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Water quality — Practices for evaluating and controlling microbiological colony count media used in water quality tests

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Qualité de l'eau — Techniques d'évaluation et de contrôle des milieux microbiologiques servant au comptage des colonies pour les essais d'évaluation de la qualité de l'eau

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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 voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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Annexes A and B form an integral part of this International Standard. Annex C is for information only.

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Introduction

The development of micro-organisms upon culture media is dependent upon a number of very important factors.

a) The proper nutrients must be available.

b) Oxygen or other gases must be available.

c) A certain degree of moisture is necessary.

d) The medium must be of the proper pH reaction.

e) Proper temperatures must be maintained.

f) The medium must be sterile and maintained free of contamination after inoculation.

g) Media must be able to be reproduced consistently with minimum variations.

h) Care should be taken to avoid plates which are too crowded.

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To ensure the reproducibility of microbiological results and to enable inter-laboratory comparison studies to be made, the preparation of microbiological media must be strictly regulated. Guidelines for ensuring the proper preparation of media which can be used with similar growth expectations from laboratory to laboratory are presented below.

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Water quality — Practices for evaluating and controlling microbiological colony count media used in water quality tests

1 Scope

This International Standard covers the comparison and evaluation of the same medium prepared from different lots of materials.

It also covers the comparison and evaluation of different media which are used for the same purpose.

It only deals with the finished product ready to be tested, and not media formulation or preparation.

This method applies to the evaluation of any solid media intended for bacteriological isolation and enumeration procedures.

2 Normative reference

The following standard contains provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the edition indicated was 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 edition of the standard indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 8199:1988, *Water quality — General guide to the enumeration of micro-organisms by culture.*

3 Productivity/selectivity testing

3.1 The general procedure is to inoculate appropriate organisms onto the media under evaluation and to compare their performance, when trying

a) to choose between various manufacturer's media;

b) to select the most appropriate medium; or

c) to assess batch-to-batch variation.

Inoculation can be either a quantitative method or simple plate-spreading by wire loop and seeding of liquid media by pipette. Alternatively, a series of natural aquatic samples with high bacterial populations may be used. This type of screening is closer to reality, but has the disadvantage that the most demanding organisms may not be present in the water sample used.

3.2 Examples of statistical analyses are given in annex A.

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4 Media preparation and tests

4.1 Application

The following tests and procedures can be applied to each lot of medium prepared from either a commercial source or in a laboratory using primary ingredients (see also ISO 8199).

4.2 Measurement of pH-value

Prepare the medium, sterilize as directed and measure the pH using an electronic pH-meter. The pH of most media should be within ±0,2 of a unit of the target value at 25 °C. Check the pH of the sterilized media, in the case of solid media after solidification.

The pH or reaction of the culture medium, expressing its hydrogen ion concentration, is very important for the growth of micro-organisms. Most micro-organisms prefer media which are approximately neutral, although some may require media which are distinctly acid.

Drift in pH or other pH problems may be caused by

— superheating;

— incomplete mixing;

— excessive sterilization;

— use of alkaline glass;

— contaminated containers;

— impure distilled water;

— repeated melting or prolonged storage, especially at high temperatures;

— hydrolysis of ingredients.

4.3 Gel strength

For agar medium, a predried plate can be checked with a wire loop. Drying methods are described in clause 6.

Improper gel strength may be due to one of the following:

— agar not in solution;

— incomplete mixing;

— incorrect proportions of dehydrated medium to volume of water;

— pH drift due to acid hydrolysis ("acid" agars e.g. malt extract agar, will hydrolyze on prolonged heating or if repeatedly melted);

— failure to compensate for dilution of agar by the inoculum.

An agar with excess gel strength may inhibit the formation of typical colonies and the usual effect is a high-domed colony, which is usually smaller than normal. The inhibitory effect of a dry agar surface may be even stronger on membranes.

4.4 Water

The water used to prepare media is very important and could be one of the major causes for incomparability between media prepared in different laboratories or even within the same laboratory. Tap water should never be used in the preparation of media as it will contain a variety of substances, varying from day to day, which may cause growth stimulation or inhibition when used in medium preparation.

Glass-distilled water is recommended for medium preparation. For critical growth studies, double glass-distilled or ultra-pure[1)] water are recommended. Water which does not meet the requirements of glass-distilled water may contain compounds which interfere with the performance of the media.

Although the pH measurement of purified water is characterized by drift, extreme readings are indicative of chemical contamination.

4.5 Glassware

Clean glassware is critical in the preparation of media. Traces of detergent or chemicals can greatly influence medium composition and growth characteristics. Because some cleaning solutions are difficult to remove completely, spot checking of clean glassware for pH reaction (especially if soaked in alkali or acid) should be carried out.

Certain wetting agents or detergents used in washing glassware may contain bacteriostatic or inhibiting substances requiring 6 to 12 successive rinsings with ultra-pure water, to remove all traces and ensure freedom from residual bacteriostatic action.

The procedure for testing these compounds, which is taken from [1], is described in annex B.

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4.6 Appearance

The colour and clarity of a prepared medium should be typical for the product. Some obvious media preparation errors are a darkening of the medium and the appearance of a precipitate. Superheating due to incomplete mixing can also cause a darkening of the medium.

Incomplete solubility of the powdered medium may be noted. This may be due to inadequate soaking, inadequate heating, incomplete mixing, use of tap water or of a container too small to allow adequate convection.

Agar media are apt to show a precipitate as a result of prolonged sterilization or heating. Repeated melting of solidified agar or maintenance of melted agar at a high temperature may likewise cause a precipitate to form in the media. Media containing agar may also form flocculant precipitates if the liquid medium remains in the water bath at 43 °C to 45 °C for longer than 30 min. The flocculant agar precipitate, however, may be dispersed by reheating the medium. Such media may also undergo nutritional impairment if held in the molten state for too long.

1) Water that can be obtained by using a device similar to a 4 cartridge Milli-Q system with membrane filtration of the final product.

The Milli-Q system is an example of a suitable system available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this system.

4.7 Chemicals

To ensure compatibility of media, reagents and solutions and reproducibility of test results, it is recommended to use the purest grade chemicals in their preparation. For instance, it is well documented that some brands of potassium dihydrogen phosphate, KH_2PO_4 contain large amounts of impurities, any one of which can have an inhibitory or growth stimulating effect on bacteria. The grade of chemical used should be carefully documented so that later formulations will be consistent with the original.

The proper dissolution of chemicals and mixing of solutions, media and reagents are important factors in ensuring homogeneity and compatibility from one batch to the next within and between laboratories.

4.8 Changes in bacterial recovery or differentiating characteristics

There are many reasons for a medium to suddenly be less efficacious than it once was. These reasons include: repeated melting; prolonged or excessive heating; incomplete mixing; charring or burning; failure to compensate for dilution of ingredients by inoculum; disturbance of the formula by the inoculum e.g. the addition of strong electrolytes or sugar solution, detergents, antiseptics, metallic poisons, protein material, etc.

The important point about some of these errors is that the medium is often, visually, the same as if it was properly prepared. Sometimes the error will be continued for months until there is a realization that either the medium is not as efficacious as it used to be, or that the samples have become less contaminated. Only constant vigilance will decrease the occurrence of this type of problem. The use of a pure culture producing a typical reaction on the medium (a positive control) is recommended.

4.9 Shelf life

The useful life of a culture medium, once prepared, depends on a number of factors and many media may safely be stored in a refrigerator for several weeks before use, although it is desirable to use them on the day of preparation for the most critical tasks. Liquid or solid media in screw-capped containers (to prevent evaporation) are more suitable for prolonged storage than media on plates or in containers plugged with cotton wool.

Prolonged storage of sterile media is not recommended unless stability has been established.

All prepared media and packaged media which have been opened should be dated for shelf life. The manufacturer's shelf life specifications should be observed for unopened dehydrated media.

5 Statistical analyses

5.1 General

Microbiologists are constantly seeking the medium which is the most suitable for selectively isolating and enumerating a target organism or population of organisms from a specific specimen or sample. The literature on this subject is very comprehensive, with many authors recommending a wide variety of media as being best under the test conditions imposed. Invariably, in these comparative studies and recommendations, the media recommended tend to have a regional following, which makes water quality comparison studies difficult, as noted in [2].

Thus, for national and international water quality comparison purposes, it is very important that one of the basic tools of water microbiology, the media, be standardized as much as possible, not only to provide consistent results, but also to enable the development of standardized procedures for enumerating specific micro-organisms.

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5.2 Principle

Aqueous samples or pure cultures (normal, stressed or injured) or dilutions of these are spread plated, pour plated or membrane filtered and the membrane filters placed on the surface of two or more types of agar medium plates to be compared using a numerically significant number of colonies on replicated plates.

For pure culture tests, the following three basic procedures are used for spread plate, pour plate and membrane filtration procedures.

a) Cultures diluted to yield countable ranges.

b) Natural aquatic samples with low bacterial populations to which a pure target organism culture is added to produce target organisms within the normal counting range.

c) Natural aquatic samples with high bacterial populations to which a pure target organism culture is added to produce target organisms within the normal counting range, with or without dilution.

For natural aqueous samples for which "colony counts" are required, the following two basic formats are used.

a) Direct spread of pour plating of up to 1,0 ml of sample or dilution of sample using appropriate procedures.

b) Concentration of sample through a membrane filter and deposition of the membrane filter on an

appropriate agar medium to obtain organisms within counting range.

5.3 Reagents and materials

Suitable for comparing the media by either the spread plate, pour plate, or membrane filtration procedure. A nutrient non-specific agar may be included in all tests to establish pure culture colony counts. Recovery of target and non-target organisms on selective media should be compared with recovery on non-selective media. Both specificity and selectivity should be verified.

5.4 Apparatus

Suitable for performing spread plate, pour plate and membrane filtration tests for target organism enumeration. Petri dishes for spread plating are approximately 100 mm. These may also be used for membrane filter procedures. However, plates varying from 50 mm to 60 mm in diameter may be more appropriate.

5.5 Sample preparation

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5.5.1 Collect fresh or salt water samples, either for total count tests or to provide background flora for target organisms, and following routine laboratory procedures, use spread or pour plate or membrane filter procedures for inoculation. Incubate the inoculated Petri dishes at an appropriate temperature during a suitable time. The water can be stored at 2 °C to 4 °C.

After incubation, examine the Petri dishes and establish the colony count. The water samples should now be used immediately for total count tests or be augmented with known volumes of target organism for use in the media comparison studies.

5.5.2 Culture the target organism for the media being compared in broth culture at an appropriate temperature. After incubation, stress the target organism by maintaining it at 2 °C to 4 °C for 24 h. Other stressing or injuring procedures which have been agreed upon may also be used.

Spread plate, pour plate or membrane filter the stressed target organism and incubate on a non-selective nutrient medium at an appropriate temperature during a suitable incubation period, which may be longer than that required by non-stressed organisms. Return the stressed organism culture to the stressing condition, i.e. between 2 °C and 4 °C.

Count both the target and non-target organism colonies, immediately following incubation, then establish the appropriate dilution for either pure culture comparison studies or for addition to water samples.

Immediately after establishing the target organism density, use the appropriate volume required and immediately proceed with pure culture and/or mixed culture tests.

6 Procedure

6.1 General

The spread plate procedures described are based on the use of 0,5 ml of liquid inoculum and predried agar plates. Agar plates to be used for membrane filter counts are not dried but, to avoid confluent growth, free water should not be present on the surface of the agar.

To dry the plates for spread plate procedures, store the freshly prepared Petri dishes plus covers upside down in the dark (if the media are light sensitive) at 30 °C to 35 °C for 15 h to 18 h.

6.2 Spread plate procedure, pure stressed culture

After preparing dilutions of pure stressed target organism so that 0,5 ml contains 50 to 200 organisms, plate out 0,5 ml of the diluted stressed organism on each medium to be compared, in random fashion, as well as on a non-selective nutrient medium. A total of five replicates should be made, plus five on the non-selective nutrient medium. Enough dilutions should be plated to give an appropriate colony number.

A bent glass rod with or without a bacteriological turntable should be used to distribute the inoculum evenly over the predried agar surface.

Replace Petri dish covers and allow the plated sample to be completely absorbed before inverting. All plates should be incubated as soon as possible after the inoculum is absorbed at the required temperature for the required time, these values being based on the target organism used in the test.

6.3 Spread plate procedure, stressed target organism and sample

Prepare dilutions so that 0,5 ml contain 400 to 500 organisms (suspension A) and 4 000 to 5 000 organisms (suspension B).

Add an appropriate volume of target organism culture to these dilutions so that 0,5 ml of augmented sample also contains approximately 50 to 200 stressed target organisms.

Plate out 0,5 ml of suspension A or B on each medium to be compared, in random fashion, as well as plating the appropriate target organism dilution (to achieve 50 to 200 target organism colonies) on non-selective nutrient media to establish baseline

counts. A total of five replicates of each medium are compared, plus five on the non-selective medium. Enough dilutions should be plated to give an appropriate colony number.

Refer to the second and third paragraphs of 6.2 for the completion of this procedure.

6.4 Pour plate procedure

The pour plate procedure, using pure stressed culture with or without sample, can be applied analogically to the spread plate procedure (6.2 and 6.3). In this technique the plates are not predried and the volume of sample and its dilutions to be plated is normally 1,0 ml.

6.5 Membrane filter procedure, stressed target organism

After preparing dilutions of pure stressed target organisms so that 1,0 ml added to 30 ml of diluent and membrane filter contains approximately 50 to 200 target organism colony forming units, use standard membrane filtration procedures and a standard membrane filter and distribute the inoculated membranes at random on the media being compared, as well as on a non-selective nutrient medium for background levels. It is also recommended that 0,5 ml of stressed target organism dilution (25 to 100 colonies) be spread plated on a non-selective nutrient medium to evaluate membrane stress. A total of at least five replicates of each medium are compared. Filter enough dilutions to give an appropriate colony number.

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Immediately after all samples are processed, invert the Petri dishes and incubate at appropriate temperatures for an appropriate time.

6.6 Membrane filter procedure, stressed target organism and sample

Prepare concentrations of water sample or dilutions of water sample so that 1,0 ml contains 400 to 500 organisms (A) and 4 000 to 5 000 organisms (B).

Add appropriate volumes of target organism culture to these concentrations or dilutions (A or B), so that 1,0 ml of water sample also contains approximately 50 to 200 stressed target organisms.

Add 1,0 ml of a mixture of water and sample-stressed organism to 30 ml of diluent and follow routine membrane filtration procedures to process the sample. Repeat the procedure as frequently as required, randomly distributing the membrane filter on the media to be compared. As an integral part of this procedure and as part of the random distribution pattern, filter the appropriate volume and concentration of the stressed target organism to yield 50 to 200 organisms per millilitre. Place the membranes on non-selective nutrient media.

At least five replicates of each medium are compared.

Refer to the last paragraph of 6.5 for the last step of this procedure.

6.7 Spread plate procedure, sample colony count

Randomly plate out 0,5 ml of sample or sample dilution, which hypothetically contain 50 to 200 organisms per ml capable of growing on the non-selective control medium, on each of the predried media to be compared. Compare five replicates of each medium.

Refer to the second and third paragraphs of 6.2 for the completion of this procedure.

6.8 Pour plate procedure, sample colony count

The pour plate procedure can be applied to evaluate the background growth analogically to the spread plate procedure (see 6.4 and 6.7).

6.9 Membrane filter procedure, sample colony count

After verifying that the volume of sample or sample dilution produces 50 to 200 colonies on a membrane filter incubated on non-selective nutrient media, use this volume combined with up to 30 ml of diluent to test the media being compared. The total volume tested should not be less than 10 ml.

Filter a sufficient number of samples and randomly place the membranes on chosen test media.

If a sample requires dilution, spread plate 0,5 ml of an appropriate sample dilution to produce 50 to 200 colonies on the agar plates used in the comparison study. The samples should be placed on randomly selected agars and included within the membrane filtration series.

Refer to 6.5 for the completion of this procedure.

7 Expression of results

7.1 After appropriate incubation periods, using a stereoscopic microscope (× 10) or other counting aid, count and record the number of typical and atypical colonies growing on each medium and each control medium.

7.2 Tabulate the counts of typical colonies and consult annex A for the appropriate statistical methods. The counts of atypical colonies give an indication of the selectivity of media.

7.3 Convert the counts into logarithms (add 1 or 0,5 to all values before conversion, if zero is observed).

7.4 Calculate the mean and the standard deviation for each medium.

7.5 If the standard deviations are approximately the same (less than a four-fold difference) then the experiment is under control. Different standard deviations can be the result of differences between media.

7.6 If only one sample is analysed, perform one-way analysis of variance for comparing the differences between media means, and if more samples are analysed, perform a two-way analysis of variance, as described in annex A. In the two-way analysis, the significance of the interaction will determine how the analysis is to be continued for the testing of differences between media (see annex A).

7.7 If the F-test is significant in the one-way analysis of variance, a more detailed analysis should be carried out.

7.8 If the decision was made *a priori* to test all new media against a reference medium, this can be done by a t-test for paired comparisons or by forming the appropriate contrasts in the analysis of variance.

7.9 If it were only planned to test if there are differences between media, then an *a posteriori* Student-Newman-Keuls test should be done to study the differences in detail.

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Annex A
(normative)

Statistical designs in method comparisons

A.1 General

Assume that a selection is to be made between m different culture media for the enumeration of a certain bacterial group or species. Basically the statistical design is the same for other comparisons, such as choices between plating techniques, incubation conditions, dilution solutions, membrane filter types, etc. Therefore, in the following, the m alternatives will simply be referred to as different "methods".

It will be assumed that the yield, i.e. the count of typical colonies, is the sole basis of evaluation and comparison of the methods.

A.2 Analysis of one sample

In the simplest instance, when a method is to be selected for the colony count of a pure culture, a simple design is sufficient. It is sufficient to make only k replicate plates in random order using m different methods. The m methods can be compared using a one-way analysis of variance (ANOVA) on the $m \times k$ counts. The calculations are illustrated in A.4.1 and A.4.2.

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In practice, it is highly unlikely that an acceptable count can always be secured from all k plates of the m methods. Some values will be missing for one reason or another. The statistical design becomes an ANOVA with unequal numbers of replicates in the different methods (see A.4.3). The value of k should always be at least 2.

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With pure cultures as test material, a one-time test may be assumed to apply generally; the result can be expected to hold for other strains of the same species.

A.3 Analysis of two or more samples

When selecting a method for the quantitative determination of a bacterial group from a mixture of species or from a natural sample, the situation is more complex. One cannot generalize about the result of a single comparison without risk. There is no *a priori* reason why the same method should be the best for all types of natural samples. The statistical design must at least be able to test whether this is the case. This can be done by applying a two-way ANOVA on the data of m methods with k replicates tested independently in s different natural samples. The most important component to test is the samples times methods (S × M) interaction. When statistically significant, it indicates significant differences in yield using different methods depending on the sample type. The mathematical analysis of such a design is illustrated in A.5. The number of replicates, k, need not be very large; two or three is quite enough. Furthermore, the experiments are independent; each different sample can be tested on a different date if necessary.

This annex does not cover means of dealing with missing values in the two-way analysis of variance. If by accident a count cannot be made, it is best to discard the whole set of data from that particular sample. The data can be replaced by testing the sample again on another occasion or by studying another sample.