Microbiology Focus

Volume 4.2, 2012

Fluka Analytical

Legionella pneumophila, an Unpredictable Pathogen

Steam from a cooling tower is one possible way that *Legionella* can be spread.

Legionnaires' Disease

By Jvo Siegrist, Product Manager Microbiology…. *ivo.siegrist@sial.com*

A Potentially Fatal Form of Pneumonia

Legionaires' disease is a dangerous and infectious pneumonia which can affect anybody, but those at highest risk are the elderly, sick, smokers, or other people with weak immune systems.

The causative organism is the *Legionella pneumophila*, which easily forms aerosols (fine airborne particles) which can be inhaled, causing infection. The natural source of *Legionella pneumophila* is all types of water, but particularly water containing algae, rust, sludge, scale, and other organic compounds. It is known that *Legionella* are able to build biofilm inside water pipes, where they are protected against antimicrobial treatments. The infected water droplets can be produced in such places as whirlpool spas or air conditioners, but they can also occur in freshwater such as rivers and ponds. However, most people exposed to *Legionella* do not become ill, and it is not possible for Legionnaires' disease to be transmitted from person to person.

The name Legionnaires' disease originated with an outbreak in Philadelphia, Pennsylvania at an American Legion convention (1976). In 1977, the new pathogen was identified and named *Legionella* (**Figure 1**). Symptoms are flu-like, with chills, muscle pains, headache, cough and high fever. Death can result from severe pneumonia. The Philadelphia outbreak sickened hundreds and resulted in 34 deaths. Additionally, *Legionella* is also the causative organism of Pontiac fever, which is a milder respiratory illness resembling acute influenza.

A serious outbreak of *Legionella pneumophila* occurred in 2009/2010 in Neu Ulm, Germany. With 64 cases and 5 deaths, it was the worst outbreak of legionellosis in Germany. Based on the molecular biological profile, it was assumed that the source was a large, wet cooling tower. About 400 incidences of legionellosis are reported in all of Germany each year.

In November 2011, a new drinking water ordinance was put into place in Germany which regulates large industrial water heating systems. Once a year a sample has to be tested, and the level of *Legionella* should be below 100 cfu per 100 mL.

Legionella pneumophila is an obligate aerobic gram-negative, rod-shaped bacterium with monopolar flagella. Often the size of the bacteria in the culture is very variable (2-20 µm, **Figure 2**), and it is commonly found in aquatic environments. The organism can survive a wide range of conditions, including temperatures from 0 to 63 °C, and pH values from 5.0 to 8.5. Because *Legionella* has cysteine as a growth requirement, it does not grow on common blood agar media. *Legionella pneumophila* primarily uses amino acids as an energy source and also needs iron (III) ions. This is probably the reason that in nature, *Legionella pneumophila* is often found together with iron bacteria and amoeba (**Figure 3**). The optimal temperature range for growth of *Legionella* is given at 25 to 50 °C.

Did you know …

that smokers are easier targets for bacteria?

According to a new study, it was observed that beneficial bacteria are inhibited in the mouths of smokers, resulting in greater susceptibility to other bacteria (Source: Science Daily). Information about the ratio of benefical flora in the lungs is not yet available, but this study may provide insight into the reason why *Legionella pneumophila* thrives in smokers' lungs.

Figure 1: Smoking person

Figure 2: Colorized scanning electron micrograph (magnification 10000X) depicted a number of gram-negative *Legionella pneumophila* bacteria. Colony was grown on BCYE medium without antibiotic addition. (Source: CDC/ Margaret Williams, PhD; Claressa Lucas, PhD; Tatiana Travis, BS, 2009).

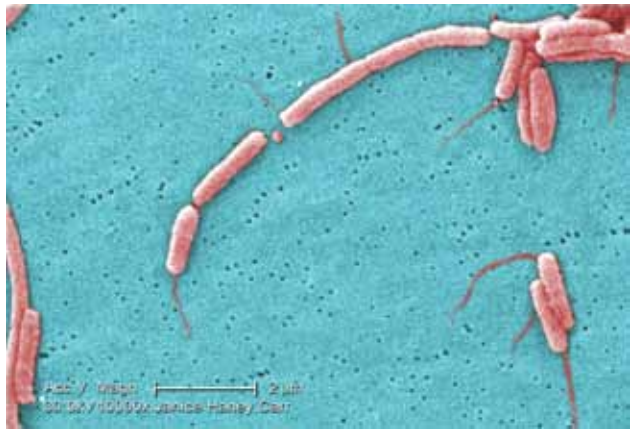


Table 1: Discs with certified reference test strains of *Legionella* (Restrictions may apply in some countries)

Vitroids™ Test Strains	Origin	Strain No.	CFU	Cat. No.
Legionella bozemanii	NCTC	11368	50000	RQC02908
Legionella pneumophilia	ATCC	12821	50000	RQC02008

Figure 3: Colorized electron micrograph depicts an amoeba, *Hartmannella vermiformis* (orange) as it entraps a *Legionella pneumophila* bacterium (green) with an extended pseudopod. After it is ingested, the *Legionella pneumophila* bacterium can survive as a symbiont within what then becomes its protozoan host. The amoeba then becomes what is referred to as a "Trojan horse", for by harboring the pathogenic bacteria, the amoeba can afford them protection, and in fact, in times of adverse environmental conditions, are able to metamorphose into a cystic-stage enabling it, and its symbiotic resident pathogens to withstand such environmental stresses. (Source CDC/ Dr. Barry S. Fields)

Kingdom: Bacteria
Division: Proteobacteria
Class: Gamma Proteobacteria
Order: Legionellales
Family: Legionellaceae
Genus: *Legionella*
Species: *pneumophila*

The traditional method to detect *Legionella* is based on buffered charcoal yeast extract (BCYE) agar. For growth, the organism requires the presence of cysteine and therefore does not grow on common blood agar media. Common laboratory procedures recommend concentrating the sample by centrifugation and/or filtration through 0.2 µm filters before inoculation. For selective isolation, antibiotics like polymyxin B, anisomycin, vancomycin, natamycin, cycloheximide, cefamandole, trimezhoprim, colistin sulphate, amphotericin B, bromo thymol blue and bromo cresol purple are added. Additionally, selectivity can be attained by applying heat or acid. Some typical media can be found in **Tables 2 and 3**.

Table 2: *Legionella* Media

Medium	Cat. No.
Legionella Agar, Base	74303
Feeley Gorman Agar	05598
Buffered Charcoal Yeast Extract (BCYE) Agar, Base	86558

Table 3: BCYE Agar based media

	Base Medium	Supplements
Legionella MWY Selective Agar:	BCYE Agar, Base	Legionella Supplement (Twin Pack; Fluka 89166), Legionella Selective Supplement IV (Fluka 94029)
Legionella GVPC Selective Agar:	BCYE Agar, Base	Legionella (GVPN) Selective Supplement (Fluka 43509), Legionella Supplement (Twin Pack; Fluka 89166)
Legionella GVPN Selective Agar:	BCYE Agar, Base	Legionella (GVPC) Selective Supplement (Fluka 61025), Legionella Supplement (Twin Pack; Fluka 89166)
Legionella BMPA Selective Agar	BCYE Agar, Base	Legionella Supplement (Twin Pack; Fluka 89166), BMPA Supplement: cefamandole 4 mg/L, anisomycin 80 mg/L, polymyxin B 80'000 IU/L
BCYE Agar without L-cysteine:	Legionella Agar Base	Legionella BCYE Supplement without L-Cysteine (Fluka 43753)

Table 4: *Legionella* Supplements

Supplement Content (per vial sufficient for 500 mL)	Legionella Supplement (Fluka 89166)	Selective Supplement IV (Fluka 94029)	GVPN (Fluka 43509)	GVPC (Fluka 61025)	BCYE Supplement w/out cysteine (Fluka 43753)	Legionella Selective Supplement (Fluka 18284)
ACES Buffer /KOH					5 g	
L-Cysteine hydrochloride	200 mg					
Ferric pyrophosphate, soluble	125 mg				125 mg	
α-Ketoglutarate					0.5 g	
Polymyxin B sulphate		25,000 Units	40,000 IU	39,600 IU		
Glycine		1.5 g	1.5 g	1.5 g		
Anisomycin		40 mg				
Vancomycin		0.5 mg	0.5 mg	0.5 mg		2.5 mg
Natamycin			20 mg			
Cycloheximide				40 mg		
Colistin sulphate						7,500 Units
Trimezhoprim						1.25 mg
Amphotericin B						1.25 mg
Bromo thymol blue		5 mg				
Bromo cresol purple		5 mg				

Figure 4: Detection and Enumeration of *Legionella* in Water acc. ISO 11731 (1998), ISO 11731-2 (2008)

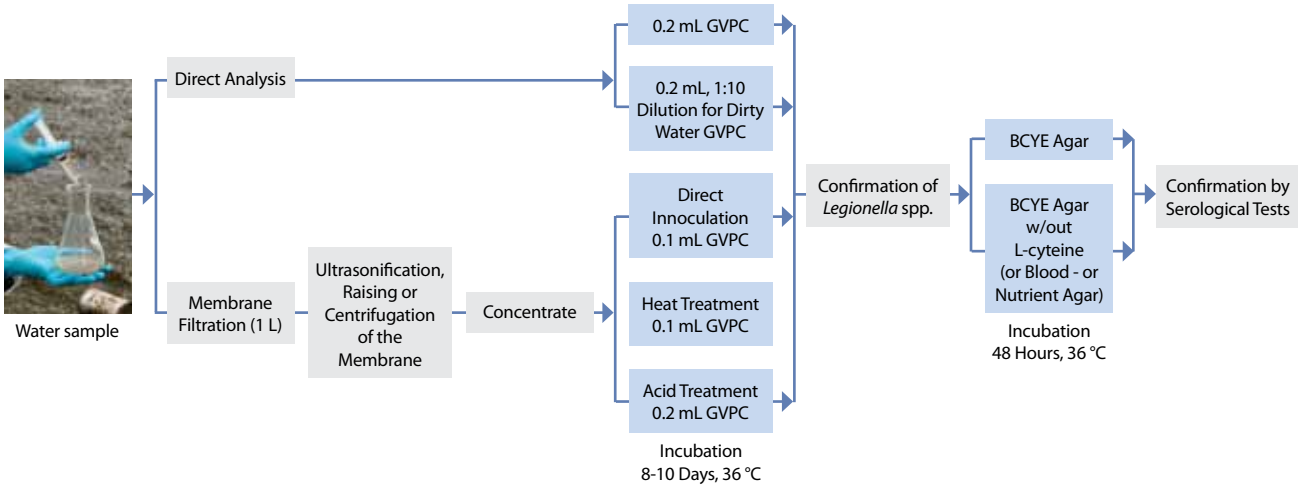


Table 5: HybriScan® Kits for Detection and Identification of *Legionella* Species

Brand	Name	Description	Cat. No.
Fluka	HybriScan***D*** *Legionella*	Detection of *Legionella*, including *L. pneumophila* in water supplies and air-conditioning systems	16593
Fluka	HybriScan***D*** *Legionella pneumophila*	Detection and identification of *L. pneumophila* in water supplies and air-conditioning systems	07190
Fluka	HybriScan***I*** *Legionella pneumophila*	Identification of *Legionella pneumophila*	49417

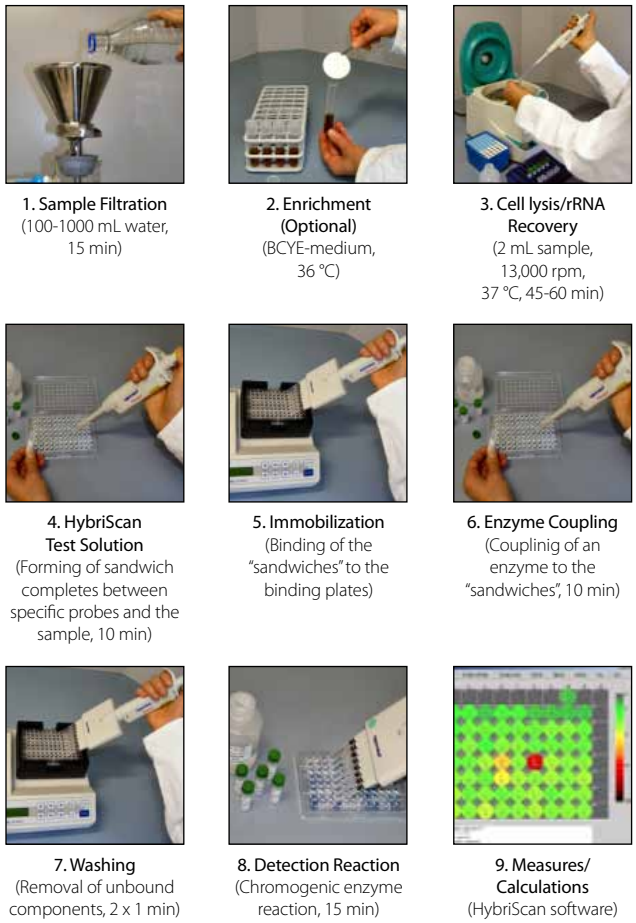
New Technology for Legionella Detection

The current procedure used to detect and identify the *Legionella* species takes a lot of time and requires several steps; therefore, new methods for the detection of *Legionella* are of great interest. A modern detection target is rRNA, which is more numerous than DNA, and is only present in living cells. The test is performed on a microtiter plate and takes less than 2.5 hours. Cell count quantification is possible with this kit using photometric methods. Compared to PCR, our system does not count dead cells, is much easier to use (see work flow in **Figure 5**), is less expensive, and is not affected by the sample matrix. Confirming its utility, our new test system won several innovation awards, and will surely become a routine method to detect *Legionella*. The rapidity, sensitivity, reliability, robustness, adaptability to sample matrix, and time-savings meet today's analytical microbiology demands.

Specificity is achieved by targeting conserved or unique rRNA sequences. A biotin-labeled capture probe is used to immobilize the target sequence on a solid support plate (streptavidin-coated microtiter plate). A digoxigenin-labeled detection probe provides an enzyme-linked optical signal read out. Detection results from application of anti-DIG-horseradish peroxidase Fab fragments. The bound complex is visualized by horseradish peroxidase substrate TMB (3,3',5,5'-tetramethylbenzidine). Photometric data are measured at 450 nm and compared with standard solutions.

For more details of our new *Legionella* detection kit, please visit *sigma-aldrich.com/hybriscan*

Figure 5: Work Flow Process of the HybriScan Legionella Kit



1. Sample Filtration (100-1000 mL water, 15 min)

2. Enrichment (Optional) (BCYE-medium, 36 °C)

3. Cell lysis/rRNA Recovery (2 mL sample, 13,000 rpm, 37 °C, 45-60 min)

4. HybriScan Test Solution (Forming of sandwich completes between specific probes and the sample, 10 min)

5. Immobilization (Binding of the "sandwiches" to the binding plates)

6. Enzyme Coupling (Couplinig of an enzyme to the "sandwiches", 10 min)

7. Washing (Removal of unbound components, 2 x 1 min)

8. Detection Reaction (Chromogenic enzyme reaction, 15 min)

9. Measures/ Calculations (HybriScan software)

Base Ingredients of Microbiology Media

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The quality of a media and its technical features depend on the base ingredients, ranging from simple sugars to peptones, salts, antibiotics, and more complex indicators.

A medium has one primary function -- to promote the growth of organisms. The components of a medium are often based upon the organism's natural habitat. For example, an organism growing on meat may require meat peptone, and an organism growing on nutrients with a high carbohydrate content may thrive on malt extract. In addition to this growth purpose, media may serve in a number of other applications, including the differentiation and identification of organisms, the selective isolation or enrichment of organisms, and the study of a certain reaction of the organism. A vast array of peptones, extracts and other additives is available to promote and sustain the growth of most organisms.

Figure 1: Diverse Ingredients Build the Base for All Kinds of Media

Proteins (Protein Hydrolysate, Amino Acids, etc.)

Although synthetic growth media are available, most media still use complex compounds, such as peptone or yeast extract, since synthetic media lack the complexity and richness of nutrients. Peptones and protein extracts are excellent natural sources of amino acids, peptides, proteins and many other growth factors. They are most often obtained by enzymatic digestion or acid hydrolysis of natural protein sources, such as animal tissues, milk, plants or microbial cultures. The range of available peptones is extensive and comprises a major role in the growth conditions of most organisms (**Table 1**).

Carbohydrates (Extracts, Sugars, etc.)

Carbohydrates are an important energy source. Mono-, di-, oligo- and polysaccharides, as well as natural extracts like rice or malt extracts, provide a versatile possibility of substrates for mold or bacteria cultures. They can also be used to make the media more selective or to identify fermentation profiles. Today, a broad range of media with chromogenic substrates is available (**Table 2**).

Biological Acids

Pyruvate, one type of biological acid, is known to promote growth and to improve the recovery rate. Other acids such as orange extracts, citric or acetic acid are also used for selective growth.

Buffering Agents

Potassium phosphates are the primary agents used for the buffering system.

Salts

Sodium chloride is used primarily for osmotic balance; however, it can also be used to make the medium more selective to halophilic and halotolerant bacteria. In addition, other salts such as lithium chloride or ammonium bismuth citrate are also used to make the medium more species specific.

Fatty Acids and Lipids

Fatty acids and lipids, such as lecithin, are necessary nutrients and a valuable source of proteins. Fluka offers egg powder and liquid sterile egg supplements, as well as pure lipids and fatty acids.

Vitamins and Trace Elements

Yeast extract, present in numerous complex media, is the most common source for Vitamin B12 (**Table 3**). Yeast extract also contains a large number of amino acids, additional vitamins, and trace elements. Some media also commonly incorporate the addition of pure vitamins and trace elements.

Selective Agents (Detergents, Bile Salts, Antibiotics, etc.)

Bile is often used as an inhibitory agent against most gram-positive bacteria. Cholates (**Table 5**), biological detergent-like compounds with anti-microbial activity, are major constituents of bile. Alternatively, SDS and other detergents are used for the same purpose. For the most part, however, selective agents are comprised of antibiotics that are often added as a mixture in supplemental vials (**Figure 1**).

Indicators and Dyes

These help to indicate biochemical properties or metabolic pathways and are vital for the identification and differentiation of organisms.

Agar

Agar is the solidifying agent in solid growth media, and its selection should be carefully considered based upon certain criteria and dependent upon the application. For example, when high transparency and brightness is needed, as in nutritional studies (Vitamin Assay Media) and sensitivity testing procedures, or when high purity and efficient diffusion of substances is essential, a highly purified agar (Fluka 05038) is recommended. For identification and differentiation, we recommend using a purified or even highly purified agar. However, when isolating a single colony, a standard quality will suffice in most cases. Typical solid media have an agar concentration of 1.0 - 1.5% to accommodate the requirements of different applications and the growth habits of target microorganisms (**Table 4**).

Table 1: Common Protein Sources for Media

Cat. No.	Description
A2427	Amicase
B4888	Beef extract
B3551	Biopeptone
53283	Brain Heart Infusion
75917	Brain Heart Infusion, Porcine*
93491	Broadbean Peptone
C7970	Casein acid hydrolysate vitamin free
22090	Casein Hydrolysate
39396	Casein Yeast Peptone
C4773	Corn gluten meal
55871	Egg powder*
93490	Fish Peptone*
49760	Gluten Hydrolysate from maize
92498	Heart extract from bovine heart*
C0501	Hy-Case® Amino
C9386	Hy-Case® SF
57462	Infusion powder from bovine heart
61300	Lactalbumin Hydrolysate
61302	Lactalbumin Hydrolysate*
03077	Liver Hydrolysate
70164	Meat Extract
C0626	N-Z-Amine® A
C1026	N-Z-Case®
18332	Peptone (vegetable)
51841	Peptone (vegetable) acid hydrolysate
19942	Peptone (vegetable), no. 1
61854	Peptone (vegetable), no. 2
77180	Peptone from animal proteins*
70173	Peptone from casein and other animal proteins
70171	Peptone from casein, acid digest*
82303	Peptone from casein, enzymatic digest
70169	Peptone from casein, pancreatic digest
70172	Peptone from casein, tryptic digest
70951	Peptone from gelatin, enzymatic digest
70176	Peptone from gelatin, pancreatic digest
P0521	Peptone from Glycine max (soybean)
93733	Peptone from meat and soybean meal*
82962	Peptone from meat, enzymatic digest
70174	Peptone from meat, peptic digest*
96174	Peptone from pea
93926	Peptone from porcine heart
83059	Peptone from potatoes
70178	Peptone from soybean meal, enzymatic digest
90765	Peptone from soybean, enzymatic digest
87972	Peptone from soybean, enzymatic digest
P6463	Peptone Hy-Soy® T
P6713	Peptone N-Z-Soy® BL 7
P4838	Peptone Primatone® HS
P4963	Peptone Primatone® RL
P5088	Peptone Primatone® RLT
68971	Peptone special
92976	Peptone special (vegetable)
77199	Peptone, mycological
P8388	Primatone®
82514	Protein Hydrolysate Amicase
82524	Protein Hydrolysate N-Z-Amine® AS*
29185	Proteose Peptone (vegetable)
P0431	Proteose Peptone Enzymatic hydrolysate
82450	Proteose-Peptone
70166	Skim Milk Powder
S1674	Soy protein acid hydrolysate
T7293	Tryptone
16922	Tryptone (vegetable)
95039	Tryptone enzymatic digest from casein
61044	Tryptone Plus
70937	Tryptose
T2813	Tryptose
12331	Tryptose (vegetable)
05138	Vegetable Extract
04316	Vegetable Extract no. 1
49869	Vegetable Extract no. 2
07436	Vegetable hydrolysate no. 2
67381	Vegetable Infusion powder
95757	Vegetable Special Infusion powder
93492	Wheat Peptone

Table 2: Carbohydrate Sources in Microbial Media Quality

Cat. No.	Description
10850	D-(-)-Arabinose
22150	D-(+)-Cellobiose
22160	D-(+)-Cellobiose octaacetate
31400	Dextrin from potato starch
31405	Dextrin from potato starch
47740	D-(-)-Fructose
48260	D-(+)-Galactose
49159	D-(+)-Glucose monohydrate
49200	alpha-D-(+)-Glucose pentaacetate
57570	myo-Inositol
70167	Malt Extract
63560	D-Mannitol
63580	D-(+)-Mannose
63582	D-(+)-Mannose
63620	D-(+)-Melezitose Monohydrate
63630	D-(+)-Melibiose
66940	Methyl alpha-D-glucopyranoside
67770	Methyl alpha-D-mannopyranoside
07915	Potato Extract
83400	D-(+)-Raffinose pentahydrate
83650	L-(+)-Rhamnose Monohydrate
84100	Sucrose
90210	D-(+)-Trehalose Dihydrate

Table 3: Yeast Extracts for Media and Fermentations

Cat. No.	Description
73145	Yeast Autolysate
92144	Yeast Extract
70161	Yeast Extract (premium quality)
09182	Yeast Extract for technological purpose
89526	Yeast Extract micro-agglomerated

Table 4: Agars for Microbiology

Cat. No.	Description
05040	Agar standard
05039	Agar purified
05038	Agar highly purified
50524	Agar anti-swarming
91411	Agar for Baird Parker Agar
05729	Agar for chromogenic media
42146	Agar for membrane filtration, low gel strength
56763	Agar high purity, low ionic content, low gel strength

Table 5: Bile Salts

Cat.No.	Description
B8381	Bile from bovine and ovine
48305	Bile Salts*
70168	Ox-bile, dehydrated, purified

Win an iPAD in the 2012 Fluka Microbiology Photography Competition

This photography competition is sponsored by Sigma-Aldrich with the aim of encouraging microbiologists to promote some aspect of their work or their field of research. The best photographic entries with the best description of the photograph's subject will win prizes such as an iPad, a Swiss army knife, a USB flashdrive, and a laser pointer.

The winning images will be published in Microbiology Focus and the best one will have the distinction of being featured on the cover.

Rules of the Competition and Conditions of Entry

1. The competition is open to all residents worldwide.
2. Entries should illustrate any microorganisms (living or dead) or a microbiologist in action at work.
3. Picture size should be at least 400 dpi and 90 x 120 mm (max 3 MB). The file format must be in jpg, tiff or pdf!
4. The entries will be judged on:
   - clarity of presentation
   - composition
   - illumination and contrast
   - congruency of subject matter and title of photograph
   - scientific interest and relevance
   - originality
5. Winning entries will be retained by Sigma-Aldrich, who will have sole rights of publication, reproduction and display.
6. Closing date for contest entries will be July 31, 2012.
7. Entries received after the closing date will not be considered. Entries received incomplete, illegible, mutilated, altered or not complying exactly with the instructions and theme may be disqualified.
8. Decisions of the judges in all matters affecting the competition will be final and legally binding.

The competition will be judged by:

Dr. Lars Fieseler
Zurich University of Applied Sciences - ZHAW
Supervisor, Department Microbiology

Prof. Mohammad Manafi
Medical University of Vienna
Head of Department for Food Hygiene

Jvo Siegrist, Sigma-Aldrich
Product Manager, Microbiology

Method of Entry

There is no entry fee, but an entry form must be completed for each entry (a maximum of two entries may be entered)

Entry forms are available from our website
sigma-aldrich.com/fluka-mibi-competition

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