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

Part 2: Validation and verification

Ten STA Dispositifs médicaux de diagnostic in vitro – Tests moléculaires multiplex pour les acides nucléiques — **Partie 2: Validation et vérification**

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

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

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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.*

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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 <u>www.iso.org/members.html</u>.

Introduction

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

The competition among reactions in multiplex molecular tests can impose more stringent requirements for sample purity, input reagents and platforms to avoid nonspecific reactions and background signal. In comparison to single target analysis, multiplex molecular tests require an increased number of controls, more complex performance evaluation/data analysis algorithms and more complex reporting of results.

Laboratories can develop assays in-house ("home-brew, laboratory-developed, in-house") or use commercially available multiplex assays involving a variety of technologies and instrument platforms. With the increase in the availability and use of multiplex molecular tests, a guideline for the development, validation, verification, control, data analysis, and implementation of multiplex molecular tests is increasingly needed. For a multiplex molecular test to reliably achieve its intended use, there should be control of the process from the acquisition of the sample and preparation of the nucleic acid for testing to the evaluation of the data and the reporting of the results. Multiplex molecular testing provides significant challenges to the laboratory with regards to appropriate validation and verification, acquisition of appropriate control materials, data analysis, and reporting. The complexity of data analysis and reporting of results is increased relative to singleplex assays. Moreover, the availability of sufficient and appropriate control and reference materials (RMs) to properly validate and verify multiplex molecular tests is a major challenge. However, the use of partial or full sequencing techniques can be useful in qualifying control materials. This document describes the recommendations for various aspects of validation and verification of the measurement by multiplex molecular tests in order to ensure reproducible performance of such tests, in developing and implementing multiplex molecular nucleic acid tests for clinical use. and ards/sist/c53e0132-88b3-4d13-be49-a097a5193e1d/so

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

Part 2: Validation and verification

1 Scope

This document gives the general requirements for validation and verification of multiplex molecular tests which simultaneously identify two or more nucleic acid target sequences of interest. This document is applicable to all multiplex methods used for examination using 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 examinations that either 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 (IVD) examination performed by medical laboratories. It is also intended to be used by laboratory customers, IVD 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". <u>ISO 21474-22022</u>

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2 Normative references

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

ISO 15189, Medical laboratories — Requirements for quality and competence

ISO 21474-1, In vitro diagnostic medical devices — Multiplex molecular testing for nucleic acids — Part 1: Terminology and general requirements for nucleic acid quality evaluation

3 Terms and definitions

For the purposes of this document, terms and definitions given in ISO 21474-1 and the following 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 <u>https://www.electropedia.org/</u>

3.1

analytical sensitivity

quotient of the change in a measurement indication and the corresponding change in a value of a quantity being measured

[SOURCE: ISO 18113-1:2009, A.3.3, modified — NOTES 1 to 4 were removed ^[1].]

3.2

diagnostic sensitivity

ability of an in vitro diagnostic (IVD) examination procedure to identify the presence of a target marker associated with a specific disease or condition

[SOURCE: ISO 18113-1:2009, A.3.15, modified —NOTES 1 to 4 were removed [1].]

4 General requirements

4.1 General

Multiplex molecular tests are IVD medical devices that detect and/or measure multiple nucleic acid sequences simultaneously, such as multiplex PCR, DNA microarray, and massive parallel sequencing-based methodologies.

In cases of multiplex molecular tests for nucleic acid, competition among individual reactions may impose more rigorous requirements for sample purity, sample input, reagents, and platforms to avoid nonspecific reactions and background signal. In comparison to singleplex analysis, multiplex molecular tests can have an increased number of controls, more complex performance evaluation/data analysis algorithms, and more complex reporting of results.

Validation activities are aimed to ensure determination of performance characteristics for intended use, which is performed in the test development process, by either the manufacturer or laboratory. The design verification and validation plan, created by manufacturers for an IVD and laboratories for an LDT, should aim to establish performance specifications such as accuracy, specificity, precision, LOD/ assay range, and cross-reactivity/interfering substance. When planning the development of a test, the intended use shall be defined, including specimen types to be used. The examination manufacturer shall specify instructions for examination processes. These shall be followed. Whenever any processes or methods are changed during the verification process and after the implementation, a laboratory shall validate the assay with the new performance characteristics. The laboratory shall perform verification activities to confirm the design input specifications required for patient testing in advance. The laboratory then performs ongoing quality assurance (QA) of the multiplex molecular tests by document control of procedures, operator training, routine quality control (QC), proficiency testing, instrument calibration, and result correlation with clinical findings.

The validity and reliability of multiplex molecular test result can be impacted by all steps of a laboratory workflow, including primary sample collection, transport, storage, and processing in the preanalytical phase as well as by the analytical phase itself.

NOTE The CLSI guideline, MM17-A ^[2] provides recommendations for various aspects of verification and validation of multiplex testing.

4.2 Laboratory requirements

The requirement for quality and competence in medical laboratories shall be as described in ISO 15189.

For assays that are not fully integrated in a single disposable or on a single platform (from sample to result), sample treatment should be carried out in separate working areas/rooms as specified in ISO 22174:2005, Clause $6^{[3]}$.

Accidental contamination of DNA and RNA can originate from aerosols containing template, such as amplicons generated from previously amplified NA (nucleic acid) targets. As a consequence, the organization of the work area in the laboratory and good practice shall be based on

- the systematic containment of the methodological steps involved in the production of the results, and
- a "forward flow" principle for sample handling.

The latter ensures that DNA and RNA in the test material remain physically segregated. Further details can be found in ISO 21571^[4].

4.3 Reagents requirements

All reagents and materials used in the analysis should be identical, or equivalent, to those specified in the method. Otherwise, all reagents and materials should be of grade relevant for molecular biology, e.g. DNase and RNase free water.

Those reagents shall be stored and used as recommended by the supplier or according to the laboratory QA specifications.

The characteristics and quality of reagents (e.g. fluorescent dye(s), buffers) and the amount of an external measurement standard, or reference material (RM), that is added to the reaction mixture should be validated/verified.

All critical reagents should be tested for functionality before inclusion in formal performance evaluations. In multiplex molecular tests, functionality should be evaluated using synthetic controls, when not feasible to establish it for all the targets using genomic specimens.

Where the examination manufacturer provides instructions for storage and use of a commercial kit for measurement, these shall be followed. Where the examination manufacturer does not provide such instructions, they shall be specified and validated by the laboratory and followed.

4.4 Apparatus and equipment

The laboratory should use properly validated (i.e. installation qualified, operationally qualified and performance qualified) and maintained equipment according to the manufacturers' instructions and the requirements given in ISO 15189. In addition to standard laboratory equipment, specific apparatus for multiplex analysis is described in the corresponding individual standards, e.g. ISO 16578 for microarray^[5].

https://standards.iteh.a/catalog/standards/sist/c53e0132-88b3-4d13-be49-a097a5193e1d/iso-Where available, calibration should be routinely performed and records maintained on equipment where performance can impact the data produced.

4.5 Reference and control materials

4.5.1 General

Whenever reference materials (RMs) are available, they shall be selected to be fit for their intended use. When RMs are not available, the alternative approaches shall be planned, developed and documented.

RMs shall be considered for the validation of multiplex molecular testing for nucleic acid. RMs can also be used for qualitative or quantitative methods, serving many different functions, such as calibration and assessment of an examination procedure. Especially for genomic analysis using massive parallel sequencing methods, genomic RMs can vary the sequence data depending on the chosen material (see <u>Annexes A</u> and <u>B</u>); therefore, RMs for such methods shall be considered for fitting to the intended use.

The laboratory should use QC materials that react to the examining system in a manner as close as possible to patient sample, as specified in ISO 15189. If these control materials are not available, it is not possible to test all the probes present in the panel for reagent acceptance or fidelity monitoring. The laboratory will adapt its arrangements notably by taking into account the clinical context.

Examination results shall be evaluated to confirm that the quality of the sample fits for the intended use. When the evaluation is performed, the use of RMs (see <u>Annexes A</u> and <u>B</u>) including nongenomic (see <u>4.5.3</u>) and genomic (see <u>4.5.2</u>) RMs shall be considered.

To validate the use of different matrices, overload assays (spiking) are necessary.

Where the manufacturer provides instructions for storage and use of RMs and control materials for measurement, these shall be followed. Where the examination manufacturer does not provide such instructions, they shall be specified and validated by the laboratory and followed.

4.5.2 Endogenous nucleic acid

Genomic DNA (gDNA) is the QC material that most closely resembles patient samples, and the most preferable, although not always the most efficient. The presence or absence of the mutations or variants in any material (e.g. immortalized human cell lines, purified gDNA, bacterial/microbial cultures, and bacterial gDNA) should be validated or verified by the laboratory for each new lot obtained before use.

Synthetic recombinant engineered or artificially constructed long DNA, e.g. recombinant artificial chromosomes, can be used as an alternative control material of gDNA (see 4.5.3). Several transcripts of housekeeping genes can be used as control materials.

NOTE Housekeeping genes include, e.g. beta-actin, GAPDH and cyclophilin.

4.5.3 Nongenomic reference materials (RMs)

Nongenomic QC materials are synthetic, recombinant, engineered, or artificially constructed, and are often composed of plasmids, due to their stability and ease of use. Nongenomic QC materials are of great value and particularly suitable for multiplex assays, since they can be engineered to contain all sequence variants in gene segments in one sample detected in an assay. Nongenomic controls should be chosen for QA purposes carefully to be sure that they are suitable for monitoring the desired system parameters. There are concerns and limitations regarding nongenomic controls. Some test systems and/or software are not designed to correctly identify multiple alleles contained in a multiplex control. Nongenomic controls have to be added to the patient material, and therefore cannot behave exactly as patient-derived material through the entire analysis process. This situation is of particular concern when there is a reasonable chance of mispriming or false-positive reporting, due to the presence of pseudogenes or other sequences normally present with high homology to the target of interest.

Nongenomic RMs can be used for not only DNA analysis but also RNA analysis.^{49-a097a5193e1d/iso-}

NOTE DNA or RNA RMs can be evaluated by establishing metrological traceability from certified RMs such as NMIJ CRM 6205-a and NMIJ CRM 6204-b.

4.6 Calibration of the analysis

In the quantitative measurement, such as for qPCR-based assays, an appropriate number of calibration points and replicates covering the range of reliable signal should be applied. The calibration influences the measurement uncertainty (MU). More details on calibration of PCR are described in ISO 20395 ^[16].

An alternative to genomic DNA or mRNA calibration RMs can be considered for use, provided that it is demonstrated to perform in an equivalent way to the genomic DNA RM and the genomic DNA extracted from the sample, or the mRNA RM and the mRNA extracted from the sample.

EXAMPLE As an alternative to genomic DNA or mRNA calibration RMs, a dilution series of a plasmid or synthetic dsDNA containing the target sequence or a plasmid or synthetic dsDNA and ssRNA can be respectively used.

4.7 Input range

Input nucleic acid should be titrated to optimize the range of concentrations, in order to ensure the detection of each target sequence in a given assay, e.g. typical ranges for PCR, arrays, and sequencing.

5 Evaluation of performance characteristics

5.1 General

The validation protocol should start with an explicit statement of the intended use, which will determine the types of samples and the performance characteristics that need to be addressed.

The validation protocol should be designed carefully to ensure that all relevant parameters are addressed as efficiently as possible. Based on design and development planning, the laboratory shall perform validation of the intended use of the test in comparison to an existing predicate device, if available. When a predicate device is not available, the laboratory shall validate the intended use by appropriately designed studies. In accordance with the validation plan, the laboratory shall describe and document relevant performance characteristics of the test in relation to the disease or other clinical context assessed by the test. The laboratory shall identify those test characteristics that are critically associated with the clinical relevance and clinical utility of the test. These characteristics shall be documented as clinical performance characteristics. For more details regarding the test using nucleic acid amplification for microbial pathogens, see ISO 17822 ^[17].

Analytical performance evaluations should test the multiplex molecular test system in its final configuration, and not in separate singleplex experiments.

Considerations specific to the multiplex molecular tests should include, but not be limited to:

- a type and heterogeneity of the specimen;
- LOD that is clinically needed;
- genetic promiscuity and instability of target sequences.

EXAMPLE Examples of genetic promiscuity and instability of target sequences are sequence heterogeneity both within the disease and across the progression of it, specificity when considering closely related target of sequence, genetic origin of acquired or inherited when considering with disease inheritance, and genetic association when considering the disease diagnosis, prognosis or treatment.

NOTE Recommendations on practical guidance for laboratories regarding validation and verification of massive parallel sequencing for genetic diseases are provided in the literature ^{[6][2][8]}.

In the verification process of a supplier method, major issues to be addressed should include as follows:

- the issue of testing all the probes present in the panel;
- the issue (or even the impossibility) of providing a number of samples that is statistically significant to conclude on the validity of the tests in particular "positive samples";
- the issue of comparing the method to an earlier method;
- the complex problem of using internal QCs for all the targets of the panel.

The selection of the analytical performance evaluation tests that are performed should include the consideration of the design or operating principles of the IVD as well as the intended use or purpose. For example, limit of quantitation (LoQ) can not be applicable for an IVD that renders a qualitative or binary result.

5.2 Analytical specificity

5.2.1 Analytical reactivity

Since analytes such as sequence variants or pathogen-specific sequences often respond differently to reagent variability, all targeted analytes shall be tested, when possible, for accurate results. The major analytes detected with selected reagent shall be identified. The information of major analytes should be considered to include in the minimum specifications for the reagents. Those analytes most