificity. To determine if quantitative molecular method is cross-reactive, a panel of similar nucleic acid sequences and/or closely related organisms should be assessed. A panel of organisms considered to be normal flora or often present in a patient specimen should be also assessed.

7.7 Interfering Substances

7.7.1 General Considerations

An interfering substance is a component of a patient specimen that alters the accurate measurement of the analyte. It is important to stress that interfering substances could contribute differently to the total analytical error for each patient sample. The source of an interfering substance can be exogenous or endogenous. Exogenous interfering substances could be due to administration of drugs, parenteral nutrition, anticoagulants, etc. In addition, certain specimen collection anticoagulants (e.g., heparin, EDTA) can also interfere with the analytical process. Endogenous interfering substances could be the result of disease (e.g., bilirubin, hemoglobin, or lipids) or more typically, inherent to the specimen itself.

For example, nontargeted nucleic acid may interfere with primer annealing, and large amounts of nucleic acid may nonspecifically interfere with reagent enzymes. Concentrations of nontarget RNA and DNA that may be tolerated by the assay without significantly effecting results should be determined. In the case of an RNA-targeted assay, homologous DNA sequences should be evaluated to determine if they interfere with the measurements of RNA.

7.7.2 Internal Control/Calibrator

The ability of real-time PCR assays to measure PCR product amplification efficiency cycle by cycle may diminish the need for use of an internal calibrator or internal control. Inclusion of an internal calibrator or control may help in identifying, but usually cannot distinguish between, suboptimal nucleic-acid extraction and the presence of interfering substance(s) as causes of a potentially inaccurate result.

7.7.3 Selecting Specimens to Identify Interfering Substances

Several approaches may be used, perhaps in combination, to identify the presence of interfering substances in specimens and to verify if they are efficiently removed or inactivated by proposed specimen processing. Adding the target analyte to a variety of specimens that contain presumptive interfering substances can be a means of identifying inhibitors. Such specimens may include serum; plasma or whole blood collected with different anticoagulants; cerebrospinal fluid contaminated with whole blood due to a traumatic lumbar puncture; and respiratory secretions containing acid polysaccharides and/or whole blood. A second approach, which may provide specific information regarding potential interference, is the addition of the purified target analyte to specimens collected from persons with a particular disease (e.g., patients with cardiac, pulmonary, liver, or kidney disease) or disorder such as hyperlipidemia; and patients undergoing treatment with certain drugs. The latter may contain relevant metabolites. A drawback of these first two methods is that more than one variable imparting interference may be present within a single specimen, and their simultaneous presence may be unrelated or unanticipated, thus confounding data analysis.

A third approach, to identify potential interfering substances, is to add the purified presumptive substance to different types of human specimens known to contain the specific analyte. In whole blood, serum, or plasma, these can include specific anticoagulants such as heparin and EDTA, hemoglobin or heme (known to inhibit certain target amplification methods at 0.8 $\mu\text{mol/L}$), bilirubin, and lipids such as triglycerides. Examples of materials, which could be added to cerebrospinal fluid to simulate traumatic taps, include whole blood, hemoglobin, or heme. Finally, when inhibition does occur, the interfering substance may not necessarily be intrinsic to the specimen, but may be due to residual substances used in specimen preparation, e.g., EDTA, detergents such as sodium dodecyl sulfate (SDS), and chaotropes like guanidinium HCl.

8 Clinical Validation

8.1 Introduction

This section provides guidance for validating the performance of a quantitative molecular method in a relevant clinical setting. Laboratory methods are used to provide information for managing patients, as well as for addressing a relevant clinical question.¹⁴ (See the most recent version of NCCLS document [C24— Statistical Quality Control for Quantitative Measurements: Principles and Definitions](#) for more information.) The usefulness of a method depends on both its analytical and clinical characteristics (i.e., sensitivity, specificity, and predictive values).

These factors are not properties of the test but are in fact properties of the clinical application. The clinical significance and utility, and therefore, the characteristics of interest, of a quantitative molecular method should be described in terms of the disease or syndrome that is the subject of the test. These

include disease prevalence; possible outcomes (including treatment options) and relative value of these outcomes; and costs of incorrect information (false-negative and false-positive results) to patients and others.

8.2 Clinical Question

The first issue in the clinical validation of a quantitative molecular method is defining the relevant clinical question. Following the current edition of NCCLS document [GP10—Assessment of the Clinical Accuracy of Laboratory Tests Using Receiver Operating Characteristics \(ROC\) Plots](#), there are three parts to defining the clinical question:

1. Characterize the subject population.
2. State the management decision to be made.
3. Identify the role of the method in making the decision.

The population and sample types should be fully described. The results for a method in a given population using a specified sample type may not be suitable for another population or even for another sample type within the same population.¹⁵ NCCLS documents [GP10—Assessment of the Clinical Accuracy of Laboratory Tests Using Receiver Operating Characteristics \(ROC\) Plots](#) and [C28—How to Define and Determine Reference Intervals in the Clinical Laboratory](#) provide considerations for the selection of a relevant population.

The management decision involves separating the clinical population into two or more subgroups based on proposed indications for use. Examples of indications for use could be diagnosing a disease or disease state associated with certain symptoms; confirming the results of another test or diagnostic method; managing patients on therapy; monitoring disease progression or resolution; or assessing prognosis.¹⁶

Quantitative molecular methods lend themselves well to some of the above, and less well to others. The laboratory should define the proposed indications for use prior to starting the clinical validation.^{15,16}

The role of the method in the management decision should be stated in a clear, unambiguous manner. This statement may include the result level, duration, or change that is considered clinically significant for the decision.

8.3 Clinical Performance

Relevant characteristics of a method are defined by its indications for use.¹⁵ While information on all characteristics of the method is desirable, the user must identify those critically associated with the clinical question (and indications for use) to ensure that they are adequately addressed by the validation. Clinical performance of a quantitative molecular method is determined by several measures including clinical (diagnostic) sensitivity, clinical (diagnostic) specificity, precision/reproducibility, measuring range, and limits of quantitation. The user must establish validation parameters for those characteristics before beginning the clinical validation. Refer to [Section 11](#) for information regarding clinical utility.

8.4 Correlation to Disease or Condition

Once test characteristics have been established, the user should ask if the test quantitates the target organism in a clinically relevant way for appropriate patients. That is, how well has the clinical question been addressed? Two measures of this are negative predictive value and positive predictive value. (See [Section 3.1, Definitions](#).) Negative predictive value is the likelihood that a patient with a test result that meets a specific criterion does not have the disease or does not fall into a particular treatment group. Positive predictive value is the likelihood that a patient with a test result that meets a specific criterion has the disease or condition or falls into a particular treatment group.

8.5 Comparison to Current Methods/Standards

Clinical validation must be performed in relation to an existing method, a quantitative standard (gold or otherwise), and/or clinical outcome. Clinical (diagnostic) evaluation of a molecular method can lead to one of three conclusions: (i) the method can be considered acceptable for routine use; (ii) the information is inconclusive or equivocal and further studies are needed; or (iii) the test performance does not meet the needs of the laboratory or is insufficient or inadequate compared to the reference method.¹⁵

NCCLS document [EP9](#)—*Method Comparison and Bias Estimation Using Patient Samples* provides guidance for evaluating the bias between two methods that measure the same analyte. NCCLS document [MM3](#)—*Molecular Diagnostic Methods for Infectious Diseases* provides a discussion of the comparison of a molecular method with a “gold standard,” or with an imperfect method. It also discusses verification in the absence of a reference method. With the advent of molecular methods, certain “gold standards” based upon growth of an agent, or upon antigen or antibody detection have been shown to be imperfect reference methods. This is especially true when quantitative molecular methods are being introduced to replace methods that may be qualitative or semiquantitative. The bias introduced by the use of imperfect reference methods has been discussed by Valenstein¹⁷ and guidance for minimizing its effects is presented in the most current edition of NCCLS document [MM3](#).

A second point to consider is that molecular methods may measure a biologically different target from the comparison method. This implies that false-positive or false-negative results, relative to the other assay, are not necessarily incorrect results. Both results could be correct with regard to their respective targets. Such cases can occur especially at the beginning of an infection, during treatment, or after treatment.

The unparalleled sensitivity of nucleic acid amplification makes direct comparisons with antigen detection or culture-based methods difficult. With HIV-1, the amount of cultivable virus in plasma may be 100 to 10,000 times less than the amount of detectable RNA. This relationship should be established for each analyte using a limited number of well-characterized specimens. In addition, quantitative molecular assays have provided new opportunities for clinical laboratories to provide meaningful information that was impractical or impossible to provide routinely using conventional culture-based assays (e.g., viral load assays for CMV, HCV, and HIV-1). Verification of many molecular assays is further complicated by the lack of commonly accepted quantitated analytical standards (see [Section 6](#)). At a minimum, method linearity, accuracy, precision, sensitivity, specificity, and subtype variation effects should be determined before the assay is used for diagnostic purposes.

Comparisons of two quantitative molecular methods should include an assessment of agreement. This is particularly important when the true values are unknown. The use of correlation coefficient alone may be misleading, because high correlation does not mean that the two methods agree. Simple methods for assessing and measuring agreement are provided by Bland and Altman.¹⁸

8.6 Outcome Measurement

Outcome measurement addresses the relative benefit to the patient of information provided by the method relative to the associated cost of performing the method. Though important in the establishment of new technologies for patient care, the discussion of outcome measurement is not within the scope of this document.

9 Reporting and Interpretation of Results

9.1 Units

Results of quantitative molecular assays can be expressed as number of molecules (copies, genome equivalents), mass (pg, ng), or international units (IU)^a of the targeted nucleic acid in a defined volume of a body fluid, number of cells, or mass of tissue. Copy number can be converted to mass by the following calculations: for a double-stranded genome multiply its length in base pairs by the approximate molecular weight of a base pair (666 g/mole) and divide that product into Avogadro's number (6.023×10^{23} copies/mole). The resulting quotient is the number of copies/g. For a single-stranded genome, multiply by the molecular weight of a base (333 g/mole). If the genome contains multiple copies of the targeted nucleic acid sequence, then the copy number may not correlate to the number of virions present in the sample. For example, infectious HIV-1 virions contain two identical copies of single-stranded RNA; therefore, the copy number must be divided by two to estimate the number of virions present in the sample.

9.2 Significant Figures

The number of significant figures used to express a result depends on the precision of the method, including how precisely the measuring instrument is calibrated. In addition, the number of significant figures resulting from multiplication or division of measured quantities is the same as the number of significant figures of the least precisely known quantity. Typical measurements are imprecise beyond three significant figures.

9.3 Transformation

Results are expressed as integers (e.g., 1000 copies/mL), in scientific notation (1.0×10^3 copies/mL), or as a logarithm₁₀ (3.0). Log₁₀ transformed data should be used for reporting quantitative molecular results, because log₁₀ transformed results better reflect biologically relevant changes in load of microorganisms that usually replicate exponentially, and because most assays exponentially amplify the target analyte. Because some clinicians are not familiar with log₁₀ transformed data, an interim approach would be to report results as both log₁₀ transformed and integer values.

9.4 Sequential Tests

When quantitative molecular assays are used to monitor response to therapy, sequential testing is performed. Updated, cumulative reports highlighting the fold-change from the last time point would be the most informative. However, problems in establishing unique patient identifiers and the limitations of laboratory information systems make cumulative reporting difficult for many laboratories. When following sequential samples on a patient, testing should be performed by the same method (e.g., PCR or bDNA), because results from different assays are not always comparable.

9.4.1 Tolerance Limit – Ensuring Biological Relevance

Viral load assays may be useful in prognosis, in decisions to treat, and in monitoring response to therapy for a number of infectious diseases (e.g., HIV-1, HBV, HCV, CMV, and EBV). The tolerance limit is the difference between two sequential results that can be considered to be significantly different. The tolerance limit for quantitative assays is the sum of the biological variation in quantitation and the intra-assay imprecision and should be included in the laboratory report. For HIV-1 quantitative assays, biological variation is approximately 0.3 log₁₀ and intra-assay variation is approximately 0.2 log₁₀. For

^a Expression of values in IU should indicate the “standard” upon which the IUs were based. Genotype or subtype limitations should be indicated.

example, changes in HIV concentrations of at least $\pm 0.5 \log_{10}$ (\pm threefold) typically reflect biologically relevant changes in the level of viral replication.

9.5 Results Outside of the Measuring Range

Results below the lower limit of quantitation, but greater than the limit of detection can be reported as detected but not quantifiable, provided the assay has been verified for both end points. The report for a “negative” result should indicate limit of detection or lower limit of quantitation, as appropriate, or another appropriate expression of the assay’s sensitivity. Results above the upper limit of quantitation may be reported as greater than the value of that upper limit. Alternately, a result within the measuring range can be generated by diluting the sample and retesting if this dilution step has been verified. If samples are diluted to bring them into the measuring range of the assay, another source of error is introduced into the assay that may affect the precision or trueness.

10 Continuing Quality Assurance

10.1 Introduction

Quality assurance encompasses all procedures and activities directed toward ensuring that a specified quality of product is achieved and maintained. The roots of quality assurance practices are in the theories and concepts of total quality management, continuing quality improvement, and the clinical laboratory’s tradition of quality control.

Testing is one of several procedures that can be used to provide information for quality assurance, but this only provides information regarding the performance of the analytical phase. Quality assurance systems must address all phases. These phases are:

- the preanalytical phase;
- the analytical phase; and
- the postanalytical phase.

The amount of ongoing quality assurance and quality control performed for quantitative molecular assays will depend on the volume and frequency of the testing, as well as the type of test employed:

- regulatory agency-cleared or -approved assay;
- “for research only” or “for investigational use only” manufacturer’s assay kit;
- adapted or changed (“off-label”) use of a manufacturer’s kit; or
- laboratory-developed test, or “home-brew” assay.

For most types of tests, quality assurance of the preanalytical and postanalytical phases is similar. In the analytical phase, those tests that are not manufacturer-developed kits, or which are adaptations of kits will need varying amounts of component quality control as well as a test of the whole analytical system. (This is assuming that verification appropriate to the type of system used has already been successfully performed. See [Sections 7](#) and [8](#) on verification and validation methods.)

10.2 Preanalytical Phase

For quantitative molecular testing, special attention must be paid to specimen collection, transport, and storage. (Refer to [Section 5](#) for specific sample handling considerations and requirements.) Since in many situations, the reported result will influence prognostic or treatment decisions, the value must be reflective of patient status, not specimen handling. There have been several studies of optimal anticoagulants, storage times, and storage and transport conditions for most common specimens and

quantified viruses.¹⁹⁻²¹ Decrease in virus concentration due to improper storage over time has also been reported.^{20,22-25} If it is suspected that specimen integrity is compromised due to specimen transport and handling, it may be necessary for a laboratory to document that certain conditions are met in a QA procedure. (i.e., by notation of remaining dry ice or requirement of shipments with temperature indicators.) This will be specific for the test and institution.

10.2.1 Reagents: Materials Handling

Quality assurance of reagents is a critical aspect of all laboratory programs but is especially important in hybridization and nucleic acid amplification, since contamination can so easily invalidate assay results. When using laboratory-developed tests, all critical reagents should be tested for composition, concentration, purity, and functionality. This information should be documented before the reagents are aliquoted and stored at the temperature experimentally determined to be optimal during verification. Components and reagents from manufacturers' assays should be checked for physical integrity upon arrival, receipt date recorded, and then promptly stored in controlled-temperature areas according to the package insert. If necessary, parallel results generated by using new reagents with current or proven reagents could be demonstrated to be comparable before the new reagents are used with clinical samples. The following section on analytical-phase quality assurance contains a discussion of suggested procedures for the individual components of quantitative molecular assays. This should be used in conjunction with NCCLS document [MM3](#)—*Molecular Diagnostic Methods for Infectious Diseases*. Issues specific to quantitative molecular testing will be addressed below.

10.2.2 Laboratory Setup/Procedures

Quantitative molecular testing generally requires the same laboratory setup and workflow as those for qualitative testing. This is discussed in the most current edition of NCCLS document [MM3](#).

10.2.3 Operator/Testing Training

Operator training for quantitative molecular assays should follow CLIA guidelines and training for qualitative methods. Specific evaluation of precision and trueness can be made by having operators test characterized panels. Additional training in areas of quality control statistics may be needed and documented. In recent years, an exam for a certified laboratory specialist in molecular biology (CLSpMB) has been implemented by the National Credentialing Agency (NCA [www.nca-info.org]).

10.2.4 Instruments/Equipment

10.2.4.1 Manufacturers' Automated Systems

Molecular methods for infectious diseases are becoming more automated. For those tests that are automated and implemented in a laboratory, the manufacturers' instructions for maintaining, checking, and calibrating the instruments should minimally be followed and documented. However, it is incumbent on laboratory directors to assess new instruments and decide if the manufacturers' directions are adequate.

10.2.4.2 Instruments Used for Laboratory-Developed Tests

A detailed discussion of quality assurance for the basic instruments in manual and semiautomatic systems is presented in the current edition of NCCLS document [MM3](#)—*Molecular Diagnostic Methods for Infectious Diseases*.

In addition to heaters, thermal cyclers, and water baths, critical instruments in quantitation procedures are the detection instruments which include but are not limited to: spectrophotometers, fluorometers, and

luminometers. These should be checked regularly (at least twice yearly) in the ranges in which the assay will measure. Consult the manufacturer of each instrument for calibrated quantitative standards that will be able to test for a specified output within the range of the assay in use.

10.3 Analytical Phase Quality Control

As stated in the beginning of this section, the amount of ongoing quality assessment performed for quantitative molecular assays will depend on several factors (assuming that proper verification has been successfully performed). A suggested minimum strategy is outlined below:

- 1) Regulatory agency-cleared or -approved test, manufacturer's test kit used according to manufacturer's instructions: Analytical quality control of the whole system with kit controls and at least one long-term independent control. The results of these controls should be recorded each time they are run. (See Section 10.3.2, Quality Control of the Whole System.)
- 2) "For research use only" or "For investigational use only" manufacturer's test kit: With each new lot of reagents, analytical quality control of the whole system with kit controls and at least one long-term independent control. (See No. 1 above regarding the long-term independent control.)
- 3) Adapted or changed ("off-label") use of a manufacturer's kit: Quality control on each new lot of the changed component may have to be performed (or certificates of analysis received) prior to analytical quality control of the whole system with kit controls and at least one long-term independent control. (See No. 1 above regarding the long-term independent control.)
- 4) Laboratory-developed test/"home-brew" assay: Quality control on each new lot of the changed component may have to be performed (or certificates of analysis received) prior to analytical quality control of the whole system with in-house run controls and at least one long-term independent control. (See No. 1 above regarding the long-term independent control.)

10.3.1 Component Quality Control

Suggestions for the quality control of new lots of critical components (laboratory-made, obtained from a manufacturer for a laboratory-developed method, or used in an adaptation of a manufacturer's test kit procedure) are discussed in NCCLS document [MM3—Molecular Diagnostic Methods for Infectious Diseases](#). Specific issues that relate to quantitation are included in the following sections. Each laboratory director is ultimately responsible for determining the importance of the components in each procedure and the amount of quality control testing that should be performed. Data from the development and verification of the assay may be useful in this determination.

For laboratory-developed tests, a QC program for each critical component should be established. When purchasing reagents, specific certificates of analysis or an analyte-specific reagent (ASR) rating may not provide sufficient QC information and may not alleviate the need for further QC testing. For example, ASR materials are supplied with the name of the reagent; the quantity or concentration; a statement of purity and quality; warnings and precautions; date of manufacture; lot number; and storage conditions and expiration date only. ASR materials may not be supplied by the manufacturer with analytical or performance characteristics claims, or instructions for use.²⁶ These must be determined by the laboratory for the assay in which the reagents are used and verified through the establishment of appropriate QC parameters. For components made by the laboratory, some quality assurance will have to be performed in-house. Below are some suggestions for both purchased and laboratory-made reagents.

10.3.1.1 Chemically Synthesized Oligonucleotides

Synthetic oligonucleotides are used in molecular methods as hybridization probes; amplification primers, as templates for amplification controls; and, particularly, for quantitative methods, as internal calibrators. When purchasing oligonucleotides, laboratories should select vendors that will supply a certificate of analysis with each shipment. When purchasing an oligonucleotide labeled as an ASR, that reagent should have been made under good manufacturing practices (GMP). Purity and concentration will be especially important in oligonucleotides used as internal calibrators and probes used as quantifiers. When the test has moved on to routine use, specific concentrations of new lots, efficiencies of label attachment, and background values should be recorded as part of ongoing quality assurance. Modifications of the procedures used in the development and verification process can be used as the basis for ongoing quality procedures.

10.3.1.2 Enzymatically Generated Polynucleotides

Double-stranded DNA (cloned sequences or fragments generated by the enzymatic digestion of genomic or plasmid DNA) or single-stranded RNA (*in vitro*-transcribed) may be generated in the laboratory. Alternatively, they may be purchased from an established vendor. Efforts should be made to eliminate or minimize the presence of vector or nontarget sequences, since this may result in nonspecific signals upon hybridization. A certificate of analysis should come with purchased material or, if made in-house, purity checks should be performed. The comments for synthetic probes regarding attachments, labels, purity, and concentration also apply.

10.3.1.3 Concentration

There are several methods used to verify and adjust concentration. Choice of method depends on the purity of the oligonucleotide being measured and the precision needed. (For example, the precision of the concentration needed for an internal calibrator will be far greater than that needed for oligonucleotides used as primers.) One simple method is to make dilutions of the oligonucleotide, read the absorbance on a spectrophotometer at 260 nm, and calculate based on the molecular weight. Oligonucleotides can be diluted in water or buffer and the absorbance read at 260 nm. At A_{260} , 1 unit indicates 50 $\mu\text{g/mL}$ of double-stranded DNA, approximately 37 $\mu\text{g/mL}$ single-stranded DNA, and 40 $\mu\text{g/mL}$ single-stranded RNA. An analysis of the phosphate content may also be appropriate. In situations where precise concentration is critical, a concordance of two or three methods may be employed.^{27,28}

In addition to these methods, laboratories use Poisson distribution procedures where the oligonucleotide is diluted to a theoretical “one copy” and tested. This method generally uses the same assay as the routine test being evaluated. When using this method, the reagents employed must be from a previously verified lot.

As international “reference standards” are introduced (see Section 6), some laboratories use them as the basis for calibration. Again, reagents used in comparative assays of new materials must be from a previously verified lot. With any of these methods, the use of independent controls over time will help assure the avoidance of drift.

10.3.1.4 Functional Quality Control to Determine Efficacy

Functional checks for oligonucleotides and polynucleotides are accomplished in the same manner as crossing over serology kits, or new lots of chemistry reagents: the old primers and probes are run in parallel with the new ones, in one run with the same samples. The samples should be a mixture of known controls and patient samples. For quantitative tests, it is particularly important to test over the measuring range. Test development and verification data should be examined to determine whether this needs to be performed on each new lot of oligonucleotides and/or polynucleotides made or purchased.

10.3.1.5 Nucleotide Labels

Nucleotides may have several types of reporter molecules or attachment molecules to link them to reporter molecules in assay systems. For some kinetic PCR methods, probes may incorporate the use of two labels, such as a fluorophor and a quencher, or two fluorophors that transfer energy. For all attachments (radioisotope labels, enzyme labels, or other molecules) the labeling efficiency must be assured; in the case of radioisotopic, enzymatic, and chemiluminescent labels, windows of usability criteria must be established and tested. For use in quantitative methods, these probe systems must be carefully monitored from lot to lot using long-term, stable testing controls at more than one point in the measuring range. Monitoring the background in negative samples is also important, because a drifting background may influence the sensitivity and specificity of the test.

10.3.1.6 Quality Control of Contamination Control Components

When evaluating reagents used in preventing carry-over of amplified material, the following issues should be considered and tested:

- 1) the added enzyme/chemical should not interfere with amplification efficiency;
- 2) the addition must not interfere with detection techniques; and
- 3) the laboratory must understand, establish, and test for the minimum and maximum efficiency of carry-over prevention.

For quantitative methods, new reagent lots may need ongoing quality control, if it has been determined in test development that the use of the decontaminating reagent affects the results (especially if it has been lot-specific).

10.3.1.7 Other Components (Nucleoside Triphosphates, Buffers)

Depending on the importance of a reagent in the amplification reaction or its probability of being contaminated with extraneous nucleic acid sequences, purity and functional checks should be performed (i.e., amplifying a series of negative samples or reagent negatives with the new reagent). Buffers and dNTPs should be subjected to functional tests when new lots arrive or are prepared, if alterations in their composition were shown to have an effect on quantitation during the development or verification process.

10.3.1.8 Amplification Controls

In quantitative target amplification methods, an internal calibrator usually serves as the amplification control. This calibrator is added at a specific concentration as determined by the development and verification procedures. Quality control of this reagent for laboratory-developed tests follows those for chemically synthesized oligonucleotides as discussed in [Section 10.3.1.1](#). In the development and verification of the assay, the use of this guideline must be checked against specimens with target values spanning the measuring range of the assay to assure that it appropriately reflects the result at all levels.

10.3.2 Quality Control of the Whole System

10.3.2.1 Daily/Ongoing Quality Control

This section discusses the issues specific to quantitative molecular methods, where NCCLS document [C24—Statistical Quality Control for Quantitative Measurements: Principles and Definitions](#) may or may not be applicable.

Some national or regional laws mandate that two different controls be tested in each run. (For a discussion and definition of what constitutes a run, refer to the current edition of NCCLS document [C24—Statistical Quality Control for Quantitative Measurements: Principles and Definitions](#).) Manufacturers of quantitative molecular tests typically include multiple controls in their kits that are lot-specific.

Some laboratories employing manufacturers' kits choose to run at least one independent control on a periodic basis. In fact, only by running an independent control can a laboratory track trends and shifts in the manner that clinical chemists have traditionally done. Using an independent run-control is critical in evaluating a laboratory's consistency using quantitative methods. The type and number of independent controls and the periodicity used will be dependent on the assay, the clinical laboratory workflow, and clinical issues. While specific measures of tracking quantitative molecular methods have not been refined, laboratories are advised to begin observing the behavior of at least one long-term, lot-independent control in their molecular quantitative assays. Decisions regarding the validity of a run based on the information of this control are the responsibility of the laboratory director.

NCCLS document [C24—Statistical Quality Control for Quantitative Measurements: Principles and Definitions](#) provides a recommendation for controls to be “of a composition similar or identical with the material being analyzed; matrix controls must be used, when available, and should mimic, insofar as possible, the unknown specimen.” This guideline also suggests that enough material for one year's testing should be aliquoted and stored.

Obtaining enough material for one year's testing may be difficult; however, units of plasma may be obtained and spiked with the target of interest, as long as the quantitative stability can be assured. Some vendors are beginning to supply control material with published target values for specific molecular methods. Depending on the stability advertised with the product, a year's supply might be purchased.

To set the value of a kit-independent control for clinical chemistry, at least 20 separate determinations should be used. In laboratories with lower volume, fewer runs can be used but no more than three values per run incorporated into the statistics. (Refer to the most current edition of NCCLS document [C24—Statistical Quality Control for Quantitative Measurements: Principles and Definitions](#).) This may be even more difficult in quantitative molecular testing, where runs are even more infrequent and more expensive. However, since critical medical decisions are made based on the numerical value reported, consistent results independent of lot must be assured. Laboratories can purchase independent controls with “target values” or develop an in-house control with a given “target value” set by four to eight determinations, and the value assigned over time.

Once the value is assigned, there are several means by which to set the limits on the independent control. A statistical approach based on the mean \pm 2 or 3 SDs, or employing Westgard's multirule analysis is useful in principle, but practically has not been tested over time. The field of quantitative molecular diagnostics is not as developed as clinical chemistry, and the implication of controls (which also are not proven at this point) being out of an “artificial range,” as well as the implications of rejecting a test on that basis is unknown. Laboratories employing manufacturers' test kits are advised to use independent controls, recording the results on Levy-Jennings charts, for general observations of trends and shifts, but decisions on rejection or acceptance of a run should be made using the manufacturers' controls and criteria. Laboratories that develop their own tests should invest the time to rigorously assign controls.

10.3.2.2 Calculating and Reporting Quality Control Data

Quantitative molecular tests for infectious diseases currently report values that can span several logs. For this reason, the values will not be “normally distributed,” which is the first criteria for parametric statistical calculations. Log₁₀ transformation will facilitate the use of parametric statistics and provide

more relevant information.^{16,29} Therefore, Levy-Jennings plots kept for long-term controls should either be graphed on log paper or the values log-transformed before plotting.

10.3.2.3 Proficiency and Performance Testing

Though regulations for participation in proficiency testing programs vary in each country, it is advised that laboratories conduct some manner of blind testing on a regular basis. In the United States, laboratories follow the specifications outlined in CLIA '88, which state that all laboratories performing moderate or high-complexity testing must enroll in CMS-certified (formally HCFA) proficiency testing for those analytes that are available. The testing requirements are: three events per year, five challenges per event. In the absence of HCFA-certified panels, laboratories must have a system for validating the accuracy and reliability of its test at least twice a year.

There are few commercially available, CMS-certified (formally HCFA) proficiency programs for quantitative molecular methods. At the time of this guideline, CAP is the only organization providing a limited number of molecular panels meeting the regulations; however, there are a larger number of organizations throughout the world which are developing and running proficiency/performance evaluations for these analytes. Some of the more widely known are: the CDC performance Evaluation Program (MPEP), the Quality Control for Molecular Diagnostics (www.qcmd.org), and the Industrial Quality Control Limited (VQC) (www.vqc.co.il). Within various countries, smaller programs are being instituted. There are no standards or regulations for the production and evaluation of these panels, so laboratories are advised to carefully evaluate a proficiency program before committing to it.

Whenever proficiency testing programs or proficiency specimens are unavailable for specific tests, laboratories should implement procedures to test for internal consistency in their results. This may be accomplished by the purchase of quantitation or seroconversion panels from vendors specializing in molecular diagnostic samples. Vendors who provide these panels usually provide only a target value that may be technique-dependent. If these panels are used for in-house proficiency where no other sources exist, several panels should be purchased, and the panels should be pretested at least once by someone other than the analyst who will perform the proficiency testing. Alternatively, if many panels are compared, results could be compared with another laboratory using the same technique and the same source.

Previously tested specimens that are blinded and arranged in a panel could also be used to test proficiency. This may be less powerful, however, because the value assigned to those samples is based on the very same test and in the same facility being tested. Some laboratories may choose to exchange samples with other laboratories, but for quantitative testing, one must choose a laboratory using the exact same technique. Some professional organizations have provided forums facilitating those wishing to set up sample exchanges.

10.4 Postanalytical Phase Quality Assurance

Since quantitative molecular tests are relatively new, there should be procedures written for the explanation of units; denominators; log-transformed vs. nontransformed values; the differences between testing periods; baselining patients; and how to handle complaints and unusual results for a patient. These are discussed in more detail in the next section. For quality assurance, all personnel should be familiar with these procedures, know where to find the information and documentation, and be able to explain them.

11 Clinical Utility

11.1 Section Goals

This section is for those who use the results of quantitative assays for nucleic acids of infectious agents, in addition to those who perform them and/or make decisions about developing or implementing such assays. The goal is to educate these audiences about considerations that pertain to the clinical utility of such assays and their results. Clinical utility is similar to what some regulatory bodies refer to as “indications for use,” which are usually listed near the end of the “intended use” section of an approved (or licensed) package insert. The assay is intended to quantify a nucleic acid (an analyte), but has utility, or is indicated for, certain clinical scenarios (e.g., “monitoring for...”).

11.2 Clinical Indications

When the clinical uses for an assay are considered, it is assumed that the assay is well understood with regard to analytical performance characteristics. Known analytical limitations (e.g., measuring range for quantifying precision with nonclinical samples) should be considered when determining clinical uses. If the assay is not yet analytically established, it should be recognized that uncharacterized aspects of analytical performance might affect its clinical performance in ways that have not been determined and may not be predictable.

For each type of indication described below (established, suggested, undefined), the clinical laboratory should consider how much data (especially to supplement those contained in the package insert) are necessary to satisfy its regulatory body. These user-generated data should be filed and maintained according to the certifier’s criteria.

The clinical laboratory should have available a list of indications for which an assay has been verified. If testing is performed for a nonverified indication, a comment to that effect should be included in the patient report. It should consider listing nonverified indications, especially when there are known safety or effectiveness concerns about such indications.

As an example, the U.S. FDA may consider testing for suggested or undefined indications to represent “off-label” use (new indication for an approved assay) or “research” or “investigational” use (any clinical indication for a commercial assay which has not been approved). When the FDA has recognized that a manufacturer has not established use for an indication that is associated with safety or effectiveness concerns, the package insert may warn, “performance has not been established” for that indication.

11.2.1 Indications Established by Data From Clinical (Diagnostic) Verification/Validation Studies

The most appropriate indications are those for which validation studies, by the manufacturer or using laboratory data, have determined the assay’s performance. Such indications may be listed in the “intended use” section of the package insert; supporting data may be found in the “performance characteristics” section.

11.2.2 Suggested Indications

Evidence from scientific literature, the user’s understanding of pathogenesis and diseases associated with the infectious agent, or biologic plausibility may suggest certain indications. For example, with a quantitative assay for monitoring drug therapy, even though nothing may be known about long-term outcome of the therapy, it is biologically plausible that decreasing plasma concentrations of nucleic acid during therapy are beneficial to the patient. The manufacturer and laboratory should be cautious, respectively, about promoting and using an assay for such indications.

11.2.3 Undefined Indications

This category pertains to the use of established assays “in search of” a disease. Hypothetical examples include testing for a recently identified agent that might be associated with a certain disease (an “uncertain” indication, such as work from one or very few laboratories in which a virus was isolated from cerebrospinal fluid [CSF] from people with a chronic neurologic syndrome). Another example could be testing for an agent that is not yet associated with any disease (an “unknown” indication, such as parvovirus B19, before it was associated with exacerbation in sickle-cell anemia, fifth disease, and other syndromes).

11.2.4 Revising Indications and Their Categories

Manufacturers, researchers, and laboratories should monitor utility by paying attention to quality assurance, to the clinicians who base decisions on assay results, and to the scientific literature. By doing so, it can be determined if clinician-users’ needs are being met and if new indications should be considered. As data accumulate (via carefully designed studies or, less desirably, via routine testing), it may be possible to revise the status of an indication to a “higher” category (e.g., from “suggested” to “established”).

11.3 Variables

In order to determine the clinical utility of a quantitative molecular assay, variables that could affect results and their interpretations should be considered. These include:

11.3.1 Microbial – Host Interactions (Biologic Variables)

11.3.1.1 Microbial Dynamics

Understanding (including rates of replication, turnover, and mutation) begins with studies of the natural history of infection. It should be realized that the concentration of microbial nucleic acid in a specimen reflects the balance between input and output. These dynamics can be altered by iatrogenic factors, such as therapy that is directed against the agent detected by the assay or therapy for other concurrent diseases. Serial testing usually has more value (i.e., leads to more interpretable information) than single-specimen testing, because results can be more validly extrapolated to serial data from studies of microbial dynamics; however, the value of serial testing should be considered potential until established by a well-designed study. (See Section 11.3.2.2.)

11.3.1.2 Microbial Variants and Mutants

Variants and mutants include (in order of decreasing genetic diversity) genetically similar serotypes or species, genotypes, and the quasispecies with which the patient is infected. Virulence may differ among serotypes or genotypes or among strains of the same type. Quasispecies diversity could be a variable that predicts the outcome of pathogenesis, independent of microbial nucleic-acid concentration. In the interpretation of quantitative molecular tests, the clinician must understand if the assay equally quantifies all subtypes and genotypes, or where the deficiencies are.

11.3.1.3 Type of Nucleic Acid Detected

Types of nucleic acid detected may have a profound effect on the performance of the assay and on the interpretation of results. Detected types can include DNA (genomic or extrachromosomal) or RNA (genomic or messenger), as well as packaged (within the microorganism or host cell) or free nucleic acid. For example, conclusions about the state of an HIV infection might be very different if testing is performed for quantified virion RNA in plasma, as opposed to proviral DNA in mononuclear cells.

11.3.1.4 Replicative Fitness of the Agent

Replicative fitness of the agent refers to a combination of virulence, inoculum size, route of inoculation, and tissue tropism. Infectivity is often defined in terms of live progeny-forming units per milliliter (colonies for bacteria, plaques for viruses), where the progeny are generated in a laboratory; these figures are hardly ever identical to the infective-dose titer that pertains to a particular human infection. When interpreting quantitative results from molecular tests, clinicians should understand that the presence of nucleic acid does not guarantee viability or infectivity. Clinical utility studies can be designed to correlate assay data with infectivity or, in the absence of such correlates, provide information to guide interpretation of results.

11.3.1.5 Host Factors

Users should be cognizant that populations on which the validation data were based may not match the patient being tested: demographic characteristics such as age, sex, and race may not be matched to an individual patient. In particular, study populations often lack children and pregnant women. Similarly, genetic background of the host or other concurrent illnesses may affect microbial dynamics or other expression (e.g., hepatitis D virus typically suppresses replication of hepatitis B virus); immunologic responses (e.g., congenital immunodeficiency or AIDS or, conversely, microbial sequestration resulting from immune response); or assay performance (e.g., interference by antibodies to the detected microorganism; interference or cross-reactivity with genetically similar infectious agents).

11.3.1.6 Patient Status

Results should be interpreted with as much knowledge as possible about the source of inoculum, duration of infection, and any previous therapy. The latter consideration should include any medication that could have a suppressive or augmenting effect on replication of the microorganism. These considerations should be integrated with understanding about the natural histories of replication and disease.

11.3.1.7 Matrix Effects

Clinical specimens usually represent a fluid that is more conveniently or less invasively collected than the microorganism's target tissues. Thus, results from serum, CSF, or urine may not accurately reflect the concentration of an organism at its site of disease or replication (e.g., hepatitis C viremia versus HCV in liver). This concern particularly applies to sequestered sites (e.g., encephalitis). Genome expression may vary among different sites of replication (e.g., abortive replication in white blood cells, with excess genomes and no infectious progeny, could lead to an artifactually high result from a quantitative assay). As noted above (see Section 11.3.1.5), the presence or absence of antibodies to the detected microorganism may affect assay performance (e.g., it may be more difficult to purify nucleic acids from immune complexes than from free microorganisms).

11.3.2 Criteria

Criteria for performing testing on a patient are critically affected by the imprecision of the assay, as determined with clinical specimens.

11.3.2.1 Number of Specimens to Collect and Test

In general, serial testing enables more detailed interpretation of results, because the course of quantified nucleic acid over time can be compared with changes in other temporally based information: symptoms and signs, laboratory and imaging data, and therapy. However, it should not be assumed that serial testing will be of value (see Sections 11.3.1.1, 11.3.2.2, and 11.3.2.4). A single data point of quantitation

can be the basis for decisions but must be interpreted more carefully, especially if assay precision is low (like that of most assays that quantify amplified nucleic acid).

11.3.2.2 Serial Testing

If serial testing is done, it is essential to understand what changes in concentration are relevant. First, one method should be used for all specimens, because values obtained from two different assays may not be comparable (see above, [Section 9.4](#)). Second, the range of “changes” due to imprecision can be determined by knowledge of precision studies for the assay. Third, awareness of clinically significant differences in concentration comes from state-of-the-art clinical knowledge. The combination of intra-assay and biologic variables is expressed as the tolerance limit (see [Section 9.4.1](#)). Fourth, when the assay yields results indicating that concentration is less than the quantifiable limit, that limit should be known (because it might be higher than a clinically significant threshold), and Poisson-distribution effects should be considered (when concentrations are very low, only a portion of samples will yield results indicating detection).

11.3.2.3 Important Effects of the Measuring Range

The measuring range has important effects on generating and interpreting results. Typically, precision is lowest around values near the ends of the measuring range. The measuring range for an ideal quantitative assay has a lower limit of “zero” or below the lowest clinically important concentration, and an upper limit above the highest clinically important concentration. If the high end of the measuring range is less than optimal, specimens with high concentrations should yield flagged results and, if possible, the assay should be configured for diluting the test specimen sufficiently to yield a result within the measuring range.

11.3.2.4 Value of Repeated Testing When Results Are at the Ends of the Measuring Range

This consideration particularly applies to less-than-quantifiable concentrations: How many such results are sufficient to determine that replication has been suppressed to a clinically insignificant level or, perhaps, eradicated? This can be assessed by reviewing data for the predictive value of a less-than-quantifiable result, as it pertains to a monitoring indication (see [Section 11.4.3](#)). In addition to the possibility of a Poisson-distribution effect on the assay itself (see [Section 11.3.2.2](#)), it is important to know the triggers for resampling (e.g., changes in symptoms, signs, imaging, or laboratory results) and, based on natural history, how often to resample if there are no such triggers. (Other laboratory results could include those for antibodies, antigens, or culture isolates pertaining to the quantified nucleic acid.) If a chronic or latent infection appears to have been eradicated, a decision about the need for resampling should also be based on knowledge of natural or iatrogenically altered history, which should include factors that influence microbial dynamics (see [Sections 11.3.1.1](#) and [11.3.2.2](#)). If a quantitative assay has repeatedly yielded less-than-quantifiable results and resampling is clinically indicated, subsequent testing should employ the most appropriate among available assays, usually that which is the most sensitive for detecting the nucleic acid of interest.

The same triggers for resampling interval apply to very high concentrations of microbial nucleic acid and to concentrations repeatedly exceeding a value that is associated with certain prognoses.

11.3.2.5 The Clinical Value of Results

This is established by conducting outcome studies, so that those pertaining to established indications have more value than suggested indications and, of course, much more value than undefined indications. The effect of false results on clinical value should be considered for each assay and each indication.

11.4 Indications Pertaining to Detection of Infection and Outcome of Infection

For indications discussed below, the usual specimen is serum or plasma. The user should note that, with reference to the many factors already discussed in this section, especially [Section 11.3.1.7](#), the concentration of microbial nucleic acid in blood might not accurately reflect replication in the target tissue(s). For this reason, “viral load” is often a misleading term.

11.4.1 Screening or Diagnostic Indications

In general, quantitative assays for microbial nucleic acids should not be used to screen populations for infection and are usually inappropriate for diagnostic indications, because they are relatively cumbersome and expensive; they yield numerical results when qualitative information is more appropriate, and current versions are often less sensitive than qualitative assays for the same analyte. This latter characteristic would lower the NPV for diagnosis, in particular. Furthermore, most such assays have not been characterized with regard to specificity for screening or clinical (diagnostic) specificity for diagnosis. Other types of assays, such as those that qualitatively detect antibodies, antigens, or nucleic acids, are usually preferred.

11.4.2 Diagnostic Indications

Several of the factors noted above ([see Section 11.3.1.5](#)) should be considered: Does detected nucleic acid represent active or latent infection and, if active, is the infection acute, chronic, or reactivated? In addition, is the pertinent microorganism associated with a disease or syndrome manifested by the patient (or is the microorganism present in the absence of disease)?

Diagnostic indications could be primary (i.e., first or only laboratory evidence of infection-associated disease), or confirmatory or “supplemental” (i.e., evidence from a second-step that has higher predictive value than a first-step assay that has already yielded positive results). Users should be aware that such indications, while feasible, have not been verified for many assays that quantify microbial nucleic acids.

11.4.3 Monitoring Indications

Monitoring indications include staging of disease, prognosis, progression of disease, and treatment efficacy. The “progression” indication could be applied to monitoring with or without therapeutic intervention. During therapy, testing is typically performed to determine effect of therapy and evidence of breakthrough resistance to antimicrobials. Based on data from clinical studies or from biologic plausibility ([see Section 11.2.2](#)), effective therapy may be indicated by (1) elimination of detectable nucleic acid when eradication is possible; (2) significant decreases in microbial concentration when eradication is not possible; or (3) stabilization of nucleic acid concentration when suppression is not possible. Ineffective therapy or possible resistance may be indicated by stable or increasing concentrations of nucleic acid. Each result represents microbial dynamics ([see Section 11.3.1.1](#)) at the time of sampling.

Few, if any, criteria have been established for associating less-than-detectable results with “permanent” eradication of the microorganism, or cure ([see Section 11.3.2.4](#)). For many chronic or latent infections, it is not even known if cure is achievable. The potential for latency or reinfection should also be considered. As discussed above, knowledge about the predictive value of less-than-quantifiable results are critical for this indication. Less-than-detectable results should be interpreted with knowledge of an assay’s limit of detection for clinical specimens and its ability to quantify at the lower end of a clinically important range. In other words, a low concentration of microbial nucleic acid may result in “less than quantifiable” with one assay and a numerical result with another, because their measuring ranges are different; examples include standard and “ultrasensitive” assays for HIV-1 RNA.

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Appendix. Technologies Used in Molecular Quantitation

NOTE: The following trade names are included in this appendix — Invader[®] Assays, Cleavase[®], eSensor[™], FRET[™] cassette. It is NCCLS policy to avoid using trade names unless the products identified are the only ones available; they serve as an example of the point illustrated in the consensus document; there is no generic description of the design and functional features of the products; and as long as the words, “or the equivalent” are added to the references. Inclusion of these trade names in no way constitutes endorsement by NCCLS. It should be understood that information on these products in this standard also applies to any equivalent products.

A1. Target Amplification Methods

A1.1 DNA Amplification Methods

A1.1.1 Quantitative Competitive PCR

Quantitative competitive PCR can be performed with DNA (e.g., cytomegalovirus) or RNA (e.g., HIV-1 or HCV) targets. For RNA targets, a reverse transcription reaction is done prior to amplification. If an RNA target is used, an RNA quantitative calibrator is preferable, thus controlling for the efficiency of both the reverse transcription and amplification reactions.

Quantitative competitive PCR utilizes a quantitative calibrator that has been constructed to contain the same primer binding sites as the target molecule, but distinguishable from it.¹ Increasing known copy numbers of the quantitative calibrator are added to replicate samples containing target DNA. The calibrator DNA competes for the primers equally with the target DNA, so that if the sample contains equal amounts of the target and calibrator DNA, the amount of amplified product of each will be equal.¹ For this technique, multiple replicates with varying targets for internal calibrators covering the linear or measuring range of the assay are required for accurate quantitation.

Quantitative PCR can also be performed by coamplifying the target molecule with a single concentration of a quantitative calibrator. Coamplification PCR uses excess primers so there is no competition between calibrator and target molecules, except when the target is present in overwhelming excess (beyond the measuring range of the assay). In this method, the calibrator has identical primer binding sites as the target, but an internal sequence has been rearranged, allowing for separate detection with probes specific for the internal sequence. Alternately, an internal portion of the calibrator can be deleted (or inserted) allowing for discrimination based on size. The calibrator is added at a known copy number prior to extraction of nucleic acid from the specimen, thus controlling for the efficiency of both nucleic acid extraction and amplification. Quantitation of nucleic acid in the original sample is determined from the following formula:²

signal from target/signal from quantitative calibrator • concentration of quantitative calibrator added • dilution factor

Both methods are competitive for substrates and targets, and both imply coamplification.

A1.1.2 Limiting Dilution PCR

Limiting dilution PCR provides another method to quantitate the number of target molecules in a given sample. A series of dilutions of the extracted specimen containing the target sequence are made. PCR is performed on the multiple aliquots of each dilution and the mean number of target molecules can be calculated based on the percentage of aliquots that are PCR positive at a given dilution.

The mean number of target molecules per reaction $[m] = -\ln [F]$, where F is the fraction of negative PCR reactions at a given dilution.³

A1.1.3 Real Time PCR (Homogeneous PCR, Kinetic PCR)

With the use of real-time methods, amplification and quantitation can be performed in a single closed tube, which minimizes the risk of amplicon cross-contamination.

A1.1.3.1 5' Nuclease PCR Assay (see Figure A1)

The 5' nuclease PCR assay detects the accumulation of specific PCR products by adding a double-labeled fluorogenic probe to the amplification reaction.⁴ This allows simultaneous or “real time” detection of the amplicons. The fluorogenic probe is an oligonucleotide that has a reporter fluorescent dye, such as fluorescein, attached to the 5' end; and a quencher dye, such as rhodamine, attached near the 3' end. The probes are 25 to 30 base pairs in length, so that when the fluorescein is excited, its fluorescent emission is quenched by the nearby rhodamine. During PCR, if the probe is hybridized to the target PCR product, it will be cleaved by the 5' to 3' nucleolytic activity of Taq DNA polymerase. The reporter dye will be separated from the quencher dye, leading to an increase in fluorescein fluorescence intensity. The increase in fluorescence indicates that the probe-specific PCR product has been synthesized. The rate of fluorescence is related to the amount of target in the sample. The amount of target is quantitated by the use of an internal calibrator that is coamplified with the target at a fixed concentration. By comparing the rate of the increase of the fluorescence intensity between the calibrator and target, the concentration of the target can be determined. Target and internal calibrator are distinguished by using different fluorescent dyes at their 5' ends.

For additional methods refer to the NCCLS document [MM3—Molecular Diagnostic Methods for Infectious Diseases](#).

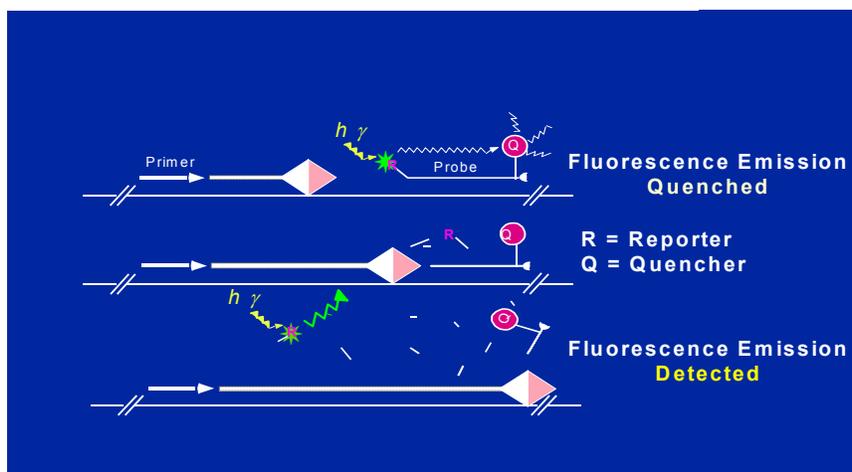


Figure A1. 5' Nuclease PCR Assay. Reprinted with permission from Roche Molecular Systems, Inc. © Copyright 1991. Roche Molecular Systems, Inc. All rights reserved.

A1.1.3.2 Continuous Fluorescence Monitoring of a Double-Strand DNA Binding Dye

PCR products can be detected continuously using a dye that preferentially binds double stranded DNA molecules. The dye is added to the reaction mix and gives a fluorescent signal that is proportional to the DNA concentration. Ethidium bromide is an example of a fluorescent dye; however, newer dyes, such as SYBR Green I, provide a stronger fluorescent signal. When the dye is unbound the fluorescence is relatively low, but when bound to double-stranded DNA molecules, the fluorescence is greatly

enhanced.^{5,6} However, the fluorescent dye will also bind to nonspecific amplification products. To improve the specificity, melting curves can be performed. The amplified product will have a characteristic melting peak at the amplified product's melting temperature (T_m), while the primer dimers and other small, nonspecific products will melt at a low temperature with broader peaks.⁷

A1.1.3.3 Fluorescence Resonance Energy Transfer (FRET)

An alternative and more specific method for the real time detection of amplified products is the use of hybridization probes based upon FRET. With this method, the accumulation of specific product can be monitored. Two probes, a donor and acceptor, are designed to bind in close proximity to the product at a location internal to the amplification primers. The 3' end of one probe is labeled with a donor fluor (usually fluorescein), and the 5' end of the acceptor probe is labeled with an acceptor fluor. The donor fluorophor is excited by an external light source and emits light that is absorbed by the acceptor fluorophor, which emits light of a different wavelength. The FRET will occur only when the two probes hybridize to the amplicon in close proximity. The amount of FRET signal is proportional to the amount of specific DNA product.^{5,8}

When glass capillaries are used in rapid-cycle DNA amplification, the optically clear sample vessel serves as a cuvette for fluorescence analysis. Instruments that combine rapid-cycle PCR with fluorescence analysis are commercially available.⁹

FRET technology may also be used for the detection of amplified RNA products.

A1.1.3.4 Molecular Beacons (see Figure A2)

Molecular beacons are single-stranded oligonucleotide probes that can monitor nucleic acid synthesis in real time.¹⁰⁻¹² The beacons possess a stem-and-loop structure. The loop portion of the molecule serves as a probe sequence that is complementary to a target nucleic acid. The stem is formed by the annealing of two complementary arm sequences that are on either side of the probe sequence. A fluorescent moiety is covalently linked to the end of one arm, and a quenching moiety is covalently linked to the end of the other arm. The stem keeps these two moieties in close proximity to each other.

When the molecular beacon is in the hairpin form (unbound to target), the molecules do not fluoresce, because the stem hybrid keeps the fluorophor close to the quencher. When the specific probe sequence in the loop hybridizes to its target, the stem hybrid dissociates and the fluorophor and quencher move apart, restoring fluorescence.

Since nucleic acid targets are in low concentration in clinical samples, molecular beacons are best used as a detection method in conjunction with target amplification. Several amplification techniques can be used in conjunction with molecular beacons such as PCR and NASBA. Quantitation is based on either the number of amplification cycles (in case of PCR) or amplification time (in case of NASBA) needed before fluorescence from the probe-target hybrid becomes detectable. Probe-target hybrid fluorescence is proportional to the logarithm of the number of target molecules in the reaction tube.

Molecular beacons technology may also be used for the detection of amplified RNA products.

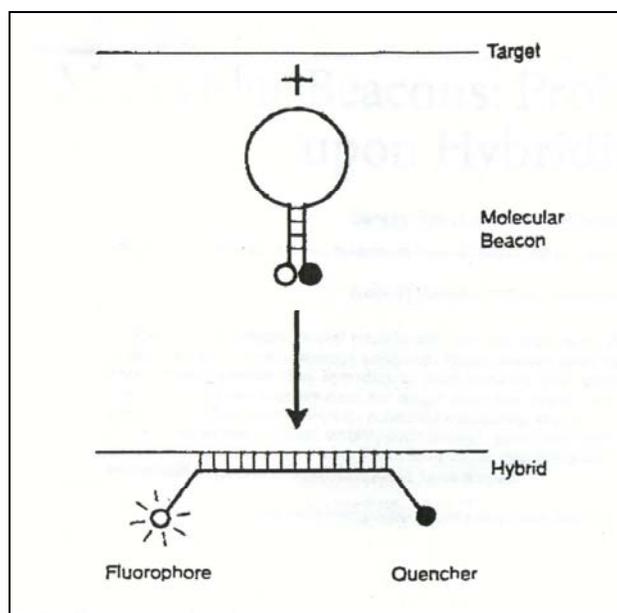


Figure A2. Molecular Beacons. Reprinted with permission from bioMerieux, Inc./bioMerieux SA.

A1.1.4 Strand Displacement Amplification (SDA)

SDA is a DNA amplification technique that consists of a target generation step followed by an exponential amplification phase that replicates the target sequence through a series of primer extension, nicking, and strand displacement steps.¹³ Quantitative SDA uses a quantitative calibrator sequence for competitive amplification that is identical to the target sequence except for an internal region that has been randomly mutagenized. The calibrator and target molecules are amplified with the same primers, but products are detected separately with specific probes. The amount of target can be determined from the ratio of the two products and the known initial level of the calibrator that was initially added to the reaction. SDA can be adapted to RNA targets by including a reverse transcriptase to generate a DNA copy of the RNA target.

A1.2 Transcription Amplification Methods

Nucleic acid sequence-based amplification (NASBA)¹⁴ and transcription mediated amplification (TMA)^{15, 16} are both isothermal RNA amplification methods. The methods are similar in that the RNA target is reverse transcribed into cDNA and then RNA copies are synthesized using an RNA polymerase. The NASBA assay utilizes AMV-RT (avian myeloblastosis virus reverse transcriptase), RNase H, and T7-RNA polymerase, while the TMA assay utilizes a RT enzyme with endogenous RNase H activity and an RNA polymerase.

For NASBA, quantitation is based on coamplification of the target molecule with three synthetic RNA calibrators of known high, medium, and low copy number that have the same primer binding sites as the target. The three quantitative calibrators are added to the sample containing target molecules prior to extraction of nucleic acid, thus controlling for nucleic acid extraction and amplification. Each of the three RNA calibrators has a unique internal sequence, allowing for separate electrochemical luminescent detection of each quantitative standard and the target amplicon with specific, labeled probes.

The RNA amplicons generated by NASBA can be quantified in real time by using molecular beacons. (See Figure A2.) Quantitation is based on the same principle as described above, but uses a single RNA calibrator. A fluorescence signal, read in a fluorescent plate reader, will be produced concomitantly with amplification due to binding of the molecular beacon to the RNA amplicon. Two molecular beacons are

used, one for the target amplicon and one for the calibrator amplicon. The use of two fluorescent dyes enables distinction of the two different molecular beacons. Kinetic analysis of the fluorescent signals reveals the respective amplification efficiency of the wild-type target and calibrator and therefore the quantity of target in the original sample.

For TMA, amplicons are detected using the hybridization protection assay. Sequence-specific oligonucleotide probes coupled to chemiluminescent acridinium ester (AE) labels are hybridized to the amplicons. After chemical degradation of the nonhybridized probes, the AE protected within the hybrid reacts under alkaline conditions to produce light, which can be detected by a luminometer. Quantitation of the target is determined by interpolation from an external calibration curve that is run in parallel with the samples containing target RNA.

A2. Signal and Probe Amplification

A2.1 Invader[®] Technology (see Figure A3)

The Invader[®] assay is an isothermal, homogenous technology platform that can detect, analyze, and quantitate DNA and RNA without target amplification.^{17,18} The assay can be run in a biplex format that can detect two distinct mRNAs or DNAs within the same sample in a single reaction.¹⁸ For DNA applications, this typically permits both alleles at a given single nucleotide polymorphism (SNP) locus to be detected in a single reaction. Alternatively, one target can be a gene of known copy number or RNA of invariant expression that acts as an internal standard. The signal from the second target can be normalized to this internal standard to allow quantitation of gene copy number or RNA expression levels; to account for errors; to permit interassay comparisons; and to simplify data analysis.

The Invader[®] reaction relies upon thermostable, structure-specific Cleavase[®] enzymes—members of a family of enzymes that cleave nucleic acid molecules at specific sites based on structure (not sequence)^{19,20} to produce and amplify a fluorescent signal in a two-step cascade.¹⁷ Although the reaction follows the same general format for both DNA and RNA targets, the specific enzyme and some reaction components vary depending on whether the target is DNA or RNA.

Figure A3 shows a schematic of a biplex Invader[®] DNA assay for SNP discrimination. Except for the base positioned to detect the SNP, the target-specific regions of the Invader[®] oligos and primary probes for the two alleles are the same. The 5' flap sequences differ for each of the two primary probes and are designed not to hybridize to any of the other components of the primary reaction. An overlapping, invasive structure forms when the primary probe and the Invader[®] oligo hybridize to their particular DNA target sequence. The Cleavase[®] enzyme recognizes the invasive structures and cleaves off the 5' flaps, releasing them as target-specific products. Because the assay operates near the melting temperature (T_m) of the primary probe, multiple probe turnovers occur without temperature cycling. Consequently, the enzyme can produce thousands of copies of cleaved 5' flaps per target sequence per hour. If the correct invasive structure does not form, i.e. (because of a genetic variation) cleavage is suppressed.²⁰⁻²²

Signal generation occurs in the secondary reaction. Its target-independent design enables the use of universal secondary reaction components for each of the targets in the primary reaction, including the cleaved 5' flaps and the fluorescence resonance energy transfer (FRET[™]) cassettes. FRET[™] cassettes are labeled with two dyes: a fluorescent donor dye and an acceptor dye that participate in FRET to quench the signal from the donor dye in uncleaved FRET[™] cassettes.²³⁻²⁵ When a released 5' flap from the primary reaction hybridizes to its respective FRET[™] cassette, a secondary invasive structure is formed. The Cleavase[®] enzyme recognizes the invasive structure and cleaves the FRET[™] cassette, separating the two dyes, and thereby generating a detectable fluorescent signal. By design, the two different FRET[™] cassettes — one for each unique 5' flap — contain spectrally distinct donor dyes, so that a specific signal is produced for each target. The enzyme can cleave thousands of FRET[™] cassettes per cleaved 5' flap per hour. Combining these sequential cleavage reactions produces 10^6 to 10^7 cleaved

FRETTM cassettes per original target sequence per hour, thus greatly amplifying the signal.²² Although cleaved and uncleaved primary probes freely associate with and dissociate from the FRETTM cassettes, signal arises only when a cleaved 5' flap and FRETTM cassette form a correct invasive structure.

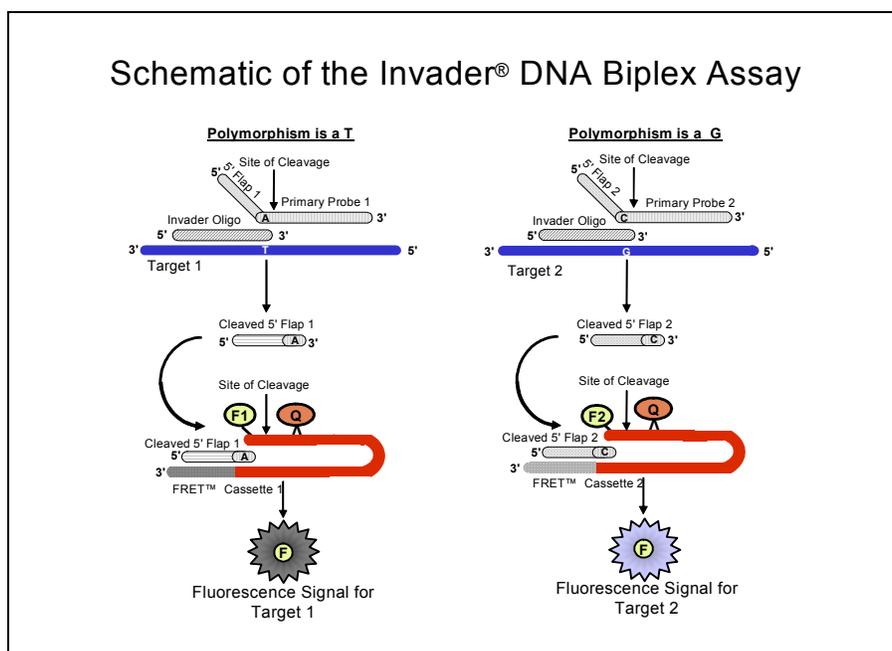


Figure A3. Invader[®] Technology

A2.2 Branched DNA (bDNA) Technology

bDNA technology is a signal amplification method, in which multiple oligonucleotide probes are bound to the target molecule through a series of hybridization events.²⁶ Alkaline phosphatase-labeled oligonucleotides are then bound to the probes, and following the addition of a chemiluminescent substrate, light emission is measured. Light emission is proportional to the number of target molecules in the original sample. The sensitivity of the bDNA assay has been increased by the addition of preamplifier probes, which allow the binding of additional bDNA molecules, and by the incorporation of the non-natural bases, isocytidine and isoguanosine, into the probe synthesis. The addition of non-natural bases minimizes nonspecific hybridization of the assay probes, thus reducing background signal. The concentration of the target molecules is determined from a quantitative calibration curve run in parallel with the specimens containing target molecules.

A2.3 Hybrid Capture (see Figure A4)

Hybrid capture technology is a signal amplification method that can be applied to the detection of DNA or RNA target molecules.²⁷⁻²⁹ A single-stranded RNA probe or cocktail of probes is added to the specimen containing denatured target DNA. After capturing the RNA:DNA hybrids to a solid support, alkaline phosphatase-conjugated antibody specific for the RNA:DNA hybrids is added to the reaction mix. Signal amplification occurs, since each captured hybrid can bind multiple conjugated antibodies and each conjugated antibody is labeled with several alkaline phosphatase molecules. After the addition of a chemiluminescent substrate, the light output is measured in a luminometer. A series of quantitative calibrators are run in parallel, allowing the generation of a quantitative standard curve that is used to determine the amount of target DNA in the specimen.

For RNA targets, a single-stranded DNA probe is hybridized to the target RNA molecules, and the RNA:DNA hybrids are detected as described above.

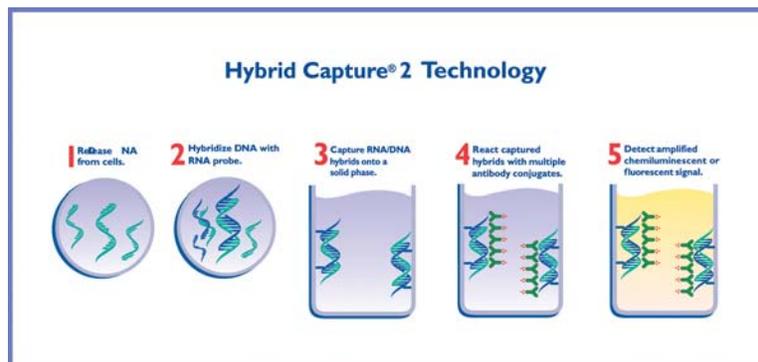


Figure A4. Hybrid Capture. Reprinted with permission from Digene Corporation, Gaithersburg, MD.

A2.4 Bioelectronic Detection of DNA and RNA Hybrids

eSensorTM DNA detection technology³⁰ rests on the specific generation of electrical current through the reversible oxidation and reduction of metal complex labels on nucleic acid targets. eSensorTM chips have electronically active gold electrodes as well as reference and auxiliary electrodes. Each electrode is coated with a specific DNA capture probe. The capture probes (~25 bases in length) are deposited on the gold electrode surfaces as a mixed monolayer of the capture probes and alkythiol molecules. The modified electrode surface inhibits nonspecific binding and blocks electrochemical signals from both unbound label and extraneous redox compounds. Bioelectronic detection proceeds via a sandwich assay whereby a nucleic acid target of interest is simultaneously bound by capture probes on the electrode surface and a second probe in the system referred to as a "signaling probe." Signaling probes are single-stranded oligonucleotide probes complementary to a different, but adjacent portion of the target than the region bound by capture probe. Signaling probes serve to label the target upon hybridization.

Covalently bound to signaling probes is the organometallic, electroactive label, ferrocene. Hybridization between target and signaling probe couples the now ferrocene-labeled target to the underlying electrode. Upon hybridization of the regions of the target complementary to the capture probe and the signaling probe, the ferrocene labels are brought into sufficient proximity to the electrode surface for detection. Application of an AC voltage to the electrode results in a reversible reduction and oxidation of ferrocenes. Electrons are transferred between the label and the electrode surface only when the target is present and hybridized by both signaling probe and capture probe.^{31,32} The current generated by this system is detectable with the electronic detection system called the eSensorTM 4800 system.

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NCCLS consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information, contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Subcommittee Responses

MM6-P: *Quantitative Molecular Methods for Infectious Diseases; Proposed Guideline*

General

1. This document seems to be fairly expansive or even verbose. Hopefully, it will be streamlined and reduced to critical information as it is reviewed outside of the drafting committee.
 - **The subcommittee edited to pare down the verbiage and minimize repeat information, while covering content required for the scope of this document.**
2. At the present time, most of the literature is from viral load monitoring of HIV treatment. However, the standard is intended to cover all infectious diseases where quantitative monitoring of the RNA or DNA of the pathogen is appropriate. For this reason, we believe it is important to avoid overly specific interpretations or recommendations, which may not be appropriate for all diseases and/or technologies.
 - **The subcommittee has been careful to avoid overly specific interpretations while at the same time trying to provide useful information. The Scope (Section 1) states that this document contains information most specific to viral diseases. If there are some specific examples where this balance may not be optimized, the subcommittee would like to know of them.**

Section 3.1, Terminology (Formerly Section 3)

3. Regarding the definition of "limit of quantitation (LOQ)" The precision of all nucleic acid amplification technology (NAT) assays goes down as the LOQ goes down, so that as improved assays are developed which have lower LOQs, it is likely that there CVs will go up. Whether the precision attained is acceptable is a question that can only be answered in the context of the clinical decision for which the assay is used.
 - **During the early work on this project, the limit of detection of most viral load assays for HIV was at about 400 copies/mL. With advancements in technology, assay sensitivity increased, and precision decreased. This was taken into consideration, and a comment on precision of these assays has been added to Section 7.2.1 which reads: "Nucleic acid amplification methods are typically much more sensitive than methods used routinely in clinical laboratories to measure other analytes. As a consequence, the measurements made with these assays may be less precise. The unparalleled sensitivity of these methods is best appreciated when the analyte concentration is expressed in molarity. For example, HIV-1 viral load assays capable of reliably measuring samples containing 400 copies/mL (approximately 10^{-19} M) may have coefficients of variation ranging from 20 to 40%."**

Section 5.1.1, Timing of Specimen Collection

4. Reference is made to the need for two pretreatment specimens taken at least two weeks apart to establish a baseline value of the analyte. This statement is taken from current guidelines for HIV patient management, and may or may not be relevant for other therapeutic situations. We believe that the sections should contain a more general statement about consideration of the need for baseline values, and the precision of the assay being used.
 - **The text has been revised to read:**

“Since quantitative molecular testing is often performed to assess patient response to specific therapy, proper timing relative to administration of the therapy is important. For HIV patients, current guidelines recommend that pretherapy specimens be tested to establish a baseline value, calculated as the average of at least two consecutive specimens tested approximately two weeks apart. Samples should be taken at the appropriate time with consideration for the biological response of the organism in order to correctly measure the magnitude of response.

Recommendations for other infectious disease pretherapy and therapy monitoring sampling will be generated from therapeutic clinical trials and practice committee guidelines.”

Section 5.2. Specimen Transport and Storage

5. Please describe the prevention of and measures to take in case of accidents during transportation and storage.

- **The text has been revised to address this comment with the addition of the statement:**

"From a safety standpoint, the handling of specimens for transport and storage should proceed with the same precautions taken for any potentially infectious materials, following current biosafety guidelines for biomedical laboratories, as well as appropriate regulatory guidelines."

Section 5.3.1. Nucleic Acid Extraction

6. Contamination of DNA in RNA samples for RT-PCR was not mentioned. I think that sometimes, in the case of RT-PCR of bacteria, the contamination of DNA causes problems.

- **The text has been revised to address this comment with an additional statement in Sections 5.3.1 and 6.1.1: "Steps should be taken to assure that residual nucleic acid is minimized if, during the verification phase of development, it has been shown to interfere with the assay or compromise the integrity of the results generated."**

Section 6.1.2. Synthetic Standards: DNA and RNA

7. In our laboratory, the positive control for PCR is made by PCR (TA) cloning. We always confirm the cloned inserted DNA sequence in the vector after PCR cloning. I believe some laboratories make positive controls for PCR using this method. If possible, please describe this method in the guideline.

- **There are no protocols provided for any methods of manufacturing controls, only considerations for their use. The subcommittee does not believe that an exception should be made in this guideline.**

Section 7.3.3. Linearity Experiment (this section has been deleted)

8. The following sentence is included: "The analyte concentration in this patient specimen should be at least 30% higher than the anticipated upper linearity of the assay." No rationale is provided for this stricture, nor can we think of one. We believe it should be removed from the section.

- **The subcommittee understands the confusion caused by this statement, and Section 7.3.3 has been removed.**

Section 7.7.2. Internal Control/Calibrator

9. There is a discussion of the variability (imprecision) "...inherent in standard PCR..." compared to real-time PCR which is claimed to be more precise as it "...operates in the geometric phase of the amplification curve." In practice, we know that this is not always true and would like to see the statements on the relative precision of standard and real-time PCR removed or made more clearly hypothetical.

- **For clarity the subcommittee has revised the section to read: "The ability of real-time PCR assays to measure PCR product amplification efficiency cycle by cycle may diminish the need for use of an internal calibrator or internal control. Inclusion of an internal calibrator or control may help in identifying, but usually cannot distinguish between, suboptimal nucleic extraction and the presence of interfering substance(s) as causes of a potentially inaccurate result."**

Section 9.3. Transformation

10. It is stated that the \log_{10} transformed data should be reported "...since viral replication is a geometric rather than linear function..." While the point is correct, we have found that many physicians (the ultimate consumers of these test results) have trouble understanding reports with log data. Accordingly, the strictures provided in this section need further consideration.

- **After careful consideration, it is the subcommittee's recommendation that the \log_{10} transformed data "should" be reported and an interim of using both values is recommended. For clarification, the text is revised to read: "Log₁₀ transformed data should be used for reporting quantitative molecular results, because \log_{10} transformed results better reflect biologically relevant changes in load of microorganisms that usually replicate exponentially, and because most assays exponentially amplify the target analyte."**

Appendix

11. The papers cited (Boeckh and Baldanti) do not provide information on the invention or development of the Hybrid Capture technique.
 - **The following reference has been added: Anthony JG, Linske-O'Connell L, Lorincz AT. Nucleic acid hybridization. In: Specter S, Hodinka RL, Young SA, eds. *Clinical Virology Manual*. Washington, DC: ASM Press. 2000:169-181.**

Summary of Delegate Comments and Subcommittee Responses

MM6-A: *Quantitative Molecular Methods for Infectious Diseases; Approved Guideline*

General

1. Since real-time PCR is very popular, it would have been useful to give a more detailed analysis of normalization, efficiency, etc. Of course this is true of Invader[®], SDA, hybrid capture in which the analysis software is treated as a black box. With real-time PCR, users actually can do some modification.
- **The subcommittee recognizes that real-time PCR is very popular and that this topic may be worthy of a full document. However, members of the subcommittee consider a detailed description of this methodology to be outside the scope of this document. The issues discussed in the document that are specific to the quantitation of nucleic acid in diagnostic testing and monitoring are applicable to real-time PCR.**

The Quality System Approach

NCCLS subscribes to a quality 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 through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS [HS1—A Quality System Model for Health Care](#). The quality 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. 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	Service & Satisfaction
Personnel	Process Control	Assessment	Facilities & Safety

MM6-A addresses the quality system essentials (QSEs) indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
					X C24-A2 C28-A2 EP5-A EP6-A EP7-A EP9-A2 EP10-A2 GP10-A MM1-A MM2-A2 MM3-A MM4-A MM5-A				EP7-A		M29-A2

Adapted from NCCLS document HS1— *A Quality 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, [GP26-A2](#) defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytical, analytical, and postanalytical. All clinical laboratories follow these processes to deliver the laboratory’s services, namely quality laboratory information.

MM6-A addresses the clinical laboratory path of workflow steps indicated by an “X.” For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the following page.

Patient Assessment	Preanalytic				Analytic		Postanalytic	
	Test Request	Specimen Collection	Specimen Transport	Specimen Receipt	Testing Review	Laboratory Interpretation	Results Report	Post-test Specimen Management
X C28-A2 MM1-A MM2-A2 MM5-A MM7-P	X MM1-A MM2-A2 MM5-A MM7-P	X C28-A2 MM1-A MM2-A2 MM3-A MM4-A MM5-A MM7-P	X C28-A2 MM1-A MM2-A2 MM3-A MM4-A MM5-A MM7-P	X MM1-A MM2-A2 MM3-A MM4-A MM5-A MM7-P	X MM1-A MM2-A2 MM3-A MM4-A MM5-A MM7-P	X C28-A2 MM1-A MM2-A2 MM3-A MM4-A MM5-A MM7-P	X C28-A2 MM1-A MM2-A2 MM3-A MM4-A MM5-A MM7-P	 MM1-A MM2-A2 MM4-A MM5-A MM7-P

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

Related NCCLS Publications*

- C24-A2** **Statistical Quality Control for Quantitative Measurements: Principles and Definitions; Approved Guideline – Second Edition (1999).** This guideline provides definitions of analytical intervals, planning of quality control procedures, and guidance for quality control applications.
- C28-A2** **How to Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline—Second Edition (2000).** This document provides guidance for determining reference values and reference intervals for quantitative clinical laboratory tests.
- EP5-A** **Evaluation of Precision Performance of Clinical Chemistry Devices; Approved Guideline (1999).** This document provides guidance for designing an experiment to evaluate the precision performance of clinical chemistry devices, recommendations for comparing the resulting precision estimates with manufacturer’s precision performance claims and determining when such comparisons are valid, as well as manufacturer’s guidelines for establishing claims.
- EP6-A** **Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline—Second Edition (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-A** **Interference Testing in Clinical Chemistry; Approved Guideline (2002).** This document provides background information and procedures for characterizing the effects of interfering substances on test results.
- EP9-A2** **Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline—Second Edition (2002).** This document addresses procedures for determining the bias between two clinical methods or devices and for the design of a method comparison experiment using split patient samples and data analysis.
- EP10-A2** **Preliminary Evaluation of Quantitative Clinical Laboratory Methods; Approved Guideline (2002).** This guideline provides experimental design and data analysis for preliminary evaluation of the performance of a measurement procedure or device.
- GP10-A** **Assessment of the Clinical Accuracy of Laboratory Tests Using Receiver Operating Characteristic (ROC) Plots; Approved Guideline (1995) (Reaffirmed 2001).** This document describes the design of a study to evaluate clinical accuracy of laboratory tests and contains procedures for preparing ROC curves; glossary of terms; and information on computer software programs. (See related publication EP10-A in Evaluation Protocols sections.)
- M29-A2** **Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline—Second Edition (2001).** Based on U.S. regulations, this document provides guidance on the risk of transmission of hepatitis viruses, and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.
- MM1-A** **Molecular Diagnostic Methods for Genetic Disease; Approved Guideline (2000).** This document provides guidance for the use of molecular biologic techniques for clinical detection of heritable mutations associated with genetic disease.
- MM2-A2** **Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays; Approved Guideline—Second Edition (2002).** This document provides guidance for conducting molecular tests of immunoglobulin and T-cell receptor gene rearrangements.
- MM3-A** **Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline (1995).** This document contains guidelines for the use of nucleic acid probes and nucleic acid amplification techniques for the detection of target sequences specific to particular microorganisms. It also addresses quality assurance, limitations, proficiency testing, and interpretation of results.

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

Related NCCLS Publications (Continued)

- MM4-A** **Quality Assurance for Immunocytochemistry; Approved Guideline (1999).** This document provides recommendations for the performance of immunocytochemical assays on cytologic and surgical pathology specimens. It is intended to promote a better understanding of the requirements, capabilities, and limitations of these diagnostic methods, to improve their intra- and interlaboratory reproducibility, and to improve their positive and negative predictive values in the diagnosis of disease.
- 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.
- MM7-P** **Fluorescence *in Situ* Hybridization (FISH) Methods for Medical Genetics; Proposed Guideline (2001).** This document addresses FISH methods for medical genetic determinations, identification of chromosomal abnormalities, and gene amplification. Recommendations for probe and assay development, manufacture, qualification, verification, and validation; instrument requirements; quality assurance; and evaluation of results are also included.

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