The determination of *Legionella* bacteria in waters samples – Method for their detection and quantification by immunomagnetic separation (IMS) and enzyme-based colorimetric method and protocol for method validation: Legipid® test.

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The determination of *Legionella* bacteria in waters samples – Method for their detection and quantification by immunomagnetic separation (IMS) and enzyme-based colorimetric method and protocol for method validation

Methods for Examination of Waters

This booklet describes the primary validation reported by the manufacturer (A) and two methods which cover respectively the protocols to be used to characterize and validate the method (B), and the detection and quantification of *Legionella* bacteria in water samples (C). Finally, Section D is dedicated to the discussion and report conclusions.

A Detection and quantification of *Legionella* spp. in water samples by immunomagnetic separation (IMS) and enzyme-based colorimetric method. Characterization and primary validation of the method reported by the manufacturer

B Detection and quantification of *Legionella* spp. in water samples by immunomagnetic separation (IMS) and enzyme-based colorimetric method Technical protocol for the verification (secondary validation) of the method

C Detection and quantification of *Legionella* spp. in water samples by immunomagnetic separation (IMS) and enzyme-based colorimetric method

D Discussion and conclusions

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D Discussion and conclusions

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About this report

The bacteria in the genus *Legionella* occur naturally in many natural environments and colonizes a variety of engineered systems that sometimes support their proliferation. They grow optimally inside protozoan hosts, such as free-living amoebae associated with biofilms that coat wet surfaces (1–3). *Legionella* is transmitted from environmental sources through contaminated water that is aerosolized and exposing those nearby via inhalation into the respiratory tract (4). Patients infected with *Legionella* can develop a milder flu-like condition called Pontiac fever or a pneumonia called Legionnaires' disease (LD); both conditions are referred to as legionellosis. LD can be fatal, with between 3 and 33 percent of infections leading to death (5–9). Those at higher risk for developing LD include the elderly, males, smokers, and especially the immunosuppressed, which case-fatality rate can reach 80% even with proper antibiotic treatment (5,10).

Even unreported, LD rates have been rising in the United States and in Europe over the past 20 years suggesting little progress in decreasing risk for *Legionella* (5,11,12). The overreliance of the urinary antigen test, which only detects *L. pneumophila* serogroup 1, coupled with the low rate of diagnostic testing, contributes to underestimation of the number of LD cases (13–17) (2). Although *L. pneumophila* is the most dominant *Legionella* species isolated from patients in North America and Europe (13,17–20), some other species can lead to disease, including *L. micdadei*, *L. bozemanii*, *L. dumoffi*, and *L. longbeachae* (6,21). In the European Economic Area (EEA), the annual notification rate increased from 1.3 per 100,000 in 2014 to 2.2 in 2018. Four countries (France, Germany, Italy and Spain) accounted for 71% of all notified cases in 2018 (22). In Spain the cases declared in 2019 add up to a total of 1,408 with a rate of 3.0 per 100,000 inhabitants (23). Meanwhile, in the United States, incidence of LD increased more than six-fold from 2000 to 2018 (24).

There is great concern about LD acquired in hospitals as settings where sizeable populations at higher risk -due to the user's age and/or health status- may be exposed, which may result in considerable mortality (25,26). Previous field studies provide knowledge about key factors associated with *Legionella* contamination in domestic hot water, among others, free chlorine and water temperature (27–29). However, the inherent complexity in water systems of large buildings such as hospitals make it difficult to pinpoint precise factors that trigger *Legionella* contamination, involving interactive effects of water temperature and flow frequency (30). Cooling towers are the most common source for large community-associated outbreaks. Colonization, survival, and

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proliferation of *L*spp. in cooling towers are necessary for outbreaks to occur. These steps are affected by the chemical and physical parameters of the cooling tower environment and disinfection strategies influences the ability of *Legionella* spp. to colonize cooling towers (31).

The mitigation of *Legionella* colonization and disinfection of water systems used in hospital settings are a key factor for controlling and preventing associated *Legionella* infections (15,32). However, environmental monitoring of *Legionella* is also fraught with difficulties in these settings, including what detection methods to use and how to interpret the data. Water systems have traditionally been monitored using culture-based methods as the gold standard, which can take many days to detect growth, making rapid decisions impossible, and can be biased toward *L. pneumophila* and a few other *Legionella* spp. (33). Furthermore, control strategies (heat treatment, chlorine-based disinfectants, and copper-silver ionization) are known to trigger *L. pneumophila* to enter a viable but non culturable (VBNC) state (34,35), which does not form visible colonies on plates but may infect different types of human macrophages and amoebae (21). These drawbacks also make it difficult to identify sources of LD outbreaks, which are not uncommon despite regulations and guidelines addressing *Legionella* contamination in water systems (36,37). Polymerase chain reaction (PCR) methods exist, but their ability to differentiate between viable and nonviable organisms is still evolving (38). A culture method specifically for *L. pneumophila* has been developed that uses a liquid-based most-probable-number (MPN) approach (Legiolert™/Quanti-Tray™, IDEXX) but generally trends higher in concentration estimations. One limitation of the reported evaluations is the lack of confirmation tests on positive wells in the tray with genetic methods (39). Therefore, it is likely that combinations of culture-based methods with rapid not growth-based methods will be used in the future to assist in developing risk estimates.

According to the reported studies on the dynamics and phenotypic plasticity of *Legionella* cell surface, the ability of *Legionella* to cause LD hinges predominantly on its cell envelope (40). These findings highlight the importance of detecting legionellae cells in their environment by considering their cell envelope as an analytical target. Hence, we used an immunomagnetic separation (IMS) technique based on the multivalent antigen-antibody interactions at the cell envelope level, thereby making this approach of high diagnostic value for a preventative purpose (41, 42).

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A Detection and quantification of *Legionella* spp. in water samples by immunomagnetic separation (IMS) and enzyme-based colorimetric method. Characterization and primary validation of the method.

A1 General

Legipid® *Legionella* Fast Detection is a third party validated commercial methods based on combined magnetic immune-separation (IMS) and enzyme-based colorimetric assay 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 is 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 revisions 11731-2004 and 2017 "Water Quality: Detection and Enumeration of *Legionella pneumophila"* in potable water, industrial water, and wastewater. The Water Management Society (WMS) - Rapid Microbiology Industry Liaison Group- has included the Legipid® test in its guides in UK, as it is also included in the regulation of Spain and Portugal. For third party validated commercial methods that fulfil the requirements given in this part, manufacturer's instructions for routine application shall be accurately followed, as is the case of Legipid® Legionella Fast Detection method.

A2 Inclusivity and exclusivity

Collection strains were obtained from American Type Culture Collection (Manassas, VA), Spanish Type Culture Collection; Valencia, Spain), and National Collection of Type Cultures, Central Public Health Laboratory (London, UK). The other referenced strains were environmentally isolated by ISO 17025 accredited laboratories. *L. pneumophila* serogroups were tested positive by PCR for *L. pneumophila*. Species of *Legionella* non-*pneumophila* were tested positive by PCR for *Legionella spp .*and negative by PCR for *L. pneumophila*.

- Inclusivity

(**a**) *Inclusivity study (Legionella pneumophila).* —Nineteen *L. pneumophila* strains of different serogroups were analyzed to determine the sensitivity and specificity of the IMS test at levels ranging from 10^3 to 10^6 CFU/L. All strains of *L. pneumophila* tested were confirmed by PCR. Seventy-one *Legionella* spp. strains other than *L. pneumophila* were

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analyzed to determine the sensitivity and specificity of the IMS test at levels ranging from 10^2 to 10^6 CFU/L. All strains of *Legionella* spp. tested were confirmed by PCR. From collection source: *L. pneumophila* sg 1 (ATCC33152), From environmental sources: *L. pneumophila* sg 12−14, *L. pneumophila* sg 4 or 5, *L. pneumophila* sg 14, *L. pneumophila* sg 8, *L. pneumophila* sg 6, *L. pneumophila* sg 2,3, *L. pneumophila* sg 3,6, *L. pneumophila* sg 3, *L. pneumophila* sg 4, *L. pneumophila* sg 5, *L. pneumophila* sg 1 or 9, *L. pneumophila* sg 1, *L. pneumophila* sg 2−15, and seventy-one *Legionella* spp. strains.

(*1*) *Methodology*. —Aliquots of 1 mL of 19 *L. pneumophila* strains were inoculated into 1 L of water. After homogenization, 10 mL samples were tested on the IMS test as described previously; other 10 mL samples were concentrated by filtration and cultured.

(*2*) *Results*. —The IMS test was found to have a reliability of 100% for detecting specific strains of *L. pneumophila* from a variety of sources including culture collection and environmental isolates.

(**b**) *Inclusivity study (Legionella* spp.*).* —Seventy-one *Legionella* spp. strains other than

L. pneumophila were analyzed to determine the sensitivity and specificity of the IMS test at levels ranging from 10^2 to 10^6 CFU/L. All strains of *Legionella* spp. tested were confirmed by PCR.

(*1*) 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. Artifi cially contaminated 1 L samples were prepared. Water samples were concentrated by fi ltration through 0.4 µm pore size, 47 mm diameter polycarbonate sterile membranes. After filtration, each membrane was directly placed in a screwcap 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 IMS testing and 1 mL for culture testing. All prepared samples were tested by IMS test following the Legipid Legionella kit. Reagents of 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 cuvette of a magnetic particle concentrator device. For each batch of analysis, a

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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.

(2) Results. —The IMS 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*.

• Exclusivity list (tested microorganisms recognized as not belonging to Legionella genus and/or being phylogenetically close).

(**a**) *Exclusivity study.* —Sixteen strains of non-*Legionella* strains were tested to determine the specificity of the kit. All the strains of *Legionella* spp. tested by the IMS test were confirmed by PCR. From collection source: *Escherichia coli, Enterococcus faecium, Clostridium perfringens, Salmonella typhi, Candida albicans, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas fluorescens, Bacillus subtilis, Pseudomonas putida, Proteus vulgaris, Listeria monocytogenes, Enterobacter cloacae, Serratia marcescens, Caulobacter spp.*

(*1*) *Methodology*. —Aliquots of 1 mL of 16 non-*Legionella* strains were inoculated into 1 L of water. After homogenization, 10 mL samples were tested on the IMS test; other 10 mL samples were concentrated by filtration and cultured.

(*2*) *Results.* — Of the 33 strains tested, 30 were correctly identified as negative by the IMS test, resulting in a specificity of 93%. *L. beliardensis, L. adelaidensis*, and an environmentally isolated *Legionella* spp. were identified as positive by the IMS test at levels of 1800, 230, and 3900 CFU/mL, respectively.

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A3 Robustness

(**a**) *Ruggedness studies.* —Ruggedness testing for this study involved variation of temperature at which the device was tested and variation of time at which it was read. Ruggedness testing additionally involved variability in stability of lots of the tests used.

(*1*) *Methodology.* —

(*a*) *Temperature study.* —IMS test reagents were maintained at 10, 20, and 30°C to mimic the potential temperature range at which the devices could be tested by the user. When devices had equilibrated to these temperatures, five negative for *L. pneumophila* (non-inoculated samples) and five positive for *L. pneumophila* [three samples inoculated with high levels of *L. pneumophila* (150 CFU/mL) and two samples inoculated with low levels of the organism (50 CFU/mL)] were tested on the devices at 10, 20, and 30°C.

(*b*) *Reading device time study.* — In this study, tests were read at 5, 15, 25, and 30 min. Five samples negative for *L. pneumophila* (non-inoculated samples) and five positive for *L. pneumophila* [three samples inoculated with high levels of *L. pneumophila* (150 CFU/mL) and two inoculated with low levels of the organism (50 CFU/mL)] were tested on the devices. IMS tests were read at 5 min intervals over a period of 30 min.

(*2*) *Results*. —The IMS test was found to function properly throughout an operational temperature range from 20 to 30°C. Additionally, when interpreting the test result in the reported temperature range, the test result should be evaluated between 5 and 30 min after stopping the reaction. In order to properly identify negative samples, the manufacturer recommends reading the device as soon as 5 min after stopping the reaction.

(**b**) *Lot-to-lot and stability study.* —The test had a shelf life of 6 months. Three different lots were tested at 0, 3, and 6 months after manufacturing.

(*1*) *Methodology*. —For each lot and time, five spiked samples were prepared with *L. pneumophila* at a level of 100 CFU/mL and tested. Five non-inoculated controls were run with these experiments.

(*2*) *Results*. —The variability of the IMS test was evaluated across three production lots. In this evaluation, no significant lot-to-lot variability was observed. The stability of the IMS test was evaluated for each lot short-term (product recently manufactured), medium term (3 months), and long-term (6 months). The IMS test demonstrated exceptional

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stability for at least 6 months. These results strongly suggest that this product has at least the 6-month stability under refrigeration conditions.

A4 Water testing and method comparison study

A4.1 General

The method comparison evaluations of the IMS assay clearly demonstrated that this method is equivalent to the ISO 11731 reference method for *Legionella* spp. determination.

A4.2 Water testing

(*1*) *Methodology*. —Intensive water testing was performed to compare the IMS method to the ISO reference method 11731 for detection of *L.* spp. in potable, natural, and industrial water samples. Inoculation levels were determined.

(*a*) *Spiking of waters*. —*L. pneumophila*, ATCC 33152, was used to inoculate the matrixes. *L. pneumophila* (ATCC 33152) was grown for 3 days on BCYE agar (buffered charcoal yeast extract) supplemented with glycine, vancomycin, polymixin, and cycloheximide (GVPC medium) to obtain exponential phase cultures. These cultures were used to inoculate water samples. Artificially contaminated samples were prepared. Samples of 2.6 L of each matrix for each level were collected and then divided into three portions; 100 mL was used to test the level of the target by the ISO method and to test the level of background flora by standard plate count of dilutions series of each type of sample, 500 mL was used to obtain five 100 mL negative portions, and 2 L was inoculated with *L. pneumophila* to obtain twenty 100 mL portions with a target of about 10–500 CFU/mL. These samples were left to equilibrate for 24 h prior to commencement of the analysis.

(*b*) *Filtration*. —Portions 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 *L. pneumophila* was eluted by vortex-mixing for 2 min. This concentrate represents the prepared sample. The volume of this prepared sample was divided into two portions: 9 mL for IMS testing and 1 mL for culture testing.

(*c*) *IMS analysis.* —

(*i*) All the prepared samples were tested by the IMS test following the IMS protocol.

(*ii*) Reagents of the IMS kit were allowed to equilibrate to room temperature before testing the samples.

(*iii*) A 9 mL volume of prepared sample was applied to the cuvette of a magnetic particle concentrator device.

(*iv*) For each batch of analysis both a negative control (without *L. pneumophila*) and a positive control (sample with 100 CFU/mL of *L. pneumophila*) were introduced.

(*v*) After analysis at room temperature, the results were observed and recorded.

(*d*) *ISO 11731 reference method analysis.* —

(*i*) After filtration, a 0.1 mL portion of prepared sample was plated onto BCYE agar supplemented with GVPC medium, incubating at 37 ± 1°C for up to 10 days in an atmosphere of air with 5% (v/v) carbon dioxide.

(*ii*) After incubation, the number of CFUs of *L. pneumophila* in the portion was estimated by multiplying that number by the concentration factor.

(*2*) *Results.* —

(*1*) This study showed 100% agreement between the IMS method and the ISO 11731 reference method.

(*2*) The IMS method produced no false negative results and four false-positive results.

A4.3 Method comparison study

(*1*) *Methodology*. —Potable water, natural water, and industrial water were collected. Water samples were tested on day of sampling for the presence of *L. pneumophila* using, in parallel, the reference culture method (ISO 11731) and the IMS test.

(*a*) The matrixes were screened before inoculation with the test organism for indigenous bacteria/*Legionella*.

(*b*) A singularly inoculated lot of each matrix was then prepared. The low and high target spike levels were the same for all matrixes: a low target level of 10–99 CFU/mL and a high target level of 100–999 CFU/mL.

(*c*) *L. pneumophila*, ATCC 33152, was used to inoculate the matrixes. *L. pneumophila* (ATCC 33152) was grown on BCYE agar supplemented with GVPC medium to obtain

exponential phase cultures. These cultures were used to inoculate water samples. These samples were left to equilibrate for 24 h prior to commencement of the analysis.

(*d*) The ISO 11731 protocol was followed for concentration of the target organisms by the filtration procedure. Because the sample filtration schemes for the reference method and the IMS method were the same, 45 samples were tested: 20 with a low target spike level of 10–99 CFU/mL, 20 with a high spike of 100–999 CFU/mL, and five uninoculated samples that served as negative controls. Samples for the ISO reference method were processed with test portions.

(*e*) *Filtration. —*

(*i*) Original water samples were concentrated by filtration through 0.4 µm pore-size, 47 mm diameter polycarbonate sterile membranes.

(*ii*) After filtration, each membrane was directly placed in a screw cap sterile container containing 10 mL of the reagent L0. Then *L. pneumophila* was eluted by vortex-mixing for 2 min. This concentrate represents the prepared sample.

(*iii*) The volume of this prepared sample is divided into two portions: 9 mL for IMS testing and 1 mL for culture testing.

(*iv*) As the same sample is tested by both the reference method and test method, confirmation of all test results was obtained from the results of the ISO 11731 reference method (paired study).

(*f*) *IMS analysis. —*

(*i*) All the prepared samples were tested by the IMS test following the IMS protocol.

(*ii*) Reagents of the IMS kit were allowed to equilibrate at room temperature before testing the samples.

(*iii*) A 9 mL volume of prepared sample was applied to the cuvette of a magnetic particle concentrator device.

(*iv*) For each batch of analysis both a negative (without *L. pneumophila*) and a positive control (sample with 104 CFU/L of *L. pneumophila*) were introduced.

(*v*) After analysis at room temperature, the results were observed and recorded.

(*g*) *ISO 11731 reference method analysis. —*

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(*i*) After filtration, a 0.1 mL portion of prepared sample was plated onto BCYE agar supplemented with GVPC medium, incubating at 37 ± 1°C for up to 10 days in an atmosphere of air with 5% (v/v) carbon dioxide.

(*ii*) After incubation, the number of CFUs of *L. pneumophila* in the original sample was estimated by multiplying that number by the concentration factor.

(*2*) *Results. —*

(a) Overall, the Chi-square value of 1.8 indicated that there was no significant difference between IMS method and the ISO 11731 reference method. The performance of the IMS test was found to be comparable to reference method.

(b) Overall, IMS method gave a false positive rate of 7% and a false-negative rate of 2%.

(*c*) The IMS test can yield a result for the presence of *L. pneumophila* in a potable water, natural water, and industrial water faster than the ISO reference method.

(*d*) When testing potable water, there were two false-negative results (one low inoculation level, one high inoculation level).

(*e*) When testing industrial water, there was one false negative result (one low inoculation level, 0 high inoculation level).

(*f*) When testing natural water, there was false-negative result (one high inoculation level).

(*g*) When testing potable water, there was one false-positive result (one low inoculation level).

(*h*) When testing industrial water, there were a total of four false-positive results (four low inoculation level).

(*i*) When testing natural water, there was one false-positive result (one low inoculation level).

(*j*) Results were clearly and easily interpreted.

(*k*) This study showed 97% agreement between the IMS method and the ISO 11731 reference method.

(*l*) The IMS method produced four false-negative results and six false-positive results.

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A4.4 Method comparison study involving both culture and PCR

(*1*) *Methodology*. —

(i) Three laboratories participated in this study. Of these laboratories, two were public health laboratories conducting *Legionella* testing in its routine work. The other one was a private laboratory also accredited for *Legionella* testing that are regularly testing water samples for clients maintaining facilities. All laboratories were experienced in the detection and isolation of legionellae by culture and PCR and demonstrated competence by their performance in external quality assurance schemes for *Legionella* isolation.

(ii) Laboratory 1 is Departamento de Microbiología General III, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, Campus Moncloa, 28040 Madrid, Spain. Laboratory 1 performed the standard ISO 11731 method and the IMS method on water samples containing target organism and different mixtures of interfering microbiota.

(iii) Laboratory 2 is Instituto de Ciencias de la Salud, Ctra. de Extremadura Km. 114, 45600, Talavera de la Reina, Spain. Laboratorio Regional de Salud Pública Consejería de Sanidad y Consumo/Comunidad de Madrid, C/ Sierra del Alquife N 8, 2 Planta, 28053 Madrid, Spain. Laboratory 2 conducted a comparison study with all three techniques: the IMS method, qPCR, and culture.

(iv) Laboratory 3 is Eurofins- Iproma, S.L, Cno.de la Raya 46, 12005 Castellón, Spain. Laboratory 3 performed the IMS method on water samples with and without different biocides.

(v) To ensure that all laboratories were able to use the immunomagnetic method reliably, a training trial was performed at the beginning of the study.

(a) **Trial with interfering microbiota**

(i) *Legionella pneumophila* serogroup 1 (ATCC 33152) was provided by Eurofins (France).

(ii) Environmental isolates of no-Legionellae microorganisms usually present at environmental water samples was used to prepare two mixtures of potentially interfering microbiota. The Mixture I consisted of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Streptococcus faecalis*. The Mixture II consisted of *Pseudomonas aeruginosa*, and Ascomycetes.

(iii) A water matrix tested negative for both methods was selected.

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(iv) Three groups of microbial samples were prepared. The first group consisted of three independent 250 ml-portions of this matrix, each spiked with 2×10^4 CFU of *Legionella pneumophila* serogroup 1. The second group consisted of three independent 250 ml-portions of this matrix, each portion spiked with 2×10^4 CFU of *Legionella pneumophila* serogroup 1 and 10^8 CFU of each microorganism belonging to the mixture I. The third group consisted of three independent 250 ml portions of this matrix, each portion spiked with 2×10^4 CFU of *Legionella pneumophila* serogroup 1, 10^8 CFU of *Pseudomonas aeruginosa* and 5×10^3 CFU of Ascomycetes, corresponding to mixture II.

(v) All 250 mL-portions were assayed by both culture and the IMS method.

(b) **Trial with biocides**

(i) Three biocides were selected for this experiment. Hypochlorite (oxidizing agent) and DBNPA (not oxidizing agent) did not need to be internalized inside the cell to act on the cell, and Mefacide (based on isothiazolinone) need to be internalized because it acts on metabolism.

(ii) Five independent experiments were conducted. For each independent experiment a suspension of *Legionella pneumophila* serogroup 1 was prepared and divided into four portions.

(iii) One portion without biocide was considered as positive control

(iv) Other three portions were mixed each one with a 1 ppm of hypochlorite, 50 ppm of DBNPA 20% and 100 ppm of Mefacide, respectively.

(v) After 1-hour contact time, both control and samples were assayed by the IMS method.

(c) **Comparative trial**

(i) Sampling. —

(1) Water samples (a total of 65) were collected from both urban and rural areas of Castilla La Mancha and Madrid (Spain). Water samples included different matrices as cooling tower, sanitary water (hot/cold), nebulizer and spa matrices.

(2) Water samples of 2 L from were collected in accordance with ISO 19458:2006 into sterile containers containing sodium thiosulphate to neutralize any residual oxidizing biocides in the water. Samples were transported to the laboratory as soon as possible and processed within 24 h of collection.

(3) Eighteen additional artificial samples were prepared by spiking *Legionella pneumophila* serogroup 1 ATCC33152 (Bioréférence, Eurofins) and were analyzed by qPCR and the IMS method.

(ii) Filtration and resuspension of cells from water samples. —

(1) Each sample was mixed well by shaking by hand then filtered through a 2.7 µm glass fiber pre-filter (Filterlab) and a 0.4 µm nylon filter (Millipore) overlapped. Prefiltration allowed separation of bacteria from bigger particles and this was discarded after filtration.
(2) The filter was then removed from the filter holder and placed with 20 ml of the diluent L0 (Biótica) in a 100 ml sterile plastic container, vigorously vortexed for 2 minutes.

(3) Each 20 ml concentrated sample was thoroughly mixed and then divided into three portions.

(4) One 10 ml portion was assayed by qPCR (Applied Biosystems) for *Legionella* spp. with internal process controls in order to assess inhibition or suboptimal reaction conditions.

(5) The second 1 ml portion was assayed by culture for *Legionella* species following ISO 11731.

(6) The third 9 ml portion was assayed by the IMS method (Legipid®, Biótica).

(7) All three techniques were applied to the same concentrated sample, so results were not affected by variety of filtration/resuspension step.

(iii) Reference culture method. —

(1) Culture procedure followed the ISO standard 11731–1:2004. 0.1-0.5 ml portions of concentrated sample were cultured onto the selective medium GVPC without pretreatments, at 36°C for 10 days.

(2) Presumptive colonies were cultivated on buffered charcoal yeast extract media, BCYE and BCYE-Cys, at 36°C during at least 2 days. Colonies grown on BCYE but not on BCYE-Cys were confirmed as Legionella. Moreover, agglutination latex test was also applied for suspicious colonies.

(iv) Immunomagnetic method. —

(1) 9 ml portion of each concentrated sample was assayed by IMS method (Legipid®, Biótica).

(2) Assays were conducted according to the instructions of manufacturer.

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(v) qPCR Legionella spp assay. —

(1) 10 ml portions of concentrated sample in falcon tubes were centrifugated at 2,000 rpm for 10 minutes, obtaining 350 µl of the supernatant. After addition of 50 µl of reaction buffer on each supernantant, two replicates of 10 µl were assayed:

(i) DNA extraction. 50 µl of lysis reagent was added on each microcentrifuge tube containing the supernatant, to facilitate the cell membrane breakage. The tubes were vortexed and then incubated at 95°C for 10 min in a thermomixer. Following incubation, tubes were left to equilibrate at room temperature for 5 minutes. The tubes were then vortexed and centrifuged at 6,000 rpm for 2 minutes and each supernatant was transferred to an eppendorf tube. Extracted DNA from concentrated samples was added to PCR mixtures immediately or stored at −20°C a maximum of 2 days.

(ii) DNA amplification. PCR tubes were placed in a model StepOne 96-well thermal cycler (Applied Biosystems). Two 20-base oligonucleotides were used as amplimers enclosing a 386-bp fragment of the 16S rRNA gene. p1.2 (59-AGGGTTGAT AGGTTAAGAGC-39) was located at positions 451 to 470, and cp3.2 (59-CCAACAGCTAGTTGAC ATCG-39) was complementary to positions 836 to 817.The amplification reactions were performed in optical microplates using a total volume of 25 µl. Ten microliter of extracted DNA was added to each well on 15 µl of PCR mix containing thermostable Taq polymerase and specific probe for Legionella spp (gen 16 s, 386 pb (451–837)). All samples were amplified in duplicate, reporting the average of the two obtained results. The reaction mixtures contained 1× TaqMan universal PCR master mix (PCR buffer, deoxynucleoside triphosphates, AmpliTaq Gold polymerase, Amp Erase uracil N-glycosylase [UNG], MgCl2; LifeTechnologies, Madrid, Spain), 300 nM of each Legionella-specific primer, 250 nM TaqMan Minor Grove Binding (MGB) Legionella-specific probe labeled with 6-carboxy fluorescein (FAM) - excitation and emission wavelengths of 495 and 515 nm respectively- , and 250 nM TaqMan Minor Groove Binding (MGB) probe labeled with VIC - excitation and emission wavelengths of 528 and 546 nm respectively-, to detect internal control of the process (IPC).

(iii) Quantification. Quantitative results were obtained by a calibration curve in the range of 10–100,000 genomic units (GU), with five levels and three replicates per level. Included in each run were three negative controls and two positive controls, and one internal positive control (IPC) for each sample. The inclusion of the IPC in each reaction avoids false negatives due to the presence of substances that inhibit PCR. The IPC signal proves that PCR reagents are working and amplifying satisfactorily. The inhibition

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is reported as partial if the IPC is inhibited in one of the replicates, and it is reported as complete if the IPC is inhibited in the two replicates.

(*2*) *Results.* —

(a) **Trial with interfering microbiota**

(i) The influence of background organisms on the determination of *Legionella* in water was investigated

(ii)Two different microbial mixtures (Microbiota I, Microbiota II) were prepared and tested negative for the IMS method. These microbial mixtures consisted mainly of background organisms usually present at water from cooling tower.

(iii) The background organisms were added to water inoculated with viable *Legionella pneumophila* serogroup 1. Previously the water matrix was tested negative for both methods.

(iv) The results suggested that the presence of a large number of background bacteria in the water sample could reduce the growth of *Legionella pneumophila*. Inhibition was more pronounced with one of the mixtures (Microbiota II) containing *Pseudomonas aeruginosa* and Ascomycetes. This preliminary study suggested the importance of the

background organisms in water for inhibition of growth of *Legionella* organisms.

(v) No significant effect was observed on the signal of the IMS method.

(b) **Trial with biocides**

(i) The loss or severe reduction in the IMS method signal after exposure of approximately 1.0×10^3 colony forming units per milliliter (CFU ml^{-1}) to 3 ppm of hypochlorite or 2,2-dibromo-3-nitrilopropionamide (DBNPA) 20% were 99% and 90 % respectively.

(ii) Reduction in IMS method signal was less evident after exposure of L. pneumophila to 100 ppm of Mefacide (5 %) than other biocides.

(iii) Biocides which are harmful to the antigens exposed at the cell envelope caused rapid loss of IMS method signal as opposed to the biocides that need to be incorporated inside the cell to work.

(iii) Results show that the IMS method distinguished between intact cells and cells damaged by biocides at level of cell envelope integrity. This could be explained because the IMS method introduced extensive washing of bacteria-beads complexes by selected

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working buffers while shaking, removing loosely bound bacteria. The number of interactions between bead surface and damaged cell surface was likely to be insufficient.

(iv) Therefore, IMS method can provide an indication of cell viability based on the integrity of the outer cell envelope, depending on the action mechanism of the applied biocide.

(c) **Comparative trial**

(i) The rate of inconclusive results found in this study for culture (36.9%) and qPCR (12.3%) confirmed the limitations of these two techniques anticipated by other studies. IMS method reduced the likelihood of inconclusive results in *Legionella* testing.

(iii) Few positive PCR or positive IMS method results were confirmed by culture (approximately 8% in both cases). This could be explained by the frequent presence of contaminating microorganisms that interfere with *Legionella* growth (36.9%), which lead to decreased sensitivity. Moreover, *Legionella* cells that are viable but non-culturable are not detected by conventional culture.

(iv) The difference in positivity rates was due largely to false-negative culture results rather than to false positive results by PCR or IMS method.

(v) Concerns remains over PCR false-positive results due to contamination with dead cells or free DNA. Moreover, eight samples (12.3%) showed complete or partial PCR inhibition. Neither DNA nor damaged envelope cells were detected by IMS method.

(vi) For natural water samples the mean log difference between quantitative PCR result (GU/L) and IMS method result (equivalent CFU l^{-1}) was 0.70 (SD = 1.02) based on 9 pairs of samples in which *Legionella* spp. were detected by both methods.

(vii) For artificial water samples, there was 8 pairs of samples for which the mean log difference was −0.05 (SD = 0.16).

(viii) This probably reflects the fact that practically all DNA target for PCR was contained into viable cells inoculated in artificial samples, which are the target of the IMS method at the same time. However, a fraction of DNA detected by PCR in environmental samples might be free or belonging to dead or damaged cells, without sanitary risk.

(ix) The results suggest that the performance of PCR and culture techniques are more influenced than the IMS by the characteristics of the water matrix (background microorganisms, inhibitory substances). Immunomagnetic separation introduced a purification step to detect target cells separated from debris or other cells

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(x) The major discrepancy of the results was observed in the dirtier samples, were also more susceptible to colonization by *Legionella*. Immunomagnetic separation of captured microbial target allowed minimizing matrix effects providing a better recovery of the *Legionella* present in the sample. As the detection step in the IMS method is always performed at the end of analysis when the captured target has been purified, the conditions of the final measurement are consistent and independent of the water matrix.

(xi) The IMS method used *Legionella*-specific polyclonal antibody-coated beads so a broader spectrum of suitable antigens on the bacterial surface can be attributed to contributing to an increase in the likelihood of detection.

(xii) Thereby it seems that the IMS method can detect an intact whole cell target more effectively in its environment. This occurs even though some of the samples (mainly from cooling towers) presented dirtiness that made handling difficult.

(xiii) IMS method could be a more reliable option, particularly in the analysis of water samples with high levels of contamination.

A4.5 Summary

(a) Overall, of a total of 474 water samples, 438 were coincident for both culture and IMS methods (263 positives, 175 negatives)

(b) 23 out of 474 ater samples were positive by IMS method and negative by culture method.

(c) 13 out of 474 water samples were negative by IMS method and positive by culture method.

(d) Sensitivity was (263/276) 95.3 %

(e) Specificity (175/198) was 88.4 %

(f) False positive rate (23/198) was 11.6 %

(g) False negative rate (13/276) was 4.7 %

(h) Efficiency (438/474) was 92.4 %

(i) Difference in the detection rate of *L. spp.* in the water samples can occur. Probable causes for unexpected results are summarized:

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(1) The culture method is a standard method for the determination of *L. spp.,* but there is a risk of underestimation in the number of cells for the following reasons:

(*i*) It can be expected that for pure exponentially growing cultures, test method and culture isolation show better comparison because the majority of cells are in a viable culturable state. In contrast, when environmental samples are tested, VBNC cells may be also detected by the test method, but these VBNC bacteria cannot grow in culture media. Especially for low levels (10–99 CFU/mL)/test portion, the proportion of VBNC target may be more significant for environmental samples, according to the available scientific reports.

(ii) L. spp. has been shown to form VBNC cells that may be responsible for the failure to culture viable *L.* spp*.* from some environmental sources. Bacteria exposed to potentially lethal environmental conditions including nutrient restriction, oxidative stress, heat, UV irradiation, osmotic stress, or sublethal concentrations of antibacterial compound undergo physiological or morphological alterations that complicate the detection and accurate enumeration of such stressed bacteria using available culture methods. However, these VBNC forms may be detected by using the test kit method that combines magnetic beads and specific anti-*L. spp.* antibodies.

(*iii*) Recovery of viable cells from environmental samples by culture isolation is usually very poor due to specific requirements of growth, overgrowth by other bacteria, and loss and damage of *Legionella* during sample preparation with acid buffer or heat treatment to eliminate or reduce the number of non-*Legionella* bacteria. For all these reasons, it is expected that the number of positive samples by the IMS test method may be higher than by culture isolation for environmental samples.

(**2**) False-negative results could be produced by operator error. In particular, if the L3 reagent has not been equilibrated at room temperature before use, color signal may not be detected before stopping the reaction. Although it is improbable, false negative results can be expected if the operator partially loses the magnetic immunoparticles during the washing steps. Obviously, if the user adequately follows the instructions of the protocol, these issues will not occur. False negatives can be produced because the probability of detection depends on the level of the target in the sample; at a low level the probability of positive detection decreases, as corresponding to a qualitative test. Finally, clumping of bacterial cells may also produce a false-negative result because a bacterial clump strongly aggregated (one aggregate can contain 200–300 cells) may not be captured by the magnetic immunoparticles, but it can be computed only as 1 CFU in the plate.

Certainly, structure of the biofilm in the environment is easy to break by conventional procedures of homogenization.

Natural environment may contain many damaged cells that do not really pose a health risk because they would have died in their natural context. However, some of these cells could be restored and recovered under the artificial conditions of a culture method, which has this recovery as the purpose instead of a quantification of the target in its environment. Procedures are directed toward the isolation of culturable colonies, in part to facilitate comparison of environmental and clinical isolates during outbreak investigations; in fact, the use of ROS scavengers (pyruvate or glutamate) can provide a greater recovery in this type of methods.

Yielding of between 20 and 1,350 Legionella cells per vesicle and 25 vesicles per day have been reported. Release of Legionella before or after of analytical steps from protozoa and/or vesicles may also cause variability on the results.

Finally, *Legionella* results depends on the sample collection and processing methods used (flushing or unflushing, filtered volume). For IMS method, it is recommended to filter at least a volume of 1000 mL, especially for flushed samples, because the target is not uniformly distributed in the water and the quantity of the target eluted from the filter depends on the volume processed.

A5 Collaborative trial

(*1*) *Methodology*. —

(a) A collaborative trial involving twelve independent laboratories was performed to evaluate the validity of the IMS by testing identical samples.

(b) The collaborative trial was designed and conducted according to internationally accepted guidelines [37,41-49]. It has been shown that concentration methods can have highly variable recovery rates, making difficult to obtain identical samples especially for low concentrations of L. pneumophila [50].

(c) Since the objective was the evaluation of the detection part of the IMS method, the tested sample simulated the concentrated sample that is habitually obtained in the laboratory from an original sample, thus avoiding the concentration phase.

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(d) In this collaborative trial, a microbiological reference material in pill format was used (BaCuanti, Labaqua, Spain). According to the manufacturer´s instructions, water samples were obtained by diluting these pills.

(e) The twelve participating laboratories received pills of L. pneumophila at four levels: (i) pills P6 and P8 as negative control, (ii) pills P1 and P3, containing a medium level of L. pneumophila, (iii) pills P2, P5 and P9, containing a high level of L. pneumophila, and (iv) pills P4 and P7, containing a low level of L. pneumophila.

(f) Each participant received a detailed protocol describing the culture technique, the immunomagnetic run, and a reporting form to record the obtained results.

(g) Samples preparation: The pills were supplied to the participating laboratories into individual sealed vials. For sample reconstitution, the safety seal was removed under aseptic conditions, the vial was opened, and 20 mL sterile distilled water were added, allowing to mix for 10 min at room temperature, gently shaking every 2 min. Sterile water was added up to a final volume of 100 mL. Three serial decimal dilutions (10^{-1},10^{-2}, and 10^{-3}) of each sample were prepared.

(i) Reference culture method: Determination of L. pneumophila by culture isolation was conducted in accordance with the ISO 11731-Part 2. Five milliliters of each sample, as well as its corresponding 10-fold serial dilutions were filtered through cellulose ester membranes (11406-47-ACN; Sartorius, Germany). The membranes were placed on the surface of the BCYE-α+GVPC medium (bioMérieux; Spain) and were incubated at 37°C, preferably in a 5% CO2 atmosphere for a period between 5 and 10 days.

(j) Immunomagnetic technique: Analysis using the IMS method was performed in accordance to the protocol described in Section C. Results were reported as presence/absence in 9 mL, and the approximate concentrations of L. pneumophila were estimated by intercalation of the end-point colour developed in the analysed sample in the colour chart provided by the manufacturer.

(k) Accordingly, samples similar to the negative control one were labelled as <LOD (limit of detection), colour between the negative control and the first colour mark corresponded to 10^2–10^3 CFU/9 mL, colour similar to the first colour mark corresponded to 10^3 CFU/9 mL, colour between first and the second colour mark corresponded to 10^3–10^4 CFU/9 mL, colour similar to the second colour mark corresponded to 10^4 CFU/9 mL, and colour darker than the second colour mark was indicative of >10^4 CFU/9 mL.

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(l) Statistical data analysis: The results reported by eleven of the twelve participating laboratories were evaluated following statistical methods described in the ISO/DIS 13528.

(ll) One laboratory was rejected due to incorrect application of the trial protocol.

(*2*) *Results*. —

(a) The results of the eleven accepted laboratories that have evaluated the IMS method.
(b) The concentrations estimated by the color chart of the IMS method were highly coincident with the reported culture results for each one of the three groups of samples prepared with certified reference material (pills) containing L. pneumophila.

(c) For the two pills used as negative control, not having L. pneumophila, this bacterium was not detected by any of the two methods (culture isolation and IMS test) in any of the participating laboratories.

(d) Coincidence between both methods was of 95.8%.

A6 Calibration function of the quantitative IMS method

(**a**) *Matrix study.* —Response curve

(1) Methodology. —A response curve was obtained measuring the relationship between the absorbance at 429 nm of the test cuvette and the target concentration in different samples of reference materials having known values tested using reference culture method ISO 11731. 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 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. 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 screwcap 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

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portions: 9 mL for test method testing and 1 mL for reference culture method testing. Samples were tested by IMS 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 (Sr) 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.

(2) 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.

By antilog transformation, the result is expressed as CFU_{eq}/volume examined as per Formula:

$X = 10^{y'}$

Agreement between test method and reference method was verified using $\log_{10}$ values and calculated as:

$y = 1.016\ x - 0.074$

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where y = log_{10} CFU/volume examined estimated by test method and x = log10 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.

A7 Limits *of detection and quantification*

(1) Methodology. — Thirty negative samples were tested. Standard deviation was calculated. According to ISO 16140 (protocol harmonized with AOAC), limit of detection (LD_{IMS}) was defined as 3 times this standard deviation and limit of quantification (LQ_{IMS}) was defined as 10 times this deviation standard.

(2) Results. — The IMS has an LD_{IMS} of 40 CFU/volume examined and an LQ_{IMS} of 60 CFU/volume examined.

A8 References

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B. Technical protocol for the verification (secondary validation) of the method

B1 General

For method verification, (secondary validation), simplified requirements can be used for the correct implementation in the laboratory of any third party fully validated method, as in the case of Legipid®.

The standard UNE-EN ISO/IEC 17025, 2017, indicates the need to "use appropriate methods and procedures for all laboratory activities" and, to do so, says that "the laboratory must verify that it can properly carry out the methods before using them, ensuring that they can achieve the required performance". The standard UNE-EN ISO 16140-1 defines the validation of a method as the "establishment of the behavioral characteristics of a method and the provision of objective evidence, demonstrating that the behavioral requirements for intended use are met". On the other hand, the UNE-EN ISO 16140-2 standard adds as an objective that validation studies "be developed by organizations dedicated to the validation of methods". The Legipid® test is a method already validated by the competent international organization AOAC-RI and it is up to the user to provide "objective evidence" that it fits the "intended use".

B2 Verification criteria

Technical criteria and requirements described in this part shall be used for the characterization and for the validation of any protocol for routine application and manufacturer´s instructions shall be accurately followed. Criteria include:

- Precision

- Recovery

- Evaluation of calibration curve: at least three ranges under intermediate reproducibility conditions.

B3 Verification of the precision, recovery and uncertainty

B3.1 General

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To carry out this verification, a protocol is proposed, which allows to confirm the operational characteristics, at least, recovery and precision (reproducibility). These parameters evaluate both the technical capability of a laboratory with the method and its robustness.

B3.2 Experimental design

Prepare separate 9 mL samples, at p- levels (p≥3) of *L. pneumophila*, derived from the primary standards provided by the manufacturer, with k-replies (k≥5) per level (direct samples). In addition, inoculate those same levels to 1-litre samples of the selected water matrices, which shall be concentrated by filtration (filtered samples). Quantify each sample according to usual IMS test protocol under intermediate precision conditions (at least on different days and/or by different operators). Record both the relative absorbances (with respect to negative control) and logarithmic concentrations (Log_{10} CFU_{eq}) obtained for each sample, both direct and filtered ones (Table 1).

Table 1—Formatting of results and calculations

Level y_i	Magnitude order	Direct samples			Filtred samples		
		$x_{i,j}$	$x'_{i,j} = \log_{10} x_{i,j}$	$y'_{i,j} = \log_{10} y_i$	$X_{i,j}$	$X'_{i,j} = \log_{10} X_{i,j}$	$Y'_{i,j} = \log_{10} Y_{i,j}$
low	10^2-10^3	$x_{1,1}$	$x'_{1,1}$	$y'_{1,1}$	$X_{1,1}$	$X'_{1,1}$	$Y'_{1,1}$
		$x_{1,2}$	$x'_{1,2}$	$y'_{1,2}$	$X_{1,2}$	$X'_{1,2}$	$Y'_{1,2}$
		$x_{1,k}$	$x'_{1,k}$	$y'_{1,k}$	$X_{1,k}$	$X'_{1,k}$	$Y'_{1,k}$
medium	10^3-10^4	$x_{2,1}$	$x'_{2,1}$	$y'_{2,1}$	$X_{2,1}$	$X'_{2,1}$	$Y'_{2,1}$
		$x_{2,2}$	$x'_{2,2}$	$y'_{2,2}$	$X_{2,2}$	$X'_{2,2}$	$Y'_{2,2}$
		$x_{2,k}$	$x'_{2,k}$	$y'_{2,k}$	$X_{2,k}$	$X'_{2,k}$	$Y'_{2,k}$
high	10^4-10^5	$x_{3,1}$	$x'_{3,1}$	$y'_{3,1}$	$X_{3,1}$	$X'_{3,1}$	$Y'_{3,1}$
		$x_{3,2}$	$x'_{3,2}$	$y'_{3,2}$	$X_{3,2}$	$X'_{3,2}$	$Y'_{3,2}$
		$x_{3,k}$	$x'_{3,k}$	$y'_{3,k}$	$X_{3,k}$	$X'_{3,k}$	$Y'_{3,k}$

y_i is the number of *L. pneumophila* CFU_{eq} per cuvette test
y'_i logarithm of y_i
$x_{i,j}$ relative absorbance at level i (i=1....p) and row j (j=1.....k)
$x'_{i,j}$ logarithm of $x_{i,j}$
k is the number of repetitions per level i (k≥5)
p is the number of levels (p≥3)

B3.3 Analysis of the results

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Calculate the mean and the standard deviation for the values of logarithmic concentrations obtained for both direct (y_i) and filtered samples (Y_i).

$$\bar{y} = \frac{\sum_{i=1}^{i=k} y_i}{k}$$

$$\bar{Y} = \frac{\sum_{i=1}^{i=k} Y_i}{k}$$

$$s_r = \sqrt{\frac{\sum_{i=1}^{i=k} y_i^2 - \left[\frac{\left(\sum_{i=1}^{i=k} y_i\right)^2}{N}\right]}{N-1}}$$

$$s_R = \sqrt{\frac{\sum_{i=1}^{i=k} Y_i^2 - \left[\frac{\left(\sum_{i=1}^{i=k} Y_i\right)^2}{N}\right]}{N-1}}$$

Where $\bar{y}$ and s_r are the mean and standard deviation of y_i , respectively (direct samples). And $\bar{Y}$ and s_R are the mean and standard deviation of Y_i respectively (filtered samples).

Calculate the **precision** (or reproducibility) according to the following Formula:

$$P = \frac{S_R}{Y_R} \cdot 100\ (\%)$$

Precision shall have a value ≤10 %.

Calculate the **recovery** according to the following Formula:

$$R = \frac{\bar{Y}}{\bar{y}} \cdot 100\ (\%)$$

Recovery shall have a value between 60-120 %.

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Calculate the **uncertainty** at each matrix and level. The uncertainty measurement of the whole method encompasses calculation of several components for each matrix and tested level:

- Uncertainty associated with strain

- Uncertainty associated with repeatability/reproducibility

- Uncertainty associated with routine

- Uncertainty associated with recovery

Proceed to the calculations stated in the table 2.

Table 2—Uncertainty components calculations

Uncertainty component	Formula
strain	$I_{strain} = \frac{s_r}{\sqrt{k}}$
reproducibility	$I_{rep} = \frac{s_R}{\sqrt{k}}$
routine	$I_{rout} = \frac{s_R}{\sqrt{k}}$; $k = 1$
recovery	$I_{rec} = \frac{\lvert \bar{Y} - \bar{y} \rvert}{\sqrt{3}}$

An uncertainty measurement is obtained from these components according to this Formula:

$$I_{measurement} = \sqrt{I_{strain}^2 + I_{rep}^2 + I_{rout}^2 + I_{rec}^2}$$

Obtain the overall expanded uncertainty by multiplying the uncertainty by a coverage factor 2:

$$I_{exp}(log) = 2 \cdot I_{measurement}$$

Expanded uncertainty shall have a maximum value of 0.3 log.

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B4 Robustness

In this instance robustness is determined through the characterization of the matrix effect. Recovery shall not be substantially affected by the type of matrix to be analyzed.

To do this, the recovery for each type of matrix to be tested shall be determined by the laboratory (e.g. cooling tower water, potable water, surface water, wastewater, etc.) and followed over time.

B5 Verification of the calibration function of the quantitative IMS method

B5.1 General

Calibration curve can easily be tested using artificially contaminated (spiked) 9 mL-samples, as equivalent to concentrated water samples. For statistical analysis, the concentrations of equivalent colony forming units (CFU_{eq}) per IMS well are expressed as decimal logarithms.

B5.2 Calibration curve verification principle

Experience has shown that the means of absorbance measurements obtained for different levels of CFU_{eq} quantities (expressed as decimal logarithms) can be represented according to a linear regression model, i.e. by a linear equation such as $y = ax' + b$. When the line parameters have been determined, it is then possible, by using the equation of this line, to calculate the number of *Legionella* CFU_{eq} present in the sample corresponding to a particular absorbance measurement. The parameters of the calibration curve are initially determined by following the validation protocol described in A.4. This curve, provided by the primary validation, predicts the concentration of CFUeq in a sample based on the relative absorbance (Abs_r) at a wavelength of 429 nm. On a Log_{10}-Log_{10} scale, it is a linear calibration curve (1), characterized by its slope and its intercept:

$$Log_{10}\ CFU_{eq} = 2.3061 \times Log_{10}\ Abs_r + 4.9815 \quad (1)$$

where slope a=2.3061 and intercept b=4.9815

Primary validation data provide confidence intervals (CI, 95 % confidence level) for both slope and intercept (Scheme 1):

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- for the slope (a) [1.83, 2.78]

- for the intercept (b) [4.53, 5.43]

Scheme 1 - Construction of confidence intervals at 95% confidence level

The statistical test used are the following:

$$\hat{\beta}_1 \pm t_{n-2,\alpha/2}\sqrt{\frac{S_R^2}{(n-1)\cdot S_x^2}}$$

$$\hat{\beta}_0 \pm t_{n-2,\alpha/2}\sqrt{S_R^2\cdot\left(\frac{1}{n}+\frac{\bar{x}^2}{(n-1)\cdot S_x^2}\right)}$$

Where S_R^2 is the variance of the residues (difference between the actual value of the dependent variable and te value for this variable estimated by the calibration curve), S_x^2 is the variance of the values for the independent variable, and n is the number of tested levels).

Albalat et al.: Journal of AOAC International Vol. 97, No. 5, 2014 – Table 2

Log_{10} Abs	x_i	0.45	-0.13	-0.75	-1.20	-1.52
Log_{10} UFC	y_i	6.14	4.39	3.50	2.13	1.47

$$\hat{y}_i = 2.3061x_i + 4.9815$$

This equation allows to obtain the estimated value of Log_{10} CFU_{eq} ($\hat{y}_i$):

$$\hat{y}_i = 2.3061x_i + 4.9815$$

Log_{10} UFC_{eq}	$\hat{y}_i$	6.02	4.68	3.25	2.21	1.48

The differences between the actual values and those estimated by the equation are called residues (e) and the resulting variance is the residual variance.

$e = y_i - \hat{y}_i$	0.12	-0.29	0.25	-0.08	-0.006

$$S_R^2 = \frac{\sum_1^5 e^2}{n-2} \qquad S_R^2 = 0.056$$

The variance of the values for the independent variable (Log_{10} Abs) is calculated:

Log_{10} Abs	x_i	0.45	-0.13	-0.75	-1.20	-1.52

$$S_x^2 = \sum_1^5 x_i^2/n - \bar{x}^2$$

$$S_x^2 = 0.637$$

The statistic Student t for 5-2 freedom degrees and confidence leve lof 0.95 is grados de libertad y nivel de confianza de 0.95 is 3.182, so the confidence interval is obtained in the following way:

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$$\hat{\beta}_1 \pm t_{n-2,\alpha/2}\sqrt{\frac{S_R^2}{(n-1)\cdot S_x^2}}$$

$$2.3061 \pm 3.182\sqrt{\frac{0.056}{(5-1)\cdot 0.637}}$$

For the slope the confidence Interval is [1.83, 2.78] $\log_{10}$; similarly, the confidence Interval for the intercept is calculated as [4.53, 5.43] $\log_{10}$

B5.3 Calibration curve evaluation protocol

The evaluation of the calibration curve shall be performed under conditions suitable for determining reproducibility (at least on different days and/or different operators).

Prepare 9 ml-samples in a range of p levels of concentrations of *L. pneumophila* CFUeq units using the primary immunodetectable standard provided by the manufacturer (ATCC33152), p being at least equal to 3, for example covering 3 magnitude orders between 10^2, 10^3, 10^4, 10^5 CFUeq units of *L. pneumophila*. At each p-level perform k repetitions of the measurement, k being at least equal to 5. Record the obtained $x_{i,j}$ values (absorbances) according to the example given in table 3.

Perform the calculations as indicated in the Table 3.

Table 3—Formatting of results and calculations

Level y_i	$y'_i = \log_{10} y_i$	$x_{i,j}$	$x'_{i,j} = \log_{10} x_{i,j}$	$T_i = \sum_{j=1}^{k} x'_{i,j}$	$m_i = \frac{T_i}{k}$
y_1	y'_1	$x_{1,1}$	$x'_{1,1}$	T_1	m_1
		$x_{1,2}$	$x'_{1,2}$		
		$x_{1,k}$	$x'_{1,k}$		
y_2	y'_2	$x_{2,1}$	$x'_{2,1}$	T_2	m_2
		$x_{2,2}$	$x'_{2,2}$		
		$x_{2,k}$	$x'_{2,k}$		
y_p	y'_p	$x_{p,1}$	$x'_{p,1}$	T_p	m_p
		$x_{p,2}$	$x'_{p,2}$		
		$x_{p,k}$	$x'_{p,k}$		

y_i is the number of *L. pneumophila* CFU_{eq} per cuvette test
y'_i logarithm of y_i
$x_{i,j}$ relative absorbance at level i (i=1….p) and row j (j=1…..k)
$x'_{i,j}$ logarithm of $x_{i,j}$
k is the number of repetitions per level i (k≥5)
p is the number of levels (p≥3)

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Calculate the total number of measurements noted N according to Formula (1):

N = k·p

B5.4 Analysis of the results

B5.4.1 Estimation of the regression curve

The regression curve is given by formula (2):

$$y' = ax' + b$$

Where *a* is the slope and *b* is the intercept.

Proceed to the following calculations in order to determine the slope a.

$\bar{y'} = \frac{1}{p} \sum_{i=1}^{i=p} y_i'$ (3)

$\bar{m} = \frac{1}{p} \sum_{i=1}^{i=p} m_i$ (4)

The estimation of the slope *a* is given by the Formula (5):

$a = \frac{\sum(m_i - \bar{m}) \cdot (y_i' - \bar{y'})}{\sum(m_i - \bar{m})^2}$ (5)

Proceed to the following calculations in order to fix the intercept point *b*:

$b = \bar{y'} - a\bar{m}$ (6)

B5.4.2 Estimate and verification of the slope and intercept

The curve (1) is accepted in the case where both the slope (a) and intercept (b) of the experimental curve are within the corresponding confidence intervals (CI). The value of

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the slope, a, shall be between 1.83 and 2.78, and the value of the intercept, b, shall be between 4.53 and 5.43. If a or b are outside of the range, the IMS system shall not be validated.

B5.5 Use of the calibration curve

For each measurement $x' = \text{Log}_{10}\ \text{Abs}_r$ of a sample, use the standard curve formula (1) to obtain $y' = \text{Log}_{10}\ y$. By antilog transformation, express the result y CFU_{eq}/volume examined as per Formula:

$$y = 10^{-y'}$$

B6 References

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C Detection and quantification of Legionella spp. in water samples by immunomagnetic separation (IMS) and enzyme-based colorimetric method.

C1 Introduction

The bacteria in the genus *Legionella* occur naturally in many natural environments and colonizes a variety of engineered systems that sometimes support their proliferation. They grow optimally inside protozoan hosts, such as free-living amoebae associated with biofilms that coat wet surfaces (1,2). *Legionella* is transmitted from environmental sources through contaminated water that is aerosolized and exposing those nearby via inhalation into the respiratory tract (3). Patients infected with *Legionella* can develop a milder flu-like condition called Pontiac fever or a pneumonia called Legionnaires' disease (LD); both conditions are referred to as legionellosis. Although *L. pneumophila* is the most dominant *Legionella* species isolated from patients, some other species can lead to disease, including *L. micdadei*, *L. bozemanii*, *L. dumoffi*, and *L. longbeachae* (4, 8-10).

The mitigation of *Legionella* colonization and disinfection of water systems used in risk settings are a key factor for controlling and preventing associated *Legionella* infections (11,12). Water systems have traditionally been monitored using culture-based methods as the gold standard, which can take many days to detect growth, making rapid decisions impossible, and can be biased toward *L. pneumophila* and a few other *Legionella* spp. (13). Furthermore, control strategies (heat treatment, chlorine-based disinfectants, and copper-silver ionization) are known to trigger *L. pneumophila* to enter a viable but non culturable (VBNC) state (14,15), which does not form visible colonies on plates but may infect different types of human macrophages and amoebae. Of particular interest is that high percentages of the *Legionella* populations in water systems cannot grow on conventional culture medium (16).

The IMS may be used used in helping to solve two shortcomings in the assessment of *Legionella*, namely: (1) underestimation of the presence and concentration of *Legionella* spp. by culture-based method because most *Legionella* cells could remain in a viable but non-culturable (VBNC) state, and (2) likewise, polymerase chain reaction-based techniques (PCR) cannot differentiate live versus dead (non-viable) cells or free DNA, so the number of Legionellae could be overestimated (17). PCR ability to differentiate between viable and nonviable organisms is still evolving (18).

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These findings suggest that the true level of *Legionella* contamination can likely be underestimated by culture and overestimated by qPCR. According to the reported studies on the dynamics and phenotypic plasticity of *Legionella* cell surface, the ability of *Legionella* to cause LD hinges predominantly on its cell envelope (19). These findings highlight the importance of detecting legionellae cells in their environment by considering their cell envelope as an analytical target. This interaction allows incorporating the effect of envelope integrity, already demonstrated to examine the effect of biocides on *Legionella* in other studies (20). The immunomagnetic separation (IMS) technique is based on the interaction antigen-antibody at the cell envelope level, thereby making this approach of high diagnostic value for a preventative purpose.

C2 Scope

This document describes a non-culturable method for detection and quantification of legionellae in water samples. It specifies general methodology and quality control requirements.

Technical details specified in this document are given for information only. Any routine protocol using this third party validated commercial test complying the manufacturer´s instructions is suitable. This methodology can be used to detect and quantify *Legionella*.

The information in this document is intended to be applied in the bacteriological investigation of all types of water. However, the intense color or suspended matter in some waters can interfere with the methods and affects its sensitivity if not special attention is paid on washing steps.

The results are expressed as the number of equivalent colonies forming units of *Legionella* spp. per litre or per volume of sample examined (volume filtered).

Users wishing to employ these methods should verify its performance under their own laboratory conditions as described in B.

C3 Definitions

Equivalent colonies forming units (CFUeq) - the number of Legionellae organisms that, when the conditions of maximum culturability and minimum aggregation were given, would produce the same mean number of colony-forming units in culture.

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***Legionella* –** a genus consisting of approximately 61 species of Gram-negative bacteria

Reference material – ready-to-use calibrated solution of immune-detectable *Legionella pneumophila* material (antigens pool or whole cells)

Working calibration solutions – *L. pneumophila* (collection strain, e.g. ATCC33152) antigens or entire cells prepared from reference material, used to verify the calibration curve or the performance parameters of the method.

C4 Principle

The detection and quantification of Legionella spp, by IMS method is carried out in three phases:

- The original sample of water is concentrated by filtration or the like, and this prepared sample is eluted and dispensed into a cuvette to be analyzed by the method;

- A suspension of magnetic particles that bind to *Legionella* is added. If *Legionella* cells are present in the prepared sample, they will bind to the antibodies immobilized on magnetic particles to form complex bacteria / particle. As these complexes may be separated by a magnet, they are easily washed and resuspended. The complexes are incubated with an anti-*Legionella* antibody conjugated with an enzyme, to form labeled complexes;

- After washing the *Legionella* / particle complexes are visualized colorimetrically, when the enzyme substrates are added.

C5 Limitations

Sensitivity of IMS for *Legionella* quantification in water samples will be dependent on the presence of suspension matter or colored materials. Due attention should be paid to manufacturer´s instructions for very dirty samples or highly colored samples.

Interaction of the immune beads with *Legionella* at the level of the cell envelope requires the expression and availability of the antigens (virulence traits) to which the antibodies bind (cell integrity), thereby providing a significance of high preventative diagnostic value. Nevertheless, *Legionella* remaining inside protozoa cannot be detected.

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C6 Health and safety

WARNING – Species of *Legionella* are pathogenic

Risk of harm is caused by the inhalation of aerosolized disease-causing strains of *Legionella* bacteria. It is therefore advisable to assess all techniques for their ability to produce aerosols.

All samples submitted for Legionella analysis should therefore be regarded as potentially contaminated with bacteria classified as “Hazard Group 2” and handled with strict adherence to the general safety precautions described for work at Containment Level 2 (CL2), to include minimizing exposure to aerosols at all times. If any doubt, carry out the work in a safety cabinet.

Based on the risk assessment of procedures undertaken during it may be necessary to carry out the examination in a microbiological safety cabinet. However, experience has determined that Legionella spp. can be handled safety by experienced operators on the open bench in a conventional laboratory conforming to containment level two.

Components, reagents and bacteria used in this method are covered by regulations and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere in this series (21).

C7 General testing conditions

The principles to be applied are as follows:

- Once received, the kit is stored between +2°C and +8°C, preferably at +4°C. The expiration of the reagents, properly stored, is **5 months from the date of manufacture**.

- Before starting the test, prepare only the amounts of reagents needed and let 30 minutes at room temperature before use. The tempered volumes must not be stored in a refrigerator again. Discard.

- The protocol includes code, lot number and expiration date, ensuring the traceability of all reagents. Ask the manufacturer for a certificate of analysis.

The user should always pay attention to the following:

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- The reagents are safe under normal conditions of use. It is unlikely to react dangerously under normal conditions of use. Avoid contact with the eyes by wearing gloves. If splashing might occur, wear safety glasses.
- The assay performance depends on strict compliance with the following practices, especially regarding to the correct execution of the protocol:
    - Do not use reagents after the expiration date.
    - Using as negative control the same eluent (L0) used in sample preparation (elution).
    - Use a negative control (L0 reagent) for each batch of test.
    - Bring test reagents (18-26 ° C) at least 30 minutes before use.
    - Shake the reagent L1 before use, to the homogeneity of the magnetic particles.
    - Run carefully washing steps (reagent L2).
- The cuvettes are disposable. Do not reuse.
- Use reference material following manufacturer´s instructions (not all reference materials commercially available are immunodetectable)

C7.1 Staff

This method should be performed by properly trained personnel, although it does not require highly skilled and well-trained staff. It is advisable to assess the competency of the staff on an on-going basis using external or internal quality control programs.

Once this has been completed the laboratory manager should formally approve the staff member competent to conduct testing. Training should be recorded and documented.

The staff shall wear laboratory coats and any gloves used shall be disposable and talc free.

C7.2 Premises

Where possible IMS facilities should be organized into up to o neighboring discrete areas (not separated rooms):

a) An area for the concentration of original water samples

b) An area for the preparations of aliquots of IMS reagents that are going to be used

c) An area for the analysis

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The IMS analysis shall be conducted at room temperature and this temperature may vary between 18–26°C, preferably not less than 21 °C.

Position both components and reagents on a horizontal surface (laboratory bench). Avoid excessive proximity between magnets. Use the positioning mat provided by the manufacturer in order to keep suitable distance among magnetic concentrators. If not, keep a distance among concentrators of at least 12 cm.

C7.3 Apparatus

C7.3.1 Membrane filtration apparatus

Suitable for filtering water samples of volume up to 1.0 litre. This apparatus normally includes a filter stand and funnel and should withstand autoclaving (alternatively sterile disposal funnels are available). The recommended diameter is 47 mm. Large apparatus are usually constructed of stainless steel.

C7.3.2 Pipettes

Use a set of pipettes 10-100µl, 100-1000µl and 1-5ml.

C7.3.3 Vortex mixer

Use a vortex for eluting the membrane filter but shaking can also be either manually or by using an ultrasonic bath.

C7.3.4 Magnetic particle concentrator

Magnetic Particle Concentrator is a module with capacity for 4 cuvettes (MP4, 4 analysis), which consists of an insertable holder, a magnetic holder, and a clamping support. Manually, it is recommended to use a maximum of 3 modules. For larger batches it is recommended a modular system that allows users to semiautomatically conduct up to 20 tests in a batch, which consists of an orbital shaker, five MP4 modules on a platform and a mat positioner.

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C7.3.4 Colorimeter

Use a colorimeter to read the absorbance at 429 nm both in control and tests. Wavelength may vary between 425-440 nm.

C7.4 Consumable

Apart from the concentration step, it is important to avoid the apparatus coming into contact with the water sample so as to prevent cross-contamination. Use preferably the consumables provided by the manufacturer.

C7.4.1 Membrane filters

Membrane filters of 47 mm diameter shall be made of polycarbonate, preferably, with a nominal porosity of 0.4 µm. Smaller pore diameter is possible, but it can hamper the filtration. For very dirty samples, consider the use a glass fiber pre-filter of 47 mm diameter and 2.7 µm pore diameter placed on top of the membrane filter.

C7.4.2 Graduated bottle with screw cap

Use a graduated bottle with screw cap for elution of the membrane filter.

C7.4.3 Vial (cuvette)

Use transparent glass 11 ml-vial, with secure pressure polyethylene (PE) lid, provided by the manufacturer, for the analysis of controls and tests. The cuvettes are disposable. Do not reuse.

C7.4.4 Semi-micro cuvette

Use disposable semi-micro cuvettes for photometer (PMMA material - 280-800nm-, pathlength of 10mm and volume of 2.5ml).

C7.5 Reagents

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C7.5.1 General

Whenever possible, all reagents shall be dispensed in appropriate volumes so as to avoid reusing the aliquots. This improves the repeatability of the method. Suitable procedures shall be used to ensure traceability of all reagents. Follow supplier´s recommendations for storage and handling of reagents.

C7.5.2 IMS reagents

Before starting the test, prepare only the amounts of reagents needed and let 30 minutes at room temperature before use. Insert the cuvettes in its support (MP4 module), as many as trials are going to be performed and another one for the negative control.

An IMS reaction generally contains the components indicated in Table 4.

Table 4—Components used in a typical IMS reaction

Component	ID	Details	Amount per reaction
Eluent	L0	Reagent to elute the microorganism from the filter membrane	10 ml
Capture reagent (immunomagnetic particles)	L1	Magnetic particles with immobilized specific polyclonal anti-*Legionella* antibody, which forms complexes with the bacteria target to separate them from the rest of the sample (other bacteria and materials or substances present in the sample)	1 ml per test [a]
Wash buffer	L2	Buffer for washing-formed complexes	18 ml
Enzyme conjugated Anti-*Legionella* antibody	L3	Specific polyclonal antibody against the bacteria target, conjugated to the enzyme horseradish peroxidase (HRP).	1 ml
Enzymatic cosubstrates	L4	Co-substrate for the conjugated HRP	1 flask for 4 reactions
Stop reagent	L5	Reagent to stop the HRP reaction	0.1 ml
Cuvette	CB	Transparent glass vial with PE lid to do the assay	1 unit

[a] before taking the relevant ml shake the original reagent bottle L1 until a completely homogeneous suspension.

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C8 Analytical procedure

C8.1 Concentration

Filter as large a volume of the original water sample as practicable (usually 1 L) to concentrate the bacteria. Record the volume (V) of the sample filtered. This is required to calculate the results (see Section 9). Performance of the method is adversely affected by small sample volumes and increase proportionally.

C8.2 Elution

Place the filter in a screw-cap sterile container containing 10 mL of L0 reagent. Vortex the screw-cap flask for 2 min to elute the target organisms. Transfer 9 mL of the supernatant into a cuvette test. The eluted sample is called prepared (pre-concentrated) sample.

C8.3 Analysis

C8.3.1 Capturing step

C8.3.1.1 General description

The IMs method includes different reagents (L0, L1, L2, L3, L4, and L5) and an easy-to-handle magnetic particle concentrator comprising a magnet for each two 10 mL glass cuvettes. A 9 mL amount of each preconcentrated sample is transferred to the kit 10 mL glass cuvette, 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 cuvette, and the supernatant is discarded by overturning the cuvettes. A control must be tested in parallel in a second cuvette.

C8.3.1.2 Protocol

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(a) Add each prepared sample (9 ml) in the corresponding cuvette (T). Then add L0 (9 ml) to the control cuvette (C) (just one control per testing batch). Apply to all cuvettes all the steps that follow.

(b) Gently shake the L1 by repeated inversions until a completely homogeneous suspension. Resuspend by gentle and repeated pipetting, and then add 1 ml in each cuvette. PLACE THE PLUGS onto cuvettes.

(c) Insert cuvette holders (CH) on the platform (P), if you have it, in such way that the cuvettes are horizontal. Stir at 80 rpm for 15 minutes on the orbital shaker (manual: on the mat in gentle sway shake 3 times every 3 minutes).

(d) Remove the cuvette holders (CH) from the platform (P), if you have one, and REMOVE AND DISPOSE THE PLUGS (place the cuvettes upright). Assemble MP4 and place in its position on the mat (TC). Wait 5 minutes to retain the magnetic particles.

(e) Discard supernatant by emptying the cuvette on the opposite side to magnets, by tipping this assembly up.

(f) Disassemble the MP4 and insert the cuvette holder (CH) upright on the platform (P) on the orbital shaker (manual: on the mat). Then add 4.5ml of reagent L2 in each cuvette.

(g) Shake WITHOUT PLUGS at 350 rpm for 10 seconds (manual: on the mat in vigorous shake swing) to resuspend the particles.

(h) Remove the cuvette holders (CH) from the platform (P) - if you have it -, assemble the MP4 and place it in its position on the mat (TC) and wait 3 minutes to retain the magnetic particles.

(i) Discard supernatant emptying the cuvettes on the opposite side to the magnets, by tipping this assembly up.

C8.3.2 Marking step

C8.3.2.1 General description

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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.

C8.3.2.2 Protocol

(a) Disassemble the MP4 and insert the cuvette holders (CH) upright onto the platform (P) in the orbital shaker (manual: on the mat). Add 1 ml of reagent L3 in each cuvette.
(b) Shake WITHOUT PLUGS at 250 rpm for 10 seconds (manual: on the mat in vigorous shake swing) and then at 80 rpm for 10 minutes (manual: gentle sway every 2 minutes for 10 minutes).

(c) Separate cuvette holders (CH) from the platform (P), if you have one, and assemble the MP4. Place them in their position on the mat (TC) and wait 3 minutes to retain the magnetic particles.

(d) Discard supernatant by pouring on the opposite side to the magnets, by tipping up the assembly.

(e) Disassemble the MP4 and insert the cuvette holders (CH) upright onto the platform (P) in the orbital shaker (manual: on the mat). Then add 4.5ml of reagent L2 in each cuvette.

(f) Shake at 350 rpm for 10 seconds WITHOUT PLUGS (manual: on the mat shake in energetic swing) to resuspend the particles.

(g) Separate cuvette holders (CH) from the platform (P), if you have it. Assemble the MP4 and place them in their position on the mat (TC). Wait 3 minutes to retain the magnetic particles.

(h) Repeat steps 4, 5, 6 and 7 (of this section B.2 MARKING STAGE) twice more

C8.3.3 Marking step

C8.3.3.1 General description

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The *Legionella* spp./magnetic bead complexes are visualized by the colorimetric reaction developed when HRP substrates are added.

C8.3.3.2 Protocol

(a) Discard supernatant by pouring on the opposite side to the magnets, by tipping up the assembly. Disassemble the MP4 and insert the cuvette holders (CH) upright onto the platform (P) in the orbital shaker (manual: on the mat).

(b) Prepare the reagent L4 (one vial for each 4 trials): unseal the protective cap and remove it. Press the piston to be fully inserted into the shutter. Shake the mixture vigorously for 10 seconds. Once the reagent L4 is prepared, it should be used immediately.

(c) Open the vial of L4 just before use. To do this remove the shutter with the piston inserted, applying light pressure with the thumb side.

(d) Immediately add 1 ml pipette to each cuvette. Shake WITHOUT PLUGS at 80 rpm for 2 minutes (manual: on the mat in gentle sway). Start shaking at 250 rpm for the first 10 seconds (manual: swing energetically) to resuspend the particles. (Note: count time from the first addition of L4)

(e) Stop shaking. Add 100 µl of L5 to each and every one of the cuvettes and shake for 5 seconds at 80 rpm (manual: on the mat in gentle sway). (Note: add reagent L5 in the same order as the L4 was added)

(f) Separate cuvette holders (CH) from the platform (P), if you have it, and reassemble the MP4. Wait 5 minutes to retain the magnetic particles.

C8.3.3 Quantitative detection

C8.3.3.1 General description

After adjusting the absorbance to zero with distilled water, measure the absorbance of the negative control (C). This absorbance should not be greater than 0.12. Then adjust

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the absorbance to zero with the control (C) before measuring the relative absorbance (A_r) of the test (T) samples. The cut-off value of relative absorbance shall be 0.04.

C8.3.3.2 Protocol

(a) Transfer 1ml of the supernatants Control (C) and Tests (T) each in its corresponding cell reading. Important note: Pipette 1ml of the supernatant from the opposite side to the magnet, being careful not to drag the particles retained by the magnet

(b) Measure absorbance at 429 nm in a cell filled with distilled water. Set the absorbance at zero.

(c) Measure absorbance at 429 nm of the supernatant of the control (C), as reference. Readjust the absorbance to zero.

(d) Measure the absorbance of the supernatant of each test (T). Immediately read: always within 10 minutes since the end of the colorimetric reaction.

Note: If the the length of the light path is not 1 cm, the corresponding correction is necessary. Follow the manufacturer's instructions of your optical reader.

C9 Calculations and expression of results

(a) Negative results — tests of samples with relative absorbance readings below the cutoff value (Ar = 0.04 units) are negative and are reported as Not Detected.

(b) Positive results — tests of samples with relative absorbance readings above the cutoff value (A_r = 0.04 units) are positive and are reported as Detected.

(c) For positive results, obtain the $\log_{10}$ of relative absorbance.

(d) Estimate the concentration of *Legionella spp* in the volume examined (filtered) entering the value of the log10 of the relative absorbance (Ar) in the following formula:

$y = 2.3061\ x + 4.9815$, where $x = \log_{10}(A_r)$ and $y = \log_{10}$ (CFU_{eq} of *Legionella* spp./ volume examined)

(e) The result is obtained by applying the inverse transformation of the logarithm:

Contamination (CFU_{eq} of *Legionella* spp./ volume examined) = 10^y

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C10 Quality Control

C10.1 General

Quality controls ensure trueness and precision of measurements carried out by a laboratory. According to the UNE-EN ISO / IEC 17025 Standard, the laboratory must establish a quality control to monitor the validity of the tests.

C10.2 Reference material and working calibration solutions

C10.2.1 Principle

The trueness of IMS measurement is ensured by the use of a quantitative material in which each vial contains a specific number of immunodetectable cells or antigens, expressed as CFU_{eq}, being available in three concentration ranges: low, intermediate and high, and obtained by the manufacturer under the test conditions.

The manufacturer shall provide a reference material which establishes a certified value of the property (CFU_{eq}) with an uncertainty for a certain confidence level (95%). The used strain to prepare these materials shall be traceable to different Type Culture Collections and can be shown to be no more than two steps from the original strain. The material shall be appropriate for method verification, and quantitative quality controls.

The reference materials must be kept as specified in manufacturer´s instructions supplied with the material itself. In the case that these instructions are not followed, their immunodetectable properties may be altered.

The cell-based reference material allows you to prepare a series of standard 9 mL samples, at three different levels of Log_{10} CFU_{eq}, with n-replies (up to 10) per level. In addition, it allows to inoculate those same levels to samples of the selected matrices, which are concentrated by filtration. The laboratory records the relative absorbances obtained for each concentration, when applying the IMS method to the samples, both in the direct samples as in the filtered ones.

The antigen-based reference material allows you to prepare a series of standard 9 mL samples, at three different levels of Log_{10} CFU_{eq}, with n-replies per level, without filtration

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step. The laboratory records the relative absorbances obtained for each concentration, when applying the IMS method to the samples and slope and intercept are calculated. Experimental values are compared with those defined as confidence intervals at 95% confidence level resulting from primary validation reported by the manufacturer.

C10.1.2 Protocol

The preparation of the material is extremely simple, as the presentation is in vial form and is supplied in a sterile plastic tube, avoiding the need for awkward, complicated manipulation.

Take the exact number of vials you need out of the freezer for each level or range. As many as replicas per level you will use. Allow defrosting and tempering at room

temperature, before preparing standard samples and analyzing them with the IMS method.

C10.1.2.1 For verification of accuracy, recovery and uncertainty

(a) Preparation of direct samples: Defrost and temper p-vials per level, as many as replicas will use (up to a maximum of 10). For each level and p-vials, take 0.5 mL of each vial and add it to each p-cuvettes of the IMS method. Bring all cuvettes to a final volume of 9 mL with L0 (i.e. add 8.5 mL of L0).

(b) Preparation of filtered samples: Defrost and temper p-vials per level, as many as replicas you're going to use (up to a maximum of 10). Take 0.5 mL of one vial and inoculate in 1000 ml of matrix, with p-replicates for each level of contamination and matrix. Homogenize for gentle and repeated inversions. Filter each inoculated sample and elute the filter according to the IMS protocol.

Note – Use cell-based reference material to prepare both direct and filtered samples as the filtration is involved.

(c) Analysis: Perform IMS method on all samples (use negative control per batch)

(d) Record the value of the absolute absorbance of negative control and the relative absorbance values (with respect to negative control) of samples.

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C10.1.2.1 For verification of the calibration curve (IMS efficiency)

(a) Preparation of working calibration samples: Defrost and temper p-vials per level, as many as replicas will use (up to a maximum of 5). For each level and p-vials, take 0.5 mL of each vial and add it to each p-cuvettes of the IMS method. Bring all cuvettes to a final volume of 9 mL with L0 (i.e. add 8.5 mL of L0).

Note – Use the antigen-based reference material to prepare the working calibration samples as no filtration is needed.

(b) Analysis: Perform IMS method on all samples (use negative control per batch)

(c) Record the value of the absolute absorbance of negative control and the relative absorbance values (with respect to negative control) of samples.

C10.1.3 Data analysis

C10.1.3.1 For verification of accuracy, recovery and uncertainty

Calculate mean and standard deviation for both direct and filtered samples. Then use the formulas described in Section B to calculate accuracy, recovery and the uncertainty of the IMS method.

C10.1.3.2 For verification of the calibration curve

By linear regression, establish the experimental values for the slope and intercept. Verifies that slope *a* lies between 1.83 and 2.78 corresponding to confidence intervals (confidence 95%) derived from primary validation reported by the manufacturer. Likewise, verifies that intercept *b* lies between 4.53 and 5.43.

If both slope and intercept lies inside the corresponding 95% confidence interval, connection with primary validation is achieved and the manufacturer´s formula can be accepted.

C11 Test Report

This test report shall contain al least the following information:

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a) the test method used

b) all the information required to identify and describe the sample

c) sampling date and conditions

d) the filtered volume of the sample

e) the results expressed as described in Section 9

f) any details not included in this Technical Specification that may have an effect on the results

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D Discussion and conclusions

D1. Discussion

Testing water for the presence of *Legionella* can be an important component of risk management for legionnaires' disease (LD) in a hospital (1,2). Nevertheless, previous studies reported low sensitivity (59%) and specificity (74%) of a 30% *Legionella* positivity as a metric based on gold-standard culture method for assessing the risk of health care–acquired LD (3). We report that *Legionella* could be better estimated by the new IMS method.

The method can be used in helping to solve three shortcomings in the assessment of *Legionella* in water, namely: (1) underestimation of the presence and concentration of *Legionella* spp. by culture-based method because other accompanying microorganism overgrow and/or most *Legionella* cells could remain in a viable but non-culturable (VBNC) state (false negative results), (2) overestimation of the presence and concentration of *Legionella* spp due to false-positive results for most probable number (MPN) culture methods with other waterborne bacteria at real-world environmental concentrations, which can trigger costly remediation and water-use restrictions, and (3) likewise, polymerase chain reaction-based techniques (PCR) cannot differentiate live versus dead (non-viable) cells or free DNA, so the number of Legionellae could be overestimated (4,5). Of particular interest is that high percentages of the *Legionella* populations in water systems cannot grow on conventional culture medium (6).

These findings suggest that the true level of *Legionella* contamination can likely be underestimated by culture and overestimated by qPCR. Furthermore, there is no consensus with regards to the concentration that will cause LD (3). Therefore, an IMS technique based on the antigen-antibody interaction at the level of cell envelope was implemented. As the antigens related with virulence mainly resides on the cell envelope, this interaction allows incorporating the effect of envelope integrity, already demonstrated to examine the effect of biocides on *Legionella* in other studies.

In agreement with other studies (7), our observations suggest that no-growth based methods should be considered when examining risk factors as determinants to *Legionella* contamination to reduce the potential exposure of people to these bacteria. The IMS method may help to prevent that many facilities might fail to mitigate when a

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true risk is present or might unnecessarily allocate limited resources to deal with a negligible risk.

Especially after recognizing *Legionella* as one possible pathogen causing co-infection among COVID-19 patients (8), a more sensitive *Legionella* monitoring should be recommended as a *Legionella* decontamination strategy.

D.2 Conclusions

Non-culturable methods with a risk-based approach are already being accepted in the regulations. For example, an EU agreement to monitor tap water for the presence of potentially lethal *Legionella* bacteria takes into consideration the emergence of newer, more effective testing methods on the revised Drinking Water Directive (DWD). In the final compromise, EU member states were left free to determine their approach to testing, as they can choose the methods, they find most appropriate for the purposes they specify in national guidelines. In Spain, the recently approved Royal Decree 487/2022 Of 21 june, By Which Establish The Hygienic Criteria For Prevention And Control Of Legionellosis, includes the possibility of using methods other than cultivation to investigate health risk. After more than 2.5 years of intense work in working group no. 12 of UNE-AENOR's CTN 100, on April 13, the UNE 1000030: 2017 Standard "Prevention and control of the proliferation and dissemination of Legionella in facilities", linked to the decree, was published and already enables the use of not-growth based methods.

Previously, these methods had also been considered in the legislation of Portugal (Despacho nº 1547/2022, 8 February) and the UK (Guides of Water Management Society, december 2018).

This report provides a new IMS not-growth based method to better assess *Legionella* contamination in the water systems, which is essential to identify relevant risk factors associated with *Legionella* contamination. The reported information suggests that this non-culturable method should be considered when examining environmental *Legionella* contamination.

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