



**Waters—Examination for *Legionella*
spp. including *Legionella*
pneumophila—Using concentration**



This Australian Standard® was prepared by Committee FT-020, Water Microbiology. It was approved on behalf of the Council of Standards Australia on 13 March 2017. This Standard was published on 10 May 2017.

The following are represented on Committee FT-020:

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

Additional Interests:

- Commercial Testing Laboratories
-

This Standard was issued in draft form for comment as DR AS 5132:2016.

Standards Australia wishes to acknowledge the participation of the expert individuals that contributed to the development of this Standard through their representation on the Committee and through the public comment period.

Keeping Standards up-to-date

Australian Standards® are living documents that reflect progress in science, technology and systems. To maintain their currency, all Standards are periodically reviewed, and new editions are published. Between editions, amendments may be issued.

Standards may also be withdrawn. It is important that readers assure themselves they are using a current Standard, which should include any amendments that may have been published since the Standard was published.

Detailed information about Australian Standards, drafts, amendments and new projects can be found by visiting www.standards.org.au

Standards Australia welcomes suggestions for improvements, and encourages readers to notify us immediately of any apparent inaccuracies or ambiguities. Contact us via email at mail@standards.org.au, or write to Standards Australia, GPO Box 476, Sydney, NSW 2001.

Australian Standard[®]

**Waters—Examination for *Legionella*
spp. including *Legionella*
pneumophila—Using concentration**

First published as AS 5132:2017.

COPYRIGHT

© Standards Australia Limited

All rights are reserved. No part of this work may be reproduced or copied in any form or by any means, electronic or mechanical, including photocopying, without the written permission of the publisher, unless otherwise permitted under the Copyright Act 1968.

Published by SAI Global Limited under licence from Standards Australia Limited, GPO Box 476, Sydney, NSW 2001, Australia

ISBN 978 1 76035 746 7

PREFACE

This Standard was prepared by the Standards Australia Committee FT-020, Water Microbiology, as a new Standard to complement AS 3896:201X, *Waters—Examination for Legionellae spp including Legionella pneumophila*. This Standard stems from a need, expressed by health authorities and other stakeholders, for a standard method for the enumeration of legionellae in waters with low background microflora in treated water systems, particularly in reticulated water systems supplying warm water systems.

Committee FT-020 did not recommend the adoption of ISO 11731:1998, *Water quality—Detection and enumeration of Legionella*, and ISO 11731-2:2004, *Water quality—Detection and enumeration of Legionella, Part 2: Direct membrane filtration method for waters with low bacterial counts* because these two ISO Standards are currently under review.

This Standard is suitable for testing water samples where low microbial loads are expected. The target water matrix for this Standard is samples from reticulated water systems where colonization by *Legionella* spp. is of public health concern. While this Standard is not restricted to this matrix, it may not be suitable for some environmental samples, such as industrial, waste and natural waters. This is because samples are concentrated and the high microbial loads in these samples could interfere with the detection of *Legionella* spp., leading to the possibility of false negative results.

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.

CONTENTS

	<i>Page</i>
FOREWORD.....	4
1 SCOPE.....	5
2 REFERENCED DOCUMENTS.....	5
3 PRINCIPLE	5
4 SAFETY PRECAUTIONS.....	6
5 CULTURE MEDIA, REAGENTS AND REFERENCE CULTURES.....	6
6 APPARATUS	7
7 SAMPLING, TRANSPORT AND STORAGE	8
8 PROCEDURE	8
9 CALCULATIONS	11
10 TEST REPORT	12
APPENDICES	
A FLOW CHART FOR <i>LEGIONELLA</i> SPP. TESTING	14
B CULTURE MEDIA AND REAGENTS.....	15
C GUIDANCE ON SAMPLING FROM BUILDING WATER SYSTEMS	22
D EXAMPLES OF TYPICAL <i>LEGIONELLA</i> COLONIES.....	24
BIBLIOGRAPHY.....	27

FOREWORD

This Standard stems from a need, expressed by health authorities and other stakeholders, for a standard method for the enumeration of legionellae in waters with low background microflora, particularly in reticulated warm water systems. Such systems have been implicated in cases and outbreaks of legionellosis. This Standard provides a tool for investigating water systems linked to legionellosis, as well as for monitoring the effectiveness of measures adopted for preventing the proliferation of legionellae in such systems.

The method is suitable for use in a laboratory equipped to carry out routine microbiological work.

Legionella pneumophila serogroup 1 causes ‘Legionnaire’s’ disease. However, some other *Legionella* are potentially pathogenic and may cause legionellosis. This Standard is designed to estimate the number of *L. pneumophila* and a range of other *Legionella* spp., the presence of which may indicate poorly managed water systems.

Some *Legionella* can be identified with commercially available systems. Identification of other species may require specialized testing that is not normally available in routine testing laboratories.

Strain characterization of *Legionella* isolates required as part of outbreak investigations is not within the scope of this Standard. This is normally carried out by public health microbiology laboratories. Other laboratories that isolate *Legionella* strains related to outbreaks should refer any *Legionella* isolates to a laboratory with *Legionella* strain characterization capability.

STANDARDS AUSTRALIA

Australian Standard

Waters—Examination for *Legionella* spp. including *Legionella pneumophila*—Using concentration**1 SCOPE**

This Standard sets out a method for isolating and estimating the number of *Legionella pneumophila* and a range of other *Legionella* spp. in water with low microbial loads. The target matrix for this Standard is water samples from reticulated water systems where colonisation by *Legionella* spp. is of public health concern. While this Standard is not restricted to this matrix, it may not be suitable for some environmental and recreational samples, such as industrial, waste and natural waters. This is because samples are concentrated and the high microbial loads in these samples could interfere with the detection of *Legionella* spp., leading to the possibility of false negative results.

This Standard has a lower limit of detection than AS 3896. Therefore, it is suitable when—

- (a) the detection of lower levels is sought; or
- (b) higher precision is desired for levels close to the lower limit of detection of AS 3896.

NOTES:

- 1 A flow chart of the procedure is shown in Appendix A.
- 2 This method will isolate *L. pneumophila* but not all other *Legionella* spp. *L. pneumophila* serogroup 1 accounts for the majority of legionellae infections.
- 3 Conditions that favour the isolation of *L. pneumophila* do not necessarily apply to some *Legionella* spp. However, treatments and media included in this method are designed to enhance the recovery of other species in addition to *L. pneumophila*.

2 REFERENCED DOCUMENTS

The following documents are referred to in this Standard:

AS

- 2031 Water quality—Sampling for microbiological analysis (ISO 19458:2006, MOD)
- 3896 Waters—Examination for *Legionella* spp. including *Legionella pneumophila*

AS/NZS

- 2243 Safety in laboratories
- 2243.3 Part 3: Microbiological safety and containment
- 4276 Water microbiology
- 4276.1 Method 1: General information and procedures (ISO 8199:2005, MOD)

3 PRINCIPLE

The sample of water is concentrated by membrane filtration. Bacteria trapped on the membrane filter are resuspended in 10 mL of diluent. The concentrated sample is cultured, directly and following heat and acid treatments, onto specified media. Presumptive *Legionella* spp. colonies then undergo confirmatory testing. Confirmed *Legionella* isolates can be speciated and typed as required.

4 SAFETY PRECAUTIONS

The safety precautions for microbiological laboratories, as described in AS/NZS 2243.3, shall be observed.

Legionella cultures on solid culture media can be handled safely by trained staff on the open bench in a conventional microbiology laboratory conforming to Containment Level 2. Infection is caused by inhalation of the micororganism and it is advisable therefore to assess all procedures for their ability to produce aerosols. If in doubt, carry out the work in a biological safety cabinet.

5 CULTURE MEDIA, REAGENTS AND REFERENCE CULTURES

5.1 Culture media

The culture media shall be as follows:

- (a) Buffered charcoal yeast extract agar (BCYE).
- (b) Buffered charcoal yeast extract agar with MWY selective supplement (MWY) or buffered charcoal yeast extract agar with GVPC selective supplement (GVPC).
- (c) Buffered charcoal yeast extract agar with BMPA selective supplement (BMPA) or buffered charcoal yeast extract agar with selective supplements (BCYE+AB).
- (d) Buffered charcoal yeast extract medium without L-cysteine (BCYE-Cys) or blood agar (BA).
- (e) Diluent: 0.1% peptone or quarter strength Ringer's solution.

NOTE: See Appendix B.

5.2 Reagents and identification systems

5.2.1 Acid buffer

The acid buffer shall be prepared using the formulation and procedure.

NOTE: See Appendix B, Paragraph B11.

5.2.2 Speciation/typing system(s)

Speciation systems, using latex agglutination, direct or indirect immunofluorescence, mass spectrometric and molecular techniques are available for identifying known serogroups and/or genotypes of *L. pneumophila* and other *Legionella* spp.

The laboratory shall ensure that the system or systems used are suitable for the laboratory's requirements for identification/typing. Fitness for purpose may be demonstrated by reference to the manufacturer's validation data or to evaluation studies published in the scientific literature.

5.3 Reference cultures

5.3.1 Positive cultures

Two positive cultures (*Legionella pneumophila* and a non-*pneumophila* sp.) shall be used. The following choice of the non-*pneumophila* preference culture [see Clause 5.3.1(b) or 5.3.1(c)] is left to the discretion of the laboratory:

- (a) *Legionella pneumophila* serogroup 1 WDCM 00107 or a culture traceable to this strain.

NOTE: WDCM—World Data Centre for Microorganisms.

- (b) *Legionella micdadei* (synonym *Tatlockia micdadei*) ATCC 33218 or CIP 103882 or NCTC 11371 or a culture traceable to one of these strains.

NOTES:

- 1 There is currently no WDCM culture reference for this strain.
 - 2 ATCC—American Type Culture Collection.
 - 3 CIP—Collection of Institut Pasteur.
 - 4 NCTC—National Collection of Type Cultures.
- (c) *Legionella bozemanæ* (synonym *Fluoribacter bozemanæ*) ATCC 33217 or NCTC 11368 or a culture traceable to one of these strains.

NOTE: There is currently no WDCM culture reference for this strain.

5.3.2 Use of reference cultures

The reference cultures are used in 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.

As a minimum, the reference cultures shall be used to provide assurance that the method has been applied properly. Each day samples are processed by this method, suspensions of the positive reference cultures are submitted to the test procedure. Growth of the positive reference cultures obtained is taken through the procedure as far as any isolate obtained from the samples, including confirmation tests.

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.

5.4 Quality control system

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.

6 APPARATUS

The apparatus shall be as follows:

- (a) Membrane filtration equipment
- (b) Membrane filters, sterile, polycarbonate or polyethersulfone, diameter 47 mm to 142 mm with rated pore size of 0.2 µm.
NOTE: See Bibliography, Reference 1.
- (c) Screw cap sterile container, with sterile glass beads (diameter 2 mm to 3 mm), sufficient to form a single layer on the bottom of the container.
- (d) Vortex mixer.
- (e) Ultrasonic water bath, suitable for ensuring that the level of diluent covering the membrane is below the level of water in the water bath.
- (f) Pipette capable of delivering the required volume, i.e. 0.1 mL.
- (g) Water bath capable of operating at 50 ±1°C.
- (h) Incubator capable of operating at 36 ±2°C.
- (i) Plate (or stereo) microscope or hand lens, 10× to 20× magnification.
- (j) Long-wave (360 nm approximately) ultraviolet lamp.

NOTE: Usual microbiological apparatus and glassware are not listed.

7 SAMPLING, TRANSPORT AND STORAGE

Sampling, transport and storage shall be carried out in accordance with AS 2031.

NOTE: Appendix C provides guidance on sampling from building water systems.

8 PROCEDURE

8.1 General

The sample is concentrated by filtration. The bacteria retained on the membrane are resuspended in diluent and this suspension (concentrate) is then used for the isolation of legionellae.

8.2 Concentration of water samples

For a general description of the membrane filtration technique, see AS/NZS 4276.1. Filtration can be achieved by vacuum filtration or positive pressure filtration or using a peristaltic pump.

The water sample shall be filtered through a polycarbonate or polyethersulfone filter [see Clause 6(b)]. The volume filtered depends on the particulate content of the water and/or the desired detection level. The volume of sample filtered shall be recorded.

NOTE: Generally, concentration of 1000 mL (1 L) of the sample is recommended. This volume is practical in terms of sampling and analysis and achieves a degree of sensitivity that allows for the detection of what are regarded as low levels of *Legionella* contamination. It also simplifies the calculations and reporting. Concentrating 1000 mL of sample and resuspending in 10 mL achieves a concentration factor of 100 and a theoretical lower limit of detection of 100 colony forming units (cfu)/L.

8.3 Resuspension of microorganisms

8.3.1 General

The membrane filter shall be removed from the stand carefully with sterile forceps so as to avoid loss of residual deposit and the microorganisms trapped on the membrane filter resuspended in 10 mL sterile diluent. There are two different procedures that can be used to resuspend microorganisms: vortexing or sonication.

8.3.2 Vortexing

The procedure shall be as follows:

- (a) Place the filter directly into a sterile screw cap container with glass beads and cut into pieces, to aid resuspension, using sterile scissors.
- (b) Add 10 mL of sterile diluent and vortex vigorously for not less than 2 min.

This concentrate represents the prepared sample.

8.3.3 Sonication

The procedure shall be as follows:

- (a) Place the filter directly into a sterile screw cap container with glass beads and cut into pieces using sterile scissors.
- (b) Add 10 mL of sterile diluent and place the container in an ultrasonic water bath for a time interval that has been verified as providing maximum recovery.
- (c) Ensure that the level of diluent covering the membrane is below the level of water in the ultrasonic water bath.

This concentrate represents the prepared sample.