INTERNATIONAL STANDARD

ISO 9308-3

First edition
1998-11-15

Water quality — Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water —

Part 3:
Miniaturized method (Most Probable Number) by inoculation in liquid medium

Qualité de l'eau — Recherche et dénombrement des Escherichia coli *et des bactéries coliformes dans les eaux de surface et résiduaires —*

Partie 3: Méthode miniaturisée (nombre le plus probable) pour ensemencement en milieu liquide

Contents

Page

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Printed in Switzerland

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.

International Standard ISO 9308-3 was prepared by Technical Committee ISO/TC 147, *Water quality,* Subcommittee SC 4, *Microbiological methods.*

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

— *Part 1: Membrane filtration method*

— *Part 2: Liquid enrichment method*

— *Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid media*

Annexes E and F form a normative part of this part of ISO 9308. Annexes A to D are for information only.

Water quality — Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water —

Part 3:
Miniaturized method (Most Probable Number) by inoculation in liquid medium

1 Scope

This part of ISO 9308 specifies a miniaturized method for the detection and enumeration of *Escherichia coli* (*E. coli*) in surface and waste water by inoculation in a liquid medium. The method is applicable to all types of surface and waste waters, particularly those rich in suspended matter.

This method is not suitable for drinking water and any other type of water for which the guideline is less than 15 counts per 100 ml.

This method is not appropriate for enumeration and detection of coliform bacteria other than *E. coli*.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard 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 3951, *Sampling procedures and charts for inspection by variables for percent nonconforming.*

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

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

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

ISO 8199, *Water quality — General guide to the enumeration of microorganisms by culture.*

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

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
Escherichia coli
E. coli
β-D-glucuronidase-positive microorganism growing at an incubation temperature of 44 °C in the specified liquid medium containing 4-methylumbelliferyl-β-D-glucuronide (MUG)

4 Principle

The diluted sample is inoculated in a row of microtitre plate wells containing dehydrated culture medium.

The microtitre plates are examined under ultraviolet light at 366 nm in the dark after an incubation period of 36 h minimum and 72 h maximum at 44 °C ± 0,5 °C. The presence of *E. coli* is indicated by a blue fluorescence resulting from hydrolysis of MUG. The results are given as most probable number (MPN) per 100 ml.

5 Apparatus

With the exception of equipment supplied sterile, the glassware shall be sterilized in accordance with the instructions given in ISO 8199.

Usual microbiological laboratory equipment, and in particular:

5.1 Apparatus for sterilization by dry heat (oven) or steam (autoclave).

5.2 Thermostatic incubator regulated at 44 °C ± 0,5 °C.

5.3 Tunnel drier or vertical laminar air flow cabinet (preferably class II).

5.4 UV observation chamber (Wood's Lamp, 366 nm).

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

5.5 Portable refractometer (optional).

5.6 pH meter with an accuracy of ± 0,1

5.7 Test tubes of dimensions 16 mm × 160 mm and 20 mm × 200 mm, or flasks with similar capacity.

5.8 8-channel multipipette, adjustable or preset, or any other system suitable for measuring and distributing 200 µl per well.

5.9 Sterile tips for multipipette.

5.10 Equipment for membrane filtration in accordance with ISO 8199, including membrane filters with a nominal pore size of 0,2 µm, for sterilization of liquid media.

5.11 Sterile microtitre plates, 96-well, 350 µl, flat bottomed, nonfluorescent.

5.12 Sterile adhesive covering strips for sealing microtitre plates.

5.13 Sterile Petri dishes, 90 mm in diameter.

6 Sampling

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

7 Culture media and diluent

7.1 General instructions

To ensure reproducible results, prepare culture medium and diluents, using either constituents of uniform quality and chemicals of recognized analytical grade, or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare them with demineralized or distilled water free from substances capable of inhibiting growth under the test conditions. If the media are not used immediately, preserve them in the dark at (5 ± 3) °C for up to one month in conditions avoiding any alterations to their composition.

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

7.2 Diluents

7.2.1 **Special diluent** (SD)

Synthetic sea salt[1)]	22,5 g
Bromophenol blue solution (optional)	10 ml
Demineralized or distilled water (7.2.2)	1000 ml

Sterilize in the autoclave (5.1) at 121 °C ± 3 °C for 15 min to 20 min.

The bromophenol blue solution is prepared by adding 0,04 g in 100 ml of 50 % ethanol. It is only used to colour the SD blue and avoid confusing it with demineralized or distilled water.

7.2.2 **Demineralized or distilled water,** free from substances inhibiting growth under the test conditions.

Sterilize in the autoclave (5.1) at 121 °C ± 3 °C for 15 min to 20 min.

7.3 Culture medium: MUG/EC medium

Composition

Tryptone	40 g
Salicin	1 g
Triton X 100®	1 g
MUG (4-methylumbelliferyl-β-D-glucuronide)	100 mg
Demineralized or distilled water (7.2.2)	1000 ml

1) A typical analysis of a commercially available and suitable synthetic sea salt is given in annex C. Other diluents, such as distilled water, can be used for *E. coli* enumeration, unless intestinal enterococci are to be enumerated from the same dilution tubes.

Successively add tryptone, salicin and Triton[2)] to one litre of water, whilst maintaining a gentle heat and magnetic stirring, then bring to the boil until completely dissolved. Allow to cool and add the fluorogenic constituent MUG, dissolved in 2 ml of *N,N*-dimethylformamide.

WARNING: *N,N*-dimethylformamide is toxic and can cause cancer. Harmful by inhalation, in contact with skin and if swallowed. Use in a chemicals fume hood.

Adjust the pH to 6,9 ± 0,2.

Sterilize by filtration with membranes of average pore size 0,2 µm (5.10).

Distribute in 96-well microtitre plates (5.11) with a volume of 100 µl of media in each well (minimum capacity 350 µl) and dehydrate immediately in a tunnel drier or laminar air flow cabinet (5.3).

The manufacturing of the medium shall meet the quality criteria given in annex E.

8 Procedure

8.1 Choice of dilutions

The number of dilutions to inoculate varies according to the presumed level of contamination of the water to be tested. Table 1 gives examples.

Table 1

Origin of sample	No. of dilutions	No. of wells/dilution	Measurement limits, bacteria/100 ml
Bathing water	2	64 wells to 1/2 32 wells to 1/20	15 to 3,5 × 10^4
Fresh surface water	4	24 wells to 1/2 24 wells to 1/20 24 wells to 1/200 24 wells to 1/2 000	40 to 3,2 × 10^6
Waste water and treatment plants	6	16 wells to 1/2 Up to 16 wells to 1/200 000	60 to 6,7 × 10^8

8.2 Preparation of dilutions

NOTE These procedures should be performed in a biological safety cabinet, as aerosols may be created by the diluting and pipetting.

8.2.1 Fresh and brackish (waste) water (salinity < 30 g/kg, measured with a refractometer (5.5) or equivalent method)

Prepare the relevant number of sterile tubes (5.7) in a rack, according to the number of selected dilutions; add 9 ml of the special diluent (7.2.1) to each tube.

Vigorously stir the sample (clause 6) in order to obtain a homogeneous distribution of the microorganisms and using a sterile pipette, immediately transfer 9 ml of this homogenized sample to the first tube containing 9 ml of diluent (1/2 dilution).

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2) Triton X 100 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. Equivalent products may be used if they can be shown to lead to the same results.

Using a fresh pipette, transfer 1 ml of this dilution (homogenized) to the second tube (1/20 dilution).

From the second tube (dilution 1/20 carefully homogenized) proceed, if necessary to another 1/10 dilution giving the dilution 1/200.

Continue as above until all the dilutions have been prepared.

8.2.2 Sea water (salinity ⩾ 30 g/kg)

Prepare the relevant number of sterile tubes in a rack, according to the number of selected dilutions; add 9 ml of demineralized or distilled water (7.2.2) to the first tube and 9 ml of the special diluent (7.2.1) to the following tubes.

Vigorously stir the sample (clause 6) in order to obtain a homogeneous distribution of the microorganisms and using a sterile pipette, immediately transfer 9 ml of this homogenized sample to the first tube containing 9 ml of diluent (7.2.2) (1/2 dilution).

Using a fresh sterile pipette, transfer 1 ml of this dilution (homogenized) to the second tube (1/20 dilution).

From the second tube (dilution 1/20 carefully homogenized) proceed, if necessary to another 1/10 dilution, giving the dilution 1/200.

Continue as above until all the dilutions have been prepared.

8.3 Inoculation and incubation of microtitre plates

8.3.1 Inoculation

Transfer the contents of the first tube of dilution to an empty, sterile Petri dish of diameter 90 mm.

Using a multichannel pipette (5.8) with eight sterile tips (5.9), distribute 200 µl into each well of microtitre plate (5.11) corresponding to this first dilution.

For subsequent dilutions (1/20, 1/200, etc.) operate in an identical manner, changing the Petri dish and the row of eight sterile tips between each dilution.

Alternatively, any other suitable system (5.8) may be used to distribute 200 µl of each dilution per well according to Table 1.

CAUTION: Beware of contamination via overflow from one well to another.

8.3.2 Incubation

Once the microtitre plate is inoculated, cover with the disposable sterile adhesive tape (5.12) provided for this purpose.

Incubate the microtitre plate in an incubator (5.2) at 44 °C ± 0,5 °C for a minimum of 36 h and a maximum of 72 h.

NOTE The microtitre plates should be handled with care, without tilting.

8.4 Reading of results

Place each microtitre plate, with the adhesive on, in the UV observation chamber (5.4).

Consider all wells in which a blue fluorescence is observed as being positive.

NOTE The reading may be carried out any time after 36 h, as the fluorescence does not alter with time.

9 Expression of results

9.1 Determination of characteristic number

For each chosen dilution note the number of positive (+) wells.

EXAMPLE 1: Bathing water

1/2	32 + out of 64
1/20	5 + out of 32

Record 32/5 as characteristic number

EXAMPLE 2: Surface water

1/2	24 + out of 24
1/20	18 + out of 24
1/200	5 + out of 24
1/2 000	1 + out of 24

Record 18/5/1 as characteristic number

EXAMPLE 3 : Waste water

1/2	16 + out of 16
1/20	16 + out of 16
1/200	12 + out of 16
1/2 000	5 + out of 16
1/20 000	0 + out of 16
1/200 000	0 + out of 16

Record 12/5/0 as characteristic number

Where three or more dilutions have been inoculated, a characteristic number of three figures, the last one 0 where possible, shall be recorded in accordance with ISO 8199.

9.2 Calculation of the MPN and its confidence interval

The MPN is a statistical estimation of the density of microorganisms, assumed to correspond to a Poisson distribution in the volumes inoculated. Confidence intervals are attached to this MPN.

Software shown in annex A or B enables the calculation of the MPN of *E. coli* per millilitre of water for each configuration of inoculations and the confidence interval at 95 %.

EXAMPLE 1: Assuming CN is the Characteristic Number, LO the Lower Limit and UP the Upper Limit:

If CN = 32/5, the software in annex A gives 7,56 *E. coli*/ml,

[LO = 5,42 − UP = 10,54],

i.e. 756/100 ml (542 to 1054).

EXAMPLE 2:

If CN = 18/5/1, the software in annex A gives 159,08/ml,

[LO = 101,99 – UP = 248,11]

EXAMPLE 3:

If CN = 12/5/0, the software in annex A gives 1724,61/ml

[LO = 1003,98 – UP = 2962,50]

If none of the wells is positive, express the result in the following form: < *n*/100 ml, *n* being the MPN for 1 positive well under the dilution conditions employed.

10 Test report

The test report shall include all details necessary for the complete identification of the sample, referring to the method used and the results.

The test report shall also mention any special phenomena observed during the test and any nonspecified or optional operations used in the method which may have altered the results.

11 Performance data

Information concerning the repeatability and reproducibility of the procedure, obtained from interlaboratory tests, is given in annex D.