Reference materials and reference measuring systems

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Table of Contents

Reference materials and reference measuring systems

Introduction

Laboratory Medicine supporting patient care and public health strategies depends amongst several other factors on the trueness and precision of measurement results. *Trueness* is particularly important since it determines the comparability of results across laboratories and measuring systems, which is crucial for establishing and implementing clinical guidelines and public health recommendations. Trueness is primarily established and maintained by commutable reference materials and reference measurement systems (1-3).

Trueness is a cornerstone for the *equivalence* of measurement results for the same measurand. Equivalence means that the differences in measured values in the same human samples do not affect the clinical interpretation (3). Equivalent results are accomplished by establishing metrological traceability of the values assigned to the calibrators for a measuring system to the highest available reference system – certified reference materials, reference measurement procedures, or harmonization reference protocols.

When different measuring systems for the same measurand do not measure the same or very closely related measurable *quantities*, proper traceability hierarchies may be difficult or impossible to establish and maintain, especially when the measurands consist of *complex and variable mixtures of chemical structures* in varying proportions, e.g., glycoproteins with multiple isoforms, variant amino acid sequences, nucleic acid sequences, and other complex molecular forms.

When the *selectivity* of a measuring system is not fit for the intended use, sample-specific *influence quantities* including disease, drugs, or other pathological conditions may lead to biased values for the intended measured quantity even though a traceability hierarchy has been established to an appropriate higher-order reference.

The property *commutability* is of special importance in laboratory medicine, where measuring systems are optimized to perform measurements directly in native patient samples without prior purification/extraction of the measurands. Therefore, the trueness of the measurement results must be ensured for measurements performed in native patient samples, and the materials used to assess trueness need also to reflect the confounding factors/matrix effects found in native patient samples.

The use of certified reference materials, reference measurement procedures, or traceability hierarchies using commutable materials - as such - does not guarantee equivalence of measurement results. Complex and variable mixtures of chemical structures characterizing a measurand, selectivity of the measuring systems, and matrix effects also play a crucial role. True equivalence of measuring systems is evidenced when used to measure the same native patient samples in actual patient care. Several publications describe various aspects of reference materials (4-10).

Reference measuring systems are used at the pinnacle of measurement hierarchies for

1. For assigning quantity values to reference materials, especially to certified reference materials
2. Value transfers in traceability hierarchies
3. Demonstrating whether there is the equivalence of different routine measuring systems claiming to measure the same quantity
4. Assessing the performance characteristics of other measuring systems, including their calibrators and reagents
5. For detecting analytical influence quantities in patient samples.

Reference measuring systems (11-16) consists of combinations of fit for the intended use certified reference materials and reference measurement methods that provide traceability - a comprehensive calibration hierarchy for the transfer of trueness to routine measuring systems (15-31). Reference measuring systems employ certified reference materials and are at the highest levels of traceability hierarchies in metrology. They are characterized by unique trueness and low measurement imprecision of results. Reference measuring systems are needed for accurate measurement results in healthcare, catering for metrological traceability, through time, distance, and different measuring systems (32-35).

J. Paul Cali pointed out already in 1973 that as manufacturers took over the production of measuring systems from the laboratories themselves, decreasing imprecision took precedence over the trueness of measurement results (33). He pointed out the need for standards, quality systems, certified reference materials, and reference measuring systems. Numerous authors supported this notion (32, 34, 35), and substantial efforts were subsequently undertaken for improved trueness (36-41).

ISO standards concerning reference materials and reference measuring systems

Numerous ISO documents define and assign property values to reference materials, including stability, homogeneity, and commutability. Some of these documents are aimed at a broad audience in metrology and are not sufficiently detailed for use in Laboratory Medicine.

- **ISO Guide 30:2015** Reference materials - selected terms and definitions. Contains the terms and definitions related to reference materials (broad audience)
- **ISO Guide 31:2015** Reference materials - Contents of certificates, labels and accompanying documentation. Describes the contents of certificates for certified reference materials, and of accompanying documents for other reference materials, respectively (broad audience)
- **ISO Guide 33:2015** Reference materials - Good practice in using reference materials (broad audience)
- **ISO GUIDE 35:2017** Reference materials — Guidance for characterization and assessment of homogeneity and stability. Provides specific guidance on technical issues and explains the concepts for processes such as the assessment of homogeneity, stability, and characterization for the certification of reference materials (broad audience)
- **ISO 17034:2016** General requirements for the competence of reference material producers (broad audience)
- **ISO 15193:2009** In vitro diagnostic medical devices. Measurement of quantities in samples of biological origin. Requirements for content and presentation of reference measurement procedures (broad audience)
- **ISO 15194:2009** In vitro diagnostic medical devices. Measurement of quantities in samples of biological origin. Requirements for certified reference materials and the content of supporting documentation (broad audience)
- **ISO 17511:2020** In vitro diagnostic medical devices — Requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials, and human samples (laboratory medicine)

- **ISO 21151:2020** In vitro diagnostic medical devices – Requirements for international harmonization protocols establishing metrological traceability of values assigned to calibrators and human (laboratory medicine)

Reference materials in laboratory medicine

Reference materials are a generic name for specialized materials used for calibration, validation, verification, and process control (42). Reference materials must be sufficiently homogeneous and stable regarding specified property values of the intended measurands(s). They must have been established to be fit for their intended use in measurements or the examination of nominal properties.

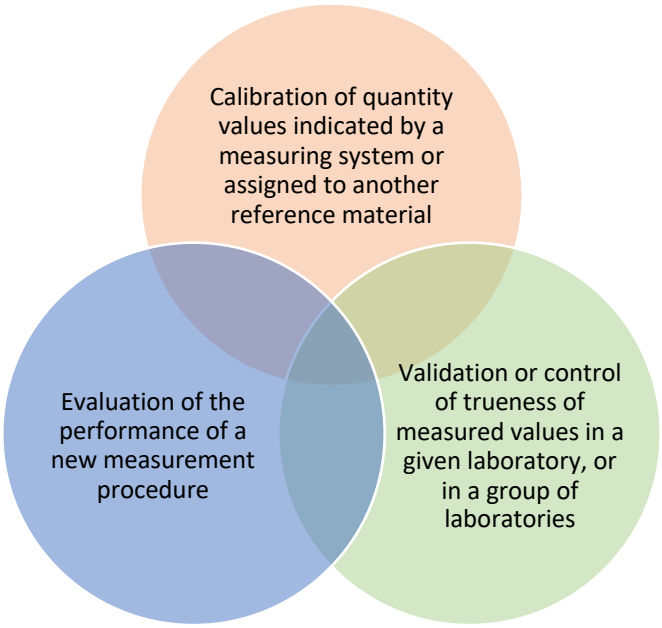

Figure 1: The three primary purposes of reference materials according to ISO 15194:2009

Primary measurement standards are either pure substances that have been measured by primary reference measurement procedures or created as an artifact chosen by convention. Measurands that consist of a single molecular form in vivo can be manufactured as such and can be made traceable to SI. When the measurand is heterogenous in vivo, it is not feasible to manufacture it as a single molecular form traceable to SI. Such measurands are a mixture of molecules post-translationally cleaved by enzymes or post-translationally enzymatically modified e.g. by glycosylation.

An example of a primary (*pure substance*) measurement standard is when a pure substance of glucose is analyzed for purity and identity by quantitative nuclear magnetic resonance (qNMR) procedure and dissolved in a known volume of pure water.

An example of primary (*conventional*) measurement standard (an artifact) (International conventional certified reference material) is "Thyroid Stimulating Hormone (TSH), Human, WHO International Standard" created from a highly purified extract of pooled human pituitaries and dissolved in a sterile solution containing 0.2% (w/v) peptidase-free human serum albumin and 1% (w/v) lactose to facilitate the dissolving of the molecules in the purified extract. The primary standard solution contains a mixture of the variants of TSH molecules occurring in vivo. A conventional measurement standard is thus an artifact chosen by a body authorized by an international agreement, commonly a "WHO International Standard." The assigned value of the conventional reference material is arbitrary and does not carry an uncertainty associated with its calibration. The uncertainty is usually considered the uncertainty of the ampoule content, which is minimal.

Certified reference materials (CRMs) represent the metrological pinnacle of reference materials. They are usually produced and issued by national or international metrology institutes and enclose a certificate stating the value of the measurand, the traceability of that value, and its uncertainty. Metrologically appropriate procedures for the production and certification of reference materials are provided, e.g., in ISO-17511:2020, ISO-21151:2020, ISO-31, ISO-34, and ISO-35.

Higher-order reference material is a *certified reference material* which

1. Meets internationally accepted quality requirements (ISO 15194:2009)
2. To which other measurement results can be referenced
3. Has a wholly established measurement uncertainty

Higher-order reference materials are usually produced and distributed by national metrology institutes (NMIs), for example, the European Commission Joint Research Centre (EU-JRC), U.S. National Institute of Standards and Technology (NIST), National Institute of Metrology in China (CN), World Health Organization (WHO), LGC Standards (UK), National Institute for Biological Standards and Control (UK), National Metrology Institute of Japan (JP), Reference Material Institute for Clinical Chemistry Standards (JP), Japanese Industrial Standards Committee (JISC), Centro Nacional de Metrología (MX).

Commercial manufacturers also produce reference materials listed in the Joint Committee of Traceability in Laboratory Medicine (JCTLM) database (https://www.bipm.org/jctlm/).

Reference materials that are not accompanied by certificates are called non-certified reference materials or simply *control materials* (43). The values of such reference

materials are not sufficiently characterized to enable their use as part of a formal traceability hierarchy.

Control materials are commonly purchased from vendors of control materials or produced in-house and are usually employed for internal quality control. The preferred term for these materials is *quality control materials* referring to their use. They are appropriate for one or more measurands and are commonly available at two different concentrations. Proficiency testing programs and organizations use reference materials given the mean consensus value of the group of measurement systems considered appropriate to group together or values allocated to the material using reference measurement methods.

Calibrators are usually purchased from the manufacturers of a corresponding measuring system where they and the measuring system constitute a coherent whole which the manufacturer is responsible for. Suppose a certified reference material is used instead of the calibrators supplied by the manufacturer. In that case, the user implicitly shoulders the responsibility of validating the measuring system since the validation performed by the producer is no longer intact even though a certified reference material has been used.

Native patient samples have usually been centrifugated to separate blood cells from the plasma or serum. Native patient samples are commutable by definition and are commonly used for trueness control between measuring systems in the same laboratory organization or during verification of new measurement systems compared to current methodologies. Furthermore, laypeople and experts believe that different measuring systems should give the same results in the same patient sample.

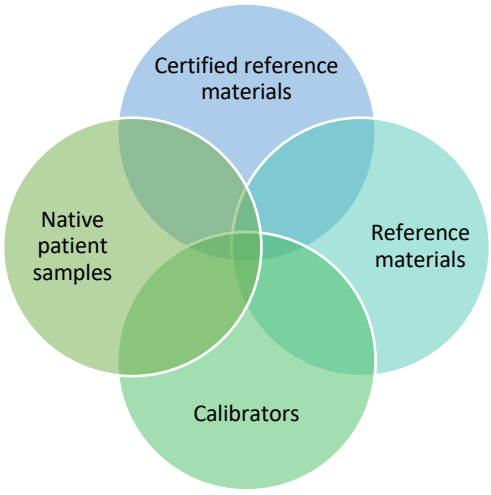

Figure 2: The four categories of reference materials. The Venn diagram illustrates that the four categories of reference materials share a part of their properties with other

reference materials and no other properties. Reference material may be turned into certified reference material using reference measurement procedures.

Purpose of reference materials

The fundamental purpose of reference materials is to establish and maintain measurement traceability, equivalence, and quality. In detail reference materials are *used for the following purposes*:

1. *Calibration* of quantity values in measuring systems
2. *Assigning values to another reference material* through measurement
3. *Validation* of trueness of measured values in a laboratory, or a conglomerate of laboratories
4. *Verification* of the results from new measuring systems
5. *Control* of trueness of measured values in a laboratory or a conglomerate of laboratories
6. Estimation of *measurement uncertainty*
7. For *proficiency testing*

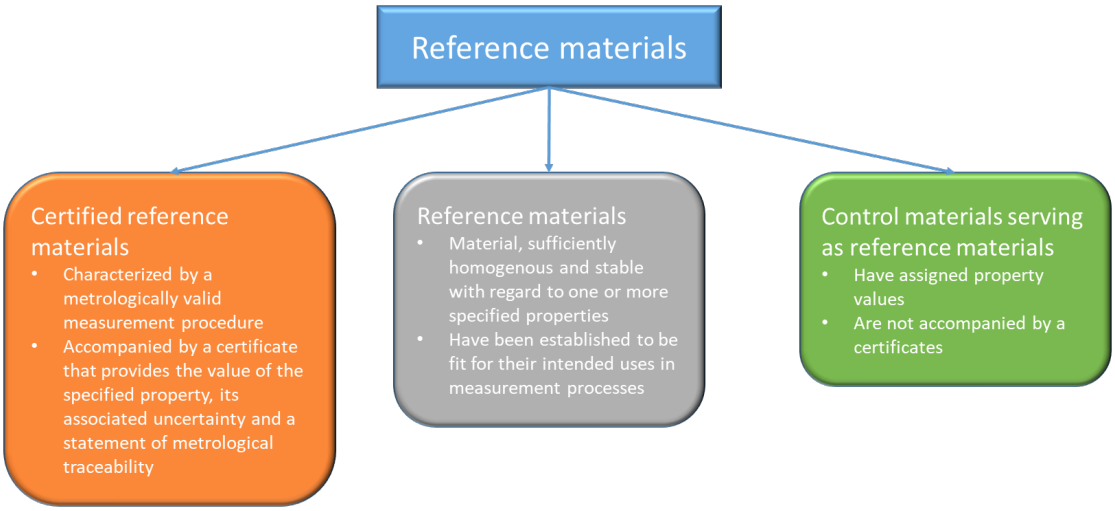

Figure 3: The three primary reference materials used in Laboratory Medicine are 1. Certified reference materials, 2. Reference Materials and 3. Control Materials. (44-47)

Since the measurands in Laboratory medicine are measured in natural matrix samples including, plasma, serum, cerebrospinal fluid, urine, etc. and the matrix commonly influence the measurement results, the *end-user in-vitro diagnostic measurement calibrators* (end-user calibrators) need to have a matrix that is as identical as native patient samples as possible.

International conventional calibrator

An international conventional calibrator is used when traceability to SI is not possible, e.g., when a pure preparation of the "analyte" is not available, the "analyte" is present in the body in multiple molecular forms, or different epitopes of the "analyte" are used by other measuring systems when measuring the "analyte." In this case, preparation of

biological material needs to be made that can be used as a standard global reference (Figure 3)

The value assigned to the international conventional calibrator has *an arbitrary value for the measurand assigned by an internationally agreed value assignment protocol*, which comprises the highest level of metrological traceability globally for the specified measurand. Therefore, the World Health Organization (WHO) frequently shouldered global responsibility.

An *international conventional calibrator* evidently must contain the measurand and should have a matrix resembling the intended human samples. This is done to make the end-user calibrators as similar as possible regarding the sample matrix and thereby increase the probability that the end user calibrators will be commutable with human samples in the measuring systems they intend to use calibrate in the intended calibration hierarchy.

An internationally agreed protocol must be used to assign the quantity value(s) to the international conventional calibrator(s). An example of a protocol is a scheme in which the mean value (after outlier removal) of the measurand in the international conventional calibrator is determined among a group of measuring systems with suitable performance characteristics. The suitability of performance characteristics is usually based on results measured for a panel of human samples. It includes measurements of imprecision, selectivity, correlation, and reduction of differences among procedures when the candidate international conventional calibrator is used to recalibrate the procedures and other essential influence quantities.

The *WHO Expert Committee on Biological Standardization (ECBS)* establishes international biological reference materials called "*International Standards (IS)*" (and previously "International Reference Preparations (IRP)") for use in Laboratory Medicine. For the first batch of these materials, an "*international unit*" is defined as an arbitrarily specified amount of the material and characterized by its specified biological activity. Subsequent batches are calibrated by interlaboratory collaborative measurements using the previous material. The batches in a series are specified by "1st IS", "2nd IS", etc. The assigned value(s) of such a reference material, even when it is highly purified, are related to a dedicated measurement procedure or other internationally agreed protocol without metrological traceability to SI units. International conventional reference materials can only be used as calibrator(s) for measuring systems if the material is developed based on a clear definition of the quantity related to the intended medical application and if the assigned value(s) of the

material have measurement uncertainty that is acceptable for calibration of measuring systems.

The *commutability of the international conventional calibrator* with human samples must be validated for a representative number of different measuring systems, consistent with the intended use of the calibrator. Before the first release of the material for its intended use, the provider of the international conventional calibrator must perform the commutability assessment. A manufacturer responsible for defining the calibration hierarchy for a particular measuring system is responsible for any additional commutability assessments needed to ensure that the selected international conventional calibrator is suitable for use with the specified measuring system, if applicable. Protocols for commutability assessment are available in CLSI EP30-A (48) and several other published recommendations (49-59).

Using a human sample-like matrix in any calibrator does not guarantee that the resulting calibrator is truly commutable.

An *international conventional calibrator* is used to calibrate the manufacturer's selected measuring system, which, when appropriately calibrated, is used to determine assigned values for the manufacturer's working calibrator.

Control materials used for proficiency testing

What proficiency testing essentially aspires to do is to quantify the equivalence of measuring system *results*. The use of certified reference materials, reference measurement procedures, or traceability hierarchies with commutable materials is no guarantee of equivalence of measurement results. It may work for measurands that consist of molecules occurring in vivo in a single form, but when complex and variable mixtures of chemical structures characterize a measurand, selectivity of the measuring systems and matrix effects of the reference materials used play a crucial role. This is the situation for probably more than half of the current biomarkers in Laboratory Medicine.

Commutable reference materials are optimal for EQA for simple molecules but not sufficient for EQA for the common complex biomarkers. If commutable reference materials are to be used in EQA for complex biomarkers, numerous samples (in the order of 80 as in harmonization schemes (60)) need to be analyzed in triplicates (80x3=240) - which of course, is unrealistic. The primary reason for the use of numerous samples is that as many of the factors influencing the results of all the measuring systems (molecular heterogeneity and post-translational modifications) need to be present in the 80 samples used to randomize/average out all possible influence factors to measure the equivalence of the measurement results correctly.

Conventional EQA using commutable samples is not possible for complex biomarkers because they contain a complex and varying mixture of the molecules representing the measurand. Such a complex and variable mixture occurring in vivo can never correctly be expressed in single commutable samples currently used for EQA.

For complex biomarkers, we must settle for the second-best – the use of stabilized materials that can measure the trueness of measuring systems compared to their peers.

A realistic alternative is using patient results and big data (61-67), thus randomizing out the effects on the extremes on the central tendency (median or average). This may not be seen as EQA but could and should – in time.

In due time, when the selectivity of measuring systems improves and we understand which epitopes of the biomarkers are related to each disease mechanism (it took 20 years of work for glycated hemoglobin), EQA for equivalence for each measurand can be made as sophisticated as a proper medical diagnosis and monitoring demands.

The matrix of reference materials

Lipids, macromolecular proteins, and water

Lipids and macromolecular proteins represent challenges in the commutability of reference materials. Unsaturated lipids are prone to oxidation on storage. Therefore, it may be tempting to extract the lipids from the materials using organic solvents, e.g., a mixture of butanol and ether separating the organic and aqueous phases by centrifugation. The organic solvents will extract the lipids and naturally change the three-dimensional configuration of lipoproteins in the remaining plasma. Furthermore, the structure of other protein macromolecules is also dependent on their interaction with the aqueous- or organic solvents they are present in. Therefore, adding organic solvents to plasma risks permanently changing the configuration of protein macromolecules, which is not reversed by re-introducing the proteins to an aqueous environment only.

Lyophilization eliminates water from the material, prevents microbial growth, and generally increases storage stability. However, water molecules are crucial for creating and maintaining the three-dimensional structures of macromolecules. When water is removed through lyophilization, the three-dimensional structure of macromolecules risks being changed. It is a substantial risk that the appropriate three-dimensional structure is re-created when the lyophilized material is dissolved in water.

Using a matrix from a single donor would be optimal for many reference materials. Usually, however, the amount of sample obtainable from a single donor is typically insufficient to create a sufficiently large batch of reference material. Therefore,

specimens from several donors usually need to be pooled, thus inherently running the risk of differentiating them from native specimen matrices. Pooled specimens, therefore, need to be evaluated for commutability as all other modified matrices.

Current routine lipid and lipoprotein analysis methods are based on enzymatic and immunologic reactions. Because pure total cholesterol (TC) or triglyceride (TG) molecules are only soluble in organic solvents, these primary standard materials are not suitable for calibrating routine assay methods. Lipoproteins can be purified, but their tertiary and quaternary structures are usually altered.

This structural alteration can affect their chemical reactivity in enzymatic and immunologic reactions. Consequently, preparing primary aqueous-based standards for lipid testing has not been practical for most assay systems.

Pooled human serum-based secondary standards are commonly used for calibration. Because of changes in lipoproteins and other serum matrix components during manufacturing, serum-based secondary standards frequently have a different chemical reactivity concerning lipid molecules than native serum specimens in routine measuring systems. Routine laboratory methods are designed to recover analytes from human serum specimens. Routine procedures do not always recover lipid analytes from matrix-modified secondary standard calibration materials. Consequently, the target values assigned to serum-based calibration materials may be adjusted to compensate for any altered reactivity due to matrix-modified materials.

Proficiency testing materials with claims of metrologically traceable target values

The manufacturer of a commutable trueness-based external quality assurance and proficiency testing material with an assigned value(s) claimed to be metrologically traceable to higher-order references (for one or more measurands) shall define, describe, and validate the relevant calibration hierarchy supporting the assigned values for each stated measurand. Where claimed by the producer, commutability of such proficiency testing materials must be demonstrated according to published recommendations (see CLSI EP30-A Characterization and qualification of Commutable Reference Materials for Laboratory Medicine and (49-51)) for representative measuring systems widely used by end-user medical laboratories. The assigned values for each measurand and the estimated total measurement uncertainty values must be determined and provided to end-users upon request.

Commutability of reference materials and calibrators

Commutability is the “property of reference material, demonstrated by the closeness of agreement between the relation among the measurement results for a stated quantity in

this material, obtained according to two given measurement procedures, and the relation obtained among the measurement results for other specified materials (42).

The concept of *commutability* was initially published in 1973 (68, 69) and used to underscore those materials for internal and external quality control programs that show properties comparable to those of patient samples. Commutability means that the relationship between two measurement procedures for patient samples would also apply to commutable reference materials. Commutability of material is evaluated by measurements using measuring systems claiming to measure the same quantity. For reference materials used to calibrate measuring techniques used in Laboratory Medicine, the samples included need to have samples from healthy and relevantly diseased individuals.

Matrix reference materials for Laboratory Medicine are usually blood, plasma, serum, urine, or other samples that have been processed to serve as reference material. Creating reference materials usually includes pooling specimens from different donors and chemically processing the pooled samples by adding analytes, preservatives, or antimicrobial agents and physically filtering, freezing, or lyophilizing the samples. Any of these treatments risk changing the characteristics of the reference materials, including the matrix, so that the results of measurements are changed in one way using a particular measuring system and, in another way, using another measuring system. Importantly, there is a possibility that the results are different compared with an authentic clinical sample with similar properties.

Challenges in the commutability of reference materials

The commutability of reference materials relative to human samples must be documented to be appropriate for its intended use at its position in the calibration hierarchy.

Measurement procedures, including those used to characterize and prepare primary (e.g., pure substance) reference materials and primary calibrators, cannot be applied to human samples when performing commutability assessments. Therefore, commutability assessment is not required for such reference materials at these levels in a calibration hierarchy.

When a reference measurement procedure for the measurand is available, the first level where commutability of reference material can be assessed is at the level where a secondary (matrix) reference material or another secondary calibrator for the manufacturer selected measurement procedure.

For subsequent steps further down the calibration hierarchy, such as at the value transfer step employing a working calibrator to calibrate the manufacturer's standing measurement procedure, the *commutability of the working calibrator must be assessed to ensure appropriate value transfers avoid bias*.

Application of non-commutable certified reference materials

Suppose a certified reference material or international conventional calibrator intended to calibrate a manufacturer's selected measurement procedure demonstrates commutability with human samples. In that case, the certified reference material may still be used as a calibrator within the calibration hierarchy for a specified measuring system for which the reference material does not demonstrate commutability to the intended human samples by the use of a correction factor or function to the assigned value of the certified reference material. If applicable, details of the use and validation of such a correction to given values of the certified reference material or other reference materials such as *International conventional calibrators* must be detailed in the documentation of the calibration hierarchy for the specified measuring system. The measurement uncertainty of values assigned to the end-user calibrator(s) must include any incremental uncertainty associated with the correction factor or function.

Alternative reference materials

In the absence of commutable certified reference materials or international conventional calibrators, rationale must be documented to select any alternative reference materials used as calibrators at each applicable stage in the calibration hierarchy. Alternative reference materials must be documented to be fit for their intended use, shall each have an assigned value with standard measurement uncertainty, and must be demonstrated to be commutable with the intended human samples in each calibration transfer step in which they are used. Technical documentation for such alternative reference materials must include relevant characteristics. Alternative reference materials include panels and pools of individual human samples, supplemented or "spiked" samples prepared in natural or artificial matrices, or other suitable materials.

Reference materials of human origin

The Clinical Standards Institute (CLSI) standards offer guidance on the appropriate selection of human sample panel members for use in a calibration hierarchy:

- CLSI EP09-A3, Measurement Procedure Comparison and Bias Estimation Using Patient Samples; Approved Guideline—Third Edition
- CLSI EP14-A2, Evaluation of Matrix Effects; Approved Guideline - Second Edition

- CLSI EP30-A, Characterization and Qualification of Commutable Reference Materials for Laboratory Medicine; Approved Guideline

Human samples are assumed to be commutable when stored under conditions that have been validated not to alter the stability of the measurand or matrix. Storage conditions can be validated for human samples with a representative panel of individual human samples for a specified measurand. Such validation of storage conditions for human sample panels can support subsequent sample panels obtained from persons with similar health/disease profiles in sustaining the calibration hierarchy for the specified measuring system, with no requirement for validation commutability of stored sample panels.

In cases where human sample panels are deployed as alternative reference materials in a calibration hierarchy for a specified measuring system, if the analyte in human samples, whether panels or pools intended as reference materials, needs to be modified by augmentation or depletion to achieve appropriate quantity values, the commutability of the modified samples must be validated.

Where sample-specific interferences or measurement procedure non-selectivity limitations are identified, individual human samples presenting with these limitations must be excluded from human sample panels intended for use as calibrators in the calibration hierarchy.

Non-commutable end-user measuring system calibrators

When *non-commutable materials* are used as end-user calibrators for a measuring system, commutable materials (for example, a panel of human samples) must be used in the calibration hierarchy to determine a correction factor or correction function to assign arbitrary values to the non-commutable measuring system calibrators to compensate for any bias due to non-commutability. If applicable, details of the use and validation of such a correction to assigned values of the non-commutable measuring system calibrators must be disclosed in the documentation of the calibration hierarchy for the specified measuring system. The measurement uncertainty of values assigned to the end-user measuring system calibrator(s) must include any incremental uncertainty associated with the correction factor or function.

Reference materials other than end-user measuring system calibrators

For reference materials other than end-user measuring system calibrators (e.g., measuring system trueness control materials), the reference materials manufacturer is responsible for validating and describing the calibration hierarchy that is the basis for any measurand values assigned to such reference materials and for documenting the status of the material's commutability with human samples (if applicable) when used

with any intended measurement procedures, including any measuring systems. Combined standard measurement uncertainty of assigned values for these kinds of reference materials for measuring systems (that are not measuring system calibrators) shall be estimated by the manufacturer and provided to end-users on request.

Nomenclature for reference materials

Varying and confusing nomenclature for reference materials has been used over the years. The International Standardization Organization (ISO) has been crucial in efforts to define concepts and terms related to manufacturing reference materials through its ISO-REMCO committee (www.iso.org/remco) (70), which also has published several ISO guides related to reference materials which will be followed here.

- ISO 30:1992 Terms and definitions used in connection with reference materials
- ISO 31:2000 Certificates of reference materials
- ISO 32:1997 Calibration in analytical chemistry and the use of certified reference materials
- ISO 33:2000 Uses of certified reference materials
- ISO 34:2009 General requirements for the competence of reference material producers
- ISO 35:2006 Certification of reference materials

Reference materials

ISO only recognizes two types of reference materials – "reference materials" and "certified reference materials." ISO defines *reference material* as "Material, sufficiently homogeneous and stable regarding one or more properties, used in calibration, assignment of a value to another material, or quality assurance" (71), and *certified reference material* as "Reference material, accompanied by documentation issued by an authoritative body and referring to valid procedures used to obtain a specified property value with measurement uncertainty and traceability" (71). Thus, certified reference materials accompany a certificate in addition to being homogenous and having established property values. The property value(s) are certified by a measuring system that establishes traceability of the property value(s) to a true value of the unit in which the property value is expressed, including an expression of its *measurement uncertainty*.

The standard ISO-15194:2009 (1) "In vitro diagnostic medical devices -- Measurement of quantities in samples of biological origin -- Requirements for certified reference materials and the content of supporting documentation" specifies the quality requirements for reference materials and their documentation (1).

Certified reference materials are higher-order materials needed at the highest metrological levels of a calibration hierarchy. Given certified reference material is supported by documentation containing sources of the material, descriptions of the

methods for allocating its concentration, measurement results, metrological traceability, measurement uncertainty, instructions for use, stability data and storage conditions, and health and safety warnings.

Types of reference materials

The two major *types of reference materials* in Laboratory Medicine are

1. Single substance reference materials.
2. Matrix reference materials

Single substance reference materials are pure chemicals or solutions of pure chemicals that have been exceptionally well characterized for purity and homogeneity. An example may be synthesized c-peptide, whose amount has been established by amino acid analysis.

Matrix reference materials in Laboratory medicine are usually natural plasma, serum, or tissue extracts containing the measurand(s) in the sample matrices intended to be measured in routine laboratories. The matrix reference materials have been characterized for the concentration of specified major, minor, or trace chemical constituents of interest. They may be prepared from matrices containing the components of interest, for example, human plasma or serum, or by adding various concentrations of the trace elements to the appropriate matrix.

Categories of calibration hierarchies for traceability in Laboratory Medicine

Measuring systems in Laboratory Medicine should report equivalent results when used for screening, diagnosing, and monitoring treatment effects. Equivalent means that proper traceability to the SI unit “amount of substance concentration,” to “international conventional reference materials” or to international harmonization reference materials (Figure 3) means that patients’ results are traceable and thereby equivalent when applied.

The essence of the traceability hierarchies is to transfer the quantity values of interest measured in the different materials in the calibration hierarchy in such a manner that the quantity measured in the end-user calibrators corresponds to the quantity measured in the native patient samples to the extent that the measurands measured around the globe provide equivalent results.

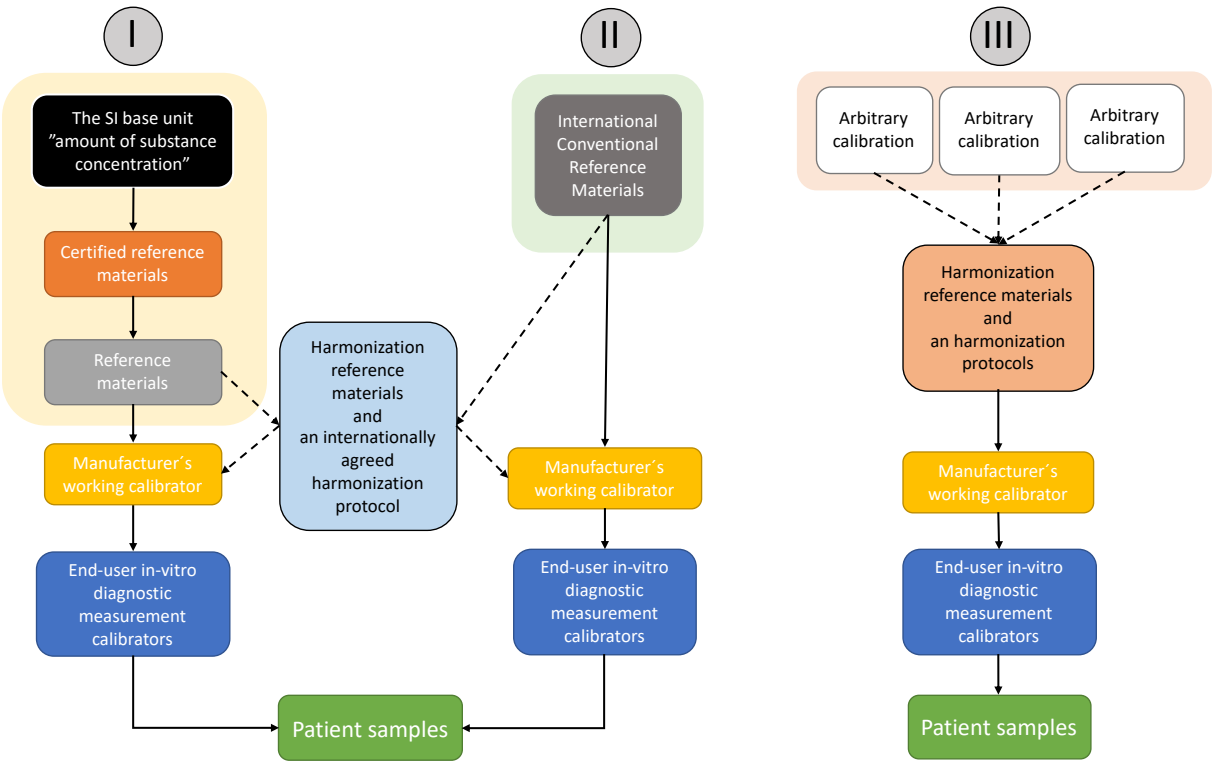

Figure 4: The three categories of calibration hierarchies for traceability in Laboratory Medicine – I. The SI base unit "amount of substance concentration" is at the top. II. With International Conventional Reference Materials at the top and III. Arbitrary calibrations based on individual manufacturers' calibrators

Selection and requirements for reference materials and calibrators

Higher-order reference materials that conform with ISO 15194

When higher-order reference materials are required for a calibration hierarchy according to ISO-17511:2020 (3), the materials that conform to ISO-15194 (1) must be used when suitable and available. Documentation of the ISO-15194 conformity status of any relevant reference materials comprising various stages in a calibration hierarchy for a measuring system must be included or referenced in the manufacturer's technical file.

The *Joint Committee for Traceability in Laboratory Medicine (JCTLM)* (https://www.bipm.org/en/committees/jc/jctlm/#resources, https://www.jctlm.org/) lists (https://www.bipm.org/jctlm/) reference materials that conform to requirements of ISO-15194.

Reference materials that do not conform with ISO 15194

When reference materials that conform with ISO-15194 are not available, or if available certified reference materials are not suitable for other reasons, for example, when commutability is not credibly established, other reference materials not fulfilling all

ISO-15194 requirements may be applied at the highest levels in a particular calibration hierarchy for a measuring system, as long as those responsible for establishing the calibration hierarchy have demonstrated and documented the fitness for the intended use and performance characteristics of the reference materials.

Selection of measuring systems for the manufacturer of reference materials

The traceability hierarchy is about transferring *values* between certified reference materials and calibrators using a sequence of appropriate measuring procedures.

Each value transfer step in a calibration hierarchy includes a defined measuring system that must fit the intended use. The rationale for selecting a measuring system at each calibration hierarchy level must be detailed in the measuring system manufacturer's documentation. It must be accompanied by appropriate supporting data demonstrating that the analytical performance characteristics of each measurement procedure used in the hierarchy are fit for the intended use.

An SI-traceable higher-order reference measuring system calibrated with a certified reference material should – if available - be used at the highest level in the calibration hierarchy. At subsequent levels, value transfer steps to assign values to commercial calibrators are introduced that deploy metrologically lower-level measurement procedures (e.g., international conventional reference measurement procedures, manufacturer's selected measurement procedures, and manufacturer's standing measurement procedures calibrated with secondary calibrators with or without a certification.

The measuring systems at each calibration hierarchy level must be identified in terms of their metrological status. Reference measuring systems comprising elements of a calibration hierarchy according to the models described in ISO-17511:2020 Field (3) and that meet the requirements of ISO-15193:2009 Field (41) is appropriately called measurement procedures *higher metrological order*. Different higher-order reference measuring systems may be deployed at various steps in the hierarchy. In the case that ISO-15193:2009 - conforming reference measurement procedures are not available, measurement procedures that do not fulfill ISO-15193:2009 requirements may still be applied in a hierarchy, for example, a manufacturer's selected measurement procedures or a manufacturer's standing measurement procedures, as long as the parties responsible for the calibration hierarchy demonstrate and document the fitness for the intended use and performance characteristics of the actual measurement procedures.

Some measurement procedures that are part of a calibration hierarchy, especially at the lower levels of a calibration hierarchy, may be based on the same principle as the end-user measuring system, e.g., a manufacturer's standing measurement procedures.

Complete descriptions of higher-order reference measurement procedures that establish traceability to SI units of measurement and conform with ISO 15193:2009 (2) are commonly published in the scientific literature.

Validation of measuring systems

The manufacturer must *validate* a claim of metrological traceability of the value assigned to the measuring system calibrator. ISO 9000 defines validation as confirmation supported by objective evidence that the requirements for a specific intended use or application have been fulfilled. ISO 9000 further defines 'objective evidence' as data that supports the existence of something. Objective evidence is obtained using observation, measurement, testing, or other appropriate means.

The most straightforward strategies for developing objective evidence of the validity of calibration traceability are for measurands with the most completely developed reference systems. The more complex validation strategies are required for calibration hierarchies supporting measurands with no existing higher-order references or harmonization protocols.

Design of studies for the validation of a claim of traceability of assigned values for end-user measuring system calibrators must be documented by the manufacturer in the *technical file* of the measuring system. The selection of a particular validation strategy for a given calibration hierarchy shall depend on the maturity and performance characteristics of the reference system for the measurement and the availability of materials and measurement procedures as needed to perform the types of studies listed below. Several validation strategies may be applied to define the measuring system's calibration hierarchy for a given calibration hierarchy. Study strategies applicable to the validation of calibration traceability claims for a measuring system include:

1. Examination of the commutability of appropriate reference materials, which preferably are certified reference materials.
2. Participation in a proficiency testing scheme(s) or other inter-laboratory comparison schemes that utilize commutable test samples, with target values preferably assigned by a reference measurement procedure or a harmonization protocol.
3. Examination of banked human samples with values previously assigned by a reference measurement procedure.
4. Compared to a higher-order reference measurement procedure, method comparison studies on a set of human samples.

5. Method comparison studies on a set of human samples with another independent measurement procedure which is not a reference measurement procedure.

Higher-order analytical controls embedded into the calibration hierarchy and value assignment measurement procedures, focusing on carefully calibrated, SI traceable measurement tools and controls (for example, balances, volumetric glassware, spectrophotometers, thermometers, ambient environmental controls, reagents with the highest available purity).

Among the validation possibilities described above, the availability of a reference measurement procedure is the most critical factor.

Among the generic validation strategies described above, #1. to #4. are focused on the output (i.e., trueness of measured values) of the specified calibration hierarchy, while the strategies in bullet 5. focus on the trueness and reproducibility of the value transfer process and procedures within the calibration hierarchy (i.e., critical steps such as volumetric and gravimetric measurement).

For guidance on the appropriate selection of human sample panel members for method comparison studies, the CLSI standards EP09-A3, EP14-A3, and EP30-A are recommended.

Test design considerations and acceptance criteria

Known influence factors/confounders affecting human sample and calibrator measurements for both the measuring system being evaluated and the reference measurement procedure must be detailed. Pre-determined acceptance criteria for validation must be derived from the measurement uncertainty specifications for the measuring system as defined in the respective calibration hierarchy for the measurand. The number of replicates of each sample was measured using the measuring system must be determined. The power to detect a bias as significant as the validation criteria is reasonably high (e.g., >80 %). In contrast, the chance of incorrectly failing the validation criteria is low (e.g., <5 %).

Selection of reference measurement laboratories

A manufacturer may select reference measurement laboratories that conform with ISO 15195 (72). Another responsible party may also be chosen to provide reference measurement services to support the implementation of a metrologically traceable calibration hierarchy. Even though they do not conform to ISO 15195:2018, the selected reference measurement laboratories need to have demonstrated competence in providing the best available measurements for the chosen measurand regarding the

metrological traceability of values measured in human samples of the types intended and within the scope of the defined calibration hierarchy. The *Joint Committee for Traceability in Laboratory Medicine (JCTLM)* (https://www.bipm.org/en/committees/jc/jctlm/#resources, https://www.jctlm.org/) lists (https://www.bipm.org/jctlm/) reference measurement procedures that conform to requirements of ISO 15195:2018.

The description of a metrologically traceable calibration hierarchy for a measuring system must include results from an investigation of the impact of influence quantities on the relevant measurement procedures at each level of the calibration hierarchy.

Documentation of reference materials

The definition of the measurand

The measurand must be defined by the following characteristics recorded in the documentation of the measuring system:

1. The name of the measurand (e.g., aspartate aminotransferase)
2. The biological system used for measurements (e.g., human plasma or human serum) includes the intended medical use of the measurement results for diagnosis, monitoring, or other decision-making.
3. The kind-of-quantity (e.g., amount-of-substance concentration).
4. The unit of measurement (e.g., µkat/L)
5. In the case of an operationally defined measurand (defined by a particular measurement protocol, measurement procedure, or a group of measurement procedures), the measurement procedures or protocols must be reported. Measurement protocols, reference materials, etc., essential for the measurand's definition, must be available from the manufacturer.

Documentation of the entire calibration hierarchy

The complete traceability calibration hierarchy must be documented from the highest metrological reference used to measure the patient samples.

The documentation must include:

1. A detailed description of the reference measuring system consists of the following
   a) The definition of the measurand and the system of units used (e.g., SI, IU, or other).
   b) The highest order measuring system is defined by a metrology institute or another mandated organization to establish the measuring system's metrologically traceable calibration.

   c) The reference materials used for calibrating the measuring system mentioned in b)
   d) The reference laboratories and laboratory networks, designated by national metrology institutes, professional bodies, accreditation bodies, or other authoritative bodies, can provide fit for the intended use examinations of the measurand in the intended human samples.
2. A *description of the calibration hierarchy* in words and illustration(s), consisting of alternating pairs of measurements and reference materials, establishing an unbroken hierarchy of value transfers, starting with the highest order reference used and ending in measured quantity values for human samples using a routine measuring system.
3. The *measurement uncertainty is the upper specification limit* for the in vitro diagnostic measuring system. The estimated combined expanded measurement uncertainty must be documented not to exceed the upper specification limit. The *combined standard uncertainty* of the final measured values on human samples for the specified in vitro diagnostic measuring system. The estimation of the combined standard uncertainty must document the combined standard uncertainty of value(s)measurement uncertainty assigned to any calibrators used to calibrate the measuring system, regardless of whether the final calibration of the measuring system is performed by the end-user of the measuring system or by the measuring system manufacturer.
4. A description of the validation study(s) supporting the claim of metrological traceability of final measured quantity values assigned to human samples, using the specified measuring system.

Documentation must be maintained in the manufacturer's technical file for the life of the measuring system of procedures and data supporting a calibration hierarchy of a measuring system for measuring a particular measurand(s) in human samples. The documentation must include the manufacturing specifications, estimated standard measurement uncertainties, materials, verification and validation studies, and the operating procedures. It must be maintained in the manufacturer's technical file at least for the life of the in vitro diagnostic measuring system.

When a measuring system or an end-user calibrator for a measuring system is modified by the manufacturer or the medical laboratory, the uncertainty of assigned values for each relevant measuring system calibrator must be confirmed or re-estimated by the entity unless valid justification is provided for why the change does not affect the measurement uncertainty.

Reference materials and calibrators

The calibrators used at each step in the calibration hierarchy must be documented to fit the intended use. The rationale for selecting each calibrator within the calibration hierarchy must be included in the in vitro diagnostic measuring system manufacturer's documentation.

For each calibrator applied in a defined calibration hierarchy for a particular measuring system (except the end-user measuring system calibrators), the following characteristics must be identified, documented, and their consistency assured in successive production lots:

1. The intended use of the material.
2. The molecular identity of the intended measurand.
3. The origin of the material (e.g., synthetic, recombinant, human, or animal).
4. The phase(s) of the material (gas, liquid, solid).
5. The state(s) of aggregation of the material (solution, suspension, lyophilized).
6. The material matrix (e.g., aqueous, other solvents, buffer, protein solution, human samples).
7. The assigned values of the measurands and their metrological traceability.
8. The expanded measurement uncertainty of the assigned values of the reference materials is included in the calibration hierarchy. The standard measurement uncertainty, which is used to calculate combined measurement uncertainty further, is the expanded measurement uncertainty, divided by the coverage factor. The probability density distribution of the assigned value the standard fate of the assigned value may be an appropriate expression for non-certified reference materials or calibrators.
9. The stability of the reference material.
10. The within-batch homogeneity of the reference material.
11. The commutability characteristics of the reference material.
12. The issuing authority of the reference material – if any (e.g., WHO, NIST, EU-JRC).
13. The status of the certificate of the reference material (non-certified, certified).

Labeling

Certified reference material has *a label* securely attached to the product packaging of an individual unit of the certified reference material.

The information provided on a label serves to identify the certified reference material and includes the producer's name, the name of the material, the producer's identification code for the material, the batch number, and relevant health and safety warnings.

The certificate

A certificate must accompany certified reference material. In addition, the certified reference material may be either include *a certification report*, or the information needed for a full certification report must be *obtainable from the produce*r of the certified reference material.

A certificate should include the items specified in ISO-Guide 31 (73).

It must at least include the following:

1. Name of the material
2. The identity of the producer and the producer's identification code for the certified reference material, including lot identification, when available
3. A general description of the material
4. The intended use of the material, including information on the commutability of the material appropriate for the intended use
5. Information about the transport of the material and instructions for appropriate conditions of storage, proper handling, and information on expected stability
6. Safety instructions
7. Instructions for proper use
8. Certified property values(s) of the material, each accompanied by a statement(s) of measurement uncertainty as appropriate
9. Information on indicative values or recommended values
10. The measurement procedure(s) used to obtain property values (including full details where the values vary depending on the measurement procedure)
11. Date of certification and period of validity, if applicable
12. Reference to appropriate certification report(s).

The certification report

The *certification report* must at least include the elements listed here:

1. Warning and safety precautions
2. Scope of application for the certified reference material
3. Terms and definitions
4. Symbols and abbreviations
5. General properties
6. Specific properties
7. Characterization
8. Intended use
9. Instructions for use
10. Certifying body
11. Dates of authorization and revision

A detailed description of the certified reference material should be provided, including the names of the quantities for which the certified reference material is intended to be used expressed as system, component, and kind-of-quantity

Concepts and terms that are potentially unfamiliar for the users of the certified reference material should be explained. Terms for kind of quantities should be detailed, e.g., in Ferard, G. et al. Compendium of Terminology and Nomenclature of Properties in Clinical Laboratory Sciences. Recommendations 2016, International Union of Pure and Applied Chemistry (74) and ISO-31 (73). If a trivial name is used, it must be given in parentheses following the appropriate standardized name the first time it appears in the text.

The appropriate application(s)

The appropriate application(s) of the reference material, including its known limitations, should be explained, for example:

- The reference measurement procedure(s) or routine measurement method(s) or measurement procedure(s) for which the certified reference material is intended
- The method(s) of measurement or measurement procedure(s) for which the certified reference material is known to be unsuitable
- The influence quantities in the certified reference material involving, for example, drugs, metabolites, additives, microbial growth
- The required pre-treatment of the certified reference material (for example, reconstitution of lyophilized material) is not performed on the biological samples.

Explanations of the general properties

Explanations of the general properties of the reference material should, for example, include

- The origin and other relevant properties of the starting material
- Relevant historical details of the starting material
- Safety aspects, including markers of infectiveness
- Details of sample preparation of the starting material
- Possible additives should be reported within the constraints of potential intellectual property rights
- The physical state of the certified reference material should be stated, for example, lyophilized serum
- The minimal portion used for analysis should be stated
- The sterilization procedure(s) used must be described
- The container and packaging must be specified
- Shelf life for the unopened container must be given
- The stability of the certified reference material must be stated
- Storage conditions for the unopened container shall be given, for example, temperature, humidity, and light. The extent of instability under the prescribed conditions must be stated. Any future check of stability must be stated
- It must be noted if the certified reference material is of restricted stability once its container is opened
- The quality systems followed during production, characterization, handling, storage, and distribution of the certified reference material must be stated.

- Any hazard associated with the certified reference material or its use must be stated, and appropriate precautions detailed.

Description of the specific properties

Description of the specific properties of the reference material should include:

- The properties of a certified reference material that influence any quantity for which a value is given must be described
- The molecular composition or biological or biochemical functional activity of each relevant component must be stated
- The quantity to which a value is assigned in the certified reference material must be specified

Documentation of the estimation of measurement uncertainty

Measurement uncertainty is preferably estimated according to the principles of the GUM (75). Regardless of whether the GUM method or a different method for estimation of the measurement uncertainty is followed, the method of statistical calculation of the measurement uncertainty must be documented and maintained in the technical file of the in vitro diagnostic measuring system calibrator at least for the life of the product.

For each in vitro diagnostic measuring system calibrator identified by a manufacturer for use in calibration of a specified in vitro diagnostic measuring system, the *measurement uncertainty* to be estimated and provided by the manufacturer of the measuring system calibrator must be determined by statistically combining the uncertainties associated with each of the sequential value assignment steps under the control of the manufacturer. In determining *measurement uncertainty*, the manufacturer must also account for the known and foreseeable uncertainties contributed by all higher-order value assignment steps in the defined calibration hierarchy, including steps, not within the manufacturer's control, such as (where applicable) the standard uncertainty of the value assigned to the highest order reference material. Estimating *measurement uncertainty* must be based on at least one representative (single) lot or batch of reagent.

Known and foreseeable variations and corresponding standard uncertainties in the specified in measuring system calibrators and reagents as well as in any intermediate reference materials and measuring systems or measurement procedures throughout the calibration hierarchy (due, for example, to factors such as but not limited to material heterogeneity and instability) must be considered.

Estimated measurement uncertainty often varies among different end-users measuring system calibrators, especially when other calibrators lots for the same measuring system have substantially different assigned values.

Specifications for maximum allowable expanded measurement uncertainty

The manufacturer must establish the maximum allowable expanded measurement uncertainty for a measuring system using the in vitro diagnostic measurement device in its intended setting with the intended human samples and at least within the measurement intervals where medical decisions are made. Specifications for maximum allowable expanded measurement uncertainty must be included in the manufacturer's documentation of the calibration hierarchy for the measuring system.

The maximum allowable expanded measurement uncertainty specification for a measuring system is the specification for the combined expanded (k=2) maximum allowable measurement uncertainty covering all steps in the calibration hierarchy, including the final measurement on human samples. Strategies for setting the maximal acceptable measurement uncertainty for a measuring system are currently widely discussed (76-80).

The maximal allowable measurement uncertainty specification Umax established by the manufacturer of the measuring system must account for the combined measurement uncertainty associated with all steps in the calibration hierarchy for the measuring system, down to and including the value assignment of end-user measuring system calibrators in addition to the expected uncertainty contribution due to routine use of the measuring system, at a minimum under repeatability conditions.

Total allowable measurement uncertainty based on biological variation is commonly used for measurands under homeostatic control. If CV_I% is the intra-individual coefficient of variation, and a coverage factor of 2 for expanded uncertainty is used (95% confidence) then

$$Umax < 2 * 0.75 * CV_I\,\%$$

Can be used as a minimum criterion for Umax.

The EFLM database on the biological variation (81) is a dependable source of information on biological variation, including CV_I %.

Information to be provided to the end-user

For assigned values of measuring system calibrators, the minimum information concerning the measurement uncertainty that the manufacturer of the calibrators must provide to the end-user on request is the numerical value assigned to the calibrator and its measurement uncertainty.

Estimates for measurement uncertainty of measuring system calibrators are sometimes presented as expanded uncertainty. Measurement uncertainty = measurement uncertainty (y) × k, usually with the coverage factor k = 2, giving a confidence level of approximately 95 %.

Dependent on local and regional requirements, medical laboratory end-users of measuring systems often use the *measurement uncertainty* value provided by the manufacturer of the in vitro diagnostic measuring system calibrator to estimate the combined measurement uncertainty of the measured value for a human specimen with the specified end-user measuring system.

Additional calibration hierarchy documentation responsibilities

The end-user measuring system calibrator(s) manufacturer must provide end-users the assigned target value, the associated metrological traceability, and measurement uncertainty for each calibrator level supplied for use with a specified measuring system.

In some cases, manufacturers of measuring systems specify end-user measuring system calibrators manufactured by a different (second or independent) manufacturer. Such independent (third party) manufacturers of measuring system calibrators must maintain the technical file supporting claims of metrological traceability of assigned values for each measurand claimed in the intended use statement for such applicable measuring system calibrator(s). Similarly, any manufacturer of an in vitro diagnostic measuring system calibrator who sells a calibrator designed for use with “other” (third party) measuring systems (with or without collaboration with the manufacturer of the measuring system) is responsible for fulfillment of all documentation requirements.

Modifications introduced by an end-user laboratory

If modifications to measuring systems are defined and implemented by a medical laboratory, full description and re-validation of the calibration hierarchy underlying the reported values for human samples when examined with the modified measuring system is the responsibility of the entity(s) that implemented the modifications.

1. When a single laboratory develops a measuring system for its use, the same laboratory is responsible for validating and describing the complete calibration hierarchy and including the results for human samples. This also applies to published measurement procedures implemented by medical laboratories.

Reference measurement procedures

A description of a reference measurement procedure should include:

1. A description of the quantity measured by the reference measurement procedure, in terms of system, component, and kind-of-quantity, including any specifications to each
2. Explanation/statement of the role of the quantity in health care, when appropriate
3. The measurement method and the rationale for its use
4. The measurement model in terms of the measurand is a function of all input quantities
5. The proper place of the reference measurement procedure in a traceability hierarchy
6. Any known limits of applicability of the measurement procedure, including limits regarding matrices of sample- and reference materials
7. Known interferences including pharmaceuticals, drugs, metabolites, and additives
8. The objectives of measurement for which the reference measurement procedure is suited
9. Established allowable modifications to the primary reference measurement procedure, e.g., as necessary to eliminate interferences or matrix effects
10. The measuring interval.

The required elements in a proper description of a reference measurement procedure according to ISO 15193:2009 are

1. Title page
2. Warning and safety precautions
3. Title of the reference measurement procedure
4. Scope
5. Measurement principle and method
6. Reagents
7. Apparatus
8. Sampling and sample
9. Preparation of measuring system and analytical portion
10. Operation of the measuring system
11. Data processing
12. Analytical reliability
13. Validation by inter-laboratory comparisons
14. Reporting
15. Quality assurance
16. Dates of authorization and revision

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