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Soil and water quality — Guidance document on designing an interlaboratory trial for validation of biotests

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National foreword

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Soil and water quality — Guidance and requirements for designing an interlaboratory trial for validation of biotests

Qualité de l'eau et du sol — Recommandations et exigences relatives à la conception d'un essai interlaboratoires pour la validation des essais biologiques

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Foreword

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

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This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization*, in collaboration with Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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Introduction

Validation of an ISO biotest standard aims to estimate the uncertainty of results by means of an interlaboratory trial. This validation program typically involves three major steps.

a) Demonstration of readiness for testing: This 1st step is an opportunity for the lead laboratory to ensure that recommended instrumentation is in place and that test organism and cell cultures have been established using performance measures to ensure healthy organisms or cultures are used in testing. These conditions are typically part of the clause/subclause entitled "Test organisms" or "Test material" in an ISO standard.

b) Demonstration of laboratory capability and transferability of the biotest: In this 2nd step, the participating laboratories aim to achieve successful control performance during this preliminary interlaboratory testing round with a reference compound added either to a solid matrix or to a liquid medium according to the biotest method to be validated. The ability to conduct the testing standard is demonstrated by fulfilling the validity criteria (e.g. variability of controls expressed as the coefficient of variation for the number of juveniles in a reproduction test) and qualifies the laboratory for the final method validation step.

c) Method validation: The 3rd step involves only laboratories who have demonstrated the expertise in conducting the ISO standard under development (step b). In case of validation of an ecotoxicological or microbiological testing method, one or two rounds of interlaboratory method validation trial using a contaminated environmental sample (or samples) are conducted and the results from each round are used to calculate the intralaboratory and interlaboratory variability of the ISO testing standard as a demonstration of method precision (Annex A). If repeated testing runs of the biotest are feasible, repeatability is determined. For the validation of ecotoxicological methods it can be useful to evaluate the correctness of the measured effect – the measurement trueness. This holds true especially for methods of which the results are reported in terms of a quantitative measurement such as a biological equivalence concentration. Obtained results are used for confirming or adjusting the validity criteria.

For the validation study, representative samples should be selected according to the intended scope of the standard (e.g. contaminated soils, amended soils, soils after remediation, waste materials, wastewaters, eluates, surface water, groundwater, sediments and extracted samples).

An overall schema of the validation process can be found in Annex D.

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Soil and water quality — Guidance and requirements for designing an interlaboratory trial for validation of biotests

1 Scope

This document aims to assist in designing and organizing trials for validation of biotests. The validation activities during the different steps of the standardization process are described. This document comprises the overall data evaluation and subsequent validation study conclusion.

This document is intended for the validation of biotests which can differ in their experimental design and endpoints. It is possible that some of the requirements of this document are not applicable to all test methods.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

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

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

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

3.1
control
material and/or matrix that duplicates all factors that can affect results except the specific condition or treatment being studied

Note 1 to entry: In toxicity tests, the control should have all the same conditions as in the treatment exposure but without the toxicant.

[SOURCE: Environment Canada 2005]

3.2
endpoint
statistically derived toxicity threshold (e.g. EC50)

[SOURCE: Environment Canada 2005, modified — The recommendation not to use the term for observed variables, such as size, is deleted]

3.3
lead laboratory
laboratory responsible for organization of the interlaboratory validation study

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3.4
measurement trueness
trueness of measurement
trueness
closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value

Note 1 to entry: The requirement for an infinite number of replicate measurements has a theoretical background. In practice a large series of test results is used to estimate the measurement trueness.

[SOURCE: ISO/IEC Guide 99:2007 2.14, modified — Notes to entry have been replaced]

3.5
performance characteristics
measures of the performance of a test under specific conditions, including its reliability and accuracy

Note 1 to entry: Performance characteristics are an indication of the test's usefulness, limitations, and relevance.

[SOURCE: OECD 2005]

3.6
precision
closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions

Note 1 to entry: Measurement precision is usually expressed numerically by measures of imprecision, such as standard deviation, variance, or coefficient of variation under the specified conditions of measurement.

Note 2 to entry: The 'specified conditions' can be, for example, *repeatability conditions* (3.10) of measurement, intermediate precision conditions of measurement, or *reproducibility conditions* (3.12) of measurement (see ISO 5725-1).

Note 3 to entry: Measurement precision is used to define measurement *repeatability* (3.9), intermediate measurement precision, and measurement *reproducibility* (3.11).

Note 4 to entry: Sometimes "measurement precision" is erroneously used to mean measurement accuracy.

[SOURCE: JCGM 200:2012]

3.7
prevalidation
initial phase(s) of a validation study

Note 1 to entry: A small-scale study intended to obtain preliminary information on the relevance and reliability of a test method. Based on the outcome of those studies, the test method protocol may be modified or optimized to reduce intra- and/or interlaboratory variability and increase accuracy in subsequent validation studies. If available, literature data may be used for this purpose.

[SOURCE: OECD:2005, modified — Reasons for performing prevalidation are not included]

3.8
reference compound
chemical for which the response of the test organism is known

3.9
repeatability
measurement *precision* (3.6) under a set of *repeatability conditions* (3.10) of measurement

[SOURCE: JCGM 200:2012]

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3.10
repeatability condition
condition of measurement, out of a set of conditions that includes the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time

Note 1 to entry: A condition of measurement is a repeatability condition only with respect to a specified set of repeatability conditions.

[SOURCE: JCGM 200:2012]

3.11
reproducibility
measurement *precision* (3.6) under *reproducibility conditions* (3.12) of measurement

Note 1 to entry: Relevant statistical terms are given in ISO 5725-1 and ISO 5725-2.

3.12
reproducibility condition
condition of measurement, out of a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects

Note 1 to entry: The different measuring systems may use different measurement procedures.

Note 2 to entry: A specification should give the conditions changed and unchanged, to the extent practical.

[SOURCE: JCGM 200:2012]

3.13
interlaboratory method validation trial
interlaboratory validation study in which all laboratories perform the same biotest using the same material under testing and the same test protocol

Note 1 to entry: The purpose of the test is to determine inter laboratory and, whenever possible, intra laboratory variability.

3.14
transferability
ability of a test procedure to be accurately and reliably performed in independent, competent laboratories

[SOURCE: OECD:2005]

4 Principle

Validation of a proposed test method is achieved through the demonstration of reasonable coefficients of intralaboratory and interlaboratory variation, which have participated in the formal method validation testing round(s) (i.e. the 3rd validation step, 5.5). Repeatability should preferentially be determined. However, on occasion, the duration or workload of the test method prevents the analysis by individual laboratories from being repeated. In case of missing experimental data obtained under repeatability conditions, it is proposed to estimate repeatability from the confidence limits of the toxic metric that integrates the variability of repeated measures of the biological variable in each treatment/dilution (see Reference [67]). If appropriate, the measurement trueness of the method can be determined in line with the method validation, in particular for methods the results of which are reported in biological equivalence (BEQ) concentrations (see ISO 23196). The final decision to include the measurement trueness in the interlaboratory method validation trial has to be taken by the lead laboratory in consultation with other experts involved in the development of the method.

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5 Requirements, design and organization of method validation testing round(s)

5.1 General

A prerequisite for the performance of an interlaboratory trial is the method development has been completed. As the aim of this kind of interlaboratory comparison is to evaluate the performance characteristics of a biotest, technical aspects are defined within an interlaboratory method validation trial procedure prepared by the lead laboratory and unique to each round of interlaboratory testing. Specific features which are recommended to be addressed by lead laboratories are summarized in Annexes F and G for terrestrial and water tests, respectively.

A lead laboratory should be able to demonstrate their level of competency for planning and conducting the interlaboratory method validation trial. For example, the laboratory proposing to lead the validation effort can state the years of experience of participating staff in conducting environmental toxicology or microbiological testing (e.g., 10 + years); provide proof of recognised quality service through a laboratory certification or accreditation program; provide proof that they have a track record in planning and conducting multi-laboratory testing studies; demonstrate in writing that their staff have a clear understanding of all steps the laboratory would be responsible for leading an inter-laboratory validation program; clearly state their willingness to provide the materials to culture or holding and acclimation organisms required to conduct the new biological testing standard prior to and during the full validation testing round; or, state their willingness to provide remote or on-site training to assist participating laboratories with preliminary steps needed to show their readiness to conduct the new testing procedure. Specific suggestions and further details on steps and capability of a lead laboratory and their staff are outlined in ISO 17043.

Within the interlaboratory method validation trial, the lead laboratory should:

— be responsible for the organization and performance of the three validation steps (5.3, 5.4, 5.5) or interlaboratory testing rounds;

— prepare an interlaboratory method validation trial procedure for each round of testing, defining all technical aspects of the performance of the biotest;

— supply the participants with the necessary instructions, test samples (e.g. contaminated soils, amended soils, soils after remediation, waste materials, wastewaters, eluates, surface water, groundwater, sediments and extracted samples) and materials selected by the lead laboratory, for each testing round;

— be responsible for the analysis and reporting of results from each testing round;

— be responsible for requesting feedback from laboratory participants on the proposed interlaboratory method validation trial procedure prior to each testing round, through an organized tele- or video conference or e-mail exchange.

An example of different roles of personnel involved in organising of the interlaboratory method validation trial can be found in ISO 5725-2:2019, Clause 6.

Unless requested otherwise by the lead laboratory, it is highly recommended to transfer all results along with all test condition parameter measurements, immediately after completion of testing for each specific testing round.

5.2 Prevalidation — Minimum requirements of test method performance

The primary step of method validation should rely on a thorough evaluation of the peer-reviewed scientific literature and other relevant and credible reports and publications providing information about the performance of the test method. This literature review should aim at identifying experimental conditions which can have an impact on the outputs of a biotest. As a result, additional laboratory experiments can be performed to refine and optimize the procedure accordingly. Special attention should be paid to the ecological relevance of organisms and biological responses used in biological, ecological and ecotoxicological testing with respect to the properties of the matrix to be considered, e.g.

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pH, organic matter, conductivity, clay content or turbidity. The interlaboratory method validation trial design should cover the range of properties appropriate for the testing matrix (e.g. water, sediment, soil) for which the biotest is relevant.

For the optimization of the biotest for ecotoxicity testing, controls have to be defined which differentiate effects of intrinsic properties of the test samples from those caused by contaminants. For example, the validation of a test with regards to its applicability in the risk assessment of contaminated sites, should include a contaminated as well as a reference soil sample. The reference soil sample should have similar intrinsic properties to the contaminated one, but contain no or negligible levels of the contaminants.

Test methods are often defined for two or more different type(s) of samples, for instance, contaminated sites, amended soils, soils after remediation, waste materials (e.g. dredged material, municipal sludge from a wastewater treatment plant, composed material, or manure, especially those for possible land disposal), wastewaters, eluates, surface water, groundwater, sediments, chemicals. Ideally, the test method is validated using the most applicable contaminant or contaminated media type. However, full validation of a method that incorporates a large variety of samples is typically not possible. It can be unnecessary to perform such a wide validation if a limited number of samples is, to the lead laboratory experts' opinion, considered representative of the most important fields of application. For example, the validation of a test method for evaluating the quality of surface waters (e.g. river water samples from a non-polluted upstream area and a downstream point affected by diffuse pollution or an effluent discharge), or the efficiency of wastewater treatment plants (e.g. using liquid samples of pre-treatment influent and/or final treated effluent) should consider the selection of samples appropriate for the application purpose.

5.3 First validation step — Demonstration of participating laboratories' readiness for testing

The first step is to demonstrate the readiness of participating laboratories to culture test organisms or cell cultures and to conduct the testing standard. It is recommended to evaluate readiness of the participating laboratories using a questionnaire or survey completed by each participant (Annex E). This questionnaire provides information to the validation trial coordinator of the lead laboratory regarding the available technical know-how, experience and resources for the culturing of test organisms and conductance of the test procedure.

5.4 Second validation step — Demonstration of laboratory capability and transferability of the biotest

The second step of interlaboratory testing is used to perform a preliminary assessment of the transferability and reliability of the test and to identify possible limitations of the test. Occasionally, interlaboratory method validation trial rounds involving experienced laboratory participants do not generate comparable data. In such a situation, the lead laboratory can further restrict method options to bring a higher degree of standardization prior to the next round of inter-lab testing or can conduct or sponsor additional method research to further standardize the culturing or testing parts of the methodology. Results of the second validation step can be used in the design of the future interlaboratory validation testing round.

At least one laboratory independent from the laboratory that developed the test method conducts the full biotest for an initial assessment and review of its interlaboratory transferability and preliminary reproducibility. Participating laboratories perform the biotest in control conditions and with a reference compound with known toxicity at a specific test concentration or known concentration range provided by the lead laboratory. Results are evaluated by the lead laboratory which can lead to optimization of the organisms' culturing and/or testing procedure. Only laboratories who pass the proposed validity criteria (e.g. variability of controls expressed as the coefficient of variation, sensitivity, biological response rate of the tested organisms in controls) should participate in the method validation testing round (see 5.5). Involvement of inexperienced laboratories is outlined in 5.5.2. Integration of these laboratories sometimes results in less precise conclusions leading to questions of test transferability. In cases where the test method fails to provide sufficient reproducibility, depending on the degree of failure, it can be considered for further optimization or can require further test method research. All

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requirements specified for the interlaboratory method validation trial in 5.5.5 are valid for the second step.

5.5 Third validation step — Method validation

5.5.1 General

The third step involves a testing round (or rounds) to achieve method validation. The interlaboratory method validation trial procedure is designed to evaluate the interlaboratory variability of the results obtained. This is done by conducting the same measurements using the full test method in each participating laboratory. Repeatability (intralaboratory variability) can be estimated if the testing and data analyses are repeated by each laboratory. An alternative approach for the estimation of repeatability using the confidence limits of the toxic metric that integrates the variability of repeated measures of the biological variable in each treatment/dilution can be found in Annex B. If the output is not a numerical value (e.g. extraction of DNA), a specific approach is needed to evaluate reproducibility (see 5.5.6).

5.5.2 Participating laboratories

The number of participants has an influence on the reliability of the statistically calculated performance data. It is suggested to obtain valid datasets from a minimum of 6 laboratories located in 3 different countries. Therefore, it is strongly recommended that a greater number of test laboratories and countries be invited and participate in interlaboratory method validation trials. Annex C provides guidance on how to proceed if a lower number of valid datasets is achieved by calculating and reporting the uncertainty of the reproducibility variance.

Participation in this kind of trials is voluntary, and each participant laboratory should be given a code (that can be communicated to the respective laboratory) to maintain anonymous the source of the validation trial datasets. If the call for participants yields an insufficient number of laboratories that are experienced with the method, assistance in applying this method should be provided by the lead laboratory (e.g. offer to train personnel at inexperienced laboratories, host a training workshop, propose a planning teleconference).

An invitation to interested interlaboratory participants shall be circulated well in advance of the interlaboratory method validation trial launch. It is recommended that the lead laboratory organizing the interlaboratory program circulate the invitation to potential participating laboratories five months prior to the start of the first round of interlaboratory testing. Laboratories should be asked to express their interest in participating within four weeks of receiving the announcement of the interlaboratory trial.

If a full validation of a method is already present, the method may be adopted without a further interlaboratory method validation trial.

5.5.3 Samples

Sample matrices shall reflect the scope of the test method (see 5.2). No further information about the expected values of estimated parameters should be given to participants. Every participant has to perform the biotest in a number of replicates specified by the interlaboratory method validation trial procedure. Each laboratory needs to receive a sufficient quantity of sample or subsamples. Samples shall be clearly labelled (e.g. number of sample or subsample, participant name, matrix, date).

If the determination of the measurement trueness is included in the validation trial a true measurement value is required. Such a true value is of theoretical nature and can only be determined by using a suitable reference material, by a reference to another, validated measurement method or by the preparation of a known reference sample. In the latter case, the environmental matrix to be assessed, for example, surface water or an eluate, is spiked with a defined amount of a reference compound with a known toxic effect potency for the method under investigation. Both the spiked and the respective un-spiked samples shall be included in the interlaboratory method validation trial where the un-spiked

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sample should produce no effect. Alternatively, synthetic or artificial environmental matrices such as synthetic sewage (see Reference [61]) can be used to produce a defined no-effect sample. In either case, the lead laboratory should verify the correct spiking and the stability of the reference compound in the environmental matrix by a pre-test.

Information on the stability and homogeneity of subsamples shall be provided along with the final date for testing initiation. Storage conditions (e.g. temperature range, protection from light) are important points to be outlined in the interlaboratory method validation trial procedure. Compliance with the critical conditions needs to be considered by the lead laboratory coordinator when shipping the samples to other countries. It is the responsibility of the organizer to choose a proper way of sample dissemination or distribution.

If the concentrations of a chemical substance are estimated within the biotest, standard solutions for checking calibration and additional information can be provided by the lead laboratory to help laboratory participants establish and conduct the biotest in their laboratories.

The lead laboratory should request that all participating laboratories ship a subsample of the test media to a common analytical laboratory under contract to the lead laboratory, for chemical confirmation of the nominal test concentration when a reference compound is used for the standard validation round.

5.5.4 Experimental design

Ecotoxicological methods allow different experimental designs which can lead to different types of statistical calculation for test endpoint estimation [e.g. LOEC (lowest observed effect concentration), ECx (effect concentration), combined approach), LID (lowest ineffective dilution) or a biological equivalence concentration (BEQ)]. The lead laboratory shall distribute detailed information about the experimental design (e.g. dilution series, number of test dilutions and controls, number of replicates per treatment) to each participating laboratory. An estimation of ECx is preferred to NOEC (no observed effect concentration) or LOEC. A statistician should be consulted at this stage but likely the lead laboratory conducts this consultation.

5.5.5 Supporting information for participants

The interlaboratory method validation trial procedure should include the following information:

— strict adherence to the validation study procedure (e.g. standard operating procedure) for the specific interlaboratory method validation trial round;

— instructions for safety precautions since potentially present contaminants in the sample can pose a risk to the laboratory staff;

— pretreatment and storage conditions of samples;

— additional instructions for performance of analysis, if necessary;

— description of the test system, exposure conditions, concentration/dilutions selection procedures, estimated variables;

— contact person (from the lead laboratory);

— schedule (deadlines for start of analysis and return of results);

— form for results of analyses (unit, number of significant digits, etc.);

— questionnaire on experimental details, especially when testing a multi-options protocol;

— list of data to be submitted, which should include: blank values, calibration data, original experimental data, culture organism health records, temperature logs, procedures used to calculate and express results, the use of controls and other performance checks or measures of validity criteria;

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— a spreadsheet for recalculation of raw data into the final output, if relevant (e.g. for calculation of microbial activities from estimated concentrations).

5.5.6 Data analysis and statistical evaluation of interlaboratory testing

All results have to meet each criterion of test validity proposed. Before statistical evaluation, the results from the participants shall also be checked for erroneous data and explainable outlying datasets. If necessary, a laboratory participant may be asked to check for errors in the transferred data.

Statistical evaluation of interlaboratory testing consists of two steps – evaluation of the individual biological observations or calculated test endpoints (e.g. calculation of microbial activity or ECx), and calculation of intra-/interlaboratory variability. Participants submit the raw data to the lead laboratory, who assures its evaluation. If intended, individual calculations performed by participating laboratories can also be submitted. It should be noted that even where the same statistical procedure (e.g. logistic regression) is used, there can be slightly different results depending on the options set for calculation and the software used. Flowcharts of dose-response modelling for estimation of NOEC/LOEC and ECx can be found in ISO/TS 20281, Reference [64] and Reference [68].

For continuous data (e.g. length, weight), the statistical evaluation should be performed according to ISO 5725-2, which describes the analysis of data consistency and outlier detection, as well as the calculation of reproducibility and repeatability. If only one test is available per laboratory, repeatability cannot be calculated according to ISO 5725-2. In this case the repeatability can be calculated according to Annex B. The decision on withdrawing outlier test outcome or laboratories from interlaboratory data analysis should be based on statistical expert opinion or a discussion with the laboratory staff involved in testing. If data do not meet criteria given by ISO 5725-2, robust statistics described in ISO 5725-5 can be more suitable. Statistical procedures for small numbers of participants are given in ISO 13528:2015, Annex D. Results reported as “less than” values are not valid and have to be excluded from the statistical evaluation of test variability.

If data does not comply with a normal distribution [counts (e.g. number of juveniles) and binomial data (e.g. if an organism is alive or dead)], appropriate statistical calculations should be applied for the estimation of standard deviation and to meet the assumptions required by the statistical technique used. In some cases, transformation (e.g. arcsin for percentage data or data in log space) results in normalization of the data, which enables the use of standard statistical procedures. See Annex A for further information. Alternatively, formulas for calculation of variability of data from a given distribution (e.g. Poisson or binomial) can be used. Statistical procedures for evaluation of ecotoxicity data can be found in references ISO/TS 20281, Reference [64], Reference [68] and Reference [69]. It is strongly advised that a statistician be consulted when challenging datasets are submitted.

If estimated, the measurement trueness is expressed as a bias, i.e. a systematic error in terms of a percentage deviation from the true measurement value. Positive values reflect an overestimation and negative values reflect an underestimation of the true value by the measurement method. Under consideration of the variability of the measurement, it can be determined if an observed bias between the average of a large series of test results, for example, from a spiked sample, and the true value is statistically significant (e.g. by analyses of variance and corresponding post hoc tests).

Control data from the method validation round can be used to confirm or adjust, if necessary, the validity criteria.

It should be noted that this guideline cannot cover all types of data obtained using biotests. The lead laboratory is responsible for such evaluation, which characterizes interlaboratory variability.

6 Assessment

A minimum of 6 valid datasets (in accordance with 5.5.2) should be considered to estimate test repeatability and/or reproducibility of the test method. It is acknowledged that different biotests can lead to differing acceptable variation coefficients of reproducibility. Based on the prevalidation step and on the test method optimization outcome, the lead laboratory and participating experts can set the acceptable values for the coefficient of variation (CV). In any case, CV ≤ 30 % is a commonly stated

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