RIDA®GENE Legionella

REF PG8005

R-Biopharm AG, An der neuen Bergstraße 17, 64297 Darmstadt, Germany
Phone: +49 (0) 61 51 81 02-0 / Fax: +49 (0) 61 51 81 02-20

1. Intended use

For *in vitro* diagnostic use. RIDA®GENE Legionella is a multiplex real-time PCR for the direct, qualitative detection and differentiation of *Legionella pneumophila* and *Legionella* spp. from human bronchoalveolar lavage fluid (BAL).

The RIDA®GENE Legionella multiplex real-time PCR is intended for use as an aid in diagnosis of respiratory infections caused by *Legionella*.

2. Summary and explanation of the test

The Genus *Legionella* belongs to the family of *Legionellaceae* and can be divided in over 40 species with more than 70 serogroups. *Legionella* are facultative, intracellular gram-negative bacteria and their infectious peak occurs during the summer months and early fall. The Genus *Legionella* belongs to the family of *Legionellaceae* and can be divided in over 40 species with more than 70 serogroups. *Legionella* are facultative, intracellular gram-negative bacteria and their infectious peak occurs during the summer months and early fall. One differentiates between community-acquired infections, travel-associated disease and hospital-acquired infections. In the US, the mortality rate of hospital-acquired *Legionella* infections is between 15 – 20%.[1,2] In Europe, 12% of all *Legionella* infections are fatal. Of the broad variety of Legionella species, two human pathogenic species are of importance. *L. pneumophila* primarily causes Legionnaire's disease and *L. longbeachae* results in Pontiac fever. Pontiac fever is an acute self-limiting influenza-like diseases, however, no pneumoniae occurs. About 7% of people infected with *Legionella* develop Pontiac fever.[3]
L. pneumophila has 16 serogroups, whereas about 70% of all Legionella infections in Europe are due to *L. pneumophila* serogroup 1. Other *Legionella* species that result in an infection are for instance *L. micdadei*, *L. bozemanii*, *L. dumoffii* and *L. longbeachae*.[4]
Legionnaire's disease is an acute respiratory infection which is caused by *L. pneumophila* in 90% of cases. In 1976, this disease was first described during a meeting of the American Legion in Philadelphia, hence the name of the disease. In 2013, two other outbreaks of Legionnaire's disease with a total of 6 fatal cases were reported in Brisbane, Australia and Reynoldsburg, Ohio. Symptoms include fever, cough (dry or sputum producing) and shivering. Other, less common symptoms are diarrhea, vomiting, bradycardia and hypnatremia.[3] People of every age can be infected, but old people as well as smokers and patients with chronic lung infection are more prone to such a disease. Even in countries with an effective health care system almost 90% of cases of Legionnaires's disease are not diagnosed since the clinical presentation can differ substantially and the disease is so rare. In addition, it is very difficult to differentiate Legionnaire's disease from other pneumonia solely on the basis of symptoms or by chest X-ray. Therefore, sensitive and specific detection methods such as real-time PCR offer an advantage for the detection of *Legionella* infections.

3. Test principle

RIDA®GENE Legionella is a multiplex real-time PCR for the direct, qualitative detection and differentiation of *Legionella* spp. and *Legionella pneumophila* from bronchoalveolar lavage fluid (BAL). After DNA isolation, amplification of gene fragments (16S-rRNA, if present) specific for *Legionella* spp. and *Legionella pneumophila* occurs.
The amplified target is detected with hydrolysis probes, which are labeled at one end with a quencher and at the other end with a fluorescent reporter dye (fluorophore). In the presence of a target the probes hybridize to the amplicons. During the extension step the Taq-Polymerase breaks the reporter-quencher proximity. The reporter emits a fluorescent signal which is detected by the optical unit of a real-time PCR instrument. The fluorescence signal increases with the amount of formed amplicons. The RIDA®GENE Legionella test contains an Internal Control DNA (ICD) as an internal control of sample preparation procedure and/or to determine possible PCR inhibition.

4. Reagents provided

Tab. 1: Reagents provided (Reagents provided in the kit are sufficient for 100 determinations)

Kit Code	Reagent	Amount		Lid Color
1	Reaction Mix	2x	1050 µl	yellow
2	Taq-Polymerase	1x	80 µl	red
D	Internal Control DNA	2x	1700 µl	orange
N	No Template Control	1x	450 µl	white
P	Positive Control	1x	200 µl	blue

5. Storage instructions

- Protect all reagents from light and store at -20 °C. All reagents can be used until the expiration date. After expiry the quality guarantee is no longer valid.
- Carefully thaw and fully defrost reagents before using (e.g. in a refrigerator at 2 - 8 °C).
- Reagents can sustain up to 15 freeze/thaw cycles without influencing the assay performance (e.g. after the first thawing separate it in aliquots and freeze immediately).
- During PCR preparation all reagents should be stored cold in an appropriate way (2 - 8 °C).

6. Additional necessary reagents and necessary equipment

The RIDA®GENE Legionella multiplex real-time PCR test is suitable for use with following extraction platforms and real-time PCR instruments:

Tab. 2: Necessary equipment (compatible)

Extraction platforms	
R-Biopharm	RIDA® Xtract
Promega	Maxwell® RSC
Real-time PCR instruments	
Roche	LightCycler® 480II, LightCycler® 480z
Agilent Technologies	Mx3005P
Applied Biosystems	ABI 7500
Bio-Rad	CFX96™
QIAGEN	Rotor-Gene Q

Note: Only use 0.1 ml tubes on the Rotor-Gene Q (QIAGEN).

If you want to use other extraction platforms or real-time PCR instruments please contact R-Biopharm at mdx@r-biopharm.de.

- RIDA®GENE Color Compensation Kit IV (PG0004) for use with the LightCycler® 480II and LightCycler® 480z
- Real-time PCR consumables (plates, tubes, foil)
- Centrifuge with a rotor for the reaction vials
- Vortexer
- Pipettes (0.5 – 20 µl, 20 – 200 µl, 100 – 1000 µl)
- Filter tips
- Powder-free disposal gloves
- PCR water (BioScience grade, nuclease-free water)

7. Precautions for users

For *in vitro* diagnostic use.

- This test must only be carried out by trained laboratory personnel. The guidelines for working in medical laboratories have to be followed.
- The instruction manual for the test procedure has to be followed.
- Do not pipet samples or reagents by mouth. Avoid contact with bruised skin or mucosal membranes.
- During handling reagents or samples, wear appropriate safety clothing (appropriate gloves, lab coat, safety goggles) and wash your hands after finishing the test procedure
- Do not smoke, eat or drink in areas where samples or reagents are being used.

- Extraction, PCR preparation and the PCR run should be separated in different rooms to avoid cross-contaminations.
- Samples must be treated as potentially infectious as well as all reagents and materials being exposed to the samples and have to be handled according to the national safety regulations.
- Do not use the kit after the expiration date.
- All reagents and materials used have to be disposed properly after use. Please refer to the relevant national regulations for disposal.

For more details see Safety Data Sheets (SDS) at www.r-biopharm.com.

8. Collection and storage of samples

8.1 DNA isolation of bronchoalveolar lavage

For DNA isolation of bronchoalveolar lavage, use a commercially available DNA isolation kit (e.g. RIDA® Xtract (R-Biopharm)) or DNA extraction system (e.g. Maxwell® RSC (Promega)). Extract DNA according to the manufacturer's instructions.

The RIDA®GENE Legionella test contains an Internal Control DNA that detects PCR inhibition, monitors reagent integrity and confirms that nucleic acid extraction was sufficient. The Internal Control DNA can either be used as PCR inhibition control or as extraction control for the sample preparation procedure and as PCR inhibition control.

If the Internal Control DNA is used only as a PCR inhibition control, 1 µl of the Internal Control DNA should be added to the Master-Mix (see Tab. 4).

If the Internal Control DNA is used as an extraction control for the sample preparation procedure and as PCR inhibition control, 20 µl of the Internal Control DNA has to be added during extraction procedure. The Internal Control DNA should always be added to the specimen-lysis buffer mixture and must not be added directly to the specimen. We also recommend to add 1 µl of the Internal Control DNA to the negative control and positive control PCR Mix.

9. Test procedure

9.1 Master-Mix preparation

Calculate the total number of PCR reactions (sample and control reactions) needed. One positive control and one negative control must be included in each assay run.

We recommend to calculate an additional volume of 10 % to compensate imprecise pipetting (see Tab. 3, Tab. 4). Thaw, mix gently and centrifuge briefly the Reaction Mix, the Taq-Polymerase, the Positive Control, the No Template Control and the Internal Control DNA before using. Keep reagents appropriately cold during working step (2 - 8 °C).

Tab. 3: Calculation and pipetting example for 10 reactions of the Master-Mix (ICD as extraction and PCR inhibition control)

Kit code	Master-Mix components	Volume per reaction	10 reactions (10 % extra)
1	Reaction Mix	19.3 µl	212.3 µl
2	Taq-Polymerase	0.7 µl	7.7 µl
	Total	**20 µl**	**220 µl**

Mix the components of the Master-Mix gently and briefly spin down.

Tab. 4: Calculation and pipetting example for 10 reactions of the Master-Mix (ICD only as PCR inhibition control)

Kit code	Master-Mix components	Volume per reaction	10 reactions (10 % extra)
1	Reaction Mix	19.3 µl	212.3 µl
2	Taq-Polymerase	0.7 µl	7.7 µl
D	Internal Control DNA	1.0 µl	11 µl
	Total	**21.0 µl**	**231.0 µl**

Mix the components of the Master-Mix gently and briefly spin down.

9.2 Preparation of the PCR-Mix

Pipette 20 µl of the Master-Mix in each reaction vial (tube or plate).

Negative control: Add 5 µl No Template Control to the pre-pipetted Master-Mix.

Note: **If the Internal Control DNA is used as extraction control for the sample preparation procedure and as PCR inhibition control, we recommend to add 1 µl of the Internal Control DNA to the PCR-Mix of the negative control.**

Sample: Add 5 µl DNA extract to the pre-pipetted Master-Mix.

Positive control: Add 5 µl Positive Control to the pre-pipetted Master-Mix.

Note: If the Internal Control DNA is used as extraction control for the sample preparation procedure and as PCR inhibition control, we recommend to add 1 µl of the Internal Control DNA to the PCR-Mix of the positive control.

Cover tubes or plate. Spin down and place in the real-time PCR instrument. The PCR reaction should be started according to the PCR instrument set-up (see Tab. 5, Tab. 6, Tab.7, Tab. 8).

9.3 PCR Instrument set-up

9.3.1 DNA real-time PCR profile

Tab. 5: DNA real-time PCR profile for LightCycler® series and Rotor-Gene Q

Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	10 sec, 95 °C 15 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.

Tab. 6: DNA real-time PCR profile for Mx3005P, ABI7500, CFX96™

Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	15 sec, 95 °C 30 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.

9.3.2 Universal real-time PCR profile

Note: The universal real-time PCR profile should only be used for DNA assays when combining RIDA®GENE DNA and RNA real-time PCR assays in one run.

Tab. 7: Universal real-time PCR profile for LightCycler® series

Reverse Transcription	10 min, 58 °C
Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	10 sec, 95 °C 15 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.

Tab. 8: Universal real-time PCR profile for Mx3005P, ABI7500, Rotor-Gene Q and CFX96™

Reverse Transcription	10 min, 58 °C
Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	15 sec, 95 °C 30 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.

9.4 Detection Channel Set-up

Tab. 9: Selection of appropriate detection channels

Real-time PCR instrument	Detection	Detection Channel	Note
Roche LightCycler® 480II	*Legionella* spp.	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required**
	ICD	533/580	
	Legionella pneumophila	618/660	
Roche LightCycler® 480 z	*Legionella* spp.	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required**
	ICD	540/580	
	Legionella pneumophila	540/610	
Agilent Techn. Mx3005P	*Legionella* spp.	FAM	**Check that passive reference option ROX is none**
	ICD	HEX	
	Legionella pneumophila	Cy5	
ABI 7500	*Legionella* spp.	FAM	**Check that passive reference option ROX is none**
	ICD	VIC	
	Legionella pneumophila	Cy5	
Bio-Rad CFX96™	*Legionella* spp.	FAM	**-**
	ICD	VIC	
	Legionella pneumophila	Cy5	
Qiagen Rotor-Gene Q	*Legionella* spp.	Green	**The gain settings have to be set to 5**
	ICD	Yellow	
	Legionella pneumophila	Red	

10. Qualitäty Control

The analysis of the samples is done by the software of the used real-time PCR instrument according to the manufacturer's instruction. Positive and negative controls have to show correct results (see Table 10, Fig. 1, Fig. 2) in order to determine a valid run.

The Positive Control for *Legionella* spp. and *Legionella pneumophila* has a concentration of 10^3 copies/µl. In each PCR run it is used in a total amount of 5×10^3 copies.

Tab. 10: For a valid run, the following conditions must be met

Sample	Assay result	IAC Ct	Target Ct
Positive Control	Positive	NA *1	See Quality Assurance Certificate
Negative Control	Negative	Ct > 20	0

**1 No Ct value is required for the IAC to make a positive call for the positive control.*

If the positive control is not positive within the specified Ct range but the negative control is valid, prepare all new reactions including the controls.

If the negative control is not negative but the positive control is valid, prepare all new reactions including the controls.

If the required criteria are not met, following items have to be checked before repeating the test:

- Expiry of the used reagents
- Functionality of the used instrumentation
- Correct performance of the test procedure

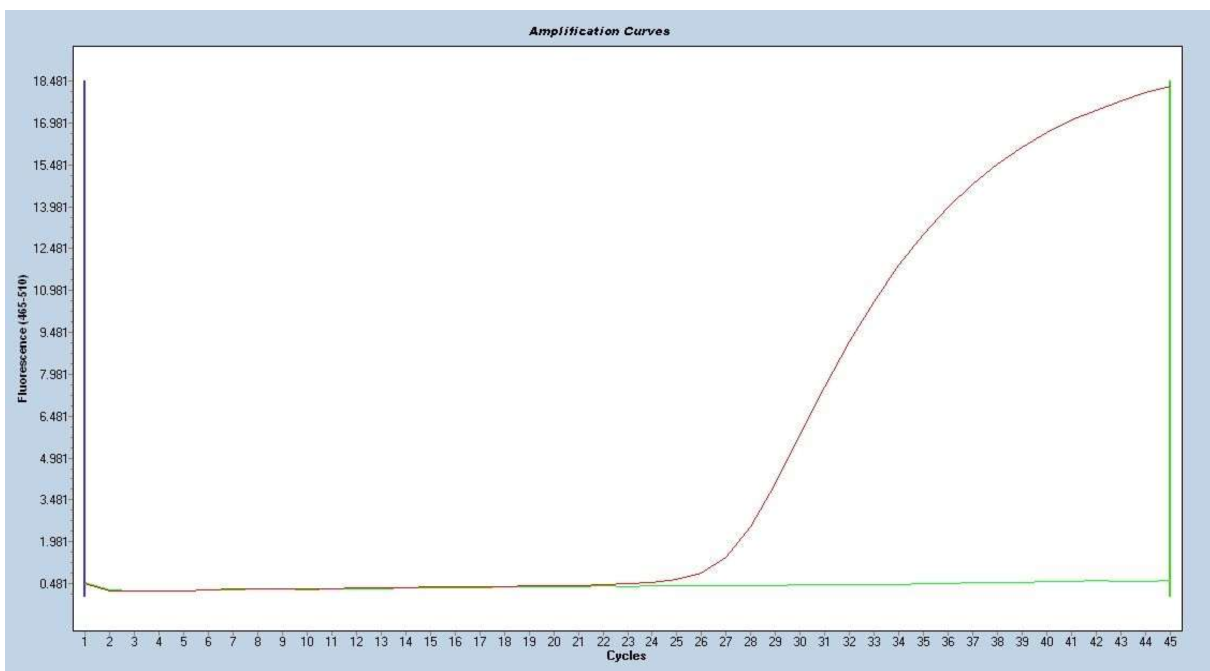

Fig. 1: Correct run of the positive and negative control (*Legionella* spp.) on the LightCycler® 480II

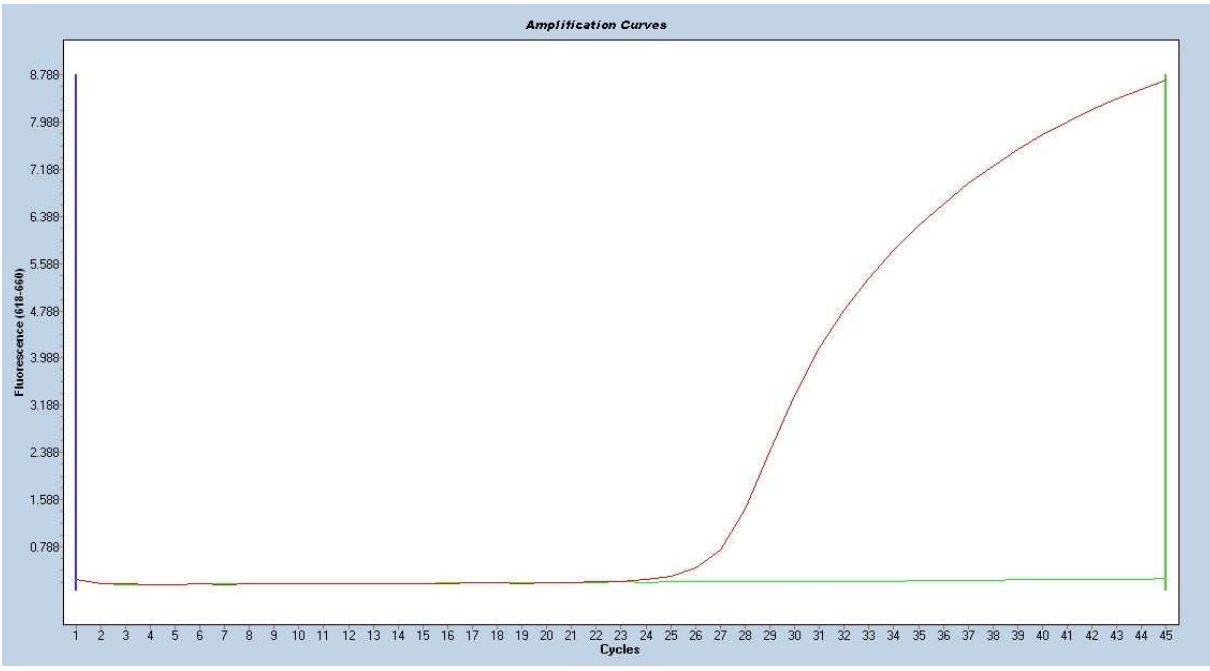

Fig. 2: Correct run of the positive and negative control (*Legionella pneumophila*) on the LightCycler® 480II

11. Result interpretation

The result interpretation is done according to Table 11.

Tab.11: Sample interpretation

Targent genes			
***Legionella* spp.**	***L. pneumophila***	**ICD**	**Ergebnis**
positive	negative	**positive/negative**	***Legionella* spp. detected**
negative	**positive**	**positive/negative**	**Invalid**
positive	**positive**	**positive/negative**	***L. pneumophila* detected**
negative	negative	**positive**	**Target genes not detected**
negative	negative	negative	**Invalid**

Legionella is detected, if the sample DNA and the Internal Control DNA show an amplification signal in the detection system.

Legionella is also detected, if the sample DNA shows an amplification signal but none for the Internal Control DNA in the detection system. The detection of the internal amplification control is not necessary because high concentrations of the amplicon can cause a weak or absent signal of the Internal Control DNA.

Legionella is not detected, if the sample DNA shows no amplification signal, but an amplification signal for the Internal Control DNA in the detection system. An inhibition of the PCR reaction can be excluded by the detection of the Internal Control DNA.

A sample is invalid, if the sample DNA and Internal Control DNA show no amplification signal in the detection system. The sample contains a PCR inhibitor. The extracted sample needs to be further diluted with PCR water (1:10) and re-amplified, or the isolation and purification of the sample has to be improved.

12. Limitations of the method

1. The result of molecular analysis should not lead to the diagnosis, but always be considered in the context of medical history and symptoms of the patient.
2. This assay is only validated for BAL samples.
3. Inappropriate specimen collection, transport, storage and processing or a pathogen load in the specimen below the analytical sensitivity can result in false negative results.
4. The presence of PCR inhibitors may cause invalid results.
5. Mutations or polymorphisms in primer or probe binding regions may affect detection of new variants resulting in a false negative result with the RIDA®GENE Legionella test.

6. As with all PCR based *in vitro* diagnostic tests, extremely low levels of target below the limit of detection (LoD) may be detected, but results may not be reproducible.
7. A positive test result does not necessarily indicate the presence of viable organisms. However, a positive result is indicative for the presence of the target genes (16S-rRNA).

13. Performance characteristics

13.1 Clinical performance

In a retrospective clinical validation study 282 extracted respiratory samples were analyzed with the RIDA®GENE Legionella test and an in-house real-time PCR assay in a laboratory in Germany (see Tab 12, Tab.13). Extraction was done using the MagNa Pure 96 and the real-time PCR was performed on the LightCycler® 480II.

Tab. 12: Correlation of the *Legionella pneumophila* results with the RIDA®GENE Legionella real-time PCR and reference in-house real-time PCR.

		In-house real-time PCR			
		Positive	Negative	Total	Comments
RIDA®GENE Legionella	Positive	51	0	51	Pos. agreement: 100 %
	Negative	0	231	231	Neg. agreement: 100 %
	Total	51	231	282	

Tab. 13: Correlation of the *Legionella* spp. results with the RIDA®GENE Legionella real-time PCR and reference in-house real-time PCR.

		In-house real-time PCR			
		Positive	Negative	Total	Comments
RIDA®GENE Legionella	Positive	70	0	70	Pos. agreement: 100 %
	Negative	0	212	212	Neg. agreement: 100 %
	Total	70	212	282	

13.2 Analytical sensitivity

The RIDA®GENE Legionella multiplex real-time PCR has a limit of detection of ≥ 10 DNA copies per reaction.

The following figures 3 and 4 show dilution series of *Legionella* spp. and *Legionella pneumophila* (each 10^5 - 10^1 DNA copies per µl) on the LightCycler® 480II.

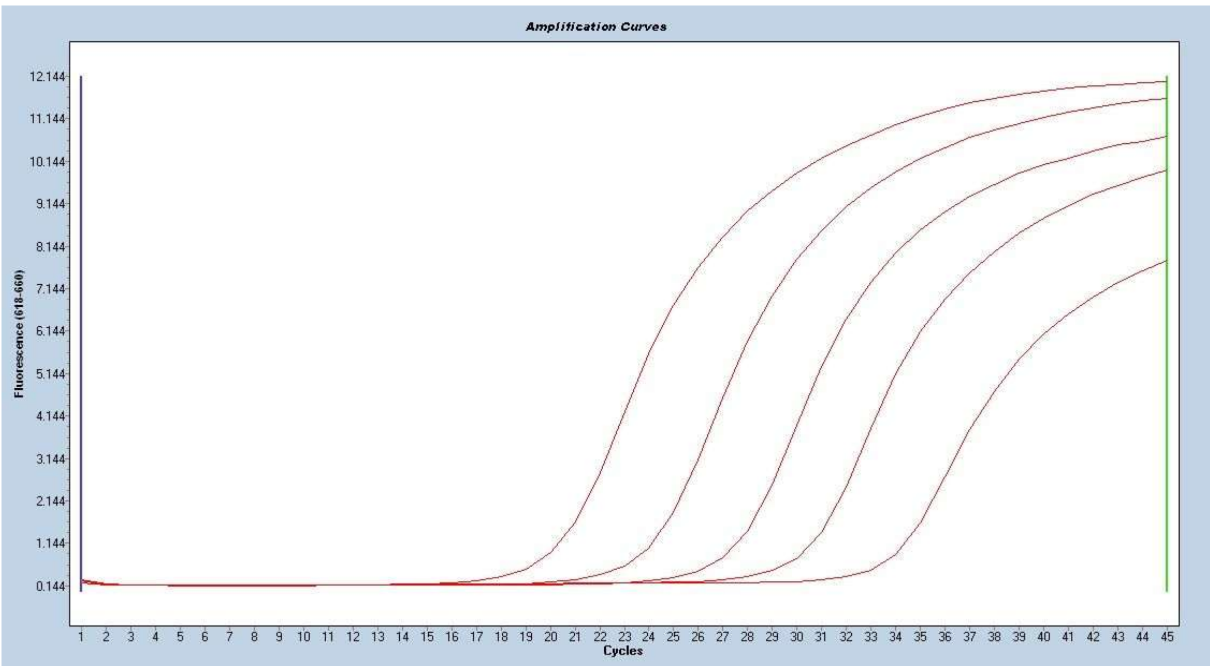

Fig. 3: Dilution series *Legionella* spp. (10^5 – 10^1 DNA copies per µl) on the LightCycler® 480II

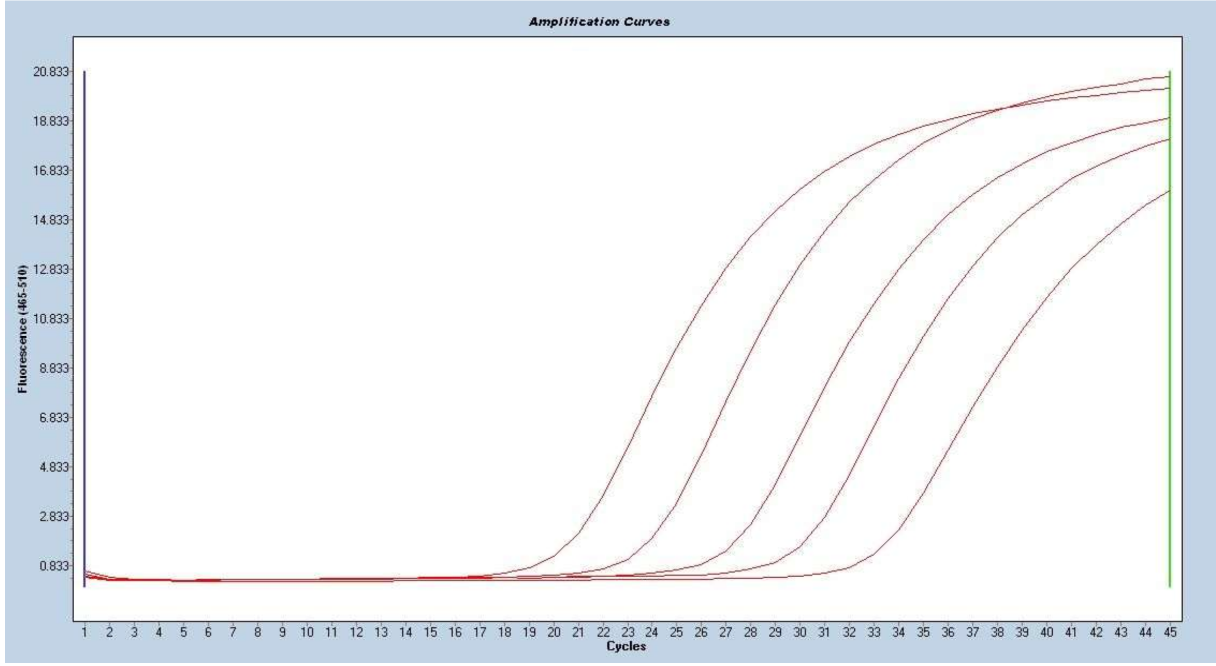

Fig. 4: Dilution series *Legionella pneumophila* (10^5 – 10^1 DNA copies per µl) on the LightCycler® 480II

The detection limit of the whole procedure depends on the sample matrix, DNA extraction and DNA concentration.

13.3 Analytical specificity

The RIDA®GENE Legionella multiplex real-time PCR is specific for *Legionella* spp. and *L. pneumophila* from human bronchoalveolar lavage. No cross-reaction could be detected for the following species (see Tab.14):

Tab. 14: Cross-reactivity testing

Adenovirus 1, human, strain Adenoid 71	-	Coxsackie virus B4, human	-	*Klebsiella pneumoniae*	-	Parainfluenza virus, serotype 3	-
Adenovirus 7, human, strain Gomen	-	Echovirus 11	-	Metapneumovirus, human	-	Parainfluenza virus 4b, human, strain CH19503	-
Bordetella parapertussis	-	Enterovirus type 71	-	*Mycoplasma fermentans*	-	Respiratory syncitial virus, human, strain Long	-
Bordetella pertussis	-	*Haemophilus influenzae*	-	*Mycoplasma hominis*	-	Respiratory syncitial virus, human, strain 9320	-
Chlamydia abortus	-	Herpes simplex virus 1, strain McIntyre	-	*Mycoplasma pneumoniae*	-	Rhinovirus Genogruppe A, human	-
Chlamydia pneumoniae	-	Herpes simplex virus 2, strain MS	-	*Neisseria meningitidis*	-	*Staphylococcus haemolyticus*	-
Chlamydia psittaci	-	Influenza virus A (PR/8/34)	-	Parainfluenza virus 1, human, strain C35	-	*Staphylococcus hominis*	-
Coronavirus 229E, human	-	Influenza virus B (B/Lee/40)	-	Parainfluenza virus 2, human, strain Greer	-	*Streptococcus pneumoniae*	-

13.4 Analytical reactivity

The reactivity of the RIDA®GENE Legionella multiplex real-time PCR was evaluated against multiple strains of *Legionella* spp. (see Tab. 15). All strains of the panel were detected by the RIDA®GENE Legionella real-time PCR.

Tab. 15: Cross-reactivity testing

Straim	Serogroup	*Legionella* spp.	*Legionella pneumophila*
Legionella pneumophila	1	**positive**	**positive**
Legionella pneumophila	2	**positive**	**positive**
Legionella pneumophila	3	**positive**	**positive**
Legionella pneumophila	4	**positive**	**positive**
Legionella pneumophila	5	**positive**	**positive**
Legionella pneumophila	6	**positive**	**positive**
Legionella pneumophila	7	**positive**	**positive**
Legionella pneumophila	8	**positive**	**positive**
Legionella pneumophila	9	**positive**	**positive**
Legionella pneumophila	10	**positive**	**positive**
Legionella pneumophila	11	**positive**	**positive**
Legionella pneumophila	12	**positive**	**positive**
Legionella pneumophila	13	**positive**	**positive**
Legionella pneumophila	14	**positive**	**positive**
Legionella anisa	-	**positive**	negative
Legionella bozemanii	-	**positive**	negative
Legionella dumoffi	-	**positive**	negative
Legionella cardiaca	-	**positive**	negative
Legionella feeleii	-	**positive**	negative
Legionella gormanii	-	**positive**	negative
Legionella hackeliae	-	**positive**	negative
Legionella jordanis	-	**positive**	negative
Legionella longbeachae	-	**positive**	negative
Legionella maceachernii	-	**positive**	negative
Legionella micdadei	-	**positive**	negative
Legionella oakridgensis	-	**positive**	negative

14. Version history

Version number	Chapter and designation
2018-07-30	Previous version
2020-10-02	General revision 13.1 Clinical performance 14. Version history 15. Explanation of symbols

15. Explanation of symbols

General symbols

IVD	For *in vitro* diagnostic use
	Consult instructions for use
LOT	Lot number
	Expiry
	Store at
REF	Article number
Σ	Number of tests
	Date of manufacture
	Manufacturer

Testspecific symbols

Reaction Mix

Taq-Polymerase

Internal Control DNA

No Template Control

Positive Control

16. Literature

1. Howden BP *et al.* Treatment and outcome of 104 hospitalized patients with Legionnaires'disease. Internal Medicine Journal. 2003, 33(11):484–488.
2. Benin AL *et al*. An outbreak of travel-associated Legionnaires' disease and Pontiac fever: the need for enhanced surveillance of travel-associated legionellosis in the United States. Journal of Infectious Diseases. 2002, 185(2):237–243.
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4. Joseph C *et al.* Surveillance of Legionnaires disease in Europe. Legionella, Washington DC, ASM Press. 2002, 311–320.