

NATIONAL STANDARD METHOD

DETECTION AND ENUMERATION OF *LEGIONELLA* SPECIES BY FILTRATION AND CENTRIFUGATION

W 12

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

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DETECTION AND ENUMERATION OF *LEGIONELLA* SPECIES BY FILTRATION AND CENTRIFUGATION

Issue no: 1.2 Issue date: 11.08.06 Issued by: Standards Unit, Evaluations and Standards Laboratory, in conjunction with the Regional Food, Water and Environmental Coordinators Forum. Page 2 of 17

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AMENDMENT PROCEDURE

Controlled document reference	W 12
Controlled document title	Detection and enumeration of <i>Legionella</i> species by filtration and centrifugation

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

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

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
2/ 11.08.06	1.1	1.2	5	Background	Updated to include the validity of ISO 11731 while it is under revision
			11	6.7.1 Confirmation of Legionella species	Heading amended to 'presumptive <i>Legionella</i> ...' and section updated to remove use of blood agar
			13	9.1 Medium	Quantitative statement added
			13	9.3 Confirmatory Test	Negative control added

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SCOPE OF DOCUMENT

The method described is applicable to relatively clean water samples, especially those from hot and cold water systems in large buildings. The method is suitable for volumes up to 1 litre.

INTRODUCTION

Background

Legionella species, the cause of legionellosis, are aquatic bacteria that are widespread in nature and have been found in water over a wide temperature range but grow best between 30°C and 40°C². Their tolerance to relatively high temperatures probably helps them to colonise some artificial water systems that are often above ambient temperatures. *Legionella* are prevalent in artificial water systems, and legionellosis is transmitted from these via aerosols and occasionally by aspiration. Cooling towers are often linked to community-acquired outbreaks, and although hot water systems are commonly incriminated in nosocomial outbreaks, a variety of other sources have been described.

Water samples may be examined for *Legionella* species during epidemiological investigations as part of a local authority, industrial, or hospital surveillance programme, or to validate new biocide treatments or other control methods. The HSE³ also recommends routine, at least quarterly, sampling of cooling towers and routine sampling of hot and cold water systems if the recommended temperature regime is not followed or has failed or if the local risk assessment suggests it is needed.

Even in hospital environments numbers of *Legionella* species are often low, rarely exceeding 1% of the total bacterial population. As a result it is usually necessary to concentrate the bacterial flora from water before using selective cultural techniques to isolate *Legionella*. However in outbreak investigations, when potential sources may have high numbers of legionellae and also be heavily contaminated with other bacteria, samples should be examined both with and without concentration. The unconcentrated samples often yield legionellae when they would not be detectable in the concentrated sample because of overgrowth by other organisms.

Concentration can be achieved by a combination of negative pressure filtration using a 47 mm diameter membrane and centrifugation as described below.

The procedures described are based on BS 6068 (ISO 11731)⁴ and other literature⁵.

Note: ISO 11731 is now in two parts. The latest part, Part 2 (BS - ISO 11731 – 2:2004 BS 6068-4.18:2004 Water quality - Detection and enumeration of *Legionella* – Part 2: Direct membrane filtration method for waters with low bacterial counts. London: British Standards Institution; 1998) is only suitable for disinfected waters with a low background count and utilises filtration followed by the direct placement of the membrane onto the selective medium. The original ISO 11731⁴ is still valid and currently under revision. When revision is completed it will subsequently be called ISO 11731 Part 1. The HPA has not adopted ISO 11731 Part 2 as one of its standard methods but will continue to follow ISO 11731 Part 1.

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1 DEFINITIONS

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

2 PRINCIPLE

The microflora in a water sample is first concentrated by negative pressure filtration through a 47 mm diameter nylon membrane (pore size 0.2 µm). Microflora retained by the filter is eluted from the filter in a suitable sterile eluent or distilled water and the eluate is further concentrated by centrifugation. A portion of the resultant suspension is subjected to treatment with acid and another portion with heat. Treated and untreated portions are inoculated onto plates of an agar medium selective for *Legionella* species and incubated. After incubation, morphologically characteristic colonies, which form on the selective medium, are regarded as presumptive colonies of *Legionella* species and counted. Presumptive colonies of *Legionella* are sub-cultured to test for their growth requirement for L-cysteine, and the count of confirmed organisms is calculated in 1 litre of sample.

3 SAFETY CONSIDERATIONS⁶⁻¹⁵

Normal microbiology laboratory precautions apply.

3.1 SAMPLE COLLECTION

N/A

3.2 SAMPLE TRANSPORT AND STORAGE

Compliance with current postal and transportation regulations is essential.

3.3 SAMPLE PROCESSING

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

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

4 EQUIPMENT

Usual laboratory equipment and in addition:

- Membrane filtration manifold
- Filter funnels (graduated)
- Pre-filter attachment (optional)
- Pyrex vacuum flask (>5 L capacity) with protective jacket or equivalent

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- Vacuum pump with moisture trap or protective filter
- Stainless steel flat-tipped forceps or equivalent
- Scissors
- Boiling waterbath (instrument steriliser)
- Centrifuge fitted with safety buckets and capable of at least 3000 x g \pm 100 x g
- Waterbath 50°C \pm 1°C
- Microbiological safety cabinet
- Incubator: 36°C \pm 1°C (with humidification and preferably 2.5% CO₂)
- Plate stereoscopic microscope with oblique incident illumination
- Ultra violet lamp (long wave length UV – 366 nm)
- Membrane filters – 47 mm diameter, 0.2 μ m pore size, polyamide (nylon)
- Stomacher bags
- Automatic pipettors and sterile pipette tips capable of delivering 0.1-10 mL volumes (optional)
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)
- Spreaders (sterile)

5 CULTURE MEDIA AND REAGENTS

Page's saline (optional)

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

1/40 Ringer's solution (optional)

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

Distilled or deionised water (optional)

Double Strength Acid buffer pH 2.2

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

0.4 mol/L Hydrochloric acid:

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

Potassium chloride (0.4 mol/L):

Add 29.8 g of potassium chloride to 1 L of water

Glycine vancomycin polymyxin cyclohexamide (GVPC) agar

Activated charcoal	2.0 g
Yeast extract	10.0 g
Potassium hydroxide	2.8 g
ACES buffer (N-2-acetamido-2-aminoethane sulphonic acid)	10.0 g
Ferric pyrophosphate	0.25 g
L-cysteine hydrochloride monohydrate	0.4 g
α -ketoglutarate monopotassium salt	1.0 g
Glycine (ammonium free)	3.0 g
Polymyxin B sulphate	80,000 IU
Vancomycin hydrochloride	1 mg
Cyclohexamide	80 mg
Agar	13.0 g
Water	1 L

pH 6.8 \pm 0.2 at 25°C

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

Buffered charcoal yeast extract agar with cysteine (BCYE+)

Activated charcoal	2.0 g
Yeast extract	10.0 g
Potassium hydroxide	2.8 g
ACES buffer (N-2-acetamido-2-aminoethane sulphonic acid)	10.0 g
Ferric pyrophosphate	0.25 g
L-cysteine hydrochloride monohydrate	0.4 g
α ketoglutarate, monopotassium salt	1.0 g
Agar	13.0 g
Water	1 L

pH 6.8 \pm 0.2 at 25°C

Buffered charcoal yeast extract agar without cysteine (BCYE-)

Activated charcoal	2.0 g
Yeast extract	10.0 g
Potassium hydroxide	2.8 g
ACES buffer (N-2-acetamido-2-aminoethane sulphonic acid)	10.0 g
α -ketoglutarate, monopotassium salt	1.0 g
Ferric pyrophosphate	0.25 g
Agar	13.0 g
Water	1 L

pH 6.8 \pm 0.2 at 25°C

2% Formal saline (optional)

Phosphate buffered saline (PBS)(Optional)

Sodium chloride	8.0 g
Potassium chloride	0.2 g

Disodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g
Water	1 L
pH 7.3 ± 0.2 at 25°C	

Reagents for immunofluorescence staining (optional)

Commercial latex agglutination test kits (optional)

6 SAMPLE PROCESSING

6.1 SAMPLE PREPARATION AND DILUTIONS

Sampling should be carried out by suitably trained personnel¹⁶. Add an excess of suitable inactivating agent (if available) to samples containing a biocide.

Water samples should be received and handled as described in National Standard Method W1: Section 5¹⁷. Samples should be transported at ambient temperature (instead of at 6°C to 18°C) and protected from sunlight. Do not mix samples that are still hot with others that are cold to avoid the cold samples being heated.

Record whether the sample originally contained biocide and, if so, details of the neutralising agent used. Oxidising biocides such as chlorine, bromine, and chlorine dioxide are easily neutralised with sodium or potassium thiosulphate. For most purposes 180 mg of sodium thiosulphate will be sufficient to neutralise up to 50 mg of chlorine in one litre of water. Also record the volume of sample processed and any details, which might affect the recovery of legionellae from the sample (for example, the presence of oil droplets).

Samples, particularly those known to have contained biocides, should be processed as soon as possible after receipt, preferably on the day of sampling. Samples should be analysed as soon as is practicable on the day of collection. In exceptional circumstances, if there is a delay, storage under the above conditions should not exceed 24 hours before the commencement of analysis.

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

6.2 FILTRATION

Mix the sample well to re-suspend any deposit.

Using polyamide membranes, filter 1 L of water following the procedures described in National Standard Method W1¹⁷. If a sample is cloudy or difficult to filter, it should be filtered in more than one stage. If a sample is very dirty or oily, centrifugation, as described in National Standard Method W13¹⁸, may be more appropriate.

6.3 PREPARATION OF SAMPLE CONCENTRATE

Pipette 10 mL of eluent (Page's saline or 1/40 Ringer's solution) into a food-grade plastic stomacher bag. Using sterile forceps, remove the filter from the manifold, and place it in one of the bottom corners inside the stomacher bag. Hold the bag so that the eluent and the membrane are in the same corner and rub the membrane between the finger and thumb of one hand for at least 30 seconds¹⁶. Label a sterile container suitable for centrifugation with the sample number and remove as much of the eluent as possible from the bag. Read the actual volume removed by the pipette and make a note on the container before dispensing the eluent into it.

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6.4 CENTRIFUGATION

Centrifuge at $3000 \times g \pm 100 \times g$ for 30 minutes ± 1 min, or $6000 \times g \pm 100 \times g$ for 10 minutes ± 1 min.

Using a sterile graduated pipette carefully remove all but 1 mL of supernatant aseptically. For example, 10 mL eluent is placed in a bag with the filter, 9.8 mL is removed by pipette and 9.8 noted on the container. Remove 8.8 mL from the container after centrifugation so that 1 mL remains. Resuspend the deposit by vortex mixing. This constitutes the final concentrate.

6.5 PRE-TREATMENT AND INOCULATION OF SELECTIVE AGAR PLATES

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

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

6.5.1 HEAT TREATMENT

Dispense 0.2 mL of the final concentrate into a sterile screw capped container and place it in a water bath at $50^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 30 minutes ± 2 minutes.

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

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

6.5.2 ACID TREATMENT

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

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

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

6.5.3 UNTREATED

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

6.5.4 STORAGE OF CONCENTRATES

Store in screw-capped containers at $6^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in the dark for up to 12 months.

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

6.6 INCUBATION AND EXAMINATION OF SELECTIVE AGAR PLATES

Allow the inoculated media to stand until the inocula have been completely absorbed, place in a moist chamber and incubate at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

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Incubation in an atmosphere of air with 2.5% CO₂ (v/v) may enhance the growth of some *Legionella* species. Incubate the plates for 10 days and examine on at least three occasions at intervals of 2 to 4 days during the incubation period.

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

6.7 COUNTING OF COLONIES

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

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

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

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

6.7.1 CONFIRMATION OF PRESUMPTIVE *LEGIONELLA* SPECIES

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

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

6.8 IDENTIFICATION AND TYPING

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

6.8.1 IMMUNOFLUORESCENCE

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

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control, and a negative control using the supplied negative control suspension.

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

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

6.8.2 LATEX AGGLUTINATION KITS

Follow the manufacturer's instructions.

7 CALCULATION OF RESULTS

Use the following to calculate the number of colony forming units (cfu) of *Legionella* species per litre in the original unconcentrated sample:

$$C = \frac{n \times v \times 1}{i \times s}$$

where:

C = cfu/litre in the original sample

n = number of colonies on the plate

v = volume (mL) of the concentrate

i = volume (mL) inoculated onto plate

s = volume (litres) of water from which the microflora were concentrated

The plate showing the maximum number of confirmed colonies should be used to estimate the number of *Legionella* species in the original sample. It is not appropriate to calculate an average count from the 3 plates as each has received a different pre-treatment and, as such, they are not replicates.

7.1 DETECTION LIMIT

In the above method the final sample concentrate from 1 litre is 1 mL and 0.1 mL is plated out onto the selective media. If every *Legionella* present in the original sample were recovered by the technique then, on average, the detection limit would be 10 cfu/L. However, in practice, organisms are lost at each stage of the process, for example, by incomplete elution from the membranes.

Note: The volume finally cultured on each plate is only equivalent to 100 mL of the original sample.

It should be possible to count up to 150 colonies on each plate, so the upper limit of enumeration for this method is 1.5×10^3 cfu per litre. Preparing decimal dilutions from the untreated stored concentrate can increase the upper limit.

8 REPORTING

Report the results using the procedure described in National Standard Method W1 Section

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9¹⁷. Express the result as the number of cfu of *Legionella* species in 1 litre of water specifying the mathematical limit of detection, the volume of sample processed, and the equivalent volume cultured.

If *Legionella* species are not detected report as:

Legionella not detected (mathematical detection limit = 10/L)

If *Legionella* species are detected report as:

a per litre

where **a** is the confirmed count

The result should give details of serological identification as *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2 – 14, or *Legionella* species other than *L. pneumophila*.

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

9 QUALITY CONTROL

9.1 MEDIUM

It is essential that each flask of BCYE or GVPC prepared be treated as a separate batch of medium. Each batch of medium should be tested for the ability to support the growth of *L. pneumophila* and *L. bozemanii* and compared quantitatively with a previous batch of medium known to support growth of *L. pneumophila* and *L. bozemanii*. Counts from aliquots of the same test suspension should fall within an established 95% confidence interval (Table 8.1 in Microbiology of Drinking Water 2002)¹⁹.

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

9.2 MEMBRANE FILTRATION

Filter 1L of sterile distilled water using the same funnel as was used for the positive control following sterilisation.

9.3 CONFIRMATORY TESTS

Positive control:

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

Negative control:

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

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10 REFERENCE FACILITIES

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

11 ACKNOWLEDGEMENTS AND CONTACTS

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

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APPENDIX: FLOWCHART SHOWING THE DETECTION AND ENUMERATION OF *LEGIONELLA* SPECIES BY FILTRATION AND CENTRIFUGATION

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



Store in the dark and examine within 24 hours



Mix sample well
(NB in outbreak investigations retain some sample for culture without concentration)



Filter



Place filter in stomacher bag and suspend in 10 mL eluent



Remove fluid and centrifuge



Remove supernatant and leave 1 mL of suspension



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



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



Incubate at 36°C and increased humidity (preferably with CO₂) for 2 to 10 days



Subculture to BCYE+ agar and BCYE- for confirmatory tests



Calculate confirmed count for *Legionella*



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

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