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Water quality — Enumeration of *Escherichia coli* and coliform bacteria —

Part 1: Membrane filtration method for waters with low bacterial background flora

Qualité de l'eau — Dénombrement des Escherichia coli et des bactéries coliformes —

Partie 1: Méthode par filtration sur membrane pour les eaux à faible teneur en bactéries

Reference number
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Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

This third edition cancels and replaces the second edition (ISO 9308-1:2000), which has been technically revised.

It also incorporates the Corrigendum ISO 9308-1:2000/Cor.1:2007.

ISO 9308 consists of the following parts, under the general title *Water quality — Enumeration of Escherichia coli and coliform bacteria*:

— *Part 1: Membrane filtration method for waters with low bacterial background flora*

— *Part 2: Most probable number method*

— *Part 3: Miniaturized method (Most Probable Number) for the detection and enumeration of E. coli in surface and waste water*

Introduction

The presence and extent of faecal pollution is an important factor in assessing the quality of water and the risk to human health from infection. Examination of water samples for the presence of *Escherichia coli* (*E. coli*), which normally inhabits the bowel of man and other warm-blooded animals, provides an indication of such pollution. Examination for coliform bacteria can be more difficult to interpret because some coliform bacteria live in soil and surface fresh water and are not always intestinal. Therefore, the presence of coliform bacteria, although not a proof of faecal contamination, may indicate failure in treatment, storage, or distribution.

Water quality — Enumeration of *Escherichia coli* and coliform bacteria —

Part 1: Membrane filtration method for waters with low bacterial background flora

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably qualified staff.

1 Scope

This part of ISO 9308 specifies a method for the enumeration of *Escherichia coli* (*E. coli*) and coliform bacteria. The method is based on membrane filtration, subsequent culture on a chromogenic coliform agar medium, and calculation of the number of target organisms in the sample. Due to the low selectivity of the differential agar medium, background growth can interfere with the reliable enumeration of *E. coli* and coliform bacteria, for example, in surface waters or shallow well waters. This method is not suitable for these types of water.

This part of ISO 9308 is especially suitable for waters with low bacterial numbers that will cause less than 100 total colonies on chromogenic coliform agar (CCA). These may be drinking water, disinfected pool water, or finished water from drinking water treatment plants.

Some strains of *E. coli* which are β-D-glucuronidase negative, such as *Escherichia coli* O157, will not be detected as *E. coli*. As they are β-D-galactosidase positive, they will appear as coliform bacteria on this chromogenic agar.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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

ISO 7704, *Water quality — Evaluation of membrane filters used for microbiological analyses*

ISO 8199, *Water quality — General guidance on the enumeration of micro-organisms by culture*

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

ISO 19458, *Water quality — Sampling for microbiological analysis*

3 Terms and definitions

For the purpose of this document, the definitions given in ISO/IEC Guide 2 and the following apply.

3.1
coliform bacteria
members of the *Enterobacteriaceae* that express β-D-galactosidase

3.2
Escherichia coli
E. coli
member of the *Enterobacteriaceae* that expresses β-D-galactosidase and β-D-glucuronidase

4 Principle

Filtration of a test portion of the sample through a membrane filter, which retains the organisms, and placement of the membrane filter on a chromogenic coliform agar plate.

Incubation of the membrane filter at (36 ± 2) °C for (21 ± 3) h.

Counting of β-D-galactosidase positive colonies (pink to red) as presumptive coliform bacteria that are not *E. coli.* To avoid false-positive results, caused by oxidase positive bacteria, for example, *Aeromonas* spp, the presumptive colonies shall be confirmed by a negative oxidase reaction.

Counting of β-D-galactosidase and β-D-glucuronidase positive colonies (dark-blue to violet) as *E. coli*.

Total coliform bacteria are the sum of oxidase negative colonies with pink to red colour and all dark-blue to violet colonies.

5 Apparatus and glassware

The following are the usual microbiological laboratory equipment.

5.1 Apparatus, suitable for sterilization by steam (autoclave), according to the instructions given in ISO 8199.

5.2 Incubator, thermostatically controlled at (36 ± 2) °C.

5.3 pH meter, with an accuracy of ±0,1 at 20 °C to 25 °C.

5.4 Equipment, for membrane filtration.

5.5 Membrane filters, composed of cellulose esters or other suitable material, usually about 47 mm or 50 mm in diameter, with filtration characteristics equivalent to a rated nominal pore diameter of 0,45 µm and, preferentially, with grid lines.

The membrane filters shall be free from growth-inhibiting or growth-promoting properties and the printing ink used for the grid shall not affect the growth of bacteria. If not obtained sterile, they shall be sterilized according to the manufacturer's instructions. Every batch of membrane filters shall be tested for its suitability for the test according to ISO 7704 especially since the use of different brands of membrane filters may result in different recovery and colour development.

5.6 Disinfected forceps, for handling of membrane filters.

6 Culture media and reagents

For the preparation of culture media and reagents, see ISO 8199 and ISO 11133. Use ingredients of uniform quality and chemicals of analytical grade (see note); follow the instructions given in Annex B.

Alternatively, use commercially available media and reagents which comply with the compositions given in Annex B and strictly follow the manufacturer's instructions.

NOTE The use of chemicals of other grades is possible providing they are shown to be of equal performance in the test.

For preparation of culture media, use distilled water or deionized water free from substances which might inhibit bacterial growth under the conditions of the test and which is in accordance with ISO 3696.

7 Sampling

Take the samples and deliver them to the laboratory in accordance with ISO 19458.

8 Procedure

8.1 Preparation of the sample

For preparation of the sample, filtration, and inoculation on isolation media, follow the instructions given in ISO 8199. Samples have to be transported and stored at (5 ± 3) °C in accordance with ISO 19458. Under exceptional circumstances, the samples may be kept at (5 ± 3) °C for up to 24 h prior to examination. In this case, the storage time has to be mentioned in the test report.

8.2 Filtration

Filter 100 ml (or other volumes, e.g. 250 ml for bottled water) of the sample to be studied using a membrane filter (5.5). The minimum volume for filtration is 10 ml of sample or dilutions thereof to ensure even distribution of the bacteria on the membrane filter.

8.3 Incubation and differentiation

After filtration (8.2), place the membrane filter on the Chromogenic Coliform Agar (CCA) (B.1), ensuring that no air is trapped underneath, invert petri dish, and incubate at (36 ± 2) °C for (21 ± 3) h.

Examine the membrane filters and count all colonies giving a positive β-D-galactosidase reaction (pink to red) as presumptive coliform bacteria that are not *E. coli*.

Count all colonies giving a positive β-D-galactosidase and β-D-glucuronidase reaction (dark-blue to violet) as *E. coli*.

To confirm the presumptive coliform bacteria that are not *E. coli*, an oxidase test has to be performed. Test preferentially all, or at least 10 pink to red colonies selected as described in ISO 8199. For this confirmation step, appropriate commercialized oxidase tests[1)] can be used.

If commercial oxidase test is not used, the oxidase-test can be performed by adding two to three drops of fresh oxidase reagent (B.2) onto a filter paper in a petri dish. The colonies which have to be confirmed are transferred onto the pretreated filter paper using a plastic or platinum inoculating loop. A positive oxidase reaction is shown by the appearance of a dark-blue colour within 30 s. This shall not be observed for coliform bacteria since they are oxidase negative.

If many colonies have grown on the membrane filter or if a presumptive colony is located next to other colonies, it might be necessary to prepare subcultures of the presumptive colonies to ensure that the oxidase test is carried out with pure cultures. It is also necessary to make subcultures if the presumptive

1) For the evaluation of the performance characteristics of the Chromogenic Coliform Agar in ANNEX C Bactident®-oxidase test has been used. Bactident® is an example of suitable product available commercially. This information is given for the convenience of the users of this International Standard and does not constitute an endorsement by ISO of this product.

colonies are too small for a reliable performance of the oxidase test. Subculture onto a non-selective agar (e.g. B.3) at (36 ± 2) °C for (21 ± 3) h.

9 Expression of results

From the numbers of confirmed colonies counted on the membrane filter (8.3), calculate the numbers of *E. coli* and coliform bacteria present in 100 ml of the sample (or other filtered volume) in accordance with ISO 8199. The count of coliform bacteria is the sum of all oxidase negative pink to red colonies plus all dark-blue to violet colonies. *E. coli* are all dark-blue to violet colonies.

10 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this part of ISO 9308 (ISO 9308-1:2014);

b) all information required for the complete identification of the sample;

c) the results expressed in accordance with Clause 9;

d) any particular occurrence(s) observed during the course of the analysis and any operation(s) not specified in this part of ISO 9308 which may have influenced the results.

11 Quality assurance

11.1 General

The laboratory shall have a clearly defined quality control system to ensure that the apparatus, reagents, and techniques are suitable for the test. The use of positive controls, negative controls, and blanks is part of the test.

11.2 Performance testing of Chromogenic Coliform Agar (CCA)

For the definition of productivity, selectivity, and specificity, refer to ISO 11133. The performance of CCA shall be tested according to the methods and criteria described in ISO 11133.

Table 1 shows the performance tests for CCA.

Table 1 — Performance testing of Chromogenic Coliform Agar

Function	Incubation	Control strains[a]	Reference-medium	Method of control	Criteria (Productivity)	Characteristic reactions
Productivity	(21 ± 3) h/ (36 ± 2) °C	*E. coli* WDCM 00013 or WDCM 00012	TSA	Quantitative	PR ≥ 0,7	Dark-blue to violet colonies
		Ent. aerogenes WDCM 00175 or *C. freundii* WDCM 00006	TSA	Quantitative	PR ≥ 0,7	Pink to red colonies
Selectivity	(21 ± 3) h/ (36 ± 2) °C	*E. faecalis* WDCM 00009	—	Qualitative	Total inhibition	—
Specificity	(21 ± 3) h/ (36 ± 2) °C	*P. aeruginosa* WDCM 00024	—	Qualitative	Growth	Colourless colonies

[a] Refer to the reference strain catalogue available (viewed 03-01-2014) on http://www.wfcc.info/pdf/WDCM_Reference_Strain_Catalogue.pdf on culture collection strain numbers and contact details.

11.3 Performance testing of oxidase test

Examples of suitable control strains are *Pseudomonas aeruginosa* WDCM 00024[Z] (positive control), *Escherichia coli* WDCM 00013[Z], or WDCM 00012[Z] (negative control).

Annex A
(informative)

Further microbiological information on coliform bacteria

In addition to expressing β-D-galactosidase, coliform bacteria are Gram-negative non-sporeforming, oxidase-negative, rod-shaped bacteria, which are capable of aerobic and facultative anaerobic growth in the presence of bile-salts (or other surface-active agents with similar growth-inhibiting properties) and which are normally able to ferment lactose with the production of acid and aldehyde within 48 h when incubated at a temperature of (36 ± 2) °C.

In addition to expressing β-D-glucuronidase, *E. coli* are coliform bacteria that are able to produce indole from tryptophan at (44,0 ± 0,5) °C within (21 ± 3) h. Therefore, in case of any doubt of *E. coli* colonies on the primary agar medium, indole test may be used as an additional confirmation. *E. coli* also give positive results in the methyl red test and can decarboxylate l-glutamic acid but are not able to produce acetyl methyl carbinol, utilize citrate as the sole source of carbon, or grow in KCN broth.

Annex B
(normative)

Composition and preparation of culture media and reagents

B.1 Chromogenic Coliform Agar (CCA)

Enzymatic digest of casein	1,0 g
Yeast extract	2,0 g
Sodium chloride	5,0 g
Sodium dihydrogen phosphate x $2H_2O$	2,2 g
Di-sodium hydrogen phosphate	2,7 g
Sodium pyruvate	1,0 g
Sorbitol	1,0 g
Tryptophane	1,0 g
Secondary alcohol ethyloxylate surfactant (CAS No. 68131–40–8)[a] (e.g. Tergitol® 15-S-7 surfactant)[b]	0,15 g
6-Chloro-3-indoxyl-β-D-galactopyranoside (Salmon-beta-D-galactosid), (CAS No. 138182-21-5)	0,2 g
5-Bromo-4-chloro-3-indoxyl-β-D-glucuronic acid, cyclohexylammonium salt monohydrate (X-beta-G-glucuronide CHX salt) (CAS No. 114162-64-0)	0,1 g
Isopropyl-β-D-thiogalactopyranoside (IPTG) (CAS No. 367-93-1)	0,1 g
Bacteriological agar (in powder or flake form)	9 g to 18 g[c]
Water	1 000 ml

[a] CAS Number/CAS Registry Number is a unique numerical identifier of the Chemical Abstracts Service (CAS) for chemical elements, compounds, polymers, biological sequences, mixtures, and alloys.

[b] Tergitol® is an example of a suitable product available commercially. This information is given for the convenience of the users of this International Standard and does not constitute an endorsement by ISO of this product.

[c] Depending on the gelling power of the agar.

Suspend the ingredients in water by heating in a boiling water bath or in free-flowing steam with frequent agitation until completely dissolved (approximately 35 min). If necessary, adjust the pH so that after heat treatment it has a value corresponding to 6,8 ± 0,2 at 25 °C. Do not autoclave, do not overheat. Dispense in petri dishes to a depth of at least 4 mm. If not for immediate use, the plates can be stored at (5 ± 3) °C in the dark and protected against evaporation for at least one month. There should be no visible moisture on the plates before use. When moisture is present, the plates should be dried for the minimum time required to remove visible moisture.

B.2 Oxidase reagent

N,N,N′,N′-Tetramethyl-*p*-phenylenediamine dihydrochloride (CAS No. 637-01-4)	0,1 g
Water	10 ml

This reagent is not stable. It shall be freshly prepared in small portions each time it is needed and has to be protected against light.

WARNING — N,N,N′,N′-Tetramethyl-*p*-phenylenediamine dihydrochloride is carcinogenic. The preparation work has to be done in a fume cupboard. Use protective gloves and avoid skin contact.

B.3 Tryptone Soy Agar (TSA)

Tryptone	15,0 g
Soya peptone	5,0 g
Sodium chloride	5,0 g
Agar (in powder or flake form)	15 g to 25 g[a]
Water	1 000 ml

[a] Depending on the gelling power of the agar.

Suspend the ingredients in water by heating in a boiling water bath or in free-flowing steam. If necessary, adjust the pH so that after autoclaving it has a value corresponding to 7,2 ± 0,1 at 25 °C. Sterilize for 15 min at (121 ± 3) °C in an autoclave. Let cool to approx. 50 °C and pour into petri dishes to a depth of at least 4 mm. If not for immediate use, the plates can be stored at (5 ± 3) °C in the dark and protected against evaporation for at least eight weeks.

NOTE Any other non-selective agar can be used for subculturing before the oxidase test, as long as it does not interfere with the oxidase test.