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Water quality — Requirements for establishing performance characteristics of quantitative microbiological methods

Qualité de l'eau — Exigences pour l'établissement des caractéristiqes de performance des méthodes microbiologiques quantitatives

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

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

This first edition of ISO 13843 cancels and replaces ISO/TR 13843:2000, which has been technically revised.

Introduction

Methods are considered microbiological when the quantitative estimate is based on counting of microbial particles either directly with the aid of a microscope or indirectly on the basis of growth (multiplication) into colonies, turbidity, a colour change or fluorescence. The principles and procedures within the scope of this document are commonly known as microscopic count, most probable number (MPN) and colony count. Most of the procedures for the determination of performance characteristics described in this document are applicable to all three types of method. However, where the procedures are not applicable, alternative suggestions are made within the body of the document or in Annexes D and E (for repeatability, reproducibility and uncertainty of counting).

Plaque counts of bacteriophages are in most respects similar to bacterial colony counts.

Some of the "newer" microbiological methods such as those utilizing fluorescent *in situ* hybridization (FISH) or polymerase chain reaction (PCR) can also be covered by this document. However, they may require special consideration, depending upon how they are used. The issues of importance in these situations include the mechanism of determining the numbers of microbes present (e.g. standard curve for qPCR or microscopic count for FISH) and the viability of the organisms detected. If such techniques are used for confirmation as part of a method then all sections of this document are relevant.

While not essential, during the characterization of microbiological methods it may be beneficial to generate data using stressed organisms. Various methods can be used to stress organisms, but the two that are most useful for water are disinfectant stress (usually chlorine injury) and nutrient depletion caused by organisms being in a low nutrient environment (i.e. drinking water and other oligotrophic waters) for a period prior to testing. The effect on some of the performance characteristics of "stressing" organisms is almost totally dependent on the type and degree of stress applied and it is inappropriate to include such detail in this document. However, there are descriptions in the literature that laboratories can follow in case they should wish to determine performance characteristics of a method with stressed cells.

Water quality — Requirements for establishing performance characteristics of quantitative microbiological methods

1 Scope

This document deals with characterization of microbiological methods. In terms of this document, characterization means the study of parameters that can be measured to describe how the method is likely to perform in a given set of conditions, which can be described as performance characteristics. The document describes procedures for the determination of performance characteristics which can be used for subsequent validation or verification of methods.

The emphasis is on selective quantitative methods and this document applies to all types of water. For methods that are not based upon direct microscopic count, colony count or most probable number, the applicability of the procedures described in this document should be considered carefully.

2 Normative references

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

ISO 17994:2014, *Water quality — Requirements for the comparison of the relative recovery of microorganisms by two quantitative methods*

3 Terms and definitions

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

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

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

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

3.1
accuracy
measurement accuracy
closeness of agreement between a measured quantity value and an assigned quantity value of a measurand

Note 1 to entry: The concept ‘measurement accuracy’ is not a quantity and is not given a numerical quantity value. A measurement is said to be more accurate when it offers a smaller measurement error.

Note 2 to entry: ‘Measurement accuracy’ is sometimes understood as closeness of agreement between measured quantity values that are being attributed to the measurand.

[SOURCE: ISO/IEC Guide 99:2007, 2.13[16], modified — “...a true quantity value” replaced by “... an assigned quantity value; Notes 1 and 2 to entry added]

3.2
analyte
component represented in the name of a measurable quantity

Note 1 to entry: In water microbiology, the analyte is ideally defined as a list of taxonomically defined species. In most cases, in practice the analyte can only be defined by group designations less accurate than taxonomic definitions.

[SOURCE: ISO 17511:2003, 3.2[14]]

3.3
analytical portion
test portion
volume of particle suspension (sample) inoculated into a detector unit (agar plate, membrane filter, test tube, microscopic grid square)

3.4
bias
measurement bias
estimate of a systematic measurement error, or the systematic difference between the quantitative assigned value and the average of measurement replicate results

3.5
categorical characteristics
method performance characteristic numerically expressed as a relative frequency based on P/A or +/- classification

3.6
colony-forming unit
CFU
colony-forming particle
CFP

organism (or cluster of organisms) with the ability to form a colony under certain specified conditions

Note 1 to entry: The term was originally introduced to convey the idea that a colony may originate not only from a single cell but from a solid chain or aggregate of cells, a cluster of spores, a piece of mycelium, etc. It mistakenly equates the number of colonies observed to the number of living entities seeded on the medium. Growth unit, viable particle, propagule and germ are terms with the same meaning but convey the original idea better and apply not only to colony count methods but also to the most probable number (MPN).

3.7
collaborative method performance
method or laboratory performance test where several laboratories join in an experiment planned and co-ordinated by a leader laboratory

Note 1 to entry: Collaborative tests are mainly of two types. Intercalibration exercises are made to allow laboratories to compare their analytical results with those of other participating laboratories.

Note 2 to entry: Method performance tests produce precision estimates (repeatability, reproducibility) out of data accumulated when several participating laboratories study identical samples with a strictly standardized method.

3.8
confirmed colony count
verified colony count
presumptive colony count corrected for false positives

Note 1 to entry: Mathematically:

$$pc = \frac{k}{n} c$$

where

c is the presumptive count;

p is the true positive rate;

n is the number of presumptive positives isolated for confirmation;

k is the number confirmed.

3.9
corroborated count
count obtained when using a secondary confirmation procedure

3.10
detection level
minimum concentration of organisms that produce evidence of growth with a probability of $P = 0{,}95$ when inoculated into a specified culture medium and incubated under defined conditions

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

3.11
detection set
combination of plates or tubes on which quantitative estimation of sample microbial concentration is based

Note 1 to entry: The detection set is the set of plates or tubes utilized for numerical estimation of a single value.

EXAMPLE Parallel plates of a suspension, plates from consecutive dilutions, 3 × 5 tube MPN system, microtitre plate.

3.12
detector
particle detector

plate of solid matrix or a tube of liquid containing a nutrient medium for counting or detecting biologically active particles

3.13
efficiency
E
fraction of colonies that are correctly assigned as positives and negatives

Note 1 to entry: Mathematically:

$$E = \frac{a+d}{n}$$

where

a is the number of typical colonies confirmed as being the target organism (true positives);

d is the number of atypical colonies confirmed as not being the target organism (true negatives);

n is the total number of colonies tested for confirmation.

3.14
false negative
result indicated by the test method to be negative which has subsequently been shown to contain the target organism

3.15
false positive
result indicated by the test method to be positive which was subsequently shown not to contain the target organism

3.16
germ
entity capable of biological activity (e.g. respiration or reproduction in a nutrient medium)

3.17
limit of determination
lowest analyte concentration per analytical portion where the expected relative standard uncertainty, equals a specified value

3.18
method-defined count
count obtained by using only the procedures in the described method

3.19
negative binomial distribution
particular "overdispersed" statistical distribution of counts

Note 1 to entry: Its variance can be expressed as $s^2 = \bar{x} + u_0^2 \bar{x}^2$ ($\bar{x}$ = mean).

Note 2 to entry: In this document, the square of the relative operational standard deviation (u_0) is substituted for the inverse of the exponent ($1/k$) of the standard equation for the negative binomial distribution.

3.20
outlier
member of a set of values which is inconsistent with other members of that set

Note 1 to entry: An extreme value which normally appears randomly in less than 1 % of repetitive tests, but more frequently if abnormal situations occur. Statistical test procedures can be used to quantify this probability.

3.21
over-dispersion
variation in excess of Poisson randomness

Note 1 to entry: Detected qualitatively by the Poisson index of dispersion and measured quantitatively by estimating the parameter u_0 (relative operational standard deviation) of the negative binomial distribution.

3.22
parallel counts
particle or colony numbers in equal analytical portions drawn from the same suspension

3.23
Poisson distribution
fully random distribution of particle numbers when sampling a perfectly mixed suspension

Note 1 to entry: The probability $P(k)$ of observing exactly k units in a test portion when the mean equals μ is calculated from

$$P(k) = \frac{u^k}{k!} e^{-\mu}$$

3.24
precision
measurement precision
closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions

Note 1 to entry: Measurement precision is usually expressed numerically by measures of imprecision, such as standard deviation, variance, or coefficient of variation under the specified conditions of measurement.

Note 2 to entry: The 'specified conditions' can be, for example, repeatability conditions of measurement, intermediate precision conditions of measurement, or reproducibility conditions of measurement (see ISO 5725-3[4]).

Note 3 to entry: Measurement precision is used to define measurement repeatability, intermediate measurement precision, and measurement reproducibility.

3.25
proportionality
agreement of observed particle counts with the volume (or dilution) of a series of analytical portions from a common root suspension

Note 1 to entry: Proportionality is evaluated as the log-likelihood ratio statistic G^2 with n-1 degrees of freedom.

3.26
recovery
general term used for the number of particles estimated in a test portion or sample, with the understanding that there is a true (although unknown) number of particles of which 100 % or less are "recovered" by the employed methodology

Note 1 to entry: Another similar term commonly used is productivity (see ISO 11133[12]).

3.27
relative recovery
ratio of colony counts obtained by two methods tested on equal test portions of the same suspension

3.28
relative operational standard deviation
u_0
operational variability, expressed as a relative standard uncertainty, associated with the technical steps of the analytical procedure

Note 1 to entry: The relative operational standard deviation is often expressed in percent.

3.29
relative operational variance
u_0^2
over-dispersion constant, the square of relative operational standard deviation

3.30
relative standard deviation
u_{rel}
estimate of the standard deviation of a population from a sample of n results divided by the mean of that sample

3.31
relative variance
u_{rel}^2
square of relative standard deviation

3.32
repeatability
measurement repeatability
measurement precision under a set of repeatability conditions of measurement

3.33
repeatability conditions
condition of measurement, out of a set of conditions that includes the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time

3.34
reproducibility
measurement reproducibility
measurement precision under reproducibility conditions of measurement

Note 1 to entry: Relevant statistical terms are given in ISO 5725-1[2] and ISO 5725-2[3].

3.35
reproducibility conditions
condition of measurement, out of a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects

3.36
robustness
insensitivity of an analytical method to small changes in procedure

Note 1 to entry: To examine the robustness it is advisable to "abuse" the method in a controlled way.

3.37
sensitivity
fraction of the total number of positive cultures or colonies correctly assigned in the presumptive inspection

3.38
specificity
fraction of the total number of negative cultures or colonies correctly assigned in the presumptive inspection

3.39
standard uncertainty
uncertainty of the result of a measurement expressed as a standard deviation

3.40
uncertainty of counting
relative standard deviation of results of repeated counting of the colonies or particles of the same plate(s) or field(s) under stipulated conditions (same person, different persons in one laboratory)

3.41
verification
performance of a second characterization by a different laboratory to confirm the results of the original characterization

4 Basic concepts

4.1 General

As far as particle statistics are concerned, microscopic counts obey the same laws as viable counts but they are, with the exception of microcolony methods, free from the biological problems associated with growth. Differential stains, specifically labelled complexes or other agents used for finding the target

do not change the basic principles. The same principles as those used with selective colony methods can be applied. For a more detailed understanding of the theory and application of the formulae used in this document, the mathematical basis for the variation encountered in all of these types of method is described in Annex A.

4.2 Characterization

The characterization of a microbiological method is largely based upon the examination and expression of the performance characteristics of that method.

Characterization is a process of providing information about the likely performance of that procedure under a specific set of circumstances. It is not the intention of this document to provide guideline values for each of the specified performance characteristics but rather to give guidance on which parameters should be determined and how best to derive them for comparative purposes. Methods that have "poor" performance characteristics may still be useful.

Characterization is an exploratory process with the aim of establishing the likely set of performance characteristics of a new, modified or otherwise inadequately characterized method. It should result in numerical and descriptive specifications for the performance and include a detailed and unambiguous description of the target of interest (such as positive colony, tube or plaque). However the values generated should not be used as limits since they may change depending on the laboratory, matrix or even specific samples.

Characterization is performed by a single laboratory in the first instance to determine the likely performance of a test method in a specific laboratory.

A collaborative method performance study can be performed as an additional step to evaluate the interlaboratory performance characteristics.

NOTE A laboratory developing an in-house method or a variant of an existing standard could carry out the steps of characterization.

It is imperative that technicians involved in the characterization of a method have considerable experience with other microbiological methods.

The performance characteristics covered by this document are listed in Table 1.

Table 1 — Performance characteristics described in the document

Parameter	Definition
Sensitivity[a, b, c]	fraction of the total positives[e] correctly assigned in the presumptive count
Specificity[a, b, c]	fraction of the total negatives[f] correctly assigned in the presumptive count
False positive rate[a, b]	fraction of positive results (e.g. typical colonies) that are subsequently shown to be due to non-target organisms
False negative rate[a, b]	fraction of negative results (e.g. atypical colonies) shown to be target organisms
Selectivity[a, b, c]	ratio of the number of target colonies to the total number of colonies in the sample volume
Efficiency[a, b]	fraction of total colonies correctly assigned in the presumptive count

[a] Required for determination of the performance characteristics.

[b] Required for single laboratory verification.

[c] Guidance specification given.

[d] Methods for interlaboratory reproducibility and precision are described in Annex F. Use of these methods should be considered when interlaboratory performance is paramount, for example when methods are being developed for regulatory compliance.

[e] Positives may be colony counts, positive reaction vessels (MPN) or cell counts.

[f] Negatives may be atypical colonies, negative reaction vessels (MPN) or cells without the specific characteristics required.

Table 1 *(continued)*

Parameter	Definition
Upper limit[a]	upper end of the working range for which the method is useful (i.e. the maximum countable colonies per plate, or other detection systems)
Repeatability[a, b, c]	precision under repeatability conditions (same operators, same operating conditions, short period of time, ...)
Reproducibility[a]	precision under intralaboratory reproducibility conditions[d]
Robustness[a]	measure of the capacity of a test to remain unaffected by small but deliberate variations in testing conditions (e.g. temperature)
Relative recovery[a]	efficiency with which a method recovers target organisms from a sample when compared to another procedure (This comparison shall be done where an alternative method for the same organism exists. Comparison with an ISO reference method is preferred.)
Uncertainty of counting[a, b]	relative standard deviation of replicate counts of the target obtained by repeated counting (plates, fields, tubes, etc.) under stipulated conditions (same person, different person, same laboratory, etc.)

[a] Required for determination of the performance characteristics.

[b] Required for single laboratory verification.

[c] Guidance specification given.

[d] Methods for interlaboratory reproducibility and precision are described in Annex F. Use of these methods should be considered when interlaboratory performance is paramount, for example when methods are being developed for regulatory compliance.

[e] Positives may be colony counts, positive reaction vessels (MPN) or cell counts.

[f] Negatives may be atypical colonies, negative reaction vessels (MPN) or cells without the specific characteristics required.

While interlaboratory reproducibility and precision do not form part of the performance characteristics described in the body of this document, in certain situations knowledge of these parameters is highly desirable. Such situations include when methods are being used for regulatory compliance or when data from a variety of laboratories are being compared for any of a number of reasons. For this reason, suggested methods to determine interlaboratory reproducibility are described in Annex F.

4.3 Verification

Verification takes place when a laboratory proceeds to implement a method developed elsewhere. Verification focuses on gathering evidence that the laboratory is able to generate performance data similar to those established in primary characterization. It is not helpful to establish limits on the various components of method characterization since these can vary dependent on many aspects of the method, type of sample and performing laboratory. The verification data should be used to establish the type and quality of data likely to be generated by the laboratory with a given procedure and any given sample type.

Typically, verification uses selected and simplified forms of the same procedures used in method characterization, but possibly extended over a longer time. Natural samples are the optimal test materials and the work need only address those aspects of the method performance that are of interest to the laboratory. The requirements for single laboratory verification are described in Clause 7.

4.4 Method comparison

Method performance consists of many aspects. There is neither a single test of method comparison nor numerical criteria for it. One method may be superior in specificity but inferior in recovery. All the collective information about robustness, precision and specificity gained during characterization tests can be used for method comparison. The methods only need to be tested in parallel for recovery comparisons.

It is necessary to apply two methods in parallel on the same samples when developing an in-house method, and also when collecting information to justify the use of an alternative method. Relative recovery studies of an alternative method against a reference method organized according to ISO 17994 involve preferably a wide range of samples and participation by a number of laboratories allowing the expansion of the sample range over large geographical areas. However, sometimes it may be necessary to verify the result of an alternative method recovery study under ecological conditions or in a geographical area not represented in the earlier collaborative trial. When a laboratory only needs to confirm the comparison result of a method already tested and officially accepted, it can take full advantage of the previous test results. The laboratory should have access to the report of the collaborative comparison. Accordingly, it should have at its disposal estimates of the mean and standard deviation of the relative difference. Formula (3) given in ISO 17994:2014, 5.4.3 can be applied to estimate the recommended number of samples. However, whatever the result of the calculation, the number of samples should not be less than thirty.

A method giving the highest recovery of confirmed target organisms is obviously the best when confirmation is required for routine use. A method giving somewhat lower recovery but not requiring confirmation may be preferable. If high false negative rates or false positive rates observed in characterization cannot be corrected by more refined target colony definitions or other procedures, the method may be deemed invalid. The comparison of two microbiological methods should include a comparison of their performance characteristics (i.e. characterization) together with a side by side comparison of recovery, using naturally contaminated or spiked samples as specified in ISO 17994.

4.5 Samples

It is a popular concept that the characterization and comparison of methods should be performed with natural samples with natural concentrations of microbes. While conceptually this is a good idea, there are exceptions under some circumstances.

Artificial samples (reference materials and spiked samples) are used in internal and external quality assurance systems to ensure the basic proficiency of the laboratories participating in method characterization exercises.

Spiking may be useful and even necessary in verification or whenever it is difficult to find natural samples with target organisms. The optimal concentration range for the characterization of microbiological methods is narrower than the projected working range. High concentrations are unnecessary. Such samples resemble pure cultures and do not put the performance of the method or of the laboratory to test.

Samples with very low bacterial content need to be studied for public health reasons but are not ideally suited for method comparisons and other characterization exercises for statistical reasons. However, their use is unavoidable in many situations. In particular, where a method seeks to identify two types of target organisms on the same plate (e.g. total coliforms and *E. coli*), low numbers of organisms are usually unavoidable.

The number and variety of samples examined need to be appropriate. Without the help of statistics, there are no objective ways of making a decision. In some instances, the first sample studied might give the answer that the method is not good enough. Usually, however, more samples are needed. Choosing too few samples may not yield representative results.

Specific guidelines on the numbers and types of samples (together with their microbial content) are given in 6.1.

5 Specifications: some guideline values

Historically, standards have provided little help for laboratories seeking to make sure that they apply the methods well and obtain valid results. What seems to be lacking is a concise presentation of what laboratories should do to verify that the method also works in their hands properly and how to distinguish between good and bad performance.