INTERNATIONAL STANDARD

ISO 9308-2

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

Part 2: Most probable number method

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

Partie 2: Méthode du nombre le plus probable

Reference number
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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

ISO 9308-2 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 9308-2:1990), which has been technically revised.

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 a body 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 a failure in treatment or ingress of water into the distribution system.

The International Organization for Standardization (ISO) draws attention to the fact that it is claimed that compliance with this document may involve the use of patents concerning Colilert-18 and Quanti-Tray and Quanti-Tray 2000 given in this document.

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

Part 2:
Most probable number method

WARNING – Persons using this part of ISO 9308 should be familiar with normal laboratory practice. This International Standard 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 part of ISO 9308 be carried out by suitably qualified staff.

1 Scope

This part of ISO 9308 specifies a method for the enumeration of *E. coli* and coliform bacteria in water. The method is based on the growth of target organisms in a liquid medium and calculation of the "Most Probable Number" (MPN) of organisms by reference to MPN tables. This method can be applied to all types of water, including those containing an appreciable amount of suspended matter and high background counts of heterotrophic bacteria. However, it must not be used for the enumeration of coliform bacteria in marine water. When using for the enumeration of *E. coli* in marine waters, a 1→10 dilution in sterile water is typically required, although the method has been shown to work well with some marine waters that have a lower than normal concentration of salts. In the absence of data to support the use of the method without dilution, a 1→10 dilution is used.

This method relies upon the detection of *E. coli* based upon expression of the enzyme β-D-glucuronidase and consequently does not detect many of the enterohaemorhagic strains of *E. coli*, which do not typically express this enzyme. Additionally, there are a small number of other *E. coli* strains that do not express β-D-glucuronidase.

The choice of tests used in the detection and confirmation of the coliform group of bacteria, 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. The test described in this part of ISO 9308 provides a confirmed result with no requirement for further confirmation of positive wells.

NOTE While this method describes the use of an enumeration device that is commercially available, the medium described here can also be used in a standard MPN format.

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

ISO/IEC Guide 2:2004, *Standardization and related activities — General vocabulary*

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

3 Terms and definitions

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

3.1
coliform bacterium
member of the *Enterobacteriaceae* that express the enzyme β-D-galactosidase

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

4 Principle

A snap pack of dehydrated medium is added to a sample of water (100 ml), or a dilution of a sample made up to 100 ml. Sample plus medium is gently shaken to ensure adequate mixing and to afford dissolution of the medium. The sample plus medium is then aseptically poured into a Quanti-Tray[1)] or Quanti-Tray/2000[1)] to enumerate up to 201 organisms or 2 419 organisms per 100 ml, respectively. Trays are sealed with a Quanti-Tray[1)] Sealer and then incubated at (36 ± 2) °C for 18 h to 22 h.

After incubation, sample wells that have a yellow colour of equal or greater intensity than that of the comparator wells are considered positive for coliform bacteria. Yellow wells that also exhibit any degree of fluorescence are considered positive for *E. coli*.

By means of statistical tables, or a simple computer program, the most probable number (MPN) of coliform bacteria and *E. coli* in 100 ml of the sample can be determined.

NOTE The yellow colouration can be seen with the naked eye and results from the cleavage of ortho-nitrophenol galactoside by the enzyme β-D-galactosidase. The fluorescence is demonstrable under ultraviolet light (365 nm) and originates from the cleavage of the molecule 4-methylumbelliferyl glucuronide (MUG) by the enzyme β-D-glucuronidase to produce the fluorescent compound methyl umbelliferone.

5 Apparatus and glassware

Use microbiological laboratory equipment and, in particular, the following:

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 Hot air oven, for dry heat sterilization.

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

5.4 Quanti-Tray[1)] sealer.

5.5 Sterile wide mouthed vessels of at least 110 ml.

1) Quanti-Tray is a trademark or registered trademark of IDEXX Laboratories, Inc. or its affiliates in the United States and/or other countries. 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.

5.6 Quanti-Tray[2] comparator.

5.7 Ultraviolet lamp, 365 nm.

5.8 Quanti-Tray[2] or Quanti-Tray/2000[2], see Annex B.

6 Culture media and reagents

6.1 Basic materials

The method utilises Colilert[3]-18 a medium based on the Defined Substrate Technology available for a 100 ml sample as a ready to use powder dispensed in snap packs. Each snap pack contains sufficient medium (2,8 g) for a single test. Medium is stored under ambient conditions (2 °C to 25 °C) out of direct sunlight and should be used before the expiry date listed on the snap pack.

The medium is composed of two components to give the final concentrations as shown in Annex C.

6.2 Diluent

For dilutions to be used with Colilert[3]-18, use only sterile, non-inhibitory, oxidant-free water (deionized or tap). The use of buffered, saline or peptone-containing diluents interferes with the performance of the test.

6.3 Antifoam B

Antifoam B is a 10 % active, water soluble suspension of silicone.

7 Sampling

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

8 Procedure

8.1 Preparation of the sample

Samples should be transported and stored at (5 ± 3) °C in accordance with ISO 19458 and analysis commenced on the day of collection or within 18 h. Under exceptional circumstances, the samples may be kept at (5 ± 3) °C for up to 24 h prior to examination.

8.2 Inoculation of media

Aseptically add a single snap pack of Colilert[4]-18 medium (2,8 g) to each 100 ml volume of sample or dilution. When the medium has completely dissolved, the sample plus medium is aseptically poured into either a

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Quanti-Tray[4] or Quanti-Tray[4]/2000 and then sealed with the Quanti-Tray[4] Sealer. Marine water samples should generally be diluted 1→10 with sterile water. In order to minimize air bubbles within wells, samples can be prepared in pre-sterilized bottles containing antifoam B. Alternatively, antifoam B can be added to each bottle using a dropper bottle. The use of either form of antifoam is optional. Alternatively, the water sample in which the Colilert[4]-18 has been dissolved can be distributed into sterile tubes for determination of the MPN using a more traditional MPN format (e.g. 1 × 50 ml and 5 × 10 ml). If a single 100 ml volume is incubated, then the method can be used as a presence/absence test for the detection of coliform bacteria and *E. coli.* If either of these latter two approaches are used, then the tubes should be pre-warmed to (36 ± 2) °C for 20 min prior to the start of incubation.

While it is recommended that marine water samples be diluted 1→10 in sterile deionized water prior to examination, it has been noted that in some geographical areas that the salt concentration of marine water is sufficiently low to allow culture without dilution. If this procedure is to be used, then validation data should be available. The salinity of marine water varies considerably and it is the responsibility of the laboratory to determine if marine water samples require dilution.

8.3 Incubation and differentiation

Incubate the inoculated Quanti-Trays[4] for 18 h to 22 h at (36 ± 2) °C for coliform bacteria and *E. coli*.

8.4 Examination of results

Examine the Quanti-Tray[4] or Quanti-Tray[4]/2000 after incubation for 18 h to 22 h and regard as positive reactions for coliform bacteria those wells that have a yellow colouration equal to or greater than the colouration of the Quanti-Tray comparator. Examine the trays under UV light (365 nm) in a dark room or in a chamber that obscures ambient light. Regard any yellow wells that also exhibit any degree of fluorescence, as positive for *E. coli*. If results are equivocal after 18 h (i.e. the yellow colouration is less than that of the comparator), incubation should be extended up to 22 h. Positive results for both coliform bacteria and *E. coli* observed before 18 h of incubation as well as negative results observed after 22 h are also valid.

9 Expression of results

From the number of wells on a Quanti-Tray[4] that are positive, the MPN/100 ml for both coliform bacteria and *E. coli* can be calculated by reference to statistical tables or by using a computer MPN generator program, see Tables B.1 and B.2.

10 Test report

This test report shall contain at least the following information:

a) the test method used, together with a reference to this part of ISO 9308;

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

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.

Annex A
(informative)

Further microbiological information on coliform bacteria

In addition to expressing β-D-galactosidase, coliform bacteria are typically 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 usually 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 within (21 ± 3) h at (44,0 ± 0,5) °C. They 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.

Some strains of *Escherichia 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.

Annex B
(normative)

The Quanti-Tray[5)] Sealer and calculation of results

B.1 General

The Quanti-Tray[5)] Sealer is a thermal sealing unit that forms a seal between wells in the Quanti-Tray. The sealer automatically distributes liquid into the wells of the Quanti-Tray or Quanti-Tray/2000. The Quanti-Tray is used when anticipated counts are below 200 cfu/100 ml. The Quanti-Tray/2000 can be used to calculate MPN values up to 2419 cfu/100 ml. When calculating MPN, the tables supplied with the trays are the reference for all counts. A simple statistical program can also be used to calculate results. If required, the MPN can be calculated manually according to the procedures given below.

B.2 Calculation of the most probable number

B.2.1 Calculation of MPN for IDEXX Quanti-Tray[5] and Quanti-Tray/2000

B.2.1.1 Quanti-Tray (51-well)

Quanti-Tray MPN was originally developed at Yale University; an additional, good example of this serial dilution MPN can be found at the U.S. Food and Drug Association in the *Bacteriological Analytical Manual* (available on BAM Appendix 2: Most Probable Number from Serial Dilutions, October 2010).

Each sample well has an approximate volume of 1,96 ml.

The overflow well will hold a minimum of 8,5 ml.

For the calculation of the Quanti-Tray MPN (Table B.1), see Equation (B.1).

$$N_{MPN} = N \cdot \ln [N/(N - X)] \quad \text{(B.1)}$$

where

N_{MPN} is the MPN;

N is the total number of wells (tubes) used in a test;

X is the number of positive wells (tubes) observed in a test.

B.2.1.2 Quanti-Tray[5)]/2000 (97-well)

Quanti-Tray/2000 MPN was originally derived as described by Reference [1].

Small wells have a mean volume of 0,186 ml.

Large wells have a mean volume of approximately 1,86 ml (ten times larger than the small wells).

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Overflow well will hold approximately 11 ml.

For the calculation of the Quanti-Tray[6] /2000 MPN (Table B.2), see Equation (B.2):

$$\sum_{i=1}^{K} \frac{V_i \, d_i \, P_i}{1 - \mathrm{e}^{-V_i \, d_i \, N_{\mathrm{mpn}}}} = \sum_{i=1}^{K} V_i \, d_i \, n_i \qquad \text{(B.2)}$$

where

d_i is the dilution factor at level i (e.g. 0,1 for 1→10 dilution);

K is the number of dilution levels;

n_i is the number of wells at level i;

N_{mpn} is the MPN;

P_i is the number of positive wells at level i;

V_i is the volume of the wells at level i.

The 95 % confidence intervals can be found at :

$$T_0 = (\ln N_{\mathrm{mpn}} - 1{,}96) \times \varepsilon(\ln N_{\mathrm{mpn}})$$

$$T_1 = (\ln N_{\mathrm{mpn}} + 1{,}96) \times \varepsilon(\ln N_{\mathrm{mpn}})$$

where

T_0 is the lower confidence interval;

T_1 is the upper confidence interval;

ε is the standard error; and

$$\varepsilon\left(\ln N_{\mathrm{mpn}}\right) = \sqrt{N_{\mathrm{mpn}}^{\,2} \sum_{i=1}^{K} \frac{V_i^2 d_i^2 n_i^2}{\mathrm{e}^{-V_i \, d_i \, N_{\mathrm{mpn}}} - 1}} \qquad \text{(B.3)}$$

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Table B.1 — 51-Well Quanti-Tray MPN

No. of wells giving positive reaction	Most probable number (MPN) per 100 ml sample	95 % Confidence limits	
		Lower	Upper
0	< 1[a]	0,0	3,7
1	1	0,3	5,6
2	2	0,6	7,3
3	3,1	1,1	9
4	4,2	1,7	10,7
5	5,3	2,3	12,3
6	6,4	3	13,9
7	7,5	3,7	15,5
8	8,7	4,5	17,1
9	9,9	5,3	18,8
10	11,1	6,1	20,5
11	12,4	7	22,1
12	13,7	7,9	23,9
13	15	8,8	25,7
14	16,4	9,8	27,5
15	17,8	10,8	29,4
16	19,2	11,9	31,3
17	20,7	13	33,3
18	22,2	14,1	35,2
19	23,8	15,3	37,3
20	25,4	16,5	39,4
21	27,1	17,7	41,6
22	28,8	19	43,9
23	30,6	20,4	46,3
24	32,4	21,8	48,7
25	34,4	23,3	51,2
26	36,4	24,7	53,9
27	38,4	26,4	56,6
28	40,6	28	59,5
29	42,9	29,7	62,5
30	45,3	31,5	65,6
31	47,8	33,4	69
32	50,4	35,4	72,5
33	53,1	37,5	76,2
34	56	39,7	80,1