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Water quality — General guide to the enumeration of micro-organisms by culture

Qualité de l'eau — Guide général 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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

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Annex A is for information only.

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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.

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Water quality — General guide to the enumeration of micro-organisms by culture

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 methods available and the criteria for the choice of a particular technique.

2 Normative references

The following standards contain provisions, which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards listed below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 3534: 1977, *Statistics — Vocabulary and symbols.*

ISO 3696: 1987, *Water for analytical laboratory use — Specification and test methods.*

ISO 5667-2: 1982, *Water quality — Sampling — Part 2: General guide to sampling techniques.*

ISO 5667-3: 1985, *Water quality — Sampling — Part 3: Recommendations for the preservation and handling of samples.*

ISO 6887: 1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

3 Principle

The general principle of these techniques consists of inoculating a known volume of a water sample into a culture medium (solid or liquid). It is assumed that on incubation each micro-organism present multiplies giving either a colony visible directly on the solid medium, or changes in appearance in 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 Safety and hygiene in the laboratory

As well as general and statutory considerations for safety in the laboratory, particular care and a high standard of personal hygiene are essential in microbiological work because pathogenic organisms may always be encountered. In addition, media not necessarily designed for pathogens may support their growth so that great care is needed in the handling and disposal of all cultures. Sound technique is the basis of safe microbiological procedures and it is important that all individuals concerned with microbiological work should have received adequate training. It is also important that the necessary laboratory equipment and facilities should conform to accepted codes of safety and good laboratory practice.

Certain precautions are essential in the laboratory not only to prevent contamination of samples and culture media but also to avoid infection hazards to personnel, namely:

— strictly observe personal hygienic precautions, keep nails short, and use hair and beard protection if necessary;

— wash hands with soap and warm water both before and after microbiological work as well as after use of the toilet;

— always wear protective clothing in the laboratory;

— sterilize all cultures by autoclaving, and sterilize or disinfect associated apparatus and materials before disposal or re-use;

— do not eat, drink or smoke in the laboratory;

— report any accident, spillage or unusual occurrence to senior personnel;

— if in doubt, ask for advice.

5 Diluents and culture media

5.1 General

Constituents of uniform quality and analytical grade chemicals should be used for the preparation of media. Alternatively, dehydrated complete media may be used. The manufacturer's instructions should be strictly followed.

The water used should be glass-distilled or demineralized and free from substances that might inhibit growth of micro-organisms under the test conditions. It should comply with the requirements of ISO 3696, Grade 3.

5.1.1 Sterilization

Diluents and culture media should be dispensed in containers suitable for sterilization by autoclaving. For most purposes, a temperature of 121 °C ± 1 °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 sterilization may be effected by filtration.

5.2 Diluents

One of the following diluents should be used.

5.2.1 Peptone diluent

Composition

Peptone	1,0 g
Water	to 1 000 ml

Preparation

The peptone should be dissolved in approximately 950 ml of water. The pH should be adjusted 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 be 7,0 ± 0,1. The solution should be made up to 1 000 ml with water, distributed and sterilized.

5.2.2 Peptone saline solution

Composition

Peptone	1,0 g
Sodium chloride	8,5 g
Water	to 1 000 ml

Preparation

The constituents should be dissolved in the water and the pH adjusted by adding sodium hydroxide solution [c(NaOH) = 1 mol/l] or hydrochloric acid [c(HCl) = 1 mol/l] so that after sterilization it will be 7,0 ± 0,1. The solution should be made up to 1 000 ml with water, distributed and sterilized.

5.2.3 Ringer's solution — quarter-strength

Composition

Sodium chloride	2,25 g
Potassium chloride	0,105 g
Calcium chloride (anhydrous)	0,12 g
Sodium hydrogen carbonate	0,05 g
Water	to 1 000 ml

Preparation

The constituents should be dissolved in the water and the solution made up to 1 000 ml.

5.2.4 Phosphate buffer solution

Composition

Potassium dihydrogenorthophosphate (KH_2PO_4)	42,5 mg
Magnesium chloride ($MgCl_2$) (see note)	190 mg
Water	1 000 ml

Preparation

a) Phosphate solution

To prepare the solution 34 g of potassium dihydrogen-orthophosphate should be dissolved in 500 ml of distilled water. The pH should be adjusted to a value of 7,2 ± 0,5 with 1 mol/l sodium hydroxide solution and the solution made up to 1 000 ml with distilled water.

NOTE — Alternatively, an equivalent amount of magnesium sulfate ($MgSO_4.7H_2O$) may be used.

b) Magnesium chloride solution

To prepare the solution 38 g of magnesium chloride should be dissolved in 1 000 ml of distilled water.

Final solution

For use, 1,25 ml of phosphate solution a) and 5,0 ml of magnesium chloride solution b) should be added to 1000 ml of distilled water. The solution should be dispensed in convenient volumes and sterilized by autoclaving at 121 °C ± 1 °C for 15 min.

5.2.5 Distilled water

Distilled water of suitable grade should be distributed and sterilized (see 5.1).

5.3 Culture media

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 between 4 °C and 10 °C for up to 1 month; before use, they should be inspected carefully for contamination, excessive evaporation, or other evidence of deterioration. Most reagents are best kept at 4°C. Culture media supplied pre-poured should be used in accordance with the manufacturer's instructions.

6 Sterilization of apparatus and glassware

Apparatus and glassware not supplied sterile should be sterilized by one of the following methods:

a) in an oven, at 160 °C ± 2 °C for 1 h (or 170 °C ± 2 °C for 40 min);

b) in an autoclave, at 121 °C ± 1 °C for a minimum of 20 min.

If membrane filters are not obtained sterile, they should be sterilized before use according to the manufacturer's instructions or, in their absence, by autoclaving at 115 °C ± 1 °C for 10 min or, for vegetative organisms, disinfected by boiling gently in distilled water for 10 min.

7 Samples

7.1 Sampling

For guidance on sampling techniques, refer to ISO 5667-2.

The prime objective is to obtain a sample which is representative, as far as possible, of the water to be examined. To achieve this, certain precautions are necessary which are common to all sampling procedures for bacteriological examination:

a) for re-use, sampling bottles should be able to withstand repeated sterilization without producing or releasing substances likely to inhibit or promote bacterial growth;

b) sampling bottles should contain an agent to neutralize any residual disinfectant in the water and should be sterile. The neutralizing agent should not affect the viability or growth of the organisms sought. For chlorine, 0,1 ml of a 1,8 % (m/m) sterile solution of sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) should be added before sterilization to each bottle for each 100 ml of capacity;

c) scrupulous care should be taken to avoid accidental contamination of the sample during collection and subsequent handling;

d) the sample should be examined as soon as possible after collection, preferably within 6 h;

e) every sample bottle should be clearly identifiable, and should be accompanied by sufficient information concerning the nature of the sample and the reasons for the examination requested.

7.2 Preservation and handling

For guidance on preservation and handling of samples, refer to ISO 5667-3.

In general, samples should be transported to the laboratory for examination as soon as possible. If there is likely to be a delay, the samples should be placed in a cool insulated container.

7.3 Preparation of test sample

Before examination, the sample should be mixed thoroughly by vigorous agitation to achieve uniform distribution of micro-organisms and, depending on the nature of the water and the bacterial content anticipated, any dilutions necessary made at this stage.

For ten-fold dilutions, 90 ml or 9 ml of the diluent should be measured out into sterile dilution bottles or tubes. Alternatively, volumes of diluent, pre-sterilized in screwcapped bottles, should be used. One or more ten-fold dilutions should be made by transferring one volume of water sample to nine volumes of diluent. With a fresh pipette or by mechanical means, the solution should be mixed thoroughly and one volume of this dilution transferred to another nine volumes of diluent. These steps should be repeated as often as required. Sufficient of each dilution for all the tests to be carried out on the sample should be prepared. For dilutions other than ten-fold, the volume of diluent to volume of sample should be adjusted accordingly.

For general guidance on the preparation of ten-fold dilutions, refer to ISO 6887; this guidance can also be applied to making dilutions with a dilution factor other than 10.

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 is inoculated by mixing with or spreading on the surface of a specified solid culture 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 sample 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 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) The sample is mixed with the medium which has previously been melted and cooled to a temperature close to that of solidification; after incubation the colonies which develop within and on the surface of the medium are counted.

b) The sample is spread over the dry surface of an agar medium and after incubation, colonies which develop on its surface are counted.

c) The sample 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 broth. 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 (see 10.2.2) as well as the nature of the micro-organisms sought (see 10.2.1), their probable concentration, and the test precision required. Indications are given in 8.2.3.1, 8.2.4.1, and 8.2.5.1 of the volumes of water samples which can be used for each method and the limits of detection are discussed in 10.1.3. In addition the precision of the methods is discussed in 10.1.2. 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 method

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 colonies formed on plates of diameter 90/100 mm is between about 25 and 300.

8.2.3.2 Inoculation

The medium required should be melted in boiling water or steam, placed in a water bath at 45 °C ± 1 °C and with sterile precautions any additional ingredients necessary added at this stage.

The Petri dishes required should be prepared and marked, any dilutions necessary made in accordance with 7.3 and, after thorough mixing, the test portions distributed into the dishes.

Each tube of melted medium should be removed in turn from the water bath, the outside of the tube dried and the neck flamed. The medium should be added to the test portion in each Petri dish without delay and mixed carefully so as to obtain a uniform distribution of micro-organisms. The plates should be left to cool on a horizontal surface and then they should be incubated in accordance with 8.2.6. Generally 15 ml of medium is used for a test portion of 1 ml or 2 ml; for larger test portions, the composition of the medium should be adjusted accordingly.

8.2.4 Spread plate method

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. The dilution should be chosen so that the expected number of colonies formed lies between about 25 and 300.

8.2.4.2 Inoculation

Plates, each containing about 15 ml of culture medium, should be prepared and marked; the surface of the medium should be dried before use. The test portion should be pipetted on to the surface of the medium and spread over the surface with a sterile glass rod or mechanical device. After absorption of the inoculum, incubation should be carried out in accordance with 8.2.6.

8.2.5 Membrane filtration method

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 which contain little particulate matter in suspension, for example drinking water. With membranes of mean pore diameter 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, however, filtration through a membrane with a mean pore diameter of 0,22 µm may be necessary. A test volume of the sample or a dilution of it should be chosen to yield less than about 100 colonies on a membrane of 47 mm or 50 mm in diameter.

8.2.5.2 Filtration

The sterile filtration apparatus should be connected to a source of vacuum. A sterile membrane should be placed, grid-side upwards, on the porous disc of the filter base, only the outer part of the membrane filter being grasped with flat-ended sterile forceps. The sterile funnel should be positioned securely on the filter base. With the vacuum stopcock turned off, one of the following should be poured or pipetted into the funnel:

a) a known volume of the sample, or dilution of it, carefully mixed;

b) the contents of a flask or bottle containing the test portion and sufficient diluent to bring the total volume to at least 20 ml to 30 ml;

c) at least 20 ml of diluent, to which the test portion, measured with a pipette, is added directly and mixed with the pipette.

The stopcock should be opened and a vacuum of about 70 kPa applied to filter the water slowly through the membrane. The stopcock should be closed as soon as the sample has been filtered.

8.2.5.3 Transfer of membrane

The funnel should then be removed and the membrane transferred 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 in a Petri dish. To avoid any confluent growth, any excess medium should be poured off, preferably before placing the membrane on the pad;

c) a Petri dish, or onto a little agar medium in a Petri dish; then the membrane should be overlaid with a molten agar medium (45 °C).

For different volumes of the same sample, the funnel may be re-used without disinfection provided that the smallest volumes are filtered first. To filter another sample, either a separate sterile apparatus should be used or for vegetative organisms the funnel disinfected, for example by immersion in boiling water for at least 1 min; alternatively, the manufacturer's instructions for disinfection should be followed. During the filtration of a series of samples the filter base need not be disinfected unless it is contaminated or a membrane is damaged.

The filtration of known polluted samples should not be alternated with those of treated water samples through the same apparatus. First all the samples of chlorinated water and those expected to give negative results should be filtered, followed by those known to be polluted. Alternatively, a separate membrane filtration apparatus can be reserved for all chlorinated samples and another for polluted samples.

8.2.6 Incubation

The inoculated plates should be inverted and placed either in an incubator or in a water-tight container in a water bath. Plates containing membranes on absorbent pads should always be placed in an air- or water-tight container to prevent desiccation of the medium.

The duration and temperature of incubation should be chosen after reference to a standard method as they depend on the micro-organism, or groups of micro-organisms, sought.

Incubation may be carried out in two stages.

Pre-incubation of variable duration, for example 2 h to 4 h at a lower temperature, 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 period. Ideally, all the plates should be placed 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

After incubation, the plates or membranes should be examined immediately. If this is not possible, they may be kept at between 4 °C and 5 °C for short periods provided that this does not affect the appearance of the colonies.

8.3.1 Colonies to be counted

For "total" counts on a non-selective medium, all the colonies should be counted. With selective and differential media, only those colonies which show the characteristic appearance of the organism sought should be counted. It is unusual for such counts to comprise organisms belonging only to one taxonomic group, but in practice this discrepancy may be accepted and the results 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 a sufficient number of them to yield a precise result. In such instances, as many colonies as possible should be examined from a given area of the plate or membrane.

8.4 Calculation of results

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 in a specified reference volume of the sample (generally 100 ml or 1 ml) according to the following equation:

$$C_s = \frac{N}{(n_1 V_1 F_1) + (n_2 V_2 F_2) + \ldots + (n_i V_i F_i)} V_s$$

where

C_s is the number of colony-forming units in the reference volume V_s of the sample;

N is the sum of all colonies counted in plates or on membranes derived from dilutions F_1, F_2, ... F_i;

n_1, n_2 ... n_i is the number of plates counted for dilution F_1, F_2 ... F_i;

V_1, V_2 ... V_i is the test volume used with dilution F_1, F_2 ... F_i;

F_1, F_2 ... F_i is the dilution used for the test portion V_1, V_2 ... V_i ($F = 1$ for an undiluted sample, $F = 0{,}1$ for a ten-fold dilution, etc.);

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

NOTE — The final count thus obtained is the weighted average of the counts from each plate.

An example of the calculation for the pour or spread plate method is as follows:

If the volume applied of the test solution (V_i) is 1 ml, and the following counts are obtained at the respective dilutions:

Dilution	Counts
10^{-2}	74 and 104 colonies
10^{-3}	9 and 15 colonies

then

$$C_s = \frac{74 + 104 + 9 + 15}{(2 \times 1 \times 0{,}01) + (2 \times 1 \times 0{,}001)} V_s$$

$$= \frac{202}{0{,}022} V_s$$

$$= 9182\ V_s$$

and if V_s is per ml,

then $C_s = 9{,}18 \times 10^3$/ml

It is desirable to report the results of such colony counts together with the 95 % confidence limits, which may be derived by application of the following formula:

$$C + 2 \pm 2\sqrt{C + 1}$$

where C is the colony count or sum of colony counts.

For example if 100 colonies are present on a membrane, then the actual number of the particular organism in 100 ml of the water sample is likely to lie between 82 and 122. However, for low counts of less than about 20 colonies, this approximate formula becomes inaccurate, as do all others; reference should then be made to Poisson tables.

9 Enumeration by inoculation in liquid media

9.1 Principle

Test portions of the water sample are inoculated into liquid medium designed to ensure the growth of a particular micro-organism or group of micro-organisms. As growth occurs throughout a liquid medium, the most probable number of the micro-organisms present in the original sample can be estimated only by the statistical procedures described below. In practice the results obtained are always imprecise and the precision can be improved only by increasing the number of samples or test portions of the sample examined.

9.2 Applications

9.2.1 Test for presence or absence of a micro-organism

After inoculation and incubation of a single test portion of the sample in a tube of liquid medium and the demonstration or otherwise of the growth of a given micro-organism in it, the only conclusion which can be drawn is the presence, or absence, of the micro-organism in this test volume.

This method can be used to check conformity with regulations expressed in terms such as: "absence of ... [designation of the micro-organism] in ... ml" but does not permit the concentration of the micro-organism to be quantified.

9.2.2 Estimation of the Most Probable Number (MPN)

The principle of the MPN technique is to inoculate multiple test portions of the same sample and/or dilutions of it, into tubes of liquid culture medium.

It is assumed that on incubation each tube which received one or more organism(s) in the inoculum will show growth with or without a characteristic change produced in the medium. Provided some of the tubes give negative results, the most probable number (MPN) of organisms in a specified volume of the sample can be estimated from the number and distribution of tubes showing a positive reaction.

A choice should be made from among the various MPN "inoculation systems" (9.3.2) available according to the expected numbers of organisms in the sample under investigation, regulatory requirements, the precision needed and any other practical considerations. The precision depends on the number of tubes inoculated with the same volume; it increases as a function of the square root of the number of tubes used, i.e. the number of tubes has to be quadrupled to double the precision. In practice, with the systems usually used, this precision is always low. On the other hand, with these systems, sensitivity, which depends on the total volume examined, is generally sufficient for practical purposes.

9.3 Procedure

The choice of the actual MPN enumeration procedure depends on similar factors to those mentioned in 8.2.2 and the following guidance is given on the procedure most applicable.

9.3.1 Test portion

Addition of the test portion should not change the composition of the medium such that significant interference occurs to the growth of the micro-organisms sought. Test portion volumes of less than, or equal to, 1 ml are normally added to single-strength media. Test portions of between 1 ml and 50 ml are normally added to equal volumes of double-strength media, provided that the sample does not contain excess inorganic salts, or toxic substances which could affect bacterial growth. For volumes greater than 50 ml, more concentrated media can be used. For special purposes, sterile dehydrated media can be dissolved in the cold (or at 30 °C) in the sample to be analysed.

This method is similar in principle to that described in clause 8 and has similar disadvantages in the presence of high concentrations of inorganic salts or toxic substances.

The disadvantages of the presence of high concentrations of minerals or toxic substances can be overcome by separating the micro-organisms from the water sample. This may be carried out by membrane filtration as described in 8.2.5 or, if the water is turbid, by addition to the sample of a filter-aid such as diatomaceous earth or a suspension of aluminium oxide. These filter aids settle out, carrying micro-organisms with them; separation is then completed by centrifugation or filtration. The membranes, diatomaceous earth, or aluminium oxide are then placed in the liquid culture medium.

9.3.2 Choice of inoculation system

Different inoculation systems are available. In the usual "symmetrical" systems, the same number of replicate tubes are used as a set for each dilution, the ratio of the volumes between the dilutions generally being 1 to 10 as shown, for example, in the MPN tables (see tables 1 and 3). Usually 3 or 5 tubes are used in each set for each dilution, although the greater the number of replicate tubes in each set the greater will be the precision of results. A minimum of 3 successive sets of tubes should be used.

Theoretically a sufficient number of dilutions should be inoculated to ensure that with the greatest volume, all the tubes will give a positive result, and with the smallest volume, all the results will be negative. In practice, however, it is not always practicable or indeed necessary to use numerous dilutions, but the nature of the water, the expected bacterial content, and a knowledge of the circumstances will usually act as a guide to selecting a suitable series of dilutions to yield 3 sets of results acceptable for calculation of the MPN value (see 9.5).

With this system and where the expected ratio between the maximum and minimum number of micro-organisms is less than about 100, or where their concentration is very low, then a simple dilution procedure for one set of tubes, as shown for example in table 2, can be used instead of a series of ten-fold dilutions.

In other inoculation systems known as "non-symmetrical", the different dilution levels do not have the same number of tubes. These systems are not as flexible as symmetrical systems and are used only to estimate numbers of micro-organisms within a well-defined range. For example, 5 tubes of liquid medium may be inoculated with known volumes of the sample or a dilution of it, with 2 additional tubes, one inoculated with the same volume of a ten-fold dilution and the other with a further ten-fold dilution, as shown in table 5.

The MPN tables show those combinations of positive and negative tube-results which are most likely to be encountered. If other less likely combinations which are not given occur in practice with a frequency greater than about 1 in 100 tests, it suggests that the statistical assumptions underlying the MPN estimate are not being fulfilled, possibly because the technique is faulty. The MPN values for combinations of positive and negative reactions not shown in the tables or for other combinations in any inoculation system may be derived by application of the following formula:

$$\text{MPN} = \frac{\text{No. of tubes} \times \text{sample reference volume (in ml)}}{\text{Volume (in ml) of sample in all tubes with negative reactions} \times \text{Volume (in ml) of sample in all tubes with positive reactions}}$$