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Water quality — Detection and enumeration of coliform organisms, thermotolerant coliform organisms and presumptive *Escherichia coli* —

Part 1:
Membrane filtration method

Qualité de l'eau — Recherche et dénombrement des organismes coliformes, des organismes coliformes thermotolérants et des Escherichia coli *présumés —*

Partie 1: Méthode de filtration sur membrane

Reference number
ISO 9308-1:1990(E)

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.

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.

International Standard ISO 9308-1 was prepared by Technical Committee ISO/TC 147, *Water quality*.

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

— *Part 1: Membrane filtration method*

— *Part 2: Multiple tube (most probable number) method*

Annexes A and B of this part of ISO 9308 are for information only.

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Introduction

The presence and extent of faecal pollution is an important factor in assessing the quality of a body of water. Examination of water samples for the presence of members of the coliform group of organisms[1], which normally inhabit the bowel of man and other warm-blooded animals, provides an indication of such pollution. As the ability of some members of the coliform group of organism to survive in water is limited, their numbers can also be used to estimate the degree of recent faecal pollution.

1) See annex A for further microbiological information relevant to water examination for the coliform group of organisms.

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Water quality — Detection and enumeration of coliform organisms, thermotolerant coliform organisms and presumptive *Escherichia coli* —

Part 1:
Membrane filtration method

1 Scope

This part of ISO 9308 specifies a method for the detection and enumeration in water of coliform organisms, thermotolerant coliform organisms and presumptive *Escherichia coli* (presumptive *E. coli*) after filtration through a membrane, subsequent culture on a differential lactose medium (see ISO 7704) and calculation of their numbers in the sample.

This method can be applied to all types of water except where the presence of suspended matter interferes with filtration or large numbers of other organisms may interfere with growth.

The choice of tests used in the detection and confirmation of the coliform group of organisms, including *E. coli*, can be regarded as part of a continuous sequence. The extent of confirmation with a particular sample depends partly on the nature of the water and partly on the reasons for the examination. In practice, the detection in water of presumptive *E. coli* as defined in 3.3 usually provides an indication of recent faecal pollution.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 9308. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 9308 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

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 5667-2:1982, *Water quality — Sampling — Part 2: Guidance on sampling techniques.*

ISO 5667-3:1985, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of samples.*

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

ISO 7704:1985, *Water quality — Evaluation of membrane filters used for microbiological analyses.*

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

3 Definitions

For the purposes of this part of ISO 9308, the following definitions apply.

3.1 coliform organisms: Organisms capable of forming colonies aerobically at either 35 °C ± 0,5 °C or 37 °C ± 0,5 °C on a selective and differential lactose culture medium with the production of acid (and aldehyde) within 24 h.

3.2 thermotolerant coliform organisms: Coliform organisms as described in 3.1 which have the same

fermentative properties within 24 h, at either 44 °C ± 0,25 °C or 44,5 °C ± 0,25 °C.

NOTE 1 As gas production is not detectable on membranes, the organisms obtained by membrane filtration are not necessarily the same as those detected by the multiple tube [most probable number (MPN)] method.

3.3 presumptive *Escherichia coli* (presumptive *E. coli*): Thermotolerant coliform organisms as described in 3.2 which also produce gas from lactose (and mannitol) as well as indole from tryptophan within 24 h, at either 44 °C ± 0,25 °C or 44,5 °C ± 0,25 °C.

4 Principle

Filtration of a test portion of the sample through a membrane which retains the organisms; placing the membrane on either a selective lactose agar culture medium or on an absorbent pad saturated with a selective liquid medium containing lactose.

Incubation of the membrane for 24 h at either 35 °C or 37 °C for the detection of coliform organisms, or alternatively at 44 °C for the presence of thermotolerant coliform organisms.

Direct count of characteristic colonies formed on the membrane; subculture of some of these colonies for confirmatory tests for gas and for indole production. Calculation of the numbers of coliform organisms, thermotolerant coliform organisms and presumptive *E. coli* likely to be present in 100 ml of the sample.

5 Diluent, culture media and reagents

5.1 Basic materials

Use ingredients of uniform quality and chemicals of analytical grade for the preparation of culture media and reagents and follow the instructions given in annex B. For information on storage see ISO 8199. Alternatively, use dehydrated complete media and follow strictly the manufacturer's instructions.

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

5.2 Diluent

For making sample dilutions, use one of the diluents recommended in annex B. Prepare the diluent according to the instructions given in annex B.

5.3 Isolation media

Use one or more of the following culture media either in solid form with agar or as a broth for saturating absorbent pads. Instructions for preparing the media are given in annex B.

5.3.1 Lactose TTC agar with Tergitol 7[2)]

5.3.2 Lactose agar with Tergitol 7[2)]

5.3.3 Membrane enriched Teepol broth[2)]

5.3.4 Membrane lauryl sulfate broth

5.3.5 Endo medium

5.3.6 LES Endo agar

5.3.7 mFC medium

5.4 Confirmatory media

Use one or more of the following.

5.4.1 Medium for gas production

Lactose peptone water.

5.4.2 Medium for indole production

Tryptone water.

5.4.3 Single-tube medium for both gas and indole production

Lauryl tryptose mannitol broth with tryptophan.

5.5 Reagents

5.5.1 Kovacs' reagent for indole

5.5.2 Oxidase reagent for the oxidase test

6 Apparatus

Usual microbiological laboratory equipment, including

6.1 Hot-air oven for dry-heat sterilization and an autoclave.

Apart from apparatus supplied sterile, glassware and other equipment shall be sterilized according to the instructions given in ISO 8199.

2) Tergitol 7, Teepol broth are examples of suitable products 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 these products.

6.2 Incubator or water bath, thermostatically controlled at either 35 °C ± 0,5 °C or 37 °C ± 0,5 °C.

6.3 Incubator or water bath, thermostatically controlled at either 44 °C ± 0,25 °C or 44,5 °C ± 0,25 °C.

6.4 pH meter.

6.5 Apparatus for membrane filtration.

6.6 Membrane filters, usually about 47 mm or 50 mm in diameter, with filtration characteristics equivalent to a rated nominal pore diameter of 0,45 µm. If not obtained sterile, they shall be sterilized according to the manufacturer's instructions.

6.7 Forceps, for handling membranes.

7 Sampling

Take the samples and deliver them to the laboratory in accordance with ISO 8199, ISO 5667-1, ISO 5667-2 and ISO 5667-3.

8 Procedure

8.1 Preparation of the sample, filtration and inoculation of media

For preparation of the sample, making dilutions, filtration and inoculation of isolation media, follow the instructions given in ISO 8199 and ISO 6887.

8.1.1 For coliform organisms, filter the required volume of the sample, or a dilution of it, through one membrane. Place on the medium chosen, ensuring that no air is trapped underneath it.

8.1.2 For thermotolerant coliform organisms, filter the required volume of the sample, or a dilution of it, through one membrane. Place on the medium chosen, ensuring that no air is trapped underneath it.

NOTE 2 The volume of sample filtered should be the same as in 8.1.1.

8.2 Incubation of membranes

8.2.1 For coliform organisms, incubate the membrane for 18 h to 24 h at either 35 °C ± 0,5 °C or 37 °C ± 0,5 °C.

8.2.2 For thermotolerant coliform organisms, incubate the membrane for 18 h to 24 h at either 44 °C ± 0,25 °C or 44,5 °C ± 0,25 °C.

NOTES

3 The same medium can generally be used for both coliform organisms and thermotolerant coliform organisms, but mFC medium should be used only at 44 °C, and Endo and LES Endo media should be used at 35 °C or 37 °C.

4 A preliminary period at a lower temperature such as 30 °C for the first 4 h of incubation is recommended to resuscitate stressed organisms, especially in the examination of drinking water.

8.3 Examination of membranes

8.3.1 Coliform organisms

Examine the membranes and count as presumptive coliform organisms all colonies, irrespective of size, which show, after incubation at 35 °C or 37 °C, the following characteristics.

— On lactose TTC agar with Tergitol (5.3.1): a yellow, orange or brick red colouration with a yellow central halo in the medium under the membrane.

— on lactose agar with Tergitol 7 (5.3.2): a yellow central halo in the medium under the membrane.

— On membrane enriched Teepol broth (5.3.3): a yellow colour extending on to the membrane.

— On membrane lauryl sulfate broth (5.3.4): a yellow colour extending on to the membrane.

— On Endo agar or broth (5.3.5): a dark red colour with a golden-green metallic sheen.

— On LES Endo agar (5.3.6): a dark red colour with a golden-green metallic sheen.

8.3.2 Thermotolerant coliform organisms

Regard as presumptive thermotolerant coliform organisms all colonies which show, after incubation at 44 °C, the same colonial characteristics as those described in 8.3.1. With mFC medium (5.3.7), such colonies are blue in colour.

8.4 Confirmatory tests

It is important to note that the counts of colonies on membranes at 35 °C or 37 °C and at 44 °C are only presumptive coliform results. Since gas production is not detected, there is also an additional presumption that the organisms forming colonies can also produce gas from lactose. For the examination of raw or partly-treated waters, this may be sufficient, but for potable supplies and other cir-

cumstances, it is important to carry out confirmatory tests, preferably on pure subcultures.

8.4.1 Subculture, incubation and examination

8.4.1.1 Coliform organisms

To confirm the membrane results, subculture each colony (8.3.1) or a representative number of them to tubes of lactose peptone water (5.4.1) and incubate at 35 °C or 37 °C for 48 h: gas production within this period confirms the presence of coliform organisms.

8.4.1.2 Thermotolerant coliform organisms and presumptive *E. coli*

For thermotolerant coliform organisms and presumptive *E. coli* on membranes, whether incubated at 44 °C or at 35 °C or 37 °C, subculture each colony (8.3.2) or a representative number of them, to tubes of lactose peptone water and tryptone water and incubate at 44 °C for 24 h. Gas production in lactose peptone water confirms the presence of thermotolerant coliform organisms, and development of a red colour at the surface of the tryptone water culture after the addition of 0,2 ml to 0,3 ml of Kovacs' reagent (5.5.1) confirms the presence of presumptive *E. coli*.

NOTES

5 The use of lauryl tryptose mannitol broth with tryptophan allows both gas and indole production to be demonstrated in a single tube.

6 The detection of presumptive *E. coli* is regarded as satisfactory evidence of faecal pollution. However, further tests for the confirmation of *E. coli* may be carried out if considered necessary (see 8.5).

7 When subculturing from colonies on the membrane to tubes of confirmatory media, it is preferable to subculture also to a plate of nutrient agar medium for the oxidase test.

8.5 Oxidase test

Some bacteria found in water may conform to the definition of coliform organisms in most respects, but are able to produce gas from lactose only at temperatures below 37 °C. They therefore give negative results in the standard confirmatory tests for coliform organisms and their presence in water is not usually regarded as significant. *Aeromonas* species, which occur naturally in water, have an optimum growth temperature in the range 30 °C to 35 °C but may nevertheless produce acid and gas from lactose at 37 °C. They are distinguishable from the coliform group by a positive oxidase reaction.

8.5.1 Carry out the oxidase test with pure subcultures of lactose-fermenting organisms, grown on nutrient agar medium, as follows:

— place 2 to 3 drops of freshly prepared oxidase reagent (5.5.2) on a filter paper in a Petri dish;

— with a glass rod, swab stick or platinum (not nichrome) wire loop, smear some of the growth on the prepared filter paper (see note 7);

— regard the appearance of a deep blue-purple colour within 10 s as a positive reaction.

NOTE 8 On each occasion that the oxidase reagent is used, control tests should be conducted with cultures of an organism known to give a positive reaction (*Pseudomonas aeruginosa*) and a negative reaction (*E. coli*).

9 Expression of results

From the numbers of characteristic colonies counted on the membranes and taking account of the results of the confirmatory tests performed, calculate the numbers of coliform organisms, thermotolerant coliform organisms and presumptive *E. coli* present in 100 ml of the sample in accordance with ISO 8199, 8.4, according to the following equation:

$$C = \frac{A \times N \times V_s \times F}{B \times V_t}$$

where

C is the confirmed colony count per 100 ml;

A is the number of colonies actually confirmed;

B is the number of colonies subcultured for confirmation;

N is the number of characteristic colonies on the membrane (8.3.1 and 8.3.2);

V_t is the test volume of water sample filtered (8.1.1 and 8.1.2);

V_s is the reference volume for expression of results (100 ml);

F is the dilution factor.

10 Test report

The test report shall contain the following information:

a) a reference to this part of ISO 9308;

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

c) the technique and isolation medium used;

d) the confirmatory media and tests used;

e) the time, temperature and conditions of incubation;

f) the results expressed in accordance with clause 9;

g) any other information relevant to the method.

Annex A
(informative)

Further microbiological information relevant to water examination for the coliform group of organisms

For routine water examination purposes, the coliform group of organisms may be described generally in microbiological, though not taxonomic, terms as follows.

Coliform organisms are Gram-negative, non-sporing, 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). They are also able to ferment lactose (and mannitol) with the production of acid, gas and aldehyde within 48 h, when incubated at a temperature of 35 °C to 37 °C.

Thermotolerant coliform organisms are coliform organisms which exhibit the same fermentative and biochemical properties when incubated at a temperature of 44 °C to 44,5 °C.

Presumptive *E. coli* are thermotolerant coliform organisms which are also able to produce indole from tryptophan.

E. coli may be regarded as presumptive *E. coli* which also give a positive result in the methyl red test and can decarboxylate L-glutamic acid, but which are not able to produce acetyl methyl carbinol, utilize citrate as the sole source of carbon or to grow in potassium cyanide (KCN) broth.

Annex B
(informative)

Culture media, reagents and diluents

Isolation media

Lactose TTC agar with Tergitol 7

Basal medium

Lactose	20 g
Peptone	10 g
Yeast extract	6 g
Meat extract	5 g
Bromothymol blue	0,05 g
Agar	16 g to 25 g[1)]
Distilled water	1 000 ml

1) Depending on the gel strength of the agar.

Dissolve the ingredients in boiling water. If necessary, adjust the pH so that after sterilization it is 7,2 ± 0,2. Distribute the medium in bottles in volumes of 100 ml and sterilize in the autoclave at 121 °C ± 1 °C for 15 min.

TTC solution

2, 3, 5-Triphenyl-tetrazolium 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 membrane filtration through a membrane of pore size < 0,2 µm.

Tergitol 7 solution

Tergitol 7	0,2 g
Distilled water	100 ml

Dissolve the Tergitol 7 in some of the water and make up to 100 ml. Sterilize in the autoclave at 121 °C ± 1 °C for 15 min.

Complete medium

Basal medium	100 ml
TTC solution	5 ml
Tergitol 7 solution	5 ml

Melt the basal medium and cool to between 45 °C and 50 °C. Add the TTC and Tergitol 7 solutions aseptically, mixing thoroughly but avoiding the formation of bubbles after each addition. Distribute in Petri dishes to a depth of about 5 mm. If not for immediate use, store at 4 °C in the dark for not longer than 10 days.

Lactose agar with Tergitol 7

Peptone	5 g
Yeast extract	3 g
Lactose	10 g
Tergitol 7	0,1 ml
Bromothymol blue	0,025 g
Agar	15 g
Distilled water	1 000 ml

Dissolve the ingredients in the water. Dispense in bottles in volumes of 100 ml and autoclave at 121 °C ± 1 °C for 15 min. The final pH is 6,9 ± 0,2. For use, melt and distribute in Petri dishes to a depth of about 5 mm. Store at 4 °C in the dark for not longer than 10 days.

Membrane enriched Teepol broth

Peptone	40 g
Yeast extract	6 g
Lactose	30 g
Phenol red [0,4 % (*m*/*m*)] aqueous solution	50 ml
Teepol 610	4 ml
Distilled water	1 000 ml

Add the peptone and yeast extract to the water and steam to dissolve. Add the lactose, phenol red and Teepol afterwards and mix gently to avoid froth. The final pH shall be 7,4 to 7,5 and it may be necessary to adjust the pH to about 7,6 before sterilization in order to achieve this. Distribute in screw-capped bottles and autoclave at 110 °C to 115 °C for 10 min.

Membrane lauryl sulfate broth

Peptone	40 g
Yeast extract	6 g
Lactose	30 g
Phenol red [0,4 % (*m*/*m*)] aqueous solution	50 ml
Sodium lauryl sulfate, high purity	1 g
Distilled water	1 000 ml

Add the ingredients to the water and mix gently to avoid froth. The final pH should be 7,4 to 7,5 and it may be necessary to adjust the pH to about 7,6 before sterilization to achieve this. Distribute in screw-capped bottles and autoclave at 110 °C to 115 °C for 10 min.

Endo medium

WARNING — Basic fuchsin is suspected of being a carcinogen.

Tryptose	10 g
Thiopeptone	5 g
Trypticase (casitone or tryptone)	5 g
Yeast extract	1,5 g
Lactose	12,5 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	4,375 g
Potassium dihydrogen phosphate	1,375 g
Sodium lauryl sulfate	0,05 g
Sodium desoxycholate	0,1 g
Sodium sulfate	2,1 g
Basic fuchsin	1,05 g
Distilled water	1 000 ml

Dissolve ingredients in the water containing 20 ml of 95 % (*V*/*V*) ethanol. Do **not** autoclave but heat to boiling, remove promptly and cool to between 45 °C and 50 °C. Final pH shall be 7,2 ± 0,2. Store finished medium at 4 °C in the dark and discard unused medium after 4 days.

NOTE 9 This medium may be solidified by the addition of 12 g per litre to 15 g per litre agar before boiling.

LES Endo agar

WARNING — Basic fuchsin is suspected of being a carcinogen.

Yeast extract	1,2 g
Trypticase (casitone or tryptone)	3,7 g
Thiopeptone	3,7 g
Tryptose	7,5 g
Lactose	9,4 g
Dipotassium hydrogen phosphate	3,3 g
Potassium dihydrogen phosphate	1,0 g
Sodium chloride	3,7 g
Sodium desoxycholate	0,1 g
Sodium lauryl sulfate	0,05 g
Sodium sulfite	1,6 g
Basic fuchsin	0,8 g
Agar	15 g
Distilled water	1 000 ml

Dissolve ingredients in the water containing 20 ml of 95 % (*V*/*V*) ethanol. Do **not** autoclave but heat to boiling, cool to between 45 °C and 50 °C and dispense in 4 ml volumes in small Petri dishes (60 mm diameter). Final pH shall be 7,2 ± 0,2. Store plates at 4 °C in the dark and discard unused medium after 2 weeks. Do not expose to direct sunlight.

mFC medium

Tryptose	10,0 g
Proteose peptone No. 3 or polypeptone	5,0 g
Yeast extract	3,0 g
Sodium chloride	5,0 g
Lactose	12,5 g
Bile salts No. 3 or bile salts mixture	1,5 g
Aniline blue	0,1 g
Distilled water	1 000 ml

Rehydrate in the distilled water containing 10 ml of 1 % rosolic acid in 0,2 mol/l NaOH. Heat the medium to the boiling point, promptly remove from heat and cool to below 45 °C to 50 °C. Do not sterilize by autoclaving. Final pH shall be 7,4 ± 0,2. Store the finished media at 2 °C to 10 °C and discard any unused medium after 96 h.

NOTES

10 This medium may be solidified by the addition of 1,2 % to 1,5 % agar before boiling.

11 Rosolic acid will decompose if sterilized by autoclaving. The stock solution should be stored in the dark at 2 °C to 10 °C and be discarded after 2 weeks, or sooner if its colour changes from dark red to muddy brown.