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Second edition
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Water quality — Requirements for the performance testing of membrane filters used for direct enumeration of microorganisms by culture methods

Qualité de l'eau — Exigences relatives aux essais de performance des membranes filtrantes utilisées pour le dénombrement direct des micro-organismes par des méthodes de culture

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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 of 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 www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 230, *Water analysis,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 7704:1985), which has been technically revised.

The main changes are as follows:

— the scope has been changed to cover the requirements for the performance testing of membrane filters used for retention and direct enumeration;

— clauses have been added for terms and definitions, microorganisms, sampling and replicates, procedure, inoculation and incubation, counting, calculation and documentation;

— the clauses referencing to culture media and diluents, test strain preparation, performance testing and procedure have been revised to align with ISO 8199 and ISO 11133;

— Annex A has been added with a diagram of the batch testing;

— Annex B has been added to give an example of a card to record the test results from batch testing and supplementary testing of membrane filters;

— Annex C has been added to describe the quantitative additional testing of membrane filters including a diagram of the procedure;

— Annex D has been added to describe the qualitative supplementary testing of membrane filters;

— Annex E has been added to give a practical example of batch testing and quantitative additional testing by the end user including a diagram of the procedure;

— the Bibliography has been added.

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Introduction

In laboratories carrying out microbiological examinations, the main objectives are to either capture, resuscitate, grow, detect or enumerate, or all, a wide variety of microorganisms. Membrane filters are used in many traditional microbiological culture techniques and are commercially available in various brands and types. Many comparison studies of membrane filters which have been reported in the literature show differences in their ability to recover bacteria from water samples, see References [22], [23], [28], [30], [31], [32], [33] and [34]. The complex manufacturing process means that the chemical composition, pore size and pore structure can vary, depending on the brands, and even on the lot of material. Furthermore, the manufacturing process can also release leachables that can potentially interfere with the recovery of microorganisms.

Thus, it is very important to standardize the performance testing of membrane filters as much as possible, not only to provide consistent results, but also to enable the development of standardized procedures for enumerating specific microorganisms.

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Water quality — Requirements for the performance testing of membrane filters used for direct enumeration of microorganisms by culture methods

1 Scope

This document specifies the requirements for the performance testing of membrane filters used for the retention followed by direct enumeration of microorganisms by culture methods.

This document is applicable to membrane filters which are used for retention followed by direct enumeration of specific microorganisms on solid media or on other devices containing media, like absorbent pads[19].

This document is not applicable for membrane filters used for concentration and elution or for qualitative methods.

These tests are applicable to the membrane filters intended for the microbiological analysis of different types of water, such as:

— drinking water, bottled water and other types of water with expected low numbers of microorganisms;

— water with expected higher numbers of microorganisms, for example, surface water and process water.

These tests are intended to demonstrate the suitability of the whole system (membrane filter together with the culture medium including the filtration step) required for the specific tests described in References [3], [6], [8], [10], [12] and [13].

This document applies to:

— manufacturers producing membrane filters;

— microbiological laboratories using membrane filters for their own testing or providing these to other end users.

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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 8199:2018, *Water quality — General requirements and guidance for microbiological examinations by culture*

ISO 11133:2014, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

ISO 11133:2014/Amd1:2018, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

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

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

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

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

3.1 General terminology

3.1.1
membrane filter
porous hydrophilic filtration matrix, composed of different polymers with filtration characteristics equivalent to its/their rated nominal pore sizes, typically ranging from 0,1 µm to 1,2 µm, which is intended to be used for the retention of microorganisms

Note 1 to entry: The membrane filter ensures the effective retention of microorganisms depending on the membrane pore size when a differential (positive or negative) pressure is applied.

Note 2 to entry: The type of membrane filter to be used for a certain microbiological method is described in the corresponding *specific standard* (3.1.7).

3.1.2
culture medium
formulation of substances, in liquid, semi-solid or solid form, which contain either natural and/or synthetic constituents intended to support the multiplication (with or without constituents for inhibition of certain microorganisms), identification or preservation of viability of microorganisms

Note 1 to entry: The culture medium to be used for a certain microbiological method is described in the corresponding *specific standard* (3.1.7), see *specific culture medium* (3.1.3).

[SOURCE: ISO 11133:2014, 3.3.1, modified — "constituents for" has been included and Note 1 to entry has been replaced.]

3.1.3
specific culture medium
culture medium (3.1.2), usually selective, as designated in a *specific standard* (3.1.7) for use with *membrane filters* (3.1.1)

EXAMPLE 1 Chromogenic coliform agar (CCA) used with membrane filters in accordance with ISO 9308-1[8].

EXAMPLE 2 Slanetz and Bartley medium used with membrane filters in accordance with ISO 7899-2[6].

3.1.4
reference medium
culture medium (3.1.2), usually non-selective, for determination of the *reference count* (3.3.8) and *supplementary quantitative testing* (3.2.2)

Note 1 to entry: The reference medium is usually a non-selective *culture medium* (3.1.2), which is different to the culture medium under test and has been demonstrated to be suitable for use in the performance testing.

EXAMPLE Tryptone soya agar (TSA) is in accordance with ISO 11133.

[SOURCE: ISO 11133:2014, 3.3.4.13, modified — Note 1 to entry has been added and "is in accordance with ISO 11133" has been added in the example.]

3.1.5
batch of membrane filter
lot of membrane filter
homogeneous and fully traceable units of *membrane filter* (3.1.1) referring to a defined amount of bulk, semi-finished product or end product, which is consistent in type and quality, and which has been produced within one defined production period, having been assigned the same batch (or lot) number

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3.1.6
batch of culture medium
lot of culture medium
homogeneous and fully traceable units of a *culture medium* (3.1.2) referring to a defined amount of bulk, semi-finished product or end product, which is consistent in type and quality, and which has been produced within one defined production period, having been assigned the same batch (or lot) number

Note 1 to entry: If the user does not define the batch or lot of culture medium used for performance testing of membrane filters as the "end product", it is important to demonstrate that the production process is sufficiently controlled to produce culture media of consistent quality (e.g. by monitoring culture media performance testing results).

[SOURCE: ISO 11133:2014, 3.1.2, modified — "medium" has been changed to "culture medium" and Note 1 to entry has been added.]

3.1.7
specific standard
International Standard or guidance document describing the microbiological analysis of different types of water for the detection or enumeration of a specific microorganism (or group of microorganisms)

3.2 Terminology of performance testing

3.2.1
batch testing
test of units of *membrane filters* (3.1.1) from a *batch of membrane filter* (3.1.5) for its/their intended use with the *specific culture medium* (3.1.3) and determination of *reference count* (3.3.6)

3.2.2
supplementary quantitative testing
test of units of *membrane filters* (3.1.1) from a *batch of membrane filter* (3.1.5) for its/their intended use with the *specific culture medium* (3.1.3), determination of *reference count* (3.3.6), test of the membrane filters on a non-selective culture medium and test of the culture medium without membrane filter

Note 1 to entry: Annex C gives detailed description of the supplementary quantitative testing.

Note 2 to entry: Supplementary quantitative testing is used when a new type of a *membrane filter(s)* (3.1.1), or a new manufacturer is tested initially, or when a problem in the day-to-day use or *batch testing* (3.2.1) of membrane filters is noticed.

3.2.3
performance of the membrane filter
response of a *membrane filter* (3.1.1) challenged by a *test organism* (3.3.3) under defined conditions

Note 1 to entry: The defined conditions are described in the *specific standard* (3.1.7) for the intended use of the *membrane filter* (3.1.1), like: *test organism* (3.3.3), *specific culture medium* (3.1.3), *reference medium* (3.1.4), *productivity* (3.2.5), *selectivity* (3.2.6), *specificity* (3.3.7), incubation time and temperature.

3.2.4
performance of the culture medium
response of a *culture medium* (3.1.2) to challenge by *test organisms* (3.3.3) under defined conditions

[SOURCE: ISO 11133:2014, 3.2.1]

3.2.5
productivity
level of recovery of a *target microorganism* (3.3.1) from the *culture medium* (3.1.2) with the *membrane filter* (3.1.1) under defined conditions

[SOURCE: ISO 11133:2014, 3.2.4, modified — "with the membrane filter" has been added to the term and the definition.]

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3.2.6
selectivity
degree of inhibition of a *non-target microorganism* (3.3.2) on or in a selective *culture medium* (3.1.2) with the *membrane filter* (3.1.1) under defined conditions

[SOURCE: ISO 11133:2014, 3.2.5, modified — "with the membrane filter" has been added to the term and the definition.]

3.2.7
specificity
demonstration, under defined conditions, that *non-target microorganisms* (3.3.2), if able to grow on the medium, do not show the same visual characteristics as the *target microorganisms* (3.3.1) on the *culture medium* (3.1.2) with *membrane filter* (3.1.1) under defined conditions

3.3 Terminology for test microorganisms

3.3.1
target microorganism
microorganism or group of microorganisms to be detected or enumerated which can be expected to grow under defined conditions

[SOURCE: ISO 11133:2014, 3.2.2, modified — "which can be expected to grow under defined conditions" has been added to the definition.]

3.3.2
non-target microorganism
microorganism that is suppressed by the medium and/or conditions of incubation or does not show expected characteristics of the target microorganism

[SOURCE: ISO 11133:2014, 3.2.3]

3.3.3
test microorganism
control strain
microorganism generally used for performance testing of *membrane filters* (3.1.1) and/or *culture media* (3.1.2)

Note 1 to entry: Test microorganisms/control strains are further defined according to their source (see 3.3.4 to 3.3.10).

3.3.4
reference strain
microorganism obtained directly from a reference culture collection and defined to at least the species level

Note 1 to entry: A reference culture collection is a culture collection which is a member of the World Federation of Culture Collections (WFCC) or the European Culture Collections' Organization (ECCO).

Note 2 to entry: A reference strain is catalogued and described according to its characteristics and preferably originating from water as applicable.

3.3.5
reference stock
set of separate identical cultures obtained by a single subculture from the *reference strain* (3.3.4) either in the laboratory or from a supplier

[SOURCE: ISO 11133:2014, 3.4.3]

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3.3.6
stock culture
primary subculture from a *reference stock* (3.3.5)

[SOURCE: ISO 11133:2014, 3.4.4]

3.3.7
working culture
subculture from a *reference stock* (3.3.5) or *stock culture* (3.3.6) or a *reference material* (3.3.9) or a *certified reference material* (3.3.10)

Note 1 to entry: Multi-strain *reference material* (3.3.9) and multi-strain *certified reference material* (3.3.10) can also be used for the preparation of working cultures.

[SOURCE: ISO 11133:2014, 3.4.4, modified — "certified or not" has been replaced with " or a *certified reference material*" Note 1 to entry has been added.]

3.3.8
reference count
inoculum level
total count of colonies on a *reference medium* (3.1.4) obtained without usage of a *membrane filter* (3.1.1)

3.3.9
reference material
RM
microbiological material containing a quantity of revivable microorganisms, sufficiently homogenous and stable with respect to the quantity of revivable microorganisms, which has been established to be fit for its intended use in a measurement process

Note 1 to entry: See ISO Guide 30[15].

Note 2 to entry: For in-house prepared quality control reference materials (QRMs), often so-called "in-house reference materials" or "internal RM", see ISO Guide 80[18] and References [23], [25], [27] and [28].

[SOURCE: ISO 11133:2014, 3.4.6, modified — the reference in Note 1 to entry has been updated and Note 2 to entry has been added.]

3.3.10
certified reference material
CRM
microbiological *reference material* (3.3.9) characterized by a metrologically valid procedure for the quantity of revivable microorganisms

Note 1 to entry: See ISO Guide 30[15].

Note 2 to entry: Metrologically valid procedures for the production and certification of RMs are given in, among others, ISO 17034,[14] ISO Guide 31[16] and ISO Guide 35[17].

Note 3 to entry: A microbiological CRM is accompanied by a certificate that provides the value of the specified quantity of revivable microorganisms, its associated uncertainty and a statement of metrological traceability. A CRM is certified only for the method/methods and media that were included in the certification process, otherwise its function is like an (ordinary) *reference material* (3.3.9).

4 Principle

4.1 General

4.1.1 Introduction

Membrane filters are used for retention followed by direct enumeration of microorganisms in water samples. Many materials, pore sizes and brands are commercially available and designed for specific

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growth purposes. In laboratories carrying out the microbiological examination of water, the main objectives are to capture and retain, resuscitate, grow, detect and/or enumerate a wide variety of microorganisms. Membrane filters which meet the required performance criteria are therefore a prerequisite for any reliable microbiological work.

Direct enumeration includes all colony-count methods following retention of the microorganisms on the membrane filters and placement of the membrane filters on solid media or on other devices containing culture media, see ISO 6461-2[3], ISO 7899-2[6], ISO 9308-1[8], ISO 11731[10], ISO 14189[12], ISO 16266[13] and Reference [19]. The direct enumeration is a quantitative method.

Sufficient performance testing should be carried out to demonstrate that a batch is 'fit for purpose' and that the filter can produce consistent results for the specific analysis. The quality of the membrane filters shall be tested together with the intended specific culture medium to demonstrate their suitability for use in a so-called "microbiological function test".

This implies that batch testing should demonstrate the suitability of the whole system (membrane filter in combination with the specific culture medium including the filtration step). It is not required to evaluate the performance of the membrane filter separately to the performance of the culture medium.

It is expected that suitable media, incubation temperature, incubation duration, incubation atmosphere and controls will be used for the specific applications. Results obtained from one species or group of microorganisms may not be valid for other groups.

Suitable specification and acceptance criteria are given in the corresponding specific standard, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F.

The testing for meeting the performance criteria is an essential part of the quality assurance procedure and appropriate documentation is necessary.

It is the responsibility of the end user to ensure all required combinations of test strains, microbiological culture media, and membrane filters have been tested before use. If testing before use is not possible due to the lability of the culture medium, parallel performance testing alongside the sample testing shall be performed.

NOTE Annex E gives a practicable example for the testing of the membrane filters by the end user.

4.1.2 Batch testing

The batch testing is required when a different batch or lot of a membrane filter is tested.

The batch testing needs to be performed for each batch of membrane filters with each batch of culture medium that is used in the quantitative analysis within the procedure of the enumeration method required. This shall be done before usage of the combination membrane filter/culture medium by the manufacturer or by the user.

Therefore, the batch testing needs to be adjusted to the procedure of the applicable standard in terms of culture media, incubation temperature and time as well as suitable test microorganism. The procedure for batch testing is described in Clause 9.

If the criteria for the batch testing of membrane filters given in the corresponding specific standard, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F are not achieved, the laboratory should assess the discrepancies between the results by supplementary testing given in 4.1.3.

4.1.3 Supplementary testing

Supplementary quantitative testing can be used when a new type of a membrane filter or a new manufacturer is initially tested, or when a problem in the day-to-day use or batch testing is noticed. The procedure for supplementary quantitative testing is described in Annex C. Supplementary qualitative testing for a membrane filter is appropriate to consider when there are discrepancies in colony appearance between different membrane filter brands, types or lots. Some qualitative aspects

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for a membrane filter used in combination with the culture medium (e.g. colony characteristics and morphology) and how they can be checked by use of scores are described in Annex D.

NOTE For the supplementary testing on homogeneity based on a microbiological culture method, see Reference [29].

4.2 Performance testing

4.2.1 Modules for batch and supplementary testing

The quantitative testing procedure of the membrane filters consists of four different modules, see Table 1. Depending upon the purpose of the testing, different modules are required:

— for batch testing of the membrane filters, modules 1 and 2 shall be tested simultaneously;

— for supplementary quantitative testing of the membrane filters, the modules 1, 2, 3 and 4 shall be tested simultaneously.

From the counts achieved in modules 1 and 2, the productivity ratio can be calculated for the membrane filter used in combination with the specific culture medium. The productivity shall reach a defined minimum limit, in accordance with the corresponding standard, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F.

Information on sampling of membrane filters for the testing is given in Clause 8.

Table 1 — Modules for batch and supplementary quantitative testing

Module no.	Testing purpose	Testing procedure	Required for	
			Batch testing	Supplementary quantitative testing
1	Determination of the reference count	Count obtained on a non-selective reference culture medium using spread plate technique without a membrane filter	x	x
2	Productivity, selectivity[a], specificity[a] of the membrane filter in its intended use	Membrane filter used with the specific culture medium using membrane filtration technique	x	x
3	Detection of inhibition of target organisms due to the membrane filter	Membrane filter used with a non-selective culture medium using membrane filtration technique	—	x
4	Detection of inhibition of target organisms due to the specific (selective) culture medium	Specific culture medium using spread plate technique without a membrane filter	—	x

[a] If applicable.

4.2.2 Absence of microbial contamination

For testing the absence of microbial contamination, an appropriate quantity of membrane filters shall be tested by incubation under appropriate conditions.

If a batch of membrane filters is specified by the supplier as sterilized in a validated sterilization cycle, a test for absence of microbial contamination by the end user is not required.

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5 Apparatus and glassware

Appropriate apparatus and glassware are stated in the corresponding specific standards or in ISO 8199.

6 Culture media and diluents

Appropriate culture media and diluents are stated in the corresponding specific standards or in ISO 8199.

For the preparation, production, storage and performance testing of culture media and diluents, follow the procedures given in the corresponding specific standards, ISO 11133 or ISO 8199. Before use, allow the agar plates to equilibrate at room temperature if stored at a lower temperature.

Appropriate culture media for estimation of the reference count are stated in the corresponding specific standards, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F.

In order to ensure the reliability of results of performance testing, the reference medium used shall be of consistent high quality. Examples of aspects to be considered by the user are given in ISO 11133:2014, 6.3.2.

7 Preparation of microorganisms for performance testing

7.1 General

This protocol requires the use of a quantified bacterial suspension (which can be a quantitative reference material) with an appropriate colony count of a target strain.

Guidance for procedures suitable for preservation and maintenance of microorganisms is stated in ISO 11133.

Appropriate microorganisms for performance testing are stated in corresponding standards, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F. Where the standards list more than one control strain for each aspect of performance testing (e.g. productivity, selectivity, specificity), the minimum number of strains to be used is indicated. Usually at least one or two strains that are considered typical target or non-target microorganisms are used in the performance testing.

A complete list of culture media and reagents used in Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*, with their names of control strains and their World Data Centre for Microorganisms (WDCM) collection numbers that should be used for testing the performance of culture media and reagents can be found in Reference [35].

NOTE 1 Well-characterized strains isolated by the laboratory can be included additionally.

NOTE 2 ISO 11133:2014, Annex J and ISO 11133:2014/Amd1:2018, Annex J provides instructions for the creation of new microbiological performance criteria, methods and control strains if these are not given in a specific standard, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F.

7.2 Reference count

7.2.1 Quantitative productivity testing

For all quantitative tests, the use of a quantified inoculum of the specified microorganisms is needed. Follow the procedures in accordance with ISO 11133:2014, 5.4.2.5 and ISO 11133:2014/Amd1:2018, 5.4.2.5. The inoculum can be prepared by the laboratory or from a RM or a CRM.

NOTE 1 For the laboratory-prepared inoculum, additional information on “Quality Control Materials” (QCMs) used for specific in-house quality control applications is given in ISO Guide 80[17].

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The reference count of the laboratory-prepared inoculum or RM is determined in relation to recovery on a non-selective reference culture medium. Follow the procedures in accordance with ISO 11133:2014, 7.2.2. Appropriate reference media are given in the corresponding specific standard or ISO 11133.

The quantitative enumeration test using membrane filters on productivity requires a level of approximately 100 colony forming units (cfu) to achieve sufficient precision, see ISO 11133:2014, Table 1. A practicable range to be used will be 80 cfu to 120 cfu. If more colonies are counted, the precision is increased.

NOTE 2 By assuming Poisson distribution in a sample, which is usually relevant, colony numbers on membrane filters from several aliquots from the sample can be added, see ISO 13843:2017, Clause A.2[11].

NOTE 3 ISO 11133:2014, Table 1 shows the 95 % confidence intervals associated with colony counts.

As guidance, the general upper limit for counting on membrane filters with a diameter of 47 mm to 50 mm can be regarded as 80 cfu, see ISO 8199:2018, 9.1.4.2.

However, for certain parameters lower, or even higher, upper limits are more appropriate, see the specific standards and NOTE 4.

Thus, more than one membrane filter can be required to reach a total of approximately 100 cfu for counting. Two membrane filters are enough with approximately 50 target colonies on each. Three and four membrane filters are necessary when about 35 and 25 target colonies, respectively, appear on each.

NOTE 4 It can be difficult to count a minimum of 50 cfu per membrane filter for bacteria that form large colonies, such as *Pseudomonas aeruginosa* (*P. aeruginosa*). Their performance tests are easier to read with three plates with approximately 30 cfu per membrane filter or four plates with approximately 25 cfu per membrane filter to reach a total of approximately 100 cfu. The opposite applies to enterococci: due to their small and defined colonies, these can often be easily counted when approximately 100 cfu or greater are present on a single membrane filter.

7.2.2 Qualitative selectivity testing

For the qualitative selectivity testing using membrane filters in combination with the specific culture medium, a suspension of the non-target microorganism containing at least 10^4 cfu is inoculated by membrane filtration. Follow the procedures in accordance with ISO 11133:2014, 5.4.2.5.1.2 and ISO 11133:2014/Amd1:2018, 5.4.2.5.1.2.

7.2.3 Qualitative specificity testing

For the qualitative specificity testing using membrane filters together with the specific culture medium, a suspension of the non-target microorganism containing at least 10^3 cfu is inoculated by membrane filtration. Follow the procedures in accordance with ISO 11133:2014, 5.4.2.5.1.3.

7.3 Preparation of a standardized test suspension using a working culture

7.3.1 General

The following guidance is given as an example of a procedure suitable for producing standardized test suspensions for the performance testing of membrane filters. These procedures are generally applicable but some microorganisms can require special conditions. For example, the culture of anaerobes may require special conditions (e.g. anaerobic atmosphere, nutritional requirements).

7.3.2 Preparation of the working culture

The test microorganism is obtained as a reference strain and stored as a reference stock. From the reference stock, a stock culture by using a solid medium is prepared and used for the preparation of a working culture. A working culture shall be prepared from the reference stock or from the stock culture as a pure stationary phase culture in a non-selective broth.

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All details for the steps of preparation and maintenance of the test microorganisms including different methods are described in detail in ISO 11133:2014, 5.4.2 and Annex B and ISO 11133:2014/Amd1:2018, 5.4.2. For *Legionella* spp. working culture and test suspension, follow the procedures in accordance with ISO 11731:2017, 11.3[10].

Although various techniques can be used, they shall be applied in a standardized way to guarantee both purity and known inoculum level.

NOTE Annex E gives an example of the preparation of the working cultures for the performance testing in accordance with ISO 9308-1[8].

For the preparation of a test suspension using reference material, see 7.4.

7.3.3 Preparation of a standardized test suspension (inoculum) for the test

From the working culture, prepare serial dilutions in a suitable diluent, for example, quarter-strength Ringer's solution, peptone salt solution, see ISO 8199:2018, Annex D. Use the most suitable dilution step for the target level of microorganisms (cfu) in a specified volume or refer to the supplier.

For every test microorganism, the suitable dilution to use as a test suspension (inoculum) should be determined from previous tests conducted under strictly standardized conditions for all steps.

Use the test suspension (inoculum) within a specified time, for example, up to 2 h at room temperature or within 24 h if stored at (5 ± 3) °C. Longer storage periods can be acceptable if the stability of the stored culture has been verified[24].

Frozen inocula can be used if it can be shown that the microorganism can survive for the chosen period.

NOTE Annex E gives an example of the preparation of the test suspensions (inocula) for the performance testing according to ISO 9308-1[8].

7.4 Preparation of a test suspension using reference material

This protocol uses RMs or CRMs to provide a stable bacterial suspension containing a known number of cfu of the target or non-target strain.

The recovery from the new batch of membrane filters used together with the specific culture medium is compared to the expected number of cfu from the RM or CRM.

The quality of the RM (including the quantity of cfu) shall be verified on the reference medium.

NOTE Reference material verification is done preferably by the manufacturer.

8 Sampling of membrane filters for testing

An appropriate quantity of membrane filters shall be tested under conditions described in the specific standard.

Manufacturers of membrane filters shall set specifications using appropriate acceptable quality limits for batch control.

NOTE 1 Information about sampling is given in the ISO 2859 series[1], the ISO/TR 8550 series[7] or other specific standards. ISO 2859-1[1] contains an introduction to the ISO 2859 series[1] attribute sampling system and provides a brief summary of the attribute sampling schemes and plans used in the different parts of the ISO 2859 series[1].

Laboratories (either those using membrane filters for their own testing or those providing these filters to other end users) shall perform the quantitative productivity testing (and either qualitative selectivity

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or specificity testing, or both, if applicable) each time the batch of membrane filters changes. For every intended use, the batch of membrane filters shall be tested with the specific batch of culture medium.

NOTE 2 The ISO 2859 series[1] specifies sampling plans with a controlled statistical power according to the size of the batch. The manufacturer's risk of being refused a valid lot according to its compliance criteria as well as the laboratory's risk of accepting an occasional poor lot are assessed in the standard.

The minimum number of membrane filters to be tested is determined by the requirements for the performance test together with the specific culture medium in accordance with corresponding specific standards, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F.

NOTE 3 Annex E gives a practical example for the testing of the membrane filters by the end user.

9 Procedure

9.1 General

The batch testing of membrane filters shall be carried out as specified in Annex A.

For the quantitative productivity testing during the batch testing, modules 1 and 2 shall be tested simultaneously; see 4.2 and Table 1.

The quantitative productivity testing requires a test suspension (inoculum) as specified in 7.2.1. The same volume should preferably be used as for the determination of the reference count by spread plating (see 9.2) and for the inoculation by membrane filtration (see 9.3). A qualitative selectivity and a qualitative specificity testing shall be included in the batch testing, if required in accordance with the requirements for the performance testing given by the corresponding specific standard or ISO 11133.

NOTE Examples of suitable reference media are given in the corresponding specific standard, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F.

Module 1 is not needed when using a CRM which is certified for its number of cfu with the specified method and culture medium; see 3.3.10, Note 3 to entry.

For the qualitative testing on selectivity and specificity during the batch testing, module 2 is tested. Module 1 is not required as these are qualitative tests. The qualitative tests on selectivity and specificity require test suspensions (inocula) as respectively specified in 7.2.2 and. 7.2.3.

For the test on absence of microbial contamination, see 9.5.

9.2 Inoculation by spread plate technique

9.2.1 General

The test portion (inoculum) containing the reference count and obtained as indicated in 7.3 or 7.4 is spread over the dry surface of a solid medium with a sterile implement. Colonies that develop on the surface after incubation are counted.

Spread-plate technique is used for modules 1 and 4; see Table 1.

Avoid any mechanical damage to the bacteria by surface-plating using the spread-plate technique with Drigalski spatula, see Reference [26].

The test portion containing the reference count should be chosen so that the expected number of colonies is achieved; see 7.2.

For a Petri dish of 90 mm diameter, the volume of the test portion containing the reference count should be from 0,1 ml to a maximum of 0,5 ml portion of the inoculum.

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Spiral platers use smaller volumes and the limit of determination will consequently be raised. Follow the manufacturer's guidance for use of spiral platers. Spiral platers may not be suitable for fungi. For more information, see ISO 7218[5].

9.2.2 Inoculation

Prepare and mark the plates required, each containing (18 ± 2) ml of culture medium for 90 mm Petri dishes. For longer incubation periods, larger volumes of culture media can be required. In this case, refer to ISO 11133 for guidance on culture medium volumes.

If necessary, dry the surface of the medium before use, as described in ISO 11133:2014, 4.5.5, and ISO 8199:2018, 9.1.3.3.

Pipette the test portion onto the surface of the medium and spread over the surface with a sterile implement, or mechanical device such as a spiral plater, avoiding the edges of the agar. Leave the plates on the bench until the inoculum is absorbed (maximum time is 15 min), then incubate the plates in accordance with 9.4.

9.3 Inoculation by membrane filtration technique

9.3.1 General

For the test on productivity, selectivity and specificity (if applicable), filter the test portion (inoculum) containing the reference count and obtained as described in 7.3 or 7.4, according to the requirements of the specific standard, using standardized procedures described in the specific standard or in ISO 8199.

Use equipment for membrane filtration and handle the filtration and placement of the membrane filter as described in the specific standard or in ISO 8199.

Membrane filtration technique is used for modules 2 and 3, see Table 1.

9.3.2 Inoculation

The test portion containing the reference count should be chosen so that the expected number of colonies will be achieved, see 7.2.

After placing the membrane filter under test and positioning the funnel on the filter base, pipette or pour the following after each other into the funnel (with the vacuum stopcock turned off) in three subsequent steps:

a) a volume of at least 10 ml of the sterile water or a suitable sterile diluent;

b) the test suspension containing the reference count, preferably the same volume as used for the determination of the reference count by surface plating;

c) approximately 10 ml of sterile water or a sterile diluent.

Open the stopcock and apply the vacuum to filter the water through the membrane filter. Close the stopcock as soon as the sample has been filtered. The funnel should be rinsed by filtering one to three portions of 10 ml to 30 ml of sterile water or diluent, while the filter is still in place, to remove organisms adhering to the funnel.

Alternatively, the inoculum can be added to a volume of at least 20 ml of the sterile water or a suitable sterile diluent. Mix well and filter the whole volume as described above.

After filtration, place the membrane filter on the culture medium, ensuring that there is no air trapped underneath and invert the Petri dish.

For membrane transfer techniques using liquid media or diluents, refer to the specific standard or ISO 8199:2018, 9.1.4.6.

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9.4 Incubation and counting

According to the method, choose the duration, temperature and special conditions (if needed) of the incubation for the plates with and without membrane filters, as given by the relevant specific standard or in ISO 8199.

Counting should be done as specified in the relevant specific standard or in ISO 8199.

9.5 Test for absence of microbial contamination

An appropriate quantity, depending on the size of the batch of membrane filters, shall be tested for absence of microbial contamination (sterility) by incubation under appropriate conditions. This can be performed by placing the membrane filter on the specific culture medium. This may be the selective culture medium used for the test or a non-selective culture medium.

NOTE For more information, see Reference [20].

Information on sampling of membrane filters for the testing is given in Clause 8.

10 Calculation, expression and interpretation of results

10.1 General

In the following subclauses, the calculation, expression and interpretation of the results are described; see productivity in 10.2, selectivity in 10.3 and specificity in 10.4 testing.

A batch of membrane filters performs satisfactorily if all the test microorganisms used perform according to the given specifications. It shall be accepted if both general and microbiological quality criteria are met.

Acceptance criteria are given in the corresponding specific standard, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F.

Either supplementary quantitative or qualitative testing, or both, can be used when a problem in the testing is noticed, see 4.1.2.

NOTE The procedure for supplementary quantitative testing is described in Annex C. The procedure for supplementary qualitative testing is described in Annex D.

10.2 Productivity testing

From the result of this quantitative testing, the productivity ratio, P_R, is determined.

P_R shall reach a defined minimum limit, see the corresponding specific standard, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F.

The productivity ratio P_R[24] is determined using Formula (1):

$$P_R = \frac{N_s}{N_o} \quad (1)$$

where

N_s is the total count of colonies obtained from one (or more) membrane filter(s) in intended use with the specific culture medium (see module 2, productivity of the membrane filter, in Table 1);

N_o is the total count of colonies obtained from one (or more) plate(s) of non-selective reference culture medium by direct inoculation without a membrane filter (see module 1, determination of the reference count, in Table 1).

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The results are accepted as valid if the following conditions are satisfied:

— each replicate shall give a positive quantitative result (target bacterial growth);

— each single reported result is included in the practicable range of analysis, see 7.2.

If the P_R exceeds 1,4, the cause shall be traced.

For using recovery from CRMs, critical difference can be used for the calculation of tolerance limits, see ISO 5725-6[2] and ISO 11133:2014, Table 1.

10.3 Selectivity testing

For the interpretation of results obtained in selectivity testing of membrane filters together with the specific culture medium, the amount of growth of non-target microorganisms after incubation is assessed as follows:

— 0 corresponds to no growth;

— 1 corresponds to weak growth (either reduction in amount of growth or colony size);

— 2 corresponds to good growth.

For selectivity tests, the degree of inhibition depends on the type of medium. The growth of non-target microorganisms shall be partly or completely inhibited; see corresponding specific standard, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F.

10.4 Specificity testing

The specificity of using membrane filters in combination with the specific culture medium is given by essential physiological characteristics to differentiate related organisms by the presence, absence and/or grade of expression of biochemical responses, by colony sizes and by morphology, see corresponding specific standard, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F.

11 Documentation of test results

11.1 Test report

The test report of the performance testing of membrane filters shall contain at least the following information:

a) the test method used, together with a reference to this document, i.e. ISO 7704:2023;

b) product name, product reference and batch number, manufacturer, material and pore size;

c) product name, product reference and batch number, manufacturer of all used media including diluents;

d) date of testing;

e) test organism(s) with identification code, for example, culture collection number or internal laboratory identification code of the test strain(s);

f) any particular occurrence(s) observed during the supplementary testing analysis and any operation(s) not specified in this document, which can have an influence on the results;

g) the results of the quantitative and, if applicable, of the qualitative testing (selectivity and specificity) and supplementary testing, see Annexes C and D;

h) reference standards and specifications / limits.

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11.2 Information provided by the manufacturer

The manufacturer or supplier of the membrane filters shall provide, on request, the microbiological growth characteristics for specific microorganisms and general information relating to the specific batch of culture medium used. Furthermore, information on ensuring homogeneity of the batch shall be provided, on request.

Manufacturers shall show that the membrane filters meet the requirements for use for quantitative enumeration as given in relevant specific standards.

11.3 Traceability

All the data from performance testing should be documented in an appropriate way and kept for a sufficient period of time according to the quality system in use. The use of control sheets for documenting and evaluating the results of the tests is recommended; see Annex B.

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Annex A
(normative)

Diagram of the procedure for batch testing

Figure A.1 shows the diagram of the procedure for the batch testing of membrane filters.

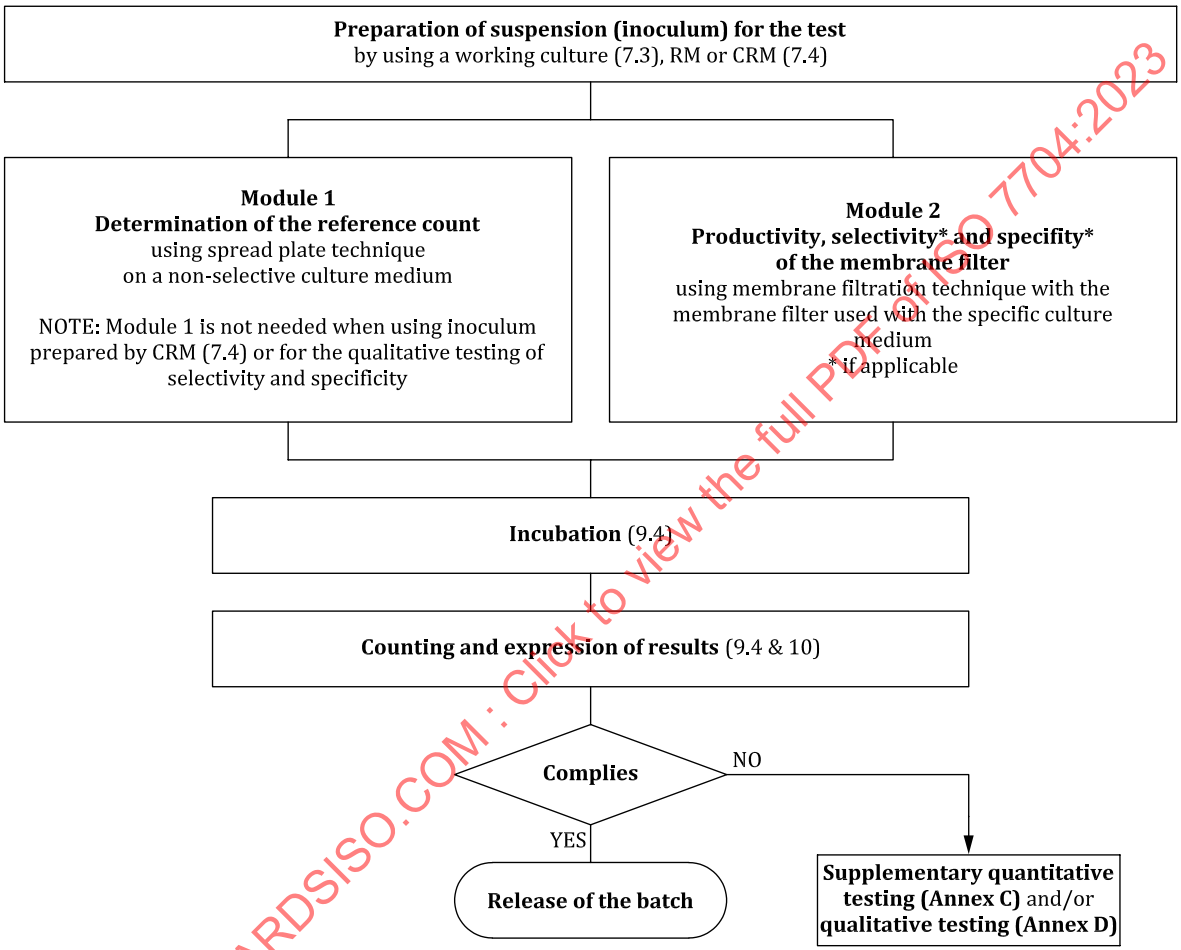

Figure A.1 — Diagram of the procedure for the batch testing

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Annex B
(informative)

Example of a card for recording test results from batch testing

Table B.1 shows an example of a card for recording test results from batch testing of membrane filters.

Table B.1 — Example of a card for recording test results from batch testing

Name/manufacturer of the membrane filter:			Batch no.:		
Non-selective reference culture medium:			Batch no.:		
Selective culture medium:			Batch no.:		
Test organism/reference material (RM):			Batch no.:		
Number of test organisms in the used sample (dilution):			cfu (µl):		
Dilutions used for the test:			Volume used for inoculation:		
Incubation temperature (°C):			Incubation time (h):		
Test started (date):			Test finished (date):		
Results					
Dilution of inoculum	Replicate		Module 1 Determination of the reference count	Module 2 Productivity of the membrane filters	
10 -	1				
	2				
			N_0:	N_s:	
Specification productivity ratio P_R[a]					
Test results productivity ratio P_R[a]					
Remarks:					
Batch of membrane filters:		Released for use with culture medium:		Batch:	
( ) yes ( ) no	Date:		Signature:		

[a] Requirement: The productivity ratio (P_R) of the membrane filtration on selective culture medium shall be in accordance with the corresponding specific standard, or ISO 11133:2014, Annex F and ISO 11333:2014/Amd1:2018, Annex F.

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Annex C
(informative)

Quantitative supplementary testing of membrane filters

C.1 Introduction

For the quantitative supplementary testing, the modules 1, 2, 3 and 4 are tested simultaneously, see 4.2 and Table 1.

It is preferred to use the same non-selective culture medium for modules 1 and 3.

C.2 Method for supplementary testing

Module 1 is not needed when using CRM, see 7.4.

NOTE 1 Examples of suitable reference media are given in the specific standard, or ISO 11133:2014, Annex F and ISO 11133:2014/Amd1:2018, Annex F.

NOTE 2 For the performance testing of the culture medium without membrane filtration use a method as described in ISO 11133.

A diagram of the procedure of supplementary testing is given in Figure C.1.

C.3 Diagram of the procedure for supplementary testing

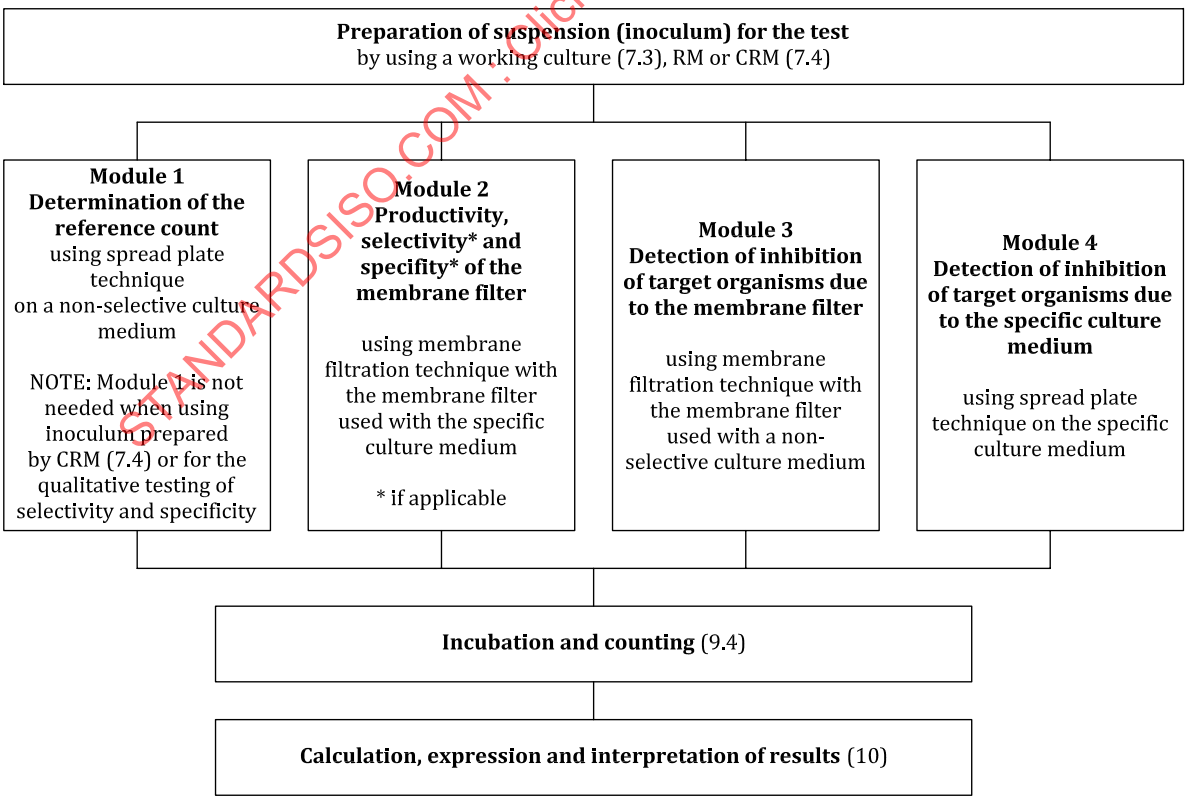

Figure C.1 — Diagram of the procedure for supplementary testing

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C.4 Calculation and interpretation of the test results

For the calculation and interpretation of results of the quantitative supplementary testing of membrane filters, the following points can be considered:

— If P_R on non-selective culture medium with membrane filter (Module 3) is not lower than P_R on selective culture medium with membrane filter (Module 2), the batch of membrane filters does not seem to have a pronounced inhibitory effect.

— If P_R on non-selective culture medium with membrane filter (Module 3) is <0,70, the batch of membrane filters can be a possible cause of growth inhibition of the control strains.

— If P_R on selective culture medium with membrane filter (Module 2) of the batch testing does not meet the specifications and productivity on non-selective culture medium with membrane filter (Module 3) shows also low P_R, the inhibitory effects of the membrane filters can be a possible cause.

— If P_R is <0,70 on non-selective culture medium with membrane filters (Module 3), this points towards inhibitory effects of the membrane filters.

— If P_R is low (<0,70 respectively <0,50, see ISO 11133:2014, 7.2.2.1.2) on selective culture medium without membrane filter (Module 4), this can also cause low P_R in the batch testing. But it is also possible that this selective culture medium tested with membrane filters shows P_R as specified and not the same degree on inhibition, see below for possible damage of the control strains using spread-plating.

— If P_R on non-selective culture medium with membrane filter (Module 3) is >1,40, inhibitory effects from damaging the test microorganism during spread-plating can be considered, see Reference [26]. For points to consider for reference culture medium, see ISO 11133:2014, 6.3.2.

Table C.1 shows examples for interpretation of the test results for one control strain according to ISO 9308-1:2014/Amd1:2016, Table 1[9].

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Table C.1 — Examples for interpretation of the test results for one control strain according to ISO 9308-1:2014/Amd1:2016, Table 1[9]

	Module no.				Comment
	1	2	3	4	
Testing purpose	Determination of the reference count	Productivity of the membrane filter in its intended use	Detection of inhibition of target organisms due to the membrane filter	Detection of inhibition of target organisms due to the specific (selective) culture medium	—
Testing procedure	Non-selective reference culture medium using spread plate technique	Specific culture medium using membrane filtration technique	Non-selective culture medium using membrane filtration technique	Specific culture medium using spread plate technique	According to ISO 9308-1[8], *Escherichia coli* (*E. coli*) WDCM 00013 can be used for quantitative productivity testing with $P_R \geq 0,7$ as test criterium.
Example	*E. coli* spread plate on TSA	*E. coli* membrane filtration on CCA	*E. coli* membrane filtration on TSA	*E. coli* spread plate on CCA	—
1	113 cfu	143 cfu $P_R = 1,27$	138 cfu $P_R = 1,22$ P_R about the same as in module 2	—	No major inhibitory effect of membrane filter.
2	113 cfu	—	72 cfu $P_R = 0,64$ (<0,70)	—	Membrane filter can have inhibitory effects on growth of microorganisms.
3	113 cfu	72 cfu $P_R = 0,64$ (<0,70)	68 cfu $P_R = 0,60$ (<0,70)	—	Inhibitory effects of the membrane filters can be a possible cause.
4	113 cfu	—	165 cfu $P_R = 1,46$ (>1,40)	98 cfu $P_R = 0,87$	Inhibitory effects from damaging the test microorganism during spread-plating can be considered.
5	113 cfu	72 cfu $P_R = 0,64$ (<0,70)	—	68 cfu $P_R = 0,60$ (<0,70)	Inhibitory effects of medium can be considered as a possible cause for low P_R in batch testing.
6	113 cfu	105 cfu $P_R = 0,93$ (≥0,70, < 1,40)	57 cfu $P_R = 0,50$ (<0,70)	68 cfu $P_R = 0,60$ (<0,70)	Membrane filter in combination with selective culture medium can work well although both compounds tested separately show inhibition of the target strains.
NOTE This table does not list counts that are irrelevant for the listed interpretation.					

As long as productivity, selectivity and specificity tests are within the specifications given in the specific standard, a low productivity in the supplementary testing does not disqualify the combination of membrane filters and culture medium. Batch testing (Module 2) can meet the specifications, although P_R of the testing of Module 3 and Module 4 are <0,7 respectively <0,5.

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Annex D
(informative)

Qualitative supplementary testing of membrane filters

D.1 Introduction

In the testing of membrane filter suitability for retention followed by direct enumeration, the recovery of the challenged microorganisms is of highest importance. The qualitative supplementary testing of membrane filters is appropriate to consider when there are discrepancies in colony appearance between different membrane filter brands/types/lots.

Some examples of the qualitative characteristics and how they can be checked by the use of scores are described. Categorical scores need to be given to each property to be able to sum up those that are appropriate for a filter for comparison against a certain reference value or against values for other filters. Ranks based on the sums can be used for an easier comparison when several filters are studied simultaneously.

D.2 Global properties

The global properties are:

a) hydrophobicity of the membrane filters, see D.4.2;

b) grid line growth inhibition, see D.4.3;

c) grid line colony proliferation, see D.4.4.

D.3 Colony type specific properties

The colony type specific properties are:

a) colony colour, see D.4.5;

b) colony irregularity, see D.4.6;

c) colony convexity, see D.4.7;

d) colony size, see D.4.8.

D.4 Categorical scores

D.4.1 General

Example scores are given below. If necessary, there can be more categories than those indicated. Most of them intrinsically are more or less subjective. To make it manageable not too many categories should be used.

Although some of the listed properties have no direct impact either on colony recovery or on the suitability of a particular membrane filter for quantitative purposes, they can be useful for the ease of reading the plates.

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D.4.2 Hydrophobicity of the membrane filters

Hydrophobic parts of the filters will not attach to the media surface, leading to reduced transport of nutrients from the medium to the filter and the possibility of reduced growth.

The scores are:

— 0 = None;

— –1 = Hydrophobic spots (small, ≤ 10 % of the filter area);

— –2 = Hydrophobic spots (large, > 10 %).

The filters are checked by the naked eye regarding the presence of hydrophobic spots.

NOTE 1 As hydrophobic spots can be wet due to capillary force, it is useful to check the hydrophobicity after approximately 10 % of the incubation time at the given incubation temperature.

NOTE 2 For more information, see Reference [21].

D.4.3 Grid line growth inhibition

The grid lines simplify the counting of colonies but their ink composition should not restrain bacterial growth or impart hydrophobicity to the membrane. An inhibiting grid line can lead to irregular colony presentation and potentially colonies almost forming two parts to avoid contact with a line, affecting the ease of counting or even leading to a too high recovery. The inhibition can even lead to deteriorated growth and a lower recovery. There shall be complete growth of the colonies over the grid lines without changes in the typical shape of the colony.

The scores are:

— 0 = None;

— –1 = Small (1 % to 10 % of colonies on lines affected);

— –2 = Large (>10 % of colonies on lines affected).

Filters are generally checked by the naked eye.

NOTE The grid line inhibition can be easier assessed by use of a binocular stereo microscope with 4× to 10× magnification.

D.4.4 Grid line colony proliferation

The grid line (imprint on membrane filters) can cause a channel in which water collects. Colonies can proliferate along the grid lines giving irregular shaped, smeared or overlapping colonies which cannot be counted correctly.

The scores are:

— 0 = None;

— –1 = Small (1 % to 10 % of colonies on lines affected);

— –2 = Large (>10 % of colonies on lines affected).

NOTE 1 Grid line growth promotion can either be an effect of the dye of the line itself but probably rather an effect of surface tension properties of the lines compared to the rest of the filter. Colonies can proliferate along the grid lines. Sometimes, it can look as if there are several colonies that will give a too high count.

NOTE 2 The grid line inhibition can be easier assessed by use of a binocular stereo microscope with 4× to 10× magnification.

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D.4.5 Colony colour

The colony colour is specified in the relevant standards or by ISO 11133:2014/Amd1:2018, Annex F.

The scores are:

— 0 = Atypical colour;

— 1 = Weak intensity of typical colour;

— 2 = Anticipated typical colour.

NOTE Colour and colour zones are not always well defined but a bit variable. Therefore, they need to be evaluated with care in relation to prior experiences and relevant definitions, for example, the basic colour itself, a colour zone or a metallic sheen.

D.4.6 Colony irregularity

Some bacterial species usually have more or less irregular colonies, while others normally are smooth.

The scores are:

— 0 = Smooth;

— –1 = Somewhat irregular;

— –2 = Very irregular.

NOTE Irregularity of colonies is somewhat arbitrary and can be caused by dust or other particles on the surface of membrane filters. Hence, interpretation of irregularity needs to be taken with care and prior experience, and the typical appearance needs to be taken into consideration.

D.4.7 Colony convexity

Usually colony convexity does not affect the recovery of colonies but needs to be considered when the colony appearance is important.

The scores are:

— 0 = Flat;

— 1 = Somewhat convex;

— 2 = Very convex.

NOTE The convexity of the colony is normally typical for a strain on a medium. However, it depends strongly on the age of the colonies and needs to be studied only within a defined time span.

D.4.8 Colony size

Colony size varies between different groups, genera, species and strains of microorganisms. The size can also vary within a bacterial strain, partly depending on when the growth of the particular colony started and on the density of colonies on the filters.

The scores (e.g. depending on strain and media) are:

— 0 = 0 mm to 1 mm;

— 1 = 1 mm to 2 mm;

— 2 = >2 mm.

NOTE The size classes need to be decided for the particular strain used and care needs to be taken when interpreting the results. Prior experience and knowledge of the strains needs to be taken into consideration.

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D.5 Combining scores

D.5.1 General

Only scores for properties showing differences between filter "types" (brands, lots etc.) need to be used in the final assessment.

The scores from all properties retained are summed up for each analytical parameter, using the scores from all properties/strains/samples used and also from each person reading a particular type of membrane filters. The average over strains/samples and persons is then calculated giving the characteristic over-all score of that filter type.

If interesting or necessary for an over-all choice of a filter type, scores can even be averaged over target organisms for several analytical parameters.

D.5.2 Comparison of filters

If several filters are compared simultaneously in one study, ranks from 1 to the number of filter types compared can be given to the filter types in accordance with the average scores obtained. The highest (most positive) score is yielding rank 1, the second highest rank 2, etc. If two or more types of membrane filters have the same score, they need to be given the same and lowest rank number in turn. The following rank needs then be the one following the number of filters that have already obtained ranks.

EXAMPLE Five types of membrane filters are studied simultaneously. One of them has the highest average score and obtains rank 1. Two types have the same, second highest score. Both of them obtain rank 2. After these follow types with second lowest and lowest score, they obtain ranks 4 and 5, respectively.

D.6 Assessment

For the assessment of the suitability of certain membrane filters, a criterion to compare against is appropriate. The criterion can be a minimum value for summarized and averaged scores both when only one membrane filter or several are examined. The criterion will depend on the particular study design, i.e. how many and which properties are studied and what scores are given for the various properties.

When several membrane filters are compared, ranks can be an alternative to select one or more suitable membrane filters.

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Annex E
(informative)

Practical example of quantitative batch testing and quantitative supplementary testing by the end user

E.1 Introduction

Performance testing for membrane filters used for a quantitative microbiological test method needs to be conducted in its intended use. For ISO 9308-1[8], this translates into testing plates of CCA with the membrane filter that is chosen to be used for the testing. Performance tests on productivity, selectivity and specificity are necessary for every combination of batches of CCA and membranes that are used together.

In this example of the performance testing of membrane filters, the test procedure does not need any confirmation step, as a working culture of a known pure single test strain is used. If different colony morphologies are observed on the plates, confirmation steps can be taken in order to ensure that there is no contamination.

ISO 9308-1:2014/Amd1:2016, Table 1[9] specifies the control strains to be used for the productivity testing. According to this Reference [8], productivity needs to be $P_R \geq 0,7$. As the method combines the enumeration of *E. coli* and other coliform bacteria, one strain for each group needs to be tested.

In this example, the performance test on productivity requires quantitative testing while qualitative testing is required for selectivity and specificity. The control strains *E. coli* WDCM 00013 and *Citrobacter freundii* (*C. freundii*) WDCM 00006 are used for the quantitative testing on productivity, *Enterococcus faecalis* (*E. faecalis*) WDCM 00009 for the qualitative testing on selectivity and *P. aeruginosa* WDCM 00024 for the qualitative testing on specificity, see Table E.1.

For a flow diagram of batch testing and supplementary testing of this example, see Clause E.9.

Table E.1 — Choice of control strains and criteria used in this example for performance testing of CCA according to ISO 9308-1:2014/Amd1:2016, Table 1[9]

Function	Control strains[a]	Method of control	Criteria[b]	Characteristic reactions[b]
Productivity	*E. coli* WDCM 00013	Quantitative	$P_R \geq 0,7$	Dark-blue to violet colonies
	C. freundii WDCM 00006	Quantitative	$P_R \geq 0,7$	Pink to red colonies
Selectivity	*E. faecalis* WDCM 00009	Qualitative	Total or partial inhibition	—
Specificity	*P. aeruginosa* WDCM 00024	Qualitative	—	Colourless colonies

[a] Refer to Reference [36] for the reference strain catalogue on culture collection strain numbers and contact details.

[b] See ISO 9308-1:2014/Amd1:2016, Table 1[9].

A practical example for the preparation of bacterial suspensions with known quantity of the control strains is described in Clause E.2. This can be used as a batch testing in lab routine functioning as daily control measures. Adaptation for a one-time batch testing procedure is also possible, for example, as an incoming control of a new batch of membrane filters to be tested with a batch of culture medium, see 4.1.1.

For each control strain, a test suspension with a known quantity of cfu has to be prepared. This is best achieved by following a strictly standardized protocol.

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The outcome of the performance testing is closely related to the physiological state of the bacteria in the test portion as well as on the suitability of the materials used.

All volumes described in this annex can be adjusted, if needed, depending on the exact protocol used (time, temperature, culture medium). Nevertheless, it is important that all plates in the different modules conducted at the same time are inoculated with the exact same volume of the test portion. This standardized performance testing allows a reliable detection of significant differences in the recovery.

For each control strain and performance test, the liquid medium and diluent need to be considered carefully. The material should be tested to give reliable results before being used in regular batch testing.

E.2 Preparation of a standardized test suspension

E.2.1 Working cultures

Take a single well separated colony of *E. coli* WDCM 00013 from the plate with the stock culture (3.3.4), for example, from TSA or blood TSA, and transfer this into 10 ml of tryptone soya broth (TSB). Using a separate tube for each test strain, carry out the same with *C. freundii* WDCM 00006, *E. faecalis* WDCM 00009 and *P. aeruginosa* WDCM 00024. Mix the tubes carefully to avoid large cell-aggregates in the broth.

Incubate for 16 h to 18 h with shaking at (36 ± 2) °C to avoid anoxic zones in the medium.

The strains will grow to stationary phase during this time. By strictly following the above protocol, the cultures will repeatedly grow to certain number of cells that is specific to each strain. Therefore, the number of dilution steps necessary for the preparation of test portions does not need to be determined for every single test, but may need to be verified for a new batch of the broth. For example, if incubation of the working cultures starts at 3 p.m. on the day of inoculation (working day 1), the cultures can be used for the preparation of the test suspension (inoculum) at 8.00 a.m. the next day (working day 2). For achieving best consistency when preparing the test suspension from the working cultures, the variation in the length of incubation time should be minimized, for example, to ±30 min.

For the preparation of the working cultures, other non-selective liquid media than TSB can be used. For each broth and control strain used, the adequate dilutions for the appropriate bacterial counts need to be determined upfront in pre-trials.

E.2.2 Test suspensions — Preparation of inoculum

E.2.2.1 For quantitative testing on productivity according to the given example, a reference count (inoculum level) of approximately 100 cfu (in total) on two plates of the reference medium (50 cfu/plate) are required for each control strain. Therefore, the test suspension should contain approximately 500 cfu/ml or 50 cfu/0,1 ml, respectively.

E. coli WDCM 00013 and *C. freundii* WDCM 00006 are tested separately.

Dilute the working cultures obtained from E.2.1 with sterile phosphate buffer solution (PBS) (e.g. 8.00 a.m. at working day 2) by following 3 steps to achieve the required reference count:

— Step 1: 0,1 ml working culture + 10 ml PBS (dilution: 1:100, dilution step: 10^{-2});

— Step 2: 0,1 ml from 10^{-2} + 10 ml PBS (dilution: 1:10 000, dilution step: 10^{-4});

— Step 3: 0,1 ml from 10^{-4} + 20 ml PBS (dilution: 1:2 000 000, dilution step: approximately10^{-6}).

Use 0,1 ml of the last suspension resulting from dilution step 3 as a test portion for the testing on productivity.

NOTE For the composition, preparation and performance testing of phosphate buffer solution, see ISO 8199:2018, Annex D.

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For the preparation of the test suspensions, other diluents than PBS can be used, see ISO 8199. For each diluent and control strain used, the adequate dilutions for the appropriate bacterial counts need to be determined upfront in pre-trials.

E.2.2.2 For the qualitative testing on selectivity according to the given example, a reference count (inoculum level) of at least 10^4 cfu is needed for each control strain. Therefore, the test suspension should contain at least 10^5 cfu/ml or 10^4/0,1 ml, respectively. For *E. faecalis* WDCM 00009, use 0,1 ml of the suspension resulting from dilution step 1 as a test portion for the testing on productivity.

E.2.2.3 For the qualitative testing on specificity according to the given example, a reference count (inoculum level) of at least 10^3 cfu is needed for each control strain. Therefore, the test suspension should contain at least 10^4 cfu/ml or 10^3/0,1 ml, respectively. For *P. aeruginosa* WDCM 00024, use 0,1 ml of the suspension resulting from dilution step 2 as a test portion for the testing on productivity.

Best standardized results can be achieved for most control strains if the test suspensions are freshly prepared from a freshly prepared working culture at each day of testing.

E.3 Performance testing for batch testing

For the performance testing of a batch of membrane filters with a batch of CCA, a batch testing is needed, see 4.1.1. It has to reach the required criteria, see Table E.1 and ISO 9308-1[8].

From the result of quantitative testing on productivity, the productivity ratio (P_R) is determined. P_R is calculated by using the count of cfu obtained with the membrane filter/culture medium combination under test (N_s obtained by using module 2) and the reference count (N_0 obtained by using module 1), see 10.2.

From the result of qualitative testing on selectivity, the degree of inhibition is determined, see 10.3.

From the result of qualitative testing on specificity, it is determined that a non-target microorganism does not show the same visual characteristics as the target microorganism, see 10.4.

E.4 Performance testing for supplementary quantitative testing

For the need of supplementary testing, see 4.1.2.

Modules 1, 2, 3 and 4 are used in parallel for supplementary testing, see Table 1 and Annex B.

Modules 3 and 4 are used to collect further information on the materials used or in search for problems with the materials whenever low or unsatisfactory productivity is observed in the batch testing procedure.

Module 3 is used to detect inhibitory effects mainly due to membrane filters.

Module 4 is used to detect inhibitory effects mainly due to specific (selective) culture medium.

E.5 Practical testing of the modules

E.5.1 General

The practical testing by using the modules is described in the following clauses.

In this example, CCA filled in plates with a diameter of 90 mm and membrane filters with a diameter of 47 mm are used.

Table E.2 summarizes the control strains, plates and their codes as described in E.5.2 to E.5.4.

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E.5.2 Module 1: Determination of the reference count from TSA by direct inoculation without membrane filter

Use 0,1 ml of the suspension resulting from step 3 for *E. coli* (see E.2.2) for the inoculation of a plate of TSA by using spread plate technique, see 9.2. Repeat this step for the inoculation of a second plate of TSA. It results in two replicates; label the plates 'A' and 'B'.

Repeat the same procedure using the suspension from step 3 of *C. freundii* and label the replicate plates 'a' and b'.

For either the qualitative selectivity or specificity testing, or both, the determination of the reference count is not required.

E.5.3 Module 2: Productivity of the membrane filters by using the membrane filters in combination with CCA

Transfer 0,1 ml of the suspension from step 3 of *E. coli* (see E.2.2) to a minimum of 10 ml of sterile saline solution as diluent and mix gently. A minimum of 10 ml sterile saline solution has to be used for an even distribution of the test microorganisms during the filtration. The volume can be higher and there is no need to measure it exactly, see 9.3.

NOTE For the composition, preparation and performance testing of saline solution, see ISO 8199:2018, Annex D. Other diluents can also be used.

Perform membrane filtration on the whole volume. Transfer the membrane filter to CCA directly afterwards. Use the same pipette to measure the volume, if possible, and repeat this step using a second membrane filter. It results in two replicates, label the plates 'C' and 'D'.

Repeat the same procedure using the suspension from step 3 of *C. freundii* (see E.2.2) and label the replicate plates 'c' and 'd'.

For the qualitative testing on selectivity, repeat the same procedure with only one plate using the suspension from step 1 of *E. faecalis* (see E.2.2) and label the plate 'I'.

For the qualitative testing on specificity, repeat the same procedure with only one plate using the suspension from step 2 of *P. aeruginosa* (see E.2.2) and label the plate 'i'.

E.5.4 Module 3: Detection of inhibition of target organisms due to the membrane filters using the membrane filters on TSA

Transfer 0,1 ml of the suspension from step 3 of *E. coli* (see E.2.2) to a minimum of 10 ml of sterile saline solution as diluent and mix gently. A minimum of 10 ml sterile saline solution has to be used for an even distribution of the test microorganisms during the filtration. The volume can be higher and there is no need to measure it exactly.

Perform membrane filtration on the whole volume. Transfer the membrane to the same culture medium as used for the determination of the reference count, in this example TSA. Use the same pipette to measure the volume, if possible, and repeat this step using a second membrane filter. It results in two replicates; label the plates 'E' and 'F'.

Repeat the same procedure with the suspension from step 3 of *C. freundii* (see E.2.2) and label the replicate plates 'e' and 'f'.

Repeat the same procedure with only one plate using the suspension from step 1 of *E. faecalis* (see E.2.2) and label the plate 'J'.

Repeat the same procedure with only one plate using the suspension from step 2 of *P. aeruginosa* (see E.2.2) and label the plate 'j'.

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