RIDASCREEN® Legionella

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1. Intended use

For *in vitro* diagnostic use. RIDASCREEN® Legionella is an enzyme immunoassay for the qualitative detection of *Legionella pneumophila* antigens in urine samples.

2. Summary and explanation of the test

The *Legionella* strain belongs to the *Legionellaceae* family and is divided into 40 species with more than 70 serogroups. Legionella bacteria are facultative, intracellular gram negative bacteria whose peak infection rate occurs in the summer and early fall. With Legionnaires' disease, a differentiation is made between externally acquired infections due to travel and nosocomial infections. In the United States, the mortality rate of nosocomial infections is between 15 - 20%.[1,2] In Europe, 12% of all cases of Legionnaire's disease are fatal. The large number of *Legionella* spp. includes two species which are important human pathogens. Infection with *L. pneumophila* mainly results in Legionnaires' disease (also known as legionellosis), and *L. longbeachae* causes Pontiac fever. Pontiac fever is an acute, self-limiting illness which resembles influenza, however without the occurrence of pneumonia. Approximately 7% of patients who suffer from Legionnaires' disease develop Pontiac fever.[3]

L. pneumophila has 16 serogroups; more than 70% of *Legionella* infections in Europa are caused by *L. pneumophila* serogroup 1. Other strains which may cause *Legionell*a infection include *L. micdadei*, *L*. *bozemanii*, *L. dumoffii* and *L. longbeachae*.[4]

Legionnaires' disease is an acute, respiratory infection which is mainly caused by *L. pneumophila*. It was first described in 1976 during a congress in Philadelphia, USA, where Legionnaires' disease was given its name. Two further outbreaks of Legionnaires' disease with a total of 6 fatalities occurred in 2013 in Brisbane, Australia and Reynoldsburg, Ohio. The symptoms are fever, coughing (dry or sputum producing) and shivering. Other less frequent symptoms are diarrhea, vomiting, bradycardia and hyponatremia.[3] Individuals of any age may become infected with Legionnaires' disease, but older persons as well as smokers and patients with chronic lung disorders are more susceptible to such an infection. Even in countries with an effective health care system, up to 90% of cases of Legionnaires' disease are not diagnosed, as the clinical symptoms are very diffuse and the illness only occurs quite rarely. In addition, it is difficult to differentiate Legionnaires' disease from other forms of pneumonia solely on the basis of symptoms or radiological examinations.

The very early detectability of soluble antigens which are specific to Legionella in the urine of patients with Legionnaires' disease makes urine an ideal examination matrix for early as well as later stages of legionellosis[5,6]. The RIDASCREEN® Legionella ELISA is especially suitable for the detection of soluble Legionella antigen in the urine of patients who are infected with *Legionella pneumophila* of serogroup 1.

3. Test principle

The RIDASCREEN®Legionella Test uses specific antibodies in a sandwich-type method. The surface of the microtiter plate is coated with polyclonal antibodies against the antigens of Le-

gionella LPS. A pipette is used to place the urine samples for examination, as well as control specimens, into the well of the microtiter plate together with biotinylated polyclonal anti-legionella antibodies (Conjugate 1) for incubation at room temperature (20–25 °C). After a washing stage, streptavidin poly-peroxidase conjugate (Conjugate 2) is added and incubated again at room temperature (20–25 °C). If Legionella antigens are present in the urine sample, a sandwich complex will form which consists of immobilized antibodies, the Legionella antigens, and the antibodies conjugated with the biotin-streptavidin-peroxidase complex. A further washing stage removes the unbound streptavidin poly-peroxidase conjugate. In positive samples, the addition of a substrate changes by the bound enzyme from a colorless solution in the wells of the microtiter plate to a blue solution. Addition of a stop reagent changes the color from blue to yellow. The extinction is proportional to the concentration of Legionella antigens in the urine sample.

4. Package Contents

The reagents in the kit are sufficient for 96 assays.

Plate	96	Microtiter plate, 12 microtiter strips (which can be divided) in a strip holder; coated with polyclonal antibodies against *L.pneumophila*
Wash	100 mL	Wash buffer, phosphate buffered NaCl solution (10x concentration); contains 0.1% thimerosal
Control | +	2 mL	Positive control, inactivated Legionella antigens; ready for use
Control | -	2 mL	Negative control; ready for use
Conjugate | 1	13 mL	Biotin-conjugated polyclonal *L.pneumophila* antibodies in stabilized protein solution; ready for use; yellow color
Conjugate | 2	13 mL	Streptavidin poly-peroxidase conjugate in stabilized protein solution ready for use; orange color
Substrate	13 mL	Hydrogen peroxide/TMB; ready for use
Stop	12 mL	Stop reagent; 1 N sulfuric acid; ready for use

5. Storage instructions for reagents

All reagents must be stored at 2 - 8 °C and can be used until the date printed on the label. If the diluted wash buffer is stored at 2 - 8 °C it can be used for a maximum of 4 weeks. Microbial contamination must be prevented. No guarantee can be accepted for the quality after expiration.of reagents is exhausted.

The aluminum bag must be opened with scissors in such a way that the clip seal is not separated. Any microtiter strips which are not required must be returned to the aluminum bag and immediately stored at 2 - 8 °C.

The colorless substrate must also be protected from direct light to prevent it from decomposing or turning blue due to auto-oxidation. Once the substrate has turned blue, it must not be used.

6. Reagents required but not provided

6.1. Reagents

- Distilled or deionized water

6.2. Accessories

- Sample vials
- Vortex mixer (optional, see 9.3.)
- Micropipette for 50 - 100 µL
- Measuring cylinder (1000 mL)
- Stop clock
- Washing device for microtiter plates or multichannel pipettes (300 µL)
- Photometer for microtiter plates (450 nm and reference filter 620–650 nm)
- Filter paper (laboratory towels)
- Waste container with 0.5% hypochlorite solution

7. Precautionary measures

For *in vitro* diagnostic only.

This test must only be carried out by trained laboratory personnel. The guidelines for working in medical laboratories must be followed. Always strictly adhere to the user instructions for this test.
Do not pipette specimens or reagents by mouth, and avoid contact with injured skin or mucous membranes. Wear personal safety equipment (suitable gloves, gown, safety glasses) when handling the specimens, and wash hands after finishing the test. Do not smoke, eat, or drink in areas where samples are being processed.
For further details, please refer to the Material Safety Data Sheets (MSDS) at www.r-biopharm.com.
The positive control in the kit contains inactivated Legionella antigen, which as with the patient specimens, must be treated as potentially infectious according to the national safety regulations.
Ensure the proper and responsible disposal of all reagents and materials after their use. Please comply with the relevant national regulations for disposal.

8. Specimen collection and storage

Urine specimens must be collected in clean standard containers and may be stored for up to 24 hours at room temperature or at 2 - 8 °C before use. In addition, storage at 2 - 8 °C for a

further 3 days is possible. If longer storage is necessary before use, the urine specimens must be stored at -20 °C.
Avoid freezing and thawing the specimen repeatedly. The urine specimens must not be collected in transport containers which contain transport media with preservatives, animal sera, metal ions, oxidizing agents, or detergents since these may interfere with the RIDASCREEN® Legionella Test ELISA.

9. Test procedure

9.1. General

All reagents and the microtiter plate Plate must be brought to room temperature (20 - 25 °C) before use. The microtiter strips must not be removed from the aluminum bag until they have reached room temperature. The reagents must be thoroughly mixed immediately before use. After use, the microtiter strips (placed in sealed bags) and the reagents must be stored again at 2 - 8 °C. Once used, the microtiter strips must not be reused. The reagents and microtiter strips must not be used if the packaging is damaged or the vials are leaking.
In order to prevent cross contamination, the samples must be prevented from coming into direct contact with the kit components.
The test should not be carried out in direct sunlight. We recommend covering the microtiter plate or sealing with plastic wrap to prevent evaporation losses.

9.2. Preparing the samples

All urine should be thoroughly mixed and can then be used for the test without dilution.
Any salt crystals which occur during storage of the urine samples must be fully dissolved by heating to 37 °C before the urine can be used in the test.
Urine which contains particles for any reason must be filtered before use.

9.3. First incubation

After inserting a sufficient number of wells in the strip holder, add 100 µL of the positive control Control | +, the negative control Control | - or the urine sample. Then add 100 µL of the biotin-conjugated antibody Conjugate | 1 and blend (by tapping lightly on the side of the plate), then incubate for 60 minutes at room temperature (20 - 25 °C).

9.4. Washing

Careful washing is important to achieve correct results and should therefore be carried out strictly according to the instructions. The incubated substance in the wells must be emptied into a waste container for disposal in accordance with official regulations. After this, tap out the plate onto absorbent paper to remove the residual moisture. Then wash the plate five times using 300 µl wash buffer each time. Make sure that the wells are emptied completely by tapping them out after each wash onto a part of the absorbent paper which is still dry and unused.

If a microplate washer or fully automated ELISA is used, ensure that the machine is correctly adjusted or request the settings from the manufacturer if necessary. Appliances delivered by R-Biopharm are already programmed with validated settings and work protocols. Only particle-free urine samples should be used in accordance with sample preparation instructions (see 9.2.) to avoid blocking the wash needles. Also ensure that all of the liquid is aspirated during each washing stage.

9.5. Second incubation

Use a pipette to fill 100 µL streptavidin poly-peroxidase conjugate Conjugate | 2 into the wells, then incubate for 30 minutes at room temperature (20 - 25 °C).

9.6. Washing

Wash as described in 9.4.

9.7. Third incubation

Fill all wells with 100 µl substrate Substrate. Then incubate the plate for 15 minutes in darkness at room temperature (20 - 25 °C). Subsequently fill all wells with 50 µL stop reagent Stop in order to stop the reaction. After blending cautiously by tapping lightly on the side of the plate, measure the extinction at 450 nm (optional: 450/620 nm). Adjust the zero point in the air, i.e. without the microtiter plate.

10. Quality control – indications of reagent expiry

For quality control purposes, positive and negative controls must be used each time the test is carried out, to ensure that the reagents are stable and that the test is conducted correctly. The test has been carried out correctly if the extinction rate (OD) for the negative control is less than 0.2 at 450 nm (less than 0.160 at 450/620 nm) and the measured value for the positive control is greater than 0.8 at 450 nm or at 450/620 nm. A value greater than 0.2 (0.160) for the negative control may indicate that washing was insufficient. Deviation from the required values, just like a turbid or blue coloration of the colorless substrate before it is filled into the wells, may indicate that the reagents have expired.
If the stipulated values are not met, the following points must be checked before repeating the test:

- Expiry date of the reagents used
- Functionality of the equipment being used (e.g. calibration)
- Correct test procedure
- Visual inspection of the kit components for contamination or leaks – a substrate solution

which has turned blue must not be used.

If the conditions are still not fulfilled after repeating the test, please consult the manufacturer or your local R-Biopharm distributor.

11. Assessment and interpretation

11.1. Calculating the cut-off

In order to establish the cut-off, 0.15 extinction units are added to the measured extinction for the negative control.

Cut-off = extinction rate for the negative control + 0.15

11.2. Test result

Assessment of the sample is positive if the extinction rate is more than 10 % higher than the calculated cut-off value.

Assessment of the sample is marginal and the test needs to be repeated if the extinction rate ranges from 10 % less to 10% greater than the cut-off value. If the repeat examination with a fresh urine sample again falls within the gray zone, the sample should be considered negative.

Samples which are more than 10 % below the calculated cut-off must be considered negative.

12. Limitations of the method

The RIDASCREEN® Legionella test detects the soluble antigen of Legionella bacteria in urine samples. It is not possible to associate the determined level of extinction with the occurrence or severity of clinical symptoms. The results obtained must always be interpreted in combination with the clinical signs and symptoms.

A positive result does not rule out the presence of other infectious pathogens.

A negative result does not rule out the possibility of a Legionella infection. Such a result may be due to intermittent excretion of the pathogen in the urine, or the amount of antigen in the sample may be too small. If the patient history gives rise to suspicion of infection with *Legionella pneumophila*, the examination should be repeated with another urine sample.

A borderline result may be due to non-homogeneous distribution of the antigens in the urine sample. In such cases the examination should be repeated with a second sample or another sample of the patient's urine should be requested for examination.

13. Performance characteristics

13.1. Test quality

A retrospective validation study with RIDASCREEN® Legionella ELISA Legionella ELISA

examined 100 urine samples. These samples were taken for routine diagnostic examination at the Legionella Reference Laboratory in Dresden, Germany, where they were then preserved at -20 °C. After thawing, the samples underwent comparative examination with RIDASCREEN® Legionella ELISA and a further commercial ELISA. The results of the study are summarized in Table 1.

Table 1: Correlation between RIDASCREEN® Legionella ELISA and a further commercial ELISA

		ELISA	
		Positive	Negative
RIDASCREEN® Legionella	Positive	59	2
	Negative	4	30

Positive match: 95.2 %
Negative match: 90.9 %

13.2. Cross reactivity

Various pathogenic organisms were investigated with RIDASCREEN® Legionella ELISA and showed no cross reactivity except for *S. aureus*. These studies were conducted with bacterial suspensions shown to have concentrations of 10^6 to 10^9 organisms per ml. Virus culture supernatants and antigens are listed accordingly. The results of that study are listed in Table 2.

Table 2: Cross reactivity with pathogenic microorganisms

Organism	Origin	Mean value [OD 450/620]
Adenovirus	Cell culture supernatant	0.005
Bacillus cereus	Culture	0.118
Campylobacter coli	Culture	0.131
Campylobacter jejuni	Culture	0.068
Candida albicans	Culture	0.043
Candida glabrata	Culture	0.023
Citrobacter freundii	Culture	0.047
Chlamydophila pneumoniae	Culture	0.003
Enterobacter cloacae	Culture	0.046
Enterococcus faecalis	Culture	0.097
Escherichia coli	Culture	0.057
Haemophilus influenzae	Culture	0.019
Influenza A/Beijing	Antigen for ELISA	0.011
Influenza A/Sydney	Antigen for ELISA	0.010
Influenza B/Harbin	Antigen for ELISA	0.020
Klebsiella pneumoniae	Culture	0.035
Mycoplasma pneumoniae	Antigen for ELISA	0.022
Parainfluenza virus	Antigen for ELISA	0.093
Proteus mirabilis	Culture	0.020
Proteus vulgaris	Culture	0.038
Pseudomonas aeruginosa	Culture	0.023
Respiratory syncytial virus	Antigen for ELISA	0.050
Serratia liquefaciens	Culture	0.066
Serratia marcescens	Culture	0.007
Staphylococcus aureus	Culture	3.579
Staphylococcus epidermidis	Culture	0.041

Organism	Origin	Mean value [OD 450/620]
Staphylococcus saprophyticus	Culture	0.020
Streptococcus agalactiae	Culture	0.049
Streptococcus pneumoniae	Culture	0.045

13.3. Precision

The reproducibility of the RIDASCREEN® Legionella ELISA was tested with six references representing the complete measurement range from weak to high positive. 40 replicates of these references were assayed to determine intra-assay reproducibility. The mean values and the variation coefficients (VC) were determined for three lots. For the inter-assay reproducibility, references from 10 different working days were assayed in duplicates, with 2 runs per day. The measurements were determined in 3 lots by 6 technicians. The inter-lot reproducibility was determined for all 3 lots. The results are shown in Table 3.

Table 3: Results for the reproducibility/precision of RIDASCREEN® Legionella ELISA

Ref.		Intra-assay			Inter-assay			Inter-lot
		Kit lot 1	Kit lot 2	Kit lot 3	Kit lot 1	Kit lot 2	Kit lot 3	Kit lots 1 - 3
1	MV	3.015	3.248	2.900	2.780	2.499	2.573	2.617
	VC (%)	2.94%	4.71%	3.88%	11.54%	12.15%	7.19%	11.73%
2	MV	2.702	2.868	2.674	2.515	2.180	2.305	2.333
	VC (%)	4.78%	8.70%	4.55%	12.63%	15.27%	8.54%	14.12%
3	MV	1.559	1.460	1.412	1.354	1.094	1.210	1.219
	VC (%)	5.99%	9.65%	5.42%	17.73%	15.94%	10.38%	18.34%
4	MV	0.838	0.701	0.725	0.672	0.513	0.587	0.591
	VC (%)	4.75%	9.72%	6.08%	19.99%	17.46%	11.67%	21.51%
5	MV	0.759	0.618	0.726	0.593	0.449	0.504	0.515
	VC (%)	11.11%	9.09%	7.41%	22.56%	21.44%	13.76%	24.10%
6	MV	0.003	0.000	0.007	0.008	0.001	0.008	0.006
	VC (%)	n/a	n/a	n/a	n/a	n/a	n/a	n/a

13.4. Analytical sensitivity

To determine the analytical sensitivity of the RIDASCREEN® Legionella ELISA the limit of blank (LoB) was analyzed in 270 assays of negative urine samples, and the limit of detection (LoD) was analyzed in 90 assays. The results of these measurements are shown in Table 4.

Table 4: Results for the analytical sensitivity of RIDASCREEN® Legionella ELISA

	MV [OD 450/620]	ng/mL
LoB	0.041	–
LoD	0.060	1.5

13.5. Interfering substances

The following list of substances showed no effects on the test results when they were blended into Legionella positive and Legionella negative urine samples in the described concentrations**:**

Human blood (10 % v/v), amoxicillin (antibiotic; 0.72 % w/v), acetaminophen (analgesic; 1.08 % w/v), cough syrup containing codeine (0.25 % v/v), albumin (0.5 % w/v), glucose (2 % w/v), ascorbic acid (vitamin C; 0.1 % w/v), bilirubin (0.02 % w/v), boric acid (0.26 % w/v), erythromycin (antibiotic; 0.06 % v/v). Levofloxacin showed a dose-related reduction of the OD values if it was mixed with the urine in a concentration corresponding to 2x to 3x the daily dose.

Appendix

Test-specific symbols:

Plate	Microtiter plate
Wash	Washing buffer
Control | +	Positive control
Control | -	Negative control
Conjugate | 1	Conjugate 1
Conjugate | 2	Conjugate 2
Substrate	Substrate
Stop	Stop reagent

References

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5. Berdal BP et al. Detectionof Legionella pneumophila antigen in urine by enzyme-linked immunospecific assay. J.Clin.Microbiol. 1979 (9): 575-578
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