

Australian/New Zealand Standard™

Water microbiology

Method 5: Coliforms—Membrane filtration method

PREFACE

This Standard was prepared by the Joint Standards Australia/Standards New Zealand Committee FT-020, Water Microbiology, to supersede AS 4276.5—1995.

The objective of this revision is to extend the applicability of the membrane filtration method when used with samples containing different levels of competing non-target bacterial flora.

The merits of ISO 9308-1:2000, *Water quality—Detection and enumeration of Escherichia coli and coliform bacteria*, Part 1: *Membrane filtration method* are acknowledged for waters containing very low numbers of background organisms. Should waters for analysis universally have a high likelihood of containing only few background organisms (e.g. high quality food processing waters, highly treated waters, etc), the ISO 9308-1:2000 method is a suitable choice. ISO 9308-1:2000 has been adopted as AS/NZS 4276.22: *Water microbiology*, Method 22: *Packaged water—Coliform bacteria and Escherichia coli—Membrane filtration method* and has been published concurrently with this Standard.

The Committee has restricted application of ISO 9308-1:2000 to packaged waters because this method is only applicable to waters with low background microflora and many waters in Australia and New Zealand contain background organisms at concentrations too high for the ISO method to be widely applicable.

Therefore, this Standard applies to testing for coliforms in all waters other than packaged waters. For packaged waters use AS/NZS 4276.22. Where waters are expected to contain low to moderate numbers of bacteria, use of membrane lauryl sulfate medium (m-LS medium) is recommended as the primary isolation medium. Where waters have a likelihood of containing high numbers of background microorganisms (e.g. some ambient untreated waters, untreated drinking waters), this Standard includes the option to employ an alternative selective medium (m-Endo medium) that is generally more inhibitory and can be expected to a greater extent to restrict the number and size of background colonies.

Coliforms are gram-negative, non-spore-forming, rod shaped bacteria capable of aerobic and facultative anaerobic growth. They metabolize lactose at 36°C, expressing 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.

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 membrane filtration, for enumerating coliforms in water other than packaged water.

Where waters are expected to contain low to moderate numbers of background bacteria, use of membrane lauryl sulfate agar (m-LS agar) is recommended as the primary isolation medium. Where waters have a likelihood of containing high numbers of background microorganisms (e.g. some ambient untreated waters, untreated drinking waters), this Standard includes the option to employ an alternative selective agar or broth (m-Endo medium) that is generally more inhibitory and can be expected to a greater extent to restrict the number and size of background colonies.

NOTES:

- 1 Membrane filtration is suitable for enumerating microorganisms only when the turbidity of the water is low.
- 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 (ISO 8199:2005, MOD)

3 DEFINITION

For the purpose of this Standard the following definition applies.

3.1 Coliforms

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

4 PRINCIPLE

A measured quantity of sample water is filtered through a $0.45 \mu\text{m}$ gridded membrane filter composed of cellulose esters. Microorganisms to be enumerated are retained on the membrane surface, being too large to pass through the pores. The membrane is placed, face upward on the surface of a differential medium, membrane lauryl sulphate or m-Endo. After incubation, presumptive colonies are enumerated and subcultured to confirmatory media to demonstrate the expression of β -galactosidase and a negative oxidase reaction.

5 CULTURE MEDIA AND REAGENTS (See Appendix B)

5.1 Membrane lauryl sulfate (m-LS) agar or broth

5.2 m-Endo agar or broth

NOTE: Sterile absorbent pads saturated with m-LS and m-Endo broth may be used, in preference to an agar medium.

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

5.4 Nutrient agar (NA)

5.5 Oxidase reagent (Kovacs')

6 APPARATUS

6.1 Apparatus and materials for membrane filtration

As described in AS/NZS 4276.1.

6.2 Incubators or water baths

Able to maintain incubation temperatures of $30 \pm 2^\circ\text{C}$ and $36 \pm 2^\circ\text{C}$.

7 REFERENCE CULTURES

7.1 *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.

7.2 *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.

7.3 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, suspensions of the positive and negative reference cultures are submitted to the test procedure. Growth of the positive reference culture obtained is taken through the procedure as far as any isolates obtained from the samples, including confirmation tests. The negative reference culture is also submitted to the confirmation tests at the same time that 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 Filtration procedure

Using the instructions for sample volume and procedure for membrane filtration described in AS/NZS 4276.1, filter an appropriate volume of sample or dilution thereof through the membrane filtration apparatus.

9.2 Resuscitation and incubation

The procedure shall be as follows:

- (a) Immediately the filtration is completed and the suction is turned off, remove the membrane from the funnel using sterilized forceps and roll the membrane, grid-side upwards, on to m-LS (5.1) or m-Endo (5.2) medium, taking care to avoid entrapping air bubbles.
- (b) Incubate the plates of m-LS and m-Endo media in an inverted position for agar and upright for broth, in a humid atmosphere, initially at $30 \pm 2^\circ\text{C}$ for 4 h followed by $36 \pm 2^\circ\text{C}$ for 16 to 18 h.

9.3 Identification of presumptive coliforms

Presumptive coliforms on m-LS medium are yellow, orange-yellow or orange-pink colonies. Plates should be counted within 15 minutes after removal from the incubator as the colonies may change colour at room temperature.

Presumptive coliforms on m-Endo medium are red to dark red in colour, mucoid or nucleated, with or without a golden metallic, metallic green or green sheen. The sheen may appear only in a central area on the periphery of the colony or cover the entire surface.

If the background growth of non-coliform organisms is greater than the coliform growth and is considered to have interfered with the recovery of all coliforms on the filter, the coliform count may be underestimated. This should be stated in the report.

Where there are presumptive coliform colonies of distinct morphologies, count each group separately.

Where spreaders occur, count each as a single colony, provided that the outer edge of each spreader can be defined; however, the count may be unreliable.

9.4 Confirmatory tests

The procedure shall be as follows:

- (a) Select a representative number of discrete colonies as described in AS/NZS 4276.1 for confirmation.
- (b) Inoculate ONPG broth (5.3) and onto NA (5.4) with the selected presumptive coliform colonies.
- (c) Incubate the cultures at $36 \pm 2^\circ\text{C}$ for 21 ± 3 h.
- (d) Examine the ONPG broth for β -galactosidase activity indicated by growth with the development of a yellow colour.
- (e) For ONPG positive cultures, check the growth on the corresponding nutrient agar plate for purity and perform a Kovacs' oxidase test (5.5).

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.