GENERON
Advanced Transfer Technologies

PATHfinder

Legionella pneumophila Dual ID assay

Cat.n. PMB15D-SG

User Guide

1 - Introduction

The genus Legionella is a pathogenic group of Gram-negative bacteria, that includes the species L. pneumophila, causing Legionellosis including a pneumonia type illness called Legionnaires' disease and a mild flu like illness called Pontiac fever.

Legionella species typically exist in nature at low concentrations, it has been found in groundwater, lakes, and streams. In the right environmental conditions, it may reproduce and reach high concentrations per liter, especially in plumbing and cooling towers

Legionella is traditionally detected by culture on buffered charcoal yeast extract (BCYE) agar. Legionella requires the presence of cysteine and iron to grow and therefore does not grow on common blood agar media used for laboratory based total viable count. After incubation for up to 10 days, suspect colonies are confirmed as Legionella if they grow on BCYE containing cysteine, but not on agar without cysteine added.

Legionella is common in many environments, including soil and aquatic systems, with at least 53 species and 70 serogroups identified. L. pneumophila is responsible for more than 90% of clinical cases, and among the 15 serogroups (SG) characterized within the species, L. pneumophila SG1 is responsible for about 85% of all cases worldwide. Given this epidemiological background, it is necessary to identify rapidly not only the presence of Legionella in water samples but simultaneously also the species and serogroups, in order to estimate the risk of legionellosis.

New techniques for the rapid detection of Legionella in water samples are emerging including the use of polymerase chain reaction (PCR). This technology can typically provide much faster results. This assay utilizes the polymerase chain reaction (PCR) to amplify a genetic target typical of the pathogen species.

Per Generon in-house validation, the LOD for PATHfinder Legionella pneumophila Serogroup 1 ID Assay was experimentally determined to be between 1 and 10 copies. DNA was extracted using Bio-Rad AquadienTM and Generon ION Force DNA Extractor FAST (Cat.#EXD001).

2 - PATHfinder Legionella pneumophila Dual ID Assay

When used along with GENERase Mastermix Colony ID (Cat.# ENG001-ID) this real-time PCR assay detects Legionella pneumophila Serogroup 1 and Legionella pneumophila Serogroup 2-15 DNA. The amplification of the target sequence is measured by the use of a specific fluorescence-labeled probe (FAM and HEX) in less than 1.5 hours.

2.1 - Assay Content

	Box 50 reactions		Box 100 reactions	
	N. vials	Volume (µl)	N. vials	Volume (µl)
PATHfinder OLIGO Mix * (OLIGOS and Probe pre-blended mix)	1	250	2	250
Positive Control	1	85	2	85
Negative Control	1	200	1	200

** reagents are supplied with a 5% of extra volume.*

We suggest to use PATHfinder Legionella pneumophila Serogroup 1 ID Assay along with the following Polymerase Enzyme Ready-to-use mastermix: GENERase Mastermix Colony ID (Cat.# ENG001-ID) or GENERase PLUS Mastermix Colony ID (Cat.# ENG002-ID). When using this GENERase PLUS an additional detection channel (HEX) becomes available to detect the Internal Amplification Control (IAC) to excluding false negative results due to a PCR inhibition.

2.2 - Storage & Expiry information

Expiry date: see date on the packaging, product validity refers to the product kept intact in its original packaging. Protect reagents from light exposure as far as OLIGO Mix reagents are photosensitive. Store frozen.

3 – Materials and equipments needed

3.1 – Isolation and colony picking

Material/Equipment	Source
BCYE agar	Generon or other Lab suppliers
Sterilized Needles/Toothpicks	Generon or other Lab suppliers

3.2 – Extraction(1)

Material/Equipment	Source
Extraction Kit	Bio-Rad Aquadien
Vortexer	Generon or other Lab suppliers
Thermal Water Bath or Heat block for 1.5 ml tubes	Generon or other Lab suppliers
Pipette sets	Generon or other Lab suppliers
Pipette tips (Barrier)	Generon or other Lab suppliers
Tube rack for 1.5 ml tubes	Generon or other Lab suppliers
2.0 and 1.5 ml micro-tubes	Generon or other Lab suppliers
Micro centrifuge for 1.5-2.0 ml micro-tubes	Generon or other Lab suppliers

3.3 – Detection via real-time PCR

Material/Equipment	Source
Real-Time PCR System (2)	Generon or other Lab suppliers
PATHfinder Legionella pneumophila Dual ID Assay	Generon (PMB15D-SG)
GENERase Mastermix or GENERase PLUS Mastermix (3)	Generon (ENG001-ID or ENG002-ID)
Optical Adhesive Seal or Optical Caps	Generon or other Lab suppliers
Optical reaction plate or Optical Tube Strips	Generon or other Lab suppliers
Micropipette sets	Generon or other Lab suppliers

(1) Equipment necessary only when ION FORCE Extractor FAST (EXD001) is used.
(2) The assay can be used with Biorad CFX and MiniOpticon, Stratagene MxSeries, ABI 7300-7500-7900-StepONE-StepONE Plus, Light Cycler 480, Eppendorff realplex, Rotor-Gene Q etc. The assay is not compatible with Roche Light Cycler I and II.
(3) GENERase PLUS Mastermix includes the IAC (Internal Amplification Control), a system to assess the presence of false negatives due to substances inhibiting PCR in your extracted DNA. In addition, the IAC is a system to check on assay performance thus confirming whether PCR reagents are working and amplifying properly.

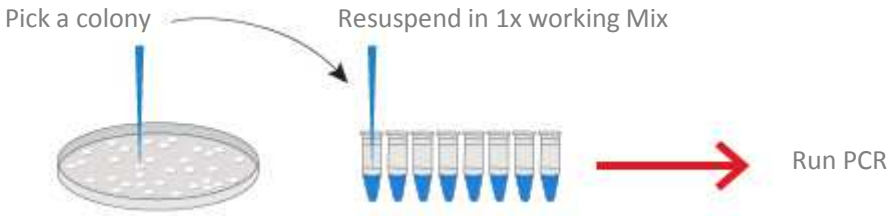
4 – DNA extraction

Each step of sample preparation (grinding, transferring, weighing, etc.) must be done according to GLP so that chance of cross-contamination between samples is minimized. It is recommended to use disposable equipment whenever possible.

This PATHfinder assay can be used for colony PCR experiments and allows the direct identification as L. *pneumophila* (either SG1 or SG2-15) of any colony isolated on GVPC/BCYE plate, to perform this technique is sufficient to pick one colony with a sterilized needle and to suspend it in 1 X Reaction Mix before running the PCR.

No DNA extraction is needed.

Colony PCR Diagram



5 – Real-Time PCR detection

5.1 – Reaction setup

I. Allow the reagents to thaw (GENERase PLUS Mastermix, PATHfinder OLIGO MIX, Positive Control and Negative Control). Vortex tubes when thawed and spin to collect contents at the bottom of the vial.

II. Mix 250 µl of PATHfinder OLIGO Mix with 500 µl of GENERase PLUS Mastermix to prepare PATHfinder Working Mastermix (WMX).

III. Vortex briefly and spin down in order to homogenize the mix.

IV. Transfer 15 µl of WMX into each well.

V. Add 5 µl of Negative Control into wells acting as negative controls.

VI. Add 5 µl of each sample into wells testing the unknown samples. When performing a Colony PCR experiment, add 5 µL of DNAse/RNAse Free Water for each well intended to be used for this technique and then pick one colony of interest with a sterilized needle from the agar plate and suspend it inside the selected well

VII. Add 5 µl of Positive Control into wells acting as positive control.

VIII. Close wells and ensure no bubbles are present at the bottom of the wells.

IX. Spin briefly optical PCR tubes or plates.

5.2 – Instrument setup

With GENERase Mastermix or GENERase PLUS Mastermix set the following parameters on your thermocycler:

I. Total Reaction volume: 20 µl

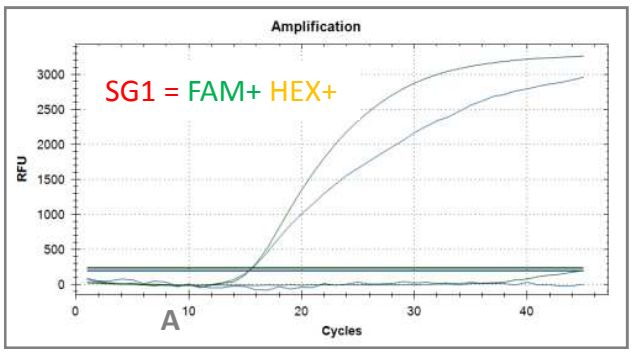
II. Fluorophores/Quenchers: Target Legionella pneumophila Serogroup 1 (FAM/BHQ1-NFQ); Legionella pneumophila Serogroup 2-15 (HEX/BHQ1-NFQ); Internal Amplification Control (Cy5/BHQ2-NFQ). Internal Amplification Control is present only in GENERase PLUS Mastermix). Depending on your thermocycler, you can replace HEX detector in the plate setting with VIC or JOE in case your own Real Time Platform does not possess the HEX reading channel.

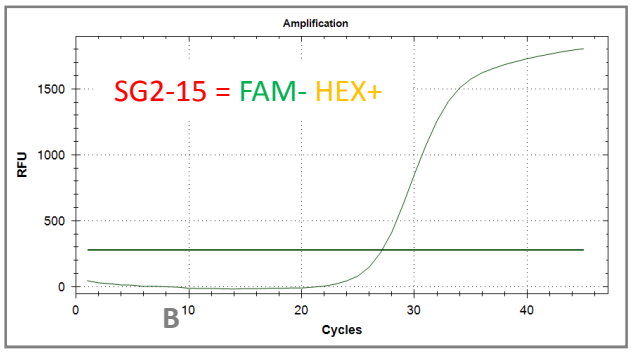
III. Thermal profile:

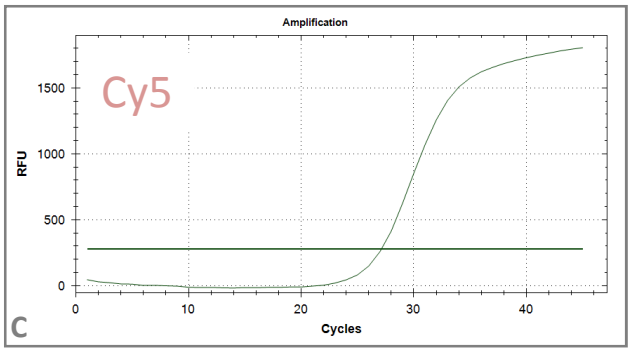
Step	T (°C)	Duration	Loops
UNG	50	10 min	1
Taq Activation	95	10 min	1
Denaturation	95	15 sec	45
Annealing/Extension + Plate Reading	60	60 sec	

5 – Data Interpretation

Results evaluation must be done according to the analysis software recommended by the Real Time PCR instrument manufacturer. After performing PCR, each individual sample is analyzed through the instrument software to produce a Cq value (quantification cycle) for each reporter dye. These values are used to determine the identity (Qualitative Test) of pathogen into the sample. See below an example of the graphics obtained for a positive Legionella pneumophila Serogroup 1 (Fig. A), a positive Legionella pneumophila Serogroup 2-15 (Fig. B) and for the IAC amplification (Fig. C)







After setting the baseline, the analysis outcome should be evaluated following the indications below.

If the following conditions are met:

TEST	L.pneumophila SG1 (FAM)	L. pneumophila SG2-15 (HEX)	Internal Amplification Control (Cy5)
Positive Control	+	+	**Not significative**
Negative Control	-	-	+

Then the possible results for any sample are:

TEST	L.pneumophila SG1 (FAM)	L. pneumophila SG2-15 (HEX)	Internal Amplification Control (Cy5)
Positive SG1	+	+	**Not significative**
Positive SG2-15	-	+	+
Not L. *Pneumophila*	-	-	+

In case of inhibition DNA isolation and purification for the sample need to be improved or you may need to dilute your sample before performing a new test. Refer to the Troubleshooting paragraph (section 8) for further suggestions.

7 – Inclusivity and Excusivity Panel

The following DNA extracts showed no amplification curve in a 15 µl total reaction volume:

Alicyclobacillus acidiphilus (DSM 14558), Alicyclobacillus acidocaldarius subsp. Caldarius (ATCC 27009), Alicyclobacillus acidoterrestris (ATCC 49025), Alicyclobacillus herbarius (DSM 13609), Bacillus cereus cereulide (DSM 4312), Bacillus cereus (ATCC 14579), Bacillus subtilis subsp. Spizizenii (ATCC 6633), Campylobacter coli (ATCC 33559), Campylobacter fetus (ATCC 27374), Campylobacter jejuni (ATCC 33560), Campylobacter laridis (lari) (ATCC 35221), Citrobacter freundi (ATCC 8090), Clostridium perfringens (ATCC 25768), Clostridium sporogenes (ATCC 3584), Cronobacter sakazakii (ATCC 29544), Edwardsiella tarda (ATCC 15947), Enterobacter cloacae (ATCC 13047), Escherichia coli (ATCC 11775), Janthinobacterium lividum (ATCC 12473), Klebsiella pneumoniae (ATCC 13883), Lactococcus lactis subsp. Lactis (ATCC 19435), Listeria innocua (ATCC 33152), Listeria ivanovi (ATCC 33090), Listeria monocytogenes (ATCC 19119), Morganella morgani subsp. Morganii (ATCC 15313), Proteus vulgaris (ATCC 25830), Providencia stuartii (ATCC 29905), Pseudomonas aeruginosa (ATCC 29914), Pseudomonas fragi (ATCC 10145), Pseudomonas syringae (ATCC 4973), Pseudomonas fluorescens (ATCC 19310), Salmonella enterica subsp. Enterica (ATCC 13525), Serratia marcescens (ATCC 13076), Shewanella putrefacens (ATCC 13880), Shigella boydii (ATCC 8071), Shigella flexneri (ATCC 8700), Shigella sonnei (ATCC 29903), Staphilococcus aureus subsp. Aureus (ATCC 12600), Vibrio aerogenes (ATCC 700797), Vibrio algynoliticus (ATCC 17749), Vibrio mytili (ATCC 51288), Vibrio parahaemolyticus (ATCC 17802), Vibrio splendidus (ATCC 33125), Vibrio vulnificus (ATCC 27562), Yersinia enterocolitica subsp. Enterocolitica (ATCC 23715), Yersinia pseudotuberculosis (ATCC 29833).

According to scientific literature, only HEX channel amplification was shown by 147 strains of Legionella pneumophila belonging to 2-15 serogroups and no amplification was shown by 50 Legionella spp. (not pneumophila) strains, whereas 257 strains of L. pneumophila SG1 gave positive amplification in both FAM and HEX channel.

Reference strains used in QC operations are:

- L. *Pneumophila SG1* (ATCC 33152) as positive control for FAM
- L. *Pneumophila SG6* DSM 25182 as positive control for HEX

8 – Troubleshooting

I. Concomitant no target nor IAC amplification, or amplification plots grossly abnormal. Possible causes and corrective actions:

- An excess of DNA in the target might inhibit the reaction and IAC may be affected due to an excess of DNA and/or PCR inhibitors. Test samples diluted 1:10 and 1:100. Please, use DNase/RNase Free Water to prepare dilutions.
- Inadequate sealing of optical caps/film caused sample evaporation. Redo the analysis using proper tools and proper optical caps/film to secure perfect sealing.
- Did not use the proper consumables. Redo the analysis and use only optical grade 96-well plates and optical adhesive seal or optical 8-well strips and caps.
- Samples were not properly prepared. Remake the sample DNA preps. Ensure that the DNA extraction method is properly performed.

II. Positive Control reactions failed to amplify, but other reactions appear correct (e.g. the IAC is amplified):

- Positive Control DNA was not added to the reaction wells. If other reactions look normal, there may be no need to repeat the run.

III. Negative Control reactions are positive:

- Contamination of the DNase/RNase Free Water vial or the PATHfinder PCR mix with PATHfinder-positive DNA. Use more care to prevent contamination while handling assay reagents and setting up assays.

In case support is needed contact Generon at: support@generon.it

9 – Disclaimers

The product is intended for research use only. Generon makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made of standard quality. If any materials are defective, Generon will provide a replacement product. Generon shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product. Please do not interchange components between assays of different lot numbers. This assay is designed to be used by laboratory personnel following the common molecular biology precautions.

Quick Reference Guide

Product Line:	PATHfinder
Part Number:	PMB15D-SG
Type:	Qualitative
Storage:	Frozen
Execution time:	about 120 minutes
Expiry date:	see date on the packaging, product validity refers to the product kept intact in its original packaging and constantly under suitable temperature conditions as mentioned above.

Assay Box Content

	Box 50 reactions		Box 100 reactions	
	N. vials	Volume (µl)	N. vials	Volume (µl)
PATHfinder OLIGO Mix (OLIGOS and Probe pre-blended mix)	1	250	2	250
Positive Control	1	85	2	85
Negative Control	1	200	1	200

All reagents are supplied with a 5% of extra volume.

Not Provided Article: GENERase PLUS Mastermix Colony ID (ENG002-ID) or GENERase Mastermix Colony ID (ENG001-ID) or equivalent. The IAC (Internal Amplification Control) is available only with GENERase Mastermix Colony ID (ENG002-ID).

Reaction Set-Up

Protect reagents from light exposure as far as OLIGO Mix reagents are photosensitive.

Before setting the analysis, we strongly advise to leave the reagents to warm up at room temperature. Vortex briefly OLIGO mix, afterwards spin to collect contents at the bottom of the vials. Spin GENERase PLUS Mastermix Colony ID (ENG002-ID) before opening it.

Prepare PATHfinder WORKING Mastermix by adding 250 µl of PATHfinder OLIGO Mix into each tube prefilled with 500 µl of GENERase PLUS Mastermix Colony ID (ENG002-ID) in order to obtain a single volume of 750 µl of PATHfinder WORKING Mastermix. Vortex briefly PATHfinder WORKING Mastermix with the aim of homogenizing the mix and excluding $MgCl_2$ gradient that could impair the results. Spin to collect contents at the bottom of the vial *(Note: label GENERase PLUS vials with target name after OLIGO Mix addition)*. Vortex briefly Positive Control and samples before proceeding further, spin to collect contents at the bottom of the vial.

Transfer PATHfinder WORKING Mastermix and samples into the plate as follows:

Reagents per well	Volume
Unknown Sample (extracted DNA or colony)	5 µl
Positive Control Negative Control	
PATHfinder WORKING Mastermix	15 µl
Final Volume	20 µl

Detector Setup

Target	Reporter Dye	Quencher Dye
Legionella pneumophila Serogroup 1	FAM	BHQ1-NFQ
Legionella pneumophila Serogroup 2-15	HEX(*)	BHQ1-NFQ
IAC (Internal Positive Control)	Cy5	BHQ2-NFQ

According to your thermocycler you can replace HEX detector in the plate setting with VIC or JOE in case your own Real Time Platform does not possess the HEX reading channel.

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Thermal cycling

Step	T (°C)	Duration	Loops
UNG	50	10 min	1
Taq Activation	95	10 min	1
Denaturation	95	15 sec	45
Annealing/Extension + Plate Reading	60	60 sec	

The thermal profile presented above was optimized for GENERase Mastermix.

Results analysis

If the following conditions are met:

TEST	L.pneumophila SG1 (FAM)	L. pneumophila SG2-15 (HEX)	Internal Amplification Control (Cy5)
Positive Control	+	+	Not significative
Negative Control	-	-	+

Then the possible results for any sample are:

TEST	L.pneumophila SG1 (FAM)	L. pneumophila SG2-15 (HEX)	Internal Amplification Control (Cy5)
Positive SG1	+	+	Not significative
Positive SG2-15	-	+	+
Not L. *Pneumophila*	-	-	+

In case of inhibition DNA isolation and purification for the sample need to be improved or you may need to dilute your sample before performing a new test. Refer to the Troubleshooting paragraph , section 8 in user manual, for further suggestions.

Warning and Precaution

Please, do not interchange components of assays with different lot numbers. This assay is designed to be used by laboratory personnel following the common molecular biology precautions (GLP).

Disclaimer

Generon s.r.l. guarantees the buyer exclusively concerning the quality of reagents and of the components used to produce the Assay. Generon S.r.l. is not responsible and cannot anyway be considered responsible or jointly responsible for possible damages resulting from the utilization of the product by the user. The user consciously and under his own responsibilities decides for the utilization purposes of the product and uses it the way he considers most suitable in order to reach his goals and/or objectives.

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The product was internally tested by our quality control. Any responsibility is waivered if the warranty of quality control does not refer to the specific product. The user is personally responsible for data that he will obtained and/or he will supply to third parties using this assay. Once the sealed package is open the user accepts all the conditions without fail; if the package is still sealed the product can be returned and the user can be refunded.