INS-002-001

Bio Lp-1 Legionella Detection Kit Cat No. BPD 2001

Bio Lp-1 Legionella Quantification Kit Cat No. BPD 3001

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1. INTRODUCTION

Legionnaires Disease (LD) is a severe and often fatal form of pneumonia which is caused by bacteria known as Legionella. It is predominantly transmitted through contaminated water systems. Older adults, smokers and immunocompromised patients are particularly susceptible to LD. Globally the incidence of LD is estimated at 180–360 cases per million inhabitants, with the rate of incidence increasing in many parts of the world[1]. Infections acquired in buildings and healthcare institutions affect approximately two million people each year resulting in 90,000 deaths and an estimated $4.5–5.7 billion per year in additional costs for patient care[2]. Current European estimates attribute an additional 16 million days spent in hospital by patients per year[3]. Within these settings, water, water distribution and premise plumbing systems have been identified as a significant source of many of Health Care Associated Infections (HCAI) and pose a significant threat to human health.

The most effective way to limit the spread of LD is to control and eliminate the bacteria from environmental reservoirs. At present the most commonly used LD tests for water rely on century old culture methodologies which require significant hands-on time, are time consuming, slow (>14 days to result) and lack specificity and sensitivity.

An alternative approach is to apply molecular based LD water testing technologies which have the capability to provide rapid (< one day) and robust results. Such approaches can be highly specific for Legionella, while also being very sensitive. Any such test should be capable of detecting all Legionella species associated with infection, while also specifically identifying all serogroups of Legionella (16 serogroups responsible for causing majority of disease) and ideally also identifying *L. pneumophila* serogroup 1 which is reported to cause ~95% of human infections.

BioProbe Diagnostics have developed a new molecular kit called Bio Lp-1, which offers advantages over currently available methods in terms of time to result, specificity and sensitivity. Bio Lp-1 provides a complete identification of Legionella to the serogroup level in a single test using molecular technologies. Bio Lp-1 is the first combined test with a capability for the rapid (< 5 h from sample in to result out), sensitive, quantitative detection and identification of Legionella spp., *L. pneumophila* and *L. pneumophila* sg1 in a water sample.

2. Bio Lp-1 ASSAY TECHNOLOGY

Bio Lp-1 technologies allow for the rapid, specific and sensitive detection and differentiation of Legionella by real-time PCR. The **Bio Lp-1 Legionella detection kit** is designed to reliably detect and differentiate between *Legionella* spp., *Legionella pneumophila* 1-15 and *Legionella*

[1] https://www.cdc.gov/legionella/surv-reporting.html
[2] Collins AS. Preventing Health Care–Associated Infections. 2008 Apr. Chapter 41.
[3] European Centre for Disease Prevention and Control: Annual Epidemiological Report on Communicable Diseases in Europe 2008

pneumophila sg1 within a single reaction. The test targets specific and unique DNA sequences within the genome.

Bio Lp-1 Legionella detection kit is user friendly, with the kit containing all necessary reagents. All PCR components are supplied in a lyophilised format, only requiring the addition of the DNA sample or control to the reaction.

The Legionella PCR products are detected using FAM, HEX and ROX labelled fluorophores quenched using ZEN/Iowa Black FQ or Iowa Black RQ.

An internal Amplification Control (IAC) is included in the lyophilised master mix. IAC DNA is amplified in parallel with the primary assay and is detected using a Cy5 fluorescent labelled probe quenched by TAO/Iowa Black RQ. An IAC is included in the reaction to indicate that the PCR is functioning correctly. It is used to reduce the risk of false negative results. The Bio Lp-1 IAC is non-competitive; therefore, the target is amplified using different primers than are primary assays.

Bio Lp-1 Legionella Quantification kit contains DNA standards for the absolute quantification of any Legionella DNA present in the sample tested.

4-plex Target assay	**Fluorophore**
Legionella spp.	FAM
Legionella pneumophila 1-15	ROX
Legionella pneumophila sg1	HEX
IAC	Cy5

This methodology allows for the detection and / or quantification of Legionella spp., Legionella pneumophila sg1-15 or Legionella pneumophila sg1 in less than 3 hours.

- This internally controlled real-time PCR tetraplex assay gives reliable and sensitive detection of Legionella from water samples.
- It can provide a complete Legionella profile in less than 3 hours.
- DNA is purified using our sensitive extraction kit that results in excellent yields of high-quality DNA.
- This kit is suitable for use on water samples such as domestic hot water, cooling tower water and mineral water.

Sampling, transport and storage of water samples is performed according to ISO 19458. After concentration by filtration, DNA is extracted from the filter using **Bio Lp-1 DNA Isolation Kit (cat # BPD 1001)**.

3. PRECAUTIONS AND RECOMMENDATIONS

- It is strongly recommended that the entire protocol be read before beginning the experiment. Strict compliance with the protocol is required.
- Store all reagents as indicated on the box and in Section 5 below.
- Reagents should not be used after the expiration date.
- Never store kit or components of the kit in proximity to samples or close to post-PCR products.
- This test should only be performed by suitably trained personnel with strict compliance with appropriate laboratory real-time PCR protocols to ensure quality results.
- It is recommended that suitable facilities are available for PCR set up, template addition and PCR operation. These facilities may be a separate room, area or biosafety cabinet.
- Laboratory equipment and/or supplies must not be moved from one facility/workstation to another.
- Change gloves regularly and whenever you suspect they may have been contaminated.
- All surfaces, equipment and workstations should be regularly decontaminated with suitable detergents or sterilising agents and rinsed afterwards using appropriate reagent such as sterile water (*Legionella* DNA free) or 70% alcohol.
- Using powdered gloves is NOT recommended as this could result in residue build-up on the sealing elements of the PCR strips. This could interfere with the data reading apparatus of the PCR instrumentation.
- A negative and positive control should be included with each PCR amplification run.
- For each quantitative PCR analysis two standards and a negative control must be included in each PCR amplification run.
- Vortex standards and controls after rehydration and before use to assure solutions are homogenous.
- All equipment and instruments used during this testing should be qualified for purpose.
- This product must not be used for *in vitro* human diagnostics.

4. KIT COMPONENTS

4.1. Bio Lp-1 Legionella Detection Kit

Reagent	**Appearance**	**Kit Component format**	**Kit Storage Temperature**
PCR assay	8 x 12-well strips (Foil wrapped)	Lyophilised	Room Temperature
Positive control DNA*	1 x 320 µl ˜ (red)	Lyophilised	Room Temperature*
Rehydration buffer	1 x 1 ml (green)	Liquid	Room Temperature
Negative control	1 x 1 ml (white)	Liquid	Room Temperature

* Store at 2-8 ˚C until rehydrated. Store at <-18 ˚C after rehydration. **Note:** Freeze thaw cycles should be avoided. If multiple uses are required, positive control DNA can be frozen in single use aliquots.
˜ Volume after rehydration (instructions for rehydration included in Section 8.1)

4.2. Bio Lp-1 Legionella Quantification Kit

Reagent	**Appearance**	**Kit Component format**	**Kit Storage Temperature**
Part A^			
PCR assay	8 x 12-well strips (Foil wrapped)	Lyophilised	Room Temperature
Part B^			
PCR Standard 1*	1 x 100 µl ˜ (red)	Lyophilised	Room Temperature
Rehydration buffer	1 x 1 ml (green)	Liquid	Room Temperature
Negative control	1 x 1 ml (white)	Liquid	Room Temperature

* Store at 2-8 ˚C until rehydrated. Store at <-18 ˚C after rehydration. **Note:** Freeze thaw cycles should be avoided. If multiple uses are required, DNA Standard can be frozen in single use aliquots.
˜ Volume after rehydration (instructions for rehydration included in Section 10.1)
^ Part A & Part B required for Legionella quantification

5. KIT STORAGE AND SHELF LIFE

All kit components (except positive control and DNA standards) should be stored at room temperature. Upon receipt of Bio Lp-1 kit, DNA standards or positive control DNA template must be stored at 2-8 ˚C until rehydration. Store at <-18 ˚C after rehydration.

Do not freeze the other reagents from the kit. Reagents stored at the correct temperature may be used until the expiration date indicated.

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED

6.1. Equipment

- PCR strip holder for PCR set up
- PCR strip holder (suitable for use in real-time PCR machine)

6.2. Laboratory Supplies

- 20 µl and 200 µl Micropipettes
- DNA/Nuclease free Filtered pipette tips suitable for 20 µl and 200 µl Micropipettes
- Unpowered gloves
- Decontamination solution e.g., 70% alcohol

6.3. Instruments and software

- LightCycler 480 System II (Roche)
- LightCycler 480 Software V1.5.1 or later (Roche)
- Exor4™

Or

- CFX96™ Touch (Bio-Rad, Cat # 1855195)
- CFX Manager™ Software Industrial Diagnostics Edition V3.1 or later (Bio-Rad, Cat # 3593893)

This product has been validated on the above platforms and software. Other PCR platforms have not been validated.

7. WATER SAMPLE COLLECTION, FILTRATION AND DNA PURIFICATION

Sampling, transport and storage of water samples should be performed according to ISO 19458. Water samples are concentrated by filtration. DNA purification is performed using **Bio Lp-1 DNA ISOLATION KIT (BPD 1001)** in accordance with the instruction provided.

Instructions for use with Bio Lp-1 Legionella Detection Kit (BPD 2001) **Go to SECTION 8**

Instructions for use with Bio Lp-1 Legionella Quantification Kit (BPD 3001) **Go to SECTION 11**

8. QUALITATIVE REAL-TIME PCR: INSTRUCTIONS FOR USE WITH BIO LP-1 LEGIONELLA DETECTION KIT

8.1. Qualitative Real-Time PCR Set-up

Note: If this is your fist time using the kit, please follow the instructions for positive control rehydration and dilution. Otherwise proceed to step i.

Positive control (red) rehydration

- Briefly spin vial.
- Add 320 µl of rehydration buffer (green) to the vial.
- Incubate for 5 mins at room temperature.
- Vortex for 10 secs followed by a brief centrifuge for 5 sec.

Positive control DNA should be stored at 2-8 ˚C until rehydrated. Store at <-18 ˚C after rehydration. **Note:** Freeze thaw cycles should be avoided. If multiple uses are required, positive control DNA can be frozen in single use aliquots.

i. Remove foil wrapping from the 12-well strips. (4 x 12 well strips are included in each foil wrap).
ii. Place strips (sufficient for all samples to be tested) in PCR strip holder.
iii. Carefully remove the caps from each strip and leave inverted on a clean surface.
iv. Add 19.2 µl of sample template to each well (in duplicate). The total volume after rehydration will be 20 µl.
v. Dilute Positive control DNA according to instructions above. Add 19.2 µl of Positive control DNA to the well for the positive control. It is recommended to run positive control in duplicate.
vi. Add 19.2 µl of nuclease free water to the well for the negative control (in duplicate).
vii. Carefully replace the caps back onto each strip.
viii. Gently tap the holder containing the 12-well strips against a flat surface to ensure the sample is at the bottom of the well.
ix. Transfer the strips to a plate vortex and vortex at medium speed (~1800rpm) for 30 s, followed by a short centrifuge step for 5 secs.
x. Transfer the strips and the holder to the real-time PCR instrument and begin run.
    - For specific instructions for PCR set-up and exporting of results for the **Bio-Rad CFX96 Touch** see section 8.2 below.

- For specific instructions for PCR set-up and exporting of results for the **Roche Diagnostics LightCycler 480 system II** see section 8.3 below.

This product has been validated on the above platforms. Other PCR platforms have not been validated.

8.2. BioRad CFX96 Touch

Bio Lp-1 Legionella Detection Kit (BPD 2001) is validated for use on the BioRad CFX96 Touch with CFX Manager™ Software Industrial Diagnostics Edition V3.1.

The following instructions outline how to set up a Bio Lp-1 Legionella Detection experiment on the BioRad CFX96 Touch.

For a more detailed description of instrument software and programming please refer to the BioRad CFX96 Touch Users Guide.

8.2.1. Performing real-time PCR experiment

8.2.1.1. Import Real-Time PCR Template

i. Turn on BioRad CFX96 Touch and associated computer.
ii. Insert USB containing the set-up files into the computer USB port.
iii. Log on to the BioRad CFX Manager software.
iv. From the home screen, select “Show Navigator”.
v. Select “Import”. Select “Bio Lp-1_Detection_BioRad_CFX96Touch_protocol file from USB. Save as protocol on platform software.
Note: This procedure only needs to be performed out the first time the experiment is performed.

8.2.1.2. Running PCR Experiment from Protocol

i. Turn on BioRad CFX96 Touch and associated computer.
ii. Log on to the BioRad CFX Manager software.
iii. Select “Bio Lp-1_Detection_BioRad_CFX96Touch_protocol”
iv. The following parameters are included in the template:
- Ensure correct fluorescence channels on the real-time PCR machine are open for reading. These channels should be compatible with FAM (465-510 nm), HEX (533-580 nm), ROX (533-610) and Cy5 (618-680) probes.
- The thermal cycling parameters are as follows:

Step	Temperature	Time	Cycles
Initial denaturation	95 ˚C	2 mins	1
Denaturation Annealing / Extension*	95 ˚C 59 ˚C	15 secs 1 min	50
Cooling	40 ˚C	10 secs	1

*A single fluorescence reading should be taken during the annealing / extension cycle.

v. Insert the 12-well strips and the holder into the real-time PCR instrument.

vi. Define the plate set-up e.g., number of samples and well positions.
vii. Fill in the required information for each well such as
    - Sample type e.g., Controls (positive or negative), standards or samples
    - Sample name or ID
viii. Save experiment.
ix. Start the experiment.
x. Once the PCR run is complete the CFX96 Touch and the CFX Manager Software will perform the data analysis automatically.
xi. For results analysis see Section 9 below.

8.3. Roche Diagnostics LightCycler 480 system II

Bio Lp-1 Legionella Detection Kit (BPD 2001) is validated for use on Roche Diagnostics LightCycler 480 System II Real-Time PCR Instrument with Roche Diagnostics LightCycler 480 Analysis Software v1.5.1.

The following instructions outline how to set up a Bio Lp-1 Legionella Detection experiment on the Roche Diagnostics LightCycler 480 System II.

For a more detailed description of instrument software and programming please refer to the Roche Diagnostics LightCycler 480 System II Users Guide.

8.3.1. Generation of LightCycler 480 System II colour compensation file for Bio Lp-1

8.3.1.1. Import Colour Compensation File Macro

i. Turn on Roche LightCycler 480 System II and associated computer.
ii. Insert USB containing the Bio Lp-1 set-up files into the computer USB port.
iii. Log on to the Roche LightCycler 480 System II software.
iv. From the home screen, select “Show Navigator”.
v. Select “Import”. Select “Bio Lp-1_Lightcycler480_CC_macro.ixo file from USB. Save as Macro on platform software.
Note: This procedure only needs to be performed once.

8.3.1.2. Running Colour Compensation Experiment from Macro

i. Turn on Roche Lightcycler 480 System II and associated computer.
ii. Log on to the Roche Lightcycler 480 System II software.
iii. From the home screen, select “New Experiment from Macro”.
iv. Select “Select “Bio Lp-1_Lightcycler480_CC_macro.ixo”
v. Select “Apply Macro”.
vi. Insert the 12-well strips and the holder into the real-time PCR instrument.
vii. Define the plate set-up.
viii. Fill in the required information for each well such as
    i. Sample type e.g., FAM, HEX, ROX, CY5 or water
    ii. Sample name or ID

ix. Select “Save button” and type experiment name. Press save.
x. Select “Start Run” to begin experiment.
xi. Results will automatically be generated once the PCR run is complete.
xii. Save experiment as Colour Compensation file.
xiii. **Note:** This procedure only needs to be performed once but must be completed before first experiment for the detection or quantification of Legionella by Bio Lp-1.

8.3.2. Performing real-time PCR experiment on Roche Diagnostics LightCycler 480 system II

8.3.2.1. Import Real-Time PCR Macro

i. Turn on Roche Lightcycler 480 System II and associated computer.
ii. Insert USB containing the set-up files into the computer USB port.
iii. Log on to the Roche Lightcycler 480 System II software.
iv. From the home screen, select “Show Navigator”.
v. Select “Import”. Select “Bio Lp-1_Lightcycler480_SystemII_macro.ixo file from USB. Save as macro on platform software.
Note: This procedure only needs to be performed out the first time the experiment is performed.

8.3.2.2. Running PCR Experiment from Macro

i. Turn on Roche Lightcycler 480 System II and associated computer.
ii. Log on to the Roche Lightcycler 480 System II software.
iii. From the home screen, select “New Experiment from macro”.
iv. Select “Bio Lp-1_Lightcycler480_SystemII_macro.ixo”
v. Select “Apply Macro”.
vi. The following parameters are included in the experimental macro
- Ensure correct fluorescence channels on the real-time PCR machine are open for reading. These channels should be compatible with FAM (465-510 nm), HEX (533-580 nm), ROX (533-610) and Cy5 (618-680) probes.
- The thermal cycling parameters are as follows:

Step	Temperature	Time	Cycles
Initial denaturation	95 ˚C	2 mins	1
Denaturation Annealing / Extension*	95 ˚C 59 ˚C	15 secs 1 min	50
Cooling	40 ˚C	10 secs	1

*A single fluorescence reading should be taken during the annealing / extension cycle.

vii. Insert the 12-well strips and the holder into the real-time PCR instrument.
viii. Define the plate set-up e.g., number of samples and well positions.
ix. Fill in the required information for each well such as
    - Sample type e.g., Controls (positive or negative), standards or samples
    - Sample name or ID
x. Select “Save button” and type experiment name. Press save.

xi. Select “Start Run” to begin experiment.
xii. Results will be generated automatically once the PCR run is complete
xiii. For results analysis see Section 9 below.

9. QUALITATIVE RESULTS ANALYSIS

9.1. Data analysis

A complete report of the experimental results will be automatically generated once the experiment is completed.

9.1.1. Primary assays *Legionella* spp, *L. pneumophila* sg 1-16, *L. pneumophila* sg 1.

The presence or absence of Legionella is determined based on the amplification of the target DNA sequence. This can be visualised on the amplification curves plotted by the PCR instrumentation software. A positive amplification has a final fluorescence that lies clearly above the background threshold.

Figure 1 is an example of one possible outcome. Other possible outcomes and results interpretation are included below in Section 9.2.

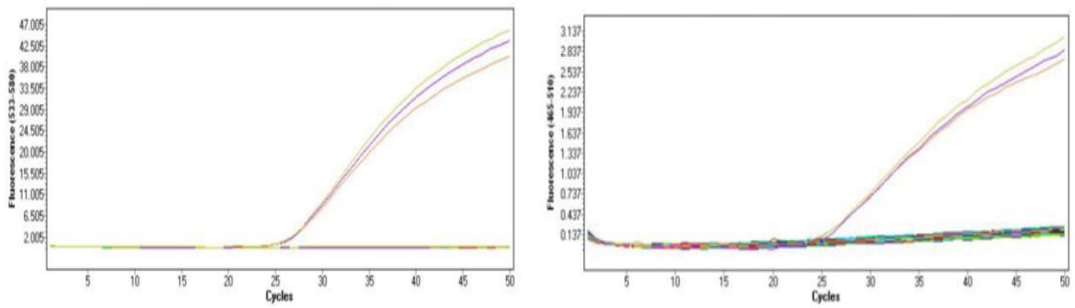

Figure 1. The amplification curves in the HEX channel (left image) and the internal control in the Cy5 channel (right image) are above the threshold, indicating the presence of *Legionella pneumophila* sg1 target DNA (HEX) and that there was no PCR inhibition.

9.1.2. IAC

In the IAC channel (Cy5) there should be an amplification curve for each control, standard and sample.

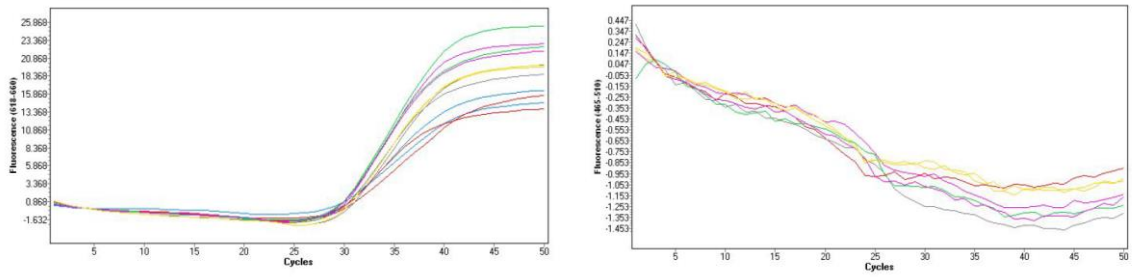

Figure 2. The amplification curves in the Cy5 channel (left image) are above the threshold indicating that the PCR reaction is performing optimally and there is no PCR inhibition in the samples. The image on the right shows no amplification in the Cy5 channel (IAC assay) indicating that there is a problem with the PCR reaction.

9.1.3. Controls

Ensure there is an amplification curve in ALL channels for the positive control and amplification in the Cy5 channel ONLY for the negative control.

9.2. Qualitative Results Interpretation

9.2.1. Summary of possible results.

Legionella spp. is detected, if the sample DNA and the Internal Control DNA show an amplification signal in the FAM and Cy5 channels.
Legionella pneumophila sg 1-16 is detected, if the sample DNA and the Internal Control DNA show an amplification signal in the FAM, ROX and Cy5 channels.
Legionella pneumophila sg 1 is detected, if the sample DNA and the Internal Control DNA show an amplification signal in the FAM, HEX, ROX and Cy5 channels.

Legionella spp, Legionella pneumophila sg 1-16 or *Legionella pneumophila* sg 1 is not detected, if the sample DNA shows no amplification signal in the FAM, HEX or ROX channels but an amplification signal for the Internal Control DNA in the Cy5 channel.

Detection of the Internal Control DNA indicates there is no inhibition of the PCR reaction. A result is invalid, if the sample DNA and Internal Control DNA show no amplification signal in the detection system. Additionally, amplification in the internal control channel (Cy5), with a higher than normal C_T value may indicate the presence of PCR inhibitors in the sample. As a guideline, in the absence of PCR inhibitors the C_T values of the internal control should range between 27-32 cycles. A C_T value above this indicates the PCR has been inhibited. It is recommended that the extracted DNA samples are diluted 1:10 with nuclease free water and the PCR should be repeated.

Note: *Legionella* may also be detected, if the sample DNA shows a strong amplification signal in the FAM, HEX or ROX channels (i.e., an amplification curve with a C_T value lower than 22)

but none for the Internal Control DNA in the detection system. The detection of the internal amplification control is not necessary because very high concentrations of the Legionella template can cause a weak or absent signal from the Internal Control DNA.

Table 1. Results interpretation table for qualitative analysis

	FAM	HEX	ROX	CY5
***Legionella* spp.**	+	-	-	+
***Legionella pneumophila* sg 1-16**	+	-	+	+
***Legionella pneumophila* sg 1**	+	+	+	+
No *Legionella* present (IAC only)	-	-	-	+
Legionella present but inhibition of IAC*	+	+	+	-
PCR inhibition	-	-	-	-

*Inhibition of IAC likely due to high concentration of Legionella DNA

10. QUANTITATIVE REAL-TIME PCR: INSTRUCTIONS FOR USE WITH BIO LP-1 LEGIONELLA DETECTION KIT AND BIO LP-1 LEGIONELLA QUANTIFICATION KIT

Note: Both BIO LP-1 LEGIONELLA DETECTION KIT AND BIO LP-1 LEGIONELLA QUANTIFICATION KIT are required in order to perform a Quantitative Legionella analysis of DNA samples.

10.1. Quantitative Real-Time PCR Set-up

Note: If this is your fist time using the kit, please follow the instructions for standard DNA rehydration and dilution. Otherwise proceed to step i.

Standard (red) rehydration

- Briefly spin vial.
- Add 360 µl of rehydration buffer (green) to the vial.
- Incubate for 5 mins at room temperature.
- Vortex for 10 secs followed by a brief centrifuge for 5 sec.

Standard dilution to use concentrations

- **For Standard 1:** (1.5×10^5 GU/19.2 µl), use directly.
- **For standard 2:** Prepare four 10-fold serial dilutions by taking 10µl of each dilution (starting with dilution 1) and adding 90µl PCR grade H_2O. Use last dilution as standard 2 (1.5×10^1/19.2 µl).
- See below table for all dilution volumes.

Dilutions	**1**	**2**	**3**	**4**	**5**
Volume from previous dilution (µl)	-	10	10	10	10
DNA free water (µl)	-	90	90	90	90
Final concentration (GU/19.2 µl)	1.5×10^5	1.5×10^4	1.5×10^3	1.5×10^2	1.5×10^1

DNA standard should be stored at 2-8 ˚C until rehydrated. Store at <-18 ˚C after rehydration.

Note: Freeze thaw cycles should be avoided. If multiple uses are required, positive control DNA can be frozen in single use aliquots.

i. Remove foil wrapping from the 12 well strips (Bio Lp-1 Legionella Detection Kit, Cat # BPD 2001). (4 x 12 well strips are included in each foil wrap).
ii. Place strips (sufficient for all samples to be tested) in PCR strip holder.
iii. Carefully remove the caps from each strip and leave inverted on a clean surface.
iv. Dilute Standard DNA according to instructions above. Add 19.2 µl of each standard control DNA to each of the wells (2 standards in duplicate) to be used for the standards.
v. Add 19.2 µl of nuclease free water to the well for the negative control (in duplicate).

vi. Add 19.2 µl of sample template to each well (in duplicate). The total volume after rehydration will be 20 µl.
vii. Carefully replace the caps back onto each strip.
viii. Gently tap the holder containing the 12-well strips 4 times against a flat surface to ensure the sample is at the bottom of the well.
ix. Transfer the strips to a plate vortex and vortex at medium speed (~1800rpm) for 30 s, followed by a short centrifuge step for 5 secs.
x. Transfer the strips and the holder to the real-time PCR instrument and begin run.
- For specific instructions for PCR set-up and exporting of results for the **Bio-Rad CFX96 Touch** see section 10.2 below.

This product has been validated on the above platform. Other PCR platforms have not been validated.

10.2. Quantitative Real-Time PCR Set-Up BioRad CFX96 Touch

Bio Lp-1 is validated for use on the BioRad CFX96 Touch with CFX Manager™ Software Industrial Diagnostics Edition V3.1

The following instructions outline how to set up a Bio Lp-1 experiment on the BioRad CFX96 Touch.

For a more detailed description of instrument software and programming please refer to the BioRad CFX96 Touch Users Guide.

10.2.1. Import Real-Time PCR Template

i. Turn on BioRad CFX96 Touch and associated computer.
ii. Insert USB containing the set-up files into the computer USB port.
iii. Log on to the BioRad CFX Manager software.
iv. From the home screen, select “Show Navigator”.
v. Select “Import”. Select “Bio Lp-1_Quantification_BioRad_CFX96Touch_protocol file from USB. Save as protocol on platform software.
Note: This procedure only needs to be performed out the first time the experiment is performed.

10.2.2. Running PCR Experiment from Protocol

i. Turn on BioRad CFX96 Touch and associated computer.
ii. Log on to the BioRad CFX Manager software.
iii. Select “Bio Lp-1_Quantification_BioRad_CFX96Touch_protocol”
iv. The following parameters are included in the template
- Ensure correct fluorescence channels on the real-time PCR machine are open for reading. These channels should be compatible with FAM (465-510 nm), HEX (533-580 nm), ROX (533-610) and Cy5 (618-680) probes.
- The thermal cycling parameters are as follows:

Step	Temperature	Time	Cycles
Initial denaturation	95 ˚C	2 mins	1
Denaturation Annealing / Extension*	95 ˚C 59 ˚C	15 secs 1 min	50
Cooling	40 ˚C	10 secs	1

*A single fluorescence reading should be read taken during the annealing / extension cycle.

v. Insert the 12-well strips and the holder into the real-time PCR instrument.
vi. Define the plate set-up e.g., number of samples and well positions.
vii. Fill in the required information for each well such as
    - Sample name or ID
    - Sample type e.g., Controls (positive or negative), standards or samples
    - Quantity of each standard (values are indicated above in section 10.1.
viii. Save experiment.
ix. Start the experiment.
x. Once the PCR run is complete the CFX96 Touch and the CFX Manager Software will perform the data analysis automatically.
xi. For results analysis see Section 11 below.

11. QUANTITATIVE RESULTS ANALYSIS

11.1. Data analysis

A complete report of the experimental results will be automatically generated once the experiment is completed.

11.1.1. Primary assays *Legionella* spp, *L. pneumophila* sg 1-16, *L. pneumophila* sg 1.

The presence or absence of Legionella is determined based on the amplification of the target DNA sequence. This can be visualised on the amplification curves plotted by the PCR instrumentation software. A positive amplification has a final fluorescence that lies clearly above the background threshold.

Figure 1 is an example of one possible outcome. Other possible outcomes and results interpretation are included below in Section 11.2.

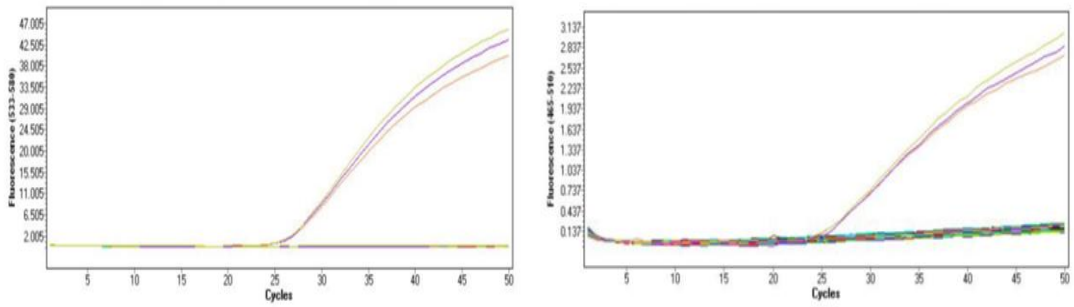

Figure 1. The amplification curves in the HEX channel (left image) and the internal control in the Cy5 channel (right image) are above the threshold, indicating the presence of *Legionella pneumophila* sg1 target DNA (HEX) and that there was no PCR inhibition.

11.1.2. IAC

In the IAC channel (Cy5) there should be an amplification curve for each control, standard and sample.

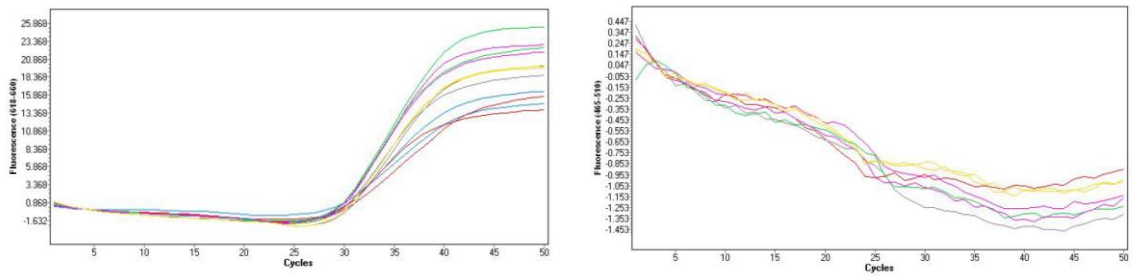

Figure 2. The amplification curves in the Cy5 channel (left image) are above the threshold indicating that the PCR reaction is performing optimally and there is no PCR inhibition in the samples. The image on the right shows no amplification in the Cy5 channel (IAC assay) indicating that there is a problem with the PCR reaction.

11.1.3. Controls

Ensure there is an amplification curve in ALL channels for the positive control and amplification in the Cy5 channel ONLY for the negative control.

11.2. Quantitative Results Interpretation

11.2.1. Summary of possible results

Legionella spp. is detected, if the sample DNA and the Internal Control DNA show an amplification signal in the FAM and Cy 5 channels.
Legionella pneumophila sg 1-16 is detected, if the sample DNA and the Internal Control DNA show an amplification signal in the FAM, ROX and Cy 5 channels.
Legionella pneumophila sg 1 is detected, if the sample DNA and the Internal Control DNA show an amplification signal in the FAM, HEX, ROX and Cy 5 channels.

Legionella spp, Legionella pneumophila sg 1-16 or *Legionella pneumophila* sg 1 is not detected, if the sample DNA shows no amplification signal in the FAM, HEX or ROX channels but an amplification signal for the Internal Control DNA in the Cy5 channel.

Detection of the Internal Control DNA indicates there is no inhibition of the PCR reaction. A result is invalid, if the sample DNA and Internal Control DNA show no amplification signal in the detection system. Additionally, amplification in the internal control channel (Cy5), with a higher than normal C_T value may indicate the presence of PCR inhibitors in the sample. As a guideline, in the absence of PCR inhibitors the C_T values of the internal control should range between 27-32 cycles. A C_T value above this indicates the PCR has been inhibited. It is recommended that the extracted DNA samples are diluted 1:10 with nuclease free water and the PCR should be repeated.

Note: *Legionella* may also be detected, if the sample DNA shows a strong amplification signal in the FAM, HEX or ROX channels (i.e., an amplification curve with a low C_T value) but none for the Internal Control DNA in the detection system. The detection of the internal amplification control is not necessary because very high concentrations of the Legionella template can cause a weak or absent signal from the Internal Control DNA.

Table 2. Results interpretation table for Legionella analysis

	FAM	HEX	ROX	CY5
***Legionella* spp.**	+	-	-	+
***Legionella pneumophila* sg 1-16**	+	-	+	+
***Legionella pneumophila* sg 1**	+	+	+	+
No Legionella present (IAC only)	-	-	-	+
PCR inhibition	-	-	-	-

Inhibition of IAC likely due to high concentration of Legionella DNA

11.3. Calculation of Legionella concentration

For the purpose of quantitative analysis, the real-time PCR software calculates the C_T value for each sample tested. These C_T values are then compared to the internal standard curve generated (DNA standards must be included in each quantitative real-time PCR run) to give absolute quantification results.

The absolute quantification results correspond to the quantity of Legionella Genome Equivalents (GE) present in 19.2 µl of the DNA extract. The value for the concentration of Legionella GE / 1 L of water sample can be calculated as follows:

X	(MQV in 19.2 µl) x D x total elution volume
	Volume of filtered sample (L) x volume of elution used in PCR reaction

X = Number of Legionella GE in 1 L of water

MQV = Mean Quantification Value - the average GE/well between replicates

D = dilution factor (only applicable if the DNA has been diluted before adding to the PCR).

12. Quality Control

The analysis of the samples using Bio Lp-1 must be performed on a real-time PCR machine which has been suitably qualified.
Positive and negative controls must show correct results (see results interpretation table) before accepting the data from the PCR machine. The Positive Control has a concentration of 10^4 copies/reaction.

If the results are questionable the following items should be checked before a complete investigation is carried out
- Confirm that the reagents are within their expiry date.
- Confirm the functionality and qualification of the instrumentation
- Ensure the test procedure has been carried out as described

PRECAUTIONS

IMPORTANT: It is essential that this Protocol is carried out by suitably trained staff.

- Do not use reagents after the kit expiry date.
- Ensure all work surfaces are regularly decontaminated using suitable solution e.g., 5% sodium hypochlorite solution or 70% alcohol.
- Gloves should be worn throughout the procedure. Gloves should be changed regularly and after suspected contact with contaminants.
- Laboratory consumables should be sterile, DNase, RNase and pyrogen free.
- Laboratory equipment should be appropriately decontaminated before each use.
- All water samples containing unknown microorganisms should be handled and disposed of as potentially infectious substances.
- Check all necessary equipment is functioning correctly before beginning experiment
- Persons using this Protocol should be familiar with good laboratory practice.

TROUBLESHOOTING

Degradation of DNA after purification

All supplied reagents and consumables are DNase free. However, DNase contamination may be introduced during the DNA extraction and purification process of certain samples. Ensure that all pipette tips and tubes used for sample processing are DNase free. Appropriate precautions during the procedure should safeguard against DNase contamination.

Ensure correct fluorescence channels on the real-time PCR machine are open for reading. These channels should be compatible with FAM, HEX, ROX and Cy5 probes.