LEGIONELLA PNEUMOPHILA DNA

Qualitative Real-Time PCR for detection of Legionella Pneumophila genome

4BShopLab

Rev. 2

Legionella pneumophila DNA

A. INTENDED USE

The **Legionella Pneumophila Qualitative** Real-Time PCR kit coded **LEPDNA.CE** is intended for the qualitative detection of Legionella Pneumophila DNA in biological samples with a simultaneous control of the amplification reaction through an **Internal Control (IC).**

The kit has been adapted for the use on the Real-Time Thermacyclers and ABI 7500 Sequence Detection System® (Software SDS version 1.3.1, Applied Biosystems™*) or MX3000P (Software MxPro version 4.01, Stratagene™***).

* Applied Biosystems is a registered trademark and ABI PRISM® is a trademark of Applera Corporation or its subsisdiaries in the US and/or certain other countries.
***Stratagene is a registered trademark

B. INTRODUCTION

Legionella pneumophila, a gram negative non spore forming motile bacillus, is the etiologic agent of Legionellosis. Clinical symphtoms can range in severity from a mild, febrile illness (Pontiac fever) to a rapid and potentially fatal pneumonia (Legionnaires' disease). L. pneumophila is a common cause of nosocomial and travel-acquired pneumonia and with the bacterial species Mycoplasma pneumoniae and Clamydia pneumoniae is one of the top three cause of sporadic, community acquired pneumonia. The first strain of legionella were isolated in 1943 and classified as Riccktesia like organism, and the genus was established in 1979 after a large outbreak of pneumonia among members of the American legion that occurred 3 years earlier. After this, a large spectrum of legionella species was classified, actually genus includes 50 species and 16 serogrups of L. pneumophila were classified. Legionellae are ubiquitous in natural and artificial water environments worldwide, and survive in a range of environmental conditions.

Laboratory diagnosis of L. pneumophila in biological specimen is typically based on either cultivation, serological, direct fluorescent antibody (DFA) staining techniques or urinary detection test. Isolation of Legionella species which has a specificity of 100%, is considered the gold standard confirming for the presence of bacteria both for biological and environmental samples. Culture diagnosis requires a special media and several days are required to obtain a positive result.

Molecular based assay such as real time PCR assays were demonstrated to be a useful tool for L.pneumophila detection because of high sensitivity, specificity, easy to use and quick method.

C. PRINCIPLE OF THE TEST

The LEPDNA.CE Kit is based on a Real Time chemistry which uses specific Primers and Probes,

Legionella pneumophila DNA, recovered from the biological sample under investigation through an extraction step, is amplified using Real Time amplification system. The amplified product is detected using a fluorescent reporter dye probe specific for a Legionella pneumophila**.**

Heterologous Internal Control (IC) serves as an Extraction/Amplification control for each individually processed specimen aiming to the identification of reaction inhibitors.

An High Positive control (CTRL-H) and a Low Positive control (CTRL-L) are supplied as controls of the PCR reaction

D. COMPONENTS

The standard format of the product code LEPDNA.CE contains reagents for 50 tests.

Component	Labelling and Contents	LEPDNA.CE 50 Reactions
A **CODED: ALL/MM** **COLOR CODE: LIGHT BLUE**	Master mix	N°2 vials / 0.4 ml
B **CODED: LEP/CB** **COLOR CODE: YELLOW**	Lyophilised Primers/Probes	N° 2 Vials (Dissolve with the volume of ALL/C indicated on the vial label)
C **CODED: ALL/C** **COLOR CODE: GRAY**	MG Water	N°2 vials /1.5 ml
NTC **CODED: ALL/NTC** **COLOR CODE: WHITE**	Negative Control	N°1 vials /1.5 ml
CTRL-H High Positive Control (1,32x10^4 Copies/ul) **CODED: LEP/CTRL-H** **COLOR CODE: VIOLET**	Lyophilised Qualitative High positive	N° 8 Vials (Dissolve with the volume of ALL/C indicated on the vial label)
CTRL-L Low Positive Control (1,32x10 Copies/ul) **CODED: LEP/CTRL-L** **COLOR CODE: PINK**	Lyophilised Qualitative Low positive	N° 8 Vials (Dissolve with the volume of ALL/C indicated on the vial label)
I.C. Internal Control **CODED: ALL/IC** **COLOR CODE: GREEN**	Lyophilised Internal Control	N° 2 Vials (Dissolve with the volume of ALL/C indicated on the vial label)
Package Insert	Instruction for Use	1

Important note: Upon request, Dia.Pro can supply reagents for 25, 100, 150 reactions, as reported below **:**

1. Component A	n°1 vial/0.4 ml	n°4 vials/0.4 ml	n°6 vials/0.4 ml
2. Component B	n°1 vial	n°4 vials	n°6 vials
3. Component C	n°1 vial/1.5 ml	n°2 vials/1.5 ml	n°3 vials/1.5 ml
4. NTC	n°1 vial/1.5 ml	n°1 vials/1.5 ml	n°1 vials/1.5 ml
5. IC	n°1 vial	n°4 vials	n°6 vials
6. CTRL-H	n°4 vial	n°4 vials	n°6 vials
7. CTRL-L	n°4 vial	n°4 vial	n°6 vial
8.Pack. insert	n° 1	n° 1	n° 1
Number of tests	**25**	**100**	**150**
Code	**LEPDNA.CE.25**	**LEPDNA.CE.100**	**LEPDNA.CE.150**

E. STORAGE AND STABILITY

The kit LEPDNAQT.CE must be stored at +2…8 °C . Once dissolved **Component B** (coded LEP/CB) and **Component IC** (coded ALL/IC) are stable for 4 months at -20°C. Once dissolved **Component positive control HIGH** and **LOW**

(coded LEP/CTRL-HIGH, LEP/CTRL-LOW) are stable for 2 weeks at -20°C. If the components are to be used only intermittently, they should be frozen in aliquots, repeated thawing and freezing should be avoided, Only one defreezing is allowed.

F. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (0.5 µl < volume <1000 µl)
2. DNA extraction kit
3. MG EtOH
4. Thermal Block
5. Microcentrifuge
6. Tube racks
7. Sterile filtered tip with aerosol barrier
8. Nuclease-Free Microtubes
9. 0,2 ml Microtubes or Pcr Microplates recommended from the Real-Time PCR instruments manufacturers
10. Disposable gloves, powder-free
11. Real-Time PCR Thermalcycler (*)
12. Absorbent paper tissues.
13. Vortex or similar mixing tools.

(*) Attention: A valid calibration of the pure dyes (Pure Spectra Component File) and of the background (Background Component File) must be done routinely.

G. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
2. The technical personnel must be deeply trained in the use of Real-Time thermalcyclers, in the manipulation of Molecular Biology reagents and skilled in the Real-Time PCR amplification protocols.
3. The kit has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
4. All the personnel involved in performing the assay have to wear protective laboratory clothes, powder-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
5. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
6. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents.
7. Components A and B are light sensitive. Protect them from strong light exposition.
8. Avoid vibration of the bench surface where the test is undertaken.
9. Upon receipt, store the kit at 2..8°C into a temperature controlled refrigerator or cold room.
10. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.
11. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
12. Avoid cross-contamination between samples by using disposable tips and changing them after each sample.
13. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
14. Do not use the kit after the expiration date stated on the external container label.
15. Treat all specimens as potentially infective. All human bronchoalveolar lavage should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
16. Store and extract specimens separately from the other reagents and use a separate room for their handling
17. Dissolve the lyophilised reagents with the correct amount, stated in the labels with Component C (Coded: ALL/C) supplied in the kit.
18. Carry on all the working operations as quickly as possible maintaining the components on ice or in a cooling block.
19. Workflow in the laboratory must proceed in an unidirectional way, beginning in the Extraction Area and moving to the Amplification and Data Analysis Area. Do not return samples, equipment and reagents to the area where previous steps have been performed.
20. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.
21. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from sample extraction procedures, has to be treated as potentially infective material and inactivated before waste. Do not put in contact the extraction waste with bleach.
22. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
23. Other waste materials generated (example: tips used for samples) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes..

H.SPECIMEN: PREPARATION AND RECOMMENDATIONS

1.Bronchoalveolar lavage (BAL) must be collected in polypropylene tubes without any addition of preservatives
2.Samples must be transported and stored +2 / +8°C for a maximum period of 3 days.
3.Do not freeze bronchoalveolar lavage samples to avoid cell lysis and the loss in titre of the bacterial DNA.
4.Haemoglobin and mucoproteins in DNA extracted from BAL samples could inihibit the amplification reaction.
5.Bronchoalveolar lavage samples must be pre-treated before DNA extraction according section M.
6.Samples have to be clearly identified with codes or names in order to avoid result misinterpretation.

I. PREPARATION OF COMPONENTS AND WARNINGS

Master Mix:

Component A. Ready to use. Mix well on vortex before use and centrifuge briefly to collect the whole volume.

WARNING: Component A is light sensitive. Protect it from strong light exposition

Primers/Probes:

Component B.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized Component B with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

WARNING: Component B is light sensitive. Protect it from strong light exposition.

MG Water :
Component C. Ready to use.

Negative Control :
NTC. Ready to use.

Positive Controls:
Component CTRL-H.
- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized CTRL-H with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

Component CTRL-L.
- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized CTRL-L with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

Internal Control:
I.C.
- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized I.C. with the volume of Component C (Code: ALL/C) indicated on the vial label .
- Keep it on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

L. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. **Micropipettes** have to be calibrated and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-5%.
2. **Extraction Device:** The LEPDNA.CE Kit is intended to be used in combination only with QIAamp DNA Minikit Code.51306 (QIAGEN) and Nucleospin Tissue kit Code: 740952 (Macherey-Nagel). The end users must strictly follow the Instruction for use supplied by the manufacturers.
3. **Real-Time Thermocyclers and Instrument Softwares.** The LEPDNA.CE Kit is intended for the use in combination only with the Real Time Thermal cyclers ABI 7500, software SDS version 1.3.1 (Applied Biosystems), and MX3000P, software MxPro version 4.01 (Stratagene).
   The end users must strictly follow the Instruments Instruction for use supplied by the manufacturers.

M. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that on the bottom of the Lyophilized components vials is present a well formed aggregate. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box.
3. Dissolve the Lyophilized Components with the appropriate amount of Component C (Molecular Grade water) as described in the proper section (I).
4. Turn the Thermalcyclers on, check settings and be sure to use the right assay protocol.
5. Follow strickly the Instruments Manual supplied by the manufacturers for the correct setting of the Real-Time Thermalcyclers.
6. Check that the micropipettes are set to the required volume.
7. Check that all the other equipment is available and ready to use.
8. In case of problems, do not proceed further with the test and advise the supervisor.

N. ASSAY PROCEDURE

The assay has to be carried out according to what reported below.

N.1 Sample Pre-Treatment

- Transfer a maximum of 1 ml of sample in a sterile tube
- Add 50 microliter per ml of N-Acetyl Cysteine mucolitic solution (Concentration:100 mg/ml)
- Vortex vigorously for 15 second
- Incubate at +2 / +8° for 10 minutes
- Vigorously shake the sample until it is completely fluid
- Centrifuge 2500 RPM for 15 minutes
- Discard the supernatants
- Proceed to DNA extraction

N.2 DNA extraction

The extraction step of the Legionella pneumophila genomic DNA has to be carried out exclusively in combination with the following kit:

Material	Description	Kit code	manufacturer
Bronchoalveolar lavage	QIAamp DNA mini kit®	51306	Qiagen™
Bronchoalveolar lavage	NucleoSpin Tissue Kit	740952	MN™

Extraction of Legionella pneumophila genomic DNA from bronchoalveolar lavage specimen has to be carried out by the end-user, according to the Manufacturer's instructions, with the following kits:

- **QIAamp DNA Mini Kit (QIAGEN)**

Important Notes: The "DNA Purification from Tissue" Protocol described in the Manufacturer's Instruction has to be applied with the following modifications:

1. *Start the protocol from step n°2a using as sample the pellet obtained as described in the section N.1 (pre-treatmentof the sample) instead of the tissue sample.*
2. *In the step n° 11 use 100 ul of Eluition Buffer instead of 200 ul.*

- **NucleoSpin Tissue Kit (Macherey-Nagel)**

Important Note: Follow the " Standard protocol for human or animal tissues and cultured cells" (Protocol 5) described in the Manufacturer's Instruction applying the following modifications:

1. *Start the protocol from step n°1 section **Cultured Cells** "Pre-Lysis" using the pellet obtained as described in the section N.1 (pre-treatmentof the sample)*

The DNA collected from the samples, not used in the run , has to be stored frozen adequately (-20°C….-80°C).
Important note: *The I.C. of the* LEPDNA.CE Kit *can be used in the isolation procedure as extraction control.*
The Internal Control Ct value for the negative samples is used to evaluate if the DNA extraction procedure has been performed correctly (see section Q).

For this application add 5 µl of I.C. to the lysis buffer and sample mixture and proceed following the instruction manual supplied by the manufacturer of the Extraction Kit.

N.3 Setting up of the reaction

LEPDNA.CE kit is intended to be used exclusively in combination with ABI 7500, software SDS version 1.3.1 (Applied Biosystems), and MX3000P, software MxPro version 4.01 (Stratagene).

N.3.*1 Preparing the PCR*

Important: *An example of dispensation scheme is reported in Section O. Please, refer to it before starting to read the instructions here below.*

- Prepare the components as described in Section I;
- Prepare the required number of reaction tubes or a 96-well reaction plate for the samples under evaluation and for the Positive controls (prepared as described in Section I).

Important note: *Use only optical tubes or microplates suggested by the Real-Time thermalcyclers manufacturers.*

- Consider that the samples, if possible, should be tested in duplicate;
- Include at least 1 tube/well for the NTC (negative control)
- Prepare the **Amplification Mix** for **Samples, NTC and positive controls (CTRL-H, CTRL-L)** as table below:

Preparation of the Amplification Mix
(I.C. as Amplification Control)

Number of Reactions		x1	x12
A	Master mix	12,5 µl	150 µl
B	Primers/probes	2 µl	24 µl
I.C.	Internal Control	0,5 µl	6 µl
Tot vol.		**15 µl**	**180 µl**

Important note: *If the Internal Control was added during the DNA isolation procedure, prepare the* ***Amplification Mix*** *for the* ***Sample,*** **NTC and positive controls (CTRL-H, CTRL-L),** *as described in the table below:*

Preparation of the Amplification Mix
(I.C. as Extraction/Amplification control)

Number of Reactions		x1	x12
A	Master mix	12,5 µl	150 µl
B	Primers/probes	2 µl	24 µl
C	MG Water	0,5 µl	6 µl
Tot vol.		**15 µl**	**180 µl**

N.3.2 Amplification procedure

- Dispense 15 ul of the amplification mix in each reaction tube or microplate well
- Add 10 ul of the **Samples, NTC, CTRL-H and CTRL-L** to the reaction tubes.
- Close firmly the reaction tubes
- Centrifuge briefly the reaction tubes at 2000 rpm
- Don't leave the reaction tubes at room temperature (RT) for more than 30 minute and at light exposure (cover the tubes).
- Load the reaction tubes in the Real-Time Thermalcycler Thermoblock Holder.
- After the setting operations described in the Sections N4 (Instrument Programming) start the Thermalcycler run.

Important note: The Components Lyophilized after dissolution in Component C (MG water) are stable no more than 3 hours kept in ice or at 2°…8° °C.
The not used volume of Component B, CTRL-H, CTRL-L and I.C. can be freeze at -20°C and used as described in Section E.

N.4 Instrument programming

For programming the instrument refer to the Instrumentation Instruction Manual provided by the manufacturers.

Important Note: *For Mx3000P set "Filter set gain settings" : ROX = x1, FAM = x8, HEX/JOE = x1. (see MxPro™ QPCR Software Instruction Manual, p.41)*

N.4.1 Thermal Profile

The thermal profile is reported in the table below:

Step	Cycle	Temp.	Time
1	1	50°C	2 min
1	1	95°C	10 min
2	50	95°C	15 sec
		60°C **(*)**	1 min

IMPORTANT NOTE: (*) step for the real time data collection

WARNING: Keep attention to set up the Real-Time Thermacycler with the correct Thermal Profile following the Instruments Manual supplied by the manufacturer.

N.4.2 Selection of the Detectors

Following the Instruction manuals for the Real-Time thermalcyclers suggested (ABI 7500 and MX3000P Stratagene) select the Detectors reported in the table here below:

Detection	Reporter	Quencher
Legionella pneumophila	FAM	Non Fluorescent
Internal Control (I.C.)	JOE	Non Fluorescent
Passive Reference	ROX	Not Present

WARNING: Keep attention to set up the Real-Time Thermacycler with the correct settings following the Instruments Manual supplied by the manufacturer.

O. ASSAY SCHEME

An example of dispensation scheme for Qualitative Analysis is reported below:

Microplate or tubes

	1	2	3	.	.
A	**CTRL-H** 1,32x10^{4}copies/ µl	Sample 6			
B	**CTRL-L** 1,32x10copies/ µl	Sample 7			
C	NTC	Sample 8			
D	Sample 1	Sample 9			
E	Sample 2	Sample 10			
F	Sample 3	Sample 11			
G	Sample 4	Sample 12			
H	Sample 5	Sample 13			

Legend: NTC = Negative Control CTRL-H, CTRL-L = Legionella pneumophila DNA Positive Controls, Sample 1,2,3 = Samples under evaluation.

P. INTERNAL QUALITY CONTROL

P.1 Pre - Analysis Settings

Before starting the analysis:

- Set the "Baseline" (the background fluorescence level) as reported here below:

"Baseline"	
ABI™PRISM® 7500 SDS	**Manual: 3-15**
STRATAGENE™ MX3000P®	**Adaptive Baseline (not use Mx4000 v1.00 to v3.00 algorithm)**

- Set manually the FAM/JOE fluorescence "Threshold"

FAM fluorescence "Threshold"	
ABI™PRISM® 7500 SDS	**0.075**
STRATAGENE™ MX3000P®	**0.075**

JOE fluorescence "Threshold"	
ABI™PRISM® 7500 SDS	**0.075**
STRATAGENE™ MX3000P®	**0.02**

P.2 Data analysis

A check is carried out on the High/Low Positive Controls any time the kit is used in order to verify whether their Ct values are as expected and reported in the table below.

ABI™PRISM® 7500 SDS/ STRATAGENE™MX3000P®	
Check FAM	**Requirements**
CTRL-H	19 ≤ Ct (Threshold Cycle) <23.5
CTRL-L	29 ≤ Ct (Threshold Cycle) <33.5

Q. INTERPRETATION OF THE RESULTS AND TROUBLESHOOTING

For each samples FAM fluorescence (positive/negative Ct value) and Internal fluorescence are assumed to validate LEP DNA detection as described in the table below:

L Pneumophila FAM	Internal Control JOE	Assay Result
SAMPLE POSITIVE	+	CORRECT
	-	CORRECT*
SAMPLE NEGATIVE	Ct < 40	CORRECT
	Ct > 40 or undetrmined	INVALID**

* High Initial concentration of L. pneumophila DNA in the sample (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal for Internal Control I.C. due to the reagents Competition.

** Problems may be occurred during the amplification step (inefficient or absent amplification) or during the extraction step (presence of inhibitors or initial sample containing an insufficient number of cells) leading to an incorrect result. The test procedure must be repeated starting from the Extraction step using a fresh sample coming from the patient.

The results obtained with this product must be interpreted taking consideration the clinical symptoms and the other laboratory parameters related to the patient conditions.

The following results are possible:

	FAM	IC	Result	CHECK
SAMPLE unknown	+	+	CORRECT RESULT *Positive*	
SAMPLE unknown	-	Ct>40 Or undet	ATTENTION ! POSSIBILITY OF: Inhibition, error in the procedure or misfunctioning of the Instruments	1. that the components have been prepared correctely 2. that no mistake has been done in the assay procedure; 3. That the selected detection dye are corrected:FAM 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube 7. That the Extraction and procedures have been executed correctly;
SAMPLE	-	+	CORRECT	

unknown			RESULT *Negative*	
CTRL-H/CTRL-L	+	+	CORRECT RESULT	
CTRL-H/CTRL-L	-	Ct>40 Or undet	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that the components have been prepared correctely 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected:FAM 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube
CTRL-H/CTRL-L	-	+	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that the components have been prepared correctely 2. that no mistake has been done in the assay procedure; 3. that the Analysis has been run with the correct Instrument settings; 4. that the kit has been stored correctly;
NTC	-	+	CORRECT RESULT	
NTC	+	+	ATTENTION ! POSSIBILITY OF: Contamination	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the work space and Instruments are decontaminated at regular intervals; 4.that the kit has been stored correctly;
NTC	+	Ct>40 Or undet	ATTENTION ! POSSIBILITY OF: Contamination	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the work space and Instruments are decontaminated at regular intervals; 4.that the kit has been stored correctly;

Important notes:

1. *Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.*
2. *When test results are transmitted from the laboratory to an informatics centre, attention has to be done to avoid erroneous data transfer*

If one of more of the problems described in the table above happen, after checking, report any residual problem to the supervisor for further actions.

R. PERFORMANCES

Evaluation of Performances has been conducted in accordance to what reported in the Internal Technical Specifications or ITS.
The performance evaluation was carried out in DiaPro's laboratories on materials supplied by the reference clinical lab.

R.1 ANALYTICAL SENSITIVITY

Analytical sensitivity may be expressed for qualitative methods as **limit of detection**.

Limit of detection (LOD): it is the lowest amount of target that can be detected by a test system with a stated probability.
For the NAT tests it is expressed as the smallest concentration of the **analyte** that tested in multiple repetitions gives a positive results.
The **limit of detection (LOD)** is determined by testing serial dilutions containing known concentrations of the analyte.
The **LOD** is the lowest concentration of analyte that can be consistently detected (e.g. in ≥ 95% of samples under routine laboratory conditions).
For the kit LEPDNA.CE the **LOD** has been determined by analysis of 24 replicates (8 replicates for three different runs) of the highest dilution of the analyte that can be detected in 100% of them.

The results are the following:

LOD Limit of Detection (p=0.05)	
ABI™PRISM® 7500 SDS	**1 copies/ µl**
STRATAGENE™ MX3000P®	**1 copies/ µl**

This means that a concentration of 1E+00 copies/ µl is always detected with the Instrument listed above

R.2 ANALITYCAL SPECIFICITY

The Analytical specificity is the ability of the method to detect only the target DNA sequence.
The analytical specificity of LEPDNA assay has been studied as follow:

1. The primer/probe Set has been choose analysing the genome target sequence with an appropriate software (LionSoft v.1.0 supplied by Biotools and Primer Express v.3.0" supplied by Applied Biosystem Inc.).
2. The primer/probe Set and the target genome sequence has been controlled by the "BLAST" software, in order to check if any of the nucleotide sequences deposited in the worldwide genomic banks has any homology with Legionella pneumophila, and by the "ClustalX" software, in order to compare the genome target sequences of the different genotypes of L. pneumophila.
3. The specificity was improved through the selection of stringent reaction conditions.
4. Samples coming from patients suffering infections due to potential interfering organisms were obtained from a reference Clinical Centre.

The results are reported in the following table:

Organism	Result
Mycobacterium sp	negative
Neisseria meningitidis	negative
Pseudomonas earuginosa	negative
Streptococco pneumoniae	negative
Mycoplasma pneumoniae	negative
Klebsiella pneumoniae	negative
Legionella longbeachae	negative
Legionella Micdadei	negative

R.3 DIAGNOSTIC SPECIFICITY AND SENSITIVITY

R.3.1 Diagnostic Specificity:

Diagnostic specificity is the probability that the device gives a negative result in the absence of the target marker. So that **true negative** sample is a specimen known to be negative for the target marker and correctly classified by the device
This parameter was studied by examining 10 bronchoalveolar lavage of L. pneumophila DNA negative

TRUE NEGATIVES	10
FALSE POSITIVES	0
TOTAL SAMPLES	10
SPECIFICITY %	**100**

On the basis of the results obtained Diagnostic Specificity of the system has been calculated ≥99%.

R.3.2 Diagnostic Sensitivity

Diagnostic sensitivity is the probability that the device gives a positive result in the presence of the target marker. So that **true positive** sample is a specimen known to be positive for the target marker and correctly classified by the device.

In the kit code LEPDNA.CE this parameter was studied by examining L. pneumophila DNA positive bronchoalveolar lavage.The samples have been studied in duplicate in the same run and then it was been calculated the percentage (%) of positive samples.

TRUE POSITIVES	10
FALSE NEGATIVES	0
TOTAL SAMPLES	10
SENSITIVITY %	**100**

On the basis of the results obtained Diagnostic Sensitivity of the system has been calculated in the 100%.

Diagnostic Sensitivity	100 %
Diagnostic Specificity	> 99.5 %

R.4 PRECISION

Precision shows the degree of the system's reliability. Every measurement procedure has an inherent random variation called "random error". Random error does not have a number value but it is determined by dispersion of measurement as standard deviation (DevST) and coefficient variation (CV%). Usually precision of an assay refers to the agreement between replicate measurements of the same material.

In the kit code LEPDNA.CE, **precision** was expressed as intra-assay variability and inter-assay variability. . CTRL-H and CTRL-L in 8 replicates were tested in the same run (intra-assay) and in three different runs (inter-assay).

On the basis of the results obtained intra and inter-assay variability were then calculated.

In absence of an established parameters in the European IVD Directive CTS we have identified the following value of acceptability for the L. pneumophila DNA:

Intra-Assay Coefficient Variation (CV%) ≤ 10%.
Inter-Assay Coefficient Variation (CV%) ≤ 10%.

S. LIMITATIONS

The user of this kit is advised to carefully read and understand this package insert. Strict adherence to the protocol is necessary in order to obtain reliable test results. In particular, accurate sample and reagent pipetting,application of a correct workflow along with careful programming of thermocycling step is essential for accurate and reproducible L.pneumiphila DNA detection.

It is recommended that confidentiality, appropriate counselling and medical evaluation be considered an essential aspect of the testing sequence.

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U.Symbols

LEGENDA			
REF	Product code		Storage temperature
IVD	In Vitro Diagnostic Device		See use instructions
LOT	Lot number		Manufacturer
	Expiry date		Number of tests
CE	CE conformity mark		Date of manufacturing

All the IVD Products manufactured by the company are under the control of a certified Quality Management System in compliance with ISO 13485 rule. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

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