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# DI-Check Legionella pneumophila kit

## MBK0081 - 100 reactions

## INTRODUCTION AND PRODUCT DESCRIPTION

*Legionella*, which is responsible for Legionellosis, is a gram- bacterium ubiquitously present at low concentration in natural aquatic ecosystems. *Legionella* can easily reach and contaminate man-made aquatic environments, such as buildings water systems, cooling towers, fountains which are important sources of infection for humans. Among the described *Legionella* species, *L. pneumophila* is one of the most important water-based bacterial pathogens in developed countries.
The reference method for enumeration of *Legionella* in water is culture, however aside from the fact that requires up to 14 days for analysis it often fails to detect legionellae in water samples. In this context, quantitative real-time PCR is an alternative tool for rapid and accurate detection or quantification of *L. pneumophila* in water samples.

The DI-Check Legionella pneumophila kit is intended for the detection and/or quantification of *L. pneumophila*. The assay is based on dual-labelled real-time PCR technology. Dye-labeled probes target unique DNA sequences specific to *L. pneumophila* and synthetic internal amplification control DNA (IAC). Target DNA, if present, is amplified by PCR and detected in real-time using fluorescent hydrolysis probe chemistry. The fluorescence signal that is generated is detected by the real-time PCR instrument and interpreted by the analysis software.
The kit provides a ready to use PCR mix highly resistant to PCR inhibitors and with an extremely stringent automatic hot-start allowing reaction assembly and temporary storage at room temperature prior to PCR amplification. This robust PCR mix contains an internal control to assess the efficiency of amplification reaction revealing the presence of inhibitory factors in the sample. Quantification of the target *Legionella* species is carried out based on a standard curve covering a concentration range of 25000–25 genome units (GU) per PCR reaction.

**Procedure overview**
The DI-Check Legionella pneumophila method allows to detect and quantify *L. pneumophila* in all types of water samples in less than four hours. The method is based on 3 steps:

- Water samples filtration using 0.45 µm polycarbonate membrane filter
- DNA extraction using DNApure Water Isolation kit (code MBK0080)
- PCR amplification for the detection or quantification of *L. pneumophila* DNA using DI-Check Legionella pneumophila kit (code MBK0081)

The performances of the kits are guaranteed when the DNA is extracted using DNApure Water Isolation kit (code MBK0080).

## CONTENTS AND STORAGE

The DI-Check Legionella pneumophila kit allows to detect up to 98 samples. For quantification test the kit contains reagent to quantify 45 samples.

| Component | Volume |
|---|---|
| L. pneumophila PCR Mix | 2 x 1200 µL |
| Standard DNA | 1 x 10 µL |
| Dilution Buffer | 5 x 1500 µL |
| Negative PCR Control | 1 x 100 µL |
| Reference Material | 2 x 50 µL |

Shipping at room temperature has no dedrimental effect on the performance of this kit. Upon arrival, store the kit at -20°C, protect from light. If properly stored, see the expiration date for the stability of the kit.

## ADDITIONAL EQUIPMENT AND MATERIAL REQUIRED

**Reagents and disposables**

- Micropipettes and filter tips,
- Vortexer,
- Micro-centrifuge,
- Powder free gloves,
- 1.5 ml tubes,
- PCR plastics (tubes or PCR plates, sealing tapes and caps) compatible with the PCR cycler to be used.

**Instruments and software**

- Applied Biosystems™ QuantStudio 96-well (Thermo Fisher Scientific),
- CFX96 Touch™ Real-Time PCR Detection System; CFX96 Touch™ Deepwell detection system (Biorad),
- Rotor-Gene Q (Qiagen),
- DI-Check Analysis Software for *L. pneumophila* Ver 2.2 (Diatheva).

For the use on different PCR cyclers please contact Diatheva.

## PRECAUTIONS AND RECOMMENDATIONS

- **For the quantification, it is necessary to test Samples, Negative PCR Control (NTC), Standards and Reference Material in duplicates at each amplification series.**
- The test must be performed by specialised, trained and authorised staff,
- Do not use reagents after the expiry date printed on the label,
- Use gloves as well as sterile pipet tips with filters. Change gloves often, especially if you suspect a possible contamination of them,
- It is suggested to provide separate and dedicated spaces, material and equipment for pre- and post-PCR amplification stages,
- Clean working space periodically with at least 5% sodium hypochlorite or another decontaminant agent.

## RELEVANT ASSAY CONTROLS

**Internal Amplification Control**

Each vial of L. pneumophila PCR Mix contain an internal amplification control (IAC) that is co-amplified with the DNA from the sample, allowing to detect the presence of PCR inhibitors in the DNA extract.

**Standard DNA**

Two vials of Standard DNA are provided to prepare the standard curve. It is provided to allow for 5 independent quantification experiments. The Standard DNA should be diluted to obtain 4 levels of *L. pneumophila* genome unit (GU): 25000, 2500, 250 and 25 GU/5 µL. It is suggested to analyse samples and standards in duplicated.

**Positive PCR Control**

Positive PCR Control contains purified *L. pneumophila* DNA ATCC33152. The Positive PCR Control should be included in each qualitative amplification series to verify the success of the amplification step ensuring the confidence in your PCR results.

**Reference Material**

The Reference Material contains purified *L. pneumophila* ATCC33152 DNA connected to the Standard Reference Material. The Reference Material is a ready to use DNA solution corresponding to a concentration of 1200 GU/5 µL, that shall be tested according to ISO/TS 12869 standard. During a quantification experiment the verification of the Reference Material genome units ensures preparation of an accurate standard curve and a correct quantification calculation.

**Negative PCR Control**

Negative PCR Control should be included in each amplification series to check for possible contamination of the assay during reaction setup. Instead of adding sample DNA to a reaction vial containing L. pneumophila PCR Mix, add the same volume of Negative PCR Control.

# PROCEDURE

## 1. SAMPLE PREPARATION

### 1.1 WATER SAMPLE FILTRATION AND DNA EXTRACTION

For sample preparation please refer to the product information of DNApure Water Isolation kit (code MBK0080). DNA sample should be mixed and centrifuged briefly prior to amplification.

### 1.2. BACTERIAL COLONY (not in the scope of NF VALIDATION)

Real-time PCR could be used for GVPC or BCYE colony confirmation.

- Dispense 100 µL of sterile water *Legionella*-DNA free in a 1.5 ml tube and dissolve the colony using a sterile loop.
- Boil the sample for 10 minutes.
- Centrifuge at 14 000 rpm for 10 minutes.
- Transfer 50 µL of supernatant in a new 1.5 ml tube taking care to do not disrupt the pellet.
- Vortex, centrifuge briefly and use 1 µL of the DNA in the real-time PCR reaction.

## 2. PCR MIX PREPARATION

### 2.1 QUALITATIVE TEST

Samples are tested in single. Include in each amplification series a Negative PCR Control (NTC- No template Control) and a Positive PCR Control (Positive Control).

- Thaw the L. pneumophila PCR Mix and Negative PCR Control. Vortex 15" and briefly spin vials in a microcentrifuge before opening.
- Aliquot 20 µL of L. pneumophila PCR mix in the PCR tubes or in the plate prepared for the experiment.

*Note: once aliquoted the L. pneumophila PCR mix may be stored for 3 h at 4°C.*

- Add 5 µL of Negative PCR Control in the corresponding tube.
- In a separate area, add 5 µL of DNA samples to be tested, into the corresponding PCR tubes or wells containing amplification mixes.
- Add 5 µL of Positive PCR Control (prepared as indicated in section 3) into the corresponding PCR tubes or wells containing amplification mixes.
- Seal hermetically the PCR tubes or plate and load into the real-time PCR instrument, following the manufacturer's instructions.

### 2.2 QUANTITATIVE TEST

It is necessary to test each Samples, Standards, Negative PCR Control (NTC) and Reference Material (Positive Control) in duplicates.

- Thaw the L. pneumophila PCR Mix and Negative PCR Control. Vortex 15" and briefly spin vials in a microcentrifuge before opening.
- Aliquot 20 µL of L. pneumophila PCR mix in the PCR tubes or in the plate prepared for the experiment.

*Note: once aliquoted the L. pneumophila PCR mix may be stored for 3 h at 4°C.*

- Add 5 µL of Negative PCR Control in the corresponding tubes.
- In a separate area, add 5 µL of DNA samples to be tested, into the corresponding PCR tubes or wells containing amplification mixes.
- Add 5 µL of standard curve dilutions (prepared as indicated in section 4) into the corresponding PCR tubes or wells containing amplification mixes.
- Seal hermetically the PCR tubes or plate and load into the real-time PCR instrument, following the manufacturer's instructions.

*Note: for PCR instrument with 96 wells block check that the reagents are at the bottom of each well, if not, briefly centrifuge at 800 x g for 1 minute.*

## 3. POSITIVE PCR CONTROL PREPARATION (only for qualitative test)

The Standard DNA could be used for the preparation of Positive PCR Control:

- Thaw one vial of Dilution Buffer and Standard DNA,
- Vortex 10" and briefly spin the vial in a microcentrifuge before opening Standard DNA,
- Pipette 998.7 µL of Dilution Buffer into a 1.5 ml tube,
- Pipette 1.3 µL of Standard DNA into the STD1 tube,
- Vortex 10" and centrifuge briefly.

*Note: Once prepared the Positive PCR Control could be stored at -20°C for 3 months.*

## 4. STANDARD CURVE PREPARATION (only for quantitative test)

The Standard DNA and Dilution Buffer reagents are provided to prepare the standard curve. In a separate area proceed with the preparation of serial dilutions to create the standard curve.

| Standard | Concentration to be entered as standard [GU/5 µL] |
|---|---|
| *25000* | 25000 GU/5 µL |
| *2500* | 2500 GU/5 µL |
| *250* | 250 GU/5 µL |
| *25* | 25 GU/5 µL |

- Thaw one vial of Dilution Buffer and Standard DNA,
- Vortex 10" and briefly spin the vial in a microcentrifuge before opening Standard DNA,
- Prepare four 1.5 ml tubes in series named from *25000* to *25*,
- Pipette 998.7 µL of Dilution Buffer into tube *25000* and 90 µL into the three tubes remained (*2500*, *250*, *25*),
- Pipette 1.3 µL of Standard DNA into the *25000* tube,
- Vortex 10" and centrifuge briefly,
- Pipette 10 µL from tube *25000* into tube *2500*,
- Vortex 10" and centrifuge briefly,
- Repeat steps, to complete the dilution series for *250* and *25*.

*Note: Once prepared the standard curve solutions could be stored at 4°C for 3 hours.*

***Continue with Section:***

**A. QuantStudio:** if you work with real-time PCR instrument QuantStudio 96-wells
**B. CFX:** if you work with real-time PCR instrument CFX96 Touch™ Real-Time PCR Detection System; CFX96 Touch™ Deepwell detection system
**C. Rotor-Gene Q:** if you work with real-time PCR instrument Rotor-Gene Q

# Section A: QuantStudio

## 5. PROGRAM SETUP

Program PCR instrument before preparing the reaction mix.
Use the following real-time PCR-protocol for the DI-Check Legionella pneumophila kit. For details on how to program the experimental protocol, see the Instrument Operator's Manual of your real-time PCR cycler:

| Step | Temperature and times | Cycles |
|---|---|---|
| Initial denaturation | 95°C<br><br>3 min | 1 X |
| Denaturation | 95°C<br><br>15 sec | 45 X |
| Annealing-extension | 60°C<br><br>90 sec | |

Fluorescence is detected during annealing-extension step on green channel (FAM dye) for *L. pneumophila* and yellow channel (VIC dye) for Internal Amplification Control.

Select ROX as passive reference dye and specify the type of probes quencher. The DI-Check Legionella pneumophila kit contains probes with a non-fluorescent quencher (NFQ).

## 6. PLATE SETUP

Define the plate set-up.

Qualitative Test: Fill in information in each well according to the table below

| **Controls and Samples** | **Task** | **Sample Name** |
|---|---|---|
| Negative PCR Control | NTC | NTC |
| Positive PCR Control | Unknown | Positive Control |
| Sample | Unknown | Assign different names to different samples |

Quantitative Test: Fill in information in each well according to the table below

| **Controls, Standards and Samples** | **Task** | **Sample Name** |
|---|---|---|
| Negative PCR Control | NTC | NTC |
| Reference Material | Unknown | Reference Material |
| Standard | Standard | 25000, 2500, 250, 25 |
| Sample | Unknown | Assign different names to different samples |

Check the instrument is ready and start the run.

## 7. DATA ANALYSIS

The analysis of the results must be done with the program of the PCR instrument, please refer to the manual for detailed information.

- Analyse the PCR run for the two fluorophores separately, positioning the threshold as indicated in the table below:

| Threshold Value | |
|---|---|
| *L. pneumophila*<br>FAM | Internal Amplification Control<br>VIC |
| 0.3 | 0.05 |

- Select "Export" function. In the opened window, please check that (*.xls) is selected in File Type, select the Location and click on the upper right "Export" function.
- Open the generated excel file. Open the "Results" sheet.
- In the Task column, for Qualitative Test assign the name "Positive control" to Positive PCR Control. For quantitative Test assign the name "Positive control" to Reference Material, moreover, for the Standard (25000, 2500, 250 and 25) assign the task "standard" also for VIC (Internal Amplification Control).
- Copy the lines from line (Well, Well position, Omit…) of the excel sheet and proceed with the DI-Check Analysis Software Ver 2.2 for *L. pneumophila* according to the section below.

## 8. INTERPRETATION AND EXPRESSION OF RESULTS

Using the DI-Check Analysis Software Ver 2.2 for *L. pneumophila* the interpretation of samples and controls will be performed automatically.

- Open the DI-Check Analysis Software Ver 2.2 for *L. pneumophila*
- Select QuantStudio function and paste the copied data, from excel file, on cell A1 in the "insert data QS" sheet of the DI-Check Analysis Software Ver 2.2 for *L. pneumophila*

• Click on "Qualitative analysis" box for Qualitative Test or "Quantitative analysis" box for Quantitative Test. The Quantitative analysis requires that the function "Selection unlock" is activated using the following password "Diatheva".

### 8.1 QUALITATIVE TEST

**a. Quality Control**

Before proceeding with the analysis of samples, check the validity of controls. If the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* shows that both controls are "OK", the operator can proceed with the result interpretation of samples. Otherwise, if controls are "KO", the PCR is invalid and must be repeated (see table below).

| **Control type** | **Control validity** | **L. pneumophila result** | **Internal Amplification Control result** |
|---|---|---|---|
| Negative PCR Control | OK | N/A* | 27≤Ct≤37 |
| | OK | Ct>Intercept value** | 27≤Ct≤37 |
| | KO | Ct<Intercept value** | Non-significant |
| Positive PCR Control | OK | 20≤Ct≤30 | Non-significant |
| | KO | 20>Ct>30 | Non-significant |

* N/A means "Not Applicable". The software indicates N/A when the fluorescence curve doesn't rise above the threshold.
**The intercept value to be considered in the contest of Qualitative analysis is Ct=43. This value is used to give an indication of the intercept value chosen during the validation.

**b. Samples**

Sample results shall be interpreted as shown in the table below

| Interpretation using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* | | | Interpretation without using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* (not in the scope of NF VALIDATION) | |
|---|---|---|---|---|
| ***Detection of L. pneumophila*** | ***Inhibition*** | ***Results and expression per Liter (GU/L)*** | ***L. pneumophila result*** | ***Internal Amplification Control result*** |
| Positive | Inhibition | L. pneumophila detected | Ct≤Intercept value* | Non-significant |
| | No Inhibition | | | |
| Negative | No Inhibition | L. pneumophila not detected | Ct>Intercept value* | 27≤Ct≤37 |
| | Inhibition-Redo test | Inhibition. Dilute the extracted DNA using Elution Buffer (MBK0080) or PCR grade water and repeat PCR amplification | Ct>Intercept value* | Ct>37 |

* The intercept value to be considered in the contest of Qualitative analysis is Ct=43. This value is used to give an indication of the intercept value chosen during the validation.

**c. Detection limit of the molecular method ($LOD_{meth}$)**

The limit of detection of the PCR step corresponds to the smallest number of genome units that provides a positive PCR result at the 90% threshold (ISO/TS 12869:2012). The LOD of the PCR step is 5 GU per 5 µL of extracted DNA. The LOD of the method ($LOD_{meth}$) depends on the volume of water sample filtered according to the following formula:

$$LOD_{meth} = \frac{5 \times F \times D}{V}$$

Where:
**F** = conversion factor GU/5 µL to GU/L. The F conversion factor of DNApure Water Isolation kit (MBK0080) is 64. For more information refer to the manual of the kit.
**V**= volume of water sample filtered expressed in litres.
**D**= DNA dilution factor (if the DNA has been diluted before PCR run).

## 8.2 QUANTITATIVE TEST

### a. Controls and Standard

Before proceeding with the analysis of the samples, check the validity of controls and Standard curve. If the software DI-Check Analysis Software Ver 2.2 for L. pneumophila shows that parameters are "OK", the operator can proceed with the result interpretation of samples. Otherwise, if parameters are "KO", the PCR is invalid and must be repeated (see table below).

| Parameter | Quality Control validity | L. pneumophila result | Internal Amplification Control result |
|---|---|---|---|
| Negative PCR Control | OK | N/A[a] | 27≤Ct≤37 |
| | OK | Ct>Intercept value[b] | 27≤Ct≤37 |
| | KO | Ct<Intercept value[b] | Non-significant |
| Slope | -3.75≤ Slope ≤-3.00 | -3.75≤ Slope ≤-3.00 | Non-significant |
| Correlation coefficient ($R^2$) | ≥0.95 | ≥0.95 | |
| Reference Material | OK | 601≤ GU/well ≤2398[c] | Non-significant |
| | KO | 601≥ GU/well ≥2398[c] | Non-significant |
| LOQ | OK | Compliant to ISO-TS 12869 standard | Non-significant |
| | KO | Compliant to ISO-TS 12869 standard | Non-significant |

[a] N/A means "Not Applicable". The software indicates N/A when the fluorescence curve doesn't rise above the threshold
[b] The intercept value to be considered in the contest of Quantitative analysis is systematically calculated in the calibration function of the amplification run.
[c] The Reference Material shall be tested according to ISO-TS 12869 standard

*The above values were determined during the method validation.*

### b. Samples

| Detection of *L. pneumophila* N GU/5 µl | Inhibition[a] | *L. pneumophila* detection Results and expression per liter (GU/L)[b] | | Note |
|---|---|---|---|---|
| N < 5 | No Inhibition | LQ | L. pneumophila not detected | Refer to paragraph 8.2.d for $LOD_{meth}$ calculation |
| | Inhibition | LQ | The sample DNA shall be diluted and PCR run repeated | |
| 5 ≤ N < 25 | No Inhibition | LQ | L. pneumophila detected below the limit of quantification | Refer to paragraph 8.2.d for $LOD_{meth}$ calculation |
| | Inhibition | LQ | The sample DNA shall be diluted and PCR run repeated | |
| 25000 ≥ N ≥ 25 | No Inhibition | L. pneumophila quantitatively detected | | Refer to paragraph 8.2.c |
| | Inhibition | The sample DNA shall be diluted and PCR run repeated | | |
| N > 25000 | No Inhibition | L. pneumophila detected above the upper limit of quantification | | Refer to paragraph 8.2.d for $UQL_{meth}$ calculation. The quantification can be obtained after DNA dilution |
| | Inhibition | The sample DNA shall be diluted and PCR run repeated | | |

[a] For the interpretation of results without using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* (not in the scope of NF VALIDATION), the Internal Amplification Control (VIC) is compliant if 27≤ Ct ≤37, (No Inhibition).
[b] Please if the sample has been diluted the Result GU/L must be multiplied by the dilution factor

### c. Calculation of *L. pneumophila* concentration in water samples

The software DI-Check Analysis Software Ver 2.2 for *L. pneumophila automatically* calculate the *L. pneumophila* GU/L by entering the filtered water volume (mL) in the column "Filtration volume" for each sample.

To calculate the amount of *L. pneumophila* in the water sample analysed without using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* (not in the scope of NF VALIDATION), it is necessary to consider (1) the volume of sample filtered in litres and (2) the *F* conversion factor. The F conversion factor of DNApure Water Isolation kit (MBK0080) is 64. For more information refer to the manual of the kit.
The instrument software automatically calculates the amount of GU of *L. pneumophila* per reaction (in 5 µL), this value corresponds to **N** parameter. To obtain the *L. pneumophila* GU contained in 1 Liter please use the following formula:

$$\textit{L. pneumophila}\ \text{Genome Units per liter} = \frac{N \times F \times D}{V}$$

Where:
N= number GU of *L. pneumophila*/5 µL (PCR result)
F = conversion factor GU/5 µL to GU/L.
V= volume of water sample filtered expressed in litres
D**=** DNA dilution factor (if the DNA has been diluted before PCR run)

Express the results in number of Genome Units of *L. pneumophila* per litre of water sample, considering two significant figures: ex. 12 312 GU/L of *L. pneumophila* is expressed as "12 000 GU/L of *L. pneumophila*".

**d. Theoretical detection limit (LODmeth, Quantification limit ($LOQ_{meth}$), and Upper quantification limit ($UQL_{meth}$) of the molecular method**

**The detection limit of the PCR step is 5 GU/**5 µL. The LOD of the method ($LOQ_{meth}$) is 320 GU/L when 1 Liter is filtered. $LOD_{meth}$ depends on the volume of water sample filtered and can be calculated according to the following formula:

$$LOD_{meth} = \frac{5 \times F}{V}$$

The limit of quantification of the PCR step is 25 GU/5 µL. The LOQ of the method ($LOQ_{meth}$) is 1600 GU/L when 1 Liter is filtered. The $LOQ_{meth}$ depends on the volume of water sample filtered and can be calculated according to the following formula:

$$LOQ_{meth} = \frac{25 \times F}{V}$$

The upper quantification limit of the method ($UQL_{meth}$) is $1.6 \times 10^6$ GU/L when 1 Liter is filtered. The $UQL_{meth}$ can be obtained with the following formula:

$$UQL_{meth} = \frac{25\ 000 \times F}{V}$$

Where:
**F** = conversion factor GU/5 µL to GU/L.
**V**= volume of water sample filtered expressed in litres

# Section B: CFX

## 5. PROGRAM SETUP

Program PCR instrument before preparing the reaction mix.
Use the following real-time PCR-protocol for the DI-Check Legionella pneumophila kit. For details on how to program the experimental protocol, see the Instrument Operator's Manual of your real-time PCR cycler:

| Step | Temperature and times | Cycles |
|---|---|---|
| Initial denaturation | 95°C<br><br>3 min | 1 X |
| Denaturation | 95°C<br><br>15 sec | 45 X |
| Annealing-extension | 60°C<br><br>90 sec | |

Fluorescence is detected during annealing-extension step on green channel (FAM dye) for *L. pneumophila* and yellow channel (HEX dye) for Internal Amplification Control.

## 6. PLATE SETUP

Define the plate set-up.

Qualitative Test: Fill in information in each well according to the table below

| **Controls and Samples** | **Content** | **Sample** |
|---|---|---|
| Negative PCR Control | NTC | NTC |
| Positive PCR Control | Positive Control | Positive Control |
| Sample | Unknown | Assign different names to different samples |

Quantitative Test: Fill in information in each well according to the table below

| **Controls, Standards and Samples** | **Content** | **Sample** |
|---|---|---|
| Negative PCR Control | NTC | NTC |
| Reference Material | Positive Control | Reference Material |
| Standard | Standard | 25000, 2500, 250, 25 |
| Sample | Unknown | Assign different names to different samples |

Check the instrument is ready and start the run.

## 7. DATA ANALYSIS

The analysis of the results must be done with the program of the PCR instrument, please refer to the manual for detailed information.

- Analyse the PCR run for the two fluorophores separately, positioning the threshold as indicated in the table below:

| Threshold Value | |
|---|---|
| *L. pneumophila*<br>FAM | Internal Amplification Control<br>HEX |
| 200 | 100 |

- Select "Export" function. In "Export All Data Sheets" select "Excel 2007" or "Excel 2003" on the bases of your pc excel version. Select the location where save the excel files.
- Open the generated "Quantification Summary" excel file. Open the "0" sheet.
- Copy the entire "0" sheet and proceed with the DI-Check Analysis Software Ver 2.2 for *L. pneumophila* according to the section below.

## 8. INTERPRETATION AND EXPRESSION OF RESULTS

Using the DI-Check Analysis Software Ver 2.2 for *L. pneumophila* the interpretation of samples and controls will be performed automatically.

- Open the DI-Check Analysis Software Ver 2.2 for *L. pneumophila*.
- Select CFX function and paste the copied "0" sheet in the "insert data CFX" sheet of the DI-Check Analysis Software Ver 2.2 for *L. pneumophila.*
- Click on "Qualitative analysis" box for Qualitative Test or "Quantitative analysis" box for Quantitative Test. The Quantitative analysis requires that the function "Selection unlock" is activated using the following password "Diatheva".

## 8.1 QUALITATIVE TEST

**a. Quality Control**

Before proceeding with the analysis of samples, check the validity of controls. If the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* shows that both controls are "OK", the operator can proceed with the result interpretation of samples. Otherwise, if controls are "KO", the PCR is invalid and must be repeated (see table below).

| **Control type** | **Control validity** | **L. pneumophila result** | **Internal Amplification Control result** |
|---|---|---|---|
| Negative PCR Control | OK | N/A* | 27≤Ct≤37 |
| | OK | Ct>Intercept value** | 27≤Ct≤37 |
| | KO | Ct<Intercept value** | Non-significant |
| Positive PCR Control | OK | 20≤Ct≤30 | Non-significant |
| | KO | 20>Ct>30 | Non-significant |

* N/A means "Not Applicable". The software indicates N/A when the fluorescence curve doesn't rise above the threshold.

**The intercept value to be considered in the contest of Qualitative analysis is Ct=42. This value is used to give an indication of the intercept value chosen during the validation.

**b. Samples**

Sample results shall be interpreted as shown in the table below

| Interpretation using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* | | | Interpretation without using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* (not in the scope of NF VALIDATION) | |
|---|---|---|---|---|
| ***Detection of L. pneumophila*** | ***Inhibition*** | ***Results and expression per Liter (GU/L)*** | ***L. pneumophila result*** | ***Internal Amplification Control result*** |
| Positive | Inhibition<br>No Inhibition | L. pneumophila detected | Ct≤Intercept value* | Non-significant |
| Negative | No Inhibition | L. pneumophila not detected | Ct>Intercept value* | 27≤Ct≤37 |
| | Inhibition-Redo test | Inhibition. Dilute the extracted DNA using Elution Buffer (MBK0080) or PCR grade water and repeat PCR amplification | Ct>Intercept value* | Ct>37 |

* The intercept value to be considered in the contest of Qualitative analysis is Ct=42. This value is used to give an indication of the intercept value chosen during the validation.

**c. Detection limit of the molecular method ($LOD_{meth}$)**

The limit of detection of the PCR step corresponds to the smallest number of genome units that provides a positive PCR result at the 90% threshold (ISO/TS 12869:2012). The LOD of the PCR step is 5 GU per 5 µL of extracted DNA. The LOD of the method ($LOD_{meth}$) depends on the volume of water sample filtered according to the following formula:

$$LOD_{meth} = \frac{5 \times F \times D}{V}$$

Where:

**F** = conversion factor GU/5 µL to GU/L. The F conversion factor of DNApure Water Isolation kit (MBK0080) is 64. For more information refer to the manual of the kit.

**V**= volume of water sample filtered expressed in litres.

**D**= DNA dilution factor (if the DNA has been diluted before PCR run).

## 8.2 QUANTITATIVE TEST

**a. Controls and Standard**

Before proceeding with the analysis of the samples, check the validity of controls and Standard curve. If the software DI-Check Analysis Software Ver 2.2 for L. pneumophila shows that parameters are "OK", the operator can proceed with the result interpretation of samples. Otherwise, if parameters are "KO", the PCR is invalid and must be repeated (see table below).

| Parameter | Quality Control validity | L. pneumophila result | Internal Amplification Control result |
|---|---|---|---|
| Negative PCR Control | OK | N/A[a] | 27≤Ct≤37 |
| | OK | Ct>Intercept value[b] | 27≤Ct≤37 |
| | KO | Ct<Intercept value[b] | Non-significant |
| Slope | -3.75≤ Slope ≤-3.00 | -3.75≤ Slope ≤-3.00 | Non-significant |
| Correlation coefficient ($R^2$) | ≥0.95 | ≥0.95 | |
| Reference Material | OK | 601≤ GU/well ≤2398[c] | Non-significant |
| | KO | 601≥ GU/well ≥2398[c] | Non-significant |
| LOQ | OK | Compliant to ISO-TS 12869 standard | Non-significant |
| | KO | Compliant to ISO-TS 12869 standard | Non-significant |

[a] N/A means "Not Applicable". The software indicates N/A when the fluorescence curve doesn't rise above the threshold
[b] The intercept value to be considered in the contest of Quantitative analysis is systematically calculated in the calibration function of the amplification run.
[c] The Reference Material shall be tested according to ISO-TS 12869 standard

*The above values were determined during the method validation.*

**b. Samples**

| Detection of *L. pneumophila* N GU/5 µl | Inhibition[a] | *L. pneumophila* detection Results and expression per liter (GU/L)[b] | | Note |
|---|---|---|---|---|
| N < 5 | No Inhibition | LQ | L. pneumophila not detected | Refer to paragraph 8.2.d for $LOD_{meth}$ calculation |
| | Inhibition | LQ | The sample DNA shall be diluted and PCR run repeated | |
| 5 ≤ N < 25 | No Inhibition | LQ | L. pneumophila detected below the limit of quantification | Refer to paragraph 8.2.d for $LOD_{meth}$ calculation |
| | Inhibition | LQ | The sample DNA shall be diluted and PCR run repeated | |
| 25000 ≥ N ≥ 25 | No Inhibition | L. pneumophila quantitatively detected | | Refer to paragraph 8.2.c |
| | Inhibition | The sample DNA shall be diluted and PCR run repeated | | |
| N > 25000 | No Inhibition | L. pneumophila detected above the upper limit of quantification | | Refer to paragraph 8.2.d for $UQL_{meth}$ calculation. The quantification can be obtained after DNA dilution |
| | Inhibition | The sample DNA shall be diluted and PCR run repeated | | |

[a] For the interpretation of results without using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* (not in the scope of NF VALIDATION), the Internal Amplification Control (HEX) is compliant if 27≤ Ct ≤37, (No Inhibition).
[b] Please if the sample has been diluted the Result GU/L must be multiplied by the dilution factor

**c. Calculation of *L. pneumophila* concentration in water samples**

The software DI-Check Analysis Software Ver 2.2 for *L. pneumophila automatically* calculate the *L. pneumophila* GU/L by entering the filtered water volume (mL) in the column "Filtration volume" for each sample.

To calculate the amount of *L. pneumophila* in the water sample analysed without using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* (not in the scope of NF VALIDATION), it is necessary to consider (1) the volume of sample filtered in litres and (2) the *F* conversion factor. The F conversion factor of DNApure Water Isolation kit (MBK0080) is 64. For more information refer to the manual of the kit.
The instrument software automatically calculates the amount of GU of *L. pneumophila* per reaction (in 5 µL), this value corresponds to **N** parameter. To obtain the *L. pneumophila* GU contained in 1 Liter please use the following formula:

$$\textit{L. pneumophila}\ \text{Genome Units per liter} = \frac{N \times F \times D}{V}$$

Where:

N= number GU of *L. pneumophila*/5 µL (PCR result)
F = conversion factor GU/5 µL to GU/L.
V= volume of water sample filtered expressed in litres
D**=** DNA dilution factor (if the DNA has been diluted before PCR run)

Express the results in number of Genome Units of *L. pneumophila* per litre of water sample, considering two significant figures: ex. 12 312 GU/L of *L. pneumophila* is expressed as "12 000 GU/L of *L. pneumophila*".

**d. Theoretical detection limit (LODmeth, Quantification limit ($LOQ_{meth}$), and Upper quantification limit ($UQL_{meth}$) of the molecular method**

**The detection limit of the PCR step is 5 GU/**5 µL. The LOD of the method ($LOQ_{meth}$) is 320 GU/L when 1 Liter is filtered. $LOD_{meth}$ depends on the volume of water sample filtered and can be calculated according to the following formula:

$$LOD_{meth} = \frac{5 \times F}{V}$$

The limit of quantification of the PCR step is 25 GU/5 µL. The LOQ of the method ($LOQ_{meth}$) is 1600 GU/L when 1 Liter is filtered. The $LOQ_{meth}$ depends on the volume of water sample filtered and can be calculated according to the following formula:

$$LOQ_{meth} = \frac{25 \times F}{V}$$

The upper quantification limit of the method ($UQL_{meth}$) is $1.6 \times 10^6$ GU/L when 1 Liter is filtered. The $UQL_{meth}$ can be obtained with the following formula:

$$UQL_{meth} = \frac{25\,000 \times F}{V}$$

Where:
**F** = conversion factor GU/5 µL to GU/L.
**V**= volume of water sample filtered expressed in litres

# Section C: Rotor-Gene Q

## 5. PROGRAM SETUP

Program PCR instrument before preparing the reaction mix.
Use the following real-time PCR-protocol for the DI-Check Legionella pneumophila kit. For details on how to program the experimental protocol, see the Instrument Operator's Manual of your real-time PCR cycler:

| Step | Temperature and times | Cycles |
|---|---|---|
| Initial denaturation | 95°C<br><br>3 min | 1 X |
| Denaturation | 95°C<br><br>15 sec | 45 X |
| Annealing-extension | 60°C<br><br>90 sec | |

Fluorescence is detected during annealing-extension step on green channel (FAM dye) for *L. pneumophila* and yellow channel (VIC dye) for Internal Amplification Control.

For Rotor-Gene Q instruments that allows the gain optimization on the acquiring channels, set the gain optimization on NTC sample (tube position 1).

## 6. PLATE SETUP

Define the plate set-up.

Qualitative Test: Fill in information in each well according to the table below

| **Controls and Samples** | **Type** | **Name** |
|---|---|---|
| Negative PCR Control | NTC | NTC |
| Positive PCR Control | Positive Control | Positive Control |
| Sample | Unknown | Assign different names to different samples |

Quantitative Test: Fill in information in each well according to the table below

| **Controls, Standards and Samples** | **Type** | **Name** |
|---|---|---|
| Negative PCR Control | NTC | NTC |
| Reference Material | Positive Control | Reference Material |
| Standard | Standard | 25000, 2500, 250, 25 |
| Sample | Unknown | Assign different names to different samples |

Check the instrument is ready and start the run.

## 7. DATA ANALYSIS

The analysis of the results must be done with the program of the PCR instrument, please refer to the manual for detailed information.

- Analyse the PCR run for the two fluorophores separately, positioning the threshold as indicated in the table below:

| Threshold Value | |
|---|---|
| *L. pneumophila*<br>FAM | Internal Amplification Control<br>VIC |
| 0.085 | 0.18 |

- In "Quantitation Results" window, click the right mouse button and select "Export to excel". Select the location where save the excel file. Export data for both fluorescent channels (FAM and VIC), two separates excel files are saved.
- Open the generated excel files.
- Copy the lines from line (No, Name, Type…) of the opened excel file obtained for the FAM dye. For the VIC dye proceed to copy the lines without line (No, Name, Type…), and proceed with the DI-Check Analysis Software Ver 2.2 for *L. pneumophila* according to the section below.

## 8. INTERPRETATION AND EXPRESSION OF RESULTS

Using the DI-Check Analysis Software Ver 2.2 for *L. pneumophila* the interpretation of samples and controls will be performed automatically.

- Open the DI-Check Analysis Software Ver 2.2 for *L. pneumophila*

- Select Rotor-Gene Q function and paste the copied FAM excel data on cell A1 of the "insert data 36" sheet of the DI-Check Analysis Software Ver 2.2 for *L. pneumophila*
- Paste the copied VIC excel data immediately below the FAM pasted data in the "insert data 36" sheet of the DI-Check Analysis Software Ver 2.2 for *L. pneumophila*
- Click on "Qualitative analysis" box for Qualitative Test or "Quantitative analysis" box for Quantitative Test. The Quantitative analysis requires that the function "Selection unlock" is activated using the following password "Diatheva".

**8.1 QUALITATIVE TEST**

**a. Quality Control**

Before proceeding with the analysis of samples, check the validity of controls. If the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* shows that both controls are "OK", the operator can proceed with the result interpretation of samples. Otherwise, if controls are "KO", the PCR is invalid and must be repeated (see table below).

| **Control type** | **Control validity** | **L. pneumophila result** | **Internal Amplification Control result** |
|---|---|---|---|
| Negative PCR Control | OK | N/A* | 22≤Ct≤32 |
| | OK | Ct>Intercept value** | 22≤Ct≤32 |
| | KO | Ct<Intercept value** | Non-significant |
| Positive PCR Control | OK | 18≤Ct≤28 | Non-significant |
| | KO | 18>Ct>28 | Non-significant |

* N/A means "Not Applicable". The software indicates N/A when the fluorescence curve doesn't rise above the threshold.

**The intercept value to be considered in the contest of Qualitative analysis is Ct=38. This value is used to give an indication of the intercept value chosen during the validation.

**b. Samples**

Sample results shall be interpreted as shown in the table below

| Interpretation using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* | | | Interpretation without using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* (not in the scope of NF VALIDATION) | |
|---|---|---|---|---|
| ***Detection of L. pneumophila*** | ***Inhibition*** | ***Results and expression per Liter (GU/L)*** | ***L. pneumophila result*** | ***Internal Amplification Control result*** |
| Positive | Inhibition | L. pneumophila detected | Ct≤Intercept value* | Non-significant |
| | No Inhibition | | | |
| Negative | No Inhibition | L. pneumophila not detected | Ct>Intercept value* | 22≤Ct≤32 |
| | Inhibition-Redo test | Inhibition. Dilute the extracted DNA using Elution Buffer (MBK0080) or PCR grade water and repeat PCR amplification | Ct>Intercept value* | Ct>32 |

* The intercept value to be considered in the contest of Qualitative analysis is Ct=38. This value is used to give an indication of the intercept value chosen during the validation.

**c. Detection limit of the molecular method ($LOD_{meth}$)**

The limit of detection of the PCR step corresponds to the smallest number of genome units that provides a positive PCR result at the 90% threshold (ISO/TS 12869:2012). The LOD of the PCR step is 5 GU per 5 µL of extracted DNA. The LOD of the method ($LOD_{meth}$) depends on the volume of water sample filtered according to the following formula:

$$LOD_{meth} = \frac{5 \times F \times D}{V}$$

Where:

**F** = conversion factor GU/5 µL to GU/L. The F conversion factor of DNApure Water Isolation kit (MBK0080) is 64. For more information refer to the manual of the kit.

**V**= volume of water sample filtered expressed in litres.

**D**= DNA dilution factor (if the DNA has been diluted before PCR run).

## 8.2 QUANTITATIVE TEST

### a. Controls and Standard

Before proceeding with the analysis of the samples, check the validity of controls and Standard curve. If the software DI-Check Analysis Software Ver 2.2 for L. pneumophila shows that parameters are "OK", the operator can proceed with the result interpretation of samples. Otherwise, if parameters are "KO", the PCR is invalid and must be repeated (see table below).

| Parameter | Quality Control validity | L. pneumophila result | Internal Amplification Control result |
|---|---|---|---|
| Negative PCR Control | OK | N/A[a] | 22≤Ct≤32 |
| | OK | Ct>Intercept value[b] | 22≤Ct≤32 |
| | KO | Ct<Intercept value[b] | Non-significant |
| Slope | -3.75≤ Slope ≤-3.00 | -3.75≤ Slope ≤-3.00 | Non-significant |
| Correlation coefficient ($R^2$) | ≥0.95 | ≥0.95 | |
| Reference Material | OK | 601≤ GU/well ≤2398[c] | Non-significant |
| | KO | 601≥ GU/well ≥2398[c] | Non-significant |
| LOQ | OK | Compliant to ISO-TS 12869 standard | Non-significant |
| | KO | Compliant to ISO-TS 12869 standard | Non-significant |

[a] N/A means "Not Applicable". The software indicates N/A when the fluorescence curve doesn't rise above the threshold

[b] The intercept value to be considered in the contest of Quantitative analysis is systematically calculated in the calibration function of the amplification run.

[c] The Reference Material shall be tested according to ISO-TS 12869 standard

*The above values were determined during the method validation.*

### b. Samples

| Detection of *L. pneumophila* N GU/5 µl | Inhibition[a] | *L. pneumophila* detection Results and expression per liter (GU/L)[b] | | Note |
|---|---|---|---|---|
| N < 5 | No Inhibition | LQ | L. pneumophila not detected | Refer to paragraph 8.2.d for $LOD_{meth}$ calculation |
| | Inhibition | LQ | The sample DNA shall be diluted and PCR run repeated | |
| 5 ≤ N < 25 | No Inhibition | LQ | L. pneumophila detected below the limit of quantification | Refer to paragraph 8.2.d for $LOD_{meth}$ calculation |
| | Inhibition | LQ | The sample DNA shall be diluted and PCR run repeated | |
| 25000 ≥ N ≥ 25 | No Inhibition | L. pneumophila quantitatively detected | | Refer to paragraph 8.2.c |
| | Inhibition | The sample DNA shall be diluted and PCR run repeated | | |
| N > 25000 | No Inhibition | L. pneumophila detected above the upper limit of quantification | | Refer to paragraph 8.2.d for $UQL_{meth}$ calculation. The quantification can be obtained after DNA dilution |
| | Inhibition | The sample DNA shall be diluted and PCR run repeated | | |

[a] For the interpretation of results without using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* (not in the scope of NF VALIDATION), the Internal Amplification Control (VIC) is compliant if 22≤ Ct ≤32, (No Inhibition).

[b] Please if the sample has been diluted the Result GU/L must be multiplied by the dilution factor

### c. Calculation of *L. pneumophila* concentration in water samples

The software DI-Check Analysis Software Ver 2.2 for *L. pneumophila automatically* calculate the *L. pneumophila* GU/L by entering the filtered water volume (mL) in the column "Filtration volume" for each sample.

To calculate the amount of *L. pneumophila* in the water sample analysed without using the software DI-Check Analysis Software Ver 2.2 for *L. pneumophila* (not in the scope of NF VALIDATION), it is necessary to consider (1) the volume of sample filtered in litres and (2) the *F* conversion factor. The F conversion factor of DNApure Water Isolation kit (MBK0080) is 64. For more information refer to the manual of the kit.

The instrument software automatically calculates the amount of GU of *L. pneumophila* per reaction (in 5 µL), this value corresponds to **N** parameter. To obtain the *L. pneumophila* GU contained in 1 Liter please use the following formula:

$$\textit{L. pneumophila}\ \text{Genome Units per liter} = \frac{N \times F \times D}{V}$$

Where:
N= number GU of *L. pneumophila*/5 µL (PCR result)
F = conversion factor GU/5 µL to GU/L.
V= volume of water sample filtered expressed in litres
D**=** DNA dilution factor (if the DNA has been diluted before PCR run)

Express the results in number of Genome Units of *L. pneumophila* per litre of water sample, considering two significant figures: ex. 12 312 GU/L of *L. pneumophila* is expressed as "12 000 GU/L of *L. pneumophila*".

**d. Theoretical detection limit (LODmeth, Quantification limit ($LOQ_{meth}$), and Upper quantification limit ($UQL_{meth}$) of the molecular method**

**The detection limit of the PCR step is 5 GU/**5 µL. The LOD of the method ($LOQ_{meth}$) is 320 GU/L when 1 Liter is filtered. $LOD_{meth}$ depends on the volume of water sample filtered and can be calculated according to the following formula:

$$LOD_{meth} = \frac{5 \times F}{V}$$

The limit of quantification of the PCR step is 25 GU/5 µL. The LOQ of the method ($LOQ_{meth}$) is 1600 GU/L when 1 Liter is filtered. The $LOQ_{meth}$ depends on the volume of water sample filtered and can be calculated according to the following formula:

$$LOQ_{meth} = \frac{25 \times F}{V}$$

The upper quantification limit of the method ($UQL_{meth}$) is $1.6 \times 10^6$ GU/L when 1 Liter is filtered. The $UQL_{meth}$ can be obtained with the following formula:

$$UQL_{meth} = \frac{25\,000 \times F}{V}$$

Where:
**F** = conversion factor GU/5 µL to GU/L.
**V**= volume of water sample filtered expressed in litres

## 8. VALIDATIONS


DI-Check Legionella pneumophila
Certificate reference No. DTV 41/01-12/19, with end of validity 04-Dec-2023
WATER ANALYSIS METHODS
The method is validated for the detection and quantification of L. pneumophila in all types of water samples, by comparison to the reference method NF T90-471 (2015) and ISO/TS 12869 (2019) and according AFNOR certification's validation protocol for the detection and enumeration of L. pneumophila by PCR.
https://nf-validation.afnor.org/

## 9. REFERENCES

1. Ballard, A. L., Fry, N. K., Chan, L., Surman, S. B., Lee, J. V., Harrison, T. G. & Towner, K. J. (2000). Detection of *Legionella* pneumophila using a real-time PCR hybridization assay. J Clin Microbiol 38, 4215–4218.

2. ISO/TS 12869:2012 Water quality — Detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR).

3. NF T90-471 (06/2015). Water quality - Detection and quantification of *Legionella* and/or *Legionella pneumophila* by concentration and genic amplification by real time polymerase chain reaction (qPCR).