

Australian/New Zealand Standard™

Water microbiology

Method 6: Coliforms, *Escherichia coli* and thermotolerant coliforms—Determination of most probable number (MPN)

PREFACE

This Standard was prepared by the Joint Standards Australia/Standards New Zealand Committee FT-020, Water Microbiology, to supersede AS 4276.4—1995, *Water microbiology*, Method 4: *Coliforms—Estimation of most probable number (MPN)* and AS 4276.6—1995, *Water microbiology*, Method 6: *Thermotolerant coliforms and Escherichia coli—Estimation of most probable number (MPN)*.

The objective of this revision is to combine the most probable number method for coliforms with that of thermotolerant coliforms and *Escherichia coli* to form a single method.

The Committee has not recommended the adoption of ISO 9308-2:1990, *Water quality—Detection and enumeration of coliform organisms, thermotolerant coliform organisms and presumptive Escherichia coli—Part 2: Multiple tube (most) probable number method*, because at the time of review the ISO method was considered to be outdated. In addition, there was a desire to align the revision of AS 4276.4—1995 and AS 4276.6—1995 employing most probable number technique with that of the membrane filtration methods, AS 4276.5 and AS 4276.7, in moving toward an enzyme-based definition and confirmation for coliforms and *Escherichia coli*.

Coliforms are gram-negative, non-spore-forming, rod shaped bacteria capable of aerobic and facultative anaerobic growth. They metabolize lactose at 36°C, express the enzyme β -galactosidase and are cytochrome oxidase negative. Coliforms comprise several species from genera within the family Enterobacteriaceae, including *Escherichia coli*, and some species of *Enterobacter*, *Klebsiella*, *Citrobacter*, *Serratia* and *Hafnia*. The Coliform group includes species of both faecal and environmental origin.

Thermotolerant coliforms are a subset of coliform bacteria that are able to metabolise lactose at 44°C. *Escherichia coli* (*E. coli*) are thermotolerant coliforms that, in addition to metabolizing lactose at 44°C, express the enzyme β -glucuronidase and produce indole from tryptophan.

The laboratory should have a clearly defined quality control system to ensure that the apparatus, culture media, reagents and technique are suitable for the test. The use of positive controls is part of this system.

The term ‘informative’ has been used in this Standard to define the application of the appendix to which it applies. An ‘informative’ appendix is only for information and guidance.

METHOD

1 SCOPE

This Standard sets out a method, using a multiple tube dilution technique, for determining the most probable number (MPN) of coliforms, *Escherichia coli* (*E. coli*) and thermotolerant coliforms in water. It is suitable for waters with high background flora and/or turbidity.

The method is suitable for non-food solid matrices. The preparation of such matrices are beyond the scope of this method (see Clause 12, Bibliography for further guidance).

NOTES:

- 1 Thermotolerant coliforms are also referred to as 'faecal coliforms' although the organisms may not be of faecal origin.
- 2 A flow diagram of the procedure is shown in Appendix A.

2 REFERENCED DOCUMENTS

The following documents are referred to in this Standard:

AS/NZS	
4276	Water microbiology
4276.1	Method 1: General information and procedures

3 DEFINITIONS

For the purpose of this Standard the following definitions apply:

3.1 Coliforms

Organisms capable of growth at $36 \pm 2^\circ\text{C}$, which express the enzyme β -galactosidase and are oxidase negative.

3.2 Thermotolerant coliforms

Organisms which are capable of producing gas from lactose-based media at $44.0 \pm 0.5^\circ\text{C}$ within 21 ± 3 hours.

3.3 *Escherichia coli* (*E. coli*)

A thermotolerant coliform that expresses the enzyme β -glucuronidase which is capable of hydrolysing the substrate 4-methylumbelliferyl- β -D-glucuronide (MUG) to produce a blue fluorescence when viewed under long wavelength ultra-violet light.

4 PRINCIPLE

Measured quantities of sample, of one or more dilutions, are distributed into a series of tubes (or bottles) of liquid medium containing lactose and bromocresol purple as an indicator of acidity. Upon incubation, each tube which received one or more of the target microorganisms in the inoculum will display characteristic growth. Presumptive positive tubes are sub-cultured onto confirmatory media.

Coliform express the enzyme β -galactosidase and are negative for cytochrome oxidase. *E. coli* is confirmed by the expression of the enzyme β -glucuronidase in EC-MUG broth incubated at 44°C . Thermotolerant coliforms are confirmed on the basis of turbidity and gas production in EC-MUG incubated at 44°C .

Providing there are some tubes that exhibit a negative reaction, the most probable number of target microorganisms in 100 mL of the sample is determined from the number and distribution of tubes displaying confirmed positive reactions.

5 CULTURE MEDIA AND REAGENTS (See Appendix B)

5.1 Improved formate lactose glutamate (IFLG) medium

5.2 MacConkey agar (MA)

5.3 Ortho-nitrophenyl- β -galactopyranoside (ONPG) broth

5.4 Nutrient agar

5.5 Oxidase reagent (Kovacs')

5.6 EC-broth with 4-methylumbelliferyl- β -D-glucuronide with fermentation tube (EC-MUG)

6 APPARATUS

6.1 Rimless bacteriological test tubes or containers of appropriate size

6.2 Pipettes or equivalent measuring devices

1 mL, 10 mL, 50 mL.

6.3 Fermentation tubes (Durham's)

6.4 Incubators or water baths

Able to maintain incubation temperatures of $36 \pm 2^\circ\text{C}$ and $44 \pm 0.5^\circ\text{C}$.

6.5 Long wave ultra-violet light (approximately 360 nm)

7 REFERENCE CULTURES

7.1 *Escherichia coli*, NCTC 9001 or ATCC 11775 or ACM 1803 or NZRM 3309 or a culture traceable to any of these strains

This microorganism produces positive reactions for coliforms, *E. coli* and thermotolerant coliforms.

7.2 *Enterobacter aerogenes*, NCTC 10006 or ATCC 13048 or ACM 4982 or NZRM 798 or a culture traceable to any of these strains

This microorganism produces positive reactions for coliforms but negative reactions for *E. coli* and thermotolerant coliforms.

7.3 *Pseudomonas aeruginosa*, NCTC 10332 or ATCC 10145 or ACM 495 or NZRM 3007 or a culture traceable to any of these strains

This microorganism produces negative reactions for coliforms, *E. coli* and thermotolerant coliforms.

7.4 Use of reference cultures

The reference cultures are used in media quality control and analytical quality control procedures relevant to this method. The purpose of the reference cultures is to demonstrate and ensure that typical positive and negative growth characteristics and test reactions are exhibited by the cultures on the media and in the tests used in this method.

As a minimum, the reference cultures shall be used to assure that the method has been used properly. Each day samples are processed by this method, the positive and negative reference cultures are inoculated into tubes of IFLG and the growth obtained is taken through the procedure as far as any cultures/isolates obtained from the samples. As appropriate, the negative reference cultures are also submitted to the confirmation tests at the same time that cultures/isolates are tested.

If the reference cultures do not give appropriate results, then an investigation shall be undertaken. Test results shall be regarded as invalid unless the investigation reveals reasons for this failure that invalidate the quality control procedures, e.g. prepared suspensions shown not to contain target bacteria.

8 SAMPLES

8.1 Laboratory samples

See AS/NZS 4276.1.

8.2 Dilutions

Dilutions of the laboratory sample shall be prepared as required, using the procedure described in AS/NZS 4276.1.

9 PROCEDURE

9.1 Presumptive test

The procedure shall be as follows:

- (a) Using the technique described in AS/NZS 4276.1, prepare dilutions, as required, of the sample of water.
- (b) Using IFLG medium (5.1), prepare sets of tubes/containers as described for the multiple tube dilution technique in AS/NZS 4276.1.
- (c) Inoculate the sets of tubes/containers with appropriate dilutions of the test sample as described for the multiple tube dilution technique in AS/NZS 4276.1.

In the testing of water, it is often necessary to inoculate 50 mL, 10 mL and 1 mL quantities of undiluted sample directly into the medium. In this case, proceed as follows:

- (i) Inoculate the 50 mL sample into 50 mL double-strength medium.
NOTE: If screw-capped bottles are used, ensure the cap is loose during incubation.
- (ii) To a set of tubes containing 10 mL of double-strength medium, add 10 mL of sample.
- (iii) To a set of tubes containing 10 mL of single-strength medium, add 1 mL of sample.
- (d) Incubate at $36 \pm 2^\circ\text{C}$.
- (e) Examine for acid production after 21 ± 3 h.
- (f) Record the presence of presumptive coliforms in tubes/containers showing production of acid as indicated by a colour change from purple to yellow.
- (g) Incubate the remaining tubes/containers that do not show production of acid for a further 21 ± 3 h.
- (h) After the incubation period record any further tubes/containers showing acid as positive for presumptive coliforms. Record cultures that do not show production of acid after 48 hours of incubation as negative for coliforms, *E. coli* and thermotolerant coliforms.

9.2 Confirmatory tests

9.2.1 *Coliforms*

The procedure shall be as follows:

- (a) Inoculate all tubes/containers that are positive in the presumptive test onto MA (5.2) plates to obtain single colonies.
- (b) Incubate at $36 \pm 2^\circ\text{C}$ for 21 ± 3 h.
- (c) Examine the plates and make a selection of target colonies which are pink to red, mucoid or non-mucoid, often with a halo of precipitation of bile salts.
If none of the colonies show the above characteristics after 21 ± 3 h of incubation, record those cultures as negative for coliforms.
- (d) Inoculate selected presumptive coliform colonies into ONPG broth (5.3), and onto nutrient agar (5.4).
- (e) Incubate the cultures at $36 \pm 2^\circ\text{C}$ for 21 ± 3 h.
- (f) Examine the ONPG broth for β -galactosidase activity, indicated by the medium changing from clear to a yellow colour.
- (g) Record cultures that do not exhibit β -galactosidase activity in ONPG broth as negative for coliforms.
- (h) For ONPG positive cultures, check the growth on the corresponding nutrient agar culture for purity and perform a Kovacs' oxidase test (5.5) on the growth.

NOTES:

- 1 Instructions on performing Kovacs' oxidase test are given in Appendix C.
 - 2 Some oxidase-positive bacteria found in water, e.g. *Aeromonas* species, are able to hydrolyse ONPG at temperatures of 36°C and below. Coliforms are oxidase-negative, and the oxidase test is used to distinguish them from these non-coliform bacteria, that may hydrolyse ONPG under the test conditions.
 - 3 Where further confirmatory tests are required consult the reference given in Clause 12.
- (i) Record those cultures which are ONPG positive and oxidase-negative as confirmed coliforms.
 - (j) Record the number of tubes from each dilution showing a confirmed coliform result.

9.2.2 *E. coli*

The procedure shall be as follows:

- (a) Inoculate the EC-MUG broth (5.6) from all the IFLG tubes/containers that are positive in the presumptive test.
NOTE: Prior to incubation discard any tubes of EC-MUG showing bubbles in the fermentation tube.
- (b) Incubate at $44 \pm 0.5^\circ\text{C}$ and examine at 21 ± 3 h.
- (c) Examine all EC-MUG broth tubes under long wave ultra-violet light (Clause 6.5) for blue fluorescence.
- (d) Record all EC-MUG broth tubes showing blue fluorescence under ultra-violet light (Clause 6.5) as confirmed for *E. coli*. Reflection of UV light off glass tubes/containers can be misinterpreted as fluorescence. Reference cultures shall be included in the examination.

NOTE: Do not discard negative tubes if they are to be examined for thermotolerant coliforms.

9.2.3 *Thermotolerant coliforms*

The procedure shall be as follows:

- (a) Examine all retained tubes of EC-MUG broth for turbidity and gas.
- (b) Record tubes that show turbidity and gas in EC-MUG broth as positive for thermotolerant coliforms.
- (c) Record tubes that do not show turbidity and gas in EC-MUG broth as negative for thermotolerant coliforms, unless they have produced a positive result for *E. coli*.

10 DETERMINATION OF THE MOST PROBABLE NUMBER (MPN) OF COLIFORMS, *E. COLI* AND THERMOTOLERANT COLIFORMS

According to the results obtained, determine the MPN of coliforms, *E. coli* and thermotolerant coliforms per 100 mL of sample using the method described in AS/NZS 4276.1.

11 TEST REPORT

The following shall be reported:

- (a) All details necessary for identification of the sample.
- (b) Reference to this Standard, i.e. AS/NZS 4276.6.
- (c) Date and time of sampling and testing.
- (d) The most probable number (MPN) of confirmed coliforms, *E. coli* and thermotolerant coliforms per 100 mL of sample.
- (e) Any circumstances or unusual observations made during the course of the tests that may have had an effect on the result.

12 BIBLIOGRAPHY

Additional information on testing for coliforms, *E. coli* and thermotolerant coliforms can be found in the following references:

EATON, A.D., CLESCERI, L.S, RICE, E.W and GREENBURG, A.E (eds). *Standard Methods for the examination of water and wastewater*, 21st ed. Washington DC: American Public Health Association, Water Environment Federation, and American Water Works Association, 2005.

Additional information on the preparation of non-solid matrices can be found in the following references:

- [1] UNITED STATES ENVIRONMENTAL PROTECTION AGENCY. Method 1680: *Fecal coliforms in biosolids by multiple-tube fermentation and membrane filter procedures (Draft)*. Washington, DC: Office of Water, January 1999.
- [2] STANDING COMMITTEE OF ANALYSTS. The Microbiology of Sewage Sludge (2003) – Part 2 – Practices and procedures for sampling and sample preparation, *Methods for the Examination of Waters and Associated Materials*. London: Environment Agency, 2003.