

STANDARDS AUSTRALIA

RECONFIRMATION

OF

AS 4276.2—1995

Water microbiology

Method 2: Culture media, diluents and reagents

RECONFIRMATION NOTICE

Technical Committee FT-020 has reviewed the content of this publication and in accordance with Standards Australia procedures for reconfirmation, it has been determined that the publication is still valid and does not require change.

Certain documents referenced in the publication may have been amended since the original date of publication. Users are advised to ensure that they are using the latest versions of such documents as appropriate, unless advised otherwise in this Reconfirmation Notice.

Approved for reconfirmation in accordance with Standards Australia procedures for reconfirmation on 18 September 2013.

The following are represented on Technical Committee FT-020:

ACT Health
Australian Society for Microbiology
Australian Water Association
Department of Health Victoria
National Association of Testing Authorities, Australia
National Measurement Institute
NSW Forensic and Analytical Science Services
PathWest Laboratory Medicine, WA
Plastics and Chemicals Industries Association
Queensland Health
Queensland University of Technology
University of Melbourne
Water Services Association of Australia

NOTES

Australian Standard®

Water microbiology

Method 2: Culture media, diluents and reagents

PREFACE

This Standard was prepared by the Standards Australia/Standards New Zealand Committee on Water Microbiology, FT/20, as part of a series of methods for the microbiological examination of waters for domestic and industrial use.

This Standard is the result of a consensus among Australian and New Zealand representatives on the Joint Committee to produce it as an Australian Standard.

The Standard provides, in part, information previously given in AS 1095.4.1.1—1981, *Microbiological methods for the dairy industry—Methods for the examination of water and air—Microbiological examination of water—Scope and general requirements*.

The formulae of the media, diluents and reagents set out in this Standard have been drawn from many sources and where possible a literature reference is given to provide more detailed information about each medium and its use.

METHOD

1 SCOPE This Standard sets out the formulations, procedures and precautions to be followed for the preparation of the culture media, diluents and reagents specified in this series of standard methods for the microbiological examination of water.

2 REFERENCED DOCUMENT The following document is referred to in this Standard:

AS

2243 Safety in laboratories (all Parts)

3 HAZARDOUS CHEMICALS Many chemicals are hazardous and care shall be taken when handling all chemicals used to make up culture media, diluents and reagents. Safety precautions and practices as detailed in AS 2243 shall be observed at all times.

4 REQUIREMENTS FOR INGREDIENTS

4.1 Agar

4.1.1 Description Agar for microbiological purposes shall be prepared especially for this use and shall be identified as bacteriological agar. Such agar, when used at an appropriate concentration in media, shall give satisfactory biological performance and shall not cause significant inhibition to the growth of microorganisms. Information on the gel strength, maximum moisture content and maximum inorganic matter content expressed as ash shall be available from the manufacturer.

4.1.2 Analysis and specifications Bacteriological agar shall comply with the following specifications:

- (a) *Gel strength* Gel strength of agar used for microbiological purposes is determined by means of the FIRA gel tester* or an instrument giving similar results. Results of such analyses shall be available from the manufacturer.

In most of the media formulations in this Standard, a satisfactory gel strength with 15 g/L agar has been assumed but this concentration may have to be varied according to the quality of the agar available.

- (b) *Gelling* Prepare a suspension of agar in water at the concentration indicated by the stated gel strength and dissolve by autoclaving at 121°C for 15 min. Cool the solution with agitation to approximately 46°C, adjust to pH 7.0 ±0.1 and allow to stand in a water bath, capable of being controlled to ±1°C, adjusted to the temperature at which the test is to be carried out in the range 39°C to 46°C. After 3 h, examine the solution for gelation by pouring through a 200 mm length tube of 5 mm internal diameter which has been preheated by immersion in water at the temperature selected for the test. The agar solution shall flow smoothly through the tube without any irregularity of flow indicative of gelling.
- (c) *pH* Prepare a 15 g/L solution of agar in water by autoclaving at 121°C for 15 min and cool to 45 ±1°C. Retain this solution at this temperature for tests (d) and (e). The pH of the solution when cooled to 45 ±1°C shall be in the range 6.0 to 7.5.
- (d) *Insoluble sediment* Pour 100 mL of the solution from (c) into a beaker with a base diameter of 65 mm, cover and allow to cool to 22.5 ±2.5°C. The amount of sediment in the beaker shall be such that, when the agar solution is used to prepare pour plates, particulate sediment will not significantly affect the counting of microbial colonies.
- (e) *Opacity* Pour some of the solution from (c) into a 10 mm spectrophotometer cell, cool to 22.5 ±2.5°C and allow to stand at this temperature for 1 h. Measure the absorbance in a spectrophotometer at 400 nm using distilled water as the blank. The optical density shall not exceed 1.00.

NOTES:

- 1 Measurements of opacity made by any other method, including the use of Brown's opacity tubes, should be related to the above method.
- 2 Bacteriological grades of agar usually remain sufficiently clear for most media. However, if greater clarity is essential, filtration of the media through cellulose pulp may be necessary.

4.2 Beef extract Any standardized brand of beef extract especially manufactured for the preparation of microbiological culture media may be used. Unless otherwise specified, the quantities of beef extract indicated in the formulations refer to beef extract powder.

4.3 Bile salts Bile salts are a standardized mixture of sodium glycocholate and sodium taurocholate, prepared from fresh ox bile, for use as a selectively inhibitory agent in media. Only standardized, commercially available dehydrated preparations especially prepared for bacteriological work shall be used.

4.4 Sugars All sugars used in the preparation of culture media shall be chemically pure and known to be suitable for bacteriological purposes.

4.5 Salts and general chemicals All salts and general chemicals used as ingredients shall be AR (analytical reagent) grade or equivalent. The nomenclature used in this document is in accordance with the Merck Index.

* JONES, N.R., 1956, *Analyst*, Vol. 83, p. 243.

4.6 Dyes All dyes used in the preparation of culture media shall be certified suitable for bacteriological purposes. This particularly applies to brilliant green used in *Salmonella* media.

4.7 Peptone A general purpose peptone is recommended for the preparation of routine bacteriological media. Any peptone which comparative tests have shown to give satisfactory results may be used.

4.8 Proteose peptone A specialized peptone prepared by papain digestion of selected fresh meat, that is used in media mainly for the production of bacterial toxins. Any proteose peptone preparation which comparative tests have shown to give satisfactory results may be used.

4.9 Yeast extract Yeast extract is the water soluble extract of lysed yeast cells. Any yeast extract shown to yield satisfactory results may be used. Unless otherwise specified, the quantities of yeast extract indicated in the formulations refer to yeast extract powder.

4.10 Water Unless otherwise specified, glass-distilled water, deionized water or water purified by reverse osmosis shall be used. The water shall be free from toxic substances.

NOTES:

- 1 Where appropriate in these methods, water of suitable quality may be referred to as 'laboratory-grade water'.
- 2 Where deionized water is used, precautions should be taken against microbial build-up in the deionizing equipment.
- 3 Routine conductivity determinations are recommended for checking the purity of laboratory-grade water.
- 4 Attention is drawn to the possibility of distillate from chlorinated water containing chlorine.

5 FILTERING

5.1 Clearing agar media For quantities greater than 500 mL, filter through paper pulp. The filter shall be preheated with warm water when hot agar is to be filtered.

5.2 Sterilization by filtration Where sterilization by filtration is specified, a Seitz filter or a membrane filter of 0.2 µm pore size or a Seitz-type filter with a sterilizing pad shall be used.

6 pH OF MEDIA The pH of media shall be read when the temperature of the medium is below 50°C. The pH can be adjusted with appropriate acid or alkali.

NOTE: When adjusting the pH before autoclaving, take into account that the pH of most media falls by about 0.2 units during autoclaving.

7 STERILIZATION It is most important that the specified time-temperature conditions of sterilization are strictly observed to ensure sterility. Overheating can also have adverse effects on culture media.

8 COMMERCIAL MEDIA In most cases dehydrated media of similar performance to those described here are available commercially, although the concentration of some ingredients may differ slightly. These commercial media should be prepared for use in accordance with the manufacturers' instructions. Several of the media may be prepared by supplementing commercial products; for example, mannitol selenite cystine broth may be prepared by the addition of cystine to commercially prepared mannitol selenite broth.

9 QUALITY CONTROL OF CULTURE MEDIA Because of batch-to-batch variation, the properties of each batch of each medium shall be verified by inoculation with the appropriate microorganisms. Selective media shall be checked for their growth-

promoting and growth-inhibiting properties with standard reference cultures known to give positive and negative results. In addition, all batches of prepared media shall be checked for sterility and pH.

10 STORAGE AND SHELF LIFE OF PREPARED MEDIA The formulation, packaging and storage conditions affect a medium's susceptibility to deterioration, both before and after it is made up for use, and experience is required to determine shelf life and performance. However, the following conditions should be observed generally to obtain optimum shelf life:

- (a) The storage temperature for the majority of prepared media is approximately 4°C.
- (b) Most liquid media will keep at 4°C for many months but some have a tendency to form deposits, especially those made up at double strength.
- (c) Media containing dyes should be kept in the dark to avoid fading.
- (d) Volume checks should be made on older stock which may be affected by evaporation.
- (e) Solid media can be preserved for many months if stored in an airtight container or in a bag.
- (f) In media where the pH is low (<5.0), softening of agar gel may take place.
- (g) Semi-solid media are better stored at room temperature unless sealed in an airtight container.
- (h) Agar plates must be stored so as to prevent contamination and dehydration. Moisture loss may be reduced by wrapping plates in plastic bags during storage at 4°C.
- (i) Minimal exposure to sunlight prevents the formation of peroxides which may affect the performance of the medium.

11 FORMULARY OF MEDIA, DILUENTS AND REAGENTS

ASPARAGINE BROTH

Ref. GEORGIA, F.R. and POE, C.F. *J. Bacteriol.* 1931, Vol. 22, p. 349.

Asparagine, DL	3.0 g
Dipotassium hydrogen phosphate, K_2HPO_4	1.0 g
Magnesium sulfate heptahydrate, $MgSO_4 \cdot 7H_2O$	0.5 g
Water	1.0 L

Dissolve all the ingredients and make up to 1 L. Check and if necessary adjust pH to 6.9 to 7.2 before sterilization. Dispense in 10 mL quantities in 16 mm OD test tubes and sterilize by autoclaving at 121°C for 15 min.

BILE AESCULIN AGAR

Ref. SWAN, A. *J. Clin. Pathol.* 1954, Vol. 1, p. 160.

Peptone	8.0 g
Bile salts	20.0 g
Ferric citrate	0.5 g
Aesculin	1.0 g
Agar	15.0 g
Water	1.0 L

Suspend the ingredients in the water and bring gently to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 min.

BISMUTH SULFITE AGAR

Ref. WILSON, W.J. *J. Hyg. Camb.* 1988, Vol. 38, p. 507.
 COOKE, G.T. *J. Path. Bacteriol.* 1952, Vol. 64, p. 559.

NOTE: The commercially available form of this medium may have special storage directions which should be observed.

Preparation of complete medium

Basal medium (see below) 100.0 mL
 Stock bismuth sulfite solution (see below) 20.0 mL
 Stock brilliant green solution (see below) 4.5 mL

Melt the basal medium and cool to 50°C. Warm the two stock solutions to 50°C, add to the basal medium, mix well and adjust pH to 7.6 ± 0.2. Dispense 20 mL quantities into sterile Petri dishes and allow the plates to set. Store inverted at 4°C and use within 5 days.

Basal medium

Beef extract 5.0 g
 Peptone 10.0 g
 Sodium chloride 5.0 g
 Agar 30.0 g
 Water 1.0 L

Dissolve the ingredients in the water by heating in a steamer, dispense 100 mL quantities into 250 mL flasks and autoclave at 121°C for 20 min.

Stock bismuth sulfite solution

Bismuth ammonium citrate scales 6.0 g
 Sodium sulfite, Na₂SO₃ 20.0 g
 Disodium hydrogen phosphate, Na₂HPO₄ 10.0 g
 D-glucose monohydrate 10.0 g
 Water 200.0 mL

Dissolve the bismuth ammonium citrate in 50 mL of boiling water and the sodium sulfite in 100 mL of boiling water. Mix the two solutions and, while still boiling the mixture, add the sodium phosphate and stir until dissolved.

Cool and add the glucose dissolved in the remaining 50 mL of water.

Store in a refrigerator.

Stock brilliant green solution

Ferric citrate scales 2.0 g
 Brilliant green, 10 g/L aqueous solution 25.0 mL
 Water 200.0 mL

NOTE: Not all batches of brilliant green dye give satisfactory performance and new batches should be checked.

BRAIN HEART INFUSION AGAR Brain heart infusion agar is prepared in the same way as brain heart infusion broth except that 15 g/L agar is included in the formulation.

BRAIN HEART INFUSION BROTH

Ref. Difco Manual. 9th ed. 1963.

Calf brain infusion (see below) 200.0 g
 Beef heart infusion (see below) 250.0 g
 Peptone or proteose peptone 10.0 g
 Sodium chloride 5.0 g

Disodium hydrogen phosphate, Na_2HPO_4 2.5 g
 D-glucose monohydrate 2.0 g
 Water to make 1.0 L

Mix the ingredients, heat to boiling with agitation to obtain complete solution, adjust pH to give a pH of 7.4 ± 0.1 after sterilization, dispense as required and sterilize by autoclaving at 121°C for 15 min.

Preparation of calf brain infusion

Finely mince 200 g of fresh calf brain, mix with 400 mL of water and store overnight in the refrigerator. Strain through muslin and express the brain residue. Skim off any fat with filter paper, boil for 15 min or steam in a steam sterilizer for 2 h, filter through paper and adjust volume to 400 mL with water.

Preparation of beef heart infusion

Finely mince 250 g of lean fresh beef or ox heart, mix with 500 mL of water and store overnight in a refrigerator. Strain through muslin and express the meat residue. Skim off any fat with filter paper, boil for 15 min or steam in a steam sterilizer for 2 h, filter through paper and adjust volume to 500 mL with water.

BUFFERED PEPTONE WATER

Ref. EDEL, W. and KAMPELMACHER, E.H. *Bull. W.H.O.* 1973, Vol. 48, p. 167.

Peptone 10.0 g
 Sodium chloride 5.0 g
 Disodium hydrogen phosphate dodecahydrate, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 9.0 g
 Potassium dihydrogen phosphate KH_2PO_4 1.5 g
 Water 1.0 L

Dissolve the ingredients in the water, adjust pH to 7.0 ± 0.1 , dispense as required and autoclave at 121°C for 15 min.

CATALASE REAGENT

Ref. MACFADDIN, J.F. *Biochemical Tests for Identification of Medical Bacteria*. 2nd ed. Baltimore: Williams and Wilkins, 1980.

FUNG, D.Y.C. and PETRISHKO, D.T. *Appl. Microbiol.* 1973, Vol. 26, p. 631.

ISENBERG, H.D. *Clinical Procedures Handbook*. Section 1.20. Washington DC: American Society for Microbiology, 1993.

Reagent

The reagent is hydrogen peroxide, H_2O_2 , 0.1% m/m aqueous solution.

Store the reagent in a dark bottle and avoid undue exposure to light. Store in a refrigerator when not in use.

Explanation of the catalase test

Catalase enzyme is involved in the breakdown of hydrogen peroxide into oxygen and water. When a catalase-positive organism is emulsified with hydrogen peroxide it releases oxygen which is observed as bubbles. The test is carried out as follows:

- (a) Using commercial capillary tubes (outer diameter 1 mm and length 67 mm) draw up the catalase reagent by capillary action to a height of about 20 mm.
- (b) Touch the centre of a colony from an 18 h to 24 h growth with the reagent-filled capillary tube.

NOTE: Do not perform the test on colonies that are more than 24 h old. The enzyme is present only in viable cultures and older cultures may give false-negative reactions.