the aim of studying the efficacy of the preparation steps, as well as, to detect possible contaminations.

PROTOCOL

1. Thaw the amplification mix (yellow cap) and the corresponding controls, blue tube (negative control) and red tube (positive control), for 5 min. on ice.
2. Add 15 µL of amplification mix (yellow cap) in each PCR reaction tube.
3. Negative Control Preparation:
   a. PCR negative control: 10 µL of water (blue cap).
   b. Negative control of the process: 10 µL of control sample.

Pipette each negative control into the corresponding reaction tubes containing the amplification mix.

4. Samples Preparation:
Pipette 10 µL of samples into the corresponding reaction tubes containing the amplification mix.
5. Positive Controls Preparation:
   a. PCR positive control: 10 µL from the control DNA (red cap).
   b. Positive control of the process: 10 µL of control sample.

With the aim of quantifying the number of *Legionella* spp. cells in the analyzed samples, a standard curve must be included. This standard curve can be prepared following the directions given in the point 11. In the case of using a robust curve, two control points must be included. Then, pipette 10 µL from a 10^{-2} dilution of control DNA (red cap) (100,000 copies) and, in another tube, pipette 10 µL from a 10^{-4} dilution of control DNA (red cap) (1,000 copies). Prepare the serial dilutions of DNA following the directions given in point 11 of this manual.

6. Proceed to the Amplification reaction of the prepared samples: a variable number of samples to detect the presence of the selected microorganim (one or several), negative controls, and positive controls.

material suitable for PCR in terms of purity, concentration and lack of inhibitors. This kit is designed for research use only.

6. ASSAY TIME TO RESULTS

. Setting-up PCR	10 min
. Amplification (thermocycling)	1 h 50 min
. Analysis and quantification	10 min
Total time	**2 h 10 min**

7. NUMBER OF TESTS

This kit is designed to perform 70 amplification reactions in a final volume of 25µL each one.

8. KIT STORAGE

- Follow the guidelines below for storing the *Legionella* spp. Quantitative Detection Kit:
- The kit is shipped with dry ice. Upon receipt, store the kit at -20 °C and protected from light. Excessive exposure to light may affect the fluorescent probes.
- The components of the unopened kit are stable until the expiration date printed in the label.
- Minimize freeze-thaw cycles by preparing aliquots.

9. SPECIFICITY

The ielab kits for *Legionella* spp. Quantitative Detection contains primers and probes specific for a *Legionella* spp. gene.

Negative for *Legionella* spp. The fluorescence emitted by the *Legionella* spp. probe is below the threshold value. The fluorescence emitted by the IPC probe is above the threshold value.

Inhibited amplification reaction. Neither the *Legionella* spp. target nor the IPC target are amplified (in both cases, the fluorescence is below the threshold value). Therefore, the reaction is not valid due to the presence of PCR inhibitors in the analyzed sample.

QUANTIFICATION

In the case of assays that include a standard curve, the kit for the quantitative detection of Legionella spp. allows to know the number of cells present in a sample. In order to avoid the need to prepare a standard curve in each amplification experiment, it is possible to work with a historic curve (robust curve) including a high number of quantification controls under reproducibility conditions. This way, when preparing the following PCR reactions, only two positive controls containing a known number of copies of Legionella spp. will be enough.

In “real-time” PCR, CT (or threshold cycle) represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. Assuming that PCR is 100% efficient, a relationship exists between threshold cycle and template concentration so that the threshold cycle decreases by one cycle as concentration of template doubles. The software generates a standard curve of CT vs. Log (starting copy number) for all standards and then determines the starting copy number of unknowns by interpolation. In the case of Legionella spp. the copy number calculated will be equivalent to the number of cells because the target is a single copy gene.

3. REAGENTS, MATERIALS AND EQUIPMENT NOT INCLUDED

Apart from the kit, the following reagents and equipment are needed to work with the ielab kits for quantitative detection of *Legionella* spp.

Reagents & Materials
Nuclease-free, sterile-filtered water Disposable gloves Pipet tips, aerosol resistant or positive-displacement Ice container Sterile microcentrifuge tubes (1.5 mL and 0.2 mL) Rack for microcentrifuge tubes (1.5 mL and 0.2 mL) Optical tubes/plates for amplification
Equipment
Benchtop microcentrifuge for 1.5 mL tubes Real-time PCR system Vortexer Pipets (2-20 µL, 20-200 µL, 200-1,000 µL)

4. KIT PRINCIPLE

The ielab kits for the quantitative detection of *Legionella spp.* provides the specific primers and probes, the appropriate reagents and the controls needed for the detection and quantification of *Legionella* spp. based on “real-time” PCR amplification, as well as the protocol and some guidelines for the correct interpretation of results.

It employs a MGB probe labelled with FAM for the specific detection of *Legionella* spp. In order to avoid an inaccurate interpretation of results (false negatives) due to the inhibition of the amplification process, the reaction mix includes an Internal Positive Control (IPC). The DNA

10. AMPLIFICATION PROTOCOL

REAGENT HANDLING RECOMMENDATIONS TO PREVENT CONTAMINATIONS

PCR assays require special laboratory practices to avoid false positive amplifications The high sensitivity of these assays can lead to amplification of a single DNA molecule. In order to get accurate results, it is important to follow these recommendations:

If possible, maintain separate work areas, dedicated equipment, and supplies for:
- Sample preparation
- PCR setup
- PCR amplification
- Analysis of PCR products

Note: Rooms can be simulated using a clean bench or PCR bench available from major laboratory suppliers. Keep the material in these areas properly labeled.

- Prepare aliquots of the kit components.
- Use sterile, nuclease-free plastic disposable materials.
- Do not bring amplified PCR products into the PCR setup area.
- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean non-powdered gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated and before leaving the work area.
- To avoid false positives due to cross contamination, do not include a positive control unless required. If a positive control is necessary, close all unknown sample tubes before pipetting the positive control.
- Open and close all sample tubes and reaction plates carefully. Try not to splash or spray PCR samples.
- It is important an accurate volume measurement with the pipettes. Use only properly calibrated pipettes.

- Keep reactions and components capped as much as possible.
- Use positive-displacement pipettes or aerosol-resistant pipette tips.
- Clean lab benches and equipment after use with freshly diluted 10% bleach solution.

POSITIVE CONTROL

Always analyze a positive control together with the samples. In order to prepare a positive control, pipette the same volume (10 µL) of the supplied DNA solution instead of the sample. It is important to firstly close the tubes containing the negative controls and then the tubes containing the unknown samples, before preparing the positive controls.

Whenever a quantitative assay is intended, it will be necessary to include a standard curve. In that case, proceed with the described protocol in point 11 of this Manual. In order to avoid the need to prepare a standard curve in each amplification experiment, it is possible to work with a historic curve (robust curve) including a high number of quantification controls under reproducibility conditions. This way, when preparing the following PCR reactions, only two positive controls containing a known number of copies of *Legionella* will be enough.

NEGATIVE CONTROL

In each PCR reaction, always analyze a negative control together with your samples. To prepare a negative control substitute the sample volume for PCR-grade water.

CONTROLS OF THE PROCESS

It is advisable to include in each PCR reaction both a negative and a positive control of the whole process. These controls are subjected to the same preparation process than the real samples, and are prepared with

fragment used as IPC consists of a recombinant plasmid that amplifies with the same primers pair than the used to amplify the *Legionella* spp. target, but as it anneals with a MGB probe labelled with VIC it is detected in a different channel. The PCR system detects the produced amplifications in “real-time”, through the increase in the fluorescence level generated by the annealing of the probes with the corresponding targets (*Legionella* spp. and IPC).

The threshold cycle (CT) represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. Assuming that PCR is 100% efficient, a relationship between threshold cycle and template concentration exists so that the threshold cycle decreases by one cycle as concentration of template doubles. The software generates a standard curve of CT vs. Log (starting copy number) for all standards and then determines the starting copy number of unknowns by interpolation.

The ielab kits for the quantitative detection of *Legionella* spp. is specifically designed to perform the amplification reaction in a final volume of 25 µL, using AbiPrism 7000, 7300, 7500 and 7900HT fast Real Time PCR Systems, StepOne and StepOne Plus Systems (Applied Biosystems), although it could be easily adapted to other conditions and/or other equipments available in the market (contact to the manufacturer).

5. APPLICATION

The ielab kits system for the quantitative detection of *Legionella* spp. has been designed for the rapid detection and quantification of *Legionella* spp. starting from any

In the PCR detection system, program the following thermocycling conditions:
1. 50ºC, 2 min,
2 95ºC, 10 min,
3. 95ºC, 15 sec, and 60ºC, 1 min x 42 cycles

11. STANDARD CURVE PREPARATION

In order to perform the quantification of *Legionella* spp. cells present in the analyzed samples, a standard curve should be included. Recommended standard curve: 6 different positive controls analyzed by quintuplicate. Follow these guidelines:

a. Thaw the DNA solution (red cap) and the nuclease free water (blue cap).

b. Label 6 Eppendorf tubes with the corresponding dilution and add 90 µL of nuclease free water.

c. Homogenize the DNA solution by softly vortexing and add 10 µL to the first tube.

d. Close the tube and homogenize by softly vortexing for 15s.

e. Add 10 µL from the first tube to the second one.

f. Proceed in the same way from step c to complete all the tubes of the serial dilution.

12. INTERPRETATION OF RESULTS

Positive for Legionella spp.. The fluorescence emitted by the Legionella spp. probe is above the threshold. The fluorescence emitted by the IPC probe can be above the threshold value; in the case there is a high amount of Legionella target in the sample, the IPC DNA cannot be amplified due to competence with the Legionella DNA, which is favoured in the amplification process.

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RESEARCH AND DEVELOPMENT

The ielab kits system for confirmation by PCR is based on the research and development of LABAQUA, S.A (www.labaqua.com), leading company in environmental diagnostics.

RELATED PRODUCTS

- *Legionella pneumophila* Quantitative Detection Kit (PN 990039)
- Water Microbial Concentration Kit (PN 990075)
- Clean Water DNA Extraction Kit (PN 990074)
- Dirty Water DNA Extraction Kit (PN 990076)
- BAControl-PCR® *Legionella pneumophila* control tablets (PN 990069)

1. GENERAL INTRODUCTION

Some species of the *Legionella* genus are causative agents of legionelosis, an infection that can be presented in two clinical manifestations: pneumonia caused by *Legionella*, known as Legionaire´s disease, and Pontiac fever. These bacteria inhabit natural water environments like rivers and lakes, but can also find a suitable ecological niche in potable water systems, cooling towers and air conditioning systems. Therefore, the presence of *Legionella* cells in bioaerosols originated in these water environments is a potential risk for human health.

The tradicional method employed to detect *Legionella* spp. is the culture isolation using a selective medium. Nevertheless, this method presents important drawbacks like excessive time to results (up to 15 days), the difficulty to isolate *Legionella* in samples with high microbiota content, and the inability to detect viable but not culturable (VBNC) cells. In order to overcome these problems, molecular methods, especially those based on the enzymatic amplification of the bacterial DNA by the Polymerase Chain Reaction (PCR), can be used. In this sense, while conventional PCR provides the results as the microorganism presence/absence, the technology of “real-time” PCR allows for the accurate quantification of the number of cells present in the sample.

The ielab kits for the quantitative detection of *Legionella spp.* has been specifically designed for the detection and quantification of *Legionella* spp. in a variety of water samples by means of the DNA amplification by “real-time” PCR.

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ielab kits

Legionella spp.

QUANTITATIVE DETECTION KIT

User Guide

70 tests

PN 990043

Version 2.0
May 2015

The amplification mix consists of a primer pair for the specific amplification of the bacterial DNA, a recombinant DNA that acts as an Internal Positive Control (IPC) and is amplified with the same primer set than the target fragment from *Legionella* spp., a TaqMan MGB (Minor Groove Binding) probe for the specific detection of *Legionella* spp., and a TaqMan probe for the IPC detection. The kit also provides the adequate controls (positive and negative) for performing the analysis, as well as the protocol and some guidelines for the correct interpretation of the results.

2. KIT CONTENTS

Cap colour	Component Name	Contents	Role
Yellow	Amplification mix	5 x 210 µL -Specific primers -TaqMan Universal Master Mix -Fluorescent probes -IPC (stable solution of plasmid DNA)	Specific amplification of *Legionella* spp. and IPC
Blue	H_2O grade PCR	1 x 1.5 mL - Nuclease-free water	Negative control
Red	Positive control	1 x 60 µL - *Legionella* spp. DNA ($1x10^6$ genomes /µL)	Positive control and standard quantification

13. APPENDIX

TROUBLESHOOTING

Problem	Possible cause	Suggestions
Amplification is not detected, neither the samples nor the positive control	- Pipetting error, lack of amplification mix - Incorrect thermocycling program	-Check the PCR setup, repeat PCR -Always include positive controls
IPC amplification not observed in negative samples	Inhibitors present in the samples	Check the samples preparation
Very low fluorescence signal	-Wrong storage of the kit -Very low DNA concentration	-Store the kit following the recommendations -Increase the starting concentration of DNA
Negative controls are positive	Cross-contamination	-Repeat PCR with fresh aliquots of all the components -Handle samples and reagents following stated criteria for preventing cross-contamination -Always prepare negative controls first, then samples, and finally, positive controls

CONTENTS

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Should any product fail to perform satisfactorily due to other reason than misuse, it will be replaced.

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