Designation: D8429 – 21

Standard Test Method for *Legionella pneumophila* in Water Samples Using Legiolert[1,2]

This standard is issued under the fixed designation D8429; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method describes a simple procedure for the detection of *Legionella pneumophila* (*L. pneumophila*) in potable water and non-potable waters (cooling towers, for example). This procedure describes a liquid culture method based on a bacterial enzyme technology. The detection of *L. pneumophila* is signaled through the utilization of a substrate present in the Legiolert reagent. *L. pneumophila* cells grow rapidly and reproduce using the rich supply of amino acids, vitamins and other nutrients present in the Legiolert reagent. Actively growing strains of *L. pneumophila* use the added substrate to produce a brown color indicator or produce turbid growth with or without brown coloration. Legiolert can detect this bacterial species at the following minimum concentrations based on the protocol employed:

1.1.1 *Potable Water:*

1.1.1.1 ≥1 organism / 100 mL at 7 days for 100 mL potable protocol.

1.1.1.2 ≥1 organism / 10 mL at 7 days for 10 mL potable protocol.

1.1.2 *Non-potable Water:*

1.1.2.1 ≥1 organism / 1.0 mL at 7 days for 1.0 mL non-potable protocol.

1.1.2.2 ≥1 organism / 0.1 mL at 7 days for 0.1 mL non-potable protocol.

1.1.3 This test method can be used for potable (drinking) waters and non-potable waters such as cooling tower waters (**1**).[3] It is the user's responsibility to ensure the validity of this test method for waters of untested matrices.

1.2 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.4 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[4]

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

D2777 Practice for Determination of Precision and Bias of Applicable Test Methods of Committee D19 on Water

D3370 Practices for Sampling Water from Flowing Process Streams

D5952 Guide for the Inspection of Water Systems for Legionella and the Investigation of Possible Outbreaks of Legionellosis (Legionnaires' Disease or Pontiac Fever)

D6503 Test Method for Enterococci in Water Using Enterolert

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this standard, refer to Terminology D1129.

3.1.2 *Legionella, n*—a bacterial genus containing over 50 species and at least 71 serogroups; abbreviated to the first initial when used relatedly with a species name, for example, *L. pneumophila*.

3.1.3 *most probable number (MPN), n*—a statistical method for determining bacterial concentration based on the Poisson distribution.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *Legionella pneumophila, n—in the context of this method*, a pathogenic gram-negative bacteria that produces any degree of brown coloration or turbid growth with or without brown coloration in the presence of the Legiolert reagent.

[1] Legiolert is a trademark of IDEXX Laboratories, Inc., Westbrook, ME.

[2] This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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[3] The boldface numbers in parentheses refer to a list of references at the end of this standard.

[4] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

3.2.2 *Quanti-Tray/Legiolert, n*—a system for the quantification of Legionella pneumophila. It consists of a Quanti-Tray Sealer PLUS[5,6] and 96-multi well Quanti-Trays that express *L. pneumophila* counts as MPN per volume of sample.

3.2.2.1 *Discussion*—For drinking water or similar waters where the volume is typically 100 mL, the system can enumerate up to 2272.6/100 mL or for small sample volumes such as the 0.1 mL protocol for non-potable water the system can enumerate up to 22726/1 mL.

3.2.2.2 *Discussion*—To increase the systems counting range a sample dilution can be utilized, but to obtain the correct MPN value and lower and upper confidence limits, these values must be multiplied by the dilution factor.

3.2.3 *snap pack, n*—a package containing Legiolert reagent for testing 100 mL sample volumes or dilutions thereof, quantitatively by means of the Quanti-Tray/Legiolert[7] system.

4. Summary of Test Method

4.1 Bacteria are grown in a defined liquid culture medium containing the Legiolert reagent.

4.2 The reagent is added to the sample, sealed in a Quanti-Tray, and then incubated, in the presence of humidity, for 7 days at either at 39 °C ± 0.5 °C or 37 °C ± 0.5 °C for potable or non-potable water samples, respectively.

4.3 *L. pneumophila* is detected if any of the Quanti-Tray wells exhibit growth indicated as a brown color change and/or turbidity.

4.4 Test results are reported as MPN per volume of sample (that is, MPN/mL).

5. Significance and Use

5.1 This test provides an easy and reliable method for the detection of *L. pneumophila* in potable and non-potable waters in 7 days.

5.2 Routine monitoring for *L. pneumophila* determines whether implemented control measures are effective, such as those outlined in a water safety program (**2**).

5.2.1 Water system management is necessary to maintain *L. pneumophila* concentrations below hazardous levels. Through routine measurement of *L. pneumophila* levels, a monitoring program can ensure that control measures are effective and implemented when necessary in response to increasing levels. Water samples may be examined for *L. pneumophila* during epidemiological investigations as part of local authority, industrial, or hospital programs, or in order to validate treatment control methods. Routine sampling could also be carried out based on risk assessments or on local, state, or federal requirements or guidelines.

6. Interferences

6.1 Hardness can interfere with the use of 100 mL of potable water sample and the addition of the Legiolert Supplement is required to prevent interference (12.2.1.3).

6.2 For non-potable, the Legiolert Pretreatment reagent is required to be added to the sample (12.2.1.4).

7. Apparatus

7.1 *Incubator,* capable of maintaining a temperature of 39 °C ± 0.5 °C (for potable water samples).

7.2 *Incubator,* capable of maintaining a temperature of 37 °C ± 0.5 °C (for non-potable water samples).

7.3 *Quanti-Tray Sealer PLUS.*

8. Reagents and Materials

8.1 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D1193, Type IV. Sterilize water by either autoclaving or by sterile filtration (0.22-micron filter filtered water).

8.2 *Legiolert Test Kit:*

8.2.1 The Legiolert reagent is a commercially available substrate provided in a snap pack format and is suitable for single samples.

8.3 *Quanti-Tray/Legiolert:*

8.3.1 For MPN results, the Legiolert regent must be used in conjunction with the Quanti-Tray/Legiolert 96-well trays. Quanti-Tray/Legiolert is commercially available, and one tray is suitable for a single sample.

8.4 *Legiolert Supplement Kit:*

8.4.1 The Legiolert Supplement is a commercially available kit containing of (*1*) a canister of dip strip tests designed to determine the total calcium/magnesium hardness in a water sample and (*2*) a 100 mL vessel containing supplement solution powder. The hardness strips are used to determine the total hardness of the sample and the supplement solution, once the powder is reconstituted with 100 mL of sterile deionized water, is added to the sample based on the determined sample's hardness.

8.5 *Legiolert Pretreatment Kit:*

8.5.1 The Legiolert Pretreatment is a commercially available kit containing of four vessels containing a dry powder to be reconstituted in sterile water.

8.6 *Quality Control Strains:*

8.6.1 IDEXX-QC *Legionella pneumophila*[8] or equivalent.

[5] Quanti-Tray is a trademark of IDEXX Laboratories, Inc., Westbrook, ME.

[6] The sole source of supply of Quanti-Tray Sealer PLUS known to the committee at this time is IDEXX Laboratories, Inc. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[2] which you may attend.

[7] The sole source of supply of Quanti-Tray/Legiolert supplies and reagents known to the committee at this time is IDEXX Laboratories, Inc. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[2] which you may attend.

[8] The sole source of supply of IDEXX-QC *Legionella pneumophila*, UN3373-WQC-LP98-009287-00, known to the committee at this time is IDEXX Laboratories, Inc. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[2] which you may attend.

8.6.2 At least one target organisms: *Legionella pneumophila*, American Type Culture Collection (ATCC) 33152/World Trade Center for Microorganisms (WDCM) 00107 or ATCC 33156/WDCM00180, or both.

8.6.3 At least one non-target organism: *Enterococcus faecalis*, ATCC 29212/WDCM 00087.

8.7 *Optional Reagents:*

8.7.1 Butterfield's/phosphate buffer.

8.7.2 Peptone water, 0.1 %.

8.7.3 Water, oxidant free, nonbuffered, sterile.

8.8 *Sterile 100 mL vessels,* with or without sodium thiosulfate. Any water containing an oxidizing agent such as chlorine must be appropriately neutralized with sodium thiosulfate.

8.9 *Sterile microtubes and sterile tubes,* capable of holding ≥1.5 mL of sample or ≥5.0 mL of sample.

9. Precautions

9.1 The analyst must observe the normal good laboratory practices and safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures and reagents and materials and while operating sterilization or other equipment.

9.2 Risk of harm is caused by the inhalation of aerosolized *L. pneumophila* and it is therefore advisable to assess all techniques for their ability to produce aerosols (**3**).

9.3 According to the CDC's Biosafety in Microbiological and Biomedical Laboratories, standard bio-safety level 2 (BSL-2) practices may be performed when routinely processing environmental water samples for Legionella (**4**). Laboratories testing for *L. pneumophila* may follow the same precautions they have in place for other BSL-2 bacteria.

10. Sampling

10.1 Collect samples as described in detail in the USEPA Microbiological Methods Manual (**5**) and in accordance with Practices D3370.

10.2 *Sample Storage, Temperature and Handling Conditions*—Ice or refrigerate samples at a temperature of 2 °C to 8 °C during transit to the laboratory. Use insulated containers to ensure proper maintenance of storage temperatures. Take care that samples containers are not totally immersed in water during transit.

10.3 *Handling Time Limitations*—Examine samples as soon as possible, after collection. Refrigerate samples that will not be processed within 48 h from the time of sample collection (**3**).

11. Quality Control Check

11.1 Check and record temperatures in incubators at least twice per day when in use, separated by at least 4 h to ensure temperature is within stated limits.

11.2 Quality control should be conducted on each new lot of Legiolert reagent. One of the following quality control procedures is recommended for each lot of Legiolert.

NOTE 1—Quality control procedure must be carried out in the absence of sodium thiosulfate.

11.2.1 *IDEXX-QC Legionella pneumophila*—Consists of three of each *Legionella pneumophila* (positive control), and *Enterococcus faecalis* (negative control).

11.2.1.1 Remove vial(s) from freezer. Equilibrate at ambient temperature for 15 minutes. Open a vial and aseptically transfer the pellet to an appropriately labeled vessel of 100 mL of sterile, nonbuffered, oxidant-free water in a sterile vessel. Swirl the sample and allow to stand for 5 minutes. The pellet should completely dissolve. After it is dissolved, mix by inverting the vessel 10 times. Use within 30 minutes of hydration. All steps must be performed at ambient temperature. Review the kit's lot activity information on the certificate of analysis to determine if a dilution of hydrated sample is required.[9]

11.2.2 For each of the American Type Culture Collection (ATCC) or World Data Centre for Microorganisms (WDCM) bacterial strains:

11.2.2.1 *Target Organisms—Legionella pneumophila*, ATCC 33152/WDCM 00107 or ATCC 33156/WDCM00180, or both, streak the culture onto a BCYE plate and incubate at 35 °C ± 0.5 °C for 48 h to 72 h.

11.2.2.2 *Non-target organism—Enterococcus faecalis* ATCC 29212/WDCM 00087; streak onto a blood agar plate and incubate at 35 °C + 0.5 °C for 18h to 24 h.

11.2.2.3 For each bacterial strain inoculate a sterile vessel containing 100 mL of sterile diluent (DI water, phosphate/Butterfield's buffer or 0.1 % peptone water) with 10^2 CFU/sample to 103 CFU/sample target organisms and 10^3 CFU/sample to 10^4 CFU/sample non-target organisms.

11.2.2.4 See 12.3.2.1 – 12.3.2.5 for Quanti-Tray/Legiolert enumeration procedure and result interpretation.

12. Procedure

12.1 *Potable Water Sample Preparation (environmental samples only):*

12.1.1 *10 mL Protocol:*

12.1.1.1 Add 90 mL of sterile diluent (DI water, phosphate/Butterfield's buffer, or 0.1 % peptone water) to a sterile vessel without sodium thiosulfate.

12.1.1.2 Add 10 mL of water sample.

12.1.1.3 Bring sample to ambient temperature.

12.1.1.4 Carefully separate one snap pack from the strip.

12.1.1.5 Tap snap pack to ensure that all of the reagent is towards the bottom of the pack.

12.1.1.6 Open the pack by snapping back the top of the score line. Do not touch the opening of the pack.

12.1.1.7 Add contents of Legiolert snap pack to sample.

12.1.1.8 Aseptically cap and seal the vessel.

12.1.1.9 Shake until contents are dissolved. Sample may remain cloudy.

12.1.2 *100 mL Protocol:*

12.1.2.1 Collect 100 mL into a sterile vessel.

12.1.2.2 Bring sample to ambient temperature.

12.1.2.3 Using a hardness dip strip (see 8.4) to determine if the sample has low or high hardness. Pad scores of 0 to 2 are

[9] IDEXX-QC Legionella pneumophila certificate of analysis is available at https://www.idexx.com/en/water.

considered as indicative of low hardness, and pad scores of 3 to 4 are considered as indicative of high hardness.

12.1.2.4 For waters of low hardness add 0.33 mL of hydrated supplement solution (see 8.4) per 100 mL of sample and add 1.0 mL of hydrated supplement solution per 100 mL of sample. For all dilutions of 10-fold or greater, the hardness test and hardness neutralization step is omitted, as sample hardness is diluted out.

12.1.2.5 Add contents of Legiolert snap pack and dissolve as directed in 12.1.1.4 – 12.1.1.9.

12.2 *Non-potable Water Sample Preparation (environmental samples only):*

12.2.1 *0.1 mL Protocol:*

12.2.1.1 Add 100 mL of sterile diluent (DI water, phosphate/Butterfield's buffer or 0.1 % peptone water) to a sterile vessel without sodium thiosulfate.

12.2.1.2 Add contents of Legiolert snap pack as directed in 12.1.1.4 – 12.1.1.8 to the 100 mL of sterile diluent.

12.2.1.3 Shake until contents are dissolved. Solution may remain cloudy.

12.2.1.4 Add 0.2 mL of Legiolert Pretreatment (see 8.5) into a sterile microtube.

12.2.1.5 Mix the non-potable sample, then add 0.2 mL of the non-potable sample to the same microtube and mix well.

12.2.1.6 Incubate the microtube at ambient temperature for 60 seconds ± 5 seconds.

12.2.1.7 Mix the microtube contents and immediately transfer 0.2 mL to the vessel containing Legiolert (see 12.3.2.1) and mix well.

12.2.2 *1.0 mL Protocol:*

12.2.2.1 Add 100 mL of sterile diluent (DI water, phosphate/Butterfield's buffer, or 0.1 % peptone water) to a sterile vessel without sodium thiosulfate.

12.2.2.2 Add contents of Legiolert snap pack as directed in 12.1.1.4 – 12.1.1.8 to the 100 mL of sterile diluent.

12.2.2.3 Shake until contents are dissolved. Solution may remain cloudy.

12.2.2.4 Add 2.0 mL of Legiolert Pretreatment (see 8.5) into a sterile tube.

12.2.2.5 Mix the non-potable sample, then add 2.0 mL of the non-potable sample to the same tube and mix well.

12.2.2.6 Incubate the tube at ambient temperature for 60 seconds ± 5 seconds.

12.2.2.7 Mix the tube contents and immediately transfer 2.0 mL to the vessel containing Legiolert (see 12.3.2.1) and mix well.

12.3 *MPN-Quanti-Tray/Legiolert Enumeration Procedure:*

12.3.1 *For Quality Control (QC) and Proficiency Test (PT):*

12.3.1.1 Aseptically add the contents of Legiolert snap pack to the sample.

12.3.1.2 Shake until the contents are dissolved. Sample may remain cloudy.

12.3.1.3 See 12.3.2.1 – 12.3.2.5.

12.3.2 *For All Test Samples:*

12.3.2.1 Aseptically pour sample/reagent mixture into a Quanti-Tray/Legiolert tray. Tap or flick the tray to remove any air bubbles.

12.3.2.2 Immediately seal the tray using the Quanti-Tray Sealer PLUS.

12.3.2.3 Incubate the sealed trays:

(1) For all potable water or quality control (QC) or proficiency test (PT) samples, incubate at 39 °C ± 0.5 °C for seven days.

(2) For all non-potable water incubate at 37 °C ± 0.5 °C for seven days.

12.3.2.4 Quanti-Tray/Legiolert must be incubated paper side down with the plastic wells facing up in an environment that will minimize Quanti-Tray /Legiolert water loss (either in high humidity or in an enclosure that traps moisture). Stack trays in alternating directions for added stability. Do not remove trays completely from incubator prior to final read.

12.3.2.5 Read results according to Table 1. Count the number of positive wells and refer to the Table 2 or use the IDEXX MPN Generator software to obtain a most probable number (MPN). Multiply results by the dilution factor for the final MPN, if applicable.

TABLE 1 Legiolert Result Interpretation

Appearance	Result
Any brown color (with or without turbidity)	Positive for *Legionella pneumophila*
Any turbidity (with or without any brown color change)	Positive for *Legionella pneumophila*
No brown color change and no turbidity	Negative for *Legionella pneumophila*

TABLE 2 Legiolert MPN Table

NOTE 1—MPN per 100 mL for a Quanti-Tray/Legiolert 96-well tray.

Number of Small Wells Positive	Number of Large Wells Positive						
	0	1	2	3	4	5	6
0	<1	1	2	4	6	8	13
1	1	2	4	5	7	10	16
2	2	3	5	7	9	12	19
3	3	4	6	8	11	15	22
4	4	5	7	9	12	17	26