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**Testing method for detection and
enumeration of *Legionella* in industrial
water and wastewater**

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Foreword

This translation has been made based on the original Japanese Industrial Standard established by the Minister of Economy, Trade and Industry through deliberations at the Japanese Industrial Standards Committee as the result of proposal for establishment of Japanese Industrial Standard submitted by Japan Industrial Water Association (JIWA)/Japanese Standards Association (JSA) with the draft being attached, based on the provision of Article 12 Clause 1 of the Industrial Standardization Law.

This Standard has been made based on **ISO 11731: 1998** *Water quality—Detection and enumeration of Legionella* for the purpose of making it easier to compare this Standard with International Standard; to prepare Japanese Industrial Standard conforming with International Standard; and to propose a draft of an International Standard which is based on Japanese Industrial Standard.

Attention is drawn to the possibility that some parts of this Standard may conflict with a patent right, application for a patent after opening to the public, utility model right or application for registration of utility model after opening to the public which have technical properties. The relevant Minister and the Japanese Industrial Standards Committee are not responsible for identifying the patent right, application for a patent after opening to the public, utility model right or application for registration of utility model after opening to the public which have the said technical properties.

JIS K 0350 series consists of following 9 parts:

- K 0350-10-10 *Testing methods for standard plate counts of heterotrophic bacteria in industrial water and wastewater*
- K 0350-20-10 *Testing methods for detection and enumeration of coliform organisms in industrial water and waste water*
- K 0350-30-10 *Testing methods for detection and enumeration of heterotrophic bacteria in industrial water and wastewater*
- K0350-40-10 *Testing method for enumeration of total bacteria in industrial water and wastewater*
- K 0350-60-10 *Testing method for detection and enumeration of sulfate-reducing bacteria in industrial water*
- K 0350-70-10 *Testing method for detection of Sphaerotilus in industrial water*
- K 0350-80-10 *Testing method for detection of iron bacteria in industrial water*
- K 0350-90-10 *Testing method for detection of sulfur bacteria in industrial water*

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Testing method for detection and enumeration of *Legionella* in industrial water and wastewater

Introduction This Japanese Industrial Standard has been prepared based on the first edition of ISO 11731 *Water quality—Detection and enumeration of Legionella* published in 1998 with some modifications of the technical contents.

The portions given continuous sidelines or dotted underlines are the matters in which the contents of the original International Standard have been modified. A list of modifications with the explanation is shown in Annex (informative).

1 Scope This part of JIS K 0350 specifies the testing method for detection and enumeration of *Legionella* in industrial water and wastewater.

NOTE : The International Standard corresponding to this Standard is as follows.

In addition, symbols which denote the degree of correspondence in the contents between the relevant International Standard and JIS are IDT (identical), MOD (modified) and NEQ (not equivalent) based on ISO/IEC Guide 21.

ISO 11731:1998 *Water quality—Detection and enumeration of Legionella*
(MOD)

2 Normative references The standards shown in attached table 1, contain provisions which, through reference in this text, constitute provisions of this Standard. The most recent editions of the standards (including amendments) given below shall be applied.

3 Terms and definitions For the purpose of this Standard, the definitions given in JIS K 0101, JIS K 0102, JIS K 0550 and JIS K 0211 and the following definitions apply.

3.1 *Legionella* Gram-negative bacillus with no spore that belongs to the genus of *Legionella*, which forms typical colonies pale to grey in colour, requiring L-cysteine when incubated in the selective medium such as GVPC α agar medium and WYO α agar medium, at the temperature of $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 5 to 7 days

Most common species is *Legionella pneumophila*.

NOTE : *Legionella* is a pathogenic bacterium and can infect a person through inhalation. Therefore, a test for *Legionella* shall be carried out by an expert with full knowledge and skills of handling pathogenic bacteria and producing and inhalation of *Legionella* containing vapor (aerosol) which may cause legionnaires' disease shall be avoided as much as possible.

4 Common matters Common matters shall be as follows.

4.1 General rules The general matters that are common to chemical analysis shall be as specified in JIS K 0050.

4.2 Water Water of A2 or A3 ⁽¹⁾ specified in JIS K 0557.

Note (1): Water purified through a still made of quartz glass or borosilicate glass-1.

4.3 Reagents Reagents shall be as follows.

- a) The reagents of finest quality specified in Japanese Industrial Standard (hereafter referred to as “JIS”) shall be used. In the absence of the reagents specified in JIS, any reagents that do not affect the result of test shall be used.
- b) For the concentration of solution of the reagents, mass concentration is expressed in g/L or mg/L and molar concentration in mol/L, unless otherwise specified.

Further, for chemical compounds, the mass of an anhydride is adopted.
- c) The concentration indicated in the parentheses listed after the names of the reagents implies that it is rough concentration.
- d) The names of reagents are based on the nomenclature of inorganic chemistry and nomenclature of organic chemistry of International Union of Pure and Applied Chemistry (IUPAC) and are in best concordance with the nomenclature of chemical compounds established by the Chemical Society of Japan and names of JIS reagents.
- e) The highest attention shall be paid when handling the reagents and waste solution, etc. according to the relevant rules and regulations.

4.4 Glassware Glassware to be used is as specified in JIS R 3503 and JIS R 3505 in general. However, in the case that special glass tool is necessary, the example of the tool shall be illustrated or described at each item. In the case that heating handling is necessary, the borosilicate glass-1 as specified in JIS R 3503 shall be utilized.

4.5 Procedures of sterilization and decontamination for tools Procedures of sterilization and decontamination for tools shall be as follows.

- a) **Dry heat sterilization** Dry heat sterilization is carried out for glassware and metal tools. Wrap tools to be sterilized with parchment paper or aluminum foil, etc. or put them in the metal container for sterilization, and then place them in a dry sterilizer and heat them for about 1 h after the inside temperature of sterilizer has reached up to approximately 170°C.
- b) **Autoclave sterilization** Autoclave sterilization is carried out for sterilization of medium, water for dilution, sample container, used medium, etc. It is carried out for those which are easy to be destroyed through dry heat sterilization, such as glassware with a relatively complex structure and thick glass bottle.

Besides, in the case that a large-sized glass bottle and flask, etc. will be autoclaved, pour a small amount of water inside. The time necessary for sterilization is 15 min to 20 min after the inside temperature of the autoclave apparatus has reached up to 121°C.

- c) **Flame sterilization** Flame sterilization is carried out for sterilizing platinum wire or nichrome wire and the mouth of a flask at the procedure of the transplant. It is also carried out for sterilizing water tap and others at sampling.
- d) **Decontamination through filtration** Decontamination through filtration is carried out for decontamination of water or solution. Filtration will be carried out through the membrane filter ⁽²⁾ with the pore size of 0.2 μm (for example, 47 mm diameter) as the filter media.

Note ⁽²⁾ : The membrane filter to be used shall be previously wrapped with aluminum foil (or parchment paper) and placed in a glass petri dish and shall be autoclaved. Commercial sterilized products may be used. The membrane filter shall be handled with tweezers.

4.6 Procedure of disinfection Procedure of disinfection shall be as follows.

- a) Disinfect the hands and the laboratory table before and after the test. For disinfection of the hands, use a cresol and soap solution (10 g/L), cationic detergent (1 g/L to 10 g/L), disinfectant ethanol (the one specified in Japanese Pharmacopoeia) or ethanol (volume percentage 80 %) [prepared using ethanol (95) specified in JIS K 8102].

The laboratory table shall be disinfected through spraying cationic detergents (10 g/L), disinfectant ethanol or ethanol (volume percentage 80 %), etc. or wiping out with a cloth infiltrated with these detergents.

- b) The tools such as used pipette, sample container and water sampler shall be immersed in the disinfectant solution such as a cresol and soap solution (30 g/L to 50 g/L) for 1 day (or autoclaved), and then rinsed with plenty of running water until the disinfectant solution will be completely removed.

5 Samples

5.1 **Sampling** The samples of water shall be collected using sample container or water sampler.

5.1.1 Tools Tools shall be as follows.

- a) **Sample container** Glass bottle with ground stopper with the volume capacity of 100 ml to 1 000 ml. The sample container shall be well rinsed in advance. Wrap the tap portion and head portion of the container with aluminum foil (or parchment paper, etc.), sterilize by dry heat or autoclave ⁽³⁾. Or sterilized polyethylene bottles for bacteria tests may be used. Be careful to keep these tools away from contamination till sampling.

Note ⁽³⁾ : In the case of sampling water that contains oxidizing material such as residual chlorine, put 20 mg to 30 mg of sodium thiosulfate pentahydrate (powdered one) specified in JIS K 8637 in the container and sterilize the container by dry heat and autoclave, etc. in advance. A commercial sterilized polyethylene bottle for microbiological examination with sodium thiosulfate pentahydrate in it may be used.

- b) **Water sampler** Heyroth sampling bottle. The water sampler shall be placed in the portable container and sterilized by dry heat. An example is shown in figure 1.

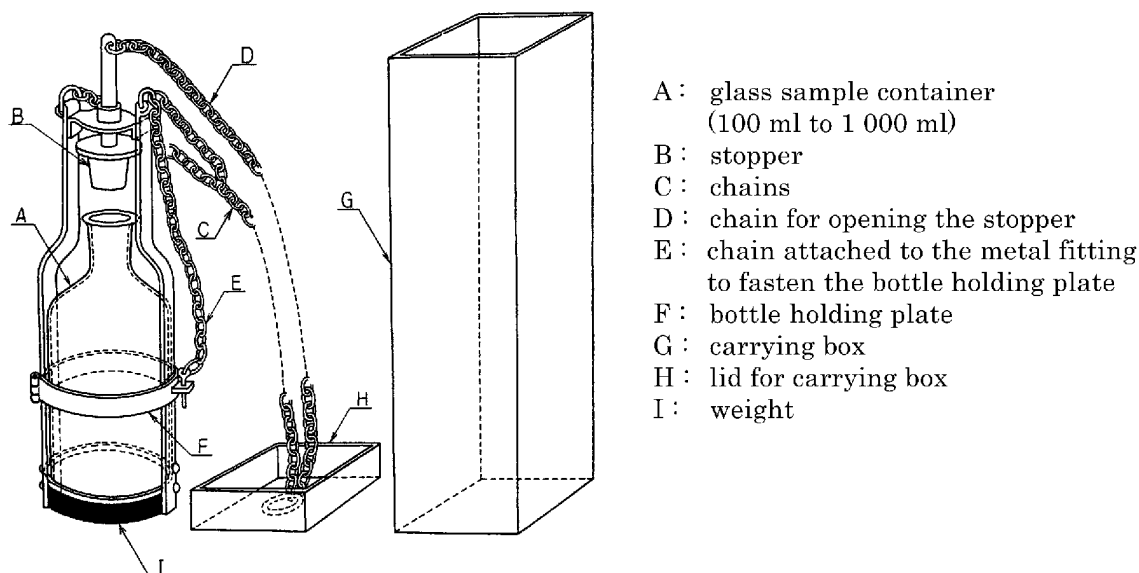


Figure 1 An example of Heyroth sampling bottle and carrying box

5.1.2 Operation procedure Sampling shall be as follows.

- a) **Sampling of the surface water** For the surface water of the lake and marsh, a river, a watercourse, an drain outlet and water tank, etc., collect the sample water with the sample container when direct sampling is possible. In this case, do not sample the water that is touched by the hand. When direct sampling is impossible, sample by using a water sampler ⁽³⁾.
- b) **Sampling of the water in the differentiated depth** The water in a certain depth shall be sampled by using a water sampler ⁽⁴⁾.

Note ⁽⁴⁾ : In the case that the sampling with Heyroth sampling bottle is impossible, the sample water is sampled with Van Dorn water sampler and transferred to the sample container.

- c) **Sampling from the water tap** In the case that the material of a water tap is proof against the flame sterilization, sterilize the water tap by flame in advance, open the tap, discard all the water inside the pipe and finally sample the sample water in the sample container.

In the case that the flame sterilization is not applicable, remove all the dirty material around the mouth of the water tap and inside of the tap in advance, disinfect with ethanol (volume percentage 80 %), etc., open the tap, discard all the water inside the pipe and finally sample the sample water in the sample container.

- d) **Sampling from the pipe and apparatus** The water is sampled according to the same procedure of c).

5.2 Handling of samples Test shall be carried out immediately after sampling. In the case that the simultaneous test is not possible, store the sample in the dark room at the temperature of 0°C to 5°C (not frozen).

6 Test method Concentrate *Legionella* in the sample through filtration with membrane filter or centrifuge, treat with low pH or with high temperature, smear on the surface of the selective medium, and incubate at the temperature of 36°C ± 1°C for 5 days to 7 days so that the colonies will be formed. If a humid colony looking pale or grey in colour is recognized there, judge it as a presumptive *Legionella* colony and count the number of the colonies.

Transfer some or whole colonies judged as presumptive *Legionella* colonies to the surface of BCYE α agar plate medium and agar plate medium without L-cysteine and incubate at the temperature of 36°C ± 1°C for at least 2 days and commonly, 5 days to 7 days so that the colonies will be formed.

If a colony is not formed in the agar plate medium without L-cysteine, and is formed only in the BCYE α agar plate medium, the colony is considered as confirmed *Legionella* colony. Count the number of the colonies decided as positive at identification tests and calculate the number of *Legionella* in the sample according to this value.

NOTE : Tests shall be in accordance with the contents of NOTE of clause 3.

6.1 Reagents Reagents shall be as follows.

- a) **Hydrochloric acid (0.2 mol/L)** Add water to 20 ml of hydrochloric acid specified in JIS K 8180 and make the final volume of 1 L.
- b) **Potassium chloride solution (0.2 mol/L)** Dissolve 14.91 g of potassium chloride specified in JIS K 8121 into distilled water to make the final volume of 1 L.
- c) **Hydrochloric acid-potassium chloride mixture solution (pH 2.2)** Mix 3.9 ml of hydrochloric acid (0.2 mol/L) and 25 ml of potassium chloride solution (0.2 mol/L) and adjust pH to 2.2 ± 0.2 using potassium hydroxide solution (1 mol/L) (Dissolve 56.1 g of potassium hydroxide specified in JIS K 8574 into the water to make the final volume of 1 L). Transfer the adjusted solution into a glass container, seal it and store in a dark place. The prepared solution shall be used within 1 month.
- d) **L-cysteine solution (36 g/L)** Dissolve 4.0 g of L-cysteine monohydrochloride monohydrate specified in JIS K 8470 into distilled water to make the final volume of 100 ml. And then decontaminate the prepared solution by membrane filtration⁽⁵⁾.

Note ⁽⁵⁾ : This prepared solution can be stored in the sterilized container at the temperature of -20°C ± 3°C for 3 months. When using, thaw the frozen solution at the room temperature.

- e) **Iron (III) pyrophosphate solution (25 g/L)** Dissolve 2.5 g of Iron (III) pyrophosphate (soluble) into distilled water to make the final volume of 100 ml. And then decontaminate the prepared solution by membrane filtration ⁽⁵⁾.

- f) **Polymyxin solution** Dissolve polymyxin B sulfate into distilled water and adjust the concentration to 10 000 international unit (i.u.)/ml ⁽⁶⁾. And then decontaminate the prepared solution by membrane filtration ⁽⁵⁾.

Note ⁽⁶⁾ : International unit (i.u.) is not the mass unit (SI unit), but is an expression of the efficacy of a given mass in an internationally integrated unit. The obtained polymyxin B sulfate (reagent) shall be adjusted according to the indicated unit on the label.

- g) **Vancomycin solution (1 g/L)** Dissolve vancomycin hydrochloride to distilled water with the concentration adjusted to 1 mg/ml. And then decontaminate the prepared solution by membrane filtration ⁽⁵⁾.
- h) **Cycloheximide solution (20 g/L)** Dissolve cycloheximide ⁽⁷⁾ into distilled water with the concentration adjusted to 20 mg/ml. And then decontaminate the prepared solution by membrane filtration ⁽⁵⁾.

Note ⁽⁷⁾: Cycloheximide has liver toxicity. Wear gloves and dust mask when handling this chemical in powder form.

- i) **Amphotericin B solution (20 g/L)** Dissolve amphotericin B into sulfinylbismethane [dimethyl sulfoxide (DMSO)] with the concentration adjusted to 20 mg/ml. And then decontaminate by membrane filtration ⁽⁵⁾.
- j) **BCYE α agar plate medium** The preparation of BCYE α agar plate medium shall be as follows ⁽⁸⁾.

- 1) Add 10.0 g of N-2-acetamide-2-aminoethane sulfonic acid (ACES) into the 500 ml of distilled water and dissolve by standing in a heated water bath (at 45°C to 50°C). Add 480 ml of potassium hydroxide solution (5.83 g/L) (Dissolve 2.9 g of potassium hydroxide specified in JIS K 8574 into distilled water to make the final volume of 500 ml) to the solution and stir the mixture.
- 2) Add 2.0 g of powder activated carbon to the solution of 1) and stir the mixture. Add sequentially to this solution, 10.0 g of yeast extract (powder), 1.0 g of 2-oxo pentanedioic acid potassium (α-ketoglutaric acid potassium) and dissolve them together. After decontamination by membrane filtration, adjust pH to 6.9 ± 0.2 using potassium hydroxide solution (0.1 mol/L) (Dissolve 5.6 g of potassium hydroxide specified in JIS K 8574 into distilled water to make the final volume of 1 L) or sulfuric acid (0.05 mol/L) (Carefully add 2.7 ml of sulfuric acid specified in JIS K 8951 to distilled water). Add and mix 15.0 g of agar (powder) specified in JIS K 8263 into the above solution, sterilize by autoclave for 15 min and then quickly cool down to about 50°C.
- 3) To this solution, add 10 ml of L-cysteine solution (36 g/L) and 10 ml of Iron (III) pyrophosphate solution (25 g/L) under the sterilized condition and stir the mixture well.