

Australian/New Zealand Standard™

Water microbiology

Method 1: General information and procedures (ISO 8199:2005, MOD)

PREFACE

This Standard was prepared by the Joint Standards Australia/New Zealand Committee FT-020, Water Microbiology to supersede AS 4276.1—1995, *Water microbiology*, Method 1: *General information and procedures*.

This Standard is an adoption with national modifications and has been reproduced from ISO 8199:2005, *Water quality—General guidance on the enumeration of micro-organisms by culture*.

The objective of this revision is to adopt ISO 8199:2005 as a joint Australian/New Zealand Standard with technical variations to the following clauses of ISO 8199:

- (a) Clause 8.3.2, colonies to be counted and confirmed.
- (b) Clause 8.4.3, case after identification or confirmation.

These variations, which are necessary for Australian/New Zealand conditions are listed in Appendix ZZ which is added at the end of the source text.

The additional requirements are included to increase the precision of the confirmed colony count.

As this Standard is reproduced from an international Standard, the following applies:

- (i) Its number does not appear on each page of text and its identity is shown only on the cover and title page.
- (ii) In the source text ‘this International Standard’ should read ‘this Australian/New Zealand Standard’.
- (iii) A full point substitutes for a comma when referring to a decimal marker.

Reference to International Standard should be replaced by reference to the following Australian/New Zealand Standard:

<i>Reference to International Standard or other publication</i>	<i>Joint Australian/New Zealand Standard</i>
ISO	AS/NZS
19458 Water quality—Sampling for microbiological analysis	2031 Selection of containers and preservation of water samples for microbiological analysis

The other international reference has not been adopted as an Australian or Australian/New Zealand Standard.

The laboratory should have a clearly defined quality control system to ensure that the apparatus, culture media, reagents and technique are suitable for the test. The use of positive controls is part of this system.

The terms ‘normative’ and ‘informative’ have been used in this Standard to define the application of the annex or appendix to which they apply. A ‘normative’ annex or appendix is an integral part of a Standard, whereas an ‘informative’ annex or appendix is only for information and guidance.

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.

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: $(-70 \pm 10) ^\circ\text{C}$; $(-20 \pm 5) ^\circ\text{C}$; $(5 \pm 3) ^\circ\text{C}$;

Incubation temperatures: $(22 \pm 2) ^\circ\text{C}$; $(36 \pm 2) ^\circ\text{C}$;

Sterilization temperatures: $(115 \pm 3) ^\circ\text{C}$; $(121 \pm 3) ^\circ\text{C}$; $(170 \pm 10) ^\circ\text{C}$;

Incubation times: $(21 \pm 3) \text{ h}$; $(44 \pm 4) \text{ h}$; $(68 \pm 4) \text{ h}$.

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 $\pm 5 \%$.

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 $(121 \pm 3) ^\circ\text{C}$ 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 $0,2 \mu\text{m}$, 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 $(121 \pm 3) ^\circ\text{C}$ for 15 min. Alternatively, the diluent can be aseptically distributed after sterilization. Store at room temperature or in a refrigerator at $(5 \pm 3) ^\circ\text{C}$ 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 [$c(\text{NaOH}) = 1 \text{ mol/l}$] or hydrochloric acid [$c(\text{HCl}) = 1 \text{ mol/l}$] so that, after sterilization (see 5.1.2), it will correspond to $7,0 \pm 0,5$ 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 [$c(\text{NaOH}) = 1 \text{ mol/l}$] or hydrochloric acid [$c(\text{HCl}) = 1 \text{ mol/l}$] so that, after sterilization (see 5.1.2), it will correspond to $7,0 \pm 0,5$ 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 [$c(\text{NaOH}) = 1 \text{ mol/l}$] or hydrochloric acid [$c(\text{HCl}) = 1 \text{ mol/l}$] so that, after sterilization (see 5.1.2), it will correspond to $7,0 \pm 0,5$ 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 [$c(\text{NaOH}) = 1 \text{ mol/l}$] or hydrochloric acid [$c(\text{HCl}) = 1 \text{ mol/l}$] so that, after sterilization (see 5.1.2), it will correspond to $7,0 \pm 0,2$ 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 $7,2 \pm 0,2$ by adding sodium hydroxide solution [$c(\text{NaOH}) = 1 \text{ mol/l}$] or hydrochloric acid [$c(\text{HCl}) = 1 \text{ mol/l}$]. 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 ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) 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 $7,0 \pm 0,2$ 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 $(5 \pm 3) ^\circ\text{C}$ 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 $(5 \pm 3) ^\circ\text{C}$. Use culture media supplied pre-poured in accordance with the manufacturer's instructions.

Pre-cool the medium to $45 ^\circ\text{C}$ to $50 ^\circ\text{C}$ 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 $(170 \pm 10) ^\circ\text{C}$ for at least 1 h (excluding pre-heating time);
- b) in an autoclave, at $(121 \pm 3) ^\circ\text{C}$ 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 $(45 \pm 1)^\circ\text{C}$ 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.

In practice, the plates will be dried by placing them with the agar surfaces downwards and with half-open lids in an incubator set at a temperature between 25 °C and 50 °C. Dry the plates until the droplets have disappeared from the surface of the lids. Do not dry any further. The agar plates can also be dried (in the same way as described above) in a laminar-flow safety cabinet (at room temperature) for 30 min to 60 min, or overnight at room temperature with the lids in place.

8.2.5 Membrane filtration technique

8.2.5.1 Test portion

The maximum volume of the test portion depends on the filterability of the water sample and on the membranes used. This technique is suitable for waters that contain little particulate or colloidal (e.g. iron) matter in suspension, for example drinking water. With membranes of mean pore size 0,45 µm, it may be possible to filter several litres of such water through a single membrane, and so achieve a high level of test sensitivity. For some organisms (like *Legionella*), however, filtration through a membrane with a mean pore size of 0,2 µm may be necessary.

A test volume of the sample or a dilution of it should be chosen so that the expected number of typical colonies formed on a membrane of 47 mm to 50 mm in diameter is between about 10 and 100. The total number of colonies on the filter (typical and non-typical) should be less than about 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.

8.2.5.2 Filtration

Connect the sterile filtration apparatus to a source of vacuum. Place a sterile membrane filter, grid-side upwards, on the porous disc of the filter base, with only the outer part of the membrane filter being grasped by flat-ended sterile 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) 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 sufficient vacuum (about 70 kPa) to filter the water through the membrane. 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.

8.2.5.3 Transfer of membrane

Remove the funnel (make sure the stopcock is closed before doing this) and transfer the membrane with sterile forceps to one of the following, ensuring that no air bubbles are trapped between the membrane and the medium:

- a) an agar medium in a Petri dish;
- b) 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 any confluent growth, pour off any excess medium, preferably before placing the membrane on the pad);
- c) a Petri dish, or on to a little agar medium in a Petri dish, subsequently overlaying the membrane with molten agar medium at $(45 \pm 1) ^\circ\text{C}$.

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 use a separate sterile apparatus or disinfect the funnel, for example by flaming. Alternatively, follow the manufacturer's instructions for disinfection.

8.2.6 Incubation

Invert the inoculated 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) to prevent desiccation of the medium (e.g. when a fan-assisted incubator is used). Plates containing membranes on absorbent pads should be placed (not inverted) in an air- or water-tight container to prevent desiccation of the medium, if necessary. Do not make higher stacks than 6 Petri dishes to ensure that all plates reach incubation temperature rapidly.

Choose the duration and temperature of incubation by consulting a standard method, as they depend on the micro-organism, or groups of micro-organisms, sought.

Incubation may be carried out in two stages, as follows:

Pre-incubation of variable duration, for example 2 h to 4 h at a lower temperature (e.g. 25 °C to 30 °C), can be used to permit resuscitation of stressed organisms, followed by a longer period of incubation at the usual temperature for the organism sought. The change may be effected either by transfer to another incubator or water bath, or by using apparatus in which the temperature is automatically changed after a given time. Ideally, place all the plates in the incubator or water bath at the same time.

Alternatively, the membranes may be 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.

8.3 Enumeration

8.3.1 General

Examine the plates or membranes immediately after incubation. If this is not possible, they may be kept at $(5 \pm 3) ^\circ\text{C}$ for short periods (e.g. a few days) provided that this does not affect the numbers, appearance or subsequent confirmation of the colonies. If the plates or membranes are stored, validate the storage period as being acceptable for the method and sample type.

8.3.2 Colonies to be counted and confirmed

For "total" counts on a non-selective medium, all the colonies are counted. With selective and differential media, only those colonies that show the characteristic appearance of the organism sought are counted. 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. In general, it is unusual for such counts to indicate the organisms belonging only to one taxonomic group, but in practice this discrepancy may be accepted and the results can be expressed as presumptive. For more precise characterization, confirmatory tests are necessary.

It may be impracticable, however, if there are numerous colonies, to confirm the identity of all of them. In such instances, examine all typical colonies from a sub-area of the plate or membrane (see also 8.4.3).

8.4 Calculation of results

8.4.1 Principle

A large part of this subclause has been taken from ISO 7218:1996/Amd 1:2001.

The methods of calculation defined below take account of the cases that occur most frequently when tests are carried out in accordance with the methods of good practice of a laboratory. Rare, special cases may occur (for example, significant discrepancy between the number of colonies on two plates with the same dilution, or

a very different ratio to that of the dilution factor between the plates of two successive dilutions), and it is therefore necessary that results obtained from counting be examined, interpreted or even refused by a qualified microbiologist.

8.4.2 General case

The calculation of results given in this subclause can be used in cases when the total number of colonies on the plates is between 10 and 200 (spread plate and membrane filtration techniques) or between 10 and 300 (pour plate technique), and where the number of typical colonies is between 10 and 150 (spread plate and pour plate techniques) or between 10 and 100 (membrane filtration technique).

Since each colony is assumed to have arisen from one micro-organism or from a single aggregate of micro-organisms, 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) by Equation (1):

$$C_s = \frac{Z}{V_{\text{tot}}} \times V_s \quad (1)$$

where

C_s is the estimate of the number of cfu or cfp in the reference volume V_s of the sample;

Z is the sum of colonies counted on plates or on membranes derived from dilutions $d_1, d_2, \dots, d_i$, or derived from separate volumes of the test portion (sample or dilution);

V_s is the reference volume chosen to express the concentration of the micro-organisms in the sample;

V_{tot} is the calculated total volume of original sample included in the plates enumerated. V_{tot} is either the sum of the separate volumes from the test portion (sample or dilution) or calculated by Equation (2):

$$V_{\text{tot}} = (n_1 V_1 d_1) + (n_2 V_2 d_2) + \dots + (n_i V_i d_i) \quad (2)$$

where

V_{tot} is the calculated total volume of original sample included in the plates enumerated;

$n_1, n_2, \dots, n_i$ is the number of plates counted for dilution $d_1, d_2, \dots, d_i$;

$V_1, V_2, \dots, V_i$ is the test volume used with dilution $d_1, d_2, \dots, d_i$;

$d_1, d_2, \dots, d_i$ is the dilution used for the test volume $V_1, V_2, \dots, V_i$ ($d = 1$ for an undiluted sample, $d = 0,1$ for a ten-fold dilution, etc.).

NOTE 1 The final result thus obtained is a function of the weighted average of the counts from each plate.

Unless otherwise stated, round off the calculated results to two significant figures, when reporting final results. 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.

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