

GUIDELINES

to **AmpliSens[®] *Legionella pneumophila*-FRT** PCR kit
for qualitative detection of *Legionella pneumophila* DNA
in the biological material, microorganism cultures, and
environmental samples as well as for quantitative detection of
Legionella pneumophila DNA in water by polymerase chain reaction
(PCR) with real-time hybridization-fluorescence detection

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® Legionella pneumophila-FRT** PCR kit for qualitative detection of *Legionella pneumophila* DNA in biological material (tracheal sputum or aspirate, nasopharyngeal and oropharyngeal swabs, bronchial washes or bronchoalveolar lavage, and autopsy material), microorganism cultures, and environmental samples (water, washes from environmental objects, biofilms, and soil) as well as for quantitative detection of *Legionella pneumophila* DNA in water by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using Rotor-Gene 3000/6000 (Corbett Research, Australia) instrument.

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) INSTRUMENTS

When working with Rotor-Gene 3000 one should use the Rotor-Gene version 6 software and the Rotor-Gene 6000 versions 1.7 (build 67) software or higher for Rotor-Gene 6000 instrument.

Hereinafter the terminology for different versions of devices and software is provided in the following order: for Rotor-Gene 3000 device / for Rotor-Gene 6000.

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with flat caps (e.g. Axygen, USA) (detection through the bottom of the tube).

Programming the thermocycler

1. Click the **New** button in the software main menu.
2. In the opened window select **Advanced** menu and the **Dual Labeled Probe/Hydrolysis probes** template. Click the **New** button.
3. Select the **36-Well Rotor** and tick the **No Domed 0.2 ml/Locking ring attached** option.
4. Click the **Next** button.
5. Select the **Reaction volume – 25 µl**. Tick the **15 µl oil layer volume** option.
6. Click the **Next** button.
7. Click the **Edit profile** button and set amplification program.

| Step | Temperature, °C | Time | Fluorescence detection | Number of cycles |
|------|-----------------|-------|------------------------|------------------|
| 1 | 95 | 5 min | – | 1 |
| 2 | 95 | 10 s | – | 10 |
| | 60 | 20 s | – | |
| | 72 | 10 s | – | |
| 3 | 95 | 10 s | – | 35 |
| | 56 | 20 s | FAM/Green, JOE/Yellow | |
| | 72 | 10 s | – | |

8. Click the **OK** button.
9. Select the **Calibrate/Gain Optimisation** button. In the opened window, click the **Calibrate Acquiring/Optimise Acquiring** button. Indicate **Min Reading 5, Max Reading 10** for both channels. In the **Tube position** column specify the number of the test tubes on which the **gain** parameter will be automatically selected. By default this is the 1st test tube in the rotor. The 1st position must contain a reaction tube with the reaction mixture. Check the **Perform Calibration. Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**. Close the **Auto Gain Calibration Setup** window. Press the **Close** button and then click **Next**.
10. Place the prepared tubes to the instrument. Select the **Start run** button. It should process amplification.
11. Name the experiment and save it to disk (results of the run will be automatically saved in this file).

As part of the thermocycling process, it's necessary to set the order of the test tubes in the carousel of the instrument. To do this, use the **Edit samples** button. For qualitative analysis name all test and control samples as **Unknown** in the **Name** column.

For quantitative analysis concentration values for DNA calibrators of *Legionella pneumophila* and DNA calibrators of Internal Control STI-338 should be entered separately. To do this, create 2 pages in the edit samples window. Page 1 is meant for entering concentration values of Internal Control STI-338 DNA. Page 2 is meant for entering concentration values of *Legionella pneumophila* DNA. Indicate names/numbers of test samples, controls, and calibrators in the **Name** column of both pages. In the **Type** column enter the **Unknown** type for test samples, the **Standard** type for DNA calibrators; set **Given Conc. Format: 1,235E+5** and **Unit: copies/ml**. In the corresponding **Given Conc.** column of Page 1 enter concentration values of IC-STI-338 DNA in LS1, LS2 and LS3 calibrators as specified in the Important Product Information Bulletin. In the corresponding **Given Conc.** column of Page 2 enter concentration values of *Legionella pneumophila* DNA in LS1, LS2 and LS3 calibrators as specified in the *Important Product Information Bulletin*.

Qualitative data analysis

Data analysis of the IC DNA amplification

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. FAM/Cycling A. Green, Show**.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window

(Quantitation analysis).

4. Select the **Linear scale** button at the right bottom of the window (if this option is activated by default then **Log scale** button will be seen).
5. In the **More Settings/Outlier Removal** window set **10 %** for the **NTC threshold** value.
6. In the **CT Calculation** menu, set **Threshold = 0.05**.
7. In the results grid (the **Quantitation Results** window) one will be able to see the **Ct** values.

Data analysis of *Legionella pneumophila* DNA amplification

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. JOE/Cycling A. Yellow, Show**.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
4. Select the **Linear scale** button at the right bottom of the window (if this option is activated by default then **Log scale** button will be seen).
5. In the **More Settings/Outlier Removal** window set **10 %** for the **NTC threshold** value.
6. In the **CT Calculation** menu, set **Threshold = 0.05**.
7. In the results grid (the **Quantitation Results** window) one will be able to see the **Ct** values.

Table 1

Boundary Ct values for control and test samples

| Sample | FAM/Green | JOE/Yellow |
|---------------------|---|--|
| | Detection of IC | Detection of <i>Legionella pneumophila</i> |
| C- | < 28 | Absent |
| PCE | < 28 | < 33 |
| NCA | Absent | Absent |
| LS3 | <30 | < 33 |
| Test samples | <28 (biological sample) <25 (environmental sample) | < 33 |

Quantitative data analysis

Data analysis of the Internal Control STI-338 DNA amplification

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. FAM/Cycling A. Green**. Check off **Page 1** and click the **Show** button.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window

(Quantitation analysis).

4. In the **More Settings/Outlier Removal** window set **10 %** for the **NTC threshold** value.
5. Select **Linear scale** button at the right bottom of the window (if this option is activated by default then **Log scale** button will be seen).
6. In the **CT Calculation** menu, set **Threshold = 0.05**.
7. In the results grid (the **Quantitation Results** window) one will be able to see the *C_t* values.
8. Calculation of the quantity of Internal Control STI-338 DNA copies in 1 ml of test sample is performed automatically using the software and the specified calibrator values. The obtained result is shown in the respective column (**Calc conc.**) of the results grid.

Data analysis of *Legionella pneumophila* DNA amplification

1. Select the **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. JOE Cycling A. Yellow**. Check off **Page 2** and click the **Show** button.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
4. In the **More Settings/Outlier Removal** window set **10 %** for the **NTC threshold** value.
5. Select **Linear scale** button at the right bottom of the window (if this option is activated by default then **Log scale** button will be seen).
6. In the **CT Calculation** menu, set **Threshold = 0.05**.
7. In the results grid (the **Quantitation Results** window) one will be able to see the *C_t* values.
8. Calculation of the quantity of *Legionella pneumophila* DNA copies in 1 ml of test sample is performed automatically using the software and the specified calibrator values. The obtained result is shown in the respective column (**Calc conc.**) of the results grid.

Calculation of results

The calculated concentration of the Positive Control of Extraction (PCE) must fall in the range specified in *Important Product Information Bulletin*.

The correlation coefficient R^2 should be greater than **0.97**.

The amplification efficiency parameter (**Efficiency**) must fall in range **0.8–1.15**.

Calculation of concentration values of *Legionella pneumophila* DNA in 1 l of water ($C_{DNA\ LP}$)

(copies/l)) is performed manually or using software enclosed in the PCR kit in accordance with following formula:

$$C_{DNA\ Lp} \text{ (copies/l)} = Q_{DNA\ Lp} / Q_{IC-STI-338} * C_{IC-STI-338} * 2$$

$Q_{DNA\ Lp}$ (copies/ml) is the calculated quantity of *Legionella pneumophila* DNA copies in 1 ml of a test sample.

$Q_{IC-STI-338}$ (copies/ml) is the calculated quantity of IC-STI-338 DNA copies in 1 ml of the internal control in a test sample.

$C_{IC-STI-338}$ (copies/ml) is the number copies of IC-STI-338 in 1 ml of the internal control (specified in the *Important Product Information Bulletin*).

2 – recalculation coefficient.