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Water quality — Detection and enumeration of *Legionella*

Qualité de l'eau — Recherche et dénombrement des Legionella

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International Organization for Standardization
Case postale 56 • CH-1211 Genève 20 • Switzerland
Internet central@iso.ch
X.400 c=ch; a=400net; p=iso; o=isocs; s=central

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 11731 was prepared by Technical Committee ISO/TC 147, *Water quality,* Subcommittee SC 4, *Microbiological methods.*

Annexes A, B, C and D of this International Standard are for information only.

Water quality — Detection and enumeration of *Legionella*

1 Scope

This International Standard describes a culture method for the isolation of *Legionella* organisms and estimation of their numbers in environmental samples.

This method is applicable to all kinds of environmental samples including potable, industrial and natural waters and associated materials such as sediments, deposits and slime.

2 Normative reference

The following standard contains provisions, which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the edition indicated was valid. All standards are subject to revision, and parties to agreements based on this International Standards are encouraged to investigate the possibility of applying the most recent edition of the standard listed below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods.*

3 Definition

For the purposes of this International Standard, the following definition applies:

3.1 *Legionella*
genus of Gram-negative organisms normally capable of growth in not less than 2 days on Buffered Charcoal Yeast Extract agar containing L-cysteine and iron(III), and forming colonies, often white, purple to blue or lime green in colour

NOTE — Some species fluoresce under long-wavelength UV light. The colonies have a ground-glass appearance when viewed with a low power stereomicroscope. With a very few exceptions, growth does not occur in the absence of L-cysteine.

4 Safety

The reagents used in this International Standard should be subject to assessment in accordance with Control of Substances Hazardous to Health.

Legionella species can be handled safely by experienced microbiologists on the open bench in a conventional microbiology laboratory conforming to Containment Level 2. Infection is caused by inhalation of the organism and it is advisable therefore to assess all techniques for their ability to produce aerosols. If in doubt, carry out the work in a safety cabinet.

5 Principle

5.1 General

Bacteria, including *Legionella* organisms, in the water sample are concentrated by membrane filtration or by centrifugation. Turbid samples can be centrifuged. To reduce the growth of unwanted bacteria, a portion of the concentrated specimen is subjected to treatment with acid and another portion with heat. Treated and untreated test portions are then inoculated onto plates of agar medium selective for *Legionella* and incubated. Samples containing sufficient numbers of *Legionella* need not be subject to concentration prior to culture.

5.2 Enumeration

After incubation, morphologically characteristic colonies which form on the selective medium are regarded as presumptive *Legionella*.

5.3 Confirmation

Presumptive colonies are confirmed as *Legionella* organisms by subculture to demonstrate their growth requirement for L-cysteine and iron. Further biochemical and serological tests are needed for species identification.

6 Culture media and reagents

6.1 General

Use chemicals of analytical grade in the preparation of media and reagents unless otherwise stated (see note 1). Alternatively, use commercially available dehydrated media and reagents. Prepare the media according to the manufacturer's instruction and add freshly prepared selective agents or growth supplements (or thaw the stored material at room temperature prior to use) at the concentrations recommended. Prepare media using glass-distilled water or water of equivalent quality complying with ISO 3696 Grade 3.

NOTE 1 The use of chemicals of other grades is permissible providing they are shown to be of equal performance in the test.

Use diagnostic serological reagents of known specificity from a known source. Do not use a reagent for which this information is not available.

NOTE 2 The possibility of cross-reactions with other organisms in environmental samples should be considered.

6.2 Culture media

6.2.1 Buffered Charcoal Yeast Extract agar medium (BCYE)

6.2.1.1 Composition

Yeast extract (bacteriological grade)	10,0 g
Agar	12,0 g
Activated charcoal	2,0 g
Alpha-ketoglutarate, monopotassium salt	1,0 g
ACES buffer (*N*-2-acetamido-2-aminoethanesulfonic acid)	10,0 g
Potassium hydroxide (KOH) (pellets)	2,8 g
L-cysteine hydrochloride monohydrate	0,4 g
Iron(III) pyrophosphate [$Fe_4(P_2O_7)_3$]	0,25 g
Distilled water	to 1000 ml

NOTE — Check manufacturer’s recommendations for concentration of agar to be added to provide adequate gelling strength.

6.2.1.2 Preparation

a) Cysteine and iron solutions.

Prepare fresh solutions of L-cysteine hydrochloride and iron(III) pyrophosphate by adding 0,4 g and 0,25 g respectively to 10-ml volumes of distilled water. Decontaminate each solution by filtration through a membrane filter with an average pore size of 0,22 µm. Store in clean sterile containers at −(20 ± 3)°C for not more than 3 months.

b) ACES buffer.

Add the ACES granules to 500 ml of distilled water and dissolve by standing in a water bath at (45 to 50) °C. To a separate 480 ml of distilled water, add all the potassium hydroxide pellets and dissolve with gentle shaking. To prepare the ACES buffer, mix the two solutions.

NOTE — ACES buffer can cause denaturation of the yeast extract if the following sequence is not followed.

c) Final medium.

Add sequentially to the 980 ml of ACES buffer, the charcoal, yeast extract and α-ketoglutarate. Prepare a 0,1 mol/l solution of potassium hydroxide (KOH) by dissolving 5,6 g in 1 litre of distilled water. Prepare a 0,1 mol/1 solution of sulfuric acid (H_2SO_4) by carefully adding 5,3 ml of H_2SO_4 to 1 litre of distilled water. Use the solutions of 0,1 mol/l potassium hydroxide or 0,1 mol/l sulfuric acid as appropriate to adjust the pH to 6,9 ± 0,2. Add the agar, mix and autoclave at (121 ± 1) °C for (15 ± 1) min (see 6.2.4, first paragraph). After autoclaving, allow to cool to (50 ± 2) °C in a water bath.

Add the L-cysteine and the iron(III) pyrophosphate solutions aseptically, mixing well between additions.

Dispense in 20 ml volumes into Petri dishes of 90 mm to 100 mm diameter. The pH of the final medium is 6,9 ± 0,4 at 25°C. Allow excess moisture on the plates to dry and store at (4 ± 2)°C in airtight containers in the dark for up to 4 weeks.

6.2.2 Buffered Charcoal Yeast extract medium without L-cysteine (BCYE – Cys)

Prepare this medium in an identical manner to BCYE (6.2.1) but omit the L-cysteine.

6.2.3 Selective medium: Buffered Charcoal Yeast Extract medium with selective supplements (GVPC medium)

NOTE — This medium is identical to BCYE except that three antibiotic supplements and glycine are added to the BCYE medium.

6.2.3.1 Selective supplements

The final concentrations in the GVPC medium shall be:

Ammonium-free glycine	3 g/l
Polymyxin B sulfate	80 000 iu/l
Vancomycin hydrochloride	0,001 g/l
Cycloheximide	0,08 g/l

6.2.3.2 Preparation of antibiotic supplements

Add the appropriate amount (usually 200 mg) of polymyxin B sulfate to 100 ml of distilled water to achieve a concentration of 14 545 iu/ml. Mix and decontaminate by membrane filtration as described in 6.2.1.2. Dispense 5,5 ml volumes into sterile containers and store at −(20 ± 3) °C. For use, thaw at room temperature.

Add 20 mg of vancomycin hydrochloride to 20 ml of distilled water, mix and decontaminate by membrane filtration (6.2.1.2). Dispense in 1 ml volumes in sterile containers and store at −(20 ± 3) °C. For use, thaw at room temperature.

Add 2 g of cycloheximide to 100 ml of distilled water and decontaminate by membrane filtration as described in 6.2.1.2. Dispense in 4 ml volumes in sterile containers and store at −(20 ± 3) °C. For use, thaw at room temperature.

NOTE — Antibiotic supplements may be stored for up to 6 months when frozen.

WARNING — Cycloheximide is hepatotoxic. Wear gloves and dust mask when handling this chemical in powder form.

6.2.3.3 Preparation of GVPC medium

Follow the instructions for preparation of BCYE medium given in 6.2.1.2, but add 3 g of ammonium-free glycine after the addition of the α-ketoglutarate and then adjust the pH to 6,9 ± 0,4.

After the addition of the L-cysteine and iron, add one volume of each of the above three antibiotic supplements (6.2.3.2) to the final medium. Mix well.

6.2.4 Quality control of media

Prolonged heating during sterilization or heating at too high a temperature shall be avoided, as it can affect the nutritional qualities of BCYE medium. Batch-to-batch variation of the ingredients of the medium (particularly α-ketoglutarate) can also affect its performance. Therefore it is essential to check the quality of each newly prepared batch of media for its ability to support the growth of *L. pneumophila* serogroup 1 within three days of incubation.

For most bacteria, it is usual to assess the suitability of culture media to support their growth by using cultures of previously isolated organisms, maintained in the laboratory. For *Legionella*s this method may be misleading, as they can easily adapt to grow on culture media that would not support the primary isolation of 'wild' strains. The following procedure is therefore recommended for assessing the suitability of GVPC selective agar medium for *Legionella* organisms.

Either

a) use plates of a previous batch of GVPC medium known to support the growth of *Legionella* together with plates from the new batch of medium and inoculate them with a water sample known to contain *Legionella* organisms, or

b) from a nationally recognized source of reference cultures, obtain a lyophilized strain of *Legionella pneumophila* serogroup 1. Reconstitute and recover as recommended, and subculture onto BYCE (6.2.1) for purity. If a type culture is not available, use a freshly isolated and confirmed strain of *L. pneumophila* serogroup 1. Stock strains of *L. pneumophila* shall be replaced after not more than 10 subcultures. After incubation, make a suspension from the resulting growth just visible to the naked eye and dispense in 1 ml volumes in sterile glycerol broth (6.3.3.4) for storage at −(20 ± 3)°C, or alternatively in Page's Saline (6.3.2.1) or distilled water for storage at −(70 ± 5) °C. Plate out one suspension of each isolate onto BCYE medium for subsequent identification and recording of the *Legionella* species and serogroup (see 9.3). For use, allow a stock suspension of one (or more) isolates to thaw at room temperature. Shake thoroughly, wait 5 min to 10 min to allow aerosols to settle, and inoculate a measured volume (e.g. 0,1 ml) onto each of two plates of GVPC medium from the batch to be tested.

After incubation, record and compare the results to ensure that the colonial morphology (9.2.6) and number of colonies are similar.

6.3 Reagents

6.3.1 Acid buffer

Prepare a 0,2 mol/l solution of hydrochloric acid (HCl) (solution A) (see note). Prepare a 0,2 mol/l solution of potassium chloride (KCl) by dissolving 14,9 g of KCl in 1 litre of distilled water (solution B). To prepare the acid buffer, mix 3,9 ml of solution A and 25 ml of solution B. Adjust to pH 2,2 ± 0,2 by addition of a solution of 1 mol/l potassium hydroxide (KOH). Store in a stoppered glass container in the dark at room temperature for not longer than 1 month.

NOTE — To prepare a 0,2 mol/l solution of hydrochloric acid, add 17,4 ml concentrated HCl (sp gr 1,18, minimum assay 35,4 %) or 20 ml concentrated HCl (sp gr 1,16, minimum assay 31,5%) to 1 litre of distilled water.

6.3.2 Diluents

6.3.2.1 Page's Saline

Composition	
Sodium chloride (NaCl)	0,120 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0,004 g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0,004 g
Disodium hydrogenphosphate (Na_2HPO_4)	0,142 g
Potassium dihydrogenphosphate (KH_2PO_4)	0,136 g
Distilled water	1000 ml

Add the chemicals to the distilled water. Allow to dissolve, mix well and autoclave at (121 ± 1) °C for (15 ± 1) min (see 6.2.4 first paragraph).

NOTE — To aid accurate preparation, it is recommended that a 10 litre volume of Page's Saline is prepared and dispensed in smaller volumes as required for autoclaving at (121 ± 1) °C for (20 ± 1) min.

6.3.2.2 Dilute Ringer's solution.

Using a commercially available preparation (usually in tablet form), prepare a 1:40 dilution of Ringer's solution. Dispense as required and autoclave at (121 ± 1) °C for (20 ± 1) min.

NOTE — This is a 1 in 10 dilution of ¼ strength Ringer's solution.

6.3.2.3 Phosphate-buffered saline (pH 7,5).

Use a commercially available preparation and reconstitute according to the manufacturer's instructions.

6.3.2.4 Formol saline.

Prepare by adding 20 ml of an 37 % (volume fraction) aqueous solution of formaldehyde to 980 ml of phosphate-buffered saline (6.3.2.3).

6.3.3 Serological reagents

6.3.3.1 Antisera to *Legionella pneumophila* and other *Legionella* species.

To identify *Legionella pneumophila,* use polyclonal or monoclonal antibody preparations capable of reacting with all known serogroups of *Legionella pneumophila*. If it is necessary to identify species other than *L. pneumophila* or serogroups of *L. pneumophila*, then use specific antisera.

6.3.3.2 Fluorescein isothiocyanate anti-rabbit conjugate (FITC conjugate)

FITC conjugates raised against rabbit serum proteins that are available commercially.

NOTE — Different conjugates are required for use with antisera raised in other animals.

6.3.3.3 Glycerol mounting medium

Use a commercially available glycerol mounting medium, or prepare by adding 1 ml of potassium phosphate-buffered saline (pH 8,5) to 9 ml of glycerol (neutral).

6.3.3.4 Glycerol broth

Dissolve 5 g of a commercially available dehydrated nutrient broth in 170 ml of distilled water and add 30 ml of glycerol. Mix well and dispense in clean, dry silica-glass bottles in volumes of 2 ml. Sterilize by autoclaving at (121 ± 1) °C for (20 ± 1) min. Store at room temperature until required.

7 Apparatus

Usual laboratory equipment including

7.1 Sterile Petri dishes with a nominal diameter of either 90 mm or 100 mm.

7.2 Incubator, capable of being maintained at (36 ± 1,0) °C.

7.3 Ultraviolet lamp, emitting light of wavelength (360 ± 20) nm.

7.4 Filter stand and funnel, suitable for filtering water volumes of 500 ml to 10 litres.

Filtration equipment shall withstand autoclaving. The filter diameter may vary from 47 mm to 142 mm. Larger filter apparatus is usually constructed of stainless steel.

7.5 Positive-pressure membrane filtration pump, peristaltic and capable of producing a flowrate of up to 3 l/min with a variable speed control. Alternatively, a compressor and pressure vessel are permissible.

NOTE — Alternatively, vacuum-assisted filtration systems for small sample volumes may be used instead of positive-pressure filtration.

7.6 Nylon or polycarbonate membrane filters, of diameter 47 mm to 142 mm with rated pore sizes of 0,22 µm or 0,45 µm.

NOTE — Although membranes of both pore sizes are used successfully for the isolation of *Legionella* organisms, the comparative efficiency is not known. Polycarbonate membranes have lower flowrates which will extend processing times if used.

7.7 Silicone tubing, with inner and outer diameters as specified by the manufacturer of the peristaltic pump (7.5) but with a wall thickness of not less than 1,5 mm.

7.8 Heat source, such as a hot plate or gas ring burner.

7.9 Centrifuge, capable of (6 000 ± 100) *g,* fitted with safety buckets.

7.10 Rotary shaker, capable of achieving at least (200 ± 5) r/min.

7.11 Ultrasound water bath, suitable for use with water samples of up to 25 ml.

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

7.13 Glassware.

Sterilize all glassware either at (170 ± 5) °C for 1 h in a hot-air oven or at (121 ± 1) °C for 20 min in an autoclave.

7.14 Microscopes.

7.14.1 Fluorescent microscope.

A binocular microscope fitted with incident fluorescent illumination. The illuminating aperture shall consist of field and aperture diaphragms and exciting light stop, an exciter filter, a dichroic beam-splitting mirror and matching suppression filter and lamp holder. The microscope shall be fitted with an oil- or water-immersion lens (at least x40) and x8 or x10 eyepieces.

7.14.2 Plate microscope, stereoscopic, with magnification of at least x30 and oblique incident illumination.

8 Sampling

8.1 Sample containers

Samples of water (generally 1 litre) shall be collected in glass, polyethylene or similar containers. If used previously, they shall be cleaned, rinsed with distilled or mains tap water and autoclaved at (121 ± 1) °C for 20 min. Containers that cannot withstand autoclaving shall instead be pasteurized either with flowing hot water (> 70 °C) or steam for a period of not less than 5 min. Smaller sterile containers shall be used for the collection of slime, deposits or sediments. Wide-necked containers for slimes, etc. shall be fitted with screw caps.

Materials from which sample containers are made should be suitable for use in contact with drinking water. The volume of sample collected depends upon the nature of water system and the purpose of the examination.

Access to some sampling points can be difficult, which can make the use of glass containers unsafe because of breakage. Plastics-wrapped glass safety containers are permissible.

Details of the origin and volume of the sample, as well as the presence and nature of any biocide, shall be recorded and given to the laboratory with the samples as an aid to examination. For both safety and analytical reasons, it is not advisable to examine samples of unknown origin or of cooling and process waters unless they are accompanied by adequate information.

8.2 Sampling in the presence of biocide

If the water sampled contains or is thought to contain an oxidizing biocide, then add an excess of an inactivating agent to the container before or at the time of sampling.

NOTE — Chlorine and other oxidizing biocides are inactivated by the addition of potassium thiosulfate or sodium thiosulfate to the container. For other biocides, the addition of a universal neutralizing agent is not yet practicable.

As a rule microbiological analysis should be commenced as soon as possible after receipt of the water sample in the laboratory, preferably on the day of sampling, particularly for samples known to contain biocides. It is, however, recognized that transport of samples to the analysing laboratory may take some time, particularly from remote sites. It is, therefore, recommended that the time interval between collection of the sample and its concentration in these circumstances is ideally 2 days and shall not exceed 5 days (see 9.1.5). The maximum time from sample collection to culture of the concentrate is 14 days.

8.3 Sample transportation

Samples should be transported at less than 18 °C but not less than 6 °C and be protected from heat and sunlight. Deliver the samples to the laboratory as soon as possible, preferably within 1 day but not more than 2 days.

9 Procedure

9.1 Samples

9.1.1 General

Liquid samples may be plated directly (see 9.2.4) if the number of *Legionella* is expected to exceed 10^5 per litre. To ensure detection of *Legionella*s below this number in liquid samples, a concentration technique will be needed. Because the number of *Legionella* in any given sample is not known, concentration techniques are usually performed.

To concentrate the organisms in water samples, either membrane filtration (9.1.2) or centrifugation (9.1.3) is permissible. For sediments, deposits or slimes, dilute and culture directly (9.1.4). Waters with high counts of non-*Legionella* bacteria may be diluted with Page's Saline (6.3.2.1) or dilute Ringer's Solution (6.3.2.2). Record volumes of sample diluted or processed.

9.1.2 Positive-pressure membrane filtration of water samples

Assemble tubing (7.7) and filter stand (7.4) and insert the membrane (7.6) in the stand if the filter membrane has not been pre-sterilized.

Sterilize the filter stand assembly (with the membrane *in situ*) by autoclaving (see for example ISO 8199).

Aseptically place sterile membrane in filter assembly if not fitted before autoclaving. Filter the water by pressure through the filter membrane (7.6.1), placed in the filter stand (7.4), using the peristaltic pump (7.5). If the sample is cloudy, turbid or coloured, consider centrifugation as opposed to filtration (9.1.3).

NOTE 1 The flowrate should be adjusted so as not to exceed the maximum specified by the manufacturer for the filter size or type.

NOTE 2 For smaller volumes (<200 ml) vacuum filtration may be used in accordance with ISO 8199.

After filtration, cut off the tab of the nylon membrane protruding outside the filter stand with sterile scissors. Remove the membrane from the stand carefully with sterile forceps so as to avoid loss of residual deposit, and place it directly in a screw cap sterile container. To wash the organisms from the membrane, add 5 ml to 25 ml of sterile diluent (6.3.2.1, 6.3.2.2) the sample filtrate or sterile distilled water, and shake vigorously for not less than 2 min. This concentrate represents the prepared sample. Filters may be cut into pieces using sterile scissors to aid elution.

Alternatively, place the container in an ultrasound tank for between 2 min and 10 min. Treat each sample individually and ensure that the level of diluent covering the membrane is below the level of water in the ultrasound tank.

NOTE 3 To ensure optimal recovery, it is recommended that a strain of *L. pneumophila* is used to optimize the time of immersion in different ultrasound tanks. See also annex A.

9.1.3 Centrifugation of water samples

After shaking to resuspend any deposit that may have settled, pour (200 ± 5) ml of each sample into sterile screw-capped centrifuge bottles of between 300 ml and 500 ml capacity. Centrifuge the bottles at 6000 *g* for 10 min or 3000 *g* for 30 min, maintaining the temperature at 15 °C to 25 °C. Remove the supernatant aseptically and discard. Resuspend the deposit in 2 ml to 20 ml of the diluent (6.3.2.1, 6.3.2.2) or in sterile distilled water. Ensure that the volume of diluent added is recorded. This concentrate represents the prepared sample.

NOTES

1 It is recommended that the supernatant is removed by vacuum, rather than decanting, to avoid disturbance and consequent loss of the deposit.

2 If a volume of water sample other than (200 ± 5) ml is used, the deposit should be resuspended in a proportionate volume of diluent.

9.1.4 Sediments, deposits and slimes

NOTE 1 This type of sample may need to be diluted to reduce the number of non-*Legionella* organisms and thereby reduce the possibility of overgrowth of the slower growing *Legionella* organisms.

Prepare dilutions of each sample in sterile diluent (6.3.2.1, or 6.3.2.2) or sterile distilled water. Mix well by shaking or by producing a vortex for not less than 2 min. Add a layer (depth of 1 cm) of sterile glass beads to the sample to aid disaggregation of the material.

With samples of slime, if the consistency allows, plate out an undiluted portion:

a) without any treatment;

b) after heat-treatment (9.2.2);

c) after acid-treatment (9.2.3).

NOTE 2 If the slime is attached to construction materials or to dry material, it should be scraped off with a sterile spatula, resuspended in a small volume of diluent (6.3.2) and then treated in the same way. As the precise procedure may vary according to the nature of the sample, all procedures should be recorded.

9.1.5 Storage of water concentrates, sediments, deposits or slimes

Preferably analyse prepared samples immediately. Samples shall be stored at (6 ± 2) °C in the dark for not longer than 14 days.

NOTES

1 For outbreak investigation or epidemiological purposes it is recommended that, after culture, unused portions of the concentrate be stored in the same way for not less than 3 months. The details and dates of sample concentration and culture may be recorded. Stressed *Legionella* organisms may recover during storage under these conditions, retesting of stored concentrates is important in investigations of outbreaks of Legionnaire's disease. Some strains of *Legionella* species can survive for months, but others may die quickly.

2 If samples have been stored or transported some distance which extends storage time, it is essential to record this on the test report, together with a comment on the affect this may have had on the result. The type of sample may also influence survival of bacteria in storage (i.e. water, sludge, mud). These effects are not known and may vary even within similar samples.

3 It is recognized that the viable count of bacteria in a water sample may change during transport or storage. Bacteria can become attached to the walls of the sample vessel, become non-culturable or die. For *Legionella*s these losses are most apparent in samples stored at between 0 °C and 6 °C. Further, the growth of heterotrophic organisms is stimulated at or above 20 °C. Thus to minimize these changes it is recommended that samples for *Legionella* analysis be stored at (6 ± 2) °C.

9.2 Culture

9.2.1 General

Divide samples, concentrated or unconcentrated, into three portions. Use one portion without any further treatment. Treat the other two portions, one with heat (9.2.2) and one with acid (9.2.3).

9.2.2 Heat treatment

Add (1 ± 0,5) ml of sample (concentrated or unconcentrated) to a sterile container and place it in a water bath (7.12) at (50 ± 1) °C for (30 ± 2) min.

9.2.3 Acid treatment

Add 1 ml to 10 ml of sample (concentrated or unconcentrated) to a screw-capped container and centrifuge in a safety bucket at 6000 *g* for (10 ± 1) min or 3000 *g* for (30 ± 1) min. Draw off the supernatant to half the original volume with a sterile pipette, and resuspend the deposit by producing a vortex or vigorous mixing. Make up to its original volume by adding acid buffer (6.3.1) and mix gently. Allow to stand for (5 ± 0,5) min.

NOTE — Alternatively, a 10x concentrated acid solution may be used, diluted 1:10 with the sample to be treated and incubated for (5 ± 0,5) min, which avoids the need for centrifugation. The volumes are not critical as long as the sample remains at pH 2,2 for the stipulated period of time.

9.2.4 Inoculation of selective media

Inoculate a plate of GVPC agar medium (6.2.3) with 0,1 ml to 0,5 ml of the untreated portion of sample (concentrated or unconcentrated) (9.2.1). Distribute the liquid inoculum over the entire surface of the plate with a sterile spreader. Record the volume of sample inoculated.

Inoculate a second plate of GVPC medium in the same way with a 0,1 ml to 0,5 ml portion of the heat-treated sample (9.2.2) as soon as possible after removal from the water bath.

Inoculate a third plate of GVPC medium in the same way with a 0,1 ml to 0,5 ml portion of the acid-treated portion immediately after acid treatment (9.2.3).

9.2.5 Incubation

Allow the inoculated media to stand until the inocula have been absorbed, then invert the plates and incubate at (36 ± 1) °C for up to 10 days. To ensure the atmosphere in the incubator is humid, place a tray of water in the bottom of the incubator. Top up this tray with fresh water (if necessary) each time the plates are examined.

NOTE — Incubation in an atmosphere of air with 2,5 % (volume fraction) carbon dioxide may be beneficial for the growth of some *Legionella*s, but it is not essential.

9.2.6 Examination of the plates

Examine the plates with a plate microscope (7.14.2) on at least three occasions at intervals of 2 days to 4 days during the 10-day incubation period, as *Legionella*s grow slowly and can be masked by the growth of other organisms. Record the number of each type of colony present.

NOTE — Colonies of *Legionella* are often white-grey-blue-purple in colour, but may be brown, pink, lime-green or deep red. They are smooth with an entire edge and exhibit a characteristic ground-glass appearance. Under ultraviolet light, (7.3), colonies of several species (*L. bozemanii, L. gormanii, L. dumoffii, L. anisa, L. cherrii, L. steigerwaltii, L. gratiana, L. tucsonensis* and *L. parisiensis*) autofluoresce brilliant white; *L. rubrilucens* and *L. erythra* appear red. Colonies of *L. pneumophila* appear dull green often tinged with yellow. The colour of fluorescence can help to differentiate colonies in samples containing different species of *Legionella*. To avoid the possibility that *Legionella* cells could be killed, plates should not be exposed to ultraviolet light for longer than is necessary. It should be noted that new species of *Legionella* may possess characteristics different to those described above.

9.3 Confirmation of presumptive *Legionella* colonies

9.3.1 Subculture on to BCYE and other media

Select at least three colonies characteristic of *Legionella* (9.2.6) from each of the GVPC plates for subculture onto plates of BYCE (6.2.1) and BCYE – Cys medium (6.2.2). Subculture each colony to plates of both media. Incubate at (36 ± 1) °C for at least 2 days. Regard as *Legionella* those colonies which grow on BCYE but fail to grow on BCYE – Cys medium. Record the results for each plate.

NOTE — Nutrient agar or blood agar medium may be used instead of BCYE – Cys medium. If no growth occurs on any of these three media then further suspect colonies should be subcultured from the original plates of selective medium. *L. oakridgensis* and *L. spiritensis* require L-cysteine and iron 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 unsupplemented media.

When the numbers of individual serogroups or species of *Legionella* are to be reported, always confirm at least three representative colonies of each colonial type of subculture (9.3.2).

As it is not always practicable to confirm by serology (9.3.2) every colony of each type that grows on the GVPC selective medium, estimate the number of presumptive *Legionella* organisms in the following way. Count the number of colonies of each 'type' on each of the three GVPC (9.2.1, 9.2.2, 9.2.3) plates for each prepared sample. Subculture two or three colonies of each 'type' onto BCYE (6.2.1) and BCYE – Cys (6.2.2) medium. Incubate at 36°C ± 1°C, for at least 2 days. Confirm by serology (9.3.2) the identity of those colonies which grow on the BCYE agar but not on the BCYE – Cys (see Annex B). This enables a confirmed count of each *Legionella* colony type to be made. (See note, 9.3.1).

9.3.2 Identification of *Legionella* species by immunofluorescence

Before performing identification tests, ensure that the growth on BCYE medium is pure by examining the morphology of the colonies (9.2.6). If necessary, subculture again to BCYE medium.

NOTE — *Legionella* species may be identified by a variety of methods. These include gas-liquid chromatography of cellular fatty acids and isoprenozel quinones, indirect immunofluorescent antibody assay, direct fluorescent antibody assay, slide agglutination, latex bead agglutination, colony blot assay based on a genus-specific monoclonal antibody and/or enzyme-linked immunosorbent assay procedures with appropriate diagnostic reagents. As an example, the immunofluorescence method is described in this International Standard. See annex C for the immunofluorescence method as an example of a serological method which can be used to identify *Legionella species*.

10 Expression of results

To estimate the number of colony-forming units (cfu) of *Legionella* in the original water sample or sediment, select from the three plates of GVPC medium (9.3) the plate showing the maximum number of confirmed colonies. Estimate the number of cfu of *Legionella* in the original sample of water by multiplying that number by the concentration factor.

NOTE — It is not appropriate to average the counts from each of the three GVPC plates, as these are not replicates.

The purpose of this International Standard is to demonstrate the presence or absence of confirmed *Legionella* organisms in a sample. Report the confirmed presence (or absence) of *Legionella pneumophila* and the presumptive presence (or absence) of other *Legionella species*. Report absence as 'not detected' in the volume examined. Ideally report the serogroup of all isolates of *Legionella pneumophila*.

11 Test report

The test report shall include the following information:

a) a reference to this International Standard;

b) all details necessary for complete identification of the sample, including the sample site, the sampling technique, the nature of the sample, the kind of water system or plant, and the sampling point;

c) volume or mass of sample examined;

d) the date and time of

   i) collection of the sample;

   ii) receipt in laboratory;

   iii) examination in the laboratory;

e) any particular occurrence(s) observed during the course of analysis which may have influenced the result;

f) the results expressed as described in clause 10.

Annex A
(informative)

Scraping the bacteria from filter membranes

A scraping method to remove *Legionella*s from the filter membranes has also been used.

The membrane is removed from the stand with sterile forceps and placed in a sterile Petri dish of a suitable size (usually a Petri dish of 60 mm in diameter for a membrane of 47 mm) containing 5 ml to 10 ml of sterile diluent or filtrate from the original sample. The membrane is then scraped with a sterile cell scraper (cell scrapers are commercially available).

The scraping is performed at least twice over the whole membrane.

Annex B
(informative)

Colony identification on BCYE – Cys

Blood agar and nutrient agar have also been used to establish the *in vitro* requirement of *Legionella*s for cysteine to be added to culture media.