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The determination of Legionella bacteria in waters and other environmental samples (2020) – Part 2 – Culture Methods for their detection and enumeration

Methods for the Examination of Waters and Associated Materials

The determination of Legionella bacteria in waters and other environmental samples (2019) – Part 2 – Culture Methods for their detection and enumeration

Methods for the Examination of Waters and Associated Materials

This booklet describes three methods for the detection, isolation and enumeration of *Legionella* bacteria in water and other environmental samples.

A Detection and enumeration of Legionella species by filtration, elution and spread plating onto a selective medium

B Detection and enumeration of Legionella species by filtration, and direct transfer onto a selective medium

C Detection and enumeration of Legionella pneumophila by a diagnostic substrate most probable number technique

Within this series there are three separate booklets dealing with the collection and processing of waters and environmental samples for the detection of *Legionella* bacteria. The other booklets are:

The determination of *Legionella* bacteria in water and other environmental samples (2005) - Part 1 - Rationale of surveying and sampling

The determination of Legionella bacteria in water and other environmental samples (2019) - Part 3 - Method for their detection and quantification by polymerase chain reaction (qPCR) and protocol for method validation

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products but serves only as an illustrative example of the type of products available. Equivalent products may be available and it should be understood that the performance of the method might differ when other materials are used and all should be confirmed by validation of the method.

Within this series there are separate booklets, each dealing with different topics concerning the microbiology of drinking water. Booklets include

The Microbiology of Drinking Water (2002)
Part 1 - Water quality and public health
Part 3 - Practices and procedures for laboratories
Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment.

The Microbiology of Drinking Water (2004)
Part 11 - Taste, odour and related aesthetic problems
Part 12 - Methods for micro-organisms associated with taste, odour and related aesthetic problems.

The Microbiology of Drinking Water (2006)
Part 9 - The isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube-most probable number techniques

The Microbiology of Drinking Water (2007)
Part 13 - The isolation and enumeration of aerobic spore-forming bacteria by membrane filtration

The Microbiology of Drinking Water (2010)
Part 2 - Practices and procedures for sampling
Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

The Microbiology of Drinking Water (2012)
Part 5 - The isolation and enumeration of enterococci by membrane filtration
Part 7 - Methods for the enumeration of heterotrophic bacteria

Contents

About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, soils (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts (established 1972 by the Department of the Environment). At present, there are seven working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical, Inorganic and physical methods, Metals and metalloids
4 Solid substances
5 Organic impurities
6 Biological, biodegradability and inhibition methods
7 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the SCA's web-page:- http://www.standingcommitteeofanalysts.co.uk/Contact.html

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary. Users should ensure they are aware of the most recent version they seek.

Rob Carter
Secretary
July 2020

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted. Numerous publications are available giving practical details on first aid and laboratory safety.

These should be consulted and be readily accessible to all analysts. Amongst such resources are; HSE website HSE: Information about health and safety at work ; RSC website http://www.rsc.org/learn-chemistry/collections/health-and-safety
"Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Biological Agents: Managing the Risks in Laboratories and Healthcare Premises", 2005 and "The Approved List of Biological Agents" 2013, produced by the Advisory Committee on Dangerous Pathogens of the Health and Safety Executive (HSE).

A Detection and enumeration of *Legionella* species by filtration, elution and spread plating onto a selective medium

A1 Introduction

Legionella species, the cause of legionellosis, are aquatic bacteria that are widespread in nature and occur in water over a wide temperature range. Multiplication has been found to occur between temperatures of 20 – 43 °C [1] and HSE note that there is a reasonably foreseeable legionella risk if the water temperature in all or some part of a water system may be between 20–45 °C[8]. Their tolerance to relatively high temperatures facilitates the colonisation of artificial water systems that are often above ambient temperatures. *Legionella* species are prevalent in artificial water systems, and the disease-causing bacteria can be transmitted from these systems to individuals via aerosols, occasionally by aspiration and on one occasion by person to person spread [2-4]. Cooling towers, hot and cold water systems, swimming or spa pools and a variety of other sources have also been commonly associated with outbreaks for which guidance has been published [5-8].

Water samples may be examined for *Legionella* species as part of surveillance programs designed to verifying risk and the effectiveness of control measures and during public health epidemiological investigations, or, or in order to validate new biocide treatment or other control methods[1]. Routine sampling should also be carried out based on Legionella Risk Assessments following national requirements/guidance [8].

When present, the numbers of legionellae are often low and rarely exceed 1 % of the total bacterial population. As a result, it is usually necessary to concentrate the bacterial flora from water samples before using selective cultural techniques to isolate *Legionella* bacteria [9]. However, in outbreak investigations, when potential sources may show high numbers of legionellae and be heavily contaminated with other bacteria, samples should be examined with and without concentration stages being used. The un-concentrated samples can yield legionellae even when legionellae would not normally be detected in the concentrated sample due to the overgrowth by other organisms.

Concentration can be achieved using filtration or centrifugation techniques, or a combination of both.

A2 Scope

This booklet describes a culture method for the detection, identification and enumeration of legionellae in water and other environmental samples, as collected using procedures described elsewhere [10].

Users wishing to employ this method should verify its performance under their own laboratory conditions[2].

A3 Definitions

For the purposes of this method, *Legionella* is defined as a genus consisting of Gram-negative bacteria normally capable of forming colonies in not less than 2 days (i.e. there is no growth in the first two days of incubation) on buffered charcoal yeast extract agar containing L-cysteine and iron(III). Colonies typically possess a ground glass-type appearance when viewed with a low powered stereomicroscope although some species of non-pneumophila *Legionella* may not display this morphology. Colonies are often white, purple to blue and can display halos of varying colours. Some species such as *Legionella anisa*, *L. bozemanii, L. cherri, L. dumoffi, L.gratiana, L. gormanii, L. parisiensis, L. steigwalti L. steelei* and *L. tusconensis* fluoresce under long-wavelength ultra-violet light.

Growth does not occur in the absence of L-cysteine, except that *Legionella oakridgensis,*and *Legionella spiritensis* (which require L-cysteine and iron(III) on primary isolation) can grow weakly in the absence of L-cysteine thereafter. Accordingly, careful comparison needs to be made of the differences in growth between supplemented and un-supplemented media, particularly during confirmatory stages.

A4 Principle

Micro-organisms, including *Legionella*, in the water sample should be concentrated by membrane filtration and/or centrifugation. To reduce the growth of unwanted micro-organisms which may inhibit the growth of *Legionella*, a portion of the concentrated specimen should be subjected to treatment with acid. In addition, a further portion of the concentrated specimen should be subjected to treatment with heat. Acid-treated, heat-treated and untreated test portions should then be inoculated onto Petri dishes containing selective agar medium (A9.5) and incubated. Samples of sediments, biofilm and other surface deposits do not require concentration but may require elution prior to culture (Appendix A). *Legionella* organisms incubated on charcoal-based media produce colonies of typical morphological appearance. There are some species of *Legionella* that do not produce typical colony morphology on primary isolation e.g. *L. longbeachae, L. cincinnatiensis*. The ground glass morphology, while on the whole accurate for *Legionella pneumophila,* is not always visible.

A5 Limitations

Not all biocides have recognised neutralising agents and where present they will therefore continue to exert their bactericidal effects after sampling. Failure to neutralise biocides may result in false negative results or an under-estimation of the number of microorganisms (e.g. *Legionella*) present in the sample.

Sample containers should therefore contain appropriate neutraliser where available [7] and a record kept of the biocide and neutralising agent used to contextualise the results of the examination made.

Other bacteria, particularly pseudomonads, or fungi present in a sample may grow on the

agar medium and either inhibit the growth of legionellae or severely mask their presence however overgrowth by other micro-organisms does not mean that the sample is negative for *Legionella*.

The enumeration of *Legionella* may be underestimated as it is generally assumed that one legionella cell produces one visible colony. The true count may however be significantly higher as clumps of *Legionella* cells also produce only one colony. The culture methods presently used for the detection of legionellae from water rarely achieve 100 % recovery, with most current practices only achieving a level of between approximately 10 - 50 %. Consequently, the true number of legionellae in water is usually higher than that detected using these methods [11].

Depending on the procedure used, this method can achieve a limit of detection that meets, or is below, the actions limits in current UK guidance (A10.2). Should even lower limits of detection be required, the approach detailed in Method B can be used.

A6 Health and safety

WARNING - Species of *Legionella* are pathogenic

Risk of harm is caused by the inhalation of aerosolised disease causing strains of Legionella bacteria it is therefore advisable to assess all techniques for their ability to produce aerosols.

All samples submitted for *Legionella* analysis should therefore be regarded as potentially contaminated with bacteria classified as “Hazard Group 2” and handled with strict adherence to the general safety precautions described for work at Containment Level 2 (CL2), to include minimising exposure to aerosols at all times[12,13]**.**

Based on the risk assessment of procedures undertaken it may be necessary to carry out the examination in a microbiological safety cabinet. However, experience has determined that Legionella spp. can be handled safely by experienced microbiologists on the open bench in a conventional microbiology laboratory conforming to containment level two.

Media, reagents and bacteria used in this method are covered by regulations[24] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere in this series[16].

A7 Storage of samples during transportation

At temperatures lower than 6 ºC *Legionella* can enter a viable but non-culturable state and at temperatures above 20 ºC proliferation is likely, therefore transport and storage at ambient temperatures - ideally 6 ºC - 20 ºC is recommended [10].

Samples should be analysed as soon as possible not exceeding more than two days, and

preferably within 24 hours.

The laboratory should however verify that their chosen transport and storage conditions are appropriate.

A8 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria described elsewhere in this series[6].

A8.1 Membrane filters for concentration and elution: Polyamide (polyethersulfone) or polycarbonate membrane filters, nominal pore size of 0.22 µm and diameter of 47 - 142 mm. Larger diameter membrane filters are more suitable for filtering larger volumes of water or samples containing more particulate matter, and withstand higher flow rates. Polycarbonate membrane filters should be used with lower flow rates but this will extend processing times.

A8.2 Vacuum-assisted membrane filtration apparatus and vacuum pump suitable for membrane filters with diameter of 47 mm and filtering volumes of water of typically up to 1 litre.

A8.3 Positive-pressure membrane filtration apparatus suitable for filtering larger volume or more turbid water samples. This apparatus normally includes a filter stand and funnel and should withstand autoclaving. The filter diameter may vary between 47 - 142 mm. Larger apparatus are usually constructed of stainless steel.

A8.4 Positive-pressure membrane filtration peristaltic pump capable of producing a flow rate of up to 3 litres per minute with variable speed control. Alternatively, a compressor and pressure vessel may be used.

A8.5 Silicone tubing with inner and outer diameters as specified by the manufacturer of the peristaltic pump.

A8.6 Centrifuge capable of achieving a centrifugal force of 6000 ± 100 g operating for 10 minutes or 3000 ± 100 g for 30 minutes. These should be operated at temperatures between 15 – 25 ^{o}C. Ideally, the rotor should be of the “swingout” variety. Also required are appropriate tubes, preferably possessing conical bottoms, and safety buckets of appropriate capacity.

A8.7 Incubator, capable of being maintained at 36.0 ± 1.0 ^{o}C. A humidified incubator is preferable, but use of carbon dioxide is optional.

A8.8 Cell scrapers. These are commercially available.

A8.9 Low power binocular microscope, with at least 6-times magnification, and illuminated from above by oblique incident light.

A8.10 Ultraviolet lamp, emitting light of wavelength 360 ± 20 nm.

A8.11 Sterile food grade plastic bags, for example stomacher bags.

A8.12 Ultrasonic waterbath.

A8.13 Water bath, capable of being maintained at (50 ± 1) °C.

A8.14 Vortex mixer.

A8.15 Screw cap sterile container, for example universal containers.

A8.16 Smooth-tipped forceps.

A8 Media and reagents

Commercial formulations of these media and reagents are readily available, but may possess minor variations in their formulation. Commercial formulations should be used and stored according to manufacturer's instructions. Laboratories may decide to produce their own media. The performance of all media and reagents should be verified prior to their use in the method[(2)]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly.

Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

A9.1 *1:40 Ringer's solution*

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride hexahydrate	0.12 g
Sodium hydrogen carbonate	0.05 g
Water to	1000ml

Dissolve the ingredients in the water and mix thoroughly. Dilute the resulting solution with water in the ratio 1:10. This solution may be dispensed as required in suitable volumes into suitable containers, capped and autoclaved at 121 ^{o}C for 15 minutes. The pH of the final solution should be 7.0 ± 0.2 [(16)] .

A9.2 *Page's saline solution*

Sodium chloride	0.12 g
Magnesium sulphate heptahydrate	0.004 g
Calcium chloride dihydrate	0.004 g
Disodium hydrogen orthophosphate	0.142 g
Potassium dihydrogen phosphate	0.136 g
Water to	1000 ml

Dissolve the ingredients in the water and mix thoroughly. The resulting solution may be dispensed as required in suitable volumes into suitable containers, capped and autoclaved at 121 °C for 15 minutes. To facilitate accurate preparation, 10 litres of Page's saline solution may be prepared and dispensed in volumes as required for autoclaving at 121 °C for 15 minutes. Alternatively, a concentrated solution may be prepared and diluted as appropriate for use[16] .

A9.3 *Buffered charcoal yeast extract agar with L-cysteine (BCYE) (17)*

N-2-acetamido-2-amidoethane-sulphonic acid (ACES) buffer	10.0 g
Potassium hydroxide	2.8 g
Activated charcoal	2.0 g
Yeast extract (bacteriological grade)	10.0 g
α-ketoglutarate, potassium salt	1.0 g
Agar	12.0 g
L-cysteine hydrochloride monohydrate	0.4 g
Iron(III) pyrophosphate	0.25 g
Water to	1000 ml

Add 0.4 g of L-cysteine hydrochloride to 10 ml of water. Filter-sterilise the solution by filtration through a membrane filter with a nominal pore size of 0.22 µm. Add 0.25 g of iron(III) pyrophosphate to 10 ml of water. Filter-sterilise the solution by filtration through a membrane filter with a nominal pore size of 0.22 µm. The sterilised solutions may be stored in clean sterile containers at – 20 (± 3) °C for up to 3 months, but should be allowed to reach room temperature before use. If these solutions are not used immediately they should be re-sterilised before addition to the medium.

Add 10 g of N-2-acetamido-2-amidoethane-sulphonic acid (ACES) buffer to 500 ml of water in a suitable container. Dissolve the buffer by standing the container in a water bath at 45 - 50 °C. Add 2.8 g of potassium hydroxide to 480 ml of water and dissolve with gentle shaking. Mix the two solutions.

The ACES buffer can de-nature the yeast extract if the following sequence is not strictly followed. Add sequentially to 980 ml of alkaline ACES buffer solution, 2 g of the activated charcoal, 10 g of the yeast extract and 1 g of the α-ketoglutarate salt. Mix well between each addition. As appropriate, use 0.1 mol l^{-1} potassium hydroxide solution or 0.1 mol l^{-1} sulphuric acid solution and adjust the pH of the resulting ACES buffer solution to 6.8 ± 0.1. Add the agar, mix well and autoclave at 121 °C for 15 minutes. After autoclaving, allow the solution to cool to 50 ± 2 °C in a water bath. Aseptically, add the L-cysteine and the iron(III) pyrophosphate solutions, mixing well between each addition. The pH of the final medium should be 6.8 ± 0.2 at 25 °C. Dispense the medium, in volumes of 20 ml, into Petri dishes, nominally between 90 - 100 mm in diameter and allow the medium to cool. Allow any excess moisture on the agar to dry. The Petri dishes may be stored at 5 ± 3 °C in airtight containers in the dark for up to 4 weeks.

The medium is heat sensitive and prolonged heating or too a high temperature during sterilisation can severely affect the nutritional qualities of the medium.

A9.4 *Buffered charcoal yeast extract agar without L-cysteine (BCYE –cys)*

This medium should be prepared as described in section A9.3 but without the addition of L-cysteine hydrochloride.

A9.5 *Buffered charcoal yeast extract agar with glycine and antibiotic supplements (GVPC)*

This medium is more selective than BCYE (A9.3). It includes glycine and antibiotics and should be prepared as described in section A9.3 with the addition of glycine before the agar is added, and the addition of the antibiotic supplements, after the addition of the L-cysteine and iron(III) pyrophosphate solutions. The glycine and antibiotic supplements should be added to the medium to give the following final concentrations:

Polymyxin B sulphate	80,000 iu l^{-1}
Vancomycin hydrochloride	1 mg l^{-1}
Cycloheximide	80 mg l^{-1}
Glycine (ammonia-free)	3 g l^{-1}

Cycloheximide is hepatotoxic and appropriate protective clothing should be worn when handling this chemical

The concentration (approximately 7000 iu mg^{-1}) of each batch of polymyxin B sulphate should be checked to ensure the correct final concentration is achieved.

Dissolve the appropriate amount of polymyxin B sulphate (about 200 mg, i.e. about 1400000 iu) in 100 ml of water. Filter-sterilise the solution through a 0.22 µm membrane filter, and dispense in suitable volumes, typically about 5.7 ml, into sterile containers. The solution may be stored at -20 ± 3°C for up to 6 months. However, the solution should be allowed to thaw to room temperature before use.

Dissolve 20 mg of vancomycin hydrochloride in 20 ml of water. Filter-sterilise the solution through a 0.22 µm membrane filter and dispense the solution, in volumes of 1 ml, into sterile containers. These containers may be stored at -20 ± 3°C for up to 6 months, but the solution should be allowed to thaw to room temperature before use.

Dissolve 2 g of cycloheximide in 100 ml of water. Filter-sterilise the solution through a 0.22 µm membrane filter and dispense the solution, in volumes of 4 ml, into sterile containers. These containers may be stored at -20 ± 3°C for up to 6 months, but the solution should be allowed to thaw to room temperature before use.

Prepare the medium, as described in section A9.3, but with the addition of glycine just before the addition of the agar. Adjust the pH of the solution to 6.8 ± 0.2. After the addition of the L-cysteine and iron(III) pyrophosphate solutions, add the antibiotic supplements solutions. The pH of the final medium, should be 6.8 ± 0.2. If the pH is higher, certain species of *Legionella* may be inhibited[8]

A9.6 *Acid buffer*

Mix together 3.9 ml of 0.2 mol l^{-1} hydrochloric acid solution with 25 ml of 0.2 mol l^{-1}

potassium chloride solution. Adjust the pH of the solution to 2.2 ± 0.2 by the addition of 1 mol l^{-1} potassium hydroxide solution. Store the resulting solution in a sealed container, in the dark, at room temperature. This solution may be stored for up to one month prior to use.

Prepare 0.2 mol l^{-1} hydrochloric acid solution by adding 17.4 ml of concentrated hydrochloric acid (specific gravity 1.18, minimum assay 35.4 %, approximately 10 M) to 1000 ml of water. Depending upon the concentration of the acid, the exact volume of acid may need to be adjusted accordingly.

Prepare 0.2 mol l^{-1} potassium chloride solution by dissolving 14.9 g of potassium chloride in 1000 ml of water.

Prepare 1 mol l^{-1} potassium hydroxide solution by dissolving 40 g of potassium hydroxide in 1000 ml of water.

This is a 2x the concentration of the Acid Buffer described in Method B (B9.6).

A10 Analytical procedure

A10.1 Water sample preparation

In the majority of testing scenarios, (for example samples for routine monitoring or commissioning) the number of legionellae in any given water sample is unknown. Concentration techniques should therefore be carried out on all liquid samples prior to analysis. If the sample is known to contain high levels of legionellae liquid samples may be inoculated directly onto the GVPC medium (A9.5).

Before an aliquot of the sample is removed from the sample submitted to the laboratory, the sample should be mixed by gentle inversions to produce a homogenised solution or suspension while minimising aerosol production. The volume of sample used in the analysis should be recorded before concentration by filtration and/or centrifugation. The final volume of any concentrated suspension should represent between a 100-fold and 1000-fold concentration of the original sample volume.

NOTE: If there is visible separation or sedimentation in the sample it may be appropriate to record this.

The volume of sample used in the analysis should be recorded (A ml) and the sample thoroughly mixed to re-suspend any deposited material. This action may generate aerosols containing *Legionella*. The sample should then be allowed to stand for an appropriate time period to allow the dispersal of any aerosol formed before the concentration procedures are carried out.

A 10.2 Factors affecting the outcome of the method

The volume of the sample analysed and the degree of concentration required depends upon the turbidity of the water, the limit of detection required, and the purpose of the sample collected. Current guidance in the UK sets alert and action limits based on enumerated counts of *Legionella* of 100 colony forming units (cfu) per litre [8]. Hence, the degree of concentration should enable at least 100 cfu in a litre of sample to be detected.

Where samples are collected from premises within which persons are at greater risk of *Legionella* infection, such as healthcare, there may be a requirement for the method of examination to have a lower detection limit [8, 18].

When a concentration step is used, micro-organisms irretrievably attached to the membrane filter or the sides of the centrifuge tube etc. reducing recovery. In addition, a proportion of the target population may be unculturable (killed or sub-lethally damaged) as a result of the selective procedures.

The resuscitation and culturability of *Legionella* is also dependent upon the inhibition of the established interference from other micro-organisms and it is for this reason the use of anti-microbial compounds in the selective media, treatment of portions of the sample with acid or heat is required.

On occasions it is necessary to retest a sample. This may be because the interference from background organisms is greater than anticipated, or the count of *Legionella* is higher than expected, or a particular type of *Legionella* is sought in outbreak investigations. Accordingly, the volume of sample collected and the volume of the final concentrate used in the examination should be sufficient to enable these tests to be carried out.

Where it is necessary to detect a small number of organisms, this may be achieved by performing two or more concentration steps. However, this will reduce the overall recovery of the organisms owing to the losses encountered at each stage.

The volume of sample collected for examination should be ideally 1000 ml. However, smaller volumes may be acceptable with lower limits of detection especially where the results of such samples are interpreted by an experienced microbiologist.

The purpose of smaller volume samples may include:

- High turbidity
- Pre flush samples of small volume outlets
- Where a larger volume would dilute the concentration of bacteria sampled

The volume examined should be recorded.

The concentration of organisms from water samples may be undertaken either by membrane filtration or centrifugation or by a combination of both techniques. Centrifugation may be necessary for water samples that are difficult to filter. This may be the case for samples that appear cloudy or turbid, contain colloidal matter or are contaminated with oils or other hydrophobic substances. Water samples with high counts of non-*Legionella* bacteria may be diluted with 1:40 Ringer's solution (A9.1) or Page's saline solution (A9.2). The volumes of sample diluted or processed should be recorded.

Concentration followed by direct membrane filter placement on the agar may be appropriate where samples are likely to contain lower levels of interfering microorganisms and a lower limit of detection is required. Filtration and direct membrane transfer obtains a lower limit of detection of 1 colony forming unit (cfu) per volume of sample analysed. This approach is documented in Method B.

With direct inoculation of the sample onto the medium with no concentration, it is possible to achieve the detection of one cfu in the volume placed on the culture medium. The volume of sample inoculated onto the medium should not normally exceed 0.5 ml. If a concentration stage is not used, the limit of detection, assuming 100 % recovery, would be approximately 5000 cfu per litre. In reality, recovery rarely reaches 100 %.

A10.3 Membrane filtration

For volumes up to 1000 ml, vacuum-filtration may be used. On occasions, more than one filter may be necessary and the eluates combined[15].

NOTE: Positive-pressure filtration is less convenient than centrifugation or vacuum-filtration and is more suitable for large volumes (typically greater than 1000 ml) and for samples with high turbidity/particulates but may lead to poorer recovery.

A10.4 Elution of samples from membrane filters

Warning - Risk of harm is caused by the inhalation of aerosolised disease causing strains of Legionella bacteria it is therefore advisable to assess all techniques for their ability to produce aerosols.

When the samples have been filtered, a suspension of the organisms retained on the filter or filters should be prepared. A volume (typically 5 – 10ml [B ml]) of sterile elution solution (A.9.2 or A.9.2) is added to the membrane filter in a sterile container. Larger volumes of elution solution may be required to ensure full coverage of the membrane filter.

A variety of elution techniques are available including physical abrasion of the surface of the membrane filter. The membrane filter may be rubbed or massaged inside a stomacher bag containing the elution solution. Alternatively, using a sterile tissue culture scraper, the surface of the membrane filter may be scraped and the organisms transferred into a small volume of sterile elution solution. In addition, the membrane filter may be vortexed, vigorously shaken or ultrasonicated in a small volume of sterile elution solution.

The techniques used to resuspend the organism from the filter must be verified as effective by the testing laboratory.

The suspension may then be cultured directly, or further concentrated by centrifugation (A.10.2.2.6) and then cultured.

A10.4.1 Elution by ultrasonication

Ultrasonication of the membrane filter is commonly used to remove the deposit from membrane filters. The membrane filter should be placed in a sterile container with sterile diluent. Care should be taken to ensure that the diluent covers the membrane. The container should be placed in an ultrasonic bath ensuring that the water is at the same height or higher than the diluent in the container. The time should be verified by the laboratory. The frequency of ultrasonic baths from different manufacturers may vary.

A10.4.2 Elution by shaking

Membranes should be placed in a sterile container with sufficient volume of diluent to cover the membrane (with or without glass beads). The container should be shaken for at least two minutes but the optimal time should be verified by the laboratory.

A10.4.3 Elution by rubbing

To a small food-grade plastic stomacher bag add the required volume of elution solution. Record the volume (B ml). Using sterile forceps, transfer the membrane filter to one of the bottom corners of the bag. From the outside of the stomacher bag, massage or rub the membrane filter for at least 30 seconds utilising the elution solution to facilitate re-suspension of the residual deposited material. The optimal time should be verified by the laboratory. All filtered material should be removed from the membrane filter to produce a well-mixed homogenised suspension. Using a sterile pipette, quantitatively transfer the suspension from the stomacher bag to a suitable container. Re-suspend the residual deposit and produce a homogenised suspension by vortex mixing the contents of the container. This suspension constitutes the final sample concentrate and, owing to the transfer procedure may be slightly smaller than the original B ml.

A10.4.4 Elution by scraping

Alternatively, using sterile forceps, remove the membrane filter from the filter stand, and place the filter in a suitably sized sterile Petri dish (usually a Petri dish 60 mm in diameter for a membrane filter of 47 mm). To the Petri dish, add a volume of elution solution and, using a sterile tissue culture scraper, remove all of the filtered material from the membrane filter by gently scraping the filter into the elution solution. The scraping should be performed at least twice over the whole membrane filter and in opposite directions. The resulting suspension should then be transferred to a sterile tube. It may be necessary to rinse the Petri dish with additional elution solution and transfer the washings to the tube. Record the volume (B ml) of the suspension, which constitutes the final sample concentrate.

A10.4.5 Elution by vortexing

The deposit may also be eluted from the membrane filter by vigorous vortex mixing. The membrane filter should be transferred directly into a suitable screw cap sterile container and, if necessary, using sterile scissors, cut the membrane filter into appropriately sized pieces. A known volume (B ml) of elution solution should then be added to the container which is vortex-mixed to produce a homogenised suspension. This suspension constitutes the final sample concentrate.

NOTE: Vortexing concentrated samples may create aerosols which are a safety risk. Refer to sections A.6 & A.10.1

A10.5 Concentration using centrifugation

Concentration by centrifugation can give much lower recovery rates for than membrane filtration technique, but may be required for samples that cannot be filtered (see A10.2). The

volume of sample (A ml, typically not less than 200 ml) used in the analysis should be shaken to re-suspend any particulate matter and a homogenised sample produced which should then be allowed to stand for an appropriate time period to allow the dispersal of any aerosol formed before being transferred to a suitable centrifuge tube. The suspension is then centrifuged at 6000 g for 10 minutes (or 3000 g for 30 minutes) whilst maintaining the temperature between 15 - 25 ºC. After centrifuging, sufficient clear supernatant liquid should be removed (and discarded). Re-suspend the residual deposit and produce a homogenised suspension by vortex mixing the contents of the centrifuge tube, the resulting final volume should be recorded. This suspension constitutes the final sample concentrate. If the centrifuge does not have the capacity to process the required volume then it may be necessary to split the sample in to multiple aliquots. The concentrates should then be combined and the final volume recorded.

A10.6 Further concentration by centrifugation

If a suspension is to be concentrated further by centrifugation, transfer the suspension from the stomacher bag or other container directly to a centrifuge tube. The final volume must be recorded. Centrifuge the suspension at 6000 g for 10 minutes (or 3000 g for 30 minutes). Carefully remove the tube from the centrifuge and using a sterile pipette, withdraw (and discard) sufficient clear supernatant liquid to leave a known volume (B ml, typically 1 - 2 ml). Re-suspend the residual deposit and produce a homogenised suspension by vortex mixing the contents of the centrifuge tube. This suspension constitutes the final sample concentrate.

A10.7 Storage of concentrates and suspensions of swabs, sediments and other deposits

To minimise changes to the viable count of bacteria in a concentrate analysis shouldn't exceed more than two days from sampling. Storage conditions should be between 6 ºC to 20 ºC and in the dark.

The laboratory should however verify that their chosen storage conditions appropriately avoid unwanted changes to the analytical result.

For outbreak investigation or epidemiological purposes, after culture, unused portions of the concentrate, sediment or biofilm suspension may be stored for future reference, but should not be stored for more than 3 months. Stored under these conditions, stressed Legionella organisms may be recovered later. The re-testing of stored concentrates may be important when investigating outbreaks of Legionnaires' disease, or for example - if high numbers of Legionella organisms or confluent growth of non-Legionella colonies are observed soon after incubation).

A10.7 Culture

Warning - Risk of harm is caused by the inhalation of aerosolised disease causing strains of Legionella bacteria it is therefore advisable to assess all techniques for their ability to produce aerosols. See section 6.

Divide samples (concentrated or un-concentrated), into three portions. Use one portion without any further treatment. Treat the other two portions accordingly, one with heat treatment and one with acid treatment.

A10.7.1 Heat treatment

Add at least 0.15 ml of sample concentrate, sample suspension or un-concentrated sample to a sterile container. Place the container in a water bath at 50 ± 1 °C for 30 ± 2 minutes.

A10.7.2 Acid treatment

For samples processed by concentration and elution a 2-times concentrated acid buffer solution (detailed in A9.6.2) may be used that is diluted with an equal volume of the concentrate or suspension. Incubate for 5 ± 0.5 minutes. The final volume inoculated onto the growth medium will need to be doubled to retain the same detection limit as for the heat and untreated portions.

A10.7.3 Inoculation of selective media

Inoculate separate Petri dishes of selective agar for each portion with a known volume (V ml, typically 0.1 - 0.5 ml) of sample or sample concentrate.

For acid treated samples it will be necessary to inoculate double the volume of sample concentrate to achieve the same level of detection as untreated or heat treated sample, or a dilution factor of 2^{-1} will need to be accounted for in the calculation of results.

With a sterile plastic “hockey stick” type spreader, distribute the liquid inoculum over the entire surface of the agar. After inoculation, the Petri dishes should be allowed to stand at room temperature until the volume of inocula has been absorbed into the agar. Larger inoculum volumes will require longer for the inoculum to absorb into the agar before the plates are inverted for incubation.

A10.8 Incubation

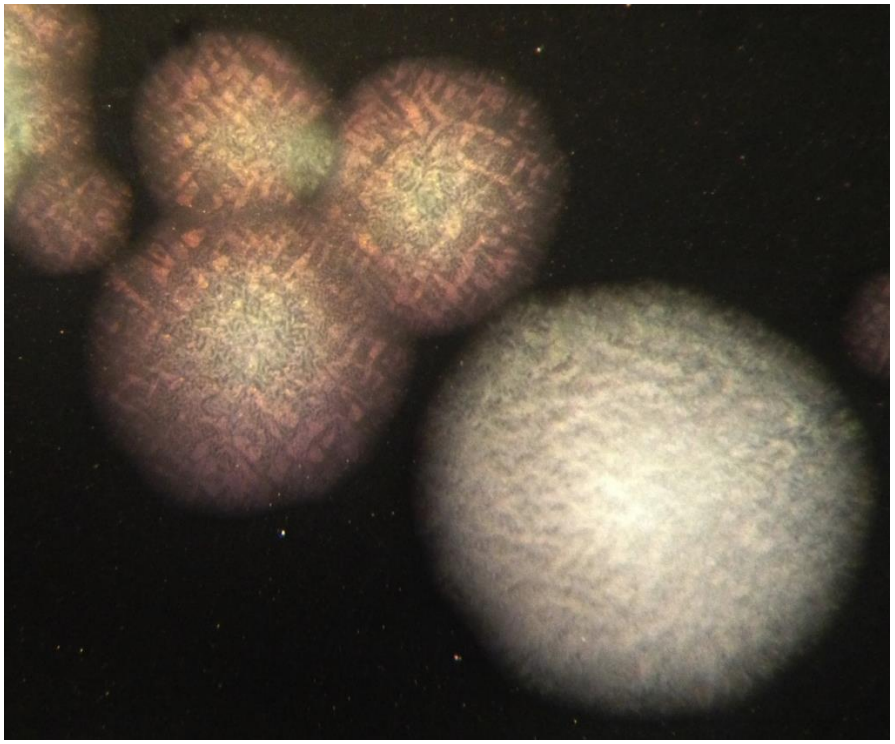
The Petri dish should then be inverted and incubated at 36.0 ± 1.0 °C for 10 days. Throughout the incubation, a humid atmosphere should be maintained. This can be achieved by a variety of methods, for example; incubation in closed containers or bags or by placing a container of water inside the incubator. The container of water in the incubator may become contaminated, for example with *Legionella* or fungi, and should therefore be periodically discarded (in an appropriate manner) or disinfected.

A10.9 Examination of plates

At suitable intervals, usually 2 - 3 days (e.g. days 3, 7 and 10), during the 10-day incubation period, the Petri dish should be removed from the incubator and the medium examined for growth. Ideally a binocular microscope with oblique incident light should be used as it can be very difficult to identify *Legionella* by naked eye. Colonies of *Legionella* are often white-grey-blue-purple in colour, but may be brown, pink, green or deep red. Colonies are smooth with an entire edge and most will exhibit a characteristic ground-glass appearance (Figure

A1). Some species of Legionellae may not produce typical ground-glass colonies on primary isolation.

Figure A1 Ground glass appearance of *Legionella pneumophila* colonies at day 4.



If, on the first examination, high numbers of *Legionella* organisms or confluent growth of non-*Legionella* colonies are observed, then the original sample or sample concentrate may be appropriately diluted (and details recorded) and the sample re-inoculated on the selective agar - however, there may be discrepancies between the results. It is, therefore, important to store (under appropriate conditions, see section A10.5) any un-used portions of the original sample or sample concentrate and to record the time between sampling and commencement of analysis of this re-inoculated sample. The subsequent test report will also need to record that analysis of the stored sample occurred after a time delay, which should be recorded.

The number of presumptive colonies conforming to the typical morphology of *Legionella* should be counted and recorded for each Petri dish incubated. If different types of colonial morphologies are observed, the number and details of each type should be recorded and confirmatory tests carried out (see Section A10.9). If a series of dilutions have been used, then, where possible, the Petri dish or Petri dishes that contain between 20 - 80 legionella colonies should be counted.

It may be helpful to examine the Petri dishes briefly under ultra-violet light particularly any samples with suspected *Legionella* as often there will be mixed populations of fluorescent and non-fluorescent species. Several species of *Legionella* (for example *Legionella anisa*) fluoresce with a brilliant blue-white colour and other species (for example *Legionella rubrilucens* and *Legionella erythra*) appear red. *Legionella pneumophila* appear dull green,

often tinged with yellow.

A10.10 Confirmation and identification

A variety of methods are available for the confirmation and identification of *Legionella* organisms. These methods may include the following but the laboratory should verify the effectiveness of the chosen approach:

- sub-culture to confirmation media
- latex agglutination tests
- MALDI-TOF
- polymerase chain reaction tests
- direct fluorescent antibody assays
- gas-liquid chromatographic methods, for example for cellular fatty acids and isoprenoid quinone compounds
- slide agglutination tests
- colony blot assays based on genus-specific monoclonal antibodies
- enzyme-linked immuno-sorbent assays using diagnostic reagents

A10.10.1 Sub-culture for confirmation

Presumptive colonies from the plate with the highest count (at least three colonies of each morphological type) and, if appropriate (if different colony morphologies are present on the different plates), at least three colonies from each of the three plates (untreated, heat treated, acid treated) should be sub-cultured into two separate Petri dishes of media (A9.3) and (A9.4). An alternative medium for the buffered charcoal yeast extract agar without L-cysteine medium (A9.4) comprises 4 % horse blood agar or nutrient agar as detailed in ISO 11731[(22)]. After incubating for 2 - 3 days at 36 ± 1.0 °C the Petri dishes should be removed from the incubator and the medium examined for growth. Colonies that only grow on the buffered charcoal yeast extract agar with L-cysteine medium (A9.3) are regarded as *Legionella* organisms, and those colonies that grow on both media are considered as non-*Legionella* organisms. It is assumed that careful picking of the colony will result in a pure subculture on both media however sometimes this may not occur. It is good practice to examine the plates under a microscope to confirm that samples not confirming at legionella are not due to erroneous plating out of a mixed isolate.

A10.10.2 Latex agglutination

Colonies that grow in the presence of L-cysteine may be further tested using commercially available antibody-based latex agglutination kits. These kits are typically able to distinguish between *Legionella pneumophila* serogroup 1, serogroups 2 –15, or may discriminate further to individual serogroup. Some kits may also identify other species of *Legionella* but the kits may not identify all Legionella species. In some cases, the identity of the colony as a specific *Legionella* organism can be confirmed. The procedures described in manufacturer's manuals should be followed.

A10.10.3 MALDI-ToF

Matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-ToF) is a rapid (~10 min) and very sensitive confirmation method[21].

Several platforms are available from different providers which work on similar principles. A small quantity of biomass (usually taken from an isolated pure colony on an agar plate) is spotted onto a target plate and overlaid with an organic matrix. Additional pre-treatment of the sample may be required prior to spotting. Laboratories should refer to the manufacturers' recommended protocols. A pulsed laser irradiates the sample, triggering desorption and ionisation. Molecules are separated by mass-to-charge ratio and detected, the target being mainly the ribosomal proteins.

By comparing this information gathered at the detector to calibration standards, it is possible to calculate the molecular mass associated with any particular peak, and measure the relative intensity of that peak. These data are then compared to a database of spectra (usually from the manufacture or an in-house database validated by the testing laboratory) and an organism identification and associated confidence score is generated.

MALDI-ToF has been used successfully to identify Legionella isolates from both environmental and clinical samples [19]. An added advantage of MALDI-ToF is the identification of *Legionella* species that are currently not identifiable to the species level by traditional confirmation techniques. However, laboratories should note that MALDI-ToF is currently unable to discriminate between different Legionella serogroups e.g. *Legionella pneumophila* serogroup 1 vs. *Legionella pneumophila* serogroups 2-14. This is a developing field and, for example, molecular typing methods for Legionella using MALDI-ToF are in development and may prove useful in public health investigations.

For confirmation of presumptive Legionella from environmental samples isolates may be tested from the primary isolation plates (A9.8) or the confirmation plates following sub-culture (A9.9.1). Multiple colonies should be confirmed as prescribed in section A9.9.1. The age of the colony may affect the accuracy of results therefore testing laboratories should refer to the manufacturer's recommended protocols. Testing laboratories should ensure that MALDI-ToF methods are appropriately verified to confirm that performance is equal to or greater than the traditional confirmation method used.

A10.10.4 DNA amplification by polymerase chain reaction PCR

DNA amplification by polymerase chain reaction (PCR) can also be used for the rapid confirmation of *Legionella pneumophila and Legionella* spp. presumptive colonies. This may be achieved by conventional end-point PCR or real-time quantitative PCR (qPCR). Specific primers (and probe for qPCR) enable an unambiguous confirmation to be achieved in just few hours. Resolution can be done by electrophoresis (classic end-point PCR) and/or fluorescent signal (qPCR).

The procedure consists of preparing a solution from each presumptive morphology in phosphate buffer saline solution (PBS) or sterile distilled water (SDW). From this solution DNA is extracted and purified by different techniques, based on physical (e.g. cycles of freezing and thawing, or bead beating), chemical (e.g. guanidine thiocyanate buffer) or enzymatic digestion. The next step consists of preparation of a reaction mix where extracted DNA is mixed in a PCR tube or plate with a master mix containing DNA

polymerase, specific primers, deoxynucleotide triphosphates (dNTPs) and probe for qPCR in an optimized reaction buffer. Finally this reaction mix is loaded into a thermocycler for the amplification to run.

Detailed procedures together with limitations, health and safety and general testing conditions for DNA amplification by qPCR are described in **Part D** of this booklet.

There are several commercial kits available for both DNA extraction and amplification, which include controls and manufacturer's validation, supporting a quick implementation of this methodology.

A10.10.5 Direct fluorescent antibody (DFA)

Direct fluorescent antibody (DFA) is an immunological technology where specific *Legionella* antigens are targeted by fluorescent dye-labelled antibodies. Then antibody – antigen complex is detected by exposing to ultra violet or blue violet light, which causes blue-green or yellow-green fluorescence.

Select colonies which grow on the buffered charcoal yeast extract agar with L-cysteine medium, do not grow on agar without L- cysteine and are regarded as *Legionella* (A10.9.1) and prepare a light suspension (1 McFarland Standard) of each colony to be tested, in 3ml of sterile distilled water, phosphate buffer saline solution or 1% neutral formalin. The suspension should be just visible to the naked eye.

Add 10µl of the culture suspension into appropriate number of wells (depending on number of reagents in the kit used) of a clean multi-well glass Polytetrafluoroethylene (PTFE) printed slide. Add approximately 10µl of positive control reagent onto one well and negative control reagent onto another one. Air dry the slide (it can be warmed to temperature between 35°C and 45°C in an incubator). Fix the prepared slide by gentle heating in a Bunsen flame or by immersion in acetone for 10 minutes followed by drying in air.

Add 5 µl of each monoclonal anti-serum to each appropriate well and 5 µl of polyvalent control reagent (including polyclonal anti-serum) to a well with positive and negative control and transfer the slide into a suitable humid container, for example a box containing damp tissue in order to prevent the slides from drying out. Cover the container to avoid exposure to light and incubate for 20 to 30 minutes at the temperature specified by the reagents manufacturer.

After incubation, wash the slides to remove excess antibodies by immersion in water or phosphate buffered saline solution. Discard the solution and repeat the process two more times with fresh water or phosphate buffered saline solution. Finally dry the slides at a temperature between 35° - 50 °C.

After drying, add a few drops of mounting medium reagent onto the test slide to hold the specimens in place between the cover slip and the slide and apply a coverslip, ensuring no bubbles are trapped beneath. Using a fluorescence microscope examine the slide applying immersion oil for a high power (100X) objective. Examine a positive and a negative control well first to make sure that the test is valid. The slide may be stored in the dark prior to examination for a maximum 24 hours.

There are commercial DFA test kits available, which include manufacturer's instruction with test procedure, quality control and interpretation of results.

A11 Calculations

Current UK guidance[5,6,7,8] sets alert and action limits for the presence of legionella in units of colony forming units per litre (cfu/L). Using the following equation, estimate the number of *Legionella* organisms expressed as cfu per litre of original water or per unit mass of sediment, taking into account the concentration factor, the proportion of concentrated sample analysed and the maximum number of colonies (for each confirmed colonial type) counted on any one of the three Petri dishes incubated:

$$Total\ cfu/L\ (x) = (UAH) \times \frac{1000}{A} \times \frac{B}{V}$$

Where:

UAH is the maximum count of <u>confirmed</u> organisms on the Petri dish using the un-treated, acid- or heat-treated process i.e the plate with the highest count is used;

$$UAH = \frac{number\ of\ colonies\ confirming}{number\ of\ presumptive\ colonies\ confirmed} \times total\ presumptive\ colonies$$

A is the volume of sample analysed (typically, 500 - 1000 ml);

B is the volume of sample concentrate (typically, 10 ml); and:

V is the volume of sample concentrate inoculated (typically, 0.1 – 0.5 ml).

It may also be necessary to take into account any dilution steps used.

For example:

The maximum number of colonies counted on the untreated sample is 45, the volume of sample taken for analysis is 1000 ml, and this volume is concentrated to 10 ml, and 0.5 ml is inoculated onto the Petri dishes, then the result, x (expressed as cfu per litre) is given by:

$$x = 45 \times \frac{1000}{1000} \times \frac{10}{0.5} = 900\ cfu\ per\ litre$$

The maximum number of colonies counted on the heat-treated sample is 21, the volume of sample taken for analysis is 500 ml, and this volume is concentrated to 10 ml, and 0.1 ml is inoculated onto the Petri dishes, then the result, x (expressed as cfu per litre) is given by:

$$x = 21 \times \frac{1000}{500} \times \frac{10}{0.1} = 4200\ cfu\ per\ litre$$

Should it be required to also report the result in the volume of sample initially analysed (e.g.

volume filtered), the following equation can be used to estimate the number of *Legionella* organisms expressed as cfu per volume of original water (cfu/vol), taking into account the proportion of concentrated sample analysed and the maximum number of colonies (for each confirmed colonial type) counted on any one of the three Petri dishes incubated. The volume analysed should also be reported:

$$Total\ cfu/vol\ (y) = (UAH) \times \frac{B}{V}$$

For example:

The maximum number of colonies counted on the heat-treated sample is 21, the volume of sample taken for analysis is 500 ml, and this volume is concentrated to 10 ml, and 0.1 ml is inoculated onto the Petri dishes, then the result, x (expressed as cfu/vol) is given by:

$$x = 21 \times \frac{10}{0.1} = 2100\ cfu/vol$$

Volume of sample analysed = 500ml

The maximum number of colonies counted on the untreated sample is 73, the volume of sample taken for analysis is 700 ml, and this volume is concentrated to 10 ml, and 0.5 ml is inoculated onto the Petri dishes, then the result, x (expressed as cfu/vol) is given by:

$$x = 73 \times \frac{10}{0.5} = 1460\ cfu/vol$$

Volume of sample analysed = 700ml

A12 Expression of results

It is not appropriate to average the number of counts obtained from each of the three Petri dishes of un-treated, acid-treated or heat-treated sample portions. These counts are different and are not replicate determinations of a single treatment process. As a minimum reporting criterion, the confirmed presence (or absence) of *Legionella pneumophila* and the presumptive presence (or absence) of other *Legionella* species should be recorded. The absence of *Legionella* species should be reported as “not detected in the volume of sample examined”. In addition, the serogroup of all isolates of *Legionella pneumophila* identified across different plates should, ideally, be reported.

A13 Quality assurance

A13.1 Media QC

Instructions for the preparation of the media should be strictly adhered to. The stability of some of the components of the media, particularly the α-ketoglutarate potassium salt, can vary between batches. It is essential therefore, that each batch of medium is checked for its

ability to support the growth of Legionella species, particularly *Legionella pneumophila* and its inability to support the growth of some representative background organisms. Example species used are *Legionella pneumophila* serogroup 1 and *Legionella anisa*. Non-target bacteria (for example Pseudomonas aeruginosa, *E. coli* and *Enterococcus faecalis*) should be tested to ensure partial or total inhibition and that the colony presentation does not resemble that of *Legionella* colonies. Petri dishes should be incubated as appropriate and colony growth should commence within two to five days of incubation. Buffered charcoal yeast extract agar with L-cysteine medium (A9.3) should be used as a ""non-selective" comparator plate for assessment of enumeration[16, 20].

Further details on Media QC refer to the Microbiology of Drinking Water Part 3 - Practices and procedures for laboratories and in BS EN ISO 11133 2014[20].

A13.2 Analytical Quality control

Instructions on the Internal Quality Control are given elsewhere in this series [16]. Pages saline (A9.1) or 1:40 Ringers (A9.2) solution should be used when reconstituting cultures of *Legionella* species, or preparing suspensions and dilutions.

A14 References

1. Schulze-Robbecke R, Rodder M, Exner M. 1987. Multiplication and killing temperatures of naturally occurring legionellas. Zentralbl Bakteriol Mikrobiol Hyg [B] 184:495-500.
2. Fitzgeorge RB, Baskerville A, Featherstone ASR. 1987. Fine particle aerosols in experimental Legionnaires disease: Their role in infection and treatment, p. 357-360. Advances in Aerobiology: Proceedings of the third international conference on aerobiology. Birkhauser Verlag, Basel.
3. Bencini MA, Yzerman EP, Koornstra RH, Nolte CC, den Boer JW, Bruin JP. 2005. A case of Legionnaires' disease caused by aspiration of ice water. Arch. Environ. Occup. Health 60:302-306.
4. Correia AM, Ferreira JS, Borges V, Nunes A, Gomes B, Capucho R, Goncalves J, Antunes DM, Almeida S, Mendes A, Guerreiro M, Sampaio DA, Vieira L, Machado J, Simoes MJ, Goncalves P, Gomes JP. 2016. Probable Person-to-Person Transmission of Legionnaires' Disease. N. Engl. J. Med. 374:497-498.
5. Anonymous. 2013. Legionnaires' disease. The control of legionella bacteria in water systems. http://www.hse.gov.uk/pubns/books/l8.htm.
6. Anonymous. 2013. Legionnaires' disease. Part 3. The control of legionella bacteria in other risk systems. http://www.hse.gov.uk/pubns/books/hsg274.htm.
7. Anonymous. 2014. Legionnaires' disease. Part 1. The control of legionella bacteria in evaporative cooling systems. http://www.hse.gov.uk/pubns/priced/hsg274part1.pdf.
8. Anonymous. 2014. Legionnaires' disease. Part 2. The control of legionella bacteria in hot and cold water systems http://www.hse.gov.uk/pUbns/priced/hsg274part2.pdf.
9. Brindle RJ, Stannett PJ, Cunliffe RN. 1987. Legionella pneumophila: comparison of isolation from water specimens by centrifugation and filtration. Epidemiol. Infect. 99:241-247.
10. Anonymous. 2008. BS 7592:2008 Sampling for Legionella bacteria in water systems. Code of practice https://shop.bsigroup.com/ProductDetail/?pid=000000000030161148.
11. Boulanger CA, Edelstein PH. 1995. Precision and accuracy of recovery of Legionella pneumophila from seeded tap water by filtration and centrifugation. Appl. Environ. Microbiol. 61:1805-1809.
12. Anonymous. 2013. The Approved List of biological agents http://www.hse.gov.uk/pubns/misc208.pdf HMSO.
13. Giglio S, Monis PT, Saint CP. 2005. Legionella confirmation using real-time PCR and SYTO9 is an alternative to current methodology. Appl. Environ. Microbiol. 71:8944-8948.
14. Monis PT, Giglio S, Saint CP. 2005. Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal. Biochem. 340:24-34.
15. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning A Laboratory Manual, NY, Cold Spring Harbor Laboratory Press.
16. Anonymous. 2017. The Microbiology of Water and Associated Materials (2017) - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, Standing Committee of Analysts, Environment Agency.

http://www.standingcommitteeofanalysts.co.uk/library/The%20Microbiology%20of%20Water%20and%20Associated%20Materials%20(2017)%20Part%203%20Practices%20and%20Procedu.pdf.
17. Edelstein PH. 1981. Improved semi-selective medium for isolation of Legionella pneumophila from contaminated clinical and environmental specimens. J. Clin. Microbiol. 14:298-303.
18. Anonymous. 2016. Health Technical Memorandum 04-01: Safe water in healthcare premises. Department of Health. https://www.gov.uk/government/publications/hot-and-cold-water-supply-storage-and-distribution-systems-for-healthcare-premises
19. Mercante JW, Winchell JM. 2015. Current and emerging Legionella diagnostics for laboratory and outbreak investigations. Clin. Microbiol. Rev. 28:95-133.
20. Anonymous. 2014 (+A1:2018). BS EN ISO 11133: Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media. https://shop.bsigroup.com/ProductDetail?pid=000000000030346864
21. Molecular Microbial Diagnostic Methods - Pathways to Implementation for the Food and Water Industries – Chapter 9: MALDI-TOF: A rapid microbiological confirmation technique for food and water analysis. Elsevier ISBN 9780124169999, Capocefalo M., Ridley E. V., Tranfield E. Y. & Thompson K. C 2015
22. Anonymous. 2017. BS EN ISO 11731: Water quality. Enumeration of Legionella. https://shop.bsigroup.com/ProductDetail/?pid=000000000030266280
23. Anonymous. 2006. BS EN ISO 19458 Water quality. Sampling for microbiological analysis. https://shop.bsigroup.com/ProductDetail/?pid=000000000030031737
24. Anonymous. 2002. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677. http://www.legislation.gov.uk/uksi/2002/2677/pdfs/uksi_20022677_en.pdf.

B Detection and enumeration of *Legionella* species by filtration, and direct transfer onto a selective medium

B1 Introduction

Legionella species, the cause of legionellosis, are aquatic bacteria that are widespread in nature and occur in water over a wide temperature range. Multiplication has been found to occur between temperatures of 20 – 43 ºC[1] and HSE note that there is a reasonably foreseeable legionella risk if the water temperature in all or some part of a water system may be between 20–45 °C[8]. Their tolerance to relatively high temperatures facilitates the colonisation of artificial water systems that are often above ambient temperatures.

Legionella species are prevalent in artificial water systems, and the disease-causing bacteria can be transmitted from these systems to individuals via aerosols, occasionally by aspiration and on one occasion by person to person spread [2-4]. Cooling towers, hot and cold water systems, Swimming or spa pools and a variety of other sources have also been commonly associated with outbreaks for which guidance has been published(5-8).

Water samples may be examined for *Legionella* species during epidemiological investigations as part of local authority, industrial, or hospital surveillance programmes, or in order to validate new biocide treatment or other control methods(1). Routine sampling should also be carried out based on Legionella Risk Assessments and based on national requirements/guidance (8).

When present, the numbers of legionellae are often low and rarely exceed 1 % of the total bacterial population. As a result, it is usually necessary to concentrate the bacterial flora from water samples before using selective cultural techniques to isolate *Legionella* bacteria (9). However, in outbreak investigations, when potential sources may show high numbers of legionellae and be heavily contaminated with other bacteria, samples should be examined with and without concentration stages being used. The un-concentrated samples (see Method A, A10.2) can yield legionellae even when legionellae would not normally be detected in the concentrated sample due to the overgrowth by other organisms.

Concentration is achieved using membrane filtration.

B2 Scope

This booklet describes a culture method for the detection, identification and enumeration of legionellae in water, as collected using procedures described elsewhere [10].

B3 Definition

For the purposes of this method, *Legionella* is defined as a genus consisting of Gram-negative bacteria normally capable of forming colonies in not less than 2 days (i.e. there is no growth in the first two days of incubation) on buffered charcoal yeast extract agar

containing L-cysteine and iron(III). Colonies typically possess a ground glass-type appearance when viewed with a low powered stereomicroscope although some species of non-pneumophila *Legionella* may not display this morphology. Colonies are often white, purple to blue and can display halos of varying colours. Some species such as *Legionella anisa, L. bozemanii, L. cherri, L. dumoffi, L.gratiana, L. gormanii, L. parisiensis, L. steigwalti L. steelei* and *L. tusconensis* fluoresce under long-wavelength ultra-violet light. Growth does not occur in the absence of L-cysteine, except that *Legionella oakridgensis*, and *Legionella spiritensis* (which require L-cysteine and iron(III) on primary isolation) can grow weakly in the absence of L-cysteine thereafter.

B4 Principle

Micro-organisms, including *Legionella*, in the water sample should be concentrated by membrane filtration. To reduce the growth of unwanted micro-organisms which may inhibit the growth of *Legionella*, the concentrated specimen should be subjected to treatment with acid. Test portion membranes should then be transferred onto Petri dishes containing selective agar medium (B9.5) and incubated. Samples of sediments, biofilm and other surface deposits are not suitable for this method but approaches described in Method A may be more suitable.

Legionella organisms incubated on charcoal-based media produce colonies of typical morphological appearance. There are some species of Legionella that do not produce typical colony morphology on primary isolation e.g. *L. longbeachae, L. cincinnatiensis*. The ground glass morphology while on the whole accurate for Legionella pneumophila is not always visible in colonies grown on a membrane filter.

The membrane filtration with direct transfer method can be used in water intended for human use (e.g. hot and cold water, water used for washing), for human consumption and for treated bathing waters (e.g. swimming pools). It is especially suitable for waters expected to contain low numbers of *Legionella* and background microorganisms. As the growth of *Legionella* may be inhibited by overgrowth of other bacteria on the membrane, the method is only suitable for waters containing low bacterial count. This method is not suitable for turbid water; samples with high bacterial counts such as Cooling Tower waters. Therefore, no centrifugation or other concentration stages should be required prior to filtration. The approaches described in Method A are more appropriate for these types of samples.

B5 Limitations

Not all biocides have recognised neutralising agents and where present they will therefore continue to exert their bactericidal effects after sampling. Failure to neutralise biocides may result in false negative results or an under-estimation of the number of microorganisms (e.g. *Legionella*) present in the sample.

Sample containers should therefore contain appropriate neutraliser where available [7] and a record kept of the biocide and neutralising agent used to contextualise the results of the

examination made.

Other bacteria, particularly pseudomonads, or fungi present in a sample may grow on the agar medium and either inhibit the growth of legionellae or severely mask their presence however overgrowth by other micro-organisms does not mean that the sample is negative for *Legionella*.

The enumeration of *Legionella* may be underestimated as it is generally assumed that one legionella cell produces one visible colony. The true count may however be significantly higher as clumps of *Legionella* cells also produce only one colony. The culture methods presently used for the detection of legionellae from water rarely achieve 100 % recovery, with most current practices only achieving a level of between approximately 10 - 50 %. Consequently, the true number of legionellae in water is usually higher than that detected using these methods (11).

Legionella oakridgensis and *Legionella spiritensis* require L-cysteine and iron(III) for primary isolation but may grow weakly in the absence of added L-cysteine thereafter. Accordingly, careful comparison needs to be made of the differences in growth between supplemented and un-supplemented media, particularly during confirmatory stages.

If high levels of interfering micro-organisms are anticipated, the approaches described in Method A may be more suitable.

B6 Health and safety

WARNING - Species of *Legionella* are pathogenic

Risk of harm is caused by the inhalation of aerosolised disease causing strains of Legionella bacteria it is therefore advisable to assess all techniques for their ability to produce aerosols.

All samples submitted for *Legionella* analysis should therefore be regarded as potentially contaminated with bacteria classified as “Hazard Group 2” and handled with strict adherence to the general safety precautions described for work at Containment Level 2 (CL2), to include minimising exposure to aerosols at all times[12,13].

Based on the risk assessment of procedures undertaken it may be necessary to carry out the examination in a microbiological safety cabinet. However, experience has determined that Legionella spp. can be handled safely by experienced microbiologists on the open bench in a conventional microbiology laboratory conforming to containment level two.

Media, reagents and bacteria used in this method are covered by regulations[24] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere in this series[15].

B7 Storage of samples during transportation

At temperatures lower than 6 ºC *Legionella* can enter a viable but non-culturable state and at temperatures above 20 ºC proliferation is likely, therefore transport and storage at ambient temperatures - ideally 6 ºC - 20 ºC is recommended [10].

Samples should be analysed as soon as possible not exceeding more than two days, and preferably within 24 hours.

The laboratory should however verify that their chosen transport and storage conditions are appropriate.

B8 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria described elsewhere in this series[6].

B8.1 Membrane filter for direct membrane transfer: cellulose nitrate or mixed cellulose esters, pore size 0.22 µm or 0.45 µm and diameter of 47 mm.

NOTE Black membrane filters may be better for observing the white Legionella colonies than lighter coloured membrane filters.

B8.2 Vacuum-assisted membrane filtration apparatus and vacuum pump suitable for membrane filters with diameter of 47 mm and filtering volumes of water of up to 1 litre.

B8.3 Incubator, capable of being maintained at 36.0 ± 1.0 ºC. A humidified incubator is preferable, but use of carbon dioxide is optional.

B8.4 Low power binocular microscope, with at least 6-times magnification, and illuminated from above by oblique incident light.

B8.5 Ultraviolet lamp, emitting light of wavelength 360 ± 20 nm.

B8.6 Smooth-tipped forceps.

B9 Media and reagents

Commercial formulations of these media and reagents are readily available, but may possess minor variations in their formulation. Commercial formulations should be used and stored according to manufacturer's instructions. Laboratories may decide to produce their own media. The performance of all media and reagents should be verified prior to their use in the method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly.

Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

B9.1 1:40 Ringer's solution

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride hexahydrate	0.12 g
Sodium hydrogen carbonate	0.05 g
Water to	1000 ml

Dissolve the ingredients in the water and mix thoroughly. Dilute the resulting solution with water in the ratio 1:10. This solution may be dispensed as required in suitable volumes into suitable containers, capped and autoclaved at 121 ºC for 15 minutes. The pH of the final solution should be 7.0 ± 0.2 (16)

B9.2 Page's saline solution

Sodium chloride	0.12 g
Magnesium sulphate heptahydrate	0.004 g
Calcium chloride dehydrate	0.004 g
Disodium hydrogen orthophosphate	0.142 g
Potassium dihydrogen phosphate	0.136 g
Water to	1000 ml

Dissolve the ingredients in the water and mix thoroughly. The resulting solution may be dispensed as required in suitable volumes into suitable containers, capped and autoclaved at 121 ºC for 15 minutes. To facilitate accurate preparation, 10 litres of Page's saline solution may be prepared and dispensed in volumes as required for autoclaving at 121 ºC for 15 minutes. Alternatively, a concentrated solution may be prepared and diluted as appropriate for use.(16)

B9.3 Buffered charcoal yeast extract agar with L-cysteine (BCYE) (17)

N-2-acetamido-2-amidoethane-sulphonic acid (ACES) buffer	10.0 g
Potassium hydroxide	2.8 g
Activated charcoal	2.0 g
Yeast extract (bacteriological grade)	10.0 g
α-ketoglutarate, potassium salt	1.0 g
Agar	12.0 g

L-cysteine hydrochloride monohydrate	0.4 g
Iron(III) pyrophosphate	0.25 g
Water to	1000 ml

Add 0.4 g of L-cysteine hydrochloride to 10 ml of water. Filter-sterilise the solution by filtration through a membrane filter with a nominal pore size of 0.22 µm. Add 0.25 g of iron(III) pyrophosphate to 10 ml of water. Filter-sterilise the solution by filtration through a membrane filter with a nominal pore size of 0.22 µm. The sterilised solutions may be stored in clean sterile containers at – 20 ± 3 ºC for up to 3 months, but should be allowed to reach room temperature before use. If these solutions are not used immediately they should be re-sterilised before addition to the medium.

Add 10 g of N-2-acetamido-2-amidoethane-sulphonic acid (ACES) buffer to 500 ml of water in a suitable container. Dissolve the buffer by standing the container in a water bath at 45 - 50 ºC. Add 2.8 g of potassium hydroxide to 480 ml of water and dissolve with gentle shaking. Mix the two solutions.

The ACES buffer can de-nature the yeast extract if the following sequence is not strictly followed. Add sequentially to 980 ml of alkaline ACES buffer solution, 2 g of the activated charcoal, 10 g of the yeast extract and 1 g of the α-ketoglutarate salt. Mix well between each addition. As appropriate, use 0.1 mol l-1 potassium hydroxide solution or 0.1 mol l-1 sulphuric acid solution and adjust the pH of the resulting ACES buffer solution to 6.8 ± 0.1. Add the agar, mix well and autoclave at 121 ºC for 15 minutes. After autoclaving, allow the solution to cool to 50 ± 2 ºC in a water bath. Aseptically, add the L-cysteine and the iron(III) pyrophosphate solutions, mixing well between each addition. The pH of the final medium should be 6.8 ± 0.2 at 25 ºC. Dispense the medium, in volumes of 20 ml, into Petri dishes, nominally between 90 - 100 mm in diameter and allow the medium to cool. Allow any excess moisture on the agar to dry. The Petri dishes may be stored at 5 ± 3 ºC in airtight containers in the dark for up to 4 weeks.

The medium is heat sensitive and prolonged heating or too high a temperature during sterilisation can severely affect the nutritional qualities of the medium

B9.4 Buffered charcoal yeast extract agar without L-cysteine (BCYE –cys)

This medium should be prepared as described in section B9.3 but without the addition of L-cysteine hydrochloride.

B9.5 Buffered charcoal yeast extract agar with glycine and antibiotic supplements (GVPC)

This medium is more selective than BCYE (B9.3). It includes glycine and antibiotics and should be prepared as described in section B9.3 with the addition of glycine before the agar is added, and the addition of the antibiotic supplements, after the addition of the L-cysteine and iron(III) pyrophosphate solutions. The glycine and antibiotic supplements should be added to the medium to give the following final concentrations:

Polymyxin B sulphate	80,000 iu l-1
Vancomycin hydrochloride	1 mg l-1
Cycloheximide	80 mg l-1
Glycine (ammonia-free)	3 g l-1

Cycloheximide is hepatotoxic and appropriate protective clothing should be worn when handling this chemical

The concentration (approximately 7000 iu mg^{-1}) of each batch of polymyxin B sulphate should be checked to ensure the correct final concentration is achieved.

Dissolve the appropriate amount of polymyxin B sulphate (about 200 mg, i.e. about 1400000 iu) in 100 ml of water. Filter-sterilise the solution through a 0.22 µm membrane filter, and dispense in suitable volumes, typically about 5.7 ml, into sterile containers. The solution may be stored at -20 ± 3 ºC for up to 6 months. However, the solution should be allowed to thaw to room temperature before use.

Dissolve 20 mg of vancomycin hydrochloride in 20 ml of water. Filter-sterilise the solution through a 0.22 µm membrane filter and dispense the solution, in volumes of 1 ml, into sterile containers. These containers may be stored at -20 ± 3 ºC for up to 6 months, but the solution should be allowed to thaw to room temperature before use.

Dissolve 2 g of cycloheximide in 100 ml of water. Filter-sterilise the solution through a 0.22 µm membrane filter and dispense the solution, in volumes of 4 ml, into sterile containers. These containers may be stored at -20 ± 3 ºC for up to 6 months, but the solution should be allowed to thaw to room temperature before use.

Prepare the medium, as described in section B9.3, but with the addition of glycine just before the addition of the agar. Adjust the pH of the solution to 6.8 ± 0.2. After the addition of the L-cysteine and iron(III) pyrophosphate solutions, add the antibiotic supplements solutions. The pH of the final medium, should be 6.8 ± 0.2. If the pH is higher, certain species of *Legionella* may be inhibited(8)

B9.6 Acid buffer

A9.6.1 To prepare the acid buffer: Mix together 3.9 ml of 0.2 mol l-1 hydrochloric acid solution with 25 ml of 0.2 mol l-1 potassium chloride solution. Adjust the pH of the solution to 2.2 ± 0.2 by the addition of 1 mol l-1 potassium hydroxide solution. Store the resulting solution in a sealed container, in the dark, at room temperature. This solution may be stored for up to one month prior to use.

Prepare 0.2 mol l-1 hydrochloric acid solution by adding 17.4 ml of concentrated hydrochloric acid (specific gravity 1.18, minimum assay 35.4 %, approximately 10 M) to 1000 ml of water. Depending upon the concentration of the acid, the exact volume of acid may need to be adjusted accordingly.

Prepare 0.2 mol l-1 potassium chloride solution by dissolving 14.9 g of potassium chloride in 1000 ml of water.

Prepare 1 mol l-1 potassium hydroxide solution by dissolving 40 g of potassium hydroxide in 1000 ml of water.

This is a 1/2 the concentration of the Acid Buffer described in Method A (A9.6).

B10 Analytical procedure

B10.1 Water sample preparation

In the majority of testing scenarios, (for example samples for routine monitoring or commissioning) the number of legionellae in any given water sample is unknown. If the sample is known to contain high levels of legionellae the sample maybe split into smaller volumes prior to filtering. The total volume of sample used in the analysis should be recorded before concentration by filtration.

Before analysis the sample should be mixed by gentle inversions to produce a homogenised solution or suspension while minimising aerosol generation. This action may generate aerosols containing *Legionella*. The sample should then be allowed to stand for an appropriate time period to allow the dispersal of any aerosol formed before the concentration procedures are carried out.

NOTE: If there is visible separation or sedimentation in the sample it may be appropriate to record this.

B 10.2 Factors affecting the outcome of the method

The volume of the sample analysed depends upon the limit of detection required, and the purpose of the investigation. Current guidance in the UK sets alert and action limits based on enumerated counts of *Legionella* of 100 colony forming units (cfu) per litre (8). Hence, the volume analysed should enable at least 100 cfu in a litre of sample to be detected. Facilities such as healthcare premises by their nature contain vulnerable patients that are considered to be in a higher risk category for *Legionella* infection. Samples from this type of facility may therefore require a method which gives a lower detection limit (18). Filtration and direct membrane transfer obtains a lower limit of detection of 1 cfu colony forming units (cfu) per volume analysed.

When a concentration step is used, micro-organisms may be lost (i.e. not fully recovered) and a proportion of the target population may be killed or sub-lethally damaged by the selective procedures used.

The recovery of *Legionella* is also dependent upon the lack of interference effects from other organisms and therefore, in addition to the use of anti-microbial compounds in the

selective media, treatment of portions of the sample with acid or heat is required. On occasions the interference from background organisms is greater than anticipated, or the count of *Legionella* is higher than expected, or a particular type of *Legionella* is sought in outbreak investigations.

The sample volume tested should be ideally 1000 ml and if a lower volume of sample is analysed e.g. due to difficulty filtering the sample, probability of high levels of *Legionella*, or a lower volume being provided, then the volume should be recorded in the report.

Where it is necessary to detect a small number of organisms, this may be achieved by filtering a sample in smaller volumes, typically 100 ml and 900 ml and transferring the membrane to separate selected agar plates (B9.5) [15]. This will reduce the risk of possible interference from background organisms, and reduce the chance of high numbers of *Legionella* organisms reaching the upper counting limit. Results can be reported from the volume that is most suitable or if the count is zero the combined volume.

B10.3 Membrane filtration

Filter volumes of water up to 1000 ml, using vacuum-filtration. On occasions, more than one filter may be necessary, and all membrane filters should be transferred to separate agar plates (B10.5).

B10.4 Acid treatment

To minimize the growth of non-Legionella bacteria, treat the sample with acid buffer (B9.6) directly in the filter funnel as follows. After filtration of the sample, add (30±5) ml of acid buffer on the top of the membrane and leave for 5 min. Remove the acid buffer by filtration through the membrane and wash the membrane by adding (20±5) ml of appropriate buffer solution (B9.1 or B9.2) and drawing this through the membrane filter, taking care that none of solution used to flush the membrane comes into contact with area of the filtration apparatus that have not previously been in contact with acid buffer.

Note: a duplicate volume of the sample can also be processed without acid treatment if required.

B10.5 Culture

Remove the funnel and transfer the membrane filter carefully with sterile forceps and place it (upside up) on a selective *Legionella* medium plate (B9.5). Ensure that no air bubbles are trapped between the membrane filter and the medium. ‘Rolling’ the membrane filter onto the medium minimises the likelihood of air bubbles becoming trapped. Cover the membrane filter with the lid of the Petri dish.

If multiple filtrations were carried out for a sample, the membrane filters must be transferred to separate agar plates.

B10.6 Incubation

The Petri dish should then be inverted and incubated at 36.0 ± 1.0 ºC for 10 days. Throughout the incubation, a humid atmosphere should be maintained. This can be achieved by a variety of methods, for example; incubation in closed containers or bags or by placing a container of water inside the incubator. The container of water in the incubator may become contaminated, for example with *Legionella* or fungi, and should therefore be periodically discarded (in an appropriate manner) or disinfected.

B10.7 Examination of plates

At suitable intervals, usually 2 - 3 days (e.g. days 3, 7 and 10), during the 10-day incubation period, the Petri dish should be removed from the incubator and the medium examined for growth. Ideally a binocular microscope with oblique incident light should be used as it can be very difficult to identify *Legionella* by naked eye. Colonies of *Legionella* are often white-grey-blue-purple in colour on the membrane (Figure B1), but may be brown, pink, green or deep red. Colonies are smooth with an entire edge and most will exhibit a characteristic ground-glass appearance throughout the colony when grown on GVPC directly. However, this appearance is limited to the edge of the colony when grown on a membrane (Figure B2). Some species of Legionellae may not produce typical ground-glass colonies on primary isolation.

Figure B1 Ground glass appearance of *Legionella pneumophila* colonies at day 7 on a black cellulose nitrate membrane

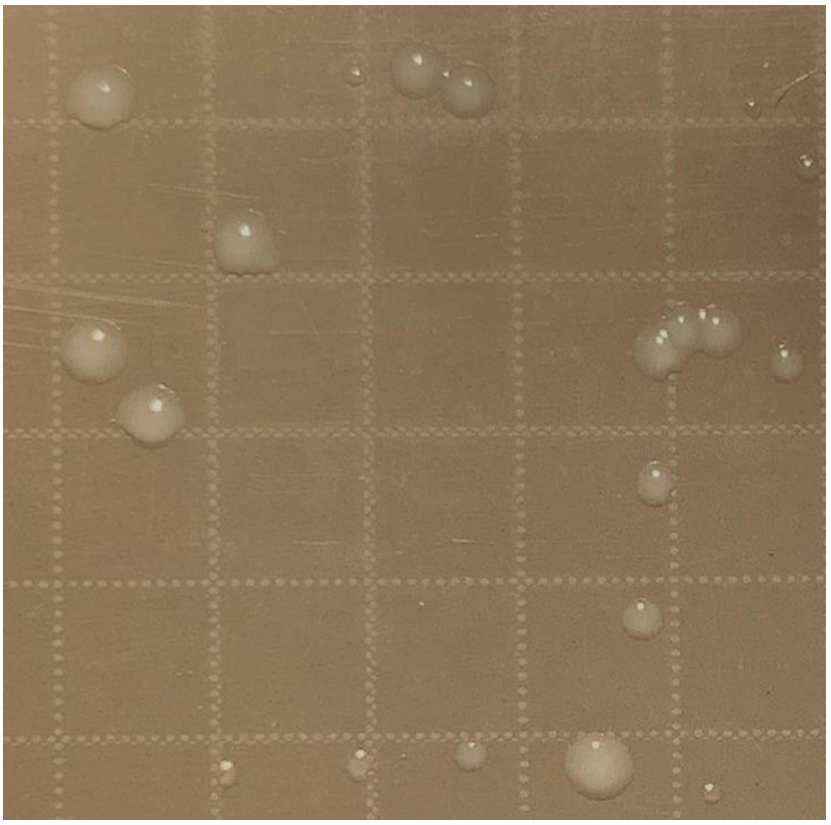
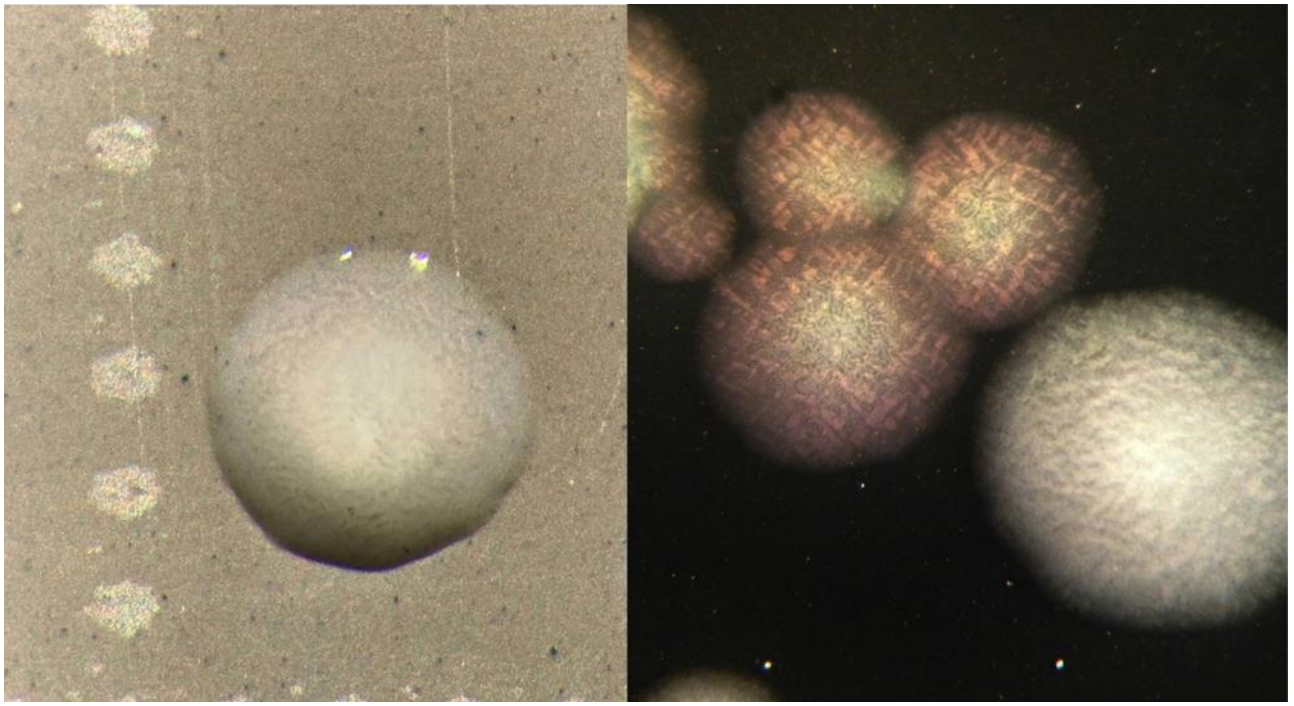


Figure B2 (Left) Ground glass appearance around the edge of *Legionella pneumophila* colonies at day 4 on a black cellulose nitrate membrane (left) compared to direct inoculation on GVPC (right).



If high numbers of *Legionella* organisms are observed on all the volumes filtered, then this should be recorded as greater than 100 colony forming units (cfu) per litre. If there is confluent growth of non-*Legionella* colonies, this should be recorded on the analytical report. For example: 'Sample overgrown with non-target organisms".

The number of presumptive colonies conforming to the typical morphology of *Legionella* should be counted and recorded for each Petri dish incubated. If different types of colonial morphologies are observed, the number and details of each type should be recorded and confirmatory tests carried out (see Section B10.9). If a series of dilutions have been used, then, where possible, the Petri dish or Petri dishes that contain between 20 - 80 *Legionella* colonies should be counted.

It may be helpful to examine the Petri dishes briefly under ultra-violet light (360±20nm). particularly any samples with suspected *Legionella* as often there will be mixed populations of fluorescent and non-fluorescent species. Several species of *Legionella* (for example *Legionella anisa*) fluoresce with a brilliant blue-white colour and other species (for example *Legionella rubrilucens* and *Legionella erythra*) appear red. *Legionella pneumophila* appear dull green, often tinged with yellow.

B10.8 Confirmation and identification

A variety of methods are available for the confirmation and identification of *Legionella* organisms. These methods may include the following but the laboratory should verify the

effectiveness of the chosen approach:

- sub-culture to confirmation media
- latex agglutination tests
- MALDI-TOF
- polymerase chain reaction tests
- direct fluorescent antibody assays
- gas-liquid chromatographic methods, for example for cellular fatty acids and isoprenoid quinone compounds
- slide agglutination tests
- colony blot assays based on genus-specific monoclonal antibodies
- enzyme-linked immuno-sorbent assays using diagnostic reagents

B10.8.1 Sub-culture for confirmation

Presumptive colonies from the plate with the highest count (at least three colonies of each morphological type) and, if appropriate (if different colony morphologies are present on the different plates), up to 5 colonies should be sub-cultured into two separate Petri dishes of media (B9.3) and (B9.4). An alternative medium for the buffered charcoal yeast extract agar without L-cysteine medium (B9.4) comprises 4 % horse blood agar or nutrient agar as detailed in ISO 11731[22]. After incubating for 2 - 3 days at 36 ± 1.0 ºC the Petri dishes should be removed from the incubator and the medium examined for growth. Colonies that only grow on the buffered charcoal yeast extract agar with L-cysteine medium (B9.3) are regarded as *Legionella* organisms, and those colonies that grow on both media are considered as non-*Legionella* organisms. It is assumed that careful picking of the colony will result in a pure subculture on both media however sometimes this may not occur. It is good practice to examine the plates under a microscope to confirm that samples not confirming at legionella are not due to erroneous plating out of a mixed isolate.

B10.8.3 MALDI-ToF

Matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-ToF) is a rapid (~10 min) and very sensitive confirmation method(21).

Several platforms are available from different providers which work on similar principles. A small quantity of biomass (usually taken from an isolated pure colony on an agar plate) is spotted onto a target plate and overlaid with an organic matrix. Additional pre-treatment of the sample may be required prior to spotting. Laboratories should refer to the manufacturers' recommended protocols. A pulsed laser irradiates the sample, triggering desorption and ionisation. Molecules are separated by mass-to-charge ratio and detected, the target being mainly the ribosomal proteins.

By comparing this information gathered at the detector to calibration standards, it is possible to calculate the molecular mass associated with any particular peak, and measure the relative intensity of that peak. These data are then compared to a database of spectra

(usually from the manufacture or an in-house database validated by the testing laboratory) and an organism identification and associated confidence score is generated.

MALDI-ToF has been used successfully to identify *Legionella* isolates from both environmental and clinical samples (19). An added advantage of MALDI-ToF is the identification of *Legionella* species that are currently not identifiable to the species level by traditional confirmation techniques. However, laboratories should note that MALDI-ToF is currently unable to discriminate between different *Legionella* serogroups e.g. *Legionella pneumophila* serogroup 1 vs. *Legionella pneumophila* serogroups 2-14. This is a developing field and, for example, molecular typing methods for *Legionella* using MALDI-ToF are in development and may prove useful in public health investigations.

For confirmation of presumptive *Legionella* from environmental samples isolates may be tested from the primary isolation plates (A9.8) or the confirmation plates following sub-culture (A9.9.1). Multiple colonies should be confirmed as prescribed in section A9.9.1. The age of the colony may affect the accuracy of results therefore testing laboratories should refer to the manufacturer's recommended protocols. Testing laboratories should ensure that MALDI-ToF methods are appropriately verified to confirm that performance is equal to or greater than the traditional confirmation method used.

B10.8.4 DNA amplification by polymerase chain reaction PCR

DNA amplification by polymerase chain reaction PCR can also be used for the rapid confirmation of *Legionella pneumophila* and *Legionella spp.* presumptive colonies. This may be achieved by conventional end-point PCR or real-time PCR (qPCR). Specific primers (and probe for qPCR) allow to obtain an unambiguous confirmation in just few hours. Resolution can be done by electrophoresis (classic end-point PCR) and/or fluorescent signal (qPCR).

The procedure consists of preparing a solution from each presumptive morphology in phosphate buffer saline solution (PBS) or sterile distilled water (SDW). From this solution DNA is extracted and purified by different techniques, based on physical (e.g. cycles of freezing and thawing), chemical (e.g. guanidine thiocyanate buffer) or enzymatic digestion. The next step consists of preparation of a reaction mix where extracted DNA is mixed in a PCR tube or plate with a master mix containing DNA polymerase, specific primers, deoxynucleotide triphosphates (dNTPs) and probe for qPCR in an optimized reaction buffer. Finally this reaction mix is loaded into the thermocycler for the amplification to run. Detailed procedures together with limitations, health and safety and general testing conditions for DNA amplification by qPCR are described in Part D of this booklet.
There are several commercial kits available for both DNA extraction and amplification, which include controls and manufacturer's validation, supporting a quick implementation of this methodology.

B10.8.5 Direct fluorescent antibody (DFA)

Direct fluorescent antibody (DFA) is an immunological technology where specific *Legionella*

antigens are targeted by fluorescent dye-labelled antibodies. Then antibody – antigen complex is detected by exposing to ultra violet or blue violet light, which causes blue-green or yellow-green fluorescence.

Prepare a light suspension (1 McFarland Standard) of each colony to be tested, that grows on the buffered charcoal yeast extract agar with L-cysteine medium, doesn't grow on agar without L- cysteine and is regarded as *Legionella* (A10.7.1), in 3 ml of sterile distilled water, phosphate buffer saline solution or 1% neutral formalin. The suspension should be just visible to the naked eye. Add 10 µl of the culture suspension into appropriate number of wells (depending on number of reagents in the kit used) of a clean multi-well glass Polytetrafluoroethylene (PTFE) printed slide. Add approximately 10 µl of positive control reagent onto one well and negative control reagent onto another one. Air dry the slide (it can be warmed to temperature between 35°C and 45°C in an incubator). Fix the prepared slide by gentle heating in a Bunsen flame or by immersion in acetone for 10 minutes followed by drying in air.

Add 5 µl of each monoclonal anti-serum to each appropriate well and 5 µl of polyvalent control reagent (including polyclonal anti-serum) to a well with positive and negative control and transfer the slide into a suitable humid container, for example a box containing damp tissue in order to prevent the slides from drying out. Cover the container to avoid exposure to light and incubate for 20 to 30 minutes at the temperature specified by the reagents manufacturer. After incubation, wash the slides to remove excess antibodies by immersion in water or phosphate buffered saline solution. Discard the solution and repeat the process two more times with fresh water or phosphate buffered saline solution. Finally dry the slides at a temperature between 35° - 50 °C.

After drying, add a few drops of mounting medium reagent onto the test slide to hold the specimens in place between the cover slip and the slide and apply a coverslip, ensuring no bubbles are trapped beneath. Using a fluorescence microscope examine the slide applying immersion oil for a high power (100X) objective. Examine a positive and a negative control well first to make sure that the test is valid. The slide may be stored in the dark prior to examination for a maximum 24 hours.

There are commercial DFA test kits available, which include manufacturer's instruction with test procedure, quality control and interpretation of results.

B11 Calculations

Current UK guidance[5,6,7,8] sets alert and action limits for the presence of legionella in units of colony forming units per litre (cfu/L). Using the following equation, calculate the number of *Legionella* organisms expressed as cfu per litre of original water, taking into account the volume of sample analysed and the number of colonies (for each confirmed colonial type) counted:

$$Total\ cfu/L\ (x) = \frac{n}{A} \times 1000$$

Where:

n *is the total number of confirmed colonies counted across all the plates analysed (if an additional sample portion was analysed without acid treatment [B10.4 NOTE] then take the highest count from the two treatments):*

$$n = \frac{number\ of\ colonies\ confirming}{number\ of\ presumptive\ colonies\ confirmed} \times total\ presumptive\ colonies$$

A is the volume of sample analysed (typically, 500 - 1000 ml);

For example,

1 L of sample was filtered onto a petri dish. The maximum number of presumptive colonies counted on the sample is 45, the number of presumptive colonies put on for confirmation is 5 and the number of colonies that have confirmed is 4. Then the result, x (expressed as cfu per litre) is given by:

$$\mathrm{n} = \frac{4}{5} \times 45$$

$$\mathrm{n} = 36$$

$$\mathrm{Total\ cfu/L\ (x)} = \frac{36}{1000} \times 1000$$

$$\mathrm{x} = 36\ \mathrm{cfu/L}$$

For example,

100 ml of sample was filtered onto a petri dish. The maximum number of presumptive colonies counted on the sample is 37, the number of presumptive colonies put on for confirmation is 5 and the number of colonies that have confirmed is 3. Then the result, x (expressed as cfu per litre) is given by:

$$\mathrm{n} = \frac{3}{5} \times 37$$

$$\mathrm{n} = 22$$

$$\mathrm{Total\ cfu/L\ (x)} = \frac{22}{100} \times 1000$$

$$\mathrm{x} = 220\ \mathrm{cfu/L}$$

Should it be required to also report the result in the volume of sample initially analysed (e.g. volume filtered), the result (n) is the total number of confirmed colonies in the volume of original water sample analysed. This is expressed in units of (cfu/vol) and represented by

the following equation. The volume analysed should also be reported:

$$Total\ cfu/vol\ (y) = n$$

For using the 100 ml example shown above;

$$n = 22\ \text{cfu/vol}$$

$$Volume\ of\ sample\ analysed = 100ml$$

B12 Expression of results

As a minimum reporting criterion, the confirmed presence (or absence) of *Legionella pneumophila* and the presumptive presence (or absence) of other *Legionella* species should be recorded. The absence of *Legionella* species should be reported as "not detected in the volume of sample examined". In addition, the serogroup of all isolates of *Legionella pneumophila* identified should, ideally, be reported.

B13 Quality assurance

B13.1 Media QC

Instructions for the preparation of the media should be strictly adhered to. The stability of some of the components of the media, particularly the α-ketoglutarate potassium salt, can vary between batches. It is essential therefore, that each batch of medium is checked for its ability to support the growth of *Legionella* species, particularly *Legionella pneumophila* and its inability to support the growth of some representative background organisms. Example species used are *Legionella pneumophila* serogroup 1 and *Legionella anisa*. Non-target bacteria (for example *Pseudomonas aeruginosa, E. coli* and *Enterococcus faecalis*) should be tested to ensure partial or total inhibition and that the colony presentation does not resemble that of *Legionella* colonies. Petri dishes should be incubated as appropriate and colony growth should commence within two to five days of incubation. Buffered charcoal yeast extract agar with L-cysteine medium (A9.3) should be used as a "non-selective" comparator plate for assessment of enumeration.[16, 20]

Further details on Media QC are given elsewhere in this series [16] and in BS EN ISO 11133:2014[20].

B13.2 Analytical Quality control

Instructions on the Internal Quality Control are given elsewhere in this series [16]). Pages saline (B9.1) or 1:40 Ringers (B9.2) solution should be used when reconstituting cultures of *Legionella* species, or preparing suspensions and dilutions.

B14 References

1. Schulze-Robbecke R, Rodder M, Exner M. 1987. Multiplication and killing temperatures of naturally occurring legionellas. Zentralbl Bakteriol Mikrobiol Hyg [B] 184:495-500.
2. Fitzgeorge RB, Baskerville A, Featherstone ASR. 1987. Fine particle aerosols in experimental Legionnaires disease: Their role in infection and treatment, p. 357-360. In Thing A (ed.), Advances in Aerobiology: Proceedings of the third international conference on aerobiology. Birkhauser Verlag, Basel.
3. Bencini MA, Yzerman EP, Koornstra RH, Nolte CC, den Boer JW, Bruin JP. 2005. A case of Legionnaires' disease caused by aspiration of ice water. Arch. Environ. Occup. Health 60:302-306.
4. Correia AM, Ferreira JS, Borges V, Nunes A, Gomes B, Capucho R, Goncalves J, Antunes DM, Almeida S, Mendes A, Guerreiro M, Sampaio DA, Vieira L, Machado J, Simoes MJ, Goncalves P, Gomes JP. 2016. Probable Person-to-Person Transmission of Legionnaires' Disease. N. Engl. J. Med. 374:497-498.
5. Anonymous. 2013. Legionnaires' disease. The control of legionella bacteria in water systems. http://www.hse.gov.uk/pubns/books/l8.htm.
6. Anonymous. 2013. Legionnaires' disease. Part 3. The control of legionella bacteria in other risk systems. http://www.hse.gov.uk/pubns/books/hsg274.htm.
7. Anonymous. 2014. Legionnaires' disease. Part 1. The control of legionella bacteria in evaporative cooling systems. http://www.hse.gov.uk/pubns/priced/hsg274part1.pdf.
8. Anonymous. 2014. Legionnaires' disease. Part 2. The control of legionella bacteria in hot and cold water systems http://www.hse.gov.uk/pUbns/priced/hsg274part2.pdf.
9. Brindle RJ, Stannett PJ, Cunliffe RN. 1987. Legionella pneumophila: comparison of isolation from water specimens by centrifugation and filtration. Epidemiol. Infect. 99:241-247.
10. Anonymous. 2008. BS 7592:2008 Sampling for Legionella bacteria in water systems. Code of practice https://shop.bsigroup.com/ProductDetail/?pid=000000000030161148
11. Boulanger CA, Edelstein PH. 1995. Precision and accuracy of recovery of Legionella pneumophila from seeded tap water by filtration and centrifugation. Appl. Environ. Microbiol. 61:1805-1809.
12. Anonymous. 2013. The Approved List of biological agents http://www.hse.gov.uk/pubns/misc208.pdf HMSO.
13. Giglio S, Monis PT, Saint CP. 2005. Legionella confirmation using real-time PCR and SYTO9 is an alternative to current methodology. Appl. Environ. Microbiol. 71:8944-8948.
14. Monis PT, Giglio S, Saint CP. 2005. Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal. Biochem. 340:24-34.
15. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning A Laboratory Manual, NY, Cold Spring Harbor Laboratory Press.
16. Anonymous. 2017. The Microbiology of Water and Associated Materials (2017) - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, Standing Committee of Analysts, Environment Agency.

http://www.standingcommitteeofanalysts.co.uk/library/The%20Microbiology%20of%20Water%20and%20Associated%20Materials%20(2017)%20Part%203%20Practices%20and%20Procedu.pdf.

17. Edelstein PH. 1981. Improved semi-selective medium for isolation of Legionella pneumophila from contaminated clinical and environmental specimens. J. Clin. Microbiol. 14:298-303.
18. Anonymous. 2016. Health Technical Memorandum 04-01: Safe water in healthcare premises. Department of Health. https://www.gov.uk/government/publications/hot-and-cold-water-supply-storage-and-distribution-systems-for-healthcare-premises
19. Mercante JW, Winchell JM. 2015. Current and emerging Legionella diagnostics for laboratory and outbreak investigations. Clin. Microbiol. Rev. 28:95-133.
20. Anonymous. 2014 (+A1:2018). BS EN ISO 11133:2014 Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media. https://shop.bsigroup.com/ProductDetail?pid=000000000030346864
21. Molecular Microbial Diagnostic Methods - Pathways to Implementation for the Food and Water Industries – Chapter 9: MALDI-TOF: A rapid microbiological confirmation technique for food and water analysis. Elsevier ISBN 9780124169999, Capocefalo M., Ridley E. V., Tranfield E. Y. & Thompson K. C 2015
22. Anonymous. 2017. BS EN ISO 11731:2017 Water quality. Enumeration of Legionella. https://shop.bsigroup.com/ProductDetail/?pid=000000000030266280
23. Anonymous. 2006. BS EN ISO 19458:2006 Water quality. Sampling for microbiological analysis. https://shop.bsigroup.com/ProductDetail/?pid=000000000030031737
24. Anonymous. 2002. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

C The detection and enumeration of *Legionella pneumophila* by a diagnostic substrate most probable number technique

This method has not been subject to widespread use or any previous verification of performance within the UK. Published data [1, 2] from multi-laboratory studies compared the results generated using this method with those obtained using the GVPC agar membrane filtration method (method B in this booklet). The studies demonstrated equivalent or better performance for the isolation of *Legionella pneumophila*. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. Information on the routine use of this method and similar methods, would be welcomed to assess their full capabilities.

C1 Introduction

Legionella species, the cause of legionellosis, are aquatic bacteria that are widespread in nature and occur in water over a wide temperature range but most frequently between temperatures of 30 - 45 °C [3]. Official statistics show that of the 1070 cases recorded in England and Wales between 2014 and 2016, 99.7% can be attributed to *Legionella pneumophila* as the primary reported cause of Legionnaires' Disease[4]. However, this is in part due to the under-diagnosis for non-pneumophila infections due to the screening techniques being optimised for *Legionella pneumophila*[20].

Their tolerance to relatively high temperatures facilitates the colonisation of artificial water systems that are often above ambient temperatures. *L. pneumophila* is prevalent in artificial water systems, and Legionnaires' Disease and other forms of legionellosis are caused by the species being transmitted from these systems to individuals via aerosols, occasionally by aspiration and on one occasion by person to person spread [5-7]. Cooling towers, hot and cold water systems, spa pools and a variety of other sources have also been commonly associated with outbreaks for which guidance has been published [8-11].

Water samples may be examined for *Legionella* species during epidemiological investigations as part of local authority, industrial, or hospital surveillance programmes, or in order to validate new biocide treatment or other control methods [3]. Routine sampling should also be carried out based on Legionella Risk Assessments and based on national requirements/guidance [11, 12].

When present, the numbers of legionellae are often low and rarely exceed 1 % of the total bacterial population. As a result, it is usually necessary to concentrate the bacterial flora from water samples before using selective culture techniques to isolate *Legionella* bacteria [13].

However, in outbreak investigations, when potential sources may show high numbers of legionellae and be heavily contaminated with other bacteria, samples should be examined with and without concentration stages being used. The un-concentrated samples can yield legionellae even when legionellae would not normally be detected in the concentrated sample due to the overgrowth by other organisms.

C2 Scope

This procedure describes a liquid culture method for the detection, identification and enumeration of *Legionella pneumophila* in potable water and other water samples, as collected using procedures described elsewhere [14].

C3 Definitions

In the context of this method, organisms which produce brown colouration through the utilisation of a substrate in a defined nutrient medium by a diagnostic enzyme, or produce turbid growth with or without brown colouration, are regarded as *L. pneumophila*.

C4 Principle

Organisms are grown in a defined liquid culture medium containing a substrate for the specific detection of *L. pneumophila*. Potable water samples are evaluated for total hardness value using a dip strip. Hardness is then neutralised with a provided supplement. The dehydrated medium is dissolved in 100 ml of hardness neutralised sample, or dilution of sample, which is then added to a 96-well reaction pouch. This is then sealed and incubated at 39 °C for 7 days in the presence of humidity. If, within the pouch, some of the wells exhibit no growth in the medium after incubation, while other wells exhibit some growth as indicated by brown colouration and/or turbidity, then the most probable number of *Legionella pneumophila* in 100 ml of sample can be estimated from appropriate probability tables, see Appendix C1. No further confirmation is required.

C5 Limitations

This method is for the detection of *Legionella pneumophila* only.

Users wishing to employ this method should verify its performance under their own laboratory conditions [15] including the limit of detection.

Not all biocides have recognised neutralising agents and where present they will therefore continue to exert their bactericidal effects after sampling. Failure to neutralise biocides may result in false negative results or an under-estimation of the number of *L. pneumophila* present in the sample.

Sample containers should therefore contain appropriate neutraliser where available [10] and a record kept of the biocide and neutralising agent used to contextualise the results of the examination made.

It can generally be assumed that the true count may be significantly higher as clumps of *L. pneumophila* cells will produce a positive reaction well, just as a single cell will. When viewed in the context of percentage recovery achieved by microbiological methods the true number of *Legionella pneumophila* in water samples is usually higher than that detected using culture methods[19].

C6 Health and safety

WARNING - Species of *Legionella* are pathogenic

Risk of harm is caused by the inhalation of aerosolised disease causing strains of Legionella bacteria it is therefore advisable to assess all techniques for their ability to produce aerosols.

All samples submitted for *Legionella* analysis should therefore be regarded as potentially contaminated with bacteria classified as "Hazard Group 2" and handled with strict adherence to the general safety precautions described for work at Containment Level 2 (CL2), to include minimising exposure to aerosols at all times[16]**.**

Based on the risk assessment of procedures undertaken it may be necessary to carry out the examination in a microbiological safety cabinet. However, experience has determined that *Legionella* spp. can be handled safely by experienced microbiologists on the open bench in a conventional microbiology laboratory conforming to containment level two.

Media, reagents and bacteria used in this method are covered by regulations[17] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere in this series[15].

C7 Storage of samples during transportation

At temperatures lower than 6 ºC *Legionella* can enter a viable but non-culturable state and at temperatures above 20 ºC proliferation is likely, therefore transport and storage at ambient temperatures - ideally 6 ºC - 20 ºC is recommended [14].

Samples should be analysed as soon as possible not exceeding more than two days, and preferably within 24 hours.

The laboratory should however verify that their chosen transport and storage conditions are appropriate.

C8 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [15] in this series. An example of the methodology for this type of method is presented and is based upon a commercially available system. Some of the equipment

listed is specific to this system and alternative systems may be available for which other equipment may be required. Other items include:

C8.1 Sterile sample bottles of appropriate volume, made of suitable material, containing sufficient sodium thiosulphate pentahydrate neutralising agent to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of a 1.8 % m/v solution of $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

C8.2 Incubator, preferably non-fan assisted, capable of maintaining a temperature of 39.0 ± 0.5°C.

C8.3 Sterile 100 ml plastic bottles as supplied by the manufacturer of the test system or suitable equivalent.

C8.4 MPN reaction pouches as supplied by the manufacturer (for example, a 96-well system) and associated heat-sealing equipment.

C8.5 Colour and fluorescence comparator as supplied by the manufacturer.

C9 Media and reagents

Various commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation and have not been included in the published data referenced in this method. Commercial formulations should be used and stored according to the manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the method [14]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality. Unless otherwise stated chemical constituents should be added as anhydrous salts.

C9.1 *Legiolert™ reagent* [18].

The reagent is a commercially available formulation provided in a blister pack format and is suitable for single samples. The reagent is a nutritionally defined formulation with a chromogenic substrate for the specific detection of an enzyme diagnostic for *Legionella pneumophila*. For MPN counts the reagent must be used in conjunction with the Quanti-Tray®/Legiolert™ 96-well reaction pouches as other Quanti-Tray® pouches do not permit the required oxygen transfer.

C9.2 *Legiolert™ supplement* [18].

The Legiolert supplement is a commercially available kit containing two items. The first item is a canister of tests in dip strip format designed to quickly determine the total calcium/magnesium hardness in the potable water sample. The second item is provided as a powder in a 100 ml vessel, which is reconstituted in sterile deionized water and is a multi-dose unit. The hardness strips are used to classify the total hardness of the sample as either

low or high, and the supplement solution is added at one of two levels based upon the determined hardness.

C10 Analytical procedure

C10.1 *Sample preparation*

The volumes, and any dilutions, of samples should be chosen so that the number of *Legionella pneumophila* per sample lies within the counting range of the MPN reaction pouch - if possible, between 1 and 2273. For treated waters, process 100 ml of the sample. When preparing dilutions use a sterile diluent (for example, deionised water, phosphate/Butterfield's buffer or 0.1 % peptone).

Using a hardness dip strip (for example, Aquadur®, Macherey-Nagel, Durën, Germany) determine whether the sample is of low or high hardness (scores of 0 – 2, i.e. ≤ 14°d, are considered as indicative of low hardness and scores of 3 – 4, i.e. > 14°d, as indicative of high hardness). For waters of low hardness, add 0.33 ml of *Legiolert*™ supplement per 100 ml of sample and for waters of high hardness, add 1.0 ml of *Legiolert*™ supplement per 100 ml of sample. For all dilutions of 10-fold or greater the hardness test and hardness neutralization step is omitted, as sample hardness is effectively diluted out.

C10.2 *Sample processing*

The sample (typically 100 ml), or appropriate dilution, is decanted into a sterile bottle. When making dilutions, sterile diluent (e.g. deionized water, phosphate/Butterfield's buffer or 0.1% peptone) should be added to a sterile vessel without sodium thiosulphate. Following the manufacturer's instructions, the contents of one blister pack of medium is then aseptically added. After capping the bottle, the contents are agitated to ensure dissolution of the medium. The contents of the bottle are then added to the MPN reaction pouch, which is then tapped or flicked gently to dislodge any air bubbles. The pouch is then sealed in the apparatus provided by the manufacturer to produce a 96-well reaction pouch. The time between the inoculation of the reaction pouch and the beginning of the incubation stage should be as short as possible and no longer than 2 hours. Each batch of tests should be accompanied by a pouch inoculated with sterile water mixed with Legiolert reagent.

Sealed MPN reaction pouches are then incubated paper-side down/well-side up at 39 ± 0.5°C for 7 days. Reaction pouches can be stacked in alternating directions for better stability. Incubators should be humidified, or the reaction pouches incubated in an appropriate container to keep in moisture. Trays may be examined during the incubation period but must not be removed completely from the incubator prior to final reading.

C10.3 *Reading of results*

After incubation, the pouch is examined and the number of wells that produce any degree of brown colouration (see Figure C1) are recorded as positive reactions. Additionally, any wells

that show turbidity with or without brown colouration are recorded as positive. The pouches may be compared to a negative blank control pouch when ascertaining colouration or turbidity.

Figure C1 Example of a 96-well MPN Quanti-Tray®/Legiolert™ 96-well reaction pouch with Legiolert™ with the 6 large wells and 24 small wells showing brown colouration for *Legionella pneumophila*

C10.4 *Confirmation tests*

This method is reported to be highly specific for *L. pneumophila*. Hence, confirmation tests are not required. If desired, positive wells contain viable *Legionella pneumophila* from which molecular typing by latex agglutination, or similar, can be carried out.

C11 Calculations

C11.1 *Confirmed Legionella pneumophila*

The MPN of *L. pneumophila* is determined by reference to appropriate probability tables, see for example Appendix C1, or by using software provided by the manufacturer. This is derived from the number of wells showing a positive brown colouration and/or turbidity. For example, if there are 6 large wells and 24 small wells showing brown colouration in the reaction pouch (as shown in Figure C1), then from Appendix C1, the MPN of *L. pneumophila* is 157 per 100 ml of sample, or diluted sample, examined. Any dilution needs to be taken into account for determination for *L. pneumophila* concentration in the original sample.

C12 Expression of results

Confirmed *L. pneumophila* counts are expressed as MPN counts per volume of sample. For drinking waters and similar waters using this method, the volume is typically 100 ml.

C13 Quality assurance

New batches of media should be tested with appropriate reference strains of target bacteria (for example, *Legionella pneumophila* NCTC 11192/ATCC 33152/WDCM 00107) and non-target bacteria (for example, *Legionella anisa* NCTC 11974/ATCC 32592/WDCM 00106 or *Enterococcus faecalis* NCTC 12697/ATCC 29212/WDCM 00087). Pouches should be incubated for 7 days at 39 ± 0.5°C. Further details are given elsewhere [14] in this series. Legiolert supplement should not be added to QC or other non-environmental samples.

C14 References

1. Sartory DP, Spies K, Lange B, Schneider S, Langer B. 2017. Evaluation of a most probable number method for the enumeration of *Legionella pneumophila* from potable and related water samples. Lett. Appl. Microbiol. **64**:271–274.

2. Spies K, Pleischl S, Lange B, Langer B, Hubner I, Jurzik L, Luden K, Exner M. 2018. Comparison of the Legiolert/Quanti-Tray MPN test for the enumeration of *Legionella pneumophila* from potable water samples with the German regulatory requirements methods ISO 11731-2 and ISO 11731. Int. J. Hyg. Environ. Health **221(7)**:1047-1053.

3. Schulze-Robbecke R, Rodder M, Exner M. 1987. Multiplication and killing temperatures of naturally occurring legionellas. Zentralbl Bakteriol Mikrobiol Hyg [B] **184:**495-500.

4. Anonymous. 2017. Legionnaires' disease in residents of England and Wales: 2016 - Official statistics. Public Health England. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/670640/Legionnaires_disease_in_England_and_Wales_2016.pdf.

5. Fitzgeorge RB, Baskerville A, Featherstone ASR. 1987. Fine particle aerosols in experimental Legionnaires disease: Their role in infection and treatment, p. 357-360. *In* Thing A (ed.), Advances in Aerobiology: Proceedings of the third internatinal conference on aerocbiology. Birkhauser Verlag, Basel.

6. Bencini MA, Yzerman EP, Koornstra RH, Nolte CC, den Boer JW, Bruin JP. 2005. A case of Legionnaires' disease caused by aspiration of ice water. Arch. Environ. Occup. Health **60:**302-306.

7. Correia AM, Ferreira JS, Borges V, Nunes A, Gomes B, Capucho R, Goncalves J, Antunes DM, Almeida S, Mendes A, Guerreiro M, Sampaio DA, Vieira L, Machado J, Simoes MJ, Goncalves P, Gomes JP. 2016. Probable Person-to-Person Transmission of Legionnaires' Disease. N. Engl. J. Med. **374:**497-498.

8. Anonymous. 2013. Legionnaires' disease. The control of legionella bacteria in water systems. http://www.hse.gov.uk/pubns/books/l8.htm.

9. Anonymous. 2013. Legionnaires' disease. Part 3. The control of legionella bacteria in other risk systems. http://www.hse.gov.uk/pubns/books/hsg274.htm.

10. Anonymous. 2014. Legionnaires' disease. Part 1. The control of legionella bacteria in evaporative cooling systems. http://www.hse.gov.uk/pubns/priced/hsg274part1.pdf.

11. Anonymous. 2014. Legionnaires' disease. Part 2. The control of legionella bacteria in hot and cold water systems. http://www.hse.gov.uk/pUbns/priced/hsg274part2.pdf.

12. Anonymous. 2019. Water Quality - Risk assessments for *Legionella* control - Code of practice. BS 8580-1:2019. https://shop.bsigroup.com/ProductDetail?pid=000000000030367524.

13. Brindle RJ, Stannett PJ, Cunliffe RN. 1987. *Legionella pneumophila*: comparison of isolation from water specimens by centrifugation and filtration. Epidemiol. Infect. **99:**241-247.

14. Anonymous. 2008. BS 7592:2008 Sampling for Legionella bacteria in water systems. Code of practice http://shop.bsigroup.com/ProductDetail/?pid=000000000030161148.

15. Anonymous. 2017. The Microbiology of Water and Associated Materials (2017) - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, Standing Committee of Analysts, Environment Agency. http://www.standingcommitteeofanalysts.co.uk/library/The%20Microbiology%20of%20Water%20and%20Associated%20Materials%20(2017)%20Part%203%20Practices%20and%20Procedu.pdf.

16. Anonymous. 2013. The Approved List of Biological Agents. HMSO. http://www.hse.gov.uk/pubns/misc208.pdf.

17. Anonymous. 2002. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677. http://www.legislation.gov.uk/uksi/2002/2677/pdfs/uksi_20022677_en.pdf.

18. IDEXX Technologies Ltd., Units 1B & 1C, Newmarket Business Park, Studlands Park Avenue, Newmarket, Suffolk, CB87ER.

19. Boulanger CA, Edelstein PH. 1995. Precision and accuracy of recovery of Legionella pneumophila from seeded tap water by filtration and centrifugation. Appl. Environ. Microbiol. 61:1805-1809.

20. Muyldermans A, Descheemaeker P, Boel A, Desmet S, Van Gasse N, Reynders M; on behalf of the National Expert Committee on Infectious Serology. What is the risk of missing legionellosis relying on urinary antigen testing solely? A retrospective Belgian multicenter study [published online December 14, 2019]. *Eur J Clin Microbiol Infect Dis*. doi:10.1007/s10096-019-03785-8

Appendix C1 MPN per 100 ml for a Quanti-Tray®/ Legiolert™ 96-well reaction pouch

Number of Small Wells Positive	Number of Large Wells Positive						
	0	**1**	**2**	**3**	**4**	**5**	**6**
0	< 1	1	2	4	6	8	13
1	1	2	4	5	7	10	16
2	2	3	5	7	9	12	19
3	3	4	6	8	11	15	22
4	4	5	7	9	12	17	26
5	5	7	8	11	14	19	31
6	6	8	10	12	16	22	36
7	7	9	11	14	18	25	42
8	8	10	12	15	20	28	47
9	9	11	14	17	22	31	53
10	10	12	15	18	24	34	60
11	11	13	16	20	26	37	66
12	12	15	18	22	28	40	72
13	13	16	19	23	30	43	79
14	14	17	20	25	32	47	85
15	15	18	22	27	35	50	92
16	16	19	23	28	37	54	99
17	17	20	24	30	39	57	106
18	18	22	26	32	42	61	113
19	19	23	27	34	44	64	120
20	20	24	29	35	46	68	127
21	21	25	30	37	49	72	134
22	23	26	31	39	51	75	142
23	24	28	33	41	54	79	149
24	25	29	34	43	56	83	157
25	26	30	36	44	59	86	164
26	27	31	37	46	61	90	172
27	28	32	39	48	64	94	180
28	29	34	40	50	66	98	188
29	30	35	42	52	69	102	196
30	31	36	43	54	71	106	205
31	32	37	45	56	74	109	213
32	33	39	46	58	76	113	222
33	34	40	48	59	79	117	231
34	35	41	49	61	81	121	240
35	36	42	51	63	84	125	249
36	37	44	52	65	87	130	258
37	38	45	54	67	89	134	267
38	40	46	55	69	92	138	277
39	41	47	57	71	95	142	287
40	42	49	58	73	97	146	297
41	43	50	60	75	100	150	307
42	44	51	61	77	103	155	318
43	45	53	63	79	105	159	328
44	46	54	65	81	108	163	339
45	47	55	66	83	111	168	350
46	48	56	68	85	114	172	361

Number of Small Wells Positive	Number of Large Wells Positive							
		0	1	2	3	4	5	6
	47	49	58	69	87	116	177	373
	48	50	59	71	89	119	181	385
	49	52	60	72	91	122	186	397
	50	53	62	74	93	125	190	410
	51	54	63	76	95	128	195	422
	52	55	64	77	97	131	200	436
	53	56	66	79	99	133	205	449
	54	57	67	80	101	136	209	463
	55	58	68	82	103	139	214	477
	56	59	69	84	105	142	219	492
	57	61	71	85	107	145	224	507
	58	62	72	87	110	148	229	522
	59	63	73	89	112	151	234	538
	60	64	75	90	114	154	239	555
	61	65	76	92	116	157	244	572
	62	66	78	94	118	160	249	590
	63	67	79	95	120	163	254	608
	64	68	80	97	122	166	260	627
	65	70	82	99	125	169	265	647
	66	71	83	100	127	172	270	668
	67	72	84	102	129	175	276	689
	68	73	86	104	131	179	281	712
	69	74	87	105	133	182	287	735
	70	75	88	107	135	185	293	760
	71	77	90	109	138	188	298	786
	72	78	91	110	140	191	304	813
	73	79	93	112	142	195	310	842
	74	80	94	114	144	198	316	872
	75	81	95	116	147	201	322	905
	76	82	97	117	149	204	328	940
	77	84	98	119	151	208	334	977
	78	85	100	121	154	211	340	1018
	79	86	101	123	156	214	346	1062
	80	87	102	124	158	218	353	1110
	81	88	104	126	161	221	359	1163
	82	90	105	128	163	225	366	1222
	83	91	107	130	165	228	372	1290
	84	92	108	131	168	232	379	1368
	85	93	110	133	170	235	386	1460
	86	94	111	135	172	239	392	1573
	87	96	112	137	175	242	399	1718
	88	97	114	139	177	246	406	1923
	89	98	115	140	180	249	414	2273
	90	99	117	142	182	253	421	> 2273

Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users wish to receive advance notice of forthcoming publications, please contact the Secretary.

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Members assisting with these methods

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