INTERNATIONAL STANDARD

ISO 9308-1

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Water quality — Detection and enumeration of *Escherichia coli* and coliform bacteria —

Part 1: Membrane filtration method

Qualité de l'eau — Recherche et dénombrement des Escherichia coli *et des bactéries coliformes —*

Partie 1: Méthode par filtration sur membrane

Reference number
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Foreword

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

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this part of ISO 9308 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 9308-1 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

This second edition cancels and replaces the first edition (ISO 9308-1:1990), which has been technically revised.

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

— *Part 1: Membrane filtration method*

— *Part 2: Liquid enrichment method*

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

Annex B forms a normative part of this part of ISO 9308. Annex A is for information only.

Introduction

The presence and extent of faecal pollution is an important factor in assessing the quality of a body of water and the risk to human health from infection. Examination of water samples for the presence of *Escherichia 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 or distribution. The identification of the strains isolated can sometimes provide an indication of their origin.

Water quality — Detection and enumeration of *Escherichia coli* and coliform bacteria —

Part 1:
Membrane filtration method

1 Scope

This part of ISO 9308 describes a reference method (Standard Test) for the detection and enumeration of *Escherichia coli* and coliform bacteria in water for human consumption. The Standard Test is based on membrane filtration, subsequent culture on a differential agar medium and calculation of the number of target organisms in the sample.

The Standard Test has a low selectivity, allowing the detection of injured bacteria. Due to the low selectivity, background growth can interfere with the reliable enumeration of coliform bacteria and *E. coli*, for example in some drinking waters, like shallow well waters, that have not been disinfected and yield a high background growth. This part of ISO 9308 is therefore especially suitable for disinfected water and other drinking waters of low bacterial numbers.

This part of ISO 9308 includes a rapid method (Rapid Test) for the detection of *E. coli* only within 24 h in water for human consumption, which can be useful in special cases when information is needed quickly. The Rapid Test is based on membrane filtration, subsequent culture under selective conditions and calculation of the number of *E. coli* in the sample.

Standard and Rapid Tests described in this part of ISO 9308 are applicable to other kinds of water provided that suspended matter or background flora does not interfere with filtration, culture and counting.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this part of ISO 9308. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this part of ISO 9308 are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO/IEC Guide 2, *Standardization and related activities — General vocabulary.*

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods.*

ISO 5667-1:1980, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes.*

ISO 5667-2:1991, *Water quality — Sampling — Part 2: Guidance on sampling techniques.*

ISO 5667-3:1994, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of samples.*

ISO 6887-1:1999, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions.*

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

3 Terms and definitions

For the purposes of this part of ISO 9308, the terms and definitions given in ISO/IEC Guide 2 and the following apply.

3.1
lactose-positive bacteria
⟨Standard Test⟩ bacteria capable of forming colonies aerobically at (36 ± 3) °C on a selective and differential lactose culture medium with the production of acid within (21 ± 3) h

3.2
coliform bacteria
⟨Standard Test⟩ lactose-positive bacteria as defined in 3.1 which are oxidase-negative

3.3
Escherichia coli
⟨Standard Test⟩ coliform bacteria as defined in 3.2 which also produce indole from tryptophan at (44,0 ± 0,5) °C within (21 ± 3) h

3.4
Escherichia coli
⟨Rapid Test⟩ bile-resistant bacteria which also produce indole from tryptophan at (44,0 ± 0,5) °C within (21 ± 3) h

4 Principle

4.1 General description of the method

The method is based on membrane filtration and consists of two parts, the reference Standard Test and the optional Rapid Test, which can be performed in parallel as described below. The Standard Test includes incubation of the membrane on a selective medium with subsequent further biochemical characterization of the typical lactose-positive colonies, leading to the detection and enumeration of coliform bacteria and *E. coli* within 2 d to 3 d. The Rapid Test consists of two incubation steps allowing the detection and enumeration of *E. coli* within (21 ± 3) h. If both tests, Standard Test and Rapid Test, are performed in parallel, the final result for *E. coli* shall be the higher of the two.

4.2 Filtration and incubation

Test portions of the sample are filtered through membranes which retain the bacteria. One membrane (Standard Test) is placed on a selective lactose agar medium which is incubated at (36 ± 2) °C for (21 ± 3) h and one membrane (Rapid Test) on a casein (tryptic digest)-containing agar medium incubated at (36 ± 2) °C for 4 h to 5 h, followed by incubation at (44,0 ± 0,5) °C for 19 h to 20 h on an agar medium containing casein (tryptic digest) and bilesalts.

4.3 Evaluation and confirmation, Standard Test

The characteristic colonies on the membrane are counted as lactose-positive bacteria. For coliform bacteria and *E. coli*, subculture is carried out of randomly selected characteristic colonies for confirmatory tests: oxidase and indole production. The numbers of lactose-positive coliform bacteria and *E. coli* likely to be present in 100 ml of the sample are counted.

4.4 Evaluation and confirmation, Rapid Test

The colonies on the membrane which are able to form indole from the tryptophan supplied in the agar medium are counted as *E. coli*. The numbers of *E. coli* likely to be present in 100 ml of the sample are counted.

5 Apparatus and glassware

Usual microbiological laboratory equipment, and in particular:

5.1 Apparatus for sterilization by steam (autoclave).

Apparatus and glassware not supplied sterile shall be sterilized according to the instructions given in ISO 8199.

5.2 Water bath and/or incubator, thermostatically controlled at (36 ± 2) °C.

5.3 Water bath and/or incubator, thermostatically controlled at (44,0 ± 0,5) °C.

NOTE For the Rapid Test, instead of the incubators 5.2 and 5.3 a programmable incubator with dual setting may be used, set to (36 ± 2) °C and (44,0 ± 0,5) °C.

5.4 pH meter, with an accuracy of ± 0,1.

5.5 Equipment for membrane filtration, in accordance with ISO 8199.

5.6 Membrane filters, composed of cellulose esters, usually about 47 mm or 50 mm in diameter, with filtration characteristics equivalent to a rated nominal pore diameter of 0,45 µm and preferably with grids.

The 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 in accordance with the manufacturer's instructions. Every batch of membranes should be tested in accordance with ISO 7704 for its suitability for the test, especially since the use of different brands of filter may result in a difference in colour development.

NOTE Green membrane filters may be helpful when using the Rapid Test for a better detection of colour development.

5.7 Forceps with rounded tips for handling membranes.

5.8 Ultraviolet lamp, wavelength 254 nm (low-pressure mercury lamp).

WARNING — UV light causes irritation of eyes and skin. Use protective glasses and gloves.

5.9 Filter pads, with a diameter of at least 47 mm.

6 Culture media and reagents

For the preparation of culture media and reagents, 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 follow strictly the manufacturer's instructions.

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

For preparation of culture media, use glass-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.

Unless specified otherwise, the prepared media are stable for at least one month if stored in the dark at (5 ± 3) °C and protected against evaporation.

7 Sampling

Take the samples and deliver them to the laboratory in accordance with ISO 5667-1, ISO 5667-2 and ISO 5667-3.

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 and ISO 6887-1. Start the examination preferably immediately after taking the samples. If the samples are kept at ambient temperatures (in the dark, not exceeding 25 °C), the examination shall begin within 6 h after taking the sample. Under exceptional circumstances, the samples may be kept at (5 ± 3) °C for up to 24 h prior to examination.

8.2 Filtration

Filter 100 ml (or higher volumes, e.g. 250 ml for bottled water) of the sample to be studied using a membrane filter (5.6.). Place the filter on the respective agar medium (8.3 and 8.4), ensuring that no air is trapped underneath.

8.3 Incubation and differentiation, Standard Test

After filtration (8.2) place the membrane on the Lactose TTC agar plate (B.1) and incubate at (36 ± 2) °C for (21 ± 3) h.

NOTE 1 Extension of the incubation time to (44 ± 4) h may result in a higher sensitivity of the test and may be especially useful for plates which do not show typical colonies after (21 ± 3) h.

NOTE 2 The use of an additional membrane filter for incubation at 44 °C may overcome the problem of background growth.

Examine the membranes and count as lactose-positive bacteria all characteristic colonies, irrespective of size, which show a yellow colour development in the medium under the membrane. For oxidase and indole tests, subculture preferentially all, or a representative number (at least ten), of the characteristic colonies obtained onto nonselective agar (B.3) and in tryptophan broth (B.2), respectively.

Incubate the nonselective agar at (36 ± 2) °C for (21 ± 2) h and carry out an oxidase test as follows.

— Place two to three drops of freshly prepared oxidase reagent (B.5.3) on a filter paper.

— With a glass rod, wooden applicator stick, plastics or platinum (not Nichrome) wire loop, smear part of the colony on the prepared filter paper.

— Regard the appearance of a deep blue-purple colour within 30 s as a positive reaction.

Incubate the tryptophan broth tube (B.2) at (44,0 ± 0,5) °C for (21 ± 3) h and examine for the production of indole by adding 0,2 ml to 0,3 ml of Kovacs' reagent (B.5.1). Development of a cherry-red colour at the surface of the broth confirms the production of indole.

Count all colonies giving a negative oxidase reaction as **coliform bacteria**.

Count all colonies giving a negative oxidase and a positive indole reaction as ***E. coli***.

NOTE 3 In special cases, the identification of coliform bacteria may be needed, e.g. to distinguish between faecal and aquatic/telluric strains.

8.4 Incubation and differentiation, Rapid Test

After filtration (8.2) place the membrane on TSA medium (B.3) and incubate at (36 ± 2) °C for 4 h to 5 h. Thereafter, place the membrane on TBA medium (B.4) and incubate at (44,0 ± 0,5) °C for 19 h to 20 h.

If desired, the two agar media can be combined into one double-layer plate (see note B.4). In that case, place the membrane on a freshly prepared double layer plate consisting of TSA (B.3) and TBA (B.4) and incubate at (36 ± 2) °C for 4 h to 5 h followed by incubation at (44,0 ± 0,5) °C for 19 h to 20 h.

After incubation, place the membrane on a filter pad (5.9) saturated with indole reagent (B.5.2) and irradiate with an ultraviolet lamp (5.8) for 10 min to 30 min depending on the colour development (see Note 1). All red colonies on the membrane filter are counted as ***E. coli***.

NOTE 1 Commercially available reagents in aqueous base may give clearer and faster results without the need for UV irradiation.

NOTE 2 Uneven distribution of colonies or the presence of high background counts may interfere with the differentiation of indole-positive colonies due to diffusion of the colour to adjacent colonies.

9 Expression of results

From the numbers of characteristic colonies counted on the membrane (8.3) and taking into account the results of the confirmatory tests performed, calculate the numbers of *E. coli*, coliform bacteria and, if necessary, lactose-positive bacteria present in 100 ml of the sample in accordance with ISO 8199. In case both tests (Standard Test and Rapid Test) are used in parallel as described for *E. coli*, the final result is the higher of the two.

10 Test report

The test report shall contain at least the following information.

a) reference to this part of ISO 9308;

b) all details necessary for complete identification of the sample;

c) the results expressed in accordance with above clause 9;

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

11 Quality assurance

Use a laboratory with a clearly defined quality control system to ensure that the materials, reagents and techniques are suitable for the test.

Annex A
(informative)

Further microbiological information on coliform bacteria

Coliform bacteria are Gram-negative non-sporeforming, oxidase-negative, rod-shaped bacteria which are capable of aerobic and facultatively 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. They also possess the enzyme β-galactosidase.

E. coli are coliform bacteria that are able to produce indole from tryptophan within (21 ± 3) h at $(44{,}0 \pm 0{,}5)$ °C. They also possess the enzyme β-glucuronidase, give a positive result in the methyl red test and can decarboxylate L-glutamic acid but are not able to produce acetyl methyl carbinol, utilise citrate as the sole source of carbon or grow in KCN broth.

Annex B
(normative)

Culture media and reagents

B.1 Lactose TTC agar with sodium heptadecylsulfate

B.1.1 Basal medium

Lactose	20 g
Peptone	10 g
Yeast extract	6 g
Meat extract	5 g
Bromothymol blue	0,05 g
Agar (in powder or flake form)	15 g to 25 g[1)]
Distilled water	1 000 ml

Dissolve the ingredients in water by heating. If necessary, adjust the pH so that after sterilization it has a value corresponding to 7,2 ± 0,1 at 25 °C. Dispense the medium into bottles, in volumes of maximum 250 ml, and sterilize in the autoclave at (121 ± 3) °C for 15 min.

B.1.2 TTC solution

2,3,5-Triphenyltetrazolium chloride (TTC)	0,05 g
Distilled water	100 ml

Dissolve the TTC in some of the water and make up to 100 ml. Sterilize by filtration through a membrane of 0,2 µm nominal pore size.

B.1.3 Sodium heptadecylsulfate solution

Sodium heptadecylsulfate (Tergitol [2)] 7)	0,2 g
Distilled water	100 ml

Dissolve the sodium heptadecylsulfate in some of the water and make up to 100 ml. Sterilize in the autoclave at (121 ± 3) °C for 15 min.

B.1.4 Complete medium

Basal medium (B.1.1)	100 ml
TTC solution (B.1.2)	5 ml
Sodium heptadecylsulfate solution (B.1.3)	5 ml

1) Depending on the gelling power of the agar.

2) Tergitol is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 9308 and does not constitute an endorsement by ISO of this product.

Melt the basal medium and cool to (50 ± 5) °C. Add the TTC and sodium heptadecylsulfate solutions aseptically, mix thoroughly but avoid the formation of bubbles after each addition. Dispense in Petri dishes to a depth of at least 5 mm. If not for immediate use, store at (5 ± 3) °C in the dark for not longer than 10 d.

B.2 Tryptophan broth

Tryptic digest of casein	10 g
L-Tryptophan	1 g
Sodium chloride	5 g
Distilled water to	1 000 ml

Dissolve the ingredients in water by heating. Dispense 3 ml per test tube. Close the tubes with cotton plugs, plastic or metal caps. Autoclave for 15 min at (121 ± 3) °C. The pH of the ready-to-use medium should be 7,5 ± 0,1 at 25 °C.

NOTE Provided that there is a sufficient amount of tryptophan in the tryptic digest of casein used, no L-tryptophan has to be added. Instead, add 10 g of additional tryptic digest of casein.

B.3 Tryptone Soy Agar (TSA)

Tryptic digest of casein	15 g
Soy peptone	5 g
Sodium chloride	5 g
Agar (in powder or flake form)	15 g to 25 g[1)]
Distilled water to	1 000 ml

Dissolve the ingredients in water by heating. Adjust the pH so that after sterilization it has a value corresponding to 7,2 ± 0,1 at 25 °C. Dispense the medium in bottles or tubes in volumes of maximum 250 ml and sterilize for 15 min at (121 ± 3) °C. Allow the medium to cool to (50 ± 5) °C and dispense in Petri dishes to a depth of at least 5 mm.

NOTE For the oxidase test, any non-selective agar (instead of TSA) which does not interfere with the oxidase test (with low content in fermentible carbohydrates) may be used.

B.4 Tryptone Bile Agar (TBA)

Tryptone	20 g
Bile salts	1,5 g
Agar (in powder or flake form)	15 g to 25 g[1)]
Distilled water to	1 000 ml

Dissolve the ingredients in water by boiling. Adjust the pH such that after sterilization it has a value corresponding to 7,2 ± 0,1 at 25 °C. Dispense the medium into bottles or tubes, in volumes of maximum 250 ml, and sterilize for 15 min at (121 ± 3) °C. Allow the medium to cool to (50 ± 5) °C and dispense in Petri dishes to a depth of at least 5 mm.

Prepare double-layer plates by pouring hot (50 ± 5) °C TSA (B.3) over a TBA plate (B.4) warmed to room temperature.

Take a quantity of TSA which gives a layer thickness of approx. 1 mm (e.g. 2,5 ml in a Petri dish with a diameter of 55 mm). Allow to solidify and dry where necessary inverted in an incubator at (36 ± 2) °C.

The double-layer plates shall be prepared freshly for every analysis (30 min to 60 min before placing the membranes on the agar plates).

B.5 Reagents

B.5.1 Kovacs' Reagent for indole test, Standard Test

p-Dimethylaminobenzaldehyde	5 g
Amyl or butyl alcohol (free from organic bases)	75 ml
Hydrochloric acid (ρ = 1,18 g/ml)	25 ml

Dissolve the aldehyde in the alcohol. Add the concentrated acid with care. Protect from light and store at (5 ± 3) °C.

NOTE The reagent should be light yellow to light brown in colour; some samples of amyl alcohol are unsatisfactory, and give a dark colour with aldehyde.

WARNING — Carry out the preparation work in an exhaust protection cabinet. Use protective gloves and avoid skin contact with *p*-dimethylaminobenzaldehyde. Amyl alcohol may irritate the mucous membranes and cause dizziness.

B.5.2 Indole reagent, Rapid Test

p-Dimethylaminobenzaldehyde	0,5 g
Hydrochloric acid, *c*(HCl) = 1 mol/l	100 ml

Dissolve the *p*-dimethylaminobenzaldehyde in the hydrochloric acid (see warning in B.5.1).

Store the reagent in a non-translucent flask at (5 ± 3) °C. The reagent should be light yellow in colour and shall not be used if the colour becomes brownish yellow.

B.5.3 Oxidase reagent

Tetramethyl-*p*-phenylenediamine hydrochloride	0,1 g
Distilled water	10 ml

This reagent is not stable and shall be freshly prepared each time it is needed.

WARNING — Tetramethyl-*p*-phenylenediamine is carcinogenic. Carry out preparation work in an exhaust protection cabinet, use protective gloves and avoid skin contact.

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