microproof® ***Legionella*** **Quantification LyoKit**

Revision A, November 2023

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

Product No. KIT230119 (LP) and KIT230120 (RP)

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

Store at 2 to 8 °C

FOR *IN VITRO* USE ONLY.

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1. What this Product Does

1.1 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.

1.2 Storage and Stability

- Store the kit at 2 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.

1.3 Kit Contents

Component	Label	Contents, Function, Storage
microproof® *Legionella* Quantification LyoKit Microplate, prefilled with 96 reactions (lyophilized)	Aluminum bag containing an 8-tube strip mat • KIT230119 has white low-profile (LP) tubes* • KIT230120 has clear regular-profile (RP) 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 carryover contamination. • Store at 2 to 8 °C in the aluminum foil 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.

***** A *Cycler Profile Compatibility Chart* is available online at www.hygiena.com/documents.

1.4 Additional Equipment and Reagents Required

- Real-time PCR instruments that have FAM, VIC/HEX, ROX and Cy5 detection channels and that are compatible with low- or regular-profile strip tubes.

  Note: If the strip tubes do not fit into your instrument, the samples must be transferred to appropriate PCR tubes or plates after resuspension of the lyophilized PCR mix.
- Sample preparation kit options (choose one):
  - foodproof® StarPrep® Two Kit (Product No. KIT230177)
  - microproof Suspension Buffer (Product No. KIT230178; recommended for colony confirmation)
- Reagent D (Product No. KIT230001, KIT230002 or KIT230003)
- Rinse Buffer (Product No. KIT230012)
- For colony confirmation: Internal Amplification Control (Product No. KIT230015)
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes
- Vortex centrifuge (choose one):
  - For PCR strips, Multispin MSC-6000 with SR-32 Rotor
  - For PCR plates, CVP-2

1.5 Applicability Statement

The microproof *Legionella* Quantification LyoKit is intended for the rapid quantitative detection of *Legionella* DNA isolated from all kinds 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 the use of Reagent D in combination with the foodproof StarPrep Two Kit.

The kit must **not** be used in diagnostic procedures.

The kit 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), Applied Biosystems™ 7500 FAST, PikoReal® 24 (Thermo Fisher Scientific) and CFX96™ (BIO-RAD).

Note: A Color Compensation Set (Color Compensation Set 5; Product No. KIT230011) is necessary and will be supplied by Hygiena® Diagnostics GmbH for users of the LightCycler 480 System I and LightCycler 480 System II. Contact Hygiena Diagnostics for further information.

2. How to Use this Product

2.1 Before You Begin

2.1.1 Precautions

Detection of *Legionella* DNA using the microproof *Legionella* Quantification LyoKit requires DNA amplification by PCR. The kit provides all the reagents required for the PCR. To achieve reliable results, the entire assay procedure must be performed under nuclease-free conditions. Follow the instructions below to avoid nuclease-, carryover- 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 carryover 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 runs to minimize the risk of carryover contamination. Use a PCR hood for all pipetting steps.

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

2.1.2 Waste Disposal

Place any waste and biohazard material potentially contaminated with pathogenic bacteria in an appropriate plastic contaminated waste bag and label as follows: CONTAMINATED waste, room number, date and initials. The bag should be autoclaved and then disposed of according to local regulations.

2.1.3 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”).

2.1.4 DNA Extraction

Hygiena Diagnostics GmbH provides sample preparation kits suitable for all kinds of foods and environmental samples (see “Additional Equipment and Reagents Required”). For more product information, visit www.hygiena.com.

2.1.5 Positive Control

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

2.1.6 Negative Control

Always run a negative control with the samples. To prepare a negative control, replace the template DNA with microproof *Legionella* Negative Control (vial 6, colorless cap). Include a negative control (e.g., PCR-grade 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

2.2.1 Program Setup

The following procedure is optimized for a real-time PCR instrument with detection channels for FAM (*Legionella pneumophila*), VIC/HEX (*Legionella* spp.), ROX (*Legionella pneumophila* serogroup 1) and Cy5 (Internal Control). Program the PCR instrument before preparing the PCR samples. Use the following real-time PCR protocol for the microproof *Legionella* Quantification LyoKit. For details on how to program the experimental protocol, refer to the Instrument Operator's Manual for 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 probe quencher as well as the use of a passive reference dye must be specified. The microproof *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. For FAM and HEX, the Filter Set Gain Setting must be modified to 'x4'.

2.2.2 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 foil bag with the silica gel packs to avoid liquid absorption.

2.2.3 *Procedure A: Qualitative Detection using External Standards*

1. Take the needed number of PCR tube strips out of the aluminum bag. Use scissors or a scalpel to cut the strips apart. Tightly seal the bag afterward 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. Carefully remove and discard the caps from the tube strips.

   Note: To avoid unwanted liquid absorption, open strips shortly before filling. Do not leave strips open for extended periods of time.
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 bring total volume to 25 µL).
   - For the negative control, add 25 µL PCR-grade H_2O (vial 6, colorless cap).
   - For the positive control, add 25 µL microproof *Legionella* Quantification Standard A (vial 2, purple cap).

   Note: To reduce the risk of cross-contamination, it is recommended to prepare one PCR tube strip at a time.
5. Seal the PCR tube strips accurately and tightly with the colorless cap strips.
6. Mix thoroughly using a vortex centrifuge.

   Note: Hygiena Diagnostics GmbH recommends vortex centrifuge Multispin MSC-6000 for PCR strips or vortex centrifuge CVP-2 for PCR plates. 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: When using any LightCycler 480 instrument, a special adapter is necessary.

   For some PCR instruments, the PCR strips must be placed in balanced order in the cycler block. For example, two strips can be placed in columns 1 and 12.

2.2.4 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 a scalpel to cut the strips apart. Tightly seal the bag afterward 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. Carefully remove and discard the caps from the tube strips.
   Note: To avoid unwanted liquid absorption, open strips shortly before filling. Do not leave strips open for extended periods of time.
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 bring the total volume to 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 microproof *Legionella* Quantification Standards A, B, C and D in duplicate to generate a standard curve (see table below).

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

* According to ISO/TS 12869:2019; 1 genetic unit (GU) ideally corresponds to 1 bacterial cell present in a sample.

Note: A typical experiment consists of 9 reactions needed for controls, plus the number of reactions needed for the 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. Then, only the microproof *Legionella* Quantification Standard A needs to be run as a calibrator. It is recommended to run Quantification Standard A in duplicate. Contact Hygiena Diagnostics GmbH for more information about generating and using an external standard curve.

5. Seal the vessels accurately and tightly with the colorless cap strips.
6. Mix thoroughly using a vortex centrifuge.
   Note: Hygiena Diagnostics GmbH recommends vortex centrifuges Multispin MSC-6000 for PCR strips or vortex centrifuge CVP-2 for PCR plates. 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: When using any LightCycler 480 instrument, a special adapter is necessary.

   For some PCR instruments, the PCR strips must be placed in a balanced order into the cycler block. For example, two strips can be placed in columns 1 and 12.

2.3 Procedure for Colony Confirmation

2.3.1 Program Setup

The following procedure is optimized for rapid confirmation of presumptive *Legionella* colonies obtained using 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 microproof *Legionella* Quantification LyoKit. For details on how to program the experimental protocol, refer to the Instrument Operator's Manual for your real-time PCR cycler:

Pre-incubation **1** cycle

Step 1: 37 °C for 4 minutes
Step 2: 95 °C for 5 minutes

Amplification 3**0** 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 probe quencher as well as the use of a passive reference dye must be specified. The microproof *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. For FAM and HEX, the Filter Set Gain Setting must be modified to ’x4’.

2.3.2 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 microproof Suspension Buffer.

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

1. Take the needed number of PCR tube strips out of the aluminum foil bag. Use scissors or a scalpel to cut the strips apart. Tightly seal the bag afterward 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. Carefully remove and discard the caps from the tube strips.

   Note: To avoid unwanted liquid absorption, open strips shortly before filling. Do not leave strips open for extended periods of time.
4. Pipetting of samples:
   - Pipet 20 µL Internal Amplification Control (Product No. KIT230015) 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 (Product No. KIT230015) into a PCR vessel and add 5 µL PCR-grade H_2O (vial 6, colorless cap).
   - For the positive control, add 25 µL microproof *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: Hygiena Diagnostics GmbH recommends vortex centrifuges Multispin MSC-6000 for PCR strips or vortex centrifuge CVP-2 for PCR plates. 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: When using any LightCycler 480 instrument, a special adapter is necessary.

   For some PCR instruments, the PCR strips must be placed in a balanced order into the cycler block. For example, two strips can be placed in columns 1 and 12.

2.4 Data Interpretation

2.4.1 General Remarks

The amplification of DNA specific for the genus *Legionella* is analyzed in the fluorescence channel suitable for VIC/HEX-labeled probe 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 the VIC/HEX (*Legionella* spp.), FAM, ROX and Cy5 (Internal Control) channels 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.

2.4.2 Procedure A – Qualitative Detection / Colony Confirmation

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

Legionella pneumophila **FAM Channel**	***Legionella*** **spp. HEX Channel**	***Legionella pneumophila*** **Serogroup 1 ROX Channel**	**Internal Control Cy5 Channel**	**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.

† Samples may contain a mixed population of *L. pneumophila* serogroup 1, serogroups 2 - 15 and other *Legionella* spp.

2.4.3 *Procedure B – Quantification of Legionella in GU/mL*

Perform quantification according to ISO 12869:2019. The microproof *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 Hygiena Diagnostics for analysis, which calculates quantitative results equivalent to ISO 12869.

$$\mathbf{result}\left[\frac{\mathbf{GU}}{\mathbf{mL}}\right] = \frac{\mathbf{result}\left[\frac{\mathbf{GU}}{\mathbf{reaction}}\right] \times \mathbf{elution\ volume}\,[\mu\mathbf{L}] \times \mathbf{recovery\ factor}}{\mathbf{PCR\ reaction\ volume}\,[\mu\mathbf{L}] \times \mathbf{sample\ volume}\,[\mathbf{mL}]}$$

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

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

$$\mathbf{result}\left[\frac{\mathbf{GU}}{\boldsymbol{Y}\ \mathbf{mL}}\right] = \frac{\mathbf{result}\left[\frac{\mathbf{GU}}{\mathbf{reaction}}\right] \times \mathbf{elution\ volume}\,[\mu\mathbf{L}] \times \mathbf{recovery\ factor} \times \boldsymbol{Y}}{\mathbf{PCR\ reaction\ volume}\,[\mu\mathbf{L}] \times \mathbf{sample\ volume}\,[\boldsymbol{Y}\ \mathbf{mL}]}$$

Example:

The following calculation is suitable for samples prepared with the foodproof StarPrep Two Kit (Product No. KIT230177), assuming filtration of 100 mL of a water sample:

- elution volume = 125 µL
- recovery factor = 1,000 µL / 700 µL Rinse Buffer = 1.43
- PCR reaction volume = 25 µL
- sample volume = 100 mL
- Y = 100 mL

$$\mathbf{result}\left[\frac{\mathbf{GU}}{\mathbf{100\ mL}}\right] = \frac{\mathbf{result}\left[\frac{\mathbf{GU}}{\mathbf{reaction}}\right] \times \mathbf{125}\,[\mu\mathbf{L}] \times \mathbf{1.43} \times \mathbf{100}}{\mathbf{25}\,[\mu\mathbf{L}] \times \mathbf{100}\,[\mathbf{100\ mL}]} = \mathbf{result} \times \mathbf{7,15}\left[\frac{\mathbf{GU}}{\mathbf{100\ mL}}\right]$$

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" must be applied to account for run-to-run variance in Cp values. This factor is calculated from the difference between the Cp of the microproof *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, VIC/HEX, ROX and Cy5. • If your instrument does not have a HEX Channel, use VIC instead.
	Pipetting errors or omitted reagents.	• Check for the 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 a 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 microproof *Legionella* Quantification LyoKit lyophilized PCR Mix at 2 to 8 °C, protected from light and moisture. • Avoid repeated freezing and thawing.
	Low initial amount of target DNA.	• Increase the amount of sample DNA. Depending on the chosen DNA isolation method, inhibitory effects may occur.
Negative control samples are positive.	Carryover contamination is present.	• 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 carryover contamination. • Add positive controls after sample and negative control reaction vessels have been sealed.
Fluorescence intensity varies.	Insufficient centrifugation of the PCR vessels. Prepared PCR mix is still in the upper part of the vessel.	• Always centrifuge reaction vessels. • Check that no air bubbles are formed or remain in tube after centrifugation.
	Outer surface of the vessel or seal is dirty (e.g., by direct skin contact).	• Always wear gloves when handling the vessel and seal.
Pellets are difficult to dissolve.	The lyophilized PCR mix started to rehydrate.	• Always store the lyophilized PCR mix in the aluminum bag with the silica gel pad. • Open the strip shortly before filling.

4. Additional Information on this Product

4.1 How this Product Works

The microproof *Legionella* Quantification LyoKit 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 FAM, HEX/VIC and ROX channels.

In case of a negative result due to inhibition of 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 microproof *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.

4.2 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 downstream from one of the primer sites 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.

4.3 Prevention of Carryover Contamination

The heat-labile Uracil-DNA Glycosylase (UNG) is suitable for preventing carryover 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 dUTP 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 microproof *Legionella* Quantification LyoKit, decontamination can be achieved with the provided reagents.

4.4 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]. However, pathogenicity potential 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].

4.5 Product Characteristics

The microproof *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 microproof *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 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 microproof *Legionella* Quantification LyoKit is 3 genomic units (GU) / PCR reaction. The limit of quantification (LoQ) of the microproof *Legionella* Quantification LyoKit is 10 GU / PCR reaction.

4.6 References

1. Bartram J, Chartier Y, Lee JV, Pond K and Surman-Lee S, eds. (2007) Legionella and the Prevention of Legionellosis. World Health Organisation Press.
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).
3. ISO 11731-2:2004 Water quality – Detection and enumeration of Legionella – Part 2: Direct membrane filtration method for waters with low bacterial counts.
4. ISO 11731:2017 Water quality – Enumeration of Legionella.
5. Collins S, Jorgensen F, Willis C and Walker J. (2015) Real-time PCR to Supplement Gold-Standard Culture-based Detection of *Legionella* in Environmental Samples. *J Appl Microbiol.* 119(4):1158 - 1169.
6. Collins S, Stevenson D, Walker J and Bennett A. (2017) Evaluation of *Legionella* Real-Time PCR Against Traditional Culture for Routine and Public Health Testing of Water Samples. *J Appl Microbiol.* 122(6):1692 – 1703.

4.7 Quality Control

The microproof *Legionella* Quantification LyoKit is function tested using the LightCycler 480 System and the Mx3005P.

5. Supplementary Information

5.1 Ordering Information

In addition to the microproof *Legionella* Quantification LyoKit, Hygiena Diagnostics GmbH offers a broad range of reagents and services. For a complete overview and for more information, visit us at www.hygiena.com or contact us via email or phone.

5.2 License Notice

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5.5 Reference Number

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R 602 45-1 (KIT230119), R 602 45-2 (KIT230120)

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.

Revision A, November 2023
Rebranding and new layout.
Change of company name and product number.
R 602 45 20 -> INS-KIT230119-20-REVA.

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