MIKROGEN
DIAGNOSTIK

Instruction for Use

*alpha*Cube PanLegionella

For qualitive *in vitro* detection of *Legionella spp.* DNA in clinical specimens and environmental samples (e.g. water samples).

REF	**830536**	**830535**
Σ	32	96

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1 Intended Use

alphaCube PanLegionella is a real-time PCR assay for the detection of *Legionella spp.* DNA in clinical specimens 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 pneumophila* 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

*alpha*Cube PanLegionella contains specific primers and dual-labeled probes for the amplification and detection of *Legionella spp.* 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, *alphaCube* PanLegionella contains a Control DNA, which is added during DNA extraction and detected in the same reaction by a differently labeled probe.

The Control DNA allows the detection of PCR inhibition and acts as control, that the nucleic acid was isolated from the clinical specimen and environmental samples.

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 or 96 reactions respectively.

Table 1: Components of *alphaCube* PanLegionella.

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. *alpha*Clean Pure RNA/DNA)
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation
- Optional: BLP-DNA (Bacterium-Like Particles, please look at chapter ‚Control DNA' for details)

6 Transport, Storage and Stability

alphaCube PanLegionella is shipped on dry ice or cool packs. All components must be stored at -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

- *alphaCube* PanLegionella 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 Instruction 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 *alphaCube* PanLegionella components of different lot numbers.

9 Sample Material

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

10 Sample Preparation

alphaCube PanLegionella is suitable for the detection of *Legionella spp.* DNA isolated from clinical specimens with appropriate isolation methods.
Commercial kits for DNA isolation such as the following are recommended:

- *alpha*Clean Pure RNA/DNA
- *alpha*Clean Mag RNA/DNA

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 the chapter 11 ‚Control DNA'.

If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the 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 samples and environmental samples

a) Control DNA or BLP-DNA used as Extraction Control:
alphaCube PanLegionella 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 the 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, thoroughly mixed (do NOT vortex the Reaction Mix but mix by pipetting up and down repeatedly), and centrifuged very briefly.

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
16.0 µl Reaction Mix	16.0 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
16.0 µl Reaction Mix	16.0 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.

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	Number of Cycles
Initial Denaturation	10 min	95°C	1
Amplification of DNA			45
Denaturation	15 sec	95°C	
Annealing	30 sec	60°C	
	Aquisition at the end of this step		
Extension	30 sec	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 *alphaCube PanLegionella*.

Real time PCR Instrument	Parameter	Detection Channel	Notes
LightCycler 480I	*Legionella spp.*	483-533	pre-installed universal CC FAM (510) – VIC (580)
	Control DNA	523-568	
LightCycler 480II	*Legionella spp.*	FAM (465-510)	
	Control DNA	HEX (498-580)	
Stratagene Mx3000P / Mx3005P	*Legionella spp.*	FAM	Gain 8 Reference Dye: None
	Control DNA	HEX	Gain 1
ABI 7500	*Legionella spp.*	FAM	Option Reference Dye ROX: NO
	Control DNA	JOE	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	*Legionella spp.*	Green	Gain 5
	Control DNA	Yellow	Gain 5

13 Data Analysis

The *Legionella spp.* specific amplification is measured in the FAM channel. The amplification of the Control DNA is measured in the VIC®/HEX/JOE™/TET channel.

Following results can occur:

- **A signal in the FAM channel is detected:**
  The result is positive, the sample contains *Legionella spp.* 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 bacterial 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 spp.* 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 C_T value of a sample differs significantly from the C_T 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.

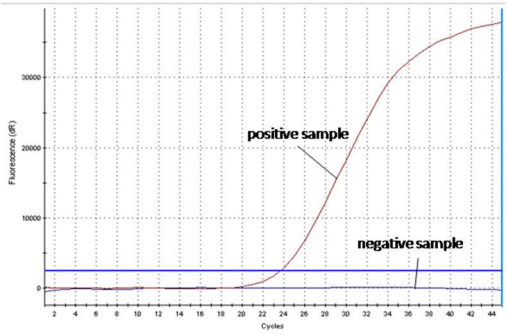

Figure 1: The positive sample shows bacteria specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

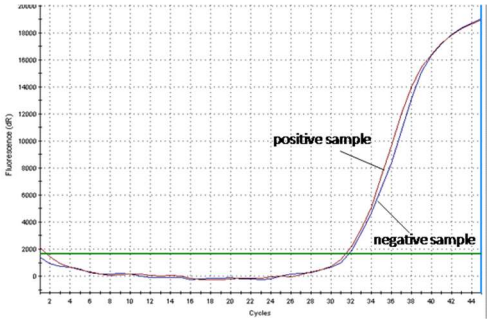

Figure 2: The positive sample as well as the negative sample show a signal in the Control DNA specific VIC®/HEX/JOE™/TET channel. The amplification signal of the Control DNA in the negative sample shows, that the missing signal in the bacteria specific FAM channel is not due to PCR inhibition or failure 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 C_T – 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 C_T of 30.

Internal Controls

All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a C_T of 33. If the internal control is above C_T 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 C_T 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 spp.* 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 *Legionella spp.* 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 ‚Procedure' on page 6.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 7).
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 ‚Transport, Storage and Stability', page 4.

Weak or no signal of the Control DNA and simultaneous absence of a signal in the bacteria specific FAM channel.	
real time PCR conditions do not comply with the protocol	Check the real time PCR conditions (page 6).
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 buffers have 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 ‚Transport, Storage and Stability', page 4.
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 *alphaCube* PanLegionella real time PCR 65 positive and 120 negative samples were tested. The diagnostic sensitivity was found to be 100% and the diagnostic specificity 100%. 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**
*alpha*Cube PanLegionella positive	65	0
*alpha*Cube PanLegionella 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 *alpha*Cube PanLegionella the limit of detection was found to be app. 2 cfu per PCR reaction. Results are shown in Table 8.

Table 8: Analytical sensitivity of *alpha*Cube PanLegionella

Concentration CFU/reaction	**CT Value**		
0.2	36.2	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	23.2	23.5
240,000	19.34	19.28	19.64
2400,000	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 spp* but not any other pathogen tested.

Table 9: Bacterial strains used for the determination of the specificity of primers and probes of the *alpha*Cube PanLegionella.

Species	**Serogroup**	**Result**
L. pneumophila	1	positive
L. pneumophila	2	positive
L. pneumophila	3	positive
L. pneumophila	4	positive
L. pneumophila	5	positive
L. pneumophila	6	positive
L. pneumophila	8	positive
L. pneumophila	9	positive
L. pneumophila	10	positive
L. pneumophila	11	positive
L. pneumophila	13	positive
L. pneumophila	14	positive
L. pneumophila	2-14	positive

Species	**Result**
L. bozemanii	positive
L. gravella feeli	positive
L. jordanis	positive
L.micdadei	positive
L.gormanii	positive
L. dumoffii	positive
Escherichia coli	negative
Salmonella enterica	negative
Shigella sonnei	negative
Listeria monocytogenes	negative
Mycoplasma pneumoniae	negative
Adenovirus	negative
Chlamydia pneumoniae	negative

18 Abbreviations and Symbols

DNA	Deoxyribonucleid Acid
PCR	Polymerase Chain Reaction
REACTION MIX	Reaction Mix
CONTROL +	Positive Control
CONTROL —	Negative Control
CONTROL DNA IC	Control DNA

REF	Catalog number
Σ	Contains sufficient for <n> test
- 18°C	Upper limit of temperature
	Manufacturer
	Use by YYYY-MM
LOT	Batch code
CONT	Content
	Consult instructions for use
IVD	*In vitro* diagnostic medical device

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19 Literature

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