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BSI Standards Publication

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

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National foreword

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The UK participation in its preparation was entrusted to Technical Committee EH/3/4, Microbiological methods.

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Qualité de l'eau - Exigences pour l'établissement des caractéristiqes de performance des méthodes microbiologiques quantitatives (ISO 13843:2017)

Wasserbeschaffenheit - Anforderungen zur Bestimmung von Leistungsmerkmalen von quantitativen mikrobiologischen Verfahren (ISO 13843:2017)

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European foreword

This document (EN ISO 13843:2017) has been prepared by Technical Committee ISO/TC 147 "Water quality" in collaboration with Technical Committee CEN/TC 230 “Water analysis” the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by January 2018 and conflicting national standards shall be withdrawn at the latest by January 2018.

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Foreword

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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.

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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.

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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]

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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

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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

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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 $s^2 = \bar{x} + u_0^2\,\bar{x}^2\,(\bar{x} = \text{mean})$.

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

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

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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

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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

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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.

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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.

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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.

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A clause of performance characteristics shall be added to all ISO standards that deal with water microbiology methods and refer to defined microbes or groups of microbes.

The format for colony count methods might include the following:

a) **Sensitivity:** Generally greater than 90 %;

b) **Specificity:** Generally greater than 80 %;

c) **Selectivity:** Results are generally not valid if selectivity is less than 10 %.

d) **Uncertainty of counting:** Individual counting uncertainty (one person) remains normally below u_{rel} = ± 0,03. Intralaboratory uncertainty of counting is below u_{rel} = ± 0,05. Intralaboratory uncertainty of counting greater than 0,1 is a certain sign of problems or difficulties.

e) **Repeatability (parallel plating):** Variation is within the Poisson distribution. If not, the extent of over-dispersion should be given.

f) **Upper limit:** For membrane filtration methods, a range that has been quoted is 0 CFU to 80 CFU while for plate count methods using a 90 mm Petri dish, the range may be 0 CFU to 300 CFU. The surface area of a 47 mm membrane filter is approximately 25 % of that of a 90 mm Petri dish. These upper limits are dependent upon the extent of background (non-target) growth, the number of different types of target organisms (e.g. total coliforms and *E. coli*) and colony size.

6 Designs for determining performance characteristics of a method

6.1 General considerations

While it is generally better to use samples that are naturally contaminated with the target organisms, in some situations this does not facilitate the determination of the performance characteristic of interest. In such situations, the use of reference materials may be appropriate. Alternatively, samples can be "spiked" with known numbers of target organisms obtained from commercial sources.

Whichever procedure is used to prepare samples, attention should be paid to adequate mixing to facilitate a random distribution of organisms. The desired number of target organisms in the sample is dependent upon the method of interest but typically a range of 10 to 60 colony forming units per test portion will be practical. The number is a compromise between a desirable higher number and the number of colonies that is practical for a membrane or plate.

6.2 Determination of sensitivity, specificity, efficiency, selectivity, false positive rate and false negative rate

6.2.1 Type of samples to be used

Appropriate samples are prepared containing 20 to 80 total colony forming units (10 to 60 CFU target organisms) per test portion. The samples are then examined by the procedure being studied. Typical and atypical colonies (i.e. those having the typical appearance of the target organism **and** those not having the typical appearance of the target organism) are counted. All typical and atypical colonies are then identified using an appropriate procedure which could include commercially available microbial identification kits, DNA sequencing or other specified procedures.

The method of preparation of samples will vary depending on the method and the types of samples typically analysed. If naturally contaminated samples are available with an appropriate level of target organisms, then these should be used. However, in many cases naturally contaminated samples are not available (e.g. for methods designed for drinking water). In such cases, laboratory-prepared spike material (using appropriate sources of target organisms such as river water or sewage) can be used to prepare samples. This has the benefit of including non-target organisms that are likely to be encountered in contaminated drinking water in a similar ratio to what may be seen in the "real world".

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The use of reference materials should be avoided because the choice of strains used directly influences the outcome of the experiment.

6.2.2 Number of samples

A minimum of twenty samples should be used from different sources. If using surface water or sewage to prepare the spike material, this should be obtained from at least three sources. It is important when performing this work to examine an adequate number of "atypical" colonies, although when the method is very selective these may be hard to find.

6.2.3 Procedure

Samples are incubated and all colonies "confirmed" according to the procedures appropriate to the method (e.g. for ISO 9308-1[10] all presumptive coliforms must be tested for the production of cytochrome oxidase). Where methods have no confirmatory procedures (e.g. ISO 9308-2[11]) described, results are recorded as described in the method with no confirmation. Colonies (or reaction vessels: wells, tubes, etc.) are recorded as positive or negative.

When determining the parameters sensitivity, specificity, false positive rate and false negative rate, it is necessary to apply a further confirmatory test to confirm or deny the results generated by the test method. This further confirmatory test is called secondary confirmation. Such methods might include commercially available identification kits, other phenotypic methods (e.g. tests for a certain trait or enzyme system), tests of chemical composition (e.g. MALDI-TOF) or molecular methods. The choice of method will influence the outcome of the data and care should be taken when selecting these procedures as they define the target. Wherever possible, the procedure used to give support to the original confirmatory test should be based on a taxonomically sound procedure or on tests that reflect the definition of the target organisms as described in the relevant standard. For example, in the characterization of a test for *Escherichia coli* isolates could be subjected to 16S ribosomal RNA sequencing to determine if they are truly *E. coli*. Alternatively, isolates could be examined by Gram staining and tested for the expression of functional cytochrome oxidase, β-D-galactosidase and β-D-glucuronidase. Organisms that are Gram negative rods, cytochrome oxidase negative and β-D-galactosidase and β-D-glucuronidase positive would be considered to be *E coli*.

6.2.4 Categorical performance characteristics

6.2.4.1 When a confirmation step is included in the method, the identification data can be divided into four categories:

a) number of typical colonies confirmed as being the target organism in the primary confirmatory test the identity of which is supported by the secondary identification test (true positives);

b) number of atypical colonies, or typical colonies that are negative in the primary confirmatory test identified as being the target organism by the secondary identification test (false negatives);

c) number of typical colonies confirmed as being the target organism by the primary confirmatory test which are subsequently shown to not be the target organism by the secondary identification test (false positives);

d) number of atypical colonies or typical colonies that are negative in the primary confirmation test which are shown by the secondary identification test to not be a target organism (true negatives).

6.2.4.2 For methods without a confirmatory procedure, the identification data can be divided into four categories:

a) number of typical colonies identified as being the target organism by an external identification test (true positives);

b) number of atypical colonies identified as being the target organism by an external identification test (false negatives);

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c) number of typical colonies identified as not being the target organism by an external identification test (false positives);

d) number of atypical colonies identified as not being the target organism by an external identification test (true negatives).

6.2.4.3 The frequencies of these categories can be conveniently expressed in a 2 x 2 diagram:

		Presumptive count		
		+	–	
Confirmed count	+	a	b	$a + b$
	–	c	d	$c + d$
		$a + c$	$b + d$	n

The total number of tests is $a + b + c + d = n$.

The sensitivity, specificity, selectivity, false positive rate and false negative rates for the target organism can be calculated as follows:

Sensitivity = $a / (a + b)$

Specificity = $d / (c + d)$

False positive rate = $c / (a + c)$

False negative rate = $b / (b + d)$

Selectivity = a / n

A further parameter, efficiency (E), which gives the fraction of colonies correctly assigned, can be calculated as $E = (a + d) / n$.

The confirmed count relates to the organisms that have been shown by some method, described in the procedure to be the target organisms. Where the method itself includes a confirmation procedure (e.g. production of indole from tryptophan for *E. coli*) then this method will suffice. Where the procedure does not include a confirmation step (e.g. ISO 9308-2[11]) then some alternative confirmation (secondary confirmation) step can be used. Such methods might include commercially available identification kits, other phenotypic methods (e.g. tests for a certain trait or enzyme system), tests of chemical composition (e.g. MALDI-TOF) or molecular methods. The choice of method will influence the outcome of the test and care should be taken when selecting a confirmation procedure as this defines the target.

NOTE For MPN methods, the same approach can be applied. The term “colonies” can be changed into “aliquots”, “typical” into “positive” and “atypical” into “negative”.

6.2.5 Worked example

The method ISO 9308-1[10] utilizes a medium containing chromogenic substrates for β-D-galactosidase and β-D-glucuronidase. Consequently, coliform colonies are coloured pink to red, *E. coli* colonies are blue to violet and non-target colonies should be colourless. No confirmation step is required for *E. coli* when using this method. For the primary characterization of the method for the recovery of *E. coli*, 16S rRNA sequencing could be used as the secondary confirmation procedure. During validation all colonies, blue/violet, pink/red and colourless would be examined using 16S rRNA sequencing.

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In a primary characterization experiment, 300 blue/violet colonies were sequenced and 285 were shown to be *E. coli.* A total of 600 pink/red colonies were sequenced and 30 shown to be *E. coli.* A total of 300 colourless colonies were sequenced and none were shown to be *E. coli.*

Table 2 shows the results from the 20 samples analysed for the primary characterization of the above-cited method.

Table 2 — Tabulation of the counts for the categorical characteristics determination

Sample	*a*	*b*	*c*	*d*
1	15	3	1	42
2	8	0	0	33
3	4	1	0	26
4	15	3	1	50
5	16	1	0	45
6	12	5	0	48
7	6	0	1	38
8	10	1	1	29
9	14	2	0	53
10	18	0	2	51
11	17	2	0	45
12	19	0	1	63
13	13	2	2	40
14	11	3	1	39
15	13	0	0	35
16	25	3	2	33
17	21	1	0	54
18	16	0	1	55
19	15	1	2	40
20	17	2	0	51
Sum	**285**	**30**	**15**	**870**

In this scenario, the following data would be generated:

		Method-defined count		
		+	–	
Corroborated count	+	285	30	315
	–	15	870	885
		300	900	1 200

The method-defined count is the count obtained when following the methods described in the original procedure. The corroborated count is that obtained after secondary confirmation.

The total number of tests (n) is 1 200.

The sensitivity, specificity, selectivity, false positive rate and false negative rates for the target organism can be calculated as follows:

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Sensitivity = a / (a + b), i.e. 285/315 = 90,5 %

Specificity = d / (c + d), i.e. 870/885 = 98,3 %

False positive rate = c / (a + c), i.e. 15/300 = 5,0 %

False negative rate = b / (b + d), i.e. 30/900 = 3,3 %

Selectivity = a / n, i.e. 285/1 200 = 23,8 %

A further parameter, efficiency (E), which gives the fraction of colonies correctly assigned, can be calculated as E = (a + d) / n, i.e. 1 155/1 200 = 96,3 %.

6.3 Determination of the upper limit and consideration of the lower limit of detection

6.3.1 Working range

The working range of a method is often specified in the original description of the procedure or defined by manufacturers when the test is a commercially available one. However, these figures may not always be accurate and can vary with different samples types.

For MPN-based methods, the working range will be determined by the sample size used and the number of reaction vessels used. The practical upper limit has been exceeded when all tubes of all dilutions are found positive.

6.3.2 Upper limit related to linearity

With colony count methods precision theoretically improves steadily with the number of target colonies observed in the detection set. The upper limits are defined by the space requirements and ensuing interactions of microbial colonies.

In practice colony count methods have an upper limit per detector which varies with the testing situation. The colony count detector (agar plate, membrane filter) becomes “clogged” or saturated for several reasons of which the number of target colonies is only one.

Every method has an upper reliable limit. It is not a clearly fixed number but a region of colony numbers where counts per plate become too uncertain to base a valid enumeration upon.

An experimental design based on a finely graded series of dilutions or volumes with replication of plating and counting provides the data for determining the upper working limit.

A carefully mixed liquid sample is prediluted to a density giving an expected colony number per plate that somewhat exceeds the assumed upper limit of the detector performance. A series of six or seven further dilutions with dilution steps 1:2 is continued from the starting suspension. Three parallel plates are seeded from each dilution.

Plates from dilutions averaging more than 20 colonies per plate are read.

The data are analysed for proportionality and over-dispersion of parallels assuming perfect randomness at every step as the basis of evaluation.

A single value of the G^2 test is of rather limited use. Performing similar proportionality tests on different samples helps determine the highest colony count where proportionality of the method is sufficient.

6.3.3 Type and number of samples to be used

The working range of a method can be determined by the use of naturally contaminated samples, pure cultures or samples spiked with contaminated material containing the target organism (e.g. sewage for enteric organisms). In the most practical choice, it should be a pure culture. Examination of a minimum of 20 samples is required.

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