



**Water analysis methods  
Analytical performances certified**

**VALIDATION CERTIFICATE FOR ANALYTICAL METHOD  
ACCORDING TO THE *Legionella* PCR VALIDATION PROTOCOL**

**Certificate No:** GEN 25/03 – 12/07

**Validation date:** 18.12.2007  
**Extension date:** 25.03.2009  
**End of validity:** 18.12.2012

**The company**      **GeneSystems**  
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is hereby authorized to refer to this AFNOR Validation certificate for the following quantitative analysis method:

**GeneSystems *Legionella* spp**

**Protocol references:**

Product name	SAP part number	New protocol reference (SAP)	Last protocol reference
Extraction Pack Environnement 1	PENVI1096	PENVI1096_01.EN	PELEG05/96_05.EN
GeneDisc <i>Legionella</i> spp 06	GLEGSP106006	GLEGSP_01.EN	GDLSP-471_08.EN
GeneDisc <i>Legionella</i> DUO 06	GLEGDUO106006	GLEGDUO_01.EN	GDLPLG-471_03.EN
Standard Calibrated DNA LP	ALEGPNE105	ALEGPNE_01.EN	SDNA-LP_04.EN

**SCOPE:** Sampling all types of waters

**RESTRICTIONS FOR USE:** None

**REFERENCE METHOD**

French Standard XP T 90-471, Detection and quantification of *Legionella* and/or *Legionella pneumophila* by concentration and genetic amplification by polymerisation chain reaction (PCR), April 2006

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## VALIDATION HISTORY

The initial validation was passed in December 2007. The following changes were made to kits in 2008:

- Changes of the silica columns format in the extraction pack (miniaturisation) to handle 48 samples simultaneously.
- Changes of the GeneDisc design making 12 sectors of analysis. PCR analyses are achieved in duplicate (2 PCR wells per target) instead of in triplicate.

A study for extending validation has been carried out by an expert laboratory as regards the following parts of the preliminary study: detection and quantification limits, linearity, optimal recovery. The extension results appear in the present version of the certificate.

The extension has been validated. The new modified kit does not replace the kit validated in 2007: the two versions are validated and will be able to be used on choice. The DNA extracts can be analysed using the Extraction Pack Environnement 1, whatever the GeneDisc design, on 2 or 3 analysis wells.

## PRINCIPLE OF THE METHOD

The GeneSystems *Legionella* method has two steps:

- a first step of microbial DNA preparation from the water sample achieved with the GeneExtract<sup>®</sup> platform and needing the use of the Extraction Pack Environnement 1,
- a second step of *Legionella pneumophila* or *Legionella* spp DNA quantification by real time PCR with the GeneDisc Cycler<sup>®</sup> instrument and GeneDisc *Legionella* spp 06 or DUO 06.

## METHOD'S OPTIMAL RECOVERY

The recovery study was carried out on 6 independent samples, with three contamination levels for each of the three different matrices (a mineral water (Evian water) as a negative control, a hot tap water and a cooling tower water).

Each of the waters have been previously tested for exemption from *Legionella's* nucleic acids. The samples have been artificially contaminated by a stock solution formed from an original *L. pneumophila* strain (ATCC33152 strain).

2007 study on the *Legionella* Extraction Pack 01:

Type of water	Contamination level examined (GU)	Average recovery by level of contamination (%)	Average recovery per water type (%)	Average bias per water type	Bias standard deviation
Evian Water	1 000	117	75	-0.19	0.25
	10 000	45			
	100 000	63			
Hot Tap Water	1 000	102	64	-0.27	0.26
	10 000	58			
	100 000	32			
Cooling Tower	1 000	84	66	-0.24	0.27
	10 000	73			
	100 000	42			

### Conclusion

The method's average recovery is greater than 60%. The method is robust as regards the different types of water: the type of water does not seem to have an impact on the bias and no inhibitions were observed.

2009 study on the *Legionella* Extraction Pack 05:

Type of water	Contamination level examined (GU)	Average recovery by level of contamination (%)	Average recovery per water type (%)	Average bias per water type	Bias standard deviation	Uncertainty (1)
Evian Water	1 000	123	97	- 0.05	0.21	0.43
	10 000	59				
	100 000	109				
Hot Tap Water	1 000	140	99	-0.07	0.25	0.57
	10 000	89				
	100 000	68				
Cooling Tower	1 000	115	84	-0.15	0.25	0.52
	10 000	80				
	100 000	58				

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

### Conclusion

The method's average recovery is greater than 25%. No inhibitions were observed. The method is robust regarding different types of water tested.

### PCR DETECTION LIMIT (LD<sub>PCR</sub>)

The *Legionella* spp GeneDisc *Premium* performances study was achieved with dehydrated DNA from *L. pneumophila* ATCC 33152, marketed by the GeneSystems company, under the SDNA-Lp part number.

2007 Study:

The *Legionella* spp GeneDisc *Premium* detection limit with DNA from *L. pneumophila* ATCC 33152 is 5 GU/PCR, that is to say 170GU/L when 1 L of water is filtered.

2009 Study:

The data was reprocessed, by taking two of the three sets of data obtained in 2007 (duplicate instead of triplicate). The *Legionella* spp GeneDisc *Premium* detection limit is 5 GU/PCR.

## PCR QUANTIFICATION LIMIT (LQ<sub>PCR</sub>)

### 2007 Study:

The quantification limit of 25 GU/PCR was tested with 30 repeated measurements from a *L. pneumophila* ATCC 33152 calibrated DNA solution.

	Target value (GU/PCR)	Target value (Log)	Average (n=30)	Bias (Log)	IC at 95% (2.t.s)	t calculated (accuracy)	Measurement Uncertainty <sup>(1)</sup>
<b>Criteria</b>					< 0.50	< 2.045	< 0.30
<b>Results</b>	25	1.39	23	0.03	0.288	2.338	0.15
<b>Conclusion</b>					Compliant	Non Compliant	Compliant

$$^{(1)}\text{Measurement uncertainty} = \sqrt{(\text{bias}^2 + (\text{standard deviation})^2)}$$

Measurements at 25 GU/PCR are repeatable but present a default in accuracy according to the Student test. In terms of measurement uncertainty, the quantification limit at 25 GU/PCR is compliant with the statistical model approved by the AFNOR T90E Committee in 2008. Results from tests on the quantification limit at 25 GU/PCR are therefore satisfactory.

### 2009 Study:

The data for 2007 was reprocessed, by taking 2 sets of data (for 2 wells) from 3 initial sets of data (for 3 wells). There were three possible combinations for calculation of the results with two wells. The results confirm that the quantification limit is repeatable and accurate up to 25 GU/wells. In terms of measurement uncertainty, the quantification limit at 25 GU/PCR complies with the statistical model approved by the AFNOR T90E Committee in 2008.

## LINEARITY

### 2007 Study:

The linearity study was carried out with five ranges of 5 *L. pneumophila* ATCC 33152 DNA concentration levels (25, 250, 2 500, 25 000 and 250 000 GU/PCR) analysed in repeatability condition.

Equation of the standard curve			
<i>slope / Efficacy</i>	<i>Acceptable domain</i>	<i>Intercept</i>	<i>Conclusion</i>
- 3.483 / 93.7%	- 4.115 < a > -2.839 75% < E < 125%	40.257	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 of regression model	2509,6.77	4.35	Compliant
F of standardisation model	0.24	3.10	Compliant

### Conclusion

The *L. spp* GeneDisc Premium standard curve complies with the acceptance criteria defined in the validation protocol.

**2009 Study:**

The linearity study was carried out with five ranges of 5 standard DNA concentration levels. Detection and amplification were carried out with the « Duo *L. pneumophila* – spp Pack»

Equation of the standard curve			
<i>slope / Efficacy</i>	<i>Acceptable domain</i>	<i>Intercept</i>	<i>Conclusion</i>
- 3.440	- 4.115 < a > -2.839 75% < E < 125%	36.448	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 of regression model	4632.6	4.35	Compliant
F of standardisation model	3.25	3.10	Non compliant

Repeatability (standard deviation < 0.12 Log) does not allow linear domain validation with the Fisher test. On the other hand, when considering the Elin linearity error (uncertainty analysis, model proposed by the AFNOR T90E Committee), the linear domain is validated between 25 and 250 000 GU of genomic DNA of *L.pneumophila* ATCC 33152.

GU Target	Uncertainty Analysis				
	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
t	2.78	2.78	2.78	2.78	2.78
Uncertainty	+/- 0.30 Log	+/- 0.32 Log	+/- 0.17 Log	+/- 0.20 Log	+/- 0.25 Log

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

**SPECIFICITY OF L. SPP PREMIUM GENEDISC**

Tests have been carried out on all of the strains listed in the AFNOR Validation protocol.

**Inclusivity Tests**

The 21 strains of *Legionella* spp and the 15 *Legionella pneumophila* serogroups have been detected.

**Exclusivity Tests**

The DNA analysis of 17 strains not belonging to the *Legionella* classification did not show the presence of crossed reactions.

## INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2007 with 16 participating laboratories. Two laboratories have not been able to give the expected results.

The purpose of this study is to assess the precision (repeatability and reproducibility) of the GeneSystems *Legionella* method:

- for the genetic amplification step alone;
- for the overall analysis (concentration, lysis, extraction, purification and genetic amplification) on characterised bacterial suspensions;
- for the whole analysis in real situation (naturally contaminated hot tap water).

### Results

	Type of samples	Calibrated DNA solution		Spiked Tap water		Natural sample
Spiking levels (GU/L)	<i>L. pneumophila</i> ATCC 33152	7 600	94 000	660	7 000	Naturally contaminated hot tap water
	<i>L. parisiensis</i> CIP 103847	8 800	85 000	1 800	16 000	
	<i>E. coli</i>			110	1 400	
Number of laboratories	participant	16	16	16	16	16
	retained	14	14	15	14	11
Homogeneity Test	Number of analyses	20	20	7	11	10
	Average (Log)	5.470	6.449	4.490	5.792	5.173
Results	Average (Log)	5.253	6.241	4.667	5.666	5.013
	Bias (Log)	0.217	0.208	0.183	0.126	0.160
	S <sub>r</sub> (Log)	0.051	0.035	0.113	0.084	0.098
	S <sub>R</sub> (Log)	0.130	0.104	0.445	0.440	0.425
	$E_r(\sqrt{\text{bias}^2 + S_r^2})$	0.223	0.211	0.215	0.151	0.188
	$E_R(\sqrt{\text{bias}^2 + S_R^2})$	0.253	0.233	0.481	0.458	0.454
	$E_{\text{Total}}(\sqrt{\text{bias}^2 + S_r^2 + S_R^2})$	0.258	0.236	0.494	0.465	0.465

In terms of repeatability (r), the standard deviation observed is 0.06 for the PCR stage (calibrated DNA solutions), 0.12 for the entire method (preparation of DNA & PCR) with artificially contaminated water samples and 0.16 for natural samples. The GeneSystems *Legionella* spp method is repeatable.

The reproducibility (R) standard deviations express the samples' degree of complexity: those obtained for the PCR analysis of calibrated DNA solutions are 0.13 whilst those corresponding to all of the analysis steps (preparation of DNA & PCR) are between 0.40 and 0.50.

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

## PRACTICABILITY

- The packaging of kits and instructions for use enables easy handling and analysis traceability.
- The duration of the different steps is compatible with a short time to result (<24H).
- The software associated with the GeneExtract<sup>®</sup> platform and the GeneDisc Cyclor<sup>®</sup> real time PCR instrument enables complete traceability.

## GENERAL CONCLUSION

The GeneSystems *Legionella spp* method performances comply with the requirements of the XP T90-471 standard.

Please send any queries concerning the performance of the validated method to  
AFNOR Certification.

You may download a summary document on the preliminary and inter-laboratory  
studies on [www.afnor-validation.com](http://www.afnor-validation.com)