SLOVENSKI STANDARD
SIST ISO 11731:1999

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Kakovost vode - Ugotavljanje prisotnosti in števila legionel

Water quality -- Detection and enumeration of Legionella

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

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Water quality — Detection and enumeration of *Legionella*

Qualité de l'eau — Recherche et dénombrement des Legionella

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Foreword

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

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

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

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

Water quality — Detection and enumeration of *Legionella*

1 Scope

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

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

2 Normative reference

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

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

3 Definition

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

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

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

4 Safety

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

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

5 Principle

5.1 General

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

5.2 Enumeration

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

5.3 Confirmation

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

6 Culture media and reagents

6.1 General

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

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

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

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

6.2 Culture media

6.2.1 Buffered Charcoal Yeast Extract agar medium (BCYE)

6.2.1.1 Composition

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

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

6.2.1.2 Preparation

a) Cysteine and iron solutions.

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

b) ACES buffer.

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

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

c) Final medium.

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

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

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

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

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

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

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

6.2.3.1 Selective supplements

The final concentrations in the GVPC medium shall be:

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

6.2.3.2 Preparation of antibiotic supplements

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

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

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

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

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

6.2.3.3 Preparation of GVPC medium

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

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

6.2.4 Quality control of media

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

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

Either

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

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

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

6.3 Reagents

6.3.1 Acid buffer

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

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

6.3.2 Diluents

6.3.2.1 Page's Saline

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

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

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

6.3.2.2 Dilute Ringer's solution.

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

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

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

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

6.3.2.4 Formol saline.

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

6.3.3 Serological reagents

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

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

6.3.3.2 Fluoroscein isothiocyanate anti-rabbit conjugate (FITC conjugate)

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

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

6.3.3.3 Glycerol mounting medium

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

6.3.3.4 Glycerol broth

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