Distribuito in ITALIA da
Li StarFish S.r.l.
Via Cavour, 35
20063 Cernusco S/N (MI)
telefono 02-92150794
info@listarfish.it
www.listarfish.it

Manual

MutaPLEX®
Legionella species
real time PCR kit

Test for the qualitative in vitro detection of Legionella species DNA in clinical specimens and environmental samples (e. g. water samples)

Valid from 2017-02-09

REF **KG191932**
KG191996

Σ 32/96

-20°C

IVD

CE

Immundiagnostik AG, Stubenwald-Allee 8a, 64625 Bensheim, Germany
Tel.: +49 6251 70190-0 Fax: + 49 6251 70190-363
e.mail: info@immundiagnostik.com www.immundiagnostik.com

Table of Contents

1 INTENDED USE

The MutaPLEX® *Legionella species* real time PCR is an assay for the detection of *Legionella species* DNA in clinical specimens (e. g. throat swabs, nasal swabs, bronchial lavage) and environmental samples (e. g. water samples).

2 PATHOGEN INFORMATION

Legionella are widespread environmental germs which occur in natural and also artificial water carrying sources, such as plumbing fixtures and potable water systems. They also are able to infect protozoans and subsequently reproduce within these organisms. Temperatures between 30 °C and 50 °C and the ability to subsequently reproduce within these organisms increase their growth. From their natural habitat, *Legionella* are sometimes placed in the man-made water systems. Consequently, *Legionella* are also prevalent in anthropogenic waters such as potable water, cooling tower reservoirs, and whirlpools.

Aerosol-generating systems such as faucets, showerheads, cooling towers, and nebulizers aid in the transmission of *Legionella* from water to air. Human inhalation of contaminated aerosols leads to *Legionella* infections and disease outbreaks. Infection from inhaling airborne water droplets or mist containing viable *Legionella*, which are small enough to pass deep into the lungs and be deposited in the alveoli, the small pockets in the lungs. The bacteria rapidly reproduce within the macrophages. Although healthy individuals may develop Legionnaires Disease, people thought to be at increased risk of infection include smokers, patients with chronic respiratory diseases and any immunosuppressed condition. Initial symptoms of Legionnaires Disease include high fever, chills, headache and muscle pain. A dry cough soon develops and most patients suffer breathing difficulty. Some patients also develop diarrhea or vomiting and can become confused or delirious. Legionnaires Disease may not always be severe; in community outbreaks, mild cases may be recognized that would probably have escaped detection except for the increased awareness of the disease. A common but less serious infection caused by *Legionella species* is an illness known as Pontiac Fever. The symptoms of Pontiac Fever are similar to those of moderate to severe influenza: headache, fatigue, fever, joint pain, muscle pain and in a small proportion of cases, vomiting and coughing. The incubation period is one to two days and the illness passes in two to seven days.

3 PRINCIPLE OF THE TEST

The MutaPLEX® *Legionella species* real time PCR kit contains specific primers and dual-labelled probes for the amplification and detection of *Legionella species* DNA in clinical specimens and environmental samples. The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification.

The fluorescence of the pathogen-specific probes is measured in the FAM channel.

Furthermore, MutaPLEX® *Legionella species* real time PCR kit contains a control DNA, which is added during DNA extraction and detected in the same reaction by a differently labelled probe.

The control DNA allows the detection of PCR inhibition and acts as control for the isolation of the nucleic acid from the clinical specimen.

The fluorescence of the control DNA is measured in the VIC®/HEX/JOE/TET channel.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 32 (KG191932) or 96 (KG191996) reactions, respectively.

Table 1: Components of the MutaPLEX® *Legionella species* real time PCR kit .

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 512 µl	2 x 768 µl
Positive control	red	1 x 50 µl	1 x 100 µl
Negative control	green	1 x 50 µl	1 x 100 µl
Control DNA	colourless	1 x 160 µl	2 x 240 µl

5 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- DNA isolation kit (e. g. MutaCLEAN® Universal RNA/DNA, KG1038, or NukEx Complete Mag RNA/DNA, KG1020)
- PCR grade water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortex mixer
- Real time PCR instrument
- Optical PCR reaction tubes with lid

- Optional: Liquid handling system for automation
- Optional: BLP-DNA (bacterium-like particles, please see chapter 11 for details).

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® *Legionella species* real time PCR-Kit is shipped on dry ice or cool packs. All components must be stored at maximum -18 °C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package.

Up to 20 freeze and thaw cycles are possible.

For convenience, opened reagents can be stored at 2–8 °C for up to 6 months.

Protect kit components from direct sunlight during the complete test run.

7 IMPORTANT NOTES

- The MutaPLEX® *Legionella species* real time PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 GENERAL PRECAUTIONS

- Stick to the protocol described in the instructions for use.
- Set up different laboratory areas for the preparation of samples and for the set up of the PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine MutaPLEX® *Legionella species* real time PCR-Kit components of different lot numbers.

9 SAMPLE MATERIAL

Starting material for the MutaPLEX® *Legionella species* real time PCR is DNA isolated or released from clinical specimens (e. g. throat swabs, nasal swabs, bronchial lavage) and environmental samples (e. g. water samples).

10 SAMPLE PREPARATION

The MutaPLEX® *Legionella species* real time PCR is suitable for the detection of *Legionella species* DNA isolated from clinical specimens with appropriate isolation methods.

Commercial kits for DNA isolation such as MutaCLEAN® Universal RNA/DNA (KG1038) are recommended.

Important: In addition to the samples, always run a water control in your extraction. Treat this water control analogous to a sample.

Comparing the amplification of the control DNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time PCR. Furthermore, possible contaminations during DNA extraction will be detectable.

Please note chapter 11 "Control DNA".

If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the DNA extraction kit's manufacturer.

11 CONTROL DNA

A control DNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the DNA isolation procedure and to check for possible real time PCR inhibition.

The bacterium-like particles (BLP-DNA) are not supplied.

DNA isolation from clinical or environmental samples

a) Control DNA or BLP-DNA used as extraction control

MutaPLEX® *Legionella species* control DNA or BLP-DNA is added to the DNA extraction.

Add 5 µl control DNA or BLP-DNA per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions. Please follow protocol A.

The control DNA must be added to the lysis buffer of the extraction kit.

b) Control DNA used as internal control of the real time PCR

If only inhibition will be checked, please follow protocol B.

12 REAL TIME PCR

12.1 Important points before starting

- Please pay attention to chapter 7 "Important Notes".
- Before setting up the real time PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.
- In every PCR run, one positive control and one negative control should be included.
- Before each use, all reagents should be thawed completely at room temperature, thouroughly mixed (do NOT vortex the reaction mix but mix by pipetting up and down repeatedly), and centrifuged very briefly.
- We recommend to keep reagents and samples at 2–8 °C (e. g. on ice or a cooling block) at all times.

12.2 Procedure

If the control DNA or BLP-DNA is used to control both, the real time PCR and the DNA isolation procedure, please follow protocol A. If the control DNA is solely used to detect possible inhibition of the real time PCR, please follow protocol B.

Protocol A

The control DNA or BLP-DNA was added during DNA extraction (see chapter 11 "Control DNA"). In this case, prepare the master mix according to table 2.

The master mix contains all of the components needed for PCR except the sample. Prepare a volume of master mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the master mix (control DNA was added during DNA extraction)

Volume per reaction	Volume master mix
16 µl Reaction Mix	16 µl x (N+1)

Protocol B

The control DNA is used for the control of the real time PCR only (see chapter 11 "Control DNA"). In this case, prepare the master mix according to table 3.

The master mix contains all of the components needed for real PCR except the sample. Prepare a volume of master mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 3: Preparation of the master mix (control DNA is added directly to the master mix)

Volume per reaction	Volume master mix
16 µl Reaction Mix	16 µl x (N+1)
0.5 µl Control DNA *	0.5 µl x (N+1)*

*The increase in volume caused by adding the control DNA is not taken into account when preparing the PCR assay. The sensitivity of the detection system is not impaired.

Protocol A and B: real time PCR set up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument.
- Pipet 16 µl of the master mix into each optical PCR reaction tube.
- Add 4 µl of the eluates from the DNA isolation (including the eluate of the water control), the positive control and the negative control to the corresponding optical PCR reaction tube (table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time PCR

Component	Volume
Master mix	16.0 µl
Sample	4.0 µl
Total volume	20.0 µl

12.3 Instrument settings

For the real time PCR use the thermal profile shown in table 5.

Table 5: real time PCR thermal profile

Description	Time	Temperature	No of cycles
Initial Denaturation	10 min	95 °C	1
Amplification of DNA			45
Denaturation	15 s	95 °C	
Annealing	30 s	60 °C	
	Aquisition at the end of this step		
Extension	30 s	72 °C	

Dependent on the real time instrument used, further instrument settings have to be adjusted according to table 6.

Table 6: Overview of the instrument settings required for the MutaPLEX® *Legionella species* real time PCR.

Real time PCR Instrument	Parameter	Detection Channel	Notes	
LightCycler 480I	*Legionella species* control DNA	483–533 523–568	Color compensation kit needed, e. g. Universal CC FAM (510) – VIC (580)	
LightCycler 480II	*Legionella species* control DNA	FAM (465–510) HEX (533–580)		
Stratagene Mx3000P/ Mx3005P	*Legionella species* control DNA	FAM HEX	Gain 8 Gain 1	Reference dye: none
ABI 7500	*Legionella species* control DNA	FAM JOE	Option reference dye ROX: NO	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	*Legionella species* control DNA	Green Yellow	Gain 5 Gain 5	

13 DATA ANALYSIS

The *Legionella species*-specific amplification is measured in the FAM channel. The amplification of the control DNA is measured in the VIC®/HEX/JOE/TET channel.

The following results can occur:

- **A signal in the FAM channel is detected:**

  The result is positive, the sample contains *Legionella species* DNA.

  In this case, detection of a signal of the control DNA in the VIC®/HEX/JOE/TET channel is inessential, as high concentrations of *Legionella species* DNA may reduce or completely inhibit amplification of the control DNA .

- **No signal in the FAM channel, but a signal in the VIC®/HEX/JOE/TET channel is detected:**

  The result is negative, the sample does not contain *Legionella species* DNA.

  The signal of the control DNA excludes the possibilities of DNA isolation failure (in case the control DNA is being used as an extraction control) and/or real time PCR inhibition. If the CT value of a sample differs significantly from the CT value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see „Troubleshooting“).

- **Neither in the FAM nor in the VIC®/HEX/JOE/TET channel a signal is detected:**

  A diagnostic statement cannot be made.

  The DNA isolation was not successful or an inhibition of the PCR has occurred. In case the control DNA was added during DNA isolation and not directly to the PCR master mix, the negative control is negative in both channels.

Figure 1 and figure 2 show examples for positive and negative real time PCR results.

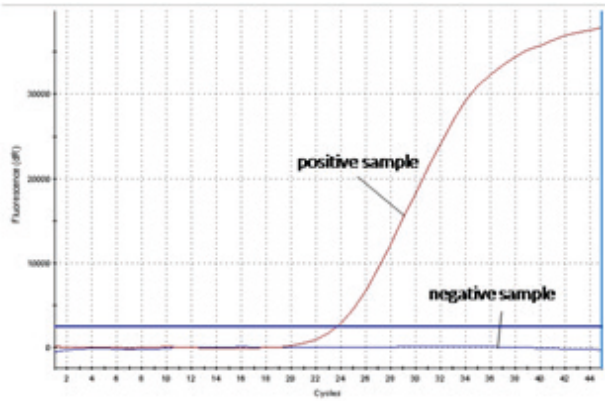

Figure 1: The positive sample shows *Legionella species*-specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

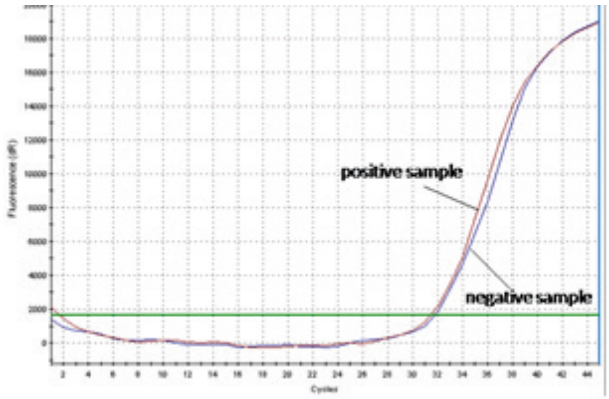

Figure 2: The positive sample as well as the negative sample show a signal in the control DNA-specific VIC®/HEX/JOE/TET channel. The amplification signal of the control DNA in the negative sample shows that the missing signal in the *Legionella species*-specific FAM channel is not due to PCR inhibition or failure of DNA isolation, but that the sample is a true negative.

14 ASSAY VALIDATION

Set a threshold as follows:

Negative controls

All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high CT – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive controls

All the positive controls must show a positive (i. e. exponential) amplification curve. The positive controls must fall below a CT of 30.

Internal controls

All internal controls must show a positive (i. e. exponential) amplification curve. The internal control must fall below a CT of 33. If the internal control is above CT 34, this points to a purification problem or a strong positive sample that can inhibit the IC. In the latter case, the assay is valid. If a water control run is performed, the IC must fall below a CT of 33.

15 LIMITATIONS OF THE METHOD

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.

A negative test result does not exclude a *Legionella species* infection.

16 TROUBLESHOOTING

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR.

No fluorescence signal in the FAM channel of the positive control

The selected channel for analysis does not comply with the protocol

Select the FAM channel for analysis of the virus-specific amplification and the VIC®/HEX/JOE/TET channel for the amplification of the control DNA .

Incorrect configuration of the real time PCR

Check your work steps and compare with chapter "Procedure".

The programming of the thermal profile is incorrect

Compare the thermal profile with the protocol (table 5).

Incorrect storage conditions for one or more kit components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter "Transport, Storage and Stability".

Weak or no signal of the control DNA and simultaneous absence of a signal in the virus-specific FAM channel

real time PCR conditions do not comply with the protocol

Check the real time PCR conditions (chapter 12).

real time PCR inhibited

Make sure that you use an appropriate isolation method (see "Sample preparation") and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffer of the isolation kit has been completely removed. An additional centrifugation step at high speed is recommended before elution of the DNA.

DNA loss during isolation process

In case the control DNA was added before extraction, the lack of an amplification signal can indicate that the DNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.

Incorrect storage conditions for one or more components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter "Transport, Storage and Stability".

Detection of a fluorescence signal in the FAM channel of the negative control

Contamination during preparation of the PCR

Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occured when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the positive control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR.

17 KIT PERFORMANCE

17.1 Diagnostic Sensitivity and Specificity

During the validation study of the MutaPLEX® *Legionella species* real time PCR kit, 65 positive and 120 negative samples were tested. The diagnostic sensitivity was found to be 100 % and the diagnostic specificity 100 % (table 7).

The positive predictive value was found to be 100 %, the negative predictive value showed to be 100 %.

Table 7: Overview of the amount of samples tested and the resulting positive and negative predictive values

	positive samples	negative samples
MutaPLEX® *Legionella species* positive	65	0
MutaPLEX® *Legionella species* negative	0	120
Sensitivity	100 %	
Specificity	100 %	

17.2 Analytical Sensitivity

In order to determine the analytical sensitivity, a suspension of *Legionella pneumophila* serogroup 1 was tested. At first, the concentration of the suspension was determined using McFarland Standard. DNA of decimal dilution series of these suspensions was isolated and tested in triplicates.

The limit of detection is defined as the concentration of *Legionella pneumophila* which can be reliably detected in all triplicates. For the MutaPLEX® *Legionella species* real time PCR kit, the limit of detection was found to be ~2 cfu (colony forming units) per PCR reaction. Results are shown in table 8.

Table 8: Analytical sensitivity of the MutaPLEX® *Legionella species* real time PCR kit

Concentration [CFU/reaction]	**CT Value**		
0.2	36.20	No CT	36.97
2.4	35.56	35.72	35.21
24	34.47	34.76	34.11
240	30.93	30.06	30.45
2,400	26.94	26.67	26.87
24,000	23.00	23.20	23.50
240,000	19.34	19.28	19.64
2,400,000	14.53	14.29	14.65

17.3 Analytical Specificity

The specificity was tested using the species listed in table 9. The primers and probes detected all *Legionella species* but not any other pathogen tested.

Table 9: Bacterial strains used for the determination of the specificity of primers and probes of the MutaPLEX® *Legionella species* real time PCR kit.

Species	**Serogroup**	**Result**
Legionella pneumophila	1	positive
Legionella pneumophila	2	positive
Legionella pneumophila	3	positive
Legionella pneumophila	4	positive
Legionella pneumophila	5	positive
Legionella pneumophila	6	positive
Legionella pneumophila	8	positive

Species	Serogroup	Result
Legionella pneumophila	9	positive
Legionella pneumophila	10	positive
Legionella pneumophila	11	positive
Legionella pneumophila	13	positive
Legionella pneumophila	14	positive
Legionella pneumophila	2-14	positive
Legionella bozemanii		positive
Legionella gravella feeli		positive
Legionella jordanis		positive
Legionella micdadei		positive
Legionella gormanii		positive
Legionella dumdoffii		positive
Escherichia coli		negative
Salmonella enterica		negative
Shigella sonnei		negative
Listeria monocytogenes		negative
Mycoplasma pneumoniae		negative
Adenovirus		negative
Chlamydophila pneumoniae		negative

18 ABBREVIATIONS AND SYMBOLS

DNA	Deoxyribonucleid Acid	BLP	Bacterium-Like Particles
CT	Cycle threshold	CONTROL DNA | IC	Control DNA
nn	not known	→REF	To be used with
PCR	Polymerase Chain Reaction	REF	Catalog number
CONTROL | –	Negative control	Σ	Contains sufficient for <n> test

CONTROL +	Positive control		Upper limit of temperature
REACTION MIX	Reaction Mix		Manufacturer
CONT	Content		Use by
	Consult instructions for use	LOT	Lot number
		IVD	In vitro diagnostic medical device

19. LITERATURE

1. Heuner K; Swanson M (editors). (2008). Legionella: Molecular Microbiology. Caister Academic Press. ISBN 978-1-904455-26-4
2. Legionella pneumophila. www.rki.de/SharedDocs/Bilder/InfAZ/Legionellen/EM_Tab_Legionella_pneumophila.html