FOR *IN VITRO* USE ONLY

microproof® *Legionella* Quantification LyoKit – 5´Nuclease –

Version 2, January 2020

PCR kit for the quantitative detection of *Legionella* spp., *Legionella pneumophila* and identification of *Legionella pneumophila* serogroup 1 using real-time PCR instruments.

Order No. R 602 45-1 / R 602 45-2

Kit for 96 reactions (lyophilized) for a maximum of 94 samples

Store the kit at 2 to 8 °C

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1. What this Product Does

Number of Tests

The kit is designed for 96 reactions with a final reaction volume of 25 µl each. Up to 94 samples (single replicate measurement) plus calibrator and negative control reactions can be analyzed per run.

Storage and Stability

- Store the kit at 2 °C to 8 °C through the expiration date printed on the label.
- Once the kit is opened, store the kit components as described in the following Kit Contents table:

Kit Contents

Component	Label	Contents / Function / Storage
microproof® *Legionella* Quantification LyoKit Microplate, prefilled with 96 reactions (lyophilized)	Aluminum bag containing a 8-tube strip mat • R 602 45-1 with white low profile tubes* • R 602 45-2 with clear regular profile tubes*	• 96 prefilled reactions (lyophilized). • Ready-to-use PCR mix containing primer and hydrolysis probes specific for DNA of *Legionella* spp., *L. pneumophila, L. pneumophila* serogroup 1 and the Internal Control (IC) as well as Taq DNA Polymerase and Uracil-DNA N-Glycosylase (UNG, heat labile) for prevention of carry-over contamination. • Store at 2 °C to 8 °C in the aluminium bag (tightly sealed). • **Protect from light and moisture!**
Quantification Standards	Vial 2 (purple cap)	• 1 x 350 µl Quantification Standard A • Contains a stabilized solution of DNA. • For use as a PCR run Quantification Standard. • Store at 2 to 8 °C.
	Vial 3 (red cap)	• 1 x 350 µl Quantification Standard B • Contains a stabilized solution of DNA. • For use as a PCR run Quantification Standard. • Store at 2 to 8 °C.
	Vial 4 (yellow cap)	• 1 x 350 µl Quantification Standard C • Contains a stabilized solution of DNA. • For use as a PCR run Quantification Standard. • Store at 2 to 8 °C.
	Vial 5 (white cap)	• 1 x 350 µl Quantification Standard D • Contains a stabilized solution of DNA. • For use as a PCR run Quantification Standard. • Store at 2 to 8 °C.
Negative Control	Vial 6 (colorless cap)	• 1 x 1 ml • Nuclease-free, PCR-grade H_2O. • For use as a PCR run negative control.
Cap strips	Plastic bag containing 8-cap strips	• 12 x 8-cap strip • For use in real-time PCR after addition of samples.

***Tube profile and instrument compatibility chart is available online: www.bc-diagnostics.com/compatibility-chart

Additional Equipment and Reagents Required

- Real-time PCR cycler suitable for detection of FAM, VIC/HEX, ROX and Cy5-labeled probes as well as for using low or regular profile strip tubes. In cases the strip tubes don´t fit for the instrument the samples have to be transferred after resuspension of the lyophilized PCR mix to appropriate PCR vessels.
- Sample Preparation Kit
  - **food**proof® StarPrep Two Kit (Order No. S 400 08)
    or
  - **micro**proof® Suspension Buffer (Order No. S 400 10; recommended for colony confirmation)
- Reagent D (Order No. A 500 02)
- Rinse Buffer (Order No. A 500 13)
- Only required for colony confirmation: Internal Amplification Control (Order No. A 500 23)
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes
- Vortex centrifuge Multispin MSC-6000 for PCR-strips (Order No. D 110 66) **with**
  - SR-32, Rotor for MSC-3000/6000 (Order No. D 110 65)

  or
  - Vortex centrifuge CVP-2 for PCR-plates (Order No. D 110 67)

Applicability Statement

The **micro**proof® *Legionella* Quantification LyoKit – 5'Nuclease – is intended for the rapid quantitative detection of *Legionella* DNA isolated from all kind of water samples that are potentially contaminated with *Legionella*. Presence and concentration of *Legionella pneumophila* and more specifically *Legionella pneumophila* serogroup 1 are assessed separately in the same reaction. DNA from dead bacteria can be excluded from analysis by use of Reagent D in combination with the **food**proof® StarPrep Two Kit.

The kit must not be used in diagnostic procedures.

The kit described in this Instruction Manual has been developed for real-time PCR instruments with FAM, VIC/HEX, ROX and Cy5 detection channels. The performance of the kit was tested with the following real-time PCR instruments: LightCycler® 480, LightCycler® 96 (Roche Diagnostics), Mx3005P®, AriaMx® (Agilent Technologies), ABI 7500 FAST, PikoReal® 24 (Thermo Fisher Scientific) and CFX96 (BIO-RAD).

Note: A Color Compensation is necessary and will be supplied by BIOTECON Diagnostics for users of the LC 480 System I and LC 480 System II (Color Compensation Set 5; Order No. A 500 15).

2. How to Use this Product

2.1 Before You Begin

Precautions

Detection of *Legionella* DNA using the **micro**proof® *Legionella* Quantification LyoKit requires DNA amplification by PCR. The kit provides all reagents required for the PCR. However, in order to achieve reliable results, the entire assay procedure must be performed under nuclease-free conditions. Follow the instructions below to avoid nuclease-, carry-over-, or cross-contamination:

- Keep the kit components separate from other reagents in the laboratory.
- Use nuclease-free labware (*e.g.* pipettes, pipette tips, reaction vials).
- Wear gloves when performing the assay.
- To avoid cross-contamination of samples and reagents, use fresh aerosol-preventive pipette tips.
- To avoid carry-over contamination, transfer the required solutions for one experiment into a fresh tube, rather than directly pipetting from stock solutions.
- Physically separate the workplaces for DNA preparation, PCR setup, and PCR to minimize the risk of carry-over contamination. Use a PCR hood for all pipetting steps.

Keep the microproof® ***Legionella*** **Quantification LyoKit lyophilized PCR Mix away from light and moisture!**

Sample Material

Use any sample material suitable for PCR in terms of purity, concentration, and absence of inhibitors. For preparation of genomic DNA from various samples, refer to the corresponding product package inserts of a suitable sample preparation kit (see *"Additional Equipment and Reagents Required"*).

DNA Extraction

BIOTECON Diagnostics provides sample preparation kits suitable for all kind of food and environmental samples (see *"Additional Equipment and Reagents Required"*).
For more product information please refer to www.bc-diagnostics.com.

Positive Control

Always run a positive control with the samples. To prepare a positive control, replace the template DNA with the provided control DNA [**micro**proof® *Legionella* Quantification Standard (vial 2, purple cap)]. The positive control serves as a calibrator in quantitative procedures.

Negative Control

Always run a negative control with the samples. To prepare a negative control, replace the template DNA with **micro**proof® *Legionella* Negative Control (vial 6, colorless cap). Include a negative control (e.g. ultrapure water) during sample preparation to monitor reaction purity and cross-contamination. This extraction control can be used as an additional negative control reaction.

2.2 Procedure for quantitative real-time PCR

Program Setup

The following procedure is optimized for a real-time PCR instrument with FAM (*Legionella pneumophila*), VIC/HEX (*Legionella* spp.), ROX (*Legionella pneumophila* serogroup 1) and Cy5 (Internal Control) detection channels. Program the PCR instrument before preparing the PCR samples. Use the following real-time PCR-protocol for the **micro**proof® *Legionella* Quantification LyoKit. For details on how to program the experimental protocol, see the Instrument Operator's Manual of your real-time PCR cycler:

Pre-incubation	**1** cycle
Step 1:	37 °C for 4 minutes
Step 2:	95 °C for 5 minutes
Amplification	**50** cycles
Step 1:	95 °C for 5 seconds
Step 2*:	60 °C for 60 seconds
Step 3:	72 °C for 60 seconds

* Fluorescence detection in step 2

Notes:

• For some real-time PCR instruments the type of the probe quencher as well as the usage of a passive reference dye has to be specified. The **micro**proof® *Legionella* Quantification LyoKit contains probes with a non-fluorescent ("dark") quencher and no passive reference dye.

• For users of the Agilent Mx3005P instrument: Click "Instrument → Filter Set Gain Settings" to open the Filter Set Gain Settings dialog box in which the gain settings may be viewed and modified. For FAM and HEX the Filter Set Gain Setting has to be modified to "x4".

Preparation of the PCR Mix

Proceed as described below to prepare a 25 µl standard reaction. Always wear gloves when handling strips or caps. Use any sample material suitable for PCR in terms of purity, concentration, and absence of inhibitors.

Note: The PCR strips must be stored in the provided aluminum bag with the silica gel pads to avoid liquid absorption.

Procedure A: Qualitative Detection

1. Take the needed number of PCR tube strips out of the aluminum bag. Use scissors or scalpel to cut the strips apart. Tightly seal the bag afterwards and store away at the recommended conditions.
2. Place the PCR tube strips containing the lyophilized reagents in a suitable PCR tube rack. Check that the reagent pellets are at the bottom of the tubes. If not, briefly centrifuge or flick the pellets to the bottom before proceeding.
3. Decap the tube strips cautiously and discard the cap strips.

Note: Do not leave strips open for extended periods of time. To avoid unwanted liquid absorption, open strips only shortly before filling.

4. Pipet 25 µl sample into each PCR-vessel:
   - For the samples of interest, add 25 µl sample DNA (if using less volume, add PCR-grade H_2O to achieve 25 µl).
   - For the negative control, add 25 µl PCR-grade H_2O (vial 6, colorless cap).
   - For the positive control, add 25 µl **micro**proof® *Legionella* Quantification Standard A (vial 2, purple cap).

Note: To reduce the risk of cross-contamination, it is recommended to prepare only one PCR tube strip at a time.

5. Seal the vessels accurately and tightly with the colorless cap strips.
6. Mix thoroughly using a vortex centrifuge.

Note: BIOTECON Diagnostics recommends vortex centrifuges Multispin MSC-6000 (Order No. D 110 66) for PCR-strips or vortex centrifuge CVP-2 for PCR-plates (Order No. D 110 67). Dedicated protocols are available for these centrifuges.

Alternatively resuspend the pellet by manual mixing. This may be achieved by cautiously pipetting the sample up and down multiple times during step 4 or flipping the tube strips after sealing while pressing down the cap strip.

7. Spin the PCR tube strips for 30 seconds at 150 – 200 x g in a suitable centrifuge.

Note: If your centrifuge exceeds 200 x g, do not centrifuge for more than 5 seconds. Avoid centrifugation forces exceeding 1,000 x g!

8. Place the samples in your PCR cycler and run the program as described above.

Note: For using any LightCycler 480 instrument, a special adapter (Order No. Z 100 24) is necessary. For some PCR instruments, the strips should be placed in balanced order in the cycler block. For example, two strips can be placed in column 1 and 12.

Procedure B: Quantitative Detection using a Standard Curve

1. Take the needed number of PCR tube strips out of the aluminum bag. Use scissors or scalpel to cut the strips apart. Tightly seal the bag afterwards and store away at the recommended conditions.
2. Place the PCR tube strips containing the lyophilized reagents in a suitable PCR tube rack. Check that the reagent pellets are at the bottom of the tubes. If not, briefly centrifuge or flick the pellets to the bottom before proceeding.
3. Decap the tube strips cautiously and discard the cap strips.

Note: Do not leave strips open for extended periods of time. To avoid unwanted liquid absorption, open strips only shortly before filling.

4. Pipet 25 µl sample into each PCR-vessel:
   - For the samples of interest, add 25 µl sample DNA (if using less volume, add PCR-grade H_2O to achieve 25 µl).
   - For the negative control, add 25 µl PCR-grade H_2O (vial 6, colorless cap).
   - For the standard curve, add 25 µl each of the four dilutions of the **micro**proof® *Legionella* Quantification

Quantification Standard	Cap Color	Concentration to be entered as standard [GU/reaction*]		
		FAM channel	HEX channel	ROX channel
A	purple	25,000	25,000	25,000
B	red	2,500	2,500	2,500
C	yellow	250	250	250
D	white	25	25	25

Standards A, B, C and D in duplicate to generate a standard curve (see table below).

* according to ISO/TS 12869:2019; 1 GU ideally corresponds to 1 bacterial cell present in the sample

Note: Therefore, a typical experiment consists of 9 reactions needed for controls, plus (n) reactions needed for the samples of interest, where (n) indicates the number of samples of interest. Since 96 reactions can be run with the kit, up to 87 samples may be analyzed quantitatively during one PCR run.

Once a standard curve has been established with a specific lot of reagents it is possible to apply this as an external standard curve to quantify samples in successive PCR runs. Only the **micro**proof® *Legionella* Quantification Standard A needs then to be added to the run to serve as a calibrator. It is recommended to add Quantification Standard A in duplicate. Please inquire at BIOTECON Diagnostics for more information on how to generate and use an external standard curve.

5. Seal the vessels accurately and tightly with the colorless cap strips.
6. Mix thoroughly using a vortex centrifuge.

Note: BIOTECON Diagnostics recommends vortex centrifuges Multispin MSC-6000 (D 110 66) for PCR-strips or vortex centrifuge CVP-2 for PCR-plates (D 110 67). Dedicated protocols are available for these centrifuges.

Alternatively resuspend the pellet by manual mixing. This may be achieved by cautiously pipetting the sample up and down multiple times during step 4 or flipping the tube strips after sealing while pressing down the cap strip.

7. Spin the PCR tube strips for 30 seconds at 150 – 200 x g in a suitable centrifuge.

Note: If your centrifuge exceeds 200 x g, do not centrifuge for more than 5 seconds. Avoid centrifugation forces exceeding 1000 x g!

8. Place the samples in your PCR cycler and run the program as described above.

Note: For using any LightCycler 480 instrument, a special adapter (Order No. Z 100 24) is necessary. For some PCR instruments, the PCR strips should be placed in a balanced order into the cycler block. For example two strips can be placed in column 1 and 12.

2.3 Procedure for colony confirmation

Program Setup

The following procedure is optimized for rapid confirmation of presumptive *Legionella* colonies obtained in microbiological culture methods (e.g. ISO 11731). It is intended for use with a real-time PCR instrument with FAM, VIC/HEX, ROX and Cy5 detection channels. Program the PCR instrument before preparing the PCR samples. Use the following real-time PCR-protocol for the **micro**proof® *Legionella* Quantification LyoKit. For details on how to program the experimental protocol, see the Instrument Operator's Manual of your real-time PCR cycler:

Pre-incubation	**1** cycle
Step 1:	37 °C for 4 minutes
Step 2:	95 °C for 5 minutes
Amplification	**30** cycles
Step 1:	95 °C for 5 seconds
Step 2*:	60 °C for 60 seconds

* Fluorescence detection in step 2

Note: For some real-time PCR instruments the type of the probe quencher as well as the usage of a passive reference dye has to be specified. The **micro**proof® *Legionella* Quantification LyoKit contains probes with a non-fluorescent ("dark") quencher and no passive reference dye.

For users of the Agilent Mx3005P instrument: Click "Instrument → Filter Set Gain Settings" to open the Filter Set Gain Settings dialog box in which the gain settings may be viewed and modified. For FAM and HEX the Filter Set Gain Setting has to be modified to "x4".

Preparation of the PCR Mix

Proceed as described below to prepare a 25 µl standard reaction. Always wear gloves when handling strips or caps. Use material from single colonies prepared with the **micro**proof® Suspension Buffer (Order No. S 400 10).

Note: The PCR strips must be stored in the provided aluminum bag with the silica gel pads to avoid liquid absorption.

1. Take the needed number of PCR tube strips out of the aluminum bag. Use scissors or scalpel to cut the strips apart. Tightly seal the bag afterwards and store away at the recommended conditions.
2. Place the PCR tube strips containing the lyophilized reagents in a suitable PCR tube rack. Check that the reagent pellets are at the bottom of the tubes. If not, briefly centrifuge or flick the pellets to the bottom before proceeding.
3. Decap the tube strips cautiously and discard the cap strips.

Note: Do not leave strips open for extended periods of time. To avoid unwanted liquid absorption, open strips only shortly before filling.

4. Pipetting of samples:
   - Pipet 20 µl Internal Amplification Control (order no. A 500 23) into each PCR-vessel.
   - Add 5 µl of suspended colony material to each well.

Note: To reduce the risk of cross-contamination, it is recommended to prepare only one PCR tube strip at a time.

5. Pipetting of controls:
   - For the negative control, pipet 20 µl Internal Amplification Control (order no. A 500 23) into a PCR-vessel and add 5 µl PCR-grade H_2O (vial 6, colorless cap).
   - For the positive control, add 25 µl **micro**proof® *Legionella* Quantification Standard A (vial 2, purple cap).
6. Seal the vessels accurately and tightly with the colorless cap strips.
7. Mix thoroughly using a vortex centrifuge.

Note: BIOTECON Diagnostics recommends vortex centrifuges Multispin MSC-6000 (D 110 66) for PCR-strips or vortex centrifuge CVP-2 for PCR-plates (D 110 67). Dedicated protocols are available for these centrifuges.

Alternatively resuspend the pellet by manual mixing. This may be achieved by cautiously pipetting the sample up and down multiple times during step 4 or flipping the tube strips after sealing while pressing down the cap strip.

8. Spin the PCR tube strips for 30 seconds at 150 – 200 x g in a suitable centrifuge.

Note: If your centrifuge exceeds 200 x g, do not centrifuge for more than 5 seconds. Avoid centrifugation forces exceeding 1000 x g!

9. Place the samples in your PCR cycler and run the program as described above.

Note: For using any LightCycler 480 instrument, a special adapter (Order No. Z 100 24) is necessary. For some PCR instruments, the PCR strips should be placed in a balanced order into the cycler block. For example two strips can be placed in column 1 and 12.

2.4 Data Interpretation

The amplification of DNA specific for the genus *Legionella* is analyzed in the fluorescence channel suitable for VIC/HEX labeled probes detection. The amplification of DNA specific for *Legionella pneumophila* is analyzed in the fluorescence channel suitable for FAM. The amplification of DNA specific for *Legionella pneumophila* serogroup 1 is analyzed in the fluorescence channel suitable for ROX. The specific amplification of the Internal Control is analyzed in the fluorescence channel suitable for Cy5.
Compare the results from channel VIC/HEX (*Legionella* spp.), FAM, ROX and channel Cy5 (Internal Control) for each sample, and interpret the results as described in the table below.

Note: For LightCycler 480, analysis must be performed with the "Fit Points" setting.

Procedure A – Qualitative Detection / Colony Confirmation

For qualitative detection compare the results from channels FAM, VIC/HEX, ROX (*Legionella*) and channel Cy5 (Internal Control) for each sample, and interpret the results as described in the table below:

Channel FAM	Channel HEX	Channel ROX	Channel Cy5	Result Interpretation
Positive or Negative	**Positive**	Positive or Negative	Positive or Negative	Positive for *Legionella* spp.
Positive	**Positive**	Negative	Positive or Negative	Positive for *Legionella pneumophila* serogroup 2-15*
Positive	**Positive**	**Positive**	Positive or Negative	Positive for *Legionella pneumophila* serogroup 1†
Negative	**Negative**	**Negative**	**Positive**	Negative for *Legionella* spp.
Negative	**Negative**	**Negative**	**Negative**	Invalid

***** Samples may contain a mixed population of *Legionella pneumophila* and other *Legionella* spp. Comparison of quantitative results from channel VIC/HEX with quantitative results from channel FAM indicates the number of non- *Legionella pneumophila* in the sample if the number of non-*Legionella pneumophila* cells exceeds the number of *Legionella pneumophila* in the sample.

† Samples may contain a mixed population of *Legionella pneumophila* serogroup 1 and serogroups 2-15. Comparison of quantitative results from channel FAM with quantitative results from channel ROX indicates the number of *Legionella pneumophila* serogroup 2-15 in the sample if the number of *Legionella pneumophila* serogroup 2-15 cells exceeds the number of *Legionella pneumophila* serogroup 1 in the sample.

Procedure B – Quantification of *Legionella* in GU/ml

Perform a quantification according to ISO 12869:2019. The **micro**proof® *Legionella* Quantification Standard is defined as GU/reaction (GU = genomic unit, amount of DNA equivalent to a single bacterial cell). The use of the calibration curve results in such a value for every sample analyzed. GU/reaction may be converted to GU/ml in a sample according to the following equation. It is recommended to use the "*Legionella* Quantification Template" provided by BIOTECON Diagnostics for analysis, which calculates quantitative results equivalent to ISO 12869.

$$\textbf{result}\left[\frac{\textbf{GU}}{\textbf{ml}}\right] = \frac{\textbf{result}\left[\frac{\textbf{GU}}{\textbf{reaction}}\right] \times \textbf{elution volume}\ [\mu\textbf{l}] \times \textbf{recovery factor}}{\textbf{PCR reaction volume}\ [\mu\textbf{l}] \times\ \textbf{sample volume}\ [\textbf{ml}]}$$

- PCR reaction volume = volume used per PCR reaction
- elution volume = final volume after sample preparation
- recovery factor = volume of Rinse Buffer recovered after washing the filter
- sample volume = initial volume used for filtration

When requiring a GU count for larger volumes (e.g. *Y* = 100 ml), use this general formula:

$$\textbf{result}\left[\frac{\textbf{GU}}{\boldsymbol{Y}\ \textbf{ml}}\right] = \frac{\textbf{result}\left[\frac{\textbf{GU}}{\textbf{reaction}}\right] \times \textbf{elution volume}\ [\mu\textbf{l}] \times \textbf{recovery factor} \times \boldsymbol{Y}}{\textbf{PCR reaction volume}\ [\mu\textbf{l}] \times\ \textbf{sample volume}\ [\boldsymbol{Y}\ \textbf{ml}]}$$

Example:
The following calculation is suitable for samples prepared with the **food**proof® StarPrep Two Kit (S 400 08), assuming filtration of 100 ml of a water sample:
- PCR reaction volume = 25 µl
- elution volume = 125 µl
- recovery factor = 1000 µl / 700 µl Rinse Buffer = 1,44
- sample volume = 100 ml
- Y = 100 ml

$$\textbf{result}\left[\frac{\textbf{GU}}{\textbf{100 ml}}\right] = \frac{\textbf{result}\left[\frac{\textbf{GU}}{\textbf{reaction}}\right] \times \textbf{125}\ [\mu\textbf{l}] \times \textbf{1.44} \times \textbf{100}}{\textbf{25}\ [\mu\textbf{l}] \times\ \textbf{100}\ [\textbf{100 ml}]} = \textbf{result} \times \textbf{7,2}\left[\frac{\textbf{GU}}{\textbf{100 ml}}\right]$$

Note: Elution volume and recovery factor depend on the respective sample preparation protocol. Use a "*Legionella* Quantification Template" tailored to the sample preparation protocol in use.

Note: When performing quantification with an external standard curve from a previous run, a "calibration factor" has to be applied to account for run-to-run variance in Cp values. This factor is calculated from the difference between the Cp of the **micro**proof® *Legionella* Quantification Standard calibrator and matching Cp values of the pre-recorded standard curve.

3. Troubleshooting

Observation	Possible Reason	Recommendation
No signal increase is observed, even with positive controls.	Incorrect detection channel has been chosen.	• Set Channel settings to FAM, HEX, ROX and Cy5. • If your instrument does not have a HEX Channel, use VIC instead.
	Pipetting errors.	• Check for correct reaction setup. Repeat the PCR run. • Always run a positive control along with your samples.
	No data acquisition programmed.	• Check the cycle programs.
No signal increase in channel Cy5 is observed, with other channels also negative.	Inhibitory effects of the sample material (*e.g.,* caused by insufficient purification).	• Use the recommended DNA sample preparation kit to purify template DNA. • Dilute samples or pipet a lower amount of sample DNA (*e.g.,* 20 µl PCR-grade H_2O and 5 µl sample DNA instead of 25 µl sample DNA).
Fluorescence intensity is too low.	Inappropriate storage of kit components.	• Store the **micro**proof® *Legionella* Quantification LyoKit lyophilized PCR Mix at 2 °C to 8 °C, protected from light and moisture.
	Low initial amount of target DNA.	• Increase the amount of sample DNA. Depending on the chosen DNA isolation method, inhibitory effects may occur.
Strong decrease of fluorescence baseline	Resuspension of lyophilized PCR mix not complete	• Always resuspend lyophilized PCR mix thoroughly.
Negative control samples are positive.	Carry-over contamination.	• Exchange all critical solutions. • Repeat the complete experiment with fresh aliquots of all reagents. • Always handle samples, kit components and consumables in accordance with commonly accepted practices to prevent carry-over contamination. • Add positive controls after sample and negative control reaction vessels have been sealed.
Fluorescence intensity varies.	Insufficient centrifugation of the PCR strips. Resuspend PCR mix is still in the upper part of the vessel.	• Always centrifuge PCR strips. • Check that no air bubbles are formed or remain in tube after centrifugation.
	Outer surface of the vessel or the seal is dirty (*e.g.,* by direct skin contact).	• Always wear gloves when handling the vessels and seal.
Pellets are difficult to dissolve.	The lyophilized PCR mix started to rehydrate.	• Store the lyophilized PCR mix always in the aluminum bag with the silica gel pad • Open Strip shortly before filling.

4. Additional Information on this Product

How this Product Works

The **micro**proof® *Legionella* Quantification LyoKit – 5'Nuclease – provides all necessary reagents and a control template for reliable interpretations of results. To ensure maximum reliability of the kit and to prevent misinterpretation of negative results due to inhibition of the amplification, an Internal Control (IC) is included. A hydrolysis probe was designed to bind specifically the IC, allowing detection in the Cy5 channel, whereas the *Legionella* DNA is detected in channels FAM, HEX/VIC and ROX. In case of a negative result due to inhibition of the amplification by the sample DNA of interest, the amplification of the IC is suppressed as well, whereas a negative result for the sample DNA of interest and amplification of the IC clearly indicates the absence of *Legionella* DNA in the sample. The **micro**proof® *Legionella* Quantification LyoKit minimizes contamination risk and contains all reagents (except for template DNA) needed for the detection of *Legionella* DNA. Primers and probes provide specific detection of *Legionella* DNA in water and other aqueous samples. The described performance of the kit is guaranteed for use on the real-time PCR instruments listed above only.

Test Principle

1. Using the kit's sequence-specific primers in a polymerase chain reaction (PCR), the PCR instrument and the supplied reagents amplify fragments of *Legionella* genomic DNA.
2. The PCR instrument detects these amplified fragments in real time through fluorescence generated by cleavage of the hybridized probe due to the 5´-nuclease activity of the Taq DNA polymerase. The probe is labeled at the 5´-end with a reporter fluorophore and at the 3´-end with a quencher.
3. During the annealing/elongation phase of each PCR cycle, the probe hybridizes to an internal sequence of the amplicon and is cleaved by the 5' nuclease activity of the Taq DNA polymerase. This cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal.
4. The PCR instrument measures the emitted fluorescence of the reporter dye.

Prevention of Carry-Over Contamination

The heat-labile Uracil-DNA N-Glycosylase (UNG) is suitable for preventing carry-over contamination between PCRs. This technique relies on the incorporation of deoxyuridine triphosphate (dUTP) during all amplification reactions, and the pretreatment of all successive PCR mixtures with the heat-labile UNG. The UNG cleaves DNA at any site where a deoxyuridine residue has been incorporated. The resulting abasic sites are hydrolyzed due to the high temperatures during the initial denaturation step, and can no longer serve as PCR templates. The heat-labile UNG is inactivated during the initial denaturation step. Native DNA (e.g., the isolated *Legionella* genomic DNA) does not contain uracil and is therefore not degraded by this procedure. Since dTTP is replaced with dUTP and UNG is included in the **micro**proof® *Legionella* Quantification LyoKit, decontamination can be achieved with the provided reagents.

Background Information

Legionellosis is a collection of infections that emerged in the second half of the 20th century, and that are caused by *Legionella pneumophila* and related *Legionella* bacteria. The severity of legionellosis varies from mild febrile illness (Pontiac fever) to a potentially fatal form of pneumonia (Legionnaires' disease) that can affect anyone, but principally affects those who are susceptible due to age, illness, immunosuppression or other risk factors, such as smoking. Water is the major natural reservoir for legionellae, and the bacteria are found worldwide in many different natural and artificial aquatic environments, such as cooling towers; water systems in hotels, homes, ships and factories; respiratory therapy equipment; fountains; misting devices; and spa pools [1]. Testing of water installations for the presence of *Legionella* spp. is therefore implemented in national standards worldwide usually according to ISO standard methods for cultural and PCR detection [2-4]. Pathogenicity potential however varies greatly among different species and serotypes. In Europe, approximately 70% of Legionella infections are caused by *L. pneumophila* serogroup 1, 20–30% are caused by other serogroups, and 5–10% are caused by non-

pneumophila species. Of the reported non-*pneumophila* infections, the causative species are predominantly *Tatlockia micdadei*, *Fluoribacter bozemanii*, *Fluoribacter dumoffii* and *Legionella longbeachae* [modified from 1]. Recently real-time PCR has been proposed as a suitable, faster, easier and more thorough alternative to cultural methods for the detection and quantification of *Legionella* spp. with the potential to simultaneously identify the presence of *Legionella pneumophila*, in particular strains belonging to serogroup 1 [5,6].

Product characteristics

The **micro**proof® *Legionella* Quantification LyoKit has been designed to fully comply with ISO/TS 12869:2019 specifications for detection of *Legionella* spp. and *Legionella pneumophila* by quantitative PCR.

Specificity: The **micro**proof® *Legionella* Quantification LyoKit inclusivity has been tested with 43 species belonging to the family Legionellaceae. This includes not only bona fide *Legionella* spp. but also *Fluoribacter* spp. and *Tatlockia* spp., which however are also referred to as *Legionella* in the medical literature. *Fluoribacter* spp. and *Tatlockia* spp. cannot be distinguished from taxonomically bona fide *Legionella* spp. by cultural methods (e.g. ISO 11731) and their detection is also required by ISO/TS 12869:2019. All tested species could be detected and precisely quantified by this kit (100% inclusivity). Likewise 28 strains belonging to *Legionella pneumophila* serogroups 2 – 15 and 38 strains belonging to *Legionella pneumophila* serogroup 1 tested positively in the designated channels.
The exclusivity was determined using 40 distinct prokaryotic and eukaryotic species. The selection included all 16 species required by ISO/TS 12869:2019.

Sensitivity: The limit of detection (LoD) of the **micro**proof® *Legionella* Quantification LyoKit is 3 genomic units (GU) / PCR reaction. The limit of quantification (LoQ) of the **micro**proof® *Legionella* Quantification LyoKit is 10 genomic units (GU) / PCR reaction.

References

1. Bartram, J., Chartier, Y., J.V., L., Ponk, K. & Surman-Lee, S.: Legionella and the Prevention of Legionellosis. World Health Organisation, 2007.
2. ISO/TS 12869:2019 Water quality -- Detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR)
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Quality Control

The **micro**proof® *Legionella* Quantification LyoKit – 5'Nuclease – is function tested using the LightCycler® 480 System (R 602 45-1) and the Mx3005P® (R 602 45-2).

5. Supplementary Information

5.1 Ordering Information

BIOTECON Diagnostics is offering a broad range of reagents and services. For a complete overview and for more information, please visit our website at www.bc-diagnostics.com.

5.2 License

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If you have questions about this or any other product of BIOTECON Diagnostics, please contact our Technical Support staff (for details see www.bc-diagnostics.com). Our scientists commit themselves to providing rapid and effective help. We also want you to contact us if you have suggestions for enhancing our product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to us and the worldwide research community.

6. Change Index

Version 1, July 2017
First version of the package insert.
Version 2, January 2020
Procedure for colony confirmation added. Format of Quantification Standard changed.

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