

# INTERNATIONAL STANDARD

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

**Part 2:**

Multiple tube (most probable number) method

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

*Partie 2: Méthode du nombre le plus probable*



Reference number  
ISO 9308-2:1990(E)

## Foreword

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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 9308-2 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<sup>1)</sup>, 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.

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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 2:

### Multiple tube (most probable number) 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*) by culture in a liquid medium in multiple tubes and calculation of their most probable numbers in the sample.

This method can be applied to all types of water, including those containing an appreciable amount of suspended matter.

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 of this part of ISO 9308, 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 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 aerobic growth at either  $35\text{ °C} \pm 0,5\text{ °C}$  or  $37\text{ °C} \pm 0,5\text{ °C}$  in a liquid lactose culture medium with the production of acid and gas within 48 h.

**3.2 thermotolerant coliform organisms:** Coliform organisms as described in 3.1 which have the same fermentative properties within 24 h, at either  $44\text{ °C} \pm 0,25\text{ °C}$  or  $44,5\text{ °C} \pm 0,25\text{ °C}$ .

**3.3 presumptive *Escherichia coli* (presumptive *E. coli*):** Thermotolerant coliform organisms as described in 3.2 which also produce indole from tryptophan within 24 h, at either  $44\text{ °C} \pm 0,25\text{ °C}$  or  $44,5\text{ °C} \pm 0,25\text{ °C}$ .

## 4 Principle

Inoculation of test portions of the sample, diluted or undiluted, into a series of tubes of a selective liquid culture medium containing lactose.

Examination of the tubes after 24 h and 48 h incubation at either  $35\text{ °C}$  or  $37\text{ °C}$ ; subculture from each tube showing turbidity with gas production into a more selective confirmatory medium and, when presumptive *E. coli* is sought, to a medium in which the formation of indole can be demonstrated.

Incubation of these confirmatory media for up to 48 h at either  $35\text{ °C}$  or  $37\text{ °C}$  for the detection of coliform organisms, and at  $44\text{ °C}$  for up to 24 h for thermotolerant coliform organisms and presumptive *E. coli*.

By means of statistical tables, calculation of the most probable numbers (MPN) of coliform organisms, thermotolerant coliform organisms and presumptive *E. coli* likely to be present in 100 ml of the sample, from the number of tubes giving positive confirmatory results.

## 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 of the following culture media. Instructions for preparing the media are given in annex A.

#### 5.3.1 Lactose broth

#### 5.3.2 MacConkey broth

#### 5.3.3 Improved formate lactose glutamate medium<sup>2)</sup>

#### 5.3.4 Lauryl tryptose (lactose) broth

## 5.4 Confirmatory media

Use one or more of the following.

### 5.4.1 Media for gas production

#### 5.4.1.1 Brilliant-green lactose (bile) broth

#### 5.4.1.2 EC medium

### 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.

**6.2 Incubator or water bath,** thermostatically controlled at either  $35\text{ °C} \pm 0,5\text{ °C}$  or  $37\text{ °C} \pm 0,5\text{ °C}$ .

**6.3 Incubator or water bath,** thermostatically controlled at either  $44\text{ °C} \pm 0,25\text{ °C}$  or  $44,5\text{ °C} \pm 0,25\text{ °C}$ .

### 6.4 pH meter.

2) Available commercially in dehydrated form as "Minerals modified glutamate medium". 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.

## 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 and inoculation of media

For preparation of the sample, making dilutions and inoculation of isolation medium with test portions, follow the instructions given in ISO 8199. For test portions of volume greater than 5 ml, use tubes containing "double strength" isolation medium.

### 8.2 Incubation of tubes

Incubate the inoculated tubes for 48 h at either  $35\text{ }^{\circ}\text{C} \pm 0,5\text{ }^{\circ}\text{C}$  or  $37\text{ }^{\circ}\text{C} \pm 0,5\text{ }^{\circ}\text{C}$ .

### 8.3 Examination of tubes

Examine the tube-cultures after incubation for 18 h to 24 h and regard as positive reactions those which show turbidity due to bacterial growth and gas formation in the inner inverted (Durham) tubes, together with acid production if the isolation medium contains a pH indicator. Reincubate those tubes which do not show any or all of these changes and examine them again for positive reactions after 48 h.

### 8.4 Confirmatory tests

It is important to note that positive reactions in tubes of isolation medium are only presumptive coliform results. It is therefore important to carry out confirmatory tests, preferably on pure subcultures.

#### 8.4.1 Subculture, incubation and examination

Subculture from each tube of isolation medium giving a positive result into one or more tubes of the confirmatory media (5.4) for gas and indole production.

NOTE 1 If the least inhibitory medium (lactose broth) is used for isolation, subculture to either of the two more selective confirmatory media [brilliant-green lactose (bile) broth or EC broth] for confirmation is recommended.

##### 8.4.1.1 Coliform organisms

To confirm the presence of coliform organisms, incubate one tube of brilliant-green lactose (bile) broth (5.4.1) at either  $35\text{ }^{\circ}\text{C}$  or  $37\text{ }^{\circ}\text{C}$ , and examine for gas production within 48 h.

##### 8.4.1.2 Thermotolerant coliform organisms and presumptive *E. coli*

To confirm the presence of thermotolerant coliform organisms, incubate another tube of EC medium (5.4.1) at  $44\text{ }^{\circ}\text{C}$  for 24 h, and examine for gas production.

To confirm the presence of presumptive *E. coli*, incubate a tube of tryptone water (5.4.2) for indole formation at  $44\text{ }^{\circ}\text{C}$  for 24 h. Then add 0,2 ml to 0,3 ml of Kovacs' reagent (5.5.1) to the tube of tryptone water: development of a red colour after gentle agitation denotes the presence of indole.

#### NOTES

2 The use of lauryl tryptose mannitol broth with tryptophan allows both gas and indole production by presumptive *E. coli* to be demonstrated in a single tube.

3 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).

4 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  $37\text{ }^{\circ}\text{C}$ . 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, interfere with the determination only at a temperature of  $37\text{ }^{\circ}\text{C}$  and below, confirmation with the oxidase test is needed only when determining coliforms.

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

- place 2 or 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 4);
- regard the appearance of a deep blue-purple colour within 10 s as a positive reaction.

NOTE 5 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 number of tubes of isolation medium and confirmatory tests giving positive reactions, calculate by reference to the statistical tables in ISO 8199, the most probable numbers of coliform organisms, thermotolerant coliform organisms and presumptive *E. coli* in 100 ml of the sample.

## 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.

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## 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-spore-forming, 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.

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## Annex B (informative)

### Culture media, reagents and diluents

#### Isolation media

##### Lactose broth

##### Double-strength medium

Peptone	10 g
Lactose	10 g
Beef extract	6 g
Distilled water	1 000 ml

Dissolve the ingredients in boiling water. Adjust the pH if necessary so that after sterilization it is  $6,9 \pm 0,2$ . Prepare single-strength medium by diluting the double-strength medium with an equal volume of distilled water.

##### MacConkey broth

##### Double-strength medium

Bile salts	10 g
Peptone	40 g
Lactose	20 g
Sodium chloride	10 g
Bromocresol (or Cresol) purple [1 % (V/V) ethanolic solution]	2 ml
Distilled water	1 000 ml

Dissolve the peptone, sodium chloride and bile salts in the water by heating and store at 4 °C overnight. Filter while still cold, add the lactose and dissolve. Adjust to pH  $7,4 \pm 0,2$  and add the bromocresol purple.

##### Single-strength medium

Prepare single-strength medium by dilution of the double-strength medium with an equal volume of distilled water or make separately using half the concentration of ingredients.

Distribute single-strength medium in 5 ml volumes and double-strength medium in 10 ml and 50 ml

volumes. Each tube or bottle used should contain an inverted fermentation (Durham) tube. Autoclave at 115 °C for 10 min.

##### Improved formate lactose glutamate medium<sup>3)</sup>

##### Double-strength medium

Lactose	20 g
L(+) Glutamic acid sodium salt	12,7 g
L(+) Arginine monohydrochloride	0,048 g
L(-) Aspartic acid	0,04 g
L(-) Cystine	0,04 g
Sodium formate	0,5 g
Dipotassium hydrogen phosphate	1,8 g
Ammonium chloride	5 g
Magnesium sulfate (MgSO <sub>4</sub> ·7H <sub>2</sub> O)	0,02 g
Calcium chloride (CaCl <sub>2</sub> ·2H <sub>2</sub> O)	0,02 g
Iron(III) citrate scales	0,02 g
Thiamine (aneurin hydrochloride)	0,002 g
Nicotinic acid	0,002 g
Pantothenic acid	0,002 g
Bromocresol purple [1 % (m/m) ethanolic solution]	2 ml
Distilled water to	1 000 ml

The medium is most conveniently prepared in quantities of 10 l or more. If it is not to be distributed in tubes immediately, the lactose and thiamine should be omitted and added immediately before dispensing. Several of the ingredients are more conveniently added as separate solutions and these should be prepared as follows.

##### Solution 1

L(+) Arginine monohydrochloride	0,4 g
L(-) Aspartic acid	0,48 g
Distilled water	50 ml

Heat to dissolve

3) Available commercially in dehydrated form as "Minerals modified glutamate medium". It may also require pH adjustment.

## Solution 2

L(-) Cystine	0,4 g
Sodium hydroxide (5 mol/l)	10 ml
Distilled water	90 ml

Heat to dissolve

## Solution 3

Nicotinic acid	0,02 g
Pantothenic acid	0,02 g
Distilled water	5 ml

Dissolve without heating

## Solution 4

Iron(III) citrate scales	0,2 g
Distilled water	10 ml

Heat to dissolve

## Solution 5

Calcium chloride (CaCl <sub>2</sub> ·2H <sub>2</sub> O)	5 g
Distilled water	100 ml
Concentrated hydrochloric acid	0,1 ml

Dissolve without heating and sterilize at 121 °C for 20 min. Keep as a stock solution.

## Solution 6

Sterile 0,1 % solution of thiamin in distilled water.

This solution is best prepared by adding the contents of an ampoule (100 mg) aseptically to 99 ml of sterile distilled water. It should be kept at 4 °C and discarded after 6 weeks.

To prepare 10 l of double-strength medium, dissolve the appropriate quantities of L(+) glutamic acid sodium salt, sodium formate, dipotassium hydrogen phosphate, ammonium chloride and magnesium sulfate in 9 l of hot distilled water. Then add the whole of solutions 1, 2, 3 and 4 and 4 ml of solution 5. Adjust the pH to 6,8 or higher if necessary, so that the final pH after completion and sterilization is 6,7. If the same equipment and meth-

ods of sterilization are used, the same change in pH should always occur on sterilization. Some preliminary trials may be necessary to establish the correct pH prior to sterilization.

After adjustment of pH, add 20 ml of 1 % ethanolic solution of bromocresol purple. Make up the final volume to 10 l with about 810 ml of distilled water. If the bulk of the medium is not required for immediate use, bottle in 500 ml volumes and autoclave at 115 °C for 10 min. For use, add the necessary amount of lactose and thiamin solution (solution 6), allow to dissolve and then distribute in 10 ml and 50 ml volumes. Each tube or bottle used should contain an inverted fermentation (Durham) tube. It is important to ensure that, after autoclaving and before use, the Durham tube is completely filled with medium. Otherwise a false positive result for gas production may be obtained. Sterilize at 115 °C for 10 min or place in a steamer at 100 °C for 30 min on three successive days.

## Single-strength medium

Prepare single-strength medium by diluting the double-strength medium with an equal volume of distilled water and distribute in 5 ml volumes in tubes containing an inverted fermentation (Durham) tube. Sterilize at 115 °C for 10 min or steam at 100 °C for 30 min on three successive days.

NOTE 6 The addition of a 0,1 % (m/m) vitamin-free acid-hydrolyzed casein may give quicker results.

## Lauryl tryptose (lactose) broth

## Double-strength medium

Tryptose	40 g
Lactose	10 g
Sodium chloride	10 g
Dipotassium hydrogen phosphate	5,5 g
Potassium dihydrogen phosphate	5,5 g
Sodium lauryl sulfate, high purity	0,2 g
Distilled water	1000 ml

Add the tryptose, sodium chloride, lactose and phosphates to the water and heat to dissolve. Add the sodium lauryl sulfate and mix gently to avoid froth. Adjust the pH to 6,8 ± 0,2. Prepare single-strength medium by diluting the double-strength medium with an equal volume of distilled water.

Distribute single-strength medium in 5 ml volumes and double-strength medium in 10 ml and 50 ml volumes. Each tube or bottle used should contain an inverted fermentation (Durham) tube. Autoclave at 115 °C for 10 min.

**Confirmatory media****Brilliant-green lactose (bile) broth<sup>4)</sup>** (for gas production)

Peptone	10 g
Lactose	10 g
Ox bile (dehydrated)	20 g
Brilliant-green (0,1 % by mass aqueous solution)	13 ml
Distilled water to	1 000 ml

Dissolve the peptone in 500 ml of distilled water. Add the 20 g of dehydrated ox bile dissolved in 200 ml of distilled water. This solution should have a pH between 7,0 and 7,5. Make up to approximately 975 ml with distilled water. Add the lactose and adjust the pH to 7,4. Add the brilliant-green solution and make up to 1 000 ml with distilled water.

Distribute 5 ml volumes in test tubes containing inverted inner fermentation (Durham) tubes and autoclave at 115 °C for 10 min.

**EC medium** (for gas production)

Tryptose or trypticase	20 g
Lactose	5 g
Bile salts mixture or bile salts No. 3	1,5 g
Dipotassium hydrogen phosphate	4 g
Potassium dihydrogen phosphate	1,5 g
Sodium chloride	5 g
Distilled water	1 000 ml

The pH should be 6,9 after sterilization. Prior to sterilization, distribute in tubes with sufficient medium to cover the inverted inner fermentation (Durham) tube at least partially after sterilization.

**Tryptone water** (for indole reaction)

Certain peptones which give satisfactory results in tests at 35 °C or 37 °C are not satisfactory for the indole test at 44 °C. Tryptone has been found satisfactory and is recommended.

Tryptone	20 g
Sodium chloride	5 g
Distilled water	1 000 ml

Dissolve the ingredients in the water and adjust to pH 7,5. Distribute in 5 ml volumes and autoclave at 115 °C for 10 min.

NOTE 7 The addition of 0,1 % (m/m) L or DL tryptophan may improve the performance of the medium.

**Lauryl tryptose mannitol broth with tryptophan**

(Single-tube medium for both gas and indole production.)

Tryptose	20 g
Mannitol	5 g
Sodium chloride	5 g
Dispotassium hydrogen phosphate	2,75 g
Potassium dihydrogen phosphate	2,75 g
Sodium lauryl sulfate	0,1 g
L(-) Tryptophan	0,2 g
Distilled water	1 000 ml

Add the tryptose, sodium chloride, mannitol, phosphates and tryptophan to the water and warm to dissolve. Add the sodium lauryl sulfate and mix gently to avoid froth. Adjust to pH  $6,8 \pm 0,2$ . Distribute in 5 ml volumes in tubes containing an inverted inner fermentation (Durham) tube. Autoclave at 115 °C for 10 min.

**Reagents****Kovacs' reagent for indole**

<i>p</i> -dimethylaminobenzaldehyde	5 g
Amyl alcohol	75 ml
Hydrochloric acid ( $\rho = 1,18$ g/ml)	25 ml

Dissolve the aldehyde in the amyl alcohol. Add the concentrated acid with care. Protect from light and store at 4 °C.

NOTE 8 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.

**Oxidase reagent**

Tetramethyl- <i>p</i> -phenylenediamine hydrochloride	0,1 g
Distilled water	10 ml

This reagent does not keep and it must therefore be freshly prepared for use in small amounts each time that it is needed.

4) This medium does not always give reproducible results and it is advisable to check its inhibitory properties beforehand.