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Accreditation for Microbiological Laboratories

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Accreditation for Microbiological Laboratories

Third edition (2023)

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Accreditation for Microbiological Laboratories

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Foreword

This third edition of the Eurachem Guide "Accreditation for Microbiological Laboratories" is a revision of the second edition published in 2013. The first edition from 2002 was produced by a joint EA/Eurachem Working Group.

The Guide focuses on the requirements of ISO/IEC 17025 [1]; however, the content should also be of use to organisations seeking accreditation or certification against the requirements of standards such as ISO 15189 [2], GLP (Good Laboratory Practice) [3], GMP (Good Manufacturing Practice) [4], and GCP (Good Clinical Practice) [5]). Specific national regulations may override the guidance given in this document. The Guide will also provide useful information for laboratories that wish to establish a quality management system but are not seeking formal recognition.

This revision mainly reflects changes that were introduced with the publication of the 2017 version of ISO/IEC 17025.

Major changes in the third edition are:

- update on recent trends in microbiology, e.g. PCR (polymerase chain reaction) techniques for the detection of microorganisms;
- addition of a list of abbreviations and symbols;
- addition of a section on risk-based thinking;
- updated sections on method verification and validation to reflect current ISO standards;
- references to the use of a decision rule;
- updated Annex A on terminology relevant to microbiology;
- new Annex C on reporting confidence intervals;
- new Annex D on estimation of uncertainty from sampling;
- the order of the sections in adherence with ISO/IEC 17025.

Symbols and abbreviations

The following symbols and abbreviations occur frequently in this guide. Other symbols and abbreviations are defined on first use.

Abbreviations		**Symbols**	
ANOVA	ANalysis Of VAriance	s_{IR}	Relative intralaboratory standard deviation
AFNOR	Association Francaise de NORmalisation/French Standardization Association	s_{QC}	Relative quality control standard deviation
AOAC	International Association of Official Analytical Collaboration	u_c	Combined relative standard uncertainty
CITAC	Cooperation on International Traceability in Analytical Chemistry	u_{conf}	Relative uncertainty due to result from confirmation
CFU	Colony Forming Unit	u_d	Relative distributional or intrinsic uncertainty due to taking a test portion of a laboratory sample
GMO	Genetically Modified Organisms	u_{matrix}	Relative uncertainty from imperfect mixing of the laboratory sample
GUM	Guide to the expression of Uncertainty in Measurement	u_o	Relative operational (technical) uncertainty
IEC	International Electrotechnical Commission	$\hat{u}_o$	One-sided upper confidence limit (UCL) for the estimate of the operational uncertainty
ILAC	International Laboratory Accreditation Cooperation	u_{samp}	Relative sampling uncertainty
ISBN	International Standard Book Number	U	Relative expanded uncertainty
MPN	Most Probable Number	U_{Max}	Upper limit of the uncertainty interval
PCR	Polymerase Chain Reaction	U_{Min}	Lower limit of the uncertainty interval
PT	Proficiency Testing		
VIM	International Vocabulary of Metrology		

Contents

1 Introduction and Scope

Microbiological testing includes sterility testing, detection, isolation, enumeration, and identification of microorganisms (viruses, bacteria, fungi, and protozoa) and their metabolites, or any kind of assay using microorganisms as part of a detection system as well as the use of microorganisms for ecological testing.

According to Reg (EU) 2017/625 for food and feed [6] and Dir (EU) 2020/2184 [7] for water intended for human consumption, laboratories in Member States shall have a quality management system in place, documented according to ISO/IEC 17025[1] and including validated, and wherever possible accredited test methods. Note that also laboratories outside the EU shall fulfil the criteria when providing certificates accompanying food.

This guide provides laboratories carrying out microbiological testing with appropriate information on how to fulfil the requirements of ISO/IEC 17025, giving detailed guidance on such requirements. Although this guide is written primarily for food, water, and environmental microbiological testing, the general principles may be applied to other areas.

2 Standards for accreditation of microbiological laboratories

Detailed references for the standards are given in the Bibliography.

Main Standards used for laboratory accreditation

ISO/IEC 17025 General requirements for the competence of testing and calibration laboratories

ISO 15189 Medical laboratories – Requirements for quality and competence

Basic standards for microbiology

ISO 7218 Microbiology of food and animal feeding stuffs – General requirements for microbiological examinations

ISO 8199 Water quality – General requirements and guidance for microbiological examinations by culture

ISO 19036 Microbiology of the food chain – Estimation of measurement uncertainty for quantitative determinations

ISO 29201 Water Quality – The variability of test results and the uncertainty of measurement of microbiological enumeration methods

ISO 16140 series, Microbiology of the food chain — Method validation

ISO 13843 Water quality — Requirements for establishing performance characteristics of quantitative microbiological methods

ISO 11133 Microbiology of food, animal feed and water — Preparation, production, storage, and performance testing of culture media

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3 Risk-based thinking

The introduction of risk-based thinking is the main change in the philosophy of the standards used for accreditation. ISO/IEC 17025 [1] provides for a broader consideration to be made by the laboratory regarding all aspects in the laboratory system and in its everyday operation.

The risk-based thinking is integrated throughout the whole standard. Reference should be made especially regarding impartiality, statements of conformity, management of non-conforming work and management reviews.

The laboratory is expected to make its considerations based on the correlation of the probability of a risk and its impact. Cases with both high impact and high probability of occurring are given much more emphasis. SWOT analysis (strengths, weaknesses, opportunities, and threats) is a useful tool – see further Eurolab Cookbook No 18 [8].

The laboratory does not need to have detailed risk management based on relevant standards e.g. ISO 31000 [9]; however, it may be useful to be aware of its basic elements. This will help the laboratory to prioritise, based upon the existing experience with nonconformities and previously required preventive actions. It is not necessary to take on board all aspects from the very beginning; initially, the laboratory may include the main aspects with adequate documentation of actions taken and comments. During the next management review, the choices will be reviewed and prompt necessary adjustments.

4 General requirements

4.1 Impartiality

The laboratory management and all personnel should be committed to impartiality; the laboratory should identify risks to its impartiality and demonstrate how it manages in cases when such risks are identified.

4.2 Confidentiality

The laboratory and all personnel should keep as confidential all information obtained or created during the performance of the laboratory activities. Particular care is required with regard to the release of such information. In case this is required by law, relevant provisions shall be followed. In other cases, the customer should be informed in advance.

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5 Personnel

5.1 Competence

Microbiological testing should be either performed or supervised by an experienced person, with a degree level qualification (or equivalent) in microbiology or a related subject. Alternative qualifications may meet requirements where a member of staff has extensive experience relevant to the laboratory's scope of accreditation. Staff should have relevant practical work experience before being allowed to work without supervision, or before being considered as experienced for supervision of accredited work. Specific national regulations may override the guidance given in this document.

5.2 Initial and ongoing competence

The laboratory management should ensure that all personnel has received adequate training for the competent performance of tests and the operation of equipment. This should include training in basic techniques, e.g. plate pouring, counting of colonies, aseptic technique, etc., with acceptability determined using objective criteria. It is important that the competence of laboratory activities is also linked to the knowledge of the evaluation of deviations. In case a method is not in regular use, it may be necessary to verify personnel performance before testing is undertaken with relevant evidence being recorded. The time interval between performance of tests, after which verification of analyst competence would be required, should be established and documented. The interpretation of test results for identification and verification of microorganisms is strongly related to the experience of the performing analyst and should be monitored for each analyst on a regular basis.

A list of competences should be available in the laboratory. Competence of personnel has to be divided into different levels of analysis such as sample pre-treatment, validation of methods, routine performance, reporting of results, quality assurance, competence of working with specific instruments, interpretations etc.

5.3 Monitoring

The competence of personnel to perform tests should be monitored and documented in relation to the results of internal and external quality control (PT). The effectiveness of the training programme and the identification of further training needs should also be evaluated based on these results.

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6 Facilities and environmental conditions

6.1 Premises

There are specific environmental requirements for the testing facilities (see 6.3). Depending on the type of testing being carried out, access to the microbiological laboratory may need to be restricted to authorised personnel. Where such restrictions are in force, personnel should be made aware of:

- the intended use of a particular area;
- the restrictions imposed on working within such areas;
- the reasons for imposing such restrictions;
- the appropriate containment safety levels.

The laboratory should be arranged so as to minimise risks of cross-contamination, where these may be significant to the type of test being performed. The ways to achieve these objectives are, for example:

- to organise the laboratory according to the 'no way back' layout principle for the samples;
- to carry out procedures in a sequential manner using appropriate precautions to ensure test and sample integrity (e.g. use of sealed containers);
- to segregate activities by space (if possible) or, where this is not practical, by time when necessary.

6.2 Safety

According to ISO 7218 [10], the laboratory design shall comply with safety requirements which will depend on the type of microorganism under test.

To this end, microorganisms are classified in four risk categories:

- **Risk category 1** – no or very low risk to the individual and to the community (e.g. non-pathogenic strain of *E. coli*);
- **Risk category 2** – moderate risk to the individual, low risk to the community (e.g. HIV, *Staphylococcus aureus*);
- **Risk category 3** – high risk to the individual, low risk to the community (e.g. yellow fever, West Nile virus, *mycobacterium tuberculosis*, SARS-CoV-2 virus);
- **Risk category 4** – high risk to the individual and to the community (e.g. Ebola virus).

Laboratory facilities are designated as: basic – Biosafety Level 1 (BSL1); basic – Biosafety Level 2 (BSL2); containment – Biosafety Level 3 (BSL3); and maximum containment – Biosafety Level 4 (BSL4). Biosafety level designations are based on a composite of the design features, construction, containment facilities, equipment, practices, and operational procedures required for working with agents from the various risk groups.

The third edition of the WHO biosafety manual [11] and the National Institute of Health (NIH) book "Biosafety in Microbiological and Biomedical Laboratories" [12] provides detailed principles and recommendations in this area. The latter details principles for Clinical Laboratory Biosafety including high-risk pathogen such as Ebola.

For examinations undertaken for the detection of microorganisms belonging to risk categories 1 and 2 it is generally considered good practice to have separate locations, or clearly designated areas, for the following:

- sample receipt and storage;
- sample preparation (e.g. a segregated location should be used for the preparation of powdery products likely to be highly contaminated);
- examination of samples, including incubation;
- maintenance of reference organisms;
- manipulation of presumptive pathogens;
- storage of culture media and reagents;
- media and equipment preparation, including sterilisation;
- sterility assessment;
- decontamination;
- cleaning of glassware and other equipment;
- storage of hazardous chemicals.

The area for washing (after decontamination) may be shared with other parts of the laboratory provided that the necessary precautions are taken to prevent transfer of traces of substances which could adversely affect microbial growth. The need for physical separation should be evaluated on the basis of the activities specific to the laboratory (e.g. number and type of tests carried out).

Laboratory equipment should not routinely be moved between areas to avoid accidental cross-contamination. In the molecular biology laboratory, dedicated pipettes, tips, centrifuges, tubes, adequate protective clothing, vials, heating blocks etc. should be located in each work area i.e. in low-medium-high DNA working environments. Where PCR primers

and probes are prepared, suitable segregation of these tasks should be ensured to minimise DNA cross-contamination. DNA amplification should be conducted in a dedicated section of the laboratory.

Space should be sufficient to allow work areas to be kept clean and tidy. The space required should be appropriate for the volume of analyses handled and the overall internal organisation of the laboratory.

Testing facilities should be appropriately ventilated and at a suitable temperature e.g. 18 - 27 °C. This may be done by natural or forced ventilation, or using an air conditioner. Where air conditioners are used, filters should be appropriate, inspected, maintained, and replaced according to the type of work being carried out.

Consideration should be given to the airflow direction, to minimise the risk of cross-contamination. Air ventilation in the laboratory should be unidirectional and in preference by "cleaning" the maximum air volume of the room; this means air intake devices should be on the opposite side of air outtake devices (and not side by side). This allows for better and healthier air quality for laboratory users.

Contamination may be prevented by addressing the following points:

- use smooth surfaces on walls, ceilings, floors, and benches. Tiles are not recommended as bench covering material;
- use concave joints between the floor, walls, and ceiling;
- whenever possible, use construction materials with low roughness, porosity, water or humidity absorption that are easy to clean and disinfect;
- place sunshades on the outside of windows. If this is not possible, ensure easy access for cleaning of internal sunshades;
- install fluid conveying pipes under bench technical areas;
- use a dust-filtered air inlet for the ventilation system;
- provide separate hand-washing arrangements, preferably non-manually controlled;
- install cupboards with a sloped ceiling on top for ease of cleaning (typically stainless steel or solid grade laminate although other materials that are easy to clean and disinfect may be considered);
- avoid rough and bare wood; ensure wooden surfaces of fixtures and fittings adequately sealed;
- arrange stored items and equipment to facilitate easy cleaning;
- ensure that the testing facilities do not contain furniture, documents, or other items except those strictly necessary for testing activities;
- ensure windows and doors can be closed when conducting the tests to minimise draughts. The ambient temperature (18 °C to 27 °C) and air quality (microorganism content, dust spreading rate, etc.) should be controlled;
- install an adequate extraction to prevent exposure to dust arising from the handling of dehydrated culture media and dusty or powdered samples;
- when tests are to be conducted in a low-contamination atmosphere, the room should be specially equipped with a clean laminar airflow cabinet and/or a safety cabinet.

This list is not exhaustive, and not all examples will apply in every situation. Ceilings, ideally, should have a smooth surface with flush lighting. When this is not possible (as with suspended ceilings and hanging lights), the laboratory should have documented evidence that they are controlled and that effective means of overcoming them are in place, e.g. a surface-cleaning and inspection programme.

Where laboratories are operating on manufacturing premises, personnel must be aware of the potential for contamination of production areas and the laboratory should demonstrate that appropriate measures have been taken to avoid any such occurrence.

In the molecular biology laboratory PCR is a sensitive detection method. Aerosols, dust, and other particles are carriers of contaminating DNA. It is therefore essential to separate in space and/or time the different stages of the analysis. In particular, provide separate dedicated areas, materials, and equipment for pre- and post-amplification stages. PCR biosafety cabinets can also be used to prepare all PCR reactions and to ensure that limited environmental and sample contamination is observed. Special care should be addressed to the DNA extraction room (before PCR) in relation to Biosafety according to WHO BSL guidelines [11].

Negative pressurised rooms or equipment are required for manipulation of Risk category 3 organisms. For Risk category 4 organisms, other means of protection should be considered.

6.3 Environmental monitoring

An appropriate programme should be devised to monitor, control, and record environmental conditions. As an example, this programme may include frequent use of air settlement plates for bacterial and fungal contaminants, (ISO 14698 [13]

and, EN 17141 [14]), as well as periodic surface swabbing (ISO 18593 [15]) for a variety of relevant microorganisms. Acceptable background counts should be assigned and there should be a documented procedure for dealing with situations in which these limits are exceeded. Analysis of data should enable trends in levels of contamination to be determined.

If room pressure and the number of air changes per hour are critical parameters for biosafety requirements or for air cleanliness (free from particles), means for monitoring and control should be provided. Measures should be taken to avoid the accumulation of dust. There should be a documented cleaning programme for the laboratory. It should take into account the results of environmental monitoring and the possibility of cross-contamination. There should be a procedure for dealing with spillages.

Protective clothing appropriate to the type of testing being performed (including, if necessary, protection for hair, beard, hands, shoes, etc.) should be worn in the microbiological laboratory and removed before leaving the area. This is particularly important in the molecular biology laboratory, where for example, movements from a high DNA load area to a low DNA load area may introduce cross-contamination.

6.4 Disposal of contaminated waste

Procedures for the disposal of contaminated materials should be designed to minimise the possibility of contaminating the test environment.

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7 Equipment

7.1 General

Equipment should be clearly identified and relevant details recorded appropriately, e.g. in data sheets. Prescriptions for maintenance and, where required, calibration, as well as the frequency should be indicated. Criteria to be fulfilled after maintenance, and, for equipment requiring calibration, acceptance/rejection criteria for the calibration should be defined and recorded. Records of such operations as well as of any event requiring repair of the equipment should be maintained. The Eurachem/CITAC Guide "Guide to Quality in Analytical Chemistry" [16] describes in more detail instrument qualification and the general requirements of ISO/IEC 17025 [1] for the management of equipment.

7.2 Maintenance

Maintenance of essential equipment should be conducted at specified intervals as determined by factors such as the frequency of use and expected risk of changes in performance outside acceptable limits (risk analysis). The frequency of maintenance should be established and recorded. Examples of maintenance of equipment and intervals are given in Annex G. Attention should be paid to the avoidance of cross-contamination arising from equipment used, for example:

- equipment should be clean and, when appropriate, sterile;
- ideally, laboratories should have a separate autoclave for decontamination. However, one autoclave is acceptable provided that adequate precautions are taken to separate decontamination and sterilisation loads. A documented cleaning programme should be in place to address both the internal and external environment of the autoclave.

Typically, the following items of equipment will be maintained by cleaning and servicing, inspecting for damage, general verification of suitability, and, where relevant, sterilising:

- general service equipment – filtration apparatus, glass or plastic containers (bottles, test tubes), glass or plastic Petri dishes, sampling instruments, wires or loops (platinum, nickel/chromium or disposable plastic);
- water baths, incubators, microbiological cabinets (laminar flow and safety cabinets), autoclaves, homogenisers, fridges, freezers;
- volumetric equipment – pipettes, automatic dispensers, spiral platers;
- measuring instruments – thermometers, hygrometers, CO_2 sensors, timers, balances, pH meters, colony counters.

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8 Reagents and culture media

8.1 Reagents

Laboratories should ensure that the quality of reagents used is appropriate for the test. They should verify the suitability of each batch of reagents critical for the test before use and during its shelf-life, using positive and negative control organisms that are traceable to recognised national or international culture collections. Commercially produced reagents must be stored and used according to the manufacturer's instructions.

8.2 In-house prepared media

The suitable performance of culture media, diluents, and other suspension fluids prepared in-house must be checked, where relevant, regarding:

- recovery or survival maintenance of target organisms;
- inhibition or suppression of non-target organisms;
- biochemical (differential and diagnostic) properties;
- physical properties (e.g., pH, volume, and sterility).

All relevant procedures are described in ISO 11133 [17]. If the laboratory uses standard published methods (e.g., ISO Standards), additional information for the performance testing of specified culture media may be found in the method documentation.

Raw materials, commercial dehydrated formulations and individual constituents should be stored under appropriate conditions. All containers, especially those for dehydrated media, should be sealed tightly. Dehydrated media that are caked or cracked, or show a colour change, should not be used. Distilled, deionised, or reverse osmosis water, free from bactericidal, inhibitory, or interfering substances, should be used for preparation unless the test method specifies otherwise.

The shelf-life of prepared media under defined storage conditions should be determined and verified. Unless otherwise specified in the method, ready-to-use culture media should be stored in a refrigerator at 5 °C ± 3 °C.

8.3 Ready-to-use-media

All media, including diluents and other suspension fluids, obtained ready-to-use or partially complete, require performance evaluation before use. Evaluation of performance in recovery or survival of target organisms and the inhibition or suppression of non-target organisms should be fully documented.

Attributes such as physical and biochemical properties should be evaluated using objective criteria.

Where the manufacturer of media purchased ready-to-use or partially complete is covered by a recognised quality management system (see ISO 9000 [18]), and the media are quality controlled according to ISO 11133 [17], relevant information needs to be reviewed for acceptability.

Checks by the user laboratory may only involve initial tests for every new manufacturer and indirect tests through internal quality control procedures. In other circumstances, quality control must be fully performed according to ISO 11133.

The laboratory needs to have adequate knowledge of the manufacturer's product specifications, which include at least the following:

- name of the media and list of components, including any supplements;
- shelf-life and the acceptability criteria applied;
- storage conditions;
- sterility check;
- check of growth of target and non-target control organisms used (with their culture collection references) and acceptability criteria;
- physical checks and the acceptability criteria applied;
- date of issue of specification.

Batches* of media should be identifiable. Each batch received should be accompanied by evidence that it meets the quality specification. The laboratory should ensure that the manufacturer will inform of any changes to the specification.

*Batch is a homogeneous and fully traceable unit of a culture medium or reagent referring to a defined amount of bulk, semi-finished product, or end-product, which is consistent in type and quality, and which has passed the requirement of production (in-process control) and performance testing and which has been produced within one defined production period, having been assigned the same number.

8.4 Labelling

Laboratories should ensure that all reagents including stock solutions, media, diluents, and other suspending fluids, are adequately labelled to indicate identity, concentration, storage conditions, date of opening, preparation date, validated expiry date and/or recommended storage period. The person responsible for the preparation of the reagent should be identifiable from records.

8.5 Reference materials

Reference materials provide essential traceability in measurements and are used, for example to:

- demonstrate the accuracy of results;
- calibrate equipment;
- monitor laboratory performance;
- validate methods;
- enable comparison of methods;
- demonstrate the quality of culture media;
- demonstrate the consistent performance of kits.

When appropriate and possible reference materials should be used in appropriate matrices.

It is recommended that reference materials are supplied from producers who are accredited against ISO 17034 [19].

8.6 Reference cultures

Reference cultures (see Annex B) are required for establishing the acceptable performance of media (including test kits), validating methods, and for assessing/evaluating ongoing performance. To demonstrate traceability, laboratories should use reference strains of microorganisms obtained directly from a recognised national or international collection, where these exist. Where traceable reference cultures are not readily available, commercial derivatives traceable to them could alternatively be used, provided that the relevant properties for the intended use have been shown by the laboratory to be equivalent.

Reference strains may be sub-cultured once to provide reference stocks. Purity and biochemical checks should be carried out in parallel as appropriate. It is recommended to store reference stocks in aliquots either deep-frozen or lyophilised. Working cultures for routine use should be primary subcultures from the reference stock (see Appendix B on the preparation of working stocks). If reference stocks have been thawed, they must not be re-frozen and re-used.

Working cultures should not be sub-cultured unless it is required and defined by a standard method or laboratories can provide documented evidence that there has been no change in any relevant property. Working stocks should not be sub-cultured to replace reference stocks. Commercial derivatives of reference strains may only be used as working cultures.

9 Metrological traceability

9.1 Calibration and verification

The laboratory should establish a programme for the calibration and verification of properties of measuring systems which directly influence the measurement results according to ISO/IEC 17025 [1]. The frequency of such calibration and verification will be justified by experience and risk analysis based on e.g. need, type and previous performance of the equipment. Further guidance about metrological traceability can be found in the relevant Eurachem Guide [20].

Whenever possible and appropriate, calibration should be performed by accredited calibration laboratories. In-house calibrations are acceptable within the provisions of ILAC P10 [21] together with policies of the national accreditation body.

The performance of the calibrated equipment should be verified before use. In addition to the scheduled verification plans, equipment should be verified against the initial values after each significant repair or modification and, if relevant, change of location. A check of equipment performance may also be part of the root cause investigation following a quality control failure.

Examples of calibration and verification intervals and typical performance checks for various laboratory instruments are given in Annex E and F respectively.

9.2 Temperature measurement devices

Temperature measuring devices (for example thermocouples and platinum resistance thermometers (PRTs) used in incubators and autoclaves) should be of an appropriate quality to achieve the accuracy required. For health and safety reasons, mercury and toluene liquid-in-glass thermometers are prohibited. The use of temperature data loggers are recommended.

Such temperature loggers may, for example, be used for monitoring storage fridges as well as freezers and incubators and water baths where acceptable tolerance around the target temperature is assured.

9.3 Incubators, water baths, ovens, freezers, and refrigerators

The stability of temperature and uniformity of temperature distribution (homogeneity) should be verified taking into account the time required to achieve equilibrium conditions in incubators, water baths, ovens and temperature-controlled rooms. Temperature stability should be verified initially, and after any repair or modification [10] regarding for example position, space between, and height of, stacks of Petri dishes ISO 7218 [10].

In atmosphere-controlled equipment (e.g. incubators) humidity and gas (mostly CO_2) content are also controlled.

Laboratories should monitor daily, or according to usage, the operation of this type of equipment and retain records.

9.4 Autoclaves, including media preparators

Autoclaves should be capable of meeting specified time, pressure and temperature tolerances. Pressure cookers fitted only with a pressure gauge are not acceptable. Sensors used for controlling or monitoring operating cycles require calibration and the performance of timers should be verified.

Based on experience, temperature sensors may be positioned within the load (e.g. in containers filled with liquid/medium) to enable location differences to be demonstrated. In the case of media preparators, where uniform heating cannot be demonstrated by other means, using two sensors, one adjacent to the control probe and one remote, would generally be considered appropriate.

Temperature monitoring may be achieved by one of the following:

- continuously using an online thermocouple;
- direct observation when in use.

In addition to directly monitoring the temperature of an autoclave, the effectiveness of its operation during a cycle may be checked by the use of chemical or biological indicators for sterilisation/decontamination.

9.5 Weights and balances

Weights and balances should be calibrated and verified at regular intervals (according to their intended use) or following any repairs or changes of location).

9.6 Volumetric equipment

Calibrations of volumetric equipment * can be performed using gravimetric methods. The bias of the delivered volume against the set volume (for several different settings in the case of variable volume instruments) should be checked. The precision of the repeated deliveries should be determined. Volumetric equipment such as automatic dispensers, dispensers/diluters, micropipettes, and disposable pipettes may all be used in the microbiology laboratory.

Verification should not be necessary for glassware that has been certified to a specific tolerance.

For 'single-use' disposable volumetric equipment, pipettes, flasks, etc., laboratories should obtain supplies from trusted companies. After initial validation of the suitability of the equipment, it is recommended that random checks are conducted.

9.7 Thermal cyclers (PCR)

Laboratories should verify temperature, ramp rate, overshoots/undershoots, and hold time. Quantitative testing of materials and items processed by thermal cyclers may also provide equivalent proof of satisfactory equipment performance.

9.8 Other equipment

Equipment such as conductivity meters, hygrometers, centrifuges, oxygen meters, pH meters, CO_2 sensors and microscopes should be verified regularly or before each use.

* The requirements for a pipette are normally set by the laboratory. ISO 8655-1 [22] e.g. recommends for a volume of 0.1 to 5 ml for piston pipettes: maximum bias of 0.8 % and maximum standard deviation of 0.3 %.

10 Selection, verification, and validation of methods

10.1 General

The validation/verification of microbiological test methods should reflect actual test conditions. This may be achieved by using naturally contaminated samples or samples spiked with a predetermined level of material (microorganism). The analyst should be aware that the spiked samples only mimic superficially the presence of the naturally occurring microorganisms. The extent of validation/ verification should cover the scope of the method.

In contrast to quantitative chemical methods, the verification and validation of quantitative microbiological methods include the determination of true and false positives and negatives, similar to the validation of qualitative methods [23]. From the results obtained, the following characteristics can be calculated: sensitivity, specificity, false positive and false negative rate, selectivity, and efficiency.

The following standards can assist laboratories in method validation and verification:

- Water matrix ISO 13843 [24], ISO/TS 12869 [25] and ISO 29201 [26];
- Food matrix ISO 7218 [10], ISO 16140 [27 – 32], ISO 17468 [33], ISO 19036 [34] and.

10.2 Selection of methods

The laboratory should use appropriate test methods to meet the specific needs and it is preferable to use standard methods, such as those published by e.g. ISO or ASTM. These methods are normally validated prior to publication as a standard method. In these cases, validation by the laboratory is not necessary. However, the laboratory needs to verify the performance of the method as detailed in ISO/IEC 17025 Clause 7.2.1.5 [1].

Non-standard methods could be used provided that they are validated by the laboratory according to ISO/IEC 17025 Clause 7.2.2.1. This refers to laboratory-developed methods, standard methods used outside their intended scope and methods described in the scientific literature or provided by manufacturers. The detailed procedure to be followed in each case varies with the nature of the method, i.e. qualitative, semi-quantitative and quantitative.

10.3 Verification

10.3.1 General

In the case of standard methods, that have been validated prior to publication, the laboratory is required to prove that it can implement them in a reliable way. The performance obtained in the verification is compared to the performance characteristics reported from the validation study. There are several standards and a procedure form NMKL describing verification and a short overview is given below, with examples from ISO standards; for details on implementation and acceptance criteria see the specific standard.

Example 1

Verification of a *quantitative method* for a water matrix according to ISO 13843 [24].

In order to verify the method the laboratory should:

- spike a minimum of five samples and determine the following performance characteristics; sensitivity, specificity, efficiency, selectivity, false positive and false negative rates;
- analyse a minimum of three samples (different sources and levels of target organisms) under repeatability conditions to obtain a set of 10 replicates per sample to determine repeatability;
- perform repeated counting of the same plate or positive tubes for MPN (Most Probable Number), to determine the uncertainty of counting (30 plates or tubes, preferably but not necessary from different samples).

For acceptance criteria for the verification, see the ISO method being used and ISO 13843.

Example 2

Verification of a *quantitative* method for a food matrix according to Table 2 ISO 16140-3 [32].

The verification of a method is undertaken in two parts:

- analyse a minimum of 10 duplicates, at various contamination levels, of a selected food matrix, which was tested in the validation study of the standard, under variable test conditions in order to determine the operational uncertainty (technical uncertainty), u_o;
- analyse artificially contaminated food items test samples in duplicate, at three levels of contamination and determine the bias – the

absolute difference in results between artificially contaminated food test samples and the mean value of the inoculum suspension. Choose an appropriate food item matrix for each food category within the scope of the accreditation.

For acceptance criteria for the verification, see the ISO method being used and ISO 16140-3 [32].

10.3.1 Verification of molecular methods

As far as the molecular methods are concerned, ISO 20836 [36], ISO 20837 [37], ISO 20838 [38] and ISO 22119 [39] provide the framework, including requirements and performance characteristics, for the various steps to perform analysis in this area, from DNA extraction and amplification to standardised thermocyclers' performance.

10.4 Validation

10.4.1 General

For quantitative microbiological test methods, the following parameters should be considered and, if appropriate, quantitatively determined: sensitivity, specificity, false positive and false negative rates, selectivity, efficiency, repeatability, intralaboratory reproducibility, the uncertainty of counting, and the limit of determination. The differences due to the matrices must be taken into account by testing different types of samples.

Qualitative microbiological test methods, where the result is expressed as detected/not detected and confirmation/identification procedures are used, should be validated by determining, if appropriate: sensitivity, specificity, false positive and false negative rates, selectivity, matrix effect, limit of detection.

10.4.2 Commercial test kits

Laboratories should retain validation data on commercial test systems (kits) used in the laboratory. This validation data may be obtained through collaborative testing and from validation data submitted by the manufacturers and subjected to third party evaluation e.g. AFNOR, NordVal, Microval, AOAC. The laboratory needs to verify test kits. If validation data are not available, or not wholly applicable, the laboratory should be responsible for completing the validation of the method.

11 Sampling and handling of test items

11.1 Sampling

It is strongly recommended that sampling be covered by quality assurance and ideally by accreditation. The following standards can assist laboratories in sampling and subsampling: ISO 19458 [40], ISO 6887 [41 – 45], ISO 7218 [10].

Sampling is performed by the analytical laboratory or as a stand-alone activity. In any case is should be based on a sampling plan and performed by trained personnel.

Sampling must be carried out aseptically using sterile equipment. If relevant, environmental conditions, for instance, air contamination and temperature, should be monitored and recorded at the sampling site. The time of sampling should be recorded.

Transport and storage should be under conditions that minimise any alteration of its microbial flora e.g. chilled or frozen where appropriate. The conditions should be monitored, and records kept. Where appropriate, responsibility for transport and storage, between sampling and arrival at the testing laboratory, should be clearly documented.

Testing of the samples should be performed as soon as possible after sampling.

11.2 Handling of test items

Microbial flora may be sensitive to factors such as temperature or duration of storage and transport, so it is important to check and record the condition of the sample on receipt by the laboratory.

The laboratory should have procedures that cover the delivery of samples and sample identification. If there is insufficient sample or the sample is in poor condition e.g. due to physical deterioration, incorrect temperature, damaged packaging, or deficient labelling, the laboratory should consult with the customer before deciding whether to test or refuse the sample.

The laboratory should record all relevant information, in particular the following:

- date and, where relevant, the time of receipt;
- condition of the sample on receipt and, when necessary, temperature;
- characteristics of the sampling operation (sampling date, sampling conditions, etc.);
- sampling plan/procedure/protocol applied, and any deviations that were made from it.

Samples awaiting test should be stored under suitable conditions to minimise changes to any microbial population present. Storage conditions should be defined and recorded.

The package of samples may be highly contaminated and should be handled and stored with care to avoid any spread of contamination.

Sub-sampling by the laboratory immediately prior to testing is part of the test method. It should be performed according to national or international standards, where they exist, or by validated in-house methods. Sub-sampling procedures should be designed to take account of the uneven distribution of microorganisms (general guidance given in ISO 6887 [41 – 45] and ISO 7218 [10]).

A procedure for the retention and disposal of samples should be written. Samples should be stored until the test results are obtained or longer, if required and if applicable, based on legislative requirements or customer's request. Laboratory sample portions that are known to be highly contaminated should be decontaminated prior to being discarded.

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12 Evaluation of measurement uncertainty

12.1 General

According to the 'International Vocabulary of Metrology – Basic and General Concepts and Associated Terms' (VIM 3) [46], a measurement result compromises a measured quantity value, the unit, and the measurement uncertainty.

The general concept is treated in the Eurachem Guide on uncertainty [47]. ISO/IEC 17025 [1] specifies detailed requirements concerning the estimation of measurement uncertainty and how it should be stated in test reports; laboratories should identify the contributions to measurement uncertainty and when estimating measurement uncertainty, all contributions that are of significance, must be taken into account, including those arising from sampling. It is also recognised that the nature of the test method can preclude rigorous estimation of measurement uncertainty; in such cases, an estimation should be made based on an understanding of the theoretical principles or practical experience of the performance of the method.

12.2 Measurement uncertainty in microbiology

The general approaches to estimating and expressing measurement uncertainty in microbiological testing, based on ISO 19036 [34] for food and ISO 29201 [26] for water, consider distinct types of uncertainty components: 1) distributional uncertainty associated with the random distribution of microorganisms, 2) technical/operational uncertainty that arises from impact of variability associated with the technical steps in the analytical procedure, 3) confirmation uncertainty and 4) matrix uncertainty.

The ISO 19036 standard is applicable to the quantitative analysis of products intended for human consumption or the feeding of animals; environmental samples in food production and food handling and samples at the stage of primary production. It is applicable to quantitative analyses using colony-count technique, MPN techniques, instrumental methods, such as impediometry, analysis for adenosine triphosphate (ATP) as well as flow cytometry and molecular methods, such as those based on the polymerase chain reaction (PCR).

ISO 29201 covers both enumeration colony counts and MPN methods and presents two different approaches to uncertainty estimation (component approach and global approach) that can be used for different matrices. Overview of the uncertainty estimations from the uncertainty components and an introduction to reporting asymmetric confidence intervals based on relative/log uncertainty are introduced in Annex C of this Guide. Also the issue of obtaining negative results when estimating operational uncertainty from duplicates is treated in the last section of Annex C.

A practical approach with several examples for estimating and expressing measurement uncertainty is given by the American Association for Laboratory Accreditation (A2LA) [48].

12.3 Uncertainty components

It is generally appropriate to base the estimate of measurement uncertainty on repeatability and intermediate precision (intra-laboratory reproducibility) data. The individual uncertainty components should be identified and demonstrated to be under control and their contribution to the variability of results evaluated. Some uncertainty components e.g. pipetting, weighing, dilution effects and incubator effects may be readily measured and easily estimated to demonstrate a negligible contribution to the overall measurement uncertainty. Other components e.g. sample stability and sample preparation cannot be measured directly. Although their contribution cannot be estimated in a statistical manner their importance to the variability of results should also be considered.

12.4 Distribution of microorganisms within matrices

It is expected that accredited microbiological testing laboratories will have a good understanding of the distributions of organisms within the matrices they test and will take this into account when sub-sampling by following good laboratory practices and/or regulatory requirements where applicable. However, it is not always practical to include this component in uncertainty estimates unless the customer's needs dictate otherwise. The principal reasons for this are:

- the uncertainty due to the distribution of organisms within the product matrix is not a function of the laboratory's performance and may be unique to individual samples tested;
- test methods should specify the sample size to be used taking into account possible poor homogeneity.

12.5 Qualitative tests

The concept of measurement uncertainty cannot be applied directly to qualitative test results such as those from detection tests or the determination of attributes for identification. Nevertheless, individual sources of variability, e.g. consistency of reagent performance and analyst interpretation, should be identified and demonstrated to be under control. Additionally, for tests where the limit of detection is an important indication of suitability, the measurement uncertainty associated with the inocula used to determine the limit should be estimated and its significance evaluated.

For quantitative measurements where the final results are expressed in a qualitative way (e.g. pass/fail), estimation of measurement uncertainty is still applicable' according to ILAC G17 [49].

12.6 Molecular methods

In the case of detection and quantification of genetically modified organisms (GMOs), measurement uncertainty is estimated according to JRC/IRMM Guidance EUR 22756 EN [50] and is further discussed in Annex C of this Guide.

12.7 Sampling uncertainty

It has become increasingly apparent that sampling is often the more important contribution to uncertainty and requires equally careful management and control. Sampling uncertainty can be estimated using duplicates as outlined in Annex D. It is recommended that contaminated samples at higher CFU be used. If the sampling uncertainty is low, it may not be possible to estimate it, and only an upper confidence limit for the sampling uncertainty may be calculated and quoted.

13 Ensuring the validity of results

13.1 Internal quality control

Internal quality control consists of all the procedures undertaken by a laboratory for the continuous evaluation of its work thus monitoring the validity of results. The main objective is to ensure the consistency of results day-to-day and their conformity with defined criteria. Guidance in ISO 8199 [51] and Nordtest 569 [52] can assist laboratories in setting up quality control.

The internal quality control programme should be planned and periodically reviewed to maintain the control of all the tests in the laboratory's scope of accreditation. The programme may include (among others) the use of:

- reference materials and quality control materials;
- functional checks of measuring and testing equipment;
- natural/spiked samples;
- blind sample(s).

The programme may also carry out:

- replicate tests;
- replicate evaluation of test results i.e. counting of colonies in petri dishes by two analysts;
- review of reported results.

For molecular methods, positive and negative and internal and external amplification controls should be used. The false positive and the false negative rates should also be documented according to ISO 22118 [53].

The internal quality control program must be adapted to the actual frequency of tests performed by the laboratory. For the daily interpretation of the control values statistical rules should be applied [51, 52]. A plot of the control values should be performed to assist in the evaluation of trends in a visual manner.

In cases a laboratory is accredited for a test which is rarely performed, a more intensive quality control should be carried out in parallel with the testing.

In any case, the laboratory should be aware of the inherent risk associated with such an approach and take all appropriate precautionary measures.

13.2 Proficiency testing

Laboratories should regularly participate in interlaboratory comparisons such as proficiency testing (PT) or external quality assessment (EQA), relevant to their scope of accreditation. Preference should be given to PT testing schemes which use appropriate matrices. ISO 22117 [54] specifies requirements and gives guidance for the organisation of PT schemes.

Participation in PT schemes is mandatory, provided that appropriate schemes are available. If this is not the case, the laboratory should participate in interlaboratory comparisons organised by a sufficient number of other laboratories on the basis of a well-documented protocol.

Although Accreditation Bodies may specify minimum participation in PT schemes, it is the responsibility of the laboratory to demonstrate that the frequency and extent of the participation is appropriate for the scope of their activities. EA-4/18 [55] may give useful support with the use of sub-disciplines, i.e. an area of technical competence defined by a minimum of one measurement technique, property and product, which are related. This facilitates the optimisation of the extent of participation in PT. Further to this, the Eurachem Guide on selection, use and interpretation of PT schemes [56] may help in the interpretation of the results from PT participation.

Laboratories are encouraged to subscribe to PT schemes accredited against ISO/IEC 17043 [57]. Other providers should only be used where the laboratory has assessed their competency based on sufficient criteria.

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14 Reporting of results

14.1 General

Reporting of results follows general and specific requirements and should include information that is necessary for the interpretation of the results.

14.2 Quantitative methods

Quantitative microbiological methods are based on counting microbial particles either directly with the aid of a microscope or indirectly based on growth (multiplication) into colonies, turbidity, a colour change, or fluorescence. With quantitative methods, results are expressed as the number of colony forming units (CFU) or as the most probable number (MPN) per volume or mass of sample. In the case of sampling from a surface, results are reported in CFU/cm^2. Results from a device with an unmeasurable surface are reported in CFU/device.

Below the limit of determination, which is 10 CFU [51], the relative standard deviation increases significantly. Below the detection level, which is on average 3 cells per volume of the material tested, the probability of positive results falls below 95 %.

Laboratories are advised to reflect this on their test reports but the recommended expression of results may differ under different standards. Table 1 presents indications on reporting results for water matrices according to ISO 8199 [51] and for food matrices according to ISO 7218 [10]. As per the customer's request and/or the legislation, measurement uncertainty for results ≥ 10 CFU should be stated in the report.

The detection level of MPN methods can be reasoned in the same way as for colony methods and has the same value of 3 particles per volume of the material tested according to ISO 13843 [24]. For very low concentrations, the distributional uncertainty associated with the random distribution of microorganisms is assumed to prevail in all suspensions of the MPN systems. In this respect, the approach presented in Table 1 may also be applicable to MPN systems.

When the expanded uncertainty is over 30 – 40 % it is recommended that *asymmetric* confidence intervals be stated instead of the expanded uncertainty for the count result in % or log units. In Annex C recommendation is given on how to report the confidence interval for the CFU and MPN methods based on the calculated measurement uncertainty. Any limitations in the estimate of uncertainty should be made clear to the customer e.g. if only the distributional uncertainty is included.

Table 1 – Expression of results in CFU/ml or per analytical test portion

Counted colonies	**Reporting of results**	
	ISO 8199 [51]	**ISO 7218 [10]**
0	Not detected or < 1	< 1
1-2	Microorganisms are present	Microorganisms present but < 4
3	Report results as an estimate	Microorganisms present but < 4
4 - 9	Report results as an estimate	Report results as an estimate
≥ 10	Report results	Report results
NOTE 1 Legislation may require different ways of reporting. NOTE 2 The volume of the inoculum/dish and the eventual dilution must be considered, e.g. 3 CFU obtained in a food sample diluted 10 times (inoculum=1 mg/dish) will be reported as: microorganisms present but < 40 CFU.		

14.3 Qualitative methods

Qualitative test results should be reported as "detected/not detected in a defined quantity of sample tested". They may also be expressed as "less than a specified number of organisms" where the specified number of organisms exceeds the detection limit of the method and this has been agreed to by the customer.

14.4 Reporting a statement of conformity – Use of a decision rule

In cases where the specification or the relevant standard does not refer to a decision rule, the laboratory should, when a conformity statement is required, document and apply the decision rule used; this rule should take into account the level of risk and be communicated and agreed with the customer – see ILAC G8 [58] as well as the relevant Eurachem Guide [59] and leaflet [60].

14.5 Reporting opinions and interpretations

If the laboratory provides opinions and interpretations of test results in reports, this should be done by authorised personnel with appropriate experience and knowledge of the specific application, as well as of legislative and technological requirements. Such opinions and interpretations should be based on the results obtained only from the tested item.

14.6 Control of data and Information management

According to ISO/IEC 17025 [1] "laboratory information management system(s)" includes the management of data and information contained in both computerised and non-computerised systems. The standards specify requirements regarding the access of the laboratory to the data and information needed to perform its activities. Furthermore, requirements are set for the collection, processing, recording, storage, and retrieval of data. All such activities should be validated for their functionality and operate within a described framework.

Annex A – Glossary of terms

Terminology for chemical, biological and clinical measurements is presented in the Eurachem Guide on terminology [61]. Below is presented terminology specific for microbiological measurements.

Limit of detection	Applied to qualitative microbiological tests: The lowest number of microorganisms that can be detected, but in numbers that cannot be estimated accurately. See also the definition in ISO 16140-1 [28].
Detection level	Minimum concentration of organisms that produce evidence of growth with a probability of P = 0.95 when inoculated into a specified culture medium and incubated under defined conditions (ISO 16140-1 [28] and ISO 13843 [24]). Note 1: The theoretical level that conforms to this definition is an average of three viable cells in an inoculum volume.
Intralaboratory reproducibility (intermediate precision)	Closeness of agreement between test results obtained with the same method on the same or similar test materials in the same laboratory with different operators using different equipment (ISO 8199 [51]). Symbol used is $s_{IR.}$
Limit of determination	Lowest analyte concentration per analytical portion where the expected relative standard uncertainty, equals a specified value (ISO 13843 [24]). NOTE: In Eurachem guidance LOQ, limit of quantification, is also used.
Negative deviation	Occurs when the alternative method gives a negative result without confirmation when the reference method gives a positive result. This deviation becomes a false negative result when the true result can be proved as being positive.
Positive deviation	Occurs when the alternative method gives a positive result without confirmation when the reference method gives a negative result. This deviation becomes a false positive result when the true result can be proved as being negative.
Reference cultures	Collective term for reference strain, reference stocks and working cultures.
Reference material	Material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in measurement or in examination of nominal properties. (VIM [46]).
Reference stock	Set of distinct identical cultures obtained from a subculture of the reference strain prepared in the laboratory or obtained from a supplier (ISO 11133 [17]).
Reference strain	Microorganisms obtained directly from a reference culture collection, i.e. a collection of cultures that is a member of the World Federation of Culture Collections (WFCC) or the European Culture Collections Organisation (ECCO), defined at least to the genus and species level, classified and described according to its characteristics and preferably derived from food, animal feed products, the food or feed production environment or water as applied (ISO 11133 [17]).
Relative recovery	Efficiency with which a method recovers target organisms from a sample when compared to another procedure. This comparison should be done where an alternative method for the same organism exists. Comparison with an ISO reference method is preferred (ISO 13843 [24], ISO 17994 [62]).
Sensitivity	The fraction of the total number of positive cultures or colonies correctly assigned in the presumptive inspection (ISO 13843 [24]).
Specificity	The fraction of the total number of negative cultures or colonies correctly assigned in the presumptive inspection (ISO 13843 [24]).

Validation	Verification, where the specified requirements are adequate for an intended use (VIM [46]). Primary validation. An exploratory process with the aim of establishing the operational limits and performance characteristics of a new, modified or otherwise inadequately characterised method. It should result in numerical and descriptive specifications for the performance and include a detailed and unambiguous description on the target of interest (positive colony, tube or plaque) (ISO 13843 [24]). Characterisation – the study of parameters that can be measured to describe how the method is likely to perform in a given set of conditions, which can be described as performance characteristics. It is an exploratory process with the aim of establishing the likely set of performance characteristics of a new, modified or otherwise inadequately characterised method. It should result in numerical and descriptive specifications for the performance and include a detailed and unambiguous description of the target of interest (such as positive colony, tube or plaque). However, the values generated should not be used as limits since they may change depending on the laboratory, matrix or even specific samples (ISO 13843). Characterisation is performed by a single laboratory in the first instance to determine the likely performance of a test method in a specific laboratory. A collaborative method performance study can be performed as an additional step to evaluate the interlaboratory performance characteristics.
Verification	Provision of objective evidence that a given item fulfils specified requirements (VIM [46]). NOTE: Verification (secondary validation) takes place when a laboratory proceeds to implement a method developed elsewhere. Verification focuses on gathering evidence that the laboratory can meet the specifications established in primary validation (adopted from (ISO 13843 [24]). Performance of a second characterisation by a different laboratory to confirm the results of the original characterisation (ISO 13843). Verification takes place when a laboratory proceeds to implement a method developed elsewhere. Verification focuses on gathering evidence that the laboratory can generate performance data similar to those established in primary characterisation. It is not helpful to establish limits on the various components of method characterisation since these can vary dependent on many aspects of the method, type of sample and performing laboratory. The verification data should be used to establish the type and quality of data likely to be generated by the laboratory with a given procedure and any given sample type. Typically, verification uses selected and simplified forms of the same procedures used in method characterisation, but possibly extended over a longer time.
Working culture	A primary sub-culture from a reference stock (ISO 13843 [24]). NOTE: Subculture from a reference stock, a primary culture or a reference material, whether certified or not.

Annex B – Reference cultures

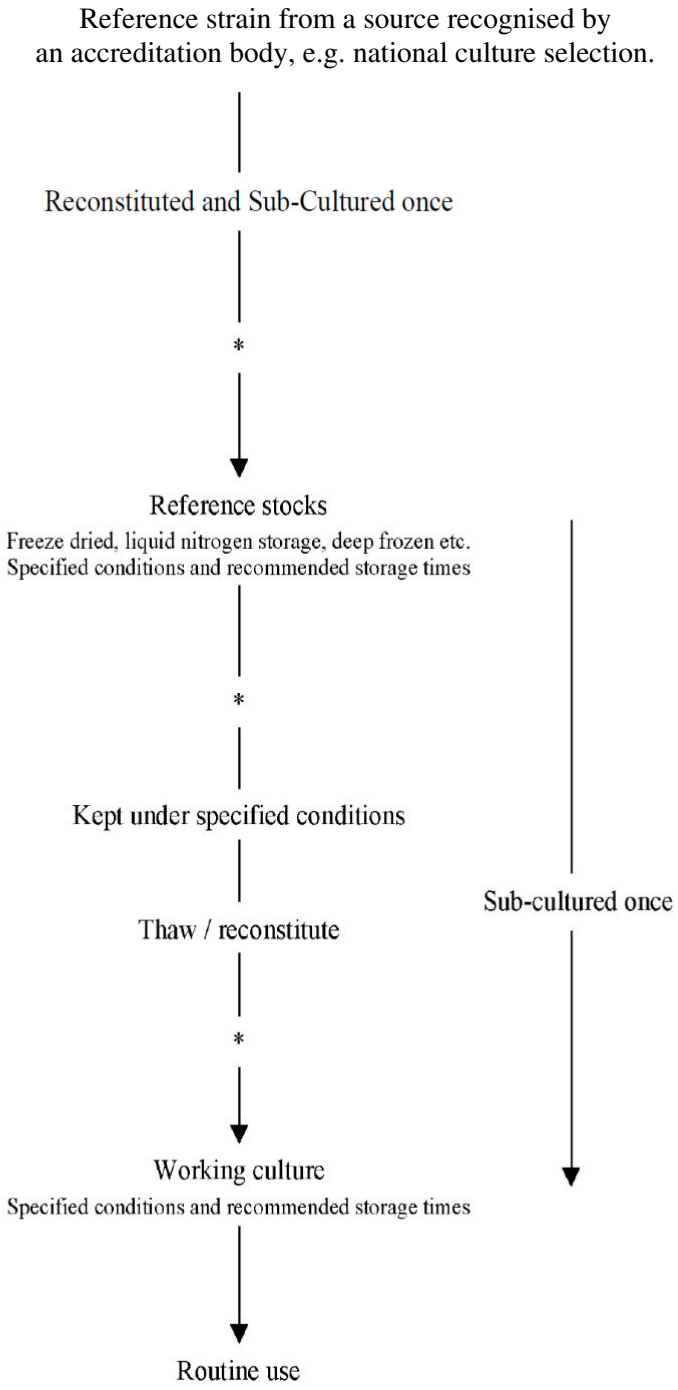

Figure B 1 – General use of reference and working cultures

*Parallel purity checks and biochemical tests as appropriate.

NOTE: All parts of the process should be fully documented, and detailed records of all steps must be maintained.

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Annex C – Reporting confidence intervals

C1 Scope

The main scope of this Annex is to briefly introduce the calculation of asymmetric confidence intervals for microbiological methods. The main references are ISO 29201 [26] and ISO 8199 [51] for water matrices, ISO 19036 [34] for food matrices and G108 for both matrices [52]. When the expanded uncertainty is over 30 – 40 % it is recommended to state *asymmetric* confidence intervals instead of just giving the expanded uncertainty for the count result in % or log units. A confidence interval for the results will be more informative for the client than just giving the result with uncertainty e.g. 50 CFU ± 42 % can also be reported as 50 [33, 76] CFU where 33 – 76 is the asymmetric confidence interval for the result 50 CFU.

C2 Introduction

In microbiology the main standard uncertainty components for the analytical uncertainty for a sample delivered to the laboratory, u_{anal}, are according to ISO 29201 [26] and ISO 19036 [34]:

1. u_o – relative operational (technical) uncertainty due to the use of the procedure;
2. u_d – relative distributional or intrinsic uncertainty due to taking a test portion of a laboratory sample. Distributional variability is the unavoidable variation without a cause that is associated with the distribution of particles in the final suspension and in the detection instrument. In microbiological suspensions it is usually believed to follow the Poisson distribution – see further Annex C in ISO 29201. Note: Normally a test portion is taken from the laboratory sample but in the case where the *whole* laboratory sample* is used for analysis u_d is set to zero;
3. u_{conf} – increase in the distributional uncertainty due to the result from confirmation.

Additional uncertainty contributions for solids and viscous fluids in particular:

u_{matrix} – relative uncertainty arising from imperfect mixing of the laboratory sample. Provided that the whole laboratory sample can be made homogeneous, u_{matrix} can be set to 0.10 $\log_{10}$ CFU/g (≈ 23 %) according to section 6.2 in ISO 19036.

Additional uncertainty from sampling:

u_{samp} – relative uncertainty due to sampling is discussed in Annex D.

In ISO 8199 [51] the only uncertainty component considered is the distributional, u_d when the operational uncertainty is not known. At low counts (< 15 CFU) the distributional uncertainty is dominating but at higher counts this will result in an underestimation of the uncertainty.

The relative uncertainty can be given in % or in units of natural (ln or $\log_e$) or common logarithms ($\log_{10}$)†.

The relative combined standard uncertainty (u_c) for a laboratory sample can be calculated from the *relevant* relative uncertainty components:

$$u_c = \sqrt{u_d^2 + u_o^2 + u_{conf}^2 + u_{matrix}^2} \qquad \textbf{(C.1)}$$

A component can be considered relevant if the size is 1/5 or more of the largest component.

* Laboratory sample is "Sample prepared for sending to the laboratory and intended for inspection or testing", from ISO19036 [34].

† Note that a given uncertainty less than 50 % can be recalculated in any of the units – see ISO 29201 [26]; e.g. an uncertainty of 20 % is approximately equal to 0.20 in natural logarithm (ln) and 0.087 in common logarithm ($\log_{10}$). The factor from natural to common logarithms is 0.4343 and from common to natural logarithms is 2.303 (ln 10).

An asymmetric 95 % confidence interval around the measured value, *n*, can be calculated according to section N3.4 of ISO 29201* from the standard uncertainty u_c given in %:

$$U_{min} = n/\exp\left(\frac{2u_c}{100}\right) \quad \textbf{(C.2)}$$

and

$$U_{max} = n \times \exp\left(\frac{2u_c}{100}\right) U_{max} = n \times \exp\left(\frac{2u_c}{100}\right) \quad \textbf{(C.3)}$$

and according to section 9.1 in ISO 19036† from u_c given in $\log_{10}$ units:

$$U_{\min} = n/10^{2u_c} \quad \textbf{(C.4)}$$

and

$$U_{\max} = n \times 10^{2u_c} U_{\mathbf{max}} = n \times 10^{2u_c} \quad \textbf{(C.5)}$$

This way of calculating an asymmetric interval has been called uncertainty factor (^{F}U) since you divide or multiply with the same factor [64]. For a standard uncertainty given in relative units (%) the uncertainty factor is:

$$^{F}U = \exp\left(\frac{2u_c}{100}\right) {}^{F}U = \exp\left(\frac{2u_c}{100}\right) \quad \textbf{(C.6)}$$

and then:

$$U_{min} = n/{}^{F}U \quad \textbf{(C.7)}$$

and

$$U_{max} = n \times {}^{F}U \quad \textbf{(C.8)}$$

For example, for n = 15 CFU and combined standard uncertainty of u_c = 30 % the uncertainty factor is $^{F}U = 1.82$. The interval can then be calculated to be $U_{min} = 8$ CFU and $U_{max} = 27$ CFU. In this Annex information is given on:

- reporting asymmetric confidence intervals based on relative uncertainty in % for colony counts common in the water sector;
- reporting asymmetric confidence intervals based on log10 units common in the food sector;
- reporting asymmetric confidence intervals for a MPN method;
- uncertainty estimation for molecular methods; and
- a possible solution to the issue when negative results are obtained in a study to estimate operational uncertainty (u_o) from duplicates.

* In most cases, absolute standard uncertainty calculated in natural logarithmic scale and the relative standard uncertainty can be assumed to be numerically equal (ISO 29201 [26]) . The natural logarithms can therefore be approximated with relative uncertainty in percent divided by 100. This is valid for a relative uncertainty < 50 %.

† ISO 19036 [34] uses an equivalent formula e.g., $U_{min} = 10^{(y-U)}$ where y = $\log_{10}$ (n) and $U = 2u_c$ in $\log_{10}$ units.

C3 Estimation of uncertainty (%) for a colony count (CFU) in water and asymmetric confidence intervals

Table C 1 gives an example of asymmetric confidence intervals based on uncertainty in % for colony counts common in the water sector with an operational uncertainty of 15 %.

Table C 1 — Estimation of uncertainty from operational (u_o) and distributional uncertainty (u_d) in units of % and calculation of a 95 % asymmetric confidence interval stating U_{min} and U_{max}

Count (CFU)	u_o (%)	u_d (%)	u_c (%)	U (%)	U_{min} (CFU)	U_{max} (CFU)
3	15	58	60	120	1	10
4	15	50	52	104	1	11
5	15	45	47	94	2	13
6	15	41	44	88	2	14
8	15	35	38	76	4	17
10	15	32	35	70	5	20
15	15	26	30	60	8	27
20	15	22	27	54	12	34
25	15	20	25	50	15	41
30	15	18	23	46	19	48
40	15	16	22	44	26	62
50	15	14	21	42	33	76
75	15	12	19	38	51	110
100	15	10	18	36	70	143
150	15	8	17	34	107	211
200	15	7	17	34	142	281
250	15	6	16	32	182	344
300	15	6	16	32	218	413
NOTE: Calculation of an asymmetric confidence interval according to equations C.2 and C.3.						

From Table C 1 it can be seen that with this specific example of an *operational uncertainty* of 15 % the distributional uncertainty is dominating in the lowest part of the working range. For example calculating the combined uncertainty using only the distributional uncertainty of 32 % for 10 CFU results in a confidence interval of [5,19]. This is very similar to the interval [5,20] given in Table C1 obtained taking into account the operational uncertainty of 15 %.

An example of how to use Table C 1 for a method with operational uncertainty u_o equal to 15 % that requires partial confirmation is presented in Table C 2 and Table C 3. The presumptive count was 25 CFU.

Table C 2 — Result before confirmation from Table C 1 – presumptive count 25 CFU

Count (CFU)	u_o (%)	u_d (%)	u_c (%)	U (%)	U_{min} (CFU)	U_{max} (CFU)
25	15	20	25	50	15	41

Confirmation: 10 colonies (n_z) out of 25 presumptive colonies (n_c) of one type on a plate method are tested. Of the 10 (n_z) colonies tested, 8 colonies (n_k) are confirmed positive. The confirmed count (n) is then 20 colonies of the 25 presumptive colonies according to the formula:

$$n = n_c \times n_k/n_z n = n_c \times n_k/n_z = \quad \textbf{(C.9)}$$

Table C 3 — Result after confirmation – confirmed count calculated to 20 CFU

Count (CFU)	u_o (%)	u_d[1] (%)	u_{conf}[2] (%)	u_c (%)	U (%)	U_{min} (CFU)	U_{max} (CFU)
20	15	20	16	30	59	11	36
[1]u_d distributional uncertainty for the 25 presumptive colonies. [2]u_{conf} calculated approximately with equation E3 (second term) in ISO 29201 [26].							

C4 Estimation of uncertainty (log_{10}) for colony count (CFU) in food and asymmetric confidence intervals

Table C 4 shows asymmetric confidence intervals based on uncertainty in log_{10} units for colony counts common in the food sector with an operational uncertainty of 35 % (0.15 log_{10}) and a matrix uncertainty of 23 % (0.10 log_{10}).

Table C 4 — Estimation of uncertainty from operational (u_o), distributional (u_d) and matrix (u_{matrix}) uncertainty[1] in log_{10} units and calculation of a 95 % asymmetric confidence interval[2] stating U_{min} and U_{max}

Count[3] (CFU)	u_o (log_{10})	u_d (log_{10})	u_{matrix} (log_{10})	u_c (log_{10})	U (log_{10})	U_{min} (CFU)	U_{max} (CFU)
3	0.15	0.25	0.10	0.308	0.618	1	12
4	0.15	0.22	0.10	0.282	0.564	1	15
5	0.15	0.19	0.10	0.265	0.530	1	17
6	0.15	0.18	0.10	0.253	0.506	2	19
8	0.15	0.15	0.10	0.237	0.474	3	24
10	0.15	0.14	0.10	0.227	0.453	4	28
15	0.15	0.11	0.10	0.212	0.425	6	40
20	0.15	0.10	0.10	0.205	0.410	8	51
30	0.15	0.08	0.10	0.197	0.394	12	74
40	0.15	0.07	0.10	0.193	0.386	16	97
50	0.15	0.06	0.10	0.190	0.381	21	120
75	0.15	0.05	0.10	0.187	0.374	32	178
100	0.15	0.04	0.10	0.185	0.371	43	235
150	0.15	0.04	0.10	0.184	0.367	64	350
200	0.15	0.03	0.10	0.183	0.366	86	464
250	0.15	0.03	0.10	0.182	0.365	108	579
300	0.15	0.03	0.10	0.182	0.364	130	694
[1]When confirmation is applied an additional uncertainty component should be included – see Table C 2, and Table C 3 and ISO 29201 [26]. [2]Intervals can be given in units of CFU or in units of log_{10} CFU; here in CFU. [3]Total number of colonies counted.							
NOTE: Calculation of an asymmetric confidence interval according to equations C.4 and C.5.							

An example of how to use Table C 4 for a validated method having an operational (technical) uncertainty, u_o = 0.15 $\log_{10}$ CFU/g and a matrix uncertainty, u_{matrix} = 0.10 $\log_{10}$ CFU/g is shown below. Test portions of 1.0 ml on one plate of each of two successive dilutions gave the following results; at 10^{-3} dilution, 102 colonies, and at 10^{-4} dilution, 8 colonies. The calculations are summarized in Table C 5 for a method with an operational uncertainty of 0.15 $\log_{10}$ CFU/g. The uncertainty interval can be calculated from u_c = 0.185 $\log_{10}$ CFU/g using Table C 4 taking into account the dilutions:

$$U_{min} = 0.43 \times 10^5 \text{ and } U_{max} = 2.35 \times 10^5 \text{ CFU/g}$$

Table C 5 — Example of estimation of uncertainty (u_c) for one sample with 2 dilutions*

Analysis	**Value**	**Unit**	**Comment**
Dilution 10^{-3}	102	CFU/g	
Dilution 10^{-4}	8	CFU/g	
Weighted mean	1.0×10^5	CFU/g	$(102 + 8)/1.1 \times 10^3 = 1.0 \times 10^5$
Weighted mean	5.0	$\log_{10}$ CFU/g	
Uncertainty	**Value**	**Unit**	**Comment**
u_o	0.15	$\log_{10}$ CFU/g	Operational
u_d	0.041	$\log_{10}$ CFU/g	Distributional, n = 102 +8
u_{matrix}	0.10	$\log_{10}$ CFU/g	Matrix
u_c	0.185	$\log_{10}$ CFU/g	Combined uncertainty $\sqrt{u_o^2 + u_d^2 + u_{matrix}^2}$
*Example from section 8.3.1 in ISO 19036 [34] where u_{matrix} is set to 0.10 for a homogeneous matrix and the distributional uncertainty in log10 units is calculated using the total number of colonies, 110 (102 + 8) using the equation $u_d = 0.4343/\sqrt{110}$.			

C5 Estimation of uncertainty for an MPN method and asymmetric confidence intervals

An example with 2 MPN results for a method with an operational uncertainty u_o of 0.071 $\log_{10}$ (≈ 16 %) is shown below. Table C 6 shows the confidence interval for the MPN method (MPN U_{min} and MPN U_{max}) and confidence intervals taking into account also the operational uncertainty.

Table C 6 — Example of estimation of combined uncertainty in $\log_{10}$ units for an MPN method from distributional uncertainty (u_d) and operational (u_o) uncertainty and calculation of a 95 % asymmetric confidence interval stating U_{min} and U_{max}

MPN	MPN U_{min}		MPN U_{max}		u_d[1]	u_o	u_c	U	U_{min}	U_{max}
	CFU	$\log_{10}$	CFU	$\log_{10}$	($\log_{10}$)	($\log_{10}$)	($\log_{10}$)	($\log_{10}$)	MPN	MPN
6.2	2.4	0.380	13.7	1.137	0.193	0.071	0.206	0.412	2	16
1986	1222	3.087	3300	3.519	0.110	0.071	0.131	0.262	1086	3636
[1]Formula $u_d = (U_{max} - U_{min})/3.92$ from ISO 29201 [26] Equation D.2										
NOTE: Calculation of an asymmetric confidence interval according to: $U_{min} = n/10^{2u_c}$ and $U_{max} = n * 10^{2u_c}$ where u_c is given in $\log_{10}$ units.										

From Table C 6, it can be seen at low counts with an operational uncertainty of 0.071 $\log_{10}$ (≈ 16 %), the distributional uncertainty is dominating, 0.193 $\log_{10}$ (≈ 44 %), and the combined uncertainty can be calculated using only distributional uncertainty. In this case, the confidence interval for 6.2 CFU with u_c = 0.193 $\log_{10}$ is [3,15]. This is to be compared with the interval given in Table C 6 which is [2,16] taking the operational uncertainty into account.

C6 Molecular methods

The estimation of measurement uncertainty for molecular methods (quantitative analyses) is within the scope of ISO 19036 [34]. Thus, calculations based on $\log_{10}$ transformed data may be preferred.

Specific standards can be consulted for methods such as PCR as they may include particular approaches to determining uncertainty. For example, in water quality, ISO/TS 12869 [25] specifies a method for detecting and quantifying Legionella spp. and L. pneumophila using PCR. A specific section is dedicated to estimating the uncertainty of the whole method, including recovery.

For the medical laboratories, Annex A in ISO/TS 20914 [63] also gives practical examples of uncertainty estimation, e.g. PCR amplification method of DNA and quantification by fluorescence emission intensity detection – determination of standard uncertainty in the $\log_{10}$ scale using test materials from internal quality control.

C7 Estimation of operational uncertainty from quality control data

The operational (technical) uncertainty can be calculated from quality control data by subtracting the square of the distributional component, u_d from the observed standard deviation s_{QC} of the results from measuring a control sample:

$$u_o = \sqrt{s_{QC}^2 - u_d^2} \qquad \text{(C.10)}$$

Example: The standard deviation for the quality control sample with a mean value of 42 CFU is 17.6 %. The distributional uncertainty for 42 CFU is 15.4 %. The operational uncertainty is then:

$$u_o = \sqrt{17.6^2 - 15.4^2} = 8.5 \qquad \text{(C.11)}$$

C8 Estimation of operational uncertainty from duplicates – the issue of negative results

The operational (technical) uncertainty can be calculated according to ISO 29201 [26] or ISO 19036 [34] from duplicate results* in a study where the experimental variations within the laboratory are maximised. The variation between the duplicates is due to both operational and distributional (Poisson) uncertainty. If the actual operational uncertainty is low, negative estimates can occur in the calculations. In this case the operational uncertainty cannot be estimated and from the study can only be reported as $u_o < x$. In Table C 7 is shown results from simulations; the one-sided confidence limit is tabulated when estimating the operational uncertainty from 10 to 100 duplicates for various CFU.

* Operational uncertainty is estimated by subtracting the mean distributional variance from the mean reproducibility variance (the variation between the duplicates), taking care that both are expressed in the same unit (section F.3.3 in ISO 29201 [26]).

Table C 7 ─ Estimated one sided confidence limit for the operational uncertainty by simulations

Number of duplicates	Median (CFU)	u_d (%)	$\hat{u}_o \hat{u}_o{}^*$ (%)
10	30	18	15
20	30	18	11
30	30	18	9
10	50	14	11
20	50	14	8
30	50	14	6
10	75	11.5	9
20	75	11.5	7
30	75	11.5	6
10	100	10	8
20	100	10	6
30	100	10	5
100	100	10	3
* $\hat{u}_o$ is based on an approximate one-sided upper confidence limit (UCL) for the estimate of the operational uncertainty when the calculated variance is zero or negative.			
NOTE: Duplicates according to Annex F in ISO 29201 Each simulation with sampling of duplicates from a distribution was repeated 2000 – 5000 times.			

Example of use of Table C 7: In a study to calculated u_o from 30 duplicates with counts between 25 and 75 CFU with a median count 50 CFU a negative result was obtained. Reading the row for number of duplicates 30 and CFU = 50 one can see that $\hat{u}_o$, the approximate one-sided upper confidence limit (UCL) for the estimate for u_o is 6 %. One can therefore conclude that u_o is ≤ 6 %; somewhere between 0 and 6 %. This max value, $\hat{u}_o$ = 6 %, can be used in the calculation of the standard uncertainty for the results.

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Annex D – Sampling uncertainty

D1 Scope

The main scope of this Annex is to briefly introduce the estimation of sampling uncertainty from duplicates following the guidance in the Eurachem Guide *Measurement uncertainty arising from sampling* [64] and Nordtest TR 604 [65]. Sampling uncertainty is not treated in the ISO standards but in Annex H in ISO 29201 [26] uncertainty due to subsampling (matrix effect) of a laboratory sample is estimated using ANOVA. In order to simplify the ANOVA calculations we use an Excel add-in, RANOVA3 [66].

D2 Introduction

When sampling is included, the measurement uncertainty consists of both sampling and analytical uncertainty*. The experimental design for estimating sampling uncertainty consists of taking several duplicate samples and each sample is analysed in duplicate. The sampling uncertainty is estimated as a difference from the total variation, u_{meas} according to Equation 3 in Nordtest 604 [65]:

$$u_{\text{samp}} = \sqrt{u_{meas}^2 - u_{\text{anal}}^2} \qquad \textbf{(D. 1)}$$

$$\text{where } u_{\text{anal}} = \sqrt{u_d^2 + u_o^2 \ldots} \qquad \textbf{(D. 2)}$$

The experimental design and calculations are well described in the Eurachem Guide [64] using the AMC add-in for Microsoft Excel RANOVA3 [66]. The minimum number of duplicate samples is eight but if the u_{samp} is lower than u_{anal} more duplicates (30-40) are recommended. The sampling uncertainty is reported in units of % for colony counts common in the water sector, and in $\log_{10}$ units that are common in the food sector.

Since the analytical uncertainty in microbiology often can be relatively high it can be difficult to estimate sampling uncertainty. When the sampling uncertainty is lower than the analytical uncertainty it is possible to obtain *negative estimates* of $u_{meas}^2 - u_{anal}^2$. The estimate is then normally reported as zero sampling uncertainty which we cannot recommend. This issue of *negative estimates* is treated in this Annex and is similar to the problem of negative results when estimating an operational uncertainty described in Annex C.

D3 Estimating sampling uncertainty

Sampling uncertainty is estimated from an experimental balanced design with duplicates given in Section 9.4.2 in the Eurachem Guide [64]. The use of Classical ANOVA assuming a lognormal distribution with the Excel add-in RANOVA [66] for estimating sampling uncertainty is discussed below.

Example – Natural spring water (contaminated) has been sampled from the same well. Ten different samples were taken in duplicate on 10 different days. Each sample C1 and C2 was tested in parallel (C1.1 / C1.2; C2.1 /C2.2 for Coliform bacteria using membrane filtration (ISO 9308-1:2014 [67]) by different operators, using the same batch of consumables and same incubator. Results are shown in Table D 1. The arithmetic mean is 35 CFU and the median is 31 CFU for these results.

* For applications with only low CFU (< 20) the standard uncertainty, u_{anal} is high so in most cases the sampling uncertainty for homogeneous sampling targets can be neglected.

Table D 1 — Test results for ten different samples taken in duplicate on 10 different days. Each sample C1 and C2 was tested in parallel, for Coliform bacteria with membrane filtration

Sample C 1		Sample C 2	
CFU $C_{1.1}$	CFU $C_{1.2}$	CFU $C_{2.1}$	CFU $C_{2.2}$
63	45	41	45
37	26	28	30
21	23	31	30
18	20	24	18
18	14	14	18
68	45	40	67
62	42	42	46
29	20	17	19
41	28	41	32
61	50	50	48

D4 Calculations

The test results in Table D 1 were given as input to RANOVA3 [66] and the uncertainties were calculated using Classical ANOVA*. The estimated uncertainties are shown below and the sampling uncertainty is given as zero indicating a negative estimate for $(u_{meas}^2 - u_{anal}^2)$.

Classical ANOVA

Mean	***35.3***		No. Targets	***10***
Total Sdev	***16.473***			
	Btn Target	Sampling	Analysis	Measure
Standard deviation	***14.299***	***0***	***8.1792***	***8.1792***
% of total variance	***75.35***	***0.00***	***24.65***	***24.65***
Expanded relative uncertainty (95%)		***0.00***	***46.34***	***46.34***
Uncertainty Factor (95%)		***1***	***1.4683***	***1.4683***

Figure D 1 — Output from AMC software RANOVA3 using data in Table D 1 as input

Assuming a lognormal distribution we use the uncertainty factor given in the RANOVA3 output to calculate the uncertainty. The *standard analytical uncertainty* in $\log_{10}$ units is calculated from the uncertainty factor ${}^{F}U$ given in Figure D 1 using the following equation:

$$u_{\text{anal.log10}} = (log^{F}U)/2 = (\log 1.47)/2 = 0.084 \quad \textbf{(D. 3)}$$

and in natural logarithms

$$u_{\text{anal}} = u_{anal.log10} \times \ln(10) = 0.084 \times 2.303 = 0.19 \quad \textbf{(D. 4)}$$

which is 19 % relative uncertainty†.

* The Excel add-in RANOVA3 calculates both Classical and Robust ANOVA. In microbiology applications the Classical ANOVA is recommended. RANOVA3 take their input as raw measurement values, but the values of the Uncertainty Factor are calculated after making a $\log_e$ transformation within the program.

† According to ISO 29201 [26] can natural logarithms be approximated with relative uncertainty in percent divided by 100 (for relative uncertainty < 50 %).

D5 Estimating upper confidence limit when sampling uncertainty is zero

In this case the sampling uncertainty is estimated to be zero. What we can say is that the expanded sampling uncertainty is probably less than a particular value. This value is the *upper confidence limit* for the estimate of zero sampling uncertainty. The AMC software can give confidence limits for the estimated expanded uncertainties in a separate output (CI RANOVA). Figure D 2 shows the confidence limits for the sampling uncertainty. Assuming a lognormal distribution the upper confidence limits for the uncertainty factor ${}^{F}U$ should be used.

Classical ANOVA No. Targets 10

Mean	35.3	Confidence limits		Confidence limits
Total Sdev	16.473	(11.945, 27.498)		
	Btn Target		Sampling	
Standard deviation	14.299	(9.2055, 26.537)	0	(0, 8.5372)
% of total variance	75.35		0.00	
Expanded relative uncertainty (95%)			0.00	(0, 48.37)
Uncertainty Factor (95%)			1	(0, 1.4932)

Figure D 2 — Output from AMC software RANOVA3 where confidence limits are added as additional information

In $\log_{10}$ units the upper confidence limit for the *standard* uncertainty can be calculated from the upper confidence limit of the uncertainty factor ${}^{F}U$ given in Figure D 2 (1.4932) using the following equation:

Upper confidence limit for the sampling uncertainty is:

$$\hat{u}_{\log} = (log^{F}U)/2 = (\log 1.49)/2 = 0.087 \quad \textbf{(D. 5)}$$

and in natural logarithms the upper relative confidence limit is

$$\hat{u}_{samp} = \hat{u}_{log} \times \ln(10) = 0.087 \times 2.303 = 0.20 \approx 20\ \% \quad \textbf{(D. 6)}$$

This estimate* is not the standard sampling uncertainty. We have estimated the sampling uncertainty as zero % and now we know that the sampling standard uncertainty is probably somewhere between 0 % and 20 %.

If we want to reduce the estimate of 20 % we need to take more duplicate samples. Using a simulation with 40 data points instead of 10 with the same experimental conditions and a mean of 35 CFU, the upper confidence limit $\hat{u}_{samp}$ will be circa 11 %.

* $\hat{u}_{samp}$ is based on an approximate one-sided upper confidence limit (UCL) for the estimate of the standard sampling uncertainty when the calculated mean variance from duplicates is zero or negative.

D6 Conclusions

Sampling uncertainty can be estimated with ANOVA using results from duplicates. However since the sampling uncertainty is calculated as a difference it is difficult to estimate a sampling uncertainty at low CFU when the analytical uncertainty is high; the uncertainty estimate obtained therefore has a high "uncertainty".

When the sampling uncertainty is lower than the analytical uncertainty an estimate of zero for sampling uncertainty can occur. We can then only report an upper confidence limit for the sampling uncertainty – in other words, the sampling uncertainty is somewhere between zero and that upper confidence limit. To address this issue the following is recommended:

1. samples with higher CFU values are recommended. Since the distributional uncertainty (that is part of the analytical uncertainty) decreases with higher CFU a lower sampling uncertainty can be estimated at higher CFU;
2. in general, 30 duplicate samples are recommended; then the upper confidence limit for the sampling uncertainty is slightly more than half the analytical uncertainty.

When the sampling uncertainty is estimated to be zero using more duplicates, e.g. 40, the contribution from sampling uncertainty can in most cases be regarded as negligible. If we wish to calculate the contribution with an analytical uncertainty of 19 % and a sampling uncertainty of 11 % or less the estimate of $\hat{u}_{samp}$, the measurement standard uncertainty (sampling + analytical) will only be 22 %. In this case, we can therefore assume $u_{\mathrm{meas}} \approx u_{\mathrm{anal}}$.

Annex E – Guidance on calibration of measuring instruments

Calibration, intended to provide traceability to the relevant SI unit(s), should be performed over the entire measurement range using appropriate measurement standards, reference procedures, and qualified personnel. Services provided by accredited calibration laboratories fulfil these requirements. All equipment should be calibrated before being put into use. The frequency of calibration will be justified by experience and risk analysis based e.g. on need, type, producer's recommendations and previous performance of the equipment. The Table below presents general guidance for the frequency of calibration.

Type of equipment	**Requirement**	**Suggested frequency**
Reference thermometers (e.g. liquid-in-glass)	Calibration Single point (e.g. ice-point) check	Every 5 years Annually
Reference thermocouples	Calibration Check against reference thermometer	Every 3 years Annually
Working thermometers & Working thermocouples	Calibration Check against reference thermometer at ice-point and/or working temperature range	Annually in the first 3 years, followed by less frequently, based on satisfactory performance Annually
Balances	Calibration over the entire range	Annually in the first 3 years, followed by less frequently, based on satisfactory performance
Calibration weights	Calibration	Every 5 years
Check weight(s)	Check against calibrated weight or check on balance immediately after calibration of the balance	Every 3 years
Volumetric glassware, including glass pipettes	Gravimetric calibration to required tolerance, unless accompanied by appropriate certificates. Glassware sterilized in a sterilizing oven should be checked regularly, even if certified.	Annually
Pipettors/ micropipettes	Calibration	Annually
Hygrometers	Calibration	Annually
pH meters	Calibration with traceable standard buffer solutions	Annually or more frequently if required
Gas analysers	Calibration	Annually
Autoclaves, media preparators	Calibration of temperature and pressure sensors if critical	Annually

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Annex F – Guidance on equipment validation and verification

This information is provided for guidance purposes and the frequency will be based on the need, type and previous performance of the equipment.

Type of equipment	**Requirement**	**Suggested frequency**
Temperature controlled equipment (incubators, baths, fridges, freezers)	(a) Define acceptability limits for stability and homogeneity of temperature (b) Determine the stability and homogeneity of temperature and compare with acceptability limits (c) Define / confirm the operating range and the corresponding alarm limits (d) Monitor temperature	(a) Initially (b) Initially, and after any repair/modification /change of location, that may have an effect on the temperature control (c) Initially and at each further occasion of evaluation (d) At least daily / at each use or by continuous monitoring and recording during the time of use
Atmosphere controlled equipment (incubators etc.)	(a) Define acceptability limits for stability and homogeneity of atmosphere composition (usually, humidity and CO_2 content) (b) Determine the stability and homogeneity of atmosphere composition and compare with acceptability limits (c) Define / confirm the operating range and the corresponding alarm limits (d) Monitor atmosphere composition	(a) Initially (b) Initially, and after any repair/modification /change of location, that may have an effect on the temperature control (c) Initially and at each further occasion of evaluation (d) At least daily / at each use or by continuous monitoring and recording during the time of use
Thermocyclers (PCR and Real time PCR)	Determine the stability and homogeneity of temperature and compare with acceptability limits to ensure performance	Initially and daily / weekly depending in the frequency and number of sample analysis
Sterilising ovens	(a) Define acceptability limits for stability and homogeneity of temperature (b) Determine the stability and homogeneity of temperature and compare with acceptability limits (c) Monitor temperature	(a) Initially (b) Initially, and after any repair/ modification /change of location that may have an effect on the temperature control (c) At least daily / at each use or by continuous monitoring and recording during the time of use

Type of equipment	Requirement	Suggested frequency
Autoclaves	(a) Establish characteristics for loads/cycles	(a) Initially, and after any repair/ modification /change of location that may have an effect on performance
	(b) Monitor temperature/time	(b) Daily/each use or by continuous monitoring and recording during the time of use
Centrifuges	Check against a calibrated and independent tachometer if the speed is critical	Annually
Safety cabinets	(a) Establish performance	(a) Initially, every year and after repair/ modification/change of location that may influence performances
	(b) Microbiological monitoring	(b) Weekly
	(c) Air flow monitoring	(d) Daily/each use
Laminar air flow cabinets	(a) Establish performance	(a) Initially, and after repair/ /change of location that may effect on performances
	(b) Check with sterility plates	(b)Weekly
Timers	Check if critical	Annually
Microscopes	Check alignment	Daily or at each use
pH meters	Check the calibration with a buffer solution not used for the calibration	Daily/each use
Balances	Check reading against check weight	Daily/each use
De-ionisers and	(a) Check conductivity	(a) Weekly
Reverse osmosis units	(b) Check for microbial contamination	(b) Monthly
Gravimetric diluters	(a) Check weight of volume dispensed	(a) Daily/each use
	(b) Check dilution ratio	(b) Daily/each use
Media dispensers	Check volume dispensed	After each adjustment or replacement
Pipettors/micropipettes	Check bias and precision of volume dispensed by gravimetric method	Regularly (to be defined by taking account of the frequency and nature of use)
Spiral platers	(a) Establish performance against conventional method	(a) Initially and annually
	(b) Check stylus condition and the start and end points	(b) Daily/each use
	(c) Check volume dispensed	(c) Monthly
Colony counters	Check against number counted manually	Annually
Centrifuges	Check speed against a calibrated and independent tachometer	Annually
Anaerobic jars/incubators	Check with anaerobic indicator	Daily/each use
Laboratory environment	Monitor for airborne and surface microbial contamination using, e.g. air samplers, settle plates, contact plates or swabs	Weekly for total count and moulds. Biannually for pathogens or as otherwise decided by the laboratory based on activities and historical trends and results

Annex G – Guidance on maintenance of equipment

This information is provided for guidance purposes and the frequency will be based on the need, type and previous performance of the equipment.

Type of equipment	Requirement	Suggested frequency
Incubators	Clean and disinfect internal surfaces	Monthly
Fridges	Clean and disinfect internal surfaces	When required (e.g. every 3 months)
Freezers, ovens	Clean and disinfect internal surfaces	When required (e.g. annually)
Water baths	Empty, clean, disinfect and refill	Monthly, or every 6 months if biocide used
Centrifuges	(a) Service (b) Clean and disinfect	(a) Annually (b) Each use
Autoclaves	(a) Make visual checks of gasket, clean/drain chamber (b) FI service (c) Safety check of pressure vessel	(a) Regularly, as recommended by manufacturer (b) Annually or as recommended manufacturer (c) Annually
Safety cabinets Laminar flow cabinets	Full service and mechanical check	Annually or as recommended by manufacturer
Microscopes	Full maintenance service	Annually
pH meters	Clean electrode	Each use
Balances, gravimetric diluters	(a) Clean (b) Service	(a) Each use (b) Annually
Stills	Clean and de-scale	As required (e.g. every 3 months)
De-ionisers, reverse osmosis units	Replace cartridge/membrane	As recommended by manufacturer
Anaerobic jars	Clean/disinfect	After each use
Media dispensers, volumetric equipment, pipettes, and general service equipment	Decontaminate, clean and sterilise as appropriate	Each use
Spiral platers	(a) Service (b) Decontaminate, clean and sterilise	(a) Annually (b) Each use
Laboratory	(a) Clean and disinfect working surfaces (b) Clean floors, disinfect sinks and basins (c) Clean and disinfect other surfaces	(a) Daily, and during use (b) Weekly or more frequently if required (c) Every 3 – 12 months depending on type of laboratory work

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Bibliography

For updates to referenced guidance and additional reading, please refer to the Eurachem Reading List on the Eurachem website, www.eurachem.org.

1. ISO/IEC 17025:2017 General requirements for the competence of testing and calibration laboratories. International Organization for Standardization. 2017. Available from: https://www.iso.org/standard/66912.html.
2. ISO 15189:2022 Medical laboratories — Requirements for quality and competence. International Organization for Standardization. 2022. Available from: https://www.iso.org/standard/76677.html.
3. Handbook: good laboratory practice (GLP): quality practices for regulated non-clinical research and development - 2nd ed. World Health Organization. 2009. Freely available from: https://fctc.who.int.
4. WHO good manufacturing practices (GMP) for pharmaceutical products: Main principles Annex 2, WHO Technical Report Series 986. 2014. Freely available from: https://cdn.who.int.
5. WHO Guidelines for good clinical practice (GCP) for trials on pharmaceutical products, . WHO Technical Report Series, No. 850, Annex 3. 1995. Freely available from: https://apps.who.int.
6. REGULATION (EU) 2017/625 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 15 March 2017. European Union. 2017. Freely available from: https://eur-lex.europa.eu
7. DIRECTIVE (EU) 2020/2184 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2020 on the quality of water intended for human consumption (recast) 2020. Freely available from: https://eur-lex.europa.eu.
8. An introduction to Risk Consideration, Cookbook No 18. Eurolab. 2021. Freely available from. https://www.eurolab.org/CookBooks/18
9. ISO 31000:2018 Risk management Guidelines. International Organization for Standardization. 2018. Available from: https://www.iso.org/obp/ui/#iso:std:iso:31000:ed-2:v1:en.
10. ISO 7218:2007 and AMD 1:2013 Microbiology of food and animal feeding stuffs General requirements and guidance for microbiological examinations. International Organization for Standardization. Available from: https://www.iso.org/standard/36534.html.
11. Laboratory Biosafety Manual 2020. Freely available from: https://www.who.int.
12. Meechan PJ, Pootts J. Biosafety in Microbiological and Biomedical Laboratories. 6th ed: Centres for Disease Control and Prevention, National Institutes of Health; 2020.
13. ISO 14698-2:2003 Cleanrooms and associated controlled environments Biocontamination control Part 2: Evaluation and interpretation of biocontamination data. International Organization for Standardization. 2003. Available from: https://www.iso.org.
14. EN 17141:2020 Cleanrooms and associated controlled environments. Biocontamination control. European Committee for Standardization. 2020. Available from: https://www.en-standard.eu.
15. ISO 18593:2018 Microbiology of the food chain Horizontal methods for surface sampling. International Organization for Standardization. 2018. Available from: https://www.iso.org/standard/64950.html.
16. V. Barwick (Ed), Eurachem/CITAC Guide: Guide to Quality in Analytical Chemistry: An Aid to Accreditation (3rd edition, 2016). ISBN 978-0-948926-32-7. Freely available from www.eurachem.org.
17. ISO 11133:2014 including AMD 1:2018 and AMD 2:2020 Microbiology of food, animal feed and water Preparation, production, storage and performance testing of culture media Amendment 2 2020. International Organization for Standardization. Available from: https://www.iso.org/standard/70354.html.

18. ISO 9000:2015 Quality management systems Fundamentals and vocabulary. International Organization for Standardization. 2015. Available from: https://www.iso.org/standard/45481.html.
19. ISO 17034:2016 General requirements for the competence of reference material producers. International Organization for Standardization. 2016. Available from: https://www.iso.org/standard/29357.html.
20. S L R Ellison and A Williams (Eds) Eurachem/CITAC Guide: Metrological Traceability in Analytical measurement (2nd ed. 2019) ISBN: 978-0-948926-34-1. Freely available from: www.eurachem.org.
21. ILAC P10:07/2020 ILAC Policy on Metrological Traceability of Measurement Results. Freely available from: https://ilac.org/?ddownload=123220.
22. ISO 8655-1:2022 Piston-operated volumetric apparatus —Part 1: Terminology, general requirements and user recommendations, International Organization for Standardization. 2016. Available from: https://www.iso.org/standard/68796.html.
23. R Bettencourt da Silva and S L R Ellison (Eds.) Eurachem/CITAC Guide: Assessment of performance and uncertainty in qualitative chemical analysis (1st edition, 2021). ISBN 978-0-948926-39-6. Freely available from https://www.eurachem.org.
24. ISO 13843:2017 Water quality Requirements for establishing performance characteristics of quantitative microbiological methods. International Organization for Standardization. 2017. Available from https://www.iso.org/standard/62979.html.
25. ISO/TS 12869:2019 Water quality Detection and quantification of Legionella spp. and/or Legionella pneumophila by concentration and genic amplification by quantitative polymerase chain reaction (qPCR). International Organization for Standardization. 2019. Available from: https://www.iso.org/standard/70756.html.
26. ISO 29201:2012 Water quality The variability of test results and the uncertainty of measurement of microbiological enumeration methods. International Organization for Standardization. 2012. Available from: https://www.iso.org/standard/45348.html.
27. ISO 16140-2:2016 Microbiology of the food chain Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method. International Organization for Standardization. 2016. Available from: https://www.iso.org/standard/54870.html.
28. ISO 16140-1:2016 Microbiology of the food chain Method validation Part 1: Vocabulary. International Organization for Standardization. 2016. Available from: https://www.iso.org/standard/54869.html.
29. ISO 16140-6:2019 Microbiology of the food chain Method validation — Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures. International Organization for Standardization. 2019. Available from: https://www.iso.org/standard/66327.html.
30. ISO 16140-5:2020 Microbiology of the food chain — Method validation — Part 5: Protocol for factorial interlaboratory validation for non-proprietary methods. International Organization for Standardization.. 2020. Available from: https://www.iso.org/standard/66326.html.
31. ISO 16140-4:2020 Microbiology of the food chain — Method validation — Part 4: Protocol for method validation in a single laboratory. International Organization for Standardization. 2020. Available from: https://www.iso.org/standard/66325.html.
32. ISO 16140-3:2021 Microbiology of the food chain — Method validation — Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory. International Organization for Standardization.. 2021. Available from: https://www.iso.org/standard/66324.html.

33. ISO 17468:2016 Microbiology of the food chain — Technical requirements and guidance on establishment or revision of a standardized reference method. International Organization for Standardization. 2016. Available from: https://www.iso.org/standard/59858.html.
34. ISO 19036:2019 Microbiology of the food chain — Estimation of measurement uncertainty for quantitative determinations. International Organization for Standardization. 2019. Available from: https://www.iso.org/standard/71899.html.
35. NMKL Procedue 32 Verification of microbiological methods. Nordic Committee on Food Analysis,. Available from: https://www.nmkl.org
36. ISO 20836:2021 Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of microorganisms — Thermal performance testing of thermal cyclers. International Organization for Standardization. 2021. Available from: https://www.iso.org/standard/75434.html.
37. ISO 20837:2006 Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for sample preparation for qualitative detection. International Organization for Standardization. 2006. Available from: https://www.iso.org/standard/39760.html.
38. ISO 20838:2006 Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for amplification and detection for qualitative methods. International Organization for Standardization. 2006. Available from: https://www.iso.org/standard/39761.html.
39. ISO 22119:2011 Microbiology of food and animal feeding stuffs — Real-time polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions. International Organization for Standardization. 2011. Available from: https://www.iso.org/standard/45035.html.
40. ISO 19458:2006 Water quality — Sampling for microbiological analysis. I Organization for Standardization. 2006. Available from: https://www.iso.org/standard/33845.html.
41. ISO 6887-1:2017 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions. International Organization for Standardization. 2017. Available from: https://www.iso.org/standard/63335.html.
42. ISO 6887-2:2017 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products. International Organization for Standardization. 2017. Available from: https://www.iso.org/standard/63336.html.
43. ISO 6887-3:2017 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products. International Organization for Standardization. 2017. Available from: https://www.iso.org/standard/63337.html.
44. ISO 6887-4:2017 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 4: Specific rules for the preparation of miscellaneous products. Organization for Standardization. 2017. Available from: https://www.iso.org/standard/63338.html.
45. ISO 6887-5:2020 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products. International Organization for Standardization. 2020. Available from: https://www.iso.org/standard/71502.html.

46. ISO/IEC GUIDE 99:2007 International vocabulary of metrology — Basic and general concepts and associated terms (VIM). International Organization for Standardization. 2007. Available from: https://www.iso.org/standard/45324.html. Freely available as JCGM 200:2008 at www.bipm.org.
47. S L R Ellison and A Williams, Quantifying Uncertainty in Analytical Measurement (eds), 3rd Edition. Eurachem. 2012. Freely available from: www.eurachem.org.
48. A2LA Guide G108 - Guidelines for Estimating Uncertainty for Microbiological Counting Methods. 2019. Freely available from: https://a2la.qualtraxcloud.com/.
49. ILAC G17:01/2021 ILAC Guidelines for Measurement Uncertainty in Testing 2021. Freely available from: https://ilac.org/publications-and-resources/ilac-guidance-series/.
50. Guidance Document on Measurement Uncertainty for GMO Testing Laboratories. European Commission, Joint Research Centre, Institute for Reference Materials and Measurements. 2009. Freely available from: https://op.europa.eu/en/publication-detail/-/publication/1485e13a-ac54-11ea-bb7a-01aa75ed71a1/language-en/format-PDF/source-144689769
51. ISO 8199:2018 Water quality – General requirements and guidance for microbiological examinations by culture. International Organization for Standardization. 2018. Available from: https://www.iso.org/standard/64151.html.
52. Handbook - Inter Quality control, Nordtest Report TR 569 (ed. 5). Nordtest. 2018. Freely available from: http://www.nordtest.info.pdf.
53. ISO 22118:2011 Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection and quantification of food-borne pathogens — Performance characteristics. International Organization for Standardization. 2011. Available from: https://www.iso.org/standard/45034.html.
54. ISO 22117:2019 Microbiology of the food chain — Specific requirements and guidance for proficiency testing by interlaboratory comparison. International Organization for Standardization. 2019. Available from: https://www.iso.org/standard/67052.html.
55. EA-4/18 G: 2021 Guidance on the level and frequency of proficiency testing participation. European Accreditation. 2021. Freely available from: https://european-accreditation.org.
56. Eurachem Guide: Selection, Use and Interpretation of Proficiency Testing (PT) Schemes by Laboratories. 2021. Freely available from: www.eurachem.org.
57. ISO/IEC 17043:2010 Conformity assessment — General requirements for proficiency testing. International Organization for Standardization. 2010. Available from: https://www.iso.org/standard/29366.html.
58. ILAC-G8: 2019 - Guidelines on Decision Rules and Statements of Conformity – A summary. 2019. Freely available from: https://ilac.org/publications-and-resources/ilac-guidance-series/.
59. A Williams and B Magnusson (Eds.) Eurachem/CITAC Guide: Use of uncertainty information in compliance assessment (2nd ed.). Eurachem. 2021. Freely available from: www.eurachem.org.
60. Eurachem information leaflet: Use of uncertainty in compliance. Eurachem. 2021. Freely available from: www.eurachem.org.
61. V Barwick and E Prichard (Eds) Terminology in Analytical Measurement - Introduction to VIM 3, 2011. Freely available from: www.eurachem.org
62. ISO 17994:2014 Water quality — Requirements for the comparison of the relative recovery of microorganisms by two quantitative methods. International Organization for Standardization. 2014. Available from: https://www.iso.org/standard/56617.html.
63. ISO/TS 20914:2019 Medical laboratories — Practical guidance for the estimation of measurement uncertainty. International Organization for Standardization. 2019. Available from: iso:std:iso:ts:20914:ed-1:v1:en.

64. M H Ramsey, S L R Ellison, P Rostron. Eurachem/EUROLAB/ CITAC/Nordtest/AMC Guide: Measurement uncertainty arising from sampling: a guide to methods and approaches (2nd edition, 2019). ISBN (978-0-948926-35-8). Freely available from http://www.eurachem.org.

65. Nordtest 604 Nordtest. Uncertainty from sampling. A Nordtest handbook for sampling planners and sampling quality assurance and uncertainty estimation. 2020. Freely available from http://www.nordtest.info/.

66. Robust ANOVA programs are available for balanced design (RANOVA) and both balanced and unbalanced designs (RANOVA3) from the Royal Society of Chemistry website. Freely available from: https://www.rsc.org

67. ISO 9308-1:2014 Water quality — Enumeration of Escherichia coli and coliform bacteria — Part 1: Membrane filtration method for waters with low bacterial background flora, International Organization for Standardization. 2014. Available from: https://www.iso.org/standard/55832.html.

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