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Water quality — General guidance on the enumeration of micro-organisms by culture

Qualité de l'eau — Lignes directrices générales pour le dénombrement des micro-organismes sur milieu de culture

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 8199 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

This second edition cancels and replaces the first edition (ISO 8199:1988), which has been technically revised.

This corrected version of ISO 8199:2005 incorporates the following major corrections:

— 5.2.5 [item b), 4th paragraph] moved the 2nd sentence, “If the solution...”, to the paragraph under “Preparation”;

— 8.4.2 (Example 1) replaced “and if V_s is per ml:” with “and if V_s is 1 ml”;

— 8.4.2 (Example 2) replaced “and if V_s is per ml:” with “and if V_s is 100 ml”;

— 8.4.3 (example) replaced “and if V_s is per ml” with “and if V_s is 1 ml”; and

— Bibliography corrected References [14], [16], [17] and [19].

The equations in 8.4.2 and 9.5.2.1 were numbered, which resulted in the following changes:

— 8.4.2 (unnumbered equations) numbered as Equation (3) and Equation (4);

— 8.4.3 (equation) renumbered as Equation (5);

— 8.4.4.1 (equation) renumbered as Equation (6);

— 9.5.2.1 (unnumbered equation) numbered as Equation (7);

— 9.5.2.2 (equation) renumbered as Equation (8);

— 9.6.2 (equation) renumbered as Equation (9); and

— 9.6.3 (equation) renumbered as Equation (10).

Several minor corrections were made, including the following:

— 8.2.3.2 (paragraph 3)	replaced “melted” with “molten”;
— 8.4.2 [under Equation (2)]	in the explanation of the symbols “d_1, d_2,..., d_i”, deleted the word “portion”;
— 9.3.3 (last paragraph, 7th line)	added “wells” after “12 × 5 ml” and “24 × 3 ml”;
— 9.5.3.2 (example)	in the equation at the end of the example, replaced “1,61/(5 ml) × 100 ml” with “(1,61/5 ml) × 100 ml”;
— 9.5.3.3 (paragraph 2, 5th line)	added parentheses around “3 or 5”;
— A.2.1, A.2.2	replaced “uncertainty in” with “uncertainty of”; and
— A.2.3.1	replaced “an accepted” with “the accepted”; and “error in” with “error of”.

Introduction

Techniques for the isolation and enumeration of micro-organisms, based on their ability to grow on specified culture media, are an important and widely used means of assessing the microbiological quality of water. The purpose of this guide is to gather in a single document the information common to the various enumeration techniques so as to avoid repetition of technical details in individual standards and to facilitate the choice of the technique most suitable for a particular problem.

Water quality — General guidance on the enumeration of micro-organisms by culture

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This standard 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 and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this International Standard be carried out by suitably trained staff.

1 Scope

This International Standard presents guidance for carrying out manipulations which are common to each technique for the microbiological examination of water, particularly the preparation of samples, culture media and apparatus. It also describes the various enumeration techniques available and the criteria for the choice of a particular technique. This International Standard is mainly intended for bacteria, yeasts and moulds. Some aspects are also applicable to viruses and parasites.

2 Normative references

The following referenced documents are indispensable for the application 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 3696:1987, *Water for analytical laboratory use — Specification and test methods*

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

3 Principle

The general principle of these techniques consists of inoculating a known volume of a water sample on or into a culture medium (solid or liquid). It is assumed that after incubation each micro-organism present multiplies, giving either a colony visible directly on the solid medium, or changes in observable properties of the liquid medium. The choice of a particular method depends not only on the nature of the micro-organisms sought, but also on the nature of the water and the reasons for the examination.

4 General

Uniformity of temperatures and (incubation) times: The following accepted ranges of temperatures and times during incubation or storage are applied, when appropriate for the intended target organism, and unless otherwise stated in the specific standard.

Storage temperatures:	(– 70 ± 10) °C; (– 20 ± 5) °C; (5 ± 3) °C;
Incubation temperatures:	(22 ± 2) °C; (36 ± 2) °C;
Sterilization temperatures:	(115 ± 3) °C; (121 ± 3) °C; (170 ± 10) °C;
Incubation times:	(21 ± 3) h; (44 ± 4) h; (68 ± 4) h.

Other times and temperatures may be specified for specific methods when necessary.

The upper incubation temperature limits are very strict (they can have large influences on the growth). The lower temperature limits may be exceeded for short periods, e.g. due to opening the door of an incubator, but recovery shall be rapid.

Tolerances on volumes and masses: Unless otherwise stated, the accepted range of any measured value is: stated value ± 5 %.

5 Diluents and culture media

5.1 General

5.1.1 Quality requirements

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

Use glass-distilled or demineralized water which is free from substances that might affect growth of micro-organisms under the test conditions. The water shall comply with the requirements of ISO 3696:1987, grade 3.

Unless otherwise stated, the ingredients are added to the volume of water instead of making up to a certain volume, as is normal in the preparation of microbiological culture media.

Before use, check the quality of the media, diluents and filters, e.g. by following the procedures described in ISO 7704, ISO/TS 11133-1 or ISO/TS 11133-2.

5.1.2 Sterilization

Dispense diluents and culture media in containers suitable for sterilization by autoclaving. For most purposes, a temperature of (121 ± 3) °C for 15 min is adequate. However, a different time and temperature may sometimes be required and details are given in each individual standard.

Alternatively, with thermolabile substances, removal of micro-organisms may be effected by filtration through a filter with a pore size of 0,2 µm, specified by the manufacturer as being suitable for “sterilization”.

5.2 Diluents

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

After preparation, distribute each solution into bottles and sterilize, e.g. by autoclaving at (121 ± 3) °C for 15 min. Alternatively, the diluent can be aseptically distributed after sterilization. Store at room temperature or in a refrigerator at (5 ± 3) °C for a maximum of 6 months. If a diluent shows any change from its normal appearance, discard it.

5.2.1 Saline solution

Composition

Sodium chloride (NaCl)	8,5 g
Water (see 5.1.1)	1 000 ml

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 (see 5.1.2), it will correspond to 7,0 ± 0,5 at 25 °C.

5.2.2 Peptone diluent

Composition

Enzymatic digest of casein (peptone)	1,0 g
Water (see 5.1.1)	1 000 ml

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 (see 5.1.2), it will correspond to 7,0 ± 0,5 at 25 °C.

5.2.3 Peptone saline solution

Composition

Enzymatic digest of casein (peptone)	1,0 g
Sodium chloride (NaCl)	8,5 g
Water (see 5.1.1)	1 000 ml

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 (see 5.1.2), it will correspond to 7,0 ± 0,5 at 25 °C.

5.2.4 Ringer's solution, quarter-strength

Composition

Sodium chloride (NaCl)	2,25 g
Potassium chloride (KCl)	0,105 g
Calcium chloride (anhydrous) ($CaCl_2$)	0,12 g
Sodium hydrogen carbonate ($NaHCO_3$)	0,05 g
Water (see 5.1.1)	1 000 ml

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 (see 5.1.2), it will correspond to 7,0 ± 0,2 at 25 °C.

5.2.5 Phosphate buffer solution

a) Phosphate solution

Composition

Potassium dihydrogen orthophosphate (KH_2PO_4)	34 g
Water (see 5.1.1) to	1 000 ml

Preparation

Dissolve the potassium dihydrogen orthophosphate in 500 ml of the 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(HCl) = 1 mol/l]. Add more water up to 1 000 ml. If the solution needs to be stored, sterilize it (see 5.1.2) before storage.

b) Magnesium chloride solution

Composition

Magnesium chloride ($MgCl_2$)	38 g
Water (see 5.1.1)	1 000 ml

Alternatively, an equivalent mass (99 g) of magnesium sulfate ($MgSO_4.7H_2O$) may be used.

Preparation

Dissolve the magnesium chloride in the water. If the solution needs to be stored, sterilize it (see 5.1.2) before storage.

c) Final solution

Composition

Phosphate solution (a)	1,25 ml
Magnesium chloride solution (b)	5,0 ml
Water (see 5.1.1)	1 000 ml

Preparation

Add the phosphate solution (a) and the magnesium chloride solution (b) to the water, dispense in convenient volumes and sterilize (see 5.1.2). The final pH should correspond to 7,0 ± 0,2 at 25 °C.

5.3 Culture media

Once a bottle of dehydrated medium (chemical) is opened, date the container and indicate a maximum storage time.

In general, most media after sterilization in sealed containers may be stored satisfactorily for several months at room temperature provided they are kept in the dark and remain sealed. Media dispensed aseptically may be stored at (5 ± 3) °C for up to 1 month, or longer if approved. Before use, inspect them carefully for contamination, excessive evaporation, or other evidence of deterioration. Most reagents are best kept at (5 ± 3) °C. Use culture media supplied pre-poured in accordance with the manufacturer's instructions.

Pre-cool the medium to 45 °C to 50 °C if heat-sensitive supplements need to be added after autoclaving.

6 Sterilization of apparatus and glassware

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

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

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

If membrane filters are not obtained sterile, sterilize them before use in accordance with the manufacturer's instructions.

7 Samples

7.1 Sampling

Take samples in accordance with ISO 19458.

7.2 Preparation of test sample

Before examination, mix the sample thoroughly by vigorous agitation to achieve uniform distribution of micro-organisms and, depending on the nature of the water and the microbial content anticipated, make any dilutions necessary at this stage.

In the case of plate counts, decimal dilutions can be used. For membrane filtration (with a smaller surface area), smaller dilution steps are recommended.

For ten-fold dilutions, measure 90 ml or 9 ml of the diluent into sterile dilution bottles or tubes. Alternatively, volumes of diluent, pre-sterilized in screwcapped bottles, can be used. 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. These steps are repeated as many times as required. Prepare sufficient volumes of each dilution for all the tests to be carried out on the sample.

For dilutions other than ten-fold, the volume of diluent to volume of sample shall be adjusted accordingly. Various approaches can be taken, i.e. 3- or 4-fold dilution series, or decimal dilution series of which both 10 ml and 30 ml volumes are filtered. Four-fold dilutions can be made as described above for ten-fold dilutions, except that, in this case, one volume of water sample is mixed with three volumes of diluent.

If the concentration of the target organism is expected to be high, hundred-fold dilution steps can be used.

For general guidance on the preparation of ten-fold dilutions, see ISO 6887-1.

8 Enumeration after inoculation of test portions of the sample in (or on) solid media

8.1 Principle

A test portion of the water sample, or a dilution, is inoculated either directly or concentrated on a membrane on the surface of a specified solid culture medium or in a molten medium so that, on incubation, micro-organisms form colonies either on or in the medium.

For practical purposes, each colony is considered to have originated from a single micro-organism or a clump of micro-organisms present in the test portion at the moment 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.

8.2 Procedures

8.2.1 General

Three main procedures may be used for the inoculation of solid media.

a) Pour plate technique.

The test portion is mixed with the medium, which has previously been melted and cooled to a temperature close to that of solidification; after incubation, the colonies that develop within and on the surface of the medium are counted.

b) Spread plate technique.

The test portion is spread over the dry surface of an agar medium and, after incubation, colonies that develop on its surface are counted.

c) Membrane filtration technique.

The test portion is passed through a membrane filter, which retains the micro-organisms sought; the membrane is then placed on an agar medium or on an absorbent pad impregnated with liquid or rehydrated medium. On incubation, colonies form on the surface of the membrane. Alternatively, for certain organisms, such as anaerobes, the membrane may be placed face downwards in a Petri dish and overlaid with molten agar medium.

8.2.2 Choice of technique

The choice of technique depends on several factors, including the physical and chemical characteristics of the water as well as the nature of the micro-organisms sought (see Annex A, Clause A.3), their probable concentration, the effective recovery of stressed or (sub-lethally) injured micro-organisms, the test precision and the sensitivity required. Indications are given in 8.2.3.1, 8.2.4.1 and 8.2.5.1 of the volumes of water samples that can be used for each technique and the limits of detection are discussed in Clause A.2. The accuracy of the techniques is also discussed in Clause A.2. The requirements of regulations may also influence the choice of technique to be used by indicating, for example, the precision desired or whether the presence or absence of an organism in a specified test volume will be sufficient.

8.2.3 Pour plate technique

8.2.3.1 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 typical colonies formed on plates of diameter 90 mm to 100 mm is between about 10 and 150. The total number of colonies on the plate (typical and non-typical) should be less than 300

(see ISO 7218). Note, however, that the total number of countable colonies depends on the size of the colonies and the number may have to be reduced for large colonies.

8.2.3.2 Inoculation

Melt the medium required in boiling water or by any other suitable process (e.g. an appropriate air incubator, a steam flow-through autoclave or a microwave oven, if the heating time/temperature combination has been validated for the medium preparation). Avoid over-heating and remove the medium as soon as it has been melted. Place the molten medium in a water bath at (45 ± 1) °C for sufficient time so that the medium will equilibrate to this temperature. It is preferable not to keep a medium molten for more than 4 h. Do not melt agar media more than once.

Prepare and mark the Petri dishes required. Make any dilutions necessary in accordance with 7.2. Distribute, after thorough mixing, the test portions into the dishes.

Remove each tube or flask of molten medium in turn from the water bath, dry the outside of the tube or flask and flame the neck. Add the medium to each Petri dish without delay, avoiding the test portion to minimize heat shock, and mix carefully so as to obtain a uniform distribution of micro-organisms. Generally, 15 ml of medium is used for a test portion of 1 ml or 2 ml; for larger test portions, adjust the concentration of the medium accordingly. Leave the plates to cool on a horizontal surface in order to allow the agar to solidify. As soon as the agar has solidified, incubate the plates in accordance with 8.2.6.

NOTE Agar preparator pourer-stacker systems can be useful in laboratories analysing large numbers of samples.

8.2.4 Spread plate technique

8.2.4.1 Test portion

For a Petri dish of between 90 mm and 100 mm diameter, the volume of the test portion of the sample, or of a dilution of the sample, should be 0,1 ml to 0,5 ml. Choose the dilution so that the expected number of typical colonies formed lies between about 10 and 150. The total number of colonies on the plate (typical and non-typical) should be less than 200. Note, however, that the total number of countable colonies depends on the size of the colonies and the number may have to be reduced for large colonies.

8.2.4.2 Inoculation

Prepare and mark plates, each containing about 15 ml of culture medium. Dry the surface of the medium before use (if necessary). Pipette the test portion on to the surface of the medium and spread over the surface with a sterile rod or mechanical device. After absorption of the inoculum, incubate the plates in accordance with 8.2.6.

For the drying of the plates the following points are of importance:

— The degree of humidity in culture media is important because optimum growth of bacteria 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 that do not spread rapidly are cultured, and the plates look dry after acclimatization, the circumstances are such that drying is not always necessary. In that 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 heating will not negatively affect the quality of the culture medium. The drying time will depend on the degree to which condensation is present in the Petri dish, but shall be kept as short as possible.

In order to avoid contamination, and if the plates are not dried in a laminar-flow cabinet, plates shall always be dried with the surface of the culture medium to be inoculated turned downwards.