



**Water analysis methods
Analytical performances certified**

**ANALYSIS METHOD VALIDATION CERTIFICATE
FOLLOWING THE *Legionella* PCR VALIDATION PROTOCOL**

Certificate No : GEN 25/04 – 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 pneumophila*

Protocol references:

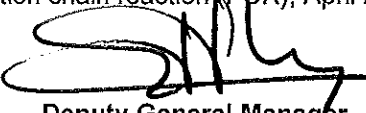
Product name	SAP part number	New protocol reference (SAP)	Last protocol reference
Extraction Pack Environnement 1	PENVI1096	PENVI1096_01.EN	PELEG05/96_01.EN
GeneDisc <i>Legionella pneumophila</i> 06	GLEGPNE106006	GLEGPNE_01.EN	GDLPN-471_07.EN
GeneDisc <i>Legionella pneumophila</i> 12	GLEGPNE112006		
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


**Deputy General Manager
Jacques BESLIN**

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 was carried out by an expert laboratory as regards the following parts of the preliminary study: detection and quantification limit, linearity and 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 water sample achieved with the GeneExtract platform[®] and needing use of Extraction Pack Environnement 1,

a second step of *Legionella pneumophila* or *Legionella* spp DNA quantification by real time PCR with the GeneDisc Cyclor[®] instrument and GeneDisc *Legionella pneumophila* 06, GeneDisc *Legionella pneumophila* 12 and GeneDisc *Legionella* 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			

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				

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

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_{PCR})

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

2007 Study:

The *Legionella pneumophila* 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:

Thirty tests were carried out in duplicate and no negative results were found. The detection limit for *Legionella pneumophila* GeneDisc Premium is 5 GU/PCR.

PCR QUANTIFICATION LIMIT (LQ_{PCR})

2007 Study:

A 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 ⁽¹⁾
Criteria					< 0.50	< 2.045	< 0.30
Results	25	1.39	23	0.03	0.288	2.338	0.15
Conclusion					Compliant	Non Compliant	Compliant

⁽¹⁾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:

Thirty analyses were achieved in duplicate in repeatability conditions.

	Target value (GU/PCR)	Target value (Log)	Average (n=30)	Bias (Log)	IC at 95% (2.t.s)	t calculated (accuracy)	Measurement Uncertainty ⁽¹⁾
Criteria					< 0.50	< 2.045	< 0.30
Results	25	1.39	23	0.03	0.390	0.118	0.208
Conclusion					Compliant	Compliant	Compliant

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

The results confirm that, the quantification limit is repeatable and accurate up to 25 GU/well when the PCR analysis is achieved in 2 wells. 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.

LINEARITY

Study 2007:

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 $\alpha = 5\%$</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. pneumophila* GeneDisc Premium standard curve is compliant 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>
-3.392	- 4.115 < a > -2.839 75% < E < 125%	38.773	Compliant
Statistical analysis of linear model			
<i>Origin</i>	<i>Value observed</i>	<i>Critical value with $\alpha = 5\%$</i>	<i>Conclusion</i>
F of regression model	10444.6	4.35	Compliant
F of standardisation model	4.13	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	0.060	-0.029	-0.070	-0.010	0.050
Standard deviation	0.028	0.017	0.058	0.025	0.023
Elin*	+/- 0.07 Log	+/- 0.03 Log	+/- 0.09 Log	+/- 0.03 Log	+/- 0.05 Log
t	2.78	2.78	2.78	2.78	2.78
Uncertainty	+/- 0.18 Log	+/- 0.09 Log	+/- 0.25 Log	+/- 0.07 Log	+/- 0.15 Log

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

SPECIFICITY of *L. PNEUMOPHILA* GENEDISC PREMIUM

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

Inclusivity Tests

15 *L. pneumophila* serogroups were detected.

Exclusivity Tests

The DNA analysis of 9 strains of *Legionella* spp and 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	5	5	5	5	5
	Average (Log)	5.401	6.489	4.338	5.365	4.798
Results	Average (Log)	5.228	6.255	4.577	5.512	4.908
	Bias (Log)	0.173	0.234	-0.239	-0.147	-0.110
	S _r (Log)	0.068	0.056	0.104	0.120	0.159
	S _R (Log)	0.134	0.110	0.414	0.493	0.414
	E _r ($\sqrt{\text{bias}^2 + S_r^2}$)	0.186	0.240	0.260	0.189	0.193
	E _R ($\sqrt{\text{bias}^2 + S_R^2}$)	0.219	0.258	0.478	0.514	0.428
	E _{Total} ($\sqrt{\text{bias}^2 + S_r^2 + S_R^2}$)	0.229	0.264	0.489	0.528	0.457

In terms of repeatability (r) the standard deviation observed is 0.06 for the PCR step (calibrated DNA solutions) and 0.12 for the whole method (DNA preparation & PCR) with spiked water samples and 0.16 for natural samples. The GeneSystems *Legionella pneumophila* method is repeatable.

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

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

PRATICABILITY

- 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® platform and the GeneDisc Cyclor® PCR instrument enables complete traceability.

GENERAL CONCLUSION

The GeneSystems *Legionella pneumophila* method performances are compliant 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