Legionella pneumophila

QUANTITATIVE DETECTION KIT

User Guide

72 tests

Cat. No. 992401 (Low profile)
Cat. No. 992400 (High profile)

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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 *L. 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 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 *L. pneumophila* have been specifically designed for the detection and quantification *of L. pneumophila* 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 *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 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. INTENDED OF USE

L. pneumophila Quantitative detection kit is designed for the rapid and specific 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. Do not use after expiration date.

3. KIT PRINCIPLE AND DESCRIPTION

The ielab kits for the quantitative detection of *L. pneumophila* provide the specific primers and probes, the appropriate reagents and the controls needed for the detection and quantification of *L. pneumophila* based on qPCR 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 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 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 (*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.

Alternatively, you may use our Quantification spreadsheet (Cat. No. 992405) that is available for free in our web site (http://www.ielab.es).

This Kits were validated following the standards ISO/TS 12869 and AFNOR NFT90-471.
If you are going to validate this method based on one of these standards, contact ielab for our support and we will explain you the parameters you have to study because it can be considered as a characterization and a secondary validation of a third party validated method. Moreover, you can also use our spreadsheet specially designed for doing this verification in an easy and quick way (Cat. No. 992405).

4. KIT CONTENTS

The ielab kits for the quantitative detection of *L. pneumophila* include the following materials and reagents detailed below:

Reagent/Material	Description	Color	Amount
L. pneumophila 8-well reaction strips	A mix of enzymes, primers, probes, buffer, dNTPs, stabilizers and IPC control in stabilized format	White	9x8-well strips
Rehydration Buffer	Solution to reconstitute the reaction master mix	Yellow	1 vial x 1.2 mL
L. pneumophila DNA standard PC1	2,500,000 GU/10 µL	Red	1 vial
L. pneumophila DNA standard PC2	250,000 GU/10 µL	Red	1 vial
L. pneumophila DNA standard PC3	25,000 GU/10 µL	Red	1 vial
L. pneumophila DNA standard PC4	2,500 GU/10 µL	Red	1 vial
L. pneumophila DNA standard PC5	250 GU/10 µL	Red	1 vial
L. pneumophila DNA standard PC6	25 GU/10 µL	Red	1 vial
Negative Control	Non-template control	Green	1 vial x 1 mL
Water RNAse/DNAse free	Water RNAse/DNAse free for DNA re-constitution	Blue	1 vial x 1 mL
Tear-off 8-cap strips	Optical caps for sealing plate during thermal cycling	Transparent	9x8-cap strips

Table 1. Reagents and materials provided with the *L. pneumophila* Quantitative Detection Kit (Cat. No. 992400 and Cat No. 992401)

5. 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 *L. pneumophila*:

Reagents & Materials
- Powder-free disposable gloves
- Micropipette tips, aerosol resistant or positive-displacement
- Ice container
- Sterile microcentrifuge tubes (1.5 mL)
- Rack for microcentrifuge tubes (1.5 mL and 0.2 mL)
- DNA extraction and purification kits

Equipment
- Benchtop microcentrifuge for 1.5 mL tubes
- qPCR system (to check compatibility see Annex I)
- Vortexer
- Micropipettes (2-20 µL, 20-200 µL, 200-1,000 µL)

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 72 amplification reactions in a final volume of 25µL each one.

8. TRANSPORT, STORAGE AND SHELF LIFE

- The kits can be shipped at 2-50ºC for a maximum of 2 weeks.
- For long term storage the kit should be stored at 2-8ºC and may be used until the expiration date indicated in the kit.
- Once Positive Controls have been resuspended, store them at -20ºC. We recommend to separate in aliquots to minimize freeze and thaw cycles.
- Keep components away from sunlight.

9. SPECIFICITY

The ielab kits for *L. pneumophila* Quantitative Detection contains primers and probes specific for a *L. pneumophila* 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.

In order to get accurate results, it is important to follow the following recommendations:

- If possible, keep 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 labelled.

- Design a unidirectional process flow. It should begin in the Sample preparation area and then move to the PCR setup and finally 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

SAMPLE CONCENTRATION

In the case of environmental samples, and following the ISO/TS 12869, 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 (Cat. 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 material from ielab (Cat. No. 990069 and Cat. No. 990083).

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

PCR PROTOCOL

Determine and separate the number of required reactions, including samples and controls.

Negative Control

In each PCR reaction, always analyse a negative control together with your samples. To prepare a negative control substitute the sample volume by the non-template control (green vial).

Positive Control

In each PCR reaction, a Positive Control must be always included in the case of a qualitative assay, and for this purpose one of the standards included in the kit, preferable with an intermediate concentration, may be used. In case of a quantitative assay a calibration curve or alternatively two positive standards, containing 25 GU and 2,500 GU, must be included as a verification of a

robust curve and considered as Positive Controls.

L. pneumophila Positive Controls contain different copies template (see in the section 4 of this Guide the kit contain), the recommendation is to open and manipulate them in a separate laboratory area away from the other components. Reconstitute the lyophilized *L. pneumophila* Positive Controls (red vials) adding 100µL of Water RNAse/DNAse free (blue vial) supplied and vortex throughly.

For storage, follow instructions included in page 4 of this document.

Standard Curve

To perform the quantification of *L. pneumophila* Genomic Units (GU) present in the analysed samples, a standard curve should be included. Recommended standard curve: Four different standards (PC3 to PC6), analysed by duplicate.

In order to avoid the need to prepare a standard curve in each amplification run, it is possible to work with a historical curve (robust curve) including a high number of quantification standards under reproducibility conditions. This way, when preparing the following PCR reactions, only two positive standards, containing a 25 GU and 2,500 GU, and analysed by duplicate will be enough.

In case of preparing a robust standard curve, we recommend to perform the six standards included in the kit, analysed by at least two analysts by triplicate and producing three independent curves each of the analysts. For this purpose, you can use our spreadsheet, specially designed for the verification of a third party validated method (Cat. No. 992404).

PCR amplification

1) Peel off protective aluminium seal from plates.
2) Reconstitute the number of wells you need. Add 15 µL of Rehydration Buffer (yellow vial) into each well.
3) Add the Negative Control. Substitute the sample volume (10 µL) by non-template control (green vial).
4) Add 10 µL of sample.
5) Close these wells with the caps provided in the kit. 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.
6) Thaw aliquoted re-suspended lyophilized Positive Controls for 5 min on ice.
7) Add the Positive Control. Add 10 µL of reconstituted DNA of *L. pneumophila* Positive Control (red vial).
   In case of a calibration curve or verification points, proceed in the same way as mentioned for all of the points. Add 10 µL of each of the points.
8) Close these wells with the caps provided in the kit.
9) Ensure that no air bubbles are present at the bottom of the PCR wells (it is possible to centrifuge briefly).
10) Load the strips in the thermocycler.
11) Set up your thermocycler.

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

1. 50ºC, 2 min
2. 95ºC, 10 min
3. 95ºC, 15 s and 60ºC 1 min x 44 cycles

12. INTERPRETATION OF RESULTS

Positive for *L. pneumophila.* 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 favoured in the amplification process.

Negative for *L. pneumophila*. 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.

Inhibited amplification reaction. 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 analysed sample.

Cross contamination. Both the fluorescence emitted by the *L. pneumophila* probe and by the IPC probe are above the threshold. Nevertheless, the negative control resulted positive as well.

In the following table, these four cases are summarised:

L. pneumophila	Internal control (IPC)	Negative control	Positive control	Interpretation
+	+/-	-	+	POSITIVE
-	+	-	+	NEGATIVE
+	+	+	+	INCONCLUSIVE (Cross-contamination)
-	-	-	-	INCONCLUSIVE (Inhibition)

Table 2. Results interpretation

QUANTIFICATION

In qPCR, 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.

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 GU present in a sample. In order to avoid the need of preparing a calibration curve in each amplification run, it is possible to work with a historical curve (robust curve) including a high number of quantification standards analysed under reproducibility conditions. This way, when preparing new PCR runs, only two positive controls containing 25 GU and 2,500 GU of *L. pneumophila* will be enough. For this purpose, you can use our spreadsheet, specially designed for the verification of a third party validated method (Cat. No. 992404).

The software included in the thermocycler use to generate a standard curve of CT vs. Log (starting copy number) for all standards and then determines the starting copy number or GU of unknowns by interpolation.

Alternatively, you may use our Quantification spreadsheet (Cat. No. 992405) that is available for free in our website (http://www.ielab.es).

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

Table 3. Troubleshooting

REFERENCES

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- Yañez, M.A, Carrasco, M.C., Barberá, V.M., and Catalán, V. (2005). Quantitative detection of *Legionella pneumophila* in Water Samples by Immunomagnetic Purification and "real time" PCR Amplification of dotA Gene. Appl. Environ. Microbiol. 71: 3433-41.
- ISO/TS 12869: Water quality-Detection and quantification of *Legionella* spp. And *Legionella pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR).
- AFNOR. NF T 90-471. Qualité de l'eau- Détection et quantification des *Legionella* et/ou *Legionella pneumophila* par concentration et amplification génique par reaction de polymerisation en chaîne en temps reel (RT-PCR).

RELATED PRODUCTS

- *Legionella* spp. Quantitative Detection Kit. High profile (Cat. No. 992402)
- *Legionella* spp. Quantitative Detection Kit. Low profile (Cat. No. 992403)
- Water Microbial Concentration Kit (Cat. No. 990075)
- Clean Water DNA Extraction Kit (Cat. No. 990074)
- Dirty Water DNA Extraction Kit (Cat. No. 990076)
- Quantification software (Cat. No. 992405)
- Validation software (Cat. No. 992404)
- BAControl-PCR *L. pneumophila* control tablets (Cat. No. 990069 and Cat. No. 990083).
- BAControl-DNA *L. pneumophila* control tablets (Cat. No. 990060).

RESEARCH AND DEVELOPMENT

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

PRODUCT WARRANTY

This product has been designed for research use only, and its performance is guaranteed in the manner described in the product literature. The purchaser must determine the suitability of the product for its particular use. ielab refuses any implicit warranty for commercialization or adaptation to particular aims. No other license is granted expressly, impliedly, or by estoppel.

In no event shall ielab be liable for incidental, special, multiple, or consequential losses or damages in connection with or arising from the purchase or use of this product.

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

NOTE TO PURCHASER

ielab kits and related products cannot be resold, altered to be sold, repackaged or employed in other commercial product manufacturing without the written approval of ielab.

ANNEX I: COMPATIBILITY OF THE MOST COMMON qPCR EQUIPMENT

Low profile strips can be used in all PCR thermocyclers equipped with low profile block, like systems listed in table below. High profile strips can be used in all PCR thermocyclers equipped with high or regular profile block, like systems listed in table. If you do not find your thermocycler in the list below, please contact with us.

LOW PROFILE BLOCK THERMOCYCLERS		HIGH PROFILE BLOCK THERMOCYCLERS	
Manufacturer	**Model**	**Manufacturer**	**Model**
ThermoFisher Scientific	7500 Fast Real-Time PCR System	ThermoFisher Scientific	7300 Real-Time PCR System
	7500 Fast Dx Real-Time PCR System		7500 Real-Time PCR System
	QuantStudio™ 12K Flex 96-well Fast		7900 HT Real-Time PCR System
	QuantStudio™ 6 Flex 96-well Fast		ABI PRISM 7000
	QuantStudio™ 7 Flex 96-well Fast		ABI PRISM 7700
	StepOne Plus™ Real-Time PCR System		QuantStudio™ 12K Flex 96-well
	StepOne™ Real-Time PCR System		QuantStudio™ 6 Flex 96-well
	ViiA™ 7 Fast Real-Time PCR System		QuantStudio™ 7 Flex 96-well
Bio-Rad	CFX96 TouchTM Real-Time PCR Detection System		ViiA™ 7 Real-Time PCR System
	Mini OpticonTM Real-Time PCR Detection System	Bio-Rad	CFX96 TouchTM Deep Well Real-Time PCR Detection System
Roche	LightCycler ®480 Real-Time PCR System		iCycleriQTM Real-Time PCR Detection System
	LightCycler ®96 Real-Time PCR System		iCycler iQTM5 Real-Time PCR Detection System
Agilent Technologies	AriaMx Real-Time PCR System		MyiQTM Real-Time PCR Detection System
			MyiQTM2 Real-Time PCR Detection System
		Eppendorf	MastercyclerTMep*realplex*
		Stratagene / Agilent Technologies	Mx3000P™ Real Time PCR System
			Mx3005P™ Real Time PCR System
		Analytik Jena Biometra	TOptical
			qTOWER 2.0

Table 4. Compatibility of qPCR equipment

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