A Field Test for Rapid Detection of *Legionella pneumophila* serogroup 1 in Water Samples

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ABSTRACT

Legionella pneumophila is the most common cause of infections caused by *Legionella* species. *L. pneumophila serogroup* 1 accounts for the majority (approximately 70 – 80%) of all reported Legionnaires' disease cases. Traditional methods for detecting *Legionella* are laboratory based and require skilled technicians. Any form of culture-based *Legionella* detection requires days before results are available, and other detection methods are typically cumbersome or expensive to perform in the field. A rapid on-site test for detecting *L. pneumophila* serogroup 1 bacteria in water samples was recently developed. The FastPath™ test detects all ten recognized subtypes of *L. pneumophila* serogroup 1. The test provides presence/absence results at a specific analytical sensitivity in 25 minutes, much like commonly known point of care medical tests. The analytical sensitivity, analytical specificity and diagnostic sensitivity have been experimentally determined. The test detects *L. pneumophila* serogroup 1 concentrations as low as 100 cells per milliliter with no sample preparation and is unaffected by other common bacteria at typical concentrations. The robustness to a range of environmental conditions and water treatment chemistries has been investigated. A sample concentration method for using the test in applications requiring even greater sensitivity has been developed.

BACKGROUND

Legionella is a genus of opportunistic pathogenic bacteria that proliferate in water systems including potable water storage and distribution systems, cooling towers and recreational waters. *Legionella* bacteria are also frequently isolated from environmental sources. *Legionellae* cause respiratory infections collectively known as legionellosis. It is generally accepted that inhalation or aspiration of aerosols containing *Legionella* is necessary for an individual to become infected.

At least 50 different species of *Legionella* have been identified. *Legionella pneumophila* is recognized as the most common cause of infections caused by *Legionella* species. The species *L. pneumophila* is comprised of 16 distinct serogroups. *L. pneumophila* serogroup 1 accounts for the majority (approximately 70 to 80 percent) of all reported Legionnaires' disease cases.[1, 2, 3] *L. pneumophila* serogroup 1 is further sub-divided into 10 distinct subtypes, with varying degrees of virulence.

Since *Legionella* bacteria cause serious disease, there are guidelines and regulations in place globally for *Legionella* monitoring and control. Risk management and control strategies typically focus on preventing exposure of people to water aerosols and reducing the concentration of *Legionella* bacteria in water systems.[4] Therefore, the ability to detect *L. pneumophila* serogroup 1 in water is an important tool for managing the risk of legionellosis.

Traditional methods for detecting and identifying *Legionella* are laboratory based and require skilled technicians. Any culture-based detection methods for *Legionella* bacteria require days or weeks to provide results and thus only provide historical views of *Legionella* contamination. Various methods such as nucleic acid amplification or epifluorescent microscopy are useful for laboratory analysis and require less time than culture, but are cumbersome and expensive to perform in the field. Another difficulty for all methods is growth inhibition or detection interferences as a function of water contamination with various chemicals or non-*Legionellae* bacteria.[5]

A rapid test for detecting *L. pneumophila* serogroup 1 bacteria in water samples was recently developed. The FastPath™ test (hereafter referred to as the "Test") is designed to detect all ten recognized subtypes of *L. pneumophila* serogroup 1. The Test is a lateral flow immunochromatographic assay that detects *L. pneumophila* serogroup 1 cell surface antigens. The Test provides presence/absence results at a specific analytical sensitivity in 25 minutes, much like commonly known point of care medical tests.

The Test was validated using cooling, spa pool and domestic waters spiked with *L. pneumophila* serogroup 1 environmental and culture-type strains. The Test is able to detect *L. pneumophila* serogroup 1 concentrations as low as 100 cells/mL with no sample preparation and is unaffected by other common water borne bacteria at typical concentrations. The robustness to a range of environmental conditions and water treatment chemistries has been investigated, as well as the reproducibility (91%) and repeatability (96%) of the visual interpretation of the test result. A sample concentration method for using the Test in applications requiring even greater sensitivity has also been developed.

This new test capability provides vital information about the status of *Legionella* contamination in a water system, targeting the most pathogenic *Legionella* organisms. Prompt and effective remedial action to reduce the risk of Legionnaires' disease can then be taken to improve microbial control and limit exposure to the water system.

TEST DESCRIPTION

The Test consists of a number of components laminated together such that when a water sample is applied to the device it flows along the Test passing through distinct regions as shown in **Figure 1**. When a water sample is first applied to the Test device, it contacts a pad containing dried reagents that enable a wide range of water samples to be processed without direct operator manipulation. The sample then contacts nanometer-sized particles of gold (which appear red in color) that have been labeled with an antibody. This antibody binds surface antigens on *L. pneumophila* bacteria. *Legionella* in the sample thus becomes colored red before passing along the strip to a line where antibodies against *L. pneumophila* serogroup 1 are bound. Any *L. pneumophila* serogroup 1 antigen in the sample become sandwiched between the line of antibodies and the red colored particles, forming a visible line across the Test. Unbound gold particles are then bound at a second line of control antibody whilst the remaining sample is drawn into an absorbent pad at the end of the device. Results are read 25 minutes after sample is applied to the Test device.

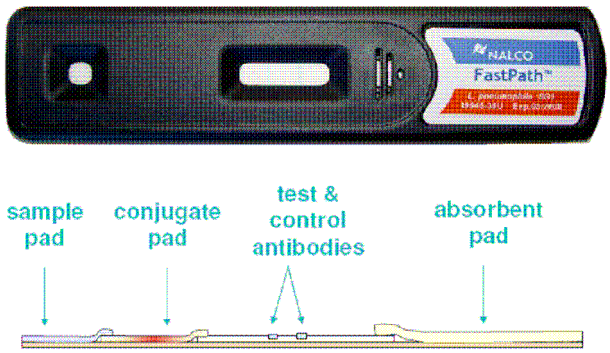

Figure 1. Picture of test device and schematic cross section of the key components.

Positive Test Result: Any Test showing two lines (no matter how light the line color) is a positive result: *L. pneumophila* serogroup 1 cells were detected in the water sample at 100 cells per mL or greater.

Negative Test Result: Any Test showing only a control line is interpreted as a negative result: *L. pneumophila* serogroup 1 cells were not detected in the sample or the number of cells was below the detection limit of the Test (100 cells per mL).

QUANTIFICATION OF TEST RESPONSE

Although the Test is not intended to provide the user with a quantitative measure of *Legionella* concentration, it is possible to determine on a relative scale the level of *L. pneumophila* serogroup 1 antigen in a given sample. This is achieved by measuring the diffuse reflectance (as optical density, OD) of the test line in a reflectometer constructed for this purpose. This instrument is useful for research and development of the Test, but is not required for field-use.

Serial dilutions of reference samples of *L. pneumophila* serogroup 1 (Health Protection Agency (UK), NTCC 12821) in deionized water and simulated cooling water were examined using the Test. The Test results were visually determined in good light by experienced operators as well as being recorded using a reflectometer.

The response from 42 Tests performed by four operators over a series of separate days is shown in **Figure 2**. For water samples tested with no concentration or processing of any kind, the visual limit of detection (analytical sensitivity) corresponds to 100 cells/mL. The United States Occupational Safety and Heath Administration (OSHA) recognizes 100 CFU/mL as action levels 1 and 2 for cooling tower and domestic water systems, respectively.[6]

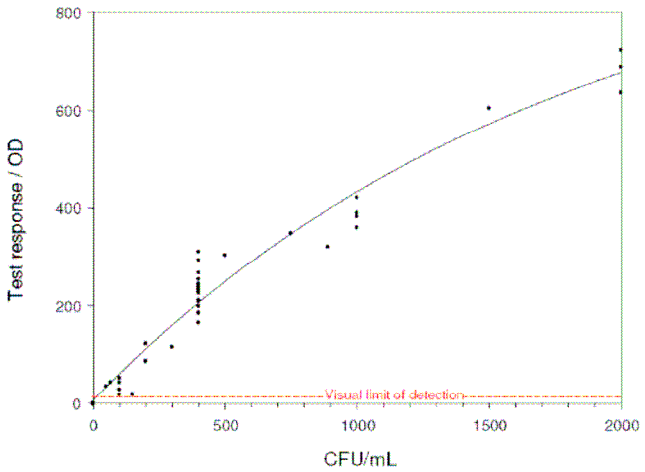

Figure 2. Graph showing relative test line intensity (OD) versus concentration of *L. pneumophila* serogroup 1. The quantified value is intended only as an objective measure during validation and should not be expected to correlate to a number of colony forming units.

ANALYTICAL SPECIFICITY

Tests were preformed using water spiked with the subtypes of *L. pneumophila* serogroup 1 listed in **Table 1**. All of these samples gave positive results, confirming the desired reactivity of the Test.

Table 1. Known *L. pneumophila* serogroup 1 subtypes detected by the Test.

***L. pneumophila* serogroup 1 subtype**	**Isolate ID**
Camperdown 1 (*)	ATCC 43113
Bellingham 1 (*)	ATCC 43111
Philadelphia 1 (*)	ATCC 33152
Allentown 1 (*)	ATCC 43106
France (*)	ATCC 43112
Olda (*)	ATCC 43109
Heysham (*)	ATCC 43107
Oxford 4032E (*)	ATCC 43110
Knoxville 1 (*)	ATCC 33153
Pontiac	NCTC 11191
Kingston 1	NCTC 11378
Benidorm W872 (*)	NCTC 12821
Benidorm O3OE	ATCC 43108

The other *L. pneumophila* serogroups and species listed in **Table 2** were prepared in water at concentrations greater than 10^9 CFU/L. Cross reactions were observed with *L. pneumophila* serogroups 4 and 7 at concentrations greater than $6x10^9$ CFU/L.

Table 2. Non-*pneumophila* serogroup 1 *Legionella* isolates tested for cross-reactivity with the Test.

Bacteria name	**Isolate ID**
L. anisa	ATCC 35292
L. boxemanii	ATCC 33217
L. dumoffii	ATCC 33279
L. feeleii	ATCC 35072
L. gormanii	ATCC 33297
L. jordanis	ATCC 33623
L. micdadei	ATCC 33218
L. oakridgensis	ATCC 33761
L. pneumophila serogroup 2	ATCC 33154
L. pneumophila serogroup 3	ATCC 33155
***L. pneumophila* serogroup 4**	**ATCC 33156**
L. pneumophila serogroup 5	ATCC 33216
L. pneumophila serogroup 6	NCTC 11287
***L. pneumophila* serogroup 7**	**ATCC 33823**
L. pneumophila serogroup 8	ATCC 35096
L. pneumophila serogroup 9	ATCC 35289
L. pneumophila serogroup 10	ATCC 43283
L. pneumophila serogroup 11	NCTC 12179
L. pneumophila serogroup 12	NCTC12180
L. pneumophila serogroup 13	NCTC 12181
L. pneumophila serogroup 14	ATCC43703
L. pneumophila serogroup 15	ATCC 35351
L. sainthelensi	ATCC 35248
L.longbeachae	ATCC 33462

Samples of the common bacteria shown in **Table 3** were prepared with concentrations greater than 10^9 CFU/L in water and analyzed using the Test. Of these bacteria, only *Staphylococcus aureus* cross-reacted with the Test. Cross-reactions were observed only at concentrations greater that 10^8 CFU/L, which is a concentration higher than would be expected in water systems.

Table 3. Non-*Legionella* bacteria tested for cross-reactivity with the Test.

Bacteria Name	**Isolate ID**
Acinetobacter calcoaceticus	NCIMB 10694
Aeromonas hydrophila	NCIMB 72
Bacillus subtillis	ATCC 6633
Burkholderia cepacia	NCIMB 8507
Citrobacter freundii	ATCC 8090
Citrobacter koseri	NCIMB 2117
Enterobacter cloacea	ATCC 35030
Escherichia coli	ATCC 25922
Klebsiella oxytoca	NCIMB 2121
Pseudomonas aeruginosa	NCTC 10332
Pseudomonas fluorescens	NCTC 10038
Pseudomonas putida	NCIMB 18
Pseudomonas stutzeri	NCIMB 568
Ralstonia pickettii	NCIMB 13142
Raoultella terrigena	NCIMB 8135
Staphylococcus aureus	**NCTC 8530**
Streptococcus pyogenes	ATCC 19615
Yersinia ruckeri	NCIMB 1315

In order to investigate whether the presence of other *Legionella* bacteria in a sample could prevent the detection of *L. pneumophila* serogroup 1, samples containing high levels of four other *Legionella* species and serogroups (**Table 4**) were spiked with 400 CFU/mL of *L. pneumophila* serogroup 1 (NCTC 12821). No interference was observed.

Table 4. Bacteria screened in combination with *L. pneumophila* serogroup 1 for inhibition. No adverse effects were detected.

Background Bacteria	
Species / serogroup	**Maximum concentration examined (CFU/L)**
L. pneumophila serogroup 4	$3.4x10^8$
L. pneumophila serogroup 10	$4.7x10^8$
L. bozemanii	$4.6x10^8$
L. Longbeachea	$7.2x10^8$

WATER CHEMISTRY COMPATIBILITY

Samples of various reference waters were spiked with *L. pneumophila* serogroup 1 before applying to the Test. Unspiked samples were also applied to the Test. No interferences were observed. The water samples used were: sterile tap water; ASTM grade II deionized water; simulated cooling water; WHO standard hard water at 460 and 1000 ppm total hardness. Water samples were also prepared by adaptation of the WHO standard formulation to give total hardness up to 5000 ppm (as calcium carbonate), and with carbonate alkalinity up to 1000 ppm, bicarbonate alkalinity at 1500 ppm in total water hardness of 3000 ppm. No interference, cross-reaction, or other adverse effect was observed in any

tests performed with these samples. The results are summarized in **Table 5** and **Table 6.**

Table 5. Reference water types **spiked with *L. pneumophila* serogroup 1**.

Water type	Result	Test Line OD
Tap water	Positive	1040
De-ionized water	Positive	1254
Cooling water	Positive	1176
460 ppm hard water	Positive	1235
1000 ppm hard water	Positive	1096
100 ppm NaS_2O_3	Positive	1218

Table 6. Negative control experiments with reference water types. (**without** *L. pneumophila* serogroup 1)

Water type	Result	Test Line OD
Tap water (1)	Negative	0
Tap water (2)	Negative	0
Tap water (3)	Negative	-3
Deionized water (1)	Negative	0
Deionized water (2)	Negative	0
Deionized water (3)	Negative	-3
Cooling water	Negative	0
460ppm hard water	Negative	0
1000ppm hard water	Negative	0
100 ppm NaS_2O_3	Negative	0

NON-OX BIOCIDE PRODUCT COMPATIBILITY

Biocides are routinely used for bacterial control in water systems and Test compatibility with common water treatment biocides was also evaluated. Biocides and biodispersants containing the following ingredients were obtained from their manufacturer: 2,2-dibromo-3-nitrilopropionamide, glutaraldehyde, methanol, 5-chloro-2-methyl-4-isothiazolin-3-one, 2-methyl-4-isothiazolin-3-one, didecyldimethylammonium chloride, isopropanol, alkyl polyglycoside, polypropylene glycol, polymeric biguanide, tetrakis hydroxymethyl phosphonium sulphate and terbuthylazine. The chemicals were diluted in sterile water at their recommended maximum dosing concentration. The solutions were then applied to the Test. The experiment was repeated after spiking the solutions with *L. pneumophila* serogroup 1 at a concentration greater than the expected limit of detection.

No interferences were detected from any of the above water treatment chemicals except polymeric biguanide and tetrakishydroxymethylphosphonium sulphate (THPS). At the recommended dosing concentration the polymeric biguanide (30 ppm active ingredient) and THPS (100 ppm active ingredient) based biocides resulted in a false positive signal on the Test. The Test should therefore not be used on water systems treated with these biocide active ingredients (biguanide or THPS).

OXIDIZING BIOCIDE COMPATIBILITY

Samples of *L. pneumophila* serogroup 1 were exposed to sodium hypochlorite at a range of temperatures and concentrations. Samples were evaluated using the Test after various contact periods. Chlorine did not interfere with the performance of the Test at concentrations less than 5 ppm of free chlorine. Chlorine does however gradually oxidize antigen in the sample over a period of time, rendering it undetectable. The rate of this reaction was observed to be dependent on both the concentration of free chlorine and the temperature of the water, with a $\log_{10}$ reduction in signal visible in as little as a few minutes with 5 ppm Cl_2 at 37 °C to several hours with 0.3 ppm Cl_2 at 5 °C. In all cases the rate of signal reduction was slower than the rate at which the oxidizing biocide killed the *Legionella* cells (as determined by culture). When the Test is used to test water samples immediately and the free oxidant residual is less than 5 ppm, then no treatment is needed to neutralize oxidant residual. If the free residual oxidant is greater than 5 ppm, or if there is delay between collecting and testing a sample, then sodium thiosulfate should be added to neutralize the residual oxidant.

OTHER TEST CONDITIONS

The robustness of the Test to a range of chemical and physical parameters was established from a D-optimal statistical experimental design to explore a range of parameters in unison. As well as investigating the effects of individual parameters, the design was structured to explore interactions between factors. All levels of each condition listed in **Table 7** were compatible with Test operation.

Table 7. Experimental conditions used to verify the operating limits of the Test.

Condition	Levels examined		
pH	5	7.5	10
Environmental temp (°C)	4	24.5	45
0.18 % Na_2SO_3	present		absent
Free chlorine / ppm	0	2.5	5
Hardness / ppm $CaCO_3$	40	270	500
Biocide pool (compatible)	present		absent
Development time / min	15	30	45

ENVIRONMENTAL SAMPLE TESTING

To further evaluate Test performance, eighteen water samples were obtained from geographically disperse locations including six samples each of cooling water, domestic (hot and cold) water, and whirlpool/spa water. Each sample was tested with a Test and confirmed as negative before spiking with an isolate of *L. pneumophila* serogroup 1. A total of fifteen *L. pneumophila* serogroup 1 isolates were used. Ten of these isolates were the type strains of the *L. pneumophila*

serogroup 1 subtypes. The other five isolates were wild isolates collected from industrial water samples and confirmed as *L. pneumophila* serogroup 1 by two independent laboratories. Each *L. pneumophila* serogroup 1 isolate was spiked into the environmental water sample at two different concentrations.

The eighteen water samples and fifteen isolates were combined in a pseudo-random pattern to yield a total of 182 experimental samples that were then analyzed in parallel via both ISO11731 culture method and using the Test. Samples were processed within 30 minutes of spiking. The ISO11731 culture method was performed to achieve an analytical sensitivity of 10 CFU/mL. The results are shown in **Table 8**. Culture results in Table 8 are reported as "Positive" at concentrations greater than 100 CFU/mL to correspond to the Test sensitivity of 100 cells per mL.

All fifteen *L. pneumophila* serogroup 1 isolates were detectable using the Test. *L. pneumophila* serogroup 1 was detectable using the Test in all 18 water samples.

The Test detected *L. pneumophila* serogroup 1 in 51 of the spiked samples where the culture method reported a result of "Not Detected" (<10 CFU/mL). The Test detected *L. pneumophila* serogroup 1 in an additional 14 of the prepared samples where the culture method reported a result of between 10 and 90 CFU/mL. These results are related to the Test property of detecting non-culturable cells.

The Test detected *L. pneumophila* serogroup 1 in 81 of the prepared samples where the culture method reported a result of greater than 100 CFU/ml. Total number of positives detected by the Test was 146.

In 16 samples both the culture method and the Test failed to detect *L. pneumophila* serogroup 1, suggesting the levels of *Legionella* spiked in these samples were below the detection limit of both methods.

In 20 samples the Test failed to detect *L. pneumophila* serogroup 1 when the culture result gave 100 CFU/mL or greater. These results can be regarded as false negatives. There was no apparent trend or correlation with the water samples or *Legionella* isolated involved.

Table 8. Comparison of culture method and FastPath Test results from spiked water samples. Note: Culture results were considered Negative when <100 CFU/mL.

Combined Results		Culture Results		
		Positive	Negative	Total
Test Results	Positive	81	65	146
	Negative	20	16	36
	Total	101	81	182

Diagnostic sensitivity is a test commonly used in assessing the performance of diagnostic devices or methods. Here it defines the percentage of occasions when the Test correctly detected the presence of *L. pneumophila* serogroup 1 cells when they were known to be present at > 100 CFU/mL. A total of 101 samples gave culture results >100 CFU/mL. 80.2% of those were also positive via the Test. Studies on other methods for detecting *Legionella* such as standard culture methods have resulted in similar diagnostic sensitivities. For example, analysis of data from the Health Protection Agency Environmental Quality Assurance Scheme revealed that 79% of samples submitted to laboratories using culture methods and known to contain *L. pneumophila* serogroup 1 are reported as positive (calculated using data from January, 1999 through November, 2006 with 10,826 laboratory reports in the HPA scheme).

Non-culturable detection rate: It is not possible to definitively define if any result obtained via the Test is a false positive or not, since there is no other technique available that is completely reliable at detecting the presence of *L. pneumophila* serogroup 1 antigen in a sample. A study consisting of 48 randomly selected water samples submitted for ISO11731 culture analysis at a central lab was performed by also analyzing each water sample using the Test. The culture and Test experiments were conducted blind of each other. 13% of the samples were positive by the Test but negative via culture. There are a number of possible explanations for these results: (a) The Test detected dead cells. (b) The culture test did not grow the cells (viable but non-culturable cells). (c) False negatives by culture. (d) Chemical or biological interference with the Test.

Chemical analysis was performed on samples to identify any likely cause of interference. No correlation could be identified between the chemical constituents of the samples and the existence of positive test results. Samples of water giving positive Test results were cultivated on nutrient agar to enrich the background microbial population, but no interfering (cross-reacting) organisms were identified. Samples of water giving unexpected (based on culture) positive results via the Test were submitted for *Legionella* analysis via polymerase chain reaction (PCR). In those samples where PCR was possible (chemicals inhibiting PCR were present in a significant proportion of the samples) *Legionella* DNA was detected in the samples, suggesting that the Test was detecting nonviable or non-culturable *Legionella pneumophila* serogroup 1 cells.

LIMIT OF DETECTION AFTER ENHANCEMENT WITH FILTRATION

A filtration method has been developed which results in a 1000-fold concentration factor, for use to achieve a detection limit lower than the standard 100 cells/mL. This method consists of passing the water sample through the walls of hollow fiber filters with a nominal pore size of 0.2 microns, such that the bacteria are concentrated within the lumen of the fiber and recovering the retained bacteria into proprietary recovery buffer. A net volumetric concentration increase of up to 1000x can thus be achieved.

To measure the improvement in analytical sensitivity achieved by this method, serial dilutions were again performed using reference samples of *L. pneumophila* serogroup 1 (Health Protection Agency (UK), NTCC 12821) in dechlorinated tap water. The water samples containing *L. pneumophila* serogroup 1 were filtered, tested using the standard Test strip, and visually examined by experienced operators. At least 20 samples were processed at each cell concentration about the suspected analytical sensitivity. In these experiments, the 91% Confidence Interval for detecting 100 cells/Liter *L. pneumophila* serogroup 1 in the original water sample was 92 – 100 %.

This improvement in analytical sensitivity is important for some regions where maintenance of *Legionella* contamination below 100 cells/mL (the standard analytical sensitivity for the Test with no sample concentration) is required. The experimentally determined analytical sensitivity using filtration may not be possible in some water systems containing high suspended solids. The practical limit of detection will sometimes be higher. Other methods, including culture, also suffer from recovery issues and problems enumerating results close to the detection limit particularly when combined with concentration processes to achieve low limits of detection.

TEST INTERPRETATION REPRODUCIBILITY AND REPEATABILITY

Analysis of the reproducibility and repeatability of the Test interpretation was performed in two experiments.

In the first experiment, 27 users (of mixed gender and age) examined 104 Tests that displayed results from water sample testing. The users were not previously experienced at reading the Tests and were asked to identify whether each Test was positive or negative based on the criterion that one visible line was negative and two visible lines constituted a positive result. Agreement (defined as only 1 or 2 users who did not read the Tests the same as remaining users) was observed with 94 of the 104 Tests (90%).

In the second phase of testing, two users were each asked to examine 167 Tests and categorize them on the same criteria. The Test order was then randomized and the users asked to evaluate them again. 96% repeatability was obtained (the user agreeing with their own analysis of a given Test) with 91% reproducibility (both users agreeing on the interpretation of the Test).

CONCLUSIONS

The Test is designed and validated for use in detecting *L. pneumphila* serogroup 1 in cooling water, domestic water and spa water samples. The operating range of the test (pH, temperature, compatibility), simplicity of use, ease of interpretation, and quick results make the test appropriate for on-site testing of water samples.

The Test is not a direct replacement for culture methods where they are recommended by legislation or guidelines. The Test is a useful tool for assessing and managing the risk of *Legionella* contamination in water systems in the context of an overall risk management program. This is especially true when time is a critical parameter in assessing or responding to the risk, and the wait of several days or weeks for other test results is problematic.

In contrast to this Test, other analytical methods require much more time for results, often after samples are collected and sent to a laboratory. Meanwhile, employees and the public may continue to be exposed to potential risk whilst waiting for test results. Even with laboratory techniques such as PCR, the time from sample collection to receiving the results is typically greater than 24 hours since samples are processed in central laboratories. This precludes any such tests from having the ability to detect *L. pneumophila* serogroup 1 in the field and take immediate corrective actions to potentially dangerous situations.

In addition to routine use as an indicator of *Legionella* colonization, the Test has particularly high utility in applications for: (1) As additional monitoring measures at high-risk sites. (2) Rapid confirmation of cleaning and disinfection. (3) Testing at remote locations where access to laboratory facilities is impractical.

The speed and ease of use provided by the Test make on-site *Legionella* testing practical for the first time, with no equipment requirement, no laboratory support and practically no training to follow the simple instructions. The quick results allow the water system operator to take immediate measures to reduce risks associated with *Legionella* contamination.

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