भारतीय मानक
Indian Standard

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पानी की गुणता — संवर्धन द्वारा सूक्ष्म जैविक परीक्षणों के लिए सामान्य अपेक्षाएँ एवं मार्गदर्शिका

(दूसरा पुनरीक्षण)

Water Quality — General Requirements and Guidance for Microbiological Examinations by Culture

(Second Revision)

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Drinking Water and Carbonated Beverages Sectional Committee, FAD 14

NATIONAL FOREWORD

This Indian Standard (Second Revision) which is identical with ISO 8199 : 2018 was adopted by the Bureau of Indian Standards, after the draft finalized by the Drinking Water and Carbonated Beverages Sectional Committee had been approved by the Food and Agriculture Division Council.

This standard was originally published in 2002 and was subsequently revised in 2012. The first revision of the standard was identical with ISO 8199 : 2005 'Water quality — General guidance on the enumeration of micro-organisms by culture'. The second revision of this standard has been undertaken to align it with the latest version of the ISO 8199 : 2018.

The text of ISO Standard has been approved as suitable for publication as an Indian Standard without deviations. Certain terminologies and conventions are, however, not identical to those used in Indian Standards. Attention is particularly drawn to the following:

a) Wherever the words 'International Standard' appear referring to this standard, they should be read as 'Indian Standard'.

b) Comma (,) has been used as a decimal marker, while in Indian Standards, the current practice is to use a point (.) as the decimal marker.

In this adopted standard, reference appear to the following International Standard for which Indian Standard also exists. The corresponding Indian Standard, which is to be substituted in its place, is listed below along with its degree of equivalence for the edition indicated:

International Standard	*Corresponding Indian Standard*	*Degree of Equivalence*
ISO 11133 Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media	IS 17383 : 2020/ISO 11133 : 2014 Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media	Identical with ISO 11133 : 2014

The technical committee has reviewed the provision of the following International Standards referred in this adopted standard and has decided that they are acceptable for use in conjunction with this standard:

International Standard	*Title*
ISO 19458	Water quality — Sampling for microbiological analysis
ISO 7704	Water quality — Evaluation of membrane filters used for microbiological analyses

In reporting the results of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2 : 1960 'Rules for rounding off numerical values (*revised*)'.

Introduction

Techniques for the detection and enumeration of microorganisms based on their ability to grow on or in specified culture media are an important and widely used means of assessing the microbiological quality of water. The purpose of this document is to gather in a single document the information common to the various techniques. This reduces repetition of technical details in individual standards and facilitates choice of the technique most suitable for a particular situation. Other guidance has been included on general topics of relevance to these techniques, such as analytical quality control, method performance characteristics and uncertainty of test results.

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Indian Standard

WATER QUALITY — GENERAL REQUIREMENTS AND GUIDANCE FOR MICROBIOLOGICAL EXAMINATIONS BY CULTURE

(Second Revision)

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

IMPORTANT — It is absolutely essential that tests conducted according to this document be carried out by suitably trained staff.

1 Scope

This document specifies requirements and gives guidance for performing the manipulations common to each culture technique for the microbiological examination of water, particularly the preparation of samples, culture media, and general apparatus and glassware, unless otherwise required in the specific standard. It also describes the various techniques available for detection and enumeration by culture and the criteria for determining which technique is appropriate.

This document is mainly intended for examinations for bacteria, yeasts and moulds, but some aspects are also applicable to bacteriophages, viruses and parasites. It excludes techniques not based on culturing microorganisms, such as polymerase chain reaction (PCR) methods.

2 Normative references

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

ISO 7704, *Water quality — Evaluation of membrane filters used for microbiological analyses*

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

ISO 19458, *Water quality — Sampling for microbiological analysis*

3 Terms and definitions

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

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

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

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

3.1
accuracy
closeness of agreement between a test result and the accepted reference value

[SOURCE: ISO 6107-8:1993, 1, modified — The note has been deleted.]

3.2
bias
difference between the expectation of the test results and an accepted reference value

[SOURCE: ISO 5725-1:1994, 3.8, modified — Note 1 to entry has been deleted.]

3.3
confirmed count
count (3.4) corrected for not confirmed *presumptive counts* (3.9) by further testing of the presumptive objects

3.4
count
<microbiology> observed number of objects such as colonies or cells determined by direct counting, or most probable number (MPN) estimation based on statistical calculation using the number of positive units or presumptive positive units in a dilution series of a *test sample* (3.16)

[SOURCE: ISO 6107-6:2004, 22, modified — "or presumptive positive units" has been added.]

3.5
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

Note 1 to entry: The theoretical level that conforms to this definition is an average of three viable cells in an inoculum volume.

[SOURCE: ISO 13843:2017, 3.10]

3.6
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

3.7
limit of determination
lowest analyte concentration per analytical portion where the expected relative standard uncertainty equals a specified value

[SOURCE: ISO 13843:2017, 3.17]

3.8
precision
closeness of agreement between independent test results obtained under stipulated conditions

[SOURCE: ISO 5725-1:1994, 3.12, modified — Notes 1 to 3 to entry have been deleted.]

3.9
presumptive count
colony *count* (3.4) or most probable number (MPN) estimate based on the number of colonies or reaction vessels that have an outward appearance that is interpreted as typical of a target organism

[SOURCE: ISO 6107-6:2004, 62, modified — "fermentation tubes" has been replaced with "reaction vessels".]

3.10
relative standard deviation
u_{rel}
estimate of the standard deviation of a population from a sample of *n* results divided by the mean of that sample

[SOURCE: ISO 13843:2017, 3.30]

3.11
repeatability
measurement repeatability
measurement *precision* (3.8) under a set of *repeatability conditions* (3.12) of measurement

[SOURCE: ISO 13843:2017, 3.32]

3.12
repeatability conditions
condition of measurement, out of a set of conditions that includes the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time

[SOURCE: ISO 13843:2017, 3.33]

3.13
reproducibility
measurement reproducibility
measurement *precision* (3.8) under *reproducibility conditions* (3.14) of measurement

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

[SOURCE: ISO 13843:2017, 3.34]

3.14
reproducibility conditions
condition of measurement, out of a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects

[SOURCE: ISO 13843:2017, 3.35]

3.15
test portion
specified quantity of the sample that is taken for analysis

EXAMPLE 0,1 ml, 1 ml, 100 ml of sample.

3.16
test sample
undiluted, diluted or otherwise prepared *test portion* (3.15) of a sample to be tested, after completion of all preparation steps such as centrifugation, filtration, homogenization, pH adjustment and determination of ionic strength

[SOURCE: ISO 6107-6:2004, 92, modified — Note 1 to entry has been deleted.]

3.17
trueness
closeness of agreement between the average value obtained from a large series of test results and an accepted reference value

Note 1 to entry: The measure of trueness is usually expressed in terms of *bias* (3.2).

[SOURCE: ISO 6107-8:1993, 63]

3.18
uncertainty of counting
relative standard deviation (3.10) of results of repeated counting of the colonies or particles of the same plate(s) or field(s) under stipulated conditions (same person, different persons in one laboratory, or different laboratories)

[SOURCE: ISO 6107-6:2004, 103, modified — The domain has been deleted.]

4 Principle

The general principle of these techniques consists of inoculating a test portion of a water sample, or resultant test sample following membrane filtration or centrifugation, on or into a culture medium (solid or liquid). It is assumed that after incubation each target microorganism present multiplies, giving either a colony visible directly on or in the solid medium or changes in the observable properties of the liquid medium. The choice of a particular culture method depends not only on the nature and numbers of the microorganisms sought, but also on the nature of the water and the reasons for the examination.

5 General measurement requirements

5.1 Uniformity of temperatures

The following accepted ranges of temperatures and their ranges for incubation or storage are applied, where appropriate for the intended target organism and unless otherwise required in the specific standard.

Storage temperatures: (−70 ± 10) °C; (−20 ± 5) °C; (5 ± 3) °C

Incubation temperatures: (22 ± 2) °C; (36 ± 2) °C; (44 ± 0,5) °C

Media tempering temperature: 44 °C to 47 °C

The upper incubation temperature limits shall be followed strictly to ensure optimal growth. The lower temperature limits may be exceeded for short periods, e.g. due to opening the door of an incubator, but recovery to the operating temperature should be rapid.

5.2 Incubation times

The following accepted ranges of incubation times are applied, where appropriate for the intended target organism and unless otherwise required in the specific standard.

Incubation times: (21 ± 3) h; (44 ± 4) h; (68 ± 4) h

5.3 Volumes and masses

Measuring equipment shall be appropriate to the required accuracy and precision. The accepted range of any measured value is ±2 % where the stated value is critical to method performance and test results, and ±5 % where the stated value has been shown not to be critical. Examples of critical values having a direct effect on test results are inoculum and diluent volumes. For tolerances relating to the mass of ingredients used to prepare culture media, refer to ISO 11133.

NOTE Critical tolerances have been set at 2 % to minimize the uncertainty of test results.

6 Diluents and culture media

6.1 General

General requirements for preparation, production, sterilization, storage and performance of culture media are given in ISO 11133.

For preparation of microbiological culture media, unless otherwise stated, add the ingredients to the volume of water, rather than make the ingredients up to a certain volume.

Before use, check the quality of the culture media, diluents, membrane filters and reagents by following the procedures described in ISO 11133 and ISO 7704 or as given in the specific standard.

For information on storage of culture media, refer to ISO 11133.

6.2 Quality requirements of ingredients

Use constituents of uniform quality and analytical grade chemicals for the preparation of media. Other grades of chemicals may be used provided they can be shown to produce equivalent results. Alternatively, dehydrated complete media or diluents may be used. Follow the manufacturer's instructions strictly.

Refer to ISO 11133 and ISO 3696[2] for further information on the quality of ingredients and the quality of water that should be used for media preparation.

6.3 Diluents

The following diluents are commonly used in water microbiology. However, other appropriate diluents may be used and this list is not exhaustive:

— saline solution;

— peptone diluent;

— peptone saline solution [maximum recovery diluent (MRD)];

— quarter-strength Ringer's solution;

— phosphate buffer solution.

Follow the formulations and the preparation, storage and performance testing instructions given in Annex D for these diluents.

7 Sterilization and decontamination

7.1 Sterilization of apparatus and glassware

Sterilize apparatus and glassware not supplied sterile by one of the following methods:

a) in an oven, operating at (170 ± 10) °C for at least 1 h (excluding pre-heating time);

b) in an autoclave, operating at (121 ± 3) °C for at least 15 min.

Some heat labile items may require sterilization by other means (e.g. ultraviolet light or irradiation) but these are not carried out in the routine laboratory.

7.2 Sterilization of consumables

Sterile disposable equipment and materials may be used instead of re-usable items (glassware, Petri dishes, pipettes, bottles, tubes, loops, spreaders, etc.) if the specifications are similar.

If membrane filters are not obtained sterile, these are usually sterilized by moist heat according to process b) described in 7.1, or by following the manufacturer's instructions.

7.3 Decontamination of glassware and materials after use

Materials for decontamination and disposal should be placed in appropriate containers, e.g. autoclavable plastic bags. Autoclaving is the preferred method for all decontamination processes (at least 30 min at 121 °C). The autoclave should be loaded in a way that favours heat penetration into the load (e.g. without over packing). Take care to loosen caps/lids and open bags to prevent dangerous pressurization of the container, which could lead to possible breakage, e.g. explosion of glass bottles.

Modern autoclaves may not require caps to be loosened, but follow the manufacturer's instructions strictly to avoid dangerous pressurization of the containers.

Alternative methods other than autoclaving may be used.

Autoclave all equipment which has been in contact with microbiological cultures (solid or liquid culture media), including re-usable containers prior to being washed.

During examination, decontamination by immersion in freshly prepared disinfectant, prepared at the correct dilution, may be used for small-sized and corrosion resistant equipment (e.g. pipettes).

Pasteur pipettes may be difficult to clean and are usually discarded after a single use.

Most disinfectants have some toxic effects. Wear gloves and eye protection when handling disinfectant and follow the manufacturer's instructions.

7.4 Waste management

The correct disposal of contaminated materials does not directly affect the quality of sample analysis, but it is a matter of good laboratory management. A system for identification and separation of waste materials and their containers should be established for:

— non-contaminated waste (e.g. uncultured water samples) that can be disposed of using general waste streams;

— scalpels, needles, knives and broken glass;

— contaminated materials for autoclaving and recycling;

— contaminated materials for autoclaving and disposal, or for disposal only if the material is to be incinerated.

Materials contaminated with risk category 3 microorganisms and their containers shall be autoclaved before they are incinerated.

8 Samples and sample handling

8.1 Sampling

Take samples in accordance with ISO 19458. Collect disinfected water samples in sample bottles containing suitable and sufficient neutralizers.

8.2 Sample preparation

8.2.1 Waters and other aqueous matrices

Clean and dirty water should be separated and processed using separate equipment in separate areas to reduce cross-contamination risk where possible. Alternatively, process batches of clean waters before dirty water.

Before examination, mix the sample thoroughly by agitation to achieve uniform distribution of microorganisms and other particles. This can be achieved by inversion of the sample or by a to-and-fro motion. Depending on the nature of the water and the microbial content anticipated, make any necessary dilutions at this stage.

For plate counts, ten-fold dilutions are usually used. For membrane filtration (with a smaller surface area), smaller dilution steps are recommended. For many most probable number (MPN) techniques, dilutions are an inherent part of the procedure. Refer to 9.2.3 for guidance on dilutions in MPN techniques. See ISO 6887-1[7] for general guidance on the preparation of serial dilutions.

For ten-fold dilutions, aseptically measure nine volumes of the diluent and one volume of the water sample into sterile dilution bottles or tubes. Alternatively, volumes of diluent pre-sterilized in screw-capped bottles are used and volumes verified after autoclaving. One or more ten-fold dilutions are made by transferring one volume of water sample to nine volumes of diluent. Mix the solution thoroughly with a fresh pipette or by mechanical means and transfer one volume of this dilution to another nine volumes of diluent. Repeat these steps as many times as required. Prepare sufficient volumes of each dilution for all the tests to be carried out on each water sample.

For dilutions of other magnitudes, the volume of diluent to volume of sample is adjusted accordingly. For example, four-fold dilutions can be made as described above for ten-fold dilutions, only in this case one volume of water sample is mixed with three volumes of diluent. Another approach is to use a ten-fold dilution series, but filter both 10 ml and 30 ml volumes.

If the concentration of the target organism is expected to be high, hundred-fold dilution steps may be used by mixing one volume of water sample with 99 volumes of diluent, but such large intervals between measurements can adversely affect the reliability of the test results.

8.2.2 Swabs

8.2.2.1 General

Swabs are sometimes used for assessing water quality, e.g. investigation of biofilms, and may be tested by both quantitative and qualitative methods.

Different types of swabs are available for specific purposes, including stick and sponge swabs, with and without neutralizing agents for any disinfectants present. Where transport times before testing are likely to be extended, special transport swabs giving some protection to the organisms present are recommended. All types of swabs should be evaluated before use, as some have been found to be inhibitory to certain microorganisms. Further guidance on the use of swabs and swabbing techniques can be found in ISO 18593[18].

NOTE Dry stick swabs are not suitable for microbiological testing because they offer no protection against dehydration or disinfectant residues for any microorganisms present during transport and storage before testing.

8.2.2.2 Stick swab preparation

Stick swabs in neutralizing solution are mixed thoroughly, either manually or by vortexing, in the solution.

Transport swabs in agar are carefully removed from the packaging and immersed in a volume (usually 10 ml) of diluent. The stick is then broken or cut off to allow thorough mixing in the sealed bottle or

tube. Mix either manually or by other means, such as vortexing, shaking or ultrasonication, to optimize the recovery of any organisms present.

In both cases, the resulting suspensions are used as the initial dilution and the results are expressed accordingly.

8.2.2.3 Sponge swab preparation

Sponge swabs are recommended for qualitative testing to ensure a representative sample is taken and a “not detected” result is more reliable based on the larger sample. These contain a volume of neutralizer sufficient to protect any organisms present during transport, which makes their use for quantitative testing problematic, unless this volume is known and can be included in subsequent calculations.

On receipt at the laboratory, a larger volume (usually 100 ml) of (pre-)enrichment broth is added to the sponge swab in the original packaging and testing continued according to the specific method.

9 Enumeration (quantitative) methods

9.1 Inoculation of test portions in (or on) solid media

9.1.1 General

A test portion of the water sample or any dilutions prepared is inoculated, either directly or concentrated on a membrane filter, on the surface of a specified solid culture medium or in a molten medium so that once incubated, microorganisms form colonies either on or in the medium.

For practical purposes, each colony is considered to have originated from a single microorganism or a clump of microorganisms present in the test portion at the time of inoculation. Taking into account the volume of the test portion and the number of colonies formed, the result can therefore be expressed as a number of colony-forming units (cfu) or colony-forming particles (cfp) in a given volume of the sample, e.g. 1 ml or 100 ml.

Three procedures are used predominantly for the inoculation of solid media and the choice of technique depends on several factors. These include the physical and chemical characteristics of the water as well as the nature of the microorganisms sought, their probable concentration, the effective recovery of stressed or (sub-lethally) injured microorganisms, and the test precision and sensitivity required. Indications are given in 9.1.2.2, 9.1.3.2 and 9.1.4.2 of the volumes of water samples that may be used for each technique.

NOTE 1 Limits of determination and the accuracy of the various techniques are discussed in A.2.

NOTE 2 The nature of the sample and organisms sought are discussed in A.3.

9.1.2 Pour plate technique

9.1.2.1 General

The test portion is mixed with the medium, which has previously been melted and cooled (tempered) to a temperature close to that of solidification, i.e. 44 °C to 47 °C, so that heat damage to organisms is minimized. After incubation, the colonies that develop within and on the surface of the medium are counted.

9.1.2.2 Test portion

The volume of the test portion of the sample, or of a dilution of the sample, can vary between 0,1 ml and 5 ml depending on the size of the Petri dish and the volume of culture medium used. The dilution should be chosen so that the expected number of colonies formed on plates of 90 mm diameter is less than 300, and the number of target colonies is greater than 10.

The acceptable maximum number of target colonies on a plate will depend on the specific method, colony size, the nature of the colonies (e.g. spreading) and the presence of non-target colonies, and may be based on the results of verification exercises. As guidance, the maximum number of colonies on a 90 mm plate (both target and non-target) is typically regarded as 300 (refer to ISO 7218[8]). Larger (such as 140 mm) dishes need separate consideration (see 9.1.7.2).

9.1.2.3 Inoculation

Melt the medium required in boiling water or by any other process (e.g. a steam flow-through autoclave or microwave oven, if the heating time/temperature combination has been verified for media preparation). Loosen caps before heating, avoid over-heating and remove the medium as soon as it has melted. Place the molten medium in a water bath or incubator at 44 °C to 47 °C for sufficient time, depending on the numbers and volumes of containers, so that the medium will equilibrate to this temperature throughout. Verify the time required for tempering agar for all quantities and volumes routinely used. Do not keep heat-sensitive molten media for more than 4 h (or the maximum time stated in the specific standard) as the quality may be reduced after this time. Do not melt agar media more than once.

Waterbaths are likely to contain microorganisms, which could be a source of contamination of molten media. Regularly changing the water and cleaning is advised to minimize this risk.

Prepare and mark the Petri dishes required with sample and other details. Make any dilutions necessary in accordance with 8.2.1. Distribute the test portions into the dishes after thorough mixing.

Remove each tube or flask of tempered medium from the water bath in turn; dry the outside of the tube or flask completely and flame the neck. Add the medium to each Petri dish without delay, avoiding the test portion to minimize heat shock, and mix carefully to obtain a uniform distribution of microorganisms. Generally, (18 ± 2) ml of medium is used for a test portion of up to 1 ml in 90 mm Petri dishes. Alternatively, if demonstrated as suitable by the laboratory, a minimum of 10 ml medium may be used if the samples are bagged for incubation. Leave the plates to cool on a horizontal surface to solidify the agar. As soon as the agar is set, incubate the plates in accordance with 9.1.5.

Agar preparators, pourers and shaker systems may be useful in laboratories analysing large numbers of samples (see ISO 7218[8]).

9.1.3 Spread plate technique

9.1.3.1 General

The test portion is spread over the dry surface of an agar medium with a sterile implement and colonies that develop on the surface after incubation are counted.

9.1.3.2 Test portion

For a Petri dish of 90 mm diameter, the volume of the test portion of the sample, or of a dilution of the sample, should be 0,1 ml to a maximum of 0,5 ml. For optimal precision, choose the dilution so that the expected number of colonies formed is less than 300, and the number of target colonies is greater than 10.

The acceptable maximum number of target colonies on a plate will depend on the specific method, the colony size, the nature of the colonies (e.g. spreading) and the presence of non-target colonies, and may be based on the results of verification exercises. As guidance, the maximum number of colonies on a 90 mm plate (both target and non-target) is typically regarded as 300 (refer to ISO 7218[8]). Larger (such as 140 mm) dishes need separate consideration (see 9.1.7.2).

Spiral platers use smaller volumes and the limit of determination will consequently be raised. Follow the manufacturer's guidance for use of spiral platers. Spiral platers may not be suitable for fungi.

9.1.3.3 Inoculation

Prepare and mark the plates required, each containing (18 ± 2) ml of culture medium for 90 mm Petri dishes, with sample and other details. For longer incubation periods (e.g. incubation periods longer than the example incubations periods stated in 5.2), larger volumes of culture media might be required. In this case, refer to ISO 11133 for guidance on culture medium volumes. Dry the surface of the medium if necessary before use as described below. Pipette the test portion onto the surface of the medium and spread over the surface with a sterile implement, or mechanical device such as a spiral plater, avoiding the edges of the agar. Leave the plates on the bench until the inoculum is absorbed (maximum time 15 min), then incubate the plates in accordance with 9.1.5.

For drying plates, the following points are important.

— The degree of humidity in culture media, because optimum growth of microorganisms will depend on the humidity conditions in and on the medium. Extensive humidity loss may lead, for example, to an increase in the concentrations of inhibitors in selective culture media and a reduction in the water activity at the surface of the medium.

— When bacteria, which do not swarm rapidly, are cultured and the plates appear to be dry after reaching ambient temperature, drying is not always necessary. In this case, drying can be omitted as it only increases the likelihood of contamination and unnecessary humidity loss.

— Select the temperature and drying time so that the likelihood of contamination is kept as low as possible and excessive heating will not reduce the quality of the culture medium. The drying time depends on the amount of condensation in the Petri dish, but shall be kept as short as possible.

Plates shall, wherever possible, be dried with the surface of the medium downwards to avoid airborne contamination.

Sufficiently dry plates can usually be obtained by placing them with lids half open in an incubator or drying cabinet set at a temperature between 25 °C and 50 °C. Dry the plates until moisture droplets have disappeared from the lids, but do not dry any further. Agar plates are also dried (positioned as described above) in a laminar flow cabinet at room temperature for 30 min to 60 min, or on the open bench but drying times may be longer. Storage of plates inverted but with the lids still in place overnight at room temperature is also effective if the plates are freshly poured and in vented Petri dishes.

9.1.4 Membrane filtration technique

9.1.4.1 General

The test portion is passed through a membrane filter, which retains the microorganisms present. The membrane filter is then placed on an agar medium. On incubation, colonies form on the surface of the membrane filter. Alternatively, for certain organisms such as anaerobes, the membrane filter can be placed face downward in a Petri dish or on a thin layer of agar and overlaid with molten agar medium.

9.1.4.2 Test portion

The maximum volume of the test portion depends on the filterability of the water sample and on the membrane filters used. This technique is suitable for waters that contain little particulate or colloidal (e.g. iron) matter in suspension, e.g. water intended for drinking. It may be possible to filter several litres of such water, achieving a high level of test sensitivity.

The test volume of the sample or dilution should be chosen so that the expected number of colonies formed on a membrane filter of 47 mm to 50 mm in diameter is less than 80, and the number of target colonies is greater than 10.

The acceptable maximum number of target colonies on a plate will depend on the specific method, colony size, the nature of the colonies (e.g. spreading) and the presence of non-target colonies, and may be based on the results of verification exercises. As guidance, based on the regarded maximum

of 300 colonies on a 90 mm plate, a membrane filter with a diameter of 47 mm would give an example maximum count of 80 colonies (target and non-target) based on the comparative surface area.

9.1.4.3 Filtration apparatus

Membrane filters with a mean pore size of 0,45 µm are commonly used in water microbiology. For some microorganisms, filtration through a membrane filter with a mean pore size of 0,2 µm may be required by the specific standard. Evaluation of the suitability of different types and batches of membrane filters before use is described in ISO 7704.

The filtration apparatus usually consists of a filtration manifold with a stopcock, membrane filter bases, membrane filter funnels, a vacuum source and a vessel to collect the filtered water. The vacuum source should be approximately 70 kPa. A vacuum that is too strong may compromise the performance of the membrane filter. The membrane filter funnels should have visible graduations that are appropriate to the volumes of sample typically analysed. Membrane filter funnels should be sterilized by autoclaving before use, or sterile single use funnels may be used as an alternative. Membrane filter funnels may be disinfected by boiling or direct flaming before use where appropriate.

When spore formers are sought (e.g. *Clostridium perfringens*), disinfection of the membrane filter funnels by boiling may not remove all spores. A separate, sterile filter funnel should be used. Alternatively, other disinfection techniques may be used if stated in the specific standard or have been verified as acceptable.

9.1.4.4 Filtration

Place a sterile membrane filter, grid-side upwards, on the porous disc of the sterile membrane filter base, taking care that only the outer edge of the membrane filter is grasped with sterile flat-ended forceps. Position the sterile funnel securely on the filter base. Pipette or pour one of the following into the funnel (with the vacuum stopcock turned off):

a) known volume of the sample, or dilution of it, carefully mixed (at least 10 ml);

b) the contents of a flask or bottle containing the test portion and sufficient diluent to bring the total volume to at least 10 ml;

c) at least 10 ml of diluent to which the test portion, measured with a pipette, is added directly and mixed with the pipette.

Open the stopcock and apply the vacuum to filter the water through the membrane filter. Close the stopcock as soon as the sample has been filtered. It may be advisable to rinse the funnel by filtering one to three 10 ml to 30 ml portions of sterile diluent, while the filter is still in place to remove organisms adhering to the funnel. Positive pressure can also be used in certain circumstances, such as testing for *Legionella* spp.

9.1.4.5 Transfer of membrane filter

Remove the funnel after closing the stopcock and transfer the membrane filter with sterile flat-ended forceps in one of the following ways, ensuring that no air bubbles are trapped between the membrane filter and the medium:

a) grid upwards onto an agar medium in a Petri dish;

b) grid downwards or upwards into a Petri dish, or onto 5 ml to 10 ml agar medium in a 50 mm or 90 mm Petri dish (for anaerobes); then overlay the membrane filter with molten agar medium (44 °C to 47 °C) as quickly as possible to avoid the membrane filter drying out and excessive contact with air.

For different volumes of the same sample, the funnel may be re-used without disinfection provided that the smallest volumes and/or the most diluted sample are filtered first. To filter another sample, either a separate sterile apparatus shall be used, or where appropriate the funnel can be disinfected,

for example, by direct flaming or immersion in a boiling water bath. Alternatively, the manufacturer's instructions for disinfection should be followed.

9.1.4.6 Membrane transfer techniques using liquid media or diluents

As an alternative to an agar plate, if stated by the relevant standard, the membrane filter may be placed grid upwards onto a sterile absorbent pad, previously saturated with a liquid medium or a dehydrated medium pad reconstituted with sterile water in a Petri dish. To avoid confluent growth, any excess liquid should be poured off, preferably before placing the membrane filter on the pad.

Certain methods, such as MPN culture and qualitative methods, may require the membrane filter to be transferred directly into a pre-enrichment or enrichment broth.

The membrane filter may also be used as part of a concentration system and may be washed (eluted) with the help of a diluent to obtain a new test portion to spread over the dry surface of an agar medium (see 9.1.3) (e.g. testing for *Legionella* spp. by membrane filtration with washing). The membrane filter should be transferred directly into the diluent then eluted and cultured as specified in the relevant standard.

9.1.5 Incubation

Invert the inoculated agar plates and place them either in an incubator or in a water-tight container in a water bath. If necessary, pack the plates in plastic bags or other containers to prevent desiccation of the medium (e.g. when a fan-assisted incubator is used). Do not stack Petri dishes more than six high to ensure that all plates reach incubation temperature rapidly, and leave space between the stacks for air circulation. However, plates may be stacked higher in specially designed racks or jars when fan-assisted incubators are used and the correct incubation temperatures for the required times have been verified.

Plates containing membrane filters on absorbent pads are placed lids uppermost in an air or water-tight container to prevent desiccation of the medium. Plates for spore-forming microorganisms above the agar surface (such as moulds or actinomycetes) shall also not be inverted.

NOTE Moulds spores can spread from Petri dishes to the surrounding dishes or the incubator when moving the Petri dishes. Sealing the lid of the Petri dish to the base can help to prevent this.

If anaerobic incubation is required, the inoculated agar plates should be placed in an anaerobic incubator, or an air-tight anaerobic jar containing an anaerobic generator of a suitable size for the volume of the jar. In addition, a suitable means of establishing that anaerobic conditions have been achieved should also be included in the jar or incubator.

Choose the duration and temperature of incubation after reference to the specific standard method as these will depend on the microorganisms, or groups of microorganisms, sought.

Membrane filters are sometimes incubated for a limited period (e.g. 2 h or 4 h) on a resuscitation medium and then transferred to another medium, which is usually selective, for further incubation.

9.1.6 Counting and confirmation from solid media

9.1.6.1 General

Examine the plates or membrane filters as soon as possible after incubation and in accordance with the specific standard. If this is not possible, they may be kept at (5 ± 3) °C for short periods (up to 24 h) provided that this does not affect the numbers, appearance or the subsequent confirmation of the colonies. The acceptable storage period shall be verified for each method and sample type.

In some cases where large (e.g. *Pseudomonas aeruginosa*) or spreading colonies are anticipated, it can be useful to examine the plates after a shorter period of incubation while discrete colonies are still visible; then continue incubation for the specified period before final counting.

9.1.6.2 Colonies to be counted and confirmed

For total counts on a non-selective medium, all colonies are counted. With selective and differential media, only those colonies that show the typical appearance of the organism sought are counted (see specific standard for description). Magnification may be used (unless otherwise stated) when colony size is small and/or when it is otherwise difficult to differentiate colonies from other particles or non-target colonies. In certain cases, it can be difficult to count the colonies (e.g. where spreading microorganisms are present). Consider spreading colonies as single colonies. If less than one-quarter of the plate is overgrown by spreading growth, count the colonies on the unaffected part and calculate, by extrapolation, the theoretical number of colonies for the entire plate. If more than one-quarter is overgrown, discard the count. Consider a chain of colonies as one colony forming unit. In practice, it is unusual for selective counts to indicate the microorganisms belonging only to one group, but this discrepancy may be accepted and the results expressed as presumptive in some cases. For more precise characterization, confirmatory tests are necessary.

When selecting colonies for confirmation, the approach adopted should take into account the purpose of the testing. For example, in investigation situations or comparison trials it may be preferable to confirm all colonies present (both target and non-target). In other situations (e.g. monitoring or research), it might be more important to establish the diversity of the target colonies present than to establish a precise confirmed count. In most testing scenarios, however, where a confirmed count is required, confirmation of all (or a randomly selected subset) of the presumptive target colonies as described in the following paragraph should be the adopted approach.

If there are numerous colonies it is impractical to confirm the identity of all of them in routine analysis. Isolate all colonies when the total number is between 1 and 10, and at least 10 colonies when the total number of colonies is greater than 10. If the presumptive colonies show only one type of morphological appearance, all presumptive colonies from a sub-area of the plate or membrane containing at least 10 colonies should be taken to avoid subjectivity in selection. If there are different presumptive colony types on the plate, each colony type should be tested for confirmation with all or a minimum of 3 colonies per type, unless stated otherwise in the specific standard.

Taking into consideration type and source (e.g. potable or non-potable water) of the sample, as well as the aim of the analysis (e.g. hygienic monitoring or scientific research), a subset of presumptive colonies can be chosen for the confirmation steps.

9.1.7 General guidance for calculation of results

9.1.7.1 General

To a large extent, 9.1.7 and 9.1.8, and the corresponding Annex C when duplicate plates are used, are based on ISO 7218:2007/Amd.1:2013[9], and have been adjusted for water microbiology techniques, such as membrane filtration, and to take account of the use of dilutions other than ten-fold dilutions. The methods of calculation below cover the cases that occur most frequently when tests are carried out in accordance with good laboratory practice. Rarely, special cases can occur (e.g. significant discrepancy between the number of colonies in two plates at the same dilution, as discussed in Annex C, or a very different ratio to that of the dilution factor between the plates of two successive dilutions). The results obtained from counting shall therefore be examined, interpreted and rejected, if necessary, by a qualified microbiologist.

9.1.7.2 General case

The calculation rules and examples given in 9.1.8, for results from single plates at each dilution, are used where the number of typical colonies on the plates is between 10 and 300 (pour plate techniques and spread plate techniques), or between 10 and 80 (membrane filtration technique).

As described in 9.1.2.2, 9.1.3.2 and 9.1.4.2, acceptable maximum numbers of both target and non-target colonies are dependent on many factors, such as the specific method, colony size and nature of the colonies (e.g. spreading), and may be based on the results of verification exercises. Example upper

counting limits for target and for total colonies may be regarded as 300 for a 90 mm plate and 80 for a 47 mm membrane filter.

When dishes with a different diameter are used, the example maximum number of colonies specified to count shall be increased or decreased in proportion to the surface area of the dishes (or membrane filters).

NOTE For poured or spread plates with a diameter of 55 mm, the maximum countable number of colonies would be 110 (equivalent to a total count of 300 cfu on a 90 mm Petri dish). For poured or spread plates with a diameter of 140 mm, the maximum countable number of colonies would be 730 (equivalent to a total count of 300 cfu on a 90 mm Petri dish).

In the various methods of calculation given in 9.1.8, account shall be taken of dishes containing no colonies, where these dishes have been retained. When a spiral plater has been used, follow the manufacturer's instructions for colony counting.

Since each colony is assumed to have arisen from one microorganism or from a single aggregate of microorganisms, the result is expressed as the number of colony-forming units (cfu) or colony-forming particles (cfp) in a specified reference volume of the sample (generally 100 ml or 1 ml).

Using duplicate plates increases precision and may enable easier detection of departures from expected colony counts or make contamination more readily apparent. When duplicate plates are used, follow the calculation rules and examples provided in Annex C.

9.1.7.3 Case with confirmation

When the method used requires identification or confirmation, all presumptive colonies should ideally be inoculated from each of the plates retained for counting colonies. When this is impractical due to large numbers, to avoid subjectivity in colony selection, all presumptive colonies from a predefined sub-area of the plate or membrane filter should be examined. If different presumptive colony types are present, examine at least 3 colonies of each presumptive colony type (see 9.1.6.2).

After identification or confirmation, calculate the confirmed result for each of the plates as follows:

a) for each colony type found, determine the proportion of presumptive colonies complying with the identification or confirmation criteria (see 9.1.8.3);

b) for each colony type, multiply the presumptive count by the proportion that has been confirmed to arrive at the confirmed count;

c) sum all of the confirmed counts.

9.1.8 Expression of results

9.1.8.1 General

The cases dealt with in this subclause are general cases:

— inoculation of one Petri dish per dilution, if at least two successive dilutions are performed;

— maximum countable number for the total colonies present adheres to the example upper colony limits described in 9.1.7.2;

— number of presumptive colonies inoculated for confirmation (see 9.1.7.3) in each dish retained complies with the criteria in 9.1.6.2.

If more than one dilution of a ten-fold dilution series is used, the ratio between the colony count of dilution d_1 and the colony count of dilution d_2 is expected to be 10 % but, in general, the colony count of dilution d_2 should not be less than 5 % or greater than 20 % of the colony count of dilution d_1. If this is not the case, the result needs to be interpreted with caution (see also ISO 14461-2[14]). For example,

if a colony count of dilution d_1 is 250, the colony count of dilution d_2 should not be less than 12 or more than 50. Special cases such as this are described in 9.1.8.6.

Round off the calculated result to two significant figures. When doing this, if the third figure is less than 5 do not modify the preceding figure; if the third figure is greater than or equal to 5, increase the preceding figure by one unit.

Express the result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 or a whole number with two significant figures.

Report the result as the number N of microorganisms per a specified reference volume (generally 1 ml or 100 ml).

9.1.8.2 Method of calculation: General case (counting of total colonies or target colonies)

For a result to be quantitatively valid, it is generally considered necessary to count the colonies on at least one Petri dish containing at least 10 colonies [total colonies, target colonies or colonies complying with confirmation criteria (see 9.1.8.3)]. Methods of calculation when Petri dishes contain lower counts are described in 9.1.8.4.

Calculate the number N of microorganisms present in the test sample as a weighted mean from usually two successive dilutions or two particular volumes from one dilution using Formula (1). More levels of dilution or more volumes from one dilution may be used:

$$N = \frac{\sum C}{\sum V} \times V_s \qquad (1)$$

where

V_s is the reference volume chosen to express the concentration of the microorganisms in the sample, in millilitres, ml;

$\sum C$ is the sum of the colonies counted on the dishes retained from successive dilutions or particular volumes from one dilution, at least one of which contains a minimum of 10 colonies;

$\sum V$ is the sum of volumes of original sample used in all dishes together, taking into account any dilutions, in millilitres, ml. This is calculated using Formula (2):

$$\sum V = (V_1 \times d_1) + (V_2 \times d_2) + \ldots + (V_i \times d_i) \qquad (2)$$

where

$V_1, V_2, \ldots, V_i$ is the volume of inoculum placed in the dish from the original sample or dilutions (e.g. the first dilution, second dilution), in millilitres, ml;

$d_1, d_2, \ldots, d_i$ is the dilution level of the first dilution, second dilution, etc. for the respective test volumes ($d = 10^0 = 1$ when the undiluted liquid test portion is retained). Often, but not necessarily, d_2 is a tenfold dilution of d_1, etc.

EXAMPLE 1 Counting of plates analysed by the pour plate technique with the reference volume $V_s = 1$ ml has produced the following results.

At the first dilution retained ($d_1 = 10^{-2}$): 168 colonies.

At the second dilution retained ($d_2 = 10^{-3}$): 14 colonies.

1 ml from each dilution is used as inoculum (V_1 and V_2 are both equal to 1).

Thus: $\sum V = \left(1 \times 10^{-2}\right) + \left(1 \times 10^{-3}\right)$

$$\sum V = 0{,}01 + 0{,}001 = 0{,}011$$

$$N = \frac{\sum C}{\sum V} \times V_s = \frac{168 + 14}{0{,}011} \times 1 = \frac{182}{0{,}011} \times 1$$

$$N = 16\,545$$

Rounding off the results, as specified in 9.1.8.1, the number of microorganisms is:

N = 17 000 or $N = 1{,}7 \times 10^4$ CFU per ml of sample.

EXAMPLE 2 Counting of plates analysed by the membrane filtration technique with the reference volume V_S = 100 ml has produced the following results.

At the first test volume analysed (100 ml): 72 colonies.

At the second test volume analysed (10 ml): 11 colonies.

Undiluted sample was used for both test volumes (d_1 and d_2 are both equal to 1).

Thus: $\sum V = (100 \times 1) + (10 \times 1)$

$$\sum V = 100 + 10 = 110$$

$$N = \frac{\sum C}{\sum V} \times V_s = \frac{72 + 11}{110} \times 100 = \frac{83}{110} \times 100$$

$$N = 74{,}45$$

Rounding off the result, as specified in 9.1.8.1, the number of microorganisms is:

N = 75 or $N = 7{,}5 \times 10^1$ per 100 ml of sample.

9.1.8.3 Method of calculation: General case with confirmation

When the method used requires confirmation, a given number A (generally 10) of presumptive colonies are confirmed from each of the Petri dishes retained for colony counting according to 9.1.6.2. After confirmation, calculate, for each of the dishes, the number a of colonies complying with confirmation criteria, using Formula (3):

$$a = \frac{b}{A} \times C \tag{3}$$

where

A is the number of presumptive colonies inoculated;

b is the number of colonies complying with confirmation criteria;

C is the total number of presumptive colonies counted on the dish.

Round off the calculated result to the nearest whole number. When doing this, if the first figure after the decimal sign is less than 5, do not modify the preceding figure; if the first figure after the decimal sign is greater than or equal to 5, increase the preceding figure by one unit.

With the following Formula (4), calculate the number N of confirmed microorganisms present in the test sample by replacing $\sum C$ by $\sum a$ in Formula (1) (9.1.8.2):

$$N = \frac{\sum a}{\sum V} \times V_s \qquad (4)$$

where $\sum a$ is the sum of the colonies complying with confirmation criteria from successive dilutions or particular volumes from one dilution, at least one of which contains a minimum of 10 colonies.

Round off the result and express it as specified in 9.1.8.1.

EXAMPLE 1 Counting of plates analysed by the pour plate technique with the reference volume V_s = 1 ml has produced the following results.

1 ml from each dilution is used as inoculum (V).

At the first dilution retained ($d_1 = 10^{-3}$): 66 colonies.

At the second dilution retained ($d_2 = 10^{-4}$): 4 colonies.

Confirmation of selected colonies was carried out:

— of the 66 colonies, 10 were tested, 6 of which complied with the criteria, hence a = 40;

— of the 4 colonies, all complied with the criteria, hence a = 4.

$$\sum V = \left(1 \times 10^{-3}\right) + \left(1 \times 10^{-4}\right) = 0{,}001\ 1$$

$$N = \frac{\sum a}{\sum V} \times V_s = \frac{40 + 4}{0{,}001\ 1} \times 1$$

$$N = 40\,000$$

Rounding off the results, as specified in 9.1.8.1, the number of microorganisms is:

N = 40 000 or $N = 4{,}0 \times 10^4$ CFU per ml of sample.

EXAMPLE 2 Counting of plates analysed by the membrane filtration technique with the reference volume V_s = 100 ml has produced the following results.

At the first test volume analysed (100 ml): 72 colonies.

At the second test volume analysed (50 ml): 30 colonies.

Undiluted sample was used for both test volumes (d_1 and d_2 are both equal to 1).

Confirmation of selected colonies was carried out:

— of the 72 colonies, 10 were tested, 4 of which complied with the criteria, hence a = 29;

— of the 30 colonies, 10 were tested, 6 of which complied with the criteria, hence a = 18.

$$\sum V = (100 \times 1) + (50 \times 1) = 150$$

$$N = \frac{\sum a}{\sum V} \times V_s = \frac{29 + 18}{150} \times 100$$

$$N = 31{,}333$$

Rounding off the result, as specified in 9.1.8.1, the number of microorganisms is:

N = 31 per 100 ml of sample.

If multiple morphological colony types are taken for confirmation (see 9.1.7.3), carry out the calculations for each morphological type and sum the results to give the total number of colonies complying with the confirmation criteria.

9.1.8.4 Method of calculation: Low counts

9.1.8.4.1 Case when one Petri dish contains fewer than 10 colonies

If the Petri dish containing the test sample (liquids) or the initial suspension (other samples) or the first dilution inoculated or retained contains fewer than 10 colonies, the results should be reported as follows:

— for 3 to 9 colonies, the result should be reported as an estimate;

— for 1 or 2 colonies, the result should be reported as presence.

Counts from 10 up to the (practical) upper limit of each method are in the optimal precision range. Precision decreases rapidly as the number of colonies decreases below 10. Thus, the lower limit of determination can be defined for a count of 10.

According to ISO 13843:2017, 3.7[13], the definition of limit of determination is the "lowest analyte concentration [x] per analytical portion where the expected relative standard uncertainty equals a specified value [RSD]". RSD is the relative standard deviation, which is calculated by dividing the estimate of the standard deviation s for a population from a sample by the mean $\overline{x}$ for that sample. Instead of RSD, the symbol u_{rel} will be used for the relative standard deviation. Thus, $u_{rel} = s/\overline{x}$.

In the case of a Poisson distribution, x is calculated by Formula (5):

$$x = \frac{1}{(u_{rel})^2} \qquad (5)$$

In microbiology, 10 is generally accepted as the lowest reliable count and therefore the lower limit of determination. The corresponding acceptable relative precision at the limit of determination of 10 can be considered as the RSD (u_{rel}), which using Formula (5) is 32 %:

$$u_{rel} = \sqrt{\frac{1}{x}}$$

$$u_{rel} = \sqrt{\frac{1}{10}} = 0{,}32 \text{ (or 32 \%)}$$

Thus, results based on counts of less than 10 should be treated as mere detection of the presence of the organism.

At the average count of 3 particles per test portion, provided that the Poisson distribution prevails, the chance of detecting the presence of the analyte (i.e. observing at least 1 colony) equals 95 %. Therefore, this average count of 3 is the detection level. This is described below.

The probability of a positive result $p(+)$ when the Poisson distribution prevails can be calculated from:

$$x = \frac{1}{(u_{rel})^2}$$

Solving the formula for x gives Formula (6):

$$x = -\ln\left[1 - p(+)\right] \tag{6}$$

where

e is the base of natural logarithms;

x is the number of particles per analytical portion.

According to the above formula, $x = -\ln(1 - 0{,}95) = -\ln(0{,}05) = 3{,}0$.

In summary, for results below the detection level, the presence of the target organism cannot be efficiently detected. For results below the limit of determination, but equal to or above the detection level, the presence of the target organism can be detected efficiently, but not quantified efficiently. Therefore, if the plate contains less than 10, but at least 3 colonies, calculate the result using Formula (7):

$$N_E = \frac{C}{V \times d} \times V_s \tag{7}$$

where

N_E is the estimated number of microorganisms per volume analysed;

C is the number of colonies counted on the dish retained, which contains a minimum of 3 colonies;

V is the volume of inoculum or test sample used in the dish, in millilitres, ml;

d is the dilution corresponding to the dilution retained (d = 1 when the undiluted liquid test portion is retained);

V_s is the reference volume chosen to express the concentration of the microorganisms in the sample in millilitres, ml.

Therefore, when 3 to 9 colonies are found, report the result as an estimated number in the volume studied.

NOTE The term "estimated number" means a less precise estimate of the true value.

If the total is 1 or 2, the precision of the result is so low that the result shall be reported as:

"Microorganisms are present in the volume studied".

If a numerical result is required by legislation, e.g. for monitoring purposes, then report an estimated numerical value with an indication of the statistical unreliability.

9.1.8.4.2 Case when the Petri dish contains no colonies

If the Petri dish containing the test sample (liquids), the initial suspension (other samples) or the first dilution inoculated or retained does not contain any colonies, report the result as follows:

"less than $\frac{1}{V \times d} \times V_s$ microorganisms per V millilitre(s)".

where

d is the dilution factor of the test sample, initial suspension or of the first dilution inoculated or retained ($d = 10^0 = 1$ where the directly inoculated test sample of liquids is retained);

V is the volume of the inoculum or test sample used in the dish, in millilitres, ml;

V_s is the reference volume chosen to express the concentration of the microorganisms in the sample, in millilitres, ml.

If information about uncertainty of measurement (imprecision) is required for the expression of the result, it is preferable to report this as:

"Not detected in the analytical test portion".

(According to the Poisson distribution, the detection level of the method is three organisms per analytical test portion).

9.1.8.5 Special cases

9.1.8.5.1 General

These two cases apply to counting of target or presumptive colonies.

9.1.8.5.2 Case 1: High background growth with target colonies

If the number of target and non-target colonies for the dish containing the test sample or a first dilution d_1 is greater than the guidance upper counting limits (e.g. 300 for spread or pour plate methods, 80 for a membrane filtration methods or any other number stated in the specific standard), with visible target colonies or confirmed colonies, and if the dish containing the subsequent test sample volume or dilution d_2 contains less than the guidance upper counting limit and no target or confirmed colony is visible, report the result as follows:

"less than $\frac{1}{V_2 \times d_2} \times V_s$ and more than $\frac{1}{V_1 \times d_1} \times V_s$ microorganisms" per the reference volume chosen.

where

d_1 and d_2 are the dilution factors corresponding to the dilution d_1 and d_2 ($d = 10^0 = 1$ where the directly inoculated test sample is retained);

V_1 is the volume of the inoculum or test sample used in the dish of the first dilution d_1, in millilitres, ml;

V_2 is the volume of the inoculum or test sample used in the dish of the subsequent dilution d_2, in millilitres, ml;

V_s is the reference volume chosen to express the concentration of the microorganisms in the sample, in millilitres, ml.

EXAMPLE 1 Counting of plates analysed by the pour plate technique with the reference volume $V_s = 1$ ml has produced the following results.

At the first dilution retained ($d_1 = 10^{-2}$): > 300 colonies on the dish, with target or confirmed colonies present.

At the second dilution retained ($d_2 = 10^{-3}$): 33 colonies, with no target or confirmed colonies present.

1 ml from each dilution is used as inoculum ($V_1 = V_2$).

The result is:

less than $\frac{1}{1\times10^{-3}}\times1$ <u>and</u> more than $\frac{1}{1\times10^{-2}}\times1$
giving less than 1 000 and more than 100 microorganisms per millilitre of sample.

EXAMPLE 2 Counting of plates analysed by the membrane filtration technique with the reference volume V_S = 100 ml has produced the following results.

At the first test volume analysed (10 ml): > 80 colonies on the dish, with target or confirmed colonies present.

At the second test volume analysed (1 ml): 21 colonies with no target or confirmed colonies present.

Undiluted sample was used for both test volumes (d_1 and d_2 are both equal to 1).

The result is:

less than $\frac{1}{1\times1}\times100$ <u>and</u> more than $\frac{1}{10\times1}\times100$
giving less than 100 and more than 10 microorganisms per 100 millilitre of sample.

9.1.8.5.3 Case 2: High background growth without target colonies

If the number of colonies for the dish containing the test sample or a first dilution d_1 is greater than the guidance upper counting limits (e.g. 300 for spread or pour plate methods, 80 for membrane filtration methods or any other number stated in the specific standard), without visible target colonies or confirmed colonies, and if the dish containing the subsequent test sample volume dilution d_2 contains less than the guidance upper counting limit and no target or confirmed colonies are visible, report the result as follows:

"less than $\frac{1}{V_2\times d_2}\times V_s$ microorganisms" per the reference volume chosen

where

- d_2 is the dilution factor corresponding to the dilution d_2 ($d = 10^0 = 1$ where the directly inoculated test sample is retained);
- V_2 is the volume of the inoculum or test sample used in the dish of the subsequent dilution d_2, in millilitres, ml;
- V_S is the reference volume chosen to express the concentration of the microorganisms in the sample, in millilitres, ml.

EXAMPLE 1 Counting of plates analysed by the pour plate technique with the reference volume V_S = 1 ml has produced the following results.

At the first dilution retained ($d_1 = 10^{-2}$): > 300 colonies on the dish, with no target or confirmed colonies present.

At the second dilution retained ($d_2 = 10^{-3}$): 33 colonies, with no target or confirmed colonies present.

1 ml from each dilution is used as inoculum ($V_1 = V_2$).

The result is:

less than $\frac{1}{1\times10^{-3}}\times1$
giving less than 1 000 microorganisms per millilitre of sample.

EXAMPLE 2 Counting of plates analysed by the membrane filtration technique with the reference volume V_S = 100 ml has produced the following results:

At the first test volume analysed (10 ml): > 80 colonies on the dish, with no target or confirmed colonies present

At the second test volume analysed (1 ml): 21 colonies with no target or confirmed colonies present.

Undiluted sample was used for both test volumes (d_1 and d_2 are both equal to 1).

The result is:

less than $\frac{1}{1 \times 1} \times 100$

giving less than 100 microorganisms per 100 millilitre of sample.

NOTE Where there are many non-target colonies, the target colonies could be masked by overgrowth and it is useful to record or report this, and request a repeat sample.

9.1.8.6 Methods of calculation: unusual, estimated and unacceptable counts

9.1.8.6.1 General

Some examples do not comply with the rules given in 9.1.8.1. If repeat analysis is not possible or appropriate, results can be calculated and expressed as indicated in 9.1.8.1 but the precision will be reduced and this shall be indicated in the test report.

9.1.8.6.2 Unexpected ratios when dilutions are used

This situation occurs when the number of colonies counted (total colonies, target colonies or presumptive colonies) is greater than 300 (or any other number stated in the specific standard) for the dish containing a first dilution d_1, with a number of colonies (total colonies, target colonies or colonies complying with the confirmation criteria) of less than 10 for the dish containing the subsequent dilution d_2.

If the number of colonies for the dish containing dilution d_1 is within the 334 to 300 interval (the upper part of the confidence interval for a colony count of 300) and the colony count of dilution d_2 is not less than 8 (lower limit of the confidence interval of 15 colonies), use the calculation method for general cases (see 9.1.8.2) using the dishes for the two dilutions retained.

NOTE The colony number of 15 corresponds to 5 % of 300.

Refer to Annex B for further guidance on confidence intervals for colony counts.

EXAMPLE 1 Colony counting has produced the following results:

— at the first dilution retained (10^{-2}): 310 colonies;

— at the second dilution retained (10^{-3}): 8 colonies.

Use the method of calculation for general cases (9.1.8.2) using the dishes for the two dilutions retained.

If the number of colonies for the dish containing dilution d_1 is greater than 334 (the upper limit of the confidence interval for a colony count of 300), and the colony count of dilution d_2 is not less than 8 (lower limit of the confidence interval of 15 colonies), only take account of the result of the count of dilution d_2 and calculate an estimated count (see 9.1.8.4.1).

EXAMPLE 2 Colony counting has produced the following results:

— at the first dilution retained (10^{-2}): more than 334 colonies in the dish;

— at the second dilution retained (10^{-3}): 9 colonies.

Report an estimated count from the colonies counted in the dish for the 10^{-3} dilution (9.1.8.4.1): $N_E = 9,0 \times 10^{-3}$.

If the number of colonies for the dish containing dilution d_1 is greater than 334 (the upper limit of the confidence interval for a colony count of 300), and the colony count of dilution d_2 is less than 8 (lower limit of the confidence interval of 15 colonies), the difference between the two dilutions is then unacceptable.

EXAMPLE 3 Colony counting has produced the following results:

— at the first dilution retained (10^{-2}): more than 334 colonies in the dish;

— at the second dilution retained (10^{-3}): 7 colonies.

The result of this count is unacceptable.

The figures corresponding to confidence intervals shall be adapted to the maximum number stated for the colony counts.

9.1.8.6.3 All dishes are above the upper counting limits

Where the counting of colonies (total colonies, target colonies or presumptive colonies) for each of the dishes for all volumes of the test sample or inoculated dilutions produces a number greater than the guidance upper counting limits (e.g. 300 for spread or pour plate methods, 80 for membrane filtration methods or any other number stated in the specific standard), report the result as follows:

"more than $\frac{C_{max}}{V \times d} \times V_s$" (in the case of total colonies or target colonies);

"more than $\frac{C_{max}}{V \times d} \times V_s \times \frac{b}{A}$" (in the case of confirmed colonies), expressed in microorganisms per the chosen reference volume

where

C_{max} is the upper limit for counting in one dish

d is the dilution of the last inoculated dilution ($d = 10^0 = 1$ where the directly inoculated test sample is retained);

V is the volume of the inoculum or test sample used in each dish, in millilitres, ml;

V_s is the reference volume chosen to express the concentration of the microorganisms in the sample;

b is the number of colonies complying with confirmation criteria;

A is the number of presumptive colonies inoculated for confirmation.

9.1.8.6.4 Only the dish with the highest dilution is countable

Where only the dish containing the last inoculated dilution or volume of test sample is countable and contains more than 10 colonies and fewer colonies (total colonies, target colonies or presumptive colonies) than the guidance upper counting limits (e.g. 300 for spread or pour plate methods, 80 for membrane filtration methods, or any other number stated in the specific standard), calculate the number N' of microorganisms present using Formula (8):

$$N' = \frac{c}{V \times d} \times V_s \quad (8)$$

where

c is the number of colonies counted in the dish;

V is the volume of the inoculum or test sample used in the dish, in millilitres, ml;

d is the dilution corresponding to the dilution retained ($d = 10^0 = 1$ where the directly inoculated test sample is retained);

V_S is the reference volume chosen to express the concentration of the microorganisms in the sample, in millilitres, ml.

Round off the result as specified in 9.1.8.

Report the result as the number N' of microorganisms per the reference volume chosen.

EXAMPLE 1 Counting of plates analysed by the pour plate technique with the reference volume $V_S = 1$ ml and inoculum volumes (V) 1 ml has produced the following results.

At the last dilution ($d = 10^{-4}$): 120 colonies on the dish.

Thus: $N' = \frac{120}{1 \times 10^{-4}} \times 1 = 1\ 200\ 000$

Rounding off the results, as specified in 9.1.8.1, gives:

$N' = 1\ 200\ 000$ *or* $N' = 1{,}2 \times 10^6$ CFU per ml of sample.

EXAMPLE 2 Counting of plates analysed by the membrane filtration technique with the reference volume $V_S = 100$ ml has produced the following results.

At the filtered volume (V) 0,1 ml: 21 colonies on the dish.

Thus: $N' = \frac{21}{0{,}1 \times 1} \times 100 = 21\ 000$

Rounding off the results, as specified in 9.1.8.1, gives:

$N' = 21\ 000$ or $N' = 2{,}1 \times 10^4$ CFU per 100 ml of sample.

9.1.8.7 Uncertainty of test results

See A.2 and ISO 29201[20] for guidance on estimation and expression of uncertainty of test results from quantitative methods.

9.2 Enumeration using a liquid medium

9.2.1 General

Test portions are inoculated into a liquid medium that is designed to support the growth of a particular microorganism or group of microorganisms. Such media are often defined also to inhibit the proliferation of non-target microorganisms.

To determine whether growth of the target microorganisms has occurred, various criteria can be used, e.g. visual detection of turbidity, gas production, colour changes, subsequent isolation of the microorganisms on a selective agar medium. The composition of the growth medium and the criteria for discriminating between a positive and a negative result are defined in the specific standards.

Using this approach, only a qualitative value can be attributed to each test portion, i.e. the result is either positive or negative. To obtain an estimate of the quantity of microorganisms that is present, it is necessary to examine several test portions and use statistical procedures to determine the MPN. These portions may be contained in tubes, bottles, or multiwell plates or microwell plates (see ISO 7899-1[10], ISO 9308-2[11] and ISO 9308-3[12]), according to the method in use.

If a selective growth medium is used, the addition of the test portion should not reduce the selective properties, thereby allowing the growth of non-target microorganisms.

NOTE In most standards, information about the compatibility of a specific matrix and the liquid medium is described in the scope, but incompatibility can be due to the biological composition of the matrix, such as heavily contaminated environmental samples. For such problematic matrices, spiking experiments using representative microorganisms can be performed to verify that the method is appropriate for the matrix.

9.2.2 Procedure

Unless otherwise stated in the specific standards, test portion volumes of less than, or equal to, 1 ml are normally added to five to ten times the volume of single-strength media. Test portions greater than 1 ml and up to 100 ml are normally added to equal volumes of double-strength media.

For volumes greater than 100 ml, more concentrated media may be used. As described in 9.1.4.6, larger test portion volumes may also be analysed using the concentration by membrane filtration and subsequent direct transfer of the membrane filter into the liquid medium for presence/absence or MPN enumeration. Depending on their size, test portions are inoculated into tubes or bottles containing the required amount of liquid medium. For small test portions, multiwell plates are also used.

For special purposes, sterile dehydrated media may be dissolved in the cold (or pre-warmed to 30 °C) sample to be tested. In addition, volumes of sample may be added to multiwell plates containing dehydrated medium.

Unless otherwise stated, the time elapsing between preparing the first dilution of a sample and inoculation of the last tube, bottle or multiwell plate should be less than 15 min.

A new sterile pipette shall be used for each dilution unless the most diluted sample is pipetted first.

9.2.3 Choice of inoculation system

9.2.3.1 General

The essence of the MPN method is the dilution of a sample to such a degree that inocula will sometimes but not always contain viable microorganisms. The outcome, i.e. the number of inocula producing growth at each dilution, will give an estimate of the initial concentration of bacteria in the sample. In order to obtain estimates over a broad range of possible concentrations, serial dilutions are used and several tubes (or plates, etc.) are incubated at each dilution. The MPN of microorganisms present in the original sample, and the precision of the estimate, can be calculated by statistical procedures on the basis of the numbers of positive and negative tubes observed after incubation.

Make a choice from the various MPN configurations available according to:

— the expected number of microorganisms in the sample under investigation;

— regulatory requirements;

— the precision needed;

— any other practical considerations.

The uncertainty of MPN test results depends on the number of positive test portions observed in a similar way as the uncertainty of a colony count depends on the number of colonies on a plate. Uncertainty changes as a function of the square root of the number of tubes used, since precision increases with increasing numbers of replicate tests; however, the number of tubes must be quadrupled to halve the uncertainty. When systems have only a few replicate tubes, the relative uncertainty will be high.

9.2.3.2 Single-dilution system

When the expected concentration of microorganisms is small or expected to vary only moderately, the most appropriate inoculation system is a single series of equal test portions. Where the expected ratio between the maximum and minimum number of microorganisms is less than about 25, 10 parallel test portions is the smallest number expected to provide useful information. 50 parallel tubes are required when the ratio is up to 200. If the actual concentration is near the extreme of the possible MPN values, then the chance of all positive or all negative tubes is probably too high.

9.2.3.3 Multiple-dilution system

9.2.3.3.1 General

When the concentration of microorganisms in the sample is unknown, or if great variation is anticipated, it may be necessary to inoculate series of tubes from several dilutions. Inoculate a sufficient number of dilutions to ensure a system with both positive and negative results. The number of dilutions to be used depends also on the calculation method used for estimating the MPN value. If MPN tables are to be used, then the configurations of the systems are restricted to those available in tables. With computer programs, the numbers of dilutions and parallel tubes are not restricted, which is important in regard to use of commercial MPN test kits.

9.2.3.3.2 Symmetric dilution system

The most commonly applied symmetric MPN system uses three or five parallel tubes per dilution. The precision obtained with these systems with small numbers of tubes per dilution is very low. Results from a three-tube design are hardly more than indications of the order of magnitude of the concentration. If more precision is required, it is recommended that five or more parallel tubes be chosen.

Alternatively, approaches using miniaturised or multiwell systems are commonly used for certain water types (e.g. surface and waste water), see ISO 7899-1[10] and ISO 9308-3[12].

9.2.3.3.3 Non-symmetric dilution system

Non-symmetric systems have different numbers of tubes at different dilution levels and should be used only to estimate numbers of microorganisms within a well-defined range. Occasionally, a tube can be lost or broken resulting in an unintended non-symmetrical system. However, some commercial test kits are based on non-symmetrical systems (see ISO 9308-2[11]). Data from such systems should be evaluated using an appropriate computer software program (see 9.2.7.4).

9.2.4 Incubation

Incubate the inoculated tubes, flasks or bottles in an incubator or in a water bath. Place multiwell plates and microwell plates in an incubator, ensuring they are adequately protected from dehydration.

Choose the duration and the temperature of incubation required by the specific standard method, as these depend on the microorganism or group of microorganisms sought.

For some microorganisms, a two-stage incubation procedure and/or a confirmation step can be necessary. Refer to the specific standards for details, but note that this can add a complication to the derivation of MPN values[22].

9.2.5 Interpretation of results

The criteria that distinguish positive from negative results vary with each microorganism or group of microorganisms and are defined in the specific standards. Using these criteria, count and record the number of positive results obtained with all the test portions derived from one sample.

9.2.6 Uncertainty of test results

See A.2 and ISO 29201[20] for guidance on estimation and expression of uncertainty of test results from quantitative methods.

9.2.7 Determination of MPN values

9.2.7.1 General

There are three different options to determine MPN values: calculating using mathematical formulae, consulting published MPN tables, or utilizing specific computer programs. Provided that these programs are based on the same statistical considerations, they are equally valid. Published MPN tables typically include 95 % confidence limits. These can also be obtained through the other two options. These three approaches are detailed below.

As MPN values are based on a statistical analysis of the number of positive reactions from a sample they are often presented in available tables to one decimal place. However, when reporting MPN values they must be quoted to their nearest whole integer.

9.2.7.2 Mathematical formulae

9.2.7.2.1 Formula for one series of tubes

The MPN value per millilitre for a single series of tubes is derived from Formula (9):

$$\mathrm{MPN} = -\frac{1}{m}\ln\left[\frac{S}{N}\right] \tag{9}$$

where

- m is the volume, in millilitres of sample in each tube of the series;
- S is the number of tubes with a negative reaction;
- N is the number of tubes in the series.

9.2.7.2.2 Precision estimates for single-dilution assays

The 95 % confidence bounds of the MPN estimate can be calculated approximately from the derived MPN and the standard deviation of the log (MPN) using the procedure of Haldane[26], as described in BAM[34]. See also Blodgett[22].

The standard deviation of the MPN is calculated using Formula (10):

$$\mathrm{SD}\left(\log_{10}\mathrm{M}\right) = \frac{\left(1-\mathrm{e}^{-\mathrm{Mm}}\right)}{2{,}303\times\mathrm{Mm}\sqrt{G\times\mathrm{e}^{-\mathrm{Mm}}}} \tag{10}$$

where

- M is MPN;
- m is the volume or mass of inoculum/tube;
- G is the number of tubes showing growth;
- e is the base of natural logarithms (e = 2,718 3).

The normal approximation to the confidence bounds is given by M ± 1,96 × SD($\log_{10}$ M).

EXAMPLE If 0,1 ml of sample (m) was inoculated into each of n = 20 tubes and gives G = 4 positive results (hence, there are 16 negative results), the MPN/ml or MPN/g is given by

$$\mathrm{M} = -\frac{1}{\mathrm{m}} \ln\left(\frac{S}{N}\right) = -\frac{1}{0{,}1} \times \ln\left(\frac{16}{20}\right) = -10 \times \ln(0{,}8) = 2{,}23$$

and the SD($\log_{10}$ M) by

$$= \frac{\left(1 - \mathrm{e}^{-\mathrm{Mm}}\right)}{2{,}303 \times \mathrm{Mm}\sqrt{G \times \mathrm{e}^{-\mathrm{Mm}}}}$$

$$= \frac{\left(1 - \mathrm{e}^{-0{,}223}\right)}{2{,}303 \times 0{,}223\sqrt{4 \times \mathrm{e}^{-0{,}223}}}$$

$$= \frac{(1 - 0{,}80)}{0{,}513\,6 \times \sqrt{3{,}2}} = \frac{0{,}20}{0{,}918\,7} = 0{,}22$$

Now, $\log_{10}$ M = 0,35 and the normal approximation to the confidence bounds is given by M ± 1,96 × SD($\log_{10}$ M).

Lower bound ($\log_{10}$ M) = 0,35 – (1,96 × 0,22) = 0,35 – 0,43 = –0,08

Upper bound ($\log_{10}$ M) = 0,35 + 0,43 = 0,78

So the lower bound (M) = antilog (–0,08) = 0,83 and the upper bound (M) = antilog (0,78) = 6,0

However, it is not necessary to make these calculations manually since they can be determined using the MPN calculator (9.2.7.4).

9.2.7.2.3 Precision estimates for symmetrical multiple-dilution assays

The $\log_{10}$ standard uncertainty of a symmetrical multiple-dilution MPN system can be obtained from Cochran's[23] approximate Formula (11):

$$\mathrm{SD} = 0{,}58\sqrt{\frac{\log_{10} f}{N}} \tag{11}$$

where

SD is the standard deviation of $\log_{10}$ MPN;

f is the dilution factor between consecutive dilutions (usually 10);

N is the number of tubes per dilution.

The upper and lower 95 % confidence bounds may be approximated, respectively, by multiplying and dividing the MPN estimate by the anti-logarithm of 2 × SD. This procedure tends to exaggerate the upper confidence limit. A more precise estimate can be obtained using the MPN calculator (9.2.7.4).

9.2.7.3 MPN tables

9.2.7.3.1 Tables for single-dilution systems

Published tables (see, for example, References [22], [27] and [30]) give the MPN values and the 95 % confidence bounds per test portion for various numbers, such as 10, 15, 20 and 25, of parallel tubes (assuming that each tube is inoculated with the same volume of a single dilution).

To express the outcome per sample reference volume, multiply the MPN and the 95 % limit values by the ratio (reference volume):(test portion volume). Do not multiply the logarithmic standard uncertainty. The reference volumes in water microbiology are usually 1 ml and 100 ml. The test portion volume corresponds to the amount of sample (in millilitres) that is present in the volume used to inoculate the tubes, e.g. 0,1 ml if 1 ml of the 10^{-1} dilution has been used.

EXAMPLE See Reference [30].

Twenty tubes of double-strength broth were inoculated with 5 ml aliquots of a ten-fold-diluted sample (0,1 ml/ml). After incubation, 16 of the tubes showed visible growth. The MPN of bacteria (as organisms per millilitre) is given in tables as 1,61 organisms per tube, which is enclosed within lower and upper 95 % bounds of 0,93 and 2,77.

Each tube received a test portion of 5 ml, which corresponds to 0,5 ml of sample. Therefore, the MPN of microorganisms in 1 ml of sample is given by:

$$\text{MPN} = \frac{1{,}61}{0{,}5} \text{ per millilitre} = 3{,}22 \text{ per millilitre}$$

with 95 % confidence bounds ranging from

— lower 2,5 % limit = $\frac{0{,}93}{0{,}5}$ = 1,9 per millilitre;

— upper 97,5 % limit = $\frac{2{,}77}{0{,}5}$ = 5,5 per millilitre.

9.2.7.3.2 Tables for multiple-dilution systems: Three successive dilutions

With symmetrical systems, it is common practice to use three successive dilutions with three, five or 10 replicates. Record the number of positive results for each set of tubes and, from the appropriate MPN table for the inoculation system used, read the MPN of microorganisms present in the reference volume of the sample.

Some tables also provide the log MPN, the SD of log MPN, the lower and upper confidence limits of the approximate 95 % confidence interval together with a rarity value and a rarity category. The rarity value (based on work by Blodgett[21][22][25]) provides a simpler approach to assessment of the likelihood that an observed result will be obtained in a test.

Some combinations of positive tubes are more likely to occur than others; for example, a combination of positive results 0 – 0 – 3 is much less likely to occur than the combination 3 – 2 – 1. To quantify this probability, the rarity index has been calculated as the ratio of two likelihood values:

$$r = \frac{L\left(\hat{\mu}\right)}{L_0\left(\hat{\mu}\right)}$$

where

$L\left(\hat{\mu}\right)$ is the likelihood of the observed result $x_1, x_2, \ldots, x_k$, of the serial dilution test;

$L_0\left(\hat{\mu}\right)$ is the likelihood if the result were most likely under a concentration μ equal to the estimate $\hat{\mu}$ of the concentration μ.

Full details of the procedure for calculation of the likelihood functions are given in Reference [28].

The rarity index is a value between 0 and 1. It is 1 if the result of the serial dilution test is most likely a concentration equal to the estimated MPN. If it is in the neighbourhood of 0 the result of the serial

dilution test is very unlikely for a concentration equal to the estimated MPN. Following the approach in References [23] and [24], three rarity categories are used:

— category 1: the MPN value would be very likely to occur if its rarity value falls within the range 0,05 to 1,00 ($0,05 \le r \le 1,00$), i.e. such a result would be likely to occur by chance on 95 % of occasions;

— category 2: the MPN value would be expected to occur only rarely if its rarity value falls within the range 0,01 to 0,05 ($0,01 \le r \le 0,05$); such a result would be likely to occur by chance with a frequency less than 5 %;

— category 3: the MPN value would be expected to occur extremely rarely if its rarity value falls within the range 0 to 0,01 ($0 < r < 0,01$); i.e. such a result would be expected to occur by chance less than once in 100 tests.

Most tables show only those combinations of results that fall within categories 1 and 2.

In any circumstance when more than three dilutions are made, all measured data values shall be used. It is not scientifically correct to select any combination of values on the premise that these values are more correct than other combinations. The results from all possible combinations of positive tubes should be recorded and the MPN calculator (9.2.7.4) used to derive MPN values.

9.2.7.4 Determination of MPN values using an MPN calculator

A MPN calculator should be used to determine MPN values from the combination of positive and negative tubes, flasks bottles or microplate wells at each dilution. Such calculators allow input of the results from all portions tested rather than restricting the use to a certain number of dilutions and replicates, as with MPN tables. The output of the calculator should include an estimate of the 95 % confidence intervals for the MPN together with an indication of the probability of occurrence of the combination of results yielding the MPN (this may be as a rarity index, rarity category, or both; see 9.2.7.3.2). A calculator that fulfils these requirements is available at: http://standards.iso.org/iso/8199/

This spreadsheet can handle up to 10 levels of serial dilution. Details of the calculations are described in Reference [28].

Some specific standards (e.g. ISO 9308-2[11]) include a MPN calculator to be used when following the standard.

The positive and negative results from all tubes or wells that have been tested at all dilutions should be used in the MPN calculator to determine the MPN value. It is not appropriate to select a subset of dilutions on which to determine the value.

The MPN calculator also yields the decimal logarithm of the MPN value, its standard deviation (SD), the lower and upper confidence limits of the approximate 95 % confidence interval together with a rarity index and a rarity category. The application of the rarity index is described in 9.2.7.3.2.

If the MPN calculator highlights a tube combination as improbable (i.e. Category 3 as described in 9.2.7.3.2), the associated MPN value should not be reported. If appropriate, the submission of a repeat sample should be requested.

10 Detection (qualitative) methods

10.1 General

Detection methods determine the presence or absence, at a given level of detection, of particular microorganisms in a given quantity of sample.

10.2 Procedure

Unless otherwise stated in the specific International Standard, mix a quantity P ml of the sample to be examined with 9 × P ml of an elective and/or a selective broth.

Alternatively, pass volumes of water samples through membrane filters, where turbidity permits, and place the membrane filters directly into an elective and/or selective broth.

To facilitate the recovery of stressed microorganisms, samples are usually pre-enriched in a non-selective broth followed by selective enrichment in a broth and isolation on selective/differential agar media.

The use of two different enrichment broths, as well as two or more selective agar media, increases method sensitivity.

Unless otherwise stated, the incubated enrichment broths may only be refrigerated after evaluation of the impact of refrigeration on the results and only if clearly stipulated in the test report.

After incubation, streak a loop of the culture obtained over the surface of a selective/differential agar medium to obtain isolated colonies.

A number (generally five per agar plate unless otherwise stated by the specific standard) of the colonies obtained after incubation is then identified using appropriate confirmation techniques. The selection of colonies for confirmation should cover representative suspect colony types.

10.3 Uncertainty of test results

Detection method results are also subject to uncertainty in that target microorganisms can be missed (false negatives or reduced test sensitivity) or non-target microorganisms can be wrongly identified (false positives or reduced test specificity). These concepts are discussed further in A.2.2, together with the level of detection, which is also an important factor in the uncertainty of qualitative results.

11 Performance characteristics of methods

Performance characteristics for microbiological testing have been defined in ISO 13843[13].

Characterization is an exploratory process with the aim of establishing the likely range of performance characteristics of a new, modified or otherwise inadequately characterized method. It is carried out by a single laboratory in the first instance to determine the likely performance of a test quantitative method.

The established performance characteristics should be quoted in International Standard test methods so that user laboratories can compare those published characteristics with their own results to verify laboratory test performance.

The performance characteristics described for characterization process are:

— categorical performances: sensitivity, specificity, selectivity, false positive rate and false negative rate;

— performances of the detection system: working range, linearity, robustness, recovery efficiency;

— uncertainty and precision when implementing the method: uncertainty of counting, repeatability, reproducibility.

Verification focuses on gathering evidence that the laboratory is able to generate performance data similar to those established in characterization. Typically, verification uses selected and simplified forms of the same procedures used in method characterization, but possibly extended over a longer time.

If alternative confirmation methods to those specified in the individual standards are to be used by a laboratory (e.g. PCR or MALDI-TOF techniques), the laboratory should ensure that the suitability of the alternative methods have been demonstrated. ISO 13843[13] notes that laboratories developing variations on standard methods could carry out the steps of characterization. In addition, the ISO 16140 (all parts)[15] series gives guidance on the general principles and the technical protocols

for the validation of alternative, often proprietary methods, including alternative methods for microbiological confirmation and typing procedures.

More information on specific performance characteristics for water test methods can also be found in Annex A and ISO 17994[17].

12 Analytical quality control

12.1 General

The application of valid methods within their specified reliable limits does not automatically ensure valid results. Analytical quality control (AQC) used in conjunction with routine analyses is necessary to monitor and control the performance of a method.

It is essential that all aspects in the enumeration and detection of microorganisms in water have appropriate method AQC in place as recommended in ISO/IEC 17025[16]. AQC is a key part of a laboratory quality assurance programme as it contributes to the reliability of results produced. Appropriate AQC shall be applied systematically to check each step of the testing process in order to ensure that a laboratory is capable of isolating, accurately identifying and enumerating target microorganisms in a sample. The key components of an AQC programme are internal quality control applied at the time of analysing the sample and external quality assurance through participation in an appropriate and preferably accredited proficiency testing scheme.

12.2 Internal quality control

12.2.1 General

Many types of internal quality control (IQC) tests are available for individual laboratories to assess the effectiveness of isolation, enumeration and confirmation procedures used for their routine samples. These are employed for different purposes and this clause details some of the options available.

All quality control samples should contain target microorganisms similar to those being sought by particular methods and, where appropriate, non-target microorganisms which will challenge test performance.

Results from many types of IQC tests are also a valuable source of data for estimating the uncertainty of routine test results in the laboratory.

12.2.2 Process controls

12.2.2.1 General

The primary IQC test uses daily process controls alongside routine testing. These consist of:

a) positive control: a strain of microorganism the same or similar to that being sought, which produces target colonies on agar (or positive reactions in broth) and, where appropriate, in confirmation tests;

b) negative control (non-target): a strain of microorganism that is capable of growing on the test medium, but produces non-target colonies (or reactions in broth) and, where appropriate, in confirmation tests;

c) negative control (inhibition): a strain of microorganism whose growth is inhibited by the test medium in use;

d) blank: a sterile sample portion that produces no colonies on agar or reaction in broth.

12.2.2.2 Replicate testing

Replicate testing of routine samples gives an indication of routine repeatability or technician performance when sufficient sample is available or requested from samplers. Duplicate data are most often used, but multiple replicates may also be considered in some situations. Results of such IQC may also be incorporated into uncertainty estimates of test results as an additional source of data.

12.2.2.3 Spiked samples

Spiked samples should be used where target microorganisms are rarely isolated during testing of routine samples. These are prepared using both target and non-target organisms or strains, as described in 12.2.2, and tested blind by all laboratory personnel. A regular programme of spiked sample is useful when training or updating technicians on routine methods, as an addition to external quality assessment tests, which can be both infrequent and expensive.

12.2.2.4 Microorganisms for internal quality control

Particulars of the microorganisms required for the performance testing of all media used in International Standards for water microbiology is given in ISO 11133:2014, Annex F, or the specific standards, and these may also be used for the other aspects of IQC discussed above.

Quality control microorganisms maintained in the laboratory shall, wherever possible, be derived from freeze-dried or other reference material generated from first-generation reference strains obtained from recognized sources (culture collections or certified suppliers of reference materials). Preparation of laboratory reference stocks and working cultures shall be in accordance with ISO 11133.

NOTE 1 In some special cases, microorganisms maintained in other ways can be used where it has been shown that the conditions specified above are unsuitable or result in poor test performance.

NOTE 2 It can also be beneficial to challenge routine test performance with wild isolates, for example, when such isolates are common in a particular water source or type of sample submitted to the laboratory.

12.2.2.5 Assessing internal quality control results

IQC is undertaken on both qualitative and quantitative methods and the results are assessed for effectiveness to highlight any problems with test performance.

Qualitative testing is used for both detection methods and confirmation tests, so results from qualitative testing are scored as correct or incorrect.

Data from quantitative method assessments may be plotted in control charts such as those first described for quality control in manufacturing by Shewhart[32][33]. Warning and action limits are determined by established methods to alert the laboratory to potential problems with precision of methodology (see ISO 11133:2014, Annex G). Such limits may also be derived from data obtained during method performance characterization as detailed in ISO 13843[13].

12.3 External quality assessment

There are accredited interlaboratory external quality assessments (EQAs) or proficiency testing (PT) schemes available for laboratories undertaking water microbiology testing. These involve examination of samples of unknown composition distributed by an independent external supplier.

Results from each distribution are assessed and continuously reviewed to determine any trends or bias; plotting consecutive results obtained by each test method will aid assessment of systematic bias.

Further details of PT by interlaboratory comparisons (ILCs) for microbiological examinations are given in ISO/TS 22117[19].

Collaborative testing by ILC has been widely applied for testing performance characteristics of chemical methods and similar systems can be useful for microbiology, particularly where no suitable EQA scheme

is available. The laboratory organizing the ILC shall have sufficient experience of preparing the test materials and clear instructions shall be given to all participating laboratories (see ISO/TS 22117[19] for other aspects of ILC).

Annex A
(informative)

Criteria for the choice of technique

A.1 General

Most culturable microorganisms in water can be enumerated by any of the four main procedures described in Clause 9 or detected as described in Clause 10. The factors affecting the choice of procedure can be classified as follows:

a) those concerned with the quality of the results (see A.2);

b) those concerned with the nature of the sample (see A.3).

This annex gives a general overview of these factors as they affect the choice of test methods to be used for particular purposes.

A.2 Factors concerning the quality of the result

A.2.1 General

The quality of results obtained from any enumeration test may be defined in terms of the following characteristics: uncertainty, accuracy (including trueness and precision) and limit of determination. Detection methods are usually assessed on the basis of the level of detection, sensitivity and specificity.

A.2.2 Uncertainty of test results

No result is perfect. It has an associated uncertainty arising from many factors including errors and imperfect reproducibility. Ideally each result should be quoted with an indication of the uncertainty, often as a ± figure or a confidence interval, so that decisions based on the result are fully informed (see ISO 29201[20]). Niemelä[31] gives further details on estimating the uncertainty of enumeration test results.

Uncertainty of counting is defined as the relative standard deviation of results of repeated counting of the colonies or particles of the same plate(s) or field(s) under stipulated conditions.

NOTE Stipulated conditions can be the same person or different persons in one laboratory, or different laboratories.

A.2.3 Accuracy

A.2.3.1 Principle

The accuracy of a result is defined as the closeness of the agreement between a test result and the accepted reference value. According to Lightfoot and Maier[29], the accuracy is inversely related to the overall uncertainty, which is the difference between the result and the true value. The overall

uncertainty of a single result is thought to consist of two types of components, systematic (i.e. bias) and random (i.e. imprecision):

UNCERTAINTY = BIAS + IMPRECISION

This is a simplification of the situation. In microbiology, there are usually also contributions to uncertainty that do not fit this classification, i.e. unexpected fluctuations that cannot be modelled mathematically. The inverse of bias is the trueness of the method. It is a characteristic of the method used for the analysis and also depends on the nature of the sample. Trueness is not related to errors in applying the method in individual laboratories. These are included in the random error of the result, introduced by variations between laboratories or within a single laboratory. The inverse of random error is the precision. Hence, the same relation as above is more commonly expressed as:

ACCURACY = TRUENESS + PRECISION

A.2.3.2 Trueness

A.2.3.2.1 General

According to ISO 3534-1[1] and ISO 6107-8[6], trueness is the closeness of agreement between the average value obtained from a large series of test results and an accepted value. ISO 3534-1[1] also indicates that the measure of trueness is usually expressed in terms of bias, where bias is defined as the difference between the expectation of the test results and an accepted reference value.

In general, for microbiological analyses, the true concentration of a component is not known and can only be used as a hypothetical aim. The systematic error can therefore not be established in an absolute manner. It can theoretically be approached by studying the same sample repeatedly, using different methods. Because microbiological methods are destructive in nature (i.e. the sample is lost during analysis), this requires well mixed test materials with randomly distributed bacteria and/or a large group of laboratories (see Lightfoot and Maier[29].

Errors can be quantitative or qualitative. Quantitative errors occur, for example, when the microorganisms enumerated do indeed belong to the group sought, but the result of the final enumeration underestimates the true number (some false negatives or reduced sensitivity). Qualitative errors occur, for example, when a number of microorganisms not belonging to the group sought are considered and counted as such, so that the final value is greater than the true value (some false positives or reduced specificity). Both types of error can occur simultaneously.

A.2.3.2.2 Quantitative errors

Some errors are independent of the enumeration procedure. In the presence of suspended particles, the microorganisms may be adsorbed and become inseparable despite vigorous agitation; this invalidates the assumption that the formation of one colony or turbidity in broth inoculated with a small volume of the sample could be derived from a single microorganism.

The action of a selective medium can be excessively inhibitory and thus interfere with the growth of not only the background microorganisms, but also the microorganisms to be enumerated.

Other errors are attributable to the nature of the water sample. Physico-chemical constituents, such as toxic substances or high concentrations of salts, can affect the culture medium and thus inhibit growth. The effect is particularly important when large volumes of test portion are used compared with the volume of the medium (e.g. MPN procedures in which double- or multiple-strength media are used, or a dehydrated medium dissolved in the sample to be tested). Membrane filtration, which separates the microorganisms from the sample, can help to overcome this problem, unless inhibiting substances are retained on the membrane filter.

Biological constituents, such as background microorganisms, can, through biological competition, interfere with the development of the microorganisms sought. This effect occurs especially in liquid

media. In other procedures where the microorganisms form colonies separate from each other, this biological competition is limited, provided there is no spreading or overcrowding.

A.2.3.2.3 Qualitative errors

Qualitative errors occur when there are differences between the definition of the microorganisms sought and the identity of those isolated. When the definition is precise, as with the individual members of a genus or species, it is exceptional for a single observation to provide sufficient information to give accurate final results, and it is often impracticable to carry out all the further tests required for identification.

Therefore, the inoculation procedures and the media used should yield as much diagnostic information as possible.

A.2.3.3 Precision

According to ISO 3534-1[1], ISO 5725-1[3] and ISO 13843[13], precision is defined as the closeness of agreement between independent test results obtained under stipulated conditions. ISO 3534-1[1] also indicates that the measure of precision is usually expressed in terms of imprecision and computed as a standard deviation of the test results. Less precision is reflected by a larger standard deviation.

General principles for the characterization of the precision of test methods have been described in ISO 5725-1[3], ISO 13843:2017[13] and ISO 16140-1[15]. Two measures of precision are described in this document: repeatability (r) and reproducibility (R). The definitions are given in 3.11 and 3.13.

NOTE A third measure of precision for assessing quantitative results in a single laboratory can be useful: intermediate precision, which is used to express intralaboratory reproducibility. This is appropriate for assessing results from all technicians in a single laboratory, in order to give a better indication of the precision component of the uncertainty of test results in that laboratory. In microbiology, truly identical test materials cannot be supplied. A random distribution of the microorganisms is the best possible objective, and the degree to which this is reached is critical for establishing r and R. Any over-dispersion in the test materials will add to the variability of the test results.

Numerous factors affect precision, but only those connected with the random distribution of microorganisms in the samples are discussed in this document.

Random distribution of the microorganisms in a sample causes imprecision in results, which is reduced by the use of a suitable inoculation system.

With the MPN procedure, the precision is increased by increasing the number of replicate tubes inoculated with each series of test portion volumes. The relative precision depends on the pattern of positive results obtained.

With any colony count procedure, precision depends on the total number of colonies counted and therefore increases as the number of plates or membrane filters cultured increases (duplicates, replicates, etc.). Precision also increases as the number of colonies counted increases up to a maximum of about 300 colonies per 90 mm plate for pour plate counts and surface plate counts, and about 80 colonies per 47 mm membrane filter for membrane filtration. Refer to 9.1.2.2, 9.1.3.2 and 9.1.4.2 for further information on example upper counting limits.

A.2.4 Detection level

A.2.4.1 Principle

According to ISO 13843[13], the detection level is defined as the particle number x (per analytical portion) where the probability of detecting the presence of the analyte equals 95 % [$p(+) = 0{,}95$].

A.2.4.2 Detection level of colony count procedures

The probability of a positive result $p(+)$ when the Poisson distribution prevails can be calculated using Formula (A.1):

$$p(+) = 1 - e^{-x} \quad \text{(A.1)}$$

Solving the formula for x gives

$$x = -\ln[1 - p(+)]$$

where

e is the base of natural logarithms(e = 2,718 3);

x is the mean number of particles per analytical portion.

EXAMPLE According to the above formula, $x = -\ln(1 - 0{,}95) = -\ln(0{,}05) = 3{,}0$. Thus, at the average count of 3 (particles per test portion), the chances of detecting the presence of the analyte equals 0,95 (provided that the Poisson distribution prevails).

The detection level is the property of suspensions and does not distinguish one method from another. It is the same for all colony count procedures.

A.2.4.3 Detection level with MPN procedures

The detection level of MPN methods can be reasoned in the same way as for the colony count methods. Irrespective of the geometrical configuration, the same average number of particles is needed in the system to ensure detection of the analyte with a chosen probability.

A.2.5 Effects of membrane filter type and batches

The types and batches of membrane filters used for methods where test samples are filtered before incubating on specific media have been shown to affect test results for these methods. Refer to specific standards for information on the types of membrane filters to be used for a particular method. Refer to ISO 7704 for information on methods to evaluate and verify the suitability of membrane filters before use.

A.3 Requirements concerning the nature of the sample

A.3.1 Nature of the microorganisms

In addition to the microorganisms to be detected, growth of other microorganisms in the same culture can also influence the choice of method.

For example, certain microorganisms are strict aerobes and their enumeration is preferably carried out by the surface spread or membrane filtration technique rather than pour plate. Other microorganisms tolerate, and sometimes prefer, a certain degree of anaerobiosis and the pour plate technique is preferable (9.1.2). If greater anaerobiosis is required to cultivate strict anaerobes and to eliminate strict aerobes, deep tubes of medium may be used or the cultures may be incubated in anaerobic jars.

Certain microorganisms cannot withstand the thermal shock resulting from the sample being mixed with molten agar at 44 °C to 47 °C and the pour plate procedure should therefore be avoided for their enumeration; this applies to numerous bacteria in lakes and other surface waters. However, the pour plate technique has some selective advantage for the detection of microorganisms that normally live at a higher temperature (e.g. faecal indicator organisms) and have greater resistance to thermal shock.

A.3.2 Constituents of water samples

Suspended matter causes interference, particularly in the membrane filtration procedure; clogging of the membrane filter limits filtration and therefore the sensitivity of the method. Partial clogging, sometimes unsuspected, can reduce nutrient exchange and prevent organisms on the membrane filter from forming colonies. This blockage can often be caused by living organisms including microplankton or even bacteria that multiply in the pores.

Sometimes large particles can be confused with colonies in the pour plate procedure. The use of magnification can help to distinguish colonies from other particles.

For very turbid waters, the MPN procedure can sometimes be the only one possible when, as is often the case, the spread plate technique does not provide sufficient sensitivity.

Soluble substances can interfere with the growth of microorganisms either by modifying the composition of the selected culture medium, or because of their toxicity. This interference occurs in particular when the volume of the sample is relatively large in relation to that of the medium to be used. Sometimes substances in the sample can react with constituents of the culture medium and adversely affect the characteristic reactions of the microorganisms sought, without interfering with their growth. An example is the change in pH of the medium following the fermentation of a newly formed fermentable sugar that is not present in the original medium. Membrane filtration can help to overcome such disadvantages.

Annex B
(informative)

Confidence intervals for colony count technique and choice of method of calculation in special cases

B.1 Confidence intervals for colony count technique

To assess the validity of results and to avoid too strict interpretations, it is necessary to estimate the uncertainty or, if not available, the confidence interval which characterizes the microbial statistical distribution in the sample.

NOTE The confidence limits calculated in this annex are based solely on the distribution uncertainty. No other uncertainty components are considered. When a value of technical uncertainty is available, the inclusion of this operational variability in the calculation leads to a wider confidence interval (see ISO 29201[20]).

When the value of the uncertainty is not available, the confidence interval δ which characterizes the microbial dispersion can be calculated using Formula (B.1) (with a 95 % probability).

$$\delta = \left[\frac{\sum c}{B} + \frac{1,92}{B} \pm \frac{1,96\sqrt{\sum c}}{B} \right] \frac{1}{d} \times V_s \qquad \text{(B.1)}$$

with

$B = V\,(n_1 + 0{,}1 n_2)$

and where

$\sum c$ is the sum of the colonies counted on all dishes retained from two successive dilutions;

V is the volume of inoculum placed in each dish, in millilitres, ml;

n_1 is the number of dishes retained at the first dilution;

n_2 is the number of dishes retained at the second dilution;

d is the dilution corresponding to the first dilution retained;

V_s is the reference volume chosen to express the concentration of the microorganisms in the sample.

EXAMPLE 1 An enumeration gave the following results (system with one dish per dilution):

— at the first retained dilution (10^{-2}): 215 colonies;

— at the second retained dilution (10^{-3}): 14 colonies.

The number of microorganisms N is calculated as follows:

$$N = \frac{\sum c}{V \times \left[n_1 + (0,1 \times n_2) \right] \times d} = \frac{215 + 14}{1 \times \left[1 + (0,1 \times 1) \right] \times 10^{-2}} = \frac{229}{0,011} = 20\,818$$

Rounding off the result, as specified in 9.1.8.1, the number of microorganisms is 21 000 or $2,1 \times 10^4$ per millilitre of sample.

With $N = 2{,}1 \times 10^4$ per millilitre, for 229 colonies counted, the confidence interval δ is:

$$\delta = \left[\frac{229}{1{,}1} + \frac{1{,}92}{1{,}1} \pm \frac{1{,}96\sqrt{229}}{1{,}1} \right] \times \frac{1}{10^{-2}}$$

$\delta = (208{,}18 + 1{,}75 \pm 26{,}96) \times 10^2$

Thus, the confidence interval limits are:

$\delta_1 = 1{,}8 \times 10^4$ and $\delta_2 = 2{,}4 \times 10^4$

EXAMPLE 2 An enumeration gave the following results (system with two dishes per dilution):

— at the first retained dilution (10^{-2}): 168 colonies and 215 colonies;

— at the second retained dilution (10^{-3}): 14 colonies and 25 colonies.

$$N = \frac{\sum c}{V \times \left[n_1 + (0{,}1 \times n_2) \right] \times d} = \frac{168 + 215 + 14 + 25}{1 \times \left[2 + (0{,}1 \times 2) \right] \times 10^{-2}} = \frac{422}{0{,}022} = 19\ 182$$

Rounding off the result, as specified in 9.1.8.1, the number of microorganisms is 19 000 or $1{,}9 \times 10^4$ per millilitre of sample.

With $N = 1{,}9 \times 10^4$ per millilitre or per gram, for 422 counted colonies, the confidence interval δ is:

$$\delta = \left[\frac{422}{2{,}2} + \frac{1{,}92}{2{,}2} \pm \frac{1{,}96\sqrt{422}}{2{,}2} \right] \times \frac{1}{10^{-2}}$$

$\delta = (191{,}82 + 0{,}87 \pm 18{,}30) \times 10^2$

Thus, the confidence interval limits are:

$\delta_1 = 1{,}7 \times 10^4$ and $\delta_2 = 2{,}1 \times 10^4$

Table B.1 gives the weighted means and confidence intervals δ for relevant numbers of colonies.

Table B.1 — Weighted means and confidence intervals δ for relevant numbers of colonies

Weighted mean of number of colonies counted on two successive dilutions	System: 1 dish per dilution	System: 2 dishes per dilution
	Confidence interval δ	Confidence interval δ
300	270 to 334	278 to 324
150	129 to 175	135 to 167
15	8 to 25	10 to 21
10	6 to 18	7 to 15
7	3 to 14	4 to 12

B.2 Choice of the method of calculation in special cases, with one dish per dilution

B.2.1 Special cases not only with low numbers

Given the confidence intervals of the maximum number of colonies required to be counted on a dish, Tables B.2 and B.3 describe the method of calculation in special cases, for a maximum number of 300 or 150 colonies per dish.

Table B.2 — Choice of the calculation method, cases with one dish per dilution, and maximum number of colonies per dish = 300

Maximum number of colonies per dish = 300 (confidence interval δ for weighted mean of 300 colonies: 270 to 334) Lower limit: 5 % of 300 = 15 colonies (confidence interval δ for weighted mean of 15 colonies: 8 to 25)		
d_1 more than 334 colonies	d_2 less than 15 colonies: two possibilities: see the 2 rows below	
more than 334 colonies.	complying with the lower limit: at least 8 colonies	method of calculation for estimated count (9.1.8.6.2) (with the special expression of result if the number is 8)
more than 334 colonies	**not complying with the lower limit: less than 8 colonies**	**method of calculation: N/A (difference between the two dilutions unacceptable)**

Table B.3 — Choice of the calculation method, cases with one dish per dilution, and maximum number of colonies per dish = 150

Maximum number of colonies per plate = 150 (confidence interval δ for weighted mean of 150 colonies: 129 to 175) Lower limit: 5 % of 150 = 7 colonies (confidence interval δ for weighted mean of 7 colonies: 3 to 14)		
d_1 more than 175 colonies	d_2 less than 7 colonies: two possibilities: see the 2 rows below	
more than 175 colonies	complying with the lower limit: at least 3 colonies	method of calculation: estimated count (9.1.8.6.2) (with the special expression of result if the number is 3)
more than 175 colonies	**not complying with the lower limit: less than 3 colonies**	**method of calculation: N/A (difference between the two dilutions unacceptable)**

B.2.2 Special cases with low numbers

The confidence intervals are given in Table B.4.

Table B.4 — Confidence intervals for enumeration with one dish per dilution, case of low numbers

Number of microorganisms[a]	**Confidence limit at 95 %**		**Error percentage for the limit**[b]	
	Lower	Upper	Lower	Upper
1	< 1	6	−97	+457
2	< 1	7	−88	+261
3	< 1	9	−79	+192
4	1	10	−73	+156
5	2	12	−68	+133
6	2	13	−63	+118

[a] Equal to the number of colonies.

[b] Compared to the number of colonies (first column).

Table B.4 *(continued)*

Number of microorganisms[a]	**Confidence limit at 95 %**		**Error percentage for the limit**[b]	
	Lower	Upper	Lower	Upper
7	3	14	−60	+106
8	3	16	−57	+97
9	4	17	−54	+90
10	5	18	−52	+84
11	6	20	−50	+79
12	6	21	−48	+75
13	7	22	−47	+71
14	8	24	−45	+68
15	8	25	−44	+65

[a] Equal to the number of colonies.

[b] Compared to the number of colonies (first column).

Annex C
(normative)

Counting and calculations with two Petri dishes per dilution

C.1 Counting of colonies

Following the period of incubation stated in the specific standard, count the colonies (total colonies, target colonies or presumptive colonies) for each dish containing less than 300 colonies (or any other number stated in the specific standard).

The different methods of calculation defined in 9.1.8 shall take account of dishes containing 0 colonies if these dishes have been retained.

When counting target or presumptive colonies, the maximum number of all target or non-target colonies present on a dish shall not exceed 300 (or any other number stated in the specific standard).

NOTE 1 In certain cases, it can be difficult to count the colonies (e.g. where spreading microorganisms are present). These cases are dealt with in the specific standards.

NOTE 2 When counting target or presumptive colonies, the description of the colonies will be given in the specific standard.

In this clause, the cases dealt with correspond to the following general cases:

— inoculation of two Petri dishes, 90 mm in diameter, per dilution;

— maximum number for counting of total colonies is 300 per dish;

— maximum number of all target and non-target colonies present on a dish when counting target or presumptive colonies is 300 per dish;

— a ten-fold dilution series is used;

— number of presumptive colonies (9.1.7.3) inoculated for confirmation, from each dish retained is 10;

— minimum number of colonies [total colonies, target colonies or colonies complying with confirmation criteria (9.1.7.3)] on at least one dish is 10.

These figures are defined in specific standards.

When dishes with a diameter different from 90 mm are used, the maximum number of colonies shall be increased proportionately to the surface area of the dishes (see 9.1.8).

The methods of calculation defined below take account of the cases that occur most frequently when tests are carried out in accordance with good laboratory practice. Rare special cases can occur (for example, significant discrepancy between the number of colonies in two dishes with the same dilution, or a very different ratio to that of the dilution factor between the dishes of two successive dilutions), and therefore results obtained from counting shall be examined, interpreted or rejected by a qualified microbiologist.

C.2 Method of calculation: General case (counting of total colonies or target colonies)

For a result to be valid, it is generally considered necessary to count the colonies on at least one dish containing a minimum of 10 colonies [total colonies, target colonies or colonies complying with the confirmation criteria (9.1.7.3)].

Calculate the number N of microorganisms present in the test sample as a weighted mean from two successive ten-fold dilutions using Formula (C.1):

$$N = \frac{\sum c}{V \times \left[n_1 + \left(0,1 \times n_2 \right) \right] \times d} \tag{C.1}$$

where

$\sum c$	is the sum of the colonies counted on all the dishes retained from two successive dilutions, and where at least one contains a minimum of 10 colonies;
V	is the volume of inoculum applied to each dish, in millilitres, ml;
n_1	is the number of dishes retained at the first dilution;
n_2	is the number of dishes retained at the second dilution;
d	is the dilution factor corresponding to the first dilution retained (d = 1 when the undiluted liquid sample is used).

Round off the results calculated to two significant figures. In order to do this, if the third figure is less than 5 do not modify the preceding figure; if the third figure is greater than or equal to 5, increase the preceding figure by one unit.

Take as the result a number between 1,0 and 9,9 multiplied by the appropriate power of 10, or a whole number with two significant figures.

Express the result as:

"number N of microorganisms per millilitre".

EXAMPLE Colony counting has produced the following results:

— at the first dilution (10^{-2}) retained: 168 and 215 colonies;

— at the second dilution (10^{-3}) retained: 14 and 25 colonies.

$$N = \frac{\sum c}{V \times \left[n_1 + \left(0,1 \times n_2 \right) \right] \times d} = \frac{168 + 215 + 14 + 25}{1 \times \left[2 + \left(0,1 \times 2 \right) \right] \times 10^{-2}} = \frac{422}{0,022} = 19\ 182$$

By rounding off the result as described above, the number of microorganisms is 19 000 or $1,9 \times 10^4$ per millilitre of sample.

C.3 Method of calculation: Case after confirmation

When the specific method used requires confirmation, a given number, A (generally 10), of presumptive colonies is inoculated from each of the dishes retained for the counting of colonies. After confirmation,

calculate, for each of the dishes, the number, a, of colonies complying with the confirmation criteria, using Formula (C.2):

$$a = \frac{b}{A} \times C \tag{C.2}$$

where

A is the number of presumptive colonies inoculated;

b is the number of colonies complying with identification or confirmation criteria;

c is the total number of presumptive colonies counted on the dish.

Round off the results calculated to the nearest whole number. In order to do this, if the first figure after the decimal sign is less than 5, do not modify the preceding figure; if the first figure after the decimal sign is greater than or equal to 5, increase the preceding figure by one unit.

Calculate the number N of identified or confirmed microorganisms present in the test sample, by replacing $\sum c$ by $\sum a$ using the formulae provided in 9.1.8.3.

Round off the result as recommended above.

Express the result as recommended in 9.1.8.3.

EXAMPLE Colony counting has produced the following results:

— at the first dilution (10^{-3}) retained: 66 and 80 colonies;

— at the second dilution (10^{-4}) retained: 4 and 7 colonies.

Testing of selected colonies was carried out:

— for 66 colonies, 10 colonies, 6 of which complied with the criteria; hence $a = 40$;

— for 80 colonies, 10 colonies, 6 of which complied with the criteria; hence $a = 48$;

— for 7 colonies, 7 colonies, 4 of which complied with the criteria; hence $a = 4$;

— for 4 colonies, all 4 of which have complied with the criteria; hence $a = 4$.

$$N = \frac{\sum a}{V \times \left[n_1 + (0,1 \times n_2) \right] \times d} = \frac{40 + 48 + 4 + 4}{1 \times \left[2 + (0,1 \times 2) \right] \times 10^{-3}} = \frac{96}{0,002\ 2} = 43\ 636$$

By rounding off the result as described above, the number of microorganisms is 44 000 or $4,4 \times 10^4$ per millilitre of sample.

C.4 Method of calculation: Estimated counts

C.4.1 Case of two dishes (test sample or initial suspension or first dilution) containing fewer than 10 colonies

If the two dishes from the test sample, the initial suspension or the first dilution inoculated or retained contain less than 10 colonies (total colonies, target colonies or colonies complying with the confirmation criteria), calculate the estimated number N_E of microorganisms present in the test sample as an arithmetical mean of the colonies counted on the two dishes using Formula (C.3):

$$N_E = \frac{\sum c}{V \times n \times d} \tag{C.3}$$

where

$\sum c$	is the sum of the colonies counted on the two dishes;
V	is the volume of inoculum applied to each dish, in millilitres, ml;
n	is the number of dishes retained (in this case, $n = 2$);
d	is the dilution factor of the initial suspension or of the first dilution inoculated or retained [$d = 1$ when the undiluted liquid product (test sample) is used].

Round off the result as recommended in C.3.

Express the result as:

"estimated number N_E of microorganisms per millilitre".

EXAMPLE Colony counting has produced the following result:

— at the first dilution (10^{-2}) retained, 8 and 9 colonies were counted.

$$N_E = \frac{8+9}{1 \times 2 \times 10^{-2}} = \frac{17}{0{,}02} = 850$$

By rounding off the result as recommended in C.3, the estimated number N_E of microorganisms is 850 or $8{,}5 \times 10^2$ per millilitre of sample.

C.4.2 Case of two dishes (test sample or initial suspension or first dilution) containing no colonies

If the two dishes from the test sample, the initial suspension or the first dilution inoculated or retained do not contain any colonies, express the result as:

"less than $1/d$ of microorganisms per millilitre".

where d is the dilution of the initial suspension or of the first dilution inoculated or retained [$d = 1$ when the undiluted liquid (test sample) is used, $d = 0{,}1$ when a 10^{-1} dilution is used, etc.].

C.5 Special cases (counting of target or presumptive colonies)

C.5.1 If the number of all target and non-target colonies for the two dishes containing a first dilution d_1 is greater than 300 (or any other number stated in the specific standard), with visible target colonies or confirmed colonies, and if, for the two dishes of the subsequent dilution d_2 containing less than 300 colonies (or any other number stated in the specific standard), no target or confirmed colony can be counted, express the result as:

"less than $1/d_2$ and more than $1/d_1$ of microorganisms per millilitre".

where d_1 and d_2 are the dilution factors corresponding to dilutions d_1 and d_2.

EXAMPLE Colony counting has produced the following results:

— at the first dilution (10^{-2}) retained: more than 300 colonies on each of the two dishes, with target or confirmed colonies present;

— at the second dilution (10^{-3}) retained: 33 and 35 colonies, with no target or confirmed colonies present.

The result expressed in microorganisms is less than 1 000 and more than 100 per millilitre of sample.

C.5.2 If the number of all target and non-target colonies for the two dishes containing a first dilution d_1 is greater than 300 (or any other number stated in the specific standard), without visible target colonies or confirmed colonies, and if, for the two dishes of the subsequent dilution d_2 containing less than 300 colonies (or any other number stated in the specific standard), no target or confirmed colony can be counted, express the result as:

"less than $1/d_2$ of microorganisms per millilitre".

where d_2 is the dilution factor corresponding to the dilution d_2.

EXAMPLE Colony counting has produced the following results:

— at the first dilution (10^{-2}) retained: more than 300 colonies on each one of the two dishes, with no target or confirmed colonies present;

— at the second dilution (10^{-3}) retained: 33 and 35 colonies, with no target or confirmed colonies present.

The result expressed in microorganisms is less than 1 000 per millilitre of sample.

C.6 Method of calculation: Special cases

C.6.1 When the number of colonies counted (total colonies, target colonies or presumptive colonies) is greater than 300 (or any other number stated in the specific standard) for the two dishes containing a first dilution d_1, with a number of colonies (total colonies, target colonies or colonies complying with the confirmation criteria) of less than 10 for the two dishes of the subsequent dilution d_2:

— if the number of colonies for each of the two dishes containing dilution d_1 is within the 324 to 300 interval (upper part of the confidence interval for a weighted mean equal to 300), use the calculation method for general cases (C.2);

— if the number of colonies for each of the two dishes containing the dilution d_1 is greater than 324 (upper limit of the confidence interval for a weighted mean equal to 300), only take account of the result of the count of dilution d_2 and then proceed with the estimated count (9.1.8.4); except when referring to a maximum number set at 300 for the counting of colonies, if the latter result is less than 7 (lower limit of the confidence interval for a weighted mean equal to 10), since the difference between the two dilutions is then unacceptable.

The figures corresponding to confidence intervals should be adapted to the maximum number stated for the counting of colonies.

EXAMPLE 1 Colony counting has produced the following results:

— at the first dilution (10^{-2}) retained: 310 and 322 colonies;

— at the second dilution (10^{-3}) retained: 8 and 12 colonies.

Follow the method of calculation for general cases using the dishes from the two dilutions retained.

EXAMPLE 2 Colony counting has produced the following results:

— at the first dilution (10^{-2}) retained: more than 324 colonies on each one of the dishes;

— at the second dilution (10^{-3}) retained: 6 and 8 colonies.

Begin estimated counting on the basis of the colonies counted on the two dishes from the 10^{-3} dilution.

EXAMPLE 3 Colony counting (when a maximum number of 300 has been set for the counting of colonies) has produced the following results:

— at the first dilution (10^{-2}) retained: more than 324 colonies on each one of the two dishes;

— at the second dilution (10^{-3}) retained: 8 and 6 colonies.

The result of this counting is unacceptable.

— If the number of colonies for each of the two dishes containing the dilution d_1 is greater than 167 (upper limit of the confidence interval for a weighted mean equal to 150), only take account of the result of the count of dilution d_2 and then proceed with the estimated count (9.1.8.4); except when referring to a maximum number set at 150 for the counting of colonies, if the latter result is less than 4 (lower limit of the confidence interval for a weighted mean equal to 8), since the difference between the two dilutions is then unacceptable.

EXAMPLE 4 Colony counting (when a maximum number of 150 has been set for the counting of colonies) has produced the following results:

— at the first dilution (10^{-2}) retained: more than 167 colonies on each one of the two dishes (upper limit of the confidence interval with a weighted mean equal to 150);

— at the second dilution (10^{-3}) retained: 8 and 6 colonies.

Begin estimated counting on the basis of colonies counted on the two dishes from the 10^{-3} dilution.

C.6.2 Where the counting of colonies (total colonies or target colonies or presumptive colonies) for each one of the dishes for all inoculated dilutions produces a number greater than 300 (or any other number stated in the specific standard), express the result as:

"more than $300/d$ (case of total colonies or target colonies) or more than $300 \times b/A \times 1/d$ (case of confirmed colonies) microorganisms per millilitre".

where

d is the dilution factor of the last inoculated dilution;

b is the number of colonies complying with the confirmation criteria among the inoculated presumptive colonies, A.

C.6.3 Where only the two dishes with the last inoculated dilution contain less than 300 (or any other number stated in the specific standard) colonies (total colonies, target colonies or presumptive colonies), calculate the number N of microorganisms present in the test sample as an arithmetical mean of the colonies counted on the two dishes, using Formula (C.4):

$$N = \frac{\sum c}{V \times n \times d} \tag{C.4}$$

where

$\sum c$ is the sum of colonies counted on the two dishes, of which at least one contains a minimum of 10 colonies;

V is the volume of the inoculum applied to each dish, in millilitres, ml;

n is the number of dishes retained (in this case, $n = 2$);

d is the dilution factor corresponding to the dilution retained.

Round off the result as recommended in C.3.

Express the result as:

"number N of microorganisms per millilitre".

EXAMPLE Colony counting has produced the following result:

— at the last dilution (10^{-4}) inoculated: 120 and 130 colonies.

$$N = \frac{120 + 130}{1 \times 2 \times 10^{-4}} = \frac{250}{0{,}000\ 2} = 1\ 250\ 000$$

By rounding off the result as recommended in C.3, the number N of microorganisms is 1 300 000 or $1{,}3 \times 10^6$ per millilitre of sample.

Annex D
(normative)

Composition, preparation and performance testing of diluents

D.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the diluents described in this annex. If diluents are prepared from dehydrated complete media/reagents or if ready-to-use diluents/media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use. The diluents listed in this annex are commonly used in water microbiology. The most appropriate diluent for specific methods should be selected to ensure that there is no impact on method performance. Certain methods may also require diluents that are not included in this annex. Refer to specific standards for guidance.

Performance testing for the quality assurance of the diluents is described in D.4.

D.2 Composition and preparation

D.2.1 Saline solution

D.2.1.1 Composition

Sodium chloride (NaCl) (CAS No. 7647-14-5)	8,5 g
Water (see 6.2)	1 000 ml

D.2.1.2 Preparation

Dissolve the ingredients in the water, if necessary by heating. Adjust the pH by adding sodium hydroxide solution [c(NaOH) = 1 mol/l] or hydrochloric acid [c(HCI) = 1 mol/l] so that after sterilization it will correspond to 7,0 ± 0,2 at 25 °C.

NOTE Ready-prepared saline solution containing 9 g of sodium chloride in 1 000ml of water (0,9 %) is also commonly available commercially. It can be used if verified as acceptable by the laboratory.

D.2.2 Peptone diluent

D.2.2.1 Composition

Enzymatic digest of casein	1,0 g
Water (see 6.2)	1 000 ml

D.2.2.2 Preparation

Dissolve the ingredients in the water, if necessary by heating. Adjust the pH by adding sodium hydroxide solution [c(NaOH) = 1 mol/l] or hydrochloric acid [c(HCl) = 1 mol/l] so that after sterilization the pH will correspond to 7,0 ± 0,2 at 25 °C.

D.2.3 Peptone saline solution [maximum recovery diluent (MRD)]

D.2.3.1 Composition

Enzymatic digest of casein	1,0 g
Sodium chloride (NaCl) (CAS No. 7647-14-5)	8,5 g
Water (see 6.2)	1 000 ml

D.2.3.2 Preparation

Dissolve the ingredients in the water, if necessary by heating. Adjust the pH by adding sodium hydroxide solution [c(NaOH) = 1 mol/l] or hydrochloric acid [c(HCI) = 1 mol/l] so that after sterilization it will correspond to 7,0 ± 0,2 at 25 °C.

D.2.4 Quarter-strength Ringer's solution

D.2.4.1 Composition

Sodium chloride (NaCl) (CAS No. 7647-14-5)	2,25 g
Potassium chloride (KCl) (CAS No. 7447-40-7)	0,105 g
Calcium chloride (anhydrous) ($CaCl_2$) (CAS No. 10043-52-4)	0,06 g
Sodium hydrogen carbonate ($NaHCO_3$) (Cas No. 144-55-8)	0,05 g
Water (see 6.2)	1 000 ml

Alternatively, the equivalent mass (0,12 g) of calcium chloride hexahydrate ($CaCl_2 \cdot 6H_2O$) may be used.

D.2.4.2 Preparation

Dissolve the ingredients in the water, if necessary by heating. Adjust the pH by adding sodium hydroxide solution [c(NaOH) = 1 mol/l] or hydrochloric acid [c(HCI) = 1 mol/l] so that after sterilization it will correspond to 7,0 ± 0,2 at 25 °C.

D.2.5 Phosphate buffer solution

D.2.5.1 Phosphate solution

D.2.5.1.1 Composition

Potassium dihydrogenorthophosphate (anhydrous) (KH_2PO_4) (Cas No. 7778-77-0)	34 g
Water (see 6.2)	1 000 ml

D.2.5.1.2 Preparation

Dissolve the potassium dihydrogenorthophosphate in 500 ml of distilled water. Adjust the pH to a value of 7,2 ± 0,2 by adding sodium hydroxide solution [c(NaOH) = 1 mol/l] or hydrochloric acid [c(HCI) = 1 mol/l]. Add distilled water to 1 000 ml. Sterilize the solution if it is to be stored before use.

D.2.5.2 Magnesium chloride solution

D.2.5.2.1 Composition

Magnesium chloride (anhydrous) ($MgCI_2$) (CAS No. 7786-30-3)	38 g
Water (see 6.2)	1 000 ml

Alternatively, the equivalent mass (81,1 g) of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) may be used.

D.2.5.2.2 Preparation

Dissolve the magnesium chloride in the water. Sterilize the solution if it is to be stored before use.

D.2.5.3 Final solution

D.2.5.3.1 Composition

Phosphate solution (a)	1,25 ml
Magnesium chloride solution (b)	5,0 ml
Water (6.2)	1 000 ml

D.2.5.3.2 Preparation

Add the phosphate solution (a) and the magnesium chloride solution (b) to the water, then dispense in convenient volumes and sterilize. The final pH shall be 7,0 ± 0,2 at 25 °C.

D.3 Sterilization and storage

After preparation, distribute each solution into bottles and sterilize [e.g. by autoclaving at (121 ± 3) °C for 15 min]. Store at room temperature or in the refrigerator at (5 ± 3) °C for a maximum of six months. Alternatively, the diluent may be aseptically dispensed after sterilization and stored in the refrigerator for a maximum of one month. Diluents shall not be used if any change from normal appearance is apparent, e.g. cloudiness in usually clear diluents.

D.4 Performance testing

The performance testing requirements for the quality assurance of the diluents is shown in Table D.1.

Table D.1 — Performance testing for quality assurance of the diluents

Diluent	Function	Incubation	Control strains	WDCM numbers[a]	Reference medium	Method of control	Criteria[d]
Saline solution	Diluent	45 min to 1 h / 20 °C to 25 °C	*Escherichia coli*[c]	00012 or 00013	TSA	Quantitative	±30 % colonies/ T_0
Peptone diluent			*Staphylococcus aureus*	00034[b]			
Peptone saline solution							
Quarter-strength Ringer's solution							
Phosphate buffer solution							

[a] Refer to the reference strain catalogue on http://www.wfcc.info for information on culture collection strain numbers and contact details; WDCM: World Data Centre for Microorganisms.

[b] Strain to be used as a minimum.

[c] Strain free of choice; one of the strains has to be used as a minimum.

[d] The count after incubation should be within 30 % of the count achieved from the diluent before incubation (therefore ±30 % of original count). Refer to ISO 11133 for further description.

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