

Legionella pneumophila QUANTITATIVE DETECTION KIT

Cat. No. 990039

Version 3 July 2021



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1. GENERAL INTRODUCTION

Legionella pneumophila is one of the main 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 pneumophila* 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 **lelab Kits** for the quantitative detection of *Legionella pneumophila* has been specifically designed for the detection and quantification of *L. pneumophila* in a variety of water samples by means of the DNA amplification by "real-time" PCR.

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 *L. pneumophila*, a TaqMan MGB (Minor Groove Binding) probe for the specific detection of *L. pneumophila*, and a TaqMan MGB 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 Environmental Master Mix 2.0 -Fluorescent probes -IPC (stable solution of plasmid DNA)	Specific amplification of Legionella pneumophila and IPC
Blue	H ₂ O grade PCR	1 x 1.5 mL - Nuclease-free water	Negative control
Red	Positive control	1 x 60 μL - <i>Legionella</i> <i>pneumophila</i> DNA 10 ⁶ 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 **lelab kits** for quantitative detection of *L. pneumophila*.

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 μ L, 20-200 μ L, 200-1,000 μ L)				



4. KIT PRINCIPLE

The **lelab Kits** for the quantitative detection of *Legionella pneumophila* provides the specific primers and probes, the appropriate reagents and the controls needed for the detection and quantification of *L. pneumophila* 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 *L. pneumophila*. 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 fragment used as IPC consists of a recombinant plasmid that amplifies with the same primers pair than the used to amplify the *L. pneumophila* 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 (*L. pneumophila* 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 **lelab Kits** for the quantitative detection of *Legionella pneumophila* is specifically designed to perform the amplification reaction in a final volume of 25 μ L, 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 **lelab Kits** system for the quantitative detection of *Legionella pneumophila* has been designed for the rapid detection and quantification of *L. pneumophila* 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 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 pneumophila 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 pneumophila* Quantitative Detection contains primers and probes specific for a *L. pneumophila* gene.

10. AMPLIFICATION PROTOCOL

10.1. 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.

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

10.3. 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 μ L of water (blue cap).
 - b. Negative control of the process: 10 μ L 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 μ L from the control DNA (red cap).
 - b. Positive control of the process: 10 μ L of control sample.

With the aim of quantifying the number of *L. pneumophila* 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 μ L from a 10⁻² dilution of control DNA (red cap) (100,000 copies) and, in another tube, pipette 10 μ L 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 microorganism (one or several), negative controls, and positive controls.



In the PCR detection system, program the following thermocycling conditions:

- 1. 50°C, 2 min,
- 2. 2 95°C, 10 min,
- 3. 95'C, 15 sec, and 60'C, 1 min x 42 cycles

11. STANDARD CURVE PREPARATION

In order to perform the quantification of *L. pneumophila* 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 $\,\mu\text{L}$ of nuclease free water.
- c. Homogenize the DNA solution by softly vortexing and add 10 μ L to the first tube.
- d. Close the tube and homogenize by softly vortexing for 15s.
- e. Add 10 μ L 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

<u>Positive for Legionella pneumophila</u>. The fluorescence emitted by the *L. pneumophila* 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.

<u>Negative for Legionella pneumophila</u>. The fluorescence emitted by the *L. pneumophila* probe is below the threshold value. The fluorescence emitted by the IPC probe is above the threshold value.

<u>Inhibited amplification reaction</u>. Neither the *L. pneumophila* 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 *L. pneumophila* 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 *L. pneumophila* 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 *L. pneumophila*, the copy number calculated will be equivalent to the number of cells because the target is a single copy gene.

13. 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 positivecontrols
IPC amplification not observed in negative samples	Inhibitors present in the samples	Check the samples preparation
Very low fluorescence signal	- Wrong storage of thekit - 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 freshaliquots 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

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C. RELATED PRODUCTS

- Legionella spp. Quantitative Detection Kit (PN 990043)
- 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)

D. RESEARCH AND DEVELOPMENT

The **lelab 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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