INTERNATIONAL STANDARD

ISO 17822

First edition
2020-11

Corrected version
2020-12

In vitro diagnostic test systems — Nucleic acid amplification-based examination procedures for detection and identification of microbial pathogens — Laboratory quality practice guide

Systèmes d'essai pour diagnostic in vitro — Modes opératoires d'examen in vitro qualitatifs fondés sur l'acide nucléique pour la détection et l'identification d'agents pathogènes microbiens — Guide pratique sur la qualité dans les laboratoires

Reference number
ISO 17822:2020(E)

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Published in Switzerland

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems.*

This first edition of ISO 17822 cancels and replaces ISO/TS 17822-1:2014, which has been technically revised. The main changes are as follows:

— Clause 4 has been updated and merged from ISO/TS 17822-1:2014.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html

This corrected version of ISO 17822:2020 incorporates the following correction:

— The title on the cover page and page 1 has been corrected to remove the part name.

Introduction

Nucleic acid amplification-based tests (NAATs) are now commonly used in in vitro diagnostic (IVD) tools in laboratory medicine for the detection, identification and quantification of microbial pathogens. The NAAT result is influenced by all steps of the entire diagnostic workflow (i.e. pre-examination, examination, post-examination). Therefore, this document considers all critical aspects of the entire diagnostic workflow when designing, developing and implementing and using a specific microbial pathogen NAAT examination.

NAAT examinations include PCR technology as well as other amplification-based technologies such as, but not limited to, loop-mediated isothermal amplification (LAMP), transcription-mediated amplification (TMA) and strand displacement amplification (SDA).

This document covers the implementation of commercially available IVD(s) into the medical laboratory routine use as well as the design, development and implementation of laboratory developed tests (LDT).

This document will address the additional specific considerations, requirements and recommendations for the detection of microbial pathogens with sampling, nucleic acid extraction, genetic heterogeneity and the laboratory containment category which is required.

Due to high analytical sensitivity of nucleic acid-based examination procedures, special attention to their design, development and use is required. This includes verification of analytical and validation of clinical performance characteristics.

In this document, the following verbal forms are used:

— “shall” indicates a requirement;

— “should” indicates a recommendation;

— “may” indicates a permission;

— “can” indicates a possibility or a capability.

In vitro diagnostic test systems — Nucleic acid amplification-based examination procedures for detection and identification of microbial pathogens — Laboratory quality practice guide

1 Scope

This document describes the particular clinical laboratory practice requirements to ensure the quality of detection, identification and quantification of microbial pathogens using nucleic acid amplification tests (NAAT).

It is intended for use by laboratories that develop, and/or implement and use, or perform NAAT for medical, research or health-related purposes. This document does not apply to the development of in vitro diagnostic (IVD) medical devices by manufacturers. However, it does include verification and validation of such devices and/or the corresponding processes when implemented and used by the laboratories.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 15189, *Medical laboratories — Requirements for quality and competence*

ISO 15190, *Medical laboratories — Requirements for safety*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
accuracy
closeness of agreement between a test result or measurement result and the true value

Note 1 to entry: In practice, the accepted reference value is substituted for the true value.

Note 2 to entry: The term “accuracy”, when applied to a set of test or measurement results, involves a combination of random components and a common systematic error or bias component.

Note 3 to entry: Accuracy refers to a combination of trueness and precision.

[SOURCE: ISO 3534-2:2006]

3.2
amplification product
amplicon
specific *DNA* (3.17) fragment produced by a DNA-amplification technology, such as the *polymerase chain reaction (PCR)* (3.34)

[SOURCE: ISO 13495:2013, 3.3.1]

3.3
analytical specificity
specificity
capability of a measuring system, using a specified measurement procedure, to provide measurement results for one or more *measurands* (3.28) which do not depend on each other nor on any other quantity in the system undergoing measurement

Note 1 to entry: Lack of analytical specificity is called analytical interference (see ISO 18113-1:2009, A.3.2[21]).

Note 2 to entry: Specificity of a measurement procedure should not be confused with clinical specificity (SOURCE: ISO 18113-1:2009, A.3.16[21]).

Note 3 to entry: VIM; JCGM 200; 2012[22] uses the term selectivity for this concept instead of specificity.

Note 4 to entry: For qualitative and semiquantitative examination procedures, analytical specificity is determined by the ability to obtain negative results in concordance with negative results obtained by the reference method.

[SOURCE: ISO 18113-1:2009, A.3.4]

3.4
biorisk
probability or chance that a particular adverse event (in the context of this document: accidental infection or unauthorized access, loss, theft, misuse, diversion or intentional release), possibly leading to harm, will occur

[SOURCE: WHO Biorisk management , Laboratory biosecurity guidance , September 2006]

3.5
biosafety
describes the containment principles, technologies and practices that are implemented to prevent the unintentional exposure to pathogens and toxins, or their accidental release

[SOURCE: WHO Biorisk management Laboratory biosecurity guidance September 2006]

3.6
biosecurity
set of preventive measures and actions to reduce the risk of intentional or unintentional transmission of infectious diseases

Note 1 to entry: Biosecurity encompasses the prevention of the intentional removal (theft) of biological materials from laboratories.

Note 2 to entry: These preventive measures are a combination of systems and practices implemented in laboratories against the use of dangerous pathogens and toxins for malicious use to prevent the spread of these biological agents.

3.7
calibration
operation that, under specified conditions, in a first step, established a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and, in second step, uses this information to establish a relation for obtaining a measurement result from an indication

Note 1 to entry: according to US Code of Federal Regulations, calibration is a process of testing and adjusting an instrument or test system to establish a correlation between the measurement response and the concentration or amount of the substance that is being measured by the test procedure (modified from 42CFR 493.1218)[20].

[SOURCE: VIM; JCGM 200; 2012]

3.8
certified reference material
CRM
reference material (RM) (3.41) characterized by a metrologically valid procedure for one or more specified properties, accompanied by a *RM* (3.41) certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability

Note 1 to entry: The concept of value includes a nominal property or a qualitative attribute such as identity or sequence. Uncertainties for such attributes may be expressed as probabilities or levels of confidence.

Note 2 to entry: Metrologically valid procedures for the production and certification of *RMs* (3.41) are given in, among others, ISO 17034[17].

Note 3 to entry: ISO/IEC Guide 99:2007[19] has an analogous definition.

[SOURCE: ISO Guide 30, 2.1.2]

3.9
clinical performance
<laboratory medicine> ability of an in vitro diagnostic examination procedure to yield results that are correlated with a specific clinical condition or physiological state in accordance with the target population and intended user

Note 1 to entry: Although sometimes referred to as diagnostic performance or clinical validity; clinical performance is the harmonized term endorsed by the Global Harmonization Task Force (GHTF) and its successor, the International Medical Devices Regulators Forum (IMDRF).

Note 2 to entry: Evaluation of clinical performance often relies on the outcome of other types of clinical examinations to define "true positive or true negative" results.

[SOURCE: GHTF/SG5/N 6:2012, 4.4.2, modified — medical device has been changed to — examination procedure and particularly has been changed to — specific.]

3.10
clinical sensitivity
diagnostic sensitivity
<laboratory medicine> ability of an in vitro diagnostic examination procedure to identify the presence of a target marker associated with a specific disease or condition

Note 1 to entry: Also defined as percent positivity in *samples* (3.44) where the target marker is known to be present.

Note 2 to entry: Diagnostic sensitivity is expressed as a percentage (number fraction multiplied by 100), calculated as 100 × the number of true positive values (TP) divided by the sum of the number of true positive values (TP) plus the number of false negative values (FP), or 100 × TP/(TP + FN). This calculation is based on a study design where only one *sample* (3.44) is taken from each subject.

Note 3 to entry: The target condition is defined by criteria independent of the examination procedure under consideration.

[SOURCE: ISO 18113-1:2009, A.3.15]

3.11
clinical specificity
diagnostic specificity
<laboratory medicine> ability of an in vitro diagnostic examination procedure to recognise the absence of a target marker associated with a specific disease or condition

Note 1 to entry: Also defined as percent negativity in *samples* (3.44) where the target marker is known to be absent.

Note 2 to entry: Clinical specificity is expressed as a percentage (number fraction multiplied by 100), calculated as 100× the number of true negative values (TN) divided by the sum of the number of true negative plus the number of false positive (FP) values, or 100 × TN/(TN + FP). This calculation is based on a study design where only one *sample* (3.44) is taken from each subject.

Note 3 to entry: The target condition is defined by criteria independent of the examination procedure under consideration.

[SOURCE: ISO 18113-1:2009, A.3.16]

3.12
complementary DNA
cDNA
single-stranded *DNA* (3.17) that is complementary to a given *RNA* (3.42) and synthesized in the presence of reverse transcriptase to serve as a *template* (3.47) for synthesis of *DNA* (3.17) copies

3.13
contamination
introduction of an undesirable substance or matter

3.14
cut-off value
quantity value used as a limit to identify *samples* (3.44) that indicate the presence or the absence of a specific disease, condition or *measurand* (3.28)

Note 1 to entry: Defines which measurement results are reported as positive and which are reported as negative.

Note 2 to entry: Measurement results near the cut-off value can be considered inconclusive.

Note 3 to entry: The selection of the cut-off value determines the *clinical specificity* (3.11) and *clinical sensitivity* (3.10) of the examination.

3.15
denaturation
physical and/or chemical treatment which results in the separation of nucleic acid double helices

Note 1 to entry: denaturation of *DNA* (3.17) results in separation of double-stranded *DNA* (3.17) into single-stranded *DNA* (3.17).

[SOURCE: ISO 21572:2013, 3.1.6 — modified, term ”denaturation of proteins” has been changed to ”denaturation”, and ” the POI or” has been deleted. Note 1 to entry has been added.]

3.16
deoxyribonucleoside triphosphate
dNTP
solution containing dATP, dCTP, dGTP, dTTP and/or dUTP

[SOURCE: ISO 22174:2005, 3.3.7]

3.17
DNA
deoxyribonucleic acid
polymer of deoxyribonucleotides occurring in a double-stranded (dsDNA) or single-stranded (ssDNA) form

[SOURCE: ISO 22174:2005, 3.1.2]

3.18
DNA polymerase for PCR
thermostable enzyme which catalyses repeated *DNA* (3.17) synthesis

[SOURCE: ISO 22174:2005, 3.4.17]

3.19
DNA sequencing
determining the order of nucleotide bases (adenine, guanine, cytosine, and thymine) in a molecule of *DNA* (3.17)

Note 1 to entry: Sequence is generally described from the 5' end.

3.20
hybridization
specific binding of complementary *nucleic acid* (3.32) sequences under suitable reaction conditions

[SOURCE: ISO 22174:2005, 3.6.3]

3.21
inhibition
reduction of amplification or interference with detection process that can lead to false negative results or reduced quantity

3.22
interfering substances
endogenous or exogenous substances in clinical specimens/*samples* (3.44) that can alter an examination result

[SOURCE: ISO 20186:2019-1, 3.15 modified]

3.23
inhouse assay
laboratory developed test
LDT
type of in vitro diagnostic test that is designed, manufactured and used within a single laboratory

Note 1 to entry: Inhouse assay/LDT needs to be validated for its intended use before putting into service.

3.24
linearity
ability of a method of analysis, within a certain range, to provide an instrumental response or results proportional to the quantity of *nucleic acid target sequence* (3.46) to be determinded in the laboratory *sample* (3.44)

Note 1 to entry: In the case of qPCR, the quantification cycle (also termed cycle threshold or crossing point) is inversely proportional to the quantity of nucleic acid target sequence. (3.46).

Note 2 to entry: The term linearity is frequently linked with the linear range of the method and refers to the ability of a method to give a response or result that is directly propotional to the concentration of the nucleic acid target sequence (3.46).

[SOURCE: ISO 16577:2016, 3.92 modified — Notes 1 and 2 to entry added; 'quantity of analyte' replaced with 'quantity of the *nucleic acid target sequence*' (3.46).]

3.25
limit of detection
LOD
measured quantity value, obtained by a given measurement procedure, for which the probability of falsely claiming the absence of a component in a material is β, given a probability α of falsely claiming its presence

Note 1 to entry: The term analytical sensitivity is sometimes used to mean detection limit, but such usage is now discouraged. See ISO 18113-1:2009, A.2.7 and A.2.8 for further information.

Note 2 to entry: In a nucleic acid-based identification examination, the lowest concentration or content of the target organism per defined amount of matrix that can be consistently detected under the experimental conditions specified in the method.

Note 3 to entry: In molecular methods and quantitative molecular methods, the lowest concentration of measurand that can be consistently detected (typically, in >95 % of samples (3.44) tested under routine clinical laboratory conditions) and in a defined type of *sample* (3.44).

Note 4 to entry: This concentration must yield an assay value that can be reproducibly distinguished from values obtained with *samples* (3.44) that do not contain the measurand.

[SOURCE: ISO 18113-1:2009, A.3.14, modified — new notes to entry added.]

3.26
limit of quantification
LOQ
lowest concentration or quantity of the *nucleic acid target sequence* (3.46) per defined volume that can be measured with reasonable statistical certainty consistently under the experimental conditions specified in the method

Note 1 to entry: Generally expressed in terms of the signal or measurement (true) value that will produce estimates having a specified coefficient of variation (CV).

[SOURCE: ISO 16577:2016, 3.91, modified — replaced, content of the analyte of interest' with 'quantity of the *nucleic acid target sequence* (3.46)', 'amount of matrix' with 'volume' and 'relative standard deviation (RSD)' with 'coefficient of variation (CV).]

3.27
mastermix
mixture of reagents needed for nucleic acid amplification, except for the target *DNA* (3.17) and the controls

[SOURCE: ISO 22174:2005, 3.4.18]

3.28
measurand
quantity intended to be measured

[SOURCE: VIM; JCGM 200; 2012]

EXAMPLE 1 Quantity of gene target measured by *PCR* (3.34) is influenced by the *amplicon* (3.2) size of the *PCR* (3.34) assay and fragment size of the *template* (3.47) (~<amplicon size).

EXAMPLE 2 *Denaturation* (3.15) of *DNA* (3.17) in a *sample* (3.44) into ssDNA influences quantification by dPCR as the two strands are partitioned separately.

Note 1 to entry: The specification of a measurant requires knowledge of the kind of quantity, including any relevant component, and the chemical entities involved.

Note 2 to entry: In the second edition of the VIM and in IEC 60050-300:2001, the measurand is defined as the 'particular quantity subject to measurement'.

Note 3 to entry: The measurement, including the measuring system and the conditions under which the measurement is carried out, might change the phenomenon, body, or substance such that the quantity being measured differs from the measurand as defined. In this, adequate correction is necessary.

[SOURCE: ISO/IEC guide 99:2007, 2.23, modified — Note 3 and examples have been modified, and Note 4 has been omitted.]

3.29
negative (PCR) control
reaction performed without target *template* (3.47)

[SOURCE: ISO 22174:2005, 3.5.6]

3.30
negative (process) control
target pathogen-free *sample* (3.44) of the collected specimen which is run through all stages of the analytical process

Note 1 to entry: The nucleic-acid based examination process typically includes *sample* (3.44) preparation, enrichment, *nucleic acid* (3.32) extraction and target amplification.

[SOURCE: ISO 22174:2005, 3.5.2, modified — Note 1 to entry modified.]

3.31
no template control
NTC
control reaction containing all reagents except the extracted test *sample* (3.44) *template* (3.47) *nucleic acid* (3.32)

Note 1 to entry: This control is used to demonstrate the absence of contaminating *nucleic acids* [3.32]. Instead of the *template* (3.47) *DNA* (3.17), for example, a corresponding volume of nucleic acid-free water is added to the reaction. The term '*PCR* (3.34) reagent control' is also sometimes used.

[SOURCE: ISO 20395:2019, 3.20]

3.32
nucleic acid
macromolecule that is the medium for genetic information or acts as an agent in expressing the information

Note 1 to entry: There are two types of nucleic acid, *DNA* (3.17) and *RNA* (3.42).

[SOURCE: ISO 22174:2005, 3.1.1]

3.33
nucleic acid extraction
separation of *nucleic acid* (3.32) from biological materials

Note 1 to entry: Generally to perform amplification and analysis of the *nucleic acid* (3.32).

3.34
polymerase chain reaction
PCR
enzymatic procedure which allows in vitro amplification of *DNA* [3.17]

[SOURCE: ISO 22174:2005, 3.4.1]

3.35
polynomial regression
least squared regression using polynomials of various orders

$Y = a + b1X$ (first-order polynomial or linear fit)

Y = a + b1X + b2X2 (second- order polynomial), and

Y = a + b1X + b2X2 + b3X3 (third- order polynomial)

3.36
PCR-quality DNA
DNA (3.17) *template* (3.47) of sufficient length, purity, and quantity for performing *PCR* (3.34)

[SOURCE: ISO 24276:2006, 3.2.3]

3.37
regulatory body approved assay
tests that are designed and developed by manufacturers and approved by regulatory body for diagnostic purposes.

EXAMPLE CE-labeled tests

Note 1 to entry: The CE marking is the manufacturer's declaration that the product meets the requirements of the applicable EC directives.

3.38
reverse transcription
RT
process of making *DNA* (3.17) from an *RNA* (3.42) *template* (3.47), using the enzymatic activity of a reverse transcriptase associated with one or more oligonucleotide primers under a suitable set of conditions

[SOURCE: ISO 16577:2016, 3.180]

3.39
modified IVD labeled assays
modified IVD labeled tests
tests that are designed and developed by manufacturers and approved by regulatory body or they meet the requirements of the applicable EC directives for diagnostic purposes but in use of the laboratories they have been changed

Note 1 to entry: Depending on the grade of change made to the original assay this assay needs to be validated again.

3.40
real time PCR
method, which combines *PCR* (3.34) and fluorescent probe detection of amplified product in the same reaction vessel

3.41
reference material
RM
material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process

Note 1 to entry: RM is a generic term.

Note 2 to entry: Properties can be quantitative or qualitative, e.g. identity of substances or species.

Note 3 to entry: Uses may include the *calibration* (3.7) of a measurement system, assessment of a measurement procedure, assigning values to other materials, and quality control.

Note 4 to entry: ISO/IEC Guide 99:2007[19] has an analogous definition (5.13), but restricts the term "measurement" to apply to quantitative values. However, Note 3 of ISO/IEC Guide 99:2007, 5.13 (VIM; JCGM 200; 2012[22]), specifically includes qualitative properties, called "nominal properties".

[SOURCE: ISO guide 30]

3.42
RNA
ribonucleic acid
polymer of ribonucleotides occurring in a double-stranded or single-stranded form

[SOURCE: ISO 22174:2005, 3.1.3]

3.43
robustness
ability of an assay to proceed optimally, despite slight variation in conditions

Note 1 to entry: to entry: Usually refers to *PCR* (3.34) in which amplification occurs despite slight changes in reaction conditions, such as *DNA* (3.17) concentration.

3.44
sample
small portion or quantity, taken from a population or lot that is ideally a representative selection of the whole

Note 1 to entry: Usually refers to *PCR* (3.34) in which amplification occurs despite slight changes in reaction conditions, such as *DNA* (3.17) concentration.

[SOURCE: ISO 16577:2016, 3.185]

3.45
sequence database
<bioinformatics> biological database consisting of *nucleic acid* (3.32) sequences, protein sequences, or other polymer sequences and associated annotation

Note 1 to entry: The annotation can relate to organism, species, function, mutations linked to particular diseases, functional or structural features, bibliographic references, etc.

Note 2 to entry: Published genome sequences can be publically available, as it is a requirement of every scientific journal that any published *DNA* (3.17) or *RNA* (3.42) or protein sequence must be deposited in a public database.

3.46
target sequence
nucleic acid target sequence
specific *DNA* (3.17) sequence targeted for detection, e.g. by *PCR* (3.34)

[SOURCE: ISO 16577:2016, 3.203]

3.47
template
strand of *DNA* (3.17) or *RNA* (3.42) that specifies the base sequence of a newly synthesized strand of *DNA* (3.17) or *RNA* (3.42), the two strands being complementary

[SOURCE: ISO 16577:2016, 3.206]

3.48
unidirectional work flow
forward work flow
<laboratory medicine> principle of material/*sample* (3.44) handling applied to ensure that the laboratory *sample* (3.44), raw and processed test portion including amplified *DNA* (3.17) remain physically segregated during the entire procedure

[SOURCE: ISO 24276:2006, 3.3.5 modified — "laboratory *sample* (3.44), raw and processed test portion" has been changed to "the primary *sample* (3.44) and the processed *sample* (3.44)", and "the whole procedure" has been changed to "the examination procedure"]

3.49
validation
confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled

Note 1 to entry: The term "validated" is used to designate the corresponding status.

Note 2 to entry: Adapted from ISO 9000:2015[31].

Note 3 to entry: See Annex B for additional information.

[SOURCE: ISO 15189:2012, 3.26]

3.50
verification
confirmation, through provision of objective evidence, that specified requirements have been fulfilled

Note 1 to entry: The term "verified" is used to designate the corresponding status.

Note 2 to entry: Confirmation can comprise activities such as performing alternative calculations, comparing a new design specification with a similar proven design specification, undertaking tests and demonstrations, and reviewing documents prior to issue.

Note 3 to entry: See Annex B for additional information.

[SOURCE: ISO 9000:2015]

4 General laboratory requirement for microbial pathogens NAAT

4.1 General laboratory risk management and biosafety requirements

The medical laboratory management shall ensure the safety and protection of laboratory staff and service personnel. The requirements of ISO 15190 apply. As many microbes are pathogenic appropriate biosafety standards shall be applied.

The nucleic acid-based examination process shall be assessed by the medical laboratory to identify risks such as failure modes, operation errors, hazards and hazardous situations. The risks to patients and laboratory workers shall be identified prior to, and mitigated during examination development. Risks shall also be reviewed, monitored and mitigated prior to and during implementation, and regularly during the life cycle of operation.

NOTE 1 The medical laboratory can also refer to ISO 35001[9].

NOTE 2 The WHO biosafety manual can be applied, WHO/CDS/EPR/2006.6[11].

NOTE 3 The general principles and risk management practices described in [8] can also be applied to medical laboratories.

NOTE 4 For general guidance for reduction of laboratory error, see also ISO 22367[40].

NOTE 5 For information about quality control planning based upon risk management principles, see also CLSI EP23[1].

4.2 General laboratory set ups for pathogen detection

General requirements for best practice in NAAT laboratory set up shall be followed and typically include separation of pre- and post-amplification rooms and potentially further modularization of laboratory steps. Specific considerations around pathogen containment shall also be adhered to along with the appropriate risk assessment.

Of note are situations where sample containment risk level is greater than that required for handling of extracted nucleic acids. Pathogens of higher risk categories will typically require lower risk category for handling extracted nucleic acid and NAAT analysis. However, it cannot be assumed that the extraction procedure will render the sample non-infectious. Therefore, safety risk assessment shall be considered.

To increase biosafety, pathogen inactivation should be achieved at the earliest possible stage. However, inactivation method needs to be confirmed and risk and safety procedures applied as appropriate.

General Laboratory setup for management and reduction of contamination.

Contamination sources can be grouped into five categories and the laboratory should be setup to minimize contamination risk from each potential source:

1) Environmental

   Derived from outside the laboratory. Not generally a problem for pathogen detection although where closely related environmental species may cause contamination risk positive pressure laboratories should be used.

   Further detailed information to unidirectional workflow and air pressure conditions are given in 7.2.2.

2) Laboratory

   Major source of contamination as a result of preparation of large amounts of nucleic acid by the laboratory. This is typically a problem when using NAATs as they function by generating large amounts of the target (amplicon) in question; this is the predominant source of any NAAT contamination. Laboratory sources can also be derived from the use of vectors containing target sequences. In pathogen detection it can also be derived from microbial culture of pathogens of interest. This can be mitigated by separation of sample preparation and test set-up from other activities where large amounts of genetic material may be present. Further risk of contamination should be reduced by ensuring task specific laboratory equipment and coats.

3) Reagents

   Derived from the fact that many NAAT reagents are recombinant in source, therefore a low-level amount of bacterial DNA may persist. This is particularly a problem when targeting orthogonal genes like 16S ribosomal RNA gene.

4) Analyst

   Not usually a major source of contamination for pathogen specific NAAT as analysts not usually carrying pathogen. Potentially a problem, where pathogen (or closely related species) can exist asymptomatically. Standard laboratory procedures (e.g. protective clothing, gloves, filter tips etc.) can mitigate source.

5) Sample

   Potential major, and frequently unrecognised, source of contamination. Where high pathogen titre sample is prepared next to low titre or negative sample and cross contamination is likely. Standard laboratory procedures (e.g. filter tips) can mitigate source.

   Standard operating procedures shall be written, implemented and staff trained to reduce the risk for contamination. This may include restricted personnel direction and forward workflow from amplicon negative to amplicon positive areas and/or use of disposable labcoats or other protective clothing. This may not apply when using closed systems which automate the nucleic acid extraction, amplification and detection as a single workflow.

   Ultimately contamination shall be monitored in an appropriate manner to determine its influence on results. Laboratory, housekeeping, and all other personnel entering the laboratory, shall be trained, and the training documented.

For further detailed information see also Clause 5.

4.3 Commercial equipment (including software programs)

Equipment intended to perform nucleic acid amplification-based examinations, including software programs necessary to perform the analysis, shall be installed, verified, calibrated and maintained according to the manufacturer's instructions for use and documented laboratory procedures.

Where applicable, integration of laboratory instruments into existing IT infrastructure shall be verified.

EXAMPLE Connectivity to databases, bioinformatic functions, etc.

If multiple instruments are potentially used for the same nucleic acid test, inter-instrument comparison shall be performed to ensure comparability of results. The laboratory shall verify any in-house developed interfaces between instrument components and shall also verify interfaces between instrument components developed by manufacturer.

4.4 Laboratory personnel

Personnel assigned to perform nucleic acid amplification-based examinations shall be qualified and trained to the level of competence required for the specific NAAT and pathogen including continuing education to maintain competency.

The qualification and training of these personnel shall be documented.

5 Planning and implementation of pathogen NAAT assay

In general the criteria of the design are listed in a design plan.

Design and development planning shall include:

1. Definition of user needs and stakeholder requirements
2. Definition of the intended medical use
3. Performance requirements and specifications and other design requirements and specifications based on the intended use
4. Product risk assessment
5. Assay design and assay component supplier qualification, this should include but is not limited to:

   Specimen collection and processing, nucleic acid extraction, nucleic acid amplification, and detection and identification of nucleic acids of the target microbial pathogen, laboratory design, work flow and laboratory practices.
6. Performing of the feasibility phase
7. Verification and validation planning
8. Verification of design specifications

   EXAMPLES Detection limit, cut-off values, analytical specificity (including cross-reactivity and interference), precision, carryover, linearity, and where appropriate, calibrator commutability and traceability of results to reference materials or reference measurement procedures.
9. Laboratory scale production process planning
10. Validation of the intended use
11. Design changes during and after the development of the examination shall be documented

Pathogen specific considerations should include, but not be limited to:

Pathogen genetic consideration (sequence heterogeneity both within infection and across species, required operational taxonomic unit (OTU), specificity when considering closely related species, genetic association when considering resistance genes). Examples are:

— Use of multiplex panels (e.g. respiratory panel)

— Latency, carriage, reference ranges, relevant for multiplex panels which may detect organisms not part of the differential diagnosis

5.1 Quality control material

5.1.1 Examination of quality control material

For the receipt of reliable data and assay results the selection and use of appropriate controls and control material is essential.

Laboratories shall determine the requirements and from those the appropriate specifications of the control materials prior to the verification/validation. The frequency of use of the selected control materials can be based on the test performance characteristics according to the verification and validation outcome.

Where appropriate, controls (e.g., a control that is capable of assessing the effectiveness of the nucleic acid extraction process) shall be identified and used to reduce the likelihood of producing an incorrect result due to inadequate performance of the whole, or part of the, examination procedure. In particular, quality assurance procedures shall be designed to minimize false-positive and false-negative results.

Internal controls, inhibition control for NAAT methods can be homologous external, heterologous external, or heterologous internal. If added before specimen preparation, the internal control can also serve as a whole process control.

Control measures shall be implemented to prevent potential failures of the two main stages of nucleic acid-based in vitro diagnostic examinations: (1) whole pre-examination workflow from sample collection up to isolated nucleic acids and (2) nucleic acid examination, including amplification and identification. The NAAT method shall have the correct controls included to ensure the overall quality and reliability of the data produced.

NOTE General guidance for qPCR and dPCR controls is given in ISO 20395:2019, 4.4[12].

The rationale for the selection of control materials and procedures shall be documented.

1. Negative controls

   The purpose of negative controls is to perform the examination without the intended target to assess for false positive results caused by either technical non specificity or contamination. At the simplest level these can be achieved as template free reactions, however more sophisticated negative controls will include nucleic acids that are likely to also be present within the sample, such as human DNA, to also assess specificity.

   Negative controls can be used to assess the whole process to provide further information on the source of false positive (e.g., cross contamination during sample handling). In this situation appropriate biological samples or synthetic surrogates can be used (e.g., blood, stock solutions), but they shall be demonstrated to be free of pathogen target nucleic acid prior to use to assess false positives.

   Additional information can be found in CLSI documents MM03[3] and MM19-A[10].

2. Positive controls

The purpose of positive controls is to assess for false negative results and evaluate the quality of the performance of the examination. Positive controls can assess the whole experimental procedure or individual sample analysis. Positive-control material may be purified nucleic acid, purified amplicons, vectors (e.g., plasmids) containing the targeted nucleic acid sequence and synthetic nucleic acid (see Table 1). Where vectors or amplified product are used they shall be diluted to a suitable concentration (≤10^7 copies/µl) prior to handling within the pre PCR setup (see Figure 1). This will reduce the likelihood of contamination from super concentrated nucleic acids.

Stock solutions containing the target pathogen can be used at an appropriate and tested dilution for use as positive control (e.g., virus diagnostic).

Concentrations of controls shall be chosen to verify test performance at relevant analytic and clinical decision points.

For qualitative procedures, a positive control (amplification or extraction) should be constructed at a concentration that is similar to the lower range of the clinical pathogen load from the sample in question.

For quantitative procedures, unless already covered by the calibration curve, a high-positive control near the upper limit of the reportable range and a low-positive control near the lower limit of detection should be used. Calibration curve can be pathogen genomic nucleic acid or equivalent (e.g., plasmids containing the targeted nucleic acid sequence) diluted serially. However, this will not evaluate the dynamic range or calibrate the whole process. Consequently it is advisable, where possible, to use the whole pathogen serially diluted in an appropriate matrix to also attempt to capture the linear dynamic range of the extraction procedure. Amplicon should not be used for standard curve construction due to potential primer dimer carry over.

If the controls are designed and made by the laboratory (specimen pool of spiked matrix, internal controls, etc.), sufficient single–use aliquots should be generated and stored to avoid repeated freeze-thaw cycles. The stability of the controls, developed by the laboratory, shall also be determined. These designed controls, shall not be used outside the determined shelf life.

Where appropriate whole process controls (e.g., controls that are capable of also assessing the effectiveness of preanalytical procedures such as extraction) should be operated. For further information see Table 1.

Extraction controls can be a batch of patient specimens containing the target nucleic acid or pathogen-free matrix spiked with the targeted pathogens. The matrix should be the same or similar to samples to be tested.

The internal and inhibition controls (i.e., a control capable of detecting the inhibition) shall mimic as much as possible the target but not be detected by the assay in question. Inhibition controls are typcially nucleic acid materials that evalute the molecular biological steps of the examination (e.g., PCR and reverse transcription).

It is advisable to use a whole pathogen where appropriate as internal control instead of only the nucleic acids. The internal control should be spiked at a known concentration in all the specimens to be tested.

The internal control should show similar characteristics regarding yield, integrity, and purity as the isolated target sequences without competing with the target nucleic acid. Additional information can be found in CLSI documents MM03[3], MM06[4] and ISO 20395[12].

Table 1 — Examples of positive and negative control material

Type of control	Control name	Typical composition	Reason for inclusion
Negative	No template control (NTC)	Water or buffer without template added to master mix	Detecting template contamination of the PCR assay at the PCR set-up stage. Detection of unintended amplification products such as amplified primer-dimers which may occur with the use of dsDNA binding dyes.
Negative	Extraction blank	Water, buffer, sample or matrix blank (as appropriate) processed alongside test sample	Detection of template contamination at the extraction stage
Negative	Reverse transcription negative (RT -ve)	RT reaction without addition of RT enzyme or with addition of denatured RT enzyme	Amplification of DNA (for RNA assay)
Negative	Specificity	DNA/RNA sample mimicking the specimen, but not containing the target template	Characterisation and monitoring of false positive rate (e.g. wild type variant when measuring mutant variant of gene)
Positive	Qualitative	Well characterised biological material or nucleic acid solution	Qualify the performance of the test run Assessing reaction specificity for genotyping assays by post-amplification melting curve analysis
Positive	Quantitative	Well characterised biological material or nucleic acid solution	As qualitative positive control; also assessing the quantitative output and efficiency of an assay or for assay calibration.
Positive	Internal Positive Control	Sample spiked with exogenous template	Confirmation that no false negative results has occurred. Can be used as individual process control (e.g. inhibition) or to evaluate the whole procedure (including extraction) depending on type of material and stage during the procedure at which it is spiked into the sample.
Source: Adapted from ISO 20395:2019			

5.1.2 Defining target sequence

The selection and qualification of nucleic acid sequences for use as amplification primers on the one hand and for use as target sequences on the other hand is a central element in the verification and validation process of nucleic acid amplification methods.

As a rule, the sensitivity of detection is determined mainly by the efficiency of primer binding to the gene target sequences. Likewise, the specificity of detection is largely dependent on the choice of the genomic region to be amplified.

The choice of the gene target shall be determined by the intended use of the test as documented in the list of requirements.

NOTE 1 Pathogen target sequences can be genomic or vector DNA, messenger RNA, ribosomal RNA or genomic RNA.

NOTE 2 For broad bacterial identification, 16 S rRNA gene and 23 S rRNA gene are routinely used, and 18 S rRNA gene and 28 S rRNA for eukaryotic pathogens.

For the multiplex assay, each target sequence should not present any homology to minimize cross reactivity.

If the target region is selected, the sequence shall be evaluated for its degree of homology with other organisms using an appropriate nucleic acid sequence database. Multiple sequence entries should be examined.

For the selection of primers, the target sequence should be known and polymorphic regions shall be avoided, unless polymorphisms are used for pathogen discrimination.

The primers should be designed according to the assay used, e.g. PCR, isothermal amplification technologies or other methods.

Design guidelines should be used, e.g. Minimum Information for Publication of Quantitative Real Time PCR Experiments[16] or Digital PCR[33] when applicable.

5.2 Verification and validation

In general, tests can be categorized into four groups:

1. IVD labeled assays/tests;
2. IVD labeled assays/tests modified by the laboratory as an unmet patient need was identified,
3. Assays/tests developed by the laboratory (LDT),
4. Commercially available research use only (RUO) tests.

Table 2 depicts possible decisions for performing verification or validation of a test.

Table 2 — Possible decisions for performing verification or validation of a test

Type of assay Performance?	What shall laboratory perform to implement assay?	
	Verification	Validation
IVD labelled assays	YES	NO
IVD labeled assays/ tests modified by the laboratory	YES	YES
Assays/tests developed by the laboratory (LDT)	YES	YES
Commercially available research use only (RUO) tests	YES	YES

6 Verification or validation of test systems

For further information see also Annex B.

6.1 Predicate assay selection by method comparison

There are two approaches to evaluate the performance characteristics of a test.

One approach consists of a comparison-of-methods study, where, specimens/samples are tested in parallel with both the developed test and a valid predicate assay.

Method comparison is applicable if a valid predicate assay is already adopted in the laboratory, or if the laboratory has access to a valid predicate assay in another clinical laboratory. It is preferred that local patient specimens/samples be used for method comparison.

As a second approach, spiked specimens/samples can be used if it is not practicable to obtain patient specimens/samples to evaluate performance characteristics, especially accuracy. Spiked specimens/samples test whether the new test can measure the analyte of interest when a known amount is present in the intended specimen matrix. Some studies might require real patient spcimens/samples depending on the risk analysis.

Spiked specimens/samples need to be well characterized and can include standards, quality control materials, proficiency testing materials, or patient specimens with known or consensus values.

7 Assay design and development of LDT

7.1 General

The planning phase of developing a LDT shall always be composed of a design phase and feasibility testing phase. During this feasibility phase, where first performance data shall be collected, troubleshooting and optimization processes shall be used to set up performance characteristics and to develop a useful validation procedure.

At the technical level, the laboratory should investigate the operational availability of necessary equipment, reagents, costs, availability of controls and reference materials needed for the assay development. Expected duration and feasibility testing for the examination procedure shall be considered for the assay design and validation.

NOTE When introducing IVD labeled assays/tests, the expected performance specifications are stated by the manufacturer. The objective of the verification plan would be to verify the test performance specifications in the specific laboratory environment.

The assay development process shall include the initial assessment (needs, intended use, test requirements), design and development planning, test development, verification, validation and the implementation into the laboratory. For further information see also ISO 20395[12].

7.1.1 Definition of customer/patient's and stakeholder needs of the intended use of the assay

The laboratory shall be aware of local regulations and ethical principles for maintaining a relationship of trust with customers, patients and the public[5].

Stakeholder needs and requirements, such as from regulatory bodies or from legislations, shall be identified and documented.

Customer/patient needs shall be identified and documented.

Collection and handling procedures as well as criteria for specimen acceptance/rejection shall be defined.

The intended use of the test, including the clinical utility, shall be validated.

The intended use should be determined by considering the following elements, including, but not limited to:

a) the purpose, benefit and use of the test (e.g., screening, diagnosis, prediction, monitoring, confirmatory);

b) the target population;

c) the use of the results in patient management,

d) specimen types,

e) collection and handling procedures,

f) criteria for specimen acceptance/rejection.

Decision shall be made whether to go on with the design and the development planning.

7.1.2 General criteria for Verification of assay

The laboratory shall verify the key performance specifications of an IVD labelled test described in the IVD manufacturer's instructions to demonstrate the performance in its own environment.For qualitative tests, positive and negative test results shall be compared to a comparable test method. For quantitative tests, there shall be a comparison of test results along the entire reportable range (accuracy) and studies of precision. In addition, the manufacturer's claims of limits of detection/ reportable range and measurement uncertainty, if applicaple, should be verified and the laboratory should verify reference intervals if indicated. For further explanation see Annex B.

If the laboratory is developing its own test (LDT) or modifying an IVD-labelled test, the laboratory shall perform a verification, establishing and demonstrating its claims of e.g. accuracy, precision, analytical sensitivity, analytical specificity (the role of interfereing substances), the reportable range, and reference intervals as clinically applicable. Data on interferreing substances can be obtained from the literature or from studies performed in other laboratories but the performing laboratory should verify the information when practicable.

The verification should occur in the laboratory where clinical testing will be performed; if a verified assay is transferred to another laboratory, the laboratory should determine that the performance characteristics have not been affected by the move or by any change in environmental conditions (temperature, humidity, etc.).

When IVD labelled tests are to be implemented in the laboratory, the performance of test conditions shall be verified.

The verification procedure (eg. verification plan, verification execution), data and the results shall be documented according to the test development process as part of the local quality management system, along with possible explanations for any discrepancies in results. The accuracy, precision, and reportable range should be reviewed and, if satisfactory, the method should be approved by the laboratory director or designee as acceptable for use as a diagnostic test prior to use in patient testing.

7.1.3 Specific criteria for verification of assay design input specifications

A risk analysis shall be performed on the potential impact of all diagnostic workflow steps on the validity and reliability of the examination.

In general, all steps of the diagnostic workflow that can have an impact on the examination result shall be specified and verified , e.g. specimen collection, transport, storage, pre-treatment, extraction, potential storage after extraction, examination steps such as amplification, data analysis, data reporting.

The risk analysis shall define the impact of any steps that cannot be controlled during the entire diagnostic workflow. Measures shall be taken to mitigate unacceptable risks identified.

The data reporting within the laboratory should be examined during the verification; if the data are further transmitted from the laboratory information system to a hospital information system, the fidelity of data transmission should be verified once the initial samples have been tested and reported.

Tests shall include appropriate controls that are capable of detecting errors in analytical steps of workflow (see 5.1).

The validation should be performed on a surrogate matrix as used in the final test and clinical samples.

Clinical samples, known to contain a defined amount of pathogens, can be diluted with appropriate target-free matrix (e.g. pooled serum samples or stool-suspensions) and samples may be spiked with the internal control.

Surrogate matrix should be spiked with the whole pathogen and the internal control.

Contamination or the introduction of interfering substances should be avoided. Constant control of potential contamination and/or interfering substances should be integrated to the test system.

For the multiplex assays, the assay should be validated in the multiplex setting it is intended to be used in, e.g. several pathogens and/or pathogen and control.

7.1.4 Validation of intended use

In accordance with the validation plan, the laboratory shall describe and document relevant performance characteristics of the test in relation to the disease or other clinical context assessed by the test.

The laboratory shall identify those test characteristics that are critically associated with the clinical relevance and clinical utility of the test. These characteristics shall be documented as clinical performance characteristics.

To measure whether the test identifies and/or quantifies the analyte in a clinically relevant way, the negative predictive value and the positive predictive value should be calculated, if appropriate.

NOTE 1 Typical clinical performance characteristics include clinical (diagnostic) specificity, clinical (diagnostic) sensitivity, measuring range and the reference interval.

The laboratory shall perform validation of the intended use of the test in comparison to an existing predicate device, if available. If a predicate device is not available, the laboratory shall validate the intended use by appropriately designed studies.

The laboratory shall clearly document the conclusions of the validation in a validation report.

For further information see 7.4.1.1 and Annex B.

7.2 Diagnostic workflow analysis for Nucleic NAAT procedure

Confidence in test results are of main importance in the fields of human diagnostics because of psychosocial, medicolegal and therapeutic consequences to patients.

Therefor screening of the test method or initial tests are indicated to detect the presence of a target organism.

Typical nucleic acid amplification-based assays will consist of a combination of steps that should include:

— Sample collection and transportation and storage

— Nucleic acid extraction and purification

— Amplification (Denaturation, Annealing, Extension)

— Detection targeting orthogonal genes

— Data analysis and interpretation.

The crucial challenge during all analytical steps of NA amplification-based assays is avoiding carryover product contamination.

7.2.1 Pre-analytical workflow requirements

Requirements for specimen collection, transport, and storage shall be specified in the instructions for use. The requirements of ISO 15189 apply.

NOTE Guidelines for “Collection, Transports, Preparation and Storage of specimens for Molecular methods” are also found in CLSI MM13[6].

Attention shall be given to potential effects of specimen collection, transport, and storage on the steps required for preparing the specimen for nucleic acid extraction.

EXAMPLES Specimen type, specimen container, criteria for specimen acceptability, specimen handling procedure to minimize changes due to nucleic acid degradation or contamination, amount required, additives required, transport conditions, storage conditions, stability factors and precautions.

The medical laboratory shall incorporate the requirements for specimen collection, transport, and storage as instructions in corresponding sections of the sample collection manual.

The specimen for NA amplification-based assay should be received and accessioned in an area that is isolated from testing area. Patient samples should be processed promptly and stored appropriately to minimize degradation of NA. Only standardised specimen containers should be accepted by the laboratory. For further information see Annex A.

Repeated freezing and thawing may result in loss of sample integrity and lower yields of NA and therefore should be avoided, if not specified and verified otherwise. Quantitative assays and detection of low target levels may be particularly affected CLSI MM19A[10].

Conditions that ensure adequate preparation and stability of the nucleic acid after nucleic acid extraction shall be specified, verified and documented in the instructions for use. The sample stability may differ when different sample types are used in tests under same conditions. Therefore the stability of each sample type needs to be tested in the given assay. Novel pathogens and unusual syndromes normally need additional studies to determine best of specimen type and volume of specimen to be used in the assay. For further information see Annex A and CLSI MM03[3].

The purity, integrity and yield of nucleic acid extracted from the sample shall be adequate for the intended uses. If sufficient nucleic acid is not present in the sample, the extraction shall be repeated using the same sample or another sample shall be collected for extraction.

For further information see Annex A and further information on nucleic acid preparation and stability is given in ISO 21571[7]. Methods for evaluating extraction efficiency are also available in ISO 20395[12][14][15].

The laboratory shall prepare and store the nucleic acid extract according to the instructions for use to ensure that the purity, integrity-and-stability are adequate to perform the examination.

7.2.2 Analytical workflow requirements

The laboratory procedures shall include at a minimum the following precautions, where applicable, to reduce the likelihood of contamination. Requirements to use separate equipment and supplies do not apply if sample preparation, amplification and detection are performed on the newer automated instruments.

a) Personal Protective Equipment (PPE) shall be used in accordance with the level of biosafety (see EP23-A[1] and EP18-A2[8]; ISO 35001[9]:

   — Dedicated laboratory coats (e.g. color-coded) should be used for the specific areas. Laboratory coats should be operator specific, should be changed when entering or leaving each area, should be changed regularly, and specific cleaning requirements should be considered. Disposable laboratory coats can also be considered. Individual hangers should be available for the lab coats that should not be overlaid.

   — Change gloves between each separate area, or additionally as appropriate to prevent cross-contamination. Long-sleeve gloves should be considered and latex free gloves are preferable to prevent allergies.

   — The operator shall wear goggles, face mask, or protecting shield, or other PPE required by the local biosafety regulations.

— Overshoes or washable laboratory shoes should be considered in different rooms where required.

b) Separate equipment (including robotics) and appropriate supplies such as filtered pipette tips for reagent preparation, specimen preparation, and post-amplification analysis shall be used.

c) To minimize cross-contamination between the samples, pulse spin is recommended for all the samples before opening of any PCR reaction tube, if the test requires this.

d) The sample or nucleic acid extract should be added last into the reaction tube after the non-sample components (such as master mix, dNTPs, primers, buffer and enzymes) in a separate area or room dedicated to template addition. (see Figure 1).

e) Keep reagent tubes capped unless in use for post-amplification manipulation of PCR amplicons (e.g., gel electrophoresis or DNA sequencing). Uncap tubes as seldom as possible; uncap only the required samples. Pulse spin PCR reaction tubes before uncapping.

f) It is recommended that transport of equipment (including IT equipment) between different working areas should be avoided. Portable items such as pipets, gloves, pens, lab books, and timers are a source of contamination and shall not be moved between working areas and shall be separately available in each working area. Furthermore, this applies to Standard Operating Procedures and other printed and writing materials such as worksheets. Any transfer of samples and assay-related material should be uni-directional (from less contaminated to most contaminated) and kept to a minimum (see Figure 1).

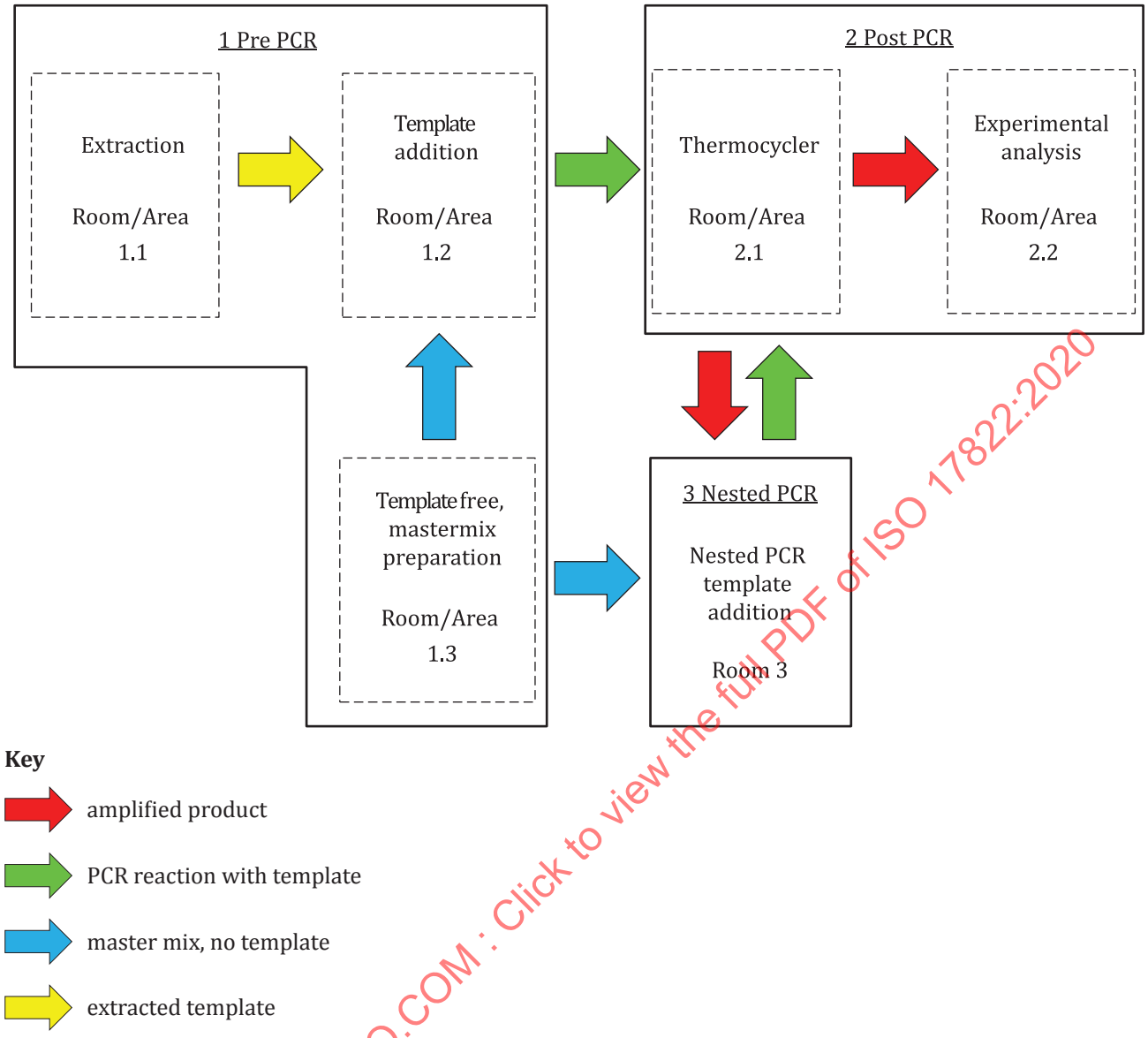

Figure 1 — Example of a unidirectional workflow by using separate rooms or working areas

A minimum of two (or where nested PCR is performed three) separate rooms shall be used for Pre PCR, Post PCR and (where necessary) Nested PCR. The respective workflow steps (indicated by dotted lines) shall be separated by dedicated areas or ideally specific rooms.

Refrigerator and freezers should not be shared among the reagent and sample preparation room/areas to avoid the storage of samples and nuclei c acid isolates at the same place as reagents. If it is needed to share them clear separation should be managed (e.g. separated in secondary containers) CLSI MM19A[10]. Pre and post amplification reagents and nucleic acids (e.g., sample and PCR products) shall be stored in separate refrigerators or freezers located in separate locations, ideally in the respective rooms/areas.

7.2.3 Post-analytical workflow requirements

This last workflow step is dedicated to the interpretation of test results of the analyzed sample and preparation of test reports. These laboratory reports may be supported by a LIS, that should be programmed to allow the use of free text and prewritten report templates.

The requirements of the test reports should meet the legal specification and should be integrated into the QMS of the laboratory. The requirements of the reporting shall be characterized in an SOP or laboratory manual.

7.3 Verification and validation performance characteristics

7.3.1 Range of detection

Laboratories shall quantitatively report only results that fall within the measurement range that has been established during the verification or validation.

The determination of the linear range within a verification study does generally not apply to qualitative NAT methods. However, concept elements can apply for semi-quantitative tests that are reported qualitatively.

The lower limit of the linear range shall be clinically relevant and acceptable for clinical use.

The upper limit of linear range should also be clinical relevant if feasible.

If a result is above the upper limit of linear range; the specimen/sample may need to be diluted in the appropriate buffer or matrix. Laboratories shall consider that weak precision will affect the assumed linearity of a test. Therefore, laboratories shall involve precision studies as part of the linearity study.

Additional replicates can be tested at the low end in order to adequately determine the lower limit of quantification.

For further detailed information see also Annex B.

Further more detailed technical guidance on NAAT validation can be found in ISO 20395[12] and CLSI MM 17[41].

To establish a linear range for laboratory-developed tests, it is recommended that laboratories use 5 to 10 concentrations across the anticipated measuring range.

To ascertain the widest possible linear range, additional samples at concentrations wider than the anticipated measuring range can be used in the same run. Although laboratories can use linear regression analysis for the data analysis if the relationship between assigned and observed values are clearly linear, log transformation PCR-based data Cq, Ct and Cp) shall be preferred.

NOTE 1 Log transformation consists of taking the log (generally base 10) of each observed value.

Laboratories shall quantitatively report only results that fall within the linear range. ISO 20395[12] gives further guidance on linearity for NAAT.

NOTE 2 Polynomial regression analysis is performed to find the polynomial function that adequately fits the data points and to assess whether there is a significant difference between linear and quadratic fits. First-, second- and third-order polynomial regression analyses can be useful.

7.3.2 Test accuracy (Trueness and Precision)

Different measures of diagnostic accuracy relate to the different aspects of diagnostic procedure: while some measures are used to assess the discriminative property of the test, others are used to assess its predictive ability. Measures of diagnostic accuracy are not fixed indicators of a test performance, some are very sensitive to the disease prevalence, while others to the spectrum and definition of the disease. Furthermore, measures of diagnostic accuracy are extremely sensitive to the design of the study. Studies not meeting strict methodological standards usually over- or under-estimate the indicators of test performance as well as they limit the applicability of the results of the study[35].

Accuracy of a test procedure expresses the closeness of agreement between the value found and the value that is accepted either as a conventional true value or an accepted reference value. The closeness

of agreement observed is the result of the systematic error (bias, expressed as trueness) and the random error (expressed by the precision of measurement).

7.3.2.1 Trueness studies

Laboratories should spread the testing over a length of time that reflects test performance under typical laboratory conditions.

Trueness studies should be conducted in accordance with the study design, statistical recommendations, and ISO 15189.

For multiplex assays, several comparative studies should be used if none of the assays in routine use covers all of the analytes of the multiplex assay. The appropriate data analysis method to establish trueness should be chosen for each individual analyte as well as for the overall multiplex assay.

The laboratory should determine and test an appropriate number of specimens.

NOTE The appropriate number of specimens per assay depends on factors such as complexity and precision of the assay, the prevalence of the target in the indicated population, the established accuracy of the reference/comparative method, the scheme used for statistical data analysis as well as the acceptable level of statistical confidence, and costs. It is recommended to test no fewer than 20 and typically 40 to 50 or more specimens.

The accuracy portion of the verification should be performed ideally on clinical samples, with an adequate number and a sample distribution representative of the clinical sample types to be tested (e.g., blood, plasma, stool, body fluids). If these are not available through an entire analytical or reporting range, clinical samples or contrived samples, known to contain a defined amount of pathogens, can be diluted with appropriate target-free matrix (e.g. pooled serum samples or stool-suspensions) and these samples may be spiked with the internal control before testing.

For defining the trueness of a test, laboratories shall interpret the obtained data graphically as well as statistically.

Both scatter diagrams with correlation and regression analysis and difference plots with calculation of 95 % limits of agreement for evaluation of method comparison data should be used.

7.3.2.2 Precision studies

Studies to determine test precision shall be done using samples with known analyte concentration, where appropriate. The samples shall be selected or prepared in a matrix as close as possible to the appropriate clinical specimens.

Test materials could include standards, quality control materials, proficiency testing samples, or patient specimens in sufficient quantity to complete the study.

Precision studies shall test the repeatability, intermediate precision and reproducibility if applicable.

Repeatability will be tested by assessing replicates of the same test material using the same method, the same equipment and by the same operator in the same laboratory over a short period of time. It can also be referred as within –batch/run or intra-assay precision.

Intermediate precision will be tested by assessing replicates of the same test material using the same method, the same equipment and by the same operator in the same laboratory over an extended period of time.

Reproducibility will be tested by assessing replicates of the same test material in different laboratories using the same method by different operators and/or different equipment. It is also known as “inter-laboratory reproducibility”.

For the precision study, the laboratory shall define an adequate length of study time and a number of specimens as appropriate for the intended use of the test.

Precision studies should be conducted in accordance with the study design, statistical recommendations, and ISO 15189.

Laboratories should consider and incorporate possibly significant sources of variation, such as different operators, multiple reagent lots, multiple instruments, into the precision study design.

For qualitative tests, the precision study shall provide an estimate of the imprecision of the method at analyte concentrations near the limit of detection.

Testing of samples at appropriate concentrations across the dynamic range of the method should be incorporated in the study design: One with an analyte concentration at the limit of detection, one with a concentration 20 % above the limit of detection, and one with a concentration 20 % below the limit of detection. The three samples could be tested in replicates up to 40.

For quantitative tests, the laboratory shall at least include a high-level sample, a low-level sample, and a sample as close as possible to the medical decision level (usually the limit of detection) in the precision study. Higher levels of variability will generally be at the low and high ends of the measuring range. If there are large differences in the precision estimates at the three levels, it can be necessary to test additional concentrations to fully describe the precision performance of the assay.

It is recommended that the precision study design will allow calculation of inter- and intra-run variation, which can then be combined to determine the total variation of the assay.

Test precision shall be expressed on the base of statistical measurements of imprecision, such as the standard deviation (SD) or coefficient of variation (CV).

NOTE Coefficient of variation (CV) is generally expressed in terms of the signal or measurement (true) value that will produce estimates having a specified coefficient of variation (CV, calculated as s\x-quer x 100 %).

For data analysis, results of the precision study shall be evaluated by the laboratory against documented acceptance criteria.

Imprecision is often expressed as the target value plus or minus two or three SDs or the target value plus or minus a percentage (e.g., target 10 %). In general, the precision around the mean value should not exceed 15 % of the CV, except at the LLOQ, where precision should not exceed 20 %. In determining acceptable performance of precision, it is recommended to construct precision profiles in which the SD or CV is plotted as a function of analyte concentration.

7.4 Analytical sensitivity / limit of detection

Laboratories shall determine the LOD for both quantitative and qualitative tests as the lowest actual concentration of an analyte in a specimen that can be consistently detected with acceptable precision. When determining analytical specificity, the laboratory shall perform cross-reactivity and interference studies.

Cross-reactive organisms include organisms which share sequence homology with the target,normal flora organisms that could concurrently be present in the specimen, and organisms that cause similar disease states or clinically relevant co-infections.

The laboratory shall conduct interference studies by analyzing the effect of potentially interfering substances added to specimens containing the nucleic acid of interest using the test method (interference screen).

Interference/cross-reactivity shall be established for each specimen type used in the test system, using potentially interfering material appropriate for the specimen matrix.

The data analysis for defining assay specificity shall follow the common statistical approaches such as paired t-test, repeated-measures test, or paired-difference test. Analysis is based on the difference

between the means of the test and control samples and the allowable error that is clinically significant for the test.

NOTE For quantitative tests, it is possible for the LOD to equal the (LOQ). This is the case if the observed bias and imprecision at the LOD meet the requirements for total error for the analyte. Most often, however, the LOD resides below the linear range of an assay and is lower than the LOQ. The LOD cannot be higher than the LOQ.

Laboratories shall determine the LOD of the test method empirically by testing serial dilutions of samples with a known concentration of the target substance in the analytical range of the expected detection limit.

Analytical sensitivity shall be determined for each type of specimen matrix that will be routinely tested in the clinical laboratory.

Laboratories shall also validate the analytical sensitivity per genotype, when reasonable.

For defining the limit of detection, laboratories shall test a statistically appropriate number of samples.

For data analysis, laboratories should use a probit analysis for empirically determining the lowest concentration of target that can be reliably detected by molecular assays.

Probit regression analysis results in probit (probability unit) values, which are converted to C95 values (concentration detectable in 95 % of the samples), indicating that the limit of detection is a definite number of copies/ml and that samples containing that concentration would be detected in 95 % of the samples tested.

For the multiplex assays, the LOD shall be determined for each analyte using the multiplex assays.

7.4.1 Validation of assay

7.4.1.1 Validation of study report

After collecting all the necessary validation data, a validation summary report shall be written by the laboratory.

This report shall include a summary of the main requirements, a complete description of the method used, a clear description of the validation study, and all the detailed analyzed results obtained during the validation tests.

This document shall record all the different steps of the workflow that was validated (e.g., pre-examination step), the reagents and conditions used and the operators involved in the study. This document will include the acceptance/rejection criteria, details concerning the incurred sample repeat/reanalysis and some information on the clinical interpretation of a test result.

Literature references shall be given to complement the local validation study results and to fill potential gaps for which direct evidence could be lacking.

Once completed, the report shall be assessed in line with the laboratory quality management system.

Finally, the laboratory director shall declare with signature and date that the test is (or is not) ready for routine clinical implementation.

NOTE 1 In certain countries, legal obligations can exist for the laboratory declaration.

NOTE 2 Guidance related to the declaration for both manufacturers and laboratories can be found in ISO/IEC 17050-1 and ISO/IEC 17050-2.

Validation of a test is a one-time process as long as the conditions under which the method was developed have not changed. Revalidation shall be performed by the laboratory if the existing method is modified in any way.

NOTE 3 Modifications can include the addition of new sample types or changes in a critical component or reagent that can affect the test performance.

7.4.1.2 Partial Validation

A partial validation can be performed when minor changes (depending on the change), that do not change the principle of the test method, were made to a method that already was fully verified. For example, if there is a change of collection device or container to the same sample type, which is not expected to have major impact on the sample itself, in such case, only a minimal validation involving testing a limited number of samples (e.g. 5 – 10) for accuracy and precision is required.

8 Implementation and use in the laboratory

Once the verification/validation process is completed, the implementation requires integrating the test into the workflow and into the quality management system of the laboratory.

A complete standard operating procedure for the routine operation of the examination should be written.

A medical laboratory that implements a validated nucleic acid-based IVD examination procedure without modification shall verify its performance before introduced into routine use.

Subsequent modifications to a validated examination process shall be validated. The requirements of ISO 15189 apply.

Before implementing a test, the laboratory should have a procedure for regular quality control and quality assurance in place (compare Table 1 and Table 2).

Quality requirements for training and competence assessment of personnel regarding shall be fulfilled by the laboratory before implementation of the newly procedure.

Adequate advisory services concerning the medical use of the newly introduced test should be provided to clinicians and other customers of the laboratory.

9 Reporting and interpretation of results

Appropriate procedures shall be implemented to ensure the timely reporting of results. Results of quantitative molecular assays should be typically reported as number of molecules (copies, genome equivalents, International Units) of the targeted nucleic acid in a defined volume of a body fluid, number of cells, or mass of tissue.

Where applicable it is recommended, that Log_{10}-transformed data should be used for reporting results from quantitative molecular tests.

The detection range of procedure should be reported along with a clear description of the units used (e.g. copies per ml of plasma).

EXAMPLE Not detected' or 'Less than the detection limit'.

Results of qualitative tests shall not be presented as "Positive" or "Negative" which could be subject to interpretation but as "Detected" or "Not detected"; "Presence" or "Absence".

The laboratory shall implement and maintain a documented procedure for the interpretation of results that ensures that results have been reviewed and authorized by qualified staff before release. The laboratory shall have a standardized procedure of tools and parameters for data analysis.

For indeterminate results, the laboratory shall have a procedure for retesting by the same or another method and/or for requesting an additional or different specimen.

Established cut-off values for analyte detection and for clinical outcomes shall be used as interpretive comments of the report.

For quantitative assays and where established cut-off values are not available, the laboratory shall indicate the validated linear range of the assay including the limit of detection and the limit of quantification in the report.

The laboratory shall define critical results for all tests that significantly impact patient management decisions and shall have a documented procedure for the notification of relevant clinical personnel, when critical results are obtained.

Any known clinically significant limitations of the test shall be indicated in the report.

NOTE For example, limitations could include cross-reactions, genotype bias and the presence of interfering substances ensuring quality of examination results.

Protocols (IVD manufacturers or LDT) shall be adhered to. Any intentional or unintentional deviations from the instructions/protocol (e.g incorrect centrifugal force during centrifugation or additional freeze-thaw cycle due to freezer failure) shall be documented and procedure for accepting associated result detailed.

10 Quality assurance procedures

Appropriate quality assurance procedures shall be implemented to ensure the quality of nucleic acid-based examination results. The requirements of ISO 15189 apply.

In particular, the quality assurance procedures shall be designed to minimize false-positive and false-negative results.

Once validated, the assay performance shall be monitored by the laboratory at regular interval during its use. It should include specific monitoring of the performance of the reagent, equipment and of the operators using various control materials. The data used during these on-going performance monitoring test shall be documented.

10.1 Performance monitoring and optimization of the assay

For the internal monitoring of the performance the laboratory shall decide on the appropriate type and frequency of use of internal and external controls to be included for both the validation process and for the routine testing process after validation. The evaluation of such controls shall be done regularly. The results shall remain within preset limits.

Internal controls should be added to the primary sample matrix (e.g. serum, plasma, culture media), and extracted, amplified, analyzed, and detected independently of the target. The internal control shall be processed in the same tube with the target.

The need to monitor the evolution and updates to genome databases especially when new findings influence test performance specifications or result interpretation is indicated.

While using internal controls is recommended; the impact of the addition of the internal control assay on the performance of the target assay shall be assessed to minimize the competition.

Ongoing performance of the parameters of the test should be checked on a regular basis. The data of this ongoing monitoring shall be interpreted and documented.

10.2 Inter-laboratory comparison

As part of the continuous quality assurance program, the laboratory shall regularly participate at appropriate external quality assessment schemes.

When external schemes are not available, the laboratory should implement internal proficiency testing programs on a regular basis.

See ISO 15189.

Before participation, the laboratory shall evaluate carefully the scope and objective of the EQA scheme as well as the method used in the scheme for identification and/or quantification of the analyte.

Internally organized or external proficiency testing programs shall be performed at least once a year.

It is recommended to perform the interlaboratory comparison program at least twice a year.

External quality controls shall be tested in the same assay run as patient samples but shall not be in the same tube or well as the sample.

Data obtained from external controls shall be evaluated statistically to monitor the test performance over time.

NOTE For example, Levey-Jennings plots of log10-transformed external control data can be used to assess test performance over time.

Annex A
(informative)

Pre-analytical consideration for sample preparation

A.1 Serum and plasma

A.1.1 Sample collection

For serum, peripheral blood is drawn using a vacuum blood collection tube that contains a serum separating gel. The tube is left at room temperature for one hour until coagulation is completed and immediately centrifuged. Coagulation accelerator, added to the collection tube, may reduce the time required for coagulation.

For plasma, EDTA-containing vacuum tubes should be used. It is recommended not to share blood specimens with those for cellular immunology or chromosomal analyses, for which blood specimens are collected in heparinized tubes.

When blood is drawn through an IVH catheter, the first several millilitres should be discarded due to the potential effect of heparin, and blood drawn after that should be used as the specimen.

Separated plasma, for which ACD (acid citrate dextrose) or CPD (citrate phosphate dextrose) are used, can also be used for nucleic acid amplification tests similarly to those treated with EDTA.

EDTA tubes are immediately and repeatedly inverted and mixed following vacuum blood collection in order to prevent blood coagulation from gradually progressing to fibrin precipitation. The collection tube that contains a reagent for neutralizing heparin should not be used, because of its inhibitory effects on nucleic acid amplification.

Formation of sediments, discoloration, and dehydration due to freezing is inappropriate and unsuitable conditions for testing. Possible causes include inappropriate storage conditions, such as long-term exposure to high temperature and repeated cycles of freeze and thaw. If the extracted nucleic acids have been appropriately stored, they can be used for re-testing. The number of freeze and thaw cycles should be minimized and recorded.

A.1.2 Sample storage and transport

It is considered that virus specimens are relatively stable in serum and plasma. After separation, serum and plasma specimens can be stored under refrigeration (2 °C to 8 °C). Some IVD manufactureres were able to demonstrate that for example the serum and plasma specimen can be stored for up to 7 days at 2 °C to 8 °C.

Freeze preservation is recommended for long-term storage. For example, specimens for DNA measurement should be stored at –20 °C or below for HBV and at –70 °C or below for hepatitis virus A (HAV), HCV, and hepatitis virus E (HEV).

Transferring the specimen into another container should be avoided. When specimens are not going to be tested immediately after centrifugation, the separated serum in the original blood collection tube should be directly frozen (–20 °C or below) and stored until use. For the hepatitis virus C (HCV) and the human immunodeficiency virus (HIV) it is recommended that the serum or plasma should be separated within the time 6 hours after blood collection, and for hepatitis virus B (HBV) it is best performed within 24 hours.