MICROBIOLOGICAL METHODS

Method Modification of the Legipid® *Legionella* Fast Detection Test Kit

***Performance Tested Method*SM 111101**

Abstract

Legipid® *Legionella* Fast Detection is a test based on combined magnetic immunocapture and enzyme-immunoassay (CEIA) for the detection of *Legionella* in water. The test is based on the use of anti-*Legionella* antibodies immobilized on magnetic microspheres. Target microorganism is preconcentrated by filtration. Immunomagnetic analysis is applied on these preconcentrated water samples in a final test portion of 9 mL. The test kit was certified by the AOAC Research Institute as *Performance Tested Method*SM (PTM) No. 111101 in a PTM validation which certifies the performance claims of the test method in comparison to the ISO reference method 11731-1998 and the revision 11731-2004 "Water Quality: Detection and Enumeration of *Legionella pneumophila"* in potable water, industrial water, and waste water. The modification of this test kit has been approved. The modification includes increasing the target analyte from *L. pneumophila* to *Legionella* species and adding an optical reader to the test method. In this study, 71 strains of *Legionella* spp. other than *L. pneumophila* were tested to determine its reactivity with the kit based on CEIA. All the strains of *Legionella* spp. tested by the CEIA test were confirmed positive by reference standard method ISO 11731. This test (PTM 111101) has been modified to include a final optical reading. A methods comparison study was conducted to demonstrate the equivalence of this modification to the reference culture method. Two water matrixes were analyzed. Results show no statistically detectable difference between the test method and the reference culture method for the enumeration of *Legionella* spp. The relative level of detection was 93 CFU/volume examined (LOD_{50}). For optical reading, the LOD was 40 CFU/volume examined and the LOQ was 60 CFU/volume examined. Results showed that the test Legipid *Legionella* Fast Detection is equivalent to the reference culture method for the enumeration of *Legionella* spp.

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The method was independently tested, evaluated, and certified by the AOAC Research Institute as a *Performance Tested Method*SM. *See* http://www.aoac.org/testkits/steps.html for information on certification.

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Scope of Method

(**a**) *Target organisms.—Legionella* spp.

(**b**) *Summary of method modification claims.*—The inclusivity of the capture and enzyme-immunoassay (CEIA). test method was extended for *Legionella* spp. and was shown to detect 90.14% of the assayed *Legionella* spp. environmental strains. The CEIA test method was evaluated and was shown to detect 64 of the 71 tested strains. Moreover, the measurement of the absorbance of the supernatant at the end of the test method, after colorimetric reaction was stopped and magnetic microspheres were retained, allowed indirect measurement of the amount of *Legionella* spp. in a sample. Relationship between the absorbance measured for the test method and the target concentration measured by ISO reference culture method 11731 was established. The test method was equivalent to the ISO 11731 reference method for detection of *Legionella* spp. at contamination levels ranging from low (1–99 CFU/mL), medium (100–999 CFU/mL), and high (1000–99 999 CFU/mL) in potable water and industrial water matrixes. The relative level of detection was 93 CFU/volume examined (LOD_{50}). For optical reading, the LOD was 40 CFU/volume examined and the LOQ was 60 CFU/volume examined.

Principle of the Method

Legipid® *Legionella* Fast Detection (Cat. No. 311-10) is a CEIA rapid test based on immunomagnetic separation by anti-*Legionella* immuno-modified magnetic beads, combined with an enzyme-linked colorimetric detection for a rapid 1 h test. Original water sample is concentrated by filtration or similar, and this prepared sample is eluted and dispensed into the test cuvette, to be analyzed by the CEIA method. A suspension of *Legionella* binding magnetic beads is added. *Legionella* cells present in the prepared sample will bind to the antibodies immobilized onto the surface of the beads, to form bacteria/bead complexes. As these complexes can be separated by a magnet, they can be easily washed and resuspended. Next, complexes are incubated with an enzyme-conjugated anti-*Legionella* antibody to form labeled complexes. After washing steps, the *Legionella*/magnetic bead complexes are visualized by the colorimetric reaction when enzyme substrates are added. A control (without target) can be tested in parallel in another control cuvette.

General Information

L. pneumophila is one of the main agents causing severe atypical pneumonia, particularly among immunocompromised people (1). Among the 52 species and 70 serogroups included in the genus *Legionella* (2), *L. pneumophila* is the major cause of sporadic outbreaks of legionellosis (91.5%), with serogroup 1 being the predominant serotype (84.2%; 3–6). Official methods for *Legionella* detection are based on cultivation in selective media (7–9), and at least 7 to 15 days are required to obtain results. Culture detection also shows other weaknesses, such as slow growth rate of the bacterium, low sensitivity, loss of bacteria viability after collection, and difficulty in isolating *Legionella* in samples contaminated with other microbes (10, 11). Colonization at human-made water systems has been associated with biofilms yielding only some free bacterial cells (12–15). Moreover, rapid fluctuations of the concentration of *L. pneumophila* at risk installations have been reported (16), as well as persistence of this pathogen in drinking water biofilms mostly in a viable but nonculturable state (17), which has also been confirmed even after treatments with chlorine used to disinfect cooling towers (18, 19). Therefore, the development of a rapid, simple, and specific detection method for *Legionella* monitoring in real time would be crucial for the efficient prevention of legionellosis. This prevention may complement current epidemiological purposes that the culture method allows (20).

Materials and Methods

Test Kit Information

(**a**) *Kit name*.—Legipid *Legionella* Fast Detection.

(**b**) *Cat. No*.—311-10-00, 01, 02, 04, and 06.

(**c**) *Ordering information*.—Biótica, Bioquímica Analítica, S.L. Parque Científico, Tecnológico y Empresarial de la Universidad Jaume I–Campus Riu Sec, Espaitec 2, planta baja, laboratorio 2, 12071–Castellón de la Plana–Spain; Tel. +34 964 108 131, Fax. +34 964 737 790, www.biotica.es

Test Kit Components

(**a**) *Reagent one (L1)*.—Magnetic particles with immobilized specific polyclonal anti-*Legionella* antibody. *Function*.—Form complexes with the bacteria target to separate them from the rest of the sample (other bacteria and materials or substances present in the sample).

(**b**) *Reagent two (L2)*.—Buffer. *Function*.—Washing-formed complexes.

(**c**) *Reagent three (L3)*.—Specific polyclonal antibody against the bacteria target, conjugated to the enzyme horseradish peroxidase (HRP).

(**d**) *Reagent four (L4)*.—Co-substrate for the conjugated HRP.

(**e**) *Reagent five (L5)*.—Reaction buffer. *Function*.—dissolving the L4 co-substrate to develop the colorimetric reaction.

(**f**) *Reagent six (L6)*.—Reagent to stop the HRP reaction.

(**g**) *Reagent seven (L0)*.—Reagent to elute the microorganism from the filter membrane.

Additional Supplies and Reagents

(**a**) *Graduated screw-cap tube*.—Deltalab (Barcelona, Spain) Cat. No. 409502G. For the elution of the filter.

(**b**) *Glass fiber filter*.—FilterLab (Barcelona, Spain) Cat. No. MFV4. For use with filtration system as a prefilter.

(**c**) *Sterile polycarbonate membrane filter*.—Sartorius, Trevose, PA; Cat. No. K04CP04700. For use with filtration system.

(**d**) *Container for residue*.—Deltalab; Cat. No. 409502G.

Apparatus

(**a**) *Magnetic particle concentrator*.—Biótica, Bioquímica Analítica, S.L., Castellón, Spain; Cat. No. 311-MP2-RA and 311-MP4-00. For the retention of immunomagnetic beads.

(**b**) *Filtration system*.—Stainless steel vacuum filtration unit, Vacuubrand, Wertheim, Germany; Cat. No. MZ 2 NT. For the preconcentration of the water sample by membrane filtration.

(**c**) *Vortex mixer*.—Wiggen Hauser, Berlin, Germany; Cat. No. 3000 ANA. To release retained material from the membrane filter.

(**d**) *Sonicator*.—Selecta (Barcelona, Spain) Cat. No. 3000512. To release retained material from the membrane filter.

(**e**) *Pipet*.—100–1000 µL.

(**f**) *Colorimeter*.—Biochrom, (Holliston, MA); Cat. No. 80-5000-03. To read the absorbance both in control and tests.

Safety Precautions

(**a**) Cultures and samples are (potentially) contaminated with pathogens (Biosafety Level 2/Advisory Committee on Dangerous Pathogens, class 2).

(**b**) Infection by *L. pneumophila* is caused by inhalation of the organism. It is advisable, therefore, to assess all techniques for their ability to produce aerosols. If any doubt, carry out the work in a safety cabinet.

General Preparation

(**a**) Assemble the concentration apparatus (filtration) according to the manufacturer's instructions.

(**b**) Remove the reagents of the kit that are going to be used from 4°C storage and allow equilibration to room temperature (18–26°C) for at least 30 min before use.

Sample Preparation

(**a**) Original water samples should be collected and concentrated (by filtration) according to standard laboratory techniques, such as those described in the ISO standard specific for the target concerned.

(**b**) Place the filter in a screw-cap sterile container containing 10 mL of L0 reagent.

(**c**) Vortex the screw-cap tube for 2 min to elute the target organisms.

(**d**) Transfer 9 mL of the supernatant into a cuvet from the test kit.

Analysis

(**a**) *Capturing step.*—(*1*) *General description.*—The kit includes different reagents (L0, L1, L2, L3, L4, L5, and L6) and an easy-to-handle magnetic particle concentrator comprising a magnet and two 10 mL glass cuvets. A 9 mL amount of each preconcentrated sample is transferred to the kit 10 mL glass cuvet, and 1 mL of L1 reagent containing *Legionella* spp. binding magnetic beads is added. Target cells will bind to the antibodies immobilized onto the surface of magnetic beads to form bacteria/bead complexes. These complexes are separated by applying a magnet to the cuvet, and the supernatant is discarded by overturning the cuvets. A control must be tested in parallel in a second cuvet.

(*2*) *Step-by-step description of the process.*—(*a*) Shake the L1 until a completely homogeneous suspension is obtained and add it up to line 1 (1 mL).

(*b*) Add the sample previously filtered and eluted up to line 3 (9 mL), being careful not let the pieces of the filter fall into the cuvet.

(*c*) Shake gently, with the lid on, once every 3 min for 15 min.

(*d*) Put the magnet as close as you can and wait 5 min to retain the immunomagnetic particles.

(*e*) Empty the cuvet carefully, making sure you do not remove the retained particles.

(*f*) Separate the magnet from the cuvet and add the L2 up to line 2 (4.5 mL).

(*g*) Shake gently without lids until the particles are suspended again.

(*h*) Put the magnet as close as you can, and wait 3 min to retain the immunomagnetic particles.

(*i*) Empty the cuvet, being careful not to remove any retained particles.

(**b**) *Marking step.*—(*1*) *General description.*—Bacteria/bead complexes are separated by a magnet, washed, and resuspended. The complexes are then incubated with an HRP-conjugated anti-*Legionella* antibody to form labeled complexes. Washing steps of these labeled complexes allow discarding of the excess HRP-conjugated anti-*Legionella* antibody.

(*2*) *Step-by-step description of the process.*—(*a*) Remove the magnet, add all the content of one of the L3 vials, and shake.

(*b*) Shake gently, without lids, every 3 min for 10 min.

(*c*) Put the magnet as close as you can and wait 3 min to retain the immunomagnetic particles.

(*d*) Discard the supernatant, being careful not to remove any retained particles.

(*e*) Separate the magnet from the cuvet and add the L2 reagent up to line 2 (4.5 mL).

(*f*) Shake gently, without lids, until the particles are resuspended again.

(*g*) Put the magnet as close as you can, and wait 3 min to retain the immunomagnetic particles.

(*h*) Repeat steps (*d*)–(*g*) twice more.

(**c**) *Detection step.*—(*1*) *General description.*—The *Legionella* spp./magnetic bead complexes are visualized by the colorimetric reaction developed when HRP substrates are added.

(*2*) *Step-by-step description of the process.*—(*a*) Add L5 reagent to a vial of L4 up to the mark of 1.3 mL (considering graduation of 0.1 mL in the vial). Shake vigorously, and set aside the dissolved mixture.

(*b*) Empty the cuvet, paying attention not to drag the captured particles.

(*c*) Separate the magnet from the cuvet and add all the contents of the dissolved mixture.

(*d*) Shake gently, without lids, until the particles are resuspended. Wait 2 min while shaking gently. If *Legionella* concentration is visually estimated, then go to the next step. If *Legionella* concentration is estimated by measuring the absorbance, then go to (**i**).

(*e*) After 2 min, if no color difference appears between color test (T) and color control (C), let color develop for 10 min before the next step.

(*f*) Add three drops of L6 and shake gently without lids. Wait 1 min.

(*g*) Bring the magnet up to the cuvet to capture the particles, and wait for 5 min.

(*h*) Compare T to C.

(*i*) Add 100 µL of L6 both in the control cuvet (C) and test cuvette (T) and shake gently. Wait for 1 min.

(*j*) Fix the magnet to the cuvets to retain the magnetic particles and wait for 5 min.

(*k*) Place the supernatants both control (C) and test (T) cuvets into corresponding reading cells. *Important note*: Pipet the supernatant from the opposite side to the magnet, being careful not to drag the particles retained by the magnet.

(*l*) Measure the absorbance at 429 nm on a cell filled with distilled water. Adjust absorbance to zero.

(*m*) Measure the absorbance at 429 nm of the supernatant of the control (C) as reference. Then adjust absorbance to zero.

(*n*) Measure the absorbance of each test (T) supernatant. Read immediately; in all cases read within 10 min.

Interpretation and Test Result Report

(**a**) *For visual interpretation.*—(*1*) The test (T) is considered positive if at 2 min or before 10 min color difference appears with the control (C).

(*a*) The test (T) has higher color than the control (C) 2 min after the beginning of the colorimetric reaction. In such case, stop the reaction following the instructions of this package insert. The general estimation of the level of *Legionella* can be obtained by comparing the test color (T) with the color chart.

(*b*) If there is no color difference 2 min after the beginning of the reaction, let the reaction progress for 8 more min (10 min in total). A positive test (T) for *Legionella* must have a stronger color than the control (C) color 10 min after the beginning of colorimetric reaction. The estimated level of *Legionella* is low, up to two orders of magnitude (10^2 CFU/test portion).

(*2*) A test (T) is considered negative if the test (T) does not have color difference with the control (C) 10 min after the beginning of the colorimetric reaction.

(**b**) *For optical reading.*—(*1*) C*ontrol value.*—After adjusting the absorbance to zero with distilled water, measure the absorbance of the control (C). This absorbance should not be greater than 0.12. Then adjust the absorbance to zero with the control (C) before measuring the absorbance of the test (T) samples.

(*2*) *Cutoff value.*—This value of absorbance is 0.04.

Table 1. Summary of environmental isolated non-*pneumophila Legionella* strains used in this study

Species[a]	Reference No.	Source	Reactivity with the test[b]
Legionella sp.	CCM 59	Environment	+
Legionella sp.	CCM 61	Environment	+
Legionella sp.	CCM62	Environment	+
Legionella sp.	1171	Environment	+
Legionella sp.	1175	Environment	+
Legionella sp.	1177	Environment	+
Legionella sp.	1195	Environment	+
Legionella sp.	1196	Environment	+
Legionella sp.	1211	Environment	+
Legionella sp.	1212	Environment	+
Legionella sp.	1215	Environment	+
Legionella sp.	1234	Environment	+
Legionella sp.	1236	Environment	+
Legionella sp.	1245	Environment	+
Legionella sp.	1246	Environment	+
Legionella sp.[c]	1247	Environment	–
Legionella sp.[c]	1258	Environment	–
Legionella sp.	1260	Environment	+
Legionella sp.	1261	Environment	+
Legionella sp.	1262	Environment	+
Legionella sp.	1265	Environment	+
Legionella sp.	1266	Environment	+
Legionella sp.	1267	Environment	+
Legionella sp.	1287	Environment	+
Legionella sp.	1298	Environment	+
Legionella sp.	1299	Environment	+
Legionella sp.[c]	1300	Environment	–
Legionella sp.	1302	Environment	+
Legionella sp.	1318	Environment	+
Legionella sp.	1333	Environment	+
Legionella sp.	1357	Environment	+
Legionella sp.	1360	Environment	+
Legionella sp.	1362	Environment	+
Legionella sp.	1363	Environment	+
Legionella sp.	1364	Environment	+
Legionella sp.	1367	Environment	+
Legionella sp.	1369	Environment	+
Legionella sp.	1371	Environment	+
Legionella sp.	1372	Environment	+
Legionella sp.	1377	Environment	+
Legionella sp.[c]	1384	Environment	–
Legionella sp.	1387	Environment	+
Legionella sp.	1388	Environment	+
Legionella sp.[c]	1389	Environment	–
Legionella sp.[c]	1390	Environment	–
Legionella sp.	1393	Environment	+
Legionella sp.	1394	Environment	+
Legionella sp.	1395	Environment	+
Legionella sp.	1402	Environment	+
Legionella sp.	1404	Environment	+
Legionella sp.	1408	Environment	+
Legionella sp.	1409	Environment	+
Legionella sp.	1410	Environment	+
Legionella sp.	1413	Environment	+
Legionella sp.	1414	Environment	+
Legionella sp.	1415	Environment	+
Legionella sp.	1417	Environment	+
Legionella sp.	1418	Environment	+
Legionella sp.	1421	Environment	+
Legionella sp.	1422	Environment	+
Legionella sp.	1423	Environment	+
Legionella sp.	1424	Environment	+
Legionella sp.	1425	Environment	+
Legionella sp.	1426	Environment	+
Legionella sp.	1427	Environment	+
Legionella sp.[c]	1433	Environment	–
Legionella sp.	1435	Environment	+
Legionella sp.	1436	Environment	+
Legionella sp.	1437	Environment	+
Legionella sp.	1438	Environment	+
Legionella sp.	1439	Environment	+

[a] Strains were environmental isolated by Microbiology Department of Iproma, an ISO 17025 analytical laboratory (Castellón, Spain).

[b] + = positive reaction; – = negative reaction.

[c] No positive reaction was observed at concentration ≤10^6 CFU.

(*3*) *Negative results.*—Test (T) with absorbance less than cutoff value are negative and reported as Not Detected.

(*4*) *Positive results.*—Test (T) with absorbance equal to or greater than cutoff value are positive and are reported as detected.

(*5*) For the positive results, perform $\log_{10}$ transformation of the absorbance.

(*6*) Estimate the concentration of the target in the volume examined by introducing the $\log_{10}$ value of absorbance into the following equation:

$$y = 2.3061\ x + 4.9815$$

where $x = \log_{10}$ absorbance and $y = \log_{10}$ CFU/volume examined.

(*7*) Result can finally be done like inverse transformation: contamination of the target, CFU/volume examined = 10^y.

Confirmation

Appropriate confirmation procedures are described in the standard ISO 11731 for the detection and enumeration of *Legionella* in water.

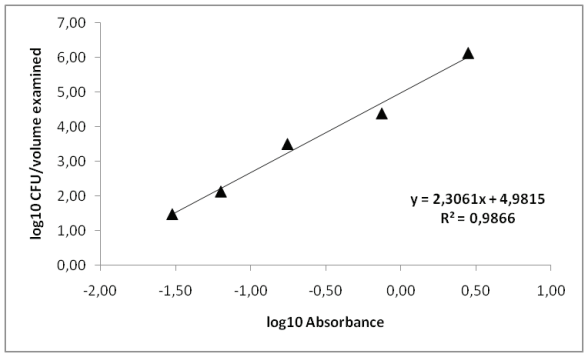

Figure 1. Calibration curve obtained when measuring the relationship between log_{10} values of absorbance (signal of the test method) and log_{10} values of concentration of the target obtained by reference method.

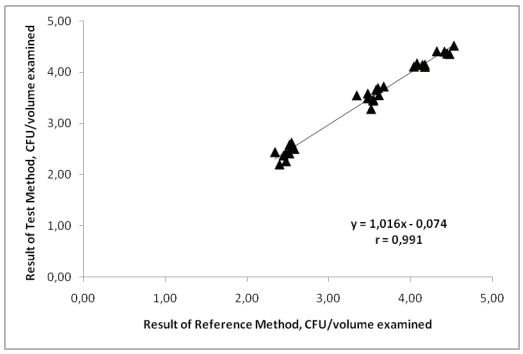

Figure 2. Verification of calibration curve obtained when plotting log_{10} values of test method against the log_{10} values of the reference method.

Independent Validation Studies

These validation studies were conducted under the AOAC Research Institute *Performance Tested Methods*SM (PTM) program. Independent validation studies were conducted by Iproma, S.L. Laboratories (Castellón, Spain), ISO 17025-accredited laboratory trained by AOAC in the PTM Study.

Extended Inclusivity

Seventy-one *Legionella* spp. strains (Table 1) other than *L. pneumophila* were analyzed to determine the sensitivity and specificity of the CEIA test at levels ranging from 10^2 to 10^6 CFU/L. All strains of *Legionella* spp. tested were confirmed by PCR.

Methodology.—*Legionella* strains were grown for 5 days on buffered charcoal yeast extract (BCYE) agar supplemented with glycine, vancomycin, polymixin, and cycloheximide [glycine, vancomycine, polymixin, and cycloheximide (GVPC) medium] to obtain post-exponential phase cultures according to ISO 11731. These cultures were used to inoculate 1 L of water samples. Artificially contaminated 1 L samples were prepared. Water samples were concentrated by filtration through 0.4 µm pore size, 47 mm diameter polycarbonate sterile membranes. After filtration, each membrane was directly placed in a screw-cap sterile container with 10 mL of the reagent L0. *Legionella* was then eluted by vortex-mixing for 2 min. This concentrate represents the prepared sample. Prepared samples were sonicated to disaggregate cell clusters. The volume of this prepared sample was divided into two portions: 9 mL for CEIA testing and 1 mL for culture testing.

All prepared samples were tested by CEIA test following the Legipid *Legionella* kit. Reagents of the Legipid *Legionella* kit were allowed to equilibrate to room temperature before testing the samples. A 9 mL volume of prepared sample (test portion) was applied to the cuvet of a magnetic particle concentrator device. For each batch of analysis, a negative control (without *Legionella*) was introduced. After analysis at room temperature, the results were observed and recorded as positive or negative (detected/not detected).

For culture testing, a 0.1 mL portion of prepared sample (three replicates) was plating onto BCYE agar supplemented with GVPC medium, incubating at 37 ± 1°C for up to 10 days in an atmosphere of air with 5% (volume fraction) carbon dioxide. After incubation, the number of CFU of *Legionella* in the portion was estimated by multiplying that number by the dilution factor.

Results.—Results for the inclusivity study are summarized in Table 1. The CEIA test was found to have a reliability of 90.14% for detecting strains of *Legionella* spp. from environmental isolates. This study showed that the Legipid *Legionella* method detected many different environmental isolates of *Legionella* spp. from a wide distribution of risk facilities. *Caution:* Certain isolates cannot be detected below 10^6 CFUs. The results obtained (64/71 positive) are explained by the serology of the family *Legionellaceae*, and in particular by the common antigens of *Legionellaceae*.

Matrix Study

Methodology.—A response curve was obtained measuring the relationship between the absorbance at 429 nm of the test cuvet and the target concentration in different samples of reference materials having known values tested using reference culture method ISO 11731 (Figure 1). Testing was performed to verify the calibration curve by comparing the test method to ISO 11731 for enumeration of *Legionella* spp. in potable and industrial

Table 2. Relationship between the signal of Legipid test method and concentration of *Legionella* spp.

Contamination level/sample, CFU/vol. examined	Legipid result by replicate No., absorbance/portion tested					Absorbance, mean	Absorbance, log_{10}	CFU/vol. examined log_{10}
	1	2	3	4	5			
1370000	2.78	2.81	2.82	2.79	2.80	2.80	0.45	6.14
24400	0.70	0.61	0.73	0.79	0.90	0.75	–0.13	4.39
3200	0.18	0.17	0.18	0.16	0.19	0.18	–0.75	3.50
135	0.07	0.06	0.05	0.08	0.06	0.05	–1.20	2.13
30	0.03	0.03	0.03	0.03	0.03	0.03	–1.52	1.47

Table 3. Summary of Legipid and ISO reference method results for the independent matrix study

Level	Sample	Log_{10} RM[a]	Log_{10} TM[b]	Mean log_{10} RM	Mean log_{10} TM	s_r mean log_{10} RM	s_r mean log_{10} TM	RSD_r, % RM	RSD_r, % TM	*P*-value from *t*-test, two-tail	*P*-value from *t*-test, one-tail	*F*-test
Potable water												
Low	1	2.34	2.44									
	2	2.52	2.41									
	3	2.48	2.43	2.44	2.35	0.07	0.11	3	5	0.17	0.08	0.40
	4	2.48	2.26									
	5	2.40	2.20									
Medium	1	3.67	3.72									
	2	3.54	3.47									
	3	3.60	3.69	3.60	3.61	0.05	0.11	1	3	0.74	0.37	0.15
	4	3.61	3.55									
	5	3.58	3.66									
High	1	4.08	4.17									
	2	4.15	4.14									
	3	4.04	4.11	4.12	4.13	0.06	0.03	1	1	0.82	0.41	0.17
	4	4.18	4.10									
	5	4.18	4.14									
Industrial water												
Low	1	2.58	2.50									
	2	2.56	2.56									
	3	2.45	2.37	2.53	2.53	0.05	0.10	2	4	0.97	0.48	0.24
	4	2.52	2.58									
	5	2.54	2.62									
Medium	1	3.34	3.54									
	2	3.52	3.28									
	3	3.48	3.49	3.47	3.47	0.08	0.12	2	3	0.95	0.48	0.45
	4	3.48	3.58									
	5	3.54	3.44									
High	1	4.53	4.51									
	2	4.32	4.40									
	3	4.41	4.40	4.44	4.41	0.08	0.06	2	1	0.41	0.20	0.73
	4	4.48	4.35									
	5	4.45	4.36									

[a] RM = Reference method.

[b] TM = Test method.

water samples. For each matrix, naturally contaminated water samples with three different contamination levels (low, medium, and high) per sample were tested. Five replicate test portions per level, per method, were tested covering the whole range of interest (Figure 2). For each contamination level per matrix, the samples were replicated by preparing five sub-samples. Each subsample was tested by both test and reference methods. Thus, 30 measurements by the test method and reference method were conducted for verification of the calibration curve, using naturally contaminated samples.

Water samples were concentrated by filtration through 0.4 µm pore size, 47 mm diameter polycarbonate sterile membranes. After filtration, each membrane was directly placed in a screw-cap sterile container with 10 mL of the reagent L0. Then *Legionella* was eluted by vortex-mixing for 2 min. The volume of each prepared sample was divided into two portions: 9 mL for test method testing and 1 mL for reference culture method testing. Samples were tested by CEIA test following the Legipid *Legionella* protocol as described above. After analysis at room temperature, the absorbance at 429 nm of the final supernatants was measured and the concentration of the target was estimated by a calibration curve. For culture testing, a 0.1 mL portion of prepared sample (three replicates) was plated onto BCYE agar supplemented with GVPC medium, and incubated at 37±1°C for up to 10 days in an atmosphere of air with 5% (volume fraction) carbon dioxide. After incubation, the number of CFU of *Legionella* in the portion was estimated by multiplying that number by the dilution factor.

A graph was plotted with the log_{10} values as bi-dimensional points for the reference and test methods for each test portion,

using the y-axis (vertical) for the test method and the x-axis (horizontal) for the reference method. The points at each level formed a discrete cluster. No outliers were detected. The Linear Regression Program in Excel was used. Repeatability (S_r) and RSD_r of replicates were calculated at each concentration of each matrix for each method using $\log_{10}$ values. A paired *t*-test was applied to determine if a significant difference between the test method and the reference method mean was detected.

Results.—Results from the method comparison study are summarized in Tables 2 and 3. The performance of the test method was found to be comparable to the reference method. The relationship between relative absorbance measured at 429 nm for the test method and the target concentration measured by ISO reference culture method 11731 was calculated as:

$$y = 2.3061x + 4.9815$$

where $y = \log_{10}$ CFU/volume examined and $x = \log_{10}$ absorbance. Linear regression resulted in a regression coefficient of r = 0.989. Agreement between test method and reference method was verified using $\log_{10}$ values and calculated as:

$$y = 1.016\ x - 0.074$$

where $y = \log_{10}$ CFU/volume examined estimated by test method and $x = \log_{10}$ CFU/volume examined obtained by reference method. Linear regression resulted in a regression coefficient r = 0.991. *P*-values from *t*-test were higher than 0.05 for each level per matrix, so no significant difference was detected. For optical reading this kit has an LOD of 40 CFU/volume examined and an LOQ of 60 CFU/volume examined.

Discussion

Inclusivity is defined as the strains, isolates, or variants of the target agent(s) that the method can detect. Considering the common antigens of *Legionellaceae* (21), extended inclusivity testing produced expected results for all assayed *Legionella* spp. strains.

Naturally contaminated water samples (potable and industrial) were examined for the estimation of the amount of *Legionella* spp. using, in parallel, the reference culture method (ISO 11731) and the Legipid test method with optical reading. When these waters were tested with *Legionella* spp. at levels ranging from low (1–99 CFU/mL) to medium (100–999 CFU/mL) to high (1000–9999 CFU/mL), no significant difference, based on *t*-test, was detected between the test method mean and ISO 11731 reference method mean. For optical reading, the LOD was 40 CFU/volume examined and the LOQ was 60 CFU/volume examined. As the absorbance reading was done at the end of the analysis, this modification had no significant impact on the protocol neither on the reagents. This modification has a practical impact limited to the detection step of the test method. After the assay is finished, the supernatant of each cuvet is transferred to a reading cell in order to measure absorbance instead of visual interpretation of the result.

Conclusions

The inclusivity data demonstrated that the test method can detect *Legionella* spp. (90.14%), besides *L. pneumophila*.

The method comparison evaluations of the Legipid test kit demonstrated that this method is equivalent to the ISO 11731 reference method for detection of *Legionella* spp. at contamination levels ranging from low (1–99 CFU/mL, medium (100–999 CFU/mL), and high (1000–99 999 CFU/mL) in potable water and industrial water matrixes.

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