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# EU Reference Laboratory for public health in the field of *Legionella*

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## Document overview

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| <b>Authors</b>                     | Sophie Jarraud, Maria Luisa Ricci, Joao Rodrigues, Markus Petzold, Camille Jacqueline, Maud Baume, Maria Scaturro, Fabiola Mancini                          |
| <b>Reviewed and agreed by ECDC</b> | External reviewers: Charlotte Michel (University of Brussels) and Claressa Lucas (US CDC)<br>ECDC: Lara Payne Hallstrom, Maximilian Riess, Cecilia Jernberg |
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## Introduction

Legionellosis is an acute bacterial infection caused by bacteria of the genus *Legionella*, most frequently *Legionella pneumophila*. *Legionella* can cause severe pneumonia known as Legionnaires' disease (LD), a mild non-pneumonic febrile illness called Pontiac fever, and extrapulmonary legionellosis. This handbook focuses primarily on LD, although some sections also include relevant information on extrapulmonary legionellosis. The clinical and epidemiological characteristics of legionellosis have been described in detail elsewhere and are summarized here.

LD is an important cause of both community-acquired and nosocomial pneumonia. Risk factors for LD include advanced age, smoking and underlying comorbidities, such as cancer, chronic lung disease, diabetes, renal disease and immunosuppression. Men are more than twice as likely to develop LD as women. The incubation period generally ranges from 2 to 10 days, although incubation periods of up to 19 days have been reported during some outbreaks. LD typically presents as pneumonia and is characterized by anorexia, malaise, myalgia, headache, chills and fever. Other common symptoms include non-productive cough, diarrhoea and abdominal pain. The disease frequently requires hospitalization and has a case fatality rate of approximately 10–15%. Mortality is higher among immunocompromised individuals, with reported case fatality rate reaching up to 39% in hospitalized patients. Most LD cases are sporadic, with a marked seasonal pattern occurring predominantly during summer and autumn; however, outbreaks are also frequently reported.

When a case of LD is identified, investigations are conducted to determine the patient's exposure history during the incubation period, typically defined as two weeks preceding symptom onset. Based on the exposure history, cases may be classified as community-acquired, domestically-acquired, occupational, nosocomial or travel-associated. Cases are generally reported to national surveillance systems following collection of epidemiological exposure data. Individual cases may also be investigated for possible temporal or geographical links with other cases. The identification of a nosocomial or domestically-acquired infection often triggers an environmental investigation of the water supply system associated with the healthcare facility or building implicated in the infection. Such investigations usually involve sampling of water and biofilms from water systems, cooling towers or other potential environmental sources. In the European Union/European Economic Area (EU/EEA), travel-associated cases may be reported through the [European Legionnaires' Disease Surveillance Network \(ELDSNet\)](#), coordinated by ECDC, which aims to identify clusters or outbreaks associated with accommodation sites worldwide. ECDC conducts EU-level surveillance with annual data collections on all LD cases diagnosed in the EU/EEA and notified by Member State. Annual retrospective data collection is also conducted on LD outbreak events. In addition to routine national and European surveillance, the Serious Cross-Border Threats to Health (SCBTH) Regulation establishes a framework for the detection and reporting of events that may pose cross-border health threats.

Genomic surveillance has the potential to complement existing epidemiological investigations by enabling higher-resolution comparison of clinical and environmental isolates, facilitating earlier detection of dispersed clusters, and strengthening evidence for cross-border transmission. As such, pilot implementation of genomic surveillance at European level through EpiPulse Cases has contributed to the objectives of the SCBTH Regulation. Microbiological investigations are therefore essential not only to identify the causative agent and, where possible, confirm the source of contamination but also to support LD surveillance at both the

national and European levels. [European technical guidelines](#) produced by ESCMID Study Group for *Legionella* Infections (ESGLI) are available to support the investigation and control of infections caused by *Legionella* species. In addition, the World Health Organization has published guidance on the [surveillance and outbreak management of water-related infectious diseases associated with water-supply systems](#), including a dedicated case study on *Legionella*.

This laboratory handbook aims to present methods, protocols and good laboratory practices to support the timely and accurate diagnosis and detection of *Legionella* in clinical and environmental specimens, as well as the identification and confirmation of infection sources. By promoting harmonised laboratory practices and strengthening laboratory capacity, it also aims to support surveillance, facilitate the detection and investigation of outbreaks, and enhance preparedness and coordinated responses to potential cross-border health threats.

## 1. EU case definition of LD

European surveillance case definitions for infectious diseases are established through EU legislation. At publication of this handbook the surveillance case definition is according to COMMISSION IMPLEMENTING DECISION (EU) 2018/945<sup>1</sup> of 22 June 2018 and stated below:

**Clinical criteria:** Any person with pneumonia

**Laboratory criteria:**

***Laboratory criteria for case confirmation:***

At least one of the following three:

- Isolation of *Legionella* spp. from respiratory secretions or any normally sterile site
- Detection of *Legionella pneumophila* antigen in urine
- Significant rise in specific antibody level to *Legionella pneumophila* serogroup 1 in paired serum samples

***Laboratory criteria for a probable case:***

At least one of the following four:

- Detection of *L. pneumophila* antigen in respiratory secretions or lung tissue for example, by DFA staining using monoclonal-antibody derived reagents
- Detection of *Legionella* spp. nucleic acid in respiratory secretions, lung tissue or any normally sterile site
- Significant rise in specific antibody level to *Legionella pneumophila* other than serogroup 1 or other *Legionella* spp. in paired serum samples

Single high level of specific antibody to *Legionella pneumophila* serogroup 1 in serum.

**Epidemiological criteria:** NA

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<sup>1</sup> <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018D0945&from=EN#page=26>

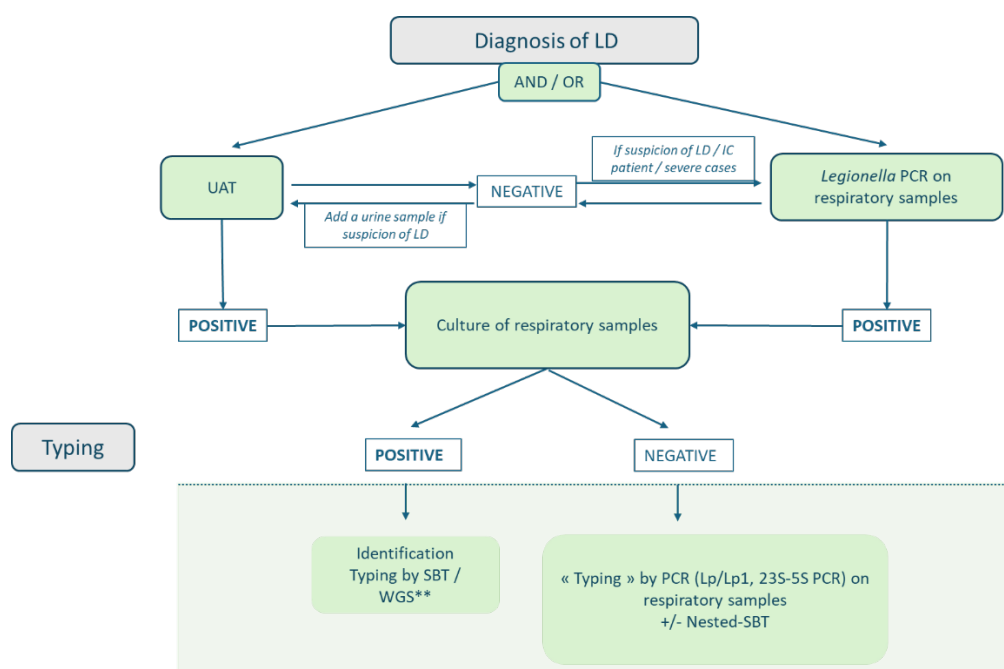
## **Diagnosis of Legionnaires' disease (LD)**

## 2. Diagnosis of Legionnaires' disease (LD)

Two methods are central to the early diagnosis of LD: urine antigen detection (UAT) and polymerase chain reaction (PCR) performed on respiratory specimens. These methods are based on different diagnostic principles. UAT detects soluble *Legionella pneumophila* serogroup 1 (Lp1) antigen excreted in the urine, whereas PCR detects *Legionella* DNA directly in respiratory specimens. Consequently, the two methods differ in their diagnostic performance, spectrum of detection, and susceptibility to factors such as the timing of specimen collection, bacterial load, prior antimicrobial treatment, and the infecting *Legionella* species or serogroup. Several studies have compared PCR and UAT results in the same patients. PCR identified 18 – 30% of symptomatic LD cases that were initially negative by UAT. Conversely, 11 – 22% of patients confirmed with *L. pneumophila* serogroup 1 (Lp1) infection detected by UAT were negative by PCR (Avni et al., 2016; Maurin et al., 2010; Mentasti et al., 2012; Peci et al., 2016; Sciurti et al., 2026).

Therefore, when LD is clinically suspected, more than one method should be considered, particularly when the initial test result is negative.

- PCR assays should be used more systematically, as they enable detection of non-Lp1 serogroups and other *Legionella* species. This is particularly important in severe cases and immunocompromised patients.
- If PCR results are negative, UAT may still be useful because of its high sensitivity for Lp1 infection and because its performance is independent of respiratory sample quality.



**Figure 1:** Laboratory algorithm for the diagnosis of Legionnaires' Disease.

Urine antigen testing (UAT) and molecular testing by polymerase chain reaction (PCR) on respiratory specimens are the main methods used for the early diagnosis of LD. Culture has little value for initial diagnosis but remains essential for epidemiological investigations and strain characterization. Abbreviations: IC patient: immunocompromised patient; NRC: national reference centre. \*\*Sequence Based Typing (SBT) and Whole genome sequence (WGS) are not covered in this version but will be in the next version of the handbook.

## 2.1. Safety

*Legionella* is classified as a Risk Group 2 microorganism according to the Directive 2000/54/EC. Because transmission occurs through the inhalation of contaminated aerosols, all procedures with the potential to generate aerosols must be carefully assessed and controlled. Specimens potentially containing *Legionella* should be handled exclusively by trained personnel using appropriate personal protective equipment (PPE), including masks, gloves and eye protection, and processed in adequately equipped laboratories using certified Class II Biohazard biological safety cabinets compliant with EN 12469 standards. In addition to ensuring operator protection, strict precautions must be taken throughout all laboratory procedures to maintain specimen integrity and prevent cross-contamination between samples or contamination from the laboratory environment.

## 2.2. Urinary antigen testing (UAT)

Most commercialized available UATs detect *L. pneumophila* serogroup 1 infections. These assays rely on the detection of antigenic lipopolysaccharide (LPS) components, whose structural and antigenic diversity forms the basis for *L. pneumophila* serogroups identification. These antigens have a molecular weight of approximatively 10kDa, are heat-stable (100°C for 30 min) and are resistant to enzymatic degradation (RB et al., 1981). A limited number of commercial assays have been developed to detect serogroups other than *L. pneumophila* serogroup 1 or other *Legionella* species, including *L. longbeachae*; however, comprehensive independent evaluations of these assays are undergoing. Some assays may target other bacterial components, such as peptidoglycan-associated lipoprotein (PAL), or antigens not specified by the manufacturer.

The diagnostic performance of UAT is particularly important given the large number of tests performed annually in Europe (ECDC, 2026). False-positive results with enzyme immunoassays (EIAs) performed on urine samples were first described in the early 1980s and have since been reported sporadically with commercially available immunochromatographic assays. False-negative results may delay appropriate treatment and adversely affect patient outcomes, whereas false-positive results may lead to incorrect case classification and compromise epidemiological surveillance. Because *Legionella* urinary antigens are heat stable, heat treatment of urine samples can be used to confirm positive results. Boiling dissociates bacterial polysaccharides from antibody complexes and reduces nonspecific assay interference, thereby improving the specificity of UAT (Birtles et al., 1990).

### 2.2.1. Specimen suitable for UAT

Freshly voided urine should be collected in a sterile container and transported promptly to the laboratory at ambient temperature. Urinary antigens remain stable for up to 1 month at room temperature and for several months at 4 °C or –20 °C (Rigby et al., 1997). Urine collected in borate tubes may also be suitable depending on the recommendations of the commercial assay manufacturer.

### 2.2.2. Material and Reagents

- Timer
- Centrifuge
- Dry bath
- Immunochromatographic test (ICT) or an enzyme-immunoassays (EIA) kits
- Detection by EIA: EIA microtiter plates and EIA reader

### 2.2.3. Pre-treatment

For any urine sample testing positive for *L. pneumophila*, regardless of the assay used, confirmatory testing is recommended to reduce the risk of false positives by:

- Retesting the urine after heat treatment (HT) (5 minutes at  $95 \pm 5$  °C), followed by centrifugation (15 minutes at  $800\text{--}1,200 \times g$  or 5 minutes at  $8,000\text{--}12,000 \times g$ ) and analysis of the supernatant.
- An alternative method consists of pre-treating urine with proteinase K ( $20 \mu\text{g}/\mu\text{l}$ ) for 10 min at  $56$  °C before analysis. Proteinase K degrades polypeptides but does not affect lipopolysaccharide urinary antigens (Deforges et al., 1999). However, to date, no large-scale single-centre or multicentre European study has validated this approach.

### 2.2.4. Available methods

*Legionella* urinary antigens can be detected using:

- Rapid immunochromatographic tests (ICT) (colorimetric, fluorescent or chemiluminescent detection);
- Enzyme-immunoassay (EIA) (comparable performance to rapid tests).

Assays should be performed according to the manufacturer's instructions. In general:

- ICTs are interpreted within 15 minutes;
- EIAs require approximately 4 hours

For some ICTs, visual interpretation may be replaced by automated optical reading, which may improve analytical sensitivity.

### 2.2.5. Interpretation of results

A diagnosis of LD may be confirmed when a patient with compatible clinical and/or radiological signs of pneumonia presents detectable soluble *Legionella* urinary antigens.

#### 2.2.5.1. Kinetics of urinary antigen excretion:

- Urinary antigens generally become detectable within 2–3 days after symptom onset
- Antigen excretion may persist despite appropriate antimicrobial therapy;
- The duration of antigen excretion varies considerably between patients, ranging from a few days to approximately 2 months on average, and up to 1 year in rare cases (Kohler et al., 1984; Sopena et al., 2002).

- Persistent antigen excretion does not indicate treatment failure but has been associated with immunosuppressive therapy (Sopena et al., 2002).
- Antibiotic treatment does not appear to significantly affect antigen excretion
- Prolonged antigen excretion may complicate the diagnosis of recurrent *Legionella* pneumonia.

#### 2.2.5.2. Specificity of UATs

False-positive reactions should be suspected when a sample tests positive before boiling but becomes negative after heat treatment. Because false-positive results are relatively uncommon and heat treatment is time-consuming, HT is generally recommended only for initially positive samples. However, laboratories may choose to systematically apply HT to all specimens provided the targeted antigen is heat-stable.

- Reported specificity after HT ranges from 97.3% to 100%, depending on the commercial assay used (Allam et al., 2021).
- In a European evaluation, all commercially available UATs except one produced false-positive results before HT, supporting the recommendation for confirmatory HT (Allam et al., 2021).
- The mechanisms underlying false-positive reactions remain poorly understood.

To date, one well-documented cross-reactive situation has been described: in kidney transplant recipients treated with rabbit anti-thymocyte globulin, rabbit antibodies excreted in urine may react with rabbit-derived assay antibodies, leading to false-positive results (Deforges et al., 1999).

#### 2.2.5.3. Sensitivity of UATs

According to two meta-analyses, the overall sensitivity of UATs ranges from 70% to 90% (Kawasaki et al., 2021; Shimada et al., 2009). Several factors influence assay sensitivity:

- **Commercial assay used:** In a European evaluation of 16 UATs, sensitivity ranged from 50% to 100% (Allam et al., 2021).
- ***Legionella* species and serogroup:** Most studies report high sensitivity (80–100%) for *L. pneumophila* serogroup 1 infections. In contrast, sensitivity for other serogroups ranges from 14% to 69% (Benson et al., 2000; Helbig et al., 1997, 2001). Recent evaluation of UATs for *L. pneumophila* LPS demonstrated that only four Lp2–14 LPS samples were detected, and only at concentrations 100-fold higher than those required for Lp1 detection (Allam et al., 2021).
- **Severity of disease:** During outbreaks, UAT positivity ranged from 88 to 100% in patients with severe disease, whereas sensitivity ranged from 40% to 53% in mild cases (Blázquez et al., 2005; Vaissière et al., 2025; Yzerman et al., 2002).
- **Origin of infection:** UATs sensitivity is generally higher in community-acquired (76 – 87%) and travel-associated cases (94%) than in nosocomial infections (44 – 46%). This difference has historically been attributed to the higher prevalence of *L. pneumophila* serogroup 1 Pontiac strains in community and travel-associated cases. However, more recent studies have not fully confirmed this hypothesis. Non-Pontiac LPS variants are generally detected at lower concentrations than Pontiac LPS (Allam et al., 2021; Ranc et al., 2017).



**Important considerations:**

Positive urinary antigen results should be interpreted in the context of compatible clinical pneumonia.

A negative UAT does not rule out *Legionella* infection. If clinical suspicion remains high, repeat **urinary antigen testing is generally not recommended**. Instead, PCR performed on a respiratory specimen should be considered to enable detection of both *L. pneumophila* and non-*pneumophila* *Legionella* species infection. Repeat antigen testing may be considered only when the initial test was performed very early after symptom onset.

A positive result confirmed after HT does not require confirmation using a second urine specimen, as antigen excretion may persist from a few days to several months.

All confirmed positive UAT results should prompt culture of respiratory specimens whenever possible in order to isolate the *Legionella* strain for epidemiological investigation and strain characterization.

### 2.3. Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR), particularly **quantitative real-time PCR (qPCR)**, has become an essential tool for the rapid and sensitive diagnosis of LD. PCR enables the direct detection of *Legionella* DNA in clinical specimens and offers significant advantages over conventional diagnostic methods, particularly for the detection of *L. pneumophila* non-serogroup 1 strains and non-*pneumophila* *Legionella* species, which are frequently undetected by the UAT. PCR should be considered complementary to UAT and culture. However, it should not replace culture, as clinical isolates remain essential for comprehensive epidemiological investigations and strain characterization.

PCR demonstrates substantially higher sensitivity than culture, particularly in patients receiving antibiotic therapy, because bacterial DNA remains detectable longer than viable organisms (Gadsby et al., 2016). In addition, PCR results are generally available within a few hours, compared with 3–10 days for culture. PCR testing is particularly indicated in the following situations:

- **(community acquired) pneumonia** with negative UAT results despite persistent clinical suspicion;
- **immunocompromised patients** who are at increased risk of infection by non-*pneumophila* *Legionella* species;
- **nosocomial pneumonia**, in which *L. pneumophila* non-serogroup 1 strains are more prevalent;
- suspected infections caused by *Legionella* species not reliably detected by UAT (e.g., *L. longbeachae*, *L. micdadei*, *L. bozemanii*).

#### 2.3.1. Specimen suitable for PCR

Specimens suitable for PCR include:

- Sputum (regardless of quality, as even poor-quality sputum may contain *Legionella*);
- Bronchoalveolar lavage (BAL) fluid;

- Tracheal or bronchial aspirates;
- Other specimens may also be considered:
  - Nasopharyngeal swabs are generally not recommended but may be used when lower respiratory tract specimens cannot be obtained (for national surveillance or outbreak investigation purposes only). A single positive nasopharyngeal swab is not considered sufficient for case confirmation (not included in EU case definition) and should be confirmed using another diagnostic method (e.g., UAT).
  - Pulmonary abscess material, particularly in immunocompromised patients;
  - Lung tissue biopsies, following appropriate tissue pre-treatment
- Extrapulmonary specimens (for diagnostic): synovial aspirates, blood cultures, excised cardiac valves, among others.

PCR performed on serum or whole blood (EDTA tube) may be considered when respiratory samples are unavailable; however, these specimen types have low sensitivity and are not recommended for diagnosis. They may nevertheless be useful in selected investigational settings. PCR is not recommended on urine samples.

### 2.3.2. *Material and Reagents*

- Refrigerator capable of maintaining a temperature of  $5 \pm 3$  °C
- Freezer capable of maintaining a temperature of  $-18 \pm 2$  °C
- Calibrated variable-volume pipettes (for delivery from 10 µL to 1,000 µL) with disposable sterile tips
- DNA extraction kits (automated or manual; e.g., magnetic bead-based or spin columns)
- Variable speed vortex
- Centrifuge for DNA extraction
- qPCR master mixes
- DNA-free water
- Target-specific primers and probes (for in-house protocols)
- Thermocycler

### 2.3.3. *Pre-treatment*

Tissue samples should be homogenized before DNA extraction. Commercially tissue grinders and compatible lysis buffers are recommended. Detailed instructions for tissue homogenization are provided in Section 2.5.3.3. Respiratory secretions, particularly sputum, are often viscous and heterogeneous. Pre-treatment with **dithiothreitol (DTT)** or N-acetylcysteine is recommended to liquefy specimens and improve extraction homogeneity. Respiratory specimens may also contain PCR inhibitors. Most commercial extraction methods are designed to reduce inhibitor carryover; dedicated inhibitor-removal kits are also commercially available.

### 2.3.4. *Technical considerations*

qPCR represents a technological evolution of conventional PCR. Unlike conventional endpoint PCR, in which amplified products are analyzed only after completion of all amplification cycles

by gel electrophoresis, qPCR enables real-time monitoring of amplicon accumulation during the amplification process. Real-time monitoring is based on fluorescence measurements performed during each thermal cycle. Two principal fluorescence detection systems are commonly used:

- DNA-intercalating dyes (e.g. SYBR Green): These dyes bind non-specifically to double-stranded DNA (dsDNA) and emit fluorescence proportional to the amount of amplified DNA. This approach is generally combined with melting curve analysis to improve specificity.
- **Sequence-Specific Probes** (e.g., TaqMan Hydrolysis Probes): These probes hybridize specifically to the target sequence and are labeled with a fluorophore (reporter) and a quencher. While the probe remains intact, fluorescence from the reporter is suppressed by the quencher. During amplification, the 5'–3' exonuclease activity of Taq polymerase cleaves the probe, separating reporter and quencher and generating a measurable fluorescence signal. A major advantage of probe-based qPCR is multiplexing capability, allowing simultaneous detection of multiple targets within a single reaction through the use of different fluorophores.

Appropriate controls must be included in each qPCR run:

- Negative control: confirms the absence of *Legionella* DNA contamination in the PCR reagents and reaction mixture.
- Extraction control: confirms the absence of contamination introduced during the DNA extraction process.
- Positive control: confirms correct amplification performance and validates the specificity of the PCR assay.
- Internal control: verifies the absence of PCR inhibitors in the tested specimens.

A wide range of commercial CE-IVD real-time PCR assays is available for the detection of *Legionella*. These assays differ in their intended use, analytical performance, and target specificity. Some assays detect the *Legionella* genus only, whereas others specifically identify *L. pneumophila* or simultaneously distinguish *L. pneumophila* from other *Legionella* species. In addition, several commercially available syndromic respiratory panels include *L. pneumophila* among multiple bacterial and viral targets. Laboratories should be aware of the target range and performance characteristics of the selected assay when interpreting results, particularly because assays limited to *L. pneumophila* will not detect infections caused by other *Legionella* species.

The sections below describe published PCR protocols for the detection of *Legionella* species, *L. pneumophila*, and *L. pneumophila* serogroup 1. These protocols may be used as a basis for the development and validation of in-house assays, where appropriate, while laboratories using commercial assays should follow the manufacturer's instructions and validation requirements.

### 2.3.5. Detection of *Legionella* species and *L. pneumophila*

This method was published in an international peer-reviewed journal and the primers listed in below are provided as an example adapted from the published protocol (Templeton et al.,

2003). However, this method has not been formally recommended by ESGLI, therefore, alternative primers or commercial kits may also be used.

#### Reagents:

- Premix Ex Taq™ (for example from Takara ®): ready to use
- ROX Dye 50X (for example from Takara ®): dilute 1/5 in water
- Probes and primers 100 µM: MIP-16S-GFP
- Plasmid “GFP DNA pUC57-2”: transformation of the pUC57-2 plasmid into DH5α bacteria (E. coli) and quantified using a Qubit assay. The plasmid is added before extraction: aliquot at the working concentration of 2 ng/µL. Maximum storage: 1 year after preparation
- Positive control: Prepared from a reference strain Lp1 Paris (CIP107629T), extracted and diluted to a final concentration on the order of 10<sup>-2</sup> ng/µL.

**Table 1** : Sequences of primers and probes for *Legionella* species and *L. pneumophila*.

| Primer / Probe | Sequence (5' - 3')                    | Final concentration (µM) |
|----------------|---------------------------------------|--------------------------|
| MIP F 100 µM   | TGGTGACTGCAGCTGTTATG                  | 10                       |
| MIP R 100 µM   | CATTGCTTCCGGATTAACAT                  | 10                       |
| Probe MIP      | FAM-CATTGGT{G}CCGAT{T}TGGGA-BHQ1      | 10                       |
| 16S F 100 µM   | AGGCTAATCTTAAAGCGCC                   | 10                       |
| 16S R 100 µM   | CCTGGCTCAGATTGAACG                    | 10                       |
| Probe 16S      | VIC-TGAGT{A}ACGCGTA{G}GA{A}TATG-BHQ1  | 10                       |
| GFP F 10 µM    | CCTGTCCTTTTACCAGACAACCA               | 1                        |
| GFP R 10 µM    | GGTCTCTCTTTTCGTTGGGATCT               | 1                        |
| Probe GFP      | Cy5-TACCTGTCCACACAATCTGCCCTTTTCG-BHQ3 | 1                        |

**Table 2** : Reaction mix for *Legionella* species / *L. pneumophila* PCR.

| Reagents             | Concentration | Final concentration | Volume for 1 reaction (µL) |
|----------------------|---------------|---------------------|----------------------------|
| Premix Takara        | 2X            | 1X                  | 12.5                       |
| Rox Dye Takara (1/5) | 50X           | 1X                  | 0.5                        |
| MIX primer 16S-MIP   | 10 µM         | 0.5 µM              | 1.25                       |
| GFP                  | 1 µM          | 0.05 µM             |                            |
| Probes MIP           | 10 µM         | 0.16 µM             | 0.4                        |
| Probes 16S           | 10 µM         | 0.16 µM             | 0.4                        |
| Probes GFP           | 1 µM          | 0.06 µM             | 1.5                        |
| H2O DNA RNA OFF      |               |                     | 3.45                       |
| Volume Mix           |               |                     | 20                         |
| DNA                  |               |                     | 5                          |
| Total volume         |               |                     | 25                         |

**Table 3** : Amplification program for *Legionella* species / *L. pneumophila* PCR.

| Target (°C) | Hold (h:mm:ss) | Ramp rate (°C/sec) | Cycle |
|-------------|----------------|--------------------|-------|
| 95          | 00:02:00       | 1.6                | 1     |
| 95          | 00:00:05       | 1.6                | 45    |
| 56          | 00:00:30       | 0.5                |       |

#### Reading:

Analysis must be performed using:

- FAM fluorescence for *Legionella* spp. PCR
- VIC fluorescence for *L. pneumophila* PCR
- CY5 fluorescence for the internal control PCR

### 2.3.6. Detection of *L. pneumophila* and *L. pneumophila* serogroup 1

This method was developed and validated by the European *Legionella* Study Group (ESCMID Study Group for *Legionella* Infections, ESGI) and was published in an international peer-reviewed journal (Mentasti et al., 2015).

#### Reagents:

- Premix Ex Taq™ (for example Takara ® but other can be used)
- ROX Dye (for example Takara ® 50X but other can be used)
- Probes and primers 100 µM: MIP-16S-GFP
- Plasmid “GFP DNA pUC57-2”: as described above
- Positive control: Prepared from a reference strain Lp1 Paris (CIP107629T)

**Table 4 :** Sequences of primers and probes for *L. pneumophila* serogroup 1.

| Primers      | Sequence (5' - 3')                        | Final concentration (µM) |
|--------------|-------------------------------------------|--------------------------|
| MIP F 100 µM | GAAGCAATGGCTAAAGGCATGC                    | 10                       |
| MIP R 100 µM | GAACGTCTTTCATTTGYTGTTCCG                  | 10                       |
| Probe MIP    | HEX-CGCTATGAGTGGCGCTCAATTGGCTTTA-BHQ1     | 10                       |
| WZM F 100 µM | CAAAGGGCGTTACAGTCAAACC                    | 10                       |
| WZM R 100 µM | CAACACCCCAACCGTAATCA                      | 10                       |
| Probe WZM    | FAM-TCTTGGGATTGGGTTGGGTTATTTTAACTCCT-BHQ1 | 10                       |
| GFP F 10 µM  | CCTGTCCTTTTACCAGACAACCA                   | 10                       |
| GFP R 10 µM  | GGTCTCTCTTTTCGTTGGGATCT                   | 10                       |
| Probe GFP    | Cy5-TACCTGTCCACACAATCTGCCCTTTTCG-BHQ3     | 1                        |

**Table 5 :** Reaction mix for *L. pneumophila* et *L. pneumophila* serogroup 1 PCR.

| Reagents             | Concentration | Final concentration | Volume for 1 reaction (µL) |
|----------------------|---------------|---------------------|----------------------------|
| Premix Takara        | 2X            | 1X                  | 12.5                       |
| Rox Dye Takara (1/5) | 50X           | 1X                  | 0.5                        |
| MIX primer           | 10 µM         | 0.5 µM              | 1.25                       |
| Probes MIP           | 10 µM         | 0.16 µM             | 0.4                        |
| Probes 16S           | 10 µM         | 0.16 µM             | 0.4                        |
| Probes GFP           | 1 µM          | 0.06 µM             | 0.15                       |
| H2O DNA RNA OFF      |               |                     | 4.8                        |
| Volume Mix           |               |                     | 20                         |
| DNA                  |               |                     | 5                          |
| Total volume         |               |                     | 25                         |

#### Reading:

The same amplification program can be used for *Legionella* spp/ *L. pneumophila* PCR and for Lp/Lp1 PCR. Analysis must be performed using:

- VIC fluorescence for *L. pneumophila*
- FAM fluorescence for *L. pneumophila* serogroup 1

- CY5 fluorescence for the internal control

### 2.3.7. Detection of *L. longbeachae*

This method was published in an international peer-reviewed journal (Cross et al., 2016). However, the method has not been recommended by ESGLI and thus other primers or commercial kits may also be used.

#### Reagents:

- PCR Probe Kit (for example QuantiNova® (Qiagen) but other can be used).
- Probe and primers 100 µM:
- Positive control: Prepared from a reference strain *L. longbeachae*, extracted and diluted to a final concentration on the order of  $10^{-2}$  ng/µL.

**Table 6 :** Sequences of primers and probes for *L. longbeachae*

| Primers  | Sequence (5' - 3')                     | Final concentration (µM) |
|----------|----------------------------------------|--------------------------|
| F 100 µM | GTACTAATTGGCTGATTGTCTTG                | 10                       |
| R 100 µM | TTCACCTCTGAGTTCGAGATGG                 | 10                       |
| Probe    | [FAM]CTGAGTATCATGCCAATAATGCGCGC-[BHQ1] | 10                       |

**Table 7 :** Reaction mix for *L. longbeachae* PCR.

| Reagents        | Concentration | Final concentration | Volume for 1 reaction (µL) |
|-----------------|---------------|---------------------|----------------------------|
| Master Mix      | 2X            | 1X                  | 10                         |
| MIX primer      | 10 µM         | 0.4 µM              | 0.8                        |
| Probe           | 10 µM         | 0.25 µM             | 0.5                        |
| H2O DNA RNA OFF |               |                     | 5.1                        |
| Volume Mix      |               |                     | 20                         |
| DNA             |               |                     | 5                          |
| Total volume    |               |                     | 25                         |

**Table 8 :** Amplification program for *L. longbeachae* PCR.

| Target (°C) | Hold (h:mm:ss) | Ramp rate (°C/sec) | Cycle |
|-------------|----------------|--------------------|-------|
| 95          | 00:02:00       | 1.6                | 1     |
| 95          | 00:00:05       | 1.6                | 40    |
| 60          | 00:00:30       | 1.6                |       |

Reading: Analysis must be performed using FAM fluorescence for *L. longbeachae*.

### 2.3.8. Characterization of *Legionella* species

This method was published in an international peer-reviewed journal (Grattard et al., 2006) and can be used on respiratory samples, environmental samples as well as on strains. However, this method has not been formally recommended by ESGLI; therefore, alternative primers or commercial kits may also be used.

#### Reagents:

- Reaction Buffer (for example Q5 ® (5X, New England Biolabs) but others can be used).
- dNTP 10mM
- Taq (for example Q5 ® (2000U/mL) but others can be used)
- Probe and primers 100 µM
- Positive control: Prepared from a reference strain *L. pneumophila* or preferably a rare species, extracted and diluted to a final concentration on the order of  $10^{-2}$  ng/µL.

**Table 9:** Sequences of primers and probes for *Legionella* species

| Primers       | Sequence (5' - 3')   | Final concentration (µM) |
|---------------|----------------------|--------------------------|
| ISR1 F 100 µM | TGAAGCCCGTTGAAGACTAC | 10                       |
| ISR2 R 100 µM | GGAAGCCTCACACTATCAT  | 10                       |

**Table 10 :** Reaction mix for *Legionella* species PCR.

| Reagents             | Concentration | Final concentration | Volume for 1 reaction (µL) |
|----------------------|---------------|---------------------|----------------------------|
| Q5 ® Reaction Buffer | 5X            | 1X                  | 10                         |
| MIX primer           | 10 µM         | 0.4 µM              | 2.5                        |
| dNTP                 | 10 µM         | 0.25 µM             | 0.5                        |
| H2O DNA RNA OFF      |               |                     | 5.1                        |
| Volume Mix           |               |                     | 20                         |
| DNA                  |               |                     | 5                          |
| Total volume         |               |                     | 25                         |

**Table 11 :** Amplification program for *Legionella* species PCR.

| Target (°C) | Hold (h:mm:ss) | Cycle |
|-------------|----------------|-------|
| 98          | 00:00:30       | 1     |
| 98          | 00:00:15       | 40    |
| 58          | 00:00:20       |       |
| 72          | 00:00:30       |       |
| 72          | 00:02:00       | 1     |

### Reading:

The amplified products are migrated on an agarose gel 2% stained with SyberSafe. Samples yielding visible amplicons are then subjected to sequencing (Sanger or WGS). For more details on bioinformatic analyses of the sequence please refer to the publication (Jacqueline et al. preprint).

### 2.3.9. Interpretation of results

While real-time PCR may be used qualitatively for pathogen detection, qPCR also enables the estimation of the initial bacterial load in a clinical specimen. The principal qPCR parameter is the Ct value (cycle threshold), also referred to as the Cq value (quantification cycle). This corresponds to the amplification cycle at which the fluorescence signal exceeds the background fluorescence threshold. Ct values are inversely proportional to the quantity of

target DNA present in the original specimen: the higher the initial concentration of bacterial DNA, the lower the Ct value. Absolute quantification (e.g., genome equivalents or copies/mL) may be achieved by generating a standard curve using serial dilutions of standards with known DNA concentrations. Concentrations in unknown clinical specimens can then be calculated based on the linear relationship between Ct values and the standard concentrations.

## 2.4. Serology

Serology has a limited role in the diagnosis of LD. Its main application is the detection of specific antibody responses against *Legionella* serogroups and species, which may support identification of the infecting strain. However, because antibody responses develop relatively late after infection, serological diagnosis is mainly retrospective and has limited value for immediate clinical management.

Serology may be considered in the following situations:

- retrospective confirmation of LD or epidemiological investigations;
- suspected LD with negative urinary antigen testing (UAT);
- cases where respiratory specimens cannot be obtained;
- diagnosis of extrapulmonary legionellosis (e.g., endocarditis).

Serological results should always be interpreted together with compatible clinical and/or radiological findings.

### 2.4.1. Specimen

Paired serum specimens are required for serological confirmation:

- an acute-phase sample collected at symptom onset;
- a convalescent-phase sample collected approximately 2–3 weeks later.

An additional sample may be collected at 5 weeks if seroconversion has not occurred. Serum samples should be stored at  $\leq -20$  °C.

### 2.4.2. Methods

#### 2.4.2.1. Indirect Immunofluorescence Assay (IFA)

IFA was the first serological method developed for the detection of antibodies against *L. pneumophila* and remains a reference method in many laboratories. It detects antibodies directed mainly against lipopolysaccharide (LPS) antigens and can be performed using mono- or polyvalent antigen preparations. Antigens may be prepared from yolk sac-derived organisms or agar-grown cultures, with most commercial reagents based on the latter approach.

#### 2.4.2.2. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA-based assays are widely used for serological screening, particularly for *L. pneumophila* serogroups 1–7. Although ELISA results generally correlate well with IFA, positive results may

require confirmation by IFA due to its higher specificity and alignment with established case definitions.

### 2.4.3. Interpretation

Diagnosis relies primarily on seroconversion between paired serum samples (confirmed case for *L. pneumophila* serogroup 1, otherwise probable). A single elevated antibody titre ( $\geq 1:256$ ) is insufficient for definitive diagnosis and generally corresponds only to a probable case classification for *L. pneumophila* serogroup 1.

Antibody kinetics should be considered when interpreting results:

- antibodies are usually detectable approximately 2 weeks after symptom onset;
- peak titres are generally reached after 4–5 weeks;
- delayed seroconversion may occur, and some confirmed cases may not develop detectable antibodies.

Antibody persistence is variable, with antibodies commonly remaining detectable for several months and, in some patients, for years. Therefore, a single positive antibody titre may reflect previous exposure rather than acute infection.

Combined detection of immunoglobulin classes (IgG, IgM, and IgA) is generally preferred, as individual immunoglobulin measurements may have limited diagnostic value. IgM may persist for prolonged periods, and some patients develop only IgG responses.

Low antibody titres ( $< 1:256$ ) may be detected in healthy individuals due to previous environmental exposure. Background seropositivity and cross-reactivity should therefore be considered, particularly when interpreting isolated positive results.

### 2.4.4. Cross-Reactions

Serological cross-reactions may occur between different *Legionella* serogroups and species, as well as with other bacterial pathogens, including *Mycobacterium* spp., *Leptospira* spp., *Chlamydia* spp., *Mycoplasma* spp., *Coxiella burnetii*, *Pseudomonas* spp., and *Bacteroides fragilis*. Cross-reactivity between *L. pneumophila* serogroups 1 and 6 has been frequently reported.

### 2.4.5. Performance

The sensitivity of serological methods is variable and generally ranges from 40% to 75%, depending on antigen preparation, assay methodology, immunoglobulin classes detected, and study population. Specificity ranges from approximately 75% to 99%, depending mainly on antigen type and assay characteristics.

## 2.5. Culture

Culture should be performed for patients with a confirmed positive UAT, a positive *Legionella* PCR result, or upon the request. Culture remains essential for epidemiological investigations, strain characterization, and antimicrobial susceptibility studies.

### 2.5.1. Specimen suitable for culture

Specimens suitable for *Legionella* culture include:

- Sputum (regardless of quality, as even poor-quality sputum may contain *Legionella*);
- Bronchoalveolar lavage (BAL) fluid;
- Tracheal or bronchial aspirates;
- Additional specimen types may also be considered:
  - Pleural fluid (pleural effusion occurs in ~30% of cases);
  - Pulmonary abscess material, particularly in immunocompromised patients;
  - Lung tissue biopsies.
- Extrapulmonary specimens (for diagnostic): synovial aspirates, blood cultures, excised cardiac valves, among others.

#### Notes on Specimen Timing and Handling:

Specimens should ideally be collected before initiation of antimicrobial therapy with intracellular activity.

*Legionella* may nevertheless still be isolated from specimens collected after several days of antimicrobial treatment.

For epidemiological investigations, respiratory specimens are particularly important because they may enable sequence-based typing (SBT) even in the absence of a cultured isolate.

A second specimen may occasionally yield *Legionella* when the initial specimen is culture-negative, although the probability is relatively low.

*L. pneumophila* remains stable at  $5 \pm 3$  °C. If immediate culture is not possible, specimens should be refrigerated and protected from desiccation. For long-term storage, specimens should be frozen at  $\leq -20$  °C.

### 2.5.2. Instruments, material and reagents

- Refrigerator maintaining a temperature of  $5 \pm 3$  °C
- Incubator (with and without 2.5% CO<sub>2</sub>) at  $37 \pm 1$  °C
- Water bath, capable of being maintained at  $50 \pm 1$  °C.
- Stereomicroscope
- UV lamp with a wavelength of  $366 \pm 20$  nm
- Calibrated variable-volume pipettes (for delivery from 10 µL to 1,000 µL) with disposable sterile tips
- Variable speed vortex
- Sterile scalpel and mortar (for biopsies) – bead-beater or Potter pestle homogenizer (with 5-10 mL capacity)
- BCYE (Buffered Charcoal Yeast Extract) medium
- BMPA medium (BCYE supplemented with cefamandole, polymyxin B, and anisomycin)
- MWY medium (BCYE supplemented with glycine, polymyxin B, anisomycin, and vancomycin)
- Blood agar medium

- Lysis agent and/or mucolytic agent (N-acetyl-L-cysteine)
- pH  $2.2 \pm 0.2$  solution
- DNA-free water

### 2.5.3. Pre-treatment

Specimen pre-treatment depends on specimen type and degree of contamination.

- Fluid or highly fluid specimens may be plated directly without dilution or pretreatment.
- Viscous specimens (e.g., sputum): pre-treatment with a mucolytic agent (N-acetyl-L-cysteine) is recommended
- Heavily contaminated specimens: acid treatment (adjust to pH  $2.2 \pm 0.2$ ,  $30 \pm 1$  min) and/or HT ( $50 \pm 1$  °C for  $30 \pm 1$  min) may be performed before inoculation.

Cell suspension prepared from biopsy specimen may also undergo HT ( $50 \pm 1$  °C for  $30 \pm 1$  min) before inoculation. If bacterial overgrowth is observed after 24 hours incubation of an initially sterile specimen, combined heat and acid treatment may subsequently be applied before repeat inoculation.

#### 2.5.3.1. Liquefaction of respiratory sample (respiratory secretion or other organic fluids) for culture

Liquefaction should be performed for respiratory specimens that are excessively viscous and difficult to process.

#### Preparation and storage of the mucolytic agent

- All manipulations should be performed in a biological safety cabinet
- The mucolytic agent should be diluted according to the manufacturer's instructions using DNA-free water when PCR testing may subsequently be performed.
- Storage
  - Undiluted mucolytic agent:  $+2$  to  $+8$ °C
  - DNA-free water: room temperature under the hood
  - Diluted mucolytic agent:  $+2$  to  $+8$ °C - prepared daily, and discarded at the end of the day. Diluted mucolytic agent should not be stored for more than 48 hours.

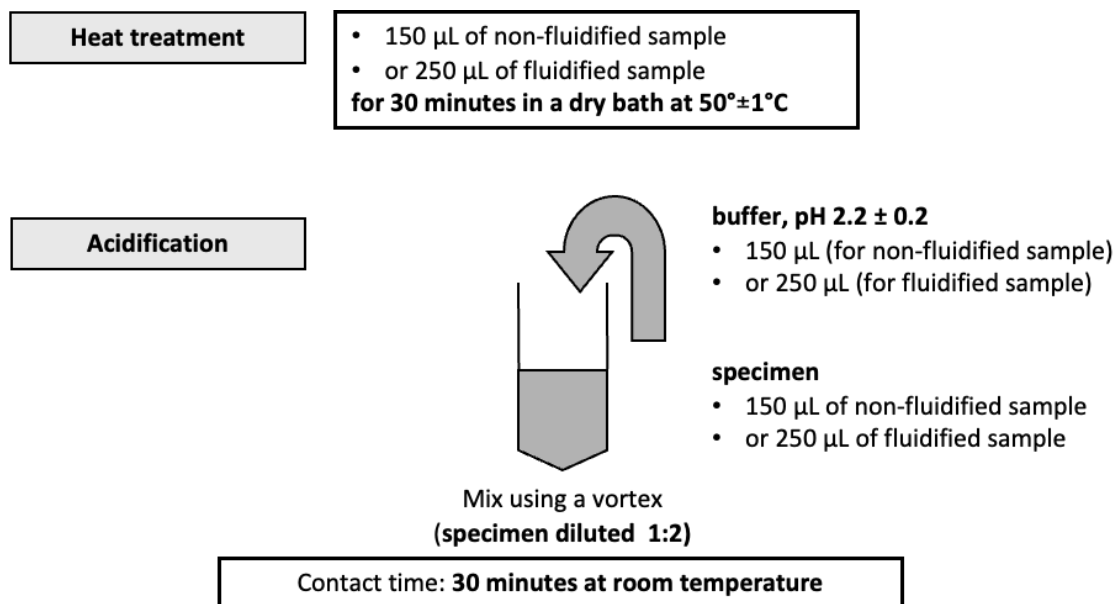
#### Liquefaction of the respiratory sample

- Add an equal volume of diluted mucolytic agent to the sample to be cultured and mix using a vortex.
- After a 15-minute contact at room temperature (DO NOT WASH), the sample is now ready to be aliquoted and/or cultured according to procedure.

#### 2.5.3.2. Acid treatment and/or heat treatment

- **Heat treatment (HT):** Incubate specimen for 30 min in a water bath at  $50^{\circ}\text{C} \pm 1$  °C. For highly contaminated specimens, HT may be followed by acidification treatment before inoculation.
- **Acidification:** Dilute specimen 1:2 in acid buffer, pH 2. Mix thoroughly and incubate at

room temperature for 30 min before inoculation.



**Figure 2:** Schematic protocol for acidification and heat treatment of respiratory samples.

### 2.5.3.3 Biopsy

- Tissue specimens must be collected aseptically in a sterile container and transported immediately to the laboratory at  $+5 \pm 3$  °C. If immediate processing is not possible, specimens should be stored at  $\leq -70 \pm 10$  °C.
- Before tissue homogenization, transfer a portion of the biopsy specimen using sterile forceps and streak directly onto selective and non-selective culture media.
- Place the tissue specimen onto a sterile Petri dish;
- Using sterile forceps and a scalpel, select a dense grey or reddish portion of tissue and cut a fragment large enough for homogenization;
- Transfer the tissue fragment into a Potter homogenizer or equivalent device;
- Add 500-1,000 µL sterile distilled water;
- Homogenize gently using the homogenizer piston;
- Remove half of the resulting cell suspension for additional processing if required;
- Inoculate both untreated and treated cell suspensions onto BCYE agar and selective media (GVPC or MWY and BMPA)
- Incubate cultures at  $37 \pm 1$  °C for approximately 10 days and inspect plates daily;
- For heavily contaminated specimens, heat treatment at 50 °C for 30 min followed by acid treatment may be performed before inoculation;
- When present, *Legionella* colonies generally become visible after  $\geq 3$  days of incubation.

### 2.5.4. Culture media (see Annex 1)

- Primary medium: BCYE agar supplemented with 0.1%  $\alpha$ -ketoglutarate.
- Selective media (recommended for contaminated specimens): BMPA and MWY

- GVPC and BCYE+AB medium: These media were originally developed for environmental water testing and are less suitable for recovery of respiratory isolates. They are therefore not recommended for primary clinical diagnosis.

**Note:**

Culture media are buffered to pH 6.9 using ACES buffer (N,N'-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid), which corresponds to the optimal pH for *Legionella* growth.

Activated charcoal is included to adsorb toxic free radicals generated during autoclaving, while yeast extract provides essential growth factors.

**2.5.5. Inoculation**

Inoculate 100 µL of non-liquified specimen or 200 µL of liquified or diluted/treated specimen onto the following media using sterile spreader:

- 3 selective media plates (preferably 1 BMPA and 2 MWY);
- 1 non-selective BCYE plate, because vancomycin and cefamandole present in selective media can inhibit certain *Legionella* species.

**Note:**

For biopsy-derived suspensions, inoculate 100–200 µL of both untreated and heat-treated suspensions onto one or more BCYE and MWY plates depending on specimen volume.

If sample volume is limited (<1 mL), prioritize inoculation of MWY plate under 2.5% CO<sub>2</sub>, followed by BMPA under 2.5% CO<sub>2</sub>. Generally, the greater the number of plates inoculated, the greater the isolation success will be.

**2.5.6. Incubation**

Culture plates should be incubated for 10 days in a humidified atmosphere to prevent agar desiccation. Recommended incubation conditions:

- at 35 ± 2°C in air: 1 MWY plate and 1 BCYE plate
- at 35 ± 2°C in 2.5% CO<sub>2</sub>: 1 BMPA plate and 1 MWY plate

**Note:**

Incubation either in ambient air or in ≤ 2.5% CO<sub>2</sub> may enhance recovery of certain *Legionella* species, although CO<sub>2</sub> supplementation is not essential. However, incubation in 5% CO<sub>2</sub> may inhibit *Legionella* growth.

If a 2.5% CO<sub>2</sub> incubator is unavailable, incubation in ambient air is preferable to incubation at higher CO<sub>2</sub> concentrations.

**2.5.7. Inspection**

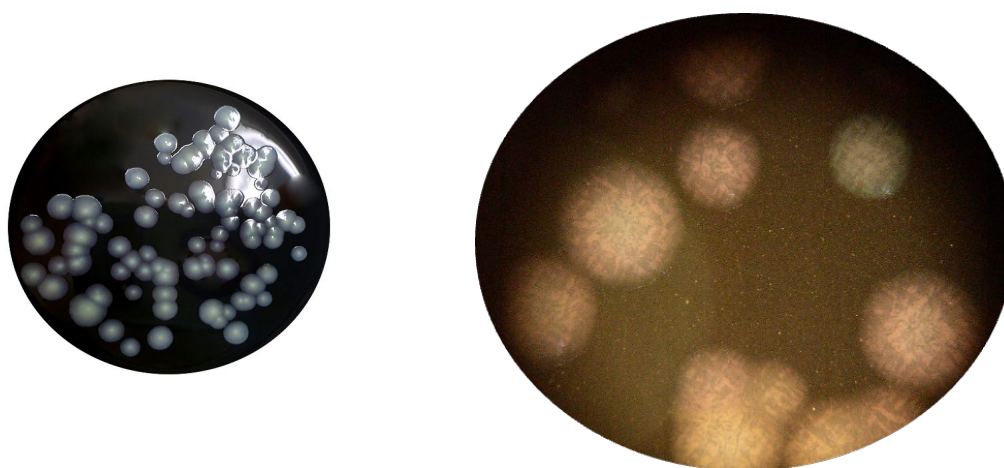
Culture plates should be examined daily on the laboratory bench. Primary isolation of *Legionella* generally requires a minimum incubation period of 72 h.

**Day 1: Monitor Contamination**

- After approximately 24 hours of incubation, assess contamination levels
- If heavy contamination is observed, perform acid and/or HT as described above.

**Day 3: Read Plates**

- Perform at least 3 readings between Day 3 and Day 10. If Day 10 occurs on a day off, the final reading should be performed on the first working day after Day 10.
- Examine all plates with a stereomicroscope (20–25x magnification) and UV illumination. Suspect colonies should be subcultured onto 1 BCYE and 1 COS plate and incubated at  $35 \pm 2^\circ\text{C}$  in 2.5%  $\text{CO}_2$ .



**Figure 3:** Aspect of *Legionella* colonies on BCYE medium.  
(A) and ground-glass appearance with a stereo microscope (B).

**2.6. Amoeba Plate Test (APT) - Amoebal co-culture**

This method exploits the ability of *Legionella* species to multiply intracellularly within amoebae, while extracellular contaminating bacteria are phagocytosed and lysed by the amoebae. Although amoebal co-culture is generally less sensitive than axenic culture, it may facilitate recovery of *Legionella* from heavily contaminated respiratory specimens (Descours et al., 2012, 2018).

APT is indicated for specimens showing:

- A positive urinary antigen and/or PCR result AND
- Negative *Legionella* culture results for at least 3 days, AND
- Very heavy contamination on BMPA and/or MWY agar

Specimens suitable for amoebal co-culture include:

- Sputum (regardless of quality, considered highly suitable);
- Bronchoalveolar lavage (BAL) fluid;
- Tracheal or bronchial aspirates.

This protocol may also be adapted for environmental specimens.

### 2.6.1. Preparation of the aliquot required for APT

Prepare for each specimen:

- A 0.5 mL aliquot (or 1 mL if the specimen was liquefied) in a 15 mL tube.
- If specimen volume is insufficient, prepare the largest possible aliquot and record the final volume used.

Storage of aliquots:

- At 4°C for up to 7 days before amoeba infection,
- At -20°C if storage exceeds 7 days.

### 2.6.2. Preparation of Culture Media

PYG (Peptone Yeast Glucose) medium and PAS (Page's Amoeba Saline) buffer should be prepared, aliquoted into 50 mL tubes, and stored at +4 °C. See Annex 2.

### 2.6.3. Amoeba Strains

The following amoeba strains may be used:

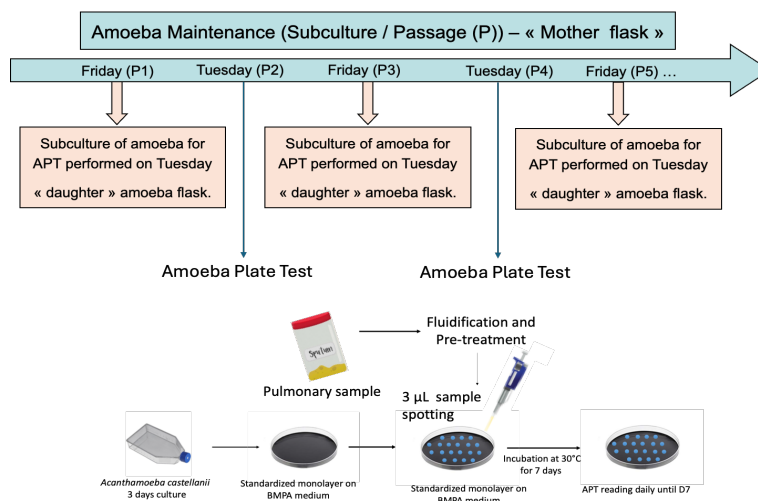
- *Acanthamoeba castellanii* (ATCC 30234)
- *Acanthamoeba polyphaga* (Linc AP-1)

### 2.6.4. APT- Protocol

*Acanthamoeba* spp. are routinely maintained axenically in PYG broth at 30 °C. Under these conditions, amoebae typically exhibit a doubling time of approximately 24 h. One- to two-week-old cultures generally contain few non-viable amoebae and are preferred for APT. Before initiating the protocol, verify that amoebae:

- grow appropriately in PYG medium at 30 °C;
- form a confluent monolayer after thawing and subculture.

The protocol may be adapted according to laboratory organization and specimen throughput.



**Figure 4:** Protocol for Amoeba maintenance and Amoeba Plate Testing.

### 2.6.4.1. Friday procedures

#### 2.6.4.1.1. Amoeba Maintenance (Mother Flask)

This procedure ensures maintenance of the amoebal stock culture. All manipulations should be performed in a microbiological safety cabinet (MSC) within a cell culture laboratory.

#### Protocol:

- Remove PYG from the 75 cm<sup>2</sup> flask
- Add 10 mL cold PAS
- Detach amoebae by tapping the flask and/or repeated aspiration and expulsion over the amoebal monolayer (do not scrape).
- Collect the PAS containing amoebae into a tube (Tube T)
- Add 12 mL PYG to the 75 cm<sup>2</sup> flask
- Record the date and passage number (e.g. P2 = 2nd passage)
- Incubate at 30°C

#### **Notes:**

Do not exceed 4 passages per flask.

At the 4th passage: Seed 3,000 amoebae (calculation in section 2.6.4.2.2) into 12 mL PYG in a new 75 cm<sup>2</sup> flask. Discard remaining material.

Continue incrementing passage numbers when changing flasks (do not restart at P1).

Do not fully tighten flask caps.

After 30 passages, thaw a new amoeba stock. Once thawed amoebae adhere to the flask, discard the previous culture (>P30).

#### 2.6.4.1.2. Preparation of “Daughter” Amoeba Flasks for APT

Using Tube T obtained from the mother flask, prepare daughter flasks according to the number of specimens planned for APT the following Tuesday.

- Establish the concentration of the Mother Flask using counting chamber with a total grid volume of 1  $\mu\text{L}$  should be used.
  - Count one large square (nine small squares).
  - Multiply the count by 9 to obtain the amoeba concentration (C) in amoebae/ $\mu\text{L}$ .
- Required volume:  $V = \text{number of amoebae required} / C$

For a 4-day incubation interval (Friday  $\rightarrow$  Tuesday): it is recommended to seed a 75  $\text{cm}^2$  flask with 3,000 amoebae in 12 mL PYG. If timing differs (e.g. day off), use the following reference values for a 75  $\text{cm}^2$  flask:

- 5 days: 300 amoebae
- 3 days: 30,000 amoebae
- 2 days: 300,000 amoebae

It is recommended to use 75  $\text{cm}^2$  flask for 2–3 specimens or to use 150  $\text{cm}^2$  flask for ~5 specimens (double all numbers).

#### 2.6.4.1.3. Subculture of the *Legionella* Control Strain

The Dot/lcm secretion system is essential for intracellular replication of *Legionella* spp. Deletion of the dotA gene abolishes intracellular multiplication. The *L. pneumophila* Paris CIP107629 $\Delta$ dotA strain is therefore used as a negative infection control during APT. The strain is used to assess amoeba viability during APT. If amoebal viability is preserved, no bacterial growth should be observed on the agar surface after incubation.

##### Protocol:

- On Friday, streak the frozen strain onto BCYE agar.
- Incubate 72 hours at 35°C with 2.5%  $\text{CO}_2$ .
- Do not exceed 4 subcultures from the frozen stock.

#### 2.6.4.1.4. Preparation of specimens

Only liquefied specimens require preparation.

##### Protocol:

- Add 5mL of DNA free water
- Vortex and spin for 10min at 3000g
- Remove supernatant
- Resuspend to a final volume of 0.5 mL DNA-free water. **The final specimen volume should not exceed the initial specimen volume.**

#### 2.6.4.2. Tuesday procedures

##### 2.6.4.2.1. Mother Flask Maintenance

Same procedure as section 2.6.4.1.1. The PAS suspension containing detached amoebae (Tube T) is discarded.

## 2.6.4.2.2. Amoeba harvesting

From the daughter flask prepared on Friday:

- Verify:
  - absence of floating amoebae macroscopically
  - viability of the amoebal monolayer microscopically.
- Remove PYG medium
- Add 10 mL of cold PAS (4°C) for a 75 cm<sup>2</sup> flask
- Detach amoebae as previously described
- Collect the suspension into a 50 mL Falcon tube
- Pool suspension if multiple flasks were used
- Discard flasks
- Count amoebae using a counting-chamber

Amoeba quantification:

- Target concentration:  $2.5 \times 10^6$  amoebae/mL
- Total amoebae required:  $N = \text{number of plates} \times 3.75 \times 10^6$
- Determine concentration (C in amoebae/ $\mu$ L) using counting-chamber as described above
- Centrifuge volume  $V = N / C$  :
  - 5 min at 500 g
  - Use multiple falcon tubes if  $V > 25$  mL
- Supernatant must be clear
- Discard supernatant
- Resuspend pellet in PAS: PAS volume (mL) = number of plates  $\times$  1.5
- Dispense 1.5 mL amoeba suspension per MWY plate
- Spread evenly with a T-shape spreader
- Dry plates under MSC until complete evaporation (approximately 1 h)

## 2.6.4.2.3. Preparation of Specimens and Control for Infection

Respiratory specimen aliquot (15 mL Falcon tube):

- Add 3 mL sterile water for osmotic lysis (*change pipette tips between specimens*)
- Vortex and centrifuge 5 min at 1,000 rpm
- Transfer supernatant into two 2 mL Eppendorf tubes
- Centrifuge 10 min at 10,000 rpm and discard supernatant
- Resuspend:
  - First pellet in 30  $\mu$ L PAS
  - Use the same 30  $\mu$ L to resuspend the second pellet

Control strain suspension (Paris  $\Delta$ dotA): Prepare a bacterial suspension at OD<sub>600</sub> ~ 1 with sterile physiological saline water.

#### 2.6.4.2.4. Amoebal Infection by APT

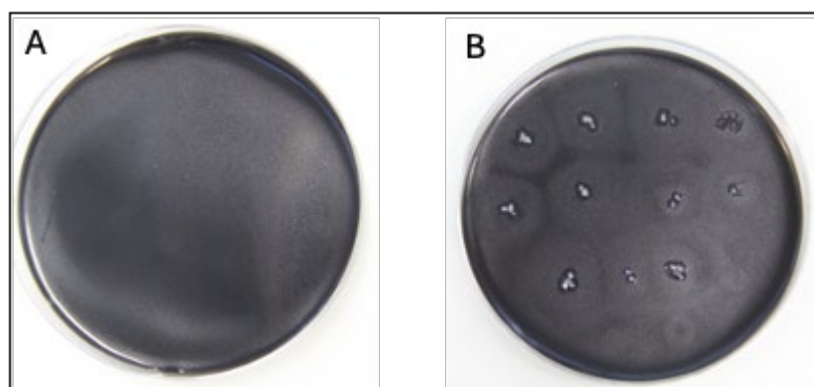
- One MWY plate per patient + one control plate
- Spot  $\geq 10$  drops of 3  $\mu\text{L}$  per specimen/control
- Adjust number of spots to avoid discarding any resuspended pellet
- Do not touch the agar surface to preserve the amoebal monolayer
- Incubate at  $30 \pm 2^\circ\text{C}$  without  $\text{CO}_2$

#### 2.6.4.3. Reading of APT Plates

- Examine plates from 72 hours post-infection until Day 10
- Perform at least 3 readings

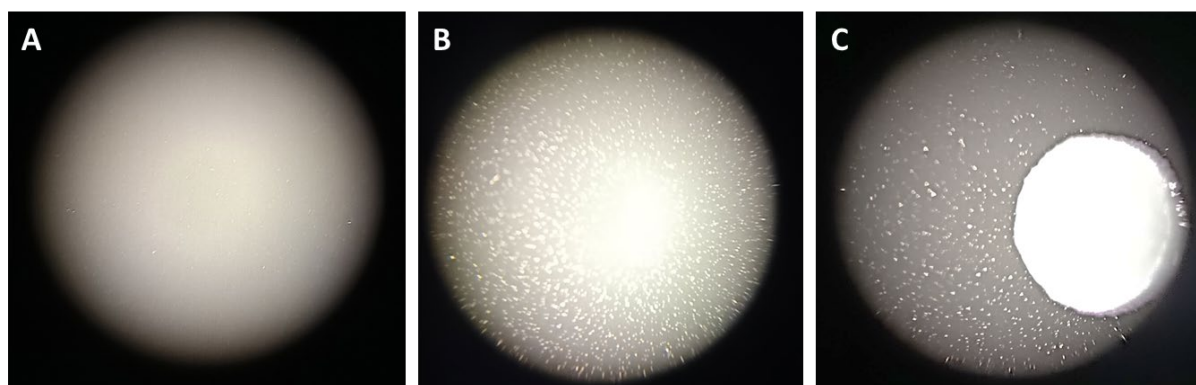
Observation is done macroscopically and using a stereomicroscope, to assess for :

- Amoebal lysis zones
- Growth of *Legionella* on agar



**Figure 5:** Example of results of APT observed macroscopically.

A) agar with amoebal monolayer and B) agar media with amoebal monolayer and *Legionella* growth.



**Figure 6:** Examples of results of APT observed using a stereomicroscope.

A) agar media without amoebal growth B) agar media with amoebal monolayer and C) agar media with amoebal monolayer and *Legionella* growth.

#### 2.6.4.4. Subculture

- Subculture any positive or suspicious spot onto: COS and BCYE
- Continue until isolated colonies are obtained.

#### 2.6.4.5. Freezing procedure of amoeba

- Mix 4 mL of fresh PYG medium with 1 mL of DMSO.
- From a 48 hours culture of amoebae:
  - Remove the culture medium and add 10 mL of PYG.
  - Gently detach the amoeba, and centrifuge the amoebal suspension.
  - Quantify the suspension using a cell counting chamber.
  - Adjust the suspension in PYG to  $10^7$  amoebae/mL.
  - Aliquot 500  $\mu$ L of this suspension into the cryotubes containing PYG + 20% DMSO.
  - Store the tubes quickly at  $-70 \pm 10^\circ\text{C}$ .

## **Detection of *Legionella* species in environmental samples**

### 3. Detection of *Legionella* species in environmental samples

#### 3.1. Sampling

##### 3.1.1. Regulatory documents and standards

Relevant standards and regulatory documents include:

- UNI EN ISO19458:2006 Water quality- Sampling for microbiological analysis
- Directive 2000/54/EC of the European Parliament and of the Council on the protection of workers from risks related to exposure to biological agents at work.
- [Legionella Sampling Procedure and Potential Sampling Sites](#)

##### 3.1.2. Safety

*Legionella* is classified as a Risk Group 2 microorganism according to Directive 2000/54/EC. Because infection occurs primarily through inhalation of contaminated aerosols, all procedures likely to generate aerosols must be carefully assessed. Samples potentially containing *Legionella* should be handled by trained personnel using appropriate PPE in adequately equipped laboratories using Class II biological safety cabinets compliant with EN 12469. In addition to personal protection, during the test, the operator must pay utmost attention to maintaining the sample's sterility, eliminating any possibility of contamination with other samples or the environment.

Personnel involved in environmental sampling should not belong to high-risk groups (people undergoing corticosteroid treatment, those with chronic respiratory conditions, diabetics, etc.) and it is recommended to:

- Wear appropriate PPE when aerosol generation cannot be avoided (e.g., in sampling operations where it is not possible to turn off cooling towers)
- Minimize aerosol formation by gently flushing the water outlets
- Avoid direct exposure to aerosols.
- Where feasible, request shutdown of cooling towers or evaporative condensers at least 20 minutes before sampling.

##### 3.1.3. Training

Reliable *Legionella* testing depends on appropriate sampling procedures. Sampling should therefore be performed or supervised by trained personnel familiar with:

- routes of *Legionella* transmission
- *Legionella* ecology
- Factors that favour its survival and growth
- environmental conditions promoting bacterial survival and proliferation
- principles of microbiological sampling and sterility

Special precautions are required to prevent cross-contamination between sampling sites, particularly during immersion sampling procedures (e.g., tanks or cooling tower basins).

Recommended precautions include:

- changing gloves between sampling points;
- disinfecting hands using 70% ethanol or isopropyl alcohol (propanol)

- avoiding contamination of the external surface of sampling containers.

If contamination of a sampling container is suspected, the container should either:

- be discarded;
- or be disinfected with 70% ethanol or isopropyl alcohol before use.

#### *3.1.4. Information of the water system to be sampled*

Before sampling, the following information regarding the water or ventilation system should be collected or updated:

- schematic diagram of the water network;
- location of the incoming water supply;
- location of hot- and cold-water storage tanks;
- identification of systems capable of generating aerosols;
- presence of stagnation zones or poorly recirculated sections;
- age of the installation;
- distribution layout of water systems at risk;
- presence and characteristics of continuous disinfection systems;
- methods used to monitor disinfectant concentrations;
- layout of air-conditioning systems at risk;
- maintenance records, including routine and corrective interventions;
- history of disinfection procedures or major maintenance work.

#### *3.1.5. Required materials*

Personnel should prepare a sampling kit containing all required materials and PPE before site visits:

- gloves, masks or respirators, protective eyewear.
- insulated transport containers;
- sampling forms or registration sheets;
- sterile sampling bottles (minimum volume: 1 L), preferably glass or polyethylene;
- sterile 5–10 L containers for low-temperature or low-contamination water systems.

Where chlorine-based disinfectants are used, sampling containers should contain sodium thiosulfate pentahydrate as specified in UNI EN ISO 19458. Where copper or silver ion disinfection systems are used, EDTA neutralization is recommended.

#### Additional materials:

- Sterile bottles (preferably glass, polyethylene, polypropylene, or other sterile plastic)
- Sterile glass or polyethylene containers for collecting deposits and scale
- Sterile plastic bags to direct the shower flow
- Sterile swabs (cotton, polyester, or other material)
- Test tubes with 2-5 mL of sterile water
- Disinfectant: 70% v/v ethanol or 70% v/v propanol, 10% sodium hypochlorite (preferably in a spray bottle)

- Sterile scalpels
- Calibrated thermometer, preferably digital with a sensitivity of 0.1°C
- Flame gun
- Resistant markers (water-based or labelled)
- Sterile forceps
- Rubber bands
- Scissors
- Flashlight
- Camera
- 70% isopropyl alcohol (propanol), preferably spray.

### 3.1.6. *Registration form*

Each sample must be uniquely identified and linked to a registration form containing all relevant sampling information. Environmental investigations should focus on artificial water systems and prioritize sampling locations based on:

- system layout;
- epidemiological information;
- risk assessment findings.

### 3.1.7. *Type of samples*

Environmental samples may include:

- domestic hot water;
- domestic cold water, particularly when temperatures exceed 20 °C;
- water from priority buildings (e.g., hospitals, accommodation sites);
- sludge or sediment from tanks and reservoirs;
- scale or encrustations from pipes and tanks;
- biofilm from pipes, taps, aerators, and showerheads;
- humidifier water from air-conditioning systems;
- cooling tower water;
- evaporative condenser water;
- air-conditioning system filters;
- humidified air samples;
- water from hot tubs and decorative fountains;
- water from respiratory therapy devices or aerosols;
- water and environmental matrices associated with spa facilities.

Water systems should be assessed from the point of supply to distal outlets (“sentinel outlets”).

#### Main sampling locations:

Typical control points include:

- incoming municipal water supply or well intake;
- cold-water storage tanks;
- hot-water storage tanks and boilers;

- locations with stagnation, sediment accumulation, or scaling;
- infrequently used outlets;
- domestic hot-water recirculation loops;
- water softeners.

Sampling should include both hot and cold-water systems. When cold-water temperatures are consistently  $\leq 20\text{ }^{\circ}\text{C}$ , the number of sampling points may be reduced based on risk assessment findings.

The number and location of sampling points should always be determined according to:

- the *Legionella* risk assessment;
- system complexity;
- previous analytical results;
- surveillance frequency.

### 3.1.8. Cooling systems with evaporative towers / evaporative condensers

Samples must be taken from the cooling basin, away from the float-controlled inlet, and/or from the hot return water line. Unless otherwise indicated by risk assessment, at least one sample per cooling system is generally sufficient.

### 3.1.9. Hot water system

Under routine surveillance conditions:

- collect at least 1 L of water;
- do not flame or disinfect the outlet before sampling;
- do not flush the outlet before sampling;
- record water temperature.

When investigating possible exposure sources:

- flush water for at least 1 min;
- stop water flow;
- disinfect the outlet internally and externally using:
  - flaming (when technically feasible),
  - 1% sodium hypochlorite,
  - or 70% ethanol;
- allow disinfectant contact for at least 60 s;
- flush again for at least 1 min to remove residual disinfectant;
- measure water temperature after stabilization;
- collect the sample.

### 3.1.10. Cold water system

Sampling of cold-water systems may follow the same procedure described for hot-water systems when investigation of internal contamination is required.

### 3.1.11. Transport and storage

Samples should be transported to the laboratory immediately after collection.

During transport:

- samples should be protected from light;
- hot- and cold-water samples should be separated;
- transport should occur at ambient temperature unless otherwise specified.

In all cases, analysis should begin no later than 24 hours after sampling.

## 3.2. Culture according to ISO 11731:2017

The methods described below are intended for the isolation and enumeration of *Legionella* spp. in water samples. Water samples may include drinking, industrial, wastewater, natural water and water-related matrices, such as biofilms, sediments. These recommendations are based on ISO 11731:2017 “Water quality – Enumeration of *Legionella*.”

### 3.2.1. Sample type

The choice of analytical method for *Legionella* enumeration depends on:

- the characteristics of the sample;
- the purpose of the analysis;
- the expected level of background flora;
- and the origin of the sample.

Recommended approaches include:

- Samples expected to contain high concentrations of *Legionella* ( $>10^4$  CFU/L) and low levels of interfering flora may be analyzed by direct plating without concentration.
- Samples with unknown *Legionella* concentrations are generally analyzed after concentration by membrane filtration and membrane washing.
- When low *Legionella* concentrations are expected and interfering flora are abundant, direct membrane plating onto selective and non-selective media may be used.
- Highly contaminated samples may require dilution prior to culture.

### 3.2.2. Material and reagents

#### Filtration equipment:

- vacuum filtration system with sterile filter holder and funnel;
- or vacuum filtration manifold equipped with sterile glass or polypropylene funnels;
- vacuum pump adjusted to approximately 500–550 mm Hg.

#### Laboratory equipment:

- Refrigerator maintained at  $5 \pm 3$  °C.
- Incubator adjustable to  $36 \pm 2$  °C.
- pH meter with sensitivity of at least 0.1 pH units.

- Calibrated micropipettes (50–1000  $\mu\text{L}$ ) with sterile tips.
- Analytical balance with sensitivity of 0.01 mg.
- Sterile graduated cylinders.
- Glass or polystyrene jars (100 mL).
- Sterile disposable L-shaped spreaders.
- Bunsen burner or equivalent device.
- Sterile 90 mm disposable Petri dishes.
- Sterile disposable tubes (5–10 mL) for dilutions.
- Sterile graduated pipettes (2–10 mL)

#### Membranes:

- For the concentration and membrane washing method: sterile polycarbonate or polyethersulfone membranes, 47 mm diameter, pore size 0.22  $\mu\text{m}$
- For the direct membrane plating method on culture media: mixed cellulose ester membranes, 47–50 mm diameter, nominal pore size 0.2  $\mu\text{m}$  or 0.45  $\mu\text{m}$ .

#### Possible diluents:

- Milli-Q purified water filtered at 0.22  $\mu\text{m}$
- Page's solution
- Ringer solution.

#### Culture media:

All media required for testing (BCYE, BCYE without cysteine, GVPC, BCYE +AB) are commercially available as ready-to-use 90 mm plates and stored at  $5 \pm 3^\circ\text{C}$  for the shelf life specified by the manufacturer. The choice of media depends on the type of water and the presumed contamination.

### **3.2.3. Pre-treatment**

#### **3.2.3.1. Untreated sample**

Without filtration or pretreatment, inoculate unconcentrated or concentrated samples directly onto BCYE and GVPC agar plates. Inoculate 200  $\mu\text{L}$  per plate and spread evenly using a sterile L-shaped spreader.

#### **3.2.3.2. Heat-treated sample**

Incubate a 1–2 mL aliquot of concentrated sample at  $50 \pm 1^\circ\text{C}$  for 30 min. After treatment, inoculate 200  $\mu\text{L}$  per plate on BCYE agar and GVPC agar.

### 3.2.3.3. *Acid-treated sample*

Prepare a 1:10 dilution by adding 500 µL of the sample to 4.5 mL of acid solution (recommended pH 2). Incubate for 5 min at room temperature. Then inoculate 200 µL per plate on BCYE agar and GVPC agar.

### 3.2.3.4. *Sample subjected to acid and heat simultaneously*

For samples containing high levels of interfering flora (e.g., cooling tower water or wastewater):

- first perform heat treatment;
- allow the sample to cool;
- then immediately perform acid treatment.

After treatment, inoculate 200 µL per plate on BCYE agar and GVPC agar.

### 3.2.4. *Direct plating*

- Mix the water sample thoroughly to detach bacteria adhering to container walls, particularly plastic surfaces. The analyzed volume and all dilutions must be recorded to allow calculation of the final concentration.
- All potable-water samples should undergo: heat treatment and acid treatment.
- Allow the inoculum to absorb onto the plates, invert plates, and incubate for 10 days at  $36 \pm 2^\circ\text{C}$  in a humid atmosphere to prevent desiccation.
- Microaerophilic incubation may be used.

Direct plating is particularly recommended for samples with high *Legionella* concentrations ( $> 10^4$  CFU/L), including as dead legs in distribution networks, stagnant water in tanks and heavily colonized pipework.

### 3.2.5. *Concentration by filtration and membrane washing*

- Filter the sample through a sterile membrane (47 mm diameter, pore size 0.22–0.45 µm)
- Using sterile forceps, place the membrane onto the sterile filter holder.
- Position a sterile cup (volume up to 250–300 mL) over the membrane. If filtration systems are used, secure the filter holder and the cup with a clamp.
- Filter the sample gradually under vacuum, preferably maintaining a pressure of about 500–550 mm Hg.
- After filtration, remove the membrane aseptically
- Place it into a sterile container containing 10 mL of diluent or same sample water
- Detach retained microorganisms by sonication for 2–10 minutes or vortexing for 2 min. When using sonication, ensure that the liquid level inside the container remains below the water level of the ultrasonic bath.
- The resulting suspension constitutes the concentrate used for inoculation.
- Incubate plates inverted for 7–10 days at  $36 \pm 2^\circ\text{C}$  under aerobic conditions in a humid atmosphere.
- According to current ISO recommendations, CO<sub>2</sub> incubation is no longer advised.

For highly contaminated samples:

- multiple membranes may be used
- the sample may be diluted before filtration
- centrifugation is not recommended as it may reduce *Legionella* recovery and should only be performed when filtration is not possible.

**3.2.6. Concentration by filtration and direct membrane plating on agar**

This method is particularly suitable for drinking-water samples.

- Divide the sample into two equal aliquots.
- Filter each aliquot separately using mixed cellulose ester membranes (47–50 mm diameter; 0.45 µm pore size).
- First membrane:
  - after filtration place the membrane directly onto BCYE agar.
- Second membrane:
  - After filtration, add 30mL of acid solution to the filtration cone with the manifold valve closed
  - Allow contact for 5 min
  - Filter acid solution
  - Rinse using 20 mL of diluent
  - Place the membrane onto BCYE +AB agar or GVPC agar.
- Incubate plates at  $36 \pm 2^\circ\text{C}$  for 10 days in a humid atmosphere.

**Note:**

Unconcentrated or concentrated sample aliquots may be stored at  $5 \pm 3^\circ\text{C}$  for up to 48h for any further analyses. This is particularly useful during outbreak investigations or when additional treatments or dilutions may be required.

**3.2.7. Plate reading and identification of *Legionella***

Plates are examined after 2, 3, 4, and 5 days of incubation. If excessive overgrowth is observed, repeat inoculation using stored sample aliquots and prepare serial 10-fold dilutions (1:10 and 1:100). This improves readability and colony isolation.

**3.2.8. Selection of colonies for confirmation**

For each plate containing presumptive *Legionella* colonies:

- select three colonies from the plate with the highest number of morphologically similar colonies;
- when different colony morphologies are present, select at least one representative colony of each type.

During outbreak investigations:

- select five presumptive colonies of identical morphology;
- and at least two colonies of each additional morphology.

Confirmation methods are described in Section 4.

### 3.2.9. Unit of measurement

Enumeration results should be expressed as colony-forming units per litre (CFU/L). The final count should be based on the plate yielding the highest number of confirmed *Legionella* colonies, whether:

- untreated;
- heat-treated;
- acid-treated;
- or subjected to combined treatment.

Results obtained before completion of the full 10-day incubation period should be reported as preliminary results. Final confirmation should be issued after completion of incubation and confirmation procedures.

## 3.3. Analysis of non-water environmental matrices

The general procedures described in sections 3.1 and 3.2 for the sample transport, storage, culture media, reagents, pretreatment and colony confirmation also apply to the environmental matrices described below.

### 3.3.1. Deposits or sediments

- Transfer 0.1 – 10 g of material into a sterile tube
- Add a volume of the Ringer's or Page's solution or sterile water corresponding to nine times the sample weight (e.g. 0.9 – 90 mL).
- Mix thoroughly with a vortex mixer.
- Inoculate 100 - 200 µL of suspension onto
  - selective and non-selective medium
  - use aliquots for untreated, HT and acid treatment according to the procedure described for water samples (3.2.3.)
- The samples can be stored at  $5 \pm 3^{\circ}\text{C}$  for up to 48h.
- Perform serial 10-fold dilution from  $10^{-1}$  to  $10^{-4}$
- Results are expressed semi-quantitatively, according to the growth distribution across dilutions:
  - Absent → no growth observed
  - Low contamination → growth observed only in the undiluted sample
  - Medium contamination → growth observed in the initial dilutions
  - High contamination → growth observed at high dilutions

### 3.3.2. *Encrustations*

- Collect material from pipes or tanks using a sterile scalpel.
- Transfer material into sterile glass or disposable containers containing 2 – 5 mL of Ringer's, Page's solution or sterile water.
- Inoculate suspension in both selective and non-selective medium
- Perform heat and/or acid treatment according to the procedure described for water samples.
- Samples may be stored at  $5 \pm 3^\circ\text{C}$  for up to 48h.
- Results are expressed qualitatively as presence/absence.

### 3.3.3. *Biofilm*

- Using a sterile swab, collect the material deposited on the internal or external surfaces (e.g. of the terminal point). Sampling should be performed before the water flow is initiated, after removal of aerator or showerheads when applicable.
- For quantitative analysis,
  - sample approximately 100 cm<sup>2</sup>.
  - place the swab into a tube containing appropriate diluent.
  - Ensure that the cotton of the swab is completely immersed in the Ringer's or Page's solution or sterile water.
  - Mix thoroughly using vortex agitation or ultrasonic bath treatment for 2 min.
  - Inoculate 100 – 200  $\mu\text{L}$  of suspension onto both selective and non-selective medium
  - Perform heat and/or acid treatment on an aliquot according to Section 3.2.3.
  - Results may be expressed as CFU/100 cm<sup>2</sup>.
- When the sampled surface area is unknown or irregular, results should be expressed semi-quantitatively per swab as described in Section 3.3.1.

Store swabs at  $5 \pm 3^\circ\text{C}$  in in sterile closed containers containing 2–5 mL diluent.

### 3.3.4. *Soil and compost*

- Suspend 5 g of compost or soil in 15 mL of diluent
- Homogenize thoroughly
- Allow suspension to settle for 10-15 minutes to sediment coarse particles.
- Mix 400  $\mu\text{L}$  suspension with the same volume of acid solution (pH 2.2).
- Incubate for 15 minutes at room temperature.
- Perform serial 10-fold dilution from  $10^{-1}$  to  $10^{-4}$
- Inoculate 100  $\mu\text{L}$  from each dilution onto two GVPC agar plates
- Incubate at  $37 \pm 1^\circ\text{C}$  and examine on days 2, 5, 7, 10 and 14.
- Results may be expressed as CFU/g

### 3.3.5. *Filter from air conditioning systems*

Analysis should be performed on filters that have been in use for a sufficient period and/or have not been recently replaced or cleaned.

- Remove the filter, or a representative section if large. Cut it into small pieces using sterile scissors, if the filter portion is too large.
- Place into a sterile plastic bag.
- Add sufficient volume of diluent to completely immerse the filter material.
- Incubate for 15 minutes at room temperature.
- Transfer suspension into a sterile tube.
- Mix thoroughly using a vortex mixer.
- Inoculate 100 – 200 µL suspension onto both selective and non-selective medium
- Use an aliquot for heat and/or acid treatment according to section 3.2.3.
- Results are expressed qualitatively as presence/absence.

#### Notes

For highly contaminated environmental matrices and water samples containing abundant interfering flora, combined heat and acid treatment may be performed as described in Section 3.2.3.4.

Combined treatment may also be applied after initial plate examination on Day 2 of incubation or when excessive contaminating growth is observed.

### 3.4. Legiolert

An alternative culture-based method, Legiolert®/Quanti-Tray® Legiolert, has been validated by AFNOR for the enumeration of *Legionella pneumophila* in environmental water samples. At the time of publication of this laboratory handbook, no other alternative culture-based methods have been validated by AFNOR for this purpose. The method detects *L. pneumophila* regardless of serogroup using a liquid culture medium. The Legiolert® assay is based on the detection of specific metabolic activity of *L. pneumophila*, resulting in a colour change of the medium from yellow to brown. The method provides results within 7 days and enables quantification of *L. pneumophila* in both potable and non-potable water samples. The method described in this section is based on the AFNOR-validated protocol "IDX 33/06-06/19 LEGIOLERT/QUANTI-TRAY LEGIOLERT". Users should follow manufacturer's instructions.

#### 3.4.1. Instruments, material and reagents

In addition to standard microbiological laboratory equipment, the following materials are required:

- Automatic Quanti-Tray™ heat sealer;
- Quanti-Tray™ multi-well pouches;
- Adjustable incubator set to  $39 \pm 0.5^{\circ}\text{C}$ , with a tray of sterile distilled water to maintain humidity;
- Adjustable 10 or 20 µL micropipettes;
- Rubber supports for Quanti-Tray™ pouches;
- Sterile plastic bottles (100 mL) or glass bottles with screw caps;
- Plastic sealing bags for Quanti-Trays™ to maintain a humid environment (optional)
- Legiolert® reagent and supplements;
- Water hardness measurement kit (e.g., Aquadu®, Macherey-Nagel)

- Hardness correction supplement;
- Paper towels and 90° ethanol.

All materials shall be stored according to the manufacturer's instructions.

The Legiolert® medium is supplied as pre-dosed dehydrated reagent vials for analysis of 100 mL samples or dilutions thereof. The reagent has a shelf life of 24 months from the date of manufacture and shall be stored at  $25 \pm 2^\circ\text{C}$ , protected from light and humidity.

For quality control, certified reference strains in accordance with UNI EN ISO 11133 are recommended. Alternatively:

- *L. pneumophila* NCTC 11192 may be used as a positive control;
- *Enterococcus faecalis* NCTC 12697 may be used as a negative control.

#### Quality control procedure:

- Remove one lenticule each of *L. pneumophila* NCTC 11192 and *E. faecalis* NCTC 12697 from storage at  $-20 \pm 5^\circ\text{C}$  and allow to equilibrate at room temperature for 15 min.
- Add 100 mL of sterile distilled water and one vial of Legiolert reagent to two sterile containers.
- Mix thoroughly and allow the reagent to dissolve for 15 minutes.
- Add one bacterial lenticule to each container.
- Allow to stand for 15 minutes and mix each suspension by shaking 10 times.
- Transfer each suspension into a dedicated Quanti-Tray® pouch.
- Seal the trays using the automatic sealer;
- Weigh and record the initial tray weights;
- Incubate for 7 days and reweigh the trays after reading;
- Weight loss shall not exceed 15%.

Appropriate personal protective equipment shall be used in accordance with the relevant safety data sheets.

#### **3.4.2. Pre-treatment**

For potable water, the analytical volume is 100 mL, either undiluted or appropriately diluted depending on the expected contamination level. For non-potable water, the analytical volume is 1 mL, either undiluted or diluted according to sample characteristics.

#### **3.4.3. Determination of water hardness**

Water hardness shall be measured prior to analysis using the manufacturer-provided colorimetric kit. Based on the colorimetric result, water is classified into four groups:

- Groups I–II: soft water;
- Groups III–IV: hard water.

Hardness correction shall be performed as follows:

- Hard water: add 1 mL hardness supplement per 100 mL reaction volume;

- Soft water: add 0.33 mL hardness supplement per 100 mL reaction volume.

#### 3.4.4. Analysis of potable water sample

- Following hardness correction, add one vial of Legiolert® reagent to 100 mL of sample or diluted sample;
- Mix thoroughly until complete dissolution of the reagent;
- Transfer the solution into a Quanti-Tray® pouch and place it into the tray holder;
- Seal the tray using the Quanti-Tray® heat sealer to distribute the sample evenly among the wells. Optionally place trays into sealed plastic pouches to maintain humidity;
- Incubate at  $39 \pm 0.5^\circ\text{C}$  for 7 days in a humidified incubator.

#### 3.4.5. Prepare non-potable water sample

- Add 100 mL sterile diluent (e.g., deionized water, phosphate buffer, or 0.1% peptone water) into a sterile container without sodium thiosulfate;
- Add one vial of Legiolert® reagent and mix thoroughly until dissolved;
- Add 2 mL of Legiolert® Pretreatment reagent into a sterile microtube;
- Add 2 mL of non-potable water sample and mix thoroughly;
- Incubate at room temperature for  $60 \pm 5$  minutes;
- Following incubation, transfer 0.2 mL of the treated sample into the Legiolert® reaction mixture and mix thoroughly;
- Transfer the final mixture into a Quanti-Tray® pouch;
- Seal immediately using the Quanti-Tray® heat sealer;
- Incubate at  $39 \pm 0.5^\circ\text{C}$  for 7 days in a humidified incubator.

#### Important considerations:

Humidity shall be maintained throughout incubation using a water tray inside the incubator.

The incubator should only be opened briefly to minimize temperature fluctuations.

Quanti-Trays® shall remain undisturbed throughout incubation, as it could result in false positive results.

Tray orientation and incubation temperature are critical for valid results.

#### 3.4.6. Interpretation of results

After incubation, count the number of positive large and small wells showing a color change from yellow to brown. MPN shall be calculated using the Quanti-Tray® MPN table for 96-well trays. Results shall be reported as:

- *L. pneumophila* MPN/100 mL.
- A negative result for potable water analysis shall be reported as: <10 MPN/L.

As Legiolert® is an AFNOR-validated method, positive results do not require additional confirmation testing.

### 3.4.7. Strain isolation

The Legiolert® method also enables recovery of viable *Legionella* strains from positive wells.

- Disinfect the paper backing corresponding to a positive well using 90% ethanol;
- Perforate the backing using a sterile pipette tip or syringe;
- Withdraw approximately 5 µL from one or several positive wells;
- Pool the collected liquid and inoculate onto BCYE agar plates;
- Incubate at  $36 \pm 2^\circ\text{C}$  in a humidified atmosphere for 2–4 days;
- Proceed with identification using standard culture-based confirmation methods.

Positive Legiolert® trays may remain viable for several months when refrigerated at  $5 \pm 3^\circ\text{C}$ , provided that the paper backing remains intact and the trays remain sealed. Once perforated, trays shall be discarded after use.

## 3.5. PCR on environmental samples

### 3.5.1. Regulatory documents and standards

- ISO/TS 12869:2019 Water quality — Detection and quantification of *Legionella* spp. and/or *L. pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR).
- UNI EN ISO19458:2006 Water quality- Sampling for microbiological analysis

### 3.5.2. Scope of the method

This method enables the detection and quantification of *Legionella* DNA in a defined volume of water using quantitative real-time PCR (qPCR). The use of qPCR is particularly valuable during clusters and outbreak investigations, where a large number of water samples must be analyzed within a short timeframe. Due to its rapidity, sensitivity and standardization potential, the use of qPCR is also recommended for routine monitoring of water system. The method detects DNA from both viable and non-viable bacteria and therefore does not directly assess bacterial culturability.

### 3.5.3. Training

Personnel performing qPCR analyses shall possess appropriate training in molecular biology techniques, particularly PCR-based methods, as well as fundamental microbiological practices. When the same operator performs both culture-based and molecular analyses:

- Separate laboratory coats shall be used for each activity;
- Dedicated micropipettes and consumables shall be reserved exclusively for qPCR procedures;
- Disposable sterile materials suitable for molecular biology applications shall be used;
- Gloves shall be worn at all times during qPCR preparation and analysis.

Strict contamination prevention procedures shall be implemented throughout the workflow.

### 3.5.4. *Work areas*

To minimize contamination risks, the laboratory should ideally include three physically separated work areas:

- Area for sample concentration and DNA extraction;
- Area for preparation of qPCR reaction mixtures;
- Area dedicated to amplification and post-amplification analysis.

Unidirectional workflow from clean to contaminated areas is strongly recommended.

### 3.5.5. *Sampling, transport and storage*

Sampling shall be performed in accordance with ISO 19458. Samples should ideally be analyzed within 24 hours of collection. Samples shall be transported either at  $5 \pm 3$  °C or  $20 \pm 5$  °C. If immediate analysis is not possible, samples shall be stored at  $5 \pm 3$  °C until processing. Repeated freeze–thaw cycles should be avoided.

### 3.5.6. *Material and equipment*

In addition to standard microbiological laboratory equipment, the following instruments are required:

- qPCR thermocycler
- vortex mixer
- mini centrifuge possibly both for plate and 0.2 µL tubes
- thermostatic water bath / heating block
- micropipettes (0.1-1000 µL)

### 3.5.7. *Sample concentration*

- Filter up to 1 L of sample. Alternative volumes may be used depending on sample characteristics and expected contamination levels, but volumes lower than 50 mL are not recommended. An adequate volume should also be selected according to the purpose of the monitoring activity and the limit of detection to be achieved.
- Record the exact filtered volume for calculation of the final result.
- Concentration shall be performed using membrane filtration under aseptic conditions
  - Recommended filters: polycarbonate membrane filter, or other materials with low protein or DNA binding capacity. Membranes containing cellulose or glass fibres should not be used.
  - Filters characteristics: 0.45 µm pore size and 47 mm diameter, placed on the filtration apparatus under aseptic conditions.

### 3.5.8. *DNA extraction and storage*

DNA extraction is performed directly from the membrane filter. Extraction methods may include physical, chemical, or enzymatic lysis. Purification may be achieved using:

- Chloroform extraction and fractional precipitation

- Ethanol or isopropanol precipitation
- Adsorption on solid matrices (resins, magnetic beads, etc.).

Commercial extraction kits may be used according to the manufacturer's instructions. Some commercial systems also allow partial removal of extracellular/free DNA prior to extraction from viable cells.

Purified DNA shall be suspended in a stabilizing solution such as buffer containing EDTA or proteins (BSA). It is recommended to analyse DNA by qPCR as soon as possible.

- If PCR is performed within 24 h, store DNA at  $5 \pm 3$  °C.
- If storage exceeds 24 h, DNA should be stored at  $-18 \pm 2$  °C.

Extracted DNA may be stored:

- at  $-20 \pm 5$  °C for up to 6 months
- at  $-70 \pm 10$  °C for extended preservation, and genome stability should be validated.

### 3.5.9. PCR amplification

To ensure analytical reliability, each qPCR run shall include:

- negative control
- positive control
- internal positive control (IPC).

The IPC is used to detect PCR inhibition and identify potential false-negative results. The IPC may be added directly into the reaction mixture, or added to the extracted DNA sample. In many commercial kits, the IPC is included within the master mix formulation. The quantity of IPC used depends on the manufacturer's recommendations and stock concentration.

#### 3.5.9.1. In-house PCR mix

Immediately before use:

- Prepare the reaction mixture for the number of samples to be analysed, plus one negative control and one positive control.
- Vortex to mix the mixture and briefly centrifuge.
- For each sample and for the negative and positive controls, dispense the required reaction mixture volume into PCR tubes or wells of a multi-well plate.
- Preparation of the PCR mix shall minimize the risk of contamination by amplified products or environmental DNA.
- Add the required volume of extracted genomic DNA according to the kit instructions.
- Close the tubes/wells carefully, place them in the thermocycler, and start the thermal cycling program.

**Table 12** : Example of PCR mix components.

| Component              | Notes                                   |
|------------------------|-----------------------------------------|
| 1. Dilution water      | Diluent                                 |
| 2. PCR buffer solution | May vary depending on the manufacturer; |

|                                |                                                                                                                                                                                                               |
|--------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 3. MgCl <sub>2</sub>           | The final MgCl <sub>2</sub> , if not present in the PCR buffer solution, concentration depends on the concentration of dNTPs, primers, probes and target DNA and must be optimized and usually is 5mM o 50mM. |
| 4. dNTPs                       | dATP, dTTP, dCTP, dGTP. A dTTP + dUTP mix and uracil-DNA N-glycosylase may also be used                                                                                                                       |
| 5. Primers                     | See section <b>3.5.9.4</b>                                                                                                                                                                                    |
| 6. Thermostable DNA polymerase | A hot-start Taq DNA polymerase should be used to avoid possible false positives                                                                                                                               |
| 7. Probes                      | See section <b>3.5.9.4</b>                                                                                                                                                                                    |

### 3.5.9.2. DNA co-precipitants and TE buffer

These are used to improve DNA recovery during the extraction process. They must be free of nuclease activity and free of sequences homologous to the PCR target gene.

For TE buffer: Dissolve Tris and EDTA in DNase and RNase-free water and adjust the pH to 8.0 with HCl.

**Table 13** : TE composition.

| Component                              | Concentration (mmol/L) |
|----------------------------------------|------------------------|
| Tris (hydroxymethylaminomethane)       | 10                     |
| EDTA (ethylenediaminetetraacetic acid) | 1                      |

### 3.5.9.4. Target sequences, primers and probes

One or more genetic targets may be amplified to detect and differentiate DNA originating from *Legionella* spp. and *L. pneumophila*. The specificity of primers and probes shall be evaluated:

- In silico, by assessing sequence homology using major nucleotide databases such as NCBI or EMBL;
- In vitro, by testing against *Legionella* spp., *L. pneumophila* strains and other bacterial species sharing similar ecological niches with *Legionella*.

A minimum panel of strains for specificity testing is provided in ISO/TS 12869.

Examples of primer and probe oligonucleotide sequences are provided below. For all primers:

- Prepare a stock solution in TE buffer diluted 1:10 to obtain a final concentration of 100 µmol/L;
- Store stock solutions at temperatures below  $-18 \pm 2^{\circ}\text{C}$ ;
- Prepare working solutions by diluting the stock solution to a final concentration of 10 µmol/L using TE buffer diluted 1:10.
- Working solutions may be stored for up to 6 months at temperatures below  $-18 \pm 2^{\circ}\text{C}$ .

**Table 14** : Sequences of primers and probes for *Legionella* spp, *L. pneumophila* et *L. pneumophila* serogroup 1.

| Primers      | Sequence (5' - 3')            | Final concentration (µM) |
|--------------|-------------------------------|--------------------------|
| 16SrRNAgeneF | 5'-GGAGGGTTGATAGGTTAAGAGCT-3' | 10                       |
| 16SrRNAgeneR | 5'-CCAACAGCTAGTTGACATCGTTT-3' | 10                       |

|              |                                               |    |
|--------------|-----------------------------------------------|----|
| 16SrRNAgeneP | 5'-FAM-AGTGGCGAAGGCGGCTACCT-3'-BHQ1           | 10 |
| LpneuF       | 5'-CCGATGCCACATCATTAGC-3'                     | 10 |
| LpneuR       | 5'-CCAATTGAGCGCCACTCATAG-3'                   | 10 |
| LpneuP       | 5'-FAM-TGCCTTTAGCCATTGCTTCCG-3'-BHQ1          | 10 |
| wzm_FP       | 5'-CAAAGGGCGTTACAGTCAAACC-3'                  | 10 |
| wzm_RP       | 5'-CAAACACCCCAACCGTAATCA-3'                   | 10 |
| wzm_HP       | 5'-FAM-TCTTGGGATTGGGTGGGTATTTTAACTCCT-3'-BHQ1 | 10 |

**Amplification:** The duration of the hot-start phase depends on the hot-start Taq DNA polymerase used for real-time PCR and must follow the manufacturer's instructions.

**Table 15 :** Example of amplification program.

| Target (°C) | Hold (h:mm:ss) | Cycle |
|-------------|----------------|-------|
| 95          | 00:03:00       | 1     |
| 95          | 00:00:20       | 43*   |
| 60          | 00:01:00       |       |

\* The number of cycles is determined as the intercept of the calibration function plus 3 cycles, in order to observe the amplification profile obtained for 1 genomic unit (GU).

### 3.5.10. qPCR commercial kits

Commercial qualitative (detected/not detected) and quantitative qPCR kits, including those providing DNA standards for quantification, primarily target:

- *L. pneumophila* through amplification of the mip gene;
- *Legionella* spp. through amplification of the 16S rRNA gene.

Commercial kits generally include a complete reaction mixture containing: reaction buffer, MgCl<sub>2</sub>, dNTPs, Taq DNA polymerase, and primers specific for *Legionella* spp. and/or *L. pneumophila*. The reaction volume and amplification conditions shall be applied according to the manufacturer's instructions.

Commercial kits validated by accredited certification bodies such as AFNOR, NordVal, MicroVal, AOAC, may be used directly to their validated scope. If commercial kits have not been validated by accredited organizations, validation may be performed according to UNI EN ISO 16140 or any other internationally recognized equivalent protocol. In the absence of kits specifically validated for water matrices, commercial kits validated for other matrices may be used provided that validation was performed by an accredited body and that validation complies with UNI EN ISO 16140-2, "Validation of a method – Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method".

### 3.5.11. Expression of results

A result is considered positive when amplification of the target sequence occurs within the positivity threshold specified by the manufacturer or, in general, when the cycle threshold (Ct) value is ≤ 35. The result is considered negative when:

- No amplification signal of the target gene is detected in the presence of successful IPC amplification

- Amplification occurs after the threshold cycle specified by the manufacturer;
- The Ct value exceeds the intercept value established according to the assay limit of detection (LOD).

**Table 16** : Expression of results depending on dilution factor.

| Number of genomic units | DNA dilution | GU/L                                    | Comment                                                                                            |
|-------------------------|--------------|-----------------------------------------|----------------------------------------------------------------------------------------------------|
| N < 1                   | 1            | $< \text{LOD} \times F/V$               | <i>Legionella</i> <sup>x</sup> not detected                                                        |
|                         | d            | $< \text{LOD} \times dF/V$              | DNA dilution due to the presence of PCR inhibitors.<br><i>Legionella</i> <sup>x</sup> not detected |
| N ≥ 1                   | 1            | <i>Legionella</i> <sup>x</sup> detected |                                                                                                    |
|                         | d            | <i>Legionella</i> <sup>x</sup> detected |                                                                                                    |

Where:

- N = mean number of genomic units (GU) per well
- F = conversion factor calculated as the ratio between the number of GU in the well and the number of GU corresponding to the detection limit per test
- d = DNA dilution factor
- V = volume (in litres) of the filtered sample
- x = specify whether *Legionella* spp. or *L. pneumophila*

If the water matrix contains substances that interfere with the polymerase reaction, the result of the procedure may be affected. In such cases, corrective measures such as sample dilution should be adopted.

#### Note

The LOD corresponds to the lowest number of genomic units that the assay is able to detect in at least 90% of the tests performed. Determination of the LOD should be carried out using at least 10 independent measurements obtained from 10 separate dilutions of a *L. pneumophila* DNA standard solution.

As reported in the literature, qPCR methods for *Legionella* detection demonstrate a negative predictive value (NPV) close to 100%. Therefore, samples yielding negative qPCR results do not require further analysis by culture.

Because no direct correlation exists between genomic units per liter (GU/L) determined by qPCR and colony-forming units per liter (CFU/L) determined by culture, samples testing positive by qPCR should subsequently be analyzed using culture methods to determine the concentration of viable *Legionella* in the sample.

#### 3.5.12. Decontamination

All reusable devices and materials must be decontaminated by immersion in, or exposure to, one of the following solutions for at least 30 minutes:

- hypochlorite solution containing 1.7% active chlorine;
- hydrochloric acid solution (1%);
- non-ionic detergent solution.

After decontamination, materials should be rinsed two to three times with filtered distilled water and sterilized at  $121 \pm 1^\circ\text{C}$  for 20 minutes. Ultraviolet (UV) radiation may be used for the decontamination of small instruments or components, such as micropipettes, forceps, tweezers, and filtration manifold filter holders.

## **Identification of colonies**

## 4. Identification of colonies

Presumptive identification of *Legionella* spp. in culture represents a critical step in the laboratory confirmation of this microorganism in **both clinical and environmental samples**. The combined use of selective and non-selective media facilitates the isolation of presumptive *Legionella* colonies, supports differentiation from background flora and enables subsequent confirmatory identification procedures.

### 4.1. Colony morphology

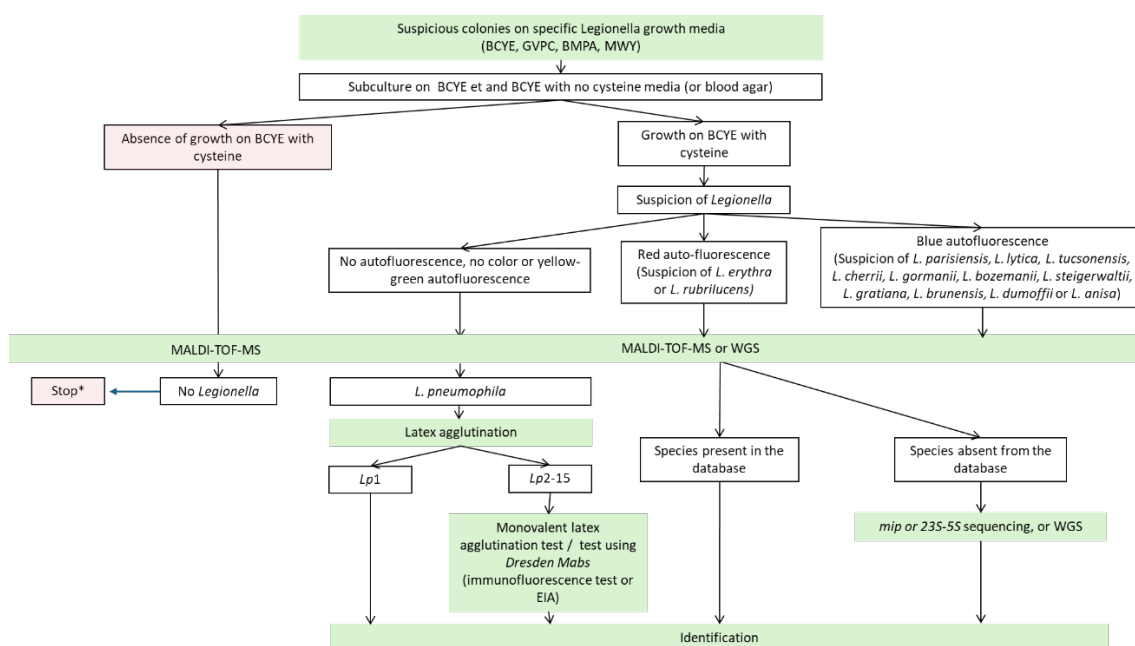
Observation of colony morphology and growth characteristics constitutes the initial screening step. Typically, *Legionella* colonies are smooth, convex, and greyish-white with entire margins. When examined under a stereomicroscope using oblique illumination, colonies may exhibit a characteristic “ground-glass” appearance. Certain *Legionella* species may produce pigmented colonies, including red, yellow-green, purple, reddish-purple, or pink coloration.

Under ultraviolet (UV) light, some species may exhibit autofluorescence:

- Bright white autofluorescence may be observed in species like *L. anisa*, *L. bozemanii*, *L. cherrii*, *L. dumoffii*, *L. gormanii*, *L. gratiana*, *L. parisiensis*, *L. steigerwaltii*, and *L. tucsonensis*,
- Red autofluorescence may be observed in *L. erythra* and *L. rubrilucens*.

Culture plates should be incubated at 35–37 °C in a humidified atmosphere for up to 10 days, although some strains may require prolonged incubation. Colony morphology is generally comparable across culture media; however, selective supplements may influence colony appearance (see Annex 1).

Presumptive colonies are confirmed by subculture onto BCYE agar supplemented with cysteine and BCYE agar without cysteine (BCYE–cys). *Legionella* colonies characteristically grow on BCYE agar but fail to grow on cysteine-free medium after incubation at  $36 \pm 2$  °C for 2–5 days.



**Figure 7:** Flowchart for *Legionella* identification.

## 4.2. Confirmation of Suspect Colonies

Presumptive identification of *Legionella* spp. from clinical and environmental isolates must always be followed by confirmatory testing using at least one validated identification method. Confirmatory methods may include:

- MALDI-TOF MS, when validated for the identification of *Legionella* species from clinical and environmental isolates;
- PCR or qPCR assays targeting the *Legionella* genus and/or specific *Legionella* species;
- latex agglutination tests for the identification of *Legionella* spp., *L. pneumophila* serogroup 1, and *L. pneumophila* serogroups 2–14;
- immunochromatographic assays;
- serological or biochemical methods (less commonly used in routine laboratories);
- whole genome sequencing (WGS) of clinical and environmental isolates, which will be further developed in future versions of this handbook.

The combined use of colony morphology, selective and non-selective culture media, and molecular or proteomic methods ensures reliable identification of *Legionella* isolates.

## 4.3. Strain storage

*Legionella* strains may be preserved using commercially available cryopreservation systems specifically designed for bacterial storage, such as bead-based systems, according to the manufacturer's instructions.

Alternatively, strains may be preserved in sterile semi-skimmed milk:

- dispense approximately 1.5 mL of fresh semi-skimmed milk into 2 mL cryogenic tubes;
- sterilize at  $115 \pm 1$  °C for 15 minutes;
- alternatively, sterilized milk may be aliquoted into sterile 5–10 mL glass tubes, stored at  $-20$  °C, and thawed immediately before use;
- once thawed, sterile milk may be stored at  $4 \pm 1$  °C for up to one week.

Bacterial growth collected from agar plates using a sterile 10 µL loop is directly suspended in the milk preparation.

Strains may also be stored in AYE (ACES-buffered yeast extract) broth supplemented with 15–20% (v/v) glycerol. Appropriate volumes of the AYE–glycerol medium should be dispensed into cryogenic tubes suitable for freezing. Bacterial biomass collected from solid culture media is suspended directly into the cryopreservation medium to obtain a homogeneous suspension before freezing.

For long-term preservation, strains should be stored at  $-70 \pm 10$  °C. Storage at  $-20 \pm 5$  °C may be used for shorter periods; however, bacterial viability is generally reduced compared with storage at lower temperatures.

## 5. Guidelines and tools

Below a few guidelines and tools related to *Legionella* diagnostic and detection available online are provided:

- Cooley L. Legionellosis (Legionnaires' disease & Pontiac fever). In: Centers for Disease Control and Prevention. CDC Yellow Book 2020. New York (NY): Oxford University Press; 2019:257–9 (<https://wwwnc.cdc.gov/travel/yellowbook/2018/infectious-diseases-related-to-travel/legionellosis-legionnaires-disease-pontiac-fever>).
- European *Legionella* Study Group (ESCMID Study Group for *Legionella* Infections, ESGLI (2017) European technical guidelines for the prevention, control and investigation of infections caused by *Legionella* species. [ESGLI European Technical Guidelines for the Prevention Control and Investigation of Infections Caused by Legionella species June.pdf](#).
- European Centre for Disease Prevention and Control. European Legionnaires' Disease Surveillance Network (ELDSNet). In: European Centre for Disease Prevention and Control [website]. Stockholm: European Centre for Disease Prevention and Control ([European Legionnaires' Disease Surveillance Network \(ELDSNet\)](#)) (includes link to ECDC Legionnaires' disease GIS tool).
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## 7. Annex 1: Composition of Culture Media for *Legionella*

The composition of the media for *Legionella* culture is also presented in the ISO norm 11731:2017. However, slight variations on the media formulations given below may be more regionally available and have been successfully used to isolate *Legionella* bacteria. Importantly, media performance may vary greatly even within lots from a single supplier (Ditommaso et al., 2020). Implementation of a robust quality control regimen is encouraged. Ideally, non-selective media should demonstrate growth of *L. pneumophila* and at least one non-*pneumophila* species, while selective media should also demonstrate inhibition of both Gram-negative and -positive bacteria.

### BCYE Medium: Buffered Charcoal Yeast Extract

BCYE is a non-selective, enriched medium essential for *Legionella* spp. growth, providing L-cysteine and  $\alpha$ -ketoglutarate, both required for bacterial metabolism. This medium serves as the reference for comparison with selective media.

Typical colony characteristics include:

- Slow onset of growth, generally visible after 3–5 days.
- Glassy or iridescent appearance, often described as “ground-glass”.
- Colour ranging from grey-white to pale blue.
- Smooth, circular margins with a slightly convex or flat surface.
- Colony diameter of 1–3 mm after 5–7 days.
- In some species emit fluorescence when exposed to UV light

The activated charcoal provides a dark background that enhances contrast and facilitates observation.

| Component                                              | Quantity |
|--------------------------------------------------------|----------|
| Yeast extract                                          | 10 g     |
| Activated charcoal                                     | 2 g      |
| Agar                                                   | 17 g     |
| ACES buffer (N-2-acetamido-2-aminoethanesulfonic acid) | 10 g     |
| L-cysteine                                             | 0.4 g    |
| Ferric pyrophosphate (soluble)                         | 0.25 g   |
| KOH 1 N                                                | 40 mL    |
| Distilled water                                        | 1,000 mL |

### BMPA medium: BCYE supplemented with cefamandole, polymyxin B, and anisomycin

BMPA combines selectivity with differential capabilities by incorporating pH indicators. It is useful for distinguishing *Legionella* from microorganisms that alter the local pH.

Typical features of *Legionella* colonies include:

- Greyish or whitish appearance, with a characteristic sheen.
- No colour change in the pH indicator (Bromocresol Purple), leaving the medium brown/purple.

- Colony size may be slightly larger than on GVPC depending on the initial bacterial load.

This medium allows rapid visual differentiation based on pH stability.

| Component                | Quantity  |
|--------------------------|-----------|
| Activated charcoal       | 2.0 g     |
| Yeast extract            | 10.0 g    |
| ACES-buffer              | 10.0 g    |
| Potassium hydroxide      | 2.8 g     |
| Iron (III) phosphate     | 0.25 g    |
| L-Cysteine hydrochloride | 0.4 g     |
| $\alpha$ -Ketoglutarate  | 1.0 g     |
| Cefamandole              | 0.004 g   |
| Polymixin B              | 80 000 IU |
| Anisomycin               | 0.08 g    |
| Agar                     | 13.0 g    |
| Distilled water          | 1,000 ml  |

### MWY medium: BCYE supplemented with glycine, polymyxin B, anisomycin, and vancomycin

MWY is one of the most visually distinctive media for presumptive identification due to its dyes and indicators.

*Legionella* colonies on MWY typically appear:

- Blue-grey or greenish-blue, often with a lighter central zone.
- Glassy or iridescent, with enhanced colour contrast.
- Circular with smooth edges.
- Surrounded by a yellow halo, due to L-cysteine utilization.

This strong chromatic contrast supports rapid and reliable screening.

| Component                | Quantity  |
|--------------------------|-----------|
| Activated charcoal       | 2.0 g     |
| Yeast extract            | 10.0 g    |
| ACES-buffer              | 10.0 g    |
| Potassium hydroxide      | 2.8 g     |
| Iron (III) phosphate     | 0.25 g    |
| L-Cysteine hydrochloride | 0.4 g     |
| $\alpha$ -Ketoglutarate  | 1.0 g     |
| Glycine                  | 3.0 g     |
| Polymixin B              | 50 000 IU |
| Anisomycin               | 0.08 g    |

|                    |          |
|--------------------|----------|
| Vancomycin         | 0.001 g  |
| Bromothymol blue   | 0.01 g   |
| Bromocresol purple | 0.01 g   |
| Agar               | 13.0 g   |
| Distilled water    | 1,000 ml |

### GVPC (BCYE with Glycine, Vancomycin, Polymyxin B and Cycloheximide)

GVPC is a selective medium commonly used for isolating *Legionella* from complex environmental samples with abundant interfering flora.

*Legionella* colonies on GVPC:

- Display a morphology comparable to BCYE, supporting presumptive identification.
- Are typically smaller, due to selective agents.
- Maintain the glassy appearance and regular margins characteristic of the genus.
- May grow more slowly than on BCYE.

GVPC is particularly useful for water system samples, cooling towers and biofilms.

| Component                | Quantity  |
|--------------------------|-----------|
| Activated charcoal       | 2.0 g     |
| Yeast extract            | 10.0 g    |
| ACES-buffer              | 10.0 g    |
| Potassium hydroxide      | 2.8 g     |
| Iron (III) phosphate     | 0.25 g    |
| L-Cysteine hydrochloride | 0.4 g     |
| $\alpha$ -Ketoglutarate  | 1.0 g     |
| Glycine                  | 3.0 g     |
| Polymyxin B              | 80 000 IU |
| Vancomycin               | 0.001 g   |
| Cycloheximide            | 0.08 g    |
| Agar                     | 13.0 g    |
| Distilled water          | 1,000 ml  |

## 8. Annex 2: Composition of Culture Media for Amoeba

### PAS (Page's amoeba saline)

PAS buffer:

- maintains amoebal viability;
- does not support amoebal replication;
- is used during co-culture with potentially contaminated specimens. There are two solutions (A and B) needed.

Solution A: Add these components and bring it 100 mL with distilled water

| Component                              | Quantity |
|----------------------------------------|----------|
| NaCl                                   | 1.2 g    |
| MgSO <sub>4</sub> · 7 H <sub>2</sub> O | 0.04 g   |
| Na <sub>2</sub> HPO <sub>4</sub>       | 1.42 g   |
| KH <sub>2</sub> PO <sub>4</sub>        | 1.36 g   |
| Distilled water                        | ~100 mL  |

Solution B: Add these components and bring it 100 mL with distilled water

| Component         | Quantity |
|-------------------|----------|
| CaCl <sub>2</sub> | 0.04 g   |
| Distilled water   | ~100 mL  |

For 1,000 mL PAS, mix 10 mL of solution A and 10 mL of solution B added by 980 mL of distilled water. Mix and use 1M KOH to adjust the pH to  $6.90 \pm 0.10$ . Autoclave it at 121°C for 20 min and store it afterwards at 4°C.

### PYG (peptone-yeast extract-glucose)

PYG is a nutrient-rich medium used for routine maintenance and propagation of amoebae. Weigh the components and bring it to a volume of 1,000 mL with PAS.

| Component         | Quantity  |
|-------------------|-----------|
| proteose peptone  | 15 g      |
| yeast extract     | 5 g       |
| glucose           | 10 g      |
| FeSO <sub>4</sub> | 3 g       |
| PAS               | ~1,000 mL |

## 9. Annex 3: List of described *Legionella* species

An updated list of all *Legionella* species can be found here: <https://psn.dsmz.de/genus/legionella>. In April 2026, that included:

|                           |                                           |
|---------------------------|-------------------------------------------|
| <i>L. adelaicensis</i>    | <i>L. massiliensis</i>                    |
| <i>L. anisa</i>           | <i>L. meanjinensis</i>                    |
| <i>L. antarctica</i>      | <i>L. micdadei</i>                        |
| <i>L. beliardensis</i>    | <i>L. moravica</i>                        |
| <i>L. birminghamensis</i> | <i>L. nagasakiensis</i>                   |
| <i>L. bononiensis</i>     | <i>L. nautarum</i>                        |
| <i>L. bozemanii</i>       | <i>L. norrlandica</i>                     |
| <i>L. brunensis</i>       | <i>L. oakridgenesis</i>                   |
| <i>L. busanensis</i>      | <i>L. osnabrueckensis</i>                 |
| <i>L. cardiaca</i>        | <i>L. parisiensis</i>                     |
| <i>L. cherrii</i>         | <i>L. pneumophila. subsp. fraseri</i>     |
| <i>L. cincinnatiensis</i> | <i>L. pneumophila. subsp. pascullei</i>   |
| <i>L. donaldsonii</i>     | <i>L. pneumophila. subsp. pneumophila</i> |
| <i>L. drancourtii</i>     | <i>L. pneumophila. subsp. raphaelli</i>   |
| <i>L. dresdenensis</i>    | <i>L. qingyii</i>                         |
| <i>L. drozanskii</i>      | <i>L. quateirensis</i>                    |
| <i>L. dumoffii</i>        | <i>L. quinlivanii</i>                     |
| <i>L. erythra</i>         | <i>L. resiliens</i>                       |
| <i>L. fairfieldensis</i>  | <i>L. rowbothamii</i>                     |
| <i>L. fallonii</i>        | <i>L. rubrilucens</i>                     |
| <i>L. feeleei</i>         | <i>L. sainthelensi</i>                    |
| <i>L. felix</i>           | <i>L. santicrucis</i>                     |
| <i>L. geestiana</i>       | <i>L. saoudiensis</i>                     |
| <i>L. genomospecies</i>   | <i>L. septentrionalis</i>                 |
| <i>L. gormanii</i>        | <i>L. shakespearei</i>                    |
| <i>L. gratiana</i>        | <i>L. sheltonii</i>                       |
| <i>L. gresilensis</i>     | <i>L. spiritensis</i>                     |
| <i>L. hackeliae</i>       | <i>L. steelei</i>                         |
| <i>L. impletisoli</i>     | <i>L. steigerwaltii</i>                   |
| <i>L. israelensis</i>     | <i>L. taurinensis</i>                     |
| <i>L. jamestowniensis</i> | <i>L. thermalis</i>                       |
| <i>L. jordanis</i>        | <i>L. tucsonensis</i>                     |
| <i>L. lansingensis</i>    | <i>L. tunisiensis</i>                     |
| <i>L. londiniensis</i>    | <i>L. wadsworthii</i>                     |
| <i>L. longbeachae</i>     | <i>L. waltersii</i>                       |
| <i>L. lytica</i>          | <i>L. worsleiensis</i>                    |
| <i>L. maceachernii</i>    | <i>L. yabuuchiae</i>                      |
| <i>L. maioricensis</i>    |                                           |