IVD For *in Vitro* Diagnostic Use

CE

Legionella pneumophila Real-TM

Handbook

Real Time PCR Kit for quantitative detection of Legionella pneumophila

REF TB50-50FRT

REF B50-50FRT

Σ **50**

NAME

Legionella pneumophila Real-TM

INTRODUCTION

Legionella pneumophila is a thin, pleomorphic, flagellated Gram-negative bacterium of the genus *Legionella*. *L. pneumophila* is the primary human pathogenic bacterium in this group and is the causative agent of legionellosis or Legionnaires' disease.

Legionella pneumophila (named in memory of the deceased veterans) is ubiquitous to acquatic environments worldwide and resided as an intracellular parasite of amoeba and protozoa provided a link between natural environment and human disease. Thus, environmental monitoring, especially of potable water, cooling towers, and related sources, is a major focus in efforts to control the spread of this disease.

Since the initial identification of 235 cases in 1976, Legionnaires disease has become recognized as the most common cause of atypical pneumonia in hospitalized patients. It is the second most common cause of community-acquired bacterial pneumonia with 25% mortality rate.

INTENDED USE

kit **Legionella pneumophila Real-TM** is a test for Real Time quantitative detection of *Legionella pneumophila* DNA in the clinical materials (sputum, aspirate from trachea, nasopharyngeal swabs, throat swabs, bronchoalveolar lavage, tissue), microorganism cultures, and qualitative detection and quantitation of *Legionella pneumophila* DNA in environmental samples (water, washes from environmental objects, biofilms scrapes, ground).

PRINCIPLE OF ASSAY

kit **Legionella pneumophila Real-TM** is a Real-Time Amplification test for the qualitative and quantitative detection of *Legionella pneumophila* in biological materials and in environmental samples.

Legionella pneumophila Real-TM kit can be used as:

- a qualitative test for *Legionella pneumophila* DNA detection in the clinical materials. During the test multiplex real-time PCR of *Legionella pneumophila* mip-gene DNA and protrombin gene DNA is performed. Protrombin gene DNA is used as endogenous internal control. *Legionella pneumophila* mip-gene DNA amplification is detected on JOE/Yellow channel, while the protrombin gene DNA amplification is detected on FAM/Green channel. Protrombin gene DNA is a part of human genome DNA and it should be present in adequate amount in DNA sample (no less then 10^3 genomes). Both improper storage conditions and poor DNA isolation process can lead to DNA degradation and loss. So, the endogenous internal control allows not only to control analysis steps, but also to estimate sample handling and storage.
- a qualitative test for *Legionella pneumophila* DNA detection in environmental samples. In this case the Internal Control (IC) is used. *Legionella pneumophila* mip-gene DNA amplification is detected on JOE/Yellow channel, while the IC DNA amplification is detected on FAM/Green channel.
- a quantitative test for *Legionella pneumophila* DNA calculation in water. In this case the Internal Control (IC) is used. *Legionella pneumophila* mip-gene DNA amplification is detected on JOE/Yellow channel, while the IC DNA amplification is detected on FAM/Green channel. To quantify *Legionella pneumophila* and Internal Control DNA copies quantitative standards are used.

⚠ For quantitation of *Legionella pneumophila* DNA in water samples, every sample must be tested two times, starting from the extraction procedure. The results is given as the average of two results.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B50-50FRT)

"Controls"

- **Legionella C+**, 0,5 ml;
- **Negative Control C-**, 1,2 ml;
- **Legionella IC**, 0,5 ml;
- **DNA-buffer**, 0,5 ml;

"Legionella pneumophila Real-TM":

- **PCR-mix-1**, 70 tubes (0,008 ml each);
- **PCR-mix-2**, 0,77 ml;
- **Standards**:
    - **QS1**, 0,06 ml;
    - **QS2**, 0,06 ml;
    - **QS3**, 0,06 ml

Contains reagents for 55 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TB50-50FRT)

"DNA-Sorb-B":

- **Lysis Solution**, 15 ml;
- **Washing Solution 1**, 15 ml;
- **Washing Solution 2**, 50 ml;
- **Sorbent**, 1,25 ml;
- **DNA-eluent**, 5 ml.

Contains reagents for 50 extractions

"Controls"

- **Legionella C+**, 0,5 ml;
- **Negative Control C-**, 1,2 ml;
- **Legionella IC**, 0,5 ml;
- **DNA-buffer**, 0,5 ml;

"Legionella pneumophila Real-TM":

- **PCR-mix-1**, 70 tubes (0,008 ml each);
- **PCR-mix-2**, 0,77 ml;
- **Standards**:
    - **QS1**, 0,06 ml;
    - **QS2**, 0,06 ml;
    - **QS3**, 0,06 ml

Contains reagents for 55 tests.

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (module n°1)
- Biological cabinet
- Vortex
- 65°C ± 2°C dry heat block
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g)
- Tube racks
- Microcentrifuge tubes, 1,5 - 2,0 ml
- Pipettes with sterile, RNase-free filters tips
- Biohazard waste container
- Disposable gloves, powderless
- Refrigerator, Freezer

Zone 2: Real Time amplification:

- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time Thermal cycler
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters

STORAGE INSTRUCTIONS

Store kit **Legionella pneumophila Real-TM** at 2-8°C. The kit can be shipped at 2-25°C but should be stored at 2-8°C immediately on receipt. Store **DNA-Sorb-B** at 2-8°C.

STABILITY

Legionella pneumophila Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

**** Only for Module No.2***

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Legionella pneumophila Real-TM can analyze DNA extracted with **DNA-Sorb-B** (supplied with the Module No.2) from:

- *Sputum, bronchial or tracheal lavage* must be treated with the following procedure*:
    - Collect sputum into 50 mL single-use PP tubes with a screw cap.
    - In a biological safety cabinet, homogenize samples after mixing with equal volume of 4% NaOH solution. (*N-acetyl-L-cysteine may be added if required in the amount of 50-70 mg per sample).* Mix intensely with a tube rotator for 5-20 minutes (depending on the density of a sample).
    - Centrifuge samples at 3000 rpm (2800-3000 g) for 15 min and carefully discard the supernatant leaving 500-1000 µl in the tube. Resuspend sediment and transfer it into a 1.5 ml tube.
    - Centrifuge samples at 12000 rpm for 5-10 min, discard the supernatant and use the same 1,5 ml sample tube for DNA isolation from sample sediment.
- *Nasopharyngeal and throat swabs:* insert the working area of the probe with cotton swab to sterile disposable tube with 500 µl of sterile saline or phosphate buffer solution (PBS). Broke off the terminal part of the probe or cut it off to allow dense closing of tube cup. Use the suspension for the DNA extraction.
- *Microorganism cultures,* suspected of *Legionella* spp: resuspend cultures in 0.5 ml of saline solution or phosphate buffer. Use 50 µl of suspension for DNA extraction
- *Tissue* (~1,0 gr) homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles and dissolved in 1,0 ml of saline water or PBS sterile (1 volume of tissue to 1 volumes of saline solution). Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml tube;
- *Water (wastewater, from water reservoir, drinking water)*: 0.5 L of water is preliminary filtered through paper filter with glass funnel. After preliminary filtration water is filtered through membrane filter with pore diameter not more than 0.45 µm. After filtration membrane filter is chopped by sterile scissors (to disposable Petri dish) and placed by sterile pincers to 1.5 ml tubes with 1 ml of saline solution. The tube is incubated at room temperature during 15-20 min, periodically mixing on vortex for ensuring of microflora transition in solution. Use 50 µl of solution for DNA extraction.

- *Washes from environmental objects* are obtained by probe with cotton swab, saturated in sterile saline solution. Working end of probe with swab is placed in tube with 1.5 ml of saline solution, another part of probe is broken off and moved away. Use 50 µl of solution for DNA extraction.
- *Biofilms scrapes from internal surface of water supply, industrial and other equipment* (for example, from tray inside air-conditioners). Scrapes of moist biofilms under water or on the water-air interface are obtained by dry sterile probe and scrapes of dried biofilms are obtained by swab, saturated in sterile saline solution. Working end of probe with swab is placed in 1.5 ml tube with 0.5 ml of saline solution, another part of probe is broken off and moved away. Use 50 µl of solution for DNA extraction.
- Ground (100 g): transfer the ground (0.4-1.0 g) to the tubes of 5 ml with tightly closable lid. Add 3 ml of saline solution in each tube, mix careful and decant 5 min. Supernatant (50 µl) is used for DNA extraction.

Specimens can be stored at +2-8°C for no longer than 48 hours, or freeze at -20°C to -80°C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the “SAMPLE COLLECTION, STORAGE AND TRANSPORT” paragraph, could be used.

Sacace Biotechnologies recommends to use the following kit:

- ⇨ **DNA-Sorb-B** (Sacace, REF K-1-1/B) for whole blood, liquor, tissue, etc;
- ⇨ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006) for cultures, water;

Please carry out DNA extraction according to the manufacture’s instruction.

Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION (reagents supplied with the Module No.2)

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for **Negative Control of Extraction** and one tube for **Positive Control** .
2. Add to each tube **10 µl** of **IC** (only for environmental samples and controls**)** and **300 µl** of **Lysis Solution**.
3. Add **100 (50) µl** of **Samples** to the appropriate tube.
4. Prepare Controls as follows:
    a. add **100 µl** of **C–** (**Neg Control** provided with the amplification kit) into the tube labeled C*neg*.
    b. add **50 µl** of **Legionella C**+ and **50 µl of C-** into the tube labeled C*pos*
5. Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 5-7 sec. If the sample is not completely dissolved it is recommended to re-centrifuge the tube for 5 min at a maximum speed (12000-16000 g.) and transfer the supernatant into a new tube for DNA extraction.
6. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 3 min at room temperature. Repeat this step.
8. Centrifuge all tubes for 30 sec at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
9. Add **300 µl** of **Washing Solution 1** to each tube. Vortex very vigorously and centrifuge for 30 sec at 5000g. Remove and discard supernatant from each tube.
10. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and centrifuge for 30 sec at 10000g. Remove and discard supernatant from each tube.
11. Repeat step 10 and incubate all tubes with open cap for 5-10 min at 65°C.
12. Resuspend the pellet in **50 µl** of **DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
13. Centrifuge the tubes for 1 min at maximum speed (12000-16000 g). The supernatant contains DNA ready for amplification. If amplification is not performed in the same day of extraction, the processed samples can be stored at 2-8°C for at maximum period of 5 days or frozen at –20°/-80°C.

PROTOCOL (total PCR reaction vol is 25 µl):

1. Prepare required quantity of **PCR-mix-1** reaction tubes for samples and controls.
2. Add **7 µl** of **PCR-mix-2** on the wax surface.
3. Add **10 µl** of **extracted DNA** to appropriate tube.
4. For each qualitative test prepare the following controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **QS3** to the tube labeled Amplification Positive Control;
5. For each quantitative test prepare the following controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **QS1** to the tube labeled QS1;
   - add **10 µl** of **QS2** to the tube labeled QS2;
   - add **10 µl** of **QS3** to the tube labeled QS3.
6. Insert the tubes in the thermalcycler.

Amplification

1. Create a temperature profile on your instrument as follows:

	Rotor-type Instruments[1]			**Plate type Instruments[2]**		
Step	***Temperature, °C***	***Time***	**Repeats**	***Temperature, °C***	***Time***	**Repeats**
1	**95**	15 min	1	**95**	15 min	1
2	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	20 s	
	72	15 s		**72**	15 s	
3	**95**	5 s	40	**95**	5 s	40
	56	20 s *fluorescent signal detection*		**56**	30 s *fluorescent signal detection*	
	72	15 s		**72**	15 s	

[1] For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)
[2] For example, SaCycler-96™ (Sacace), CFX96/iQ5™ (Biorad), Mx3000P/3005P™ (Agilent); LineGeneK® (Bioer)

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

Legionella pneumophila is detected on JOE/Yellow channel, while the protrombin gene DNA (Internal Control) is detected on FAM/Green channel.

Qualitative test:

The analysis results are considered valid, only if the control samples results comply the following:

Results for controls

Control	Stage for control	Ct channel FAM /Green	Ct channel JOE/Yellow	Interpretation
C-	DNA isolation	Pos (< 30)	Neg	OK
C+	DNA isolation	Pos (< 30)	Pos (< 30)	OK
DNA-buffer	Amplification	Neg	Neg	OK
QS3	Amplification	Pos (< 33)	Pos (< 33)	OK

Quantitative test:

The analysis results are considered valid, only if the control samples results comply the following:

Results for controls

Control	Stage for control	Ct channel FAM (Green)	Ct channel JOE (Yellow)	Interpretation
C-	DNA isolation	Pos	Neg	OK
C+	DNA isolation	Pos	Pos	OK
DNA-buffer	Amplification	Neg	Neg	OK
QS1	Amplification	Pos	Pos	OK
QS2	Amplification	Pos	Pos	OK
QS3	Amplification	Pos	Pos	OK

Calculate the concentration of *Legionella pneumophila* DNA in control and samples according to the following:

$$C_{L.pn.\ DNA}\ (cop/L) = K_{L.pn.\ DNA}/K_{IC} \times C_{IC} \times 2$$

- $C_{L.pn.\ DNA}$ (cop/L) – quantity of *Legionella pneumophila* DNA copies in 1 L of water;
- $K_{L.pn.\ DNA}$ (cop/ml) – calculated quantity of *Legionella pneumophila* DNA copies in 1 ml of sample;
- K_{IC} (cop/ml) - calculated quantity of IC DNA copies in 1 ml of sample;
- C_{IC} (cop/ml) - quantity of IC DNA copies in 1 ml of IC according to Data Card,
- 2 – adjustment for sample filtration.

1. The calibration curve correlation coefficient R^2 must be more than 0.97.
2. The Efficiency value must be in range: 0.85 – 1.15.

ANALYTICAL CHARACTERISTICS

ANALYTICAL SPECIFICITY

Analytical specificity of the primers and probes was validated with complex negative samples. They did not generate any signal with the specific Legionella primers and probes.

ANALYTICAL SENSITIVITY

The analytical sensitivity of the **Legionella pneumophila Real-TM** kit was valuated using the Standard DNA of the Legionella pneumophila. This Standard was serially diluted in the DNA-buffer and tested in 3 replicates 20 times. The analytical sensitivity of the kit **Legionella pneumophila Real-TM** was not less than 1000 copies/ml.

Target region: mip-gene

TROUBLESHOOTING

1. Weak (Ct > 35) or no signal of the IC (Fam/Green channel) for the Neg. Control of extraction and samples.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting of the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and select for the IC detection the fluorescence channel reported in the protocol.
    - The IC was not added to the sample (only for environmental samples and controls**)** during the pipetting of reagents.
        - ⇒ Make attention during the DNA extraction procedure.
2. Weak (Ct > 35) or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
3. Joe (Yellow) signal with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips between tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at		Expiration Date
	Manufacturer		Consult instructions for use
C–	Negative control of Extraction	**NCA**	Negative Control of Amplification
C+	Positive Control of Amplification	**IC**	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* Rotor-Gene™ is a registered trademark of Qiagen
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* MX 3000P/3005P® is a registered trademark of Agilent Technologies
* LineGeneK® is a registered trademark of Bioer

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