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In vitro diagnostic medical devices — Multiplex molecular testing for nucleic acids —

Part 1: Terminology and general requirements for nucleic acid quality evaluation

Dispositifs médicaux de diagnostic in vitro — Tests moléculaires multiplex pour les acides nucléiques —

Partie 1: Terminologie et exigences générales pour l'évaluation de la qualité 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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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

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

A list of all parts in the ISO 21474 series can be found on the ISO website.

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

The first generation of in vitro diagnostics (IVD) medical devices for nucleic acid-based molecular tests have been focused on detection or quantitation of a single nucleic acid sequence (e.g., viral RNA, mRNA or genomic DNA) within a clinical specimen. By comparison, a multiplex molecular test simultaneously measures multiple nucleic acid sequences of interest in a single reaction. The development and clinical use of multiplex IVD medical devices are rapidly expanding with technological advances and new elucidation of the clinical significance of many biomarkers.

The measurement of multiple analytes of interest in a clinical specimen is generally performed by the following successive (or simultaneous) steps. After specimen collection, transport and storage, nucleic acids are extracted, with or without a subsequent purification procedure. The nucleic acid is then quantified, and its quality evaluated (if necessary), diluted (if necessary) and subjected to multiplex molecular test(s). Multiplex molecular tests in current clinical use detect DNA or RNA targets using various techniques, such as multiplex PCR examinations, microarrays, mass array or massive parallel sequencing-based methodologies.

Although quality aspects of nucleic acids for single target molecular analysis (such as singleplex PCR) has been described[1][2], this cannot necessarily be applied to multiplex molecular tests. Due to the inherent competition for more than one nucleic acid target in a multiplex assay, these assays are usually more sensitive to the isolated nucleic acid quality and quantity than single target assays. The variability of each specimen in biological, physical and chemical properties can influence the performance of multiplex assays to a larger degree than single target assays, potentially leading to unreliable results and hampering patient care. Thus, sample quality evaluation should require additional considerations for multiplex molecular tests.

The collection, transport and preparation of specimens for medical laboratory use has been addressed in national and international efforts in general including ISO/TS 20658 “Medical laboratories—Requirements for collection, transport, receipt and handling of samples”[3], “Guideline for the Quality Management of Specimens for Molecular Methods; The Procurement, Transport, and Preparation of Specimens” (Japan, JCCLS)[4] and “Guideline for the Quality Management of Specimens for Molecular Methods (Part 2) New Technologies and Sample Quality Control (Japan, JCCLS)”[5], and more specifically for different biological specimen types in the series of ISO 20166, 20184, and 20186[6][7][8].

This document describes the terminology and general quality requirements for nucleic acid used in multiplex molecular tests, in order to ensure reproducible performance of such tests.

NOTE Guidelines, requirements, and performance criteria laid down in this document, are intended to ensure that comparable, accurate and reproducible results are obtained in different laboratories.

In vitro diagnostic medical devices — Multiplex molecular testing for nucleic acids —

Part 1: Terminology and general requirements for nucleic acid quality evaluation

1 Scope

This document provides the terms and general requirements for the evaluation of the quality of nucleic acids as the analytes for multiplex molecular tests, which simultaneously identify two or more nucleic acid target sequences of interest. This document is applicable to all multiplex molecular methods used for examination using in vitro diagnostic (IVD) medical devices and laboratory developed tests (LDTs). It provides information for both qualitative and quantitative detection of nucleic acid target sequences.

This document is intended as guidance for multiplex molecular assays that detect and/or quantify human nucleic acid target sequences or microbial pathogen nucleic acid target sequences from human clinical specimens. This document is applicable to any molecular in vitro diagnostic examination performed by medical laboratories. It is also intended to be used by laboratory customers, in vitro diagnostics developers and manufacturers, biobanks, institutions and commercial organizations performing biomedical research, and regulatory authorities. This document is not applicable to metagenomics.

NOTE An examination procedure developed for a laboratory's own use is often referred to as a "laboratory developed test", "LDT", or "in-house test".

2 Normative references

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

ISO 15189:2012, *Medical laboratories — Requirements for quality and competence*

3 Terms and definitions

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

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

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

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

3.1
accuracy
closeness of agreement between a measured quantity value and a true quantity value of a measurand

Note 1 to entry: The term accuracy, when applied to a set of test results, involves a combination of random components and a common systematic error or bias component (ISO 3534-2:2006, 3.3.1).

[SOURCE: ISO/IEC Guide 99:2007, 2.13, modified — "NOTE 1", "NOTE 2" and "NOTE 3" have been deleted, and new "Note 1 to entry" has been added.]

3.2
algorithm
set of rules or calculations applied to test data that generate an interpretable or reportable result

3.3
allele
<genetics> any of several forms of a gene that is responsible for hereditary variation

Note 1 to entry: An allele can also be defined as:

1) one of the alternate forms of a polymorphic DNA sequence that is not necessarily contained within a gene;

2) one of the alternative forms of a gene that may occupy a given locus.

3.4
allelic ratio
ratio of a specified *allele* (3.3) to the total number of *alleles* (3.3), normally expressed as a fraction

Note 1 to entry: For example, if a specific *allele* (3.3) represents 40 % of the total *alleles* (3.3) found at a given locus, the allelic ratio is 0,4.

Note 2 to entry: Allelic ratio is synonymous with allele frequency.

3.5
analyte
component represented in the name of a measurable quantity

[SOURCE: ISO 17511:2020, 3.1, modified — The example has been deleted.]

3.6
chemical purity
degree of contamination with chemical substances that influences the multiplex analysis

Note 1 to entry: The purity of nucleic acid for PCR is absence of interfering organic and protein components carried through from the extraction step, as well as contaminating nucleic acids.

3.7
DNA microarray
DNA chip
solid substrate where a collection of probe DNA arranged in a specific design is attached in a high-density fashion directly or indirectly, that assays large amounts of biological material using high-throughput screening methods

[SOURCE: ISO 16578: 2013, 3.3]

3.8
documented procedure
specified way to carry out an activity or a process that is documented, implemented and maintained *interlaboratory comparison* (3.13)

3.9
evaluation method
method of evaluating the quality specified for nucleic acid

3.10
expiry date
expiration date
upper limit of the time interval during which the performance characteristics of a material stored under specified conditions can be assured

Note 1 to entry: Expiry dates are assigned to *IVD reagents* (3.16), calibrators, control materials and other components by the manufacturer based on experimentally determined *stability* (3.38) properties.

[SOURCE: ISO 18113-1:2009, 3.17, modified — "Note 2 to entry" and "Note 3 to entry" have been deleted.]

3.11
external measurement standard
reference standard
material or substrate prepared for testing the compatibility of the methods of multiplex analysis, whose property value is derived as a consensus value based on collaborative experimental work under the auspices of a scientific or engineering group

Note 1 to entry: This is commonly targeted at the multiplex molecular analysis.

Note 2 to entry: Reference material can be used as an alternative of external measurement standard.

[SOURCE: ISO 16578:2013, 3.9, modified — "Note 1 to entry" and "Note 2 to entry" have been added.]

3.12
intended use
intended purpose
objective intent of an IVD manufacturer regarding the use of a product, process or service as reflected in the specifications, instructions and information supplied by the IVD manufacturer

[SOURCE: ISO 18113-1:2009, 3.31, modified — "Note 1 to entry" and "Note 2 to entry" have been deleted.]

3.13
interlaboratory comparison
organization, performance and evaluation of measurements or tests on the same or similar items by two or more laboratories in accordance with predetermined conditions

[SOURCE: ISO/IEC 17043:2010, 3.4]

3.14
in vitro diagnostic instrument
IVD instrument
equipment or apparatus intended by a manufacturer to be used as an *IVD medical device* (3.15)

[SOURCE: ISO 18113-1:2009, 3.26, modified — "Note 1 to entry" has been deleted.]

3.15
in vitro diagnostic product
in vitro diagnostic medical device
IVD medical device
reagents, instruments, and systems intended for use in the diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae

[SOURCE: 21CFR809.3 of the US Federal Food, Drug and Cosmetic Act]

3.16
in vitro diagnostic reagent
IVD reagent
chemical, biological, or immunological components, solutions or preparations intended by the manufacturer to be used with an *IVD medical device* (3.15)

[SOURCE: ISO 18113-1:2009, 3.28, modified — "Note 1 to entry" has been deleted.]

3.17
laboratory developed tests
LDTs
type of in vitro diagnostic devices that are intended for clinical use and are designed, manufactured and used within a single laboratory

Note 1 to entry: It is often referred to as a "in-house test".

[SOURCE: CLSI QSRLDT]

3.18
limit of detection
LOD
measured quantity value, obtained by a given measurement procedure, for which the probability of falsely claiming the absence of a component in a material is β, given a probability α of falsely claiming its presence

Note 1 to entry: IUPAC recommends default values for α and β equal to 0,05.

Note 2 to entry: This is for LODs when the tests are evaluating the presence or absence of multiple *analytes* (3.5) rather than a *multivariable molecular test* (3.26).

Note 3 to entry: Limit of detection, LOD, is alternatively defined as 1) the lowest quantity of a nucleic acid that can be sequenced reliably and distinguished from its absence typically within a stated confidence limit; 2) the minimum detectable allelic fraction in a given sample.

[SOURCE: CLSI MM09 2014]

3.19
limit of detection for microarray platform
limit of detection for multiplex molecular test platform
LODP
lowest relative quantity of the *external measurement standard* (3.11) (or reference material) that can be consistently detected experimentally at a 95 % confidence level, given a known (determined/estimated) number of copies and/or concentration of the *external measurement standard* (3.11) (or reference material)

Note 1 to entry: This is commonly targeted at the multiplex molecular analysis.

Note 2 to entry: LODP can be used as a performance indicator replaced by *limit of detection* (3.18) for multiplex analysis.

[SOURCE: ISO 16578:2013, 3.1, modified — "Note 1 to entry" and "Note 2 to entry" have been added.]

3.20
massive parallel sequencing
methodology that enables high-throughput DNA sequencing using the concept of processing a very large number of molecules in parallel

Note 1 to entry: For example but not limited to the technologies with miniaturized and parallelized platforms for sequencing of thousands to millions of short reads (≈50 to 400 bases), or polymerase-based real-time DNA sequencing platform enabling long read (mean length ≈10,000–15,000 bases).

3.21
microRNA
17 to 25 nucleotide-long single strand RNA relating to post transcriptional expression regulation

3.22
multiple sequences of analyte(s)
constituent of a sample with multiple sequences of nucleic acid measured simultaneously

Note 1 to entry: This includes extracted nucleic acid and that before and/or after amplification in case of nucleic acid amplification-based assay.

3.23
multiplex molecular test
in vitro diagnostic test that simultaneously evaluates sequence identity and/or amounts of multiple, namely two or more nucleic acid targets of interest in a single run of the assay, such as *multiplex PCR* (3.25), multiple hybridization detection, microarray and *massive parallel sequencing* (3.20) based methodologies

Note 1 to entry: "Multiplex" is defined as "those in which two or more targets are simultaneously detected through a common process of sample preparation, target or signal amplification, *allele* (3.3) discrimination, and collective interpretation. (CLSI/MM17-A[24]).

Note 2 to entry: Targets of interest is defined as detection targets of interest and exclude the control material from being a target.

3.24
multiplex molecular test quality nucleic acid
nucleic acid template with appropriate property that ensures the measurement by a *multiplex molecular test* (3.23) such as that of sufficient length, quantity, *chemical purity* (3.6), *structural integrity* (3.40), and presence of nucleic acid sequence of interest

3.25
multiplex PCR
PCR technique that employs multiple pairs of primers combined within a single reaction mixture to produce multiple amplicons simultaneously

[SOURCE: ISO 16577:2016, 3.117]

3.26
multivariable molecular test
molecular test that combines the values of multiple variables using an interpretation function to yield a single, patient-specific result including "classification," "score" and/or "index"

Note 1 to entry: This is usually based on a platform of multiplex molecular tests.

Note 2 to entry: This is intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment or prevention of disease.

Note 3 to entry: The term "multivariable" as used in statistics implies the evaluation of multiple outcomes rather than using multiple variables to evaluate a single outcome.

3.27
pathogen
infectious agent that causes diseases in its host

Note 1 to entry: Pathogen includes some virus, viroid, prion, bacterium, fungus, or parasite.

[SOURCE: ISO 15714:2019, 3.1.2, modified.]

3.28
PCR quality DNA
DNA template of sufficient length, quantity, *chemical purity* (3.6), and *structural integrity* (3.40) to be amplified by PCR

[SOURCE: ISO 24276:2006, 3.2.3, modified — "quantity" is added.]

3.29
preanalytical phase
pre-examination processes
processes that start, in chronological order, from the clinician's request and include the examination request, preparation and identification of the patient, collection of the primary sample(s), and transportation to and within the laboratory, isolation of analytes, and end when the analytical examination begins

[SOURCE: ISO 15189:2012, 3.15, modified — The words "isolation of analytes" have been added.]

3.30
primary sample
specimen
discrete portion of a body fluid 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: 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 ISO and CEN documents, a specimen is defined as "a biological sample derived from the human body".

Note 3 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]

3.31
range of reliable signal
ability (within a given range) to provide results that are directly proportional to the concentration and/or copy number of the *external measurement standard* (3.11) (or reference material)

Note 1 to entry: This is used mostly for quantitative but not qualitative tests.

Note 2 to entry: Linear range or analytical measurable range is also used.

[SOURCE: ISO 16578:2013, 3.2, modified — "Note 1 to entry" and "Note 2 to entry" have been added.]

3.32
reportable range
region of the genome in which sequence of an acceptable quality can be covered by the laboratory test

Note 1 to entry: The reportable range is also defined as "the range of test values over which the relationship between the instrument, kit, or system's measurement response is shown to be valid" (US CFR 493).

3.33
reference range
reportable sequence variations the assay can detect that are expected to occur in an unaffected population

Note 1 to entry: A reference range is also defined as a set of values that include upper and lower limits of a laboratory test based on a group of otherwise healthy people.

3.34
RT
reverse transcription
synthesis of DNA from an RNA template using a reverse transcriptase enzyme combined with an RT-primer in the presence of deoxyribonucleoside triphosphate

[SOURCE: ISO 22174:2005, 3.3.1]

3.35
RT-PCR
method consisting of two reactions, a *reverse transcription* (3.34) of RNA to DNA and a subsequent PCR

[SOURCE: ISO 22174:2005, 3.4.2]

3.36
RT-PCR quality RNA
RNA template of sufficient length, quantity, chemical purity and structural integrity suitable for *reverse transcription* (3.34) and PCR

[SOURCE: ISO 22174:2005, 3.2.4, modified.]

3.37
sample
one or more parts taken from a primary sample

[SOURCE: ISO 15189:2012, 3.24, modified — The example has been deleted.]

3.38
stability
ability of an *IVD medical device* (3.15) to maintain its performance characteristics within the limits specified by the manufacturer

Note 1 to entry: Stability applies to:

— *IVD reagents* (3.16), calibrators and controls, when stored, transported and used in the conditions specified by the manufacturer;

— Reconstituted lyophilized materials, working solutions and materials removed from sealed containers (when prepared, used and stored according to the manufacturer's instructions for use).

Note 2 to entry: Stability of an *IVD reagent* (3.16) or measuring system is normally quantified with respect to time:

— in terms of the duration of a time interval over which a metrological property changes by a stated amount;

— in terms of the change of a property over a stated time interval.

[SOURCE: ISO 18113-1:2009, 3.68, modified — "Measuring instruments or measuring systems after calibration "in the "Note 1 to entry" and "Note 3 to entry" have been deleted.]

3.39
specimen stability
resistance of a specimen to quality change during long-term storage

[SOURCE: ISO 23833: 2013, 5.5.10, modified — The text "changes in chemical composition during electron bombardment, i.e. the resistance to change of the intensity of the relevant characteristic X rays observed during the time the specimen is exposed to the electron beam" has been replaced with "quality change during long-term storage".]

3.40
structural integrity
degree of preservation of nucleic acid reflecting the original state

3.41
validation
confirmation, through the provision of objective evidence, that the requirements for a specific *intended use* (3.12) 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, modified — "Note 1 to entry" and "Note 3 to entry" have been deleted.]

3.42
verification
confirmation, through provision of objective evidence, that specified requirements have been fulfilled

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

Note 2 to entry: Confirmation can comprise activities such as

— performing alternative calculations,

— comparing a new design specification with a similar proven design specification,

— undertaking tests and demonstrations, and

— reviewing documents prior to issue.

[SOURCE: ISO 9000:2015, 3.8.12, modified — "Note 1 to entry" and "Note 2 to entry" have been reworded.]

4 General considerations

4.1 General

4.1.1 Pre-analytical phase considerations

For general statements on quality management systems of medical laboratory and in particular on specimen collection and handling, see ISO 15189:2012, 4.2, 5.4.4, 5.4.7 and ISO/TS 20658[3]. The requirements on laboratory equipment, reagents, and consumables according to ISO 15189:2012, 5.3 shall be followed; ISO 15189:2012, 5.5.1.2 and 5.5.1.3 can also apply.

The pre-analytical phase generally consists of the following workflow:

— Sample collection, storage and transport;

— Pre-treatment of the sample;

— Nucleic acid extraction and purification.

These pre-analytical factors greatly impact the quality of a sample and subsequent test results. Details for pre-analytic aspects relevant to molecular assays have been described[6][7][8][25][26][27][28][29][30].

One of the major pre-analytical variables causing a strong impact on analytical test results is post-collection changes of analyte profile (RNA, DNA) by biological causes, such as gene inductions, gene down regulations, apoptosis etc. These effects for example depend on the duration of warm (warm ischemia) and cold ischemia and the ambient temperature before formalin fixation. Such usually remain unrecognized in analytical tests but are a major source for wrong or unreliable analytical test results[6][7][8][30]. Thus, the transport of the tissue before storage or fixation in formalin should be made in the shortest time and possibly at low temperature or under vacuum.

Multiplex molecular tests are IVD tests or medical devices that measure two or more nucleic acid sequence of interest simultaneously. A sample with a high degree of quality should be procured for multiplex molecular tests to ensure it is fit for purpose.

4.1.2 Specimen quality considerations

Specimen source, collection, preparation, extraction, and purification should be validated and verified (if necessary) in order to ensure nucleic acid quality is appropriate for all analytes or targets to be detected within the assay. A panel of targets for multiplex assays includes both high and low prevalence targets.

The variability of the biological, physical and chemical properties in each specimen can influence the quality of the nucleic acid obtained, and thus the performance of multiplex molecular assays between different specimens. However, it is not practical to evaluate all such influences in each specimen. Thus, the laboratory should ensure that each clinical specimen is procured in a manner so as to avoid such variability in properties. When it cannot be avoided, the influence of variability in properties should be evaluated by an appropriate method, such as including an assessment of internal controls in the assay.

4.1.3 Nucleic acid quality considerations

Multiplex molecular test quality nucleic acid is defined as a nucleic acid template with appropriate properties that ensures measurement by a multiplex molecular test. While general considerations on nucleic acid quality evaluation are shared by singleplex and multiplex tests, there are unique considerations for multiplex molecular tests due to the potential for interference or interactions between the multiple targets and/or test components.

The quality of isolated nucleic acid from a specimen depends on a number of factors including, but not limited to, the quantity, the chemical purity, the length and the structural integrity of the nucleic acids, and the abundance of the target of interest. Its influence should be considered in regard to assay parameters such as LOD and linear range of each analytical method.

The quality of nucleic acid for multiplex molecular test should be evaluated by appropriate methods, based on the measurement method to be used. Quality evaluations should define the representative profile of isolated nucleic acid as determined by length, quantity, chemical purity, and structural integrity, or the detection or quantity of the representative genes (for example, internal control genes).

Process control materials should be used to monitor both the pre-analytical and analytical processes where appropriate and available. Such materials should be used to monitor nucleic acid extraction and purification procedures. Internal controls should be considered to determine the nucleic acid quality of each target sequence.

NOTE The CLSI MM17-A guideline provides recommendations for various aspects of verification and validation of multiplex testing[24].

4.2 Multiplex molecular test quality nucleic acid and evaluation

4.2.1 Evaluation of nucleic acid quality for multiplex molecular tests

“PCR quality DNA” is described in ISO 22174, ISO 16577 and ISO 20395. In ISO 16577, it is described as a DNA template of sufficient length, quality and structural integrity to be amplified by PCR, and “RT-PCR quality RNA”, also described in ISO 22174, is an RNA template of sufficient length and quantity suitable for reverse transcription and PCR. These cannot be applied to the various measurement methods of multiplex molecular tests including PCR or RT-PCR-based methods, microarray, and massive parallel sequencing.

Considering that multiplex molecular test is a molecular biological technique capable of simultaneous detection of multiple nucleic acid sequence even of nucleic acids with shorter length, the evaluation methods for multiplex molecular test quality nucleic acid suitable for each measuring system shall be developed.

The nucleic acid quality should be determined prior to use in multiplex molecular tests. The method by which nucleic acid quality is determined will depend on several factors such as the multiplex method to be used, the known or anticipated quantity of nucleic acid present in the sample and the nucleic acid to be analysed (DNA or RNA). The user shall choose the most appropriate approach, depending on the subsequent multiplex molecular test to be used.

In order to determine quantity, concentration, purity and potential degradation of nucleic acid in a sample, evaluation by spectrophotometric and/or fluorometric methods and/or gel or capillary electrophoresis can be used. The nucleic acid quality should be estimated based on the size distribution shown by electrophoresis of nucleic acids, the detection or quantity of representative genes (for

example, internal control genes including house-keeping genes). Spiked internal controls (i.e. samples to which the analyte of interest has been added in a precisely known amount) can be used to determine the nucleic acid quality of each target sequence to determine the process control. There are methods available to evaluate the quality of nucleic acid present in a solution, as described in Annexes A to D.

EXAMPLE 1 The quality of RNA can be evaluated by electropherogram by means of the ratio of 28S:18S ribosomal RNA of sample containing total RNA, the RNA integrity number (RIN value), or RNA integrity score (RIS)[31][32].

While ribosomal RNA based quality metrics are popular for those conducting RNA analysis, they may not always be useful for evaluating the quality of messenger RNA[33].

EXAMPLE 2 A260/A280 and A260/A230 ratios can be used to assess the purity of RNA

When multiplex molecular tests are designed to detect or quantify targets of different lengths it should be ensured that the nucleic acid template is of sufficient quality as to be amplifiable across the target size range. This can be achieved by use of a PCR reaction with primer sets that produce fragments assessing the lower and upper target size range. Quality of the nucleic acid template suitable for the assay can then be estimated based on the size distribution of the resultant PCR fragments using capillary or gel electrophoresis.

For certain multiplex molecular tests, a critical aspect to ensuring quality of the nucleic acid sample is to determine if the nucleic acid of interest is present. For example, the multiplex analysis of a low abundance nucleic acid target from a specific source tissue or organism should ensure that the sample contains nucleic acid from that tissue/organism. This confirmation may be a component included within the multiplex test design or can be an external assay performed prior to the use of the nucleic acid sample for multiplex assay.

NOTE To improve detection of viable pathogens, simultaneous measurement for ribosomal RNA or messenger RNA can be used. Another approach could be considered to treat bacteria with DNA intercalators that penetrate inactivated cells and inhibit PCR amplification but are excluded from viable cells.

The nucleic acid sample shall have a sufficient quantity of nucleic acid sequence of interest, which is determined by the variant and population of interest. When multiplex molecular tests are designed to detect or quantify different sequences of interest, sufficient amount required in a given subsequent measurement method to fit for purpose shall be ensured. Measurement of the sequence of target with lower prevalence relative to the others shall be ensured.

EXAMPLE For multiplex ligation-dependent probe amplification (MLPA), two denaturation fragments (D-fragments) are used to indicate poor DNA denaturation due to salt contaminants in the DNA sample.

4.2.2 Evaluation of nucleic acid quantity

Appropriate methods for evaluating nucleic acid quantity shall be selected that are fit for purpose of the test design of the multiplex molecular test.

Quantitation of the purified nucleic acid for analysis in a multiplex molecular test should measure the quantity of the target genetic element, relative to the quantity of a specific reference, appropriate calibrants and controls in order to compare the relative values of multiple variables. In this case, the principle of quantitation is to determine the ratio (expressed as a percent) of two DNA or RNA target sequences; i.e. a sequence of interest and a control gene or material (such as internal control genes, reference materials). This confirmation may be a component included within the multiplex test design or can be an external assay performed prior to the use of the nucleic acid sample for multiplex assay.

5 Procedure for preparation of nucleic acid

5.1 General

The nucleic acid extraction method employed shall be appropriate to obtain the quality and quantity of nucleic acid required for the subsequent analysis.

Evaluation of nucleic acids should be performed for their suitability in the multiplex molecular test system in its final configuration, rather than suitability in separate singleplex reactions of which the multiplex test is comprised.

5.2 Preparation of samples

5.2.1 General

In the pre-analytical phase, there are many stages of specimen handling including collection, fixation, storage, transport, preparation, and processing. Since these pre-analytical factors greatly impact the quality of a sample and subsequent test results, the appropriate handling of specimen shall be ensured[6][7][8][30].

As the number of targets of interest increases in a multiplex assay, false negative results for certain sequences can become more problematic. In particular, the quality as it pertains to the target with the lowest abundance within the nucleic acid sample should be assessed, as competition for reaction components can affect rare targets more significantly than more abundant targets.

EXAMPLE 1 In multiplex tests for detection of human papilloma virus subtypes in cervical infection, a poor-quality specimen from uterine cervix, such as mucous without a sufficient number of human cells, can occur during specimen collection. This can result in a false negative test due to an inadequate specimen. To validate a negative test result in samples that are potentially sub-optimal, simultaneous detection of a human genome sequence can be used as an internal assay control.

EXAMPLE 2 In multiplex tests for somatic variations in cancer, relative decrease in the targets of interests can occur when non-neoplastic cells such as inflammatory infiltrates or endothelial cells for example are larger in the proportion than the neoplastic cells, resulting in underestimates or false negative results for the target sequence.

EXAMPLE 3 In multiplex tests for detection of microbial pathogens in respiratory tract infection, poor quality nucleic acid due to a high viscosity of specimen can occur during collection and preparation of respiratory specimens, resulting in false negative tests for a species with a low abundance. This can be minimized by dissolving the specimen in NALC (*N*-acetyl-L-cysteine) and semi-alkali-proteases.

As the number of targets of interests increases, false positive results without clinical relevance becomes more problematic. Thus, the clinical specimen should be collected and handled in a manner to avoid a false positive result due to contamination of such an influence. When it cannot be avoided, the influence should be assessed, with an appropriate method such as using the quantitative measurement with cut-off values.

EXAMPLE 1 In multiplex tests for detection of bacterial pathogens in blood stream infection, contamination can occur during blood collection, resulting in false positive tests for normal human skin flora such as coagulase-negative staphylococcus. This can be reduced by aseptic techniques at blood collection, including antisepsis of skin, hand hygiene and pre-packaged kits. Contamination of non-viable bacteria can also occur in blood after antimicrobial treatment, resulting in positive tests for such bacteria. This can be reduced by avoiding blood collection before or right after antimicrobial administration.

EXAMPLE 2 In multiplex tests for detection of mycobacterium species in respiratory tract infection, contamination can occur during collection of respiratory specimens, resulting in positive tests for normal environment, such as *M. gordonae*, *M. chelonae* or *M. simiae.* This can be reduced by use of a sterile collection device such as bronchoscopy.

5.2.2 Consideration on tissue preparation

When methods based on enzymatic amplification reactions are used for multiplex molecular tests, inhibitors present within tissue specimens may interfere with the detection or quantification of low-level targets of interest relative to more abundant targets. Therefore, inhibitors shall be minimized as much as possible, to an extent that ensures the measurement of each sequence of interest.

The presence and proportion of tumour cells should be evaluated to ensure interpretation of the test result, and to determine whether the enrichment of tumour cells is needed to an extent that ensures the measurement of each sequence of interest.

For FFPE specimens, formalin fixation treatment is accompanied by the fragmentation and chemical modification of nucleic acids, and therefore it is desirable to perform the formalin fixation treatment in an appropriate fixative reagent (i.e. standard buffered formalin solution), at a low temperature and in the shortest possible time[2][6][30].

NOTE The pre-examination workflows of various specimen types such as FFPE, frozen tissue and blood are described in the series of ISO 20166, ISO 20184 and ISO 20186[6][7][8].

NOTE Neutral buffered formalin (NBF) is normally used for the process of fixation.

NOTE DNA obtained from older formalin-fixed paraffin embedded blocks (e.g., >3 years) often shows evidence of deamination of cytosine to uracil, which leads to C:G to T:A transition in DNA sequences. Treatment with uracil-*N*-glycosylase can eliminate uracil-containing DNA molecules in such a sample. Alternatively, assessment of transition to transversion ratios in sequence data from massively parallel sequencing assays can be used to detect significant deamination.

When working with small volume tissue specimens, such as those obtained by fine needle aspiration, stochastic bias should be considered as the number of genome equivalents present in the sample can be insufficient to consistently detect variants or organisms with low genome or allele abundance.

During formalin fixation and tissue preparation for multiplex detection of somatic cancer variants, cross-over contamination of tissue from one patient into the tissue preparation of another patient can occur. This can result in a positive test result for the patient in question, although the nucleic acid sequence originates from a different patient sample. Care should be taken to minimize this risk, by handling tissue on a clean surface and by using single-use devices or consumables (e.g. blade, pad and container). If cross-over contamination is unavoidable, then multiplex assays should be designed with appropriate controls to detect tissue cross-contamination.

NOTE The inclusion of polymorphic genomic regions (such as those used in forensic assays) within the target regions of massively parallel sequencing assays can be used to assess the presence of more than one patient genome within samples being sequenced.

5.2.3 Nucleic acid extraction and purification

The nucleic acid extraction or purification method shall be selected by taking into account the influences of specimen types and matrices on each multiplex method to which the extracted or purified nucleic acid will be subjected.

When measuring using certain high sensitivity multiplex methods, potential contamination of nucleic acid from reagents, columns and plastic ware can cause false positive results. Use of appropriately designed and produced materials for these types of assays should be considered to minimize nucleic acid contamination. Also, potential cross contamination of samples can cause false positive results. Protocol of handling and processing specimens/samples should be established, and it shall be documented and followed in order to minimize the influence of such a cross-contamination.

When low yields of nucleic acid are anticipated from a specimen, special plastic ware should be used to reduce nucleic acid binding to minimize sample loss, such as by use of low-adsorbing plastic ware.

NOTE Some tube materials bind nucleic acids. Polyallomer tubes as reagent vessels absorb less DNA compared to standard polypropylene microfuge tubes which absorb as much as 100 ng DNA. Detergent such as 0,02 % Polyethylene glycol sorbitan monolaurate in every reaction step reduces adsorption of DNA to tube walls[30].

When a multiplex molecular test is designed to detect or quantify nucleic acid targets, a reduced extraction efficiency can cause loss of certain nucleic acid targets leading to false negative results on testing. Care shall be taken to ensure reproducible efficiency of extraction for all nucleic acids targets to be tested in multiplex assays.

Potential contamination of nucleic acids with inhibitors can also cause a false negative result in multiplex assays. Efficient removal of inhibitors should be performed in order to prevent this occurrence.

NOTE When bead beating is used for fungal cell walls or tissue, aggressive extraction tends to lead to fragmentation of nucleic acids.

5.2.4 Quality evaluation method

In order to evaluate the quality of nucleic acids, there are several methods such as absorption spectra to determine quantity, absorption ratio of 260 nm (A260) to 280 nm (A280) to assess purity, and gel electrophoresis to assess fragment length or integrity[31]. Those values, however, do not appropriately indicate the sample quality for multiplex molecular tests, such as an A260/A280 ratio in the evaluation of fragments for sequencing.

NOTE In all sample preparation protocols for massive parallel sequencing, the starting material is the DNA in the form of e.g. isolated genomic DNA, reversed-transcribed cDNA or immunoprecipitated chromatin. To convert this into a sequenceable library, the source DNA is fragmented, polished, size selected, adapter ligated and quantified. DNA concentration and quality of fragments for sequencing using an A260/A280 ratio (for example 2,00) as indicator of sample purity is not appropriate, since the leftover primers, free nucleotides and improperly adapted fragments are indistinguishable from the desired, productive fragments. Instead, other methods such as use of an intercalating fluorescent dye are needed to measure double-stranded DNA specifically.

A260/280 ratio can give valuable information. If the A260/280 ratio is outside of range specified by the analytical test, the sample should be discarded. For example, a value <1,60 can be a strong indication that potentially interfering compounds such as protein, phenol, guanidine or other reagents are present in that sample. A 260/280 ratio can also be useful to determine the purity of the PCR product after cleaning up to remove excess primers/ssDNA, etc.

NOTE The A 260 value is also used to measure the yield of total nucleic acid. This can be used to determine the contamination of genomic DNA when this value is unexpectedly high. For example, when HIV virions are measured from plasma, a high A 260 value can be an indicator of genomic DNA contamination originating from white blood cells.

Methods to directly evaluate the fragmentation status of DNA include direct confirmation by electrophoresis, evaluation of the PCR amplification of DNA fragments of known length (such as internal control genes β-globin or vitamin D receptor, or a reference material spiked in), or evaluation of amplification reactions using the delta Cq (quantification cycle, also known as Cycle threshold, Ct or crossing point, Cp) value in quantitative real-time PCR.

Fragment lengths for sequencing can be estimated from size separation on capillary electrophoresis or a set of cloned and Sanger-sequenced fragments.

To evaluate RNA fragmentation, methods include directly measuring the RNA conformation using denatured agarose gel electrophoresis. Furthermore, the integrity of the extracted RNA can be evaluated by the ribosomal RNA (rRNA) and internal control genes expression including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or beta-actin. When these genes are also to be used for normalization purposes they shall be validated to ensure they are appropriate for the specific test. For RNA quality evaluation, RIN value or RIS and/or the ratio of 18S unit to 28S unit of ribosomal RNA resulted from electrophoresis are available.

NOTE RIN and ribosomal RNA based quality metrics analyses are not always useful indicator for natural degradation of messenger RNA, especially in FFPE samples. For RNA from FFPE, the use of paraffin-embedded RNA metric (PERM) algorithm based on a formula that approximates a weighted area-under-the-curve analysis of an electropherogram of the extracted RNA is suggested to be better than RIN[34].

NOTE A goal of RNA sequencing is to determine the RNA content of a sample with quantitative accuracy. RNA sequencing is achieved through isolation of the RNA fraction of interest (or selective removal of unimportant fractions), conversion into double-stranded cDNA and use in sequencing reactions.

The use of an internal control carried throughout the extraction, amplification and detection phases can be a useful tool in quality evaluation of nucleic acids, estimating effects of inhibitors, and evaluating efficacy of extraction methods. The internal control can be designed to specifically detect each target

of interest. Multiplex molecular tests with such internal controls can be used to assess the amount of particular templates in a sample, for pathogen identification, genotyping, or multiplex mutation analysis.

NOTE In PCR, a multiplex internal amplification control can be used, which contains forward and reverse primer binding regions for multiple targets of interest.

Annex A
(informative)

Evaluation of RNA Integrity

Evaluation methods for RNA integrity by using capillary electrophoresis format include the RNA integrity number, RIN, and the RNA integrity score, RIS[2][31][32]. RIN is obtained with an electrophoresis and is used for an assessment of RNA intactness by showing a detailed picture of the size distribution of RNA fragments[2][35]. A software algorithm has been designed that is capable of assessing RNA quality better than ribosomal ratios. Automatic systems combined with microfluidic chips, voltage-induced size separation and fluorescence detection are widely used for the electrophoresis. RNA molecules are stained with an intercalating dye and detected by means of the system. RIN data is also analysed from electropherograms in the system. In electropherograms and gel-like image, the degradation of rRNA is reflected by a shift towards shorter fragment sizes. The RIN of sample is assigned in the range from 10 (intact) to 1 (totally degraded). In electropherograms and gel-like image, the degradation of rRNA is reflected by a shift towards shorter fragment sizes. As another method for RNA integrity, QIAxcel (Qiagen) system measurement unit for RNA provides RIS using high-resolution capillary electrophoresis with a different software algorithm from RIN.

Annex B
(informative)

Evaluation of DNA Integrity

The DNA Integrity Number (DIN) algorithm was developed, in order to provide an objective and standardized tool for reliable integrity assessment of DNA. DIN determines the fragmentation of a genomic DNA sample by assessing the distribution of signal across the size range and applies an automatically calculated number. To provide a numerical assessment, the samples are assorted according to their signal distribution to a scale of DIN 1 to 10. A high DIN indicates highly intact gDNA, and a low DIN a strongly degraded gDNA sample[2][36].

Annex C
(informative)

Use of PCR to assess amplifiable DNA from FFPE samples

DNA extracted from formalin-fixed tissues is fragmented and also contains DNA lesions that are the sources of sequence artefacts. In particular, extensive fragmentation significantly reduces the amount of amplifiable templates available for PCR amplification. A second major problem related to FFPE DNA is the occurrence of sequence artefacts, i.e. apparent sequence changes that are not present in the original sample.

Fragmentation is the common form of DNA damage found in formalin-fixed tissues. Fragmentation of DNA in formalin-fixed tissues has been shown to be increased with longer storage time and lower pH of formalin used in tissue fixation. Thus, the same quantity of FFPE DNA from different samples can contain significantly different amounts of amplifiable templates, depending on the degree of fragmentation damage.

Formaldehyde-induced crosslinks of DNA reduce the stability of double-stranded DNA, resulting in a partial denaturation of DNA. Fragmentation of DNA in formalin-fixed tissues has been shown to be increased with longer storage time and lower pH of formalin used in tissue fixation[37]. Formaldehyde is readily oxidized to formic acid in the reaction with atmospheric oxygen. The formation of formic acid reduces the pH of formalin. Formalin thus is buffered to maintain a neutral pH level. The N-glycosidic bonds of the purine bases to the sugar backbone are susceptible to hydrolysis at low pH, generating abasic sites in the DNA.

Among the sequence artefacts detected in FFPE DNA, transitional C:G>T:A variants are the most frequent type of SNVs. Sequence artefacts are more readily detectable when low copy numbers of FFPE DNA are tested, as is often the case in amplicon-based protocols. Such artefactual C:G>T:A variants can be markedly reduced after treatment of FFPE DNA with UDG before PCR amplification, indicating that uracil lesions are a major source of artefactual C:G>T:A variants in FFPE DNA. High levels of artefactual C:G>T:A SNVs are found at CpG dinucleotide sites in FFPE DNA, strongly indicative of deamination of 5-methylcytosine bases.

The quality of FFPE DNA for DNA-based analysis can be evaluated by the PCR reaction with primer sets that produce short and long sized fragments (100, 200, 300 and 400 bp) from non-overlapping target sites in a particular gene such as GAPDH. Samples can be classified based on the largest of possible PCR products detected, namely 100, 200, 300 and 400 bp.

Minimizing sequence artefacts is crucial for the accurate detection of actionable mutations in formalin-fixed clinical tissues. Suggested strategies for the minimization of sequence artefacts are summarized in Table C.1[38].

Table C.1 — Strategies for minimization of sequence artifacts from FFPE DNA

Step	Strategy
DNA extraction	Assessment of tumour purity and identification of tumour-enriched areas by a pathologist. Macro dissection or coring of the tumour-enriched areas. Use of sufficient tissue, whenever possible, to ensure that a sufficient quantity of DNA is isolated for subsequent molecular testing. Heat treatment to remove formaldehyde-induced crosslinks and to facilitate subsequent tissue digestion with proteinase. Extended proteinase K treatment to digest tissue and to remove proteins cross-linked to DNA.
DNA assessment	Evaluation of double-stranded DNA quantity using fluorometry. Quantification of amplifiable templates using qPCR or digital PCR, especially for massively parallel sequencing. Use amplicons sizes that correspond to the mean amplicon size of the sequencing assay.
Sample library preparation	In vitro removal of uracil prior to PCR amplification of FFPE DNA. Using assays generating short amplicons to increase the number of templates for PCR. Capture-based target enrichment allowing the recognition of the initial templates in sequence reads using their unique start and end sites. Using primers specific for each strand of the DNA template in amplicon-based target enrichment approach. Molecularly tagging DNA templates for identification of sequence artefacts.
PCR amplification	Use of specific DNA polymerases (e.g. Pfu and KAPA) that have low bypass efficiency over DNA lesions such as uracil and abasic sites. Use a high-fidelity DNA polymerase to reduce polymerase errors.
Validation of sequence variants from amplicon-based MPS	Running each test in duplicate so that separate pools of templates are used. Using orthogonal methods for clinically actionable mutations.