1. **Purpose:** This document establishes guidelines for assigning validation levels to methods based on national and international standards. The four validation levels defined in this document are based on a number of factors including the number of laboratories involved in the validation process, the number of matrices, number of validation samples analyzed, etc. Consistent method validation protocols for chemical, biological and radiochemical methods do not exist among member Agencies and networks of the ICLN. Therefore, no basis exists for one network to determine the validation status of another network's methods other than the laborious task of reviewing that network's method validation protocol and relevant method validation data for each method of interest. As this approach is far too burdensome to be practical, this document provides guidance which allows each member ICLN network to uniformly assign validation levels to their methods. These uniformly assigned validation levels will allow an ICLN network to rapidly assess, in a timely manner, the validation status of another network's methods and thus enable the network to evaluate the potential use of another network's methods for their specific needs.

2. **Scope:** This document establishes guidelines for assigning validation levels for methods. However, it does not establish a common method validation protocol for ICLN member agencies and networks. In addition, this document does not create a new definition for method validation but rather relies on definitions of method validation from national and international standards.

3. **Outline of Validation Procedure**:

3.1 Method validation is a process by which a laboratory confirms by examination and the provision of objective evidence that the particular requirements for specific use are fulfilled. It serves to demonstrate that:

3.1.1 The method can detect, identify, and potentially measure the amount of (quantify) an analyte(s):

- in all matrices to be analyzed; and
- with a demonstrated sensitivity, specificity, accuracy, trueness, reproducibility, ruggedness, and precision to ensure that results are meaningful and appropriate for decision making by the receiving network.

3.1.2 The method will function reliably for its intended purpose as defined by participating networks.

3.2 The method developer validates a method by conducting experiments to determine or verify a number of specific performance characteristics that serve to define and quantify method performance.

4. References and Resources:

4.1 Food and Drug Administration, Laboratory Manual, ORA Laboratory Procedure, Volume II, ORA-LAB 5.4.5, Methods, Method Verification and Validation.
https://www.fda.gov/media/73920/download
https://www.fda.gov/science-research/field-science-and-laboratories/field-science-laboratory-manual

4.2 International ANS/ISO/IEC Guide to the Expression of Uncertainty in Measurement, 1995.
https://www.iso.org/standard/50461.html
https://www.bipm.org/en/publications/guides/

4.3 International vocabulary of metrology -- Basic and general concepts and associated terms (VIM), ISO/IEC Guide 99:2007 (replaces ISO Guide 99:1993).
https://www.iso.org/standard/45324.html

4.4 Foundations of Clinical Research, Applications to Practice, Leslie Gross Portney, Mary Watkins, Appleton & Lange, 1993.

4.5 Validation and Peer Review of U.S. Environmental Protection Agency Chemical Methods of Analysis, Forum on Environmental Measurements, October 14, 2005.

4.6 Protocol for the Design, Conduct, and Interpretation of Method-Performance Studies, *Pure and Applied Chemistry*, 67, No. 2, 1995, 331-343.

4.7 Harmonized Guidelines for Single-laboratory Validation of Methods of Analysis, *Pure and Applied Chemistry*, 2002, 74, 835 - 855.

5. Specific Procedure(s):

5.1 Prior to submitting methods to the involved network:

5.1.1 The intended use of the method should be defined

5.1.2 The intended use should be aligned with network capabilities.

5.1.3 A study plan for method validation should be submitted for approval to the involved network before starting the validation procedure or if the validation is in progress.

5.1.4 A required written procedure may include all the following elements:

- intended use and criteria for use;
- assay principle and safety precautions;
- acceptable sample types, sample collection method, preparation; preservation, storage, and transportation conditions;
- description of reagents supplied or directions for preparation and the quality control of the preparation procedure;
- description of method quality controls to be used;
- detailed instructions on how to perform the method;
- detailed instructions on how to interpret and report the result;
- instructions on data processing and calculations (e.g., peak integration, signal to noise ratio, ion ratio and others);
- outlining acceptance criteria for any decision making (e.g., acceptable vs. not acceptable: retention time, ion ratio, signal to noise ratio; analyte: detected vs. not detected, confirmed vs. not confirmed and others);
- any limitations or critical points of the method that are known or suspected;
- a summary of the performance characteristics of the method; and,
- a statement on the target uncertainty of measurements for each analyte (analytical goal for accuracy) in order for the values to be fit for their intended purpose.

5.2 Methods can be validated whenever any of the following occur:

5.2.1 submission of a new method to the involved network for inclusion as an official network method;

5.2.2 expansion of the scope of an existing network method to include additional analytes or new matrices;

5.2.3 modification of a network method's range beyond validated levels; or,

5.2.4 modification of a network method that may alter its performance specifications. This includes changes to the fundamental science of an existing method, equivalence issues such as substitutions of reagents/apparatus, or changes to some instrumental parameters. Because it is difficult to predict the results of any change, all but the most trivial of changes should be evaluated for effects on method performance.

5.3 Performance specifications required to validate a method:

5.3.1 Performance specifications that may be determined to validate a method will vary depending on the intended use, the type of method being validated, and the degree to which it has previously been validated. All methods submitted must have all the proper controls and the parameters for calibrating and operating the method instrumentation included in the written procedure.

5.3.1.1 Typical validation characteristics that may be considered are the following: (See Glossary for definitions of these characteristics in Appendix 1)

Characteristics of quantitative methods:

- Method uncertainty;
- Minimum quantifiable concentration (MQC);
- Detection limit (See MDL and MDC);
- Applicable analyte concentration range;
- Accuracy;
- Trueness;
- Precision;
- Analytical specificity;
- Linearity; and/or,
- Critical Points/Ruggedness/robustness.

Characteristic for qualitative methods:

- Reliable identification of an analyte at some target level(s);
- Sensitivity;
- Specificity;
- Limit of detection (See Detection limit, MDL, MDC);
- Ruggedness/robustness;
- Clinical sensitivity (if human specimens are used in the assay); and/or,
- Clinical specificity (if human specimens are used in the assay).

5.3.1.2 Validation tools (Ref ORA-LAB SOP# 5.4.5)

The following tools may be used to demonstrate the ability to

meet method specifications of performance:

- Blanks: Use of various types of blanks enables assessment of how much of the result is attributable to the analyte in relation to other causes.
- Reference materials and certified reference materials with typical interferences expected: Use of known materials can be incorporated to assess the accuracy of the method, as well as obtain information on interferences.
- Fortified (spiked) materials and solutions: Use to understand that spiked recovery may not be truly representative of recovery from naturally incurred analytes.
- Repeatability: Use replicate analyses to provide a means of checking for changes in precision in an analytical process which could adversely affect the results.
- Statistics: Use statistical techniques to evaluate accuracy, trueness (or bias) precision, linear range, limits of detection and quantification, and measurement uncertainty.

5.3.1.3 General validation protocol guidance

The following provides guidelines that may be used to determine method performance characteristics:

- Take quantitative measurements (e.g., determine limit of quantification (LOQ), linear response (minimally need LOD)).
- Prepare and analyze spiked blanks and matrix samples of known concentration utilizing one to three different concentration levels: low, medium, high based on the intended use of the method. These samples are carried through the complete sample preparation procedure, extraction, and analytical steps of a particular method. Matrix effects also can be assessed with these samples. Accuracy or bias and precision are calculated from these results; data also will evaluate robustness of the method resulting from changes in the sample matrix. (Note: Proper certified reference materials and reference standards are used when available.)
- Assure that adequate sample replicates are performed and compare results from replicate measurements of each analyte.
- Analyze blanks (reagent and matrix) and compare these results

to the reported limit of detection.

- Evaluate interferences, including spectral, physical, chemical, or memory by analyzing samples containing various suspected interferences in the presence of the object being measured.

5.3.1.4 Validation of methods (original, new, or modified), may include, but not be limited to, matrix extensions and platform changes.

- In cases where the sample preparation and/or the extraction procedure/analytical method is modified from the existing test procedure and protocol, the new method may demonstrate that the modifications do not adversely affect the precision and accuracy or bias of the data obtained.
- In order to implement the modified method, the standard or existing method is first performed. The modified method is then verified against the original method validation protocol as defined in section 5.3.1.3.
- For original or new methods the authors may pick a validation level that is suitable for their situation as defined in section 5.4.
- Statistical methods are employed to verify performance between the original validated and new method sample means and to determine the degree of accuracy. (For example: The t-test assesses whether the means of two groups are statistically different. The t-test is to be less than or equal to the t-critical value. The F-test is used to determine the significance of difference between two sample variances. The F value is to be less than or equal to the F-critical value.)

5.4 Levels of Validation

5.4.1 The individual network is responsible for determining the level of validation that is acceptable for use on the particular method.

5.4.2 This section references four validation levels:

Level One: The method may be tested in one laboratory for one or more analytes and one or more matrices. The laboratory would select a limited number of key characteristics to evaluate the method performance (see tables 1, 2, and 3 for specific details for level 1 validation).

Level Two: The method may be validated in a single laboratory (see tables 1, 2, and 3 for specific details for level 2 validation). This is similar to the Multi-Agency Radiological Laboratory Analytical Protocols tiered project method validation approach for radio analytical methods.

- "Wherever possible and practical a laboratory may use a method of analysis that has had its performance characteristics evaluated through a collaborative trial conforming to an international protocol." Page 844 *Pure and Applied Chemistry*, 2002, 74.

- "Single-laboratory validation requires the laboratory to select appropriate characteristics for evaluation from the following: applicability, selectivity, calibration, accuracy, precision, range, limit of quantification, limit of detection, sensitivity, and ruggedness." Page 845 *Pure and Applied Chemistry*, 2002, 74.

Level Three: The method may be validated by testing the applicable performance characteristics from a single laboratory validation study using two to seven labs with one or more matrices (see tables 1, 2, and 3 for specific details for level 3 validation).

Level Four: The method may be tested using the criteria for a full collaborative study. The study may examine bias, recoveries, applicability, interference, method comparison, calibration procedures, and all applicable performance characteristics examined in a single laboratory validation (see tables 1, 2, and 3 for specific details for level 4 validation).

- "For a single type of substance at least five materials (test samples) must be used; only when a single level of specification is involved for a single matrix may this minimum required number of materials be reduced to three." Page 334, *Pure and Applied Chemistry*, 67, No. 2, 1995.

- "At least eight laboratories may report the results for each matrix; only when it is impossible to obtain this number may the study be conducted with fewer, but with an absolute minimum of five laboratories." Page 335, *Pure and Applied Chemistry*, 67, No. 2, 1995.

- The entire protocol is outlined in the article "Protocol for the Design, Conduct, and Interpretation of Method-Performance Studies," *Pure and Applied Chemistry*, 67, No. 2, 1995, 331-343.

Table 1. ICLN Validation Recommendations for Chemistry Methods

Originating Laboratory Study	Level One: Urgent Usage/ Matrix Extension	Level Two: Single Lab Validation	Level Three: Independent Lab Validation	Level Four: Collaborative Study[a,b]
Number of matrices	1 or more as the situation requires	1–5	1–5	At least 5
Number of sources	3–5 as the situation requires	3–5 as the situation requires	3–5 as the situation requires	3–5 as the situation requires
Number of participating laboratories	1	1	2–7	8 (quantitative) to 10 (qualitative)
Number of minimum analyte level	1 spike level and 1 matrix blank	2 spike levels and 1 matrix blank	2 spike levels and 1 matrix blank	3 spike levels and 1 matrix blank
Replicates per matrix tested at each level	2 (quantitative) 2 (qualitative)	2 (quantitative) 4 (qualitative)	2 (quantitative) 6 (qualitative)	2 (quantitative) 6 (qualitative)

[a]*Pure and Applied Chemistry*, 67, No. 2, 1995, 331-343.
[b]AOAC International, "AOAC Peer-Verified Methods Program Manual on Policies and Procedures", AOAC international 1998.

Table 2. ICLN Validation Recommendations for Microbiological Methods

Originating Laboratory Study	Level One: Urgent usage	Level Two: Single lab Validation	Level Three: Independent Lab Validation[k]	Level Four: Collaborative Study[i,j]
Number of strains of target organism (inclusivity)[a]	1 to 5[b]	30[b]	30[b]	50[b]
Number of strains of non-target organism (exclusivity)	1 to 5[c]	30[c]	30[c]	30[c]
Number of matrices	1 or more[d]	1 or more[d]	1 or more[d]	Up to 20 matrices[d]
Number of analytes level/matrix	Set level based on intended use and 1 uninoculated control	One inoculated level[e], one at 1 log higher, and 1 uninoculated control	One inoculated level[e], one at 1 log higher, and 1 uninoculated control	One inoculated level[e], one at 1 log higher (optional), and 1 uninoculated control
Replicates per matrix[f]	2 or more	6 or more	6 or more	at least 10
Aging of inoculated samples prior to testing	No	No	Yes[g]	Yes[g]
Addition of competitor strain[h]	Normal background flora	In 1 matrix at +1 log > analyte at fractional positive[d] analyte level	In 1 matrix at +1 log > analyte at fractional positive[d] analyte level	In 1 matrix at +1 log > analyte at fractional positive[d] analyte level
Comparison to recognized method	No	Yes, if available	Yes, if available	Yes, if available

[a] For bacteriological methods at 10^3 CFU/ mL (g) following the method protocol.
[b]Select agent organisms may have limited strain availability for inclusion and exclusion studies. An attempt may be made to obtain necessary strains when possible.
[c] For bacteriological methods at 10^3 CFU/mL, non-target organisms grown in a non-selective rich medium.

[d]Depends on applicability of method.
[e]Must be adjusted to achieve fractional positive results (one or both methods give 40–90% positive results).
[f] Except for Level 1, must run six replicates per matrix, with as many different matrix sources as possible, up to six. Additional replicates may be required based on the specific method requirements for bias, precision, uncertainty, etc.
[g]Period of aging depends on food being tested.
[h]An appropriate competitor is one that gives similar reactions in enrichment and detection systems.
[i]*Pure and Applied Chemistry*, 67, No. 2, 1995, 331-343.
[j]AOAC International, "AOAC Peer-Verified Methods Program Manual on Policies and Procedures", AOAC international 1998.
[k]OIE Terrestrial Manual. Chapter 1.1.6. Principles and methods of validation of diagnostic assays for infectious diseases. World Organisation for Animal Health (OIE) [ISBN 978-92-95108-18-9] 2018.

Table 3. ICLN Validation Recommendations for Radiological Methods

Originating Laboratory Study	Level One: Urgent Usage Matrix Extension	Level Two: Single Lab Validation	Level Three: Independent Lab Validation	Level Four: Collaborative Study[a,b]
Number of matrices	1 or more as the situation requires	1 or more as the situation requires	1 or more as the situation requires	1 or more as the situation requires
Number of sources	3–5 as the situation requires	3–5 as the situation requires	3–5 as the situation requires	At least 5
Number of participating laboratories	1	1	2–7	8 (quantitative) to 10 (qualitative)
Number of minimum analytes level/matrix	1 spike level and 1 matrix blank	2 spike levels and 1 matrix blank	2 spike levels and 1 matrix blank	3 spike levels and 1 matrix blank
Replicates per matrix tested at each level	2 (quantitative) 2 (qualitative)	2 (quantitative) 6 (qualitative)	2 (quantitative) 6 (qualitative)	2 (quantitative) 6 (qualitative)

[a]*Pure and Applied Chemistry*, 67, No. 2, 1995, 331-343.
[b]AOAC International, "AOAC Peer-Verified Methods Program Manual on Policies and Procedures", AOAC international 1998.

APPENDIX 1. Glossary

(Note: This listing of definitions is taken from the sources in the reference section with slight modifications.)

Action Level: ICLN level of concern for an analyte that must be reliably identified or quantitated in a sample.

Accuracy: A measure of the degree of conformity of a value generated by a specific procedure to the assumed or accepted true value. It includes precision and bias.

Analytical Batch: An analytical batch taken within 24 hours that consists of samples that are analyzed together with the same method sequence and same lots of reagents and with the manipulations common to each sample within the same period or in continuous sequential periods.

Analyte: Specific component of a test sample measured.

Applicability: The validated analytical method provides data that can resolve a particular scientific issue in a specified matrix.

Bias: The difference between the expectation of the test results and an accepted reference value. Note: Bias is the total systematic error as contrasted to random error. There may be one or more systematic error components contributing to the bias. A larger systematic error difference from the accepted reference value is reflected by a larger bias value.

Calibration: The set of operations that establish, under specific conditions, the relationship between values of quantities by a measuring instrument or measuring system or values represented by a material measure or a reference material and the corresponding values realized by standards.

Certified Reference Material (CRM): Reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure that establishes metrological traceability to an accurate realization of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence (slightly modified from VIM04).

Confirmatory Method: A method that provides an unequivocal confirmation of the identity of the analyte and may also confirm the quantity present. Confirmatory methods are the most definitive and frequently are based on combined chromatographic and mass spectrometric techniques, such as liquid chromatography – mass spectrometry (LC/MS). Such methods when used for confirmation of analyte identity should provide reliable structural information within established statistical limits. When the confirmatory method does not provide quantitative information, the quantification result of the original quantitative method should be verified by analysis of replicate test portions using the original quantitative method or a suitably validated alternative quantitative method.

Control Chart: A graphical representation of data taken from a repetitive measurement or process. Control charts may be developed for various characteristics (e.g., mean, standard deviation, range, etc) of the data. A control chart has two basic uses: as a tool to judge if a process was in control and as an aid in achieving and maintaining statistical control. For applications related to radiation detection instrumentation or radiochemical processes, the mean (center line) value of a historical characteristic (e.g., mean detector response), subsequent data values, and control limits are placed symmetrically above and below the center line displayed on the control chart.

Fractional Recovery: Validation criterion that is satisfied when an unknown sample yields both positive and negative responses within a set of replicate analyses. The proportion of positive responses should fall within 25 and 75% and should ideally approximate 50% of the total number of replicates in the set. A set of replicate analyses are those replicates analyzed by one method (either candidate or reference). Only one set of replicates per matrix is required to satisfy this criterion.

Laboratory: An entity that performs tests and/or calibrations. When a laboratory is part of an organization that carries out activities additional to sample preparation, testing, and calibration, the term laboratory refers only to those parts of that organization that are involved in the sample preparation, testing, and calibration process. A laboratory's activities may be carried out at a permanent, temporary, or remote location.

Limit of Detection (LOD): Lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value. It is the lowest concentration level that can be determined statistically different from a blank at a specified level of confidence. It is determined from the analysis of sample blanks and samples at levels near the expected LOD (see ISO 11843, CLSI EP17).

Limit of Quantification (LOQ): Lowest amount or concentration of analyte that can be quantitatively determined with an acceptable level of uncertainty, also referred to as the limit of determination.

Linearity: Defines the ability of the method to obtain test results proportional to the concentration.

Matrix: All the constituents of the test sample with the exception of the analyte.

Matrix Blank: A quality control sample of a specified amount of matrix that does not contain the analyte of interest.

Matrix Spike: An aliquot of a sample prepared by adding a known quantity of target analytes to a specified amount of matrix and subjected to the entire analytical procedure to establish if the method or procedure is appropriate for the analysis of a particular matrix.

Method Blank: Quality control sample that does not contain the analytes of interest but is subjected to all sample processing operations including all reagents used to treat the samples.

Method Detection Limit (MDL): Lowest amount or concentration of analyte that a specific method can statistically differentiate from analyte-free sample matrix. This is dependent on sensitivity, instrumental noise, blank variability, sample matrix variability, and dilution factor.

Minimum Detectable Concentration (MDC): An estimate of the minimum true concentration of analyte that must be present in a sample to ensure a specified high probability (usually 95%) that the measured response will exceed the detection threshold (i.e., critical value), leading one to conclude correctly that the analyte is present.

Minimum Quantifiable Concentration (MQC): The smallest concentration of analyte whose presence in a laboratory sample ensures the relative standard deviation of the measurement does not exceed a specified value, usually 10%.

Performance Characteristics: Method characteristics defined and measured for every analyte and specific type of sample matrix listed in the scope of the fully optimized procedure.

Precision: Degree of agreement of measurements under specified conditions. The precision is described by statistical methods, such as a standard deviation or confidence limit. See also Random Error. Repeatability expresses the precision under the same operating conditions over a short period. Intermediate precision expresses within-laboratory variations, such as different days, different analysts, and different equipment. Reproducibility expresses the precision between laboratories.

Qualitative Method: A method that identifies analyte(s) based on chemical, biological, or physical properties. Most qualitative methods are or can be made at least “semi-quantitative” to provide rough estimates of amount present.

Quantitative Method: A method that provides quantitative information which may be used to determine if analytes in a particular sample exceed an action limit, but do not provide unequivocal confirmation of the identity of the residue. Such methods which provide quantitative results must perform in good statistical control within the analytical range that brackets the action limit.

Random Error: The irreproducibility in making replicate measurements resulting from random changes in experimental conditions that affects the precision of a result. The distribution of random errors usually follows a Gaussian-shaped "bell" curve. See also Precision.

Range: The interval of concentration over which the method provides suitable precision and accuracy.

Recovery: Proportion of incurred or added analyte that is extracted and measured from the analytical portion of the test sample.

Reference Material: A material or substance, with one or more property values, that are sufficiently homogenous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.

Reference Standard: A standard, generally having the highest metrological quality available at a given location in a given organization, from which measurements are made or derived. Note: A reference standard is a recognized national or international traceable product provided by a standards producing body, such as the National Institute of Standards and Technology (NIST).

Repeatability: The closeness of the agreement between the results of successive measurements of the same measurand carried out under the same conditions of measurement.

Ruggedness or Robustness: The ability of a method to resist changes in test results when subjected to minor deviations in experimental conditions of the procedure. Ruggedness testing examines the behavior of an analytical process when subtle small changes in the environment and/or operating conditions are made, akin to those likely to arise in different test environments.

Screening Method: A qualitative or semi-quantitative method intended to identify or detect the presence (or absence) of an analyte in a sample which may exceed an action limit established by a competent authority.

Selectivity: The capability of a method to discriminate between the analyte of interest and other components of the sample including matrix components.

Sensitivity: The lowest concentration that can be distinguished from background noise or the smallest amount of a substance or organism that can accurately be measured by a method or test system is the analytical sensitivity. However, sensitivity is commonly defined as the slope of the calibration curve at a level near the LOQ. For assays that will be used to test human clinical specimens, the method's analytical sensitivity is distinct from the method's clinical diagnostic sensitivity. Clinical diagnostic sensitivity is the percentage of persons who have a given condition who are identified by the method as positive for the condition (high analytical sensitivity does not guarantee acceptable diagnostic sensitivity).

Sensitivity: The change in the response of a measuring instrument divided by the corresponding change in the stimulus.

Source: The origin of a test sample. A sample matrix may have variability due to its source. For example, a water sample may have variable characteristics, and therefore, may show method results variability, depending on whether the sample source is drinking water, ground water, surface water, or wastewater. Different food sources are defined as different commercial brands. Different water sources could be from different areas of a reservoir. Different plant or soil sources could be samples from the different areas of a

plot or field. Different sediment sources could be samples from different areas of a water body.

Specificity: Analytical specificity is the ability of a method to measure one particular analyte in the presence of components that may be expected to be present. For methods that will be used to test human clinical specimens, the method's analytical specificity is distinct from the method's clinical diagnostic specificity. Clinical diagnostic specificity is the percentage of persons who do not have a given condition who are identified by the method as negative for the condition.

Standard Reference Material (SRM): A certified reference material issued by the National Institutes of Standards and Technology (NIST) in the United States for specific chemical or physical purposes. It is issued with a certificate that reports the results of the characterization and indicates the intended use of the material (www.nist.gov/SRM).

Systematic Error: A form of measurement error, where error is constant across trials. This may also be referred to as bias.

Target Level: The level at which an analyte can be reliably identified or quantified in a sample.

Trueness: The degree of agreement of the expected value from a measurement with the true value or accepted reference value. This is related to systematic error (bias).

Uncertainty: The parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand. (VIM, 1993)

Validation: The process intended to demonstrate that a method is fit-for-purpose. This means that in the hands of a properly trained analyst using the specified equipment and materials, and following the procedures described in the method, reliable and consistent results can be obtained within specified statistical limits for the analysis of a sample.

Verification: The confirmation by examination and provision of the objective evidence that specified requirements have been fulfilled.

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