BS EN ISO 11731:2017

BSI Standards Publication

Water quality — Enumeration of *Legionella*

bsi.

National foreword

This British Standard is the UK implementation of EN ISO 11731:2017. It is identical to ISO 11731:2017. It supersedes BS EN ISO 11731-2:2008 and BS 6068-4.12:1998 (dual numbered as ISO 11731:1998), which are withdrawn.

BSI, as a member of CEN, is obliged to publish EN ISO 11731:2017 as a British Standard. However, attention is drawn to the fact that during the development of this European Standard, the UK committee voted against its approval as a European Standard.

The standard has many potential variations and interpretations, not only between the method options specified but also within each specified method. This will lead to differences in approach being taken by different laboratories, which are all theoretically valid interpretations of this standard.

The criteria for the selection of the method variants are based on prior knowledge of the sample characteristics or are based on the sample matrix or type. This assumes that prior knowledge of the sample type is available, which may not always be the case for samples received by laboratories from third parties. In addition, this assumes that samples within each of the water type categories have a similar constituency or that the constituency of individual samples may not change between sampling events (for example in periodic routine sampling).

The standard brings together the procedures described in ISO 11731:1998 and EN ISO 11731:2008. There are also additional requirements for some method variations within the standard that were not previously required, such as additional media requirements. Instructions on the choice of approach are described in Annex J and are summarised in Figure J.1. This shows 10 method variations which consist of 14 potential procedures and three media types, with the selection of method being based on sample matrix and constituency.

In respect to sampling and transport of samples for *Legionella* analysis, the UK committee believes that ISO 11731:1998 and EN ISO 11731:2008 are currently more appropriate. The action level in UK guidance is 100 cfu/l. To detect this level reliably requires that the portion inoculated onto the selective isolation medium would be equivalent to at least 30 ml of the original water sample.

Additionally, the user should be aware that the acid treatment step (for the membrane filtration with washing procedure) is now a 1 in 10 dilution which may affect the theoretical limits of detection of the method. This may therefore not match the action levels stated in national guidelines. This standard also does not definitively specify an incubation time but gives a range of 7 to 10 days. A note has been added to subclause 8.4.6 to identify that natural samples may require the full 10 days.

The UK participation in its preparation was entrusted to Technical Committee EH/3/4, Microbiological methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

Published by BSI Standards Limited 2017

ISBN 978 0 580 79531 2

ICS 07.100.20

Compliance with a British Standard cannot confer immunity from legal obligations.

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 30 June 2017.

Amendments/corrigenda issued since publication

Date	Text affected

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN ISO 11731

June 2017

ICS 07.100.20

Supersedes EN ISO 11731-2:2008

English Version

Water quality - Enumeration of Legionella (ISO 11731:2017)

Qualité de l'eau - Dénombrement des Legionella (ISO 11731:2017)

Wasserbeschaffenheit - Zählung von Legionellen (ISO 11731:2017)

This European Standard was approved by CEN on 12 February 2017.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

Ref. No. EN ISO 11731:2017 E

European foreword

This document (EN ISO 11731:2017) has been prepared by Technical Committee ISO/TC 147 "Water quality" in collaboration with Technical Committee CEN/TC 230 “Water analysis” the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by December 2017, and conflicting national standards shall be withdrawn at the latest by December 2017.

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

This document supersedes EN ISO 11731-2:2008.

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Endorsement notice

The text of ISO 11731:2017 has been approved by CEN as EN ISO 11731:2017 without any modification.

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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

This second edition of ISO 11731 cancels and replaces ISO 11731:1998 and ISO 11731-2:2004, which have been technically revised.

Introduction

After the first recognized outbreak of Legionnaires' disease in 1976, the isolated bacterium was named *Legionella pneumophila*. Legionellae are widely found in natural and artificial aquatic environments, soils, composts and can cause legionellosis. Legionellae can grow intracellularly in protozoa like *Acanthamoeba castellanii*, *Hartmannella* species or *Naegleria* species. At least 61 different *Legionella* species have been described. In 26 of these species, some strains infecting humans have been reported. *Legionella pneumophila* can be subtyped into at least 15 different serogroups; nine other species also can be subtyped into at least two separate serogroups. Monitoring for legionellae is important for public health reasons to identify environmental sources which can pose a risk of legionellosis, such as evaporative cooling towers, hot- and cold-water distribution systems in buildings and associated equipment such as spa pools, dental units, air conditioning units, etc. Monitoring is also important for validation of control measures and ongoing verification that controls remain effective.

INTERNATIONAL STANDARD ISO 11731:2017(E)

Water quality — Enumeration of *Legionella*

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

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably qualified and competent staff.

1 Scope

This document specifies culture methods for the isolation of *Legionella* and estimation of their numbers in water samples.

These methods are applicable to all kinds of water samples including potable, industrial, waste and natural waters. These methods can be used for water related matrices, e.g. biofilms, sediments, etc.

Not all *Legionella* species are culturable; therefore, the methods described in this document do not recover all species of *Legionella*.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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, *Water for analytical laboratory use — Specification and test methods*

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

ISO 8199, *Water quality — General guidance on the enumeration of micro-organisms by culture*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

ISO 19458, *Water quality — Sampling for microbiological analysis*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
Legionella
genus of microorganisms normally capable of growth on buffered charcoal yeast extract (BCYE) agar containing L-cysteine and iron(III) salts

Note 1 to entry: For a more detailed description of *Legionella* species, see Annex A.

4 Principle

4.1 General

Legionellae in the water sample are concentrated by membrane filtration, diluted or directly plated depending on the origin/characteristics of the sample. The desired level of detection can vary depending on (inter)national legislation and the reason for sampling or investigation. Samples expected to contain high numbers of legionellae, such as those obtained during outbreak investigations, can be processed with and/or without the concentration steps. To reduce the growth of the concentrated non-target bacteria, which can interfere with the recovery of the target legionellae, portions of the water samples are also subjected to heat treatment, acid treatment or a combination of both treatments.

Dilution is necessary when high concentrations of *Legionella* and/or other bacteria are expected. Separate portions of the diluted sample should be pre-treated; one with heat and a second with acid solution or, in case of extremely contaminated samples, with a combination of acid solution and heat before culturing on selective media.

Treated and/or untreated portions of the sample are transferred onto plates of the chosen culture medium selective for *Legionella* and incubated.

NOTE Mechanical treatment of the sample can enhance the recovery of *Legionella*.

4.2 Examination

After incubation, morphologically characteristic colonies on the selective culture media are regarded as presumptive *Legionella*.

4.3 Confirmation

Presumptive colonies are confirmed as *Legionella* by subculture to demonstrate their growth requirement for L-cysteine and iron(III).

NOTE If species and serotype identification are requested, further tests are needed (see Annex G). These tests are not part of the standardized methods described in this document.

5 Apparatus and glassware

Usual laboratory equipment and in particular:

5.1 Sterile Petri dishes.

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

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

5.4 Membrane filtration equipment, suitable for filtering water volumes of 10 ml up to 1 000 ml.

5.5 Membrane filter.

5.5.1 Membrane filter for concentration and elution, polycarbonate or polyethersulfone membrane filters, diameter 47 mm to 142 mm with rated pore sizes of 0,2 µm; see Reference [6]. These types of membrane filters are used for concentration followed by a washing procedure.

5.5.2 Membrane filter for direct placing on culture media, membrane filters from cellulose nitrate or mixed cellulose esters, diameter 47 mm to 50 mm with rated pore sizes of 0,2 µm or 0,45 µm. These

types of membrane filters are used for direct placing onto the culture media after filtration. Filters shall be evaluated prior to use in accordance with ISO 7704.

NOTE Black membrane filters contrast better with the white *Legionella* colonies than light-coloured membrane filters.

5.6 pH meter, with an accuracy of ± 0,1 at 20 °C to 25 °C.

5.7 Vortex mixer.

5.8 Ultrasonic water bath, suitable for ensuring that the level of diluent covering the membrane filter is below the level of water in the water bath.

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

5.10 Glassware, sterilized according to ISO 8199.

5.11 Dissection microscope, stereoscopic, with magnification of at least 4× and with oblique incident illumination.

NOTE Also, a hand lens (magnification at least 4×) can be used.

5.12 Disinfected forceps, for handling of membrane filters.

NOTE Forceps with round ends are generally used in order not to damage the membrane during handling.

5.13 Screw cap sterile container, with or without sterile glass beads. To ensure maximum removal of the legionellae from the membrane filter, sterile glass beads (diameter 2 mm to 3 mm) can be added to the sterile container. Add sufficient glass beads to the sterile container just enough to cover the bottom of the container.

6 Culture media and reagents

Use chemicals of analytical grade in the preparation of culture media and reagents unless otherwise stated (see the Note). Prepare the culture media and reagents according to the instructions given in Annexes B, C and D. Prepare culture media using distilled or demineralized water, which is free from substances that might affect growth of microorganisms under the test conditions. The water shall comply with the requirements of ISO 3696, grade 3.

Alternatively, use commercially available culture media and reagents prepared and used according to the manufacturer's instructions.

NOTE Chemicals of other grades can be used, providing they are shown to be of equal performance in the test.

6.1 Culture media.

See Annex B.

6.1.1 Buffered charcoal yeast extract (BCYE) agar.

See B.1.

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

See B.2.

NOTE Blood agar (see B.6), nutrient agar (see B.7) or tryptone soy agar (see B.8) can be used instead of BCYE–cys agar.

6.1.3 Buffered charcoal yeast extract agar with selective supplements (BCYE+AB).

See B.3.

6.1.4 Glycine vancomycin polymyxin B cycloheximide (GVPC) agar.

See B.4.

6.1.5 Modified Wadowsky Yee (MWY) agar.

See B.5.

6.2 Diluents.

See Annex C.

6.2.1 Page's saline.

See C.1.

6.2.2 Diluted Ringer's solution.

See C.2.

6.3 Acid solution.

See Annex D.

7 Sampling

Carry out sampling, transport and storage of the samples in accordance with ISO 19458. Take care not to expose the samples to adverse temperature conditions (e.g. freezing or overheating).

NOTE The use of insulated containers is helpful in this regard.

8 Procedure

8.1 Samples

Due to the complex nature of different sample matrices, the laboratory shall determine the appropriate method for each sample type. The decision matrix is provided in Annex J to determine which appropriate method shall be undertaken. Annex J describes the requirements and provides additional options.

In order to ensure the detection of legionellae from water samples, a concentration technique by membrane filtration (see 8.2.2 or 8.2.3) will be required in most cases. Where the concentration of legionellae is expected to be greater than 10^4 colony forming units per litre (cfu/l), direct plating of the unconcentrated sample can also be carried out. For highly contaminated samples, dilute (refer to Annex C for suitable diluents) and use direct plating before and after the pre-treatment (see 8.3). Record volumes of sample diluted or processed and which pre-treatment(s) has (have) been applied.

When the number of legionellae in any given sample is not known, concentration techniques are usually performed. Therefore, follow the procedure described in 8.2.2 or 8.2.3.

8.2 Concentration of water samples

8.2.1 General

For a general description of the membrane filtration technique, see ISO 8199. Filtration can be done by vacuum filtration or positive pressure filtration.

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

NOTE The procedure for water related matrices (swabs, sediment, etc.) is described in Annex I.

8.2.2 Membrane filtration and direct placing of the membrane filter on culture media

Filter the water sample (without treatment, after acid treatment and, if required, after heat treatment) through a cellulose nitrate or mixed cellulose esters membrane filter (5.5.2). The acid treatment can also be done directly on the membrane filter in the funnel (see 8.3.2). The volume filtered depends on the particulate content of the water or the desired detection level. The filtered volume of the sample shall be recorded. Carefully remove the membrane filter from the stand with disinfected forceps (5.12) and place it (right-side up) directly on the culture media, ensuring that no air bubble is trapped underneath.

NOTE Where concentration by filtration is not possible (e.g. due to a high level of deposit), the sample can be concentrated by centrifugation (see Annex F).

8.2.3 Membrane filtration followed by a washing procedure

Filter the water sample through a polycarbonate or polyethersulfone membrane filter (5.5.1). The volume filtered depends on the particulate content of the water or the desired detection level. The filtered volume of the sample shall be recorded. Remove the membrane filter from the stand with disinfected forceps (5.12). Work carefully to avoid loss of residual deposit. Place the membrane filter (right-side down) in a screw cap sterile container with or without sterile glass beads (5.13). To wash the microorganisms from the membrane filter, add 5 ml to 10 ml of sterile diluent (see Annex C) or sample, and shake vigorously using a vortex mixer (5.7) for at least 2 min. Alternatively, place the container (5.13) in an ultrasonic water bath (5.8) for a time interval that has been verified to determine the optimum time interval for maximum recovery. Ensure that the level of diluent covering the membrane is below the level of water in the ultrasonic water bath.

This concentrate represents the prepared sample. Record the volume of the concentrate. Membrane filters may be cut into pieces using sterile scissors to aid elution.

Divide the concentrate into three portions. Use one portion untreated, one portion for treatment with heat (see 8.3.1) and one portion for treatment with acid solution (see 8.3.2).

NOTE 1 Alternatively, the scraping or rubbing technique can be used for removal of the bacteria from the membrane filter (see Annex E).

NOTE 2 Where concentration by filtration is not possible (e.g. due to a high level of deposit), the sample can be concentrated by centrifugation (see Annex F).

NOTE 3 An additional membrane filtration can be used for the acid pre-treatment directly on the membrane filter in the funnel.

8.3 Sample pre-treatment

8.3.1 Heat treatment

Add the sample (concentrated or unconcentrated) to a sterile container and place it in a water bath (5.9) at (50 ± 1) °C for (30 ± 2) min. Small volumes (≤ 5 ml) should be used to ensure a short period until the desired temperature is reached. If many samples are treated together or large sample volumes are treated or thick-walled containers are used, monitor the temperature in a separate container similar to that used for the sample. The time starts when the required temperature is reached. Large sample volumes or thick-walled containers should be cooled to avoid overheating after being removed from the water bath.

8.3.2 Acid treatment

Dilute one volume of the sample (concentrated or unconcentrated) with nine volumes of the acid solution (see Annex D), mix well and leave it for (5,0 ± 0,5) min. If the diluted acid treated sample is used for the calculation of the final concentration of *Legionella* species in the sample, the dilution should be factored. Volumes greater than 0,1 ml can be plated to decrease the limit of detection.

Acid treatment can also be done directly on the membrane filter in the funnel. Transfer around 30 ml acid solution (see Annex D) onto the membrane filter. Leave it for (5 ± 0,5) min and remove the acid solution by filtration. Wash the membrane filter with at least 20 ml of the diluent (see Annex C). It is important that the diluent does not rinse the surface of the funnel that had not been in contact with the acid solution.

8.4 Culture

8.4.1 General

The choice of the method used for the enumeration of *Legionella* species depends on the origin/characteristics of the sample and the reason of sampling or investigation. An assumption shall be made about the expected concentration of interfering microorganisms based on experience or origin of the sample. Also, the desired lower limit of detection level needs to be considered. A decision matrix for choosing an appropriate method is described in detail in Annex J.

Depending on the desired detection level, it is possible to use more than one plate of the different culture media mentioned in the following subclauses.

8.4.2 Samples with a high concentration of *Legionella* species and a low concentration of interfering microorganisms

Plate the sample directly if the number of *Legionella* is expected to exceed 10^4 cfu/l. Inoculate and spread 0,1 ml to 0,5 ml of the sample on one plate of BCYE agar (see B.1) and one plate of BCYE+AB agar (see B.3). Record the inoculated volume.

8.4.3 Samples with a low concentration of *Legionella* species and a low concentration of interfering microorganisms

8.4.3.1 Direct placing of membrane filter on culture media after membrane filtration

Filter the sample (see 8.2.2) and place the untreated membrane filter directly on one plate of BCYE agar (see B.1). The membrane filters treated with acid solution according to 8.3.2 are placed on one or more of the selective or highly selective plates of BCYE+AB agar (see B.3) or GVPC agar (see B.4) or MWY agar (see B.5).

8.4.3.2 Plating after membrane filtration with washing procedure

Inoculate and spread 0,1 ml to 0,5 ml of each concentrated portion of the sample (untreated, heat treated and acid treated) from the membrane filtration with washing procedure (see 8.2.3) on one plate of BCYE agar (see B.1) and on one or more of the selective or highly selective plates of BCYE+AB agar (see B.3) or GVPC agar (see B.4) or MWY agar (see B.5). Record the inoculated volume.

8.4.4 Samples with a high concentration of interfering microorganisms

Culture samples with a high concentration of interfering microorganisms unconcentrated (direct), concentrated (see 8.2) or diluted (1:10). Divide each subsample into three portions. Use one portion untreated, the second portion for treatment with heat (see 8.3.1) and the third portion for treatment with acid solution (see 8.3.2). Inoculate and spread 0,1 ml to 0,5 ml of each portion of the subsamples on one plate of GVPC agar (see B.4) or one plate of MWY agar (see B.5). Record the inoculated volume.

8.4.5 Samples with an extremely high concentration of interfering microorganisms

Culture samples with an extremely high concentration of interfering microorganisms unconcentrated and diluted (1:10 and 1:100) after a pre-treatment with a combination of heat and acid. For the combined treatment first, the heat treatment (see 8.3.1) is done followed by the acid treatment (see 8.3.2). It is important to cool the heat-treated sample to room temperature before the acid treatment is done. Prepare dilutions directly after the acid treatment in sterile diluent (see Annex C).

Mix well by shaking, using a vortex mixer (5.7) or an ultrasonic water bath (5.8). If necessary, add a layer (just enough to cover the bottom of the container) of sterile glass beads to the sample to aid disaggregation of the material. Inoculate and spread 0,1 ml to 0,5 ml of each portion on one plate of GVPC agar (see B.4) or one plate of MWY agar (see B.5). Record the inoculated volume.

8.4.6 Incubation

Allow the inoculated plates to stand until the inoculated volume has been absorbed, then invert the plates and incubate at (36 ± 2) °C for 7 d to 10 d. Create a humid atmosphere to prevent desiccation of the plates, for example, by placing in an enclosed container.

NOTE Validation data using artificially spiked samples have demonstrated no difference in counts between incubation for 7 d and 10 d. Natural samples containing wild strains of *Legionella* can, however, require the full incubation period of 10 d to present growth.

8.4.7 Examination of the plates

Inspect the plates for the first time either on day 2, 3, 4 or 5 followed by a final inspection at the end of the incubation period. This is to identify samples where overgrowth has occurred. The final quantitative result is not available until the end of the incubation period. For the range of quantitative determination, see Table H.1. As *Legionella* grows slowly and can be masked by the growth of other microorganisms, it is also recommended to use the dissection microscope with oblique incident illumination (5.11). Record the number of each type of presumptive *Legionella* colony present.

In case of outbreak investigations, it is advisable for samples expected to have a high concentration of interfering microorganisms to check the plates on day 2 to determine if dilutions are needed. Be aware of the potentially negative impact of the conservation of the concentrate or sample for a period of two additional days.

Colonies of *Legionella* are white-grey in general but can also appear in other colours. They are smooth with an entire edge and exhibit a characteristic ground-glass appearance. Under an ultraviolet lamp (5.3), colonies of several species (*L. anisa*, *L. bozemanii*, *L. cherrii*, *L. dumoffii*, *L. gormanii*, *L. gratiana*, *L. parisiensis*, *L. steigerwaltii* and *L. tucsonensis*) autofluoresce brilliant white; *L. erythra* and *L. rubrilucens* 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 or damaged so that they are not recoverable,

plates should not be exposed to ultraviolet light for a longer time than is necessary. It should be noted that new species of *Legionella* might possess characteristics different to those described above.

8.5 Confirmation of presumptive *Legionella* colonies on culture media: BCYE agar and BCYE–cys agar

Subculture from the plate(s) showing the highest counts of presumptive colonies of *Legionella* (see 8.4.7) per water volume. When there is only one colony type, pick three presumptive colonies. If more morphological different types of presumptive colonies of *Legionella* are growing on the plate, take at least one colony from each type. Subculture onto a plate of BCYE agar (see B.1) and a plate of BCYE–cys agar (see B.2 or alternative media as described in the Note in 6.1.2). Be careful not to carry over any culture media with the colony and first inoculate a plate of BCYE–cys agar and then a plate of BCYE agar. Incubate at (36 ± 2) °C for 2 d to 5 d. Regard as *Legionella* those colonies which grow on the plate of BCYE agar (see B.1) but fail to grow on the plate of BCYE–cys agar (see B.2). Record the results for each plate. If the initial subcultures do not confirm as *Legionella*, analyse further subcultures of presumptive *Legionella* colonies from another type of plate (culture media or sample treatments).

L. oakridgensis and *L. spiritensis* require L-cysteine and iron(III) for primary isolation but sometimes 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 culture media.

NOTE Alternative procedures can be used to confirm the isolate as *Legionella* species providing the suitability of the alternative procedure is verified.

For special situations like outbreak investigations, at least five presumptive colonies if only one morphology is present, or two presumptive colonies for each type of morphology shall be confirmed. If identification of the *Legionella* colonies is to be carried out (see Annex G) and included on the test report, all typical morphologies present on any of the plates should be confirmed and the identifications reported.

9 Expression of results

To estimate the number of colony forming units of *Legionella* in the original water sample, select the plate or set of plates (from the same culture medium) showing the maximum number of confirmed colonies per water volume. Take the dilution into account. Do not average the counts from different methods, treatments or culture media, as these are not replicates.

Calculate the number of *Legionella* colony forming units in the original sample (see examples shown below) per litre, in accordance with ISO 8199, as follows.

— Direct plating: $C_s = \frac{a}{V_{tot}} \times V_s$

— Membrane filter on plate: $C_s = \frac{a}{V_{tot}} \times V_s$

— Filtration with washing procedure (indirect filtration): $C_s = \frac{a \times V_c}{V \times V_{tot}} \times V_s$

— Plating after dilution: $C_s = \frac{a \times V_s}{V_{dil}} \times Df$

where

C_s is the number of *Legionella* in cfu/l;

a is the number of calculated confirmed *Legionella* colonies

$$a = \frac{\text{fraction positive confirmed}}{\text{fraction total confirmed}} \times \text{total count}$$

V_c is the (concentrated) sample volume in millilitres, ml;

V is the sample volume inoculated per plate or set of plates (from the same culture medium) in millilitres, ml;

V_{tot} is the total tested sample volume in millilitres, ml;

V_s is the reference volume chosen to express the concentration of the microorganisms in the sample (normally 1 000 ml);

V_{dil} is the diluted sample volume inoculated per plate or set of plates (from the same culture medium) in millilitres, ml;

Df is the dilution factor.

The purpose of this document is to demonstrate the number of confirmed *Legionella* in a sample. Report the confirmed number of *Legionella* colony forming units in accordance with ISO 8199. Report absence as "not detected" in the volume examined and indicate the detection limit calculated according to the procedure used (see Table J.1).

10 Test report

The test report shall contain at least the following information:

a) the test method used (in conjunction with Annex J), together with a reference to this document, i.e. ISO 11731;

EXAMPLE Further specification, in conjunction with Annex J, can be done as follows: [Matrix A; Procedure 8, 9, 10; Medium A and Medium C — GVPC].

b) all details necessary for complete identification of the sample, such as the sample site with a unique identifier, so that, if necessary, a repeat sample can be taken from the exact location and sample point [e.g. building location (address and postcode), floor and room number, exact location of outlet (wash hand basin in room)], the sampling technique, the nature of the sample, the kind of water system or plant, the temperature and the biocide concentration;

c) the date and time of collection of the sample, the date of receipt of the sample, and start and end of the examination, any particular occurrence(s) observed during transport, storage and the course of analysis which might have influenced the result (e.g. particulates in the sample);

d) the maximum volume used in the analysis;

e) the results expressed in accordance with Clause 9.

11 Quality assurance

11.1 General

The laboratory shall have a clearly defined quality control system to ensure that the apparatus, reagents and techniques are suitable for the test. The use of positive controls, negative controls and blanks is part of the test.

11.2 Performance testing of *Legionella* culture media

For the definition of productivity, selectivity and specificity, refer to ISO 11133. The performance of the different *Legionella* culture media shall be tested according to the methods and criteria described in ISO 11133. Preparation of the working cultures and test suspensions is described in 11.3. For plates of BCYE agar (see B.1), only productivity can be tested. For plates of BCYE–cys agar (see B.2 or alternative media as described in the Note of 6.1.2), only test for its inability to support growth with one of the *L. pneumophila* strains is mentioned in Table 1.

11.3 Preparing working culture and test suspension for performance testing

Working cultures shall be prepared from a reference stock or stock culture of a *Legionella* strain. Reconstitute and recover as recommended, and subculture onto one or several plates of BCYE agar (see B.1) for purity. After incubation, make a suspension in sterile glycerol broth (see G.2.4) 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 (see C.1) or distilled water for storage at (–70 ± 10) °C or other appropriate freezing culture media and store at (–20 ± 5) °C or (–70 ± 10) °C as appropriate. Plate out one suspension of each isolate onto *Legionella* culture medium for subsequent identification and recording of the *Legionella* species. For use, allow a working culture of one (or more) isolates to thaw at room temperature. Shake thoroughly, wait 5 min to 10 min to allow aerosols to settle. If necessary, dilute to achieve a test suspension with the desired number of microorganisms in a specified volume in accordance with ISO 11133.

Table 1 — Control strains and performance criteria

Culture media	Function	Incubation	Control strains	WDCM numbers [a]	Reference media	Method of control	Criteria	Characteristic reactions
BCYE–cys agar, nutrient agar, blood agar, tryptone soy agar	Confirmation	2 d to 5 d/ (36 ± 2) °C	*Legionella pneumophila*	00107[b] 00180	BCYE agar	Qualitative	No growth	
GVPC agar, BCYE+AB agar, MWY agar	Productivity	2 d to 5 d/ (36 ± 2) °C	*Legionella pneumophila*	00107[b] 00180	BCYE agar	Quantitative	$P_R \geq 0,5$	White-grey-blue-purple colonies with an entire edge and exhibiting a characteristic ground-glass appearance
		5 d to 10 d/ (36 ± 2) °C	*Legionella anisa*	00106				
	Selectivity	3 d/ (36 ± 2) °C	*Enterococcus faecalis*[c]	00009 or 00087	—	Qualitative	Total inhibition	—
			Escherichia coli[c]	00012 or 00013	—	Qualitative	Total or partial inhibition (0 to 1)[d]	—
BCYE agar	Productivity	2 d to 5 d/ (36 ± 2) °C	*Legionella pneumophila*	00107[b]	Media batch BCYE agar already validated	Quantitative	$P_R \geq 0,7$	White-grey-blue-purple colonies with an entire edge and exhibiting a characteristic ground-glass appearance

[a] Make reference to the reference strain catalogue available on http://www.wfcc.info for information on culture collection strain numbers and contact details.

[b] Strains to be used as a minimum.

[c] At least one strain of *Enterocoocus faecalis* (WDCM 00009 or WDCM 00087) and at least one strain of *Escherichia coli* (WDCM 00012 or WDCM 00013) have to be used.

[d] "0" means "no growth" and "1" means "weak growth".

Annex A
(informative)

Legionella species

The genus *Legionella* belongs to the taxonomic order *Legionellales*, which includes the families *Coxiellaceae* and *Legionellaceae*. Three different genera have been proposed for the *Legionellaceae*: *Legionella*, *Fluoribacter*, and *Tatlockia*. However, the latter two generic names have never been widely used or accepted, and the single genus *Legionella* is almost universally used to describe all species.

Legionellae are small Gram-negative bacilli with fastidious growth requirements. Proteins rather than carbohydrates are used as an energy source. *Legionellae* are obligate aerobes and grow at temperatures ranging from 20 °C to 42 °C.

The amino acid L-cysteine is required for the (initial) growth of *Legionella* species from environmental and clinical sources. In addition, *L. oakridgensis* and *L. spiritensis* require L-cysteine and iron(III) for primary isolation but can grow weakly in the absence of added L-cysteine thereafter. Soluble iron(III) is required for optimal growth and for the initial isolation of the bacterium from both clinical and environmental sources. Iron(III), L-cysteine, α-ketoglutarate, and charcoal-containing yeast extract agar buffered with an organic buffer (BCYE agar) is the preferred growth culture medium for clinical isolation. Clinically important, *Legionella* species grow best at 35 °C in humidified air on plates of BCYE agar, usually in 2 d to 5 d after inoculation of plates. Of the *Legionella* species, *L. lytica* is not able to grow on BCYE agar, but it can be cultivated using co-cultivation with amoeba.

At least 61 different *Legionella* species have been described. In 26 of these species, some strains infecting humans have been reported. *L. pneumophila* contains at least 15 different serogroups; nine other species contain two different serogroups, with the remaining species containing only one serogroup each. *L. pneumophila* serogroup 1 caused the 1976 Philadelphia outbreak and is the cause of 70 % to 90 % of all cases of Legionnaires' disease for which there has been a bacterial isolate. Like *L. pneumophila*, other *Legionella* species are widely distributed in aquatic habitats and soil. Recovery of these species is generally less frequent and technically more demanding than recovery of *L. pneumophila*. 25 *Legionella* species other than *L. pneumophila* have been documented to cause human infection (see Table A.1) based on isolation from clinical material. Isolates of the other species are limited to water and soil, although several of them have been implicated in human infection based on seroconversion in the absence of isolation.

When grown on charcoal-yeast extract agar, *Legionella* species produce a water-soluble, extracellular compound that fluoresces yellow-green on exposure to long-wave ultraviolet light (360 ± 20) nm. Several species exhibit blue-white or red autofluorescence under long-wave ultraviolet light. Differentiation of the common species is most conveniently made in the laboratory by direct fluorescent antibody staining of the isolates. Identification of *L. pneumophila* serogroups, and other *Legionella* species, is often much more difficult and is best conducted by specialized reference laboratories. Determination by using molecular techniques is the definitive method, especially for the less common strains.

Table A.1 — *Legionella* species associated with disease

L. anisa	*L. erythra*	*L. longbeachae*	*L. pneumophila*
L. birminghamensis	*L. feeleii*	*L. lytica*	*L. sainthelensi*
L. bozemanii	*L. gormanii*	*L. maceachernii*	*L. steelei*
L. cardiaca	*L. hackeliae*	*L. micdadei*	*L. tusconensis*
L. cincinnatiensis	*L. jordanis*	*L. nagasakiensis*	*L. wadsworthii*
L. clemensonensis	*L. lansingensis*	*L. oakridgensis*	
L. dumoffii	*L. londiniensis*	*L. parisiensis*	
NOTE In addition, *L. waltersii* has been detected by polymerase chain reaction (PCR) from a clinical sample.			

Annex B
(normative)

Culture media

B.1 Buffered charcoal yeast extract (BCYE) agar

B.1.1 Composition

Yeast extract (bacteriological grade)		10,0 g
Agar		12,0 g
Activated charcoal		2,0 g
α-ketoglutarate, monopotassium salt	(CAS No. 58485–42–0)	1,0 g
ACES buffer (N-2-acetamido-2-aminoethanesulfonic acid)	(CAS No. 7365–82–4)	10,0 g
Potassium hydroxide (KOH) (pellets)	(CAS No. 1310–58–3)	2,8 g
L-cysteine hydrochloride monohydrate	(CAS No. 7048–04–6)	0,4 g
Iron(III) pyrophosphate [$Fe_4(P_2O_7)_3$]	(CAS No. 10058–44–3)	0,25 g
Water (see Clause 6)		to 1 000 ml

Check manufacturer's recommendations for concentration of agar to be added to provide adequate gelling strength. All the water used for the preparation of culture media and supplements shall comply with the requirements of ISO 3696, grade 3 (see Clause 6).

B.1.2 Preparation

a) Cysteine and iron(III) 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 water. Sterilize each solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. 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 water and dissolve by standing in a water bath at 45 °C to 50 °C. To a separate 480 ml of water, add all the potassium hydroxide pellets and dissolve with gentle shaking. To prepare the ACES buffer, mix the two solutions.

c) Final culture medium

Add sequentially to the 980 ml of ACES buffer the charcoal, yeast extract and α-ketoglutarate.

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

Prepare a 0,1 mol/l solution of potassium hydroxide (KOH) by dissolving 5,6 g in 1 l of water. Prepare a 0,1 mol/l solution of sulfuric acid (H_2SO_4) by carefully adding 5,5 ml of H_2SO_4 (95 % to 98 %) to 1 l of 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 6,8 ± 0,2. Add the agar, mix and autoclave at (121 ± 3) °C for (15 ± 1) min. After autoclaving, allow to cool to (48 ± 3) °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 culture 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 3 months.

B.2 Buffered charcoal yeast extract agar without L-cysteine (BCYE–cys)

Prepare this culture medium in an identical manner to BCYE agar (see B.1) but omit the L-cysteine.

Allow excess moisture on the plates to dry and store at (5 ± 3) °C in airtight containers in the dark for up to 3 months.

B.3 Selective culture medium: Buffered charcoal yeast extract agar with selective supplements (BCYE+AB)

B.3.1 Selective supplements

The final concentrations of the selective supplements in BCYE+AB agar shall be the following:

Polymyxin B sulfate	(CAS No. 1405–20–5)	80 000 IU/l
Sodium cefazolin	(CAS No. 27164–46–1)	0,009 g/l
Pimaricin (syn Natamycin)	(CAS No. 7681–93–8)	0,07 g/l

NOTE This culture medium is identical to BCYE agar (see B.1) except that three antibiotic supplements are added to the BCYE agar.

B.3.2 Preparation of antibiotic supplements

Add the appropriate amount of polymyxin B sulfate to 100 ml of water to achieve a concentration of 14 545 IU/ml. Sterilize the solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. Dispense in 5,5 ml volumes into sterile containers and store at (–20 ± 3) °C for not more than 3 months. For use, thaw at room temperature.

Add 180 mg of sodium cefazolin to 20 ml of water and sterilize the solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. Dispense in 1 ml volumes into sterile containers and store at (–20 ± 3) °C for not more than 3 months. For use, thaw at room temperature.

Add 1,75 g of pimaricin to 100 ml of water and sterilize the solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. Dispense in 4 ml volumes into sterile containers and store at (–20 ± 3) °C for not more than 3 months. For use, thaw at room temperature.

B.3.3 Preparation of BCYE+AB agar

After the addition of the L-cysteine and iron(III) pyrophosphate solutions, add one volume of each of the above three antibiotic supplements (see B.3.2) to the final culture medium. Mix well.

Dispense in 20 ml volumes into Petri dishes of 90 mm to 100 mm diameter. The pH of the final culture 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 3 months.

B.4 Highly selective culture medium: Glycine vancomycin polymyxin B cycloheximide (GVPC) agar

B.4.1 Selective supplements

The final concentrations of selective supplements in the GVPC agar shall be the following:

Ammonium-free glycine	(CAS No. 56–40–6)	3 g/l
Polymyxin B sulfate	(CAS No. 1405–20–5)	80 000 IU/l
Vancomycin hydrochloride	(CAS No. 1404–93–9)	0,001 g/l
Cycloheximide	(CAS No. 66–81–9)	0,08 g/l

NOTE This culture medium is identical to BCYE agar (see B.1) except that three antibiotic supplements and glycine are added to the BCYE agar.

B.4.2 Preparation of antibiotic supplements

Add the appropriate amount of polymyxin B sulfate to 100 ml of water to achieve a concentration of 14 545 IU/ml. Sterilize the solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. Dispense in 5,5 ml volumes into sterile containers and store at (–20 ± 3) °C for not more than 3 months. For use, thaw at room temperature.

Add 20 mg of vancomycin hydrochloride to 20 ml of water and sterilize the solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. Dispense in 1 ml volumes into sterile containers and store at (–20 ± 3) °C for not more than 3 months. For use, thaw at room temperature.

Add 2 g of cycloheximide to 100 ml of water and sterilize the solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. Dispense in 4 ml volumes into sterile containers and store at (–20 ± 3) °C for not more than 3 months. For use, thaw at room temperature.

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

B.4.3 Preparation of GVPC agar

Follow the instructions for preparation of BCYE agar given in B.1, 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(III) pyrophosphate solutions, add one volume of each of the above three antibiotic supplements (see B.4.2) to the final culture medium. Mix well.

Dispense in 20 ml volumes into Petri dishes of 90 mm to 100 mm diameter. The pH of the final culture 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.

B.5 Highly selective culture medium: Modified Wadowsky Yee (MWY) agar

B.5.1 Selective supplements

The final concentrations of selective supplements in the MWY agar shall be the following:

Ammonium-free glycine	(CAS No. 56–40–6)	3 g/l
Polymyxin B sulfate	(CAS No. 1405–20–5)	50 000 IU/l
Vancomycin hydrochloride	(CAS No. 1404–93–9)	0,001 g/l
Anisomycin	(CAS No. 22862–76–6)	0,08 g/l
Bromothymol blue	(CAS No. 76–59–5)	0,01 g/l
Bromocresol purple	(CAS No. 115–40–2)	0,01 g/l

NOTE This culture medium is identical to BCYE agar (see B.1) except that three antibiotic supplements, two indicators and glycine are added to the BCYE agar.

B.5.2 Preparation of antibiotic supplements

Add the appropriate amount of polymyxin B sulfate to 100 ml of water to achieve a concentration of 10 000 IU/ml. Sterilize the solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. Dispense in 5 ml volumes into sterile containers and store at (–20 ± 3) °C for not more than 3 months. For use, thaw at room temperature.

Add 20 mg of vancomycin hydrochloride to 20 ml of water and sterilize the solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. Dispense in 1 ml volumes into sterile containers and store at (–20 ± 3) °C for not more than 3 months. For use, thaw at room temperature.

Add 0,1 g of anisomycin to 10 ml of ethanol (see Clause 6) and sterilize the solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. Dispense in 0,8 ml into sterile containers. Use a fresh solution of anisomycin.

Add 100 mg of bromothymol blue to 100 ml of water and sterilize the solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. Dispense in 10 ml volumes into sterile containers and store at (5 ± 3) °C for a maximum of 1 year.

Add 100 mg of bromocresol purple to 100 ml of water and sterilize the solution by filtration through a sterile membrane filter with an average pore size of 0,2 µm or lower pore size rating. Dispense in 10 ml volumes in sterile containers and store at (5 ± 3) °C for a maximum of 1 year.

B.5.3 Preparation of MWY agar

Follow the instructions for preparation of BCYE agar given in B.1, 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(III) pyrophosphate solutions, add 5 ml of the polymyxin B sulfate, 1 ml of the vancomycin hydrochloride, 0,8 ml of the anisomycin solutions and 10 ml of both indicators (see B.5.2) to the final culture medium. Mix well.

Dispense in 20 ml volumes into Petri dishes of 90 mm to 100 mm diameter. The pH of the final culture 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.

B.6 Blood agar

B.6.1 Composition

Agar		15,0 g
Nutrient substrate (heart extract and peptones)		20,0 g
Sodium chloride	(CAS No. 7647–14–5)	5,0 g
Blood (e.g. horse or sheep blood)		50 ml
Water		to 1 000 ml

Check manufacturer's recommendations for concentration of agar to be added to provide adequate gelling strength. All the water used for the preparation of culture media and supplements shall comply with the requirements of ISO 3696, grade 3 (see Clause 6).

B.6.2 Preparation

Suspend the ingredients, with exception of the blood, in water and mix well. If necessary, adjust the pH so that, after autoclaving, it has a value corresponding to 6,8 ± 0,2 at 25 °C. Autoclave at (121 ± 3) °C for (15 ± 1) min. After autoclaving, allow to cool to (48 ± 3) °C in a water bath. Add 50 ml blood, mix well and pour into Petri dishes to a depth of at least 4 mm. If not for immediate use, the plates can be stored at (5 ± 3) °C in the dark and protected against evaporation for up to 4 weeks.

B.7 Nutrient agar

B.7.1 Composition

Agar		15,0 g
Meat extract		1,0 g
Enzymatic digest of peptone		5,0 g
Sodium chloride	(CAS No. 7647–14–5)	5,0 g
Water		to 1 000 ml

Check manufacturer's recommendations for concentration of agar to be added to provide adequate gelling strength. All the water used for the preparation of culture media and supplements shall comply with the requirements of ISO 3696, grade 3 (see Clause 6).

B.7.2 Preparation

Suspend the ingredients in water and mix well. If necessary, adjust the pH so that after autoclaving, it has a value corresponding to 6,8 ± 0,2 at 25 °C. Autoclave at (121 ± 3) °C for (15 ± 1) min. After autoclaving, allow to cool to (48 ± 3) °C and pour into Petri dishes to a depth of at least 4 mm. If not for immediate use, the plates can be stored at (5 ± 3) °C in the dark and protected against evaporation for at least 8 weeks.

B.8 Tryptone soy agar (TSA)

B.8.1 Composition

Enzymatic digest of casein		15,0 g
Enzymatic digest of soy or soybean meal		5,0 g
Sodium chloride	(CAS No. 7647–14–5)	5,0 g
Agar		15,0 g
Water		to 1 000 ml

Check manufacturer's recommendations for concentration of agar to be added to provide adequate gelling strength. All the water used for the preparation of culture media and supplements shall comply with the requirements of ISO 3696, grade 3 (Clause 6).

B.8.2 Preparation

Suspend the ingredients in water and mix well. If necessary, adjust the pH so that after autoclaving, it has a value corresponding to 6,8 ± 0,2 at 25 °C. Autoclave at (121 ± 3) °C for (15 ± 1) min. After autoclaving, allow to cool to (48 ± 3) °C and pour into Petri dishes to a depth of at least 4 mm. If not for immediate use, the plates can be stored at (5 ± 3) °C in the dark and protected against evaporation for at least 8 weeks.

Annex C
(normative)

Diluents

C.1 Page's saline

C.1.1 Composition

Sodium chloride (NaCl)	(CAS No. 7647–14–5)	0,120 g
Magnesium sulfate (-heptahydrate) [$MgSO_4 \cdot 7H_2O$]	(CAS No. 10034–99–8)	0,004 g
Calcium chloride (-dihydrate) [$CaCl_2 \cdot 2H_2O$]	(CAS No. 233–140–8)	0,004 g
Disodium hydrogen phosphate [Na_2HPO_4]	(CAS No. 7558–79–4)	0,142 g
Potassium dihydrogen phosphate [KH_2PO_4]	(CAS No. 7778–77–0)	0,136 g
Water (see Clause 6)		1 000 ml

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

NOTE To aid accurate preparation, a 10 l volume of Page's saline can be prepared and dispensed in smaller volumes as required for autoclaving at (121 ± 3) °C for (20 ± 1) min.

C.2 Diluted Ringer's solution

Use a 1 in 10 dilution of ¼ strength Ringer's solution as described in ISO 8199.

C.3 Phosphate-buffered saline (PBS) [pH 7,5]

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

C.4 Sterile tap water

Use sterile tap water that has shown not to have an effect on the recovery of *Legionella*.

Annex D
(normative)

Acid solution

Prepare a 0,2 mol/l solution of hydrochloric acid (HCl) by adding 17,4 ml concentrated HCI (ρ = 1,184, minimum assay 37 %) or 20 ml concentrated HCI (ρ = 1,16, minimum assay 31,5 %) to 1 l of water (solution A).

Prepare a 0,2 mol/l solution of potassium chloride (KCl) by dissolving 14,9 g of KCl in 1 l of water (solution B).

To prepare the acid solution, 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 suitable glass container in the dark at room temperature for not more than 1 month.

Annex E
(informative)

Scraping or rubbing the bacteria from membrane filters

A scraping method to remove *Legionella* from the membrane filters can also be used.

The membrane filter is removed from the stand with disinfected 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. Record the used volume. The membrane is then scraped a few times with a sterile cell scraper (commercially available).

Annex F
(informative)

Centrifugation technique

If there is so much deposit in the sample that concentration by filtration is not possible, the centrifugation technique can be used. Recovery rates for this method are usually much lower than in the other techniques described in this document. Recovery rates are the best with swing out rotors as the deposit is restricted to the bottom of the tube, making it easier to remove the supernatant without disturbing the deposit. Recovery with angle rotors is erratic and lower than in the other techniques in this document.

After shaking to re-suspend any deposit that possibly has 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 6 000 g for 10 min or 3 000 g for 30 min, maintaining the temperature at 15 °C to 25 °C. Remove the sealed centrifuge head with the bottles contained inside to a safety cabinet before opening the rotor. This avoids exposure to aerosols if a bottle has broken during centrifugation. Remove the supernatant aseptically and discard. Re-suspend the deposit in 2 ml to 10 ml of the diluent (see Annex C). Ensure that the volume of diluent added is recorded. This concentrate represents the prepared sample. It is recommended that the supernatant is removed by vacuum, rather than decanting, to avoid disturbance and consequent loss of the deposit. If necessary, less sample volume may be used with adjustment to volume of diluent for re-suspension.

Annex G
(informative)

Indirect immunofluorescent antibody assay for the identification of *Legionella* species

G.1 General

Legionella species can 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, enzyme-linked immunosorbent assay procedures with appropriate diagnostic reagents, matrix assisted laser desorption/ionization time of flight (MALDI-TOF), polymerase chain reaction (PCR) and DNA sequencing. In this annex, the immunofluorescence method is described as an example of serological method, which can be used to identify *Legionella* species.

G.2 (Serological) reagents

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

NOTE Serological cross reactions with other microorganisms in environmental samples can sometimes occur.

G.2.1 Antisera to *L. pneumophila* and other *Legionella* species

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

G.2.2 Fluorescein isothiocyanate anti-rabbit conjugate (FITC)

Use FITC raised against rabbit serum proteins that are available commercially. Different conjugates are required for use with antisera raised in other animals.

G.2.3 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).

G.2.4 Glycerol broth

Dissolve 5 g of a commercially available dehydrated nutrient broth in 170 ml of water (see Clause 6) 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 ± 3) °C for (20 ± 1) min. Store at room temperature until required.

G.2.5 Formol saline

Prepare by adding 20 ml of a volume fraction of 37 % aqueous solution of formaldehyde to 980 ml of phosphate-buffered saline (see C.3).

G.3 Preparation of antigens

Using pure growth of the *Legionella* isolate onto plates of BCYE agar (see 8.5), prepare a test antigen (sample) by emulsifying several colonies in formol saline (see G.2.5).

For some reagents, the use of formol saline may not be appropriate, in which case, the colonies can be emulsified in water (see Clause 6) and the suspension inactivated at 60 °C for (60 ± 5) min. It is essential to verify which method of preparation is applicable.

Dilute the test antigen in phosphate-buffered saline (PBS) (see C.3) to give a suspension, which is faintly turbid when viewed with the naked eye.

This corresponds to approximately 1×10^5 cells/ml to 1×10^6 cells/ml and to an optical density of 0,1 using a spectro-photometer at a wavelength of 395 nm with a cuvette of 10 mm optical path length or McFarland nephelometer standard 0,5 diluted 1:100.

Prepare a *Legionella* control antigen in the same way from a reference strain obtained from a recognized culture collection (e.g. National Collection of Type Cultures) cultured on BCYE agar. Store the reference strains frozen as described in ISO 11133.

G.4 Preparation of slides

Use polytetrafluoroethylene-coated slides with multiple wells of diameter 3 mm. Add 5 µl of each antigen (see G.3) to separate wells and allow to dry. Fix either by gentle heating in a Bunsen flame or by immersion in acetone for 10 min.

G.5 Preparation of antiserum (antibody)

Dilute the *Legionella* antiserum (see G.2.1) to the recommended working titre according to the supplier's instructions. Add sufficient diluted antiserum to cover each well containing antigen. Place the slides in a plastic or glass box containing damp tissue or blotting paper to prevent drying.

Cover the box with a lid and incubate at (36 ± 2) °C for (30 ± 1) min.

NOTE Replicates of the same test antigen (sample) can be examined on one slide, but to avoid risk of cross-contamination by reagents or samples, it is advisable to use separate slides for different test antigens (samples).

G.6 Rinsing of slides

Immerse the slides in phosphate-buffered saline (see C.3) in a rotary mixer and wash gently for (5 ± 1) min; replace with fresh phosphate-buffered saline and wash again for (5 ± 1) min; repeat wash once more for (5 ± 1) min.

G.7 Application of conjugate

Dilute the fluorescein isothiocyanate anti-rabbit conjugate (FITC) (see G.2.2) to the working titre, following the supplier's instructions. Add 5 µl of the diluted FITC to each well and incubate in the moist box at (36 ± 2) °C for (30 ± 1) min. Rinse the slides as described in G.6.

G.8 Mounting the slides

Add to each slide sufficient (usually two to three drops) glycerol-mounting medium (see G.2.3) to spread under a clean cover slip big enough to cover all the wells. Ensure that no air bubbles are trapped between the slide and the cover slip.

G.9 Examination of slides

Examine the slides using an epifluorescent microscope. Regard cells which fluoresce bright green with the species-specific antiserum as confirmed *Legionella* species. Repeat the test with fresh reagents if no fluorescence occurs with the control antigen.

If control antigens do not fluoresce, check the working strength of the antisera by preparing two-fold serial dilutions and titrating these against the control antigen(s).

If cross-reactions or inconclusive results occur, isolates can be sent to a reference laboratory for further examination. The method of packaging for transport should conform to relevant safety regulations.

Annex H
(informative)

Performance data

Several trials were undertaken to determine the performance characteristics of this procedure (see Clause 8). Results from an intralaboratory trial and three interlaboratory studies are summarized in Table H.1 and Table H.2. In the intralaboratory trial, which took place at Vitens Laboratory in Leeuwarden (the Netherlands), the performance parameters such as range of quantitative determination, robustness of incubation time, counting uncertainty, precision, identification (categorical performance characteristics) and recovery have been determined. The data are summarized in Table H.1.

The samples used for the determination of the performance characteristics are mainly potable water and water derived from cooling towers. Data for the determination of the performance characteristics were collected in accordance with ISO 13843[1]. The identification procedures show a high sensitivity, specificity, selectivity and efficiency, with low false-positive and false-negative rates. These performance characteristics were derived in a laboratory, which has a high level of experience in determining *Legionella* species in water samples. It may be necessary for laboratories to carry out their own secondary validation to determine their own performance characteristics.

The robustness of the incubation time was assessed using 147 counts obtained, after 7 d and 10 d of incubation, from three different laboratories. As the robustness determination is based on data from the interlaboratory trial, they may not be representative for non-spiked natural samples.

Three different *Legionella* species (*L. anisa*, *L. gormanii*, and *L. pneumophila*) and four different culture media were used for the calculation of the recovery. BCYE agar was used as the non-selective culture medium and BCYE+AB agar, GVPC agar and MWY agar were used as the selective culture media. The lowest average recovery was found for *L. gormanii* on GVPC agar (64 %).

In March 2015, an interlaboratory study with 27 laboratories from 10 countries was performed (Austria, Cyprus, Finland, Germany, Italy, Netherlands, Spain, Switzerland, United Kingdom, United States) to investigate the precision of the method. Samples used in the interlaboratory trial were spiked with different *Legionella* species and different concentrations of interfering microorganisms. Samples were analysed using direct plating, membrane filtration with direct placing of the membrane filter on the culture media, membrane filtration followed by a washing procedure and plating after dilution of the sample. The different pre-treatments of the sample were also carried out where applicable. Culture media were provided to all participants, therefore the participants used the same batches of culture media.

Due to a change in the acid treatment used as a pre-treatment step for the analyses for *Legionella*, a second interlaboratory study was carried out in June 2015. In this interlaboratory study, 8 laboratories from 3 countries participated (Austria, Germany, the Netherlands). Samples used in the interlaboratory trial were spiked with different *Legionella* species and different concentrations of interfering microorganisms. Samples were analysed using membrane filtration with direct placing of the membrane filter on the culture media, membrane filtration followed by a washing procedure and plating after dilution of the sample. The different pre-treatments of the sample were also carried out where applicable.

The repeatability of the second interlaboratory study varied in a range of 18,0 % to 29,5 % compared to a range of 30,1 % to 35,3 % obtained in the first study. The performance of the reproducibility was better in the second interlaboratory study. The reproducibility in the second study varied from 46,0 % to 54,0 % compared to 56,4 % to 78,5 % in the first interlaboratory study. This may be due to the lower number of participants and shorter sample transport distances in the second study.

A third interlaboratory study was necessary to calculate the performance characteristics of the method using membrane filtration with direct placing of the membrane filter on the culture media. In this interlaboratory study that was carried out in June 2016, 10 laboratories from 4 countries participated

(Austria, Germany, Netherlands, United Kingdom). The two samples used in this interlaboratory trial were spiked with different *Legionella* species. Samples were analysed using membrane filtration with direct placing of the membrane filter on the culture media. The pre-treatment of the sample with acid solution was also carried out where applicable.

In the third interlaboratory study, of the sample spiked with *L. pneumophila*, the repeatability was 51,1 % and the reproducibility was 65,9 %. The other sample containing *L. anisa* did not show growth on the membrane filter at seven laboratories. There was growth observed at three laboratories with a maximum concentration of 900 cfu/l. One of the laboratories also carried out the direct plating method for this sample. The concentration found with the direct plating method was 36 000 cfu/l. This shows that, in this case, the membrane filter strongly influenced the growth of *L. anisa* on the membrane filter.

The data are summarized in Table H.1 and Table H.2.

Table H.1 — Parameters for performance characteristics of the intralaboratory trial

Intralaboratory trial		
Range of quantitative determination	— Single strain without interfering microorganisms between 10 cfu/plate and 300 cfu/plate. — Counts including interfering microorganisms 10 cfu/plate to 150 cfu/plate. — For the membrane filter technique with direct placing of the membrane filter on the plate 10 cfu/plate to 80 cfu/filter. — Always take into account that the growth of other microorganisms on *Legionella* culture media can strongly influence the growth of *Legionella* and that the range of quantitative determination has to be adapted to the specific situation.	
Robustness of incubation time[a]	No significant difference in counts between 7 d and 10 d of incubation (n = 147).	
Robustness of the method using direct membrane filtration technique	Membrane filters, used for the method of placing the membrane filter directly onto the culture media, have a significant influence on the recovery of *Legionella*.	
Counting uncertainty [relative standard deviation (RSD)]		
Repeatability (within laboratory)	4,10 %	
Reproducibility (within laboratory)	5,40 %	
Precision (RSD) — direct plating		
Repeatability (within laboratory)	4,15 %	
Reproducibility (within laboratory)	8,23 %	
Precision (RSD) — membrane filter on plate		
Repeatability (within laboratory)	5,09 %	
Reproducibility (within laboratory)	11,86 %	
Precision (RSD) — filtration with washing procedure		
Repeatability (within laboratory)	6,04 %	
Reproducibility (within laboratory)	[b]	
Identification (categorical performance characteristics) (n = 1 067)[c]	*Confirmation by subculturing on BCYE agar and BCYE–cys agar*	*Confirmation by PCR*
Sensitivity	99,0 %	98,6 %
Specificity	95,3 %	97,5 %
False-positive rate	3,3 %	1,8 %
False-negative rate	1,4 %	2,1 %
Selectivity	57,2 %	58,1 %
Efficiency	97,5 %	98,1 %
Recovery	> 64 %	

[a] From interlaboratory trial.

[b] The average relative operational standard deviation in intralaboratory reproducibility conditions observed during the characterization process was not significantly different from zero (ideal case).

[c] The performance characteristics of the identification were also calculated based on the confirmation by PCR. Besides the confirmation of suspicious colonies by subculturing on BCYE agar and BCYE–cys agar, these colonies were also confirmed by an ISO/IEC 17025[2] accredited in-house PCR method. The small differences of the identification were caused by target and non-target colonies that were overgrowing each other.

Table H.2 — Parameters for performance characteristics of the interlaboratory trial

Interlaboratory trial	
Precision (RSD) — direct plating, low concentration of interfering microorganisms (see 8.4.2) (*n* = 24)	
Repeatability	35,3 %
Reproducibility	56,4 %
Precision (RSD) — membrane filter on plate, low concentration of interfering microorganisms (see 8.4.3.1) (*n* = 10)	
Repeatability	51,1 %
Reproducibility	65,9 %
Precision (RSD) — filtration with washing procedure, low concentration of interfering microorganisms (see 8.4.3.2) (*n* = 14)	
Repeatability	22,5 %
Reproducibility	49,5 %
Precision (RSD) — direct plating and plating after dilution, high concentration of interfering microorganisms (see 8.4.4) (*n* = 16)	
Repeatability	29,5 %
Reproducibility	54,0 %
Precision (RSD) — filtration with washing procedure, high concentration of interfering microorganisms (see 8.4.4) (*n* = 74)	
Repeatability	30,1 %
Reproducibility	78,5 %
Precision (RSD) — direct plating and plating after dilution, extremely high concentration of interfering microorganisms (see 8.4.5) (*n* = 8)	
Repeatability	18,0 %
Reproducibility	46,0 %

Annex I
(informative)

Pre-treatment of water related matrices

Water related matrices include, for example, swabs, sediments, biofilms, filter material from water treatment filters (sand, granular activated carbon, etc.).

Pre-treatment of the water related sample depends on the type of sample. In this annex, examples are described of commonly used water related matrices.

EXAMPLE 1 Swab: For a semiquantitative analysis, swab a certain area (e.g. approximately 10 cm × 10 cm = 100 cm^2). The swab will be transferred into a tube with an appropriate diluent. Be sure that the cotton of the swab is completely immersed in the diluent. Mix thoroughly with the help of a vortex mixer or by means of an ultrasonic water bath for 2 min. The suspension is then further used in the procedure for water related matrices. Results can be expressed as number per 100 cm^2. If the area swabbed is not known, e.g. in case of irregular surface, record the number per swab.

EXAMPLE 2 Sediments or other solid material: Aseptically transfer from 0,1 g to 10,0 g of material into a tube with a volume of the appropriate diluent in millilitres nine times the value number of the weight in grams (e.g. from 0,9 ml to 90,0 ml). Mix thoroughly with the help of a vortex mixer or by means of an ultrasonic water bath for 2 min. The suspension is then further used in the procedure for water related matrices.

Annex J
(normative)

Decision matrix

The choice of the method used for the enumeration of *Legionella* species depends on the origin/characteristics of the sample and the reason of sampling or investigation. Once the desired lower limit of detection level for a sample is known, this will play an important role in the selection of the most appropriate method.

The decision matrix (see Figure J.1) summarizes all possible types of water (samples), methods, treatments and culture media. Clause 8 and the text of this annex give more background information. The following steps shall be followed (Table J.3 shows examples).

The **first step** is to determine the origin and characteristics of the sample. In most cases, this information can be derived from the customer. Choose at least one of the different possibilities which are the following:

— Water with an expected low concentration of interfering microorganisms, e.g. potable water:

  — **and** an expected high concentration of *Legionella* species → the sample shall be analysed by the procedure described in 8.4.2;

  — **and** an expected low concentration of *Legionella* species → the sample shall be analysed by one of the procedures described in 8.4.3.

— Water with an expected high concentration of interfering microorganisms, e.g. evaporative cooling tower, process water, water from air washers chambers, water from dental units → the sample shall be analysed by the procedure described in 8.4.4.

— Water with an expected extremely high concentration of interfering microorganisms or microorganisms that only will be removed from the samples with a combined heat/acid treatment, e.g. waste water, surface water → the sample shall be analysed by the procedure described in 8.4.5.

The **second step** is to determine the desired lower limit of detection level and to select one or more method(s). Examples of the lower limit of detection level per method are shown in Table J.1. Be aware that different methods have advantages and disadvantages (see Table J.2).

The **third step** is the selection of the required treatment(s). Besides the required treatment(s), optional treatment(s) can be carried out.

The **fourth step** is the selection of the required culture media. Besides the required culture media, optional culture media can be used.

Table J.1 — Lower limit of detection level per method

Direct plating (see 8.4.2)	**Membrane filter on plate (see 8.4.3.1)**
$C_s = \frac{a}{V_{tot}} \times V_s$ where C_s is the number of *Legionella* in cfu/l; a is the number of calculated confirmed *Legionella* colonies $a = \frac{\text{fraction positive confirmed}}{\text{fraction total confirmed}} \times \text{total count};$ V_{tot} is the total tested sample volume in millilitres, ml; V_s is the reference volume chosen to express the concentration of the microorganisms in the sample (normally 1 000 ml). Example detection limit: in case of 0,1 ml sample inoculation, the detection limit is 10 000 cfu/l.	$C_s = \frac{a}{V_{tot}} \times V_s$ where C_s is the number of *Legionella* in cfu/l; a is the number of calculated confirmed *Legionella* colonies $a = \frac{\text{fraction positive confirmed}}{\text{fraction total confirmed}} \times \text{total count};$ V_{tot} is the total tested sample volume in millilitres, ml; V_s is the reference volume chosen to express the concentration of the microorganisms in the sample (normally 1 000 ml). Example detection limit: in case of 10 ml filtered sample, the detection limit is 100 cfu/l.
Filtration with washing procedure (see 8.4.3.2)	**Plating after dilution (see 8.4.4 and 8.4.5)**
$C_s = \frac{a \times V_c}{V \times V_{tot}} \times V_s$ where C_s is the number of *Legionella* in cfu/l; a is the number of calculated confirmed *Legionella* colonies $a = \frac{\text{fraction positive confirmed}}{\text{fraction total confirmed}} \times \text{total count};$ V_c is the (concentrated) sample volume in millilitres, ml; V is the sample volume inoculated per plate or set of plates (from the same culture medium) in millilitres, ml; V_{tot} is the total tested sample volume in millilitres, ml; V_s is the reference volume chosen to express the concentration of the microorganisms in the sample (normally 1 000 ml). Example detection limit: in case of 500 ml filtered sample washed in 5 ml water and 0,1 ml inoculation, the detection limit is 100 cfu/l. If there is overgrowth on the “heat treated” and “untreated” plates and no detectable *Legionella* on the “acid treated” plate, the example detection limit is increased by a factor of 10.	$C_s = \frac{a \times V_s}{V_{dil}} \times Df$ where C_s is the number of *Legionella* in cfu/l; a is the number of calculated confirmed *Legionella* colonies $a = \frac{\text{fraction positive confirmed}}{\text{fraction total confirmed}} \times \text{total count};$ V_s is the reference volume chosen to express the concentration of the microorganisms in the sample (normally 1 000 ml); V_{dil} is the diluted sample volume inoculated per plate or set of plates (from the same culture medium) in millilitres, ml; Df is the dilution factor. Example detection limit: 1 ml heated sample treated with 9 ml acid. In case of 10 times dilution and 0,1 ml treated sample inoculation, the detection limit is 1 000 000 cfu/l. If there is overgrowth on the “heat treated” and “untreated” plates and no detectable *Legionella* on the “acid treated” plate, the example detection limit is increased by a factor of 10.

Table J.2 — Advantages and disadvantages of the different methods

Method	Advantages	Disadvantages
Direct plating	Easy to count Good recovery	High limit of detection
Membrane filter on plate	Easy method Low limit of detection	Difficult to count (due to overgrowth of interfering microorganisms) Influence of the membrane filter
Filtration with washing procedure	Easy to count Low limit of detection	Lower recovery (compared with direct plating method and membrane filter on plate method) Time-consuming
Plating after dilution	Easy to count Good recovery	High limit of detection Time-consuming

			Step 1								
						Water or water derived from water related matrices e.g. swabs, biofilm, sediments					
			Matrix A			**Matrix B**			**Matrix C**		
			Water with low background (see 8.4.2 and 8.4.3) e.g. potable water			**Water with high background** (see 8.4.4) e.g. cooling tower, process water, water from air washers chambers, water from dental units			**Water with extremely high background**[a] (see 8.4.5) e.g. waste water, surface water		
			Step 4								
			Culture media								
Step 2	***Step 3***	**Procedure**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
Direct plating	Without treatment	*1*	R	R	O		O	R			
	Heat treatment	*2*	O	O	O		O	R			
	Acid treatment	*3*	O	O	O		O	R			
	Combination of heat/acid treatment	*4*					O	O		O	R
Membrane filter on plate	Without treatment	*5*	R	O	O						
	Heat treatment	*6*	O	O	O		O	O			
	Acid treatment	*7*	O	R[b]			O	O			
Filtration with washing procedure	Without treatment	*8*	R	R[b]			O	R			
	Heat treatment	*9*	R	R[b]			O	R			
	Acid treatment	*10*	R	R[b]			O	R			
Plating after dilution	Without treatment	*11*	O[c]	O[c]	O[c]		O[c]	R[c]			
	Heat treatment	*12*	O[c]	O[c]	O[c]		O[c]	R[c]			
	Acid treatment	*13*	O[c]	O[c]	O[c]		O[c]	R[c]			
	Combination of heat/acid treatment	*14*					O[c]	O[c]		O[d]	R[d]

Culture media

A: BCYE agar (see B.1).

B: Selective BCYE agar [BCYE+AB agar (see B.3)].

C: Highly selective culture media [GVPC agar or MWY agar (see B.4 or B.5)].

Key

R required

O optional

[a] For this type of water, both methods (direct plating and plating after dilution) are required.

[b] Choice of culture media B or C.

[c] With dilution 1:10.

[d] With dilution 1:10 and 1:100.

NOTE 1 For the different matrices above, some examples are described (e.g. potable water). It is possible, based on the experience of the laboratory, that the examples can be covered by another matrix using one or more pre-treatment methods.

NOTE 2 For the different matrices, the shorter expression is used above: "water with low background" (= an expected low concentration of interfering microorganisms), "water with high background" (= an expected high concentration of interfering microorganisms), and "water with extremely high background" (= an expected extremely high concentration of interfering microorganisms).

NOTE 3 The cells in "grey" can be used for a more detailed way of reporting: *Reference to this document (ISO 11731) [Matrix A; Procedure 1; Media A and B].*

Figure J.1 — Decision matrix

Table J.3 — Four examples for illustration of the decision matrix

Example 1	
Step 1: Determine the origin and expected characteristics of the sample and choose one of the water types from the two top rows in the decision matrix.	**Sample 1**: Water with a low concentration of interfering microorganisms and a high concentration of *Legionella* species
Step 2: Choose one of the methods from the first column, based on the desired lower limit of detection and on what is shown as required in the decision matrix.	**Method**: Direct plating
Step 3: Select the treatment(s), based on what is shown as required in the decision matrix.	**Treatment**: Without treatment **Optional treatment** (additional, if desired): Heat treatment and/or acid treatment
Step 4: Select the culture media, based on what is shown as required in the decision matrix.	**Required media**: BCYE agar and BCYE+AB agar **Optional medium** (additional, if desired): GVPC agar or MWY agar
Detailed reporting in case of Example 1	*Reference to this document (ISO 11731)* *[Matrix A; Procedure 1; Media A and B]*
Example 2	
Step 1: Determine the origin and expected characteristics of the sample and choose one of the water types from the two top rows in the decision matrix.	**Sample 2**: Water with a low concentration of interfering microorganisms and a low concentration of *Legionella* species
Step 2: Choose one of the methods from the first column, based on the desired lower limit of detection and on what is shown as required in the decision matrix.	**Method**: Membrane filter on plate
Step 3: Select the treatment(s), based on what is shown as required in the decision matrix.	**Treatment**: Without treatment and acid treatment
Step 4: Select the culture media, based on what is shown as required in the decision matrix.	**Required media**: BCYE agar for the sample without treatment and a choice between either "BCYE+AB agar" or "GVPC agar or MWY agar" for the acid-treated filtered sample
Detailed reporting in case of Example 2	*Reference to this document (ISO 11731)* *[Matrix A; Procedure 5 (Medium A) and Procedure 7 (Medium C — MWY)]*
Example 3	
Step 1: Determine the origin and expected characteristics of the sample and choose one of the water types from the two top rows in the decision matrix.	**Sample 3**: Water with a high concentration of interfering microorganisms
Step 2: Choose one of the methods from the first column, based on the desired lower limit of detection and on what is shown as required in the decision matrix.	**Method**: Filtration with washing procedure
Step 3: Select the treatment(s), based on what is shown as required in the decision matrix.	**Treatment**: Without treatment, heat treatment and acid treatment
Step 4: Select the culture medium, based on what is shown as required in the decision matrix.	**Required medium**: GVPC agar or MWY agar **Optional medium** (additional, if desired): BCYE+AB agar
Detailed reporting in case of Example 3	*Reference to this document (ISO 11731)* *[Matrix B; Procedure 8, 9 and 10; Medium C — GVPC]*

Table J.3 *(continued)*

Example 4	
Step 1: Determine the origin and expected characteristics of the sample and choose one of the water types from the two top rows in the decision matrix.	**Sample 4**: Water with an extremely high concentration of interfering microorganisms
Step 2: Choose the two required methods from the first column, based on the desired lower limit of detection and on what is shown as required in the decision matrix.	**Method**: Direct plating and plating after dilution
Step 3: Select the treatment(s), based on what is shown as required in the decision matrix.	**Treatment**: Combination of heat and acid treatment
Step 4: Select the culture medium, based on what is shown as required in the decision matrix.	**Required medium**: GVPC agar or MWY agar **Optional medium** (additional, if desired): BCYE+AB agar
Detailed reporting in case of Example 4	*Reference to this document (ISO 11731)* *[Matrix C; Procedures 4 and 14; Medium C — MWY]*

Bibliography

[1] ISO 13843, *Water quality — Requirements for establishing performance characteristics of quantitative microbiological methods*

[2] ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

[3] Bopp C.A. et al. Isolation of *Legionella* spp. from environmental water samples by low-pH treatment and use of a selective medium. *J. Clin. Microbiol.* April 1981, **13** (4), pp. 714–719

[4] Dennis P.J. et al. *Comparison of isolation methods for Legionella spp. Legionella, Proceedings of the 2nd International Symposium*. American Society for Microbiology, 1984. pp. 294–296

[5] Ditommaso S. Recovery of *Legionella* species from water samples using an internal method based on ISO 11731: suggestions for revision and implementation. *Diagn. Microbiol. Infect. Dis.* 2011, **70** pp. 200–206

[6] Smith L. Comparison of membrane filters for recovery of legionellae from water samples. *Appl. Environ. Microbiol.* January 1993, **59** (1), pp. 344–346

[7] Wadowsky R.M., & Yee R.B. Glycine-containing selective medium for isolation of Legionellaceae from environmental specimens. *Appl. Environ. Microbiol.* November 1981, **42** (5), pp. 768–772

This page deliberately left blank

British Standards Institution (BSI)

BSI is the national body responsible for preparing British Standards and other standards-related publications, information and services.

BSI is incorporated by Royal Charter. British Standards and other standardization products are published by BSI Standards Limited.

About us

We bring together business, industry, government, consumers, innovators and others to shape their combined experience and expertise into standards -based solutions.

The knowledge embodied in our standards has been carefully assembled in a dependable format and refined through our open consultation process. Organizations of all sizes and across all sectors choose standards to help them achieve their goals.

Information on standards

We can provide you with the knowledge that your organization needs to succeed. Find out more about British Standards by visiting our website at bsigroup.com/standards or contacting our Customer Services team or Knowledge Centre.

Buying standards

You can buy and download PDF versions of BSI publications, including British and adopted European and international standards, through our website at bsigroup.com/shop, where hard copies can also be purchased.

If you need international and foreign standards from other Standards Development Organizations, hard copies can be ordered from our Customer Services team.

Copyright in BSI publications

All the content in BSI publications, including British Standards, is the property of and copyrighted by BSI or some person or entity that owns copyright in the information used (such as the international standardization bodies) and has formally licensed such information to BSI for commercial publication and use.

Save for the provisions below, you may not transfer, share or disseminate any portion of the standard to any other person. You may not adapt, distribute, commercially exploit, or publicly display the standard or any portion thereof in any manner whatsoever without BSI's prior written consent.

Storing and using standards

Standards purchased in soft copy format:

- A British Standard purchased in soft copy format is licensed to a sole named user for personal or internal company use only.
- The standard may be stored on more than 1 device provided that it is accessible by the sole named user only and that only 1 copy is accessed at any one time.
- A single paper copy may be printed for personal or internal company use only.

Standards purchased in hard copy format:

- A British Standard purchased in hard copy format is for personal or internal company use only.
- It may not be further reproduced – in any format – to create an additional copy. This includes scanning of the document.

If you need more than 1 copy of the document, or if you wish to share the document on an internal network, you can save money by choosing a subscription product (see 'Subscriptions').

Reproducing extracts

For permission to reproduce content from BSI publications contact the BSI Copyright & Licensing team.

Subscriptions

Our range of subscription services are designed to make using standards easier for you. For further information on our subscription products go to bsigroup.com/subscriptions.

With **British Standards Online (BSOL)** you'll have instant access to over 55,000 British and adopted European and international standards from your desktop. It's available 24/7 and is refreshed daily so you'll always be up to date.

You can keep in touch with standards developments and receive substantial discounts on the purchase price of standards, both in single copy and subscription format, by becoming a **BSI Subscribing Member**.

PLUS is an updating service exclusive to BSI Subscribing Members. You will automatically receive the latest hard copy of your standards when they're revised or replaced.

To find out more about becoming a BSI Subscribing Member and the benefits of membership, please visit bsigroup.com/shop.

With a **Multi-User Network Licence (MUNL)** you are able to host standards publications on your intranet. Licences can cover as few or as many users as you wish. With updates supplied as soon as they're available, you can be sure your documentation is current. For further information, email subscriptions@bsigroup.com.

Revisions

Our British Standards and other publications are updated by amendment or revision.

We continually improve the quality of our products and services to benefit your business. If you find an inaccuracy or ambiguity within a British Standard or other BSI publication please inform the Knowledge Centre.

Useful Contacts

Customer Services
Tel: +44 345 086 9001
Email (orders): orders@bsigroup.com
Email (enquiries): cservices@bsigroup.com

Subscriptions
Tel: +44 345 086 9001
Email: subscriptions@bsigroup.com

Knowledge Centre
Tel: +44 20 8996 7004
Email: knowledgecentre@bsigroup.com

Copyright & Licensing
Tel: +44 20 8996 7070
Email: copyright@bsigroup.com

BSI Group Headquarters

389 Chiswick High Road London W4 4AL UK

bsi.