INTERNATIONAL STANDARD

ISO 11731-2

First edition
2004-05-01

Water quality — Detection and enumeration of *Legionella* —

Part 2: Direct membrane filtration method for waters with low bacterial counts

Qualité de l'eau — Recherche et dénombrement des Legionella —

Partie 2: Méthode par filtration directe sur membrane pour les eaux à faible teneur en bactéries

Reference number
ISO 11731-2:2004(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published in Switzerland

Contents

Page

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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 11731-2 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

ISO 11731 consists of the following parts, under the general title *Water quality — Detection and enumeration of* Legionella:

— *Part 2: Direct membrane filtration method for waters with low bacterial counts*

The general method will be the subject of a future Part 1 of ISO 11731.

Water quality — Detection and enumeration of *Legionella* —

Part 2:
Direct membrane filtration method for waters with low bacterial counts

WARNING — Persons using this part of ISO 11731 should be familiar with normal laboratory practice. This part of ISO 11731 does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This part of ISO 11731 describes a monitoring method for the isolation and enumeration of *Legionella* organisms 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*. As the growth of *Legionella* may be inhibited by overgrowth of other bacterial colonies on the membrane, the method is only suitable for waters containing low bacterial counts.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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

ISO 8199:—[1)], *Water quality — General guidance on the enumeration of micro-organisms by culture*

ISO 11731:1998, *Water quality — Detection and enumeration of Legionella*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
Legionella
genus of Gram-negative bacteria normally capable of growth in no 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. Growth does not occur in the absence of L-cysteine with the exception of *L. oakridgensis* and *L. spiritensis*. *L. oakridgensis* and *L. spiritensis* require L-cysteine and iron for primary isolation but can grow weakly in the absence of added L-cysteine thereafter.

1) To be published. (Revision of ISO 8199:1988)

4 Safety

The reagents used in this part of ISO 11731 should be subject to assessment in accordance with control 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 any 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. After filtration, the filter is treated with acid buffer added directly into the funnel to reduce the growth of non-*Legionella* organisms. The filter is subsequently transferred onto a plate of agar medium selective for *Legionella* and incubated. Samples expected to contain sufficient numbers of *Legionella* need not be subjected to concentration prior to culture (9.1).

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. Species identification may not be considered necessary for routine monitoring but is indispensable in outbreak situations.

NOTE *L. pneumophila* serogroup 1 is the causative agent of most legionellosis cases and is therefore considered the most “critical” type of *Legionella* to be found in the water system. Since increasing numbers of cases of legionellosis caused by other serogroups of *L. pneumophila* and other *Legionella* species are being described, even the presence of other *Legionella* species in water is considered a potential risk.

6 Culture media and reagents

6.1 General

Use chemicals of analytical grade in the preparation of media and reagents unless otherwise stated. Alternatively, use commercially available dehydrated media and reagents. Prepare the media according to the manufacturer's instruction and add freshly prepared (or thaw the stored material at room temperature prior to use) selective agents or growth supplements at the concentrations recommended. Prepare media using glass distilled water or water of equivalent quality complying with ISO 3696:1987, Grade 3. Other grades of chemicals may be used providing they can be shown to produce the same results.

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
α-Ketoglutarate, monopotassium salt	1,0 g
ACES buffer (*N*-2-acetamido-2-aminoethane sulfonic 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	1 000 ml

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

6.2.1.2 Preparation

6.2.1.2.1 Cysteine and iron solutions

Prepare fresh solutions of L-cysteine hydrochloride and iron(III) pyrophosphate by adding the 0,4 g and 0,25 g respectively to 10 ml volumes of distilled water. Decontaminate each solution by filtration through a cellulose ester membrane filter with an average pore size of 0,2 µm. Store in clean, sterile containers at (−20 ± 5) °C for no more than 3 months.

6.2.1.2.2 ACES buffer

Add the ACES granules to 500 ml of distilled water and dissolve by standing in a water bath at 45 °C 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.

6.2.1.2.3 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 l of distilled water. Prepare a 0,1 mol/l solution of sulfuric acid (H_2SO_4) by carefully adding 5,3 ml of H_2SO_4 (ρ = 1,84, of 95 % to 98 % purity) to 1 l of distilled water. Use the solutions of 0,1 mol/l potassium hydroxide or 0,1 mol sulfuric acid as appropriate to adjust the pH to 6,8 ± 0,2. Add the agar, mix and autoclave at (121 ± 3) °C for (15 ± 1) min (6.2.4). After autoclaving allow to cool to (50 ± 2) °C in a water bath.

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

Dispense in 20 ml volumes into Petri dishes of 90 mm to 100 mm diameter. Petri dishes of 60 mm may also be used for incubating the membranes (see 9.1 and 9.2). The pH of the final medium is 6,8 ± 0,2 at 25 °C. Allow excess moisture on the plates to dry and store at (5 ± 3) °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

Natamycin may be used instead of cycloheximide.

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 ± 5) °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 ± 5) °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 ± 5) °C. For use, thaw at room temperature.

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 the 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 6,8 ± 0,2.

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 has to 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 3 d 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* 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.

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.

b) Alternatively, 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 BCYE medium (6.2.1) for purity. If a reference 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 in sterile glycerol broth (6.2.5) from the resulting growth so that it is just visible to the naked eye. Dispense in 1 ml volumes for storage at (-20 ± 5) °C. Alternatively, use Page's saline (6.3.2) or distilled water for storage at (-70 ± 10) °C or other appropriate freezing media and store at (-20 ± 5) °C or (-70 ± 10) °C as appropriate. Plate out one suspension of each isolate onto BCYE medium for subsequent identification and recording of the *Legionella* species and serogroup (9.4). 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 (9.4) and number of colonies are similar.

6.2.5 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 volumes of 2 ml. Sterilize by autoclaving at (121 ± 3) °C for (20 ± 1) min. Store at room temperature until required.

6.3 Reagents

6.3.1 Acid buffer

Prepare a 0,2 mol/l solution of hydrochloric acid (HCl) (Solution A). Prepare a 0,2 mol/l solution of potassium chloride (KCl) (Solution B). To prepare the acid buffer mix 3,9 ml of Solution A and 25 ml of Solution B. Adjust to pH $2,2 \pm 0,2$ by addition of a 1 mol/l solution of potassium hydroxide (KOH). Store in a stoppered glass container in the dark at room temperature for no longer than 1 month.

6.3.1.1 Solution A: 0,2 mol/l HCl

Add 17,4 ml of concentrated HCl (ρ = 1,18, minimum assay 35,4 %) or 20 ml HCl (ρ = 1,16, minimum assay 31,5 %) to 1 l distilled water. Sterilise by autoclaving at (121 ± 3) °C for (15 ± 1) min.

6.3.1.2 Solution B: 0,2 mol/l KCl

Dissolve 14,9 g of KCl in 1 l of distilled water. Sterilise by autoclaving at (121 ± 3) °C for (15 ± 1) min.

6.3.2 Page's saline

Sodium chloride (NaCl)	0,120 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0,004 g
Calcium chloride ($CaCl_2 \cdot 2\ H_2O$)	0,004 g
Disodium hydrogenphosphate (Na_2HPO_4)	0,142 g
Potassium dihydrogenphosphate (KH_2PO_4)	0,136 g
Distilled water	1 000 ml

Add the chemicals to the distilled water. Allow to dissolve, mix well and autoclave at (121 ± 3) °C for (15 ± 1) min.

To aid accurate preparation, prepare a 10 l volume of Page's saline and dispense in smaller volumes as required for autoclaving at (121 ± 3) °C for (15 ± 1) min.

7 Apparatus

Usual laboratory equipment including

7.1 Petri dishes, with a nominal diameter of either 90 mm to 100 mm or 60 mm.

7.2 Incubator, capable of being maintained at (36 ± 2) °C.

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

7.4 Vacuum filtration system, with vacuum pump, tubing, filter stand and funnel suitable for filtering water volumes of 10 ml to 1 l. Filter stand and funnel shall withstand autoclaving.

7.5 Black nitrocellulose membrane filters, diameter 47 mm or 50 mm with nominal pore sizes of 0,45 µm.

NOTE White membrane filters have successfully been used for the isolation of *Legionella*. The appearance of *Legionella* colonies is, however, different compared to black membrane filters.

The quality of membrane filters may vary from brand to brand or even from batch to batch. It is therefore advisable to check the quality on a regular basis, in accordance with ISO 7704[2)].

7.6 Sterile forceps, with rounded ends.

7.7 Glassware

Except for disposable glassware which is delivered sterile, sterilize all glassware in accordance with the instructions given in ISO 8199.

7.8 Low power binocular microscope, with magnification of at least × 6 illuminated from above by oblique incident light.

2) ISO 7704, *Water quality — Evaluation of membrane filters used for microbiological analyses*

8 Sampling

8.1 General

The volume of sample collected depends upon the nature of water system and the purpose of the examination.

Details of the origin and volume of the sample, the temperature of the water at the time of sampling 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 that will include information about the chemical additives used in or likely contaminants that are present as a result of the process.

8.2 Sample containers

Samples of water (generally 1 l) 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 ± 3) °C for 15 min.

Materials from which sample containers are made should be suitable for use in contact with drinking water.

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

8.3 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 appropriate 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 thiosulphate or sodium thiosulphate to the container.

8.4 Sample transportation and storage

Deliver the samples to the laboratory as soon as possible preferably within 1 d but not more than 2 d.

If analysed the same working day, transport samples at ambient temperature protected from sunlight. Otherwise cool samples, ideally (5 ± 3) °C, during transport. Hot water samples should be cooled directly after sampling.

As a rule microbiological analysis should be commenced as soon as possible after arrival in the laboratory, preferably on the day of sampling, particularly 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 filtration in these circumstances is ideally within 24 h and should not exceed 2 d. Store the samples at (5 ± 3) °C.

9 Procedure

9.1 Membrane filtration of water samples

Filter 10 ml to 1 000 ml of the water sample. The volume filtered may depend on the particulate content of the water. Make a note of the volume of sample filtered. To minimize the growth of non-*Legionella* bacteria, treat the sample with acid buffer (6.3.1) directly in the filter funnel as follows. After filtration of the sample, add (30 ± 5) ml of acid buffer on top of the membrane and leave for 5 min. Remove the acid buffer by filtration

through the membrane and wash the membrane with (20 ± 5) ml of Page's saline (6.3.2) or other corresponding buffer.

It is important to take care that none of the water used to flush the membrane comes into contact with areas of the filtration apparatus that have not previously been in contact with acid buffer. Otherwise water droplets that have not been into contact with the acid buffer might be flushed on the membrane which may result in increased background flora.

NOTE Filtration of large volumes of sample can lead to enrichment of toxic substances on the membrane filter. A decrease in recovery rate with increasing filtration volumes can indicate the presence of inhibitory substances.

Carefully remove the membrane from the stand with sterile forceps (7.6) and place it (upside up) directly on a BCYE-agar (6.2.1) or GVPC-agar (6.2.3) plate ensuring that no air bubble is trapped underneath.

9.2 Incubation

Incubate the plates upside down at (36 ± 2)°C for 10 d. To ensure the incubation atmosphere is humid, either place a tray of water in the bottom of the incubator and top up this tray with fresh water (if necessary) each time the plates are examined or incubate the plates in plastic bags or use closed containers.

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

9.3 Examination of the plates

Examine the plates, preferably with a binocular microscope (7.8), on at least two occasions starting at day three or four during the 10 d incubation period, as *Legionella* grow slowly.

Record the number of presumptive *Legionell*a colonies present.

The growth of *Legionella* species can be inhibited or masked by the growth of other organisms. If inhibition is suspected, or there is a heavy background growth, smaller volumes or dilutions of the sample should be re-examined.

Colonies of *Legionella* on black media or black membranes are often white-grey-blue-purple in colour, but can 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 blue/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.

NOTE 1 *Legionella* colonies growing on a membrane filter on the agar grow more slowly and usually to a smaller size than colonies growing directly on the agar surface. Some species other than *L. pneumophila* can fail to grow on the membranes.

NOTE 2 *Legionella* colonies on white membrane filters have a different appearance from those growing against a black background (medium or black membrane filters).

9.4 Confirmation of presumptive *Legionella* colonies: Subculture to BCYE-Cys

Select at least five colonies characteristic of *Legionella* (9.3) at random for each sample for subculture onto plates of BCYE (6.2.1) and BCYE-Cys medium (6.2.2). If different types of presumptive *Legionella* colonies are present make sure to select at least two colonies from each type. Subculture each colony onto plates of both media. Incubate at (36 ± 2) °C for at least 2 d. Regard as *Legionella* those colonies which grow on BCYE but fail to grow on BCYE-Cys medium. Record the results for each plate.

Nutrient agar or blood agar medium may be used instead of BCYE-Cys medium.

In outbreak investigations, the target organisms may be a minority of the *Legionella* population. Therefore it is necessary to ensure that the colonies selected for confirmation include at least one representative of each colony type of presumptive *Legionella*. This may mean that more than five colonies have to be selected. It may ultimately be necessary to select all colonies if the target is not detected in those sub-cultured initially.

L oakridgensis and *L. spiritensis* require L-cysteine and iron for primary isolation but can 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 according to ISO 11731.

10 Expression of results

The purpose of this part of ISO 11731 is to estimate the number of *Legionella* present or demonstrate the absence of *Legionella* in the sample. Report the confirmed estimated number of *Legionella* present, as CFU of *Legionella* species in the volume examined, and report the absence of *Legionella* as “not detected” in the volume examined.

11 Test report

The test report shall include the following information:

a) a reference to this part of ISO 11731 (ISO 11731-2:2004);

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

c) volume of sample examined;

d) the water temperature at the time of sampling;

e) the date and time of

   1) collection of the sample;

   2) receipt in laboratory;

   3) examination in the laboratory;

f) the results expressed as described in Clause 10;

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

ICS 07.100.20

Price based on 9 pages