

NATIONAL STANDARD METHOD

# DETECTION AND ENUMERATION OF *LEGIONELLA* SPECIES IN BIOFILMS AND SEDIMENTS

W 15

Issued by Standards Unit, Evaluations and Standards Laboratory  
Centre for Infections

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## Suggested citation for this document:

Health Protection Agency (2006). *Detection and enumeration of Legionella species in biofilms and sediments*. National Standard Method W 15 Issue 1. [http://www.hpa-standardmethods.org.uk/pdf\\_sops.asp](http://www.hpa-standardmethods.org.uk/pdf_sops.asp).

**DETECTION AND ENUMERATION OF LEGIONELLA SPECIES IN BIOFILMS AND SEDIMENTS**  
Issue no: 1.2 Issue date: 11.08.06 Issued by: Standards Unit, Evaluations and Standards Laboratory, in conjunction with the Regional Food, Water and Environmental Coordinators Forum. Page 2 of 15

Reference no: W 15i1.2

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# AMENDMENT PROCEDURE

|                               |  |
|-------------------------------|--|
| Controlled document reference | W 15   |
| Controlled document title     | Detection and enumeration of <i>Legionella</i> species in biofilms and sediments |

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from [standards@hpa.org.uk](mailto:standards@hpa.org.uk).

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

| Amendment Number/ Date | Issue no. Discarded | Insert Issue no. | Page | Section(s) involved                             | Amendment  |
|------------------------|---------------------|------------------|------|---|--|
| 2/<br>11.08.06         | 1.1                 | 1.2              | 5    | <b>Background</b>                               | Updated to include the validity of ISO 11731 while it is under revision                                |
|                        |                     |                  | 10   | <b>6.5.1 Confirmation of Legionella species</b> | Heading amended to 'presumptive <i>Legionella</i> ...' and section updated to remove use of blood agar |
|                        |                     |                  | 12   | <b>9.1 Medium</b>                               | Quantitative statement added   |
|                        |                     |                  | 12   | <b>9.2 Confirmatory tests</b>                   | Negative control added   |

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# DETECTION AND ENUMERATION OF *LEGIONELLA* SPECIES IN BIOFILMS AND SEDIMENTS

## SCOPE OF DOCUMENT

This procedure describes a quantitative method for isolating strains of *Legionella* from biofilm or sediment samples.

## INTRODUCTION

### Background

*Legionella* species, the cause of legionellosis, are aquatic bacteria that are widespread in nature and have been found in water over a wide temperature range but grow best between 30°C and 40°C<sup>2</sup>. Their tolerance to relatively high temperatures probably helps them to colonise some artificial water systems that are often above ambient temperatures. *Legionella* are prevalent in artificial water systems, and legionellosis is transmitted from these via aerosols and occasionally by aspiration. Cooling towers are often linked to community-acquired outbreaks, and although hot water systems are commonly incriminated in nosocomial outbreaks, a variety of other sources have been described.

Water samples may be examined for *Legionella* species during epidemiological investigations as part of a local authority, industrial, or hospital surveillance programme, or to validate new biocide treatments or other control methods. The HSE<sup>3</sup> also recommends routine, at least quarterly, sampling of cooling towers and routine sampling of hot and cold water systems if the recommended temperature regime is not followed or has failed or if the local risk assessment suggests it is needed.

Even in hospital environments numbers of *Legionella* species are often low, rarely exceeding 1% of the total bacterial population. In contrast, the numbers in biofilm and sediments can be much higher, and samples may need to be diluted prior to analysis. The procedures described are based on BS 6068 (ISO 11731)<sup>4</sup>.

Note: ISO 11731 is now in two parts. The latest part, Part 2 (BS -ISO 11731 – 2:2004 BS 6068-4.18:2004 Water quality - Detection and enumeration of *Legionella* – Part 2: Direct membrane filtration method for waters with low bacterial counts. London: British Standards Institution; 1998.) is only suitable for disinfected waters with a low background count and utilises filtration followed by the direct placement of the membrane onto the selective medium. The original ISO 11731<sup>4</sup> is still valid and currently under revision. When revision is completed it will subsequently be called ISO 11731 Part 1. The HPA has not adopted ISO 11731 Part 2 as one of its standard methods but will continue to follow ISO 11731 Part 1.

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# 1 DEFINITIONS

For the purpose of this method *Legionella* is defined as a genus of Gram-negative organisms normally capable of growth in not less than 2 days on buffered charcoal yeast extract (BCYE) agar containing L-cysteine and a source of iron. They form colonies, often white, purple to blue, or lime green in colour, which may fluoresce under long wavelength ultraviolet light. The colonies have a ground glass or opalescent appearance when viewed with a low-power plate microscope. Growth does not occur in the absence of cysteine.

# 2 PRINCIPLE

A suspension of biofilm or sediment sample is prepared in sterile diluent or water. A portion of this suspension remains untreated while another portion is subjected to treatment with acid, and another portion to treatment with heat.

Treated and untreated portions are inoculated onto plates of an agar medium selective for *Legionella* species and incubated. After incubation, morphologically characteristic colonies, which form on the selective medium, are regarded as presumptive colonies of *Legionella* species and are counted. Presumptive colonies of *Legionella* are sub-cultured to test their growth requirement for L-cysteine.

# 3 SAFETY CONSIDERATIONS<sup>5-14</sup>

Normal microbiology laboratory precautions apply.

## 3.1 SAMPLE COLLECTION

N/A

## 3.2 SAMPLE TRANSPORT AND STORAGE

Compliance with current postal and transportation regulations is essential.

## 3.3 SAMPLE PROCESSING

- Although *Legionella* are Hazard Group 2 organisms, shaking or vortex mixing of the concentrates should be performed in a microbiological safety cabinet conforming to EN12469:2000 Biotechnology - Performance criteria for microbiological safety cabinet
- Centrifuge buckets should be opened within a microbiological safety cabinet. If no leakage has occurred, tubes should be removed carefully so as not to disturb any deposit. If leakage has occurred, it should be dealt with according to normal laboratory safety procedures

***The above guidance should be supplemented with local COSHH and risk assessments***

# 4 EQUIPMENT

Usual laboratory equipment and in addition:

- Glass beads
- Vortex mixer
- Microbiological safety cabinet
- Waterbath at 50°C ± 1°C
- Incubator at 36°C ± 1°C (with humidification and preferably 2.5% CO<sub>2</sub>)

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- Plate stereoscopic microscope with oblique incident illumination
- Ultra violet lamp (long wavelength UV – 366 nm)
- Automatic pipettors and sterile pipette tips capable of delivering 0.1-10 mL volumes (optional)
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)
- Spreaders (sterile)

## 5 CULTURE MEDIA AND REAGENTS

### *Page's saline (optional)*

|                                 |        |
|---------------------------------|--------|
| Sodium chloride                 | 120 mg |
| Magnesium sulphate pentahydrate | 4 mg   |
| Calcium chloride dihydrate      | 4 mg   |
| Disodium hydrogen phosphate     | 142 mg |
| Potassium dihydrogen phosphate  | 136 mg |
| Water                           | 1 L    |
| pH 6.8 ± 0.2 at 25°C            |        |

### *1/40 Ringer's solution (optional)*

|                            |        |
|----------------------------|--------|
| Sodium chloride            | 225 mg |
| Potassium chloride         | 11 mg  |
| Calcium chloride anhydrous | 12 mg  |
| Sodium bicarbonate         | 5 mg   |
| Water                      | 1 L    |

### *Distilled or deionised water (optional)*

### *Double Strength Acid buffer pH 2.2:*

Mix 39 mL of 0.4 mol/L HCl with 250 mL of 0.4 mol/L potassium chloride solution. Adjust to pH 2.2 ± 0.2 by addition of a solution 1 mol/L potassium hydroxide (KOH). Store in a stoppered glass container in the dark at room temperature for not longer than 1 month.

### *0.4 mol/L Hydrochloric acid:*

Add 34.8 mL of concentrated HCl (specific gravity 1.18, minimum assay 35.4%, approx. 10 molar) to 1 L of water. The exact volume of acid may need to be adjusted according to the purity.

### *Potassium chloride (0.4 mol/L):*

Add 29.8 g of potassium chloride to 1 L of water

### *Glycine vancomycin polymyxin cyclohexamide (GVPC) agar*

|  |        |
|--|--------|
| Activated charcoal                                       | 2.0 g  |
| Yeast extract  | 10.0 g |
| Potassium hydroxide                                      | 2.8 g  |
| ACES buffer (N-2-acetamido-2-aminoethane sulphonic acid) | 10.0 g |
| Ferric pyrophosphate                                     | 0.25 g |
| L-cysteine hydrochloride monohydrate                     | 0.4 g  |

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|  |           |
|--|-----------|
| $\alpha$ -ketoglutarate monopotassium salt | 1.0 g     |
| Glycine (ammonium free)                    | 3.0 g     |
| Polymyxin B sulphate                       | 80,000 IU |
| Vancomycin hydrochloride                   | 1 mg      |
| Cyclohexamide                              | 80 mg     |
| Agar                                       | 13.0 g    |
| Water                                      | 1 L       |
| pH 6.8 $\pm$ 0.2 at 25°C                   |           |

Note: GVPN containing natamycin at 50 mg/L in place of cyclohexamide can be used as an alternative

*Buffered charcoal yeast extract agar with cysteine (BCYE+)*

|  |        |
|--|--------|
| Activated charcoal                                       | 2.0 g  |
| Yeast extract  | 10.0 g |
| Potassium hydroxide                                      | 2.8 g  |
| ACES buffer (N-2-acetamido-2-aminoethane sulphonic acid) | 10.0 g |
| Ferric pyrophosphate                                     | 0.25 g |
| L-cysteine hydrochloride monohydrate                     | 0.4 g  |
| $\alpha$ ketoglutarate, monopotassium salt               | 1.0 g  |
| Agar   | 13.0 g |
| Water  | 1 L    |
| pH 6.8 $\pm$ 0.2 at 25°C                                 |        |

*Buffered charcoal yeast extract agar without cysteine (BCYE-)*

|  |        |
|--|--------|
| Activated charcoal                                       | 2.0 g  |
| Yeast extract  | 10.0 g |
| Potassium hydroxide                                      | 2.8 g  |
| ACES buffer (N-2-acetamido-2-aminoethane sulphonic acid) | 10.0 g |
| $\alpha$ -ketoglutarate, monopotassium salt              | 1.0 g  |
| Ferric pyrophosphate                                     | 0.25 g |
| Agar   | 13.0 g |
| Water  | 1 L    |
| pH 6.8 $\pm$ 0.2 at 25°C                                 |        |

2% Formal saline (optional)

*Phosphate buffered saline (PBS)(Optional)*

|                                |        |
|--------------------------------|--------|
| Sodium chloride                | 8.0 g  |
| Potassium chloride             | 0.2 g  |
| Disodium hydrogen phosphate    | 1.15 g |
| Potassium dihydrogen phosphate | 0.2 g  |
| Water                          | 1 L    |
| pH 7.3 $\pm$ 0.2 at 25°C       |        |

Reagents for immunofluorescence staining (optional)

Commercial latex agglutination test kits (optional)

## 6 SAMPLE PROCESSING

### 6.1 SAMPLE PREPARATION

Sampling should be carried out by suitably trained personnel<sup>15</sup>. Add an excess of suitable inactivating agent (if available) to samples containing a biocide.

Samples of deposits and biofilms should be received and handled as described for water in National Standard Method W1: Section 5<sup>16</sup>. Samples should be transported at ambient temperature (instead of at 6°C to 18°C) and protected from sunlight.

Samples should be stored at room temperature and protected from the light until processed.

### 6.2 PREPARATION OF BIOFILM AND SEDIMENT SUSPENSIONS

Prepare a 1:10 and a 1:100 suspension of the sample in sterile diluent or water. If the sample cannot be pipetted, prepare the suspensions by weighing out the original sample, ie 1:10 (w/v).

Mix well by shaking or vortex mixing. To assist in mixing, add approximately 1 cm<sup>3</sup> of sterile glass beads to the sample. If the consistency of the initial sample permits, it can be used neat as well as diluted for further testing.

### 6.3 PRE-TREATMENT AND INOCULATION OF SELECTIVE AGAR PLATES

The concentrate is cultured in three ways: directly without further treatment; after heat pre-treatment; and after acid pre-treatment. Treatment and culturing procedures are described below.

Note: In outbreak investigations the unconcentrated original sample should also be cultured in the same manner. This applies particularly to samples from cooling towers and other non-potable waters.

#### 6.3.1 HEAT TREATMENT

Dispense 0.2 mL of the prepared suspension into a sterile screw-capped container and place it in a water bath at 50°C ± 1°C for 30 minutes ± 2 minutes.

Mix the heated sample well, and spread 0.1 mL over the whole surface of a GVPC or GVPN plate using a sterile spreader.

If a delay in spreading is likely, for example when handling large numbers of samples, cool the sample rapidly after heating.

#### 6.3.2 ACID TREATMENT

Dispense 0.2 mL of the test suspension into a sterile screw capped container. Add an equal volume of acid buffer at pH 2.2 and leave for 5 minutes ± 30 seconds.

Immediately spread 0.2 mL of treated sample on to the whole surface of a GVPC or GVPN plate.

When more than one sample is to be treated process in batches of no more than 5 adding the acid buffer at 1 minute intervals.

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### 6.3.3 UNTREATED

Prepare dilutions if necessary. Inoculate 0.1 mL of suspension onto the surface of a GVPC plate and spread using a sterile spreader.

### 6.3.4 STORAGE OF CONCENTRATES

Store in screw-capped containers at 6°C ± 2°C in the dark for up to 12 months.

Note: Re-testing of stored concentrates may be important in the investigation of outbreaks of Legionnaires' disease.

## 6.4 INCUBATION AND EXAMINATION OF SELECTIVE AGAR PLATES

Allow the inoculated media to stand until the inocula have been completely absorbed, place in a moist chamber and incubate at 36°C ± 1°C.

Incubation in an atmosphere of air with 2.5% CO<sub>2</sub> (v/v) may enhance the growth of some *Legionella* species. Incubate the plates for 10 days and examine on at least three occasions at intervals of 2 to 4 days during the incubation period.

If there is a heavy growth of non-*Legionella* colonies on a plate they can suppress the growth of any *Legionella* species present. If a heavy background growth is observed after incubation for 2 to 4 days a portion of the remaining stored concentrate should be diluted 100-fold and re-cultured.

## 6.5 COUNTING OF COLONIES

After incubation, examine colonies under a plate microscope with oblique incident light. Colonies of *Legionella* species have a typical 'ground-glass', pearly iridescent appearance. Characteristic colonies are smooth with an entire edge and often have a white or grey-blue-purple colour, but may be brown, pink, lime green or deep red.

Count all presumptive *Legionella* colonies on plates containing up to 150 colonies.

Autofluorescence can be an aid to identification when a sample contains several species of *Legionella*. Therefore, also examine plates under ultraviolet light (366 nm). Colonies of *Legionella bozemanii*, *Legionella gormanii*, *Legionella dumoffii*, *Legionella anisa*, *Legionella cherrii*, *Legionella steigerwaltii*, *Legionella gratiana*, *Legionella tucsonensis* and *Legionella parisiensis* fluoresce brilliant white, *Legionella rubrilucens* and *Legionella erythra* appear red. The latter species may only show fluorescence when grown at lower temperatures or left at room temperature for 48 hours. *Legionella pneumophila* does not autofluoresce and appears dull green or yellow.

Suspect colonies of each colony type present should be counted and recorded from each pre-treatment method, and a selection subjected to confirmatory tests.

### 6.5.1 CONFIRMATION OF PRESUMPTIVE *LEGIONELLA* SPECIES

Select three characteristic *Legionella* colonies from each of the GVPC agar plates. Subculture these to BCYE+ agar and BCYE- agar.

Place in an incubator at 36°C ± 1°C for at least 2 days. Colonies, which grow on BCYE+ but fail to grow on BCYE- are presumptive *Legionella* species. A confirmed count of each *Legionella* colony type is then calculated and recorded.

## 6.6 IDENTIFICATION AND TYPING

*Legionella* species can be identified by a variety of methods including immunofluorescence or latex agglutination. For routine purposes it is sufficient to identify strains to the level of *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2 - 14 or *Legionella* species other than *L. pneumophila*. During epidemiological investigations, isolates should be retained and sub-cultures sent to the reference laboratory for full identification and typing. As many as 30 colonies per plate may need to be retained

### 6.6.1 IMMUNOFLUORESCENCE

Ensure that the growth of *Legionella* species from a culture 2 to 4 days old is pure by examination of the colonial morphology (sub-culture to BCYE if necessary). Prepare a faintly turbid suspension of 3 colonies of *Legionella* in 1 to 2 mL of 2% formal saline (if this is inappropriate for the reagent supplied, follow the manufacturers method). Dilute if necessary in phosphate buffered saline. Prepare a positive control suspension in the same manner using *L. pneumophila* NCTC 12821 as a serogroup 1 control, *L. pneumophila* serogroup 6 NCTC 11287 as a serogroups 2-14 control, *L. micdadei* NCTC 11371 as a *Legionella* species control, and a negative control using the supplied negative control suspension.

Label PTFE multi-well slides using a separate slide for each of the controls and each strain to be identified. Follow the supplier's instructions for fixation and staining of the test antigen suspensions and controls.

Mount the slides by adding sufficient mountant to spread under a clean coverslip large enough to cover all wells (usually 2 to 3 drops). Ensure that no air bubbles are trapped between the slide and the coverslip. Examine the slides using the x 50 water immersion objective on the epifluorescent microscope. Regard cells that fluoresce bright green with the species-specific antibody as positive. Strains that show cross reactivity may be sent to the reference laboratory after discussion with them if it is considered appropriate.

### 6.6.2 LATEX AGGLUTINATION KITS

Follow the manufacturer's instructions.

## 7 CALCULATION OF RESULTS

Calculate the presumptive count per gram or mL for *Legionella* species as follows:

$$\text{Presumptive count} = \frac{\text{Number of colonies}}{\text{Volume on plate}} \times \text{Dilution factor}$$

Calculate the number of confirmed *Legionella* species per gram or mL as follows:

$$\text{Confirmed count} = \frac{\text{No. of colonies confirmed}}{\text{No. of colonies tested}} \times \text{Presumptive count}$$

## 8 REPORTING

If no colonies of *Legionella* species are present on the 10<sup>-1</sup> dilution, report as:

< 1.0 x 10<sup>2</sup> cfu/g or mL

If *Legionella* are detected at counts of 100 or greater per gram, report the count to 1 decimal point expressed to the power of 10 in the form of:

a x 10<sup>b</sup> cfu/g or mL

where **a** is the cfu count (to 1 decimal point) and **b** represents the appropriate power of ten.

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Round counts up if the last figure is 5 or more and down if the last figure is 4 or less: eg

1920 cfu /g reported as  $1.9 \times 10^3$  cfu/g

235,000 cfu/g reported as  $2.4 \times 10^5$  cfu/g

Any factors which may have an effect on the final result such as high levels of pseudomonads should be included in the interpretation and on the report.

## 9 QUALITY CONTROL

### 9.1 MEDIUM

It is essential that each flask of BCYE or GVPC prepared be treated as a separate batch of medium. Each batch of medium should be tested for the ability to support the growth of *L. pneumophila* and *L. bozemanii* and compared quantitatively with a previous batch of medium known to support growth of *L. pneumophila* and *L. bozemanii*. Counts from aliquots of the same test suspension should fall within an established 95% confidence interval (Table 8.1 in Microbiology of Drinking Water 2002 )<sup>17</sup>.

As some pseudomonas strains can also suppress the growth of legionellae on GVPC, the ability of the medium to suppress background organisms should also be determined. This can be achieved by preparing decimal dilutions of *Pseudomonas aeruginosa* NCTC 10662 and inoculating each onto a GVPC and a BCYE agar plate to give a target for counting of between 10 and 50 colonies. The medium is acceptable if at least a one log reduction is achieved on the GVPC. This verification step is only required for in-house media or commercially prepared media where the certificate of performance does not show evidence of inhibition of pseudomonads.

### 9.2 CONFIRMATORY TESTS

Positive control:

Inoculate a GVPC agar plate with either *L. pneumophila* serogroup 1 NCTC 12821 to give discrete colonies or 0.1 mL of a stored concentrate, which has previously been found to contain *Legionella* species.

Negative control:

Inoculate a GVPC agar plate with *Pseudomonas aeruginosa* NCTC 10662. Process all controls alongside routine test samples.

## 10 REFERENCE FACILITIES

In instances such as outbreaks, isolates may be referred to the Health Protection Agency, Atypical Pneumonia Unit for full serological identification and for subtyping of *L. pneumophila* serogroup 1 after discussion with them if it is considered appropriate.

## 11 ACKNOWLEDGEMENTS AND CONTACTS

This National Standard Method has been developed, reviewed and revised by the Water Working Group for Standard Methods ([http://www.hpa-standardmethods.org.uk/wg\\_water.asp](http://www.hpa-standardmethods.org.uk/wg_water.asp)). The contributions of many individuals in clinical bacteriology laboratories and specialist organisations who have provided information and comment during the development of this document, and final editing by the Medical Editor are acknowledged.

The National Standard Methods are issued by Standards Unit, Evaluations and Standards Laboratory, Centre for Infections, Health Protection Agency London.

For further information please contact us at:

Standards Unit  
Evaluations and Standards Laboratory  
Centre for Infections  
Health Protection Agency  
Colindale  
London  
NW9 5EQ  
E-mail: [standards@hpa.org.uk](mailto:standards@hpa.org.uk)

**DETECTION AND ENUMERATION OF *LEGIONELLA* SPECIES IN BIOFILMS AND SEDIMENTS**

Issue no: 1.2 Issue date: 11.08.06 Issued by: Standards Unit, Evaluations and Standards Laboratory, in conjunction with the  
Regional Food, Water and Environmental Coordinators Forum. Page 13 of 15

Reference no: W 15i1.2

This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency

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Email: [standards@hpa.org.uk](mailto:standards@hpa.org.uk)

# APPENDIX: FLOWCHART SHOWING THE DETECTION AND ENUMERATION OF *LEGIONELLA* SPECIES IN BIOFILMS AND SEDIMENTS

Transport to laboratory in suitable containers at ambient temperature out of direct sunlight



Store at ambient temperature in the dark and examine within 24 hours of collection



Prepare 1:10 and 1:100 dilutions



Mix using a vortex mixer



Divide into 3 portions - untreated, heat-treated and acid treated



Pretreat as appropriate and plate 0.1 – 0.2 mL (depending on treatment) onto GVPC agar



Incubate at 36°C and increased humidity (preferably with CO<sub>2</sub>) for 2 to 10 days



Subculture to BCYE+ agar and BCYE- for confirmatory test



Calculate confirmed count for *Legionella*



Refer to the reference laboratory for serology and species identification if necessary

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