AS 4276.17.2:2016

Australian Standard®

Water microbiology

Method 17.2: Spores of *Clostridium perfringens*—Estimation of most probable number (MPN) using the multiple tube dilution technique

PREFACE

This Standard was prepared by the Standards Australia Committee FT-020, Water Microbiology, to supersede AS/NZS 4276.17.2:2000, *Water microbiology*, Method 17.2: *Spores of sulfite-reducing anaerobes (clostridia) including Clostridium perfringens—Estimation of most probable number (MPN) using the multiple tube dilution technique*.

The objectives of this revision are—

(a) to incorporate an additional more selective medium [oleandomycin polymyxin sulphadiazine perfringens (OPSP) agar] for testing of samples with a greater proportion of competing microflora and to incorporate other technical variations;

(b) to incorporate culture media, reagents and to remove reference to AS 4276.2;

(c) to update reference cultures; and

(d) to update the references.

There is no ISO Standard for estimation of most probable number (MPN) using the multiple tube dilution technique for spores of *Clostridium perfringens*.

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.

FOREWORD

The spores of sulfite-reducing anaerobes such as *Clostridium perfringens* are widespread in the environment. They are present in human and animal faecal material, in waste water and in soil. Unlike *Escherichia coli* and other coliform bacteria, spores of *Clostridium perfringens* survive in water for months as they are more resistant to the action of chemical agents (e.g. chlorination) and physical conditions (e.g. heat) than vegetative forms and may be used as indicators of water quality.

As *Clostridium perfringens* is associated with faecal contamination, it can indicate remote or intermittent pollution in the absence of other faecal indicator microorganisms.

Monitoring of *C. perfringens* has proven useful for the assessment of the quality of water resources and to check the stages of water treatment to evaluate treatment efficiency.

METHOD

1 SCOPE

This Standard sets out a method for determining the most probable number (MPN) of spores of *C. perfringens* in water, using a multiple tube dilution technique, with a confirmation procedure for identifying *C. perfringens*.

Refer to AS/NZS 4276.1 for a description of the general procedure for estimating the MPN of microorganisms.

MPN procedures may be used for the examination of all water samples and are particularly applicable for samples containing sufficient suspended material to make filtration procedures impractical.

NOTE: A flow diagram of the procedure is shown in Appendix A.

2 REFERENCED DOCUMENTS

The following documents are referred to in this Standard:

AS		
2031	Water quality—Sampling for microbiological analysis	
5013	Food microbiology	
5013.16	Method 16:	Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of *Clostridium perfringens*—Colony-count technique
AS/NZS		
4276	Water microbiology	
4276.1	Method 1:	General information and procedures(ISO 8199:2005, MOD)

3 DEFINITIONS

For the purposes of this Standard the following definitions apply:

3.1 Presumptive *C. perfringens*

Bacteria that produce all shades of black or grey to yellow-brown colonies, even if the colour is faint, after anaerobic incubation at 36 ±2°C or 44 ±1°C for 21 ±3 h, on tryptose-sulfite-cycloserine (TSC) agar at 44 ±1°C or oleandomycin polymyxin sulphadiazine perfringens (OPSP) agar at 36 ±2°C or 44 ±1°C.

3.2 Confirmed *C. perfringens*

Anaerobic bacteria that produce characteristic colonies on TSC or OPSP agar, capable of growth at 44 ±1°C, and possess the enzyme acid phosphatase.

4 PRINCIPLE

A series of dilutions are inoculated into the differential reinforced clostridial medium (DRCM) in accordance with the MPN method in AS/NZS 4276.1. Tubes are incubated anaerobically at 44 ±1°C for 48 ±4 h. A loopful of broth from each blackened DRCM tubes is subcultured onto TSC or OPSP medium to obtain isolated colonies for confirmation.

This method consists of the following stages:

(a) A heating procedure to eliminate non-spore-forming microorganisms from the test sample.

(b) A multiple tube dilution cultural procedure to selectively detect H_2S producing anaerobic microorganisms in the heat treated sample.

(c) A confirmation procedure to identify *C. perfringens* amongst the H_2S producing microorganisms.

Incubation at 44°C increases the selectivity of the test for *C. perfringens*.

The MPN is determined by reference to an MPN table and the result is reported as the most probable number per 100 mL.

5 MEDIA, REAGENTS, AND REFERENCE CULTURES

5.1 Culture media (see Appendix B)

5.1.1 *Differential reinforced clostridial medium (DRCM)*

DRCM should be distributed in screw-capped bottles instead of tubes, with sufficient depth of medium to ensure anaerobiosis during incubation.

5.1.2 *Tryptose sulfite cycloserine (TSC) agar*

5.1.3 *Oleandomycin polymyxin sulphadiazine perfringens (OPSP) agar*

5.1.4 *Columbia blood agar or other suitable nutrient-rich agar*

5.1.5 *Columbia blood agar with neomycin (0.01%)*

5.1.6 *Acid phosphatase reagent*

5.2 Reference cultures

5.2.1 *Positive culture*

C. perfringens ATCC 13124, NCTC 8237, WDCM 00007 or a culture traceable to any of these strains.

5.2.2 *Negative culture*

C. sporogenes ATCC 19404, WDCM 00008 or a culture traceable to any of these strains.

5.2.3 *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 growth characteristics and test reactions are exhibited by the cultures on the media and in the tests used in this method.

When testing a sample of water by this standard method, a culture of the positive reference microorganism (5.2.1) shall be submitted to the test procedures at the same time to demonstrate and ensure that typical growth characteristics and test reactions are exhibited. The negative reference microoganism (5.2.2) is used in addition to the positive reference microorganism for the acid phosphatase test.

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 that invalidate the quality control procedures, e.g. prepared suspension shown not to contain target bacteria.

NOTE:

ATCC—American Type Culture Collection

NCTC—National Collection of Type Cultures

WDCM—World Data Centre for Microorganisms

6 APPARATUS

6.1 Screw cap containers

Suitable for the volume of water to be tested.

6.2 Pipettes or equivalent measuring devices

1 mL, 10 mL, 50 mL

6.3 Anaerobic incubators/jar

With appropriate equipment and materials to generate an atmosphere of approximately 90% hydrogen and 10% carbon dioxide.

6.4 Incubators or water bath

Able to maintain at 36 ±2°C, 44 ±1°C and 60 ±2°C.

7 SAMPLES

Sample holding and storage times shall be in accordance with AS 2031.

8 TEST PROCEDURES

The procedure shall be as follows:

(a) Heat the sample to 60 ±2°C in a waterbath and maintain this temperature for 15 ±1 min. The volume heated should be greater than the volume to be analysed. The temperature should be monitored by placing an appropriate thermometer in a reference bottle of the same size as the sample bottle and containing the same volume of water at the same initial temperature as the sample being treated. The time taken to reach 60 ±2°C shall not exceed 15 min and can be minimized by ensuring the water in the water bath is circulated to maximize heat exchange.

(b) Using DRCM heated by bringing to the boil or steaming for 5 minutes to drive off oxygen and cooled immediately before use, prepare and inoculate bottles with appropriate volumes dilutions of the heated sample, as described for the MPN method in AS/NZS 4276.1. Top-up with single strength medium so as to leave a minimal amount of air space.

(c) Incubate the bottles at 44 ±1°C for 48 ±4 h. A positive reaction is shown by blackening of the medium.

NOTE: Large volumes of culture in sealed bottles may explode. Bottles may be sealed in plastic bags in order to contain any breakage or spillage during incubation. Alternatively, the bottles may be incubated anaerobically with the caps loosened and with sufficient headspace to avoid explosions.

9 CONFIRMATION OF *C. PERFRINGENS*

9.1 Waters with low levels of competing spore forming bacteria

The procedure shall be as follows: Streak a loopful of broth from blackened DRCM tubes onto TSC agar plates to obtain single colonies and incubate anaerobically at 44 ±1°C for 21 ±3 h.

9.2 Waters with high levels of competing spore forming bacteria

The procedure shall be as follows: Streak a loopful of broth from blackened DRCM tubes onto OPSP agar plates to obtain single colonies and incubate anaerobically at 36 ±2°C for 21 ±3 h.

When interpretation of results becomes problematic due to interference from spreading micro-organisms or unacceptable confirmation rates (presumptive *C. perfringens* colonies not confirming), or when samples have a history of problematic results, OPSP agar may be incubated at 44 ±1°C.

NOTE: Increasing the selectivity of a procedure may not recover some strains of the target microorganism.

9.3 Selection of colonies

The procedure shall be as follows:

(a) *C. perfringens* produces black or grey to yellow-brown colonies on TSC and OPSP agar. Since the black colour of the colonies rapidly fades and finally disappears under aerobic conditions, the plates have to be counted within 30 min after completion of the anaerobic incubation. If a number of anaerobic jars are used, the plates should be checked jar by jar or in portions if the incubation was performed in an anaerobic incubator.

(b) The target colonies are subcultured onto Columbia blood agar or a suitable nutrient-rich agar with or without blood (e.g. Columbia agar base, tryptone soya agar). For waters with high background counts or where other sulfite reducing bacteria may make it difficult to isolate *C. perfringens* (e.g. spreading colonies), the use of Columbia blood agar (or a suitable nutrient rich agar containing blood) with neomycin (0.01%) may be beneficial.

(c) Incubate anaerobically at 44 ±1°C for 21 ±3 h.

NOTE: Non-selective media are preferred when undertaking confirmation testing. Generally, there are some strains of a target microorganism that are inhibited by a given selective agent.

9.4 Biochemical confirmation

9.4.1 *Acid phosphatase test*

The procedure shall be as follows: Pre-warm acid phosphatase reagent to approximately 36°C for 30–60 minutes prior to use. Add 2 to 3 drops of the acid phosphatase reagent onto filter paper and spread colonies, grown anaerobically on Columbia blood or a suitable nutrient rich agar plate, onto the phosphate reagent soaked paper. A purplish colour developing within 3 to 4 min is considered as a positive reaction.

9.4.2 *Alternative biochemical tests*

Confirmation may be performed in accordance with AS 5013.16.

9.5 Confirmed *C. perfringens*

C. perfringens produces growth and blackening in DRCM at 44 ±1°C, produces black or grey to yellow-brown colonies on TSC or OPSP agar, even if the colour is faint, and possesses acid phosphatase or produces reactions as described in AS 5013.16.

10 CALCULATIONS

Calculation of results shall be carried out as described in AS/NZS 4276.1.

11 REPORT

The report shall contain the following information:

(a) All details necessary for the complete identification of the sample.

(b) Date of testing.

(c) Reference to this Australian Standard, i.e. AS 4276.17.2.

(d) The MPN of confirmed spores of *C. perfringens* per 100 mL of sample.

(e) Any circumstances or conditions that may have influenced the results.

12 BIBLIOGRAPHY

Information given in the following literature was taken into account in the preparation of this Standard:

1 HANDFORD, PM. A new medium for the detection and enumeration of *Clostridium perfringens* in foods, *J. Appl. Bact.*, 1974, Vol. 37. pp. 559–570.

2 HARMON, SM, KAUTTER, DA and PEELER, JT. Improved medium for enumeration of *Clostridium perfringens. Appl. Microbiol*., 1971, Vol. 22, pp. 688–692.

3 ISO 14189:2013, *Water quality—Enumeration of Clostridium perfringens—Method using membrane filtration*

4 LOWBURY, EJL and LILLY, HA, A selective plate medium for *Cl. welchii*. *Journal Of Pathology And Bacteriology*, 1955, Volume 70, Issue 1, pp. 105–109.

5 Report on Public Health and Medical Subjects No. 71, 1994, *Methods for the Examination of Waters and Associated Materials. The Microbiology of water*, Part 1: *Drinking water*, pp. 78–80, HMSO.

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7 SARTORY, DP, WALDOCK, R, DAVIES, CE and FIELD, AM. Evaluation of acid phosphatase as a confirmation test for *Clostridium perfringens* isolated from water. *Lett. Appl. Microbiol*., 2006, 42, pp. 418–424.

8 UENO, K, FUJII, H, MARUI, T, TAKAHASHI, J, SUGITANI, T, USHIJIMA, T and SUZUKI, S. Acid phosphatase in *Clostridium perfringens*. A new rapid and simple identification method., *Jpn J Microbiol*, 1970, Vol. 14, pp. 171–173.

9 WOHLSEN, T, BAYLISS, J, GRAY, B, BATES, J and KATOULI, M. Evaluation of an alternative method for the enumeration and confirmation of *Clostridium perfringens* from treated and untreated sewages. *Lett Appl Microbiol*., 2006 May, 42(5), 438–44.

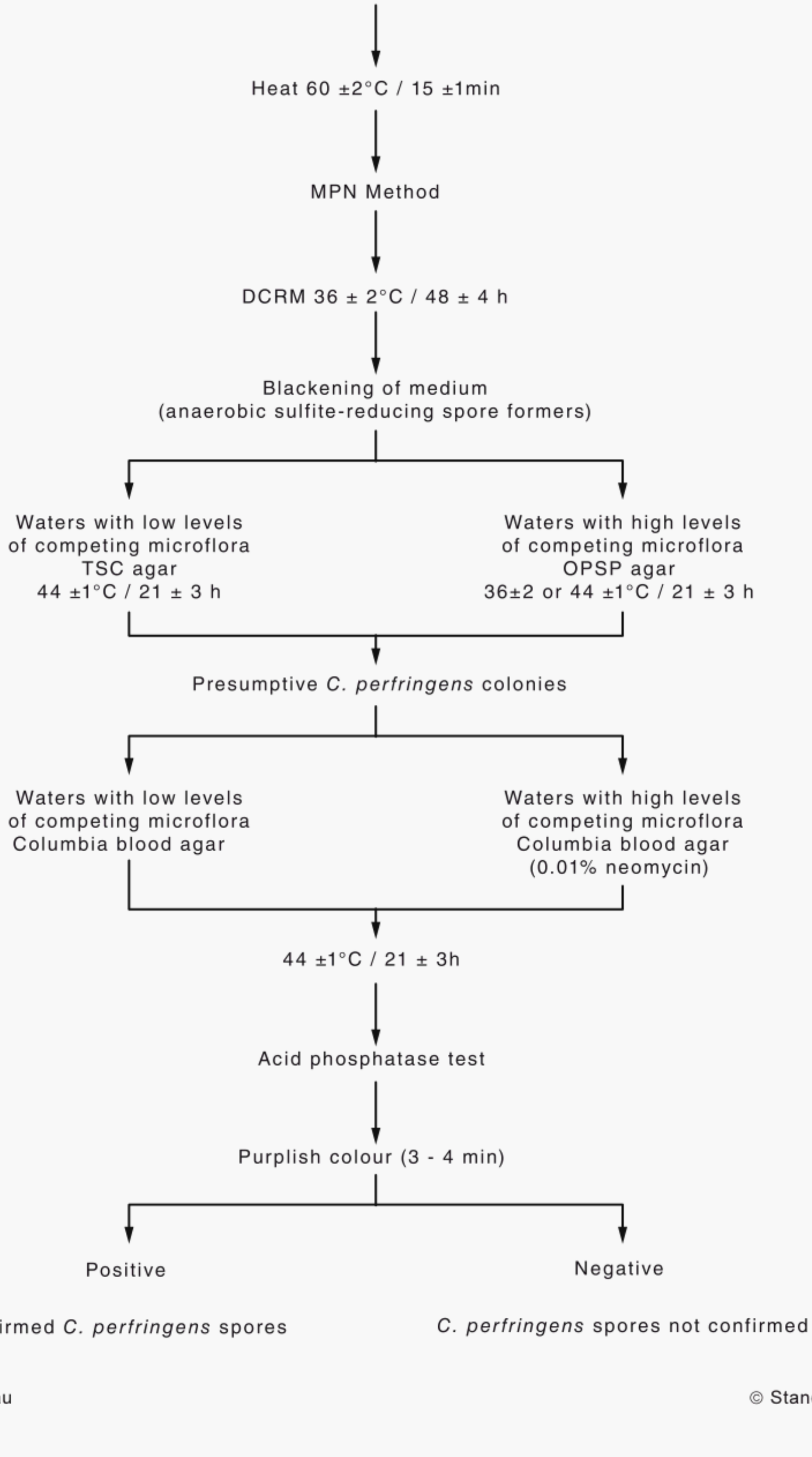
10 GIBBS, A and HIRSCH, A. *J. Appl. Bact*., 1956, Vol. 19, pp. 129–141.

11 Report on Public Health and Medical Subjects No. 71 1994.

APPENDIX A

FLOW CHART OF MPN EXAMINATION PROCEDURES FOR SPORES OF CLOSTRIDIA AND *CLOSTRIDIUM PERFRINGENS* IN WATER

(Informative)



APPENDIX B
CULTURE MEDIA
(Informative)

B1 SCOPE

This Appendix sets out the formulations and procedures for preparing the culture media specified in this Standard.

NOTES:

1 Equivalent formulations of the culture media and supplements listed in this Appendix are commercially available.

2 Freshly prepared culture plates which are used for the detection of H_2S production, will usually give a more distinct reaction than those which have been stored in the presence of oxygen.

B2 DIFFERENTIAL REINFORCED CLOSTRIDIAL MEDIUM (SINGLE STRENGTH)

Reference:

GIBBS, A and HIRSCH, A. *J. Appl. Bact*., 1956, Vol. 19, pp. 129–141.

Formulation

Tryptone .....5.0 g
Peptone .....5.0 g
Meat extract .....8.0 g
Yeast extract .....1.0 g
Glucose .....1.0 g
L-cystein HCI .....0.5 g
Sodium acetate .....5.0 g
Sodium disulfite .....0.5 g
Ferric ammonium citrate .....0.5 g
Sodium resazurin .....0.002 g
Water .....to 1.0 L

Dissolve the ingredients in water, adjust pH to 7.1 ±0.2, dispense into bottles, autoclave at 121 ±1°C/15 min. Store at 21°C.

NOTE: Use double quantities of ingredients in 1.0 L water for double strength medium.

B3 TRYPTOSE SULFITE CYCLOSERINE (TSC) AGAR

Reference:

HARMON, SM, KAUTTER, DA and PEELER, JT. *Appl. Microbiol*. 1971, Vol. 22, p. 688.

B3.1 TSC base agar

Tryptose .....15.0 g
Soy peptone .....5.0 g
Yeast extract .....5.0 g

Sodium metabisulfite .....1.0 g

Ferric ammonium citrate .....1.0 g

Agar.....20 g

Distilled water ..... to 1.0 L

B3.2 TSC selective agent (per 1 L TSC base)

D-cycloserine, filter-sterilized 1% aqueous solution ..... 40 mL

A 1% D-cycloserine aqueous solution is prepared by dissolving 1 g of D-cycloserine in 100 mL of distilled water and then filter sterilized. D-cycloserine powder is stored at –20 ±5°C. Aqueous solutions of D-cycloserine can be stored in aliquots at –20 ±5°C for up to 4 weeks and thawed just prior to use. Alternatively, aliquots can be stored at –70 ±10°C for a maximum of 12 months.

NOTE: If a commercial selective supplement is used, it will need to be reconstituted and used in accordance with the manufacturer's instructions.

B3.3 Preparation of complete TSC agar

Dissolve all the ingredients for the base agar in the distilled water, adjust to pH 7.6 ±0.1 and autoclave at 121 ±1°C/10 min. Allow to cool to 50°C and aseptically add the selective agent. Mix well and pour into sterile plates. These plates can be stored anaerobically at 5 ±3°C for up to 7 days.

Alternatively, because of the short shelf life and the need for anaerobic storage: when plates are required, solidified TSC base agar can be melted and cooled, followed by selective agent addition at the required rate for the complete medium.

B4 OLEANDOMYCIN POLYMYXIN SULPHADIAZINE PERFRINGENS (OPSP) AGAR

Reference:

HANDFORD, PM. A new medium for the detection and enumeration of *Clostridium perfringens* in foods, *J. Appl. Bact*., 1974, Vol. 37, pp. 559–570.

B4.1 OPSP base agar

Tryptose..... 15.0 g

Soy peptone .....5.0 g

Yeast extract .....5.0 g

Ferric ammonium citrate .....1.0 g

Sodium metabisulphite.....1.0 g

Sulphadiazine (10% (w/v) solution in 1N NaOH) ..... 1.0 mL

Agar.....20.0 g

Distilled water ..... 1.0 L

B4.2 OPSP selective agents (per 1 L OPSP base)

Oleandomycin phosphate, filter-sterilized 50 µg/mL aqueous solution ..... 10.0 mL

Polymyxin B, filter-sterilized 1000 IU/mL aqueous solution ..... 10.0 mL

Concentrated stock solution of olendomycin is prepared by dissolving 0.5 g of oleandomycin phosphate in 100 mL of distilled water (0.5% = 5000 µg/mL) and then filter sterilized. The diluted solution (50 µg/mL) is prepared by diluting the concentrate 1 in 100 in sterile deionized water.

Concentrated stock solution of polymyxin B is prepared by dissolving 500 000 IU of polymyxin B sulphate in 5 mL distilled water (100 000 IU/mL) and then filter sterilized. The diluted solution (1000 IU/mL) is prepared by diluting the concentrate 1 in 100 in sterile deionized water.

The concentrated stock solutions can be stored for up to 2 months and the diluted solutions for up to 1 week at 5 ±3°C.

NOTE: If commercial selective supplements are used, they will need to be reconstituted and used in accordance with the manufacturer's instructions.

B4.3 Preparation of complete OPSP agar

Dissolve all ingredients for the base agar in the distilled water and bring gently to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Allow to cool to 50°C and aseptically add the selective agents. Mix well and pour into sterile plates. These plates are stored anaerobically at 5 ±3°C for up to 7 days.

Alternatively, because of the short shelf life and the need for anaerobic storage: when plates are required, solidified OPSP base agar can be melted and cooled, followed by selective agent addition at the required rate for the complete medium.

The antibiotic concentrations in the complete medium are: sulphadiazine 0.1 mg/mL, oleandomycin phosphate 0.5 µg/mL, and polymyxin B 10 IU/mL.

B5 COLUMBIA BLOOD AGAR

Ellner PD, Stoessel CJ, Drakeford, E and Vasi, F. (1966) Tech. Bull. Reg. Med. Techn. 36. No. 3, reprinted in Amer. J. Clin. Path. (1966) 45. 502-504.

Pancreatic digest of casein .....10 g

Proteose peptone ..... 5 g

Yeast extract ..... 5 g

Beef heart digest ..... 3 g

Corn starch ..... 1 g

Sodium chloride ..... 5 g

Agar .....15 g

Distilled water ..... to 1.0 L

Blood—Defibrinated ..... 50.0 mL

Add all ingredients except the blood to 1 L of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C and add the sterile defibrinated blood. Mix well and pour into sterile dishes. Check final pH is pH 7.2 ±0.2 at 25°C.

B6 COLUMBIA BLOOD AGAR WITH NEOMYCIN (0.01%)

Reference:

LOWBURY, EJL and LILLY, HA, A selective plate medium for Cl. welchii. Journal of Pathology And Bacteriology, 1955, Vol. 70(1), pp. 105–109 (this reference only relates to the concentration of neomycin).

Prepare Columbia blood agar (B6) up to the point before 50 mL sterile defibrinated blood is added. Add 10 mL of a filter-sterilized 1% aqueous solution of neomycin with the blood to 1 L of the medium cooled to 50°C. Mix well and pour into sterile dishes. Check final pH is pH 7.4 ±0.2 at 25°C.

A 1% neomycin aqueous solution is prepared by dissolving 1 g of neomycin sulfate in 100 mL of distilled water and then filter sterilized.

B7 ACID PHOSPHATASE REAGENT

Reference:

STANDING COMMITTEE OF ANALYSTS, The Microbiology of Drinking Water (2010) – Part 6 – Methods for the isolation and enumeration of sulphite-reducing clostridia and Clostridium perfringens by membrane filtration. Methods for the Examination of Waters and Associated Materials. Environment Agency, England & Wales. 2010.

1-naphthylphosphate disodium salt.....0.4 g

Fast blue B salt [o-dianisidine bis (diazotized) zinc double salt].....0.8 g

Acetate buffer (pH 4.6 ±0.2) ..... 20.0 mL

Prepare the acetate buffer by dissolving 0.3 mL glacial acetic acid and 0.4 g sodium acetate in deionized water and make up to 100 mL. The final pH should be 4.6 ±0.2.

Dissolve the ingredients in the acetate buffer and allow to stand for 60 ±5 min at 5 ±3°C to allow to precipitate. Then filter the solution through a fluted filter to remove the precipitate. Store prepared solution at 5 ±3°C for no longer than two weeks. If precipitation occurs filter once more before use.

NOTE: Instead of 1-naphthylphosphate disodium salt, 1-naphthylphosphate monosodium salt may be used.

CAUTION: FAST BLUE B SALT IS TOXIC AND MAY CAUSE CANCER—TAKE APPROPRIATE PRECAUTIONS WHEN WEIGHING OUT, PREPARING AND DISCARDING THE REAGENT.

This Australian Standard® was prepared by Committee FT-020, Water Microbiology. It was approved on behalf of the Council of Standards Australia on 4 February 2016 and published on 12 April 2016.

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