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Research Article

MICROBIOLOGICAL METHODS

Validation of MICA *Legionella* for Enumeration of *Legionella pneumophila* in Sanitary Waters and Cooling Tower Waters: AOAC *Performance Tested Method*^{SM} 032201

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Abstract

Background: Frequent testing for *Legionella* concentration in water is required by most health risk monitoring organizations worldwide. Domestic hot water and cooling tower water networks must be regularly controlled to prevent Legionnaires' disease, a potentially deadly lung infection. MICA *Legionella* is the fastest culture-based detection method for all serogroups of *Legionella pneumophila*, with automatic enumeration in 48 h and no need for confirmation.
Objective: This study compares the performance and robustness of MICA *Legionella* with the reference method ISO 11731:2017 for the enumeration of culturable *L. pneumophila*.
Methods: MICA *Legionella* and ISO 11731:2017 results were compared for domestic hot water and cooling tower water. Inclusivity and exclusivity were tested on reference and environmental strains. Ruggedness, lot-to-lot consistency, and stability of the reagents kit were also studied.
Results: Enumeration of *L. pneumophila* by MICA *Legionella* was statistically equivalent to ISO 11731:2017 in both matrixes. In cooling tower waters, MICA *Legionella* showed better sensitivity than ISO 11731:2017. It presented a 94% sensitivity and a 97% specificity.
Conclusion: MICA *Legionella* is a highly sensitive and specific method for culturable *L. pneumophila* enumeration. It presents, in 48 hours, equivalent or better results than ISO 11731:2017. Its protocol is robust to variations. Its reagents kit is stable for up to 18 months.
Highlights: MICA *Legionella* is a robust and reliable method for the enumeration of culturable *L. pneumophila* in domestic and cooling tower water. It reduces significantly the number of sample pretreatments required in ISO 11731:2017. Automatic identification and enumeration of *L. pneumophila* microcolonies eliminates the requirement to have skilled analysts and limits the results variability. It also greatly reduces the time to results to 48 h instead of 7–10 days with ISO 11731:2017 while providing statistically equivalent results.

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General Information

Legionnaires' disease is a potentially fatal lung infection due to pathogenic bacteria that develop in hot water systems and cooling tower systems. According to the Center for Disease Control an Prevention (CDC) (1), about one out of every ten people who gets sick with Legionnaires' disease will die due to complications from their illness (2); for those who get Legionnaires' disease during a stay in a healthcare facility, about one out of every four will die (3). The infection is contracted via inhalation of small aerosols of contaminated water. The vast majority of cases are due to *Legionella pneumophila* (4, 5), mostly from serogroup 1, but also from other serogroups (8 to 15% of the infections by *L. pneumophila* are due to serogroups other than serogroup 1; 5–8). Importantly, Legionnaires' disease is commonly diagnosed by a urinary antigen test specific to *L. pneumophila* serogroup 1, leading to underdiagnosis for other serogroups (5, 8). The number of infections is increasing every year due to climate change and increased population density in urban areas; it has, for example, increased by 220% in Europe and by 550% in the United States since 2005 (9, 10). Moreover, the number of cases is potentially greatly underestimated (4).

Regular monitoring of the presence of *L. pneumophila* in hot water and cooling tower systems is the major strategy used to limit the occurrence of outbreaks. It is required (or at least highly recommended) by most health risk monitoring organizations worldwide (11–17). However, the standard, culture-based methods, such as ISO 11731:2017, require up to 10 days to determine the presence of this bacterium in a water system (18). This delay considerably limits the frequency of the tests. It also means that the effectiveness of any treatment can only be known 10 days after the treatment, leading to shutdowns of water systems for longer than needed. Additionally, this standard method is time-consuming and needs expert-trained technicians for identification of *Legionella*, leading to interpretation differences between technicians depending on their experience (19). Another drawback of ISO 11731:2017 is the number of different pretreatment and culture plates it requires. As the *Legionella* culture plates are not highly selective, pretreatment of the sample with acid and heat shock is often necessary to reduce the number of interfering organisms. The plating of the different combinations of these pretreatments on different dilutions of the sample requires several plates per sample, which weighs on the time and cost of the analysis. Methods allowing fast and reliable detection and quantification of *L. pneumophila* would greatly improve the risk management and have a major impact on the incidence of legionellosis (20).

Principle

MICA *Legionella* is a detection method allowing detection of culturable microcolonies of *L. pneumophila* at 48 h of growth instead of 10 days in the standard procedures such as ISO 17731:2017. The original water sample is concentrated by membrane filtration as in the standard procedure. The membrane is then laid over a drop of culture supplement on a standard selective *Legionella* agar plate [Glycine, Vancomycin, Polymyxin B, Cycloheximide (GVPC)]. This culture supplement contains Diamidex's patented molecule, a precursor of legionaminic acid coupled with the bio-orthogonal azido group (pLeg-N3; 21). Legionaminic acid is a specific component of the O-antigen of *L. pneumophila*, so the molecule will be specifically internalized by growing *L. pneumophila* and integrated in their O-antigen on the surface of the cells. After 48 h of bacterial growth, the membrane is transferred onto a drop of tagging solution containing a fluorescent molecule that will bind by click chemistry specifically onto the bio-orthogonal azido group, i.e., the labelled *L. pneumophila*. This specific fluorescent tagging allows the CFU to be automatically detected at the microcolony stage by solid-phase cytometry using the MICA microcolony counter which will perform a high-resolution scan of the membrane. The MICA *Legionella* AI (artificial intelligence) analyzer then uses multiple parameters to specifically identify *L. pneumophila* microcolonies (as low as 2 CFU per test portion) and gives a result as a concentration of *L. pneumophila* in the original sample. For a better reproducibility, the MICA software provided with the microcolony counter provides a step-by-step protocol guide, including control of the incubation times and reagent traceability. The guidance and analysis by the software allows the MICA *Legionella* method to be used by anyone.

This validation study was conducted under the AOAC Research Institute *Performance Tested Method*^{SM} program and follows the AOAC INTERNATIONAL *Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* (22). Method developer studies were conducted in the laboratories of Diamidex, and included the inclusivity/exclusivity study, matrix studies for all claimed matrixes, product consistency and stability studies, and robustness testing. The independent laboratory study was conducted by Q Laboratories (MicroVal expert lab, accredited ISO 17 025) and included a matrix study for cooling tower water.

Scope of Method

(a) *Target organisms*.—*L. pneumophila*, all serogroups
(b) *Matrixes*.—Hot domestic/tap water and cooling tower water
(c) *Summary of validated performance claims*.—The MICA *Legionella* for *L. pneumophila* is a simple and fast kit, which detects and counts only *L. pneumophila* bacteria capable of being cultivated (similar to regulatory procedure NF T90-431 or ISO 11731:2017) in samples of select environmental and domestic waters. The sensitivity (inclusivity) of MICA *Legionella* was found to be 94% and specificity (exclusivity) 97%. Performance of the kit is equivalent to ISO 11731:2017 for the enumeration of *L. pneumophila* in hot domestic water and can be better than ISO 11731:2017 on cooling tower water.

Definitions

(a) *Repeatability standard deviation* (s_r).—Standard deviation of replicates for each strain at each concentration of each matrix for each method.
(b) *Bias*.—Bias is the difference between the candidate method mean result and the true value or reference method value, [$mean_{candidate}$ – known spike] or [$mean_{candidate}$ – $mean_{reference}$].
(c) *Selectivity*.—Ability of the method to detect analyte without interference from matrix or other components with similar behavior.
(d) *Sensitivity*.—Probability of the method giving a positive response when the sample is truly without analyte.
(e) *Specificity*.—Probability of the method giving a negative response when the sample is truly without analyte.
(f) *Repeatability*.—Precision where independent test results are obtained with the same method on equivalent test items in

the same laboratory by the same operator using the same equipment within a short interval of time.

(g) *RSD*.—The ratio of the standard deviation to the mean, often reported as a percentage.

(h) *Confidence interval (CI)*.—A confidence interval displays the probability that a parameter will fall between a pair of values around the mean. Confidence intervals are calculated at the 90 and 95% levels.

(i) *Statistical equivalence*.—The acceptance criterion for statistical equivalence is that the 90% CI of the bias between the methods falls within −0.5, 0.5.

Materials and Methods

MICA Legionella Test Kit Information

(a) *Kit name*.—MICA *Legionella* Detection Kit.

(b) *Cat. No*.—00 917 (for laboratories) or 00 916 (includes sterile distilled water, pipet tips, etc. for non-laboratory customers).

(c) *Ordering information*.—DIAMIDEX, Grand Luminy Technopole, Zone Luminy Entreprise Biotech, Case 922, 163 Avenue de Luminy, 13288 Marseille Cedex 09, France, contact@diamidex.com, tel +33 (0)6 61 93 49 29.

MICA Legionella Test Kit Components (Figure 1)

(a) *Filtration membranes*.—48 mm diameter, with a tab for orientation. PVDF (polyvinylidene fluoride), white, 0.45 µm pore size.

(b) *Reagent A*.—Freeze-dried culture supplement containing Diamidex's patented molecule for specific labelling of *L. pneumophila*. Storage between +4°C and +8°C.

(c) *Reagent B*.—Freeze-dried tagging solution. Storage between +4°C and +8°C.

(d) *Reagent C*.—pH 2 Solution for acid treatment of the sample, as in ISO 11731:2017.

(e) *Reagent D*.—Sterile water for washing Reagent C.

(f) *Fiberglass pads*.—Provide a proper surface for the tagging step.

(g) *Concentrated wash buffer*.—11X Concentrated buffer to wash away excess tagging solution. Storage between +4°C and +8°C.

(h) *Sterile distilled water vials, 10 mL*.—For hydration of the membrane during the scan.

(i) *Labels*.—For printing the sample QR codes.

Additional Supplies and Reagents for the MICA Legionella Test Kit

(a) *Sterile distilled water*.—Available as part of the all-in-one version of the MICA *Legionella* detection kit, Cat. No 00 916.

(b) *Agar plates*.—GVPC agar plates, Thermo Scientific Oxoid, Cat. No. PO5074A for the internal studies; Hardy Diagnostic buffered charcoal yeast extract (BCYE) selective agar plates with GPCV, ref. W169, for the external study. Other agars can also be used, such as KANTO CHROMagar (ref. 717592-1), Bio-Rad (Cat. No. 3563717) or Liofilchem (Cat. No. 10128).

(c) *Disinfection solution*.—Effective against *Legionella* and non-corrosive for the equipment, for example, hydrogen peroxide at 6% or ethanol at 70%. An adequate disinfectant can be supplied as part of the all-in-one version of the MICA *Legionella* detection kit.

Apparatus for MICA Legionella (Figure 2)

(a) *MICA microcolony counter with reading cassettes*.—Diamidex, Cat. No. 00 877.

(b) *MICA Legionella software*.—Diamidex, Cat. No. 01 037.

(c) *Microcolony counter accessory set*.—Barcode scanner, label printer, USB hub and touch pen. Diamidex, Cat. No. 01 108.

(d) *MICA washing bench*.—Diamidex, Cat. No. 00 755.

(e) *MICA tagging tray*.—Diamidex, Cat. No. 00 721.

Figure 1. Packaging of the kit reagents. Packaging of the all-in-one version of the MICA *Legionella* detection kit

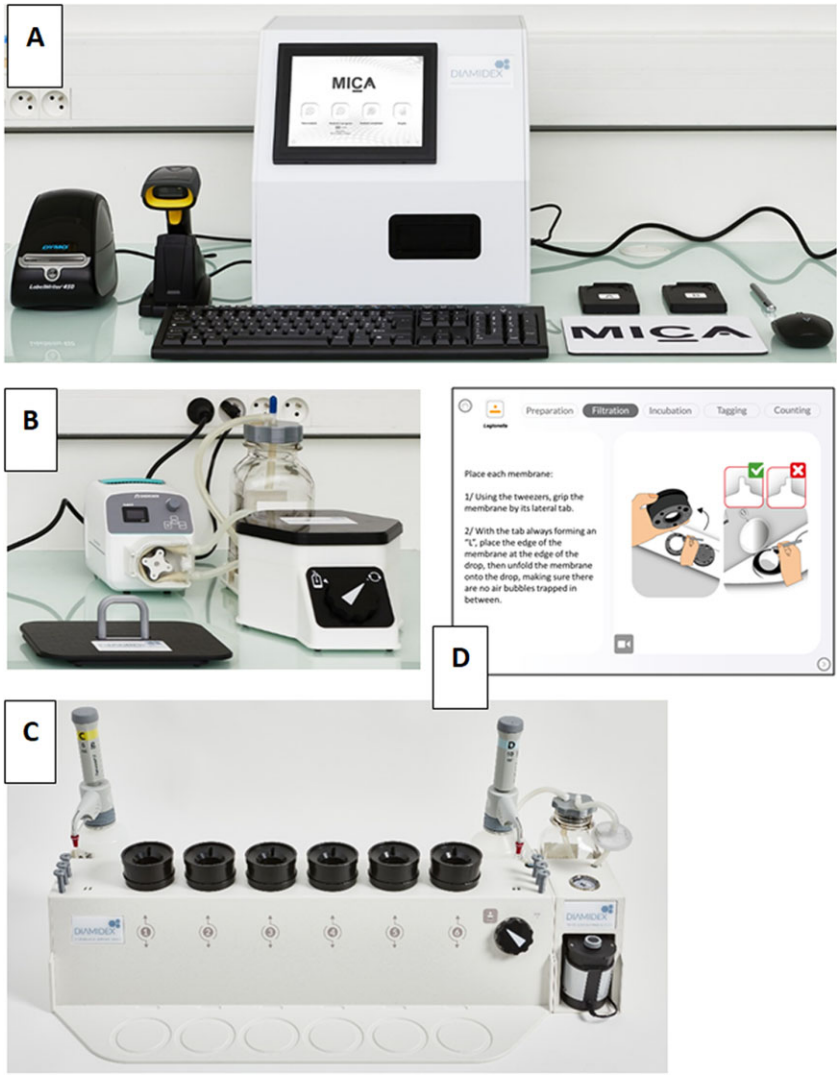

Figure 2. Specific apparatus. (A) MICA microcolony counter and its accessories (label printer, scanning gun, reading cassettes). (B) MICA tagging tray (lower left) and washing bench. (C) MICA filtration manifold (other types of filtration manifolds can also be used). (D) Screenshot from the step-by-step protocol in the MICA software.

(f) *MICA Petri dish holders*.—Diamidex, Cat. No. 01 002.
(g) *Filtration manifold with filtration units and pump*.—Up to six filtering positions, filtration units containing at least 20 mL Diamidex, Cat. No. 00 878 (or equivalent).
(h) *Incubator*.—Suitable for cultures, capable of maintaining 37 ± 1°C, Diamidex, Cat. No. 00 903 (or equivalent).
(i) *Incubator*.—Suitable for heat treatment, capable of maintaining 52 ± 1°C, Diamidex, Cat. No. 01 183 (or equivalent).
(j) *Incubator*.—Suitable for tagging incubation, capable of maintaining 30 ± 1°C, Diamidex, Cat. No. 00 913 (or equivalent).
(k) *Precision pipets*.—Capable of dispensing 500 and 700 µL, Diamidex Cat. No. 00 904 and 00 905 (or equivalent).
(l) *Filtered micropipette tips*.—To use with the precision pipets.
(m) *Tweezers*.—Suitable for handling membranes, Diamidex Cat. No. 00 821 (or equivalent).
(n) *Dispensers*.—Capable of dispensing 5 and 10 mL volumes, resistant to low pH, Diamidex Cat. No. 01 030 and 01 031 (or equivalent).
(o) *Refrigerator*.—Capable of maintaining 2–8°C, Diamidex Cat. No. 00 883 (or equivalent).

Reference Materials

Bacterial strains for this study were obtained from ATCC (23), DSMZ (24), the Pasteur Institute (25), or characterized by the CNR-L (26). All strains used in this study are listed in Tables 1 and 2.

General Preparation for MICA Legionella

Apart from the apparatus setup, the MICA software guides the user through the entire protocol, from the preparation of the reagents to the final results. Step-by-step instructions (Figure 2, panel D) with videos are available to ensure that mistakes are prevented and to provide traceability of the process. This also includes countdowns for all incubations, scanning of the QR codes or barcodes of all reagents and samples, as well as alerts when the reagents are used up or out of date.

(a) Assemble the filtration manifold according to the manufacturer's instructions.
(b) Assemble the MICA washing bench according to the manufacturer's instructions

(c) Set up the temperature of the incubators at least 30 min ahead of time to allow for equilibration at the desired temperature (culture incubator at 37°C, heat treatment incubator at 52°C if necessary, tagging incubator at 30°C).
(d) Rehydrate Reagents A and B from the MICA *Legionella* test kit according to the manufacturer's instructions.

Sample Preparation for a MICA Legionella Analysis

(a) Using the MICA software, enter the requested information for each water sample to be analyzed.
(b) Scan or enter the batch numbers requested by the MICA software for the following items: GVPC agar plates, vial A, vial B, membrane filters, and bottle C (pH 2).
(c) Print a label for each sample to be analyzed and attach it to the bottle.
(d) Print a second label for each sample and attach it to the corresponding GVPC agar plate.
(e) Filter 20 mL of the test sample using the MICA filtration membrane.
(f) Apply a pH 2 treatment: add 5 mL Reagent C (pH 2 solution) over the filtration membrane, incubate for 5 mins at room temperature (countdown on the software), eliminate Reagent C by filtration, then add 10 mL sterile distilled water to wash away the pH 2 solution and eliminate by filtration.

Analysis of the Sample with MICA Legionella

(a) Labelling and culture step.
  (1) Put a 500 μL drop of Reagent A onto a GVPC plate.
  (2) Lay the filtration membrane (filtered bacteria facing up) over the drop of Reagent A.
  (3) Arrange the GVPC plate inverted in the numbered Petri dish holder indicated by the software.
  (4) For cooling tower water samples only: incubate the plate upside down at 52°C for 45 min.
  (5) Incubate the plate upside-down for 48h. This step allows for the formation of microcolonies of *L. pneumophila* and their labelling by Diamidex's patented molecule.
  (6) Follow the prompts in the software to decontaminate the equipment.
(b) Tagging step.
  (1) After incubation is complete, remove the indicated numbered Petri dish holder from the incubator and scan the label on each plate.
  (2) Lay a fiberglass pad into the tagging tray and soak it with 700 μL Reagent B.
  (3) Lay the filtration membrane (with the microcolonies facing up) over the soaked fiberglass pad.
  (4) Incubate for 15 min at 30°C, following the countdown on the software. This step tags the microcolonies with a fluorescent molecule, via a click-chemistry reaction to bind the fluorescent molecule to the Diamidex-patented molecule bound to the bacteria.
  (5) During the 15 min incubation, prepare the washing bench: pour into the trough a vial (50 mL) of concentrated wash buffer and 500 mL sterile distilled water, then start the pump of the washing bench at 50 rpm (rotations per min).
  (6) At the end of the 15 min incubation, transfer the filtration membrane onto the washing bench (microcolonies still facing up), and allow for 15 min of washing to eliminate excess fluorescent molecules (countdown on the software).
  (7) Lay three drops of sterile distilled water onto a reading cassette and transfer the filtration membrane onto the cassette, microcolonies facing up, taking care to avoid air bubbles under the membrane.
  (8) Read the membrane on the cassette with the MICA microcolony counter.
  (9) Transfer the membrane to its respective GVPC plate and dispose of according to laboratory procedures for decontaminating biohazardous waste or disinfect with bleach before disposal.
  (10) Follow the prompts in the software to decontaminate the equipment.
  (11) The result is displayed on screen in the MICA software as CFU/L. Confirmation is unnecessary.

Calculations, Interpretation, and Test Result Report of MICA Analyses

The AI analyzer integrated in the MICA software automatically identify microcolonies of *L. pneumophila* on the membrane based on a multi-parametric analysis and directly gives a concentration of *L. pneumophila* in the water sample (in CFU/L). No human interpretation or calculation is needed, reducing the inter-user variability, and giving more reproducible results. The results are stored in the MICA software and can be accessed at any time there or exported as a csv file or as pdf analysis reports. Traceability sheets are also available for each analysis.

Enumeration of L. pneumophila following ISO 11731:2017

Briefly, for ISO 11731 analyses of hot domestic water, each test portion is split into three parts. One part, 0.2 mL, is plated without treatment on a GVPC plate. The other two parts, respectively 10 and 100 mL, are concentrated by filtration; the filtration membrane is then covered by 5 mL pH 2 solution and incubated for 5 min; after filtering out the pH 2 solution, the membrane is rinsed with 10 mL sterile distilled water; finally, the membrane is transferred onto a GVPC agar plate. For analyses of cooling tower water, each test portion is split into three parts. Two parts, respectively of 0.02 and 0.2 mL are directly plated by spreading onto GVPC agar plates. The other part, 50 mL, is concentrated by filtration and resuspended in sterile phosphate buffer. The concentrate obtained is then split into three parts. One part (0.1 mL) is plated untreated onto a GVPC plate, another part is incubated at 50°C for 30 min before plating 0.1 mL onto a GVPC plate, and the last part is diluted by half with pH 2 solution and incubated for 5 min at room temperature before plating onto a GVPC plate. For both sample types, all GVPC plates are then incubated at 37°C and read at 3 and 7 days. Suspected *Legionella* colonies are confirmed by a latex agglutination test (Oxoid). According to ISO 11731, for each sample test portion, the plate giving the highest density per liter is used for the final result.

For the independent laboratory study (on cooling tower water only), the sample is divided into three portions: one portion is direct plated (0.1 mL) onto GVPC agar; the second portion is used for a 1:10 dilution in pH 2 acid treatment solution prior to plating; and the third portion is used for heat treatment at 50 ± 1°C for 30 ± 0.5 min prior to plating. Additionally, 50 mL of bulk inoculated sample is filter concentrated and the filter washed in 5 mL phosphate-buffered saline (pH 7.5) and plated

untreated, acid treated, and heat treated, as previously described. All GVPC plates are incubated at 36 ± 2°C for 7 to 10 days. The plates are observed for suspect *L. pneumophila* colonies at Day 4 and on the final day of incubation. Typical colonies are enumerated, and the results recorded.

Sampling of Matrixes for the Matrix Study

The sample of cooling tower water and the sample of hot domestic water matrix were collected following ISO 19458 (27)

Cooling tower water samples were too small individually, so they were pooled from different locations to obtain mixed samples of the required volume. All samples were screened using ISO 11731 and no natural *Legionella* contamination was found.

Artificial Sample Contamination

Due to the low frequency of water samples contaminated by *L. pneumophila*, all samples in this study are artificially contaminated. A stock culture stored at −70°C is streaked onto a GVPC agar plate. The plate is then incubated at 37°C for 3 to 4 days. A *Legionella* liquid medium is prepared freshly (10 g/L yeast extract, supplemented with BCYE supplement SR0110A from Oxoid) and inoculated from the agar plate. This liquid culture is grown overnight at 37°C with shaking at 160 rpm before diluting at the appropriate concentration in the water matrix. Serial dilutions of the liquid culture are plated onto GVPC agar plates to determine the theoretical inoculation concentration in the water matrix.

For the independent laboratory matrix study, the matrix is artificially contaminated with *L. pneumophila* serogroup 1, ATCC 33152. The culture is propagated on BCYE agar from a stock culture stored at −70°C. The BCYE agar plate is incubated at 37 ± 1°C for 72–96 h before transferring a single colony to Legionella enrichment broth (Sigma Aldrich) and incubating statically at 37 ± 1°C for 96 ± 4 h. Serial dilutions of the culture are prepared to achieve the target concentrations.

Statistical Analysis for This Study

To allow reliable statistical analysis as well as a clear graphical presentation of the results, all results, initially in CFU/L, are converted to $\log_{10}$, with an offset of +1 to accommodate the zeros in the data set. When comparing results obtained from two different methods, they are considered significantly different if the 95% confidence interval of the bias extends outside of the −0.5 to +0.5 range, according to the recommendations of AOAC.

Results and Discussion

Inclusivity

To determine the sensitivity of MICA *Legionella*, 35 different strains of *L. pneumophila* were tested with MICA *Legionella* (Table 1). Approximately 10^3 to 5×10^3 cells were used to artificially contaminate 20 mL test portions of sterile phosphate buffer. The artificially contaminated test portions were processed with MICA *Legionella* and the results were compared with the theoretical inoculation density (Table 1). Out of the 35 tested *L. pneumophila* strains, 33 (94%) were correctly detected, covering all serogroups. The only two exceptions were from *L. pneumophila* serogroup 7 (strains Nos.19 and 23) for which four other strains were properly detected. Serogroup 7 is a very poorly represented serogroup both in infection cases and in the environment (6, 28, 29). Lower identification of this serogroup can be explained by an atypical composition of the O-antigen of this serogroup (21).

Exclusivity

To determine the specificity of MICA *Legionella*, 16 non-*pneumophila Legionella* strains and 13 non-*Legionella* strains, chosen among possible water background flora, were tested with MICA *Legionella* (Table 2). Approximately 10^4 to 5×10^4 cells were used to artificially contaminate 20 mL test portions of sterile phosphate buffer. The artificially contaminated test portions were processed with MICA *Legionella* (Table 2). Results are summarized in Table 2.

Out of 29 tested *Legionella* non-*pneumophila* and background water-borne organisms, 28 (97%) correctly produced negative results, while only one produced a positive result. This strain, *Legionella norrlandica* (strain No. 48), was isolated in 2015 from the biopurification system of wood processing plants in Sweden. It is closer to *L. pneumophila* than the other known *Legionella* non-*pneumophila* species and contains most of the virulence genes of *L. pneumophila*, in particular its cell wall structure (30), which explains its detection by MICA *Legionella*. It is classified as a class-2 pathogen, as is *L. pneumophila*, and its presence in the water systems should be treated as is the presence of *L. pneumophila*. Thus, getting a positive result for this strain is more of an advantage than a trouble as its presence should lead to the same treatment as *L. pneumophila*.

Method Developer Matrix Study

The results of MICA *Legionella* were compared with that of the standard reference method ISO 11731:2017 on two different matrixes: domestic hot water and cooling tower water. The hot domestic water matrix did not contain background flora growing on GVPC at 37°C, while the cooling tower water contained 7×10^6 CFU/L of background flora growing on GVPC at 37°C. Artificial contamination of the matrixes was performed using liquid cultures of *L. pneumophila* serogroup1 (strain No. 5) and *L. pneumophila* serogroup 6 (strain No.15), respectively, for the cooling tower water and the hot domestic water, at low level (≈10^3 CFU/L), medium level (≈10^4 CFU/L) and high level (≈10^5 CFU/L). The theoretical inoculation density was estimated by plating serial dilutions of each culture. MICA *Legionella* and ISO 11731:2017 analyses were both started on the day of the inoculation. Five test portions of each contamination level and of the uncontaminated matrixes were tested with both methods.

Results in CFU/L are converted to $\log_{10}$ before statistical analysis and comparison. They are summarized in Table 3 and Figure 3 and detailed in Supplementary Table 1.

From domestic hot water, both methods showed very low standard deviation on positive samples, ranging from 0.01 to 0.1 log unit for both methods, indicating a very good reproducibility of the methods. Importantly, the correlation of the results of the two methods is very high (correlation coefficient $R^2 = 0.99$, Figure 3, panel A), indicating that MICA *Legionella* gives similar results to ISO 11731:2017 on domestic hot water.

From cooling tower water, ISO 11731:2017 shows a very high variability: the standard deviation on positive samples ranges from 0.18 to 1.6 log unit, with two false-negative results on the low-level contamination, due to background flora growth over the entire agar plates. On the other hand, MICA *Legionella* results

Table 1. Inclusivity strains and results

No.	Serogroup	*L. pneumophila* strain[a]	MICA result, $\log_{10}$ *L. pneumophila*/L	Numeration by plating, $\log_{10}$ CFU/L	Bias[b]	Detection[c]
1	1	ATCC 33152	5.29	6.25	−0.96	+
2	1	CIP 107629	4.88	4.76	0.13	+
3	1	CIP 108286	5.32	4.56	0.77	+
4	1	CIP103854T	4.90	6.23	−1.34	+
5	1	CIP105349	5.24	5.11	0.13	+
6	1	Environmental D131	5.20	5.01	0.19	+
7	1	Environmental D138	5.08	4.67	0.40	+
8	1	Environmental D139	5.13	4.72	0.41	+
9	1	Environmental D17	5.14	4.79	0.34	+
10	1	Environmental D20	5.01	4.75	0.26	+
11	2	CIP103856	4.95	5.05	−0.10	+
12	3	CIP103857	5.17	5.30	−0.13	+
13	4	Environmental D15	5.14	4.94	0.20	+
14	5	Environmental D16	5.07	4.72	0.36	+
15	6	Environmental D18	5.30	5.15	0.15	+
16	6	Environmental D47	4.21	5.35	−1.14	+
17	6	Environmental D48	5.07	4.94	0.13	+
18	6	Environmental D46	5.03	4.78	0.25	+
19	7	CIP 103861	0.00	5.03	−5.03	−
20	7	Environmental D52	4.96	4.73	0.23	+
21	7	Environmental D53	2.51	4.79	−2.28	+
22	7	Environmental D54	4.74	4.89	−0.15	+
23	7	Environmental D50	0.00	4.77	−4.77	−
24	7	Environmental D51	4.90	4.86	0.05	+
25	8	Environmental D122	5.14	4.91	0.23	+
26	9	CIP103863	5.06	5.08	−0.02	+
27	10	Environmental D124	4.51	4.80	−0.29	+
28	11	Environmental D125	5.50	4.65	0.85	+
29	12	Environmental D169	5.49	4.62	0.87	+
30	13	DSM 25225	5.23	5.19	0.04	+
31	14	CIP 103869	5.06	5.01	0.06	+
32	15	Environmental D129	4.80	4.67	0.13	+
33	2–14	Environmental D140	5.25	5.01	0.24	+
34	4,5,8,10	Environmental D141	5.09	4.87	0.22	+
35	6	Environmental D142	5.22	4.95	0.27	+

[a] All strains are *L. pneumophila*. Strain numbers preceded by ATCC are from the ATCC collection, by CIP from the Pasteur Institute collection, by DSM from the DSMZ collection. Environmental strains were characterized by the CNR-L.
[b] Calculated as the difference between the MICA result and the numeration by plating.
[c] A strain is considered as being detected if the MICA reading gives a positive result (>100 CFU/L).

show low standard deviations ranging only from 0.16 to 0.25 log units, without any false negatives. Comparison of each method with the theoretical inoculation level of the cooling tower water (Figure 3, panels C and D) shows that MICA *Legionella* provides results closer to the theoretical inoculation level than ISO 11731:2017 ($R^2 = 0.99$ vs $R^2 = 0.80$).

It is striking that the new MICA *Legionella* method performs better than the gold standard ISO 11731:2017 on this more complex matrix, but it is easily explained. Indeed, with such a matrix containing a high amount of background flora, when the plates are read for ISO 11731:2017 after 3 to 10 days of incubation they are often covered up on large parts by the background flora, hiding an unknown number of *Legionella* colonies. In contrast, when the plates are read for MICA *Legionella* after only 48 h of incubation, the background flora has not yet grown as much and they hide only a few parts of the plates. Thus, unlike ISO 11731:2017, MICA *Legionella* is not affected by the abundant background flora often found in cooling tower waters and gives more reliable results than ISO 11731:2017 on this type of matrix.

Independent Laboratory Matrix Study

An independent laboratory study was conducted on the most complex of the two types of matrixes: cooling tower water. The matrix was artificially contaminated with *Legionella pneumophila* serogroup 1 ATCC 33152, originally isolated from a human, at the following target concentrations: 5×10^2, 10^3, 10^4, 10^5, and 10^6 CFU/L. Prior to inoculation, the cooling tower water was dosed with liquid chlorine and thoroughly homogenized to achieve a level of 0.1 ppm (parts per million, mg/L).

For the MICA *Legionella* test portions, 500 mL was prepared for each contamination level and the uninoculated level. A 20 mL volume was taken for each of the five replicates from the 500 mL bulk sample for analysis. For the ISO 11731:2017 test portions, 500 mL was prepared for each contamination level and the uninoculated level. A 50 mL volume was taken for each of the five replicates from the 500 mL bulk sample for analysis in addition to the aliquots required for direct plating and pre-treatment.

Table 2. Exclusivity strains and results

No.	Species	Strain[a]	MICA result, $\log_{10}$ (Lp/L + 1)[b]	Numeration by plating, $\log_{10}$ (CFU/L + 1)	Detection[c]
36	*Legionella anisa*	CIP103870T	0.00	6.64	–
37	*Legionella bozemanii*	Environmental D25	0.00	6.42	–
38	*Legionella cincinnatiensis*	Environmental D31	0.00	6.76	–
39	*Legionella feeleii*	HL 0418 4001 D26	0.00	7.82	–
40	*Legionella geestiana*	DSM-21217	0.00	8.06	–
41	*Legionella gormanii*	Environmental D30	0.00	6.61	–
42	*Legionella jordanis*	Environmental D27	0.00	7.01	–
43	*Legionella longbeachae*	Environmental D29	0.00	6.64	–
44	*Legionella maceachernii*	Environmental D23	0.00	7.66	–
45	*Legionella micdadei*	Environmental D24	0.00	8.02	–
46	*Legionella moravica*	DSM-19234	0.00	6.61	–
47	*Legionella. nagasakiensis*	DSM-24727	0.00	8.04	–
48	*Legionella norrlandica*	DSM-105104	5.20	6.98	+
49	*Legionella spiritensis*	DSM-19324	0.00	7.07	–
50	*Legionella tucsonensis*	Environmental D28	0.00	6.93	–
51	*Legionella waltersii*	DSM-21908	0.00	6.94	–
52	*Acinetobacter baumanii*	ATCC 19606	0.00	6.18	–
53	*Bacillus cereus*	ATCC 14579	0.00	6.48	–
54	*Candida albicans*	ATCC 10231	0.00	5.57	–
55	*Citrobacter freundii*	ATCC 8090	0.00	6.34	–
56	*Escherichia coli Migula*	ATCC 8739	0.00	6.25	–
57	*Enterococcus faecelis*	ATCC 19433	0.00	6.07	–
58	*Listeria monocytogenes*	ATCC 35152	0.00	7.84	–
59	*Pseudomonas aeruginosa*	ATCC 10145	0.00	6.36	–
60	*Pseudomonas fluorescens*	ATCC 13525	0.00	7.05	–
61	*Staphylococcus aureus*	ATCC 6538	0.00	6.04	–
62	*Staphylococcus epidermidis*	ATCC 12228	0.00	6.38	–
63	*Klebsiella pneumoniae*	ATCC 13883	0.00	6.16	–
64	*Salmonella typhimurium*	ATCC 13311	0.00	6.47	–

[a] Strain numbers preceded by ATCC are from the ATCC collection, by CIP from the Pasteur Institute collection, by DSM from the DSMZ collection. Environmental strains were characterized by the CNR-L.
[b] For exclusivity strains the result given by MICA *Legionella* should be 0.
[c] A strain is considered as being detected if the MICA reading gives a positive result (>100 CFU/L).

Table 3. Matrix study results

Matrix	Artificial contamination density, CFU/L	n[a]	MICA		ISO				
			Mean, $\log_{10}$ CFU/L	s_r[b]	Mean, $\log_{10}$ CFU/L	s_r	Bias[c]	95% CI[d]	90% CI[d]
Domestic hot water	0	5	0.000	0.000	0.000	0.000	0.000	(0.000, 0.000)	(0.000, 0.000)
	Low ($\approx 10^3$)	5	2.701	0.094	2.864	0.080	−0.163	(−0.316, −0.010)	(−0.268, −0.059)
	Medium ($\approx 10^4$)	5	4.046	0.048	3.755	0.034	0.291	(0.218, 0.364)	(0.241, 0.341)
	High ($\approx 10^5$)	5	4.988	0.014	4.731	0.107	0.257	(0.122, 0.391)	(0.154, 0.360)
Cooling tower water	0	5	0.000	0.000	0.000	0.000	0.000	(0.000, 0.000)	(0.000, 0.000)
	Low ($\approx 10^3$)	5	2.817	0.248	1.801	1.650	1.016	(−1.056, 3.088)	(−0.691, 3.522)
	Medium ($\approx 10^4$)	5	3.664	0.209	4.202	0.295	−0.538	(−0.987, −0.089)	(−0.844, −0.232)
	High ($\approx 10^5$)	5	4.820	0.160	4.624	0.181	0.196	(−0.104, 0.496)	(−0.009, 0.401)
Cooling tower water[e]	0	5	0.000	0.000	0.000	0.000	0.000	(0.000, 0.000)	(0.000, 0.000)
	Very low ($\approx 5 \times 10^2$)	5	3.090	0.289	4.311	0.216	−1.22	(−1.60, −0.84)	(−1.53, −0.92)
	Low ($\approx 10^3$)	5	4.117	0.059	4.181	0.165	−0.064	(−0.27, 0.14)	(−0.23, 0.10)
	Medium ($\approx 10^4$)	5	4.136	0.087	4.451	0.164	−0.315	(−0.52, −0.11)	(−0.48, −0.15)
	High ($\approx 10^5$)	5	5.007	0.050	5.146	0.136	−0.139	(−0.31, 0.03)	(−0.27, −0.008)
	Very high ($\approx 10^6$)	5	5.558	0.238	6.363	0.049	−0.805	(−1.06, −0.55)	(−1.00, −0.60)

[a] n = Number of replicates.
[b] S_r = Repeatability standard deviation.
[c] Bias: difference of mean (MICA−ISO).
[d] CI = Confidence interval of the bias.
[e] Independent laboratory matrix study.

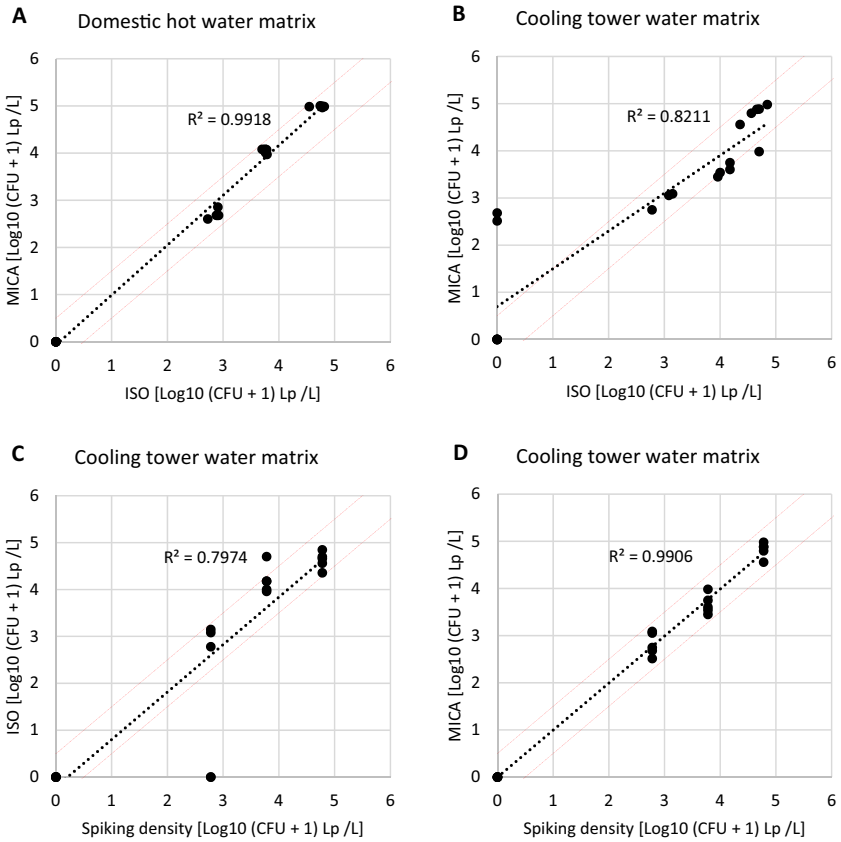

Figure 3. Internal matrix study results. In all panels, the red lines show the −0.5 to +0.5 interval around the perfect correlation. Dotted lines: tendency curve. (A) Comparison of the MICA and ISO results on the domestic hot water matrix. (B) Comparison of the MICA and ISO results on the cooling tower water matrix. (C) Comparison of the ISO results with the theoretical inoculation (as determined by plating serial dilutions of the culture used for inoculation) in cooling tower water matrix. (D) Comparison of the MICA results with the theoretical inoculation in cooling tower water matrix.

Results are summarized in Table 3 and Figure 4 and detailed in Supplementary Table 1. The 90% confidence interval of the bias between the two methods fell between −0.5 to 0.5 $\log_{10}$ for each concentration indicating equivalence between the two methods. The repeatability (s_r) calculated as SD, of the Diamidex MICA *Legionella pneumophila* kit and the reference method was determined for the cooling tower matrix.

The MICA *Legionella pneumophila* kit proved to be a more rapid, reliable, and sensitive culture method when compared to the ISO 11731:2017 reference standard for enumeration of *L. pneumophila* in cooling tower water. The results of the statistical analysis using the difference of means with calculated 90/95% confidence intervals indicated equivalence between the MICA *Legionella pneumophila* kit and the reference standard in three of the five artificial contamination levels analyzed: low, medium, and high. For the very low and very high concentration levels the results of the statistical analysis demonstrated a statistically significant increase in the sensitivity of the MICA *Legionella* method over the ISO 11731 culture method.

Robustness

To assess the robustness of the MICA *Legionella* method, variations of three key parameters were tested (Table 4) and the analysis results compared with the recommended conditions (*see* Supplemental Information for details).

The results proved that MICA *Legionella* is resilient to most tested variations of the protocol. Nonetheless, to prevent the risk of deviation from the recommended parameters, the MICA software does not allow shorter culture or labelling times (the most impactful variations) and gives a warning for any incubation exceeding the tolerance margin. Thus, the combination of the protocol resilience with the guidance provided by the software ensures that the MICA *Legionella* performance is highly robust.

Test Kit Consistency and Stability

The product consistency and stability studies were conducted together. Three lots are tested at time point 0 for the consistency study. Kits from each lot are then stored at 25°C for the accelerated stability study and at 4°C for the real-time stability study (see details in Supplemental Information).

At time point 0, all three tested lots give similar results, with no significant difference from the inoculation density, indicating excellent reproducibility of the test kit.

Both the accelerated and real-time stability studies demonstrate that the test kit is stable up to 18 months at 4°C. Further time points (24 months, maybe more) of the real-time study will be performed on time to check for a potential longer stability than initially expected.

Conclusions

Since its discovery in 1976, *Legionella pneumophila* has been considered an important pathogen that should be monitored in

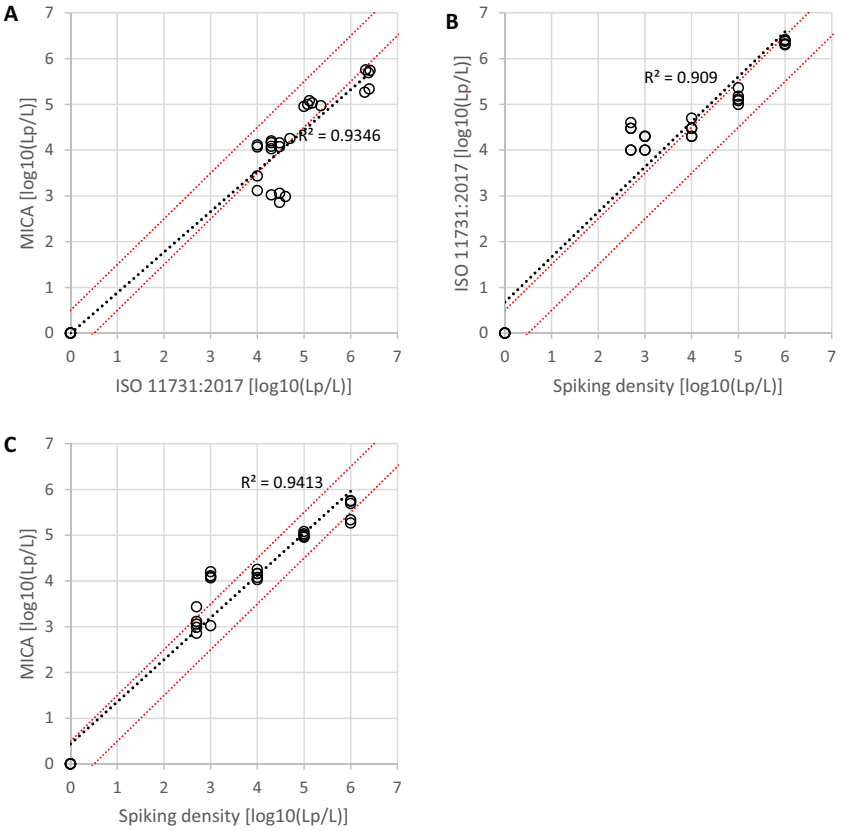

Figure 4. Independent laboratory matrix study results on cooling tower water. In all panels, the dotted red lines (smaller dots) show the −0.5 to +0.5 interval around the perfect correlation. Dotted black lines (bigger dots): tendency curve. (A) Comparison of the MICA and ISO results. (B) Comparison of the ISO results with the theoretical inoculation level. (C) Comparison of the MICA results with the theoretical inoculation level.

Table 4. Parameters of the robustness study

Parameter	Recommended range	Tested range
Culture incubation duration, h	48–49	46—50
Culture incubation temperature, °C	36–38	35—39
Tagging incubation duration, min	15–17	10–30

domestic hot water and cooling tower water. Several detection methods have been developed, but the gold standard remains a culture method, as in ISO 11731:2017. However, this method has important issues, such as the long time-to-result, the high amount of human time and number of culture plates required, as well as the high level of training for the technicians. These issues can be addressed by the development of new, culture-based detection methods that must achieve the same performance level as the standard method while allowing for a shorter result delay (ideally 24 to 48 h) and rely as little as possible on human skills (20). Indeed, a short result delay allows a better reactivity both in the case of a contamination and in the case of a successful disinfection of the water system, leading to lower sanitary risks, lower use of sanitizers, and shorter shutdown events; a low requirement of human skills allows for better reproducibility and reliability of the results and makes it easier to implement the method directly on site instead of relying on expert laboratories. Diamidex developed MICA *Legionella* to answer this (Table 5).

Table 5. Comparison of the advantages of the MICA and ISO method

	ISO 11731	MICA *Legionella*
Performance level	High	High
Culture-based method	Yes	Yes
Enumeration in CFU/L	Yes	Yes
Time to results	Up to 10 days	2 days
Number of different pretreatment processes	Up to 4	1
Number of different sample volumes to be analyzed	Up to 3	1
Number of plates to be read	Up to 9	1
Plate reading	Manual	Automatic
Confirmation step needed	Yes	No
Impact of background flora	High	Low
Risk of human mistakes	High (human identification, counting, and calculation)	Low (automatic identification, counting and calculation, step-by-step user guidance)
Requirement for skilled analysts	Yes	No
Analysts training time	6 months	1 day

As shown in this study, MICA *Legionella* can detect all serogroups of *L. pneumophila* and does not wrongly recognize other species. The protocol proved robust to variations and,

additionally, the MICA *legionella* software reduces the risk of deviations from the protocol by providing a step-by-step protocol and control of incubation time. Furthermore, the final result does not rely on human interpretation, but instead on automatic identification of microcolonies of *L. pneumophila* by the AI analyzer and automatic calculation of contamination density in the original water sample, thus reducing both the required human time and skills and the risk of human mistakes. Another advantage is the use of a single culture plate without extra confirmation steps instead of up to nine initial plates plus extra confirmation plates for the standard method, which not only reduces the waste but also further reduces the human time and skills needed for the analysis (Table 5).

When compared to ISO 11731:2017, MICA *Legionella* gives in 48 h equivalent results to the standard method in 10 days for a simple matrix (hot sanitary water). On complex matrixes (cooling tower water), MICA *Legionella* performs better than the standard method, thanks to the shorter culture incubation time that makes it less sensitive to background flora interference at reading time. Another advantage of this low sensitivity to background flora is that the volume of analyzed sample can be higher for MICA *Legionella* than for ISO 11731:2017, which leads to a lower LOD. In the present study, the LOD of the ISO method on the complex matrix was 1000 CFU/L, while the LOD of MICA *Legionella* was 100 CFU/L. Moreover, the LOD of MICA *Legionella* could be further lowered by increasing the filtered volume.

Importantly for a routine analysis method, the MICA *Legionella* test kit is reproducible from lot-to-lot and is stable at the recommended storage temperature (4°C) for a long time, up to 18 months according to the stability study.

Altogether, MICA *Legionella* can be considered as a reliable and fast alternative to the standard methods for enumeration of *L. pneumophila* in hot domestic water and cooling tower water and has been granted PTM certification.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Supplemental Information

Supplemental information is available on the *J. AOAC Int.* website.

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