

# Summary

## Report

### **AFNOR validation of the Genesystems method for the detection and quantification of *Legionella spp.* in waters**

- CAE Central Laboratory

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Reference method : XP T 90-471

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# **PART 1: AFNOR validation of the Genesystems method for the detection and quantification of *Legionella spp* in waters**

## **1. PURPOSE OF THE STUDY**

The present study looks at the AFNOR validation of the GeneSystems *Legionella spp.* method for the detection and quantification of *Legionella spp* by PCR in waters.

It has been carried out in accordance with the “validation protocol for detection kits and counting of *Legionella* and *L.pneumophila* by concentration and genetic amplification using polymerase chain reaction (PCR) (Revision 0 adopted by AFNOR certification on 26.09.2006)”. The modifications defined at the meeting of 07 September 2007 were also taken into account for this study.

Phases 1 and 2 enable an expert laboratory to study performances notified by the supplier. The recovery, detection and quantification limits of PCR, quantification linearity, detection and quantification inclusivity and exclusivity as well as feasibility are examined in particular. Phase 3 includes an interlaboratory study to evaluate the fidelity (repeatability and reproducibility) of the method and the supplier’s protocol from a statistical analysis of the results obtained.

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### **1.1. STANDARD OF VALIDATION**

The AFNOR validation protocol is based on the criteria, experiments and calculation methods defined in the XP T 90-471 standard (April 2006) relating to the counting of *Legionella* and *L.pneumophila* by concentration and genetic amplification by polymerase chain reaction (PCR).

### **1.2. PRINCIPLE OF GENESYSTEMS METHOD**

The GeneSystems *Legionella* method meets the requirements of the AFNOR XP T90-471 experimental standard, “detection and quantification of *Legionella spp.* and/or *Legionella pneumophila* from concentration and genetic amplification by polymerase chain reaction (PCR)”.

The GeneSystems *Legionella* method has two steps:

- a first step of preparing microbial DNA from the water sample achieved with the GeneExtract® platform,
- and a second quantification step for DNA from *Legionella pneumophila* or *Legionella spp* by PCR in real time with the GeneDisc Cyclo instrument®.

The water sample is filtered onto a polycarbonate membrane 0.45 µm. The filter on which the microorganisms present in the water have been retained is inserted into a lysis tube containing a detergent. The lysis tube is then incubated for 20 min in an ultrasound bath and then for 10 min in a bain-marie at 100°C. This mechanical and chemical lysis allows for the release of DNA from the legionella. The lysate is then clarified by filtration on to a molecular sifter (Filter column) and then purified by adsorption on a silica gel (silica column). The DNA purification protocol is achieved by filtration under vacuum. The DNA is eluted from the silica column in an elution buffer volume of 200 µL and 37 µL is used to achieve the PCR analysis with the GeneDisc Cyclers.

The GeneExtract® is a semi-automated platform patented by GeneSystems. It allows for the extraction and purification of DNA from 5 water samples and a negative control of the GeneSystems method (sterile water). It offers the advantage of uniting all of the apparatus necessary for the extraction and purification of DNA, according to the chosen protocol, on a single and unique platform: water sample filtration rack, sonotrodes and bains-maries rack intended for cellular lysis and a filtration rack for the purification of DNA on the silica column. These different sectors are controlled by an integrated software which also assures total samples' traceability (the identification and position of 6 samples, identification of the operator, extraction pack batch number, date and time of handling). The different steps are displayed on a touch screen as the protocol progresses. The operator is thus guided and can follow procedural progress.

The GeneDisc is a ready to use PCR consumable patented by GeneSystems. It is composed of 36 reactional chambers divided into 6 analysis sectors. Each analysis sector (1 sample to be analysed per sector) is linked to 6 reactional chambers *via* microchannels. The GeneDisc's 36 reactional chambers are pre-loaded in reagents by the GeneSystems Production Department: primers, probes, internal inhibition controls, positive crossed controls.

The GeneDisc Cyclers® is a Real Time PCR instrument patented by GeneSystems. It enables the amplification and quantification of DNA from the legionella in the GeneDiscs in less than an hour. Fluorescence measurements are analysed automatically to give an immediate result. The GeneDisc Cyclers assures total traceability of the samples and analyses. The GeneDiscs bar code reader selects the analysis program automatically. For each GeneDisc batch, a calibration GeneDisc, corresponding to the PCR amplification of standard genomic DNA of *L. pneumophila* ATCC33152, is analysed quantitatively. The GeneDisc Cyclers software automatically calculates the Ct, standard curve parameters and the second degree polynomial representing the: Inhibition controls fluorescence amplitude curve = f (Ct *L. pneumophila*). This polynomial is indicative of the sensitivity of the internal inhibition control in competition and in the presence of genomic DNA from *L. pneumophila* (outside of inhibition).

The parameters obtained with GeneDisc calibration linked with Master Mix are saved and applied to all GeneDiscs from the same batch, on a given GeneDisc Cyclers, in accordance with the PCR series definition which follows the XP T90-471 standard.

In the same experimental conditions as those for GeneDisc calibration, the inhibition percentage for the internal inhibition controls is determined by the software so as to indicate to the operator the dilution factor to apply if necessary for each sample analysed (d5 or d10). If the PCR reaction is not inhibited, the software calculates the Ct automatically and converts it into GU of *Legionella* / L according to the volume of water filtered.

The results analysis standardisation is effective owing to automatic analysis achieved by the software integrating algorithms and necessary standard curves. At the end of the reaction, the GeneDisc Cycloer analysis software gives a direct result in Genomic Units per litre (GU/L) corresponding to the number of *Legionella* per litre in the analysis sample, taking into account the filtered volume and the DNA dilution factor. The amplification curves and all analysis parameters are displayable and are saved in the memory for analyses traceability.

The GeneExtract® and the GeneDisc Cycloer® work with consumables such as: a *Legionella* DNA extraction pack (Legionella Extraction Pack 01) and pack of GeneDiscs® (*Legionella* spp Premium GeneDisc Pack).

## 2. PRELIMINARY STUDY RESULTS

### 2.1. DETERMINATION OF OPTIMAL RECOVERY

The recovery has been evaluated so as to check the method's compliance. It has been evaluated for 6 independent samples, three different matrices and three different concentration levels. 54 samples have been analysed in total.

Matrices tested:

- hot tap water sampled in the laboratory\*
- TAR water\*\*
- Evian mineral water\*

\* exempt from *Legionella nucleic acids*

\*\* exempt from *Legionella pneumophila nucleic acids*

Concentration levels assessed: 1000, 10 000 and 100 000 GU/250 ml.

Inoculum: *L. pneumophila* culture (ATCC 33152 T strain)

The *L. pneumophila* stock solution was titrated by direct lysis and microscopic counting after marking of the cells in DAPI.

The analyses were achieved over 3 days.

- Uncertainty associated with direct lysis

	<i>Legionella spp.</i>		<i>L. pneumophila</i>		DAPI*
	Mean	Standard deviation	Mean	Standard deviation	Mean
<b>Day 1</b>	<b>8.02</b>	0.138	<b>7.96</b>	0.084	<b>8.28</b>
<b>Day 2</b>	<b>8.80</b>	0.104	<b>8.40</b>	0.161	<b>8.81</b>
<b>Day 3</b>	<b>8.54</b>	0.473	<b>8.57</b>	0.299	<b>8.96</b>

\* The counting after DAPI marking is obtained from the average from the number of bacteria counted over 20 fields.

- Study of the optimal recovery from the GeneSystems *Legionella method*

Type of water	Level of concentration examined	Recovery average obtained (%)	Recovery average per type of water (%)	Average from bias obtained (Log)	Bias standard deviation
<b>Evian water</b>	1 000	117	75	-0.19	0.25
	10 000	45			
	100 000	63			
<b>ECS</b>	1 000	102	64	-0.27	0.28
	10 000	58			
	100 000	32			
<b>TAR</b>	1 000	84	66	-0.24	0.27
	10 000	73			
	100 000	42			

#### CONCLUSION

The average recovery obtained are much greater than 25%. No inhibitions were observed on testing. The method is robust: no observations of significant difference in recovery according to the type of water analysed.

## 2.2. DETECTION LIMIT

30 independent DNA solutions from a concentration estimated at 5 GU/well were tested. The amplification and detection were carried out on the consumable intended for the detection of *Legionella spp* (*Legionella spp. Premium* GeneDisc pack – Ref. GDLSP-471).

### CONCLUSION

The detection limit is validated at 5 GU/well for the two *Premium* GeneDisc packs, in accordance with the performances notified by the supplier.

The gross results are detailed in appendix 1

## 2.3. QUANTIFICATION LIMIT

A DNA solution estimated at 25 GU/well was analysed 30 times, in accordance with repeatability requirements. The amplification and detection were carried out on the “*Legionella spp. Premium* GeneDisc pack” kit.

### Results

	Target value	Target value (log)	Value average measured (n = 30)	Bias (log)	Confidence interval at 95% (2.t.s)	Accuracy test (t calculated)	uncertainty measurement*
<b>Validation criteria</b>					< 0.50	<2.045	<0.30
<b>Results obtained</b>	25	1.39	23	0.03	0.289	2.609	0.15
<b>Conclusion</b>					compliant	Non compliant	compliant

Measurement uncertainty =  $2 \times \sqrt{(\text{bias}^2 + \text{standard deviation}^2)}$

### CONCLUSION

For the GeneDisc *Premium* pack, the quantification limit is repeatable at 25 GU/well but presents an accuracy default with the Student test. In terms of measurement uncertainty, it complies with the new statistical model, accepted at the last T90E group meeting (09/10/2007) and in the technical study.

## 2.4. DETERMINATION OF LINEARITY

5 independent standard curves were achieved from 5 tubes of standard genomic DNA from *L. pneumophila* ATCC33152, marketed by Genesystems (SDNA-Lp). The linearity study was completed for values of 25, 250, 2 500, 25 000, 250 000 GU/well.

- Results

Equation of the standard curve			
<i>Slope/Efficacy</i>	<i>Acceptable domain</i>	<i>Intercept</i>	<i>Conclusion</i>
-3.483 / <b>94.9%</b>	-4.115 < a < -2.839 75% < E < 125%	37.616	compliant
Statistical analysis of linear model			
<i>Origin</i>	<i>Value observed</i>	<i>Critical value With <math>\alpha = 5\%</math></i>	<i>Conclusion</i>
F regression	2375.3	4.35	compliant
F model error	<b>0.18</b>	3.10	compliant

	Uncertainty analysis – model undergoing evaluation (XP T90-471)				
<b>GU Target</b>	<b>25</b>	<b>250</b>	<b>2500</b>	<b>25000</b>	<b>250000</b>
<b>Log Target</b>	<b>1.40</b>	<b>2.40</b>	<b>3.40</b>	<b>4.40</b>	<b>5.40</b>
<b>Average bias</b>	-0.007	-0.007	0.011	0.027	-0.024
<b>standard deviation</b>	0.035	0.071	0.048	0.037	0.021
<b>Elin*</b>	+/- 0.04 Log	+/- 0.07 Log	+/- 0.05 Log	+/- 0.05 Log	+/- 0.03 Log
<b>Uncertainty</b>	+/-0.08	+/-0.14	+/-0.10	+/-0.10	+/-0.06

\*Elin =  $\sqrt{(\text{bias}^2 + \text{standard deviation}^2)}$

### CONCLUSION

The linear domain is validated between 25 and 250 000 GU of genomic DNA from *L.pneumophila* ATCC 33152 for the *Legionella* spp GeneDiscs Premium pack.

## 2.5. INCLUSIVITY AND EXCLUSIVITY

Inclusivity tests were carried out on DNA extracts so as to obtain approximately 100 GU per well.

Exclusivity tests were carried out on DNA extracts so as to obtain a minimum of 10 000 GU per well.

- Bacterial strains not belonging to the *Legionella spp* classification.

<b>Bacterial strain</b>	<b>Genedisc Premium pack <i>Legionella spp.</i></b>
<i>Aeromonas hydrophila</i>	Absence
<i>Alcaligenes faecalis</i>	Absence
<i>Bacillus subtilis</i>	Absence
<i>Burkholderia cepacia</i>	Absence
<i>Clostridium perfringens</i>	Absence
<i>Enterobacter aerogenes</i>	Absence
<i>E.coli</i>	Absence
<i>Flavobacterium flavobacter</i>	Absence
<i>Klebsiella oxytoca</i>	Absence
<i>Listeria monocytogenes</i>	Absence
<i>Proteus vulgaris</i>	Absence
<i>Pseudomonas aeruginosa</i>	Absence
<i>Pseudomonas fluorescens</i>	Absence
<i>Pseudomonas putida</i>	Absence
<i>Serratia marcescens</i>	Absence
<i>Stenotrophomonas maltophilia</i>	Absence
<i>Xanthomonas</i>	Absence

- Bacteria strain belonging to the *Legionella* spp. classification

<b>Bacterial strain</b>	<b>Premium Genedisc pack <i>Legionella</i> spp.</b>	<b>PCR Quantification (Log GU / PCR well)</b>
<i>L.anisa</i>	Presence	1.75
<i>L.birminghamsis</i>	Presence	1.77
<i>L.bozemanii1</i>	Presence	1.90
<i>L.bozemanii 2</i>	Presence	2.14
<i>L.cherrii</i>	Presence	1.98
<i>L.cincinnatiensis</i>	Presence	2.22
<i>L.dumofii</i>	Presence	1.88
<i>L.erythra 2</i>	Presence	1.81
<i>L.feeleii1-2</i>	Presence	1.93
<i>L.gormanii</i>	Presence	2.13
<i>L.hackeliae 1-2</i>	Presence	1.85
<i>L.jordanis</i>	Presence	2.10
<i>L.lansingensis</i>	Presence	1.88
<i>L.longbeachae1-2</i>	Presence	1.94
<i>L.maceachernii</i>	Presence	2.15
<i>L.micdadei</i>	Presence	2.13
<i>L.oackridgensis</i>	Presence	2.05
<i>L.pariensis</i>	Presence	1.85
<i>L.sainthelensis1-2</i>	Presence	2.06
<i>L.tucsonensis</i>	Presence	1.71
<i>L.wadsworthii</i>	Presence	2.07
<i>L.pneumophila s1</i>	Presence	1.90
<i>L.pneumophila s2</i>	Presence	2.06
<i>L.pneumophila s3</i>	Presence	2.19
<i>L.pneumophila s4</i>	Presence	2.11
<i>L.pneumophila s5</i>	Presence	1.72
<i>L.pneumophila s6</i>	Presence	1.90
<i>L.pneumophila s7</i>	Presence	1.75
<i>L.pneumophila s8</i>	Presence	2.12
<i>L.pneumophila s9</i>	Presence	1.89
<i>L.pneumophila s10</i>	Presence	1.79
<i>L.pneumophila s11</i>	Presence	1.78
<i>L.pneumophila s12</i>	Presence	2.00
<i>L.pneumophila s13</i>	Presence	1.49
<i>L.pneumophila s14</i>	Presence	2.02
<i>L.pneumophila s15</i>	Presence	1.74

## 2.5. PRATICABILITY

The 18 criteria defined in the AFNOR validation protocol were studied.

### REAGENTS PACKAGING

The reagents are present in the following packaging.

- *Legionella* Extraction Pack 01 (water samples)

Items are given on the packaging and page 2 of the instructions for use

The extraction reagents are:

- ✓ "Lysis buffer".
- ✓ "Washing buffer".
- ✓ "Linkage buffer" useable after adding an "extra Linkage buffer".
- ✓ "Washing Buffer 1".
- ✓ "Washing Buffer 2".
- ✓ "Elution buffer".
- ✓ Filter columns.
- ✓ Silica columns.

- Genedisc *Premium* pack

Items are given on the packaging and page 2 of the instructions for use

There are two Genedisc *Premium* packs:

- ✓ "*Legionella* spp Genedisc *Premium* Pack".
- ✓ "*Legionella pneumophila* Genedisc *Premium* Pack".

Each Genedisc *Premium* pack contains:

- ✓ The "reactional mix" used at the time of the PCR reaction  
(6 tubes: 1 tube / GeneDisc)
- ✓ 6 "Genediscs"

### REAGENTS' VOLUME

The volume for reagents to be used is indicated on the "*Legionella* Extraction Pack 01" instructions for use (water samples).

### COMPONENTS STORAGE CONDITIONS AND PRODUCTS SHELF LIFE.

The storage temperature is indicated on the packs and on page 3 of the extraction pack instructions for use and on page 1 of the Genedisc *Premium* pack instructions for use.

The Silica Columns "Silica COLUMNS" as well as the Genediscs *Premium* Pack must be kept in a refrigerator (5°C ± 3°C). All other components can be stored at ambient temperature (15°C-30°C).

The shelf life is indicated on the Packs, as well as on each component of the Packs. Out of date GeneDiscs are not recognised by the GeneDisc Cyclor and can therefore not be used in error.

### USAGE METHODS AFTER FIRST USE

The reagents are used until exhaustion with respect of the shelf life.

### NEEDS IN TERM OF EQUIPMENT AND SPECIFIC FACILITIES

The material and necessary consumables are indicated on page 2 in each of the instructions for use for the two packs.

Safety requirements are indicated on page 3 of the extraction Pack instructions for use.

## REAGENTS READY FOR USE OR TO BE RE-FORMED

### ✓ **Extraction pack:**

When the *Legionella* Extraction Pack 01 is first used, the following solutions must be prepared :

- "Binding buffer"
- "Washing buffer 2"
- "Elution Buffer"

Reagents preparation is described on page 3 of the pack's instructions for use. The other reagents are ready for use.

### ✓ **GeneDisc Premium pack:**

The reagents are ready for use. However a standard curve must be validated in advance for each GeneDiscs GDLSP-471 or GDLP-471 batch number. For this, it is necessary to reconstruct a DNA tube calibrated at 250 000GU.

## TRAINING TIME FOR OPERATOR NOT INITIATED INTO THE METHOD

Initial training for a technician is 2 days.

## REAL HANDLING TIME

Step	Time necessary for 6 samples
Filtration	Between 5 to 30 minutes according to the type of water
DNA extraction	1h45
PCR	15 min of preparation / PCR duration: 55min
Results analysis	10 minutes

## TIME LIMIT FOR OBTAINING RESULTS

### • **Minimum time limit:**

5h for 5 samples. The result can be delivered in JO.

In the event of inhibition, the duration is increased by 1h30.

### • **Achievement of PCR after extraction:**

Analysis can be interrupted after extraction. The extract is thus preserved at  $-20^{\circ}\text{C} \pm 3^{\circ}\text{C}$  if the PCR analysis is not carried out within 6 hours after the extraction. This enables analysis organization optimisation.

## OPERATOR'S QUALIFICATION TYPE

Technician.

## ANALYSIS RESULTS TRACEABILITY

Results are preserved in computer files and/or on paper. Computer files cannot be modified by the operator. Steps other than PCR are traced in the documents anticipated by the laboratory. The GeneDisc Cyclo<sup>®</sup> and GeneExtract<sup>®</sup> are equipped with bar code readers enabling analysis traceability (batch, date, operator, samples identification).

## LABORATORY MAINTENANCE

Maintenance was not carried out by the laboratory.

Annual maintenance is carried out by Genesystems: thermal metrology, optics and biological validation.

## MINIMUM VOLUME TO PIPETTE

The minimal volume to pipette is 20  $\mu\text{l}$  and in the event of inhibition, it is 10 $\mu\text{l}$ .

## STABILITY OF REAGENTS AND RANGES

The shelf life and stability, are indicated on the pack. Storage conditions are described on the Packs. The different reagents from the “*Legionella* Extraction Pack 01” are aliquoted for the execution of two series of assays.

## UNG

Contamination avoidance guideline are on page 8 of the “*Legionella* Extraction Pack 01” instructions for use and page 3 of the “GeneDisc Pack Premium” instructions for use.

Indeed, prevention goes by decontamination of modules, filtration accessories and respect for Good Laboratory Practices.

The “blank method” guarantees, among others, an absence of DNA contamination at the time of analysis.

## PROTECTION OF REAGENTS FROM UV

The reagents are kept in their original packaging (opaque in the light).

## EXTERNAL QUANTITATIVE PCR CONTROL

An amplification of a standard DNA with origins different to that used for the range and pre-loaded in sector 6 of the disc is achieved for each disc. (cf. Page 2 of the “GeneDisc Premium Pack”) instructions for use.

## CONTROL FOR THE ABSENCE OF INHIBITORS

The presence of the PCR inhibitor is checked in each DNA extract on each analysis.

Two internal inhibition controls are present in each analysis sector. This is due to calibrated oligonucleotides including specific primers from *Legionella* spp.

These controls are amplified at the same time as the samples.

(Cf. Page 2 of the “Premium GeneDisc Pack instructions for use”).

# 3. RING TRIAL

## 3.1. AIM

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This study aims to assess the fidelity (repeatability and reproducibility) of kits marketed by Genesystems for the detection of *Legionella* spp. All of the laboratories participating in this test use Genesystems technology.

## 3.2. TEST PLAN AND ANALYSES PERFORMED

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### 3.2.1. TEST PLAN

These tests have three parts which correspond to different matrices:

- Part I: DNA solution from two extracts of *L. parisiensis* (CIP 103847) and *L.pneumophila* (ATCC 33152).

- Part II: distribution water doped in *L. parisiensis* (CIP 103847), *L.pneumophila* (ATCC 33152) and *E .coli* (CIP 106878).
- Part III: naturally contaminated hot tap water.

The legionella strains used are cultivated on GVPC agar-agar, *E .coli strains, are cultivated in nutritive broth. The L.pneumophila* (ATCC 33152) come from commercial pellets and *E .coli* (CIP 106878) come from the Central Laboratory collection for *L. parisiensis* (CIP 103847).

*Legionella spp* has been detected for the three part.

Two spiking levels were achieved in Phase I. The first spiking level was contained in the A tubes and the second spiking level was contained in the B tubes.

In part II, two spiking levels were also achieved. The first spiking level was contained in the C flasks and the second spiking level was contained in the D flasks.

For part III, the samples were contained in the E flasks.

### 3.2.2. CONTROL OF THE QUALITY OF THE MATERIALS

The different samples (A, B, C, D and E).were controlled by batch out of the 10 flasks tested over the desired analysis period.

## 3.3. RESULTS

### 3.3.1. CONTROL OF THE QUALITY OF THE MATERIALS

Batch control demonstrated that all of the flasks tested (A, B, C, D and E) were homogenous and stable over the desired analysis period

### 3.3.2. REPEATABILITY AND REPRODUCIBILITY

The table which follows gives a summary of the main information for the *Legionella spp* parameter.

#### Valeurs de fidélité - *Legionella spp* :

Parameter	<i>Legionella spp</i>	<i>Legionella spp</i>	<i>Legionella spp</i>	<i>Legionella spp</i>	<i>Legionella spp</i>
Sample	A	B	C	D	E
Number of laboratories retained	14	14	15	15	11
Initial	16	16	16	16	16
M = General average (n/l)	186 546	1 795 000	70 756	708 257	149 673
Sr (n/l)	20 123	146 896	25 698	100 706	36 279
SR (n/l)	55 991	486 895	65 645	686 326	141 448
CVr (%)	10,8%	8,2%	36,3%	14,2%	24,2%
CVR (%)	30,0%	27,1%	92,8%	96,9%	94,5%
M = General average (in log)	5,253	6,241	4,667	5,666	5,013
Sr (in log)	0,051	0,035	0,113	0,084	0,098
SR (in log)	0,130	0,104	0,445	0,440	0,425
CVr (%)	1,0%	0,6%	2,4%	1,5%	2,0%
CVR (%)	2,5%	1,7%	9,5%	7,8%	8,5%

With:

- $M$  = general average: sum of all non eliminated data, divided by the number of non eliminated data
- $s_r$ : repeatability standard deviation
- $s_R$ : reproducibility standard deviation
- $CV_r$ : repeatability variation coefficient ( $=s_r/M$ ), expressed in %
- $CV_R$ : reproducibility variation coefficient ( $=s_R/M$ ), expressed in %

Several conclusions can be drawn from this study.

The first conclusion relates to the results average obtained by all of the laboratories compared to the average value estimated by the laboratory operator (homogeneity test). The maximal difference between these two values is 0.25 Log.

Repeatability standard deviations show that the overall method is repeatable since the maximal value obtained is 0.113 Log.

Finally, the reproducibility standard deviations express the samples' degree of complexity. Indeed, the maximal standard deviation obtained for the analysis of DNA solutions is 0.130 Log and it can reach 0.445 for the samples taking into account all of the analysis step (DNA preparation and amplification).

#### **CONCLUSION**

This data is compliant with the performances notified by the supplier.

## 3. CONCLUSION

Results from the preliminary study, achieved by the Specialist Laboratory, and the inter-laboratory study demonstrate that the Genesystems method performances for the detection of *Legionella* spp comply with the requirements of the XP T 90-471 standard.

After this validation, the Genesystems method received certification number GEN 25/03-12/07 from AFNOR Validation. The GeneSystems *Legionella* spp. method can be applied to any type of water, without restrictions on use.

# **PART 2: Extended AFNOR validation of the Genesystems method for the detection and quantification of *Legionella spp* in water analysis**

## **1. PURPOSE OF THE STUDY**

The present study looks at extending the AFNOR validation of the GeneSystems *Legionella spp* method for the detection and quantification of *Legionella spp* by PCR in waters.

It has been carried out in accordance with the “validation protocol for detection kits and counting of *Legionella* and *L.pneumophila* by concentration and genetic amplification using polymerase chain reaction (PCR) (Revision 0 adopted by AFNOR certification on 26.09.2006)”. The modifications defined at the meeting of 07 September 2007 were also taken into account for this study.

Phases 1 and 2 enable an expert laboratory to study performances notified by the supplier. The extraction recovery, detection limits and PCR step quantification as well as the method’s practicability were examined in particular. It was agreed, in accordance with the technical study, that inclusivity and exclusivity of detection and quantification as well as phase 3 (inter-laboratory study) were not necessary, account taken of modifications contributed to the method.

### **1.1. STANDARD OF VALIDATION**

The AFNOR validation protocol is based on experimental plan criteria and calculation method defined in the XP T 90-471 standard (April 2006) relating to the counting of *Legionella* and *L.pneumophila* by concentration and genetic amplification with polymerase chain reaction (PCR).

## 1.2. GENESYSTEMS METHOD PRINCIPLE

The GeneSystems *Legionella* method is based on the use of the following kits:

- Bacterial DNA extraction:
  - o *Legionella* Extraction Pack 05 (Instructions PELEG05-96\_04.F and PELEG05-96\_01.EN)
  - o *Legionella* Extraction Pack 06 (Instructions PELEG06-48\_01.F and PELEG06-48\_01.EN)
- Detection and quantification by PCR in real time:
  - o *Legionella* spp. GeneDisc Pack 12 sectors (Instructions GDLSP-471-12\_01.F and GDLSP-471-12\_01.EN)
  - o *Legionella pneumophila* GeneDisc Pack 12 sectors (Instructions GDLPN-471-12\_01.F and GDLPN-471-12\_01.EN)
  - o Duo *Legionella pneumophila*-spp. GeneDisc Pack (Instructions GDLPLG-471\_01.F and GDLPLG-471\_01.EN)

The purpose of the extension is the miniaturisation of silica columns, enabling 48 samples to be analysed simultaneously (*Legionella* Extraction Pack 05) as well as the PCR analysis achieved in duplicate (*Legionella* spp. 12 sectors GeneDisc Pack and Duo *Legionella pneumophila*-spp. GeneDisc Pack).

The *Legionella* Extraction pack 05 contains all consumables and reagents necessary to carry out the filtering of water samples, cellular lysis and DNA purification.

DNA extraction is based on a mechanical lysis of cells in the presence of detergent, followed by DNA purification by adsorption on mini silica columns. The DNA preparation protocol is managed by the GeneExtract.

*Legionella* DNA is then quantified by real time PCR assisted by the *Legionella* GeneDiscs Packs.

The PCR GeneSystems *Legionella* test rests on genetic amplification in real time by PCR from a specific nucleic sequence of the *Legionella* spp classification or the *L. pneumophila* type. Detection is possible with the use of TaqMan<sup>®</sup> probes marked by a fluorophore (FAM or ROX). When the amplicon is elongated, the probe is cleaved and the fluorophore, physically separated from the Quencher, gives off fluorescence. This fluorescence is measured directly by the GeneDisc Cyclor optical module.

The DNA/Master Mix mixture is loaded in a GeneDisc sector and depressed in 6 reactional chambers preloaded in reagents (primers, probes, DNA) for each analysis. The composition in the 6 reactional chambers of each sector of the different types of GeneDiscs in the method is indicated in the following tables:

GDLPN-471-12		FAM detection	ROX detection
Analysis sector no	PCR wells no		
1-11	1	Internal inhibition control	-
	2	<i>L. pneumophila</i> analysis	Negative control -
	3	<i>L. pneumophila</i> analysis	-
12	1	<i>L. pneumophila</i> analysis	Negative control
	2	Internal inhibition control	-
	3	External quantitative control	-

GDLSP-471-12		FAM detection	ROX detection
Analysis sector no	PCR wells no		
1-11	1	-	Internal inhibition control
	2	Negative control	<i>Legionella spp</i> analysis
	3	-	<i>Legionella spp</i> analysis
12	1	Negative control	<i>Legionella spp</i> analysis
	2	-	Internal inhibition control
	3	-	External quantitative control

GDLPLG-471		FAM detection	ROX detection
Analysis sector no	PCR wells no		
1-5	1	Lp internal inhibition control	-
	2	<i>L. pneumophila</i> analysis	-
	3	<i>L. pneumophila</i> analysis	Negative control -
	4	Negative control	<i>Legionella spp</i> analysis
	5	-	<i>Legionella spp</i> analysis
	6	-	L.g internal inhibition control
6	1	<i>Legionella spp</i> analysis	Negative control
	2	Lp internal inhibition control	-
	3	<i>L. pneumophila</i> analysis	Negative control
	4	Lg internal inhibition control	-
	5	Lg external quantitative control	-
	6	Lp external quantitative control	-

Lp: *L. pneumophila* Lg: *Legionella spp*

A calibration GeneDisc, corresponding to the PCR amplification of standard genomic DNA from *L. pneumophila* ATCC33152, is quantitatively analysed for each GeneDisc batch. The GeneDisc Cycler software calculates the Ct, equation parameters of the standard curve and the second degree polynomial automatically representing the curve: Inhibition controls fluorescence amplitude = f (Ct *L. pneumophila*). This polynomial expresses internal inhibition controls sensitivity and competition in the presence of genomic DNA from *L. pneumophila* (outside of inhibition).

The parameters obtained with GeneDisc calibration linked with Master Mix are saved and applied to all GeneDiscs from the same batch, on a given GeneDisc Cyclor, in accordance with the PCR series definition which follows the XP T90-471 standard.

In the same experimental conditions as those for GeneDisc calibration, the inhibition percentage for the internal inhibition controls is determined by the software so as to indicate to the operator the dilution factor to apply if necessary for each sample analysed (d5 or d10). If the PCR reaction is not inhibited, the software calculates the Ct automatically and converts it into GU of *Legionella* / L according to the volume of water filtered.

## 2. PRELIMINARY STUDY RESULTS

### 2.1. DETERMINATION OF RECOVERY

The recovery was assessed to verify the method's compliance. It was assessed for 6 independent samples, for three different matrices and for three different concentration levels. 54 samples were analysed in total. The recovery was assessed with the «*Legionella pneumophila* GeneDisc pack» (Ref. GDLPN-471-12 HT *L. pneumophila*).

Matrices tested:

- hot tap water sampled in the laboratory\*
- TAR water\*\*
- Evian mineral water\*

\* exempt from *Legionella nucleic acids*

\*\* exempt from *Legionella pneumophila nucleic acids*

Concentration levels assessed: 1000, 10 000 and 100 000 GU/250 ml.

Inoculum: *L. pneumophila* culture (ATCC 33152 T strain)

The *L. pneumophila* stock solution was titrated by direct lysis and microscopic counting after marking of the cells in DAPI.

The analyses were achieved over 3 days.

- Uncertainty associated with direct lysis

	<i>L. pneumophila</i>		DAPI*
	Mean	Standard deviation	Mean
<b>Day 1</b>	<b>8.39</b>	0.17	<b>7.7</b>
<b>Day 2</b>	<b>8.34</b>	0.03	<b>7.8</b>
<b>Day 3</b>	<b>8.55</b>	0.19	<b>7.9</b>

\* The counting after DAPI marking is obtained from the average from the number of bacteria counted over 20 fields.

- Study of the optimal recovery from the GeneSystems *Legionella* method

Type of water	Level of concentration examined	Recovery average obtained (%)	Recovery average per type of water (%)	Average from bias obtained (Log)	Bias standard deviation (S <sub>R</sub> )	Uncertainty <sup>(1)</sup>
Evian water	1 000	123	97	- 0.05	0.21	0.43
	10 000	59				
	100 000	109				
ECS	1 000	140	99	-0.07	0.25	0.57
	10 000	89				
	100 000	68				
TAR	1 000	115	84	-0.15	0.25	0.52
	10 000	80				
	100 000	58				

<sup>(1)</sup> Uncertainty =  $(2x\sqrt{((\text{bias})^2 + (S_R)^2)})$

### CONCLUSION

The average recovery obtained is greater than 25%. No inhibitions were observed at the time of these tests. The method is robust vis-à-vis the different types of water analysed, with the average recovery obtained for each matrix not being significantly different.

## 2.2. DETECTION LIMIT

To assess the *Legionella spp* detection limit (*Legionella spp*. GeneDisc pack), data obtained from the previous study was used and processed according to the new protocol (final result calculated from an average from 2 sets of data).

### CONCLUSION

The detection limit is validated at 5 GU/well for the *Legionella spp* GeneDisc pack, in compliance with performances notified by the supplier.

## 2.3. QUANTIFICATION LIMIT

To assess the *Legionella spp* quantification limit (*Legionella spp*. GeneDisc pack), the data obtained from a previous study was used and processed according to the new protocol (final result calculated from an average from 2 sets of data).

Three combinations for calculating results are possible. The results from these three processings are expressed in "Result 1", "Result 2", "Result 3".

	Target value	Target value (log)	Value average measured (n = 30)	Bias (log)	Confidence interval at 95% (2.t.s)	Accuracy test (t calculated)	uncertainty measurement*	Conclusion
<b>Validation criteria</b>					< 0.50	<2.045	<0.30	
<b>Result 1</b>	25	1.39	23	0.07	0.293	0.157	0.179	compliant
<b>Result 2</b>	25	1.39	23	0.09	0.369	0.125	0.216	compliant
<b>Result 3</b>	25	1.39	24	0.11	0.339	0.136	0.179	compliant
<b>Previous preliminary study performance</b>	25	1.39	23	0.03	0.289	2.609	0.15	
<b>Conclusion</b>					compliant	compliant	compliant	

Measurement uncertainty =  $2 \times \sqrt{(\text{bias}^2 + \text{standard deviation}^2)}$

## CONCLUSION

For the “*Legionella* spp. GeneDisc Pack”, the quantification limit is repeatable and accurate at 25 GU/well. In terms of measurement uncertainty, it complies with the new statistical model in the XP T90-471 standard currently being revised.

## 2.4. DETERMINATION OF LINEARITY

Linearity has been evaluated by analysing 5 rows achieved from standard DNA from *L. pneumophila* ATCC33152 supplied by Genesystems (Ref. SDNA-Lp).

The linearity study was carried out for values 25, 250, 2 500, 25 000, 250 000 GU/well.

Detection and amplification were achieved with the “Duo *L.pneumophila* - spp. Pack”.

- Results

Equation of the standard curve			
Slope/Efficacy	Acceptable domain	Intercept	Conclusion
- 3.440	-4.115 < a < -2.839 75% < E < 125%	36.448	Compliant
Statistical analysis of linear model			
Origin	Value observed	Critical value With $\alpha = 5\%$	Conclusion
F regression	4632.6	4.35	Non compliant
F model error	3.25	3.10	Non compliant

Uncertainty analysis – model undergoing evaluation (XP T90-471)					
GU Target	25	250	2500	25000	250000
Log Target	1.40	2.40	3.40	4.40	5.40
Average bias	0.068	-0.028	-0.061	-0.066	0.087
standard deviation	0.083	0.112	0.014	0.027	0.029
Elin*	+/- 0.11 Log	+/- 0.12 Log	+/- 0.06 Log	+/- 0.07 Log	+/- 0.09 Log
Uncertainty	+/- 0.30 Log	+/- 0.32 Log	+/- 0.17 Log	+/- 0.20 Log	+/- 0.25 Log

\*Elin =  $\sqrt{(\text{bias}^2 + \text{standard deviation}^2)}$

## CONCLUSION

Taking Elin into account, the linear domain is validated between 25 and 250 000 GU of genomic DNA from *L.pneumophila* ATCC 33152 for the “Duo *L.pneumophila* - spp. Pack”. Too good a repeatability (standard deviation < 0.12Log) does not allow the linear domain to be validated by the Fisher test.

## 2.5. INCLUSIVITY AND EXCLUSIVITY

Inclusivity and exclusivity tests were not carried out in accordance with the technical study.

## 2.6. PRATICABILITY

The 18 criteria defined in the AFNOR validation protocol were studied.

### REAGENTS PACKAGING

The reagents are present in the following packaging.

- **Legionella Extraction Pack 05**

Items are given on the packaging and page 2 of the instructions for use

The extraction reagents are:

- ✓ "Lysis buffer".
- ✓ "Linkage buffer" useable after adding an "extra Linkage buffer".
- ✓ "Washing Buffer 1".
- ✓ "Washing Buffer 2".
- ✓ "Elution buffer".
- ✓ 8 mini silica columns strips.

- **Legionella GeneDisc Pack**

Information on the components is given on the packaging and on page 2 of the instructions for use.

There are two GeneDisc packs:

- ✓ "*Legionella* spp GeneDisc Pack".
- ✓ "*Legionella pneumophila* GeneDisc pack"  
(Ref. GDLPN-471-12 HT L. pneumophila).

Each GeneDisc pack contains:

- ✓ The "reactional mix" used on PCR reaction (6 tubes: 1 tube / GeneDisc)
- ✓ 6 "GeneDiscs"

- **Duo *Legionella pneumophila* – spp GeneDisc Pack**

Items are given on the packaging and page 2 of the instructions for use

Each GeneDisc pack contains:

- ✓ The "reactional mix" used on PCR reaction (6 tubes: 1 tube / GeneDisc)
- ✓ 6 "GeneDiscs"

## REAGENTS' VOLUME

The reagents' volume to be used is indicated in the "*Legionella* Extraction Pack 05" instructions for use.

## COMPONENTS STORAGE CONDITIONS AND SHELF LIFE OF PRODUCTS

The storage temperature is indicated on the packs as well as in the instructions for use: on page 3 of the extraction pack and on page 1 of the GeneDisc pack.

The GeneDisc Packs must be kept in a refrigerator (5°C ± 3°C). All other components can be stored at ambient temperature (15°C-30°C).

The shelf life is indicated on the Packs, as well as on each constituent of the Packs.

## USAGE METHODS AFTER FIRST USE

The reagents are used until exhaustion with respect of the shelf life.

## NEEDS IN TERM OF EQUIPMENT AND SPECIFIC FACILITIES

The necessary material and consumables are indicated on page 3 of each set of instructions for use for the two GeneDiscs packs and on page 2 of the extraction pack instructions for use.

Safety measures are indicated on page 2 of the Extraction pack instructions for use.

## REAGENTS READY FOR USE OR TO BE RECONSTRUCTED

- ✓ **Extraction pack:**

When first using the *Legionella* Extraction Pack 05, the following solutions must be prepared:

- "Binding buffer"
- "Washing buffer 2"

Reagents preparation is described on page 3 of the pack's instructions for use. The other reagents are ready for use.

✓ **GeneDisc pack and DUO:**

The reagents are ready for use. However, a standard curve must be validated beforehand for each GeneDiscs GDLSP-471-12 or GDLP-471-12 or GDLPLG-471 batch number. To do this, it is necessary to reconstruct a DNA tube calibrated at 250 000GU.

**TRAINING TIME FOR OPERATOR NOT INITIATED INTO THE METHOD**

Initial training for a technician is 2 days.

**REAL HANDLING TIME**

Step	Time necessary for 8 samples
Filtration	Between 5 to 30 minutes according to the type of water
DNA extraction	1h15
PCR	15 min of preparation / PCR duration: 55min
Results analysis	10 minutes

**TIME LIMIT FOR OBTAINING RESULTS**

• **Minimum time limit:**

2h15 for 5 samples (GeneDiscs DUO Legionella pneumophila – spp.), 2h15 for 11 samples (GeneDiscs Pack Legionella or Legionella pneumophila) or 3h15 for the 12 samples GeneDiscs Pack (Legionella and Legionella pneumophila pack). The result can be issued on J0. In the event of inhibition, the duration is increased by 1h30.

• **Achievement of PCR after extraction:**

Analysis can be interrupted after extraction. The extract is thus preserved at -20°C ± 3°C if the PCR analysis is not carried out within 6 hours after the extraction. This enables analysis organization optimisation.

**OPERATOR'S TYPE OF QUALIFICATION**

Technician.

**ANALYSIS RESULTS TRACEABILITY**

The results are preserved in computer files and/or on paper. Steps other than PCR are traced in documents expected at the laboratory. GeneDisc Cyclor® and GeneExtract® are equipped with bar code readers enabling analysis traceability (batch, date, operator, samples identification).

**LABORATORY MAINTENANCE**

Maintenance was not carried out by the laboratory. Annual maintenance is carried out by Genesystems : thermal metrology, optics and biological validation.

**MINIMUM VOLUME TO PIPETTE**

The minimal volume to pipette is 20 µL.

**STABILITY OF REAGENTS AND RANGES**

The shelf life and stability are indicated on the pack. Storage conditions are described on the Packs.

**UNG**

Advice for the prevention of contamination is indicated on page 8 of the “Legionella Extraction Pack 05” instructions for use and on page 3 of the “GeneDisc Pack” instructions for use.

Indeed, prevention goes by the decontamination of filtration accessories and respect for Good Laboratory Practices.

The “blank method” guarantees, among others, an absence of DNA contamination at the time of analysis.

### REAGENTS' UV PROTECTION

The reagents are kept in their original packaging (opaque in the light).

### EXTERNAL QUANTITATIVE PCR CONTROL

An amplification of a standard DNA with origins different to those used for the range and pre-loaded in sector 12 of the 12 sector disc (and in sector 6 for DUO discs) is achieved for each disc. (cf. Page 2 of the "GeneDisc Pack" instructions for use).

### CONTROL FOR THE ABSENCE OF INHIBITORS

The presence of the PCR inhibitor is checked in each DNA extract on each analysis. An internal inhibition control is present in each analysis sector. It consists of calibrated oligonucleotides including specific primers of *Legionella* spp or *L. pneumophila*. These controls are amplified at the same time as the samples. (Cf. Page 2 of the "GeneDisc Pack" instructions).

## 3. RING TRIAL

In accordance with the AFNOR technical team, the ring trial was not carried out for this method's extended validation.

## 3. CONCLUSION

The results from the Expert Laboratory demonstrate performances compliant with the requirements of the XP T 90-471 standard as regards the evaluation of the Genesystems method for the detection of *Legionella* spp.

After this validation, the Genesystems method received certification number GEN 25/03-12/07 from AFNOR Validation. The GeneSystems *Legionella* spp. method can be applied to any type of water, without restrictions on use.