

Primerdesign™ Ltd

# Legionella\_spp (all species)

23S/5S Intergenic Spacer Region

genesig® Advanced Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

# Introduction to Legionella\_spp (all species)

Legionella is a genus of Gram-negative bacterium. This genus contains over 50 species, of which there are 2 which cause pathogenesis in humans; these being *L. pneumophila* and *L. longbeachae*. All of these bacteria can cause Legionellosis, which can be a relatively common cause of nosocomial and community acquired pneumonia.

*Legionella pneumophila* is a flagellated bacterium which is a non-encapsulated, aerobic bacillus of approximately 2µm in length with a single, polar flagellum. The genome of this bacterium is over 3Mbp long and encodes for around 3000 genes. There are several serotypes of *L. pneumophila*, of which serotype 1 is responsible for 70-90% of cases.

The natural environment of *L. pneumophila* is hot and moist with a supply of nutrition from the sediment. These environmental features can be found in storage facilities for drinking water leading to infection of individuals by inhalation. After inhalation *L. Pneumophila* avoid the defenses of the upper respiratory tract and target the alveolar. Here, macrophages and epithelial cells can be infected by the bacteria via phagocytosis. The generated phagosome does not fuse with the lysosome and so the bacteria avoid destruction. After several hours within the host cell, endosome enclosure allows bacterial multiplication. The bacteria express genes known to be involved in necrosis and exit the host cell and spread intracellularly.

*L. pneumophila* infection is known as legionellosis which can cause Legionnaires' disease or Pontiac fever. Legionnaires' disease initially presents in fever, headache, aching joints, lack of energy or tiredness that can progress into, cough, diarrhea, nausea, abdominal pain and eventually pneumonia, which can lead to coma and death. Pontiac Fever can cause symptoms including fever, headache, tiredness, loss of appetite, muscle and joint pain, nausea, coughing but usually desists within 5 days without medical treatment.

*Legionella micdadei* is the second most commonly isolated Legionella species and is responsible for approximately 60% of Legionella pneumonias other than those caused by *L. pneumophila*. The pneumonia caused is a variant of Legionnaires' disease called Pittsburgh Pneumonia and is thought to occur mainly in immunocompromised patients.

The natural environment of *Legionella longbeachae* is in damp soil and is known to infect humans via inhalation of particles from contaminated soil or compost. This bacterium is thought to cause pneumonia in people with diseases of the immune system.

# Specificity

The Primerdesign genesig Kit for Legionella\_spp (all species) (Legionella\_spp) genomes is designed for the in vitro quantification of Legionella\_spp genomes. The kit is designed to have a broad detection profile. Specifically, the primers represent 100% homology with over 95% of the NCBI database reference sequences available at the time of design.

The dynamics of genetic variation means that new sequence information may become available after the initial design. Primerdesign periodically reviews the detection profiles of our kits and when required releases new versions.

The 23S/5S intergenic spacer gene, is the ideal target to achieve a broad based detection profile within this family. The primers and probe sequences in this kit have 100% homology with over 95% of reference sequences in the NCBI database based on a comprehensive bioinformatics analysis.

If you require further information, or have a specific question about the detection profile of this kit then please send an e.mail to [enquiry@primerdesign.co.uk](mailto:enquiry@primerdesign.co.uk) and our bioinformatics team will answer your question.

# Kit contents

- **Legionella\_spp specific primer/probe mix (150 reactions BROWN)**  
FAM labelled
- **Legionella\_spp positive control template (for Standard curve RED)**
- **Internal extraction control primer/probe mix (150 reactions BROWN)**  
VIC labelled as standard
- **Internal extraction control DNA (150 reactions BLUE)**
- **Endogenous control primer/probe mix (150 reactions BROWN)**  
FAM labelled
- **RNase/DNase free water (WHITE)**  
for resuspension of primer/probe mixes
- **Template preparation buffer (YELLOW)**  
for resuspension of internal control template, positive control template and standard curve preparation

## Reagents and equipment to be supplied by the user

### Real-time PCR Instrument

#### Extraction kit

This kit is recommended for use with genesig Easy DNA/RNA extraction kit. However, it is designed to work well with all processes that yield high quality RNA and DNA with minimal PCR inhibitors.

#### oasig™ lyophilised or Precision® PLUS 2X qPCR Master Mix

This kit is intended for use with oasig or PrecisionPLUS2X qPCR Master Mix.

#### Pipettors and Tips

#### Vortex and centrifuge

#### Thin walled 1.5 ml PCR reaction tubes

## Kit storage and stability

This kit is stable at room temperature but should be stored at  $-20^{\circ}\text{C}$  on arrival. Once the lyophilised components have been resuspended they should not be exposed to temperatures above  $-20^{\circ}\text{C}$  for longer than 30 minutes at a time and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances.

If a standard curve dilution series is prepared this can be stored frozen for an extended period. If you see any degradation in this serial dilution a fresh standard curve can be prepared from the positive control.

Primerdesign does not recommend using the kit after the expiry date stated on the pack.

## Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and DNA integrity (An internal PCR control is supplied to test for non specific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative-control, replace the template DNA sample with RNase/DNase free water.

## Dynamic range of test

Under optimal PCR conditions genesig Legionella\_spp detection kits have very high priming efficiencies of  $>95\%$  and can detect less than 100 copies of target template.

## Notices and disclaimers

This product is developed, designed and sold for research purposes only. It is not intended for human diagnostic or drug purposes or to be administered to humans unless clearly expressed for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. During the warranty period Primerdesign genesig detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired. PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applied Biosystems Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc., and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation.

## Trademarks

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The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG. BI, ABI PRISM®, GeneAmp® and MicroAmp® are registered trademarks of the Applied Biosystems (Applied Biosystems Corporation). BIOMEK® is a registered trademark of Beckman Instruments, Inc.; iCycler™ is a registered trademark of Bio-Rad Laboratories, Rotor-Gene is a trademark of Corbett Research. LightCycler™ is a registered trademark of the Idaho Technology Inc. GeneAmp®, TaqMan® and AmpliTaqGold® are registered trademarks of Roche Molecular Systems, Inc., The purchase of the Primerdesign™ reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-La Roche Inc.

# Principles of the test

## Real-time PCR

A Legionella\_spp specific primer and probe mix is provided and this can be detected through the FAM channel.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the Legionella\_spp DNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of qPCR platforms.

## Positive control

For copy number determination and as a positive control for the PCR set up, the kit contains a positive control template. This can be used to generate a standard curve of Legionella\_spp copy number / Cq value. Alternatively the positive control can be used at a single dilution where full quantitative analysis of the samples is not required. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers and probes for detecting the target Legionella\_spp gene worked properly in that particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. This can be achieved by handling this component in a Post PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

## Negative control

To validate any positive findings a negative control reaction should be included every time the kit is used. For this reaction the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run.

Legionella\_spp DNA is known to be highly prevalent within the air and environment generally and the negative control may therefore give a late positive signal due to environmental contamination. The interpretation of results section of this handbook gives guidance on how to interpret results where environmental contamination is evident.

### **Internal DNA extraction control**

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co-purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and qPCR for the control DNA also indicates that PCR inhibitors are not present at a high concentration.

A separate primer and probe mix are supplied with this kit to detect the exogenous DNA using qPCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the *Legionella\_spp* target DNA even when present at low copy number. The Internal control is detected through the VIC channel and gives a Cq value of 28+/-3.

### **Endogenous control**

To confirm extraction of a valid biological template, a primer and probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel and it is NOT therefore possible to perform a multiplex with the *Legionella\_spp* primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

# Resuspension protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps.

- 1. Pulse-spin each tube in a centrifuge before opening.**  
This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.
- 2. Resuspend the primer/probe mixes in the RNase/DNase free water supplied, according to the table below:**  
To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in water	Volume
<b>Pre-PCR pack</b>	
Legionella_spp primer/probe mix (BROWN)	165 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
Endogenous control primer/probe mix (BROWN)	165 µl

- 3. Resuspend the internal control template and positive control template in the template preparation buffer supplied, according to the table below:**  
To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in template preparation buffer	Volume
<b>Pre-PCR heat-sealed foil</b>	
Internal extraction control DNA (BLUE)	600 µl
<b>Post-PCR heat-sealed foil</b>	
Legionella_spp Positive Control Template (RED) *	500 µl

\* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

# DNA extraction

The internal extraction control DNA can be added either to the DNA lysis/extraction buffer or to the DNA sample once it has been resuspended in lysis buffer.

**DO NOT add the internal extraction control DNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.**

- 1. Add 4µl of the Internal extraction control DNA (BLUE) to each sample in DNA lysis/extraction buffer per sample.**
- 2. Complete DNA extraction according to the manufacturers protocols.**



# qPCR detection protocol

1. **For each DNA sample prepare a reaction mix according to the table below:**  
Include sufficient reactions for positive and negative controls.

Component	Volume
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Legionella_spp primer/probe mix (BROWN)	1 µl
Internal extraction control primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	3 µl
<b>Final Volume</b>	<b>15 µl</b>

2. **For each DNA sample prepare an endogenous control reaction according to the table below (Optional):**  
**This control reaction will provide useful information regarding the quality of the biological sample.**

Component	Volume
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Endogenous control primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
<b>Final Volume</b>	<b>15 µl</b>

3. **Pipette 15µl of each mix into individual wells according to your qPCR experimental plate set up.**
4. **Prepare sample DNA templates for each of your samples.**
5. **Pipette 5µl of DNA template into each well, according to your experimental plate set up.**  
For negative control wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl.
6. **If a standard curve is included for quantitative analysis, prepare a reaction mix according to the table below:**

Component	Volume
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Legionella_spp primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
<b>Final Volume</b>	<b>15 µl</b>

## 7. Preparation of standard curve dilution series.

- 1) Pipette 90µl of template preparation buffer into 5 tubes and label 2-6
- 2) Pipette 10µl of Positive Control Template (**RED**) into tube 2
- 3) Vortex thoroughly
- 4) Change pipette tip and pipette 10µl from tube 2 into tube 3
- 5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control ( <b>RED</b> )	$2 \times 10^5$ per µl
Tube 2	$2 \times 10^4$ per µl
Tube 3	$2 \times 10^3$ per µl
Tube 4	$2 \times 10^2$ per µl
Tube 5	20 per µl
Tube 6	2 per µl

## 8. Pipette 5µl of standard template into each well for the standard curve according to your experimental plate set up.

The final volume in each well is 20µl.

## qPCR amplification protocol

Amplification conditions using oasis or PrecisionPLUS 2X qPCR Master Mix.

	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x50	Denaturation	10 s	95 °C
	DATA COLLECTION *	60 s	60 °C

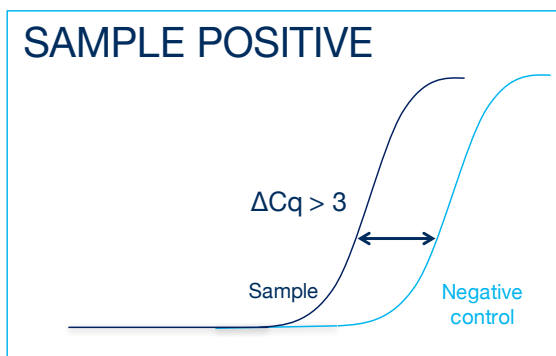
\* Fluorogenic data should be collected during this step through the FAM and VIC channels

# Interpretation of results

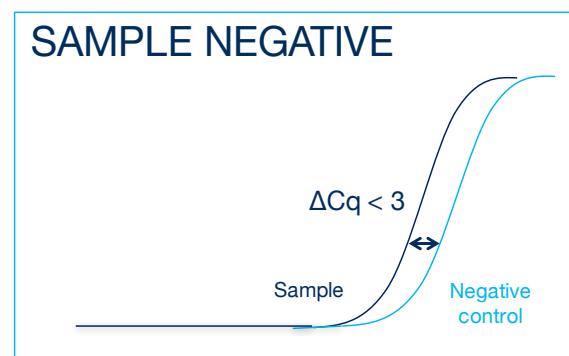
Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	+ / -	+	-	<b>POSITIVE QUANTITATIVE RESULT</b> calculate copy number
> 30	+	+	-	<b>POSITIVE QUANTITATIVE RESULT</b> calculate copy number
> 30	-	+	-	<b>POSITIVE QUALITATIVE RESULT</b> do not report copy number as this may be due to poor sample extraction
-	+	+	-	<b>NEGATIVE RESULT</b>
+ / -	+ / -	+	≤ 35	<b>EXPERIMENT FAILED</b> due to test contamination
+ / -	+ / -	+	> 35	*
-	-	+	-	<b>SAMPLE PREPARATION FAILED</b>
+ / -	+ / -	-	+ / -	<b>EXPERIMENT FAILED</b>

Positive control template (RED) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised

\*Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 3 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 3 Cq earlier than the negative control then the positive sample result is invalidated and a negative call is the correct result.

**Internal PCR control**

The C<sub>q</sub> value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. C<sub>q</sub> values of 28±3 are within the normal range. When amplifying a *Legionella\_spp* sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

**Endogenous control**

The signal obtained from the endogenous control primer and probe set will vary according to the amount of biological material present in a given sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.