Implementation and Validation of a Detection and Quantification Method for *Legionella* spp. and *Legionella pneumophila* through qPCR according to the norm ISO 12869

Nuno Duarte Alves Ferreira
Master in Applications in Biotechnology and Synthetic Biology
Department of Biology / Department of Chemistry and Biochemistry
Faculty of Sciences of the University of Porto
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Internship Report carried out as part of the Master in Applications in Biotechnology and Synthetic Biology

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Supervisor
Maria Adelaide Ferreira da Silva Rocha, Lab Coordinator, Municipal Company for Waters and Energy of Porto

Co-supervisor
Ana Paula de Campos Mucha, Auxiliary Invited Professor at the Faculty of Sciences of the University of Porto / Principal Investigator at CIIMAR – Interdisciplinary Centre of Marine and Environmental Research

Porto. Águas e Energia do Porto

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Resumo

A qualidade da água é um fator crucial na saúde pública. Devido à evolução da legislação ao nível nacional e europeu relativamente ao tratamento e monitorização da água, a qualidade desta tem vindo a melhorar ao longo das últimas décadas em Portugal. Em resposta à iniciativa de cidadãos Right2Water, a Comissão Europeia reexaminou a diretiva 98/83/CE, o que resultou numa atualização da legislação relativa à qualidade da água ao nível da União Europeia.

Entre os limites legais definidos para agentes patogénicos no Decreto-lei n. º 69/2023, que transcreve a diretiva europeia previamente mencionada, estão incluídos limites para bactérias do género *Legionella*. Estas bactérias encontram-se altamente disseminadas no ambiente, mas só representam um risco significativo para a saúde pública se estiverem presentes em sistemas de água artificiais. Sabendo isto, a deteção e quantificação efetiva destas bactérias é essencial para a proteção da saúde pública. Entre os métodos descritos como ferramentas aceitáveis para este propósito no Decreto-lei n. º 69/2023 estão as técnicas de PCR em conformidade com a norma ISO 12869.

Este projeto foi desenvolvido no laboratório de análise das Águas e Energia do Porto, que se encontra acreditado para um conjunto variado de atividades desde 2000. O objetivo principal deste projeto foi a implementação e validação no laboratório de análise de um método de PCR quantitativo para a deteção e quantificação de *Legionella* spp. e *L. pneumophila* em água para consumo. Este objetivo evoluiu, sendo a sua forma final a implementação e validação de um método de PCR quantitativo para a deteção de *Legionella* spp. e *L. pneumophila* em várias matrizes de água. Ao longo do projeto, foram também feitas contribuições consideráveis para o objetivo das Águas e Energia do Porto de acreditar este mesmo método.

Palavras-chave: *Legionella* spp.; *Legionella pneumophila*; biologia molecular; validação; validação secundária/verificação; acreditação; reação em cadeia da polimerase quantitativa (qPCR); qualidade de água

Abstract

Water quality is a key factor in public health. Thanks to the evolution of Portuguese and EU-wide legislation regarding water treatment and monitoring, water quality in Portugal has significantly improved over the last few decades. As a response to the Right2Water citizen's initiative, the European Commission re-examined the Directive 98/83/CE, resulting in a thorough updating of the legislative body on water quality at the EU level.

Among the legal limits for specific pathogens set by the Decree-law n. º 69/2023, which transcribes the previously mentioned EU directive, were ones for bacteria of the genus *Legionella*. The latter are widely disseminated in the environment but only pose a significant risk for public health when present in man-made water systems. Knowing this, their effective detection and quantification is essential for the protection of public health. Among the methods described as accepted tools for this purpose in Decree-law n. º 69/2023, are PCR techniques in conformity with the norm ISO 12869.

This internship was developed at Waters and Energy of Porto analysis laboratory, which has been accredited since 2000 for a varied set of activities. The main goal of this internship was the implementation and validation of a quantitative PCR method for the quantification and detection of *Legionella* spp. and *L. pneumophila* in potable water (with this goal having been expanded to other water matrices) at the analysis laboratory. This objective evolved, with its final form being the implementation and validation of the detection of *Legionella* spp. and *L. pneumophila* in various water matrixes. Large contributions were also made towards Waters and Energy of Porto's goal of accrediting the detection of *Legionella* spp. and *L. pneumophila* through this method.

Keywords: *Legionella* spp.; *Legionella pneumophila*; molecular biology; validation; secondary validation/verification; accreditation; quantitative polymerase chain reaction (qPCR); water quality

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FCUP	FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO
ISO	INTERNATIONAL ORGANIZATION FOR STANDARDIZATION
AEDP	ÁGUAS E ENERGIA DO PORTO / WATERS AND ENERGY OF PORTO
IPAC	INSTITUTO PORTUGUÊS DE ACREDITAÇÃO / PORTUGUESE INSTITUTE OF ACCREDITATION
ECDC	EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL
ELDSNET	EUROPEAN LEGIONNAIRES' DISEASE SURVEILLANCE NETWORK
EWGLI	EUROPEAN WORKING GROUP FOR LEGIONELLA INFECTIONS
QPCR	QUANTITATIVE POLYMERASE CHAIN REACTION
ERSAR	ENTIDADE REGULADORA DOS SERVIÇOS DE ÁGUAS E RESÍDUOS / REGULATORY ENTITY FOR WATER SERVICES AND WASTE
EU	EUROPEAN UNION
EEA	EUROPEAN ECONOMIC AREA
WHO	WORLD HEALTH ORGANIZATION
IAC	INTERNAL AMPLIFICATION CONTROL
PI	PROCEDIMENTO INTERNO / INTERNAL PROCEDURE
GU	GENOMIC UNIT
CFU	COLONY FORMING UNIT

1. Introduction

1.1. Context and goals

The quality of water intended for human consumption, among other purposes, is a key factor in public health. The consumption and/or use of low-quality water can easily lead to adverse health effects. Thanks to the evolution of laws regarding water treatment and monitoring, water quality levels in Portugal have significantly improved over the last few decades. This development is a result of not only legislative bodies that, since the 90s, have ensured the transposition of European directives regarding water quality, but also the continuous improvement of the model for water quality regulation, which has enabled the improved control of various parametric values pertaining to the water provided to consumers, and the detection followed by correction of situations that carry a risk for public health[1].

As a response to the Right2Water citizen's initiative, the European Commission re-examined the Directive 98/83/CE[2]. As a result, it became evident that improvements to the current legislative body were needed. Among them were improvements regarding the ease of access to water quality data for consumers and other clients, better monitoring of what materials and products are allowed to come into contact with water intended for consumption and the promotion of more modern practices, better suited than those common at the time in order to minimize water loss and/or contamination resulting from lackluster maintenance of water infrastructure, etc. Another result of this re-examining was the conclusion that the list of parametric values tracked at the time for water intended for general use needed to be expanded and updated[3].

Among the new parametric values set by the Decree-law n. º 69/2023 were legal limits for bacteria of the genus *Legionella*. This decree also mandates that the main analysis method be culture-based, such as those defined in the norm ISO (International Organization for Standardization) 11731:2017. However, equivalent methods are also accepted as complementary tools, such as the PCR-based methods defined ISO 12869:2019, fast culture methods, among others[1,4,5]. Bacteria of the *Legionella* genus are both pathogenic and widely disseminated in the environment. However, they only pose a significant risk for public health when present in man-made water systems[6]. Given the threat they pose, their effective detection and quantification is essential for outbreak prevention and protection of public health.

This internship was performed in the Water and Energy of Porto (Águas e Energia do Porto - AEdP) analysis lab, under the local supervision of the Lab Coordinator Eng. Adelaide Rocha. Dr. Ana Paula Mucha assumed the role of university-affiliated co-supervisor for the Faculty of Sciences of the University of Porto.

The initial goal of this internship was to implement and validate the real time detection and quantification of *Legionella pneumophila* and *Legionella* spp. in water through quantitative Polymerase Chain Reaction (qPCR) as defined in ISO 12869. However, AEdP opted to advance with the accreditation only of the qualitative capabilities of this method, as they were the ones most useful in the current legal context. Accomplishing this objective involved the analysis of a wide variety of norms, protocols and other literature related to the goal at hand, as well as the elaboration of new internal documents to structure and summarize all relevant information for future use at AEdP. The validation process involved very thorough and structured work, including strict statistical analysis of the results, and will be given a central focus in this report.

Finally, this internship was an opportunity to witness and directly participate in and contribute to (when permissible) the daily routine of an accredited lab, and all which that entails in terms of requirements and practices. Therefore, it was a highly enriching experience even beyond the core work of the internship.

1.2. AEdP: Waters and Energy of Porto

Waters and Energy of Porto (Águas e Energia do Porto - AEdP) are a local business entity, possessing statutory, administrative and financial autonomy, formed in 2006, whose social capital is fully held by the Municipal Council of Porto (Câmara Municipal do Porto). Its headquarters can be seen in figure 1.

AEdP's role centers on the integrated and sustainable management of the urban cycle of water in the Porto Municipality (Município do Porto). To this initial role, design and implementation of the municipal energy strategy was later added, conferring to AEdP a critical role in the management of multiple essential resources. The following activities are included in AEdP's responsibilities: distribution of potable water, wastewater drainage and treatment, drainage of rainwater, general water management, maritime front management, municipal-level energy management and the promotion of education related to the environment and sustainability.

AEdP distinguishes itself as one of the largest Portuguese entities in the business of providing not only water but also wastewater sanitation, serving 160 476 clients and the

populational equivalent of 500 000 inhabitants. Every day, on average, 45 130 m^3 of water are provided to the inhabitants of Porto.

The analytical control of water quality is assured by AEdP's analysis laboratory, which is accredited by the Portuguese Institute of Accreditation (Instituto Português de Acreditação - IPAC) according to ISO/IEC 17025:2017[7]. 25 063 analyses of the distributed water are performed per year, 18 747 of them in the public network and 6 320 in property networks. This thorough and constant monitoring of water quality ensures that the highly demanding water quality standards imposed by Portuguese and European legislation are all respected. The indicator Safe Water (Água Segura), corresponding to the percentage of potable water that meets all required criteria, stands at 99,74% at the time of writing.

Regarding sanitation, AEdP collects 57 754 m^3 of wastewater daily, which are then subjected to up to tertiary treatment in 2 wastewater treatment plants (Freixo and Sobreiras). The monitoring of the pre and post treatment waters from the wastewater treatment plants corresponds to 424 samples and a total of 1 897 assays: 1 689 physical-chemical parameters and 208 bacteriological parameters. AEdP is also responsible for the drainage of rainwater, for the management of 85 km of water lines, the management of bathing waters, Porto's maritime front and education, focused on the environment and sustainability, through the Water Pavillion (Pavilhão da Água)[8].

Figure 1. The headquarters of AEdP.

1.3. AEdP's analysis laboratory

To guarantee the quality of the water that it provides and monitors, AEdP resorts to its analysis laboratory. This lab has been accredited for a varied set of activities since 2000 and is currently accredited by IPAC according to the norm ISO/IEC 17025. It is the mission of the AEdP analysis laboratory to guarantee the analytical control of the entire water cycle, having as their core principles impartiality, independence, integrity and innovation, with the aim of fomenting trust in the sustainable use of water.

The main activities of the lab consist, but are not limited to, the control of the quality of potable water and wastewater. It also guarantees the analytical control of bathing water and sand, within the scope of the Blue Flag Program and of the water that flows in Porto's smaller streams in the scope of the Plan for Appreciation and Rehabilitation of the Porto Municipality's Water Lines (Plano de Valorização e Reabilitação das Linhas de Água do Município do Porto).

It is through the previously mentioned accreditation of AEdP that its credibility is attested, its technical competence recognized and the whole water quality control process made transparent. This transparency is a contributing factor for the safeguarding of water quality and therefore public health. Currently, the lab is accredited to perform 16 different microbiology assays, listed on table 1. The AEdP Lab also integrates the list of qualified labs emitted by the Regulatory Entity for Water Services and Waste (Entidade Reguladora dos Serviços de Águas e Resíduos - ERSAR), following the specifications of Decree-law n. º 69/2023[9]. The lab can be seen in figure 2.

Figure 2. AEdP's analysis laboratory.

Table 1. All microbiology assays AEdP's analysis laboratory is currently accredited for.

Matrix	Assay	Assay method source
Potable water	*Clostridium perfringens* quantification; Filtering membrane	ISO 14189
Potable water & pools	Search & quantification of *Pseudomonas aeruginosa*; Filtering membrane	ISO 16266
Potable water & pools	Quantification of coliform bacteria; Chromogenic media & filtering membrane	ISO 9308-1
Potable water & pools	Quantification of *Escherichia coli*; Chromogenic media & filtering membrane	ISO 9308-1
Potable water & pools	Search & quantification of coagulase producing *Staphylococcus*; Filtering membrane	NP 4343
Potable water & pools	Search & quantification of *Staphylococcus*; Filtering membrane	NP 4343
Potable water, natural fresh water (superficial and underground) & pools	Enumeration of viable microorganisms – n. º of colonies at (36±2) ºC; Incorporation	ISO 6222
Potable water & natural fresh water (superficial and underground)	Enumeration of viable microorganisms – nº of colonies at (22±2) ºC; Incorporation	ISO 6222
Potable water, natural fresh water (superficial and underground & pools	Search & quantification of *Enterococcus*; Filtering membrane	ISO 7899-2
Potable water, natural fresh water (superficial and underground) & natural saline water (bathing)	Quantification of *Enterococcus*; Miniaturized method (NMP)	ISO 7899-1
Natural saline waters (bathing)	Quantification of *Escherichia coli*; Miniaturized method (NMP)	ISO 9308-3
Potable water, natural fresh water (superficial and underground), natural saline water (bathing) & wastewater (treated and untreated effluents)	Search of *Salmonella* spp. except *Salmonella typhi* and *paratyphi*; Filtering membrane	PIB 7
Potable water, natural fresh water (superficial and underground), natural saline water (bathing) & wastewater (treated and untreated effluents)	Quantification of coliform bacteria; Enzymatic substrate method (NMP)	ISO 9308-2
Potable water, natural fresh water (superficial and underground), natural saline water (bathing) & wastewater (treated and untreated effluents)	Quantification of *Escherichia coli*; Enzymatic substrate method (NMP)	ISO 9308-2
Sand	Detection & quantification of fecal *Enterococcus*; Miniaturized method	PIB 21
Sand	Detection & quantification of *Escherichia coli*; Enzymatic substrate method (NMP)	PIB 22

1.4. *Legionella*

This report focuses on bacteria of the genus *Legionella* (Legionellae), the single member of the family Legionellaceae. The first isolates of *Legionella*, although not classified as such at the time, were obtained by Tatlock in 1943, followed by Jackson et al. in 1947[10]. The genus *Legionella* was only established in 1979, following an outbreak of pneumonia at an American Legion convention that occurred 3 years prior, and which was found to have been caused by an at the time unknown bacterium, *L. pneumophila*[11,12].

There are more than 60 *Legionella* species and approximately 70 serogroups currently identified. Around 30 of the latter have been confirmed to be pathogenic in humans[13,14]. *L. pneumophila* is particularly important, as it is responsible for approximately 90% of the confirmed cases of *Legionella* infection worldwide (*L. pneumophila* serogroup 1 is responsible for the majority of the cases associated with this species) followed by *L. longbeachae* and *L. bozemanii*[15]. According to one study, despite being responsible for the overwhelming majority of 95% of *Legionella* clinical isolates, *L. pneumophila* has been shown to correspond to a much more conservative 28% of environmental isolates. This disparity is thought to be driven by an elevated virulence of the *L. pneumophila* strains compared to the remainder of the genus[16].

Legionellae are Gram-negative bacilli that range from 0.3 to 0.9 μm in width and 2 to 20 μm in length, are strictly aerobic and have remarkably fastidious growth requirements. Most *Legionella* colonies present with a characteristic ground glass look and some also display a somewhat purple tint, as can be seen in figure 3. Legionellae utilize amino acids as their carbon source[17]. For their culture in a laboratory environment, media supplemented with both iron salts and L-cysteine must be utilized. In nature, to satisfy their nutritional requirements, Legionellae obtain the molecules they require from other organisms through parasitism[18,19]. *L. pneumophila* proliferates in a temperature range of 20–48 °C, but the optimal temperature for bacterial growth is 35 °C[20].

Legionella is ubiquitous in aquatic environments[21,22], although exceptions exist such as *L. longbeachae*, typically isolated from potting soils and composts[23,24]. However, it is most commonly through the contamination of man-made water systems that *Legionella* transmission occurs[6,25]. Despite the oligotrophic nature of these systems, such as water distribution networks, cooling and climatization systems, among others, *Legionella* survives and proliferates.

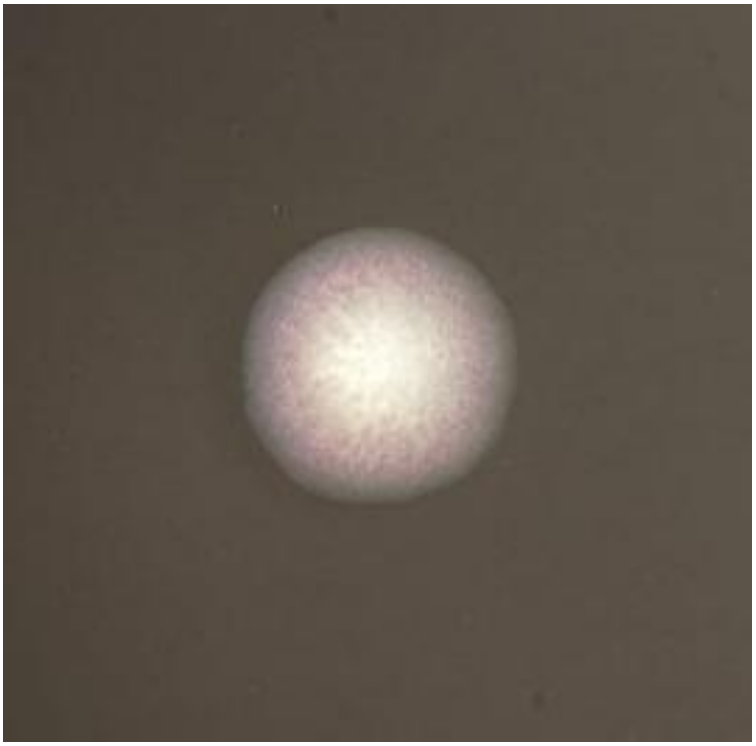

Figure 3. A wild-type *Legionella* colony grown on Buffered Charcoal Yeast Extract.

Some system elements can promote the proliferation of *Legionella*, such as dead-end loops, water stagnation, periods of non-use/construction and water temperatures within the previously mentioned interval favorable for *Legionella*[21,26,27]. Among these systems some possess the capability to aerosolize water (i.e. cooling towers, showers, air conditioning, dental equipment, etc.) and therefore are capable of generating airborne *Legionella* particles which can be inhaled, the typical pathway for infection to occur[21,28].

Legionella's life cycle is not only a key factor in its capability to survive in the often-harsh conditions present in man-made water systems but also its capacity to infect humans, and so it will be described in the following section.

1.5. *Legionella*'s Life Cycle

As previously mentioned, *Legionella* is ubiquitous in aquatic environments[29]. In these, *Legionella* can exist in the planktonic phase, in biofilms and as a parasite within ameba (preferred host) and protozoa[28]. Biofilms and hosts are integral to *Legionella*'s ability to survive in highly oligotrophic conditions, such as the artificial water systems in which they become a threat, but through what mechanisms? Ameba and protozoa are fundamental for *Legionella* since they function as hosts in nature, providing them with a stable, safe and nutrient rich environment inside which they can replicate. But what are biofilms, and what is their role in the survival of *Legionella*?

Microorganisms can organize themselves into biofilms, three-dimensional structures built by the microorganisms on interfaces. Biofilms are mostly composed of a polymer matrix secreted by microorganisms that allow them and other biofilm participants to stay attached to interfaces and in proximity to each other, while also creating an environment separate and shielded from external conditions. Within these structures, microorganisms can cooperate and even specialize/differentiate into different roles that improve the robustness of the overall community[30]. *Legionella* benefits greatly from biofilms for multiple reasons: within them, it can find respite from various environmental stresses (such as disinfection attempts); they can provide *Legionella* with easy access to, for example, amoebae hosts (these highly favor biofilms as a source of their prey, bacteria); multiple bacterial and algal species have been shown to be possible nutrient sources for *Legionella* not currently inside a host[31].

To succeed as a parasite, *Legionella* resorts to the ability to differentiate into two highly specialized forms, depending on its current needs: the replicative and transmissive forms. The transmissive form of *Legionella* is present in the planktonic phase and biofilms, where it can invade hosts/be phagocyted. When this occurs, it halts the evolution of phagosomes by blocking their fusion with lysosomes, after which it establishes inside them conditions ideal for replication[32]. In these inert phagosomes, the conditions are met for legionellae to begin repressing transmissive traits and expressing replicative ones, and so legionellae adopt the replicative form. Eventually, as the number of legionellae grows and a deterioration of conditions within the replicative compartment occurs (such as nutrient depletion), the legionellae begin to shift back to the transmissive form. When the host cell suffers lysis, transmissive legionellae are released, and the cycle begins anew[33,34]. Figure 4 contains a simplified overview of *Legionella*'s lifecycle.

The nature of *Legionella* as a parasite of ameba and protozoa is particularly important, since their strategy for infection of their conventional hosts is a functional strategy against certain human cells, to the point *Legionella* can function as an accidental parasite[35,36] in humans. But how does infection by *Legionella* occur and manifest?

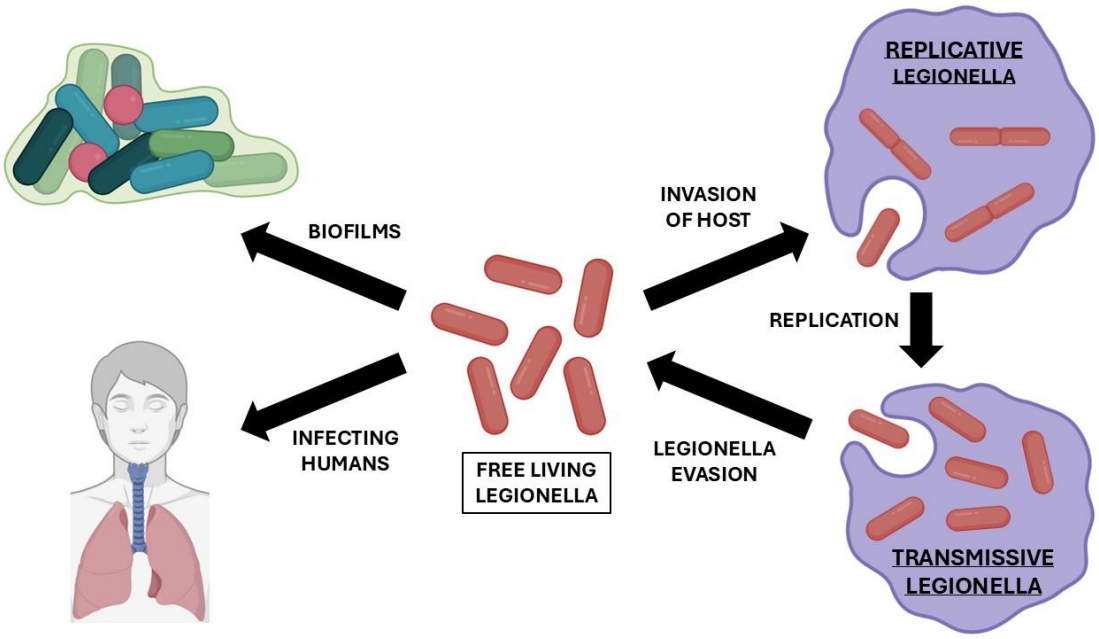

Figure 4. The lifecycle of *Legionella*. Figure produced with BioRender.

1.6. Legionellosis

Legionellosis is the generic term utilized to describe infections by bacteria of the genus *Legionella*. As previously mentioned, *Legionella* is considered an opportunistic parasite in humans, as its evolution as a parasite of amoebas and protozoans inadvertently resulted in it being capable of infection human phagocytes such as macrophages. In fact, the behavior of *Legionella* is remarkably similar between human and its normal hosts, but in particular for alveolar macrophages and respiratory epithelial cells[35,36]. Human infection is in a way a dead end, as the bacteria cannot propagate from infected humans to elsewhere. Human to human infection is exceedingly uncommon[37,38]. Legionellosis can range from a mild, febrile illness (Pontiac fever) to a rapid, severe and potentially fatal pneumonia (Legionnaires' disease). Less common but also possible are extrapulmonary syndromes that occur when legionellae spread from the respiratory system to the body or enter it through another pathway[39]. Table 2, sourced from the World Health Organization (WHO) summarizes the main characteristics of the 2 most common manifestations of *Legionella* infection.

Table 2. The symptoms of *Legionella* infection. Table sourced from a WHO report [39].

Characteristic	Legionnaire's disease	Pontiac fever
Incubation period	2-10 days, rarely up to 20 days	5 hours – 3 days (most commonly 24-48 hours)
Duration	Weeks	2-5 days
Cause-fatality rate	Variable depending on susceptibility; in hospital patients, can reach up to 40-80%	No deaths
Attack rate	0.1-5% of the general population 0.4-14% in hospitals	Up to 95%
Symptoms	• Often non-specific • Loss of strength (asthenia) • High fever • Headaches • Nonproductive, dry cough • Sometimes expectoration blood-streaked • Chills • Muscle pain • Difficulty in breathing, chest pain • Diarrhea (25-50% of cases) • Vomiting, nausea (10-30% of cases) • Central nervous system manifestations, such as confusion and delirium (50% of cases) • Renal failure • Hyponatraemia (serum sodium <131 mmol/litre • Lactate dehydrogenase levels >700 units/ml • Failure to respond to beta-lactam antibiotics or aminoglycosides • Gram stain of respiratory specimens with numerous neutrophils and no visible organisms	• Influenza-like illness (moderate to severe influenza) • Loss of strength (asthenia), tiredness • High fever and chills • Muscle pain (myalgia) • Headache • Joint pain (arthralgia) • Diarrhea • Nausea, vomiting (in a small proportion of people) • Difficulty breathing (dyspnea) and dry cough

The typical pathway for *Legionella* infection is through the inhalation of contaminated aerosols, generated from contaminated waters or potting mixes/compost (as previously mentioned for *L. longbeachae*). This allows legionellae to reach the lungs, where they bypass the host's defense mechanisms and parasitize the local phagocyte population. Long-term smoking, chronic lung disease, organ transplants, age over 50, diabetes, chronic respiratory or heart disease, weakened immune system (glucocorticoid treatment, AIDS, etc.), lung cancer and heavy consumption of alcoholic beverages, kidney failure, among others are all risk factors for *Legionella* infection[40–42].

Regarding the diagnosis of *Legionella* infection, culture of the bacteria from samples such as alveolar lavages, sputum, blood, lung biopsies, etc. is one possibility[43]. Direct fluorescent antibody staining, serologic diagnosis, urine antigen detection and detection of nucleic acids are all available as alternatives[19,28]. It should be noted that there is no vaccine available for any species of *Legionella*. Regarding treatments, Pontiac fever requires none, as it tends to resolve itself spontaneously without the need for medical attention or antibiotics. Legionnaires' disease, on the other hand, is a much more severe condition and so antibiotic-based treatment should be started as soon as possible. However, *Legionella* does not respond to the standard set of antibiotics used against pneumonia. This only makes it more important that *Legionella* outbreaks and infection cases be identified rapidly so that all victims can be administered the appropriate treatment as quickly as possible.

It should be noted that many cases of *Legionella*-caused pneumonia are not identified as such, and so the disease is likely severely underreported. Many such cases are treated as pneumonia with an unknown cause and are resolved before a more specific diagnosis is reached. Furthermore, in 2021, only 11% of cases were culture-confirmed, likely leading to an underestimation of cases caused by non-*L. pneumophila* species, as non-culture-based tests are more geared towards that specific species[44,45].

1.7. Legionnaires' disease: European & Portuguese perspective

Legionnaires' disease was first reported in Portugal in 1979 and has belonged to the transmissible disease Obligatory Declaration List (Declaração de Doença Obrigatória) since 1999 as specified in Portaria n. º 1071/98, from the 31st of December[46,47]. The notification of the communal network has also been required since 1999, as specified in the Decision No 2119/98/CE of the 24th of December in 1998[48]. Up until 2010, Legionnaires' disease data at the European level were managed by EWGLI (European Working Group for Legionella Infections), but that responsibility has been transferred to ELDSNet (European Legionnaires' Disease Surveillance Network), which belongs to the ECDC (European Center for Disease Prevention and Control). In 2021's annual Epidemiological Report on Legionnaires' disease by the ELDSNet, the highest ever rate of notification of Legionnaires' disease cases was verified, at 2.4 cases per 100 000 people. The majority of cases are thought to have been community acquired. 19 outbreaks were reported across the EEA (European Economic Area) by its members[44]. Chart 1 displays the distribution of cases of Legionnaires' disease in the EU/EEA from 2017 up to 2021.

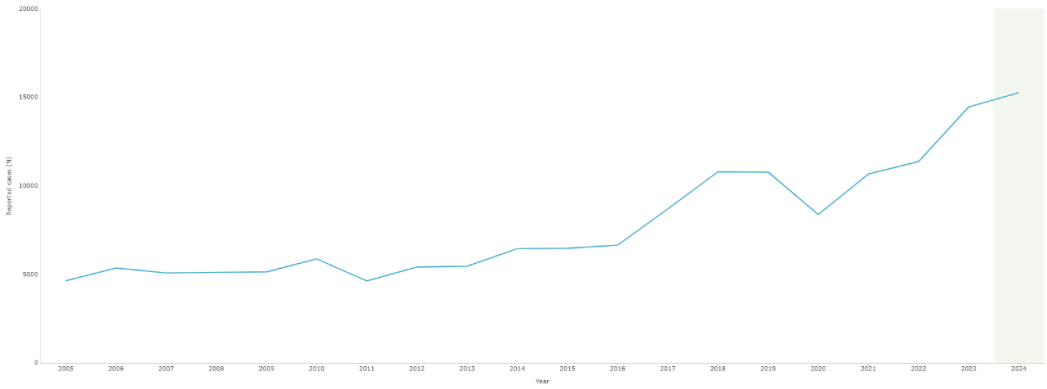

Chart 1. Reported cases of Legionnaires' disease in the EU from 2005 to 2024. Sourced from the ECDC[46].

Regarding Portugal in specific, in 2004 the National Program for the National Integrated Epidemiological Surveillance of Legionnaires' Disease (Programa Nacional de Vigilância Epidemiológica Integrada da Doença dos Legionários) was established, with the stated goal of reinforcing the epidemiological monitoring of Legionnaires' Disease through the following actions: increase in diagnosis and notification; lab diagnosis optimization; increase the number of strains isolated from clinical cases of *Legionella* infection; improvement of the epidemiological investigation of cases, promotion of the environmental study and isolation of environmental strains following disease cases; environmental strand isolation promotion; promotion of the molecular characterization of isolated strains of *Legionella*, of environmental origin; comparison of the typification of the clinical strains with those of environmental origin. Despite the best efforts of the General Directorate for Health (Direção Geral de Saúde) and its partners in fighting Legionnaires' disease, multiple outbreaks have occurred since the 2000s. Chart 2 displays the number of reported Legionnaires' disease cases in Portugal, from 2006 to 2024.

Chart 2. Reported cases of Legionnaires' disease in Portugal from 2006 to 2024. Sourced from the ECDC[46].

Of particular note is the outbreak in Vila Franca de Xira, in November of 2014, responsible for the spike visible in 2014 on chart 2. At the time, it was the 3rd largest outbreak of Legionnaires' disease ever recorded worldwide (it remains the largest ever recorded in Portugal), with a total of 403 cases, 377 confirmed and 26 deemed likely. A total of 14 deaths occurred among these cases. *L. pneumophila* serogroup 1 was the pathogen involved in this outbreak with its source being a wet cooling system from a local factory[49]. In 2017, at the Hospital São Francisco Xavier in Lisboa, an outbreak occurred resulting in 61 infections, 22 with severe sequels and a total of 10 deaths. This outbreak was determined to be caused by inadequate maintenance of the hospital's cooling system[50]. Since then, smaller outbreaks have been registered in, for example, Vila do Conde e Póvoa do Varzim[51]. 2024 was also the year with the highest ever number of registered cases since 2014, continuing a rising trend, as can be seen in charts 1 and 2.

1.8. *Legionella* Prevention & Control

Although it is considered clean, the water distributed by companies like AEdP all over the world is not sterile. Given the previously mentioned capability of *Legionella* to infiltrate, survive and even thrive in man-made water systems, constant efforts must be made to minimize the risk that *Legionella* transmission through the various water systems that humans employ poses.

To this end, smart design of water systems is key. Design choices that minimize the occurrence of conditions that favor *Legionella* growth should be prioritized (i.e. minimize stagnant waters, unnecessary water storage, etc.). After a system is built, it should be adequately monitored and maintained.

One way to achieve adequate monitoring and maintenance is water safety plans, the strategy recommended by the WHO[39]. The development of such plans can be divided into 3 main phases: system assessment, in which the system in focus is examined and risks/hazards are identified and characterized. Monitoring, where control measures are identified and monitored, followed by the validation of the water safety plan. Finally, in the management and communication phase, programs that indirectly support the risk management effort are developed, protocols for the management of the water system are established and procedures for communication and documentation involving the water system are established. When designing such systems, these methods should be considered to make the systems as receptive as possible to their deployment. The methods used in the prevention of *Legionella* proliferation and elimination range from

maintaining water temperatures that discourage microorganism growth to disinfection with various substances, UV sterilization, filtration, etc. A table, sourced from a WHO report, on the most common methods for the prevention and elimination of *Legionella* in water systems, is provided under Attachment 3[39].

1.9. *Legionella* Analysis Methods

Currently, there are multiple methods available for the detection and quantification of *Legionella*. They can be split into 3 categories: culture methods, associated with ISO 11731; molecular biology methods, associated with ISO 12869; and enzymatic methods. We will now elaborate on all these methods to make their advantages and disadvantages clear, and why the AEdP analysis lab has decided to implement a molecular biology method on top of the culture method currently the last stage of accreditation.

1.9.1. Culture-based Methods & ISO 11731

The culture of bacteria from samples followed by specific tests to identify relevant species and/or strains is the classical method for the detection of bacteria. ISO 11731 defines a culture-based *Legionella* enumeration approach for water samples. Although the approach defined in ISO 11731 has proven reliable and can be paired with serologic tests for the differentiation of *Legionella* spp. and *L. pneumophila* serogroups, a major downside is the time investment required. Legionellae require multiple days in order to achieve proper levels of growth, and given that the method defined by ISO 11731 requires two separate rounds of growth on solid media, the culture method takes quite a lot of time to output results that, if obtained sooner, could be much more helpful in preventing or minimizing the effects of a potential outbreak. The lack of a true early warning with this method means that when detection finally occurs, an outbreak has had time to worsen considerably. Another downside of the culture method is its inability to detect species of *Legionella* that do not grow well in the media utilized for this purpose, which might include species of *Legionella* that could be pathogenic to some degree.

1.9.2. Molecular Biology Methods & ISO 12869

A reaction from the domain of molecular biology, PCR allows for the exponential enzyme-catalysed amplification of DNA, from minimal amounts of a DNA template, in a very precise manner. PCR utilizes natural and/or modified thermal-resistant DNA polymerases, the enzymes responsible for DNA synthesis in nature, to achieve this. The reagents required for a PCR are, other than the enzyme itself: the DNA sequence to be amplified (a template); primers (short single strand DNA sequences that bind to the sequence to be replicated and serve as a starting point for replication);

deoxyribonucleotides (dNTPs, the building blocks of DNA) and Mg^{+2}, an important cofactor of DNA polymerases. In PCR, a cycle of 3 steps, each performed at very specific temperatures maintained by specialized equipment (a thermal cycler) enable the duplication of DNA in each cycle, therefore an exponential growth rate over multiple cycles. These steps of PCR are: denaturation, the opening of the DNA double helix; annealing, the binding of primers to DNA; and extension, where DNA duplication synthesis occurs).

A type of probes, single-strand DNA fragments labelled with a fluorophore reporter and fluorophore quencher, can be used to improve the utility of PCR. These DNA fragments only fluoresce when bound to their complementary strand, as otherwise the quencher reduces the signal produced by the reporter. Fluorescence in the bound state can be exploited to detect & quantify DNA in PCRs, assuming it behaves in a directly proportional manner to the amount of DNA. Figure 5 illustrates this concept. As replication cycles occur, fluorescence gradually increases as more probes bind to the DNA being synthesized. Eventually, after enough cycles, fluorescence exceeds a fluorescence baseline which is defined in a way that attempts to eliminate all background noise. This baseline is of great importance because of its role in threshold cycle (C_t), which can be defined as the number of PCR cycles (denaturation and amplification) required to replicate the initial target DNA copies, so that the DNA concentration meets the requirements for exceeding the fluorescence baseline for a given fluorescence channel. C_t is the actual metric/result we get from a qPCR experiment.

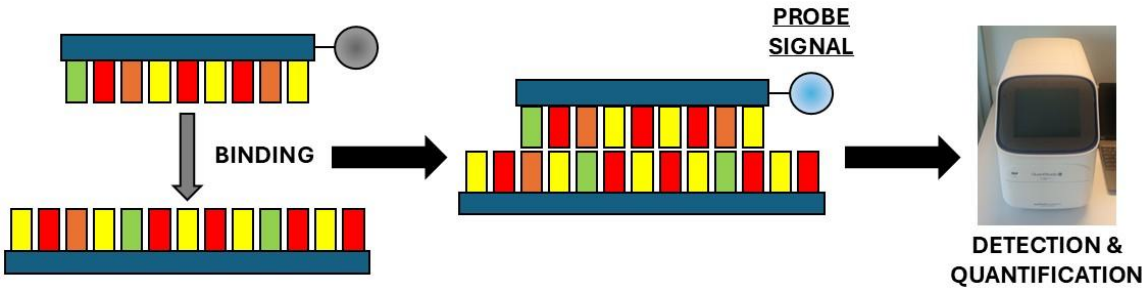

Figure 5. The utility of fluorescent DNA probes in PCR for real time DNA detection.

With all of this in mind, quantitative PCR (qPCR), can be described as the amplification of specific DNA fragments, to which bind highly specific fluorescent probe/s, allowing real time monitoring with specialized equipment, followed by DNA quantification by employing a calibration curve. The intensity of fluorescence is proportional to the amount of DNA and so, by preparing a set of standards with known DNA concentrations and determining their C_t alongside other samples, an equation that relates the C_t of the

standards to the initial the quantity of DNA can be obtained, making it possible to calculate the initial quantity of DNA in samples from their C_t. Figure 6 summarizes the workflow for the detection of bacteria in water through qPCR. It begins with sample collection, followed by its enrichment/concentration in the targeted species, the extraction of the DNA and finally the qPCR itself.

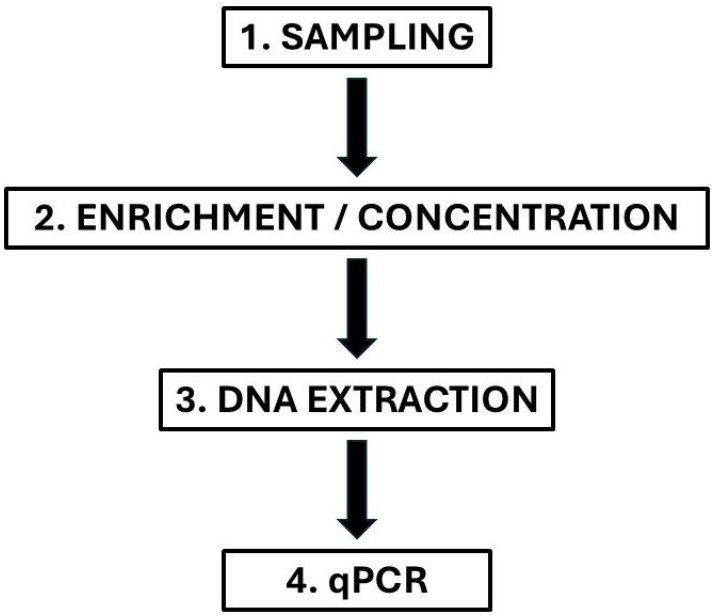

Figure 6. Generic workflow for the detection and/or quantification of bacteria in water through qPCR.

In the case of the *Legionella* qPCR detection and quantification protocol defined in ISO 12869, instead of working with DNA mass, the unit utilized is genomic units (GUs), as in complete bacterial genomes or DNA fragments that can be equated/related to them in some proportion.

The brevity of the qPCR protocol is a big advantage relative to the culture methods. Results can be obtained much faster, in a matter of hours, rather than days involved in the culture-based protocol. PCR is also much more effective than the culture-based alternatives at detecting non-cultivable *Legionella*. Furthermore, PCR techniques are still evolving in many ways, such as falling costs, improving capabilities and ease of automation. However, the downsides for PCR-based methods are also considerable. The reagents and equipment involved are expensive and staff knowledgeable on the subject is required for the execution of the protocols and interpretation of the results.

Regarding our choice of qPCR protocol in this internship, a multiplex (multiple simultaneous amplification targets, *Legionella* spp. and *L. pneumophila*) kit, manufactured by Bioside, was chosen. A commercial kit was chosen over developing an

in-house protocol for the sake of ease of use and heavily cutting down on the work required for the validation of the protocol, as protocols already validated by third parties involve a simplified version of validation, verification. The concept of validation will be expanded upon later in this report. We settled on a multiplex kit over individual kits for *Legionella* spp. and *L. pneumophila* due to the similar overall price and twice as fast (relatively two having to execute two separate protocols on the same sample) nature of the multiplex kit.

PCR can also be used to complement culture methods in another manner. It can be used to confirm that colonies identified through culture methods are indeed *Legionella* spp. or *L. pneumophila* when other tests such as serological tests are inconclusive.

Note on the utility of detection and quantification of *Legionella* through qPCR in Portugal

Portuguese legislation establishes no limits in GU/volume for *Legionella* spp. or *L. pneumophila*[1]. It also does not establish any correlation between *Legionella* GUs and *Legionella* CFUs (colony forming units). It does, however, specify that any detection of *L. pneumophila* should be treated as a situation of elevated risk[52]. In practical terms, this means that if the execution of the qPCR protocol reveals the presence of any *Legionella*, the culture-based method will have to be performed to obtain results that fit the current legal requirements, but an early warning can be emitted. However, if the PCR detects no *Legionella*, the same laws accept PCR results as valid, and one can avoid having to perform the culture method. This is because a negative PCR result carries with it the certainty of the absence of the target DNA/its presence in very low levels. In turn, this means that *Legionella* cells are, at most, present in very reduced amounts or absent. AEdP's intent at the time of writing is to utilize capability of PCR to detect *Legionella* spp. and *L. pneumophila*, as way to check for their presence before having to commit to the culture method. The quantification feature is currently an extra, with its validation being more of a futureproofing effort, in case Portuguese legislation evolves in a direction that makes it more useful to the company.

1.9.3. Enzymatic Methods

There are other methods for the detection and/or enumeration of *Legionella*, beyond those codified in the 2 previously mentioned ISO norms. Legiolert and Legipid® are two such methods available commercially.

Legiolert can detect all serogroups of *L. pneumophila* at concentrations of 1 cell per 100 ml. This method takes advantage of the consumption by *L. pneumophila* of a certain substrate, which is metabolized into a brown colour indicator that can be then tracked to

detect the presence of *L. pneumophila*. A water sample, diluted or not, is mixed with a nutritive supplement and sealed in a Quanti-Tray bag, a sealable bag 96 many wells. To read the results, positive (brown) wells are tallied up, and by referring to a most probable number (MPN) table (while also considering dilutions), one can quantify the *L. pneumophila* in the initial sample. This method is much faster than typical culture-based (spread plate) methods (such as te one described in ISO 11731). It is a liquid culture test, so isolates can be stored and/or serotyped. The test requires much less hands-on time than traditional culture methods and the results are easy to read. No confirmation steps are required, and literature points to it being both more sensitive and resistant to competing organisms than traditional spread plate methods. It is currently widely used by all kinds of laboratories, and thorough testing of the method is available in literature [53]. The fact Legiolert can only detect *L. pneumophila* is a downside in the current Portuguese legal context, as legislation asks for *Legionella* spp. results as well.

Legipid® is another enzymatic method. It is also quite fast when compared to culture methods, execution being measured in hours rather than days. In Legipid®, the water sample is filtered, with *Legionella* then being removed from the filter, followed by the addition into the resulting sample of antibody covered magnetic micro-bits. These antibodies bind to *Legionella* spp. making it easier to isolate them by capturing the magnetic bits. Then, another antibody for *Legionella* spp. is added. This antibody is fused with an enzyme that allows a colorimetric reaction to occur and so, when the enzyme's substrate is added, the colour resulting from the reaction can be compared with a colour chart to determine the approximate order of magnitude of *Legionella* CFUs present in the sample, or their absence. It is the dual antibody/magnetic nature of this assay that earns it the classification of immunomagnetic capture and enzyme immunoassay[54,55].

1.10. The implementation and validation of Molecular Biology methods

A large component of this internship was the validation of the *Legionella* qPCR protocol implemented during the internship. In that context, I contributed to the establishment at AEdP of Internal Procedures (PIs) for the implementation, validation and quality control of future Molecular Biology methods. I also participated in the design and establishment of PIs for the recording and presentation of results. Validation is a complex process, defined by the competent authorities as the means through which a method/assay is deemed adequate for its intended purpose according to legislation, norms and specific client requirements. Validation, in the context of molecular biology assays, involves the

monitoring of performance characteristics such as the inclusivity and exclusivity of probes and primers, calibration of the quantitative phase of qPCR, evaluation of detection and quantification limits and evaluation of recovery, among others. According to necessary degree of rigor for the validation, and the type of assay, either normalized or internal, this process can be classified as Primary Validation (for internal methods or those equivalent) or Secondary Validation/Verification (for normalized methods).

1.10.1. Primary Validation

Primary validation is applied to non-normalized methods, those classified as internal according to the IPAC DRC005 Guideline[56], in other words, methods developed by the lab itself or that have been modified. In this process, operational limits are established, and the performance characteristics of the method are studied for the moment of its implementation and periodically whenever justified. It is fundamental that this process results in numeric and descriptive specifications of the method's performance. For molecular biology methods (including the detection and quantification of *Legionella* through qPCR that concerns this report), the process of primary validation should reflect the study and resulting conclusions associated with the following performance characteristics, if applicable:

- Primer and probe inclusivity and exclusivity;
- Calibration of the function of the quantitative phase of the PCR;
- Limit of quantification of the qPCR, LQ_{qPCR};
- Limit of detection of the qPCR, LD_{qPCR};
- Recovery;
- Robustness;
- Uncertainty of measurement of the whole method;
- Accuracy:
    - Repeatability;
    - Intralaboratory reproducibility/intermediate precision;
    - Interlaboratory reproducibility/interlaboratory assays;
- Uncertainty surveys.

1.10.2. Secondary Validation/Verification

Secondary validation or verification is applicable to standardized/normalized methods, as defined by ISO and the IPAC DRC005 Guideline. Normalized methods are those that follow the relevant norms specifications regarding not only execution but also performance characteristics. The objective of the verification process is to demonstrate

that a lab possesses the competence to meet the specifications and characteristics associated with the standardized/normalized method as it is implemented, but also periodically whenever justified. This process is simplified relatively to primary validation. The verification process for molecular biology methods is very similar to primary validation, as the only omitted study is that of the inclusivity and exclusivity of probes and primers, accompanied by a reduction in the scale of the validation tests comparatively to Primary Validation.

During this internship it was the verification process which was performed, as AEdP resorted to a commercial kit and its accompanying protocol. The overarching method had already been validated by kit manufacturer according to ISO 12869. The following section will go over various criteria and concepts relevant to the implementation and validation in more detail. Note that although ISO 12869 specifies the inclusivity and exclusivity testing is not necessary for verification, some degree of testing was still done at AEdP to ensure the method's functionality.

1.10.3. Samples

Samples of natural origin with adequate levels of the target-microorganism/s should be utilized in the evaluation of a method's capacity to deal with real world conditions (for qPCR, this occurs during the study of robustness). However, this is not always possible during validation, as natural samples with the desired characteristics are not always available or adequate. And so, the contamination of molecular grade water and/or samples with, for example, bacterial reference material/standards is necessary at times. One such situation is when samples with tightly controlled parameters are necessary. You can also introduce contaminants, both biological and non-biological in nature in a very controlled manner to artificial samples. For example, this allows for a rigorously controlled study of the method's effectiveness when faced with specific interferents.

1.10.4. Accuracy, precision and trueness

The terms accuracy, precision and trueness/veracity are used in this report as they are defined by ISO 5725-1[57]. Accuracy can be defined as the displacement of results from a reference value, due to random as well as systematic effects. Trueness and precision are two descriptors of accuracy. Trueness corresponds to the proximity of the mean of a large set of results to a reference value and can be evaluated through interlaboratory studies. Precision on the other hand, refers to the closeness between test results obtained under a specific set of conditions. The evaluation of precision is done through the study of repeatability and intermediate precision.

1.10.5. Repeatability

Repeatability is a measure of precision, studied under so called repeatability conditions. These include the use of the taking of the same measurement/use of the same procedure, done by the same operator, with the same equipment, under the same conditions, in the same location and repeated in a short period of time. The study of repeatability is a key step in the process of the study of accuracy.

1.10.6. Intralaboratory reproducibility/intermediate precision

Intermediate precision, also known as intralaboratory reproducibility, is essential for the validation of methods as a very robust measure of precision. It involves the collection of data for the latter in a manner that more correctly depicts day-to-day method performance variability. This means collecting method performance data with the variation of a few conditions that will vary in day-to-day operation, such as the operator and time. For example, in one step of validation, data associated with 5 calibration curves is gathered under intermediate precision conditions. This entails splitting the work over at least two days, with 3 operators participating in one day and 2 in another. By collecting data in this manner, very robust data on method performance can be obtained. This practice extends to almost every faced of validation.

1.10.7. Interlaboratory reproducibility/interlaboratory studies

Another powerful tool to study a method characteristics and a laboratory's capacity to execute it is the interlaboratory study, the results of which can be used as a metric for veracity. This type of study consists in the analysis of artificial samples prepared by the study promoter at multiple labs, each following their standard method. This is followed by the mathematical analysis of the pooled results from all the participating laboratories to evaluate the veracity of their results. The main criterion used to evaluate the quality of interlaboratory study results is the *z* score. The z score for a given value is calculated through the division of the difference between the lab result and the assigned value by the standard deviation. A *z* score module (absolute value) lower than or equal to 2 is considered acceptable, one between 2 and 3 is considered questionable and one equal or higher than 3 is considered unacceptable. In this case, the correct determination detection/no detection of *Legionella* spp. and/or *L. pneumophila* will be the main criteria of interest, as our goal is the validation of only detection (qualitative), something that cannot be evaluated with a *z* score. However, quantitative data will shall be sent to the study promoter as acquiring some insights into our proficiency at *Legionella* spp. quantification is still valuable.

1.10.8. Primers & Probes: Inclusivity & Exclusivity

Primers and probes are key components of any PCR method. Their usage must result in the expected results for supposed positive/negative controls for the method to have any utility at all, in other words, primer binding and DNA amplification must, ideally, occur for and only for *Legionella* spp. or *L. pneumophila* DNA, depending on the primer set, and no other species. This is confirmed by running inclusivity and exclusivity tests on a large set of *Legionella* and non-*Legionella* bacterial species, in other words, running the PCR protocol on all of them to check for the expected positive (amplification) or negative (no amplification) result. The list of required bacterial species is provided in Attachment 2. On paper, this step is not to be performed in verification efforts, as it becomes a role of the manufacturer. However, as previously mentioned, AEdP decided to still do some degree of inclusivity and exclusivity testing to ensure the method's functionality.

1.10.9. Verification of the calibration function of the quantitative PCR phase

It is not easy to apply calibration to the whole qPCR method. In this section, only the calibration of the quantitative phase is evaluated. This does not mean that applying the same characterization to the whole method through a similar approach is impossible, only that the norm does not require it. It could be achieved, for example, by utilizing artificially contaminated water. Calibration shall be performed for both the *Legionella* spp. and *L. pneumophila* systems. As previously mentioned, qPCR protocols as defined in ISO 12869 use a calibration curve, estimated through linear regression from measurements taken from executing the protocol on a range of samples with known DNA concentrations, to then calculate the amount of DNA in other samples. After obtaining the curve formula, its quality is analysed through the following criteria:

- Efficiency, which assesses PCR yield, and must fall within the 75% to 125% interval for the system to be validated;
- Linear regression performance, in the form of accuracy of linearity, E_{lin}, which must be smaller than or equal to 0,15 for all levels for the system to be validated.
- The expanded uncertainty of the linearity, U_{lin}, is also calculated.

ISO 12869 demands that at least 5 ranges prepared under intermediate precision conditions shall be evaluated in this step for the verification efforts. This criterion is not applicable to qualitative methods (only detection, no quantification).

1.10.10. Verification of the PCR limit of quantification, LQ_{qPCR}

The LQ_{qPCR} is the lowest acceptable limit of quantification for a qPCR system, typically 25 GU/PCR well due to the sampling distribution (Poisson distribution) over all the tests

performed on a sample. This limit shall correspond to the first level of the calibration range mentioned in the previous section and is considered verified if the lack of accuracy at the limit of quantification, E_{LQ}, is less than or equal to the critical value of 0.15, which was obtained from experimental data. Verification of this limit involves the preparation of 5 samples at the target $\mathrm{LQ}_{\mathrm{qPCR}}$, followed by their quantification and statistical analysis of the results. Since the verification criteria overlap with those of the first level in the calibration curve verification, success in verifying the latter will also verify the LQ_{qPCR}. This criterion is not applicable to qualitative methods.

1.10.11. Verification of the PCR limit of detection, LD_{qPCR}

LD_{qPCR} corresponds to the smallest number of GUs that results in a positive PCR result (detection) in at least 90% of runs (for example, 5 GU/PCR well) for 10 or more samples under intermediate precision conditions. Note that in this report, positive/negative qPCR results are used as synonyms for detection/no detection.

1.10.12. Recovery study

Recovery measures the capacity of the method to “recover” DNA from a sample. Its calculation involves the quantification of DNA in various artificially contaminated samples, prepared from the spiking of DNase/RNase free water with dilutions of a mother suspension of *Legionella*, which is also quantified with the qPCR protocol to obtain a reference value with which to compare the quantification results for the samples. The final decimal logarithm of the average recovery value for all samples must fall between -0,6 and 0,3 for the qPCR system to be verified. For qualitative methods, this step is simplified to the simple confirmation of *Legionella* detection in contaminated samples to some degree (such as 90% of all tested samples, the criterion is left to the lab to choose, within the boundaries of common sense).

1.10.13. Robustness

In this context, robustness is determined through the characterization of the matrix effect on recovery. As in, the effects of the various possible sample matrices on recovery (which should not be substantial). To determine robustness, estimate recovery for each relevant sample matrix (you may track it over time with control charts). To estimate recovery, refer to the previous section’s protocol. The limits of acceptability remain identical. As it is for recovery, for qualitative methods, this step is simplified to the simple confirmation of *Legionella* detection in all contaminated samples.

1.10.14. Measurement uncertainty for the whole method

The measurement uncertainty (overall expanded uncertainty) of the whole method encompasses method accuracy, with the approach in this case being based on the analysis of the recovery values. Bias is estimated from the average recovery value for all the matrices, while precision is estimated from the variance of the recovery, from the values obtained during validation and all the monitoring that follows. Lysis recovery is included in this uncertainty, and so it is possible and even recommended in ISO 12869 that the lysis protocol chosen be compared with others to ensure a lack of bias in this stage. A recovery measurement is obtained from two PCR measurements (global method and direct lysis). So, the uncertainty is overestimated. It is also possible to estimate uncertainty through interlaboratory studies. This criterion is not applicable to qualitative methods.

1.10.15. Uncertainties survey

The calculation of uncertainties is essential for the validation and accreditation of methods. There are two types of uncertainty applicable to this internship, analytical and of measurement. The former consists in uncertainties pertaining to the analysis part of the method, while the latter includes both the analysis and sampling contributions. However, this is not always feasible, such as for qualitative methods. In this case IPAC, the competent authority in Portugal on these matters, dispenses the need for the calculation of uncertainties, but requests that this be replaced with an uncertainties survey. This entails the listing of all possible sources of uncertainty for a given method, and their collection in an organized manner such as a fish spine chart, with the one associated with this internship being available as Chart 3.

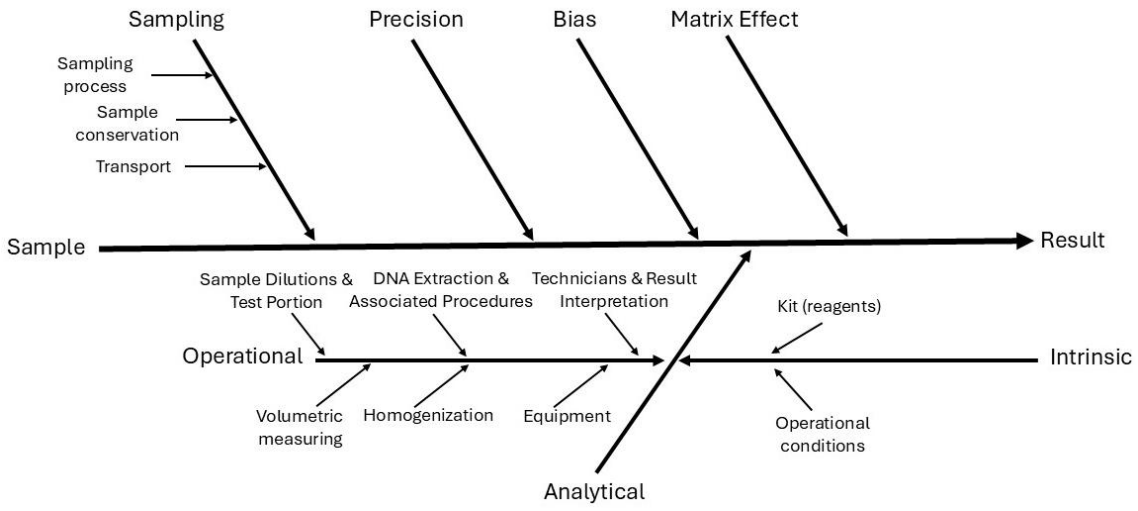

Chart 3. Fish spine chart of all known uncertainty sources for the method employed in this internship.

1.10.16. Operator qualification

Ensuring that all operators can correctly perform a method is essential to ensure the quality and reliability of a lab's results for that method. The way this is achieved is through two sets of criteria: one for the initial evaluation of qualification, and one for qualification maintenance. These criteria shall be set in a manner that ensures the full capacity of the operator to perform the method if they are met. They can also be split into different groups, associated with different parts of a method. If an operator meets the criteria for a specific part of the method, they are allowed to perform it but not the rest.

1.10.17. A brief overview of IPAC accreditation requirements

For assay labs seeking accreditation in Portugal, DIC006[58] is the form that must be filled in and sent to IPAC. This document is built on the back of ISO/IEC 17025, which continues to function as the normative reference for labs seeking accreditation and DRC005, which delineates procedure for lab accreditation. To fill out this form, you must define in detail what type of accreditation are you requesting and what you are accrediting. The three types of accreditation request are: accreditation concession, for a non-accredited lab; extension of accreditation, to add something to the list of methods the lab is accredited to perform; for normative alterations, to maintain accreditation in some situations of norm change/update. In the case of accreditation extension, which is the option applicable to this internship, you must provide the following as attachments to the filled-in DIC006:

- The means through which you will pay for you application;
- A list of personnel with their roles and type of contract;
- Examples of emitted assay bulletins;
- An internal audit report and the resulting plan of correction/corrective measures;
- The method's technical protocols;
- Results of method validation;
- Procedures and calculations of uncertainty estimates, when applicable;
- Results of interlaboratory studies and/or measuring audits, that you have participated in within the last 3 years;
- A list of standards and measuring equipment, calibrating entities and the frequency of calibration;
- When applicable, internal calibration procedures, the list of standards and/or equipment and their traceability, uncertainty calculations and results of interlaboratory comparisons.

Regarding the method to be accredited, first define the product or material being analysed, which, for this internship's purpose, is potable water and other water matrices. Then, define the parameter to be analysed and, when applicable, its measuring range and its uncertainty. Then, define the parameter which is to be analysed and, when applicable, its measuring range and its uncertainty. For AEdP, this parameter would be the detection/no detection of *Legionella* spp. In the next section, indicate the main norm, normative document or normative procedure that describes in detail the assay you are seeking accreditation for. Afterwards, you must list main equipment to be used in that assay. Finally, you must define the category of the place the assay is performed in, for which there are 3 options: 0, for assays performed in permanent lab installations; 1 - assays performed outside of the permanent lab installations or in mobile labs; 2 - assays performed both in and out of the permanent lab installations.

After this request is submitted, the accreditation seeking lab will have to be audited by IPAC and go through a process of correction of all non-conformities relative to all the relevant norms to receive the accreditation status it requested.

1.11. Quality Control

As is defined in ISO 12869, quality control is what ensures the trueness and precision of the measurements carried out by a laboratory. The quality control specific to molecular biology assays has multiple components, such as: multiple controls to evaluate method performance on each run; a set of three standards to ensure PCR trueness; control charts, to monitor the performance of the method over time (regarding multiple features such as PCR efficiency, calibration curve characteristics, etc); and interlaboratory studies. All of these quality control components are crucial in ensuring the quality of the qPCR results. The latter three will now be given an introduction:

1.11.1. qPCR's triple standards of trueness

The trueness of the real-time qPCR measurements is ensured by three different standards:

- A primary standard;
- A working calibration from which to prepare the standards used to build the calibration curve necessary for every run;
- A reference material connected to the primary standard, used in every PCR run.

The primary standard, sourced from a trusted provider, such as the Centre National de Référence des Légionelles in Lyon, France is a very accurately quantified DNA solution,

backed by a certificate. It serves the purpose of ensuring the quality of the working solutions utilized to build the calibration curve. This purpose is achieved through a rigorous comparison of the primary standard and the working calibration solution, which for commercial kits, is a responsibility of the manufacturer. In the context of this internship, the working solutions are not provided to us as solutions but as pre-dosed lyophilised pellets. This introduced unique challenges to verification and quality control which will be explained later in this report. The reference material fulfils a critical role in qPCR. It is an accurately quantified DNA solution backed by a certificate. A sample of it shall be included in all PCR runs, undiluted, allowing us to monitor both the accuracy and stability of the methods quantification capabilities. Primary standards can also fulfil the role of reference material, which was the case in this internship.

1.11.2. Control charts

These charts can be defined as those on which some statistical measure of a series of samples is plotted, in a particular order, to steer the process with respect to that measure. The capacity to monitor method performance, over time, in function of specific characteristics is extremely important for the success of long-term quality control efforts. There are various types of control charts, but for our purposes the most appropriate are Shewhart control charts. This variety of control chart is designed to facilitate the task of differentiating between process performance variation caused by random causes and systematic causes. The former are the expected and unavoidable variations in method performance in its day-to-day use due to causes such as operator variability, environmental condition fluctuations, etc. The latter, however, include all systematic errors that can consistently shift results in one direction. Chart 4 is an example of a Shewhart control chart.

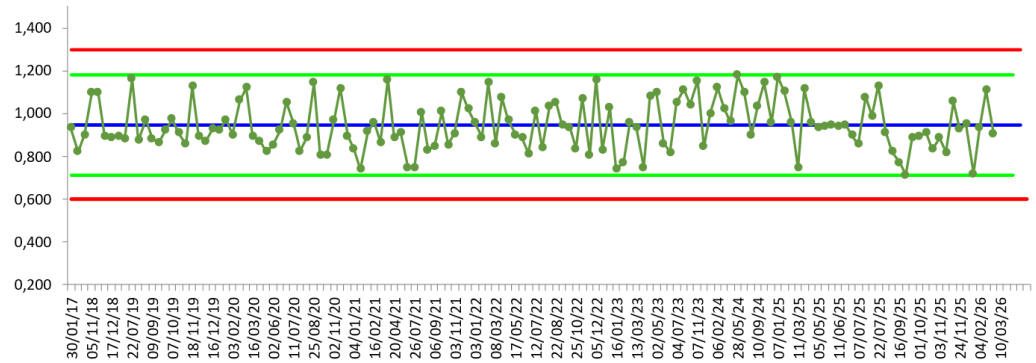

Chart 4. An example of a Shewhart control chart. The centre line is blue, while the upper and lower action/control limits are in red. The upper and lower warning limits are in bright green, while the data points themselves are plotted in a darker shade of green.

Control charts typically plot time in the X axis and the data on the Y axis. Their core elements are:

- The data itself, as individual points each associated with some moment in time;
- The centre line, a continuously updated average of the tracked parameters;
- The upper and lower action/control limits, typically defined by 3 times the tracked parameter's standard deviation. This limit is chosen as the probability of a method which is in control/stable outputting values that fall outside this limit is very low (0,27%).
- The upper and lower warning limits, typically defined by 2 times the tracked parameter's standard deviation. These limits, although not as binding as the previous, are still important, as the chance of a process in control outputting data that lies outside them is still low and, if that occurs, a closer look at the process is recommended.

Another important feature of control charts is how they make it easier to monitor trends in a process's output. For example, 7 sequential data points or more for which a consistent increase/decrease trend is verified are most likely symptomatic of some unwanted underlying trend in the processes' performance.

qPCR characteristics such as PCR efficiency, recovery, among others, are to be tracked using control charts. A more concrete look at the implementation of control charts in this internship will be done under the Methods section.

1.11.3. Equipment maintenance, calibration and performance monitoring

The principles of quality control permeate all activities done in accredited labs. Materials like standards and samples (ex: DNA standards and extracts) and equipment such as thermal blocks, laminar flow hoods and thermal cyclers are all subject to strict quality control and maintenance to ensure they behave within the manufacturing specifications and relevant norm requirements. For thermal cyclers, calibration procedures and acceptable deviations from ideal behaviour are defined in the equipment manuals and ISO 20836[59]. Other equipment, such as centrifuges and thermal blocks, must be calibrated and monitored as well.

DNA material, like standards and sample DNA extracts, shall also be subject to stability studies if they are to be stored. DNA can only be stored and used for as long as a study ensures it remains stable. Although they were not undertaken during this internship, these studies will have to be done to ensure that no significant degradation occurs for

the expected storage intervals. Handling of DNA should also be done in a manner that minimizes its time spent in the hydrated form (rehydration of only parts of a lyophilizate if applicable) and minimizes the number of freezing/unfreezing cycles (aliquoting is a good strategy).

2. Equipment and methods

2.1. Necessary equipment, lab Conditions and good practices

A lab that wishes to validate and become accredited to perform a specific qPCR method must meet various requirements in terms of equipment, lab conditions and good practices by its operators. The one stand-out equipment is, of course, the thermal cycler. qPCR requires an advanced thermal cycler as real-time fluorescence monitoring is a necessary function. Regarding lab conditions, there are various factors, such as an obligatory separation of the phases of the qPCR protocol in terms of space. This implies performing the filtration in one area, the DNA extraction in another and finally an area dedicated to the reaction and reaction products. For this purposes, laminar flow hoods each count as a separate space. This finely defined spatial separation greatly diminishes the risk of contamination between the various steps of the qPCR protocol. Lab coats are also subject to some restrictions, as separate coats must be utilized for each work area.

Sterilization of filtration ramps by flaming, although effective against microorganisms, might not fully destroy all DNA. Disposable cups are a less contamination prone option but result in increased waste production in an industry which already outputs considerable volumes of waste for its scale. For the cleaning of work surfaces too, normal methods might not suffice. Dilute hypochlorite solutions (bleach) can thoroughly eliminate DNA contamination. Alternatively, proprietary formulations for DNA/RNA elimination are available on the market. Whatever choices they end up making, labs should verify that their chosen methods of decontamination are effective.

2.2. Sampling

The sampling process is crucial to ensure the quality of all subsequent steps, including the lab analysis that this report focuses on. And so, to ensure that the analysis results of the qPCR detection and quantification of *Legionella* are valid and representative, all good practices regarding sample collection and handling must be respected. A sample must both be collected in the correct manner and both transported and stored in a way that its integrity, at least pertaining to the elements intended to be analyzed, is wholly preserved.

ISO 12869 indicates that sampling is to be performed according to ISO 19458 requirements. In the case of water meant for human consumption, the sample collection bottle must contain a solution of sodium thiosulfate at 1,8% (m/m), in the proportion of 0.1 ml for each 100 ml of sample, in order to neutralize residual chlorine in the sample (commonly present due to disinfection procedures). Unless specified otherwise, all

sampling bottles mentioned in the following sections are both sterile and contain the thiosulfate levels previously defined. Once collected, samples can be stored at environmental temperature if they are to be processed in the same day. Otherwise, they should be stored at 5 ± 3 ºC (monitoring of the temperature is required). Even with cold storage, samples must still be processed within 48 hours of sampling. The standard volume of sample collected is 1000 ml, but smaller samples (i.e. 500 ml) can be collected if the planned analysis does not require a whole litre. Next, more specific potable water sampling procedures for *Legionella* will be elaborated on.

2.2.1. Water Taps

The technician begins by labelling the sample bottle and disinfecting his hands with 70% ethylic alcohol. Sterilize the tap (i.e. flaming, which is preferred, or treatment with hypochlorite/70% ethylic alcohol). If the goal is to collect the water provided to the tap, open the tap and let it flow for 5 to 10 seconds at max flow, then reduce the flow (this step eliminates influences from the sterilization procedure). Then, open sample bottle with the cap being kept face down to minimize the risk of contamination and collect the sample, taking care to leave around 10% empty space in the bottle to allow for proper homogenization. Immediately after collection, the sample bottle is closed and homogenized through light agitation.

Measurements of sample water temperature and free chlorine levels are taken. If the tap provides both hot and cold water, the sampling procedure should be done for both streams, first hot then cold. To help ensure the integrity of the samples until analysis, they are transported in thermal containers, protecting them from both heat and direct exposure to sunlight.

If the goal is to collect the water that is consumed (worst case scenario), open the tap without previous sterilization and begin the collection of the sample into the bottle, following the previous procedure for the remainder of the process.

2.2.2. Showers (including biofilm collection)

The technician begins by identifying his sample bottle and disinfecting his hands with 70% ethylic alcohol. The showerhead is placed in a sterilized plastic bag, which had one of its corners previously cut with a scissor disinfected with 70% ethylic alcohol. The sample bottle is opened next to the shower (once again keeping the lid facing down to minimize the risk of contamination) and is kept in a titled position while it is filled to around half capacity with the initial water flow. After this initial collection phase is over, the showerhead is dismantled, allowing for the collection of biofilm samples from its internal

structure to be collected with a sterile swab. The swab is introduced to the sample bottle by cutting off the section utilized in the swabbing with a scissor sterilized with 70% ethylic alcohol. The showerhead is reassembled, and the remainder of the flask is filled (10% empty space shall be left), closed and gently homogenized.

Finally, measurements of sample water temperature and free chlorine are taken. The samples shall be stored and carried in thermal containers to protect them from heat and light. If a showerhead can't be disassembled to obtain internal biofilm samples, the full sample is recovered from the initial water flow. This exception to standard procedures must be noted down by the sampling staff in the appropriate digital and physical records.

2.2.3. Circuit water

If the goal is to collect water from the general circuit (i.e. thermal accumulator in the case of a shower), a terminal like a tap or shower should be removed, and water left to flow until the temperature is constant. Afterward, sample collection is performed, and in this way, it should be representative of the water provided to the dependent endpoints. This collection requires the same plastic bag technique utilized in shower sample collection to minimize the formation of aerosols at the uncapped terminal.

2.3. qPCR quantification of *Legionella* – complete method

The qPCR kits utilized in this internship, manufactured by Bioside, were developed and validated according to ISO 12869. The main kit can detect *Legionella* spp. and *L. pneumophila* but also has quantitative capabilities for *Legionella* spp. if a complementary kit is acquired. The complete list of kits we utilized is as follows:

- *qualyfast*® DNA Extraction kit I [Fast DNA Extraction kit from clear water];
- *qualyfast*® free DNA inactivator [Kit for elimination of free DNA contaminating water samples];
- *qualyfast*® Legionella – Quantified Standards [Standards Kit for quantification of Legionella species];
- *qualyfast*® Legionella [qPCR detection Kit of Legionella species and L. pneumophila with Inhibition Amplification Control].

2.3.1. DNA Extraction & Free DNA Inactivation (*qualyfast*® DNA Extraction kit I & *qualyfast*® free DNA inactivator):

The DNA Extraction kit was designed for the fast extraction of DNA from legionellae in clear water samples (low interferent counts). The protocol begins with the concentration of the sample through a filter membrane. Post filtration, this membrane is subject to

treatment with heat, various buffers and physical force (vortex and centrifuging), lysing the legionellae and allowing for the recovery of their DNA in a supernatant. The DNA inactivation kit expands on this process, preceding the lysis step with the digestion of all free DNA. This inactivation avoids the contamination of the qPCR that will follow with DNA from dead and/or unviable cells, which are not relevant for our purposes.

Begin by filtering the water sample through a filter membrane (0.45 µm pores – 47 mm in diameter) provided in the extraction kit in a vacuum-driven filtration ramp. Filter as much of the sample as you can up to 1 litre but, for more corpusculated samples, half a litre is a good stopping point. It should be noted that in the AEdP, the sterility of the filtration ramps is ensured by thorough flaming of the filtration cups with ethanol, both before and after they are utilized. Then, fold and place the filter inside a 1.5 mL tube. To this tube add 2 µL of the DNA enzyme mix, 20 µL of DNA Buffer I, 200 µL of DNA Buffer II and 4 µL of Bioside® Enzyme mix (most reagents provided with the kits, any reagent not provided will be marked as such). Vortex the tube at the maximum available speed for 5 minutes. After this, confirm that the filter is completely immersed in solution and incubate for 10 minutes at 37 ºC, followed by 15 minutes at 65 ºC. Vortex the Bioside® Buffer for 1 minute, then add 200 µL of it to the tube. Follow this up by incubating the tube for 15 minutes at 95 ºC. Centrifuge the tube at 10 000 g for 1 minute at room temperature and then, finally, transfer the resulting supernatant into a clean 1.5 mL tube (leave behind the filter and any pellet/non-clear liquids).

The DNA-carrying supernatant can be stored at -20 ºC but should be immediately used after thawing. It is recommended to dilute the DNA samples 3, 10 or even 100-fold for use in real time PCR. This is to prevent inhibition of the PCR reaction by excessive DNA concentrations and/or PCR inhibitors originating from the sample. For clear waters, a 1:3 dilution is recommended, while for more corpusculated waters a 1:10 dilution might be preferable. A 1:100 dilution can be tried if the 1:10 dilution still displays inhibitory effects.

2.3.2. qPCR detection of *Legionella* spp. and *L. pneumophila* (*qualyfast*® Legionella & *qualyfast*® Legionella – Quantified Standards):

Following the preparation of the DNA samples from water in the previous section, proceed with the detection and quantification of *Legionella* spp. and *L. pneumophila* through qPCR. The chosen qPCR kit can detect both *Legionella* spp. and *L. pneumophila* simultaneously, saving time by eliminating the need to perform two separate PCR protocols. The kit provides stripes of PCR tubes with pre-aliquoted PCR mixes in the form of pellets. Also provided are positive control PCR tubes, in which the pellet includes

L. pneumophila DNA. Finally, a DNA free solution is included for the purposes of preparing the positive and negative controls. To prepare the negative control, add 15 µl of DNA free solution to a standard PCR tube from the kit and up-and-down to resuspend the pellet while also homogenizing the resulting solution. To prepare the positive control, do the same but on a positive control PCR tube instead. Finally, to prepare samples for a run, add 15 µl of the sample (diluted to a level of your choice) to the kit's standard PCR reaction tube and homogenize in the same manner as previously indicated. The thermal cycler protocol indicated by the kit is available in table 3.

Table 3. The thermal cycler protocol provided with the *qualyfast*® Legionella kit.

Step	Temperature	Duration	Nº Cycles
Initial denaturation	95.0 ºC	10 min	1x
Denaturation	95.0 ºC	15 s	50x
Annealing & Extension	60.0 ºC	30 s	

This protocol has the capacity for the simultaneous amplification of an Internal Amplification Control (IAC) alongside the other targets, a feature useful for highlighting possible inhibition effects present in the DNA samples. The functionality of this control will be thoroughly explained later in this report. The kit is rapid, sensitive and highly specific regarding its targets. The IAC, *Legionella* spp. probe and *L. pneumophila* probe correspond to different fluorescence channels, VIC/HEX, FAM and CY5 respectively. Knowing this, a basic table for the resolution of PCR results is as follows:

Table 4. Guide for resolution of qualitative (detection) results for the qPCR protocol. Provided by Bioside.

Target FAM ***Legionella* spp.**	**Target CY5** ***L. pneumophila***	**Target HEX** **IAC**	**RESULT**
+	+	+ / -	Presence of *L. pneumophila*
+	-	+ / -	Presence of *Legionella* spp.
-	-	+	Absence of *Legionella*
-	-	-	PCR inhibition, inconclusive

While *qualyfast*® Legionella by itself only allows for the detection of *Legionella*, quantitative PCR becomes possible with an additional kit, *qualyfast*® Legionella – Quantified Standards. This kit includes strips of standards, each with 4 tubes containing different levels of *L. pneumophila* DNA (12500, 1250, 250 and 25 GU), all of them prepared from a working solution connected to a primary standard. Do note that these standards do not include the *L. pneumophila* qPCR system or the IAC. To utilize this part

of the kit, prepare the standards by adding 15 µl of DNA free solution to each of them and homogenize them through up-and-down. Run the PCR reaction with these standards, alongside the samples and controls and, in the end, build the calibration curve from the C_t results for the standards so that you can calculate the number of GU/PCR well, followed by the GU/sample volume. Basic resolution of results still follows the guidelines set on Table 4.

2.3.3. Expression of results

For both qualitative and quantitative detection, the results shall be presented in a manner that respects the specifications laid out in ISO 12869. Tables 5 and 6, sourced from this norm, describe the rules for the expression of results for qualitative and quantitative qPCR respectively. In the context of this internship, quantitative detection applies only to *Legionella* spp., while qualitative detection can be used for both *Legionella* spp. and *L. pneumophila*. Note that ISO 12869 suggests that the criteria for the acceptance/rejection of *Legionella* spp. detection in qPCR be that a sample displays a C_t lower than or equal to that of the intersect value for the qPCR calibration curves for the same system. Also important is for all controls to behave as expected. Figures 7 through 10 are 2 displays of appropriate qPCR amplification curve behavior and 2 displays of anomalous qPCR amplification curve behavior, respectively.

Table 5. Expression of qualitative results, as defined in ISO 12869.

Number *N* GU/PCR well	**DNA dilution**	**Reported result** GU/l	**Comment**
N < 1	1	$< \frac{LD_{qPCR}F}{V}$	*Legionella*[a] not detected
	d	$< \frac{LD_{qPCR}dF}{V}$	DNA dilution due to the presence of PCR inhibitors; *Legionella*[a] not detected
N ≥ 1	1	*Legionella*[a] detected	
	d	*Legionella*[a] detected	

Where:

- ***N*** is the average number of GU/PCR well;
- **LD_{qPCR}** is the qPCR limit of detection;
- ***F*** is the conversion factor (No. of GU per well to No. of GU per test portion);
- ***d*** is the DNA dilution factor;
- ***V*** is the volume, in litres, of samples filtered;
- **a** According to the PCR system, specify *Legionella* spp. or *L. pneumophila*;

Table 6. Expression of quantitative results, as defined in ISO 12869.

Number *N* GU/PCR well	**DNA dilution**	**Reported result** GU/l	**Comment**
$N < 1$	1	$< \frac{LD_{qPCR}F}{V}$	*Legionella*[a] not detected
	d	$< \frac{LD_{qPCR}dF}{V}$	DNA dilution due to the presence of PCR inhibitors; *Legionella*[a] not detected
$1 < N < LQ_{qPCR}$	1	$< \frac{LQ_{qPCR}F}{V}$	*Legionella*[a] detected below the limit of quantification
	d	$< \frac{Lq_{qPCR}dF}{V}$	DNA dilution due to the presence of PCR inhibitors; *Legionella*[a] detected below the limit of quantification
$N > LQ_{qPCR}$	1	$\frac{NF}{V}$	*Legionella*[a] quantitatively detected.
	d	$\frac{NdF}{V}$	DNA dilution due to the presence of PCR inhibitors; *Legionella*[a] quantitatively detected
$N > C$	0	$> \frac{CF}{V}$	*Legionella*[a] detected above the limit of quantification[b]
	d	$> \frac{CdF}{V}$	DNA dilution due to the presence of PCR inhibitors; *Legionella*[a] detected above the limit of quantification[b]

Where:

- ***N*** is the average number of GU/PCR well;
- **LD_{qPCR}** is the qPCR limit of detection;
- **LQ_{qPCR}** is the limit of quantification;
- ***C*** is the upper value of the calibration range (12 500 GU);
- ***F*** is the conversion factor (No. of GU per well to No. of GU per test portion);
- ***d*** is the DNA dilution factor;
- ***V*** is the volume, in litres, of samples filtered;
- **a** Specify the target, *Legionella* spp. or *L. pneumophila*;
- **b** In this case, the quantification can be obtained after DNA dilution.

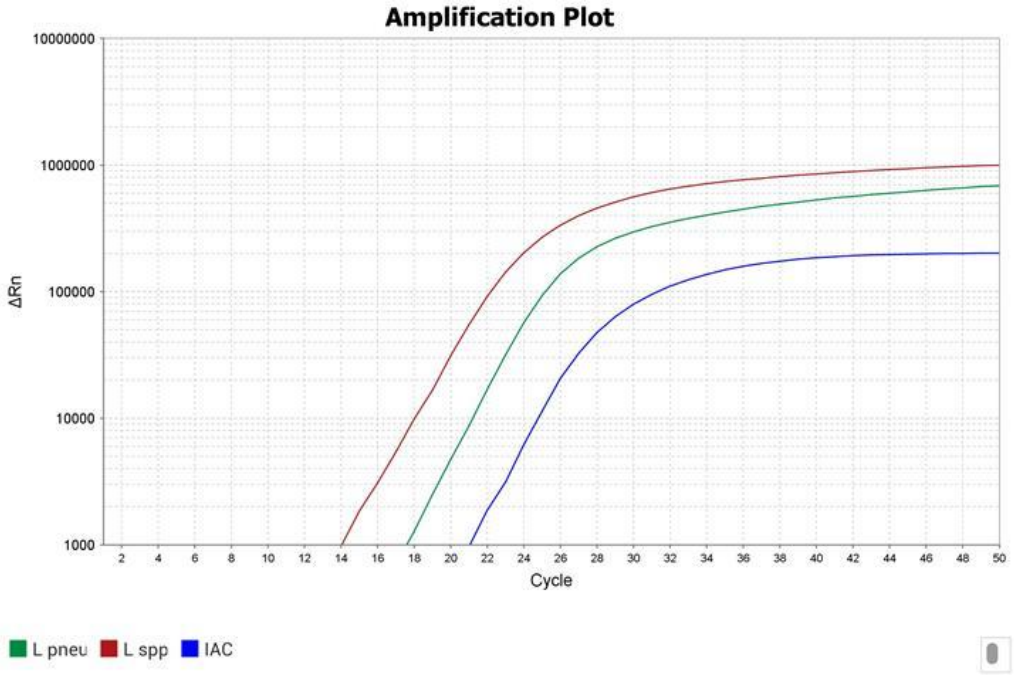

Figure 7. Expected amplification curves for a *L. pneumophila* sample. Both the curves associated with the *Legionella* species (red/green) and the IAC curve (blue) have the ideal sequence of an exponential phase followed by plateauing.

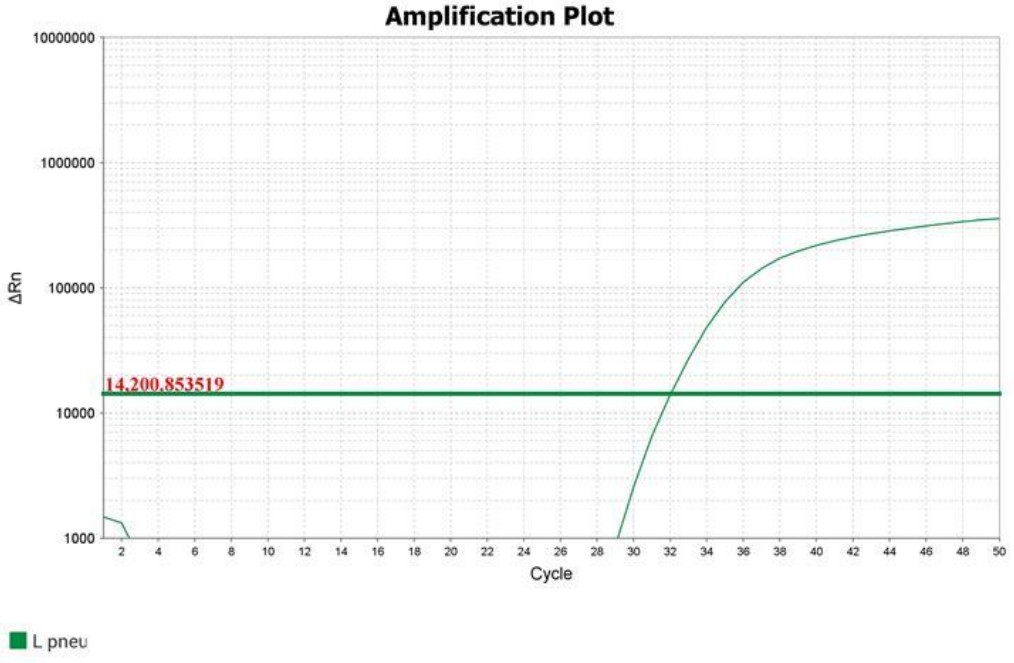

Figure 8. An ideal amplification curve for *L. pneumophila*, singled out. Of note is the clear intersection of the baseline during the exponential amplification phase. This would be considered a positive result for *L. pneumophila*.

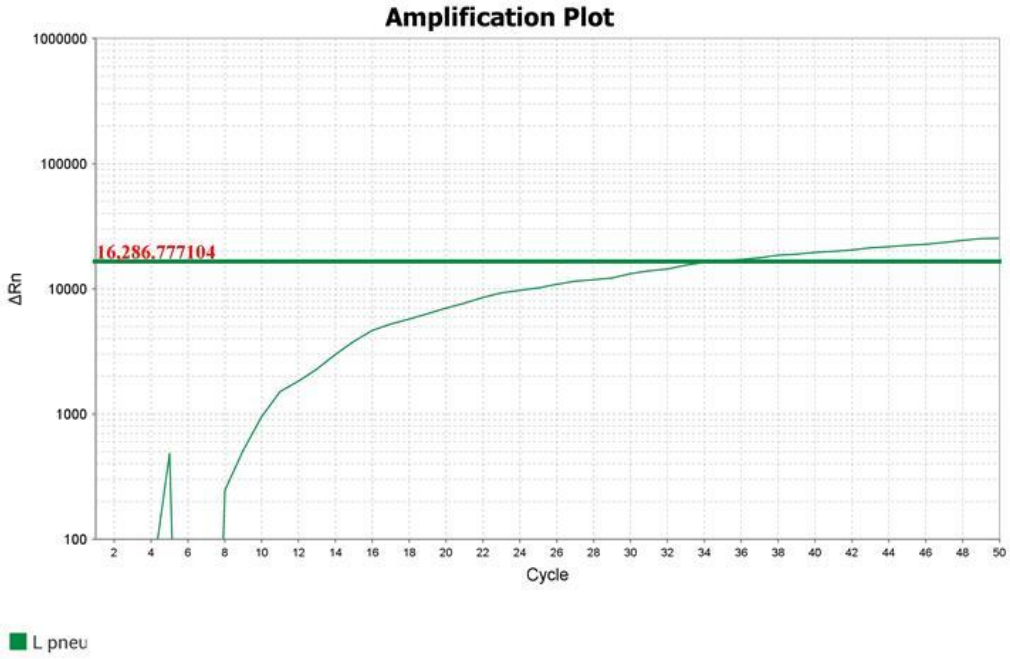

Figure 9. An *L. pneumophila* amplification curve with anomalous behaviour. The curve only surpasses the baseline in the plateau phase and is quite noisy all throughout. Therefore, this would be considered a negative result.

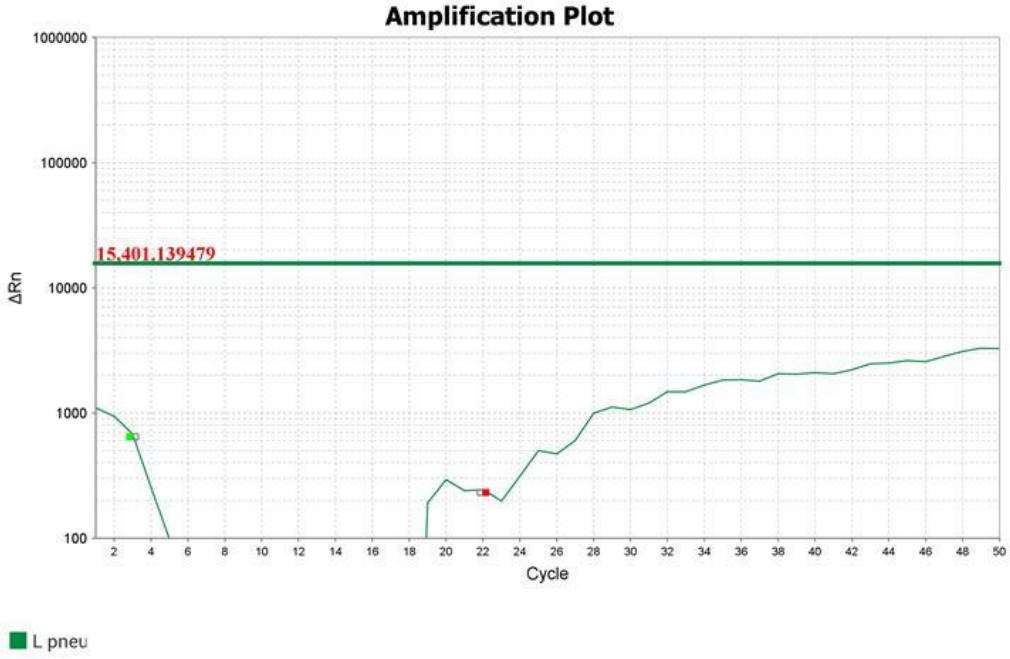

Figure 10. A very anomalous *L. pneumophila* amplification curve. Although it seems some amplification is occurring, it is so noisy and weak that the baseline is not intercepted. Therefore, this would be considered a negative result.

2.4. Implementation and validation of Molecular Biology methods

The following section will go over the diverse methodologies (both in terms of experimental design and statistics treatment of results) involved in the implementation and validation of the qPCR method. Do note that although many steps of the verification of the method quantitative capabilities regarding *Legionella* spp. and qualitative capabilities regarding *L. pneumophila* were undertaken, in the end, only the detection of *Legionella* spp. and *L. pneumophila*. will be accredited. An example of the complete pipeline for the qualitative processing of a sample and results analysis with the *qualyfast*® Legionella kit is available later in this report.

2.4.1. Inclusivity and exclusivity testing

As previously mentioned, although ISO 12869 norm specifies that inclusivity and exclusivity testing can be omitted in verification, AEdP decided to perform some degree of inclusivity/exclusivity testing, for at least 5 different bacterial species. This testing can be done by extracting DNA from bacterial suspensions of the different species, followed by running the qPCR protocol on the DNA extracts to verify that the results are as expected (be that negative or positive).

Practical implementation

On a 1st attempt, 5 different bacterial reference material in sterile water were prepared, with the chosen species being: *L. anisa* (WDCM 00106), *L. pneumophila* (WDCM 00107), *S. aureus* (WDCM 00034), *P. aeruginosa* (WDCM 00024) and *E. coli* (WDCM 00024). Although *S. aureus* is not in the species list (Attachment 2) provided in ISO 12869, AEdP believed it important to test its compatibility with the *qualyfast*® Legionella kit due to its frequent appearance in samples. DNA was extracted from 100 µl of each suspension, with the resulting extracts being subject to qPCR.

On a 2nd attempt, DNA was extracted again from the previous non-*Legionella* species and the qPCR repeated for both the previous and the new extracts DNA. Finally, on a 3rd attempt, DNA was extracted once again from *S. aureus* but also *S. epidermidis* (WDCM 00132) and *E. aerogenes* (WDCM 00175) and the extracts subjected to qPCR. *S. epidermidis* is also not included in the ISO 12869 list of species for exclusivity testing but was included as a generic *Staphylococcus* for reasons which will be elaborated on later in this report. Certificates for all the bacterial reference materials are available in the Attachments 6 through 12.

2.4.2. Verification of the calibration function of the quantitative PCR phase

Estimation of the calibration curve

The evaluation of the calibration features shall be performed under intermediate precision. To obtain a calibration curve, begin by preparing a range of p levels of *L. pneumophila* GUs per PCR well, with p being equal to or higher than 4. The first point in the concentration range shall correspond to the limit of quantification ($\mathrm{LQ_{qPCR}}$). Quantify the whole range through qPCR. k repetitions must be performed, with k being equal to or higher than 5. Record and analyse the resulting values as indicated in Table 7. Perform this step of validation under intermediate precision conditions (different ranges on different days and/or by different operators).

Table 7. Initial calculations required for the evaluation of the calibration function.

Nível x_i	x_1 = LQ_{qPCR}	x_2 = 10 LQ_{qPCR}	x_3 = 100 LQ_{qPCR}	x_4 = 1 000 LQ_{qPCR}	x_p	Totals
$x_i' = \log_{10}(x_i)$	x_1'	x_2'	x_3'	x_4'	x_p'	
$y_{i,j}$ (k repetições)	$y_{1,1}$	$y_{2,1}$	$y_{3,1}$	$y_{4,1}$	$y_{p,1}$	
	$y_{1,2}$	$y_{2,2}$	$y_{3,2}$	$y_{4,2}$	$y_{p,2}$	
	$y_{1,k}$	$y_{2,k}$	$y_{3,k}$	$y_{4,k}$	$y_{p,k}$	
$T_i = \sum_{j=1}^{k} y_{i,j}$	T_1	T_2	T_3	T_4	T_p	$T_G = \sum_{i=1}^{p} T_i$
$m_i = \frac{T_i}{k}$	m_1	m_2	m_3	m_4	m_p	
$x_i' T_i$	$x_1' T_1$	$x_2' T_2$	$x_3' T_3$	$x_4' T_4$	$x_p' T_p$	$\sum_{i=1}^{p} x_i' T_i$

Where:

x_i is the nº of GUs of *L. pneumophila* per PCR well (the given x_i values merely function as an example);

x_i' is the decimal logarithm of x_i ;

$y_{i,j}$ is the value of C_t for the level i ($i = 1 \ldots p$) and the row j ($j = 1 \ldots k$);

k is the number of repetions per level i ($k \geq 5$);

p is the number of levels ($p \geq 4$).

Then, the total number of measurements, N, is calculated according to the following formula:

$$N = kp$$

With this out of the way, one can begin estimating the regression curve, which shall follow by the following formula:

$$y = \mu_{C_t} = ax' + b$$

Where μ_{C_t} is the average of the C_t values.

Plot the points with coordinates (x'_1, m_1), (x'_p, m_p) to visually verify if these are satisfactorily along the curve, If this result is satisfactory, proceed with the following calculations:

$$\sum_{i=1}^{p} x'_i = k\left(x'_1 + x'_2 + x'_3 + x'_4 + \cdots + x'_p\right)$$

$$\sum_{i=1}^{p} {x'_i}^2 = k\left({x'_1}^2 + {x'_2}^2 + {x'_3}^2 + {x'_4}^2 + \cdots + {x'_p}^2\right)$$

Proceed with the following calculations in order to estimate the slope of the curve, a. The variance of x'_i, V_{x_i} is given by the following formula:

$$V_{x'_i} = \frac{\sum {x'_i}^2 - [(\sum x'_i)^2]/N}{N-1}$$

The covariance of $x'y$, $\sigma_{x'y}$ is given by the formula:

$$\sigma_{x'y} = \frac{\sum x'_i T_i \left(\sum_i^{x'_i} T_G /N\right)}{N-1}$$

The estimation of the slope, a, is given by the following formula:

$$a = \frac{\sigma_{x'y}}{V_{x'_i}}$$

Next, we will proceed with the calculation of the intercept point, b. This is the remaining element required to fully defined the calibration curve's equation. The curve passes through the average point whose coordinates are, on the abscissa given by the formula:

$$\overline{x'} = \frac{\sum x'}{N}$$

And on the ordinate, given by the formula:

$$\overline{y} = \frac{T_G}{N}$$

Consequently, through some reorganization of the curve equation:

$$\overline{y} = a\overline{x'} + b$$

$$b = \overline{y} - a\overline{x'} = \frac{T_G}{N} - a\frac{\sum x'}{N}$$

Analysis of the calibration curve

With the curve equation now available, begin by estimating and analysing the efficiency, e, of the PCR reaction. Efficiency is calculated from the slope, a, through the following formula:

$$e = 100\left(10^{-1/a} - 1\right)$$

Efficiency should fall within the interval of 75% and 125%. Consequently, the value of the slope, a should fall between -4,115 and 2,839. This range is wide enough to encompass all possible qPCR systems on all thermal cyclers. For a given qPCR protocol performed routinely on the same thermal cycler, efficiency is an important quality control parameters that should have acceptance limits defined through statistical analysis/control charts and be thereafter monitored.

The linear regression shall satisfy the following accuracy requirement for each level of the curve (criteria that include bias and precision), with the main criteria being:

$$E_{lin} \leq 0{,}15$$

Where E_{lin} is the accuracy of the linearity expressed as a decimal logarithm.

To calculate this value, proceed with the calculations given in table 8. If $E_{lin\,i} \leq 0{,}15$ for whatever level of i then linearity is verified for the whole domain. If one of the $E_{lin\,i}$ values is above the critical value of 0,15 then the curve cannot be verified altogether. Examination of the bias values and standard deviations can show whether the error is caused by precision issues (s_i' too high) or bias issues ($\overline{x_i'} - x_i'$ too high).

In the case of qualitative PCR validation, the validation of the calibration curves can be omitted.

Table 8. Bias, precision, accuracy and uncertainty of linearity calculations.

Estimated x_i level	x_1	x_2	x_3	x_4	x_p
Theoretical x_i'	x_1'	x_2'	x_3'	x_4'	x_p'
$x_{i,j}'$	$x_{1,1}'$	$x_{2,1}'$	$x_{3,1}'$	$x_{4,1}'$	$x_{p,1}'$
	$x_{1,2}'$	$x_{2,2}'$	$x_{3,2}'$	$x_{4,2}'$	$x_{p,2}'$
	$x_{1,3}'$	$x_{2,3}'$	$x_{3,3}'$	$x_{4,3}'$	$x_{p,3}'$
	$x_{1,4}'$	$x_{2,4}'$	$x_{3,4}'$	$x_{4,4}'$	$x_{p,4}'$
	$x_{1,k}'$	$x_{2,k}'$	$x_{3,k}'$	$x_{4,k}'$	$x_{p,k}'$
$x_i' = \frac{\sum x_{i,j}'}{k}$	$\overline{x_1'}$	$\overline{x_2'}$	$\overline{x_3'}$	$\overline{x_4'}$	$\overline{x_p'}$
Bias, $\overline{x_i'} - x_i'$	$\overline{x_1'} - x_1'$	$\overline{x_2'} - x_2'$	$\overline{x_3'} - x_1'$	$\overline{x_4'} - x_4'$	$\overline{x_p'} - x_p'$
$s_i' = \sqrt{\frac{\sum_{j=1}^{k} {x_{i,j}'}^2 - [(\sum_{k=1}^{k} {x_{i,j}'}^2)^2 / k]}{k-1}}$	s_1'	s_2'	s_3'	s_4'	s_p'
$E_{lin\ i} = \sqrt{{s_i'}^2 + (\overline{x_i'} - x_i')^2}$	$E_{lin\ 1}$	$E_{lin\ 2}$	$E_{lin\ 3}$	$E_{lin\ 4}$	$E_{lin\ p}$
$U_{lin\ i} = E_{lin\ i} t_{k-2}$	$U_{lin\ 1}$	$U_{lin\ 2}$	$U_{lin\ 3}$	$U_{lin\ 4}$	p

Theoretically:

x_i' is the decimal logarithm of x_i;

$x_{i,j}'$ are the values calculated from $y_{i,j}'$ utilizing the standard curve;

$\overline{x_i'}$ is the average of $x_{i,j}'$;

s_i' is the standard deviation of $x_{i,j}'$ with $k-1$ degrees of freedom;

E_{lin} is the accuracy of linearity;

U_{lin} is the expanded uncertainty of linearity;

t_{k-2} is the value given by the Student table for $k-2$ degrees of freedom for a risk of 5% (Attachment 1).

Use of the calibration curve

For each measurement $y = C_t$ of a sample, use the standard curve formula that follows to obtain x' by inverse calibration:

$$x' = \frac{y - b}{a}$$

Where k values of x' are obtained if k measurements of the same sample are taken. Calculate the average of x' ($\overline{x'}$) and the associated standard deviations, according to the formula specified in Table 6. Then, by antilog transformation, express result as x GU/l:

$$x = 10^{\overline{x'}}$$

Important note regarding day-to-day operations

It should be noted that in day-to-day lab work, software tools such as Excel can perform these calculations (ex: linear regression or standard deviation) directly. For this to be permissible however, requires thorough verification of the software's capability to provide results consistently identical to those obtained in the more step-by-step calculations.

Practical implementation

Five calibration ranges were prepared under intermediate precision conditions, one per operator (5 operators), three on the 1st day and two on the 2nd. In this case, preparation was simply utilizing the pre-dosed calibration ranges included in the *qualyfast*® Legionella kit, which only require resuspension of the reagents in DNA-free water to be ready to use. Each calibration range included 25, 250, 1250 and 12500 GU/PCR well reactions. Additionally, operator 5 collected data corresponding to another two calibration ranges on two other days. This was done due to the outlier nature of some of their original calibration range points. The resulting data was then analysed to check for compliance with the validation criteria.

2.4.3. Verification of the PCR limit of quantification, LQ_{qPCR}

To begin, prepare k separate dilutions ($k \geq 10$) at the target LQ_{qPCR} from a solution of *L. pneumophila* DNA derived from a primary standard/a standard connected to it. Then, quantify each solution according to the lab's chosen protocol and under intermediate precision conditions. The targeted LQ_{qPCR} may not be lower than 25 GU/well for single analysis, 15 for duplicates and 10 for triplicates.

After quantifying all samples, proceed with the calculation of standard deviation according to the following formula:

$$s = \sqrt{\frac{\sum_{j=1}^{k} x_i'^{2} - \left[\frac{\left(\sum_{k=1}^{k} x_i'^{2}\right)^{2}}{k}\right]}{k - 1}}$$

Where:

- x_i' is the decimal logarithm of the number of GUs of L. pneumophila, calculated by inverse calibration from the CT values and Ct values and the calibration curve formula;
- k is the number of measuraments.

Then, calculate the bias with the following formula:

$$\overline{x_i'} - \log_{10}(x)$$

Where x is the theoretical value of the targeted LQ_{qPCR}.

Finally, calculate the accuracy at the limit, E_{LQ}:

$$E_{LQ} = \sqrt{s^2 + \left[\overline{x_i'} - \log_{10}(x)\right]^2}$$

Where:

- E_{LQ} is the accuracy at the limit of quantification;
- s is the standard deviation of the x_i' vales obtained from the k measuraments.

If $E_{LQ} \leq 0{,}15$, then the targeted limit of quantification is verified. Otherwise look for the possible causes of this problem (values too low, outliers, etc).

You can also calculate the uncertainty at the limit of quantification, U_{LQ} ,using the following formula:

$$U_{LQ} = E_{LQ} \times t_{tab}$$

Where:

- U_{LQ} is the uncertainty at the limit of quantification;
- t_{tab} is the Student table value (at 5% risk, for $k - 1$ degrees of freedom);

For verification, the number of necessary LQ_{qPCR} data points is reduced to 5, which means that the analysis done for the purposes of calibration function validation satisfies all the criteria for LQ_{qPCR} validation if it is successful. In the case of qualitative PCR validation, the validation of this limit can be omitted.

Practical implementation

Verification of compliance regarding this criterion was done as a part of calibration curve verification because, as previously mentioned, the criteria overlap with those of the previous verification step.

2.4.4. Verification of the PCR limit of detection, LD_{qPCR}

To verify the LD_{qPCR}, prepare over 2 days 10 or more PCR reactions at the targeted LD_{qPCR} from the working solution/primary standard of *L. pneumophila* DNA. Then, run the qPCR reaction and verify that for at least 90% of the solutions, the result is positive.

Practical implementation

20 PCR reactions at the targeted LD_{qPCR}, 5 GU/PCR well, were prepared over 2 days and by 2 operators from an *L. pneumophila* DNA standard connected to the primary standard and then analysed in the same qPCR run. The resulting data was then analysed to check for compliance with the validation criteria.

2.4.5. Study of recovery

Begin by preparing a mother suspension of *L. pneumophila*. This is achieved by resuspending multiple *L. pneumophila* (for example, 5) colonies that are less than 72h old in a tube containing 2 ml of a saline medium (ex: Ringer solution or tryptone salt). It is recommended to measure the optical density of the suspension at 600 nm. An optical density of 0,5 at this wavelength corresponds to approximately 10^9 *L. pneumophila* cells per ml. Before proceeding to the next steps, homogenize the suspension vigorously for 30 seconds. Set aside 3 aliquots of the mother suspension for quantification through direct DNA extraction followed by qPCR.

The next step involves creating spiking suspensions from the mother suspension, which are to be obtained by successive tenfold dilution of the mother suspension in the same salty medium. Then, inoculate samples (100 ml to 1 l) with a minimum volume of 100 µl of the spiking suspensions (designated V_{pe}). This should be done for two different levels of spiking dilutions (ex: 10^{-3} and 10^{-5} dilutions of the mother suspension). The inoculated samples must then go through the whole protocol (filtration, extraction, qPCR).

The quantification of the mother solution and spiked samples shall be carried out in the same day, in the same amplification series.

The formula for calculating recovery is the following:

$$\log_{10}(\eta_x) = B - A + D + \log_{10}\left(\frac{1000}{V_{pe}}\right)$$

Where:

- $\log_{10}(\eta_x)$ is the decimal logarithm of recovery for sample x;
- A is the reference value for the concentration of mother suspension, expressed as a decimal logarithm of the number of GUs/ml;
- V_{pe} is the volume of the spiking suspension, in µl;
- B is the value measured from the spiked sample, expressed as a decimal logarithm of the number of genome units per sample;
- D is the decimal logarithm of the dilution factor between the mother suspension and the spiked suspensions, e.g. D is 3 for a 10^{-3} dilution.

This procedure shall be carried out at least 10 times for each spiked level under intermediate precision conditions. From the resulting dataset, an average recovery and standard deviation shall be calculated.

The decimal logarithm of the average recovery should fall between -0,6 and 0,3. The values obtained during this verification step can be the values utilized to introduce control charts that will then be used to monitor recovery over, therefore ensuring process stability.

In the case of qualitative recovery studies, the results are detection/no detection and calculations are not involved. The acceptance criterion is left to each lab to decide, within the boundaries of common sense, and ours landed on a minimum 90% detection rate (the same criteria used in LD_{qPCR} evaluation).

Practical implementation

We began by preparing a mother suspension of *L. pneumophila* by resuspending 5 colonies in 2 ml of Ringer solution. 3 100 µl aliquots of this mother suspension were set aside. From this initial suspension, 1 ml was taken and diluted in 9 ml of Ringer. This dilution process was repeated 4 more times, resulting in spiking suspensions that are dilutions of growing factors of 10 of the mother suspension (-1 to -5). The -3 and -5

dilutions were each used to spike 10 one litre samples of sterile water (20 total), by taking 100 µl of the spiking suspension and adding it to each of the sterile water samples. The spiked samples were then subject to the entire DNA extraction procedure, alongside the mother suspension aliquots set aside initially. The spiking and DNA extractions were done over multiple days and with the participation of multiple operators to guarantee intermediate precision conditions. In the end, all DNA extracts were quantified in the same PCR run.

After this first study, since the goal of the internship had shifted to the validation of qualitative capabilities, the recovery and study efforts were combined. The second study is discussed in the following section.

2.4.6. Robustness

As previously stated, for qPCR, robustness is determined through the characterization of the matrix effect (i.e. the effects of different types of samples on the process), and so is measured in this context through the effects of the matrix on recovery. The criteria are identical to those for recovery.

Practical implementation

Robustness was evaluated through 2 separate studies. In the first, we performed the quantitative analysis of recovery on 4 different potable water sources, with 2 samples being analysed per source, by 2 operators, over 2 days (a total of 8 samples). This study was done in an identical manner to the 1st recovery study, using the -3 and -5 spiking solutions. The 1st robustness study's samples were analysed in the same qPCR run as the 1st recovery study's samples.

The second robustness study attempt was only qualitative in nature, doubled as a recovery study and involved a total of 8 samples. The sources were water for reutilization (2 samples), potable water (2 samples), sprinklers (1 sample), wells (1 sample) and ornamental fountains (2 samples). The spiking levels chosen for this study were -1 and -2.

2.4.7. Measurement uncertainty of the whole method

To calculate the expanded uncertainty of the whole method, you shall utilize the recovery values obtained in the previous section, with the calculations used available in table 9, is specified in ISO 12869.

Table 9. Calculation of uncertainty from recovery values.

Sample Nº	Matrix	Level tested	Sample recovery
$x = 1 \ldots n$ where n is the total number of samples for all matrices and all levels together	Sterile water	Level 1 (e.g. 1 000 GU) Level 2	η_x
	Hot sanitary water	Level 1 Level 2	
	Air cooling water	Level 1 Level 2	
	Etc.	Level 1 Level 2	
Average recovery ($\overline{\eta}_x$)			$\frac{\sum_{x=1}^{n} n_x}{n}$
Variance (s^2)			$s^2 = \frac{\sum_{x=1}^{n} \eta_x^2 - [(\sum_{x=1}^{n} \eta_x)^2/n]}{n-1}$
Overall expanded uncertainty, $U_{overall}$			$U_{overall} = 2 \times \sqrt{\eta_x^{-2} + s^2}$

Practical implementation

This component of verification was not done due to the unavailability of data. In the first round of recovery/robustness studies, quantitative in nature, only 1 type of matrix was analysed and much of the resulting data was rejected, making the final dataset inappropriate for this type of analysis. The second recovery/robustness study was qualitative in nature and so the results were wholly incompatible with this type of analysis.

2.4.8. Repeatability & Intralaboratory reproducibility

The study of repeatability & intralaboratory reproducibility was performed with data from the LD_{qPCR} validation study, for both the participating operators, as they produced enough data points for this analysis to be done for each of them. For each operator's dataset (repeatability) and the global dataset (intralaboratory reproducibility), the average C_t and its corresponding standard deviation and relative standard deviation were calculated.

2.4.9. Interlaboratory reproducibility

At the time of writing, the laboratory has participated in 1 interlaboratory study for *Legionella* PCR methods. This study was the Legionella PCR Single round – March 2026, promoted by IELAB, in which a total of 5 sample vials were analysed. Vials A1, A2, B1 and B2 contained lyophilized bacteria, while sample C was lyophilized DNA. Vial

A1, A2, B1 and B2 were rehydrated with 4ml of sterile water and left to incubate at room temperature for 10 minutes, while shaken gently every 2 minutes through successive inversions. Then, the rehydrated contents of vials A1 and B1 were each added to its own bottle containing 500 ml of sterile water, with the resulting mix being used to wash the sample tubes to ensure a full transfer of the contents. These contaminated water samples were then subject to the standard concentration and DNA extraction protocol, followed by PCR analysis in triplicate. The results were submitted in GU/500ml an C_t.

Samples A2 and B2 were analysed through direct lysis (no filtration), with an 100 µl of each being subject to the adjusted direct lysis protocol that was previously explained. The resulting DNA was analysed with the standard PCR protocol in triplicate. The results were submitted in GU/4ml and C_t.

To vial C, 500 µl of DNAse-free sterile water were added, followed by 10 mins of incubation at room temperature. This mixture was then homogenized with a micropipette (up-and-down). Then, 3 successive ten-fold dilutions were prepared from the suspension, with each dilution being directly analysed with the standard qPCR protocol in triplicate. The results were submitted to the study organizer in C_t form and as GU/sample volume.

2.5. Quality control methods

2.5.1. Whole method/PCR performance monitoring controls

Positive/negative whole-method controls VS positive/ negative PCR controls

The positive whole method control consists in a required monthly assessment of recovery, as that process was previously described, to monitor its evolution over time. For qualitative methods, this control becomes a simple assessment of detection/no detection for an artificially contaminated sample, once a month. The negative whole method control involves running the complete procedure on a *Legionella*-DNA free sterile water. This control must be carried out for every filtration series. These whole method controls allow for monitoring of contamination in the complete process.

However, a positive and negative control exclusive to the PCR component of the protocol also exist. The positive control is a PCR reaction in which *L. pneumophila* DNA is included. The negative control, also known as the no template control, should be utilized in every PCR run, to verify that there is no DNA contamination during PCR and the preparation of the reaction mixes. The negative control could be used for this purpose, but utilizing the additional no template control prepared specifically for this step allows to detect contaminations originating from the PCR reaction preparation stage, avoiding the

unnecessary investigation of the entire method. A no template control where amplification is detected implies contamination occurred and requires the whole PCR series to be re-evaluated.

Internal amplification control (IAC)

The internal amplification control (IAC), also referred to as inhibition control or internal inhibition control, is essential as the method of assessment of the presence or absence of PCR inhibitors in the DNA samples. This control shall be added to the sample extract and is either the target itself or another plasmid/oligonucleotide. In this internship, this control is included in the pre-prepared PCR reaction mixes provided with the kit.

If general inhibition of PCR occurs in a sample, it will influence the IAC amplification curve, and by comparison with the positive/negative control's IAC curves you can verify that inhibition occurred. For methods previously validated by a third-party, IAC should be evaluated as indicated by the manufacturer. Bioside's criteria, available in this report in table 4, are compliant with ISO 12869 and the more generalist ISO 16099. Beyond that, Bioside also specifies that IAC amplification associated with C_t values greater than 40 shall be treated as a definitive sign of inhibition. In more practical terms, if the IAC C_t of a sample is close to that of the negative control, where no inhibition occurs, then one can conclude that it is unlikely PCR inhibition occurred for that sample.

LD_{qPCR} control

This control consists in the inclusion of a PCR reaction with a GU level corresponding to the LD_{qPCR} in every run. Although not obligatory, it is a recommended control, as it allows for the monitoring of the stability of a method's qualitative capabilities close to their operational limit.

2.5.2. Control Charts

ISO 12869 recommends that various qPCR parameters be tracked over time with control charts to ensure process stability. These are PCR efficiency, recovery/robustness and the value of the reference material. As of the date of submission for this report, not enough data has been gathered regarding these parameters to build proper control charts for them. However, there are another parameter which labs should track although that fact is not explicitly mentioned in ISO 12869, for which enough data was gathered during this internship to build some viable Shewhart control charts. These parameters were fluorescence baselines for used in this internship's chosen qPCR protocol. The LD_{qPCR} control behaviour should also be tracked but not enough data has been collected as of report submission to build a proper control chart.

Regarding the fluorescence baseline for each fluorescence channel, set automatically by the thermal cycler software. A good qPCR requires a baseline high enough to supress all noise but not exceedingly high either. A baseline which is too low might trick the software into registering noise peaks as C_t values, while a baseline which is too high will likely result in distorted C_t values (increased relatively to an ideal quantification). Ideally, data corresponding to 10 baselines for each fluorescence channel should be collected before establishing the 1st control charts. The same logic applies to the IAC C_t control charts. These control charts are available in the "Results and discussion" section of this report.

2.5.3. Equipment calibration, maintenance and performance monitoring

As previously mentioned, thermal cyclers must be rigorously controlled and maintained. ISO 20836 goes over in detail on how the thermal performance testing of these machines must be done. This is a process typically done by companies specializing in these various maintenance matters and accredited by the local competent authorities, in Portugal's case, IPAC. Thermal cyclers with fluorescence monitoring capabilities, like the one used during this internship, must also have this feature calibrated from time to time. This is a highly streamlined process that can be done by the labs themselves, and which was done during this internship. Doing it simply requires buying and preparing (unfreeze, vortex and centrifuge) specific calibration plates for features such as ROI, Uniformity, Background and various fluorescent dyes, loading these into the thermal cycler and following the simple instructions on screen.

Thermal block calibration and monitoring is done on two fronts: one, its calibration by an accredited entity, done for all temperature setpoints that will be utilized; and two, the use of a temperature probe (which must also be calibrated) in conditions that mimic those of the method's incubation vessel, a 1.5 ml tube, to ensure that the incubation timer only begins once the contents of the tube reach the desired temperature. For centrifuges, their rotation speed shall be calibrated, a process also delegated to an accredited entity.

2.6. Qualitative sample processing pipeline and results analysis guidelines

Begin by performing the complete DNA extraction and qPCR protocol on a sample. Alongside the sample, an extraction blank (whole method negative control) shall be processed as well. In the PCR section, both a positive and negative control specific to this phase shall be included. Ideally, include a limit of detection control (PCR reaction loaded with 5 GU) to ensure the stability of the LD_{qPCR}. With the results available, first

ensure that the sample's IAC results (in the VIC channel) are as expected, in other words, verify that the corresponding amplification curve and C_t are similar to those of the controls. Also verify that all controls presented with the expected behaviour.

With this criterion met, you can move on to evaluating the samples amplification curve in the FAM channel, corresponding to *Legionella* spp. If the curve is present, verify that it has the appropriate behaviour and, if so, you can move on C_t-based analysis. Refer to figures 7 through 10 for examples of expected/anomalous amplification curve behaviour. If the C_t is higher than the detection criteria ($C_t \leq 37$) or non-existent, then we declare that the level of GU/litre in the sample is lower than the methods effective limit of detection for a given sample volume, as is described in table 6. This critical value of 37 is an overestimation of the *y*-axis intercept value of the calibration curves collected during the internship, which should correspond to 1 GU/PCR well. This criterion was set in the manner recommended in ISO 12869. Presence of *L. pneumophila* is evaluated in a similar manner through the channel CY5.

If the C_t is lower than or equal to the detection acceptance criteria C_t of 37, then we can declare that *Legionella* spp. is detected and move on to culture methods while putting out an early warning. If IAC analysis indicates inhibition occurred in a sample for which that inhibition might have caused a negative result, dilute the DNA extracts and repeat the PCR to try and counteract the effects of possibly present PCR inhibitors. IAC inhibition in cases where *Legionella* spp. and/or *L. pneumophila* are still amplified do not interfere with the emission of a warning.

3. Results and discussion

3.1. Inclusivity and exclusivity testing

The experimental data obtained in this part of the verification process is available in table 10. As a result of the first study attempt, the method's expected behaviour for *Legionella* spp. and *L. pneumophila* was confirmed. In other words, the *L. anisa* sample tested positive for only *Legionella* spp. while *L. pneumophila* sample tested positive for both *Legionella* spp. and *L. pneumophila*. However, the DNA extracts of *S. aureus* tested positive for *Legionella* spp. while *E. coli* and *P. aeruginosa* tested positive for both *Legionella* spp. and *L. pneumophila*. At the time, we thought this was most likely a result of the contamination of the samples. Despite this, the PCR negative/positive and whole-method controls had the expected results, with no identifiable contamination. At the time, we believed that this behaviour and the difference in the *Legionella* amplification patterns between *S. aureus* and the other 2 unwanted positives (*Legionella* spp. only versus *L. pneumophila* as well) could be explained by a lower sensitivity in the *L. pneumophila* system and/or differing degrees or sources of contamination. Although for *E. coli* and *S. aureus* one of their C_t values was slightly greater than the detection criteria of $C_t \leq 37$, the fact any amplification occurred at all and so close to the limit was problematic.

On the second attempt, new DNA extractions were done for the 3 species with unexpected results. A qPCR was performed for both the 3 old and 3 new DNA extractions. This time, *P. aeruginosa* and *E. coli* tested negative for *Legionella* as expected, with the old extractions still displaying the effects of contamination. The new extraction of *S. aureus* however, surprised us by once again testing positive for *Legionella* spp. This time, all the controls also behaved as expected with no sign of contamination. With this new information, we hypothesized that *S. aureus* or perhaps *Staphylococcus* in general possessed a DNA sequence with some complementarity to the primers of the method we employed. Sadly, we could not investigate this directly as the primer sequence is not disclosed.

In an attempt to resolve this problem, we performed a third study, including *S. aureus* once again but also *S. epidermidis,* to check if the unwanted amplification problem was general to *Staphylococcus*. Another species, *E. aerogenes*, was also tested to get even more exclusivity data. The latter species tested negative as expected, and this time, so did *S. aureus*. However, *S. epidermidis* tested positive for *Legionella* spp. We concluded that the *qualyfast*® Legionella kit *Legionella* spp. primers might have some level of unspecific affinity for *Staphylococcus*. Do note that, as previously mentioned, no

Staphylococcus species are on the obligatory testing list for inclusivity and exclusivity provided by ISO 12869. Further studies must be done to clarify this matter, as it is of great operational importance. These studies shall include testing greater dilutions of the offending species' DNA extracts to check if the unwanted amplification is an effect of elevated DNA levels, and the comparison of culture and qPCR results for samples containing *Staphylococcus* to determine if the unwanted amplification occurs for "natural" levels of *Staphylococcus*. More studies on *Legionella* species will be done as well to better ensure the capacity for *Legionella* spp. detection. These additional studies were not performed during the internship due to a lack of reagents, since the *qualyfast*® Legionella kit was out of stock.

Table 10. Results of the inclusivity and exclusivity testing.

Species	Study Attempt	*Legionella* spp. Amplification	*Legionella* spp. C_t	*L. pneumophila* Amplification	*L. pneumophila* C_t	IAC C_t
L. pneumophila	1	YES	15,572	YES	16,897	23,460
L. anisa	1	YES	19,127	NO		23,748
S. aureus	1	YES	37,918	NO	46,642	22,602
P. aeruginosa	1	YES	34,702	YES	34,825	23,620
E. coli	1	YES	35,590	NO	41,261	24,130
+ Control	1	YES	26,290	YES	22,398	26,037
- Control	1	NO		NO		24,257
S. aureus	1 (REPEAT)	YES	36,084	NO		24,007
P. aeruginosa	1 (REPEAT)	YES	34,017	YES	36,245	23,739
E. coli	1 (REPEAT)	YES	33,466	YES	35,195	23,981
S. aureus	2	YES	37,368	NO		23,903
P. aeruginosa	2	NO		NO		23,675
E. coli	2	NO		NO		23,741
+ Control	2	YES	26,195	YES	24,033	26,037
- Control	2	NO		NO		24,794
S. aureus	3	NO		NO		24,291
E. aerogenes	3	NO		NO		23,965
S. epidermidis	3	YES	33,956	NO		24,266
+ Control	3	YES	25,931	YES	24,141	26,287
- Control	3	NO		NO		23,770

3.2. Verification of the calibration function of the quantitative qPCR phase/verification of the qPCR limit of quantification, LQ_{qPCR}

The experimental data obtained in this part of the verification process is available in table 11. The data from the 5th operator's 3rd attempt was the one used for that operator in the final calculations, as it was the one that met all the verification criteria. By performing a linear regression with the averaged-out data from all the operators, the calibration curve in chart 5 was obtained, with a slope value of approximately -3,590. This falls within the -4,115 and -2,839 interval of acceptability for PCR reaction efficiency. The R^2 of the curve is 0,9989 is also acceptable, as it is greater than the common criteria of 0,995.

Table 11. Experimental C_t results for the purposes of verifying the calibration curve.

Calibration Curve Data	25 GU	250 GU	1250 GU	12500 GU
Operator 1	30,535	27,072	24,470	21,374
Operator 2	30,493	27,505	24,177	21,123
Operator 3	31,068	28,188	25,216	21,219
Operator 4	31,173	27,617	25,140	21,236
Operator 5 - 1	31,000	28,137	25,135	21,825
Operator 5 - 2	32,331	28,384	26,238	22,625
Operator 5 - 3	31,366	27,641	24,940	21,638
Theoretical Log_{10}(GU/PCR well)	1,40	2,40	3,10	4,10

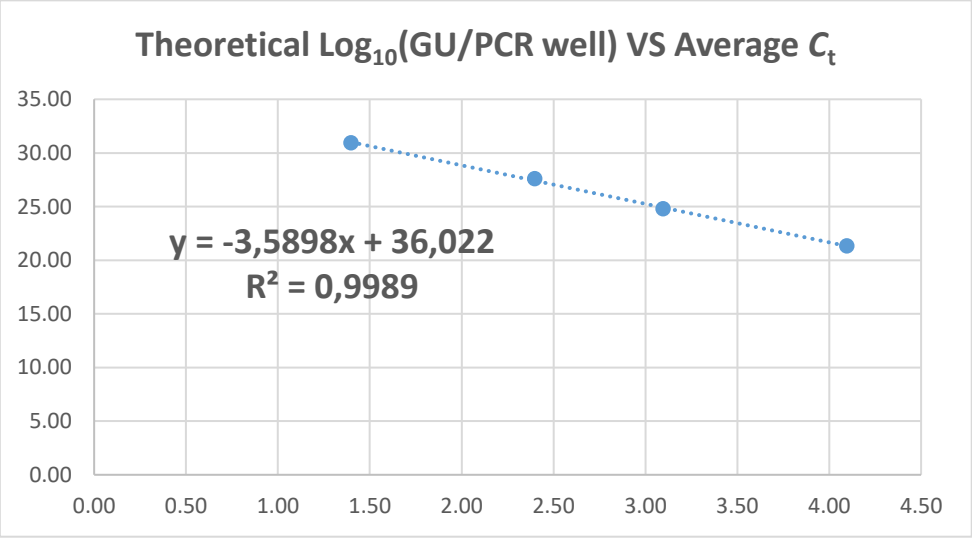

Chart 5. Plot and linear regression of the decimal logarithm of the number of GU per PCR well versus the average C_t result for each level.

Afterwards, statistical analysis of the table 11 dataset was done, but only after its conversion into GU/PCR well values by using the calibration curve. The results of this analysis are available in table 12. For all GU/PCR well levels, the accuracy criteria of $E_{lin} \leq 0,15$ is respected. Therefore, the calibration function has passed all requirements for verification. As previously mentioned, in verification, the criteria for LQ_{qPCR} verification are the same as those of the 1st point of the calibration in calibration function verification, and so the LQ_{qPCR} is now also verified.

Table 12. Results of the statistical analysis of the table 11 dataset, after the conversion of C_t values into the equivalent GU/PCR well values.

Statistical Analysis	**25 GU**	**250 GU**	**1250 GU**	**12500 GU**
Standard Deviation	0,1093	0,1110	0,1249	0,0557
Bias	0,0214	-0,0531	0,0324	-0,0007
E_{lin}	0,1114	0,1231	0,1290	0,0557
U_{lin}	0,3544	0,3916	0,4106	0,1772

The first two attempts at the statistical analysis component of the calibration curve's function verification failed due to some results deviating too far from the theoretical values. A couple more attempts at data collection by operator 5 provided a set of calibration curve data points good enough to meet ISO 12869's criteria. A comparison of the accuracy of linearity and PCR efficiency when using the different operator 5 results can be seen in table 13. Despite being small, the fluctuations of C_t for operator 5's calibration curve data had considerable effects in the accuracy of linearity. PCR efficiency, on the other hand, appears to be less sensitive to these minute variations, most likely due to the linear regression process.

Table 13. Comparison of the calibration function statistical analysis results for the different operator 5 attempts.

	Operator 5 Attempts		
	Attempt 1	**Attempt 2**	**Attempt 3**
PCR Efficiency	91,05	90,38	89,92
$E_{lin\ 25}$	0,0964	0,2085	0,1114
$E_{lin\ 250}$	0,1522	0,1540	0,1231
$E_{lin\ 1250}$	0,1367	0,2237	0,1290
$E_{lin\ 12500}$	0,0784	0,1786	0,0557

3.3. Verification of the qPCR limit of detection, LD_{qPCR}

The data resulting from the LD_{qPCR} verification experiments is available in table 14. The criterion for verification is for detection to occur in at least 90% of samples, for a sample size of at least 10, under intermediate precision conditions. This was met for the *Legionella* spp. system, for which a 100% success rate was verified (20 out of 20 samples). However, for *L. pneumophila*, the success rate was only 85% (17 out of 20 samples). Note that internally (at AEdP) the detection/no detection criterion for this study was not $C_t \leq 37$, as the LD_{qPCR} was evaluated before its definition. However, even if that criterion is used, this data still represents in a 90% success rate for the *Legionella* spp. system, and so verification of the *Legionella* spp. qPCR system endures.

Given the fact that no PCR inhibition was detected through IAC in any of the samples, and other results obtained during this internship, we suspect that the *L. pneumophila* qPCR system might be less sensitive and therefore have a higher effective limit of detection than the *Legionella* spp. qPCR system, which would explain the standalone *Legionella* spp. detection for *L. pneumophila* samples in cases of extreme dilution. Further attempts at the validation of the LD_{qPCR} will have to be done for the *L. pneumophila* qPCR system in order to reach the validation criteria of 90% positive results on the LD_{qPCR} with it. The study repetition was not done during the internship due to a lack of reagents, since the *qualyfast*® Legionella kit was out of stock.

Table 14. Experimental results of the LD_{qPCR} verification study.

Operator	Sample	*Legionella* spp. amplification	*Legionella* spp. C_t	*L. pneumophila* amplification	*L. pneumophila* C_t	IAC C_t
1	1	YES	35,273	YES	36,011	24,382
	2	YES	35,396	YES	42,071	24,227
	3	YES	33,788	YES	36,191	24,099
	4	YES	37,038	NO		24,268
	5	YES	36,658	YES	38,003	23,951
	6	YES	33,927	YES	37,358	24,424
	7	YES	34,627	YES	36,019	24,316
	8	YES	36,082	NO		24,895
	+ Control	YES	25,920	YES	25,920	26,126
	- Control	NO		NO		24,820
2	1	YES	36,487	YES	34,074	23,731
	2	YES	35,100	YES	36,760	24,316
	3	YES	34,509	YES	36,760	24,111
	4	YES	35,316	YES	35,472	24,352

Operator	Sample	*Legionella* spp. amplification	*Legionella* spp. C_t	*L. pneumophila* amplification	*L. pneumophila* C_t	IAC C_t
2	5	YES	34,702	YES	36,809	23,415
	6	YES	33,609	YES	37,824	23,706
	7	YES	33,599	YES	35,199	24,633
	8	YES	36,219	YES	36,566	24,854
	9	YES	37,600	YES	38,239	24,041
	10	YES	36,838	NO		24,862
	11	YES	34,807	YES	37,056	24,556
	12	YES	34,151	YES	37,497	24,540
	+ Control	YES	26,191	YES	24,212	26,317
	- Control	NO		NO		24,886

3.4. Study of recovery & robustness

For the 1st recovery and robustness studies, which were quantitative, all extracted DNA was analysed in the same qPCR run, and the experimental results are available in table 15. Alongside the samples, a set of standards was also analysed, allowing for the linear regression into the calibration curve that can be seen in chart 6. This was the curve used to quantify *Legionella* spp. in the samples for the purposes calculating recovery.

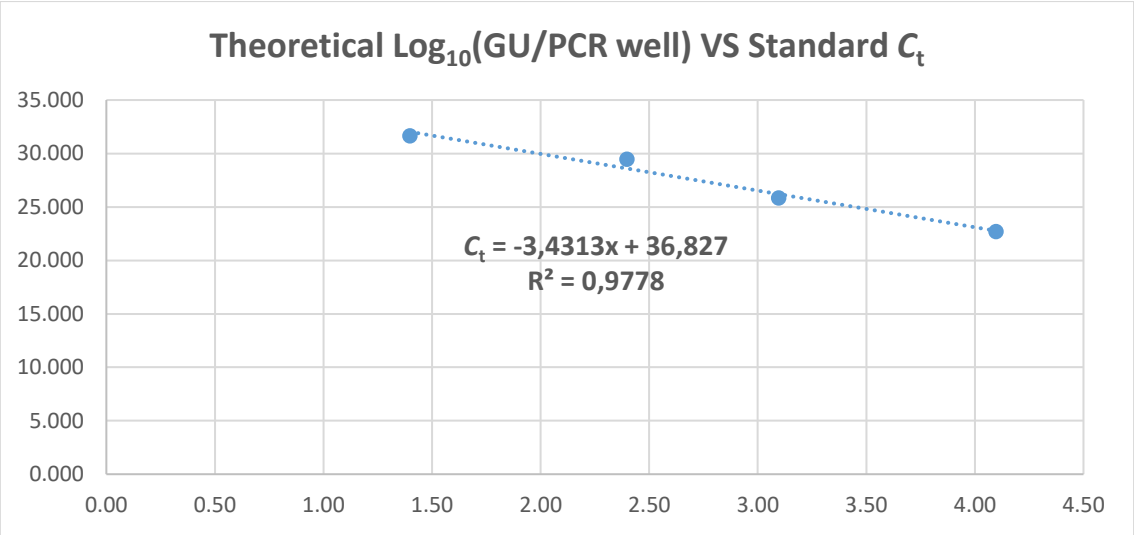

Chart 6. Plot of the calibration equation for the standards included in the PCR reaction batch for the 1st attempt of the recovery and robustness study.

Table 15. Experimental results for the 1st recovery and robustness studies.

Sample Type	Operator	Contamination Level	*Legionella* spp. Amplification	*Legionella* spp. C_t	*L. pneumophila* Amplification	*L. pneumophila* C_t	IAC C_t
MOTHER ALIQUOT 1	1		YES	19,120	YES	21,256	23,675
MOTHER ALIQUOT 2	1		YES	19,136	YES	21,389	23,879
MOTHER ALIQUOT 3	1		YES	19,533	YES	21,746	23,907
Robustness	1	-3	YES	32,305	YES	34,159	23,786
Robustness	1	-5	NO		NO		23,044
Robustness	2	-3	YES	30,722	YES	32,122	23,459
Robustness	2	-5	YES	27,798	YES	29,748	23,405
Recovery	1	-3	YES	30,973	YES	32,362	23,797
Recovery	1	-5	YES	34,345	NO		23,433
Recovery	2	-3	YES	30,404	YES	31,987	23,513
Recovery	2	-5	YES	34,382	NO		23,920
Recovery	3	-3	YES	31,770	YES	32,631	23,855
Recovery	3	-5	YES	36,867	YES	37,292	23,375
Recovery	4	-3	YES	30,632	YES	31,976	23,790
Recovery	4	-5	YES	35,927	YES	36,939	22,040
Recovery	5	-3	YES	30,945	YES	32,072	23,879
Recovery	5	-5	YES	37,835	NO		23,443
Recovery	1	-3	YES	32,797	YES	34,407	23,626
Recovery	1	-5	NO		NO		23,802
Recovery	2	-3	YES	31,625	YES	32,824	23,961
Recovery	2	-5	YES	36,917	NO		24,009
Recovery	3	-3	YES	30,171	YES	31,515	24,025
Recovery	3	-5	YES	38,588	NO		25,466
Recovery	4	-3	NO		NO		
Recovery	4	-5	NO		NO		27,143
Recovery	5	-3	YES	33,861	YES	34,600	24,828
Recovery	5	-5	NO		NO		29,346
Robustness	1	-3	YES	30,458	YES	31,808	23,188
Robustness	1	-5	YES	46,507	YES	37,488	23,627
Robustness	2	-3	YES	29,197	YES	30,640	23,348
Robustness	2	-5	YES	39,212	NO		23,756
25 GU	1		YES	31,630			
250 GU	1		YES	29,454			
1250 GU	1		YES	25,827			
12500 GU	1		YES	22,687			
+ Control	1		YES	26,086	YES	23,683	25,186
- Control	1		NO		NO		24,171
Quantitative Control	1		YES	31,091	YES	32,014	24,679

It is immediately noticeable that a multiple of the -5 contamination level samples failed to display amplification in the case of *Legionella* spp. and even more so for *L. pneumophila*. This means that not only were these samples under the LQ_{qPCR}, but they were also below the LD_{qPCR}. Therefore, this level is easily excluded as a failure. We believe that this was caused by the -5 level at times corresponding to samples frequently diluted beyond the limit of detection. Although, just like for all the other studies, the results were not internally evaluated using the $C_t \leq 37$ criterion, if it is applied then even more -5 level samples would not correspond to detection of *Legionella*. There were also multiple samples with high IAC C_t, and for which reduced or no *Legionella* detection occurred. We believe this inhibition was caused by inhibitors pulled into the DNA extracts through pipetting errors.

The amount of GU in the mother suspension and all the -3 contamination level samples was calculated and from that, decimal logarithm of the individual recoveries and final average recovery were obtained for the -3 contamination level. The results if this analysis are available in tables 16 and 17. No mathematical analysis was done for the robustness data as AEdP decided to only validate the qualitative features of the method before that was done. These initial robustness results were pooled with those of the 2nd study.

Since the acceptability interval for the average of the decimal logarithm of recovery for a given contamination level is -0,6 to 0,3 we can say that the -3 level, with an average decimal logarithm of recovery value of -0,65, does not pass the verification criteria. Therefore, we concluded that attempt at verification of recovery and robustness was not successful on this first attempt for any contamination level.

In parallel to the robustness and recovery studies, a quantitative standard of *L. pneumophila* DNA was included in order to guarantee that the method's quantitative capabilities were functioning properly. Acquired from Bioside, this standard had a concentration of 5 to 9 GU/µl of the *qualyfast*® Legionella kit reference material. It is designed to be used without dilution and so, for a 15 µl reaction, its certificate specifies that for *Legionella* spp. a mean result of 73 GU/PCR, within an interval of 66 to 80 GU/PCR well, is expected (Attachment 4). Given the obtained C_t value and the calibration curve, this standard was calculated to have 46,94 GU/PCR well, a result solidly outside the interval defined in the certificate. Assuming the quality of the standard and its quantification, this result raises some questions on the fidelity of our capacity for quantification of *Legionella* spp. GU with the *qualyfast*® Legionella kit. Further studies with quantification standards will have to be performed to clarify this matter and resolve possible issues with the quantification capabilities of the method.

Table 16. Calculation of the decimal logarithm of the number of GU per millilitre of mother suspension.

Mother Suspension Aliquot	*Legionella* spp. C_t	Average C_t	GU/PCR well	GU/ml	Log_{10}(GU/ml)
1	19,12	19,26	131438	$1,38*10^8$	8,14
2	19,14				
3	19,53				

Table 17. Calculation of the decimal logarithm of recovery for each -3 contamination level sample, followed by the calculation of the average of the decimal logarithm of recovery.

Operator	C_t	Log_{10}(GU/Sample)	Log_{10}(Recovery)
1	30,97	3,64	-0,50
2	30,40	3,80	-0,34
3	31,77	3,40	-0,74
4	30,63	3,74	-0,41
5	30,94	3,64	-0,50
1	32,80	3,10	-1,04
2	31,62	3,45	-0,69
3	30,17	3,87	-0,27
4			
5	33,86	2,79	-1,35
Average Log_{10}(Recovery)			-0,65

On a second attempt, we studied recovery and robustness simultaneously but only from a qualitative perspective, following the same experimental protocol as the previous study but for using -1 and -2 dilutions of the mother suspension instead. The combined results of the first and second recovery studies are available on table 18. The various water matrix samples were processed in different days, always within 24 hours of sampling, accompanied by an extraction blank every time.

In this 2nd study the sprinkler, well and potable water samples all passed the recovery/robustness test for both the *Legionella* spp. and *L. pneumophila* qPCR systems. Although the 1st study's potable water results are subpar, they reinforce confidence in the method's ability to recover even vestigial amounts of *Legionella* DNA as signalled by their high C_t values paired with no inhibition detectable through the IAC.

For the ornamental fountains and water for reutilization samples only the -2 contamination level samples partially passed the test. We believe the lack of *Legionella* amplification was caused by interferents in these samples. The resolution of this problem will involve performing the qPCR on their DNA extracts again, but with a greater dilution. We conclude that, once the ornamental fountain and water for reutilization samples have been retested, enough data will be available to ensure the method's recovery and robustness capabilities (verification of the recovery and robustness of the method is close to completion). The final evaluation of these matrixes was not performed during the internship due to a lack of reagents, as the *qualyfast*® Legionella kit was out of stock.

Table 18. Complete list of results for the 1st and 2nd recovery studies.

Matrix Type	Operator	Spiking Suspension Level	*Legionella spp.* Amplification	*Legionella* spp. C_t	*L. pneumophila* Amplification	*L. pneumophila* C_t	IAC C_t
Potable Water	1	-3	YES	32,305	YES	34,159	23,786
Potable Water	1	-5	NO		NO		23,044
Potable Water	2	-3	YES	30,722	YES	32,122	23,459
Potable Water	2	-5	YES	27,798	YES	29,748	23,405
Potable Water	1	-3	YES	30,458	YES	31,808	23,188
Potable Water	1	-5	YES	46,507	YES	37,488	23,627
Potable Water	2	-3	YES	29,197	YES	30,640	23,348
Potable Water	2	-5	YES	39,212	NO		23,756
1st + Control	1		YES	26,086	YES	23,683	25,186
1st - Control	1		NO		NO		24,171
Potable Water	1	-1	YES	23,499	YES	26,553	23,942
Potable Water	1	-2	YES	26,343	YES	28,843	23,859
Water for Reutilization	1	-1	NO		NO		
Water for Reutilization	1	-2	YES	35,15	NO		
Well	1	-1	YES	21,165	YES	28,501	23,819
Sprinkler	1	-2	YES	30,721	YES	24,163	26,610
Ornamental Fountain	1	-1	NO		NO		
Ornamental Fountain	1	-2	YES	27,27	YES	29,732	23,643
2nd + Control	1		YES	25,931	YES	24,131	26,287
2nd - Control	1		NO		NO		
Extraction Blank 1	1		NO		NO		23,668
Extraction Blank 2	1		NO		NO		23,382
Extraction Blank 3	1		NO		NO		24,092

3.5. Precision: repeatability & intralaboratory reproducibility

Repeatability and intralaboratory reproducibility were studied based on data obtained during the LD_{qPCR} study (table 14). Table 19 contains the results of that analysis. OGC002[60] sets a generic limit of 5% for the variation coefficient/relative standard deviation in the context of precision evaluation. This limit is flexible and can be adapted to method being evaluated, if that adaptation is done in a justified and logical manner. In this internship, we decided to keep the 5% limit, which was successfully respected for both repeatability and intralaboratory reproducibility. Repeatability must be studied in the future for the remainder of the operators that might perform the method.

Table 19. Results of the analysis of repeatability and intralaboratory reproducibility study.

	C_t Mean	**Standard Deviation**	**Relative Standard Deviation (%)**
Operator 1 Repeatability	35,35	1,20	3,40
Operator 2 Repeatability	35,24	1,29	3,65
Intralaboratory Reproducibility	35,29	1,22	3,46

3.6. Interlaboratory reproducibility/interlaboratory study

The interlaboratory study was a success on the qualitative front, but generated uncertainties regarding the quantitative capabilities of the method. For all samples, which were spiked with *L. pneumophila* as indicated by t, both *Legionella* spp. and *L. pneumophila* were correctly detected, as can be seen in Table 20. However, for samples A2 and B2 the *z* score is quite inadequate, as its module is much higher than 3 for both samples. Sample C was the standout good result, with a *z* score module solidly lower than 2. All *z* score module values are available in table 21. It should be noted that the interlaboratory study organizer noted that the quantitative results are low confidence due to the very reduced number of study participants, which means participation in further quantitative studies would be required for the proper verification of the method's quantitative features.

Overall, this study lends more confidence to our capacity to correctly ascertain the presence/absence of *Legionella* spp. and/or *L. pneumophila* but points us towards the need for a careful study of the quantitative capabilities of the method if those become useful at a later date. Given our present goal of validating only qualitative capabilities,

this study was still an overall success. At the time of writing, the lab has signed up for another *Legionella* PCR methods interlaboratory study and is awaiting the arrival of the study samples.

Table 20. Results of the evaluation of AEdP's interlaboratory study qualitative results by IELAB. For all samples, *L. pneumophila* was correctly identified as being present.

Sample	Presence/Absence of *Legionella spp.*	Presence/Absence of *L. pneumophila*	Qualitative Evaluation
A1	PRESENCE	PRESENCE	ACCEPTABLE
A2	PRESENCE	PRESENCE	ACCEPTABLE
B1	PRESENCE	PRESENCE	ACCEPTABLE
B2	PRESENCE	PRESENCE	ACCEPTABLE
C	PRESENCE	PRESENCE	ACCEPTABLE

Table 21. Results of the evaluation of AEdP's interlaboratory study quantitative results by IELAB.

Sample	*z* score module	*z* score Acceptability
A1	NOT AVAILABLE	
A2	6,4	UNNACEPTABLE
B1	NOT AVAILABLE	
B2	6,6	UNNACEPTABLE
C	0,1	ACCEPTABLE

3.7. Control charts

Three control charts were prepared, one for each of the monitored fluorescence channels FAM, CY5 and VIC, which correspond respectively to *Legionella* spp., *L. pneumophila* and IAC. They are available in this report as charts 7 through 9. For the method implemented during this internship, these charts can serve as the foundation for future efforts of baseline monitoring at AEdP's analysis laboratory. For all charts, no data points fell outside the warning or control limits, a strong sign of baseline stability. In the IAC baseline chart, an initial upwards trend of 5 data points is visible, but AEdP internal protocols specify that only trends of 6 more points require corrective action. No trends are visible for the other 2 control charts. Given these results, we can conclude that the baselines behaved in an adequate manner. Note that, for all charts, UCL/LCL and UWL/LWL stand for, respectively, the upper/lower control (or action) limits and upper/lower warning limits. The mean is calculated from each chart's plotted data points.

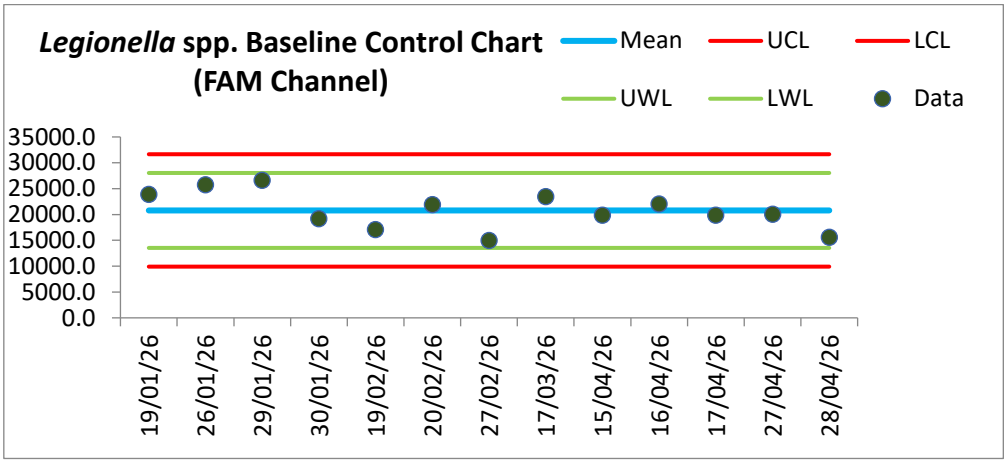

Chart 7. Shewhart control chart for the FAM fluorescence channel (*Legionella* spp.).

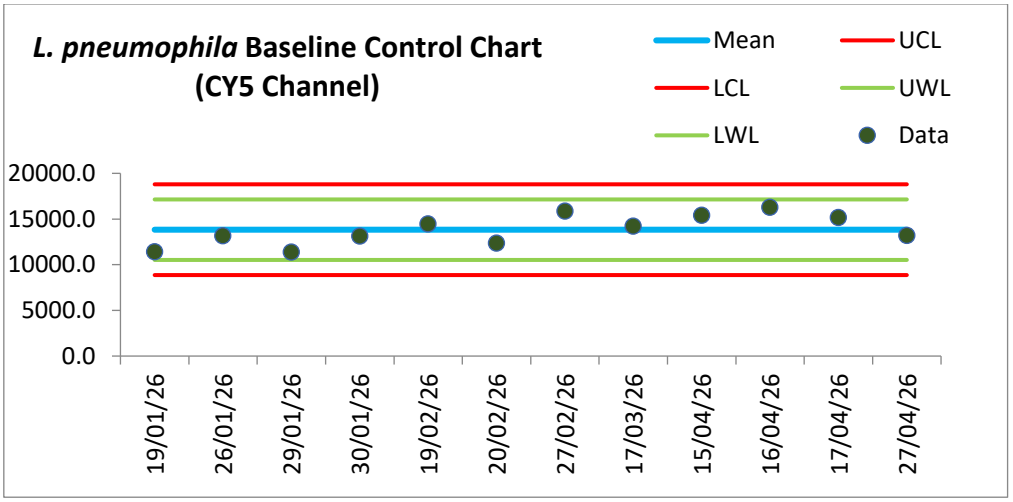

Chart 8. Shewhart control chart for the CY5 fluorescence channel (*L. pneumophila*).

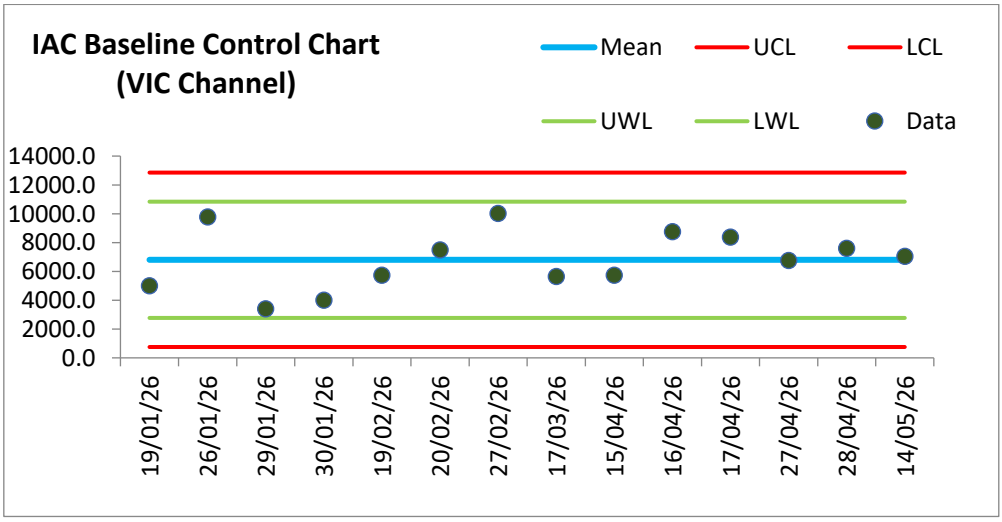

Chart 9. Shewhart control chart for the VIC fluorescence channel (IAC).

3.8. PCR as a confirmation tool for culture methods

PCR also has some utility as a tool to confirm the identity of supposed *Legionella* spp. or *L. pneumophila* colonies identified through culture methods and other tools. For example, as a backup in case of inconclusive serological tests, a PCR protocol can be used to reinforce results. The inconclusive colonies can be resuspended and their DNA directly extracted. Not only is this application explicitly allowed in Portuguese legislation, it was also successfully exploited multiple times during this in the context of parallel projects/daily work at AEdP.

3.9. Comments on the functionality/difficulty of validation of the *qualyfast*® Legionella kit

During this internship, we verified that not all commercial kits are very well suited for an easy validation/verification process. ISO 12869 was first published in 2012, at which time PCR kit reagents were mostly still provided in bulk, in separate vials, in lyophilized form when possible. This norm has been updated, but only in minor ways. In a lot of modern PCR kits, including the one used in this internship, reagents are provided pre-dosed in lyophilized form pre-loaded into the PCR tubes/plates. Users of these kits do not have access to the working solution used by the manufacturer to prepare the quantification standards and positive controls. Despite reducing the probability of reagent dosing mistakes/contamination, this also makes validation harder, as the lab performing this task will have to buy separate standards from the manufacturer/obtain a DNA solution connected to the primary standard from elsewhere or, if all else fails, acquire a primary standard of *L. pneumophila* DNA and ensure the quality of their own, in-house DNA extracts through a rigorous comparison with of the latter with the former. In other words, kits should be slightly adjusted to increase compatibility with ISO validation requirements or, alternatively, ISO should re-evaluate ISO 12869 given the current trends in commercial kits and, within the bounds of rationality, adjust the validation requirements.

3.10. Culture-based methods VS PCR-based methods: is qPCR actually a good tool to minimize workloads/response time?

The intent of this internship was to provide AEdP with a tool to reduce their response time in *Legionella* detection, which would be of great use in situations of outbreak risk, but also reduce workloads by, in theory, avoiding the need to perform the full culture method for many samples. Although the first point still stands, as our qPCR protocol showed itself to be doable from beginning to end in approximately 3 hours, the second practical use of qPCR has been questioned during this internship. The problem is that

the qPCR method is both extremely sensitive and capable of detecting non-culturable strains. This might mean that, in some cases, qPCR will detect *Legionella* not easily detectable in culture methods, because of its presence in extremely low-levels and/or the strains not being easily culturable. This fear is backed up by a few experiments done in parallel with this internship, in which samples processed for the qPCR protocol were also subject to AEdP's culture *Legionella* protocol. In all cases, when detection of *Legionella* through qPCR occurred, no growth of any *Legionella* colonies was identified. Further studies should be undertaken to verify how common this disconnect between the culture and PCR methods is, to achieve a better understanding of qPCR's utility to AEdP.

3.11. Commentary on the EU-wide *Legionella* approach

Portugal's parametric limit of *Legionella* in water is defined in Decree-law n. º 69/2023[1], as 1000 CFU/L of water, in the context of predial networks. This is a direct transposition of the EU Directive n.º 98/83/CE [2]. This relatively "high" limit contrasts with, for example*, E. coli* and fecal Enterococcus, for which the acceptable parametric value is absence. There are some who argue that this limit might be too lax, letting through *Legionella* levels that are high enough for outbreaks to occur[61]. Although most known cases of legionellosis are caused by *L. pneumophila*, for which Portuguese legislation decrees that any level of detection shall be treated as a situation of elevated risk[52], the pathogenicity of other *Legionella* species should not be disregarded. Having experienced how commonly *Legionella* spp. is found in various sources during this internship, we believe that a renewed discussion on *Legionella* and its control might result in tangible public health benefits.

We also believe that the discussion on what methods are allowed to be used in the detection and quantification of *Legionella* in the EU should be revisited. Although culture-based methods have been the microbiological and public health golden standard for many decades, molecular biology methods have in many ways surpassed them, particularly in speed and sensitivity. Although there is no easy to establish correlation between CFUs and GUs, we believe that a long-term effort to standardize and allow molecular biology methods results to be emitted as the final result might greatly speed up and reduce waste production (no need for a culture-based confirmation step) in its various use cases, such as water or food quality control, without compromising public health in the process.

4. Conclusion

I would highlight as this internship's key takeaway the amount of effort and knowledge involved in validation and accreditation processes. This includes the study of a large body of norms and legal documents, paired with strict and thorough experimentation to study the performance characteristics of the methods being evaluated. This type of work reinforces the importance of highly educated graduates in all industries where quality control meets complex technologies, since they can better comprehend, plan and perform all parts of the validation and accreditation processes.

Inclusivity and exclusivity testing was successful, with sufficient data being collected on various species. The *Staphylococcus* results are concerning, but further studies might be able to dispel worries about the kit's possible susceptibility to unspecific and undesirable DNA amplification.

The verification of the calibration curve function and LQ_{qPCR} was successful, with all criteria being met for the *Legionella* spp. system. The validation of the LD_{qPCR} however, was only successful for the *Legionella* spp. qPCR system at the time of writing. More studies are required to validate the LD_{qPCR} for the *L. pneumophila* qPCR system.

Recovery and robustness for both the *Legionella* spp. and *L. pneumophila* was validated for potable water, wells and sprinkler from a qualitative perspective. The remaining matrixes, however, will require further studies to collect the necessary data for validation. If the validation of quantification becomes a goal, new quantitative studies will have to be done for all matrixes.

The study of precision was successful, with both repeatability and intralaboratory reproducibility results being within limits. However, reproducibility data still must be collected for the remaining operators. Interlaboratory reproducibility results were also good from a qualitative perspective, but the quantitative results raised some doubts regarding capacity to quantify *Legionella* spp. in samples with the *qualyfast*® Legionella kit. If the verification of quantification becomes a goal, the laboratory should participate in more interlaboratory studies.

To conclude, the implementation and verification efforts should be considered a success. All of ISO 12869's verification criteria have been met at AEdP with the *qualyfast*® Legionella kit for *Legionella* spp. in potable water. Although verification of capabilities of the kit for *L. pneumophila* and for a couple water matrixes are still underway, given what has been discussed in this report, we do not believe these remaining tests will pose any

considerable difficulties. Regarding accreditation, the march towards that goal is well under way, with documentation and data being prepared and organized for an eventual internal audit, a key first step towards accreditation of the method by IPAC.

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Attachments

Attachment 1. Specific Student distribution; risk of α = 5% for a bilateral test

α	0,05
$1-\alpha/2$	0,975
1	12,706
2	4,303
3	3,182
4	2,776
5	2,571
6	2,447
7	2,365
8	2,306
9	2,262
10	2,228
11	2,201
12	2,179
13	2,160
14	2,145
15	2,131
16	2,120
17	2,110
18	2,101
19	2,093
20	2,086
25	2,060
30	2,042
40	2,021
50	2,009
60	2,000
100	1,984
∞	1,960

Attachment 2. Bacterial species and strains for inclusivity & exclusivity testing according to ISO 12869

Inclusivity list (microorganisms to test for a *Legionella* spp. method): *L. anisa* (e.g. WDCM 00106), *L. birminghamensis*, *L. bozemanii* 1 and 2, *L. cherrii*, *L. cincinnatiensis* (e.g. CIP 103875), *L. dumoffii*, *L. erythra* 2, *L. feeleii* 1 and 2, *L. gormanii*, *L. hackeliae* serogroup1 (e.g. CIP 103844) and serogroup 2, *L. jordanis*, *L. lansingensis* (e.g. ATCC 43751), *L. longbeachae* 1 and 2, *L. maceachernii*, *L. micdadei* (e.g. ATCC 33218), *L. oackridgensis*, *L. parisiensis* (e.g. CIP 103847), *L. pneumophila* serogroups 1 to 15 (e.g. *Legionella pneumophila* serogroup 1 WDCM 00107; *Legionella pneumophila* serogroup 6 ATCC 33215), *L. sainthelensi* 1 and 2, *L. tucsonensis*, *L. wadsworthii* (e.g. CIP 103886).

Inclusivity list (microorganisms to test for a *L. pneumophila* method): 15 serogroups from the species.

Exclusivity list (tested microorganisms recognized as not belonging to *Legionella* genus and/or being phylogenetically close). At least the following list shall be tested: *Aeromonas hydrophila*, *Alcaligenes faecalis* (e.g. CIP 60.80), *Bacillus subtilis* (e.g. CCM 1999), *Burkholderia cepacia*, *Clostridium* spp., *Enterobacter aerogenes*, *Escherichia coli* (e.g. WDCM 00003), *Flavobacterium* spp., *Klebsiella oxytoca* (e.g. WDCM 00179), *Listeria monocytogenes* (e.g. WDCM 00109 or WDCM 00020), *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Serratia marcescens*, *Stenotrophomonas maltophila*.

Exclusivity list (tested microorganisms recognized as not belonging to *L. pneumophila* species): *L. anisa* (e.g. WDCM 00106), *L. bozemanii*, *L. dunmofii*, *L. gormanii*, *L. jordanis*, *L. longbeachae*, *L. micdadei* (e.g. ATCC 33218), *L. parisiensis* (e.g. CIP 103847), *L. tucsonensis*.

Attachment 3. Strategies for control and elimination of *Legionella*[39]

Method	Advantages	Disadvantages
Keeping temperature <20 ºC	• Simple, effective and easily monitored • Little significant growth of *Legionella*	• Only really applicable to potable water systems
Keeping temperature >50 ºC	• Simple, effective and easily monitored	• Does not eliminate legionellae • Requires circulation temperature to be near 60 ºC • Difficult to maintain temperatures in old systems • Requires protection against scalding
Periodic flushing with hot water at 50-60 ºC (usually an essential part of control by high temperature, above)	• Simple, effective and easily monitored	• Not applicable in cold-water systems • Requires protection against scalding • Must be maintained and inspected to achieve consistent control • Recolonization within days
Dosing with sodium hypochlorite	• Proven, effective disinfection technique • Simple to use • Relatively cheap	• Formation of trihalomethanes • Needs protection (e.g. carbon filter) for dialysis patients • Toxic to fish • Affects taste and odour • Not stable, particularly in hot water • Increases corrosion of copper
Dosing with monochloramine	• More persistent than chloramine • Simple to use in mains distributions • Penetrates into biofilms	• Needs protection (e.g. carbon filter) for dialysis patients • Toxic to fish • Affects rubber components • No commercial kit available for dosing small water systems
Dosing with chlorine dioxide	• Proven disinfection technique • Simple to use	• Formation of chlorite • Needs protection (e.g. carbon filter) for dialysis patients • Safety considerations (depending on method of generation)
Dosing with hydrogen peroxide	• Simple to use	• Weak disinfectant • Suspected of mutagenicity
Copper and silver ionization	• Effective when prescribed concentrations are maintained	• Frequent monitoring of copper and silver needed • Pretreatment needed (depending on effect of pH and hardness) • Increased concentrations of copper and silver in water
Anodic oxidation	• Disinfection demonstrated	• Pretreatment needed (depending on effect of pH and hardness) • Effect on *Legionella* biofilms not known
Ultraviolet disinfection	• Proven disinfection technique • Simple to use	• Effective only at point of application, no downstream control (no residual) • Not suitable for turbid waters • No effect on biofilm formation
Ultrafiltration at point of entry to the building or system	• Physical disinfection barrier • Effective removal of biomass and particles	• No inactivation of *Legionella* downstream of filter within system • Effect on formation of biofilms and sediment not known
Point-of-use filters	• Physical barrier • Easy to install (may require some modification of the outlet) • Suitable for hot and cold-water systems • Good for use in systems exposing high-risk patients	• Only suitable at point of use • Must be replaced regularly • Particulates in water may reduce flow and operational life • Expensive
Pasteurizing with heat or flushing	• Disinfection barrier • Useful as short-term remedial measure • Simple to apply in hot water installation	• Transient effect on *Legionella* • No limitation of biofilm formation • Scalding risk
Non-oxidising biocides	• Proven technique for cooling systems	• Not suitable for potable water systems • Most not applicable to spa pools • Resistant populations may develop • Need to alternate two different biocides • Often concentrations cannot be readily monitored • Difficult to neutralize for sampling purposes

Attachment 4. Certificate for the *qualyfast® L. pneumophila* reference material

qualyfast®

LEGIONELLA
REFERENCE MATERIAL

PRODUCT IDENTIFICATION

Product name	**qualyfast® Legionella Reference Material**
Expiration date (YYYY-MM)	2026/11
Lot	A100260

QUALITY CONTROL PROTOCOL USED

qualyfast® Legionella Reference Material is connected with the primary standard *Legionella pneumophila* DNA, Certified in Genome Units (GU) number (Legionelles Centre National de Reference Lyon, France). The calibration is performed by Real time PCR Test. The quantity is shown in ANNEX 1.

Result: **Conform.**

Signature:

Quality Assurance Manager

Date:
10 April, 2026

BIOSIDE

Annex 1

Batch A100260

Reference Material of *Legionella* species and *Legionella pneumophila*

Add 150 µl DNA free solution before use. The concentration of the material is 5-9 Genomic Unit / µl
Aliquot 15 µl of the reference material in a detection tube of **qualyfast® Legionella** kit.
Following the data obtained during quantification with primary standard *Legionella pneumophila* DNA.
Certified in Genome Units (GU) number (Legionelles Centre National de Reference Lyon, France).

Target	Mean Quantity	Range	M.U.
Legionella spp.	73	66-80	Genomic Unit / Reaction
Legionella pneumophila	126	113-139	Genomic Unit / Reaction

Attachment 5. Certificate of validation for the *qualyfast*® Legionella kit

BIOSIDE

May 25, 2023

To whom it may concern,

The following information is intended to provide specific details regarding the Bioside product, ***qualyfast*® Legionella**.

The inhibition control in ***qualyfast*® Legionella** kit has been done according to the requirements detailed in Clause 10.6 of the ISO/TS 12869.

***qualyfast*® Legionella** has been validated according to the requirements detailed in Clause 9 of ISO/TS 12869:2019 Water quality – Detection and quantification of Legionella spp. and/or Legionella pneumophila by concentration and genic amplification by quantitative polymerase chain reaction (qPCR), as described in the following table.

Characteristics	Study design	Outcomes
Inclusivity and exclusivity of primers and probes (according with Clauses 7.3.2 and 9.2 of ISO/TS 12869)	The specificity of the primers and probes is checked by homology research using appropriate softwares in NCBI Genbank and EMBL Nucleotide sequence database, and by testing on strains of *Legionella*, *L. pneumophila* and strains of microorganisms likely to be found in the same ecological niches as legionella. Microorganisms tested for the verification of inclusivity are: *L. anisa*, *L. birminghamensis*, L. *bozemanii* 1 and 2, *L. cherrii*, *L. cincinnatiensis*, *L. dumoffii*, *L. erythra* 2, *L. feeleii* 1 and 2, *L. gormanii*, *L. hackeliae* 1 and 2, *L. jordanis*, *L. lansingensis*, *L. longbeachae* 1 and 2, *L. maceachernii*, *L. micdadei*, *L. oakridgensis*, *L. parisiensis*, *L. pneumophila* 1 to 15, *L. sainthelensi* 1 and 2, *L. tucsonensis*, *L. wadsworthii*. Microorganisms tested for the verification of exclusivity are: *Aeromonas hydrophila*, *Alcaligenes faecalis*, *Bacillus subtilis*, *Burkholderia cepacia*, *Clostridium*, *Enterobacter aerogenes*, *Escherichia coli*, Flavobacterium, *Klebsiella oxytoca*, *Listeria monocytogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Serratia marcescens*, *Stenotrophomonas maltophila*.	All the *Legionella* and *L. pneumophila* sequences analyzed by NCBI and EMBL softwares, show 100% homology with the ***qualyfast*® Legionella** primers and probes. No primer/probe combinations generate cross-reaction with the non-target sequences. For strains not belonging to the genus *Legionella*, no amplification product was detected by the real time PCR by both targets: *Legionella* spp. and *L. pneumophila*. *L. pneumophila* target show no amplification also for all the *Legionella* not *pneumophila* strains. *Legionella* spp. target show amplification for all the *Legionella* strains tested. *L. pneumophila* target show amplification for all the serogroups of *L. pneumophila* tested.

BIOSIDE S.R.L.
Via Albert Einstein, snc – Località Cascina Codazza c/o Parco Tecnologico Padano, 26900 Lodi (LO), ITALY
P.IVA/C.F.: 08559840965 - **www.bioside.it**

BIOSIDE

Characteristics	Study design	Outcomes
Verification of the calibration function of the quantitative PCR phase (according with Clause 9.3 of ISO/TS 12869)	Five independent series using ***qualyfast*®** **Legionella Quantified Standards kit** and five independent series using ***qualyfast*®** **L. pneumophila Quantified Standards kit** starting from 25000 to 25 GU/PCR were prepared on different days, by different operators. Quantified Standards kits are connected to primary reference material *L. pneumophila* (WDCM 00107) from Legionelles centre National de reference, by comparation of three series obtained with reference material and three series obtained with the Quantified Standards kits.The calibration function was calculated by linear regression model of threshold cycles (Ct) measured for each amplification vs. the $\log_{10}$ copy number for each standard dilution. The measured quantity for each dilution level was determined in retrospect by using the formula: logx=(Ct-b)/a, where a is the slope, b is the y-intercept, and x is the quantity. The accuracy of linearity (E_{lin}) for the entire calibration range was calculated using the formula: $E_{lin}=\sqrt{(SD)^2 + (bias)^2}$.	For *Legionella* spp. target the calibration function calculated resulted to be: y=-3,30x + 36,82 corresponding to a validated qPCR efficiency of 100,53 %. For *Legionella pneumophila* target the calibration function calculated resulted to be: y=-3,24x + 37,81 corresponding to a validated qPCR efficiency of 103,73%. All the values are within the defined limits: efficiency between 75% and 125% and slope between -4,115 and -2,839. The accuracy of linearity (E_{lin}) for the entire calibration range was calculated and all values are less than the critical value of 0,15.
Verification of the PCR limit of quantification LQ_{qPCR} (according with Clause 9.4 of ISO/TS 12869)	The limit of quantification (LQ) corresponds to the first level of the calibration range (25 GU) and is the smallest number of GU that can be quantified. 20 dilutions series were tested to determine the standard deviation, the bias, the accuracy (E_{LQ}) and uncertainty (U_{LQ}) at the LQ.	E_{LQ} at the LQ (25 GU/PCR) were determined to be less than the critical value of 0,15$\log_{10}$ both for *Legionella* spp. target and *L. pneumophila* target (0,10 and 0,09 respectively).
Verification of the PCR limit of detection LD_{qPCR} (according with Clause 9.5 of ISO/TS 12869)	The limit of detection (LD) is defined as the smallest number of GU per PCR that provides a qPCR positive result (amplification) in at least 90% of cases. The verification of the PCR limit of detection (LD_{qPCR}) was done with 20 measurements from 10 separate dilutions prepared from a DNA solution of *L. pneumophila* (WDCM 00107).	The limit of determination is 5 GU/PCR both for *Legionella* spp. target and *L. pneumophila* target. For both *Legionella* spp. target and *L. pneumophila* target 20/20 measurements are positive, so LD is confirmed for both targets in 100% of cases. All Ct values were lower than the intercept.

BIOSIDE

Characteristics	Study design	Outcomes
Recovery method and Robustness (according with Clauses 9.6 and 9.7 of ISO/TS 12869)	The recovery was evaluated on sterile water and the robustness was evaluated on eight different artificially contaminated water samples (potable water, hot sanitary water, surface water, two different waste waters, two different cooling tower waters, sulfurous water) at two different spiked levels (1000 and 100000 GU/L). The contamination was made with *L. pneumophila* (WDCM 00107). For each level of concentration, 10 separate spiked samples were prepared, on different days and by several operators and were processed both with ***qualyfast*® DNA Extraction kit I** and ***qualyfast*® DNA Extraction kit II**.	Using ***qualyfast*® DNA Extraction kit I**, the average recovery per level and per matrix have a value between -0,32 log_{10} and -0,1 log_{10}. Using ***qualyfast*® DNA Extraction kit II**, the average recovery per level and per matrix have a value between -0,35 log_{10} and -0,05 log_{10}. Results were therefore in line with conforming criteria: value between -0,6 log_{10} and +0,3 log_{10}, equivalent to efficiency comprise between 25% and 199%.
Measurement uncertainty of the whole method (according with Clause 9.8 of ISO/TS 12869)	The uncertainty measurement of the method is evaluated by the analysis of the recovery values. The bias was estimated by the average recovery value for all the matrices. The precision was estimated through the recovery variance, using all values obtained during the validation of the method, the robustness study and the recovery study.	Using ***qualyfast*® DNA Extraction kit I**, $U_{overall}$ calculated starting from the average recovery value of -0,17 and from the variance value of 0,10 resulted to be 0,73. Using ***qualyfast*® DNA Extraction kit II**, $U_{overall}$ calculated starting from the average recovery value of -0,18 and from the variance value of 0,09 resulted to be 0,71. The values obtained is within the expected limits.

Sincerely,
Michela Savoldi Boles
Chief Technical Officer

Attachment 6. Certificate of the *L. anisa* reference material (WDCM 00106)

INFORME DE ANÁLISIS

ielab

BAControl-5

(material referencia, MR)

CECT
Vniversitat de València
Material trazable a cepas CECT*

Productor

ielab Calidad, S.L.
C/ Dracma, 7
Pol. Ind. Las Atalayas
03114 Alicante (España)
Tlf: +34 966 10 55 01

Descripción

Código: 990537
Microorganismo: *Legionella anisa* L6839 trazable a CECT 8177 (correspondiente con ATCC 35292; WDCM 00106), con un pase desde la cepa de reserva de referencia de la Colección de Cultivos Tipo
Lote nº: PLA06025
Fecha de preparación: 13-feb.-2025
Fecha de caducidad: 10-mar.-2026
Formato: pastilla liofilizada

Pruebas de autenticidad de la Cepa de Colección de Cultivo empleada

Métodos moleculares

Información de seguridad

Grupo de riesgo 2

Condiciones de conservación

Conservar a -20±5ºC

Uso previsto

Controles internos de calidad en términos de precisión (control de proceso, gráficos de control y control de calidad de medios de cultivo). Material especialmente indicado para ensayos con filtros de policarbonato (anterior ISO 11731-1).

Condiciones de reconstitución

(indicadas en la Guía Rápida de Uso)
Diluyente: Agua destilada estéril
Volumen: 20 mL
Tiempo de reconstitución: 10 minutos

Condiciones de ensayo del productor

Laboratorio: Un laboratorio siguiendo criterios ISO 17025
Diluciones: Hasta 10^3
Volumen analizado: 3 mL
Técnica: Filtración
Temperatura de incubación: 36 ± 2º C
Tiempo de incubación: 96 ± 4h
Medio de cultivo: Agar GVPC
Membrana filtración: Ésteres de celulosa (0.45 µm)

Controles de calidad en las condiciones de ensayo descritas

Contaminación cruzada: No detectada
Homogeneidad: Homogéneo(u_H=0.035 log)
Estabilidad: Estable(u_E=0.029 log)

Resultados obtenidos en el volumen de reconstitución

Porcentaje de lote analizado: 15%
Número de ensayos: 56
Valor obtenido: 2.59×10^5 ufc/pastilla
Intervalo de confianza 95%:
6.71×10^4 - 9.98×10^5 ufc

Alicante, 10 de marzo de 2025

Raquel Múrtula Corbí
Directora técnica área de microbiología.

Fabricado cumpliendo los requerimientos de la norma ISO 17034

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Attachment 7. Certificate of the *L. pneumophila* reference material (WDCM 00107)

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BAControl-5

(material referencia, MR)

CECT
VNIVERSITAT DE VALÈNCIA
Material trazable a cepas CECT*

Productor

ielab Calidad, S.L.
C/ Dracma, 7
Pol. Ind. Las Atalayas
03114 Alicante (España)
Tlf: +34 966 10 55 01

Descripción

Código: 990477
Microorganismo:*Legionella pneumophila (sg 1)* L2949 trazable a CECT 7109 (correspondiente con ATCC 33152; WDCM 00107), con un pase desde la cepa de reserva de referencia de la Colección de Cultivos Tipo
Lote nº: PLP04025
Fecha de preparación: 13-feb.-2025
Fecha de caducidad: 10-mar.-2026
Formato: pastilla liofilizada

Pruebas de autenticidad de la Cepa de Colección de Cultivo empleada

Métodos moleculares

Información de seguridad

Grupo de riesgo 2

Condiciones de conservación

Conservar a -20±5ºC

Uso previsto

Controles internos de calidad en términos de precisión (control de proceso, gráficos de control y control de calidad de medios de cultivo). Material especialmente indicado para ensayos con filtros de policarbonato (anterior ISO 11731-1).

Condiciones de reconstitución

(indicadas en la Guía Rápida de Uso)
Diluyente: Agua destilada estéril
Volumen: 20 mL
Tiempo de reconstitución: 10 minutos

Condiciones de ensayo del productor

Laboratorio: Un laboratorio siguiendo criterios ISO 17025
Diluciones: Hasta 10^3
Volumen analizado: 1 mL
Técnica: Filtración
Temperatura de incubación: 36 ± 2º C
Tiempo de incubación: 96 ± 4h
Medio de cultivo: Agar GVPC
Membrana filtración: Ésteres de celulosa (0.45 µm)

Controles de calidad en las condiciones de ensayo descritas

Contaminación cruzada: No detectada
Homogeneidad: Homogéneo(u_H=0.042 log)
Estabilidad: Estable(u_E=0.027 log)

Resultados obtenidos en el volumen de reconstitución

Porcentaje de lote analizado: 15%
Número de ensayos: 56
Valor obtenido: 1.07×10^6 ufc/pastilla
Intervalo de confianza 95%:
2.64×10^5 - 4.37×10^6 ufc

Alicante, 10 de marzo de 2025

Raquel Múrtula Corbí
Directora técnica área de microbiología.

Fabricado cumpliendo los requerimientos de la norma ISO 17034

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Attachment 8. Certificate of the *E. coli* reference material (WDCM 00013)

INFORME DE ANÁLISIS

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BAControl-5

(material referencia, MR)

CECT
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Material trazable a cepas CECT*

Productor

ielab Calidad, S.L.
C/ Dracma, 7
Pol. Ind. Las Atalayas
03114 Alicante (España)
Tlf: +34 966 10 55 01

Descripción

Código: 990095
Microorganismo:*Escherichia coli* V6 trazable a CECT 434 (correspondiente con ATCC 25922; WDCM 00013), con un pase desde la cepa de reserva de referencia de la Colección de Cultivos Tipo
Lote nº: PEC14015
Fecha de preparación: 17-ene.-2025
Fecha de caducidad: 11-feb.-2026
Formato: pastilla liofilizada

Pruebas de autenticidad de la Cepa de Colección de Cultivo empleada

Batería de pruebas bioquímicas

Información de seguridad

Grupo de riesgo 2

Condiciones de conservación

Conservar a -20±5ºC

Uso previsto

Controles internos de calidad en términos de precisión (control de proceso, gráficos de control y control de calidad de medios de cultivo).

Condiciones de reconstitución

(indicadas en la Guía Rápida de Uso)
Diluyente: Agua destilada estéril
Volumen: 20 mL
Tiempo de reconstitución: 10 minutos

Condiciones de ensayo del productor

Laboratorio: Un laboratorio siguiendo criterios ISO 17025
Diluciones: Hasta 10^2
Volumen analizado: 1 mL
Técnica: Filtración
Temperatura de incubación: 36 ± 2º C
Tiempo de incubación: 21 ± 3h
Medio de cultivo: Agar CCA (coliforms chromogenic agar)
Membrana filtración: Ésteres de celulosa (0.45 µm)

Controles de calidad en las condiciones de ensayo descritas

Contaminación cruzada: No detectada
Homogeneidad: Homogéneo(u_H=0.024 log)
Estabilidad: Estable(u_E=0.040 log)

Resultados obtenidos en el volumen de reconstitución

Porcentaje de lote analizado: 15%
Número de ensayos: 56
Valor obtenido: 8.71×10^4 ufc/pastilla
Intervalo de confianza 95%:
3.31×10^4 - 2.29×10^5 ufc

Alicante, 11 de febrero de 2025

Raquel Múrtula Corbí
Directora técnica área de microbiología.

Fabricado cumpliendo los requerimientos de la norma ISO 17034

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Attachment 9. Certificate of the *E. aerogenes* reference material (WDCM 00175)

INFORME DE ANÁLISIS

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CECT
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Material trazable a cepas CECT*

BAControl-5

(material referencia, MR)

Productor

ielab Calidad, S.L.
C/ Dracma, 7
Pol. Ind. Las Atalayas
03114 Alicante (España)
Tlf: +34 966 10 55 01

Descripción

Código: 990381
Microorganismo: *Klebsiella (Enterobacter) aerogenes* V533 trazable a CECT 684 (correspondiente con ATCC 13048; WDCM 00175), con un pase desde la cepa de reserva de referencia de la Colección de Cultivos Tipo
Lote nº: PEA02104
Fecha de preparación: 04-oct.-2024
Fecha de caducidad: 18-nov.-2025
Formato: pastilla liofilizada

Pruebas de autenticidad de la Cepa de Colección de Cultivo empleada

Batería de pruebas bioquímicas

Información de seguridad

Grupo de riesgo 2

Condiciones de conservación

Conservar a -20±5ºC

Uso previsto

Controles internos de calidad en términos de precisión (control de proceso, gráficos de control y control de calidad de medios de cultivo).

Condiciones de reconstitución

(indicadas en la Guía Rápida de Uso)
Diluyente: Agua destilada estéril
Volumen: 20 mL
Tiempo de reconstitución: 10 minutos

Condiciones de ensayo del productor

Laboratorio: Un laboratorio siguiendo criterios ISO 17025
Diluciones: Hasta 10^3
Volumen analizado: 1 mL
Técnica: Filtración
Temperatura de incubación: 36 ± 2º C
Tiempo de incubación: 21 ± 3h
Medio de cultivo: Agar CCA (coliforms chromogenic agar)
Membrana filtración: Ésteres de celulosa (0.45 µm)

Controles de calidad en las condiciones de ensayo descritas

Contaminación cruzada: No detectada
Homogeneidad: Homogéneo(u_H=0.062 log)
Estabilidad: Estable(u_E=0.042 log)

Resultados obtenidos en el volumen de reconstitución

Porcentaje de lote analizado: 15%
Número de ensayos: 56
Valor obtenido: 6.33×10^5 ufc/pastilla
Intervalo de confianza 95%:
1.83×10^5 - 2.19×10^6 ufc

Alicante, 18 de noviembre de 2024

Raquel Múrtula Corbí
Directora técnica área de microbiología.

Fabricado cumpliendo los requerimientos de la norma ISO 17034

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Attachment 10. Certificate of the *P. aeruginosa* reference material (WDCM 00024)

INFORME DE ANÁLISIS

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CECT
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Material trazable a cepas CECT*

BAControl-5

(material referencia, MR)

Productor

ielab Calidad, S.L.
C/ Dracma, 7
Pol. Ind. Las Atalayas
03114 Alicante (España)
Tlf: +34 966 10 55 01

Descripción

Código: 990512
Microorganismo:*Pseudomonas aeruginosa* V659 trazable a CECT 110 (correspondiente con ATCC 10145; WDCM 00024), con un pase desde la cepa de reserva de referencia de la Colección de Cultivos Tipo
Lote nº: PPA40250
Fecha de preparación: 21-dic.-2024
Fecha de caducidad: 28-ene.-2026
Formato: pastilla liofilizada

Pruebas de autenticidad de la Cepa de Colección de Cultivo empleada

Batería de pruebas bioquímicas

Información de seguridad

Grupo de riesgo 2

Condiciones de conservación

Conservar a -20±5ºC

Uso previsto

Controles internos de calidad en términos de precisión (control de proceso, gráficos de control y control de calidad de medios de cultivo).

Condiciones de reconstitución

(indicadas en la Guía Rápida de Uso)
Diluyente: Agua destilada estéril
Volumen: 20 mL
Tiempo de reconstitución: 10 minutos

Condiciones de ensayo del productor

Laboratorio: Un laboratorio siguiendo criterios ISO 17025
Diluciones: Hasta 10^2
Volumen analizado: 3 mL
Técnica: Filtración
Temperatura de incubación: 36 ± 2º C
Tiempo de incubación: 44 ± 4h
Medio de cultivo: Agar Pseudomonas CN
Membrana filtración: Ésteres de celulosa (0.45 µm)

Controles de calidad en las condiciones de ensayo descritas

Contaminación cruzada: No detectada
Homogeneidad: Homogéneo(u_H=0.026 log)
Estabilidad: Estable(u_E=0.046 log)

Resultados obtenidos en el volumen de reconstitución

Porcentaje de lote analizado: 15%
Número de ensayos: 56
Valor obtenido: 6.37×10^4 ufc/pastilla
Intervalo de confianza 95%:
1.86×10^4 - 2.18×10^5 ufc

Alicante, 28 de enero de 2025

Raquel Múrtula Corbí
Directora técnica área de microbiología.

Fabricado cumpliendo los requerimientos de la norma ISO 17034

Attachment 11. Certificate of the *S. aureus* reference material (WDCM 00034)

INFORME DE ANÁLISIS

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BAControl-5

(material referencia, MR)

CECT
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Material trazable a cepas CECT*

Productor

ielab Calidad, S.L.
C/ Dracma, 7
Pol. Ind. Las Atalayas
03114 Alicante (España)
Tlf: +34 966 10 55 01

Descripción

Código: 990105
Microorganismo:*Staphylococcus aureus* V459 trazable a CECT 435 (correspondiente con ATCC 25923; WDCM 00034), con un pase desde la cepa de reserva de referencia de la Colección de Cultivos Tipo
Lote nº: PSA10104
Fecha de preparación: 14-oct.-2024
Fecha de caducidad: 27-nov.-2025
Formato: pastilla liofilizada

Pruebas de autenticidad de la Cepa de Colección de Cultivo empleada

Batería de pruebas bioquímicas

Información de seguridad

Grupo de riesgo 2

Condiciones de conservación

Conservar a -20±5ºC

Uso previsto

Controles internos de calidad en términos de precisión (control de proceso, gráficos de control y control de calidad de medios de cultivo).

Condiciones de reconstitución

(indicadas en la Guía Rápida de Uso)
Diluyente: Agua destilada estéril
Volumen: 20 mL
Tiempo de reconstitución: 10 minutos

Condiciones de ensayo del productor

Laboratorio: Un laboratorio siguiendo criterios ISO 17025
Diluciones: Hasta 10^3
Volumen analizado: 1 mL
Técnica: Filtración
Temperatura de incubación: 36 ± 2º C
Tiempo de incubación: 44 ± 4h
Medio de cultivo: Agar Baird Parker
Membrana filtración: Ésteres de celulosa (0.45 µm)

Controles de calidad en las condiciones de ensayo descritas

Contaminación cruzada: No detectada
Homogeneidad: Homogéneo(u_H=0.042 log)
Estabilidad: Estable(u_E=0.064 log)

Resultados obtenidos en el volumen de reconstitución

Porcentaje de lote analizado: 15%
Número de ensayos: 56
Valor obtenido: 5.54×10^5 ufc/pastilla
Intervalo de confianza 95%:
8.82×10^4 - 3.48×10^6 ufc

Alicante, 27 de noviembre de 2024

Raquel Múrtula Corbí
Directora técnica área de microbiología.

Fabricado cumpliendo los requerimientos de la norma ISO 17034

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Attachment 12. Certificate of the *S. epidermidis* reference material (WDCM 00132)

INFORME DE ANÁLISIS

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BAControl-5

(material referencia, MR)

CECT
VNIVERSITAT DE VALÈNCIA
Material trazable a cepas CECT*

Productor

ielab Calidad, S.L.
C/ Dracma, 7
Pol. Ind. Las Atalayas
03114 Alicante (España)
Tlf: +34 966 10 55 01

Descripción

Código: 990334
Microorganismo: *Staphylococcus epidermidis* V596 trazable a CECT 232 (correspondiente con NCTC 11047; WDCM 00132), con un pase desde la cepa de reserva de referencia de la Colección de Cultivos Tipo
Lote nº: PSE40075
Fecha de preparación: 30-nov.-2024
Fecha de caducidad: 07-ene.-2026
Formato: pastilla liofilizada

Pruebas de autenticidad de la Cepa de Colección de Cultivo empleada

Batería de pruebas bioquímicas

Información de seguridad

Grupo de riesgo 2

Condiciones de conservación

Conservar a -20±5ºC

Uso previsto

Controles internos de calidad en términos de precisión (control de proceso, gráficos de control y control de calidad de medios de cultivo).

Condiciones de reconstitución

(indicadas en la Guía Rápida de Uso)
Diluyente: Agua destilada estéril
Volumen: 20 mL
Tiempo de reconstitución: 10 minutos

Condiciones de ensayo del productor

Laboratorio: Un laboratorio siguiendo criterios ISO 17025
Diluciones: Hasta 10^3
Volumen analizado: 1 mL
Técnica: Filtración
Temperatura de incubación: 36 ± 2º C
Tiempo de incubación: 44 ± 4h
Medio de cultivo: TSA (Triptona soja agar)
Membrana filtración: Ésteres de celulosa (0.45 µm)

Controles de calidad en las condiciones de ensayo descritas

Contaminación cruzada: No detectada
Homogeneidad: Homogéneo(u_H=0.077 log)
Estabilidad: Estable(u_E=0.062 log)

Resultados obtenidos en el volumen de reconstitución

Porcentaje de lote analizado: 15%
Número de ensayos: 56
Valor obtenido: 4.25×10^5 ufc/pastilla
Intervalo de confianza 95%:
5.72×10^4 - 3.16×10^6 ufc

Alicante, 7 de enero de 2025

Raquel Múrtula Corbí
Directora técnica área de microbiología.

Fabricado cumpliendo los requerimientos de la norma ISO 17034

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comercial@ielab.es
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