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In vitro diagnostic test systems — Requirements and recommendations for detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by nucleic acid amplification methods

Systèmes d'essai pour diagnostic in vitro — Exigences et recommandations pour la détection du coronavirus 2 associé au syndrome respiratoire aigu sévère (SARS-CoV-2) par des méthodes d'amplification des acides nucléiques

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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).

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This document was prepared by Technical Committee ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems*, in collaboration with Technical Committee ISO/TC 276, *Biotechnology*.

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.

Introduction

Coronaviruses are enveloped RNA viruses that are broadly distributed in the animal kingdom. They have been identified in humans, other mammals, and birds. Coronaviruses were named because the spike proteins known to facilitate viral attachment and cell entry appear like a halo on the virus surface when viewed under an electron microscope. Coronaviruses are roughly spherical with a diameter ranging from 118 nm to 136 nm. The coronavirus genome, which ranges from 26 kb to 32 kb, is the largest among all RNA viruses, including RNA viruses that have segmented genomes. Until 2019, six coronaviruses have been associated with human diseases:

— severe acute respiratory syndrome-related coronavirus (SARS-CoV),

— Middle East respiratory syndrome coronavirus (MERS-CoV),

— human coronavirus 229E (HCoV-229E),

— human coronavirus OC43 (HCoV-OC43),

— human coronavirus NL63 (HCoV-NL63), and

— human coronavirus HKU1 (HCoV-HKU1)[1].

In 2019, a cluster of patients presenting with a respiratory disease were shown, by sequencing, to be infected with a novel coronavirus[2]. The coronavirus associated with this cluster was subsequently named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses[3]. SARS-CoV-2 is the seventh coronavirus known to infect humans. The disease caused by SARS-CoV-2 was designated as coronavirus infectious disease 2019 (COVID-19) by the World Health Organization (WHO)[4].

The host range for SARS-CoV-2 is not yet fully defined. SARS-CoV-2 is a beta-coronavirus. The receptor for SARS-CoV-2 is the angiotensin-converting enzyme 2 (ACE2). ACE2 is a cell-surface, zinc-binding carboxypeptidase involved in regulation of cardiac function and blood pressure. It is expressed in epithelial cells of the lung and the small intestine, which are the primary targets of SARS-CoV-2, as well as the heart, kidney, and other tissues.

SARS-CoV-2 replicates in the upper and lower respiratory tracts and is transmitted by droplets and aerosols and most likely other contact with asymptomatic and symptomatic infected persons. The basic reproduction number (R_0) of the original variant is between 2 and 3, but significantly more contagious variants have developed. The median incubation period is 5,7 (range 2 to 14) days[5]. Similarly to SARS and MERS, superspreading events have been reported, with a dispersion parameter (kappa) estimated at 0,1. Most infections are uncomplicated, and 5 % to 10 % of patients are hospitalized mainly due to pneumonia with severe inflammation. However, complications include respiratory and multiorgan failures. Risk factors for the complicated disease increase with age and include hypertension, diabetes, chronic cardiovascular and chronic pulmonary diseases, and immunodeficiency.

Clinical management of COVID-19 and control of infections and spread of SARS-CoV-2 require effective and efficient in vitro diagnostics. There are a number of tests and kits in use for the detection of SARS-CoV-2 and the number of methods will continue to increase. Acceptable design, development and establishment of quality SARS-CoV-2 diagnostics based on nucleic acid detection methods is critical to ensure COVID-19 infection control. Establishing indices for conducting comprehensive quality evaluation of these methods and kits both during development and in routine application will ensure the accuracy of the test results and support epidemic prevention and control. This document provides requirements and recommendations to consider for the quality practice of SARS-CoV-2 nucleic acid amplification methods.

In vitro diagnostic test systems — Requirements and recommendations for detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by nucleic acid amplification methods

1 Scope

This document provides requirements and recommendations for the design, development, verification, validation and implementation of analytical tests for detecting the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) using nucleic acid amplification. It addresses pre-examination, examination and post-examination process steps for human specimens.

This document is applicable to medical laboratories. It is also intended to be used by in vitro diagnostic developers and manufacturers, as well as by institutions and organizations supporting SARS-CoV-2 research and diagnostics.

This document does not apply to environmental samples.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

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

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

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

3.1
severe acute respiratory syndrome coronavirus 2
SARS-CoV-2
virus that causes coronavirus infectious disease 2019 (COVID-19)

3.2
specimen
primary sample
discrete portion of a body fluid, breath, hair or tissue taken for examination, study or analysis of one or more quantities or properties assumed to apply for the whole

Note 1 to entry: The Global Harmonisation Task Force (GHTF) uses the term specimen in its harmonized guidance documents to mean a sample of biological origin intended for examination by a medical laboratory.

Note 2 to entry: In some countries, the term “specimen” is used instead of “primary sample” (or a subsample of it), which is the sample prepared for sending to, or as received by, the laboratory and which is intended for examination.

[SOURCE: ISO 15189:2012, 3.16[6] modified — Note 2 to entry was removed and Note 3 to entry was renumbered as Note 2 to entry.]

3.3
sample
one or more parts taken from a *primary sample* (3.2)

EXAMPLE A volume of serum taken from a larger volume of serum.

[SOURCE: ISO 15189:2012, 3.24[6]]

3.4
reverse transcription
RT
process of making complementary DNA [*cDNA* (3.6)] from an *RNA* (3.20) *template* (3.22), 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[7], modified — Replaced "DNA" with "complementary DNA (cDNA)".]

3.5
deoxyribonucleic acid
DNA
polymer of deoxyribonucleotides occurring in a double-stranded (dsDNA) or single-stranded (ssDNA) form

[SOURCE: ISO 22174:2005, 3.1.2[8]]

3.6
complementary DNA
cDNA
single-stranded *DNA* (3.5), complementary to a given *RNA* (3.20) and synthesised in the presence of reverse transcriptase to serve as a *template* (3.22) for *DNA* amplification

[SOURCE: ISO 20395:2019, 3.5[9]]

3.7
analytical specificity
capability of a measuring system, using a specified measurement procedure, to provide measurement results for one or more measurands 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).

[SOURCE: ISO 18113-1:2009, A.3.4[10]]

3.8
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 0,05, given a probability of 0,05 of falsely claiming its presence

[SOURCE: ISO/IEC Guide 99:2007, 4.18[11], modified — "β, given a probability α" was replaced by "0,05, given a probability of 0,05" and Notes 1 to 3 to entry were deleted.]

3.9
verification
provision of objective evidence that a given item fulfils specified requirements

[SOURCE: ISO/IEC Guide 99:2007, 2.44[11], modified — EXAMPLES 1 to 3 and Notes 1 to 6 to entry were deleted.]

3.10
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 word "validated" is used to designate the corresponding status.

[SOURCE: ISO 9000:2015, 3.8.13[12], modified — Notes 1 and 3 to entry were deleted and Note 2 to entry was renamed Note 1 to entry.]

3.11
amplicon
specific *DNA* (3.5) fragment produced by a DNA-amplification technology, such as the *polymerase chain reaction (PCR)* (3.12)

[SOURCE: ISO 13495:2013, 3.3.1[13]]

3.12
polymerase chain reaction
PCR
enzymatic procedure which allows in vitro amplification of *DNA* (3.5) or *RNA* (3.20)

[SOURCE: ISO 22174:2005, 3.4.1[8], modified — "or RNA" added to the end of the definition and "in vitro" has been unitalicized in accordance with the ISO House Style.]

3.13
reference material
material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in measurement or in examination of nominal properties

[SOURCE: ISO/IEC Guide 99:2007, 5.13[11], modified — Notes 1 to 8 to entry and EXAMPLES 1 to 5 were deleted.]

3.14
pseudo-virus
virus or virus-like particle that can integrate the envelope glycoprotein of another virus to form a virus with an exogenous viral envelope, and the genome retains the characteristics of the retrovirus itself

3.15
digital PCR
dPCR
procedure in which nucleic acid *templates* (3.22) are distributed across multiple partitions of nominally equivalent volume, such that some partitions contain *template* and others do not, followed by *PCR* (3.12) amplification of target sequences and detection of specific *PCR* products, providing a count of the number of partitions with a positive and negative signal for the target template

Note 1 to entry: Nucleic acid target sequences are assumed to be randomly and independently distributed across the partitions during the partitioning process.

Note 2 to entry: The count of positive and negative partitions is normally based on end point detection of *PCR* products following thermal cycling, however real-time *qPCR* (3.16) monitoring of *PCR* product accumulation is additionally possible for some dPCR platforms.

[SOURCE: ISO 20395:2019, 3.10[9]]

3.16
quantitative real-time PCR
qPCR
enzymatic procedure which combines the in vitro amplification of specific *DNA* (3.5) or *RNA* (3.20) segments with the detection and quantification of specific *PCR* (3.12) products during the amplification process

Note 1 to entry: While the *PCR* is producing copies of the relevant *DNA* sequence, the fluorescent marker fluoresces in direct proportion to the amount of *DNA* present, which can theoretically be back-calculated to infer the original amount of that particular *DNA* present in a *sample* (3.3) prior to initiation of *PCR*.

[SOURCE: ISO 20395:2019, 3.25[9], modified — "RNA" was added.]

3.17
quantification cycle
C_q
quantitative real-time PCR (qPCR) (3.16) cycle at which the fluorescence from the reaction crosses a specified threshold level at which the signal can be distinguished from background levels

Note 1 to entry: Quantification cycle is a generic term which includes cycle threshold (C_t), crossing point (C_p), take off point and all other instrument specific terms referring to the fractional cycle which is proportional to the concentration of target in the qPCR assay.

Note 2 to entry: The quantification cycle is based either on a threshold applied to all *samples* (3.3) or on a regression analysis of the signal, for each sample.

Note 3 to entry: The quantification cycle is a measure with poor reproducibility and cannot be used when comparing kit performance.

Note 4 to entry: Laboratory based considerations sometimes lead to selection of a cut-off for the cycle number. The cut-off cannot be chosen not to have a detrimental influence on available *limit of detection* (3.8).

Note 5 to entry: C_q does not apply for *digital PCR* (3.15) and isothermal amplification methods.

[SOURCE: ISO 20395:2019, 3.8[9], modified — Notes 3 to 5 to entry have been added.]

3.18
clinical specificity
diagnostic specificity
ability of an in vitro diagnostic examination procedure to recognize the absence of a target marker associated with a particular disease or condition

Note 1 to entry: Also defined as "percent negativity" in *samples* (3.3) 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* 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[10]]

3.19
clinical sensitivity
diagnostic sensitivity
ability of an in vitro diagnostic examination procedure to identify the presence of a target marker associated with a particular disease or condition

Note 1 to entry: Also defined as "percent positivity" in *samples* (3.3) 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 (FN), or 100 × TP/(TP + FN). This calculation is based on a study design where only one sample 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[10]]

3.20
ribonucleic acid
RNA
polymer of ribonucleotides occurring in a double-stranded or single-stranded form

[SOURCE: ISO 22174:2005, 3.1.3[8]]

3.21
calibrator
measurement standard used for calibration

[SOURCE: ISO 20395:2019, 3.4[9], modified — Note 1 to entry and the EXAMPLE were deleted.]

3.22
template
strand of *DNA* (3.5) or *RNA* (3.21) that specifies the base sequence of a newly synthesized strand of *DNA* or *RNA*, the two strands being complementary

[SOURCE: ISO 16577:2016, 3.206[7]]

3.23
saliva
whole saliva
bio-fluid of the mouth composed mainly of secretion originating from the three major salivary glands (parotids, submandibular and sublingual glands) and from salivary glands present in the oral cavity

[SOURCE: ISO 4307:2021, 3.15[14]]

3.24
reverse transcription polymerase chain reaction
RT-PCR
process that combines *RT* (3.4) and *PCR* (3.12) to allow amplification of *cDNA* (3.6) target as a route to detect *RNA* (3.20) *templates* (3.22)

Note 1 to entry: This can be conducted using various formats. A popular approach uses real time *PCR* instrumentation which simultaneously conducts the *PCR* and the analysis; this is described as reverse transcription quantitative PCR [RT-*qPCR* (3.16)].

Note 2 to entry: Adapted from ISO 20395:2019, 3.31[9].

3.25
in vitro diagnostic medical device
IVD medical device
device, whether used alone or in combination, intended by the manufacturer for the in vitro examination of *specimens* (3.2) derived from the human body solely or principally to provide information for diagnostic, monitoring or compatibility purposes and including reagents, *calibrators* (3.21), control materials, specimen receptacles, software, and related instruments or apparatus or other articles

[SOURCE: ISO 17511:2020, 3.21[15]]

3.26
pre-examination processes
processes that include preparation and identification of the patient, collection of the primary *specimen(s)* (3.2), transportation to and within the medical laboratory, and isolation of *RNA* (3.20)

Note 1 to entry: Pre-analysis or pre-analytics are synonymous with pre-examination.

Note 2 to entry: Adapted from ISO 15189:2012, 3.15[6].

3.27
limit of quantification
LOQ
lowest concentration or content of the analyte of interest per defined amount of *matrix* (3.31) 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 relative standard deviation (RSD).

[SOURCE: ISO 16577:2016, 3.91[7]]

3.28
positive PCR control
reliable source of well-characterized positive *sample* (3.3) material, containing intact target nucleic acid sequences for *PCR* (3.12)

[SOURCE: ISO 16577:2016, 3.150[7], modified — Note 1 to entry was deleted.]

3.29
internal inhibition control
material acting as an internal control and obtained during the amplification reaction of the target fragment by adding *DNA* (3.5) or primers

Note 1 to entry: This material is clearly different from the target fragment.

Note 2 to entry: Adapted from ISO 16577:2016, 3.82[7].

3.30
laboratory developed test
LDT
test developed (or modified) and used within a single laboratory to carry out testing on *samples* (3.3), where the results are intended to assist in clinical diagnosis or to be used in making decisions concerning clinical management

Note 1 to entry: Laboratory developed test needs to be validated for its intended use before putting into service.

Note 2 to entry: Adapted from ISO 17822:2020, 3.23[16].

3.31
matrix
components of a material system, except the analyte

[SOURCE: ISO 15193:2009, 3.6[17]]

3.32
matrix effect
influence of a property of the *sample* (3.3), independent of the presence of the analyte, on the measurement and thereby on the measured quantity value

[SOURCE: ISO 15194:2009, 3.7[18], modified — Notes 1 to 2 to entry and the EXAMPLE were deleted.]

3.33
no template control
NTC
control reaction containing all reagents except the extracted test *sample* (3.3) *template* (3.22) nucleic acid

Note 1 to entry: This control is used to demonstrate the absence of contaminating nucleic acids. Instead of the template DNA, for example, a corresponding volume of nucleic acid free water is added to the reaction. The term "PCR reagent control" is also sometimes used.

[SOURCE: ISO 20395:2019, 3.20[9]]

3.34
loop-mediated isothermal amplification
LAMP
strategy for achieving isothermal *DNA* (3.5) amplification by utilizing two or three uniquely designed primer sets and a polymerase with high strand displacement activity

[SOURCE: ISO 16577:2016, 3.94[7]]

4 Overview

4.1 SARS-CoV-2

4.1.1 General

The process of SARS-CoV-2 molecular detection testing using the nucleic acid amplification test (NAAT) typically includes pre-examination and examination steps. The pre-examination steps include collection of clinical specimens, transport, storage, sample lysis, and nucleic acid extraction and concentration. Examination steps include reverse transcription (cDNA synthesis) and an appropriate amplification method. In addition, post-examination steps such as management of waste and reporting of the test results are included.

Quality attributes for the NAAT-based detection processes include, but are not limited to, evaluation of the performance of a suitable extraction procedure, evaluation of test reagents to meet minimum test criteria, a comprehensive evaluation of the analytical specificity, limit of detection (LOD) of the assay, and evaluation of the stability of the reagents.

The technical procedure to evaluate the quality attributes is shown in Figure 1, including the whole process evaluation and the key analytic performance evaluation.

NOTE The nucleic acid extraction part of quality evaluation is not always needed for NAATs where only one nucleic acid extraction method is used or when the extraction method is an integral part of the workflow.

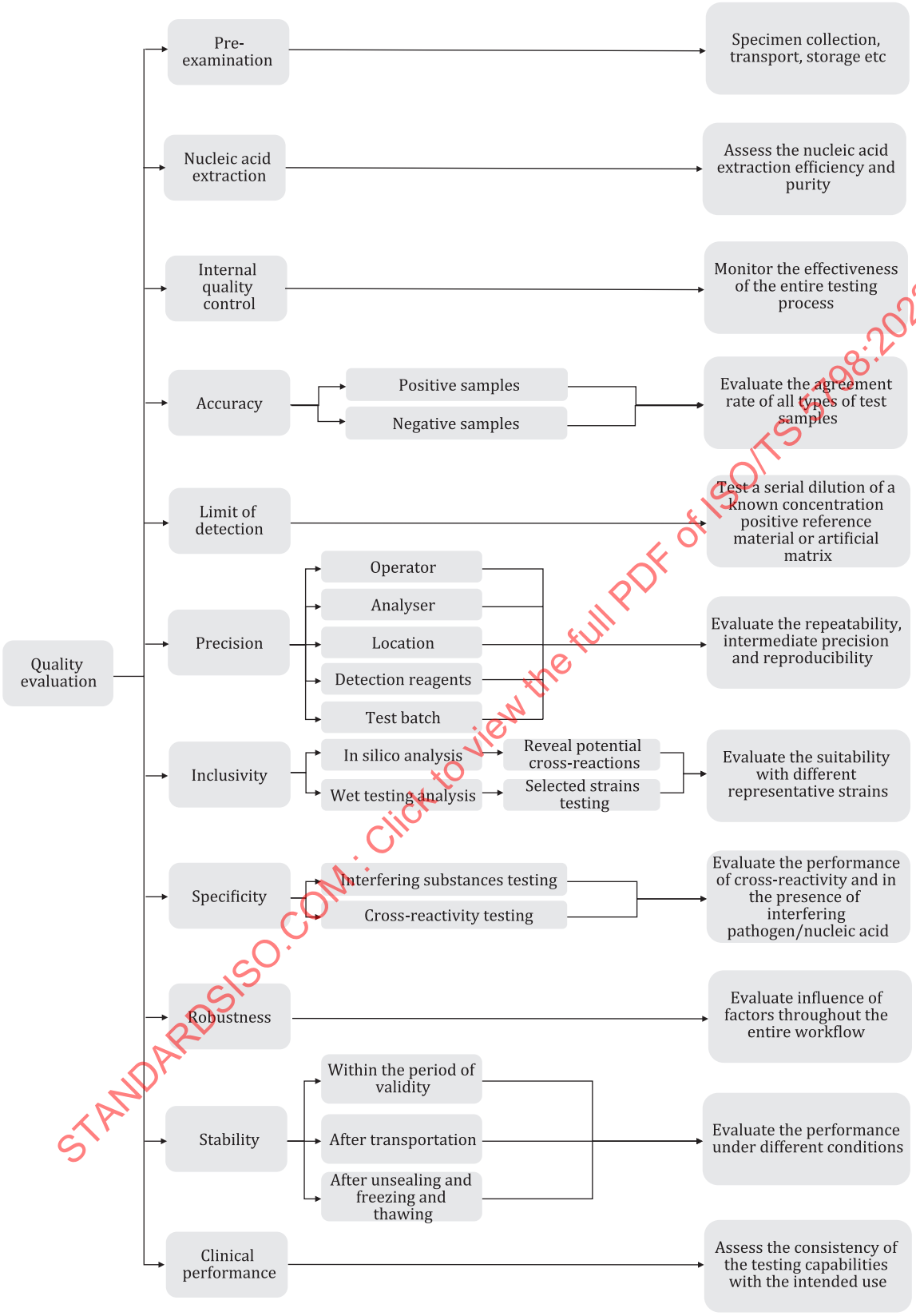


Figure 1 — Workflow of quality evaluation of SARS-CoV-2 detection method based on the nucleic acid amplification test (NAAT)

4.1.2 Pre-examination

For the detection of SARS-CoV-2, during the pre-examination work process, the following general considerations should be taken into account:

a) Appropriate personal protective equipment (PPE) should be used.

b) Specimen type selection: SARS-CoV-2 mainly infects the respiratory system; specimen selection should be determined with reference to the characteristics of SARS-CoV-2-related exposure or infection.

c) Specimen collection: depending on the selected specimen types, clinical specimens should be obtained according to standardized sampling requirements.

d) Specimen packaging: specimen packaging should take into account appropriate biosafety practices.

e) Specimen transport and storage: during the transport and storage process, the impact on the degradation of viral nucleic acid should be taken into account.

f) Inactivation of SARS-CoV-2: before testing in the laboratory, initial processing (before inactivation) of all specimens should take place in a validated biosafety cabinet (BSC) or primary containment device. If initial procedures involve manipulation of a primary specimen (e.g. dilution with inactivating reagent), they should be included in assay verification and validation.

NOTE Further detailed information on pre-examination parameters can be found in 6.5.1.

4.1.3 Examination — Overview

4.1.3.1 General

During laboratory testing of SARS-CoV-2 nucleic acid, the following general considerations should be taken into account:

a) Appropriate PPE should be used for all examinations.

b) Separate equipment, single use disposables, or both should be used for all activities to avoid cross-contamination.

c) Sample extraction, reaction reagent preparation, and amplicon handling should be conducted in separate laboratory rooms.

The need for separate rooms can be somewhat reduced by use of commercially available assays, closed-tube methods, and automated instruments. The fully automated methods require only one room or dedicated zone for laboratories using only commercially available kit-based assays. Closed-tube methods are methodologies where the amplification and analysis are performed in a single tube without the need to transfer the PCR products for further analysis.

d) Unless used for post-amplification steps, opening tube caps should be avoided as much as possible.

e) To avoid cross-contamination, it is recommended to avoid movement of the instrument or sharing equipment in different work areas.

f) For detection methods using conventional NAAT techniques, partition requirements for a NAAT laboratory should be strictly followed when performing the tests.

g) The dUTP and Uracil-DNA Glycosylase (UDG) may be included in the reaction mix to eliminate amplicon contamination.

h) Temporary storage and disposal of waste during sample testing should be considered.

i) A biobanking infrastructure can be considered if specimen archiving is intended to enable, for example, preservation of valuable biological materials. Further information on quality and competence of biobanks can be found in ISO 20387[19].

j) Laboratories should always establish the information system for the collection, processing, recording, reporting, storage or retrieval of examination and pre-examination data and relevant information.

4.1.3.2 Metrological traceability

Assay performance design-input characteristics should be verified with established reference materials. The materials should be traceable to verified new coronavirus reference materials (e.g. the WHO standard on SARS-CoV-2[20]) or appropriate equivalent or the assays should be validated according to external quality assurance schemes. Reference materials should be stored and handled appropriately and where commercially available, according to the supplier's instructions. Reference materials from different sources should be quantitatively traceable, i.e. the test results of reference materials from different sources should be consistent. Further information on metrological traceability can be found in ISO 17511[15].

The laboratory can prepare its own reference materials for feasibility and use of reagents (in-house reference material) using the following indications:

a) substances that can be used to formulate reference materials include materials of the same type or that have the same ingredients as the test sample, as well as inactivated new coronavirus or pseudo-virus;

b) inactivated viruses or prepared pseudo-viruses should be used after concentration determination by a reference measurement procedure employing a verified quantitative method such as digital PCR (dPCR) to ensure the accuracy of the results.

The storage conditions and storage time of in-house prepared reference materials should be specified and verified to avoid the loss of target nucleic acid. dPCR or qPCR can be used for this purpose. The reference material should be stored at an appropriate temperature, and if commercially available, at the temperature specified by the supplier and should be used prior to the expiration date, otherwise it will affect the test results.

4.1.3.3 Positive threshold

For PCR-based nucleic acid amplification methods, the positive threshold refers to the critical C_q value used as a limit to determine the presence of the target nucleic acid and defines which measurement results are reported as "detected" and which are reported as "not detected". The appropriate setting of the positive threshold value determines the clinical specificity and clinical sensitivity of the examination. Confirmation of the increased amplification signal can be used for result interpretation. The C_q value should be comparable with the shape of the amplification curve. A well designed and optimized SARS-CoV-2 PCR should yield no C_q signal where no SARS-CoV-2 RNA is present. Any C_q cut-off that differentiates between the negative and positive result should be verified with clinical specimens.

High C_q values have been applied to rule out very low signals that can be due to very low viral quantity or non-specific amplification. However, C_q values obtained by different laboratories should not be compared directly, unless the results are harmonized, e.g. by using a certified reference material.

4.1.3.4 Packaging

Reagent kits, whether commercially obtained or in-house produced, should only be used by the laboratory if the packaging is intact and labelled clearly (e.g. product name, batch code, contents, expiry date, storage condition).

4.1.3.5 Instructions for use (IFU)

IFU of both commercially obtained and in-house produced reagent kits should detail the intended use and handling procedures for application. The IFU contents should include target user, application area, interpretation of the test results, handling procedures and precautions.

4.1.4 Post-examination

Because SARS-CoV-2 is pathogenic, appropriate biorisk management procedures shall be followed under all circumstances and the following procedures should be properly performed after laboratory testing:

a) preservation and processing of remaining clinical specimens;

b) disposal of discarded samples after amplification;

c) provision of appropriate test report after laboratory testing: the content of the test report should contain at least the following information: essential specimen information, type of test specimen, test method, specimen collection time, storage and transport duration and conditions, report issuance time, information of judgement and interpretation of test results, information of person reviewing the result and authorizing release;

d) cleaning and decontamination of laboratories including rooms used and equipment;

e) storage and disposal of amplification product after amplification.

Further guidance for biorisk management can be found in ISO 35001[21].

4.2 Nucleic acid amplification methods

4.2.1 Reverse transcription qPCR (RT-qPCR)

RT-qPCR is a test method for SARS-CoV-2 RNA that combines reverse transcription (RT) with qPCR to enable detection and quantification once a user applied calibration step is performed. RT generates a complementary DNA (cDNA) copy of the RNA sequence enabling qPCR to be performed. qPCR-amplified cDNA is measured (typically with a fluorescent readout) following each cycle. The fluorescence can be incorporated using non-specific intercalating dyes or specific oligonucleotide hybrid probes that contain fluorophores. The latter tends to be the more common use for SARS-CoV-2 testing due to the additional analytic specificity provided by the addition of a third oligonucleotide.

Currently, the most commonly used method for SARS-CoV-2 nucleic acid detection is the hydrolysis fluorescent probe technique (which contains fluorophore and quencher). The diagnostic RT-qPCRs typically target the ORF1ab, RdRp, S, E or N gene(s) of the SARS-CoV-2 genome (see Figure 2). The detection of SARS-CoV-2 nucleic acid using RT-qPCR technique requires a qPCR instrument.

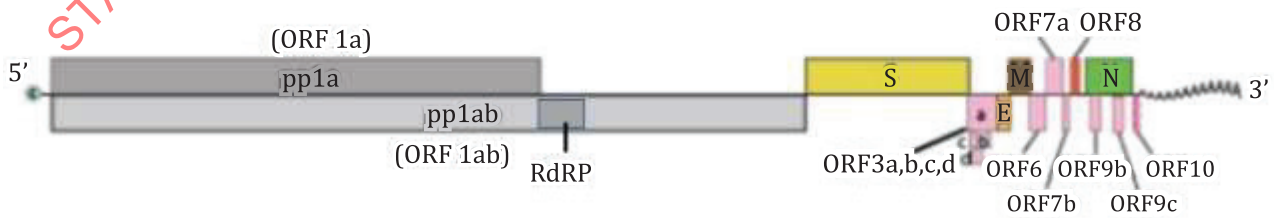


Figure 2 — SARS-CoV-2 genome[22]

If the SARS-CoV-2 cDNA is present in the sample, the primers and probe specifically bind to the target region and a new nucleic acid sequence is generated, primed off the respective primers that bind the complementary DNA strands frequently between 60 bp and 150 bp apart. The complementary molecule

is generated, and the exonuclease activity of the polymerase digests the hydrolysis probe separating the fluorophore from a quencher also attached to the probe. Liberating the fluorophore from its quencher allows fluorescence to be detected. As the number of reaction cycles increases, the concentration of labelled target nucleic acid sequence in samples increases, and so does the fluorescent signal. An increase in fluorescent signal through the fluorescence monitoring system coincides with detection of the SARS-CoV-2 nucleic acid sequence in the sample. With the addition of a reference calibrator with known concentration for the RT-qPCR, the relationship between C_q value and calibrator copies can be established according to the gradients of each standard to set the standard curve, and SARS-CoV-2 nucleic acid can be quantified.

4.2.2 Reverse transcription digital PCR (RT-dPCR)

RT-dPCR uses the same reagents as RT-qPCR, but applies limiting dilution, end-point PCR, and Poisson statistics, without using a calibrator. The template is randomly distributed at limiting dilution into discrete partitions (generated using oil in water droplets or prefabricated wells), some of which contain no template and others contain one or more templates. The partitions are amplified to end point and then counted to determine the number and fraction of positive partitions, from which the concentration is estimated by modelling as a Poisson distribution. Consequently, quantification is less affected by poor amplification efficiency and inhibitors of amplification that can be present in samples, i.e. bias resulting from repeatability and reproducibility errors. The reaction mixture can be divided into tens of thousands of nanolitre partitions during the process. This technology has been used for analysis of absolute viral load from clinical samples and has been demonstrated to provide high reproducibility[23] [24].

Further guidance on the use of dPCR can be found in ISO 20395[9].

4.2.3 Isothermal amplification methods

While PCR-based approaches are most widely used for confirmatory SARS-CoV-2 diagnosis, isothermal amplification methods offer an alternative strategy for detecting the SARS-CoV-2 virus and identifying infected patients. Isothermal amplification methods can be used without expensive thermocycling equipment and are often applied in combination with other diagnostic solutions that apply novel detection strategies such as CRISPR or DNA sequencing.

Examples of isothermal amplification methods can be found in Annex A. Readers should review published literature to best determine the most suitable methods, including potential advantages and limitations, for their intended use.

5 Laboratory requirements

5.1 General

Laboratories carrying out SARS-CoV-2 nucleic acid testing should take into account relevant requirements for biorisk and biosafety levels, laboratory setup, use of equipment and personnel protection. Laboratory equipment includes, for example, hardware and software of instruments, measuring systems, and laboratory information systems.

The laboratory should be aware of relevant national, regional, and international ethical requirements, laws, and regulations related to the collection and use of the specimens.

When using specimens derived from patients, even for quality control purposes, the laboratory shall take into account the considerations in this subclause.

5.2 Biosafety requirements

5.2.1 Laboratory area

Safe detection of SARS-CoV-2 requires a working area in the laboratory designed for preventing aerosol contamination. The use of suitable BSCs and separated dedicated work areas can be taken into consideration. Laboratories used for NAAT detection of SARS-CoV-2 should be set up to an appropriate biosafety level. At least biosafety level 2 is recommended. In addition, at least three physically separate rooms should be available in the laboratory for detection of SARS-CoV-2 by NAAT, one for reagent preparation, one for sample processing and the other for NAAT.

5.2.2 Risk control

The laboratory should conduct a local risk assessment to ensure it is competent to safely perform the intended testing with appropriate risk control measures. Infection risks of handling specimens associated with SARS-CoV-2 by laboratory services teams should be assessed to scrutinize the potential hazards arising from the work procedures. Thorough risk assessments of all work sequences, laboratory equipment, and workplace environments should be completed. Aerosol-generating procedures (mixing and transfer steps) should be performed in BSCs.

Although external lysis buffer of the common RNA extraction kits is possibly effective in inactivating the SARS-CoV-2 without heat or other additional means, any surface or material known to be, or potentially contaminated by biological agents during laboratory operations should be correctly disinfected to minimize infectious risks. All technical procedures should be performed in a way that minimizes the generation of aerosols and droplets. Appropriate PPE should be provided for laboratory work. Competence in proper donning and doffing of PPE accompanied by hand hygiene techniques is of the utmost importance for infection control.

Personnel analysing samples potentially containing viable SARS-CoV-2 viruses should be aware of authorities and regulations applicable to handling of these samples.

All residual risk levels of the potential hazards identified should be within the acceptable level.

Further guidance for biorisk management can be found in ISO 35001[21].

5.2.3 Personal protective equipment (PPE)

SARS-CoV-2 is highly contagious. Laboratory staff should wear PPE appropriate to the level of risk such as protective suits, gloves (cover all) and shoe covers. Face shields and appropriate face masks plus eye protection are highly recommended. Respiratory protection is of particular importance for the handling of suspected SARS-CoV-2 specimens and samples, especially when procedures that can create aerosols and droplets are performed. Local biosafety requirements for handling disposal and use and removal of protective equipment should be made aware to relevant personnel.

NOTE 1 General guidance on biorisk management and safe practices in medical laboratories can be found in ISO 15190[25] and ISO 35001[21].

Further information is provided in the WHO Laboratory Biosafety Manual, and it should be regularly checked for updated biosafety information regarding SARS-CoV-2 on the WHO or the US Centers for Disease Control and Prevention (CDC) websites[1)].

NOTE 2 ISO 22609[26] describes test methods for resistance of medical face masks against penetration by synthetic blood.

5.3 General laboratory set-up

A unidirectional workflow should be observed in the laboratory, to reduce the chances for contamination to occur. Materials, supplies or equipment from the sample preparation room should

1) WHO: www.who.int, CDC: www.cdc.gov

not be taken into the reagent preparation room. Similarly, nothing from the amplification and product detection room should be taken into the sample preparation room or the reagent preparation room. Further information can be found in ISO 17822[16].

Those recommendations should be applied taking into account the laboratory equipment and facilities after benefit risk analysis.

5.4 Instrumentation

Differences among manufacturers and models of instruments can lead to variable detection conditions and amplification efficiency when SARS-CoV-2 nucleic acid detection reagents are used in the corresponding nucleic acid amplification method. To establish suitability of reagents for multiple models, applicable performance should be demonstrated with various instruments if these are specified and claimed in the IFU.

5.5 Laboratory personnel

Clinical laboratory operators should be specifically instructed and trained in all relevant standard operating procedures (SOPs) as appropriate in the detection methods and procedures for SARS-CoV-2, NAAT techniques, in vitro diagnostic procedures, the applicable laboratory information system and the interpretation of results, with special attention paid to the highly infectious nature of SARS-CoV-2. In addition, specific training on relevant knowledge of biosafety and managing molecular biological laboratories should be performed as appropriate, including the handling of SARS-CoV-2 specimens, and the mechanisms and proper use of PPE (donning and doffing).

6 Design and development

6.1 Customer, patient and stakeholder needs

Customer, patient and stakeholder needs are defined and documented depending on intended use. Collecting and documenting these needs and requirements are essential for defining the design input specifications as the next development step. The purpose, benefit and use of diagnostic testing for SARS-CoV-2 RNA in respiratory specimens support decision-making for clinical, infection control or public health management, along with policy-oriented surveillance purposes. The target population ranges from individual primary care or hospital care patients, vulnerable peoples in healthcare facilities, targeted populations at risk in the community for detection, tracking and isolation to those without signs or symptoms of respiratory infection (e.g. monitoring work force or school populations).

Apart from real-time use for medical or public health case management and transmission control, virus detection tests are used for policy-oriented surveillance purposes to monitor the epidemiologic situation in terms of incidence and prevalence of infection and disease. This use includes prevalence surveys and sentinel surveillance programmes in the community, primary care or hospital care patient populations.

Specimens for diagnostic tests for SARS-CoV-2 can be taken from the upper (e.g. nasopharyngeal swabs, oropharyngeal swabs, saliva) or lower respiratory tract, e.g. sputum, tracheal aspirate, bronchoalveolar lavage (BAL). Data comparing the accuracy of RT-PCR testing suggest that test clinical sensitivity and LOD can vary by type of specimen and disease progression[27]. The manufacturers' instructions for intended use(s) for appropriate specimen types should always be consulted. More information is provided in 6.5.1 and should always be consulted.

6.2 Intended use of analytical test

The intended use of dedicated SARS-CoV-2 analytical tests is based on the customer and stakeholder needs and requirements. Analytical tests for SARS-CoV-2 RNA in respiratory specimens can support decision-making for clinical treatment, infection control or public health management. SARS-CoV-2

detection for diagnosis of patients with COVID-19-like symptoms is essential for patient care, triage and isolation in healthcare facilities.

SARS-CoV-2 detection can also be used for screening close contacts for asymptomatic infection and disease as part of contact tracing or outbreak investigations. The manufacturers' and providers' instructions should be consulted for intended use(s).

Two other important aspects of detection assays are their rapidity and ease of use. Tests that can be performed at the point-of-care are called point-of-care tests (POCT).

6.3 Institutional guideline strategy

6.3.1 Laboratory developed tests (LDTs) versus in vitro diagnostic medical devices (IVD medical devices)

Laboratory developed tests (LDTs) are non-commercial in vitro test methods performed in laboratories following a scientifically sound protocol after internal performance verification and validation in accordance with their quality assurance system based on international clinical laboratory quality standards. RT-PCR test methods targeting SARS-CoV-2 viral RNA are frequently used among other various RNA based in vitro methods for diagnosing suspected cases of SARS-CoV-2 (see Annex A). These PCR tests can be automated by using robotic molecular platforms for high-throughput batch processing of clinical specimens.

The "intended purpose" of an IVD medical device refers to the use for which the device is intended according to the data supplied by the manufacturer on the labelling, in the IFU, and in promotional materials.

6.3.2 Emergency use authorization

Guidance from relevant authorities should determine the criteria for authorization for emergency use of analytical tests that lack the required governmental, regulatory, agency approvals and clearances or in absence of an adequate supply of such products. Manufacturers pursuing emergency use authorization from relevant authorities, e.g. WHO, US Food and Drug Administration (FDA), in response to SARS-CoV-2-related health care efforts, should evaluate those criteria prior to providing the product to the marketplace for emergency use.

6.4 Clinical strategy

An assessment of overall clinical needs and stakeholder requirements is important to identify and develop the intended use. Good laboratory practices that produce accurate results are important to ensure that laboratory testing benefits the public health response.

Faced with community transmission over large areas, laboratories should be prepared for a significant increase in the number of specimens that need to be tested for SARS-CoV-2. Testing constraints should be investigated, and prioritization will be required to assure the highest public health impact of reducing transmission using available resources.

As part of the measures to contain the spread of SARS-CoV-2, a number of actions should be undertaken to scale up the testing capacity and ensure adequate quality of tests locally and internationally, including:

a) assessment of common approaches in national testing strategies (e.g. individual specimen or specimen pool testing);

b) discussion of best practices and development of guidance on performance evaluation and conformity assessment of tests;

c) provision of reference materials, certified reference materials, quality control material or research grade materials or all of these and common methods for the comparison of devices;

d) sharing of information on the performance of tests;

e) additional dialogue with industry and national competent authorities;

f) ensuring appropriate labelling to ensure the acceptability of test devices;

g) coordination of supply and demand.

6.5 Design and development planning

6.5.1 Pre-examination of respiratory specimens for SARS-CoV-2 testing

6.5.1.1 General

This subclause focuses on the respiratory tract as the primary target of infection with SARS-CoV-2 virus. Respiratory specimens for SARS-CoV-2 can include nasopharyngeal swabs, throat (oropharyngeal) swabs, anterior mid-turbinate nasal swabs, sputa, bronchial-alveolar lavage fluid and salivary specimens. Appropriate specimen handling is critical to ensure specimen integrity and the accuracy of qualitative or quantitative for both types of nucleic acid detection. Specimens should be collected using all appropriate biosafety guidelines. Information regarding potential interfering substances (e.g. nasal or other medications) should be reviewed. Specimens should be collected, transported, and stored properly prior to testing. If extended transport time to the testing laboratory is expected, the collection devices should be constructed such as to provide for stabilization of viral RNA. Inappropriate sample handling can result in nucleic acid degradation and false negative results (CLSI-MM13A)[28]. It is critical that laboratory personnel are trained to understand the importance of maintaining the integrity of the sample received for testing.

6.5.1.2 Specimen collection

Correctly performed sampling from the respiratory tract can have a profound effect on the viral load detected, which is also known to change dramatically in the course of the infection[4]. It is important, therefore, to ensure that the sampling technique includes both recovery of fluid and epithelial cells of the respiratory tract that have been infected by the SARS-CoV-2 virus to improve the capture of viral RNA[29][30]. It is expected this will improve the sensitivity of the nucleic acid tests described herein. It is also essential that personnel collecting the respiratory specimens are well-trained to ensure that specimen collection accurately reflects the sampling site. Well-documented information on sampling of the respiratory tract has been complicated because of the need for rapid determination of potential SARS-CoV-2 positive cases. Available information on other viral pathogens (e.g. influenza, measles) can provide useful information on the best swabs or other systems for sampling[31][32].

Information accompanying collected specimens, e.g. patient demographics, in-patient or out-patient, date and time of collection, is highly recommended in order to track patients with positive results. These data should be recorded in the laboratory information system. This will support isolation protocols as required and assist public health personnel to track others who might have been in contact with patients who have a positive result. Specimen types, amounts required, appropriate containers, criteria for specimen acceptability, transport, storage conditions and duration, and factors to minimize nucleic acid degradation or contamination should be considered.

In out-patient settings, available data suggest that flocked nylon swabs show the best clinical sensitivity compared to standard dacron swabs for fluid absorption and adherence of epithelial cells for pathogen recovery[33]. In recent studies which were often not well standardized, and subject to many variables, nasopharyngeal swabs have shown the best clinical sensitivity (90 % to 95 %) and good specificity. Oral pharyngeal (throat) specimens can have lower clinical sensitivity (80 % to 90 %) depending on sampling techniques and standardization[34]. For various reasons, if a nasopharyngeal specimen cannot be collected, a combined nasal-mid-turbinate specimen and a throat swab specimen (if possible), or aspirate specimens can provide good virus recovery[32], and gargling for collection of

salivary specimens can be effective[35]. The criteria for quantitative virus recovery should take into account the intended use of the test result.

NOTE In a meta-analysis of collection studies of three specimen types (naso-pharyngeal swab, oropharyngeal swab, and sputum) from over 3 000 patients[36], sputum was shown to have the best clinical sensitivity followed by nasopharyngeal swabs and then oropharyngeal swabs.

Training in specimen collection should always be conducted due to the importance of specimen quality. CLSI MM13-A [28] can be referenced as an appropriate resource.

Before the specimens are collected, information that identifies the source of the specimen and the patient (e.g. a barcode) should be affixed to the collection tubes. The collection of different specimen types should be performed according to corresponding standard procedures. The recommended sampling methods are given in 6.5.1.3.

6.5.1.3 Specimen types

6.5.1.3.1 Nasopharyngeal swabs

Sampling personnel should gently support the subject's head, hold the swab with one hand and insert it into the nostril, and then, slowly push the swab backward along the base of the inferior nasal passage. Because the nasal passage is curved, excessive force should be avoided to prevent traumatic bleeding. When the swab tip reaches the posterior wall of the nasopharyngeal cavity, it should be gently rotated (the rotation should be paused in the case of reflex cough). Then, the swab should always be removed slowly to avoid breaking off the swab in the nasal cavity. The swab is immersed in a viral transport medium or equivalent stabilizing solution. After discarding the end of the swab at the break point, the tube cap is tightened. A nasopharyngeal swab and throat swab can be placed in the same tube of transport medium if required.

6.5.1.3.2 Throat (oropharyngeal) swabs

The patient should slightly raise the head, open the mouth widely, and say "ah" to expose the bilateral pharyngeal tonsils. It is highly recommended to rub the swab over both tonsillar pillars back and forth with mild force at least three times, and avoid touching the tongue, teeth, and gums. Then the swab tip is immersed in a viral transport medium.

6.5.1.3.3 Nasal swabs

Anterior nasal swabs can be used for testing for SARS-CoV-2, so long as they are verified, but it is recommended they not be used alone[32]. False negatives can occur more frequently in screening environments if personnel collecting the specimens are not well trained to ensure that at least both nares are sampled, and that sufficient fluid and cells are collected. If nasopharyngeal swabs are not possible, then a combined throat and nasal swab can be acceptable[37].

Collection of a nasal-mid-turbinate specimen has greater clinical sensitivity than an anterior nasal swab[38]. The swab should be inserted into the nostril to the level of the mid-turbinate, rotated several times for 10 s to 15 s and then the process repeated with the same swab in the other nostril. Then, the swab tip is immersed in a viral transport medium. In some areas where there is significant circulation of SARS-CoV-2, and limited trained personnel to perform the testing, self-collection using nasal swabs can be done so long as explicit collection instructions are provided with testing kits to reduce the likelihood of false negative test results.

6.5.1.3.4 Upper respiratory (salivary) rinses and saliva

Recent data has suggested that respiratory rinses can provide adequate samples for qPCR testing. These are sometimes called "gargle and spit" samples. Available evidence suggests that these can be useful for self-collection in outpatients and in children as an alternate to nasopharyngeal specimens with equivalent clinical sensitivity, at least in that population[35]. Other data[39] suggest that direct saliva collection is not necessarily as effective as a nasopharyngeal swab for collection of specimens.

Saliva naturally contains microorganisms and extraneous substances (e.g. food debris), which make the composition of saliva more complex and unique among patients and donors. This bears a risk of SARS-CoV-2 degradation including loss of viral RNA copies during the pre-examination workflow. This can impact the examination performance. The stability of the SARS-CoV-2 RNA copies during the pre-examination process is, therefore, an important prerequisite for reliably performing SARS-CoV-2 examinations from saliva.

Preliminary information suggests that saliva directly spit into collection devices without prior gargling can also be used for larger scale screening purposes, particularly in younger age groups. Collection of saliva in devices with suitable SARS-CoV-2 RNA stabilization solutions can, therefore, be an appropriate solution. Therefore, it is highly recommended also to specify, verify and validate the pre-examination workflow steps during the development of a SARS-CoV-2 test. This includes, but is not limited to, the saliva collection device choice, the collection process, transport to and within the testing laboratory and storage duration and conditions as well as the SARS-CoV-2 RNA isolation where required.

6.5.1.3.5 Respiratory tract aspiration fluid

A catheter with collector head should be used with a negative pressure pump to aspirate mucus from the nasopharynx or aspirate respiratory secretions from the trachea. The collector head is inserted into the nasal cavity or trachea. Negative pressure is then applied, and the catheter is slowly withdrawn with a rotation movement. A soft catheter with a thumbhole can be used to control the suction at the base of the catheter. Sample preserving media are used to collect the aspirated mucus. Then the collector should be rinsed once with 3 ml of sampling solution (a paediatric catheter can be connected with a 50 ml syringe). The collected sample volume should be 2 ml to 3 ml.

6.5.1.3.6 Deep cough sputum

Before the sputum specimen is collected, it is recommended to always determine whether the patient is able to cooperate with deep expectoration. The patient is instructed to rinse the mouth with normal saline 2 times to 3 times (those wearing dentures should remove the dentures first). After the patient is asked to exert a deep cough, the coughed-up sputum is transferred into a collector (tube). Collecting saliva should be avoided. The collected sample volume should be 2 ml to 3 ml.

6.5.1.3.7 Bronchial lavage fluid

The collector head is inserted into the trachea (~30 cm deep) through the nostril or tracheal socket. After injecting 5 ml of normal saline, negative pressure is turned on, and then the collector head is rotated and withdrawn slowly. The aspirated mucus is collected in a viral transport medium or equivalent stabilizing solution, and the collector is rinsed once with normal saline (a paediatric catheter can be connected with a 50 ml syringe). The collected sample volume should be 2 ml to 3 ml.

6.5.1.3.8 Alveolar lavage fluid

After local anaesthesia, a fibre-optic bronchoscope is inserted into the trachea of the right middle lobe or left lingular segment through the mouth or nose and then the pharynx. After its top is tapped into the bronchial opening, sterilized normal saline is slowly added through the tracheal biopsy hole, 30 ml to 50 ml each time, the total volume is 100 ml to 250 ml, and should not exceed 300 ml. Alveolar lavage fluid is immediately aspirated using appropriate negative pressure (100 mmHg equivalent to 13,3 kPa is generally recommended), and the total recovery rate should be ≥ 30 %. The recovered fluid contains ~10 ml of secretions from terminal bronchi and alveoli. After the potentially contaminated prior portion is discarded, approximately 2 ml to 3 ml of the remaining portion should be collected using viral transport media or equivalent stabilizing solution.

6.5.1.3.9 Pooled respiratory specimens

When there is a need for high-throughput SARS-CoV-2 testing in a low prevalence population, pooling of respiratory screening specimens can be helpful to provide more rapid throughput of specimens in order to provide more rapid turnaround of population and case reporting. For this purpose, the

importance of properly collected specimens as detailed above cannot be overstated. Available evidence suggests that pooling can be most effective and potentially increase throughput by a factor of 5-fold when the prevalence of the disease in the population being tested is low (5 % to < 10 %) and the number of pooled specimens is fewer than 10. Depending on the testing strategy, it can be preferable to pool after extraction from each primary specimen (to avoid cross-contamination) for testing. Risk of false negativity increases significantly when viral RNA concentration is low in the pooled samples. In these populations, pools of about five samples, if negative, can all be considered negative and reported as not detected. If any pool is positive, the individual samples in the pool should be re-tested prior to reporting[40].

It is possible to pool between 5 to 10 respiratory specimens either from individual specimen collection devices or from a pool of swabs in the same device[41]. If it is clear that the entire pool is negative (from the RT-qPCR test results) then individual results from the pool can be reported as not detected. If there is a suggestion that one or more samples within the pool are positive, then repeat specimens are required from each patient within that pool of specimens to determine which of the specimens was positive. This can result in delays in reporting individual positive result(s) within the pool. This technique should be verified and clinically validated to ensure that within-pool and between-pool samples perform in the same manner in RT-qPCR tests and that testing individual samples in the pool at the same time provides the same test results.

6.5.1.3.10 Alternative methods for respiratory specimen collection

The current publication of this document was developed based on knowledge of collection methods that have been validated and verified in clinical studies for their intended use to recover SARS-CoV-2 strains from the collection sites identified above. As the pre-examination technology develops it is expected that alternative methods, which are more acceptable for collection from some groups (e.g. children), can be utilized. Such techniques, which are then verified, and then validated for their intended use by manufacturers, and then used in peer reviewed studies with proven high clinical sensitivity and specificity, will become acceptable for routine use.

6.5.1.3.11 Other specimen types

Other specimen or sample types such as blood plasma, urine, and faeces, collected from COVID-19 patients have been reported to contain detectable SARS-CoV-2 RNA[42] although the accuracy of some extra-oral specimens for SARS-CoV-2 testing has been called into question[43]. If a laboratory includes such specimens in their diagnostic pipeline, their collection should employ usual practices with notification to the laboratory of suspected or confirmed infection. Facility outflow sewage can be effective, particularly in long-term care facilities, to identify the burden of SARS-CoV-2 virus in that environment.

NOTE Regular inspection of waste water or sewage (prior to treatment) for SARS-CoV-2 RNA can provide useful early information about the potential burden of infection in various environments (e.g. residential, places where persons concentrate), or increased transmission of viral mutants[44].

6.5.1.3.12 Collection period during infection

For testing of different specimen types, the collection times (in the course of infection) in Table 1 are given.

Table 1 — Recommended collection time

Specimen type	Recommended collection time
Nasopharyngeal swabs Throat (oropharyngeal) swabs Anterior mid-turbinate nasal swabs Upper respiratory (salivary) rinses	These specimens are all upper respiratory and are recommended for testing in early-stage infections, especially in asymptomatic or mild cases.

Table 1 *(continued)*

Specimen type	Recommended collection time
Respiratory tract aspiration fluid Deep cough sputum Bronchial lavage fluid Alveolar lavage fluid	Lower respiratory specimens are recommended if collected later in the course of the COVID-19 disease, in hospitalized patients, or in patients with negative upper respiratory sampling and there is a strong clinical suspicion of COVID-19.
Blood (plasma)	It is recommended to collect blood specimens from patients with significant acute phase infection within seven days after onset of symptoms. Plasma testing is also recommended for patients with chronic infections or severe diseases to estimate disease severity and monitor treatment response[45][46][47].
Conjunctival swabs	Conjunctival swab collection is recommended in suspected SARS-CoV-2 cases presenting symptoms of eye infection.

6.5.1.4 Transportation

Unless otherwise specified in manufacturer's instructions, respiratory specimens should be sent to the laboratory within 2 h to 4 h after collection for testing, as long as the specimens are not transported in stabilizing solution verified for different transport conditions and durations. If the shipping time is likely to be more than 4 h, specimens should be placed into a viral transport medium or equivalent stabilizing solution and immediately placed, as appropriate, on refrigerant gel packs or at 2 °C to 8 °C (refrigeration) for transport to the testing laboratory, if not verified differently. If delivery will be delayed for more than 3 d to 4 d, as can occur in remote locations, the specimen should be frozen at −70 °C, if not verified differently. If not available, specimens can be stored at −20 °C. During specimen transportation, repeated freezing and thawing should be avoided as far as possible to prevent degradation of viral nucleic acid.

Data on stability of RNA in specimen collection transport tubes at this time suggest that SARS-CoV-2 RNA has good long-term stability. Recent data indicates that SARS-CoV-2 RNA maintains stability for at least 48 h and up to a week at 4 °C without degradation, and for months at freezer temperatures in various matrices such as lysis buffers and buffered saline[48][49].

Transport of specimens within national borders should take into consideration applicable national regulations. International transport of potential SARS-CoV-2 virus containing specimens should take into consideration, for example, the UN Model Regulations, and any other applicable regulations depending on the mode of transport being used. More information can be found in the WHO guidance documents[50][51].

All materials transported within and between laboratories should be placed in secondary packaging, to minimize the potential for breakage or a spill. Specimens leaving the BSC should be surface decontaminated. The WHO biosafety video series can be referenced[52].

Patient specimens from suspected or confirmed cases of COVID-19 should be transported as UN3373, "Biological Substance Category B", when they are transported for diagnostic or investigational purposes. Viral cultures or isolates should be transported as Category A UN2814, "infectious substance, affecting humans"[53]. All specimens being transported (whether UN3373 or UN2814) should have appropriate packaging, labelling, and documentation, as described in the documents referenced in this subclause.

6.5.1.5 Specimen receipt and storage

Laboratory recipients should be properly trained and have the ability to handle emergency situations such as container damage and sample leakage. After specimens are received, the transport boxes should always be disinfected with a disinfectant before opening, and the secondary containment plastic bags should also always be disinfected. The secondary containment plastic bags should be opened in a BSC to remove the specimens. The name, gender, age, number and test item of the specimen should be verified as well as the status of specimens and the abnormalities of the specimens. All information should be recorded in the laboratory information system.

Specimens should be stored in containers with adequate strength, integrity, and volume to contain the specimen, and that are leakproof when the cap or stopper is correctly applied. Plastic containers that are free of any biological material on the outside of the packaging, should always be used whenever possible. In addition, containers should always be correctly labelled, marked and correspondingly, laboratory system recorded to facilitate identification. They should be made of an appropriate material for the type of storage required. Inactivation methods should be properly validated whenever an inactivation step is used, before the storage of specimens. The specimens should be stored at the appropriate conditions or at −70 °C if no IFU or recommendations on storage condition are available. Extracted nucleic acid should be stored at appropriate verified conditions or, in the absence of such specifications, −70 °C or below.

6.5.1.6 Archiving

6.5.1.6.1 Archiving information

After specimens are received, the specimen information should be recorded and archived in a timely manner. The information should be recorded in the laboratory information system and the content should include, but is not limited to:

a) unique laboratory identification;

b) patient personal information (at least two identifiers, including name, unique health care number, date of birth);

c) specimen information (source including, for example, type of swab, sampling date, sample volume if important, whether the sample status is screening or for clinical diagnosis);

d) clinical information (e.g. clinical symptoms, epidemiological information).

The archived information should always be properly stored to minimize deterioration, preserve confidentiality and prevent loss.

6.5.1.6.2 Archiving of specimens or samples

Information on specimen collection devices and sample storage devices, whether they stabilize or inactivate viral specimens or samples, should be available. This is important for retrospective analysis, e.g. sequencing (allows virus cultivation), as well as biosafety measures for storage (see also 6.5.1.4).

6.5.1.7 Specimen processing including RNA isolation

It is common practice to extract and isolate viral RNA before RT and detection by PCR. This process typically also concentrates the RNA solution potentially providing improvements to analytical specificity and LOD. Extraction is a critical step for purification and particularly to remove PCR inhibitors that can disrupt the reaction leading to false negative results.

NOTE ISO 20395[9] can be referred to for general methods of measuring total concentration and assessing the quality of nucleic acid.

To verify whole procedure performance, the procedure (e.g. extraction efficiency, reagents, PCR performance) should be compared to other existing assays used for extracting viral RNA from similar specimens or samples, to ensure the smooth progress of the downstream PCR based detection. This should include using a dilution series approach to determine the LOD and, where necessary, limit of quantification (LOQ). This can also include comparing the influence of different extraction methods on analytical sensitivity.

For kits that include nucleic acid extraction components, the product description should offer technical guidance for nucleic acid extraction and describe how the efficiency of nucleic acid extraction was verified. For example, known interference factors in the sample extraction process and their possible impact on the subsequent amplification process should be stated.

Assays are available that do not contain nucleic acid extraction components, and the user is expected to provide this aspect of the procedure. For such assays, the product description should fully describe or specify a suitable extraction process (kit) and indicate the nucleic acid extraction efficiency and expected analytical performance.

Direct addition amplification kits are available that work without a separate extraction step (samples are added directly to the reaction).

The extraction of nucleic acid can be evaluated in accordance with relevant ISO standards, e.g. the ISO 20184 series[55] and the ISO 20186 series[56]. Assessment of the nucleic acid extraction and purity should be done by performing tests using inactivated SARS-CoV-2 virus in a specimen matrix that mimics clinical specimens.

Sample processing for extraction in the laboratory should be performed strictly in line with device instructions to ensure claimed performance and quality. The following points should be given particular attention:

a) Correct temperature and volume of reagents and samples in nucleic acid extraction are important for efficiency of extraction and performance of downstream PCR tests. The recommended nucleic acid extraction method should be used and operated strictly in line with manufacturer's instructions.

b) Operations of reaction mix preparation and program setting should proceed in line with the manufacturer's instructions. Otherwise, there is a possibility that the performance of the examination is negatively impacted, and unexpected results can be produced in testing.

c) Technicians responsible for result interpretation should be trained on reading the results correctly, from calibrating the results as needed or addressing any complicated situations in line with the manufacturer's instructions.

In addition, the operator should avoid any conditions that can lead to degradation of nucleic acid (e.g. prolonged exposure to ambient temperature, enzyme deactivation caused by direct contact with hands). It is generally recommended to keep reaction components on crushed ice unless the manufacturer instructions explicitly require a different temperature.

6.5.2 Examination design specifications (analytical test specifications)

6.5.2.1 Qualitative and quantitative assays

6.5.2.1.1 Qualitative testing

Qualitative testing is appropriate for situations where only negative or positive determination of the samples is needed, without requiring measurement of viral loads. It is unnecessary to use reference materials of known concentration gradients to plot the standard curves. Instead, only the final signal intensity is needed, and the obtained results can be a negative or positive description of the samples. It is suitable for the screening and testing of subjects with suspected infection.

qPCR and isothermal amplification are frequently used for qualitative evaluation. Isothermal amplification methods are referenced in Annex A. Readers should review published literature to best determine the most suitable methods, including potential advantages and limitations, for their intended use.

6.5.2.1.2 Quantitative testing

Quantitative testing is appropriate for situations where measurement of the viral loads in samples is needed. For qPCR detection, reference materials of known concentration gradients should always be used to plot the standard curves. Meanwhile, fluorescence signal intensity of the samples should always be recorded in real time so that the fluorescence signals during exponential growth period can be quantitatively analysed. The obtained results are specific values, such as virus loads. It is suitable

for observation of the therapeutic efficacy or disease progression, determination of disease severity and scientific research.

qPCR and dPCR are applicable for quantitative evaluation. Isothermal amplification methods are referenced in Annex A. Readers should review published literature to best determine the most suitable methods, including potential advantages and limitations, for their intended use.

6.5.2.2 Selection of nucleic acid amplification technology

Laboratories should select the appropriate amplification technique according to the testing requirements and environment. Following RT where appropriate (see 4.2.1), the characteristics of each amplification technique are as follows:

a) qPCR is suitable for situations where qualitative evaluation or quantitation of the samples is needed. It is always recommended to provide a fluorescent quantitative PCR instrument and a stable energy supply. qPCR provides high analytical sensitivity and high throughput, and is simple to operate, but the reaction time is relatively long.

b) dPCR is suitable for situations that require absolute quantitation of the samples. Because dPCR requires only a small sample volume, it is also suitable for precious samples or samples with severe nucleic acid degradation. It is always recommended to provide a dedicated dPCR instrument to analyse and read samples, a reaction chip or droplet generator, and a stable energy supply. Despite the low LOD, the throughput depends on specific instrument systems. In addition, the operation is more complicated, and the reaction time is relatively long.

c) Isothermal amplification is suitable for situations that require qualitative evaluation of the samples. A thermal cycler and a stable energy supply are not necessarily needed. It is characterized by low LOD, low throughput, short reaction time, and is easy to operate.

6.5.2.3 Quality control concept and material

6.5.2.3.1 Material and environmental control

The claimed conditions and duration for storage and transportation of IVD reagents should be determined by evidence of stability studies. It is recommended to store the samples and RNA-based calibrator materials as per manufacturer's instructions or, if no instructions are provided, at –18 °C or –70 °C for no more than one week or six months, respectively.

All reagents used should be free of DNase or RNase, unless otherwise specified.

All reagents should be handled, stored, transported or disposed of according to the applicable requirements and laboratory standards. Date of arrival and date of unsealing should be recorded.

Name, concentration, date of preparation and storage conditions of all the lab-made reagents should be tagged on the reagents.

The reagents should be sterilized by autoclaving after manufacturing, when needed. For those reagents which cannot be autoclaved, use of filtration for sterilization is recommended.

Repeated freezing and thawing the reagents should be avoided. The reagent should always be maintained on ice when thawed. The freeze and thaw cycles should be determined according to the key performance indices described in the instructions or after sufficient laboratory verification.

Extracted RNA and related calibrator material is prone to degradation, therefore the NAAT tests should be carried out immediately, as long as they are not verified differently.

The water used should be suitable to enable the required performance for the examination (e.g. no interfering compounds, nuclease free water, reduced ion content).

6.5.2.3.2 Quality control set-up

Controls are always recommended to monitor the performance of the developed method and are ideally used in each amplification and detection run. A negative control should always be used to monitor whether there is contamination for the whole process. A positive PCR control should always be used to monitor the detection performance. An internal inhibition control is recommended to monitor the specimen collection, handling, RNA isolation, and RT-qPCR process. When using controls in each run is not possible (e.g. POC systems where each run consists of a single sample), they should be tested when a reagent lot or batch is changed, or alternatively at a pre-defined time interval (e.g. daily or weekly).

6.5.2.4 Performance parameters

The performance parameters requiring an assessment include, but are not limited to, the confirmation of positive determination cut-off value, the confirmation of lower LOD, and the applicability in different instrument systems or models (if any). The stability parameters include, but are not limited to, for example, sample stability evaluation and reagent stability evaluation.

Diagnostic performance should be confirmed through testing a statistically significant number of samples with known clinical diagnosis results using appropriate evaluation methods (e.g. ROC curve). The confirmed diagnostic performance should pass validation using a certain number of samples with known clinical diagnostic results regarding detection of infection.

After the positive determination cut-off value is confirmed, it is recommended to confirm the minimum detectable concentration of the kit using a reference material of known SARS-CoV-2 concentration to evaluate the lower LOD for the kit. Further information can be found in 7.2.2.

If the kit is suitable for different instrument systems, such as qPCR instruments and nucleic acid extraction methods from different manufacturers, it is recommended to assess different models and evaluate their equivalence. Further information on the evaluation of sample and reagent stability can be found in 6.5.2.9.

ISO/TS 16393[57] provides guidance for developing qualitative (binary) PCR test methods.

6.5.2.5 Trueness (recovery, accuracy, matrix effects)

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 statistical recommendations and ISO 15189[6].

The selection of test sample size should comprehensively consider the complexity and precision requirements of the established methods, statistical protocol for data analysis and acceptable statistical confidence level. It is recommended that no fewer than 20 samples be tested.

During accuracy studies, all test specimen and sample types defined in the design input specifications should be included (e.g. pharyngeal swabs, nasal swabs, alveolar lavage fluid, sputum, whole blood, stool, anal swabs) unless otherwise justified. If clinical specimens and samples cannot be obtained, simulated samples may be used. They should be composed of SARS-CoV-2-negative sample matrix spike with appropriate SARS-CoV-2 target (e.g. pseudo-virus containing internal reference sequences or SARS-CoV-2 target sequences) and can be prepared in-house or obtained from commercial sources.

In order to determine test accuracy, laboratories should interpret the obtained data using graphical and statistical presentations.

Data analysis and comparison should include use of relevant scatter plot and regression analysis and describe the method for calculating 95 % confidence intervals.

6.5.2.6 Precision (repeatability, intermediate precision, reproducibility)

It is always recommended to evaluate the main variables that can affect test precision, including detection reagents (including nucleic acid extraction components), the analyser, operator, location, test round and other factors.

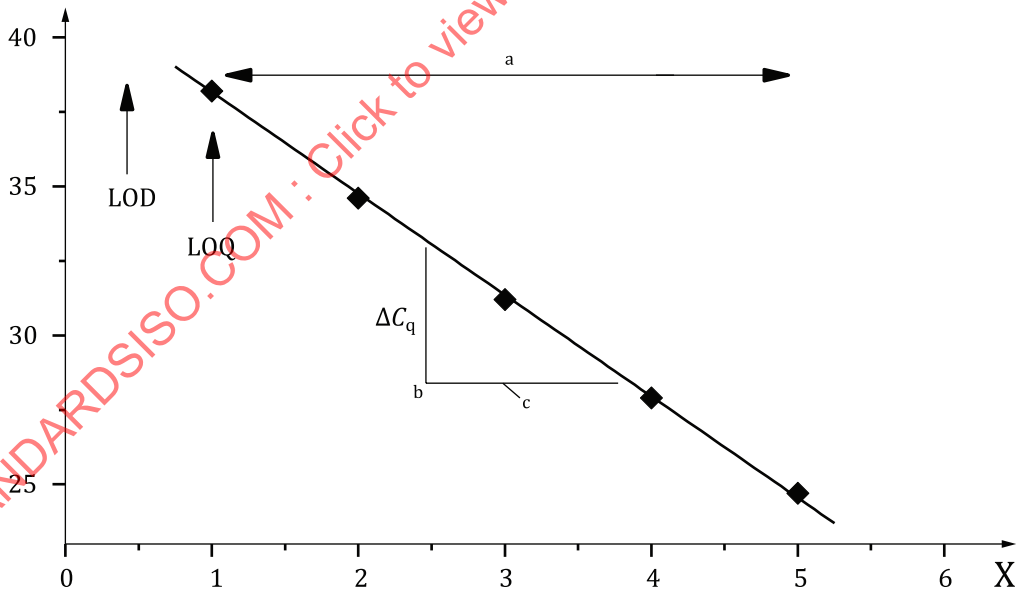
Precision studies should test the repeatability, intermediate precision and reproducibility, if applicable (see 6.6.7).

Testing of samples used for precision evaluation can include standards, quality control materials, proficiency testing samples, or patient specimens, in sufficient quantity to complete the study. For example, spike-in sample prepared with materials, which is sufficiently homogeneous and stable with respect to pre-measured characteristic values, can be used for this purpose. Those materials can spike-in to the samples including patient specimens or extracted nucleic acid samples before amplification or both if the amplification of both materials can be discriminated. Single spike-in material should be considered for monitoring all processes of precision evaluation test, namely from the steps of nucleic acid extraction to detection. The sample selection should contain at least three levels: negative, positive sample near LOD, and (medium or strong) positive samples. Appropriate precision requirements should be set according to the product characteristics.

Test precision should be expressed on the basis of statistical measurements of imprecision, e.g. standard deviation (SD).

6.5.2.7 Performance of an analytical test

The performance of an analytical test is characterized by several parameters. These parameters should be evaluated using a dilution series of a traceable positive standard to measure the response curve. An example for measurements is shown in Figure 3 for a test applying qPCR.



Key

X log10 concentration
LOD limit of detection
LOQ limit of quantification
[a] Linear range.
[b] Sensitivity.
[c] $\Delta\log_{10}$(conc).

Figure 3 — Schematic response curve for a qPCR test

The LOQ is the lowest concentration that can be measured with reasonable statistical certainty consistently under the experimental conditions specified for the analytical test. The LOQ sets the lower limit for the linear range. The sensitivity is the slope in the linear range. In case the slope is negative, the absolute value of the slope should be reported as sensitivity. A minimum of three varying concentrations should be included in sensitivity studies.

The LOD of the assay should be determined utilizing the entire test procedures of the kit from specimen preparation, nucleic acid extraction, to detection for each clinical specimen type or matrix claimed. The LOD sets the lower limit for the working range. The upper limit of the working range is given by concentrations at which significant anomalies in sensitivity are observed.

Representative SARS-CoV-2 samples of a clinically relevant type strain should be used to determine the LOD. The LOD should also be verified using samples of other different SARS-CoV-2 strains, including genetic variants.

If a variety of matrix specimen types are applicable, e.g. sputum, BAL fluid, nasopharyngeal swab, it is recommended to conduct an LOD study for each claimed specimen type separately unless otherwise justified since variations in sampling and extraction efficiency can influence LOD.

6.5.2.8 Specificity interferences

The analytical specificity is assessed through testing both interfering substances and potentially cross-reactive microorganisms.

The influence of interfering substances is usually already known for routine PCR detection reagents or reagents with established extraction methods before application in testing a new pathogen. However, their specificity against the potentially cross-reactive organisms can be tested. For other NAAT products such as isothermal amplification or deployment of new extraction methods, potential interferences should always be evaluated.

Potential interfering substances can include, but are not limited to, blood clot, nasal spray (benzolin, hydroxymethozoline, sodium chloride with preservatives), nasal medicine (beclomethasone, dexamethasone, flunisolone, triamcinolone acetonide, budesonide, mometasone, fluticasone), anti-allergy medicine (histamine hydrochloride), anti-viral medicine (interferon - α, zanamivir, ribavirin, oseltamivir, pramivir, lopinavir, litonavir, abidol), antimicrobials (levofloxacin, azithromycin, ceftriaxone, meropenem, tobramycin) and human genomic DNA and RNA.

It is recommended that interference samples be tested in triplicate to avoid random errors caused by a single test. For cross-reaction studies of pathogens, it is recommended to prepare test samples by mixing the culture isolates into a sample buffer, in order to avoid directly using the pathogen culture.

Cross-reactivity of pathogens should be evaluated through testing. In cases when wet testing cannot be conducted, in silico analysis (use of bioinformatics to aid assay design and/or predict performance) should be conducted to assess the homology between the testing microorganisms and the primers and probe(s) of assay. The analysis should include multiple representative strains of all testing pathogens for each organism whenever possible. It is recommended to refer to a) to e) in this subclause.

If in silico analysis reveals ≥ 80 % homology between the cross-reactive microorganisms and the assay test primers and probe(s), the following actions should be taken:

a) a microbial interference study should be conducted between SARS-CoV-2 and the microorganisms that the test primers and probe(s) have homology to; or

b) as an alternative to the microbial interference study, a justification study should be performed as to why (e.g. amount of primers and probe(s) included in the master mix) the performance of the device is not impacted by the presence of microorganisms bearing ≥ 80 % homology with the test primers and probe(s); or

c) an explanation should be given as to why the in silico results are clinically irrelevant (e.g. low prevalence of MERS-CoV).

Testing of near-neighbour species and strains as well as testing of organisms whose infection produces symptoms similar to those observed at the onset of COVID-19, should be conducted by in silico and by wet testing. The testing organisms and strains should be included, but not limited to, those indicated below.

The following pathogens and nucleic acids are examples for testing cross-reactivity for avoiding false positive results in known negative specimens or false negative results in known positive specimens in SARS-CoV-2 examination:

a) regional human coronavirus (HKU1, OC43, NL63 and 229E), SARS-CoV-1 and MERS coronavirus;

b) other respiratory viruses, such as H1N1 [new H1N1 influenza virus (2009), seasonal H1N1 influenza virus], H3N2, H5N1, H7N9, Influenza B Yamagata, Victoria, Respiratory syncytial virus A and B, Parainfluenza virus 1, 2 and 3, rhinovirus A, B, and C, Adenovirus 1, 2, 3, 4, 5, 7, and 55, Enterovirus A, B, C, and D, Human metapneumovirus;

c) EB virus, Measles virus, Human cytomegalovirus, Rotavirus, Norovirus, Mumps virus;

d) *Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila, Bordetella pertussis, Haemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Klebsiella pneumoniae, Mycobacterium tuberculosis*;

e) *Aspergillus fumigatus, Candida albicans, Candida glabrata, Cryptococcus neoformans*;

This information applies to manufacturers or LDTs and should appear in the technical communications.

6.5.2.9 Productivity (e.g. speed, hazards)

Manufacturers of SARS-CoV-2 NAAT IVD should meet the same requirements as those for other IVDs, including but not limited to production environment of the factory, facilities and equipment, production management, quality control system, risk management system, and production capacity.

It is pre-supposed that the production environment, facilities and equipment, production management and quality control systems of the plant meet international quality standards, e.g. ISO 13485[58] or country specific standards, e.g. Chinese GMP, US QSR (21 CFR 820), Japanese Ministry of Health, Labour and Welfare Ordinance No. 169.

6.5.3 Design risk management

The design risk analysis shall specifically address how to mitigate the risk that SARS-CoV-2 variants are not detected by the developed examination. A typical PCR assay design begins with computational (in silico) identification of a unique region (signature) that can support the binding of primer and probe sequences for target-specific amplification as a means of detecting the presence of the target organism. This step is followed by wet lab testing of the primers and probes using reverse transcribed RNA and performance-optimization of selected assays. In addition, extensive testing of the assay in the intended clinical matrix is recommended to evaluate assay parameters, such as LOD, clinical sensitivity, and specificity. The clinical sensitivity and specificity of the assay are experimentally determined using a set of target strains (inclusivity), near-neighbour strains (exclusivity), and matrix-relevant organisms (background). Assay performance also should always be measured in assay-specific matrices (i.e. respiratory tract swab, sputum and saliva). Often, assays are computationally designed using a set of available genomic or gene sequences at that time and then experimentally validated for signature presence in all available samples of the target organism (inclusivity panel) and validated for signature absence in many other samples that do not contain the target (exclusivity panel and matrix panel). Detection assays are typically designed using all sequences available at that time, which may, however, change during product application (see 10.1).

NOTE 1 Verification of in silico nucleotide identity match, termed as inclusivity analysis, is also a component of the performance criteria of SARS-CoV-2 diagnostic assays by the US FDA as well as the European Commission.

NOTE 2 This information applies to manufacturers or LDTs and appears in the technical specification.

6.6 Optimization of reagents and methods

6.6.1 Selection of SARS-CoV-2 target sequences

It is recommended that SARS-CoV-2 specific sequences, published by WHO or other reputable genetic sequence sources (e.g. GISAID[59]), be used for the identification of an assay's target regions and the development of primers for detection.

For laboratories independently selecting target regions, regular checks against sequence databases should be performed to determine whether a modification or change is required. For selected SARS-CoV-2 target sequence regions, the specificity should be fully verified, including comparative analysis between homologous species.

NOTE These recommendations apply to manufacturers or LDTs, but this information does not appear in the technical notices.

6.6.2 Potential impact of variants of concern (VOCs) on the quality of NAAT diagnostic methods for detecting SARS-CoV-2

As long as the genetics of the circulating variants are known, it is relatively straightforward to determine likely impact by comparing the new sequence with that of the primer and probe. Genetic variants not known at the time of test design can require separate validation for patient care (see 10.1). While sequence changes in the primer and the probe binding region can lead to no effect, primers and probes are not usually knowingly designed with such sequence mismatches, and identification of such variants can indicate a need to redesign an assay (either by targeting a different genetic region that does not differ or by incorporating degeneracy in the assay to account for multiple genetic sequences). This is because such changes can reduce performance or result in failure of the molecular step leading to false negative results[60][61]. Performing a test that targets multiple genetic regions (usually in a multiplex format) can mitigate against false negative results occurring due to changes at a specific genetic region. However, as such changes can be cumulative, occurring together in the same variant, it is always recommended that test developers are cognisant of the fact that such genetic changes can impact on test performance[60]. Furthermore, if SARS molecular diagnostics becomes incorporated into tests that measure multiple possible pathogens (e.g. a viral respiratory panel including Influenza sub-types and respiratory syncytial virus), the ability to target multiple regions of the same virus can be limited.

Test developers should monitor primer and probe complementarity within circulating variants in the geographical regions where the tests are being used. Where the genetics of a new variant can be predicted to affect the assay performance, developers should alert those using the test of such an eventuality. The urgency with which this information is determined and disseminated is especially relevant for situations like those caused by SARS-CoV-2, because timely and effective management of the pandemic can depend on the most accurate use of diagnostic tests. This can be compromised if the test in question is negatively impacted by new VOCs that are rapidly spreading across a population of a given region and delay the ability to implement mitigating interventions to stop the spread.

NOTE As primer and probe sequences represent only a part of a successful test solution, many IVD providers opt to disclose primer and probe sequences[62] as this enables wider and more rapid determination of the likely impact of a given genetic change on the diagnostic tests.

6.6.3 Selection of amplification methods

According to laboratory environment, conditions and facility compatibility, amplification methods suitable for the laboratory should be selected, including, but not limited to, fluorescent PCR, dPCR, isothermal amplification, and other amplification methods.

6.6.4 Design and selection of primers

Primer sequences published by WHO are recommended for testing.

For laboratories determining and selecting SARS-CoV-2 target regions for their assay design, the amplification primers for the selected target region should be screened and should take into consideration the design factors of primer length, GC content and dissolution curve to benefit from optimal annealing and avoid formation of primer dimers.

NOTE These recommendations apply to manufacturers or LDTs.

6.6.5 Optimization of the reaction system

In development of the reaction system, the PCR components used in the reaction system (including but not limited to amplification enzyme, reagents, and primers) should be thoroughly examined by experimental processes to determine the optimal component balance. In addition, for the selected amplification protocol, the efficacy and stability of the amplification conditions should be fully considered to establish efficient amplification reaction conditions.

NOTE These recommendations apply to manufacturers or LDTs.

6.6.6 Determination of cut-off values

Cut-off values should be determined according to the intended use and used as a limit to identify samples that indicate the presence or the absence of a specific disease, condition or measurand.

For samples used to determine the cut-off values, clinical samples should be selected. The samples should represent varying concentrations of the target from negative, borderline positive, up to strongly positive. In addition, the cut-off should be verified for various lots.

These recommendations apply to manufacturers and LDTs. The user should be aware that national or local requirements regarding ethical consideration can apply, particularly when using clinical samples for evaluation of the accuracy of a test method or assay.

NOTE 1 Cut-off value defines which measurement results are reported as positive, and which are reported as negative.

NOTE 2 Measurement results near the cut-off value can be considered inconclusive.

NOTE 3 The selection of the cut-off value determines the clinical specificity and clinical sensitivity of the examination.

Cut-off values should be determined based on a specific confidence interval with an associated power and coefficient of variance.

6.6.7 Verification and validation of test design

6.6.7.1 General

During the development of SARS-CoV-2 nucleic acid test kit, the performance and stability parameters of the kit should be evaluated against a predefined verification and validation plan. For the detection of SARS-CoV-2, clinical specimens from targeted patient populations should be selected for testing in order to ensure the accuracy of test results, and an appropriate number of samples should be selected for testing according to the study requirements (see 6.5.2.5).

The verification study for precision should allow calculation of inter- and intra-run variation, which can then be combined to determine the total variation of the assay (see 6.5.2.6). Repeatability should be tested by assessing replicates of the identical test items (e.g. specimen matrix, contrived samples, artificial matrix) suitable for the examination workflow, the same equipment and by the same operator in the same laboratory over a short period of time. It is recommended to perform repeatability tests over a defined period of time as described in the verification plan reflecting design input specifications. It can also be referred as within-lot, run or intra-assay precision.

Where required, intermediate precision should be tested by assessing replicates of the identical test items (e.g. specimen matrix, contrived samples, artificial matrix) suitable for the examination workflow, the same equipment and by the same operator in the same laboratory over an extended period of time as described in the validation plan.

Reproducibility should be verified by assessing replicates of the identical test items (e.g. specimen matrix, contrived samples, artificial matrix) suitable for the examination workflow by different operators and different equipment. It is also known as "interlaboratory reproducibility".

Verification of analytical specificity should reflect test specimens prepared at the highest clinically relevant level of organism (6.5.2.8). Test samples can be prepared by spiking cultured isolates into negative clinical matrix or from clinical specimen confirmed positive by authorized methods. The cross-reaction should be verified at concentrations observed in clinical infections for the microorganism.

6.6.7.2 Stability of reagents

Stability studies should be performed to determine the shelf life of the reagents or LDT reagents. These studies should ensure claimed performance characteristics are met during the claimed shelf life.

The reagents should be subjected to real or simulated shipping conditions as part of the shelf life study. The conditions investigated should include the extremes of conditions (e.g. temperature, humidity, vibration, pressure) to which the reagents are likely to be exposed to during transport and storage.

In the case of limited time for emergency use, not all studies will have been completed. A plan for completion of the studies should be made, and the stability study can be continued after the product is launched.

In practical operation, reference materials with known concentration of analytes should be used in stability tests.

The shelf life of reagents should be determined based on ISO 23640[63]. It is always recommended to test reference materials using the reagents before and after transportation in claimed condition to assess the reagent stability after transportation.

Freeze-thaw stability should be determined for reagents that can be exposed to multiple freeze-thaw cycles during use. It is always recommended to test the reference materials using reagents that have been exposed to repeated freezing and thawing to determine the maximum number of freeze-thaw cycles that can occur without affecting performance of the reagents. Performance should be assessed after the reagents have been exposed to various storage and transport conditions with different duration, taking into consideration also the local routes, transport means, and transit used to supply the reagents.

6.6.7.3 Feasibility studies of in-house tests

The planning phase of developing an LDT should always be composed of a design phase and feasibility testing phase. During this feasibility phase, performance data should be collected first, then troubleshooting and optimization processes should be used to set up performance characteristics and to develop a useful validation procedure, as described in ISO 17822[16].

6.6.7.4 Diagnostic accuracy and validation

Manufacturers should assess what information SARS-CoV-2 NAAT can deliver for medical and public health decision-making and how to verify and validate that the test performance is fit for purpose. The criteria for assay performance include, but are not limited to, LOD, analytical specificity, reportable range, robustness, interferences, clinical sensitivity and clinical specificity.

Prior to the availability of full clinical validation, manufacturers data should always be reviewed to assess molecular detection assays suitable for emergency use by authorities in according with specific procedure.

7 Verification for patient care

7.1 General

Performance verification for patient care is a process to confirm that the performance of the approved IVD reagent by a regulatory body (e.g. FDA cleared or approved, or CE labelled) can be used for patient care. Before carrying out performance verification, the laboratory environment, facilities and the local quality management system should be fully considered to meet the requirements of the test.

NOTE Further information on verification of assays applying nucleic acid amplification-based examination procedures is given in ISO 17822:2020, Annex B[16].

Performance verification should take place in the laboratory that will carry out SARS-CoV-2 nucleic acid testing. If the verified analysis is transferred to another laboratory, it is recommended to determine that the relevant performance characteristics of this testing method have not been affected by environmental changes.

For approved IVD reagents, the purpose of verification is to confirm that reagents or products function according to the key performance characteristics described to prove the suitability in the end user's environment.

The evaluation indices for performance verification include, but are not limited to, accuracy, sensitivity, analytical specificity, LOD, and precision.

The types of specimens used for performance verification can be clinical specimens or artificial simulation samples (matrix effects need to be considered). During verification of precision, the test samples should include at least three concentrations, i.e. negative, borderline positive, moderate or strong positive. Performance verification methods for relevant indices are listed in 6.5.2.

7.2 Confirmation of analytical performance characteristics

7.2.1 Accuracy

Ideally, clinical specimens or samples with known examination results should be used to evaluate the accuracy of a test method or assay. For qualitative detection methods, i.e. agreement rates with negative and positive samples, the accuracy should be assessed from the underlying quantitative result and summarized according to the specific range of detection from the quantitative method.

For detection methods covering multiple sample types, all types of test samples should be included at the same time. In situations where samples cannot be obtained, non-target clinical samples or contrived samples can be selected as the dilution matrix, and then pseudo-virus of known concentrations of target sequences can be added for testing.

Accuracy tests should also take into account the selection of test sample size and the determination of accuracy results (see 6.5.2.5). The number of test specimens should be adequate to ensure statistical data analysis with acceptable level of confidence. It is recommended to test no fewer than 20 and typically 40 to 50 or more specimens. Users of clinical samples for evaluation of the accuracy of a test method or assay should be aware of necessary ethical considerations under national or local jurisdiction.

7.2.2 Limit of detection (LOD)

The LOD should be determined utilizing the entire test system from specimen preparation, nucleic acid extraction, to detection for each clinical specimen type and matrix claimed, unless otherwise justified. A variety of commercially available products are available for use in establishing the LOD of the assay if cell culture virus cannot be used due to the required biosafety level.

In order to ensure statistical validity, a sufficiently large specimen matrix volume is recommended to pool individual negative donor specimens to generate a sufficiently large volume of a homogeneous

matrix. Alternatively, an artificial matrix can be used if it can be demonstrated that this performs equivalently to the patient clinical matrix.

LOD confirmation should be performed on enough samples to be statistically valid and include template control (or negative control) and known low concentration samples. More information on statistical analysis of NAAT tests is available in ISO 20395[9].

The performance specifications are constantly evolving, thus, the provider of the test should be aware of and monitor the recommendations set out by local competent authorities.

The LOD should be determined for each claimed specimen type.

The LOD should be reported in copies/ml or IU/ml[20]. C_q (C_t or C_p) should not be used to report LOD, unless otherwise justified.

NOTE These recommendations apply to manufacturers or LDT. The LOD, analytical specificity, robustness, clinical performance can be evaluated systematically and can be included in the datasheet.

7.2.3 Inclusivity

The inclusivity should include in silico analysis and wet testing analyses, when possible. In silico analysis should include multiple representative strains from, for example, the GenBank or GISAID sequence database[59] for each organism. If in silico analysis reveals other potential cross-reactants, it is highly recommended to carefully review the alignment and determine, based on the positions of the homologous stretches and mismatches, whether additional cross-reactivity or interference or both types can be present and can affect specificity.

For wet testing analysis, representative SARS-CoV-2 samples from different sources should be used. The selected strains should be tested at a concentration near the LOD. It should be ensured that all strains from different sources can be accurately detected.

7.2.4 Specificity

Specificity should be evaluated by testing that includes the presence of interfering substances and for cross-reactivity with common respiratory pathogens. For the detection of SARS-CoV-2 nucleic acid, the interfering substances include interference introduced by, for example, sampling disposables and sample state itself (including interference of various potential drugs).

Details regarding interfering substances and cross-reactive pathogens are described in 6.5.2.8.

7.2.5 Robustness

Varying factors that can impact the assay performance and stability should be evaluated. The influence of specimen types, transport media brands, reagent volumes, contaminants, instrumentation, operating temperature among other factors should be considered. Exploring this will ensure the possible challenges to the test performance and should be documented for consideration and evaluation by the user.

Robustness of testing should fully consider the influencing factors during detection of different amplification methods.

Robustness studies are designed to challenge the system under conditions of stress to identify potential deficiencies and determine the robustness of the product.

The influence of the following factors on expected results should be evaluated: sample and reagent volume, handling and operating temperature.

For new instrumentation to be used, compatibility with the IVD reagent should be validated and the following should be taken into account: ruggedness (including the effect of vibration from other instruments), impact of environmental contaminants (e.g. dust, moisture, mold) on equipment (e.g. optics), impact of power supply fluctuation.

Studies investigating the impact of sample volume should ideally be conducted on all claimed specimen types.

7.3 Clinical evidence

Clinical evaluation is the assessment and analysis of data generated from studies for validating the clinical intended use of the product, and the verification of clinical performance. Clinical evidence is the combined information from the clinical data and its evaluation. A manufacturer should have clinical evidence to support any clinical claims.

The clinical performance, in general, should be ideally evaluated for each claimed clinical specimen type. Specimens should be tested in a blinded fashion, e.g. positive and negative samples should be mixed and blinded to the end user, the end user should also be blinded to the results of any comparator method testing.

For clinical evaluation, natural clinical specimens should be selected to test. Repeat sampling from individuals should be avoided. Ideally, prospective specimens should be tested. If a prospective study is not feasible, an alternative can be to test retrospectively collected specimens from patients, accompanied by basic information, e.g. the specimen collection date, date of onset of symptoms (if present and known), tests used to identify SARS-CoV-2 positive specimens.

The number of clinical specimens tested should meet the basic statistical requirements and percent agreement should be calculated in comparison to a reference method to evaluate the clinical performance of the product.

8 Validation for patient care

8.1 General consideration

When laboratories carry out SARS-CoV-2 nucleic acid detection using in-house-developed assays or modified commercially available SARS-CoV-2 nucleic acid detection IVD assays (e.g. modifying the use conditions or testing off-label specimen types), performance of the analytical test should always be verified and its intended use be validated to be suitable for patient care. Laboratories should assess the information SARS-CoV-2 NAAT can deliver for medical and public health decision-making (see 6.6.7.4).

NOTE Further information on validation of assays applying nucleic acid amplification-based examination procedures is given in ISO 17822:2020 Annex B[16].

The purpose of performance validation is to evaluate whether the assay can meet the corresponding clinical applications, that is, to assess whether the assay can meet the test needs of intended clinical use.

8.2 Clarification of the intended use

The intended use should be determined by taking the following factors into account, including but not limited to:

a) purpose, benefit and use of the test (e.g. screening or diagnosis); whether the result is qualitative, semi-quantitative or quantitative;

b) target population;

c) specimen types;

d) collection and processing procedures;

e) specimen acceptance and rejection criteria.

8.3 Performance with clinical specimens or samples

Clinical specimens or samples with known examination results should be tested in accordance with the intended use to assess the ability of the test to yield results that correlate with the presence or absence of SARS-CoV-2 infection and the consistency with which the method produces those results.

The assays should be reassessed in light of the risk of mutations during viral evolution. As with any pathogen, SARS-CoV-2 genetic VOCs, which differ temporally and geographically, can occur that will impact the performance of a NAAT assay. The resultant phenotypic changes can lead to differences in the viral burden or tropism during the course of infection that can be advantageous or deleterious to a diagnostic pipeline. Consequently, the impact of VOCs should be monitored as part of routine test evaluation. Furthermore, as the final molecular step in the NAAT process, it works by directly measuring the genetic material, and any genetic changes that occur at the binding sites of the primers or probes also impact on assay performance. Reassessment should include in silico cross-reactivity with human genes, genes of other members of family *Coronaviridae*, and other respiratory viruses or bacteria.

The clinical performance, in general, should be ideally evaluated for each claimed clinical specimen type, and the test developers should establish the corresponding statistical analysis methods to describe the results.

The selection of clinical sample size should meet the basic statistical requirements and the clinical performance should be consistent with the pre-defined acceptance criteria. The underlying analysis and the number of individuals included in clinical testing should be described.

9 Design transfer to production

Each organization should have procedures to perform and document design transfer to ensure that device design is correctly translated into production specifications and that processes will consistently yield a product that meets requirements. The design and development outputs should be verified as suitable for manufacturing and production capability, including availability of components and materials, required production equipment, training of operators, before becoming final production specifications.

It is recommended that the design translation activities should transfer every specific process into a design requirement that relates to product realization.

10 Implementation and use in the laboratory and reporting of results

10.1 Implementation and use in the laboratory

Once the validation and verification process are completed, the testing should always be integrated into work procedures and the laboratory quality management system for implementation. The assays should be reassessed in light of the risk of mutations during viral evolution. As with any pathogen, SARS-CoV-2 genetic VOCs, which differ temporally and geographically, can occur and impact the performance of a NAAT assay. The resultant phenotypic changes can lead to differences in the viral burden or tropism during the course of infection that can be advantageous or deleterious to a diagnostic pipeline. Consequently, the impact of VOCs should be monitored as part of the routine test evaluation. Furthermore, as the final molecular step of the NAAT procedure function by directly measuring the genetic material, any genetic changes that occur where the primers or probes bind, can also impact on assay performance for clinical specimens. Reassessment should include in silico cross-reactivity with human genes, genes of other members of family *Coronaviridae* and other respiratory viruses or bacteria.

NOTE Further information on validation and verification of assays applying nucleic acid amplification-based examination procedures is given in ISO 17822:2020 Annex B[16].

A complete set of SOPs should be compiled for daily operation of the test methods.

When testing procedures for IVD reagents are modified by a laboratory, reagent performance should be fully validated prior to implementation for routine use.

In case of subsequent modifications to the validated testing procedures, the modified procedures should be confirmed before implementation and use in the laboratory.

Before implementing the test, the laboratory should formulate regular quality control procedures.

Before implementing the new procedures, the laboratory should complete the quality requirements for training and competence assessment of relevant personnel.

Clinicians and other clients of the laboratory should be provided with adequate consulting services for newly introduced test methods for medical use.

10.2 Reporting and interpretation of results

Appropriate procedures should be implemented to ensure timely reporting of the results and robust data maintenance. The results of quantitative molecular analysis should usually be reported as copies of a copy-based unit (e.g. copies, international units) per unit volume (e.g. volume of collected specimen, transport medium, or body fluid).

The detection range of assay should be reported and the units used (including denominator) clearly stated (e.g. genome copies, international units, per ml samples, µl extract).

EXAMPLE "not detected" or "below the limit of detection".

While quantitative metrics are useful for SARS-CoV-2 NAAT assay development, optimization and continued performance monitoring, the actual clinical value of a given quantitative measurements of SARS-CoV-2 RNA remains unclear. The use of copy based (genome copies, international units) quantitative measurements should be applied with caution when managing patients. Furthermore, as a given C_q (C_t or C_p) value can differ in magnitude between laboratories by > 1 000 fold[64], this unit is unsuitable to be used to stratify individual patients or as reference units to set analytical targets for other in vitro diagnostic tests.

Qualitative test results should not be expressed as "positive" or "negative", rather they should be expressed as "detected" or "not detected". The laboratory should always 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 should always have a standardized procedure of tools and parameters for data analysis. For indeterminate results, the laboratory should always have a procedure for retesting by the same or another method or for requesting an additional or different specimen. Additionally, the limits of detection and the cut-off values for the assay, along with the clinical picture are all considerations for result interpretation.

For quantitative analysis, the laboratory should indicate clinically important test limits by any combination of cut-off values, effective range of linearity, limits of detection and limits of quantification for ease of interpretation of the results. The laboratory should define critical results for all tests that significantly influence the decisions on patient management. When such results are obtained, notification of relevant clinical personnel should occur through a reporting process.

Interpretation of results from in vitro diagnostic assays should be performed in accordance with the manufacturer's IFU by personnel, where allowed by the manufacturer. The PCR positivity threshold can be adjusted to ensure reduction of false SARS-CoV-2 results caused by noise signals.

All result reports should be designed to deliver and include the relevant interpretation considerations and notes about the testing limits as it relates to the method of detection.

The result where SARS-CoV-2 was detected should be reported to public health authorities.