Legionella spp.

QUANTITATIVE DETECTION KIT

Part No. 990043

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CONTENTS

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. This bacterium inhabits 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 traditional 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. 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 or quantitative PCR (qPCR) allows for the accurate quantification of the number of cells present in the sample.

The **ielab kits** for the quantitative detection of *Legionella* spp. have been specifically designed for the detection and quantification of *Legionella* spp. in a variety of water samples by means of the DNA amplification by qPCR.

The amplification mix consists of a primer set for the specific amplification of the bacterial DNA, a recombinant DNA that acts as an Internal Positive Control (IPC) and that it is amplified with the same primer set that the target fragment from *Legionella* spp., a TaqMan MGB (Minor Groove Binding) probe for the specific detection of *Legionella* spp., and a TaqMan MGB probe for the IPC detection. The kit also provides the appropriate 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 Environmental MasterMix 2.0 - 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 aprox.10^5 copies/1µL**	Positive control and standard quantification
-	8-well reaction strips	9 strips of 8 reaction tubes	Ready to use in the thermocycles (low or high profile)

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 and materials:
  - Nuclease-free
  - sterile-filtered water
  - Disposable gloves
  - Pipet tips
  - aerosol resistant or positive displacement
  - Ice container
  - Sterile microcentrifuge tubes (1.5mL and 0.2 mL)
  - Rack for microcentrifuge tubes (1.5mL and 0.2 mL)
  - Optical tubes/plates for amplification
- Equipment:
  - Pipets (2-20 µL, 20-200 µL, 200-1000 µL)
  - Benchtop microcentrifuge for 1.5 mL tubes
  - Real-time PCR system
  - Vortex

4. KIT PRINCIPLE AND DESCRIPTION

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. 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 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 an 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 equipment 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 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 30 min
- Analysis and quantification: 10 min

Total time: 1h 50min

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 it at -20±5°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.

10. 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. 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.

- Design a unidirectional process flow. It should begin in the Sample preparation area and then move to the PCR amplification space. Do not return samples, equipment, and reagents to the area in which the previous step was performed.
- 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 tubes 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.

11. PROTOCOL

1. SAMPLE CONCENTRATION

In the case of environmental samples, it is recommended to include a water sample concentration step. This process can be performed following "in house" protocols, standard procedures, or commercial kits. **ielab** offers you the Water Microbial Concentration Kit (Part No. 990075) specially designed for this purpose.

The calculation and monitoring of efficiency of this stage is always important independently of the protocol used.

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

For this purpose, you may use reference materials from **ielab** (Part No. 990069 and Part No. 990083).

2. DNA EXTRACTION

Perform the sample preparation by an "in- house" protocol, following a standard method or using a commercial one. In this last case, manufacturer´s instruction must be followed.

ielab offers also you two different extraction kits, one designed for "clean" water samples (Part No. 990074) and another especially developed for "dirty" samples (Part No. 990076).

3. 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 (blue cap).

4. 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 12 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.

5. PCR AMPLIFICATION

Thaw the amplification mix (yellow cap) and the corresponding controls, negative control (blue cap) and positive control (red tube), for 5 minutes on ice.

1. Add 15 µL of amplification mix (yellow cap) in each PCR reaction tube.
2. 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.
3. Samples Preparation:
   Pipette 10 µL of samples into the corresponding reaction tubes containing the amplification mix.
4. 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 (see point 12 of this document). In the case of using a robust curve, two control points must be included.

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

12. 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 a

standard curve with 6 levels of different concentration analyzed by quintuplicate. Follow these guidelines:

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

13. 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 favored in the amplification process.**

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.

14. APPENDIX

A. 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	-Store the kit following the recommendations
	Very low DNA concentration	-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**

B. REFERENCES

* Catalán, V., Moreno, C., Dasi, M.A., Muñoz, C., and Apraiz, D. (1994). Nested Polymerase Chain reaction for detection of Legionella pneumophila in water. Res. Microbiol. 145: 603-610.

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* Fliermans, C.B., Cherry, W.B., Orrison, L.H., Smith, S.J., Tison, D.L., and Pope, D.H. (1981). Ecological distribution of Legionella pneumophila. Appl. Environ. Microbiol 41: 9- 16.

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C. RELATED PRODUCTS

- *Legionella pneumophila* Quantitative Detection Kit (Part No. 990039)
- Water Microbial Concentration Kit (Part No. 990075)
- Clean Water DNA Extraction Kit (Part No. 990074)
- Dirty Water DNA Extraction Kit (Part No. 990076)
- BAControl-PCR® *L. pneumophila* control tablets (Part No. 990069)
- BAControl-DNA® *L. pneumophila* quantitative DNA (Part N. 990060)

D. 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.

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