



# FAST DETECTION OF LEGIONELLA PNEUMOPHILA IN COOLING TOWERS BY IMMUNOMAGNETIC MICROSPHERES

By Guillermo Rodríguez<sup>1</sup>, Fabrice Sultan<sup>2</sup>,  
Begoña Bedrina<sup>1</sup> and Inmaculada Solís<sup>3</sup>

**▷SUMMARY**

Legionnaires' disease is a severe form of pneumonia caused by bacteria of the genus *Legionella*. It is a notifiable disease worldwide, with relevant clinical aspects, such as a fatality rate of 12-15 %, easily extended up to 30-50% in immuno-compromised patients. Predominant species responsible for illness is *L. pneumophila*, a virulent pathogen which is responsible for 90-98 % of the cases. But Legionnaires' disease is not only a serious threat to the public health system. Although the disease is preventable if persons are not exposed to environments which harbor *L. pneumophila*, this microorganism is extremely successful at persisting in man-made aquatic environments from which they can be transmitted to humans. Cooling towers, hot tubs, showerheads, whirlpools and spas, public fountains, in the home, offices, hotels, hospitals, among other locations. The WHO, suggest that Water Safety Plans (WSP) must be adopted for *Legionella* risk assessment in these environments. In this context, detection of *L. pneumophila* in environmental samples also represents a considerable challenge for public health. Rapid tests are essential to quickly take on the right preventive and corrective actions, periodically verifying if WSP is correctly implemented and functional. Cost-effective, reliable and simple tests are a key issue for monitoring water quality and prevention in the outbreaks of *Legionella* infections.

Culture based-methods require long incubation times (12-15 days) due to slow growth rate of the bacterium, and the results can be under-estimated because *L. pneumophila* can enter into a state viable but non-culturable (VBNC). This physiological state still represents a public health hazard. Moreover, it is difficult to isolate *Legionellae* in environmental samples containing high levels of other microbiota. Numerous PCR-based methods have been developed as an alternative to culture isolation. However, it fails to discriminate between live and dead bacteria and results can be over-estimated due to the persistence of DNA in the environment after cells have lost viability or died. PCR-based methods require bacterial lysis, special instrumentation and trained personnel.

## “Cooling towers are known to be one of potential sources of harboring, amplifying and disseminating *L. pneumophila*”

Cooling towers are known to be one of potential sources of harboring, amplifying and disseminating *L. pneumophila*. In order to minimize potential health hazards associated with *L. pneumophila*, the excessive growth of this bacterium must be prevented in cooling towers. Composition of water in cooling towers can be very complex, including substances that may cause a loss of culturability in the cells, so they can not recovered by culture techniques. Moreover, this water can contain substances, such as heavy metals, inhibiting polymerase reaction in the PCR. Immunosensing techniques addressed to detect intact cells can be a good approach to restrict detection to the viable cells. The surface integrity of a bacterial cell is a well-accepted criteria for characterizing viable forms and distinguishing them from damaged and surface-compromised cells. Concentrated samples can be analyzed by immunosensing formats, avoiding time-consuming culturing. In this context, the development of biosensors and quick assays for *L. pneumophila* detection based on immunomagnetic microspheres appears as a very interesting field. In this study, kit Bioalarm *Legionella* based on Estapor magnetic microspheres is applied to environmental monitoring of cooling towers, as an efficient immunosensing alternative overcoming drawbacks of the above mentioned techniques. The methodology used is enzyme-linked immunomagnetic colorimetry.

**▷ENVIRONMENTAL SAMPLES**

Water samples were collected from several industrial cooling towers. This water represents very complex matrices, with unstable effects on results obtained by current analytical methods. According to standard ISO 11731 for Detection and Enumeration of *Legionella*, sample was obtained concentrating 1 liter of water using 0.40 µm polycarbonate filter membranes. Water samples were also analyzed by culture technique and PCR technique.

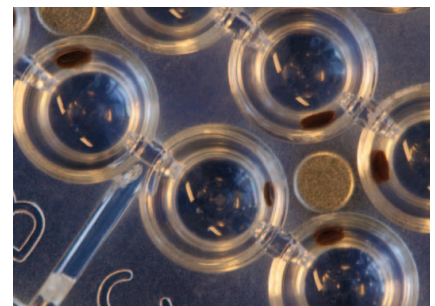
**▷ESTAPOR® MICROSPHERES**

Estapor® microspheres are super-paramagnetic material, colloiddally stable in the absence of a magnetic field. Exposed to a magnetic field, a total separation of the microspheres is achieved rapidly and completely. The iron oxide material is uniformly distributed in the polystyrene matrix. In this study, an antibody against *Legionella pneumophila* has been immobilized by covalent coupling onto the surface of Encapsulated Magnetic Microspheres, reference EM1-100/40. *Figure 1* was obtained with these magnetic microspheres (size: 1 µm and ferrite:45%). The Estapor® Magnetic Microspheres are, in fact, superparamagnetic materials. The magnetization of the microspheres increase with the applied magnetic field and falls back to zero when the field is removed. Neither hysteresis nor residual magnetization is observed and that has a very practical advantage on the immunosensing format, because when the field is removed, the microspheres demagnetize and re-disperse easily. This property allows efficient washing steps, low background and good reproducibility.

This immuno-activated material was used in the separation of the captured cells, present in filtered water samples taken from a cooling tower, in industrial facilities. High binding capacity, fast magnetic response time, good colloidal stability, and a fast recovery of dispersion after attraction, are critical benefits reported by this material to construct quick immunoassays for microbial detection.

**▷KIT BIOALARM® LEGIONELLA**

The Kit Bioalarm *Legionella* utilizes magnetic microspheres for rapid determination of *L. pneumophila* (50 minutes), providing effective separation, concentration, and measurement of this target in waters. Recently, Kit →



▲Figure 1: Magnetic Microspheres reference EM1-100/40

## “Legionnaires’ disease is a severe form of pneumonia caused by bacteria of the genus Legionella”

Bioalarm has been validated by expert laboratories accredited for Legionella analysis. It is designed for the use by any laboratory, company or organization that needs Legionella’s early detection in environmental samples, for the efficient prevention, alertness and control of potential sources of risk. The magnetic microspheres with antibodies anti-legionella in the reaction compositions specifically bind with the *L. pneumophila* cells present in the water sample. A negative control (without *L. pneumophila*) was also analyzed in parallel. An easy to handle micro-device (MD) enables the user to approach and remove a magnet to the basin containing the reaction mixture, as shown in figure 2. This allows the retention and suspension of the particles during the analysis, for the capture, separation and concentration of *L. pneumophila*. All reactions were performed with this micro-device at room temperature.

The procedure (figure 3) entails the capture of *L. pneumophila* by immunomagnetic microspheres (IMM), labeling with enzyme-conjugated antibody in a sandwich format, and subsequent colorimetric analysis after a brief reaction with enzyme substrates. This kit is the result of a project enhancing both procedure and compositions, to obtain reduced non-specific binding, high colloidal stability, low bacterial aggregation, and inhibited bacterial enzymes that may interfere with reading reaction. If the basin of the

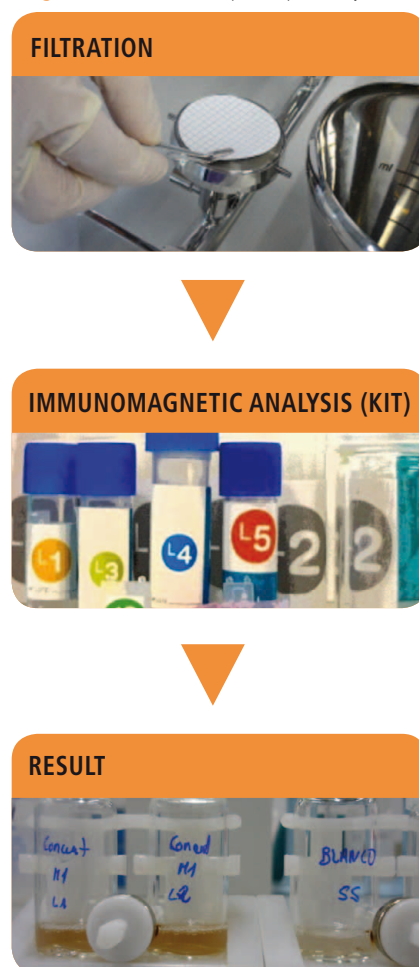
sample turns out to be more colored than the basin of the control, the result is considered positive. Then, this color is visually compared to a color card to estimate the order of magnitude of the quantity of *L. pneumophila* that would expect to be obtained by culture: 102, 103, 104, or more than 104cfu/L. In this way, the user know his fulfillment of the in force legislation, based on a rapid screening of the samples. Approximately in 50 minutes, one person can analyze between 10-20 samples, without training or special instrumentation.

Enumeration of bacterial cells also may be conducted using a validated calibration curve. In this case, the liquid can be placed in the reading chamber of the spectrophotometer. Absorbance both of the sample and negative control were measured. Normalized absorbance was introduced into calibration curve and quantity of *L. pneumophila* was determined.

### ▷ RESULTS

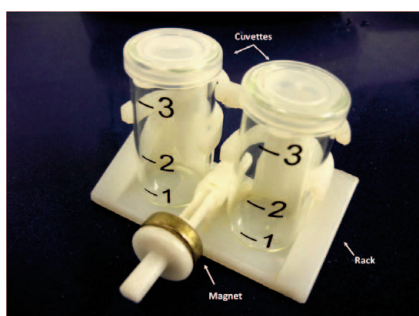
Six environmental samples collected from different cooling towers, were examined for the presence of *L. pneumophila*. Each sample was divided into three portions, a first portion analyzed by culture method, a second portion analyzed by PCR method, and a third portion analyzed by Kit Bioalarm. This third portion was divided into ten identical portions as replicates. The detection limit was considered the level of *L. pneumophila* in which nine portions of the ten ones were visually positive. Results are shown in figure 4. Under conditions of this experiment, the detection limit of the Kit Bioalarm obtained by comparison with culture was 280cfu/L. It is interesting that this detection limit allows the use of this method to rapidly monitoring the degree of the mandatory regulations.

▼ Figure 3: Workflow of the *L. pneumophila* analysis



Kit Bioalarm can minimize false-positives results compared to PCR because it is based on antigen-antibody interaction which is depending on surface integrity, so damaged surfaces are less reactive than integrate surfaces, and give rapid results compared to the current time-consuming culture method, also minimizing false-negatives due to the detection of viable but non-cultivable cells.

In other experiment, a water sample systematically positive by PCR but negative by culture was also analyzed by the three methods: culture, PCR and Kit Bioalarm. Culture revealed the →



▲ Figure 2: Micro-device (MD) for easy to handle magnetic microspheres

Water samples from cooling towers		Kit Bioalarm		
Culture (cfu/L)	PCR	No. of Positive	No. of Negatives	Positives (%)
280	Not defined	9	1	90
340	+	10	0	100
1600	+	10	0	100
2800	+	10	0	100
3400	+	10	0	100
27800	+	10	0	100

(\*) Ten replicates for each sample

▲ Figure 4: Comparison of culture, PCR and Kit Bioalarm results.

## “Outbreaks of Legionnaires’s disease have been traced to a wide variety of environmental water sources”

presence of background organisms, inhibiting growth of Legionellae in the plate. PCR was positive, suggesting that *L. pneumophila* was present but in a form that is not detectable by culture technique. Recovery by culture of *L. pneumophila* in spiked samples of this water was not possible, confirming loss of cultivability. Chlorine disinfection treatment did not alter the qPCR results, confirming that PCR assay lack the ability to discriminate between living and not living *L. pneumophila* cells. This water “culture - / PCR +” was also analyzed by the Kit Bioalarm (figure 5). The result was positive, with a color corresponding to an order of magnitude

of 100cfu/L. Moreover, final liquid was placed in the reading chamber of a spectrophotometer. Both absorbance of the sample and a negative control were measured. According to calibration algorithm, reported absorbance was corresponding to an equivalent concentration of 600cfu/L.

Finally, chlorine disinfection treatment did reduce obtained color to 50%, but did not reduce PCR result, demonstrating that kit may be used as a good indicator of the risk associated with *L. pneumophila*.

### ▷ CONCLUSION

Outbreaks of Legionnaires’s disease have been traced to a wide variety of environmental water sources. The vast majority of cases are due to *L. pneumophila*, capable to achieve infective level in only a few days. Over the last few years, the reported incidence has steadily increased. Immunosensing approach based on magnetic microspheres appears as a very interesting alternative to the culture for detecting intact cells of *L. pneumophila*, without training or special instrumentation.

In this study a test that allows an efficient surveying and environmental monitoring of *L. pneumophila* is presented. Kit Bioalarm, based on Estapor Magnetic Microspheres, represent a powerful tool for this purpose. <sup>16</sup>

▼ Figure 5 Detection of viable but non cultivable *L. pneumophila* by Kit Bioalarm.

METHOD	RESULT
Kit Bioalarm	0,10
PCR	+
Culture	-

### ▷ REFERENCES

References available on request (magazine@iirme.com)

### ▷ AUTHOR INFORMATION:

1. Biótica, Bioquímica Analítica, S.L., Parque Científico Tecnológico y Empresarial de la Universidad Jaume I- Edificio de Investigación I, 2ª Planta, Castellón-Spain
2. Merck Chimie SAS, 94126 Fontenay-sous-Bois, France, France
3. Iproma, S.A., Camino de la Raya, No. 46, Castellón-Spain.

